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Analysis of the biosynthesis of altertoxins in *Alternaria alternata*

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Publikationsliste

Aus dieser Arbeit sind folgende Publikationen entstanden:

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Weitere Publikationen, die nicht direkt mit dem Thema dieser Arbeit verknüpft sind:

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Shen, L.*, Gao, J.*, Wang, Y., Li, X., Liu, H., & Zhong, Y. (2021) Engineering the endoplasmic reticulum secretory pathway in *Trichoderma reesei* for improved cellulase production. *Enz. Microb. Tech.*, 152, 109923.

Li, Y., Sun, T., Guo, D., Gao, J., Zhang, J., Cai, F., Druzhinina, I., Fischer, R., Shen, Q. & Yu, Z. (2021) Comprehensive analysis of the regulatory network of blue-light-regulated conidiation and hydrophobin production in *Trichoderma guizhouense*. *Env. Microbiol.*, 23(10):6241-6256.

Yu, Z., Gao, J., Igbalajobi, O.A., Skoneczny, M., Sienko, M., Maciejewska, A.M., Brzywczy, J. & Fischer, R. (2021) The sulfur metabolism regulator MetR is a global regulator controlling phytochrome-dependent light responses in *Aspergillus nidulans*. *Sci. Bullet* 66(6):592-602.

Igbalajobi, O.A., Gao, J. & Fischer, R. (2020) The HOG pathway plays different roles in conidia and hyphae during virulence of *Alternaria alternata*. *Mol. Plant. Micr. Interact*. 33(12): 1405-1410.

* Autoren haben in gleichem Maße zu der Arbeit beigetragen

Abbreviations

SM	Secondary metabolite
PKS	Polyketide synthase
NRPS	Non-ribosomal peptide synthetase
PQ	Perylene quinone
1,8-DHN	1,8-dihydroxynaphthalene
YWA1	2,5,6,8-tetrahydroxy-2-methyl-2,3-dihydro-4H-naphtho (2,3-b) pyran-4-one
AT4HN	2-acetyl-1,3,6,8-tetrahydroxynaphthalene
T4HN	1,3,6,8-tetrahydroxynaphthalene
T3HN	1,3,8-trihydroxynaphthalene
ATX	Altertoxin
ALP	Alterperylenol
STP	Stemphyperylenol
AOH	Alternariol
AME	Alternariol monomethyl ether
TEN	Tentoxin
TLC	Thin-layer chromatography
WT	Wild type
ROS	Reactive oxygen species
LC-HRMS	Liquid chromatography–high-resolution mass spectrometry
MS/MS	Tandem mass spectrometry
MAPK	Mitogen-activated protein kinase
AH	Aerial hyphae
SH	Substrate hyphae

Contents

Summary

The filamentous fungus *Alternaria alternata* is one of the most widespread contaminants of food and feed, and a weak plant pathogen. It produces a great diversity of secondary metabolites, many of which are generally recognized as phytotoxins and mycotoxins, with alternariol and its derivatives as prominent examples. *A. alternata* is a black mold that produces dihydroxynaphthalene (DHN) melanin in its cell walls of spores and hyphae. The DHN-melanin biosynthetic gene cluster in *A. alternata* contains only three genes: the polyketide synthase-encoding gene *pksA*, the 1,3,8-trihydroxynaphthalene (T3HN) reductase-encoding gene *brm2,* and the transcription factor-encoding gene *cmrA*. The scytalone dehydratase-encoding gene *brm1* is found elsewhere in the genome. Compared to the well-characterized DHN-melanin biosynthetic pathway in *Aspergillus fumigatus*, the pathway for DHN melanin biosynthesis in *A. alternata* is less well studied. *A. alternata* also produces several secondary metabolites of the perylene quinone (PQ) family, such as altertoxins (ATX I-III). Some PQs are phytotoxic but exhibit some anticancer activity. On the other hand, ATX II is a powerful mutagen that causes DNA strand-breaks, and is therefore a serious threat to human health. The biosynthesis route of PQs is still unclear. Here, I show that the DHN-melanin biosynthetic pathway shares most of the enzymes with the pathway for PQs. Intriguingly, DHN-melanin was synthesized mainly in spores and aerial hyphae, whereas PQs were formed in substrate hyphae.

A. alternata pksA was heterologously expressed in *Aspergillus oryzae*, and the heptaketide YWA1, the hexaketide AT4HN and the pentaketide T4HN were identified as products of the enzyme reaction. I characterized two α-hydrolyses, AygA and AygB, required for DHN melanin biosynthesis. PQs biosynthesis was independent of the two enzymes. Promoter-reporter assays showed that *aygA* and *aygB* are expressed in spores and aerial hyphae, where DHN melanin is synthesized, but not in substrate hyphae, where PQs are formed. These results suggest that T4HN is probably

synthesized directly in substrate hyphae and then used to produce PQs. In spores and aerial hyphae, YWA1 and AT4HN are hydrolyzed to T4HN by AygA and AygB, which boosters the concentration of T4HN for DHN melanin biosynthesis. I also identified a new T4HN reductase, Brm3, catalyzing the formation of scytalone from T4HN. Furthermore, Brm1, Brm2 and Brm3 were required to produce PQs.

Next, I performed a feeding experiment to determine the intermediate for PQs formation in the DHN-melanin biosynthetic pathway. When different amounts of 1,8-DHN were fed to the *pksA*-deletion strain, the production of PQs, altertoxin I (ATX I), altertoxin II (ATX II) and alterperylenol (ALP), was recovered. Melanization of the *⊿pksA* strain was also recovered in liquid culture in the presence of 1,8-DHN. 1,8-DHN is hence the last common intermediate for melanin and PQs biosynthesis.

Several laccase-encoding genes were deleted separately or in combination using the CRISPR/Cas9 knock-out technology. Four laccases, LccB-D and LccF, were required for DHN melanin production, but not for PQs biosynthesis. The dimerization enzyme catalyzing the reaction of 1,8-DHN to PQs remains to be identified.

Depletion of the regulator CmrA resulted in a brownish mutant strain, unlike the pale or pinkish *pksA*-deletion strain. In fact, the genes *pksA*, *aygB*, *brm2* and *lccD* were still expressed. The transcription factor CmrA strictly controls the expression of *aygA*, *brm1*, *brm3*, *lccB* and *lccF*. PQs were not detected in the *cmrA*-deletion strain. Promoter-reporter assays showed that *pksA* is not expressed in substrate hyphae, suggesting that CmrA controls the production of PQs through controlling the expression of *pksA* and/or the dimerization enzyme-encoding gene.

The PQs biosynthesis pathway in *A. alternata* is an example of how spatial regulation of gene expression of one biosynthetic pathway can lead to different secondary metabolites.

Zusammenfassung

Der Fadenpilz *Alternaria alternata* ist einer der am weitesten verbreiteten Kontaminanten von Lebens- und Futtermitteln und ein schwacher Pflanzenpathogen. *A. alternata* produziert eine große Vielfalt von Sekundärmetaboliten, von denen viele als Phytotoxine und Mykotoxine anerkannt sind, wobei Alternariol und seine Derivate die prominentesten Beispiele sind. *A. alternata* ist ein Schwarzschimmelpilz, der in den Zellwänden seiner Sporen und Hyphen Dihydroxynaphthalin (DHN) als Melanin produziert. Das Gencluster, das für die DHN-Melanin-Biosynthese in *A. alternata* kodiert, enthält nur drei Gene, nämlich das Polyketid-Synthase-kodierende Gen *pksA*, das 1,3,8-Trihydroxynaphthalin (T3HN)-Reduktase-kodierende Gen *brm2* und das Transkriptionsfaktor-kodierende Gen *cmrA*. Das für die Scytalon-Dehydratase kodierende Gen *brm1* ist an anderer Stelle im Genom kodiert. Im Vergleich zum gut charakterisierten DHN-Melanin-Biosyntheseweg in *Aspergillus fumigatus,* ist der Weg der DHN-Melanin-Biosynthese in *A. alternata* nur unzureichend erforscht. *A. alternata* produziert auch mehrere Sekundärmetabolite, die zur Familie der Perylenchinone (PQ) gehören, wie Altertoxine (ATX I-III). Einige PQs sind phytotoxisch und haben krebshemmende Wirkung. ATX II hingegen hat starke mutagene und DNA-Strang-brechende Eigenschaften, was eine ernsthafte Bedrohung für die menschliche Gesundheit darstellt. Der Biosyntheseweg von PQs ist noch unklar. In dieser Studie habe ich nachgewiesen, dass der DHN-Melanin-Biosyntheseweg die meisten Enzyme mit dem Weg für PQs teilt. Interessanterweise wird DHN-Melanin vor allem in Sporen und Lufthyphen gebildet, während PQs vor allem in Substrathyphen synthetisiert werden.

A. alternata pksA wurde in *Aspergillus oryzae* heterolog exprimiert und die Produkte mittels Massenspektrometrie identifiziert. Das Heptaketid YWA1, das Hexaketid AT4HN und das Pentaketid T4HN sind vermutlich Produkte von PksA. Ich habe zwei α-Hydrolysen AygA und AygB charakterisiert, die für die DHN-

Melaninbiosynthese erforderlich sind. Ohne AygA und AygB wurden dennoch PQs gebildet. Ein Promotor-Reporter-Assay zeigte, dass AygA und AygB in Sporen und Lufthyphen, wo DHN-Melanin synthetisiert wird, exprimiert werden, nicht aber in Substrathyphen, wo PQs entstehen. Diese Ergebnisse deuten darauf hin, dass T4HN wahrscheinlich direkt in den Substrathyphen synthetisiert und dann zur Herstellung von PQs verwendet wird. In Sporen und Lufthyphen werden YWA1 und AT4HN durch AygA und AygB zu T4HN hydrolysiert, was die Konzentration von T4HN für die DHN-Melaninbiosynthese erhöht. Ich habe außerdem eine neue T4HN-Reduktase Brm3 identifiziert, die die Umwandlung von Scytalon zu T4HN katalysiert. Außerdem waren Brm1, Brm2 und Brm3 für die Produktion von PQs erforderlich.

Als nächstes wurde ein Fütterungsexperiment durchgeführt, um das Zwischenprodukt für die PQs-Bildung im DHN-Melanin-Biosyntheseweg zu bestimmen. Der *pksA*-Deletionsstamm wurde mit unterschiedlichen Mengen an 1,8-DHN gefüttert. Die Produktion von PQs, Altertoxin I (ATX I), Altertoxin II (ATX II) und Alterperylenol (ALP) wurde dadurch wieder möglich. Auch die Melanbildung des △*pksA*-Stammes war in Flüssigkultur wieder hergestellt. 1,8-DHN ist damit das letzte gemeinsame Zwischenprodukt für die Biosynthese von Melanin und PQs.

Mehrere Laccase-kodierende Gene wurden einzeln oder in Kombination mit Hilfe der CRISPR/Cas9-Knock-out-Technologie deletiert. Vier Laccasen, LccB-D und LccF, waren für die DHN-Melaninproduktion, aber nicht für die PQs-Biosynthese erforderlich. Das Dimerisierungsenzym, das die Reaktion von 1,8-DHN zu PQs katalysiert, wurde noch nicht entdeckt.

Die Abwesenheit von CmrA führte zu einem bräunlichen Mutantenstamm, im Gegensatz zu dem blassen oder rosafarbenen *pksA*-Deletionsstamm. Tatsächlich wurden die Gene *pksA*, *aygB*, *brm2* und *lccD* immer noch exprimiert. Demgegenüber kontrolliert der Transkriptionsfaktor CmrA strikt die Expression von *aygA*, *brm1*, *brm3*, *lccB* und *lccF*. Dementsprechend wurden in dem *cmrA*-Deletionsstamm keine PQs mehr gebildet. Ein Promotor-Reporter-Assay zeigte, dass *pksA* in den Substrathyphen nicht exprimiert wird, was darauf hindeutet, dass CmrA die Produktion von PQs durch die Kontrolle der Expression von *pksA* und/oder des Dimerisierungsenzym-

kodierenden Gens reguliert.

Die Analyse der PQs Biosynthese in *A. alternata* ist ein Beispiel dafür wie eine räumliche Regulation der Expression der Gene eines Sekundärmetabolitbiosyntheseweges die Komplexität der gebildeten Sekundärmetabolite erhöht.

1. Introduction

1.1 Secondary metabolism in fungi

Fungi are widespread in the natural environment. About 12 million fungal species were estimated on earth recently, but only about 100,000 of them were described (Wu *et al.*, 2019). To adapt to changing environments, fungi have evolved to degrade many different kinds of substrates, many of which are polymers. They are degraded by various enzymes, which the fungi secrete into the surroundings. In addition to enzymes, most fungi produce a large number of small chemical compounds called secondary metabolites (SMs) to defend themselves against competitors living in the same habitat or protect themselves from predators (Keller, 2019). Among fungal SMs, penicillin produced by *Penicillium* spp. is the most famous one and shows antibiotic activity against bacteria, and the widespread use of penicillin as an antibiotic saved tens of millions of lives all over the world during World War II and ever since (Gardner, 1940; Keller *et al.*, 2005). Another example of secondary metabolites produced for protection is xanthones. Liu *et al*. proved that Hülle cells, nursing the overwintering fruiting bodies of *Aspergillus nidulans*, produce xanthones to avoid being eaten by fungivorous animals (Liu *et al.*, 2021). In addition, many fungi are considered as pathogens, and SMs are crucial virulence factors when plants, humans or animals are infected (Macheleidt *et al.*, 2016; Rokas *et al.*, 2018). For instance, ACR-toxin, one of the host-specific toxins (HSTs) from *Alternaria* sp., enters plant mitochondria and interrupts its oxidative phosphorylation to cause damage to plant cells (Meena & Samal, 2019). Oxalic acid secreted by the phytopathogen *Sclerotinia sclerotiorum* could induce cell death in host plant tissues (Kabbage *et al.*, 2013). Gliotoxin modulates the immune response and causes apoptosis in animals and humans infected with *Aspergillus fumigatus* (Scharf *et al.*, 2012). Many SMs may also be harmful to humans if they are ingested with the food. For instance, aflatoxins produced by *Aspergillus flavus* and *Aspergillus parasiticus* can cause carcinoma formation through inducing DNA

mutagenicity and suppressing the immune system in humans and animals (Yu, 2012). However, fungal SMs behave as a “double-edged sword”. This means, in addition to causing damage to other organisms, SMs originating from fungi may also be used as pharmacological drugs to treat human and animal infections (e.g., penicillin, griseofulvin) or diseases (e.g., lovastatin) and as fungicides for plant protection (e.g., strobilurins) (Duke *et al.*, 2010; Greco *et al.*, 2019; Keller, 2019; Nofiani *et al.*, 2018).

Fungal SMs are divided into four groups based on the gene whose protein product synthesizes the SM precursors, namely non-ribosomal peptides (e.g., penicillin, gliotoxin and cyclosporin), indole alkaloids (e.g., ergopeptides and fumitremorgens), terpenes (e.g., aristolochene, trichothecene T2 toxin and gibberellin) and polyketides (e.g., aflatoxin, lovastatin, alternariol and xanthones (Keller *et al.*, 2005; Liu *et al.*, 2021; Wenderoth *et al.*, 2019). However, some fungal SMs are believed to be the products of a polyketide synthetase and non-ribosomal peptide synthetase hybrid enzyme (e.g., tenuazonic acid, equisetin)(Kato *et al.*, 2015; Yun *et al.*, 2015). And hybrids of a polyketide synthase and a terpenoid synthase also catalyze the formation of some fungal SMs, with arthrosporols as examples (Song *et al.*, 2017). The genes involved in fungal SMs biosynthesis are typically organized in clusters in the genome, such as the penicillin biosynthesis gene cluster in *Penicillium* sp., the aflatoxin biosynthesis gene cluster in *Aspergillus* sp. and DHN-melanin biosynthesis gene cluster in *A. fumigatus* (Bhatnagar *et al.*, 2003; Laich *et al.*, 2002; Perez-Cuesta *et al.*, 2020). However, it is not rare that the genes involved in DHN melanin biosynthesis are not all clustered in the genome like in many plant pathogens (e.g., *Cercospora beticola*, *Bipolaris maydis*, *Botrytis cinerea,* and *Alternaria alternata*)(Ebert *et al.*, 2019).

1.1.1 Melanins

Melanins are a family of heterogeneous biopigments formed by oxidatively polymerizing phenolic or indolic precursors, and they are ubiquitously found in all kingdoms (Cao *et al.*, 2021; Upadhyay *et al.*, 2016). In brief, there are five types of

melanin, namely eumelanin (black), neuromelanin (black), pheomelanin (yellow and red), pyomelanin (red-brown), and allomelanin (black and grey- or dark-green) **(Fig. 1)**. Eumelanin, pheomelanin, and neuromelanin, which derives from tyrosine, are mainly found in animal tissues. However, pyomelanin deriving from the catabolism of tyrosine and allomelanin produced from derivatives of acetate, malonyl-CoA, or catechol are primarily found in microorganisms and plants (Cao *et al.*, 2021; Gessler *et al.*, 2014). Melanins are not essential for growth or reproduction but have a pivotal role in helping organisms to survive in the environment. In animals, melanins are used for camouflage and photoprotection. In fungi, melanins contribute to resisting environmental stress such as UV irradiation, desiccation, and oxidative stress. In plants, the formation of melanins is always associated with injuries caused by infection of microbes and damaging through climate conditions or physical cuttings (Varga *et al.*, 2016).

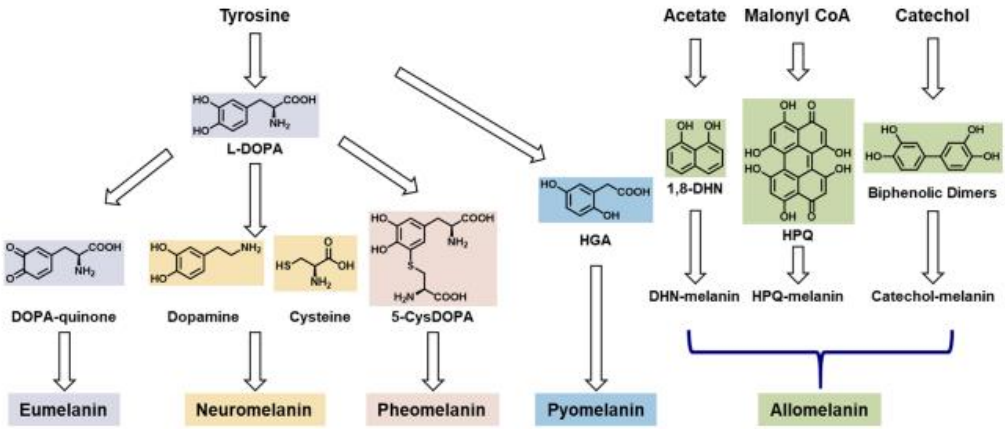


Fig. 1: Types of melanin and the simplified biosynthetic pathways in all-natural kingdoms. Taken from (Cao *et al.*, 2021).

In fungi, all types of melanin except neuromelanin have been identified, but pyomelanin and DHN-melanin are the two common ones whose biosynthesis pathways have been well characterized. In fungi, like *A. fumigatus*, the genes for tyrosine degradation are clustered, and the formation of pyomelanin shares the pathway with tyrosine degradation **(Fig. 2A)**. The degradation of tyrosine starts with the catalyzation of tyrosine to 4-hydroxyphenylpyruvate by tyrosine aminotransferase

(Tat). Then 4-hydroxyphenylpyruvate dioxygenase (HppD) catalyzes 4-hydroxyphenylpyruvate to form homogentisate through oxidative decarboxylation. Next, homogentisate is oxidized into 4-maleylacetoacetate by homogentisate dioxygenase (HmgA), followed by isomerization into 4-fumarylacetoacetate by maleylacetoacetate isomerase (MaiA). Eventually, 4-fumarylacetoacetate is hydrolyzed into acetoacetate and fumarate by fumarylacetoacetate hydrolase (FahA). However, homogentisate accumulates when the function of HmgA is lost, which leads to the formation of pyomelanin. In *A. fumigatus,* all the genes involved in DHN-melanin biosynthesis are clustered **(Fig. 2B)**. In detail, first, malonyl-CoA and acetyl-CoA are used by polyketide synthase PksP to generate the heptaketide napthopyrone YWA1. Then, YWA1 is hydrolyzed to 1,3,6,8-THN by a-hydrolase Ayg1, which, in turn, is reduced to scytalone by 1,3,8-trihydroxynaphthalene reductase Arp2. Next, scytalone is dehydrated to 1,3,8-THN by scytalone dehydratase Arp1, followed by reduction to vermelone through Arp2. Finally, vermelone is dehydrated to 1,8-DHN by copper oxidase Abr1, which is polymerized by laccase Abr2 to form DHN-melanin. MAP kinase pathways regulate the balance between these two melanins. The absence of MpkB enhances the phosphorylation of MpkA and then up-regulates the expression of genes for DHN-melanin biosynthesis. At the same time, the activity of HmgA is significantly stimulated, which leads to a decrease in pyomelanin formation (Manfiolli *et al.*, 2019). The biosynthesis of different types of melanin may be correlated with varying stages of the development of fungi. For instance, pyomelanin can protect the cell wall of hyphae from reactive oxygen species (ROS). Nevertheless, DHN-melanin is required for the cell wall integrity of spores and hyphae and protects fungi from UV irradiation and drying (Gessler *et al.*, 2014). In the phytopathogen *A. alternata*, the production of DHN-melanin is inhibited by pyomelanin synthesis (Fernandes *et al.*, 2021).

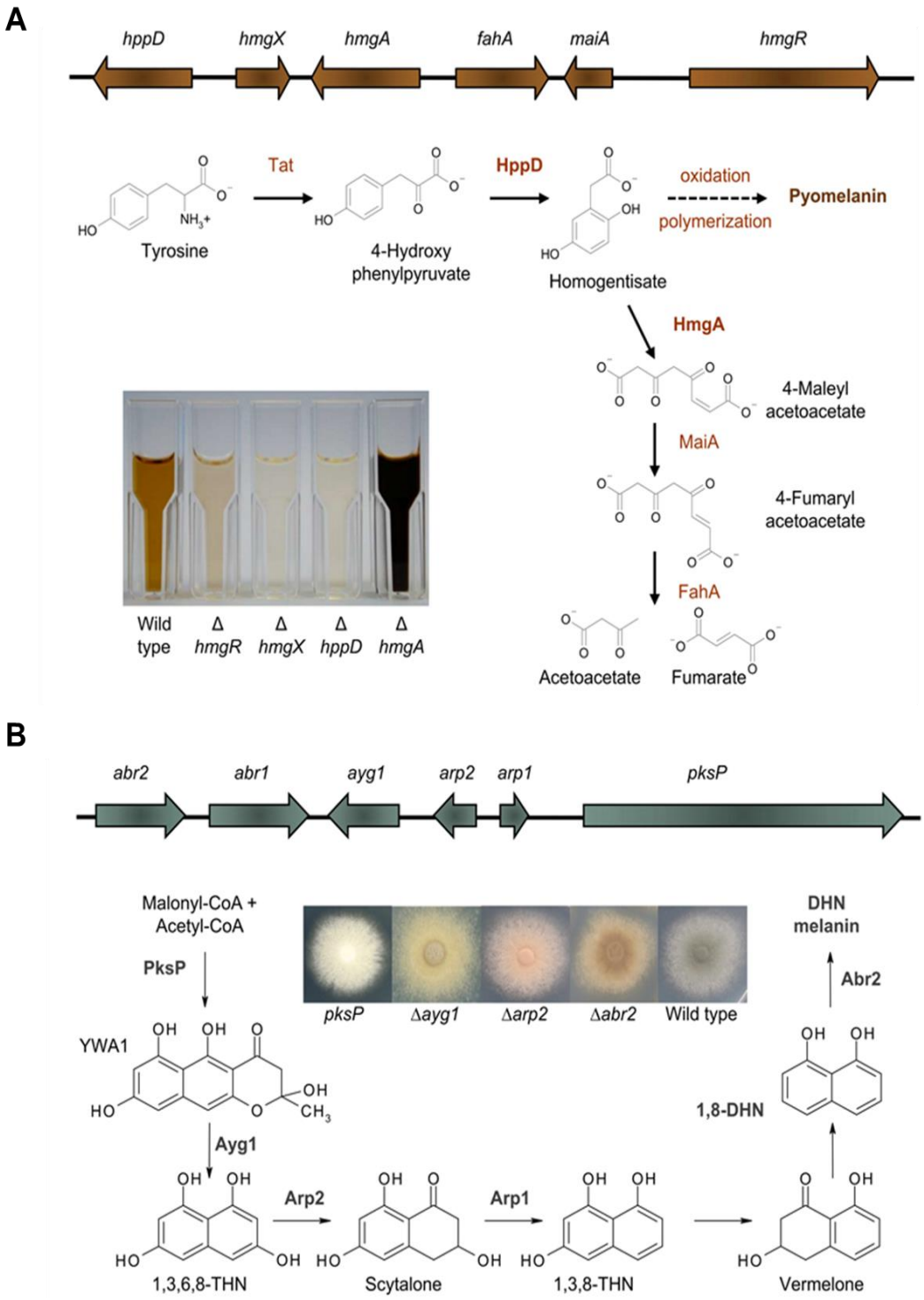


Fig. 2: Pyomelanin and DHN-melanin biosynthesis in *A. fumigatus*. (A) Scheme of the pyomelanin biosynthetic gene cluster and the biosynthetic pathway. Tat, tyrosine aminotransferase; HppD, 4-hydroxyphenylpyruvate dioxygenase; HmgA, homogentisate dioxygenase; MaiA, maleylacetoacetate isomerase; FahA, fumarylacetoacetate hydrolase. Supernatants in the cuvettes represent pyomelanin produced by different *A. fumigatus* strains incubated in minimal medium supplemented with tyrosine. **(B)** Scheme of the DHN-melanin biosynthetic gene cluster and the biosynthetic pathway. PksP, polyketide synthase; Ayg1, a-

hydrolase; Arp2, 1,3,8-trihydroxynaphthalene reductase; Arp1, scytalone dehydratase; Abr1, copper-oxidase; Abr2, laccase. Taken from (Heinekamp *et al.*, 2013).

Many fungi produce DHN melanin on hyphae and/or spores, but genomic rearrangements of genes for melanin production have been well studied only in some of them. Currently, clustering of all the genes involved in DHN-melanin biosynthesis is merely found in *A. fumigatus*, *A. clavatus,* and *Neosartorya fischeri* (Valiante *et al.*, 2016). Some melanin-related genes form one or two small gene clusters in many other fungi, while the remaining genes are scattered in the genome. For instance, two DHN-melanin biosynthetic gene clusters are identified in *Botrytis cinerea*. The polyketide synthase gene *Bcpks12* forms a small gene cluster with the transcription factor *Bcsmr1*. And another gene cluster is comprised of polyketide synthase gene *Bcpks13*, scytalone dehydratase gene *Bcscd1*, two T3HN reductase genes *Bcbrn1* and *Bcbrn2*, and two transcription factor genes *Bcztf1* and *Bcztf2*. The α-hydrolase gene *Bcygh1* is not located elsewhere in the genome **(Fig. 3)**. In *B. cinerea*, polyketide synthases BcPks12 and BcPks13 catalyze the initial step of DHN-melanin biosynthesis in sclerotia and conidia, respectively. The transcription factors BcCMR1 and BcZTF1/BcZTF2 control the formation of DHN-melanin in sclerotia and conidia, respectively (Schumacher, 2016). In *A. alternata*, the polyketide synthase gene *PksA*, the T3HN reductase gene *brm2*, and the transcription factor gene *cmrA* are clustered, with the scytalone dehydratase gene *brm1* scattered in the genome **(Fig. 3)**. Other genes required for melanin formation need to be further characterized.

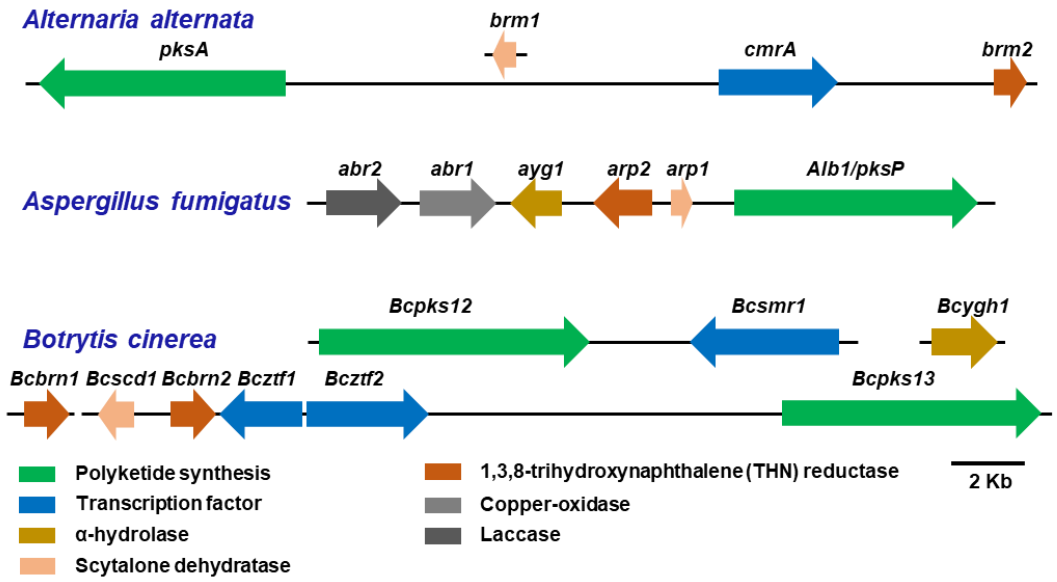


Fig. 3: Synteny and rearrangements of DHN-melanin biosynthetic gene clusters in *A. alternata*, *A. fumigatus* and *B. cinerea*.

1.1.2 Perylene quinones

Perylene quinones (PQs) are a family of polyketide-derived photosensitizers formed through phenol-coupling reactions. In nature, many fungi, particularly phytopathogenic fungi, secrete PQs as toxins to damage host cells through absorbing light energy and generating ROS during infection. A prominent example of PQs is cercosporin, which was well characterized since being first isolated in 1957 (Kuyama & Tamura, 1957). Cercosporin, mainly produced by *Cercospora* sp., is a deep red pigment (Yamazaki *et al.*, 1975), and the cercosporin toxin biosynthesis (*CTB*) gene cluster is required to produce it (Choquer *et al.*, 2005). De Jonge *et al*. reported that the *CTB* gene cluster in *Cercospora* sp. is ancient, and was transferred to several fungal plant pathogens via horizontal gene transfer. For instance, the apple pathogen *Colletotrichum fioriniae* can also produce cercosporin (De Jonge *et al.*, 2018). In addition to causing plant disease, cercosporin is determined to be universally toxic to mice, bacteria, and fungi (De Jonge *et al.*, 2018; Yamazaki *et al.*, 1975). Remarkably, many SMs of the PQ family produced by different plant pathogenic fungi share the same backbone structure with cercosporin,

namely, phleichrome (*Cladosporium phlei*), hypocrellin A (*Shiraia bambusicola*), and elsinochromes A-D (*Elsinoë fawcettii*) **(Fig.4)**(Ebert *et al.*, 2019). Besides, these PQs show significant antitumor activity and have promising potential for further drug development (Kočišová *et al.*, 1999; Ma *et al.*, 2003; So *et al.*, 2018).

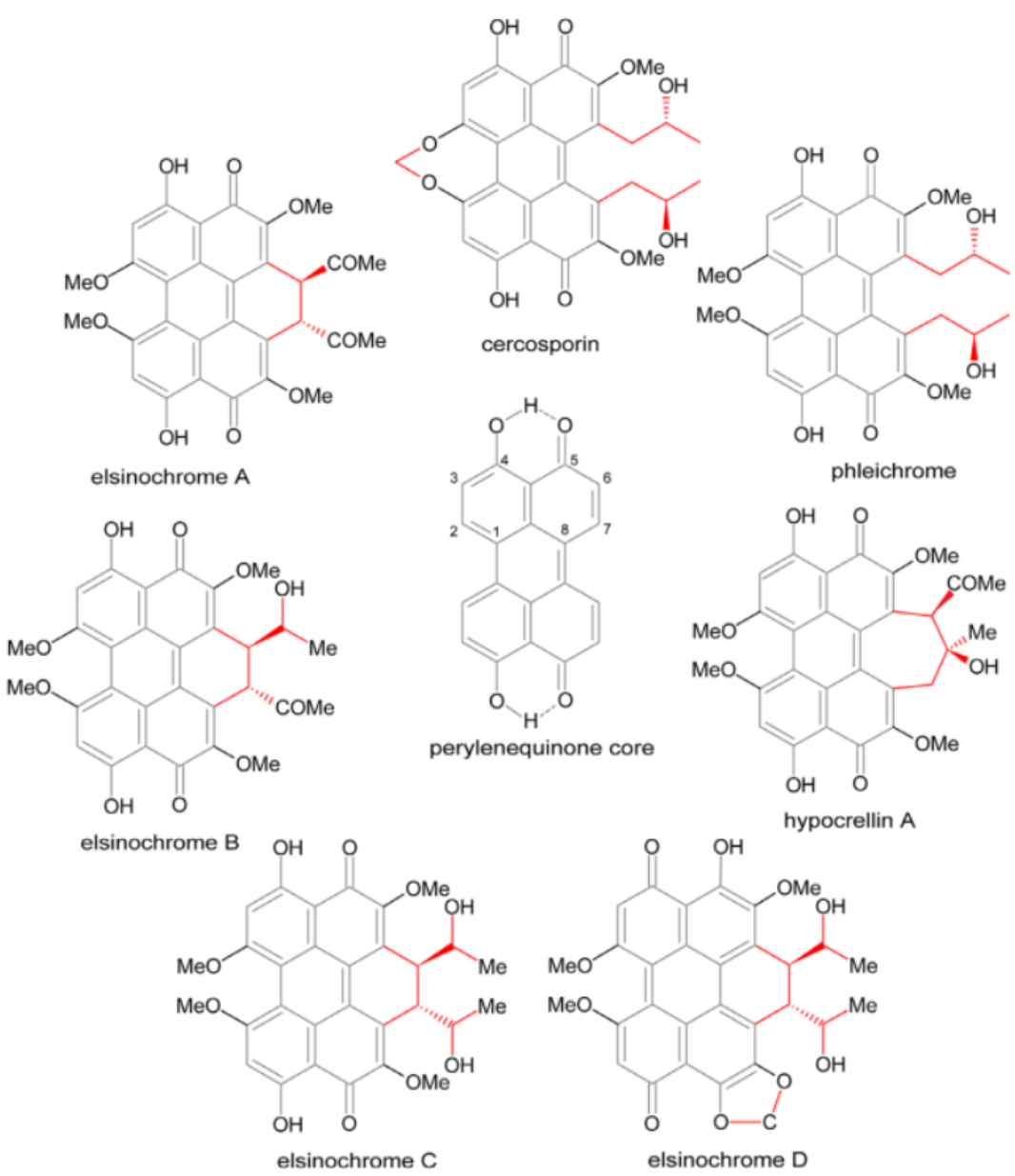


Fig. 4: Perylene quinones share the mutual backbone structure. Various side chains attached to the backbone are shown in red. Taken from (Ebert *et al.*, 2019).

Fungi of the *Alternaria* genus also produce phytotoxins of the PQ family, such as altertoxins I-VII (ATX I-VII) and some other derivatives **(Fig. 5)**(Pinto & Patriarca, 2017; H. Wang *et al.*, 2022). Among these derivatives, stemphyperylenol and stemphyltoxin

III are also produced by *Stemphylium botryosum* (Arnone *et al.*, 1986). *Alternaria* is a common food contaminant worldwide; thus, consumption of these compounds such as ATXs represents a severe threat to human health. However, regulations for controlling ATXs in food are still missing. ATX II was reported to be a potent mutagen and DNA strand-breaking agent in cultured mammalian cells, exhibiting at least 50-fold more vigorous mutagenic activity than the *Alternaria* mycotoxins alternariol (AOH) and its derivative, alternariol monomethyl ether (AME)(Fleck *et al.*, 2012). ATX II introduces two covalent deoxyguanosine adducts when incubated with DNA *in vitro* (Soukup *et al.*, 2020). On the other hand, some ATXs have anticancer activity as well. For instance, ATX II is a highly effective and specific compound against Ewing sarcoma, an aggressive bone and soft tissue cancer in children and adolescents (Robles *et al.*, 2021). ATX I-III and V significantly inhibited the replication of the HIV virus, showing the potential to treat AIDS caused by HIV (Bashyal *et al.*, 2014). Although some research has been done on the toxicity and anticancer activity of *Alternaria* PQs, especially ATX II, it is still unknown how these compounds are biosynthesized. The gene clusters involved in cercosporin and elsinochrome formation have been characterized to some extent (De Jonge *et al.*, 2018). Some research suggested that elsinochrome biosynthesis may share the pathway with DHN-melanin production (Griffiths *et al.*, 2018; Liao & Chung, 2008). However, later it was reported that the routes of PQs and DHN-melanin formation are separate pathways in *C. betia* and *E. fawcettii* (Ebert *et al.*, 2019). Hence, the production of PQs is still debatable and not solved, although many of such chemicals are recognized as virulence factors in plant pathogenic fungi, as mycotoxins or novel anticancer drugs.

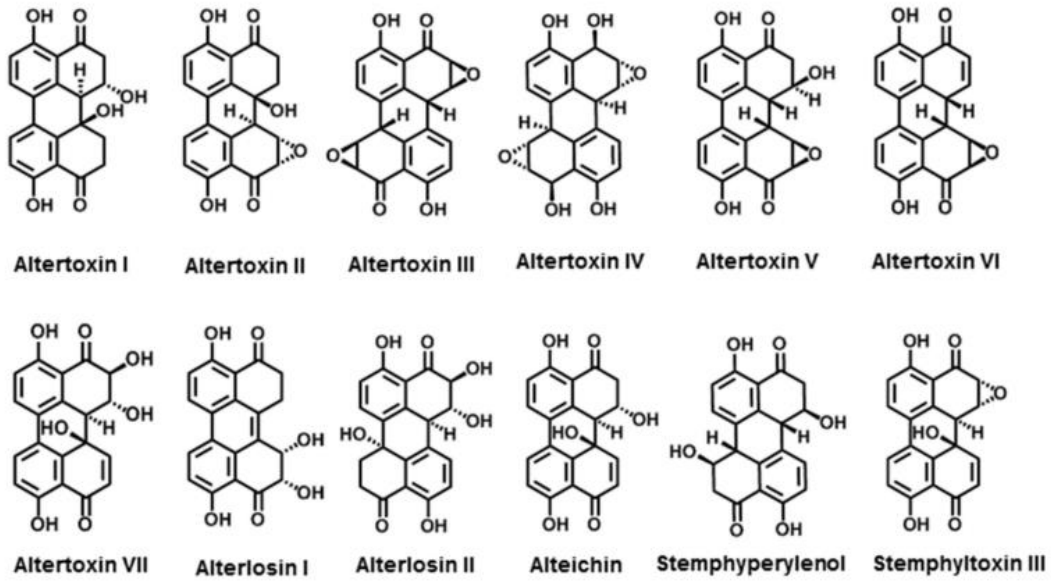


Fig. 5: Chemical structures of perylene quinones produced by the genus *Alternaria* and *Stemphylium botryosum*. Taken from (H. Wang *et al.*, 2022).

Since PQs produced by diverse plant pathogenic fungi are harmful to microorganisms, especially fungi that cannot produce PQs, one interesting question is how these fungi can survive from toxic PQs synthesized by themselves. It is well known that genes required for fungal SMs formation are typically arranged in a gene cluster. To answer that question, more attention was given to the content of the gene clusters. There are always some genes in the gene cluster whose products seem to be unnecessary for the biosynthesis of SMs. Nowadays, it is well defined that they play vital roles in the self-protection from their chemicals. Generally, three strategies are adopted to achieve this self-defense **(Fig.6)**(Keller, 2015). (i) The gene encoding the target of SMs is duplicated in the gene cluster. For example, the polyketide metabolite lovastatin produced by *A. terreus* can disrupt the fungal cholesterol biosynthesis pathway by inhibiting the critical enzyme HMG-CoA reductase (Chamilos *et al.*, 2006). To reduce this toxicity on itself, the lovastatin gene cluster in *A. terreus* encodes a second HMG-CoA reductase to increase the target pool of lovastatin (Nyilasi *et al.*, 2013). (ii) Chemical modification of functional groups to detoxify SMs. For instance, in *A. fumigatus*, the thioredoxin reductase GliT, encoded in the gliotoxin gene cluster, modifies the gliotoxin structure by catalyzing the sulfhydryl group, the functional group

of gliotoxin, to form a disulfide and then generate a less-toxic compound (Saleh *et al.*, 2018). (iii) Encoding transporters to pump SMs out of the cell. These transmembrane proteins are the most common way used by fungi to reduce damages caused by their SMs (Keller, 2015). The disruption of GliA, a transporter encoded in the gliotoxin gene cluster in *A. fumigatus*, reduced the accumulation and tolerance to gliotoxin (Wang *et al.*, 2014). Similarly, when a transporter encoding gene, which was not believed to be related to the known fasaric acid (FA) gene cluster, was deleted, *Fusarium oxysporum* showed a lower ability to secrete, accumulate, and resist FA (Crutcher *et al.*, 2015).

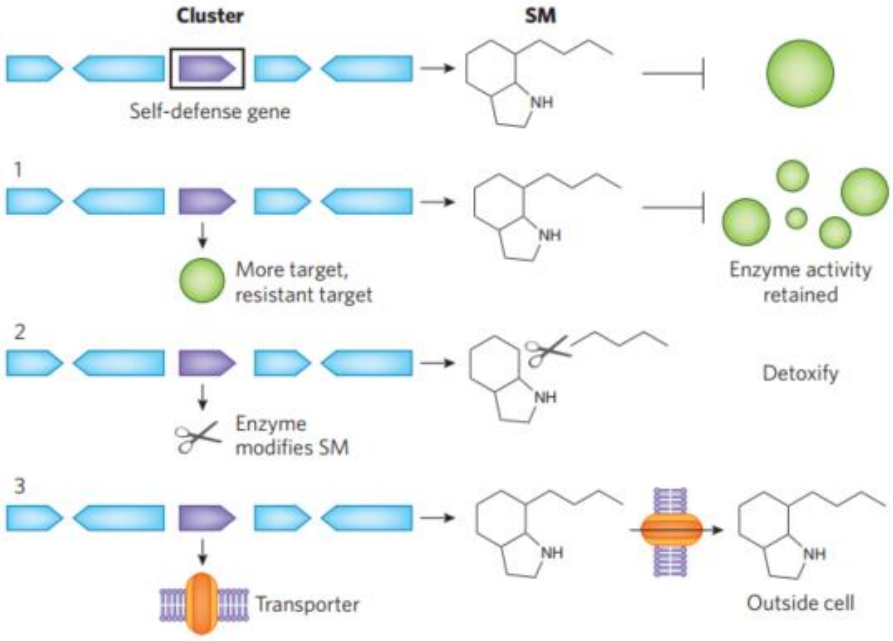


Fig. 6: Strategies of self-defense of toxic SMs in fungi. 1) Increasing target pool through duplicating target encoding genes. 2) Detoxification of SMs by chemically modifying functional groups important for binding the target. 3) Exporting SMs out of the cell via transporters. Taken from (Keller, 2015)

It is intriguing that the deletion of the major facilitator superfamily transporter encoding gene *CTB4* in *C. nicotianae* led to reduced cercosporin accumulation but didn not influence the self-defense against the toxicity of cercosporin (Choquer *et al.*, 2007). However, the disruption of a transporter encoding gene *CFP*, out of the *CTB* gene cluster, in *C. kikuchii* resulted in a significant reduction in the accumulation and tolerance of exogenous cercosporin (Callahan *et al.*, 1999). It is still unclear how PQs-

producing fungi protect themselves from their toxic compounds. One possibility is that many transporters with the redundancy of function are used to safeguard fungi against toxic PQs. Furthermore, other PQs-producing fungi like *Alternaria solani* and *Cladosporium fulvum* have resistance to cercosporin, suggesting a similar way to defend against PQs in fungi secreting PQs (Daub, 1987).

1.1.3 Subcellular localization of enzymes involved in fungal secondary metabolites biosynthesis

Compared to studies on identifying gene clusters for fungal SMs, less research has been done about the cellular compartmentalization of biosynthesis steps. Enzymes required for the biosynthesis of fungal SMs could reside in the cytosol, peroxisome, and other delimited membrane-bound organelles, like vacuoles, vesicles, endosomes and toxisomes (Keller, 2015; Kistler & Broz, 2015). Compartmentalizing biosynthesis of three different groups of fungal SMs, the polyketide aflatoxin, the non-ribosomal peptide penicillin, and the terpene trichothecene, has been well characterized **(Fig.7)**. The aflatoxin biosynthesis is initiated in peroxisomes, organelles known for generating hydrogen peroxide, transferring ROSs to less-toxic compounds, and oxidizing fatty acids to produce acetyl-CoA. Then, endosomes are generated through multiple fusion events, such as incorporating materials originating from the endoplasmic reticulum and ribosome-associated vesicles. Eventually, an aflatoxisome, containing a large number of aflatoxins, is obtained by fusing endosomes, and aflatoxin is secreted into the environment in an unidentified manner **(Fig. 7A)**. Vacuoles are acidified organelles with various functions, such as protein turnover, Ca^{2+} and amnio acids storge, and regulating pH and osmotic pressure (Veses *et al.*, 2008). Penicillin synthesis originates from transporting L-cysteine and L-valine from the vacuole to its cytoplasmic side, where they are used to produce the following intermediate. And then, the intermediate is released and further modified by the enzyme in the cytoplasm. Finally, the biosynthesis of penicillin is completed in peroxisomes. One transporter on the

peroxisome is also required to form penicillin, but how penicillin is released into the environment is still unknown **(Fig. 7B)**(Herr & Fischer, 2014). Similar to penicillin, the biosynthesis of trichothecene also starts in the vacuole, followed by multiple vesicle fusion events. And in the end, trichothecene is synthesized in the toxisome. One transporter encoded in the gene cluster is supposed to secrete trichothecene outside of the cell **(Fig. 7C)**.

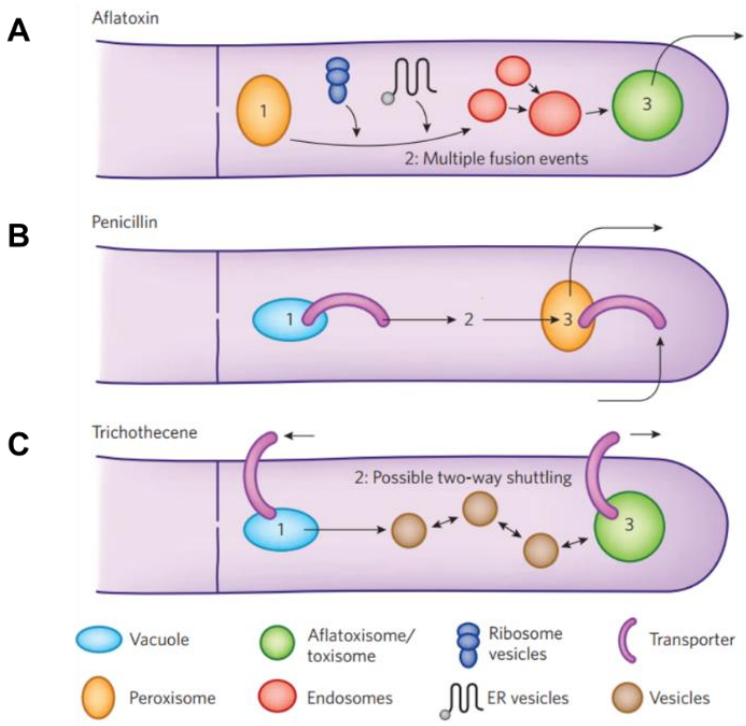


Fig. 7: Subcellar trafficking models for the biosynthesis of aflatoxin (A), penicillin (B), and trichothecene (C). ER, endoplasmic reticulum. Taken from (Keller, 2015)

The subcellular compartmentalization of biosynthesis of DHN melanin in fungi has been revealed to some extent. In *A. fumigatus*, the early melanin enzymes without secretion signals, including the polyketide synthase Alb1/PksP, the α-hydrolases Ayg1, the scytalone dehydratases Arp1 and the 1,3,8-trihydroxynaphthalene reductases Arp2, are recruited into endosomes in an atypical secretory way. Then, the late melanin enzymes having secretion signals, including the copper oxidase Abr1 and the laccase Abr2, accumulate in the cell wall to complete the biosynthesis of DHN melanin **(Fig. 8A)**. In *B. cinerea*, the formation of T4HN is catalyzed by the polyketide synthase

BcPKS12 or BcPKS13 and the α-hydrolases BcYGH1 in peroxisomes. Then, T4HN is reduced to scytalone by the 1,3,8-trihydroxynaphthalene reductases BcBRN1/2 in endosomes. After being exported from endosomes to the outside of the cell, scytalone is modified by different enzymes step by step, and finally, DHN melanin is synthesized in the cell wall **(Fig. 8B)**. Although the compartmentation of biosynthesis of DHN melanin in fungi was initially thought to be conserved, it appeared to be different in *B. cinerea* suggesting that the biosynthesis in fungi is more complicated than expected.

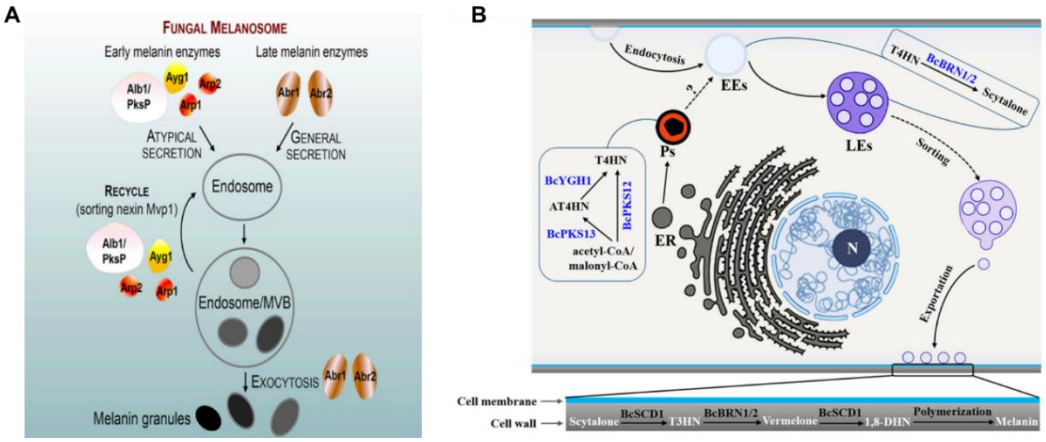


Fig. 8: Subcellular compartmentalization models for the biosynthesis of DHN melanin in *A. fumigatus* (A) and *B. cinerea* (B). Polyketide synthases: Alb1/PksP, BcPKS12/13; α-hydrolases: Ayg1, BcYGH1; scytalone dehydratases: Arp1, BcSCD1; 1,3,8-trihydroxynaphthalene reductases: Arp2, BcBRN1/2; Abr1: copper oxidase; Abr2: laccase. N, nucleus; ER, endoplasmic reticulum; Ps, peroxisome; EEs, early endosomes; LEs, late endosomes. Taken from (Chen *et al.*, 2021; Upadhyay *et al.*, 2016).

1.1.4 Regulation of secondary metabolism

The genes required for the biosynthesis of fungal SMs are typically clustered in the genome. One or more transcription factors encoding genes are often included in the gene clusters, which play positive roles in the expression of other genes of the cluster and thus induce the formation of SMs. For instance, the transcription factor encoding gene *aflR* found in many *Aspergillus* sp., like *A. nidulans*, *A. parasiticus*, and *A. flavus*, plays a vital role in the production of the carcinogenic compounds sterigmatocystin and

aflatoxins (Woloshuk *et al.*, 1994; Yu *et al.*, 1996). The regulator AflS residing next to AflR could improve the biosynthesis of aflatoxin by interacting with AflR to enhance the AflR activity (Chang, 2003). Another example is the transcriptional regulator CTB8 encoded in the cercosporin gene cluster in *C. nicotianae*. Disruption of the *CTB8* gene eliminated or significantly reduced the expression of all of the core genes in the gene cluster (Chen *et al.*, 2007). It also occurs that in-cluster transcription factors may activate another gene cluster on a different chromosome. For instance, the induced expression of the regulatory gene in a silent gene cluster awakened this 'sleeping' gene cluster, but also, activated the expression of another gene cluster on a different chromosome in *A. nidulans* (Bergmann *et al.*, 2010). Furthermore, specific regulators in a conserved gene cluster may be functionally distinct through the loss/gain of transcription factor binding sites on the target genes in different fungi. In contrast to the subsequent activation of the *xan* gene cluster through overexpressing the in-cluster transcription factor XanC in *A. fumigatus*, only one gene in the putative *xan* gene cluster was upregulated when *PexanC*, the homolog of *xanC* in *Penicillium expansum*, was overexpressed. That led to the failure of activating xanthocillin production. However, the *cit* gene cluster was activated, and more citrinin was synthesized in *P. expansum* (Wang *et al.*, 2021).

Except for the specific transcription factors encoded in the gene clusters, the broad-domain regulators, which are known for regulating fungal development, can globally control the biosynthesis of SMs responsive to general environmental stimuli, such as light, temperature, carbon and nitrogen sources, and pH **(Fig. 9)**. One good example is the discovery of how light coordinates sexual development and sterigmatocystin production through the velvet complex (VeA/VelB/LaeA) in *A. nidulans* (Bayram *et al.*, 2008). Both, the sexual fruiting-body formation and sterigmatocystin biosynthesis are positively controlled by the *velvet* gene *veA*, which is mainly expressed in the dark (Kato *et al.*, 2003). Deletion of the *velvet*-like gene *velB* generated a similar phenotype to that of the *veA*-deletion strain. A histone methyltransferase-encoding gene *laeA* regulates a large proportion of the secondary metabolism. Without light exposure, VeA bridges VelB to LaeA to influence the

activation of SM gene clusters. In *A. alternata*, the biosynthesis of alternariol and altertoxins is stimulated under blue light (Igbalajobi *et al.*, 2019; Pruß *et al.*, 2014). Like light, temperature is also an environmental cue that frequently changes. RNA-sequencing assays suggested that the velvet complex could regulate SM gene clusters in a temperature-dependent manner in *Aspergillus* (Lind *et al.*, 2016). The production of the toxic PQ cercosporin is strictly dependent on visible light and significantly blocked at high temperatures in the genus *Cercospora* (Daub & Chung, 2009). The linkage of light and temperature to regulate SM gene clusters is still unclear. There is some evidence that the phytochrome FphA is involved in temperature sensing in *A. nidulans* (Yu *et al.*, 2019). In nature, fungi survive in the environment with a wide pH range. The *Aspergillus* transcription factor PacC up-regulates genes expressed in alkaline conditions and down-regulates genes expressed in acidic conditions (Peñalva *et al.*, 2008). On the other hand, PacC controls the biosynthesis of fungal SMs, such as penicillin, sterigmatocystin and aflatoxin in *Aspergillus* and Patulin in *Penicillium expansum* (Chen *et al.*, 2018; Espeso *et al.*, 1993; Keller *et al.*, 1997). Nutrient sources, such as carbon and nitrogen, are essential for growth of fungi. Carbon and nitrogen consumption are controlled by the C2H2 zinc finger transcription factor CreA and the GATA transcription factor AreA, respectively. CreA is well known for mediating carbon catabolite repression (CCR). When the preferred carbon source, like glucose, is supplied, CreA downregulates the expression of enzymes required to utilize hardly assimilable carbon sources such as lignocellulose and sucrose. Furthermore, CreA is also involved in regulating SM gene clusters in fungi. For instance, the production of aflatoxin in *A. flavus* and penicillin in *P. chrysogenum* is highly repressed by glucose-inducing CCR. Yet, the biosynthesis of citrinin in *P. citrinum* is induced (Ruiz-Villafán *et al.*, 2022). Fungi preferentially use easily assimilated nitrogen sources, such as ammonium and glutamine, instead of secondary nitrogen sources, such as nitrate and urea. This process is mediated by AreA and its co-repressor NmrA (Tudzynski, 2014). Like CCR mediated by CreA, the SM gene cluster is regulated by AreA-mediated nitrogen metabolite repression. For instance, AreA controls the biosynthesis of gibberellins and bikaverin in *Fusarium fujikuroi* (Teichert *et al.*, 2006). Interspecies

communication occurs in fungal-bacteria, fungal-fungal and fungal-animal interactions. To protect themselves, fungi secrete some small molecules such as effectors and SMs when competing with other microorganisms for nutrients and defending against animals for predation. Spt-Ada-GcnE acetyltransferase (SAGA) and ADA co-activator complex influence chromatin remodeling and control the expression of host genes, such as SM gene clusters. For instance, the regulation of the *ors* gene cluster, for orsellinic acid biosynthesis in *A. nidulans,* is mainly dependent on the activity of GcnE when interacting with the bacterium *Streptomyces rapamycinicus* (Netzker *et al.*, 2015). Except for the stimuli mentioned above, the redox status and iron starvation also play vital roles in regulating SM gene clusters. The bZIP transcription factor Yap1 balances the intracellular redox state and links the oxidative stress response to secondary metabolism. The iron-sensing transcription factor HapX represses iron-consuming pathways under iron starvation conditions. Meanwhile, the absence of HapX leads to misregulation of the secondary metabolism. The HAP complex is comprised of HapB, HapC, HapE and HapX. The disruption of the HAP complex reduces the tolerance to oxidative stress, suggesting cross-talk between the redox status and iron starvation (Liu *et al.*, 2005; Ridenour & Bluhm, 2014).

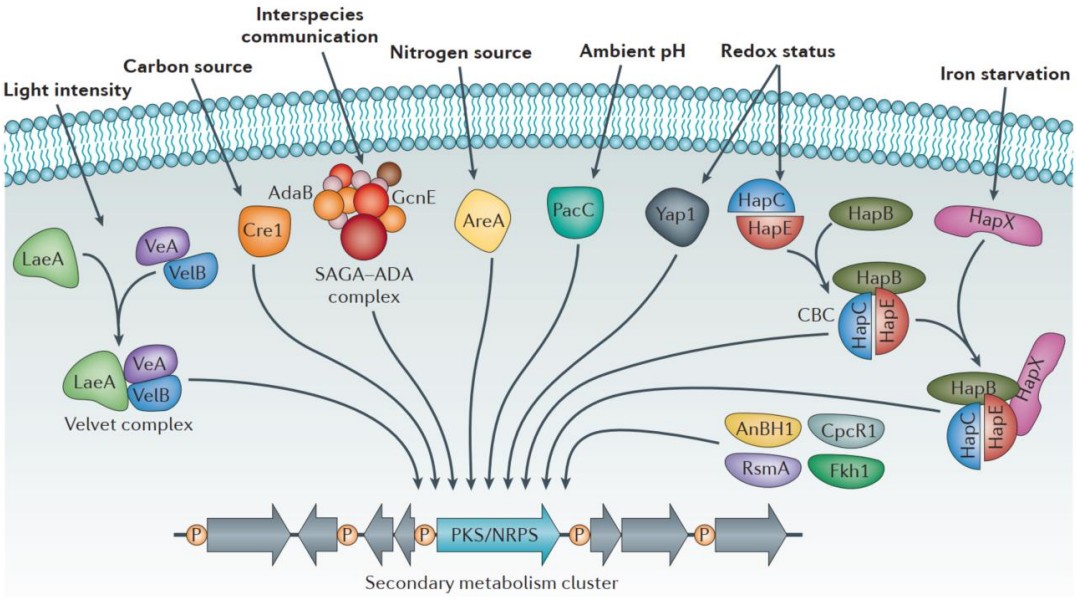


Fig. 9: Global regulation of secondary metabolism gene clusters in fungi. Velvet complex, VeA/VelB/LaeA; Cre1, the C2H2 zinc finger transcription factor, mediating carbon catabolite

repression; SAGA-ADA complex, Spt-Ada-GcnE acetyltransferase and ADA complex; AreA, the GATA transcription factor, mediating nitrogen metabolite repression; PacC, the pH-dependent transcription factor; Yap1, the bZIP transcription factor; HapX, the iron-sensing transcription factor; the HAP complex, HapB/HapC/HapE/HapX; PKS, polyketide synthetase; NRPS, nonribosomal peptide synthetase. Taken from (Brakhage, 2013).

1.2 *Alternaria alternata*

1.2.1 Characteristics and lifestyle of *A. alternata*

A. alternata is a filamentous fungus and is ubiquitously present in the atmosphere, soil, and various rotting plant materials (Estiarte *et al.*, 2016; Wang *et al.*, 2016). This fungus is characterized by the production of large spores, which are dark, and multicellular, with both transverse and longitudinal septa **(Fig. 10A)**(Dauda *et al.*, 2022). The black mold *A. alternata* is a weak plant pathogen **(Fig. 10B)**. It could cause severe post-harvest contaminations of grain (wheat, maize, and rice), fruits (apple, citrus, and strawberries), and vegetables (tomato, onions, and garlic), which has caused considerable economic losses for farmers and the food industry throughout the world (Chain, 2011). Generally, *A. alternata* is regarded as an asexual fungus, even though still carrying a functional *MAT1* gene. The sexual form, teleomorph, has not yet been observed in nature (Meng *et al.*, 2015). In the life cycle of *A. alternata*, aerial spores released from *Alternaria* brown spots on plant tissues infect young leaves, fruits, and stems of the plant, followed by colonizing and developing symptoms, and in the end, form new *Alternaria* brown spots (Hou *et al.*, 2016). Furthermore, *A. alternata* is considered as an allergen, and exposure to *A. alternata* is related to asthma symptoms (Salo *et al.*, 2006).

Fig. 10: Morphology of *A. alternata* colonies and spores and plants infected by *A. alternata*. (A) 10^4 spores of *A. alternata* were incubated on mCDB medium at 28 °C for 3 days (I); 5 X 10^4 spores of *A. alternata* were spread on mCDB medium and incubated at 28 °C for 7 days (II); spores of *A. alternata* (III, https://en.wikipedia.org/wiki/Alternaria_alternata). (B) As a plant pathogen, *A. alternata* could infect and cause damage to many plant tissues (e.g., fruits, stems, and leaves), such as tomatoes (I, https://apps.extension.umn.edu/garden/diagnose/plant/vegetable/tomato/fruitspots.html), apple (II, https://www.epicgardening.com/alternaria-leaf-spot/) and tobacco leaves (III, http://ephytia.inra.fr/en/D/2380).

1.2.2 Secondary metabolites produced by *A. alternata*

A. alternata may produce more than 70 secondary metabolites, many of which are considered as phytotoxins or mycotoxins. Toxins produced by plant pathogens are classified as specific host-selective and non-specific toxins. *A. alternata* could be divided into at least seven pathotypes, and each of them produces a host-specific toxin (HST) when infecting particular plant tissues, namely AK-toxin (Japanese pear), AM-toxin (apple), AF-toxin (strawberry), AAL-toxin (tomato), ACT-toxin (tangerine), ACR-toxin (lemon) and AT-toxin (tobacco) (Tsuge *et al.*, 2013). The deficiency of producing host-selective toxins leads to the reduced virulence of *A. alternata* pathotypes. The

non-specific toxins mainly include tenuazonic acid (TeA), alternariol (AOH), alternariol methyl ether (AME), Altertoxins I-III (ATX I-III), and tentoxin (TEN) (Meena & Samal, 2019). Although the SMs produced by *A. alternata* have been identified a long time ago, how they are synthesized still needs further study. The biosynthesis of host-specific toxins has been characterized to some extent. For instance, the biosynthesis of the AAL-toxin, ACT-toxin, and ACR-toxin is completed by three different polyketide synthase encoding genes, *ALT1*, *ACTTS3*, and *ACRTS2,* respectively (Izumi *et al.*, 2012; Miyamoto *et al.*, 2010; Zhu *et al.*, 2008). The cyclic peptide AM-toxin is synthesized via a non-ribosomal peptide synthetase encoding gene *AMT1* (Johnson *et al.*, 2000). Regarding the biosynthesis of non-specific toxins, recently, it has been revealed that a NRPS-PKS hybrid enzyme AaTAS1 is required for the biosynthesis of TeA, and the major facilitator superfamily protein AaMFS1 functions as efflux transporter of TeA (Sun *et al.*, 2022). The polyketides AOH and its derivative AME are synthesized through the *pksI* gene cluster, and only the PksI enzyme is sufficient for the production of AOH (Wenderoth *et al.*, 2019). Two clustered genes, encoding a NRPS TES and a cytochrome P450 protein P450, respectively, catalyze the biosynthesis of TEN (Li *et al.*, 2016). The synthesis of polyketides, PQ family toxins, ATX I-III, and other derivatives is still unknown compared to other PQ family toxins such as cercosporin and elsinochrome, whose biosynthesis pathways have been partially characterized.

1.3 Objectives of this work

A. alternata is a significant food contaminant and produces many different mycotoxins with altertoxins (ATXI-III) and other perylene quinones as prominent examples. Consumption of food contaminated by these toxins is a severe threat to human health. However, to date, the biosynthesis of these toxic chemicals is still controversial and has not been solved. This project aims to determine the biosynthesis pathway required for altertoxins production and to reveal how they are regulated.

2. Results

2.1 Double use of *A. alternata* polyketide synthase A (PksA) for DHN-melanin and perylene quinones (PQs) biosynthesis

In a previous study, 10 putative polyketide biosynthesis gene clusters (*pksA-J* gene cluster) were identified in *A. alternata* (Saha *et al.*, 2012). Later, it was revealed that the *pksI* gene cluster is responsible for the biosynthesis of the mycotoxins AOH, AME, and other derivatives (Wenderoth *et al.*, 2019). These polyketides are readily produced after *A. alternata* WT strain is incubated on mCDB medium for 7 days and can be detected as prominent blue bands in TLC analyses under 254 nm UV light. Deletion of *pksI* led to the disappearance of these blue bands. However, four yellow bands, which means new secondary metabolites (SMs), became obvious **(Fig. 11A)**. In order to study which *pks* gene cluster is required for the production of these SMs, other polyketide synthase-encoding genes were deleted one by one in the *pksI*-deletion strain using the CRISPR/Cas9 knock-out technology. The yellow bands disappeared when the *pksA* gene was deleted in the *⊿pksI* strain **(Fig. 11A)**. This was surprising, because PksA catalyzes the formation of the black pigment DHN melanin in *A. alternata* (Kimura & Tsuge, 1993; Wenderoth *et al.*, 2017). The critical role of PksA in the biosynthesis of DHN-melanin was confirmed again as the *⊿pksA/I* strain appeared indeed pale or pinkish, similar to the *⊿pksA* strain, and in contrast to the black WT and the *⊿pksI* strain **(Fig. 11B)**. To further verify that these yellow SMs were produced through the DHN-melanin biosynthetic pathway, 30 mg/l tricyclazole, the enzyme activity inhibitor of T4HN reductase, was added to mCDB medium to grow the *pksI*-deletion strain. Tricyclazole inhibits the reduction of 1,3,6,8-THN to scytalone and the reduction of 1,3,8-THN to vermelone and thereby suppresses the biosynthesis of DHN melanin. TLC analysis showed that the *pksI*-deletion strain lost the ability to produce the yellow SMs after 7 days of cultivation on mCDB medium containing tricyclazole (30 mg/l), but instead, several other new SMs were produced **(Fig. 11A)**.

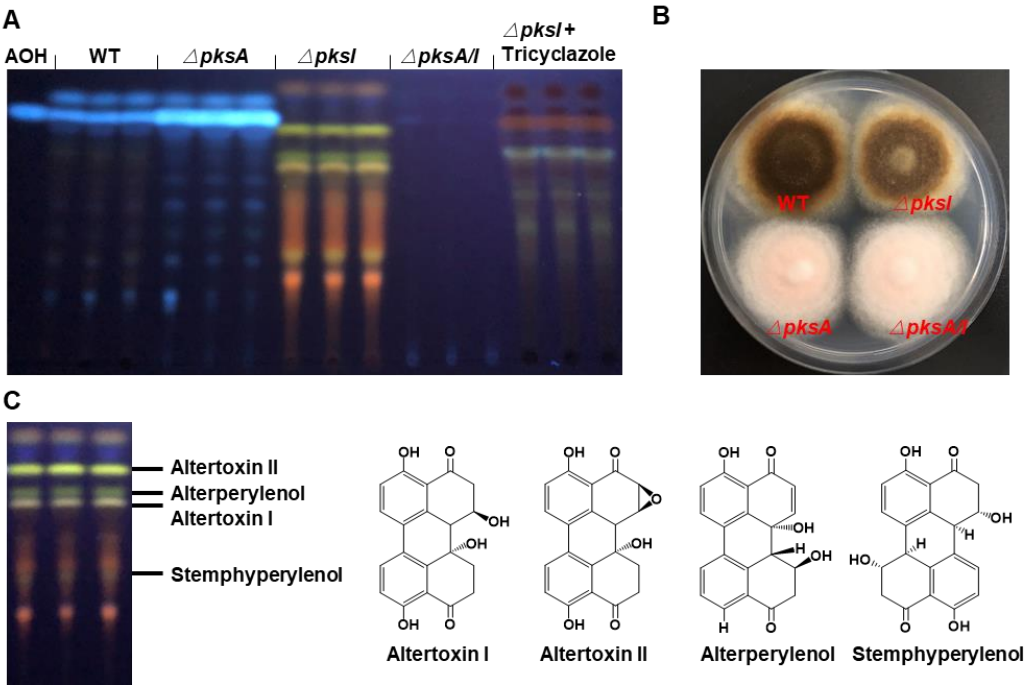


Fig. 11: Polyketide synthase A (PksA) is required for the formation of DHN-melanin and perylene quinones (PQs). (A) Thin-layer chromatographic (TLC) analysis of extracts from wild type (WT) (parental strain ATCC 66981), single mutant strains △*pksA* and △*pksI*, and the double mutant strain △*pksA/I* cultivated on mCDB medium or mCDB medium supplemented with 30 mg/ml tricyclazole at 28°C for 5 days. 5 X 10^4 of spores of each strain were spread evenly on the plate. An alternariol (AOH) standard was utilized for comparison. **(B)** Growth of WT and mutant strains on mCDB medium at 28°C for 5 days. **(C)** Annotation of the yellow bands detected in the TLC analysis in **(A)**. Chemical structures of PQs altertoxin I-II, alterperylenol, and stemphyperylenol.

Next, to further confirm that PksA is responsible for the production of DHN melanin and the yellow SMs, the native *pksA* gene amplified from wild-type genomic DNA was used to re-complement the *pksA/I* double-deletion strain. Melanization of the complemented strain was rescued but not fully restored **(Fig. 12A)**. TLC analysis showed that the complemented strain produced less yellow SMs as compared to the *pksI*-deletion strain **(Fig. 12B).** Taken together, we discovered that *A. alternata* PksA is required for DHN melanin and several new SMs.

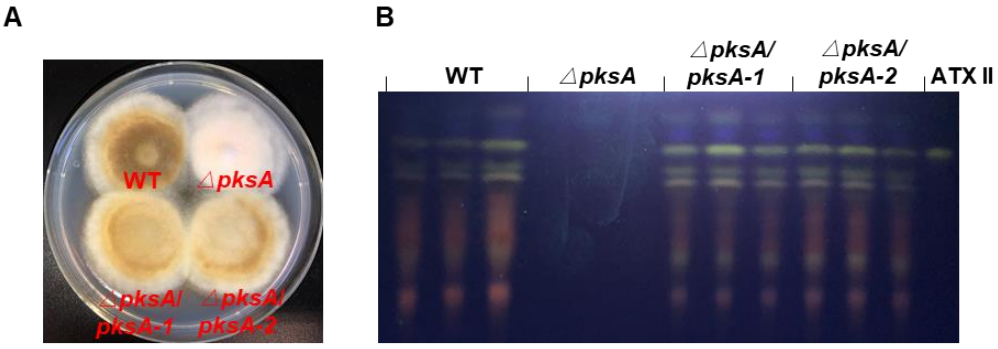


Fig. 12: Melanization and PQs production of the *pksA*-deletion strain were rescued by complementation with the native *pksA* gene. (A) Growth of the wild-type (WT) strain, the *pksA*-deletion strain and the *pksA*-complemented strains on mCDB medium at 28°C for 5 days. **(B)** TLC analysis of extracts from WT and the *pksA*-deletion strain, and the *pksA*-complemented strains cultivated on mCDB medium at 28°C for 5 days. 5 X 10^4 of spores of each strain were spread evenly on the plate. An altertoxin II (ATX II) standard was used for comparison.

Furthermore, we noticed that when *pksA* was deleted in the WT strain, meaning that these yellow SMs were not synthesized, more AOH was produced in the mutant strain (the Δ*pksA* strain). And when the AOH biosynthesis pathway was absent (the Δ*pksI* strain), more yellow SMs were produced **(Fig. 11A)**. Acetyl-CoA and Malonyl-CoA are two common precursors used by PKSs to synthesize polyketide-type SMs. The AOH biosynthesis pathway probably competes for these two compounds with the yellow SMs biosynthesis pathway.

In order to detect the nature of the yellow SMs, the bands were scratched from the TLC plates respectively. A mixture of acetonitrile and water was used to extract the compounds, followed by being measured through LC-HRMS. Comparison with reference standards allowed the assignment of altertoxin II (ATX II), alterperylenol (ALP), and altertoxin I (ATX I), to the respective bands on the TLC plate. As a stemphyperylenol (STP) standard is not available, the presence of STP was confirmed by m/z, MS/MS fragmentation behavior and database search (Stack & Prival, 1986)**(Fig. 11C)(Table 1)**.

Table 1: Detailed information for the identification of PQs ATX II, ALP and ATX I. Molecular formula, retention time, detected ions, accurate mass of the most abundant ion and mass deviation are compared between the reference standards and the respective biological samples

from TLC plates.

Name	Molecular Formula		Retention time [min]	Detected Ions	Accurate Mass of Most Abundant Ion [*m/z*]	Mass Deviation [ppm]
ATX II	$C_{20}H_{14}O_6$	reference standard	23.09	[M-H]- *, [M+Cl]-	349.0719	0.40
		sample	23.10	[M-H]- *	349.0718	0.11
ALP	$C_{20}H_{14}O_6$	reference standard	20.97	[M-H]- *; [M+Cl]-; [M+FA-H]-	349.0719	0.40
		sample	20.96	[M+H]+; [M-H]- *; [M+Cl]-; [M+FA-H]-	349.0717	-0.17
ATX I	$C_{20}H_{16}O_6$	reference standard	20.69	[M-H]- *; [M+Cl]-; [M+FA-H]-	351.0877	0.82
		sample	20.67	[M+H]+; [M-H]- *; [M+Cl]-; [M+FA-H]-	351.0874	-0.03

* Most abundant ion

As our following experiments were mainly performed with the *pksI*-deletion strain, this strain was considered as the WT strain from now on. In order to study the regulation of DHN-melanin and PQs biosynthesis, the timing was analyzed first. The WT strain was cultivated on solid mCDB medium for 1-7 days. After 4 days of incubation, the WT strain was obviously melanized **(Fig. 13A)**. However, the formation of ATX II, ALP and ALP, was clearly detected after 2 days of incubation, and after 3

days of cultivation, the amounts of these three PQs remained unchanged. The production of STP was clearly detected after 3 days of incubation, and after 5 days of cultivation, the quantities of STP remained constant **(Fig. 13B)**. These results showed that the biosynthesis of PQs occurs before *A. alternata* is melanized. And it is possible that the biosynthesis of DHN-melanin and the production of PQs are differentially regulated, although at least PksA should be required in both pathways.

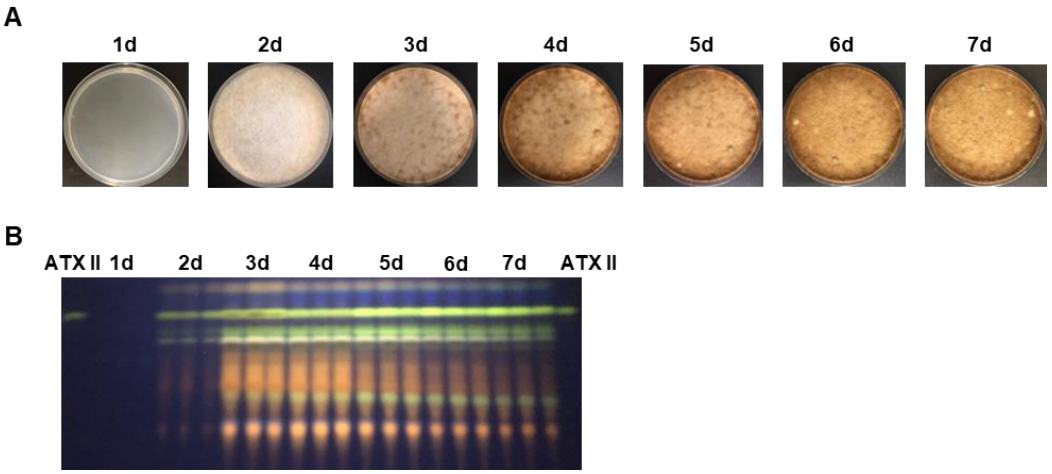


Fig. 13: Timing of DHN-melanin and PQs biosynthesis of WT strain (Δ*pksI*). (A) Growth of WT cultivated on mCDB medium at 28°C for 1-7 days. 5 X 10^4 spores were spread evenly on the mCDB plate **(B)** TLC analysis of extracts from WT strain after 1-7 days of growth on mCDB medium at 28°C. An altertoxin II (ATX II) standard was used for comparison.

2.2 Analysis of the DHN-melanin gene cluster and synteny in *A. alternata*

Although the DHN-melanin biosynthesis pathway has been well studied in many fungi, some aspects are still not revealed in *A. alternata* due to several limitations, such as a few genes existing in the *pksA*-gene cluster and low efficiency of gene knock-out strategies (homologous recombination) in the past. The DHN-melanin biosynthesis pathway in *A. alternata* was compared with that in *A. fumigatus* **(Fig. 14)**. Although the polyketide synthases responsible for DHN-melanin formation from different fungi display high similarity, three different products, namely, the heptaketide YWA1, the hexaketide AT4HN and the pentaketide T4HN, are synthesized. For instance, YWA1 is the product of *A. fumigatus* Alb1/PksP and *A. nidulans* WA; BcPKS12 and BcPKS13

catalyze the formation of T4HN and AT4HN respectively, in the plant pathogenic fungus *Botrytis cinerea*; and WdPks1p produces AT4HN in *Wangiella dermatitidis*. YWA1 and AT4HN should be hydrolyzed to T4HN, and then the biosynthesis of DHN melanin could continue. In *A. alternata*, the product of PksA needs to be explored. Based on the unclear product of *A. alternata* PksA, it is worth detecting whether a α-hydrolase encoding gene exists in the *A. alternata* genome. The biosynthesis of DHN-melanin will be more easily completed if fungal PKSs produce T4HN. In *A. fumigatus*, both the reduction of T4HN to scytalone and the reduction of T3HN to vermelone are catalyzed by Arp2. The orthologs of *A. fumigatus* Arp2, such as Brm2 in *A. alternata*, THR1 in *Bipolaris oryzae* and Brn2 in *B. cinerea* catalyze the same reactions. However, except for THR1, another T4HN reductase, THR4 is also involved in convertingT4HN to scytalone, but not the conversion of T3HN to vermelone in *B. oryzae*. The ortholog of THR4 in *A. alternata* should be further detected. The last aspect that needs to be discovered in *A. alternata* is the enzyme mediating the polymerization of 1,8-DHN to DHN melanin. Laccases, such as Abr2 in *A. fumigatus*, PbrB in *Talaromyces marneffei* and LAC2 in *Colletotrichum orbiculare*, were proven to polymerize 1,8-DHN to DHN melanin. These results suggested that laccases are good candidates to catalyze the enzymatic polymerization of DHN in *A. alternata*.

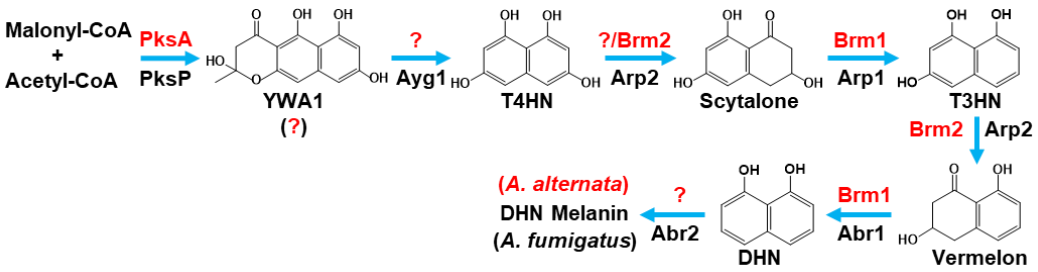


Fig 14: Comparison of the DHN-melanin biosynthetic pathways in *A. fumigatus* and *A. alternata*. Enzymes required for DHN-melanin production in *A. alternata* are exhibited in red, and the orthologs in *A. fumigatus* in black. YWA1, 2,5,6,8-tetrahydroxy-2-methyl-2,3-dihydro-4H-naphtho[2,3-b]pyran-4-one; T4HN, 1,3,6,8-tetrahydroxynaphthalene; T3HN, 1,3,8-trihydroxynaphthalene; DHN, 1,8-dihydroxynaphthalene.

2.3 Hexaketide AT4HN, heptaketide YWA1 and pentaketide T4HN are products of *A. alternata* PksA

In order to explore the product of *A. alternata* PksA, the maltose-inducing promotor *amyB(p)* was used to express *pksA* in *Aspergillus oryzae* heterologously. There are two advantages that *A. oryzae* is an excellent host for determining the product of PksA. One is that *A. oryzae* does not produce endogenous SMs under laboratory conditions, which could disturb product identification. And another one is that, except for polyketide synthase, no other genes involved in DHN-melanin biosynthesis, such as α-hydrolase-encoding gene, scytalone dehydratase-encoding gene and T4HN reductase, are absent in *A. oryzae*. That means the primary product of *A. alternata* PksA should not be further modified. *A. alternata pksA* was successfully expressed in *A. oryzae*, and the mutant strains were confirmed by PCR (data not shown). Compared to the *A. oryzae* WT strain, the mutant strains *AapksA-1* and *AapksA-2* appeared black, secreted a brownish pigment, and grew weakly on MPY medium after 5 days of incubation **(Fig. 15A)**. TLC analysis indicated that the mutant strains *AapksA-1* and *AapksA-2* secreted numerous colorful SMs compared with the *A. oryzae* WT strain **(Fig. 15B)**. The retardation of growth was probably caused by the products of *A. alternata* PksA.

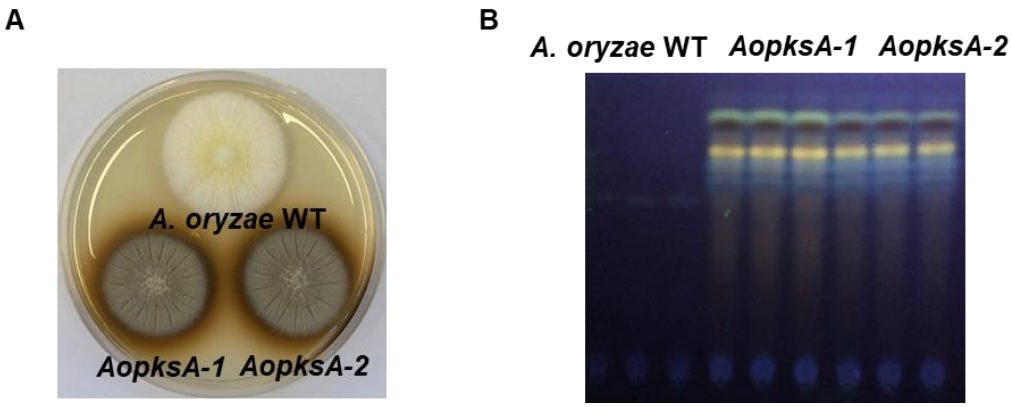
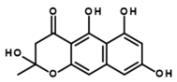
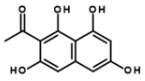
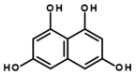
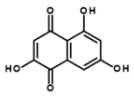
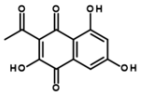
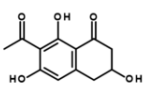


Fig. 15: *A. alternata* PksA was heterologously expressed in *Aspergillus oryzae*. (A) Growth of *A. oryzae* WT and *A. oryzae* expressing *A. alternata pksA* (*AopksA-1* and *AopksA-2*) on MPY medium for at 28°C 3 days. **(B)** TLC analysis of extracts from *A. oryzae* WT and transgenic strains grown on MPY medium at 28°C for 3 days.

To determine the products of *A. alternata* PksA, liquid chromatography-high resolution mass spectrometry (LC-HRMS) was performed to analyze the extracts from the mutant strains *AapksA-1* and *AapksA-2* with that from the *A. oryzae* wilt type as control (collaboration with R. Schumacher and M. Doppler, University of Natural Resources and Life Science, Vienna, Austria). Then, the mass over charge (*m/z*) traces for YWA1, AT4HN and T4HN as well as some related derivatives were examined in the resulting chromatograms **(Table 2)**. Through comparison with reference standards, the extracted ion chromatogram (EIC) peaks of YWA1 and AT4HN were consistently detected in the extracts from both mutant strains, but that of T4HN was not. However, a careful inspection of EICs for closely related compounds suggested the existence of flaviolin, the auto-oxidation product of T4HN. Flaviolin was typically identified when T4HN was present, consistent with its presence in the T4HN standard in this study (Fujii *et al.*, 1999; Fujii *et al.*, 2004). Furthermore, we also identified an analogue oxidation product of AT4HN (sum formula $C_{12}H_8O_6$) and a potential acetylated scytalone (sum formula $C_{12}H_{12}O_5$) in the extracts from the mutant strains *AapksA-1* and *AapksA-2* but not in the wild-type extract sample.

Table 2: Compounds isolated from crude extracts of the *A. oryzae* strain expressing *A. alternata* PksA

Name	Molecular Formula	Structure	Retention time [min]	Ion	Accurate Mass [*m/z*]	Mass Deviation
2,5,6,8-tetrahydroxy-2-methyl-2,3-dihydro-4H-naphtho[2,3-b]pyran-4-one (**YWA1**)	$C_{14}H_{12}O_6$		18.92	[M-H]-	275.0562	0.32
2-acetyl-1,3,6,8-tetrahydroxynaphthalene (**AT4HN**)	$C_{12}H_{10}O_5$		15.18	[M-H]-	233.0454	-0.63
1,3,6,8-tetrahydroxynaphthalene (1,3,6,8-THN or **T4HN**) *	$C_{10}H_8O_4$		12.23	[M-H]-	191.0351	0.62
Flaviolin	C10H6O5		16.71	[M-H]-	205.0173	0.26
AT4HN oxidation product	C12H8O6		24.43	[M-H]-	247.0249	0.36
Acetylated scytalone derivative (AT4HN+2H)	$C_{12}H_{12}O_5$		16.92	[M-H]-	235.0611	-0.41

* not found in samples

In order to further confirm the annotation of PksA products, liquid chromatography-high resolution mass spectrometry/tandem mass spectrometry (LC-HRMS/MS) was performed to re-measure the presumed PksA products. Product ion spectra of an authentic T4HN standard were used for comparison. The result suggested that the Pks candidates YWA1 and AT4HN shared the same intact bicyclic phenolic structure with T4HN as several fragments were concurrently detected in their MS/MS spectra (*m/z* 65, 81, 103, 119, 123, 146, 147, 149). However, the putative flaviolin shared a different core structure with T4HN as its primary fragment ions (*m/z* 89; 133, 135 and 177) were missing in the T4HN standard. These fragment ions were also observed in the putative AT4HN oxidation product, suggesting structural similarity of these two presumed oxidation products. Many fragments in the T4HN reference standard (*m/z* 65, 81, 103, 105, 123, 146, 149) and in the presumed AT4HN (*m/z* 118, 172, 190) were also present in the MS/MS spectra of the acetylated scytalone derivative, suggesting their structural similarity and approving the annotations.

2.4 Two α-hydrolases, AygA and AygB, are involved in DHN melanin biosynthesis, but not in PQs production

The hexaketide AT4HN and the heptaketide YWA1 formed by fungal PKSs should be deacetylated to the pentaketide T4HN by α-hydrolase, and then, DHN melanin biosynthesis could continue. The heterologous-expression experiment in *A. oryzae* determined AT4HN as the main product of *A. alternata* PksA, with small amounts of YWA1 and T4HN. Thus, there should be an α-hydrolase encoding gene existing in the genome for DHN melanin biosynthesis in *A. alternata*. Protein sequences of Ayg1 from *A. fumigatus* and WdYg1P from *W. dermatitidis* were used to search the *A. alternata* ATCC 66981 genome database for orthologues genes (https://mycocosm.jgi.doe.gov/Alalte1/Alalte1.home.html). The BLAST results identified two putative α-hydrolase-encoding genes, *aygA* (transcript ID 115293) and *aygB* (transcript ID 105009). The derived proteins AygA and AygB shared 40.9 % and 53.2 % amino acids sequence identity with *A. fumigatus* Ayg1, and 59.8% and 44.2%

amino acids sequence identity with *W. dermatitidis* WdYg1P, respectively **(Fig. 16)**. cDNA sequencing confirmed that one 80 bp intron at position 106 in the 1343 bp long open reading frame of the *aygA* gene. No intron was identified in the 1212 bp long open reading frame of the *aygB* gene. The coding regions of *aygA* and *aygB* code for a 421 and a 404 amnio acids long protein, respectively.

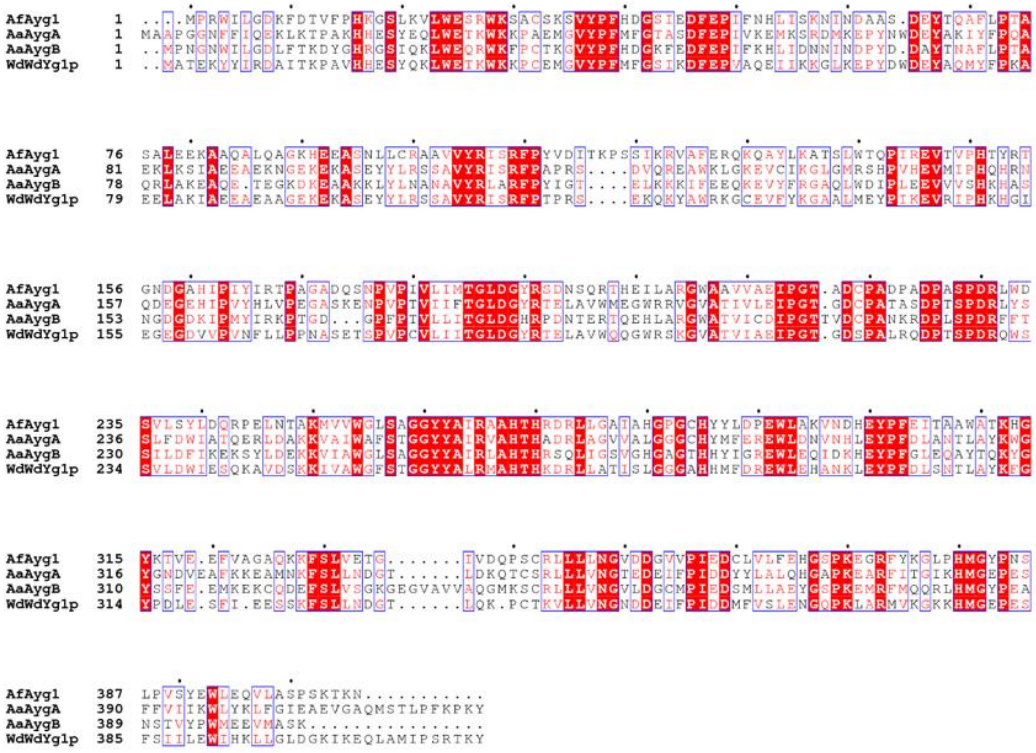


Fig. 16: Alignment of AygA and AygB of *A. alternata* with Ayg1 of *A. fumigatus* and WdYg1p of *Wangiella dermatitidis*.

To determine the functions of AygA and AygB on the DHN melanin biosynthesis, *aygA* and *aygB* were deleted, using the CRISPR/Cas9 knock-out technology. Compared to WT, the *⊿aygA* strain showed a deficiency in melanin production, whereas the *⊿aygB* strain exhibited no noticeable difference. To further investigate the role of AygB in DHN melanin formation, an *aygA* and *aygB* double-deletion strain was constructed. The double-deletion strain appeared even less brown, but resembling the *⊿aygA* strain **(Fig. 17A)**. These results suggested that both AygA and AygB were probably required for DHN melanin biosynthesis, with AygA playing a more significant

role. AygA could compensate for the deficiency of DHN-melanin production caused by the absence of AygB; in contrast, the role of AygA could not be replaced by AygB. Furthermore, we used TLC analysis to explore the roles of AygA and AygB in PQs biosynthesis. Deletion of *aygA* and/or *aygB* did not influence PQs production, different from the negative affection on the formation of DHN melanin **(Fig. 17B)**. As mentioned above, YWA1, AT4HN and T4HN are the products of *A. alternata* PksA. It is possible that the *aygA*- and *aygA/B*-deletion strains still produce DHN melanin using a small amount of T4HN produced directly by PksA or T4HN originating from the hydrolysis of YWA1. To test this hypothesis, WT, *aygA*-, *aygB*- and *aygA/B*-deletion strains were incubated on mCDB medium containing tricyclazole (30 mg/l). Biosynthesis of melanin was prevented in all strains **(Fig.18A)**. On the other hand, the TLC assay showed that no strain produced PQs **(Fig.18B)**. Taken together, these results suggest that probably *A. alternata* uses two routes to synthesize DHN melanin in nature. One is that YWA1 and AT4HN are produced by PksA, then hydrolyzed to T4HN by AygA and AygB. And afterward, T4HN is used to produce DHN-melanin. Another route is that T4HN is produced directly by PksA and then used to synthesize DHN-melanin. It seems that T4HN produced directly by PksA is used to form PQs, explaining that the absence of *aygA* and/or *aygB* does not influence the production of PQs. Furthermore, we questioned the biological meaning of the different performances of AygA and AygB on DHN melanin and PQs biosynthesis. I speculated that the expression of *aygA* and *aygB* is probably spatially regulated. In detail, T4HN produced directly by PksA is used to produce PQs in substrate hyphae where *aygA* and *aygB* are not expressed. And AygA and AygB are produced in spores and aerial hyphae and then involved in the hydrolyzing of YWA1 and AT4HN to T4HN, which boosts the concentration of T4HN and provides enough precursor for melanin biosynthesis.

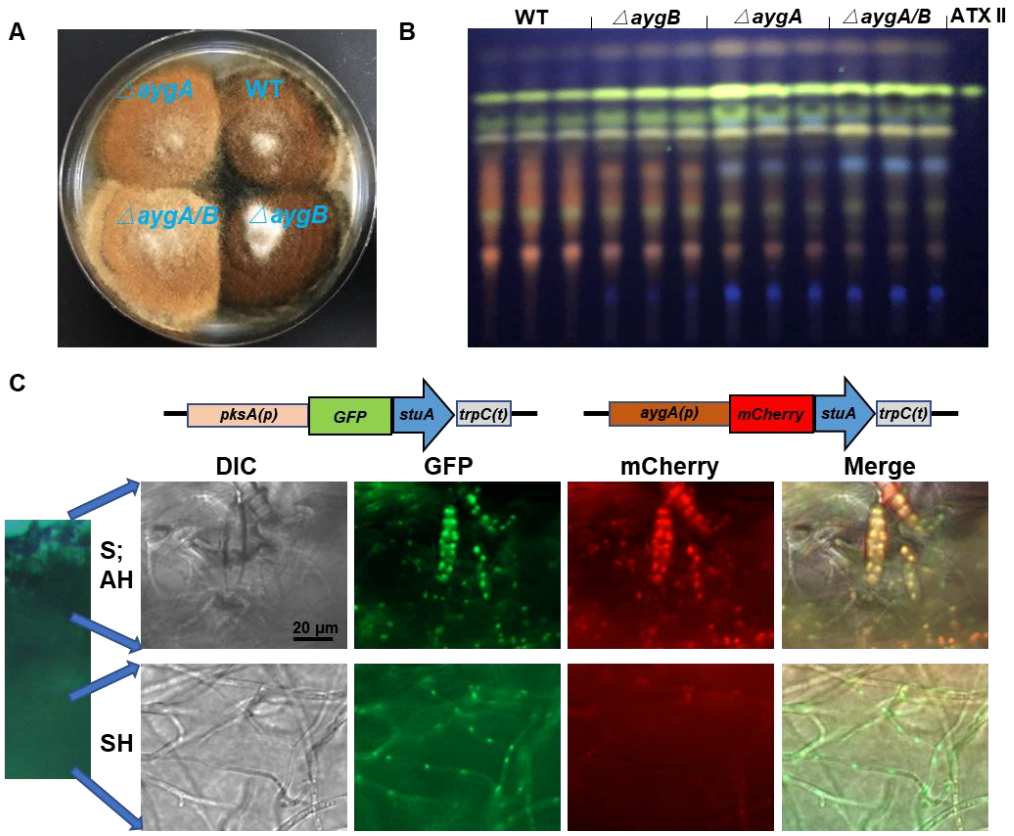


Fig. 17: α-hydrolases AygA and AygB are required for DHN melanin production in spores and aerial hyphae but not for PQs biosynthesis in substrate hyphae. (A) Growth of wild type (WT) strain, single mutant strains Δ*aygA* and Δ*aygB*, and double mutant strain Δ*aygA*/*B* on mCDB medium at 28 °C for 5 days. **(B)** TLC analysis of extracts from WT and mutant strains cultivated on mCDB medium at 28 °C for 5 days. 5 X 10^4 of spores of each strain were spread evenly on the mCDB plate. An altertoxin II (ATX II) standard was used for comparison. **(C)** Observation of the expression of *pksA* and *aygA* in spores, aerial hyphae and substrate hyphae through a promoter-reporter assay. The cassette of *pksA(p)::gfp::stuA::trpC(t)* was transformed into WT protoplasts together with the cassette of *aygA(p)::mCherry::stuA::trpC(t)* with hygromycin-resistance gene as selection marker. 5 X 10^4 of spores of the mutant strain were spread evenly on mCDB plate and cultivated at 28 °C for 3 days. The fluorescent signals were detected with a fluorescence microscope. Scale bar, 20 μm. S, spores; AH, aerial hyphae; SH, substrate hyphae.

In order to test this hypothesis, a promoter-reporter assay was performed based on the expression of the fluorescent protein GFP or mCherry (Yu *et al.*, 2021). The *pksA* promoter was used to express a chimeric protein consisting of the NLS-containing part of the transcriptional factor StuA and the green fluorescent protein GFP, together with the *aygA* promoter used to express another chimeric protein consisting

of the NLS-containing part of the transcriptional factor StuA and red fluorescent protein mCherry. When these two chimeric proteins were expressed, they would shuttle into nuclei and make them fluorescent. This allows spatial and timely resolution of the expression of the two genes, whose promoter were used **(Fig. 17C)**(Toews *et al.*, 2004). Microscopic analysis indicated that both, GFP and mCherry signals, were strongly detected in spores and aerial hyphae, whereas a GFP signal was only detected in substrate hyphae. That means, in spores and aerial hyphae, both *pksA* and *aygA* were expressed. However, in substrate hyphae, only the expression of *pksA* appears to be required, without the expression of *aygA*. When the *aygB* promoter was used for expressing the chimeric protein consisting of the NLS-containing part of the transcriptional factor StuA and red fluorescent protein mCherry, we observed the same phenomenon **(Fig. 19)**.

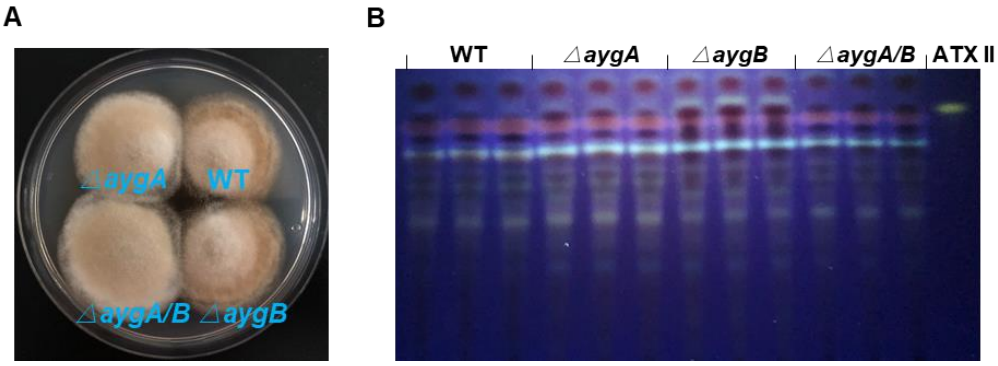


Fig. 18: The production of DHN-melanin and PQs is blocked by tricyclazole in aygA- and/or aygB-deletion strains. (A) Growth of wild type (WT) strain, single mutant strains Δ*aygA* and Δ*aygB*, and double mutant strain Δ*aygA*/*B* strains incubated on mCDB medium supplemented with 30 mg/l tricyclazole at 28°C for 5 days. **(B)** TLC analysis of extracts from WT and mutant strains on mCDB medium supplemented with 30 mg/l tricyclazole at 28°C for 5 days. An altertoxin II (ATX II) standard was utilized for comparison.

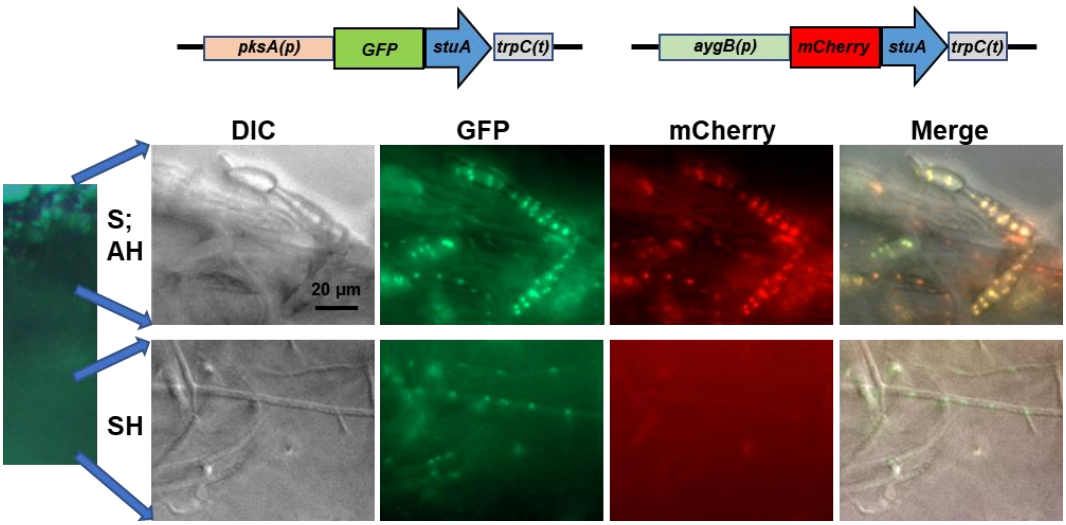


Fig. 19: Observation of the expression of *pksA* and *aygB* in spores, aerial hyphae and substrate hyphae through a promoter-reporter assay. The cassette of *pksA(p)::gfp::stuA::trpC(t)* was transformed into WT protoplasts together with the cassette of *aygB(p)::mCherry::stuA::trpC(t)* with hygromycin-resistance gene as selection marker. 5 X 10^4 of spores of the mutant strain were spread evenly on mCDB plate and cultivated at 28 °C for 3 days. The fluorescent signals were detected with a fluorescence microscope. Scale bar, 20 µm. S, spores; AH, aerial hyphae; SH, substrate hyphae.

To verify that DHN melanin and PQs are synthesized in different mycelial parts, the *pksI*-deletion strain was cultivated on mCDB medium for 3 days. A razor blade was used to collect melanized spores and aerial hyphae carefully into a 1.5 ml Eppendorf tube. The mCDB agar medium containing the colorless substrate hyphae was collected into another 1.5 ml Eppendorf tube, followed by TLC analysis **(Fig. 20A)**. PQs were clearly detected in the medium containing substrate hyphae but not in the spores and aerial hyphae **(Fig. 20B)**. The same experiment was performed to check the possibility that AOH was formed only in some hyphal parts. The WT strain (ATCC 66981) was incubated on mCDB medium for 5 days. AOH was mainly detected in substrate hyphae, with some AOH being measured in spores and aerial hyphae. A previous study proved that AOH is the dominant SM after 5 days of incubation on mCDB solid medium (Wenderoth *et al.*, 2019). And in this study, the separation of spores and aerial hyphae from substrate hyphae was not perfect. Thus, the AOH measured in the spores and aerial hyphal fraction was probably due to contamination with substrate hyphae. We

also incubated the WT (ATCC 66981) strain using liquid mCDB medium in submerged conditions for 3, 5 and 7 days. Intriguingly, PQs were detected earlier than AOH and were the dominant SMs **(Fig. 20C)**.

Collectively, these results prove that DHN melanin and PQs biosyntheses are spatially regulated in *A. alternata*. The achievement of this regulation is reflected in the spatial expression of *aygA* and *aygB*. In substrate hyphae, T4HN produced by PksA is probably used to synthesize PQs. And in spores and aerial hyphae, AygA and AygB are expressed and hydrolyze YWA1 and AT4HN to promote DHN melanin production.

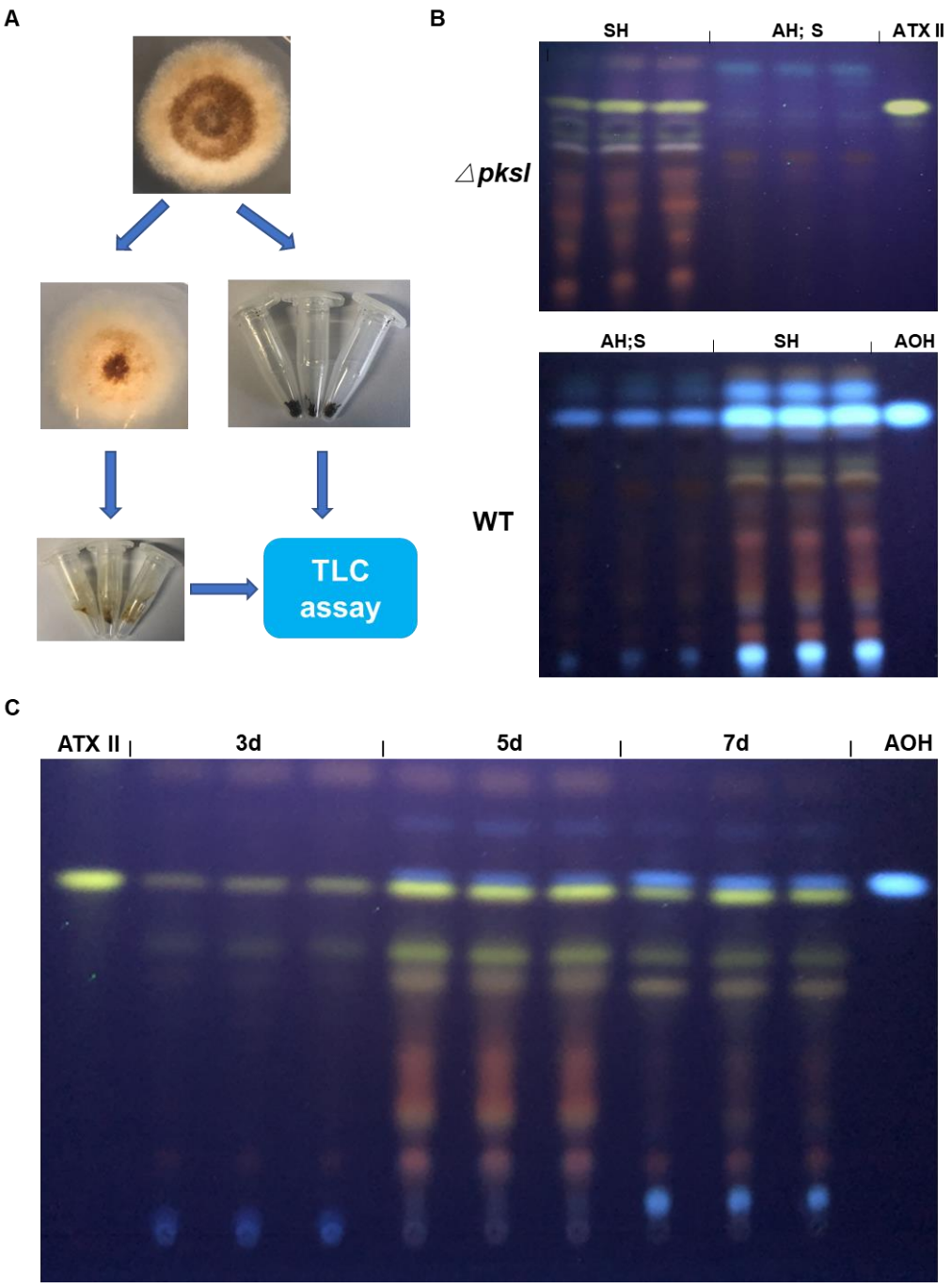


Fig. 20: The biosynthesis of PQs and AOH occurs in substrate hyphae and not in spores and aerial hyphae. (A) Separation of spores/aerial hyphae and substrate hyphae from an *A. alternata* colony. 10^4 spores of *A. alternata* were spotted onto a mCDB plate and cultivated at 28 °C for 3 or 5 days. Spores together with aerial hyphae were collected into a 1.5 ml Eppendorf tube with a razor blade. mCDB agar containing substrate hyphae was collected into another 1.5 ml Eppendorf tube. **(B)** TLC analysis of extracts from spores/aerial hyphae and substrate hyphae from Δ*pksI* and wild type (WT) strains. An altertoxin II (ATX II) standard and an alternariol (AOH) were utilized for comparison. S, spores; AH, aerial hyphae; SH, substrate hyphae. **(C)** TLC analysis of extracts from WT strain incubated in the submerged culture at 28 °C for 3, 5 or 7 days.

2.5 Except for Brm2, a new T4HN reductase Brm3 participates in the biosynthesis of DHN-melanin and PQs

In *B. oryzae*, two T4HN reductases, THR1 and T4HR1, catalyze the reaction of T4HN to scytalone, with T4HR1 as the ortholog of Brm2 from *A. alternata*. In order to investigate the putative ortholog of THR1 in *A. alternata*, protein sequences of Arp2 from *A. fumigatus*, Brm2 from *A. alternata* and THR1 from *B. oryzae* were used to search the *A. alternata* ATCC 66981 genome database (https://mycocosm.jgi.doe.gov/Alalte1/Alalte1.home.html). A new putative T4HN reductase-encoding gene was identified and named brm3 (transcript ID 112254). The derived proteins Brm3 shared 49.8%, 48% and 90.4 % amino acids sequence identity with *A. fumigatus* Arp2, *A. alternata* Brm2 and *B. oryzae* THR1, respectively **(Fig. 21)**. No intron was identified in the 807 bp long open reading frame of the *brm3* gene based on cDNA sequencing. The coding region of *brm3* codes for a 269 amnio acids protein.

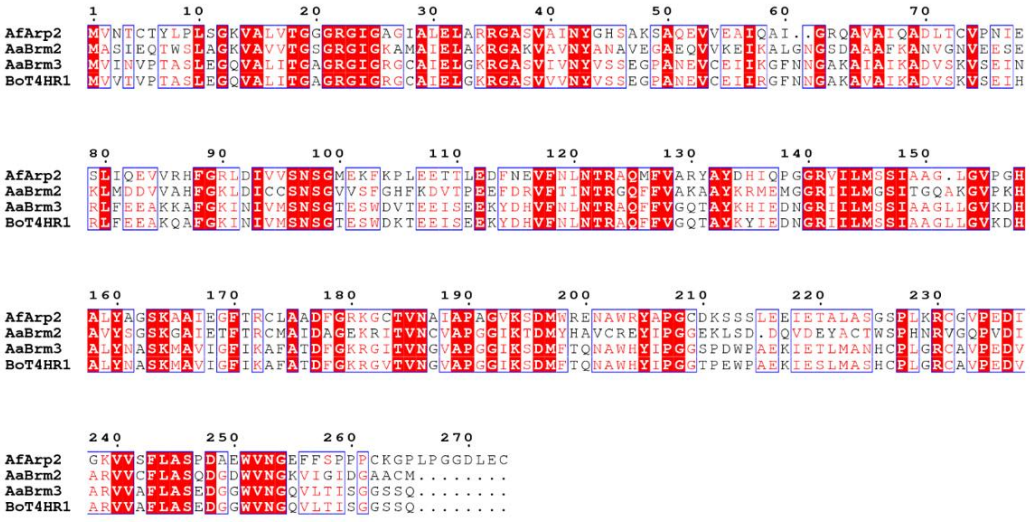


Fig. 21: Alignment of Brm2 and Brm3 of *A. alternata* with Arp2 of *A. fumigatus* and T4HR1 of *B. oryzae*.

Brm2 is characterized to be required for the formation of DHN melanin in *A. alternata*. Here, we generated a *brm2*-deletion strain in WT and confirmed the role of Brm2 in DHN melanin biosynthesis **(Fig. 22A)**. Brm2 is also involved in PQs production

(Fig. 22B). To detect the role of Brm3 in DHN melanin and PQs formation, we deleted the *brm3* gene in WT and the *brm2*-deletion strain using the CRISPR/Cas9 knock-out technology. The absence of Brm3 had no influence on the production of DHN melanin on mCDB medium, whereas the *⊿brm2/3* strain appeared light orange, different from the brownish *△brm2* strain **(Fig. 22A)**. That indicated the involvement of Brm3 in DHN melanin formation in *A. alternata*. However, when these mutant strains were cultivated on mCDB medium supplemented with tricyclazole (30 mg/l) for 14 days, the WT and the *△brm2* strain displayed the same phenotype (brownish colonies), and the *△brm3* and *△brm2/3* strains had the same phenotype (orange colonies) **(Fig. 22A)**. These results suggest that different from Brm2, Brm3 only catalyzes the conversion of T4HN to scytalone, and the activity of Brm3 could not be inhibited by tricyclazole. Furthermore, the *⊿brm2/3* strain did not produce PQs, whereas the *⊿brm3* strain produced more PQs than the WT strain **(Fig. 22B)**.

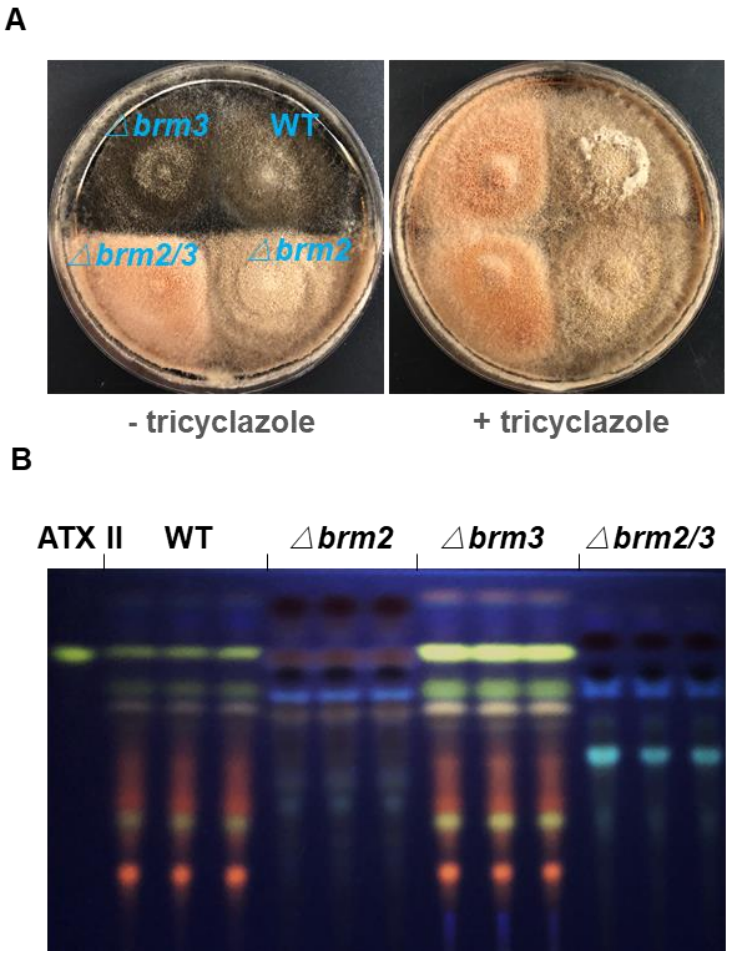


Fig. 22: Two T4HN reductases, Brm2 and Brm3, are required for DHN-melanin and PQs production. (A) Growth of wild type (WT), single mutant strains △*brm2* and △*brm3*, and double mutant strain △*brm2/3* on mCDB medium with/without tricyclazole (30 µg/ml) at 28 °C for 2 weeks. **(B)** TLC analysis of extracts from WT strain, single mutant strains △*brm2* and △*brm3*, and double mutant strain △*brm2/3* incubated on mCDB medium at 28 °C for 5 days. An altertoxin II (ATX II) standard was utilized for comparison.

2.6 Scytalone dehydratase, Brm1, is required for PQs biosynthesis

Brm1 catalyzes the conversion of scytalone to T3HN and the conversion of vermelon to 1,8-DHN in the DHN-melanin biosynthetic pathway in *A. alternata*. To test the possibility that Brm1 is involved in PQs formation, we generated a *brm1*-deletion strain using the CHRISPR/Cas9 knock-out technology. The *⊿brm1* strain looked slightly brownish, confirming its role in DHN-melanin production **(Fig. 23A)**. Depletion of Brm1 resulted in the production of several other SMs, but not PQs **(Fig. 23B)**.

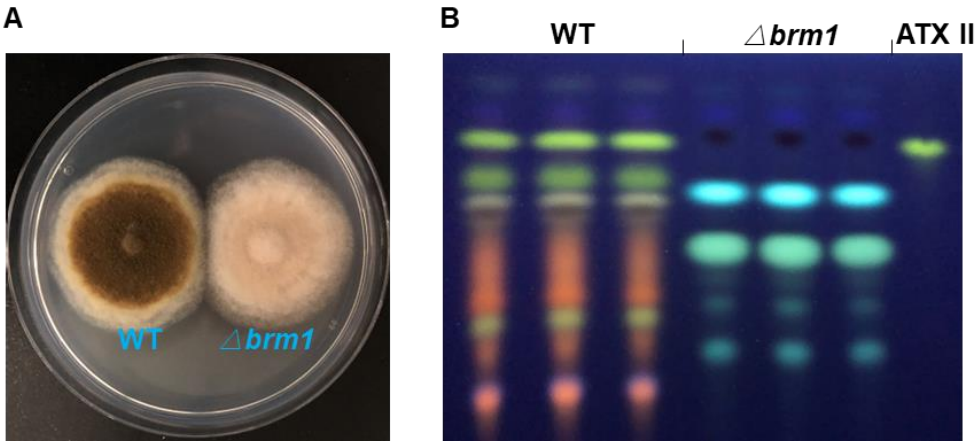


Fig. 23: Scytalone dehydratase Brm1 is involved in DHN-melanin and PQs biosynthesis. (A) Growth of the wild type (WT) strain and the mutant strain ∆*brm1* incubated on mCDB medium at 28 °C for 5 days. **(B)** TLC analysis of extracts from WT and the *brm1*-deletion strain cultivated on mCDB medium at 28 °C for 5 days. An altertoxin II (ATX II) standard was used for comparison.

2.7 1,8-DHN is the last common intermediate for DHN melanin and PQs formation

As we mentioned before, depletion of Brm1 and Brm2 resulted in the disruption of PQs production in *A. alternata*. Both, the conversion of scytalone to T3HN and the conversion of vermelon to 1,8-DHN are achieved by Brm1 **(Fig. 14)**. Thus, vermelon and 1,8-DHN are two optional precursors for the synthesis of PQs in the melanin biosynthetic pathway. Since vermelon could not be purchased, we tested the possibility that 1,8-DHN is the intermediate used for synthesizing PQs. We incubated the *pksA*-deletion strain on mCDB supplemented with different amounts of 1,8-DHN (0, 0.01, 0,02, 0.03 and 0.04 mg/ml) for 5 days and then performed TLC analysis **(Fig. 24A)**. mCDB medium supplemented with 0.02 mg/ml 1,8-DHN, not used to cultivate the ⊿*pksA* strain, was utilized as one negative control. TLC analysis showed that the PQs (ATX II, ATX I and ALP) were obviously measured, with their amounts increasing when more 1,8-DHN was supplemented (0.01-0.03 mg/ml) **(Fig. 24B)**. However, when 0.04 mg/ml 1,8-DHN was added to mCDB medium, lower amounts of PQs were produced, probably due to toxic side effects of 1,8-DHN restricting growth. The melanization of the *pksA*-deletion strain was rescued after 5 days of incubation in mCDB liquid medium supplemented with 0.02 mg/ml 1,8-DHN under shaking conditions **(Fig. 25)**. These results show that 1,8-DHN is the last common intermediate to form DHN melanin and PQs in *A. alternata*.

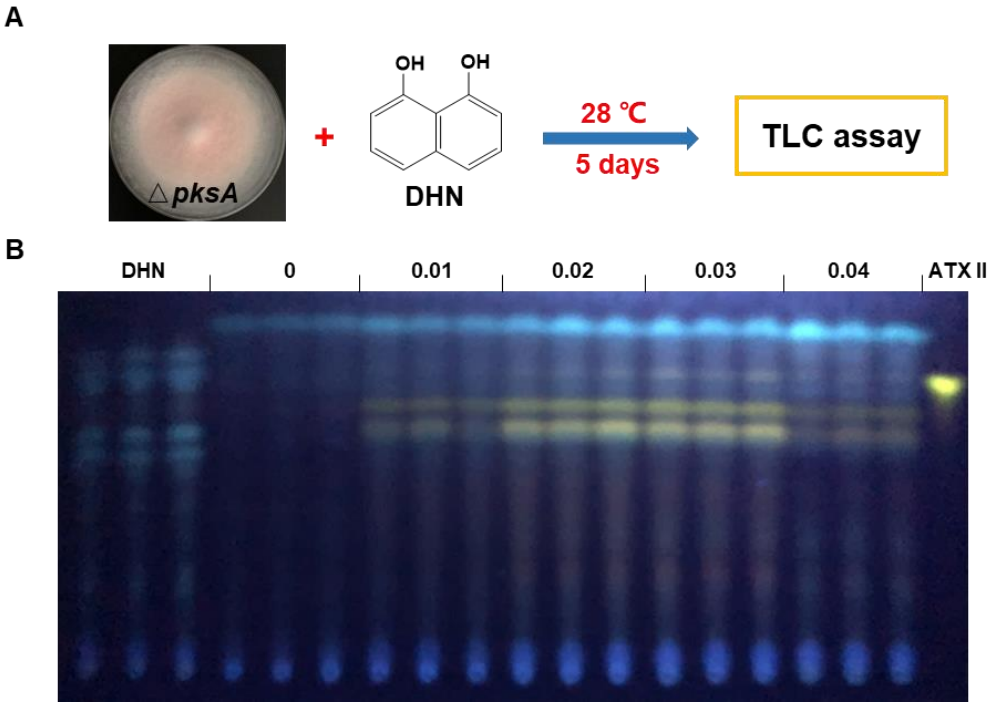


Fig. 24: 1,8-DHN is the intermediate utilized (here utilized is ok) for synthesizing PQs. (A) Scheme of the feeding experiment of the *pksA*-deletion strain with 1,8-DHN. The mutant strain Δ*pksA* was incubated on mCDB medium supplemented with different amounts of 1,8-DHN (0, 0.01, 0.02, 0.03 and 0.04 mg/ml) at 28 °C for 5 days respectively. mCDB medium supplemented with 0.02 mg/ml 1,8-DHN without cultivating the *pksA*-deletion strain was regarded as a negative control. **(B)** TLC analysis of extracts from the different culture conditions. An altertoxin II (ATX II) standard was used for comparison. 1,8-DHN, 1,8-dihydroxynaphthalene.

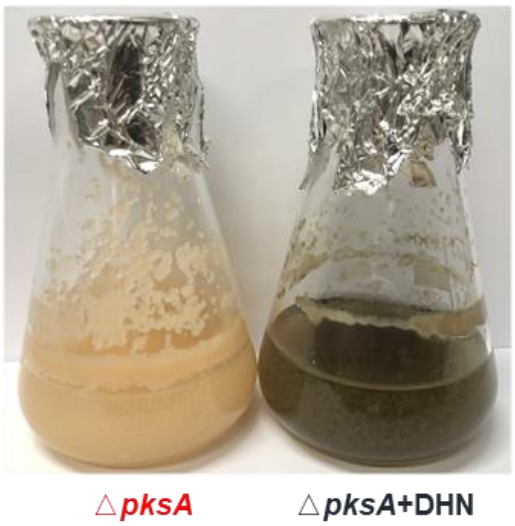


Fig. 25: Melanization of the *pksA*-deletion strain was rescued through feeding with 1,8-DHN. 10^5 of spores of the mutant strain Δ*pksA* were cultivated in liquid mCDB medium

with/without 1,8-DHN (0.02 mg/ml) in a flask at 28 °C at 180 rpm for 5 days. 1,8-DHN, 1,8-dihydroxynaphthalene.

2.8 Four laccases, LccB-D and LccF, are required for melanin biosynthesis, but not for PQ production

The cooper oxidase laccase is generally considered to play a key role in DHN-melanin biosynthesis through polymerizing 1,8-DHN in fungi. Several examples show that laccases are also required for the biosynthesis of fungal mycotoxins by mediating phenol-coupling reactions, such as cercosporin in *Colletotrichum beticola*, viriditoxin in *Aspergillus viridinutans* and *Paecilomyces variotiin*, and sporandol in *Chrysosporium merdarium* (De Jonge *et al*., 2018). Therefore, there should be one or more laccases responsible for the formation of DHN melanin, or perhaps involved in PQs biosynthesis. Protein sequences of known laccases from diverse fungi were used to search the *A. alternata* ATCC 66981 genome database (https://mycocosm.jgi.doe.gov/Alalte1/Alalte1.home.html), and seven putative laccase-encoding genes, named *lccA-G* were identified. The *lccA* gene (Transcript ID 112523) is comprised of 1,963 bp with one 226 bp intron at position 1,504. The *lccB* gene (Transcript ID 114657) is comprised of 2,104 bp with four introns (49 bp, 53 bp, 138 bp, 52 bp) at positions 358, 608, 1,061 and 1,633 respectively. The *lccC* gene (Transcript ID 115320) is comprised of 1,847 bp with one 50 bp intron at position 229. The *lccD* gene (Transcript ID 114031) is comprised of 1,864 bp with three introns (61 bp, 63 bp, 54 bp) at positions 261, 468 and 737, respectively. The *lccE* gene (Transcript ID 111569) is comprised of 2,321 bp with two introns (49 bp, 49 bp) at positions 765 and 1,101 respectively. The *lccF* gene (Transcript ID 110245) is comprised of 1822 bp with two introns (89 bp, 47 bp) at positions 97 and 572 respectively. The *lccG* gene (Transcript ID 116332) is comprised of 2,070 bp without any intron. The derived proteins of *lccA-G* consist of 579, 604, 599, 562, 741, 562 and 690 amino acids, respectively.

As a first step to test which of the seven laccases may be involved in melanin

and/or PQs biosynthesis, expression analyses were performed. To this end *A. alternata* WT strain was also incubated on mCDB medium or mCDB medium supplemented with tomato puree (400 g/l). Compared to growth on mCDB medium, WT is hyper-melanized when grown on mCDB medium containing tomato puree **(Fig. 26A)**. Hence, laccases involved in melanin formation should be induced under such growth conditions. Then, we calculated the melanin contents of WT under the two conditions and found that the melanin content of WT in mCDB medium containing tomato puree was around three-fold higher than that in mCDB medium **(Fig. 26B)**. However, TLC analysis showed that the quantity of PQs produced on mCDB medium containing tomato puree was significantly reduced compared with that on mCDB medium **(Fig. 26C)**. These results suggest that the expression of laccases involved in melanin biosynthesis should be up-regulated, and the laccases involved in PQs production should be down-regulated when grown in the presence of tomato puree. Next, we performed quantitative real-time RT-PCR analysis using these two kinds of media to determine the expression level of the laccase-encoding genes. Transcript levels of *lccB*, *lccE* and *lccF* were remarkably upregulated (23.8, 8.6 and 5.8 times higher, respectively) and the transcript level of *lccD* was seriously downregulated (44.1 times lower) in the presence of tomato puree. In contrast, *lccA* and *lccG* were barely expressed when *A. alternata* were cultivated under either condition. Only a small difference was observed in the expression of *lccC* **(Fig. 26D)**. Taken together, these results suggest that LccB, LccE and LccF are probably involved in DHN melanin biosynthesis, and LccD could be required for PQs production.

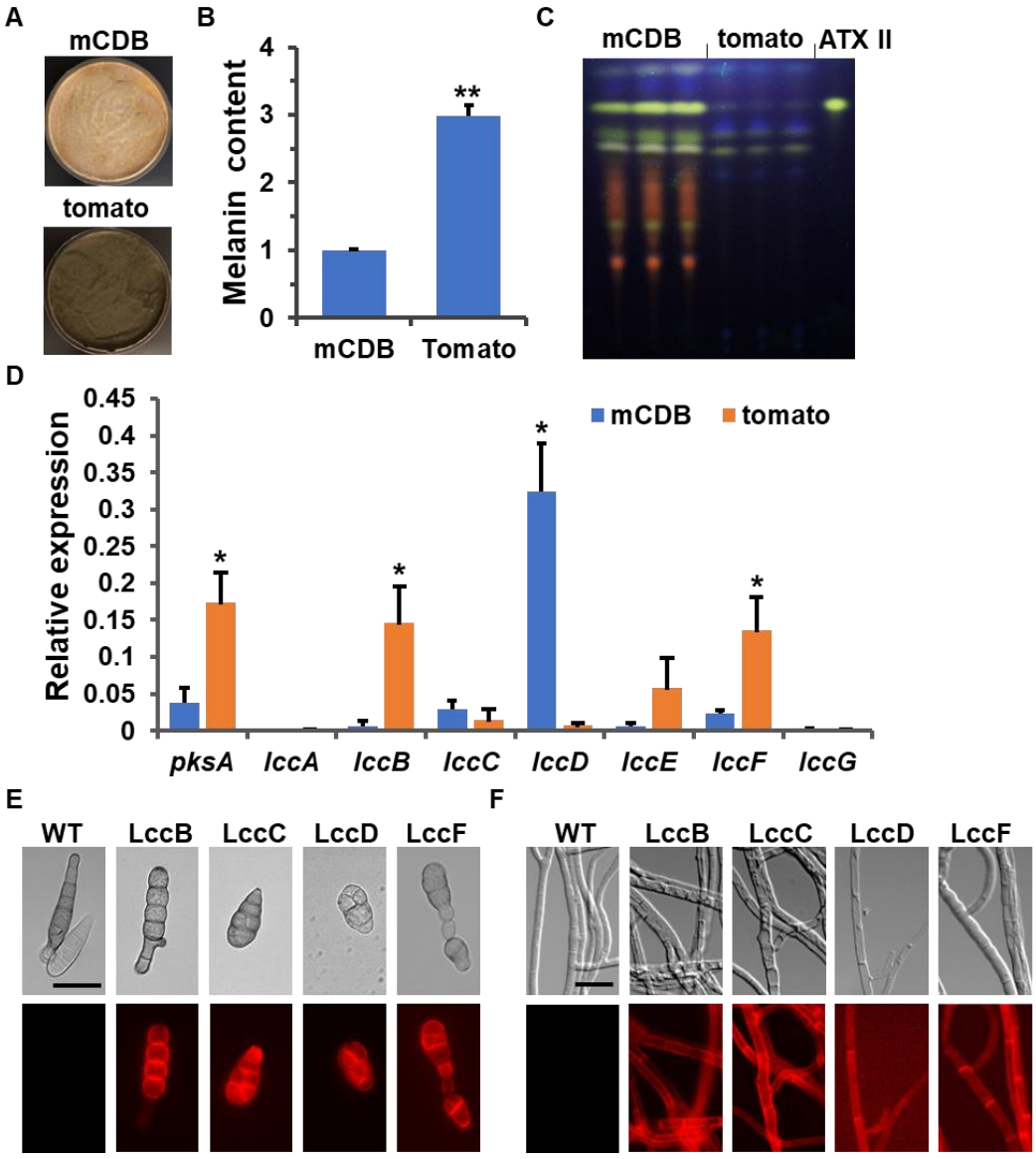


Fig. 26: Four laccases, LccB-D and LccF, are potentially involved in DHN-melanin or PQs biosynthesis. (A) Growth of wild type (WT) on mCDB medium supplemented with/without tomato puree (400 g/l). 5 X 10^4 spores were spread evenly on the plates and cultivated at 28 °C for 5 days. **(B)** Quantification of the melanin content of WT cultivated on mCDB medium supplemented with/without tomato puree (400 g/l) at 28°C for 5 days. **(C)** TLC analysis of extracts from WT incubated on mCDB medium supplemented with/without tomato puree (400 g/l) at 28 °C for 5 days. An altertoxin II (ATX II) standard was utilized for comparison. **(D)** Expression analysis of seven putative laccase-encoding genes (*lccA-G*) by quantitative real-time PCR in WT strain incubated on mCDB medium supplemented with/without tomato puree (400 g/l) at 28 °C for 5 days. *h2b* was selected as the endogenous control for gene expression analysis. This experiment was performed in three biological replicates. The error bar represents the standard deviation. Statistical analysis was done with Student's test, *, $p \leq 0.05$; **, $p \leq 0.01$. **(E)** Subcellular localization of LccB-D and LccF in spores. Scale bar, 10 µm. **(F)** Subcellular localization of LccB-D and LccF in aerial hyphae. Scale bar, 20 µm. Tomato, mCDB medium

supplemented with 400g/l tomato puree.

To further explore the functions of laccases in *A. alternata*, protein domains of these putative laccases were predicted using InterPro (https://www.ebi.ac.uk/interpro/). Except for LccE, a potential signal peptide at the N-terminus is identified in the other six laccases, LccA-D, LccF and LccG, respectively. Furthermore, LccA and LccG harbor a transmembrane domain close to their C-terminus **(Fig. 27)**. We compared the protein domains of known laccases required for DHN melanin production from different fungi and found that all of them contain a signal peptide without having a transmembrane domain **(Fig. 28)**. The laccases for melanin biosynthesis are secreted from cells and stay in the cell walls of spores and/or aerial hyphae, where 1,8-DHN or other precursors are polymerized to melanin. In *A. alternata*, LccB, LccC, LccD and LccE are supposed to be good candidates required for DHN melanin formation. All four laccases were labeled with mCherry at the C-terminus and expressed using their native promoters. They all located in the cell walls and septa of spores and aerial hyphae **(Fig. 26E**, **F)**, suggesting their requirement for melanin formation.

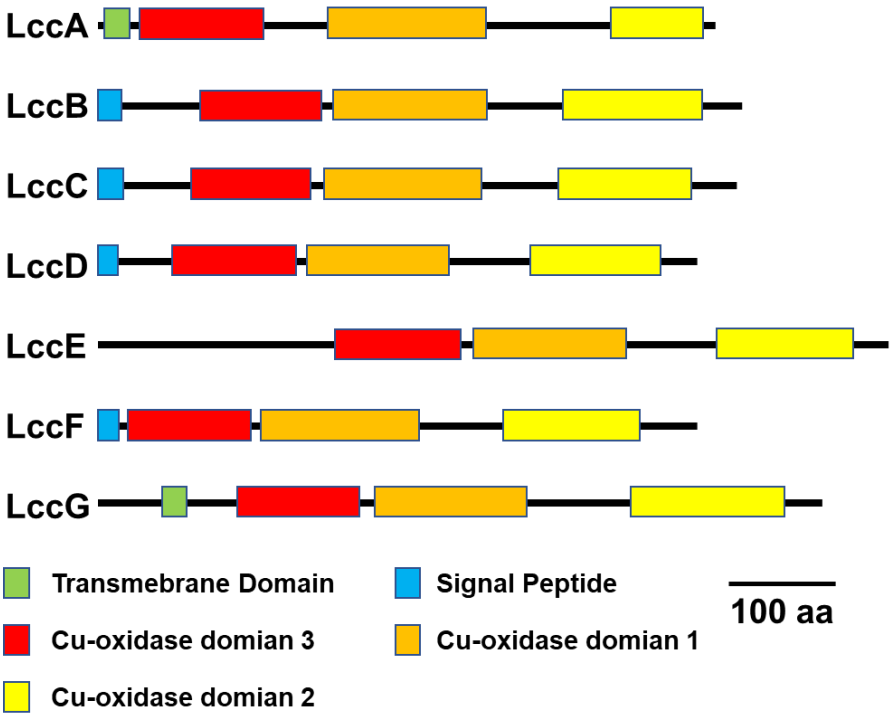


Fig. 27: Comparation of protein domains of LccA-G from *A. alternata*.

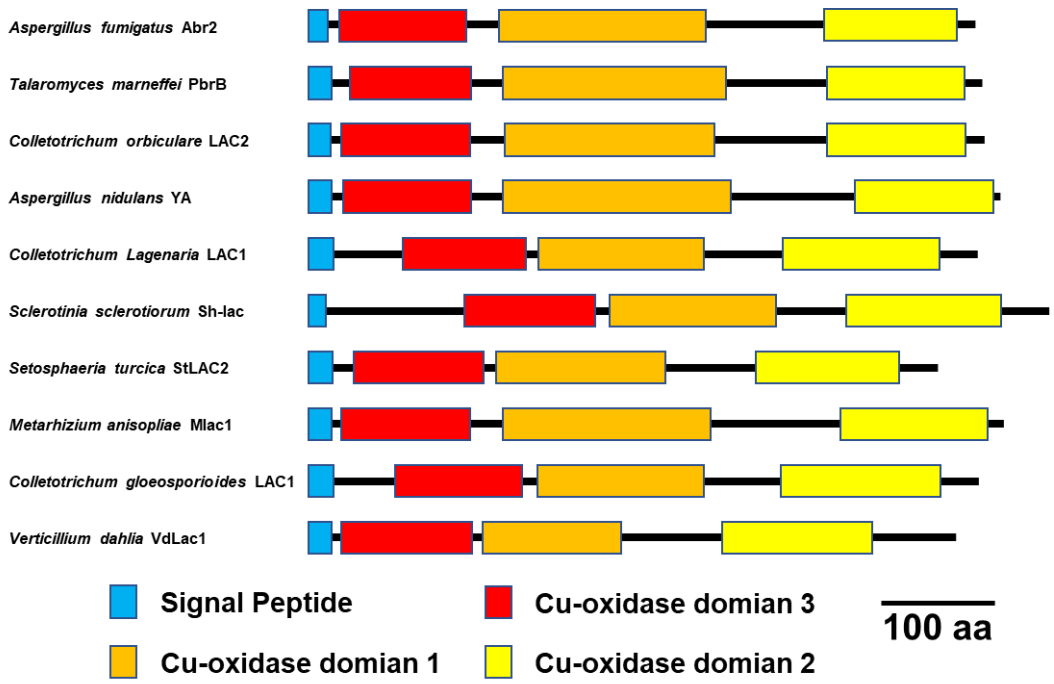


Fig. 28: Comparation of protein domains of laccases involved in melanin biosynthesis from different fungi.

To determine the roles of these four laccases, we generated *lccB*-, *lccC*-, *lccD*- and *lccF*-deletion strains in WT using the CRISPR/Cas9 knock-out technology. *lccB*-, *lccC*-, *lccD*- and *lccF*- deletion strains displayed no noticeable difference on melanin production compared to WT after 3 and 5 days of cultivation on mCDB medium. However, *lccB*- and *lccF*- deletion strains produced less melanin than WT after 3 days of incubation on mCDB medium supplemented with tomato puree (400 g/l). But, neither of the mutants showed a difference in melanin formation compared to WT after 5 days of cultivation **(Fig. 29A)**. The depletion of LccB, LccC, LccD and LccF had no influence on the biosynthesis of PQs **(Fig. 29B).** In addition, to further explore functions for these four laccases, we generated *lccC*/*D*- and *lccB*/*F*- double deletion strains. The melanization level of these two mutants was dramatically decreased after 3 and 5 days of incubation on mCDB medium or mCDB medium supplemented with tomato puree (400 g/l) **(Fig. 29C)**. In contrast, no noticeable difference was detected in the formation

of PQs in both mutant strains **(Fig. 29D)**.

These results indicate that all of the investigated laccases (LccB, LccC, LccD and LccF) are required for DHN-melanin formation to some extent, with probably redundant functions. However, none of the four laccases seem to play a role in the formation of PQs.

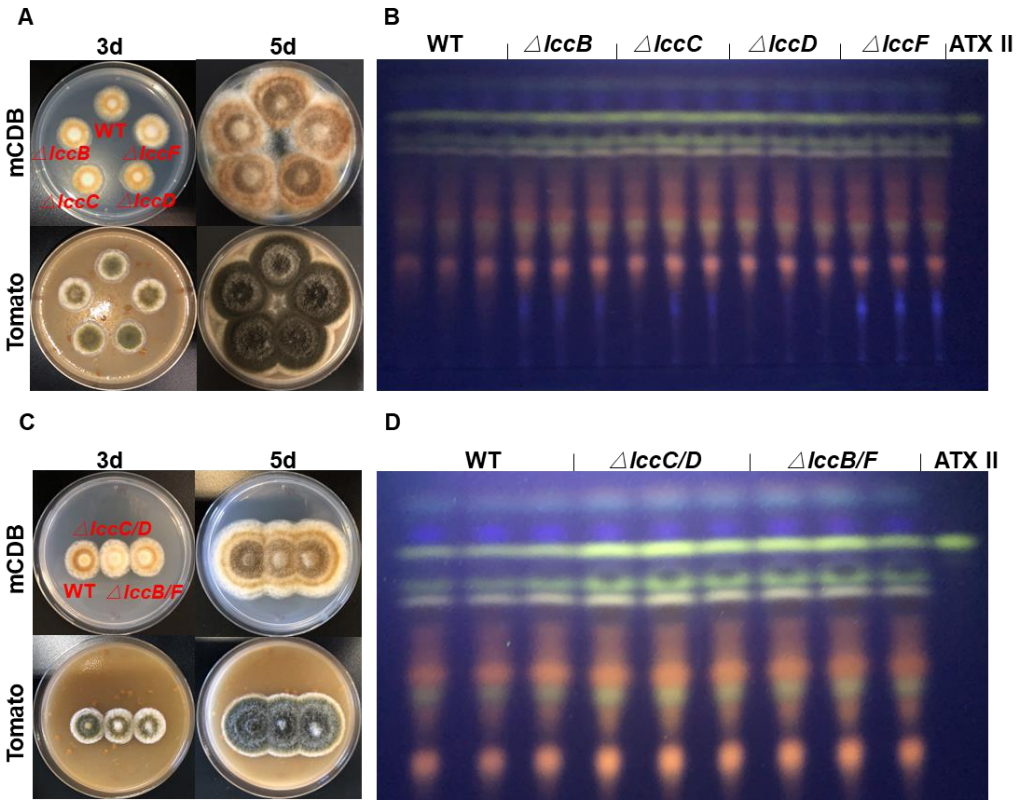


Fig. 29: Four laccases, LccB-D and LccF, are required for melanin biosynthesis but not for PQs production. (A) Growth of wild type (WT) strain, the mutant strains *⊿lccB*, *⊿lccC, ⊿lccD and ⊿lccF* on mCDB medium supplemented with/without tomato puree (400 g/l) at 28 °C for 3 or 5 days. **(B)** TLC analysis of extracts from WT strain, and mutant strains *⊿lccB*, *⊿lccC, ⊿lccD and ⊿lccF* incubated on mCDB medium at 28°C for 5 days. **(C)** Growth of WT strain, and mutant strains *⊿lccC*/*D and ⊿lccB*/*F* on mCDB medium supplemented with/without tomato puree (400 g/l) at 28 °C for 3 or 5 days. **(D)** TLC analysis of extracts from WT strain, and mutant strains *⊿lccC*/*D and ⊿lccB*/*F* incubated on mCDB medium at 28°C for 5 days. An altertoxin II (ATX II) standard was utilized for comparison.

2.9 Regulation of the DHN-melanin and PQs biosynthesis pathway by the transcription factor CmrA

In this study, except for these four known genes, namely *pksA*, *brm1*, *brm2* and *cmrA*, we discovered that another seven genes, namely *aygA*, *aygB*, *brm3*, *lccB*, *lccC*, *lccD* and *lccF*, scattered in the genome, also participate in the production of DHN melanin in *A. alternata*. In addition, PksA, Brm1, Brm2 and Brm3 are involved in the formation of PQs. To explore the roles of the transcription factor CmrA in DHN melanin and PQs production, we generated a *cmrA*-deletion strain using the CRISPR/Cas9 knock-out technology. The ⊿*cmrA* strain looked brownish **(Fig. 30A)**, different from the pinkish ⊿*pksA* strain **(Fig.11B)**, suggesting that DHN melanin biosynthesis is controlled by other cues or transcription factors. However, the ⊿*cmrA* strain could not produce PQs **(Fig. 30B)**. To detect whether genes for DHN melanin and PQs biosynthesis are all controlled by CmrA, we performed quantitive real-time RT PCR (qPCR) to measure their expression levels. Except for *lccC*, transcript levels of the other nine genes were significantly downregulated in the ⊿*cmrA* strain compared to WT after 5 days of incubation on mCDB medium. Specifically, CmrA strictly controls the expression of *aygA*, *brm1*, *brm3*, *lccB* and *lccF*, but only partially the expression of *pksA*, *aygB*, *brm2* and *lccD* (**Fig. 30C**). This result suggested a rather complicated regulation of DHN melanin production and other cues or transcriptional factors may also play a role. Next, we performed promoter-reporter assays to check the spatial expression of *pksA* in the ⊿*cmrA* strain. The cassette of *pksA(p)::stuA::gfp::trpC(t)* was constructed and transformed into WT and the ⊿*cmrA* strain. The microscopic assay showed that green fluorescent signals were only detected in nuclei of substrate hyphae in WT, but in nuclei of spores and aerial hyphae in both WT and the ⊿*cmrA* strain **(Fig. 30D)**. This result indicates that CmrA strictly controls the biosynthesis of PQs through influencing the expression of *pksA* and/or the dimerization enzyme encoding gene in substrate hyphae.

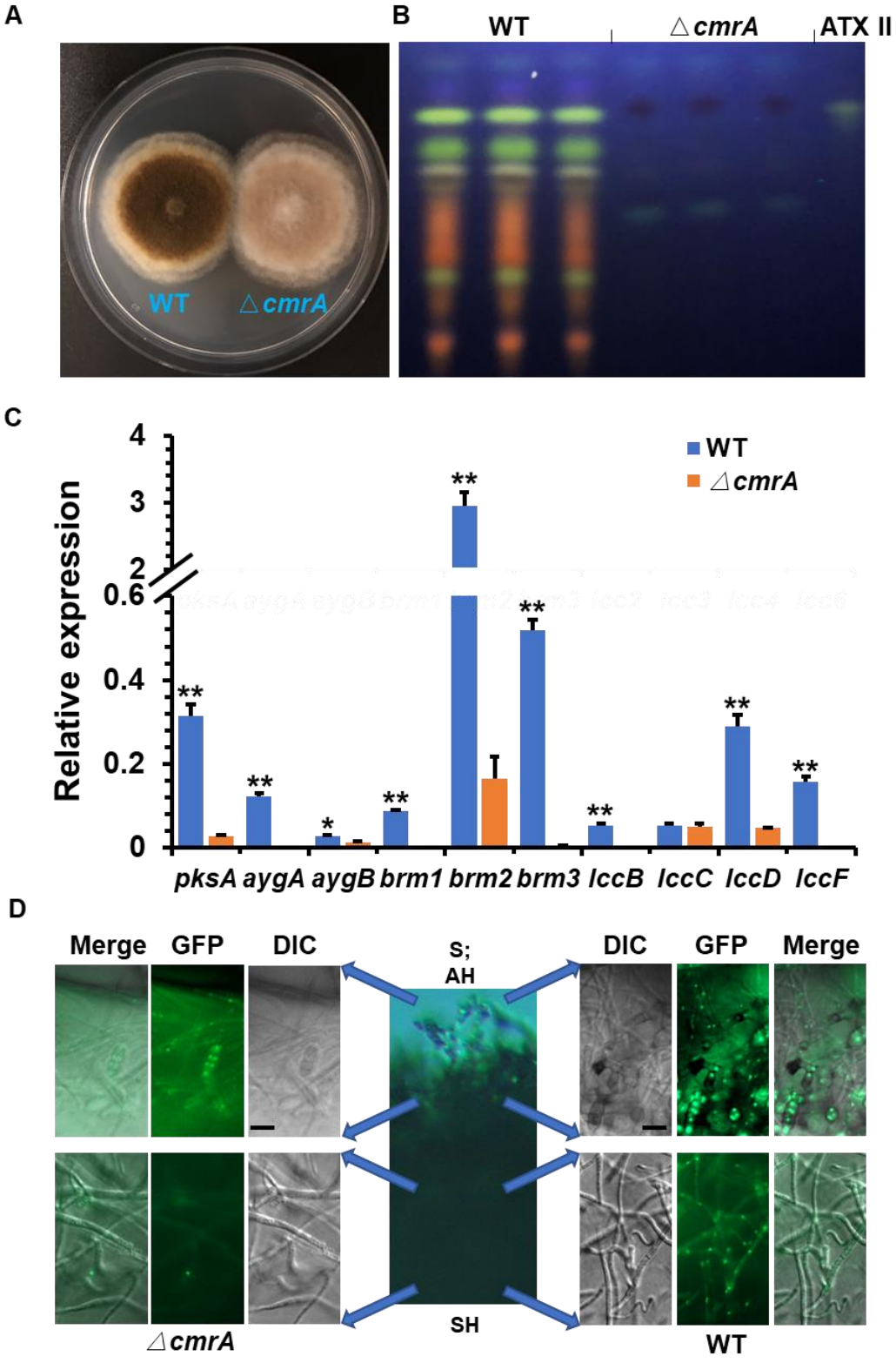


Fig. 30: The biosynthesis of PQs in substrate hyphae is strictly controlled by the transcription factor CmrA. (A) Growth of the wild type (WT) and the *cmrA*-deletion strain on mCDB medium at 28 °C for 5 days. **(B)** TLC analysis of extracts from WT and the *cmrA*-deleted strain grown on mCDB medium at 28°C for 5 days. An altertoxin II (ATX II) standard was utilized

for comparison. **(C)** Expression analysis of genes required for DHN-melanin and/or PQs production by quantitative real-time PCR in WT and the *cmrA*-deleted strain incubated on mCDB medium at 28°C for 5 days. *h2b* was selected as the endogenous control for gene expression analysis. This experiment was performed in three biological replicates. The error bar represents the standard deviation. Statistical analysis was done with Student's test, *, $p \leq 0.05$; **, $p \leq 0.01$. **(D)** Observation of the expression of *pksA* in spores, aerial hyphae and substrate hyphae through a promoter-reporter assay. The cassette of *pksA(p)::GFP::stuA::trpC(t)* was transformed into WT and the *cmrA*-deletion strain, respectively, with the hygromycin-resistance gene as a selection marker. 5 X 10^4 spores of the respective mutant strain were spread evenly on the mCDB agar plate and cultivated at 28 °C for 3 days. The fluorescent signals were detected using fluorescence microscopy. Scale bar, 20 μm. S, spores; AH, aerial hyphae; SH, substrate hyphae.

2.10 *A. alternata* PksA is located in peroxisomes

The enzymes required for secondary metabolites biosynthesis may reside in different subcellular compartments. In order to determine the subcellular localization of *A. alternata* PksA, an ORF fragment of GFP was inserted before the stop codon of *pksA* using the CRISPR/Cas9 knock-in strategy **(Fig. 31A)**. Afterwards, a plasmid encoding mCherry targeted to peroxisomes (SKL) was transformed into the transgenic strain with the GFP-tagged PksA. Green and red fluorescent signals were clearly detected and overlapped, suggesting that PksA is located in peroxisomes **(Fig. 31B)**. This result is consistent with the peroxisomal localization of Pks12/13 in *B. cinerea* but unlike the endosomal localization of Alb1/PksP in *A. fumigatus* (Chen *et al.*, 2021; Upadhyay *et al.*, 2016).

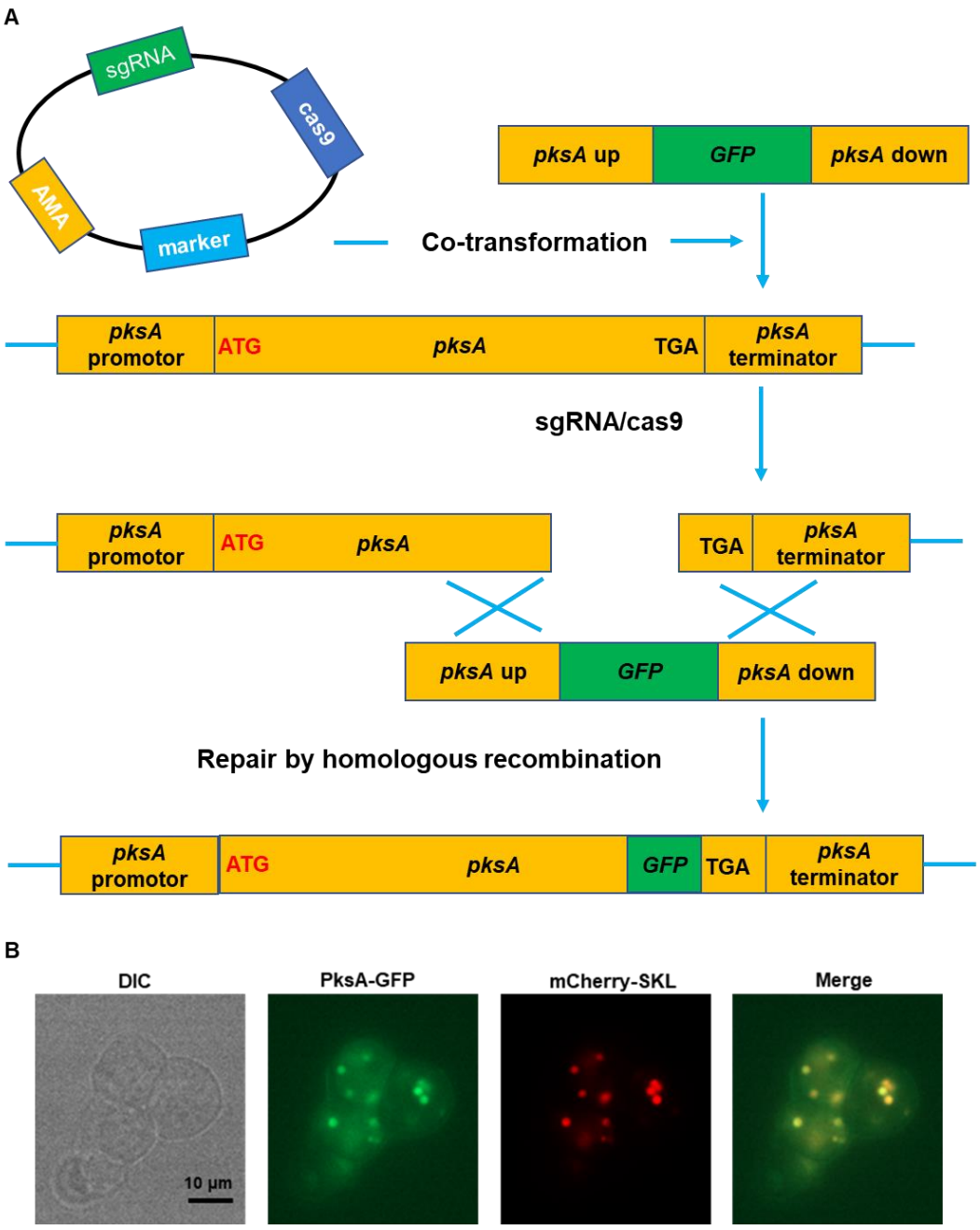


Fig. 31: Subcellular localization of *A. alternata* PksA. (A) The CRISPER-Cas9 plasmid containing one protospacer sequence near the stop codon of *pksA* and the chimeric cassette *pksA*(L)-*GFP*-*pksA*(R) were co-transformed in *A. alternata* wild type (WT) strain. Cas9 enzyme cut the *pksA* gene sequence near the stop codon under the guide of sgRNA. Then the cutting side was repaired, and the *GFP* sequence was inserted inside the *pksA* gene through homologous recombination. **(B)** Subcellular localization of PksA in *A. alternata* spores. GFP was fused to the C-terminal region of PksA protein, and the peroxisomal targeting sequence SKL was fused to the C-terminal region of mCherry protein. Scale bar, 10 µm.

2.11 Regulation of the biosynthesis of PQs

2.11.1 Mitogen-activated protein kinases MpkA and MpkB negatively regulate the biosynthesis of PQs

Mitogen-activated protein kinases (MAPK) are essential for the transduction of environmental signals and participate in diverse cellular processes in fungi. The MAPK protein MpkA/SLT2 plays a crucial role in maintaining cell wall integrity, melanin accumulation, conidial formation, hyphal elongation and production of a host-specific toxin in *A. alternata* (Yago *et al.*, 2011). The MAPK protein MpkB/FUS3 is required for conidiation, vegetative growth, melanin biosynthesis, salt tolerance and pathogenicity (Lin *et al.*, 2010). In order to determine any influence of MpkA and MpkB on PQs biosynthesis, *mpkA*- and *mpkB*-deletion strains were constructed via the CRISPR/Ca9 knock-out strategy. Both mutant strains showed growth defects compared to WT on mCDB medium. However, the depletion of MpkA led to lower-melanin accumulation, while the hyphae were more melanized in the *mpkB*-deletion strain **(Fig. 32A)**. These phenomena are consistent with those described before. And compared to the WT strain, *mpkA*- and *mpkB*-deletion strains produced less or no PQs **(Fig. 32B)**, meaning that both MpkA and MpkB positively regulate the formation of PQs.

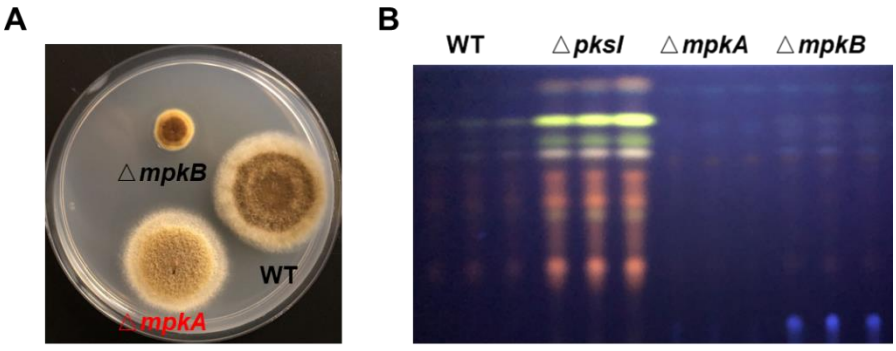


Fig. 32: The absence of MpkA and MpkB caused growth retardation and loss of the biosynthesis of PQs. (A) Development of the WT and mutant strains on mCDB medium at 28 °C for 5 days. **(B)** TLC analysis of extracts from WT and mutant strains grown on liquid mCDB medium in submerged condition at 28 °C for 4 days.

2.11.2. The bZIP transcription factor AtfA positively regulates the biosynthesis of PQs

The high-osmolarity and stress-activated MAP kinase, HogA plays a central role in light and stress signaling in *A. alternata* (Igbalajobi *et al.*, 2020; Igbalajobi *et al.*, 2019; Lin & Chung, 2010). Phosphorylation of HogA activates the bZIP transcription factor AtfA to control conidiation, oxidative stress response, conidia germination and secondary metabolism. The absence of HogA led to the accumulation of some yellow bands detected under UV light, which were proven to be PQs in this study (Igbalajobi *et al.*, 2019). In order to reveal the influence of AtfA on the biosynthesis of PQs, the *atfA*-deletion strain was generated through the CRISPR/Cas9 knock-out strategy. Compared to the WT strain, the *atfA*-deletion strain showed a reduction of growth to some extent **(Fig. 33A)**. The absence of AtfA caused a significant decrease in spore formation **(Fig. 33B)**. However, the *atfA*-mutant strain produced more PQs, comparable to the *hogA*-mutant strain **(Fig. 33C)**. This result suggests that that HogA negatively regulates the biosynthesis of PQ by activating the bZIP transcription factor AtfA.

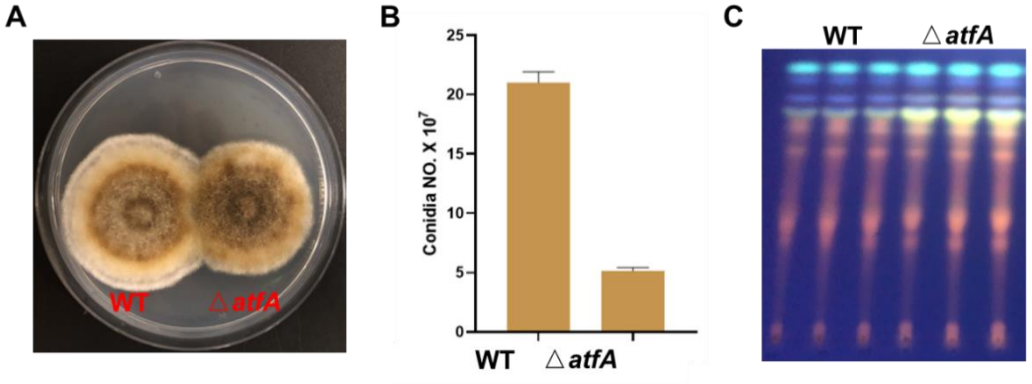


Fig. 33: Absence of the bZIP transcription factor AtfA caused growth reduction, less spore formation and more PQs production. (**A**) The WT and atfA-deletion strains grew on mCDB medium at 28 °C for 5 days. (**B**) Quantification of spores produced by the WT and *atfA*-deletion strains grown on mCDB medium for 7 days at 28 °C. (**C**) TLC analysis of extracts from WT and *atfA*-deleted strain grown on mCDB medium for 7 days at 28 °C.

2.11.3 Velvet complex proteins VeA and LaeA negatively regulate the biosynthesis of PQs

In order to determine the influence of the velvet complex (VeA/VelB/LaeA) on the biosynthesis of PQs, *veA*- and *laeA*-deletion strains were constructed via the CRISPR/Cas9 knock-out strategy, respectively. Both mutant strains showed a reduction in growth, sporulation, and melanization compared to the WT strain **(Fig. 34A)**. However, the production of PQs was significantly improved in the *veA*- and *laeA*-mutants compared to the WT strain **(Fig. 34B)**. This result suggested the biosynthesis of PQs is negatively regulated by the velvet complex proteins VeA and LaeA.

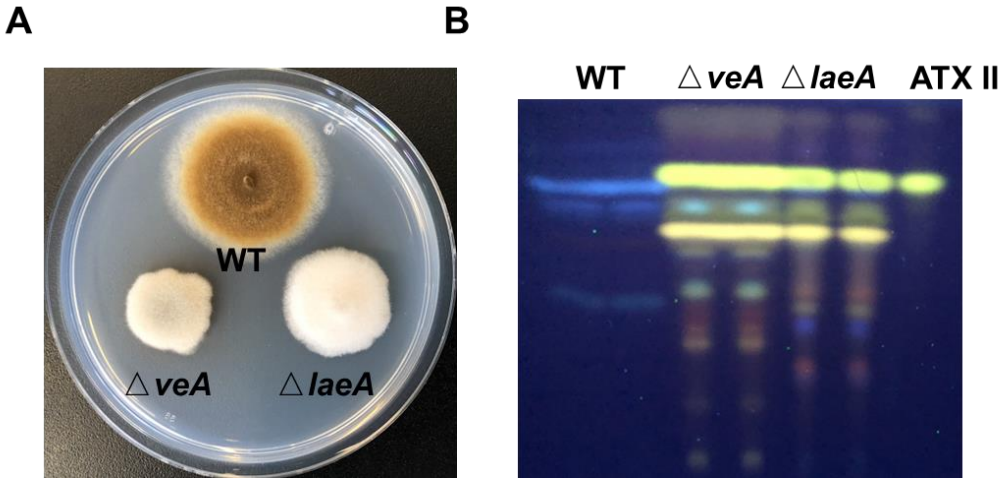


Fig. 34: The absence of the velvet complex proteins VeA and LaeA causes growth and sporulation deficiency but produces more PQs. (A) Growth of WT and mutant strains on mCDB medium at 28 °C for 5 days. **(B)** TLC analysis of extracts from WT and mutant strains grown on mCDB medium for 5 days at 28 °C.

2.12 Search for the dimerization enzyme required for PQs biosynthesis

I found that 1,8-DHN is the last common intermediate utilized for PQs and DHN-melanin biosynthesis in *A. alternata* **(Fig. 24 and 25)**. One remaining question is the dimerizing enzyme. Many mutant strains (P450 proteins and laccases encoding genes-deletion strains) were constructed to test a role in the formation of PQs, but

unfortunately, all of them still produced PQs. The enzyme dimerizing 1,8-DHN to produce PQs is therefore still missing. If reverse-genetic approaches do not help to discover the dimerizing enzyme, other methods such as enzyme purification or forward-genetic approaches must be established. Another possibility could be extensive genome-wide expression analyses. The deletion of *hogA*, *atfA*, *veA* and *pksI* boosted the biosynthesis of PQs to different extents in mCDB liquid medium in submerged conditions compared to WT. In contrast, the *pksI*/*cmrA* double mutant strain failed to produce PQs under the same condition. Both, the PQs super-producers, *hogA*-, *atfA*-, *veA*- and *pksI*-deletion strains could not synthesize PQs anymore when grown on mCDB liquid medium containing tomato puree (400 g/l) **(Fig. 35A)**. Mycelia of these strains grown on mCDB liquid medium or mCDB liquid medium containing tomato puree (400 g/l) were collected to extract RNA, followed by RNA-seq. The aim of the study was the detection of commonly up-regulated genes in the *hogA*-, *atfA*-, *veA*- and *pksI*-deletion strains compared to WT strain grown on mCDB medium. Likewise, we want to find common down-regulated genes of *hogA*-, *atfA*-, *veA*- and *pksI*-deletion strains compared to WT strain grown on mCDB medium containing tomato puree (400 g/l) and *pksI*/*cmrA*-deletion strain compared to *pksI*-deletion strain grown on mCDB medium. The dimerization enzyme encoding gene required for PQs should be present both, in the group of up-regulated genes and in the group of down-regulated genes **(Fig. 35B)**. The analysis of the RNA-seq data is still ongoing and will not be displayed in detail here.

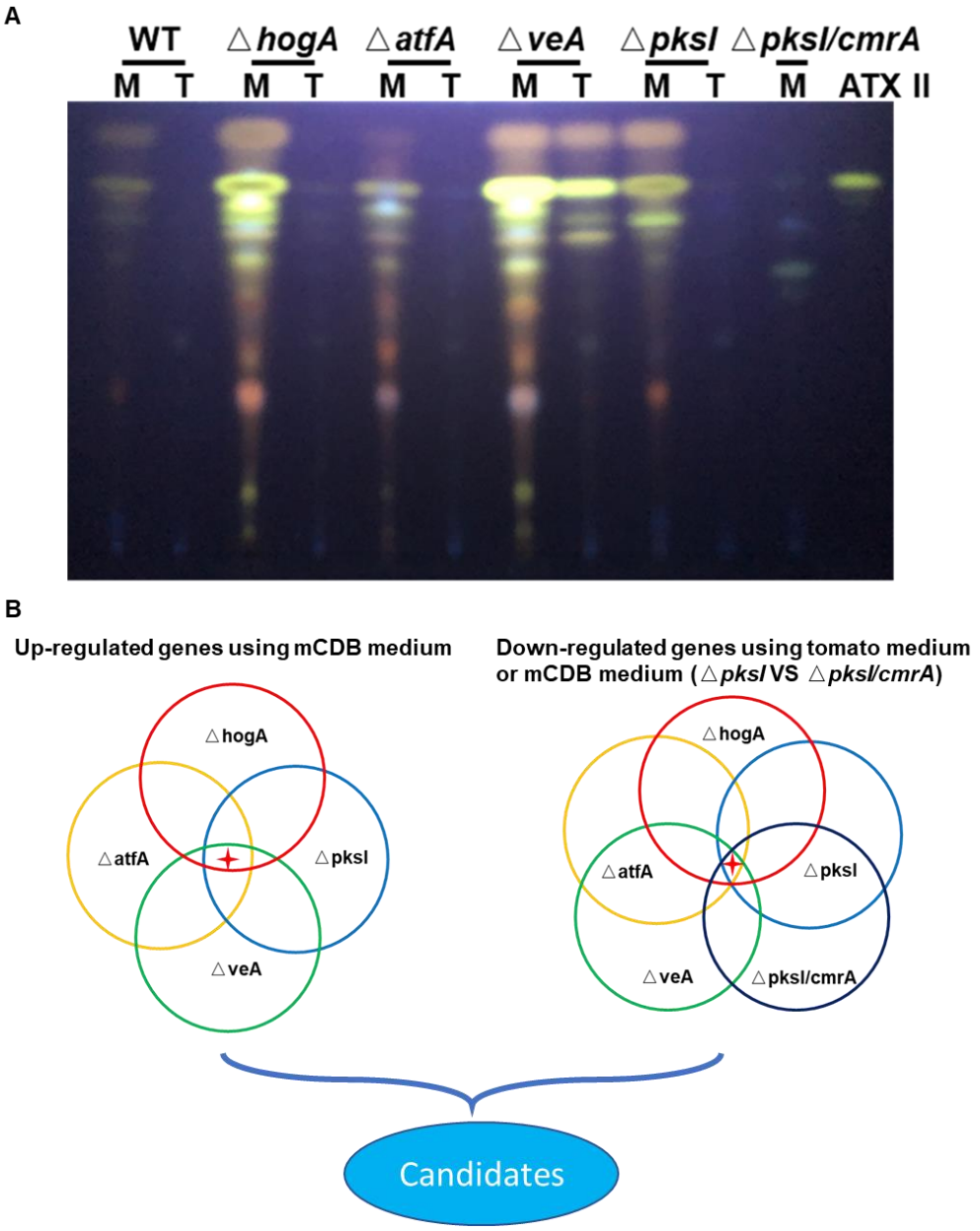


Fig. 35: RNA-seq was performed to identify candidates for the dimerization enzyme encoding gene required for PQs biosynthesis. (A) TLC analysis of extracts from WT and *hogA*-, *atfA*-, *veA*-, *pksI*- and *pksI*/*cmrA*-deleted strains grown on mCDB liquid medium in submerged condition for 3 days at 28 °C; TLC analysis of extracts from WT and *hogA*-, *atfA*-, *veA*-, *pksI*-deleted strains grown on mCDB liquid medium containing tomato puree (400 g/l) in submerged condition for 3 days at 28 °C. Altertoxin II (ATX II) standard was used for comparison. M, liquid mCDB medium; T, liquid mCDB medium containing tomato puree (400 g/l). **(B)** Scheme of RNA-seq strategy to find out the potential genes required for the biosynthesis of PQs.

3. Discussion

3.1 The promiscuity of compounds synthesized by PksA contributes to the spatial production of DHN-melanin and PQs in *A. alternata*

Large numbers of SMs secreted by fungi have been characterized as phytotoxins or mycotoxins, which cause tremendous losses on farms and represent a severe threat to human health (Adeyeye, 2016; Berestetskiy, 2008; da Rocha *et al.*, 2014; Ismaiel & Papenbrock, 2015). However, many SMs also possess antibiotic activity or inhibit enzymatic functions that are applied to treat human diseases, such as penicillin and lovastatin (Folkers *et al.*, 1990; Herrell, 1945; Jones *et al.*, 1994). Genes required for SM biosynthesis are typically arranged nearby in the genome and form gene clusters. The expression of SM gene clusters may be constantly modulated by special environmental conditions and many corresponding products remain to be discovered. To dig out new potential antibiotics or pharmaceuticals, diverse strategies, such as imitating natural conditions and heterologously expressing presumed gene clusters, have been used to induce the expression of endogenous gene clusters which are silent under laboratory conditions (Caesar *et al.*, 2020; Clevenger *et al.*, 2017; Netzker *et al.*, 2015; Stroe *et al.*, 2020).

Fungal spores and/or hyphae are always melanized to defend against UV irradiation or oxidative stress in the environment (Eisenman & Casadevall, 2012). DHN-melanin is a typical dark pigment in many fungi such as *A. fumigatus*, *B. cinerea* or *A. alternata*. In *A. fumigatus*, all the genes required for DHN-melanin formation are clustered in the genome. In *B. cinerea*, two polyketide synthase-encoding genes, *pks12* and *pks13*, generate two different gene clusters together with other related genes involved in melanin biosynthesis, and catalyze the production of DHN-melanin in sclerotia and conidia, respectively. The α-hydrolase-encoding gene *Bcygh1*, whose derived protein hydrolyze AT4HN to T4HN, belongs to neither of the two gene clusters. In *A. alternata*, not all the genes required for DHN-melanin production had been

characterized. The DHN-melanin biosynthesis gene cluster contains only three genes: the polyketide synthase-encoding gene *pksA*, T3HN reductase-encoding gene *brm2* and transcription factor-encoding gene *cmrA*, and the scytalone dehydratase-encoding *brm1* was located elsewhere in the genome **(Fig.3)**. In this study, we discovered many other genes required for DHN-melanin biosynthesis in *A. alternata*, namely two α-hydrolase-encoding genes *aygA* and *aygB*, a T4HN reductase-encoding gene *brm3*, as well as four laccase-encoding genes *LccB-D* and *LccF.* They are scattered in the genome **(Fig. 36A)**. We also revealed that the biosynthesis of PQs (ATXs and other related compounds) shared most of the pathway with the production of DHN melanin. DHN melanin tends to be produced in spores and aerial hyphae, while the biosynthesis of PQs is completed in the substrate hyphae **(Fig. 36B)**. Although the polyketide synthases required for DHN-melanin biosynthesis are highly conserved in fungi, three different compounds, namely the hexataketide YWA1, the heptaketide AT4HN and the pentaketide T4HN, were identified as their respective products (Schumacher, 2016). YWA1 and AT4HN need to be hydrolyzed to T4HN first, and afterwards, the DHN-melanin biosynthesis can continue. As *A. oryzae* has a very limited capacity for producing SMs, it is commonly used as a host to detect putative chemicals produced by fungal PKSs (Wenderoth *et al.*, 2019; Yu *et al.*, 2021). The expression of *A. alternata pksA* was induced in *A. oryzae* using maltose as a carbon source, and YWA1, AT4HN and T4HN were suggested as the products of the derived protein PksA with AT4HN as the main one. The spatial regulation of the biosynthesis of DHN-melanin and PQs is probably achieved by the promiscuity of compounds synthesized by PksA in *A. alternata*. In our study, we proved that AygA and AygB were especially required for the biosynthesis of DHN-melanin. Thus, a small amount of T4HN may be produced directly by PksA, which drives the pathway to the biosynthesis of PQs in substrate hyphae. However, in spores and aerial hyphae, except for T4HN, AT4HN and YWA1 are also synthesized and further hydrolyzed to enhance the concentration of T4HN and drive the pathway to the production of DHN-melanin. As the products of PksA were determined using *A. oryzae* as a heterologous host, it's still unclear the native products in spores, aerial hyphae and substrate hyphae in *A. alternata*. If T4HN is the sole

product in substrate hyphae, that could probably be achieved by structure modification of PksA through specific interacting proteins. If AT4HN and YWA1 are also produced in substrate hyphae, their application should be further detected. When the *Colletotrichum lagenarium pks1* was expressed in *A. oryzae*, except for the main product T4HN, small amounts of the tetraketide orsellinic acid and the pentaketide isocoumarin were also measured. That suggests that the production of the hexataketide YWA1 and the heptaketide AT4HN is specific for *A. alternata* PksA in our expression experiment and not due to the further modification by *A. oryzae* enzymes (Fujii *et al.*, 1999).

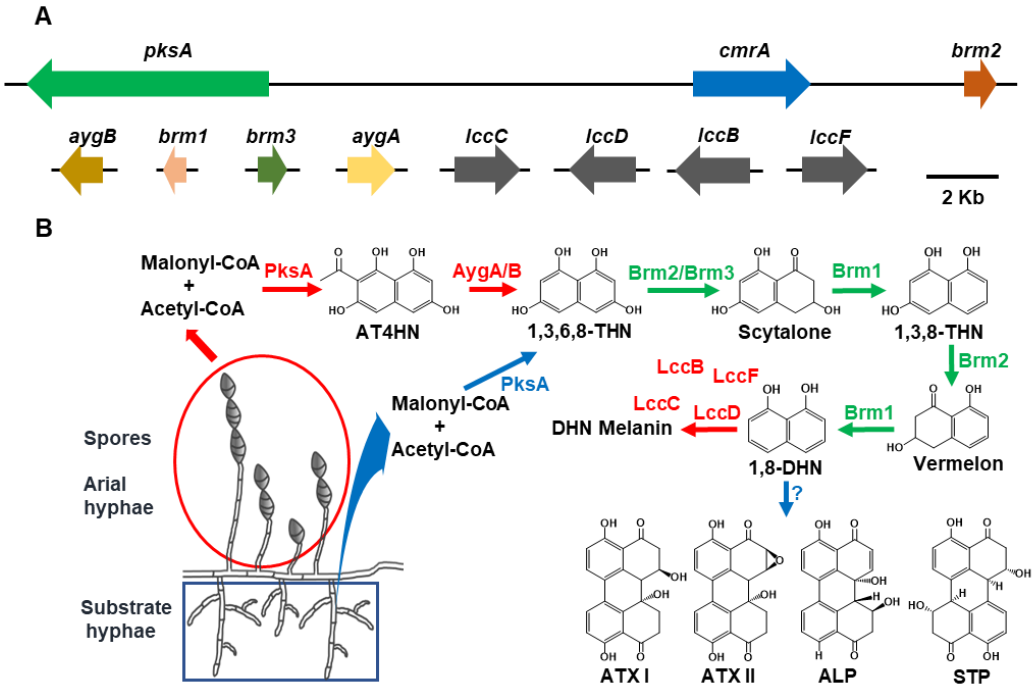


Fig. 36: Scheme of the DHN-melanin and PQ biosynthetic pathways in *A. alternata*. (A) Synteny and rearrangements of the DHN-melanin and PQs biosynthetic gene cluster in the genome in *A. alternata*. Three genes, namely polyketide synthase-encoding gene *pksA*, 1,3,8-trihydroxynaphthalene (T3HN) reductase-encoding gene *brm2* and transcription factor-encoding gene *cmrA* are clustered in the genome; other genes required for DHN-melanin and/or PQs production, such as α-hydrolase-encoding genes *aygA* and *aygB*, scytalone dehydratase encoding gene *brm1*, 1,3,6,8-tetrahydroxynaphthalen (T4HN) reductase-encoding gene *brm3*, and laccase-encoding genes *lccB-D* and *lccF*, are scattered and located elsewhere in the genome. **(B)** Spatial regulation of DHN-melanin and PQs production in *A. alternata*. In spores and aerial hyphae, AT4HN is mainly synthesized by PksA as the initial step, and then the pathway goes to the production of DHN melanin. However, in substrate hyphae, PksA catalyzes the formation of T4HN directly, and following the pathway is switched to the

biosynthesis of PQs. α-hydrolase AygA and AygB are required for DHN-melanin biosynthesis but not PQs production. The enzyme dimerizing 1,8-DHN to form PQs remains to be further discovered. Enzymes required for DHN-melanin biosynthesis are exhibited in red; enzymes involved in PQs production are exhibited in blue, and enzymes shared by the formation of DHN-melanin and PQs are exhibited in green. ATX I-II, altertoxin I-II; ALP, alterperylenol; STP, stemphyperlenol.

3.2 Functional redundancy of enzymes required for DHN-melanin biosynthesis

Functionally redundant proteins are involved in various cellular processes in the three kingdoms of life, such as transporter proteins mediating detoxification of self-made or exogenous toxins, G-protein-coupled receptors mediating stress responses and transcription factors responding to pathogens (Affeldt *et al.*, 2014; Xu *et al.*, 2006; Zwiers *et al.*, 2003). In this study, we proved that some enzymes show a reductant function in the DHN-melanin biosynthetic pathway in *A. alternata*, such as two α-hydrolases AygA and AygB hydrolyzing AT4HN and/or YWA1 to T4HN, two T4HN reductases Brm2 and Brm3 reducing T4HN to scytalone and four laccases LccB-D and LccF polymerizing 1,8-DHN to DHN-melanin. Isoenzymes may be produced to react with different compounds. For instance, as discussed before, AT4HN was the main product in the heterologous expression experiment of *A. alternata* PksA and YWA1 was only a minor compound. The absence of AygA caused a reduction in melanin production but the absence of AygB had no effect. These results suggest that AT4HN and YWA1 are probably hydrolyzed to T4HN by AygA and AygB in *A. alternata*, respectively. This speculation is supported by the fact that AygA shares more identical amino acids with *W. dermatitides* WdYg1 whose function has been proven to hydrolyze AT4HN to T4HN, and AygB shares more identical amino acids with *A. fumigatus* Ayg1 whose function is to hydrolyze YWA1 to T4HN.

In addition, the functional redundancy of enzymes guarantees the subsequent formation of DHN-melanin in changing environments. For instance, the absence of Brm3 had no impact on DHN-melanin biosynthesis. But the lack of Brm2 and Brm3 caused a melanin-deficient phenotype different from that of the *brm2*-deletion strain,

suggesting that both Brm2 and Brm3 perform the reduction of T4HN to scytalone in *A. alternata*. When Brm2 activity is blocked for some reasons, such as being inhibited by chemicals or down-regulated, Brm3 remedies the role of Brm2 on the reduction of T4HN to scytalone, but not the reduction of T3HN to vermelone. The functional redundancy of T4HN reductases is also observed in other melanized fungi, such as BcBrn1/2 in *B. cinerea* and THR1/T4HR1 in *Bipolaris oryzae* (Schumacher, 2016; Tanaka *et al.*, 2015).

Another example is that laccases exert a redundant function in DHN-melanin biosynthesis. The absence of LccB or LccF in *A alternata* showed a lower ability to produce DHN-melanin on mCDB medium supplemented with tomato puree but not on mCDB medium. And the deficiency of DHN-melanin biosynthesis was observed on both media when LccB/F or LccC/D were missing. Perhaps the requirement of special laccases for DHN-melanin production is stimulated by a particular environment. Or in order to respond to the harsh environments, laccases work together to produce DHN-melanin fast. As for the promiscuity of *A. alternata* PksA, here is another possibility that under certain conditions, YWA1 or T4HN are the main products of PksA to synthesize DHN-melanin with minor amounts of AT4HN.

3.3 Candidates for a dimerization enzyme required for PQs biosynthesis

Perylene quinones (PQs) are a group of well-known secondary metabolites produced by many plant pathogens, such as cercosporin produced by *Cercospora* spp. and elsinochromes by *E. fawcettii*. These compounds share a common backbone core and are structurally related **(Fig. 4)**. The fungus *S. botrysum* also produces a few PQs named stemphyltoxin I-IV, whose chemical structures are more similar to that of ATX I-II and ALP, as the modifications of the side chain in cercosporin and elsinochromes are not detected. This suggests that *S. botrysum* probably performs a parallel pathway for synthesizing stemphyltoxins with that for ATXI-II and ALP production in *A. alternata*. Except for the known PQs synthesized in *A. alternata*, namely ATX I-III, ALP, STP,

xanalteric acid I and II, as well as alterlosin I and II, recently, many more PQs with related chemical structures were isolated from cultures of *Alternaria* species (Bashyal *et al.*, 2014; Pang *et al.*, 2018; Zhao *et al.*, 2019). They are named altertoxin IV-XII, 7-epi-8-drooxyaltertoxin, stemphytriol, 6-epi-stemphytriol, cladosporol I and alternatone A, respectively **(Fig. 37)**. Up to date, their biosynthetic pathway has not yet been characterized. However, it is speculated that the intermediate of the DHN-melanin biosynthetic pathway T3HN was utilized to produce alternatone A (Zhao *et al.*, 2019). In this study, we revealed that the biosynthesis of PQs (ATX I, ATX II, ALP and STP) shares most of the pathway with the production of DHN-melanin with 1,8-DHN as the last common intermedia. Since so many PQs with similar chemical structures of ATX I, ATX II, ALP and STP have been identified in *Alternaria* spp. and other fungi, the DHN-melanin biosynthetic pathway likely participates in producing more SMs. In other words, many melanized fungi could be able to form PQs.

To discover which fungi can produce PQs using 1,8-DHN or other intermediates originating from DHN-melanin biosynthetic pathway, it is necessary to identify the dimerizing enzyme that catalyzes the coupling of two molecules of 1,8-DHN to PQs. Such phenol coupling reactions are typically performed by three different classes of enzymes, namely cytochrome P450 enzymes, flavin-dependent monooxygenases and laccases (Fürtges *et al.*, 2019). For instance, the cytochrome P450 monooxygenase ClaM is involved in anthraquinone dimerization to synthesize cladofulvin in *Cladosporium fulvum* (Griffiths *et al.*, 2016). And the laccase Av-VirL is required for the biosynthesis of viridotoxin by mediating the phenol-coupling process (Fürtges *et al.*, 2019). In our study, several laccases encoding genes were deleted in the genome, and some double-deletion strains were generated. Unexpectedly, all the laccase-mutant strains still produced PQs like the parental strain. Therefore, it appears unlikely that laccases are required for the biosynthesis of PQs in *A. alternata*, although it cannot be excluded that other laccase could perform the dimerization reaction or several laccases have a redundant function in the biosynthesis of PQs. The combination of a laccase with a fasciclin domain-containing protein was proven to be crucial for the production of the biaryls sporandol from *Chrysosporium merdarium* (Thiele *et al.*, 2020).

A fasciclin-encoding gene is frequently found nearby laccase-encoding genes in the cercosporin biosynthetic gene cluster, and the combination of derived proteins was speculated to be required for cercosporin production (Ebert *et al.*, 2019). In *A. alternata*, a conserved fasciclin protein was identified and displayed 35.2% identical amnio acids compared to the *C. beticola* protein. However, the absence of this protein did not influence the biosynthesis of PQs. At the moment, it seems that the dimerization enzyme cannot be identified by reverse-genetic approaches. However, we generated three MAP kinases-deletion strains, two velvet complex proteins-deletion strains and one bZIP transcription factor-deletion strain in *A. alternata*, which show different abilities to synthesize PQs. We found one kind of medium that inhibited the formation of PQs. RNA-seq is being performed based on these conditions to search for the dimerization enzyme, anticipating that it is regulated at the transcriptional level in the same way as the other PQ biosynthetic enzymes.

Although purified PQs have been considered as phytotoxins to cause a delay in the root growth of plants *in vitro*, currently, we cannot solve the question if PQs are used as phytotoxins when *A. alternata* infects plants *in vivo*, as mutants in the PQ biosynthesis also have defects in DHN melanin production. Hence, if we would detect differences in virulence we cannot decide if that is due to the lack of PQs or to the lack of melanin. However, we should be able to produce unambiguous results once the dimerizing enzyme has been identified. That should be specific only for PQ biosynthesis and corresponding mutant strains can be used for virulence studies.

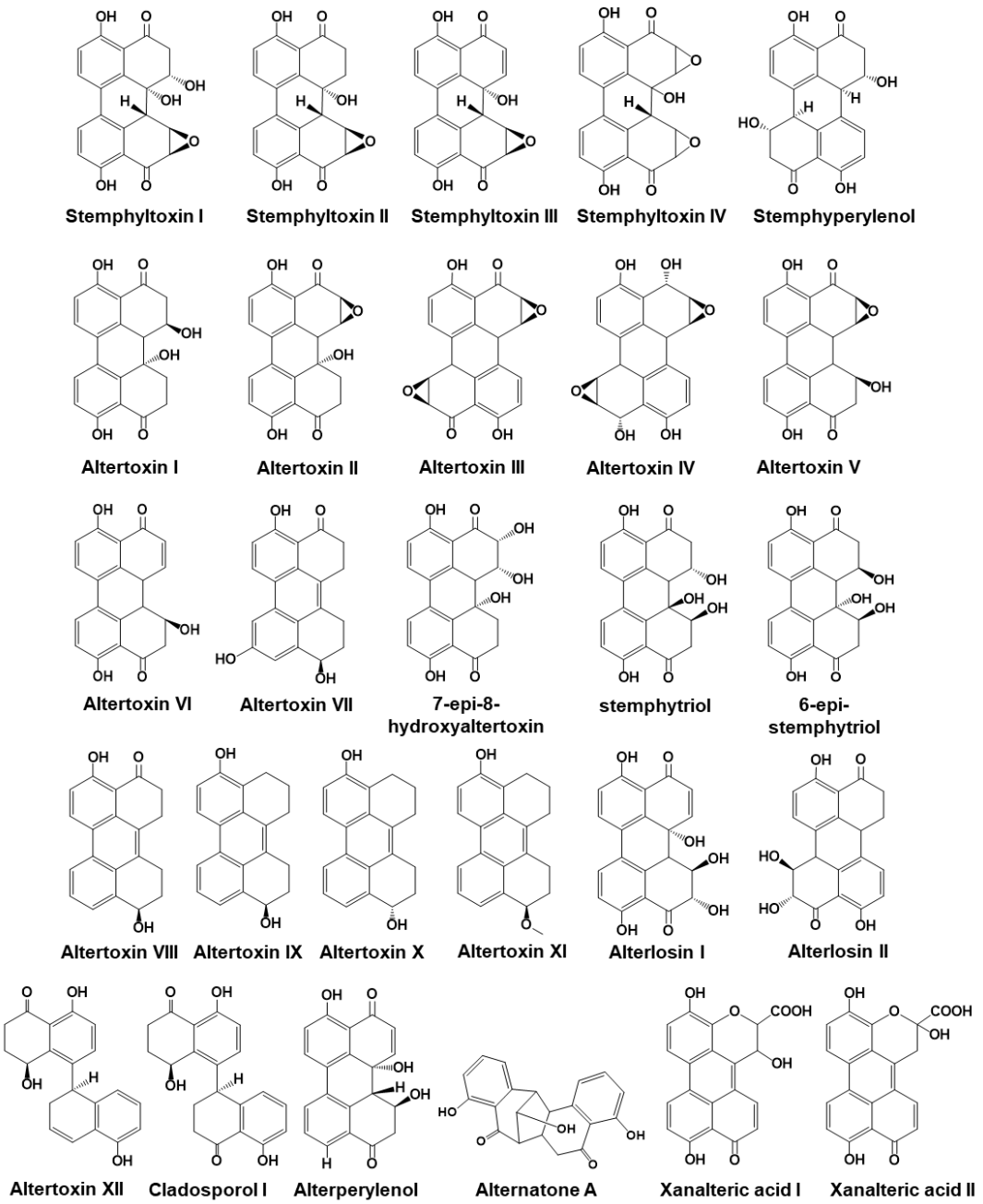


Fig. 37: Chemical structures of PQs probably originating from the DHN-melanin biosynthesis pathway in fungi.

3.4 MAP kinases and velvet complex proteins regulate the production of DHN-melanin and PQs in different ways

This study revealed that DHN melanin and PQs are spatially produced in different parts of the mycelia in *A. alternata*, although most enzymes are shared in the two pathways.

The transcription factor CmrA in the *pksA* gene cluster strictly regulated the biosynthesis of PQs through controlling the expression of the *pksA* gene in substrate hyphae, and perhaps also the expression of the dimerization enzyme-encoding gene. However, the production of DHN melanin is controlled by CmrA only to a certain extent, as many genes involved in DHN melanin are still expressed in the *cmrA*-deletion strain, with the expression of *pksA* gene in spores and aerial hyphae as an example. These results suggested the production of DHN melanin and PQs should be controlled in a complicated but accurate manner.

First, we studied the impact of mitogen-activated protein kinases (MAPK) MpkA and MpkB on the biosynthesis of DHN melanin and PQs. In *A. fumigatus*, a Slt2 ortholog, MpkA phosphorylates MADS-box transcription factor RlmA through physical interaction to control the expression of genes involved in DHN-melanin production (Fabri *et al.*, 2020; Valiante *et al.*, 2016). Likewise, several genes required for the production of gliotoxin were downregulated in the *mpkA*-deletion strain, causing a decrease in gliotoxin levels (Jain *et al.*, 2011). However, in liquid culture, the absence of MpkA led to the accumulation of pyomelanin (Valiante *et al.*, 2009). We generated a *mpkA*-deletion strain in *A. alternata*. The mutant strain showed deficiency in DHN melanin production, consistent with the phenotype of the same defective strain described before (Yago *et al.*, 2011). On the other hand, lower amounts of PQs and AOH were detected in the *mpkA*-deletion strain compared to WT. In *A. fumigatus*, the depletion of the Fus3 ortholog, MpkB, significantly increased the production of DHN-melanin (Perez-Cuesta *et al.*, 2020). Recently, it has been reported that the absence of *Pox*MK1, the Fus3 ortholog in *Penicillium oxalicum*, induced the expression of genes in the DHN-melanin biosynthetic gene cluster and thus enhanced the production of DHN-melanin (Ma *et al.*, 2021). In addition, highly melanized hyphae were observed in *A. alternata Fus3*-deletion strains (Lin *et al.*, 2010), consistent with the phenomenon of the mutant strain (*mpkB*-deletion strain) in this study. These results suggest a conserved function of the FUS3 orthologs on the biosynthesis of DHN-melanin in fungi. However, the role of MpkB in the production of SMs is different in different fungi. For instance, deletion of *mpkB* in *A. fumigatus* reduced the production of gliotoxin (Frawley

et al., 2020b). The depletion of MpkB results in block of aflatoxin formation but an improvement of several SMs biosynthesis, such as leporin B and aspergillicins (Frawley *et al.*, 2020a). Although MpkB was considered to play a non-prominent role in producing host-specific toxin in *A. alternata* (Lin *et al.*, 2010), the biosynthesis of PQs and AOH was highly reduced in *mpkB* mutant strain. The absence of MpkB induced the biosynthesis of DHN melanin, but reduced the production of PQs. In addition, our previous study showed that the lack of MAP kinase HogA boosted the production of DHN melanin and PQs (Igbalajobi *et al.*, 2019).

The velvet complex is composed of the methyltransferase LaeA and the velvet proteins VeA and VelB. The general functions of the complex are conserved in filamentous fungi to coordinate development and secondary metabolism in response to light (Bayram *et al.*, 2008; Schumacher *et al.*, 2015). LaeA is typical to positively control the regulation of secondary metabolism, as the absence of LaeA usually results in a decrease or elimination of several SMs in various fungi, such as penicillin in *A. nidulans* and *P. chrysogenum*, gliotoxin in *A. fumigatus* and aflatoxin in *A. flavus* (Bok & Keller, 2016). However, the function of LaeA varies on the regulation of mycelial pigmentation. For instance, the depletion of LaeA resulted in the failure of mycelial pigmentation in *A. fumigatus* and *A. nidulans (*Bok & Keller, 2004). In contrast, mycelial melanization was enhanced when *Chlae1* was deleted in the plant pathogen *Cochliobolus heterostrophus,* and repressed through overexpression of *Chlae1* (Wu *et al.*, 2012). In *A. alternata*, the *laeA*-deletion strains generated in two subspecies were less-pigmented, consistent with our mutant strain *⊿laeA* (Estiarte *et al.*, 2016; Takao *et al.*, 2016). Depletion of LaeA resulted in the reduction of AOH and AME production and host-specific toxin AAL-toxin. In this study, we found that the biosynthesis of PQs was strongly induced when LaeA was missing. The expression of the velvet protein VeA predominately occurs in the dark, and VeA bridges VelB and LaeA to form the velvet complex, with VelB and LaeA interacting with the N- and C-terminus of VeA respectively. Then, the complex assembles in the nucleus to regulate development and secondary metabolism. The transport of LaeA into the nucleus does not require the complex, whereas nuclear localization of VelB depends on VeA (Sarikaya Bayram *et*

al., 2010). The mutation of nucleotide T to A in the initiation codon ATG of the *veA*-open reading frame (ORF) generates a partial loss-of-function VeA1 protein (Kim *et al.*, 2002). Recently, Wang *et al*. revealed that VeA1, but not VeA, was responsible for the universal SM alteration in both *A. nidulans* and *A. fumigatus* during co-cultivation of the endophytic fungus *Epicoccum dendrobii* (G. Wang *et al.*, 2022). Similar to that of laeA, the role of VeA is also different with respect to melaninzation in different fungi. For instance, the production of DHN melanin is increased in the absence of BcVel1, an ortholog of VeA, in *B. cinerea* (Schumacher *et al.*, 2015). In contrast, the *veA*-deletion strain is less-pigmented compared to WT in *P. expansum* (El Hajj Assaf *et al.*, 2018). In *A. alternata*, depletion of VeA resulted in the reduction of melanin production to some extent (Estiarte *et al.*, 2016; L. Wang *et al.*, 2022), consistent with the phenomenon of the mutant strain *⊿veA* in this study. And the biosynthesis of AOH, AME and TeA was significantly decreased (L. Wang *et al.*, 2022). However, we found that the production of PQs was dramatically increased in our *veA*-deletion strain. Collectively, in *A. alternata*, the velvet complex proteins LaeA and VeA downregulate the production of DHN melanin but upregulate the biosynthesis of PQs.

Taken together, these results suggest that, although most of the DHN-melanin and PQs pathways are shared, the regulation of DHN melanin and PQs biosynthesis should be separate and more complicated than anticipated. Future studies are required to unravel the complex regulation and interconnection of the different pathways and to decipher the natural functions for this.

4. Materials and Methods

4.1 Chemicals and equipment used in this study

The chemicals used in this study were purchased from the company Roth (Karlsruhe), Roche (Mannheim), Sigma Aldrich (Seelze), Sigma (Taufkirchen)， Invitrogen (Karlsruhe), Applichem (Darmstadt) and Serva Feinbiochemica (Heidelberg). DNA polymerases for PCR, restriction endonucleases and makers for DNA were produced by New England Biolabs (Frankfurt) and Fermentas (St-Leon-Rot). Other chemicals were indicated in the text. The equipment used in this study is listed in **Table 3**.

Table 3: Equipment used in this study

Equipment	Type	Manufacturer
Thermocycler	Labcycler & Gradient	SensoQuest GmbH
Heating block	Thermo mixer 5436	Eppendorf, Germany
pH meter	Hanna HI 208	Hanna, Romania
Weighing instrument	Kern 440-47N Sartorius R200D	Sartorius, Göttingen Kern, Germany
Real-time PCR	MyiQ™ Single Color Real-Time PCR Detection System	Bio-Rad, USA
Autoclave	Systec 3850 ELV Systec VE-75	Systec GmbH, weteenberg Systec GmbH, weteenberg
Digital camera	Canon PowerShot G15	Canon, Japan
Dry Oven	Model 30-1060	Memmert GmbH, Germany
Microscopy	Axio Imager. Z1 Nikon Eclipse E200	Carl Zeiss Microimaging GmbH, Germany Nikon Instruments Europe BV, Amsterdam. Netherlands

Spectrophotometer	ND-1000	NanoDrop, USA
	JASCOV-550	JASCO GmbH, Germany
Centrifuge	5415 R	Eppendorf, Hamburg
	5415 D	Eppendorf, Hamburg
	Universal 320R	Hettich, Tuttlingen
Shaker/incubator	Heraeus-Inscubators	Kerdro, Langenselbold
	series 6000	Infors AG, Switzerland
	HT Infors	Edmund Buhler GmbH,
	Kelinschutller KM-2	Tubingen
Gel image	FastGene ® FAS-V	NEPON Genetics
	Imaging System	EUROPE GmbH
UVGL-25 Compact UV Lamp	4 Watt | 254/365 nm	Cambridge, UK

4.2 Microbiology methods

4.2.1 *Escherichia coli* cultivation and transformation

Frozen aliquots of competent cells were taken out from the freezer (-80 °C), thawed on ice for 5-10 min, mixed with plasmid DNA and then incubated on ice for 10 min. The cells were heat-shocked at 42 °C for 1 min and then transferred on ice for 2 min. Finally, the cells were spread evenly on a LB plate supplemented with ampicillin (100 µg/ml) for selection and incubated overnight at 37 °C. Colonies were picked and cultured in test tubes overnight at 37 °C. The positive clone was determined by digesting the isolated plasmids with a restriction enzyme. Medium used for *E. coli* cultivation is listed in **Table 4**.

Table 4: *E. coli* growth medium

Medium	Ingredients (1 liter)

LB	10 g Tryptone; 10 g Yeast extract; 5 g NaCl; pH 7.5
Antibiotic (1000X)	100 mg/ml ampicillin

4.2.2 *A. alternata* transformation

Fresh spores were harvested from a mCDB culture plate and inoculated into 100 ml of mCDB medium for overnight cultivation at 28 °C and 180 rpm. The mycelia were harvested by filtering, washed once with NaCl solution and then digested in a Kitalase (Wako Chemicals) suspension (150 mg in 15 ml of NaCl solution) for 1h with soft shaking at 100 rpm and 30 °C. The quality and quantity of protoplast were checked using microscopy. Protoplasts were separated from cell fragments by filtering through two layers of Miracloth (Millipore) and precipitated at 2,430 rpm for 10 min at room temperature. The Kitalase solution was discarded and the protoplasts were washed once with ice-cold 0.7 M NaCl by repeating the centrifugation step at 4 °C. And then, the protoplasts were resuspended in 200 µl of STC solution. 5 µg of plasmid DNA were added to the protoplasts, followed by incubation on ice for 10 min. DNA uptake was induced with a heat shock at 42 °C for 5 min, and after a 10 min of incubation step on the ice, 1 ml of 40% PEG 4000 solution was added to the protoplasts, followed by a 20 min of incubation at room temperature. The suspension was mixed with 50 ml of warm regeneration medium and split into a Ø 15 cm petri dish. After overnight incubation at 28°C, the transformation plate was overlayed with 40 ml of warm regeneration medium containing HygB (80 µg/ml) and again incubated at 28 °C until colonies were formed.

Table 5: Solutions or media used for *A. alternata* transformation

Buffer or medium	Composition
NaCl solution	0.7 M NaCl
STC	1 M sorbitol, 50 mM $CaCl_2$, 50 mM Tris-HCl, pH 8.0
40% PEG 4000 solution	Polyethylene glycol 4000 [PEG 4000], 50

	mM Tris-HCl [pH 8.0] and 50 mM $CaCl_2$
Regeneration medium	34.3% saccharose, 0.5% yeast extract and 0.5% Casein hydrolysate, 0.75% agar
Antibiotic (1000X)	80 mg/ml hygromycin

Table 6: Medium and stock solution used for *A. alternata* cultivation

Medium or Stock	Ingredients (1 liter)
mCDB	50 ml 20X Salt stock solution, 40 g glucose, 1 g Yeast extract, pH 5.5
20X Salt stock solution	20 g $NaNO_3$, 5 g NH_4Cl, 20 g KH_2PO_4, 5 g NaCl, 10 g $MgSO_4$ 7XH2O, 0.2 g $FeSO_4$ 7XH_2O, 0.2 g $ZnSO_4$ 7XH_2O

4.2.3 *A. oryzae* transformation

A. oryzae strain NSAR1 was grown on mCD medium for 7 days at 28 °C. Fresh spores were harvested and inoculated overnight in liquid mCD medium at 28°C and 180 rpm. The mycelium was filtered through Miracloth and washed with NaCl solution. The mycelium was digested in a *Trichoderma harzianum* lysing enzyme (Novozymes) for 1h with soft shaking at 30 °C and 100 rpm. The quality and quantity of protoplast were checked using microscopy. Protoplasts were separated by filtering through two layers of Miracloth and precipitated for 5 min at 4 °C. The lysing enzyme solution was discarded. Protoplasts were resuspended in 100 µl of solution 1. 3 µg of plasmid DNA were added to the protoplasts and incubated on ice for 5 min. 1 ml solution 2 was added to the protoplasts, followed by a 20 min of incubation at room temperature. The suspension was mixed with 15 ml of warm CZDS soft-top agar and poured onto a Czapek-Dox plate supplemented with 1M sorbitol, arginine (10 ml Arginine solution per 1 l medium) and methionine (5 ml Methionine solution per 1 l medium). and they were incubated at 28°C until colonies were formed.

Table 7: Solutions or media used for *A. oryzae* transformation

Buffer or medium	Composition
NaCl solution	0.8 M NaCl
Solution 1	0.8 M NaCl, 10 mM $CaCl_2$, 50 mM Tris-HCl, pH 7.5
Solution 2	40% PEG 4000, 0.8 M NaCl, 50 mM Tris-HCl [pH 7.5] and 50 mM $CaCl_2$
CZDS soft-top Agar	3.5% Czapek Dox broth, 0.7% agar and 1M sorbitol
Czapek-Dox Agar	3% sucrose, 0.3% $NaNO_3$, 0.1% KH_2PO_4, 0.05% KCl, 0.05% $MgSO_4$ $7XH_2O$, 0.001% $FeSO_4$ $7XH_2O$, 1.5% agar, pH 7.3
Czapek Dox Agar + Sorbitol	3% sucrose, 0.3% $NaNO_3$, 0.1% KH_2PO_4, 0.05% KCl, 0.05% $MgSO_4$ $7XH_2O$, 0.001% $FeSO_4$ $7XH_2O$, 1.5% agar, 1M sorbitol, pH 7.3
mCD	1.5% Glucose, 0.4% Yeast extract, 0.1% KH_2PO_4, 0.05% NaCl, 0.05% $MgSO_4$ $7XH_2O$, 0.001% $FeSO_4$ $7XH_2O$, 1.5% agar, pH 7.0
Arginine solution	0.5 M arginine
Methionine solution	0.5 M methionine

Table 8: *Alternaria alternata* and *Aspergillus oryzae* strains used in this study

Strain	Genotype or description	Source
Alternaria alternata		
ATCC 66981	Parental strain	Christopher Lawrence, Virginia Tech, Blacksburg, VA, USA
⊿pksA	*pksA* deleted in the parental strain	Wenderoth *et al*., 2017
SMW35	*⊿pksI*, considered as wild type	Wenderoth *et al*., 2019
SJG9	*⊿lccD*	This study
SJG10	*⊿lccC*	This study
SJG11	*⊿lccB*	This study
SJG14	*⊿lccF*	This study
SJG21	*⊿brm3*	This study
SJG22	*⊿brm2/3*	This study
SJG25	*⊿aygB*	This study
SJG26	*⊿aygA*	This study
SJG27	*⊿aygA/B*	This study

SJG28	*⊿pksA*	This study
SJG29	*⊿cmrA*	This study
SJG31	*⊿atfA*	This study
SJG32	*⊿mpkA*	This study
SJG33	*⊿mpkB*	This study
SJG37	*PpksA::gfp::stuA::TtrpC,* checking the expression of *pksA* in the wild type strain.	This study
SJG38	*PpksA::gfp::stuA::TtrpC* checking the expression of *pksA* in *⊿cmrA* strain	This study
SJG39	*PpksA::gfp::stuA::TtrpC PaygA::mcherry::stuA::TtrpC* checking the expression of *pksA* and *aygA* in the wild type strain.	This study
SJG40	*PpksA::gfp::stuA::TtrpC PaygB::mcherry::stuA::TtrpC* checking the expression of *pksA* and *aygB* in the wild type strain.	This study
SJG42	*PlccB::lccB::mCherry::TtrpC*	This study
SJG43	*PlccC::lccC::mCherry::TtrpC*	This study
SJG44	*PlccD::lccD::mCherry::TtrpC*	This study
SJG45	*PlccF::lccF::mCherry::TtrpC*	This study
SJG48	*⊿laeA*	This study
SJG57	*⊿brm1*	This study
SJG58	*⊿brm2*	This study
SJG59	*⊿lccC/D*	This study
SJG60	*⊿lccB/F*	This study
SJG62	*⊿pksA* complemented with *Alternaria alternata pksA*	This study
SJG63	*pksA(p)::pksA::gfp::pksA(t) gpdA(p)::mCherry-SKL::trpC(t)*	This study
SJG64	*⊿veA*	This study
Aspergillus oryzae		
NSAR1	*niaD^{-}, sC^{-}, ⊿argB, adeA^{-}*	Russel J. Cox, Leibniz Universität Hannover
SJG61	*amyB(p)::pksA::amyB(t); adh1(p)::adh1(t); gpdA(p)::gpdA(t); enoA(p)::enoA(t); ampR; adeA; URA3;* expressing *A. alternata pksA* in *A. oryzae*	This study

4.2.4 Culture conditions

A. alternata and *A. oryzae* strains used in this study are listed in **Table 8**. *A. alternata* strains were grown on modified Czapek Doth broth (mCDB) medium or on mCDB medium supplemented with tomato puree (400 g/L), tricyclazole (30 µg/ml) or 1,8-DHN (0, 0.01, 0.02, 0.03, 0.04 mg/ml) at 28 °C for 1-14 days. *A. oryzae* strain NSAR1 was grown on mCD medium at 28 °C for 5 days. For metabolites expression, *A. oryzae* strains were incubated on MPY medium at 28 °C for 5 days.

4.3 Molecular biological methods

4.3.1 Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) was performed according to manufacturer's protocols with Q5 or Phusion polymerase from New England Biolabs (NEB, Frankfurt am Main, Germany). Oligonucleotides synthesis was performed by Eurofins Genomics (Ebersberg, Germany) with the final concentration of 0.2 µM in the PCR reaction. And in the PCR reaction, ~ 10 ng plasmid DNA or ~ 100 ng *A. alternata* genomic DNA was used as a template. dNTPs were purchased from Roth (Karlsruhe, Germany) with the final concentration of 200 µM in the PCR reaction. The PCR program included a pre-denaturation step for 3 min at 98 °C, followed by a denaturation step for 20 s at 98 °C; the annealing step included 5 cycles at 63 °C for 20 s, 5 cycles at 61 °C for 20 s, and 25 cycles at 59 °C for 20 s. the elongation step was performed at 72 °C and the time is changeable according to the length of the amplified fragments (2 kb/min). The primers used in this study are listed in **Table 9.**

Table 9: Oligonucleotides used in this study

Oligonucleotide	Sequence 5' → 3'
CRISPY_fw_2.0/3.0	GGTCATAGCTGTTTCCGCTGA
CRISPY_re_TtrpC	CTTCAATATCAGTTAACGTCGAGCCAAGAGCGGATTCC

kurz/PtrpC	
CRISPY_fw_PtrpC	GACGTTAACTGATATTGAAGGAG
CRISPY_re_TtrpCl ang/Vect	GTCTCGGCTGAGGTCTTAATTCTAGAAAGAAGGATTACC TCTAAAC
CRISPY re 2.0	TGATTCTGCTGTCTCGGCTG
Protospacer cmrA 1 fw	GTCCGTGAGGACGAAACGAGTAAGCTCGTCTCTACAGC CAGGCTCTCTCCGTTTTAGAGCTAGAAATAGCAAGTTAAA
HH cmrA 1 rev	GACGAGCTTACTCGTTTCGTCCTCACGGACTCATCAGTC TACACGGTGATGTCTGCTCAAGCG
Protospacer cmrA 2 fw	GTCCGTGAGGACGAAACGAGTAAGCTCGTCGTCGCCAC CAGGTTTTCCGAGTTTTAGAGCTAGAAATAGCAAGTTAAA
HH cmrA 2 rev mit PtrpC	GACGAGCTTACTCGTTTCGTCCTCACGGACTCATCAGGT CGCCATCGATGCTTGGGTAGAATAGG
cmrA-ko-test-fw	ACGTACTCGAGCCTCGAGAAA
cmrA-ko-test-rev	TCTCTACACGGGAACCACCTAC
Protospacer lccB 1 fw	GTCCGTGAGGACGAAACGAGTAAGCTCGTCGAGGTTCC GTGGAAAGAAACGTTTTAGAGCTAGAAATAGCAAGTTAAA
HH lccB 1 rev	GACGAGCTTACTCGTTTCGTCCTCACGGACTCATCAGGA GGTTCGGTGATGTCTGCTCAAGCG
Protospacer lccB 2 fw	GTCCGTGAGGACGAAACGAGTAAGCTCGTCCTCGTCTG AGATTAAGGCCAGTTTTAGAGCTAGAAATAGCAAGTTAAA
HH lccB 2 rev mit PtrpC	GACGAGCTTACTCGTTTCGTCCTCACGGACTCATCAGCT CGTCATCGATGCTTGGGTAGAATAGG
LccB-ko-test-fw	CGGCTCTGCAGACTACCCA
LccB-ko-test-rev	TGCATTCTGCATGATGCGCT
Protospacer lccC 1 fw	GTCCGTGAGGACGAAACGAGTAAGCTCGTCCGTATGAA CAGATTGTCAAGGTTTTAGAGCTAGAAATAGCAAGTTAAA
HH lccC 1 rev	GACGAGCTTACTCGTTTCGTCCTCACGGACTCATCAGCG TATGCGGTGATGTCTGCTCAAGCG
Protospacer lccC 2 fw	GTCCGTGAGGACGAAACGAGTAAGCTCGTCTCAAGAAT GGGTGCGACGAGGTTTTAGAGCTAGAAATAGCAAGTTAA A
HH lccC 2 rev mit PtrpC	GACGAGCTTACTCGTTTCGTCCTCACGGACTCATCAGTC AAGAATCGATGCTTGGGTAGAATAGG
LccC-ko-test-fw	CCAACTGACATGCAGCAATAAGC
LccC-ko-test-rev	CGAGGTATTGAAGGCATGAACGT
Protospacer lccD 1 fw	GTCCGTGAGGACGAAACGAGTAAGCTCGTCCGACCCAG AGCAGCCTGTGAGTTTTAGAGCTAGAAATAGCAAGTTAA

	A
HH lccD 1 rev	GACGAGCTTACTCGTTTCGTCCTCACGGACTCATCAGCG ACCCCGGTGATGTCTGCTCAAGCG
Protospacer lccD 2 fw	GTCCGTGAGGACGAAACGAGTAAGCTCGTCACGAGTCA TACTGAAGCGGTGTTTTAGAGCTAGAAATAGCAAGTTAAA
HH lccD 2 rev mit PtrpC	GACGAGCTTACTCGTTTCGTCCTCACGGACTCATCAGAC GAGTATCGATGCTTGGGTAGAATAGG
LccD-ko-test-fw	CAGTTTGAGGCACGACGACG
LccD-ko-test-rev	GATGATGTGCGTGAGCTTAGCT
Protospacer lccF 1 fw	GTCCGTGAGGACGAAACGAGTAAGCTCGTCTTATTCCTA GTCACGACCAAGTTTTAGAGCTAGAAATAGCAAGTTAAA
HH lccF 1 rev	GACGAGCTTACTCGTTTCGTCCTCACGGACTCATCAGTT ATTCCGGTGATGTCTGCTCAAGCG
Protospacer lccF (2) fw	GTCCGTGAGGACGAAACGAGTAAGCTCGTCGCAGGAAA GAAGAAGTCGAGGTTTTAGAGCTAGAAATAGCAAGTTAA A
HH lccF (2) rev mit PtrpC	GACGAGCTTACTCGTTTCGTCCTCACGGACTCATCAGGC AGGAATCGATGCTTGGGTAGAATAGG
LccF-ko-test-fw	GCTATTAGTCGCTGAATGCATGGG
LccF-ko-test-rev	CCTTGCCGCTGAATCTGATGC
Protospacer AygA 1 fw	GTCCGTGAGGACGAAACGAGTAAGCTCGTCCCAAGAGA AGCTGAAGACTCGTTTTAGAGCTAGAAATAGCAAGTTAAA
HH AygA 1 rev	GACGAGCTTACTCGTTTCGTCCTCACGGACTCATCAGCC AAGACGGTGATGTCTGCTCAAGCG
Protospacer AygA 2 fw	GTCCGTGAGGACGAAACGAGTAAGCTCGTCGTATAAGCT GTTTGGTATTGGTTTTAGAGCTAGAAATAGCAAGTTAAA
HH AygA 2 rev mit PtrpC	GACGAGCTTACTCGTTTCGTCCTCACGGACTCATCAGGT ATAAATCGATGCTTGGGTAGAATAGG
AygA-ko-test-fw	TGATGCCTCTTCGTCAATTCATAG
AygA-ko-test-rev	CTTGAATTTTCCTTGCACGACG
brm1_crispy_fw	GTCCGTGAGGACGAAACGAGTAAGCTCGTCCGAATTGA AGCCTACGTTCGGTTTTAGAGCTAGAAATAGCAAGTTAAA
brm1_crispy_re	GACGAGCTTACTCGTTTCGTCCTCACGGACTCATCAGCG AATTCGGTGATGTCTGCTCAAGCG
brm1_crispy_fw2	GTCCGTGAGGACGAAACGAGTAAGCTCGTCACTTCATC GGCGGTACACGGGTTTTAGAGCTAGAAATAGCAAGTTAA A
brm1_crispy_re2	GACGAGCTTACTCGTTTCGTCCTCACGGACTCATCAGAC

	TTCAATCGATGCTTGGGTAGAATAGG
Brm1_test_fw	GCCATGGAATGCTTTGCTTATG
Brm1_test_re	CGCCCATGTTGTACCAGATG
pksA_crispy_fw	GTCCGTGAGGACGAAACGAGTAAGCTCGTCCCGACCAG TACCCGCTCCTGGTTTTAGAGCTAGAAATAGCAAGTTAAA
pksA_crispy_re	GACGAGCTTACTCGTTTCGTCCTCACGGACTCATCAGCC GACCCGGTGATGTCTGCTCAAGCG
pksA_crispy_fw2	GTCCGTGAGGACGAAACGAGTAAGCTCGTCACTGGTCA ACTCTGGTGAGAGTTTTAGAGCTAGAAATAGCAAGTTAAA
pksA_crispy_re2	GACGAGCTTACTCGTTTCGTCCTCACGGACTCATCAGAC TGGTATCGATGCTTGGGTAGAATAGG
brm2_crispy_fw	GTCCGTGAGGACGAAACGAGTAAGCTCGTCGACATGGA GCCTTGCCGGCAGTTTTAGAGCTAGAAATAGCAAGTTAA A
brm2_crispy_re	GACGAGCTTACTCGTTTCGTCCTCACGGACTCATCAGGA CATGCGGTGATGTCTGCTCAAGCG
Brm2_test_fw	GCCGATTTCTAGGACACTCC
Brm2_test_re	GGTCACAGTAAGAGTGGATGG
Protospacer brm3 1 fw	GTCCGTGAGGACGAAACGAGTAAGCTCGTCAGGGGCTG CGCCATCGAGCTGTTTTAGAGCTAGAAATAGCAAGTTAAA
HH brm3 1 rev	GACGAGCTTACTCGTTTCGTCCTCACGGACTCATCAGAG GGGCCGGTGATGTCTGCTCAAGCG
Protospacer brm3 2 fw	GTCCGTGAGGACGAAACGAGTAAGCTCGTCGAGGACGG CGGTTGGGTTAAGTTTTAGAGCTAGAAATAGCAAGTTAAA
HH brm3 2 rev mit PtrpC	GACGAGCTTACTCGTTTCGTCCTCACGGACTCATCAGGA GGACATCGATGCTTGGGTAGAATAGG
brm3-ko-test-fw	GTGCTGCTGGTACAGTTTGAAGT
brm3-ko-test-rev	GTTTCTCTGGAAGTCGGGCATAC
Protospacer AygB 1 fw	GTCCGTGAGGACGAAACGAGTAAGCTCGTCTCTTGGCG ACCTCTTCACAAGTTTTAGAGCTAGAAATAGCAAGTTAAA
HH AygB 1 rev	GACGAGCTTACTCGTTTCGTCCTCACGGACTCATCAGTC TTGGCGGTGATGTCTGCTCAAGCG
Protospacer AygB 2 fw	GTCCGTGAGGACGAAACGAGTAAGCTCGTCGGTGTATC CGTGGATGGAAGGTTTTAGAGCTAGAAATAGCAAGTTAA A
HH AygB 2 rev mit PtrpC	GACGAGCTTACTCGTTTCGTCCTCACGGACTCATCAGGG TGTAATCGATGCTTGGGTAGAATAGG
AygB-ko-test-fw	CGCAAACGCTAGCCCGTC

AygB-ko-test-rev	TTCGGCTCAGTGGCCACTT
lccA_rt_fw	GTGCAGGCGATGGACAATTC
lccA_rt_re	CCGCAGCAGGTAGATTGAT
lccB_rt_fw	TGCCTTCGAGTCTGACAACC
lccB_rt_re	ATCTCAGACGAGCGCTCAAG
lccC_rt_fw	CATCATCGATGGTCCTGCCA
lccC_rt_re	CACGGGTAGAATGCTGTGCAA
lccD_rt_fw	GAATACTGGTTGGAGCTCACC
lccD_rt_re	CCAGTCTGCAATGAGGGTTG
lccE_rt_fw	CTCAGCTTGCGCAGTGCAAGA
lccE_rt_re	GTGCCATTGGAGCGGAAATC
lccF_rt_fw	AAGCTGTCTGGCTCTGTTCC
lccF_rt_re	CTTGGTGCTTGCATCCTTCG
lccG_rt_fw	CGACTCTGGAGCCTGTCAT
lccG_rt_re	CTCGCGAGAGTCTCCAATC
pksA_rt_fw	TCGCCTCTGACTGGTGAAA
pksA_rt_re	TGGTAAGGGCAGTGCAGAA
cmrA-qPCR-fw	CTAACTGGACCCCATCGTG
cmrA-qPCR-rev	GAGACTCGCCACTACTAGAC
AygA-qPCR-fw	GCTGAAGACTCCGGCAAAG
AygA-qPCR-rev	GCATATTCGTCCCAGTTATAGG
Brm1-qPCR-fw	CAGTCGGCATGCTACGAG
Brm1-qPCR-fw	GCCTAGGACGGCAGGATC
Brm2-qPCR-fw	GGCCGTGGTATCGGAAAG
Brm2-qPCR-rev	CTCCTCGACGTTGCCAAC
Brm3-qPCR-fw	GCTTCCGTCATCGTCAACTAC
Brm3-qPCR-rev	GCTCATGACGATGTTGATCTTGC
AaltH2B_RT_fw	ACAAGAAGAAGCGCACCAAG
AaltH2B_RT_re	CGTTGACGAAAGAGTTGAGAAT
AygB-qPCR-fw	CAGAAATTGTGGGAACAACGTTG
AygB-qPCR-re	TGTATGCATCGTCATACGGATC
pksA-re-fw	GTTTTTCAGCAAGATTTAATGGCTCGGCAGCTCTTGTAA
pksA-re-rev	CCACTAGCATTACACTTTTACTAGATCGTGCCAGACAAAGC
pksA-m-fw	TCCTTTGCGGCACTGTCGAGGTTGTCGAGTCCACCAACG
pksA-m-rev	GCGTTGGTGGACTCGACAACCTCGACAGTGCCGCAAAG
pksA-AO-fw	AAGAGTCAGTCAGTCTTAATATGAACGTCCTCATTTTCGG

	AGA
pksA-AO-rev	AGATCGACTGACTGACTTTATTATAGCTTGAGACCTTGCTGAATG
AygA-re-fw	GTTTTTCAGCAAGATTTAATAAGCGAACCAGAAGGCTGC
PaygA-mCherry-rev	TTATCCTCCTCGCCCTTGCTGCTTGAATCAGTCTTCTCTCGTTATAG
mCherry-PaygA-fw	GAGAGAAGACTGATTCAAGCAGCAAGGGCGAGGAGGATAA
StuA-TtrpC-rev	GGGATCGGTCGGCATCTACTTCATGCAGCCCAAGAGGAAC
TtrpC-stuA-fw	GTTCCTCTTGGGCTGCATGAAGTAGATGCCGACCGATCCC
TtrpC-pJet-rev	CCACTAGCATTACACTTTTATGGGGGGAGTTTAGGGAAAGA
pJet-PaygB-fw	CCACTAGCATTACACTTTTAGAAAGCGACGAGGAGCGAA
PaygB-mCherry-rev	TTATCCTCCTCGCCCTTGCTCATGATGTGAATATGGATATATCGCTC
mCherry-PaygB-fw	ATATCCATATTCACATCATGAGCAAGGGCGAGGAGGATAA
PpksA-pJet-fw	GTTTTTCAGCAAGATTTAATGGTACTTGGCCGAGGAAAC
PpksA-gfp-rev	AGTTCTTCTCCTTTACTCATGATGGCAAGGGTATAAAAGAATTAATAAAAA
gfp-PpksA-fw	TCTTTTATACCCTTGCCATCATGAGTAAAGGAGAAGAACTTTTCAC
pJet-lccB-fw2	GTTTTTCAGCAAGATTTAATCTTCAGCCGTGTCATCAAGG
LccB-GA5-rev	CCCGCACCCGCACCCGCACCTAGCCCAGAGTCTCCCGG
GA5(lccB)-mCherry-fw	TCCCGGGAGACTCTGGGCTAGGTGCGGGTGCGGGTGCGGGTGCGGGTGCGAGCAAGGGCGAGGAGGATAA
mCherry-TtrpC-rev	GGGATCGGTCGGCATCTACTTCAAGCGTAATCTGGAACATCGTATG
pJet-lccC-fw	GTTTTTCAGCAAGATTTAATACCAGTGGATCGGAATACGG
LccC-GA5-rev	CCCGCACCCGCACCCGCACCGATACCAGAGTCAGTTTGGTTGT
GA5(lccC)-mCherry-fw	ACCAAACTGACTCTGGTATCGGTGCGGGTGCGGGTGCGGGTGCGAGCAAGGGCGAGGAGGATAA
pJet-lccD-fw	GTTTTTCAGCAAGATTTAATAGAAGGCGAAGCCAGAAGTG
LccD-GA5-rev	CCCGCACCCGCACCCGCACCAACACCGGAGTCATCCTG

	C
GA5(lccD)-mCherry-fw	AGCAGGATGACTCCGGTGTTGGTGCGGGTGCGGGTGCGGGTGCGGGTGCGAGCAAGGGCGAGGAGGATAA
pJet-lccF-fw	GTTTTTCAGCAAGATTTAATATTGCAGGCAGGATTGTCCG
LccF-GA5-rev	CCCGCACCCGCACCCGCACCCTCAATCAAAGCGCCCCG
GA5(lccF)-mCherry-fw	TGCGGGGCGCTTTGATTGAGGGTGCGGGTGCGGGTGCGGGTGCGAGCAAGGGCGAGGAGGATAA
Protospacer PksA fw	GTCCGTGAGGACGAAACGAGTAAGCTCGTCCTAGGCAAGCTCATTCAGCAGTTTTAGAGCTAGAAATAGCAAGTTAAA
HH PksA rev	GACGAGCTTACTCGTTTCGTCCTCACGGACTCATCAGCTAGGCCGGTGATGTCTGCTCAAGCG
Gfp-pksA-fw	ACACTAGGCAAGCTCATTCAGAGTAAAGGAGAAGAACTTTTCACTG
Gfp-pksA-rev	AGCTTATAGCTTGAGGCCTTGTTTGTATAGTTCATCCATGCCATG
pksA-L-fw	GTCGAGGGTGTTTCCCGC
pksA-L(gfp)-rev	AAAAGTTCTTCTCCTTTACTCTGAATGAGCTTGCCTAGTGTGG
pksA-R(gfp)-fw	GCATGGATGAACTATACAAACAAGGCCTCAAGCTATAAGCTTCG
pksA-R-rev	GGATTTACGCCAGCGGCA
PgpdA-pJet-fw	GTTTTTCAGCAAGATTTAATAATCATCCTTATTCGTTGACCAAGAAT
PgpdA-mCherry-rev	TTATCCTCCTCGCCCTTGCTCATTGTGATGTCTGCTCAAGCGG
mCherry-PgpdA-fw	CCGCTTGAGCAGACATCACAATGAGCAAGGGCGAGGAGGATAA
mCherry (SKL)-rev	GGGATCGGTCGGCATCTACTTCATAGCTTAGAAGCGTAATCTGGAACATCGTATG
TtrpC-mCherry (SKL)-fw	ACGATGTTCCAGATTACGCTTCTAAGCTATGAAGTAGATGCCGACCGATCCC
Protospacer laeA 1 fw	GTCCGTGAGGACGAAACGAGTAAGCTCGTCCCTCGCTCCAATGGTCACGAGTTTTAGAGCTAGAAATAGCAAGTTAAA
HH laeA 1 rev	GACGAGCTTACTCGTTTCGTCCTCACGGACTCATCAGCCTCGCCGGTGATGTCTGCTCAAGCG
Protospacer laeA 2 fw	GTCCGTGAGGACGAAACGAGTAAGCTCGTCCATTGGGAGGTGGTACAACCGTTTTAGAGCTAGAAATAGCAAGTTAAA
HH laeA 2 rev mit PtrpC	GACGAGCTTACTCGTTTCGTCCTCACGGACTCATCAGCATTGGATCGATGCTTGGGTAGAATAGG

laeA-kotest-fw	GAAGGGACAGGGAGATGGAAAG
laeA-kotest-rev	GCAGACTCCCTTGTTCTGCTC
Protospacer MpkA 1 fw	GTCCGTGAGGACGAAACGAGTAAGCTCGTCCGAGCGCTACAATGTGACCAGTTTTAGAGCTAGAAATAGCAAGTTAAA
HH MpkA 1 rev	GACGAGCTTACTCGTTTCGTCCTCACGGACTCATCAGCGAGCGCGGTGATGTCTGCTCAAGCG
Protospacer MpkA 2 fw	GTCCGTGAGGACGAAACGAGTAAGCTCGTCCAAGAAGCCTTCAACCAGGGGTTTTAGAGCTAGAAATAGCAAGTTAAA
HH MpkA 2 rev mit PtrpC	GACGAGCTTACTCGTTTCGTCCTCACGGACTCATCAGCAAGAAATCGATGCTTGGGTAGAATAGG
MpkA-kotest-fw	GCTCGCAGCGCTAGGC
MpkA-kotest-rev	GATGCGACACCCGTGCAG
Protospacer MpkB 1 fw	GTCCGTGAGGACGAAACGAGTAAGCTCGTCTCCGCATTGCACAAGCCGTCGTTTTAGAGCTAGAAATAGCAAGTTAAA
HH MpkB 1 rev	GACGAGCTTACTCGTTTCGTCCTCACGGACTCATCAGTCCGCACGGTGATGTCTGCTCAAGCG
Protospacer MpkB 2 fw	GTCCGTGAGGACGAAACGAGTAAGCTCGTCTGACCCAGATGACGAGCCGAGTTTTAGAGCTAGAAATAGCAAGTTAAA
HH MpkB 2 rev mit PtrpC	GACGAGCTTACTCGTTTCGTCCTCACGGACTCATCAGTGACCCATCGATGCTTGGGTAGAATAGG
MpkB-kotest-fw	CTCCCAGGCACCACCAAAG
MpkB-kotest-rev	CGGGTAGTGCGTATGCATCC
Protospacer atfA 1 fw	GTCCGTGAGGACGAAACGAGTAAGCTCGTCCCCCATCGCTGCCGCCGTCGGTTTTAGAGCTAGAAATAGCAAGTTAAA
HH atfA 1 rev	GACGAGCTTACTCGTTTCGTCCTCACGGACTCATCAGCCCCATCGGTGATGTCTGCTCAAGCG
Protospacer atfA 2 fw	GTCCGTGAGGACGAAACGAGTAAGCTCGTCAGCCCTGATGAACAGGAAGAGTTTTAGAGCTAGAAATAGCAAGTTAAA
HH atfA 2 rev mit PtrpC	GACGAGCTTACTCGTTTCGTCCTCACGGACTCATCAGAGCCCTATCGATGCTTGGGTAGAATAGG
atfA-kotest-fw	ACCAGCATCGCGACCAGA
atfA-kotest-rev	TCGCCTCTGTAACGCCAGT
veA_doko_test_fw	TAGAGCTGCAGAGGCTGCA
veA_doko_test_rev	CTAGGGGTTGACAACGCAC

4.3.2 DNA Purification

After the digestion of plasmid DNA or PCR, the samples were mixed with 6X DNA loading dye, and loaded on the 1% agarose gel supplemented with 2% Midori Green Advance DNA Stain (NIPPON Genetics EUROPE GmbH). 1 kb DNA ladder was used as the standard marker of DNA size and loaded on the gel in the meantime. After running at 100V for 30 min, DNA fragments were examined using the FastGene® Imaging System (NEPON Genetics EUROPE GmbH). The purification of DNA fragments was done using the Zymoclean Gel Recovery Kit (Zymo Research, USA) based on the manufacturer's protocols. The concentration of the purified DNA fragments was detected by NanoDrop 1000 (NanoDrop, USA).

Table 10: Solutions used for DNA agarose gel electrophoresis

Solution	Composition
50X TAE buffer	40 mM Tris-acetate, 1Mm EDTA, pH 8.0
6X Loading dye	0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol

4.3.3 DNA digestion, cloning and sequencing

Plasmids (0.5-1 µg) were digested with restriction endonucleases based on the manufacturer's protocol. The restriction enzyme digestion was performed in a total volume of 10 µl with 0.3 µl restriction enzyme and the sample was incubated at 37 °C for 20-30 min. Cloning of plasmid DNA was performed using the NEBuilder HiFi DNA Assembly kit. The molar ratio of insert to vector was 1:1 in a total volume of 20 µl. And the Builder mixture was incubated at 50 °C for 30 min. After the ligation, the sample was transformed into competent cells directly. The DNA fragments used for sequencing were amplified with Q5 polymerase. DNA sequencing was performed by Eurofins Genomics (Ebersberg, Germany).

4.3.4 CRISPR/Cas9 plasmid construction for gene deletions

For the design of the deletion constructs, two protospacer sequences per gene were chosen to produce two different sgRNAs concurrently from the respective constructs. The strategy is based on our previous work. To insert two different protospacer sequences into a linearized pFC332 vector, two chimeric PCR fragments obtained through fusing two small PCR fragments respectively were combined in a NEBuilder reaction (New England Biolabs, Frankfurt, Germany). In detail, two small PCR fragments (f1 and f2), to insert the first protospacer sequence, were amplified using pFC334 as a template; another two small PCR fragments (f3 and f4), to insert the second protospacer sequence, were amplified using pAK4 as template. Next, two chimeric fragments F1 and F2 were obtained through combining f1/f2 and f3/f4 in a fusion-cloning step and then used to insert two different protospacer sequences into the pFC332 vector. The primers, which contain the variable regions used to generate the sgRNA gene fragments, were obtained from MWG Eurofins and are listed in **Table 9**. The fragments were amplified from pFC334 and pAK4 with proofreading polymerase Q5 (NEB) by a touchdown PCR program (denaturation, initial step for 3 min at 98 °C; all following denaturation steps for 20 s at 98 °C; annealing, 5 cycles at 67 °C for 20 s, 5 cycles at 65 °C for 20 s, 25 cycles at 63 °C for 20 s; and elongation, 10 s at 72 °C). The chimeric fragments were amplified by a fusion-PCR program (denaturation, initial step for 3 min at 98 °C; all following steps are 30 cycles; denaturation, 20 s at 98 °C; annealing, 20s at 63 °C; and elongation, 25s at 72 °C). Standard reaction mixture volumes were 50 µl, including 1 U Q5 reaction buffer, 200 µM deoxynucleotide triphosphates (dNTPs), 0.5 µM primers, 1 U Q5, and 100 ng of plasmid DNA or PCR fragments. Plasmid pFC332 was linearized using *Pac*I and assembled with the PCR fragments, following the NEBuilder protocol. *Escherichia coli* transformation and plasmid isolation were done according to standard protocols.

4.3.5 Plasmid and chimeric cassette construction for PksA tagging

A CRISPR-Cas9 *knock-in* strategy was used for PksA GFP tagging. For the design of the *knock-in* construct, one protospacer sequence near the stop codon of *pksA* was chosen. To insert the protospacer sequence into the linearized pFC332 vector, two PCR fragments, amplified with pFC334 as a template, were combined in a NEBuilder reaction (New England Biolabs, Frankfurt, Germany). The chimeric cassette *pksA*(L)-*GFP*-*pksA*(R) was obtained through a fusion-PCR program. In detail, two 600-bp fragments of the upstream or downstream region of the Cas9-cutting site in the *pksA* gene, *pksA*(L) and *pksA*(R), were amplified using *A. alternata* genomic DNA as a template. GFP was amplified using pBV1 as template. Finally, the chimeric cassette *pksA*(L)-*gfp*-*pksA*(R) was obtained in a fusion-cloning step. The oligonucleotides are listed in **Table 9**.

4.3.6 Plasmid construction for peroxisomal labeling

The *gpdA*(*p*) and *trpC*(*t*) fragments were amplified from plasmid pBV1. The *mCherry-SKL* fragment was amplified from plasmid pVW05. The chimeric fragment was obtained by a combination of *gpdA*(*p*), *mCherry-SKL* and *trpC*(*t*) fragments in a fusion-cloning step and then cloned into the linearized vector pJet-*hph* (hygromycin B phosphotransferase gene) in a NEBuilder reaction. The primers are listed in **Table 9**.

4.3.7 Heterologous expression of *A. alternata pksA* plasmid construction

The ORF of the *pksA* gene was amplified by PCR using the primers ‘pksA-AO-fw/rev’ (**Table 9**) and *A. alternata* genomic DNA as a template. The PCR fragment was cloned into the linearized vector pTYGSade2.0 (**Table 10**) in a NEBuilder reaction.

4.3.8 Laccase-tagging plasmids construction

The construction of laccase-tagging plasmids was based on the strategy of tagging

laccase Abr2 in *A. fumigatus* (Upadhyay *et al*., 2016). The *lcc(p)-lcc* (laccase encoding gene promoter and ORF sequences) fragment was amplified using *A. alternata* genomic DNA as a template. The GA5-*mCherry* fragment was amplified from the plasmid pVW05. The *trpC*(*t*) fragment was amplified from the plasmid pBV1. The chimeric fragment *lcc*(*p*)-*lcc*-GA5-*mCherry-trpC*(*t*) was obtained by the combination of *lcc(p)-lcc*, GA5-*mCherry* and *trpC*(*t*) fragments in a fusion-cloning step and then cloned into the linearized vector pJet-*hph* (hygromycin B phosphotransferase gene) in a NEBuilder reaction. The primers used to generate the *lcc*(*p*)-*lcc*-GA5-*mCherry-trpC*(*t*) fragment are listed in **Table 9**.

4.3.9 Complementation of *A. alternata pksA* plasmid construction

The *pksA* gene sequences containing 1 kb upstream of ORF (promotor), ORF, and 1 kb downstream of ORF (terminator) were amplified by PCR using *A. alternata* genomic DNA as a template. The PCR fragment was cloned into the linearized vector pJet-*hph* in a NEBuilder reaction. The primers are listed in **Table 9**.

4.3.10 Promoter-reporter plasmids construction

The *pksA*(*p*), *aygA*(*p*) and *aygB*(*p*) (promotor sequences of the PksA, AygA and AygB encoding genes) fragments were amplified using *A. alternata* genomic DNA as template. *mCherry-stuA-trpc*(*t*) and *gfp-stuA-trpc*(*t*) fragments were amplified using pVW05 and pBV1 as templates respectively. *pksA*(*p*)-*mCherry-stuA-trpc*(*t*)*, aygA*(*p*)-*gfp-stuA-trpc*(*t*) and *aygB*(*p*)-*gfp-stuA-trpc*(*t*) were obtained by the combination of *pksA*(*p*)/*aygA*(*p*)/*aygB*(*p*) and *mCherry-stuA-trpc*(*t*)/*gfp-stuA-trpc*(*t*) fragments in a fusion-cloning step and then cloned into the linearized vector pJet-*hph* in a NEBuilder reaction. The primers are listed in **Table 9**.

4.3.11 Plasmid DNA extraction from *E. coli*

The plasmids used for transformation were extracted from *E. coli* cells with the NucleoSpin Plasmid EasyPure kit (MACHEREY-NAGEL, Düren) based on the manufacturer's protocols. And an alkali-lysis method was used for the quick miniprep of plasmid DNA (Sambrook et al., 1989). *E. coli* was incubated in 2 ml LB medium supplemented with ampicillin (100 µg/ml) overnight at 180 rpm, at 37 °C. Cells were collected into a 1.5 ml microtube by centrifuged for 1 min at 13,000 rpm. The pellet was resuspended in 200 µl Cell-Suspension buffer by vortex or pipetting. And then, 200 µl of Cell-lysis buffer was added, mixed gently and kept at room temperature for 2 min. 200 µl of Potassium-Acetate buffer was added into the mixture, mixed by inversion 2-5 times, and incubated on the ice for 10 min. Then, the mixture was centrifuged at 13,000 rpm for 10 min. Afterward, 500 µl of supernatant was transferred into 500 µl isopropanol (-20 °C) in a new 1.5 ml micro tube, inverted for 4-6 min, and kept on ice or in the fridge for 10 min. And then, the sample was centrifuged at 13,000 rpm for 10 min at 4 °C. The supernatant was discarded, and 500 µl of 70% ethanol was added to the pellet and centrifuge at 13,000 rpm for 5 min at 4 °C. The supernatant was discarded, and the pellet was dried at room temperature for 20 min. The dried pellet was resuspended in 20 µl of TE buffer by shaking at 68 °C for 10 min.

Table 11: Solutions used for Plasmid-Minipreparation

Solution	Composition
Cell-Suspension buffer	5 ml 1M Tris-HCl (pH 7.5), 2 ml 0.5 M EDTA (pH 8.0), 10 mg RNase in 100 ml buffer
Cell-Lysis buffer	0.2 M NaCl, 1% SDS
Potassium-Acetate buffer	1.5 M Kac, pH 4.8
TE buffer	10 mM Tris-HCl (pH 7.5), 1mM EDTA (pH 8.0)

Table 12: Plasmids used in this study

Name	Description	Reference

pFC332	*tef1*(p)*::cas9::tef1*(t); *gpdA*(p)*::gpdA*(t); *hph; ampR*; AMA1	Uffe H. Mortensen, Technical University of Denmark
pFC334	*tef1*(p)*::cas9::tef1*(t); *gpdA*(p)*::sgRNA-AnyA::gpdA*(t); *Afpyr4; ampR*; AMA1	Uffe H. Mortensen, Technical University of Denmark
pAK4	pJET1.2 blunt + *trpC*(p)*::sgRNA-AnyA::trpC*(t)	This work
pJET1.2 blunt	Cloning Vector, Component of CloneJET PCR Cloning Kit	Thermo Fisher Scientific, USA
pTYGSade2.0	*amyB*(p)::*amyB*(t); *adh1*(p)::*adh1*(t); *gpdA*(p)::*gpdA*(t); *enoA*(p)::*enoA*(t); ampR; adeA; URA3	Colin M. Lazarus, University of Bristol
pJG3	*tef1*(p)*::cas9::tef1*(t); *gpdA*(p)*::atfA-sgRNAcassette1::gpdA*(t)*::trpC*(p)*::atfA-sgRNAcassette2::trpC*(t); *hph*; *ampR*	This work
pJG10	*tef1*(p)*::cas9::tef1*(t); *gpdA*(p)*::lccD-sgRNAcassette1::gpdA*(t)*::trpC*(p)*::lccD-sgRNAcassette2::trpC*(t); *hph*; *ampR*	This work
pJG11	*tef1*(p)*::cas9::tef1*(t); *gpdA*(p)*::lccC-sgRNAcassette1::gpdA*(t)*::trpC*(p)*::lccC-sgRNAcassette2::trpC*(t); *hph*; *ampR*	This work
pJG16	*tef1*(p)*::cas9::tef1*(t); *gpdA*(p)*::lccB-sgRNAcassette1::gpdA*(t)*::trpC*(p)*::lccB-sgRNAcassette2::trpC*(t); *hph*; *ampR*	This work
pJG19	*tef1*(p)*::cas9::tef1*(t); *gpdA*(p)*::lccF-sgRNAcassette1::gpdA*(t)*::trpC*(p)*::lccF-sgRNAcassette2::trpC*(t); *hph*; *ampR*	This work
pJG28	*tef1*(p)*::cas9::tef1*(t); *gpdA*(p)*::brm3-sgRNAcassette1::gpdA*(t)*::trpC*(p)*::brm3-sgRNAcassette2::trpC*(t); *hph*; *ampR*	This work
pJG30	*tef1*(p)*::cas9::tef1*(t); *gpdA*(p)*::aygB-sgRNAcassette1::gpdA*(t)*::trpC*(p)*::aygB-sgRNAcassette2::trpC*(t); *hph*; *ampR*	This work
pJG31	*tef1*(p)*::cas9::tef1*(t); *gpdA*(p)*::aygA-sgRNAcassette1::gpdA*(t)*::trpC*(p)*::aygA-sgRNAcassette2::trpC*(t); *hph*; *ampR*	This work
pAK1	*tef1*(p)*::cas9::tef1*(t); *gpdA*(p)*::pksA-sgRNAcassette1::gpdA*(t)*::trpC*(p)*::pksA-*	This work

	sgRNAcassette2::trpC(t); *hph*; *ampR*	
pJG32	*tef1*(p)*::cas9::tef1*(t); *gpdA*(p)*::cmrA-sgRNAcassette1::gpdA*(t)*::trpC*(p)*::cmrA-sgRNAcassette2::trpC*(t); *hph*; *ampR*	This work
pMW89	*tef1*(p)*::cas9::tef1*(t); *gpdA*(p)*::brm1-sgRNAcassette1::gpdA*(t)*::trpC*(p)*::brm1-sgRNAcassette2::trpC*(t); *hph*; *ampR*	This work
pMW90	*tef1*(p)*::cas9::tef1*(t); *gpdA*(p)*::brm2-sgRNAcassette1::gpdA*(t)*::trpC*(p)*::brm2-sgRNAcassette2::trpC*(t); *hph*; *ampR*	This work
pJG33	pJET1.2 blunt + *hph*	This work
pJG35	*tef1*(p)*::cas9::tef1*(t); *gpdA*(p)*::mpkA-sgRNAcassette1::gpdA*(t)*::trpC*(p)*::mpkA-sgRNAcassette2::trpC*(t); *hph*; *ampR*	This work
pJG36	*tef1*(p)*::cas9::tef1*(t); *gpdA*(p)*::mpkB-sgRNAcassette1::gpdA*(t)*::trpC*(p)*::mpkB-sgRNAcassette2::trpC*(t); *hph*; *ampR*	This work
pJG37	*tef1*(p)*::cas9::tef1*(t); *gpdA*(p)*::pksA-sgRNAcassette3*; *hph*; *ampR*	This work
pJG38	*pksA*(p)*::gfp::stuA::*trpC(t); *hph*; *ampR*	This work
pJG40	*aygA*(p)*::mCherry::stuA::*trpC(t); *hph*; *ampR*	This work
pJG41	*aygB*(p)*::mCherry::stuA::*trpC(t); *hph*; *ampR*	This work
pJG43	*lccB*(p)::*lccB*::*mCherry*::*trpC*(t); *hph*; *ampR*	This work
pJG44	*lccC*(p)::*lccC*::*mCherry*::*trpC*(t); *hph*; *ampR*	This work
pJG45	*lccD*(p)::*lccD*::*mCherry*::*trpC*(t); *hph*; *ampR*	This work
pJG46	*lccF*(p)::*lccF*::*mCherry*::*trpC*(t); *hph*; *ampR*	This work
pJG47	*amyB*(p)::*pksA*::*amyB*(t); *adh1*(p)::*adh1*(t); *gpdA*(p)::*gpdA*(t); *enoA*(p)::*enoA*(t); ampR; adeA; URA3	This work
pJG48	*pksA*(p)*::pksA::pksA*(t); hph; *ampR*	This work
pJG49	*tef1*(p)*::cas9::tef1*(t); *gpdA*(p)*::laeA-sgRNAcassette1::gpdA*(t)*::trpC*(p)*::laeA-sgRNAcassette2::trpC*(t); *hph*; *ampR*	This work
pJG59	*gpdA*(*p*)*::mCherry-SKL::trpC*(*t*) ; hph; *ampR*	This work
	tef1(p)*::cas9::tef1*(t); *gpdA*(p)*::veA-sgRNAcassette1::gpdA*(t)*::trpC*(p)*::veA-sgRNAcassette2::trpC*(t); *hph*; *ampR*	

4.3.12 Isolation of genomic DNA from *A. alternata*

A. alternata strain was incubated on mCDB plate for 2-3 days at 28 °C. Half of the fresh colony was collected, put into a 2 ml microtube, and disrupted to small pieces. 750 µl of extraction buffer was added into the microtube and mixed thoroughly by the vortex. The sample was incubated at 65 °C for 60 min in a thermos mixer via gently shaking. After adding 375 µl of 3 M KAc solution, the sample was inverted 4-5 times and incubated on ice for 30 min, then centrifuged at 13,000 rpm, at 4 °C for 30 min. 700 µl of the supernatant was transferred into a new 1.5 ml microtube, followed by adding 800 µl of isopropanol (-20 °C), mixing gently and incubating for 30 min at -20 °C. The mixture was centrifuged at 4 °C at 13,000 rpm for 15 min. The supernatant was discarded, and the pellet was washed with 500 µl of 70% ethanol by centrifuging at 13,000 rpm at 4 °C for 3 min. The supernatant was discarded, and the pellet was dried at 68 °C for 15 min. And then, 50 µl of TE buffer was used to resuspend the dried pellet.

Table 13: Solutions used for *A. alternata* genomic DNA extraction

Solution	Composition
Extraction buffer (100 ml)	10 ml 1M Tris-HCl (pH 7.5), 10 ml 0.5 M EDTA (pH 8.0), 10 ml 10% SDS, 10 ml 5 M NaCl, 60 ml dH_2O, 10 mg RNase in 100 ml buffer
3 M KAc solution (100 ml)	60 ml 5 M KAc, 11.5 ml acetic acid, 28.5 ml dH_2O
TE buffer	10 mM Tris-HCl (pH 7.5), 1mM EDTA (pH 8.0)

4.3.13 RNA isolation and quantitative real-time PCR

A. alternata spores (10^5) were inoculated in 10 ml of mCDB or mCDB containing tomato puree (400 g/L) in a Ø 3.5 cm Petri dish and incubated for 5d at 28 °C. Subsequently, the mycelia were harvested and frozen immediately in liquid nitrogen. Frozen mycelia were ground into powder, and total RNA was isolated using the Fungal RNA Extraction Kit (Omega). RNA samples were quantified and treated with TURBO

DNA-free kit (Invitrogen) to remove DNA contaminations. After the treatment, the isolated RNA was diluted to 50 ng/µl. SensiFAST SYBR & Fluorescein One-Step Kit from Bioline (Luckenwalde) was used for quantitative real-time PCR. Each reaction mixture of 20 µl contained 1 µl of reverse transcriptase (RT) enzyme, 0.16 µM primers, and 100 ng of total RNA. The program included 10 min of the reverse transcription reaction at 50 °C, followed by 5 min at 95 °C to inactive the reverse transcriptase and 40 PCR cycles (10 s at 95 °C and then 30 s at 59 °C). After each PCR, melting curve analyses were performed to show the specific amplification of single DNA segments and the absence of nonspecifically amplified DNA. The histone gene *H2B* was used to normalize the results of each gene. Each expression level is the average of three biological replicates. The error bar means the standard deviation for the replicates. The oligonucleotides used in this study are listed in **Table 9**.

4.3.14 Microscopy

For microscopic analysis, *A. alternata* strains were grown on mCDB medium for 3-5 days at 28 °C. For the promotor-reporter assay, a slice of agar containing spores, aerial hypha and substrate hypha was obtained by trimming the *A. alternata* colony using a razor blade. And then, it was placed on a microscope slide and covered with a coverslip. Conventional fluorescence images were captured using an EC Plan-Neofluar 100x/1.3 Oil or 40x/0.75 objective with an AxioImager Z.1 and AxioCamMR (Zeiss).

4.4 Biochemistry methods

4.4.1 Melanin content measurement

Fresh spores of wild type strain were inoculated in 20 ml of liquid mCDB medium or mCDB medium containing tomato puree (400 g/L) in a Ø 15 cm petri dish for 7 days at 28 °C. Mycelia of respective strains were collected and frozen using liquid nitrogen.

The frozen mycelia were ground into powder, suspended in 5ml of 1 M NaOH solution, and then boiled at 100 °C for 2h. The pH value of the solution was adjusted to 2.0 with 5 M HCl, and the acidified solution was centrifuged at 10,000 x g for 20 min. The resulting melanin (supernatant) was dissolved in 2% NaOH, and then the solution was measured using a spectrophotometer for absorbance at 459 nm (Babitskaia *et al.*, 2000).

4.4.2 Extraction and analysis of secondary metabolites by this-layer chromatography

To extract secondary metabolites, *A. alternata* and *A. oryzae* strains were grown for 1-7 days on their respective production medium at 28 °C. Four disks from each plate were excised with the back of a blue pipette tip and extracted by shaking with 1 ml of ethyl acetate for 1 h. After centrifuged at 13,000rpm for 10 min, 700 µl of supernatant containing secondary metabolites of each sample was taken into a 1.5 ml microtube and dried at room temperature, followed by dissolving secondary metabolites again using 70 µl of ethyl acetate for further TLC analysis or dissolving in 1 ml ACN:H_2O (1:1 (v/v)) for further LC-HRMS analysis.

For the qualitative analysis of the *A. alternata* extracts, 10 µl of crude extract was put on TLC plates coated with 0.25 mm silica gel as stationary phase (Pre-coated TLC plates SIL G-25, Macherey-Nagel, Düren, Germany). The mobile phase for separation of the metabolites consisted of 50% of toluol, 40% of ethyl acetate, and 10% of formic acid. The secondary metabolites were visualized using UV light at 254 nm.

4.4.3 LC-HRMS analysis of *A. alternata* and *A. oryzae* samples

To analyze *A. alternata* secondary metabolites, the respective bands were extracted and analyzed with LC-HRMS. The bands were visualized with UV light (254nm), scratched off the TLC plates, and collected in reaction tubes. To obtain more intense signals, five bands were pooled for each metabolite. 500µl of a 1:1 (v/v) mixture of

MS grade acetonitrile (ACN, HiPerSolv Chromanorm, HPLC gradient grade, VWR, Vienna, Austria) and water (ELGA Purelab Ultra-AN-MK2 system, Veolia Water, Vienna, Austria) was added, mixed and kept at room temperature for 30 minutes. After filtration (0.2µm PTFE, 13mm syringe filters, VWR international), the extracts were transferred in glass vials for measurement.

To search for initially released PksA polyketide products dried *A. oryzae* extracts were dissolved in 1ml ACN:H_2O (1:1 (v/v)) and further diluted (1:10000, (v/v)) for Fullscan LC-HRMS measurement. For MS/MS spectra generation 1:1000 (v/v) dilutions were prepared.

LC-HRMS measurements were carried out with a Vanquish UHPLC system coupled to a QExactive HF Orbitrap via a heated ESI source operating in polarity switching mode (all Thermo Fisher Scientific, Bremen, Germany) applying the chromatographic method described previously (Sauerschnig *et al.*, 2018). Full scan mass spectra were recorded with a resolving power setting of 120 000 (FWHM) at *m/z* 200 and a mass range of *m/z* 100 to 1000. MS/MS fragmentation was carried out using an inclusion list of the defined targets, and collision energy was adapted for the respective target (either stepped CE (20, 45, 70) or CE 70, 80 or 90. For identification reference standards of ATX I, ATX II and ALP. T4HN was obtained from MedChemExpress.

Data were evaluated with the vendor software Xcalibur. Metabolites stated as identified were compared and confirmed with the authentic reference standards and classified as level 1 according to the metabolomics standards initiative. Other described compounds are annotated with level 3.

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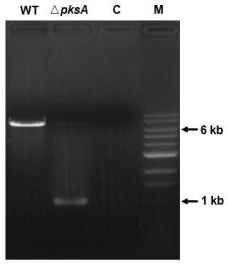
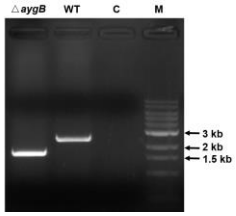
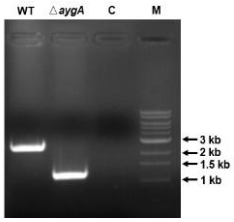
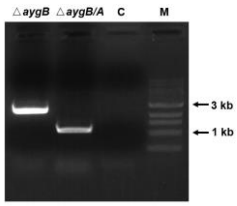
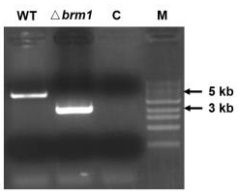
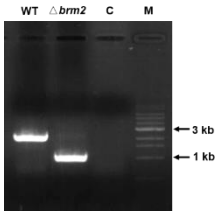
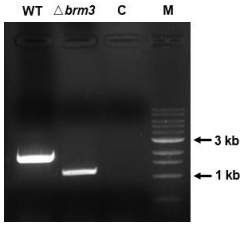
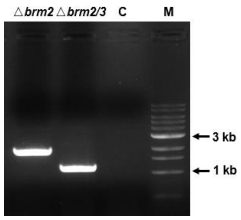
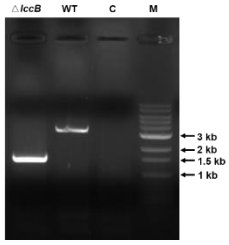
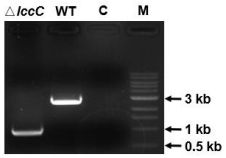
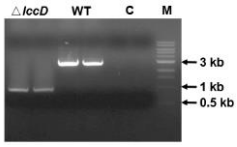
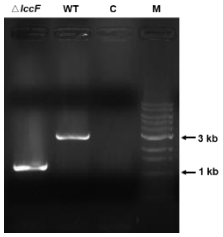
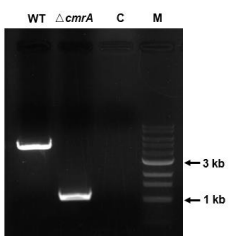
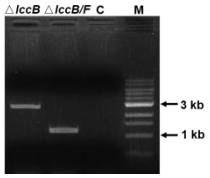
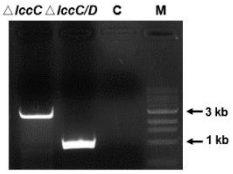
Appendix

Proof of mutant strains

Mutants	PCR Testing Results	Sequencing Results	Deletion Region
△*pksA*		*pksA* ATGAACGTCCTCATTTTCGGAGATCAAACCGCCGACCAGTACCCGCTCCTGCGG △*pksA* ATGAACGTCCTCATTTTCGGAGATCAAACCGCCGACCAGTACCCGCTC---------- *pksA* *****AACTGGTCAACTCTGGTGAGAAGGTTGAGAACCTC**********AAGCTATAA △*pksA* ----------AGAAGGTTGAGAACCTC**********AAGCTATAA	5965-bp deletion: 5965 bp of the ORF
△*aygB*		*aygB* ATGCCTAACGGAAACTGGATTCTTGGCGACCTCTTCACAAAGGACTACGGACAT △*aygB* ATGCCTAACGGAAACTGGATTCTTGGCGACCTCTTCA---------- *aygB* *****ACGGTGTATCCGTGGATGGAAGAGGTTATGGCGAGTAAGTAGATATCTGGT △*aygB* ----------AGATATCTGGT	1173-bp deletion: 1173 bp of the ORF
△*aygA*		*aygA* ATGGCAG******ATGAGTCTTACGAACAGCTTTGGGAGACGAAGTGGAAGAAGCCT △*aygA* ATGGCAG******ATGAGTCTTACGAAC---------- *aygA* *****GGCTGTATAAGCTGTTTGGTATTGAGGCCGAAGTCGGGGC******AAGTACTAA △*aygA* ----------GGGGC******AAGTACTAA	1218-bp deletion: 1218 bp of the ORF
△*aygA* (△*aygB/ aygA double mutant)*		*aygA* ATGG******ACTATGAGTCTTACGAACAGCTTTGGGAGACGAAGTGGAAGAAGCCT △*aygA* ATGG******ACTA---------- *aygA* *****GGCTGTATAAGCTGTTTGGTATTGAGGCCGAAGTCGGGGC******AAGTACTAA △*aygA* ----------TGAGGCCGAAGTCGGGGC******AAGTACTAA	1226-bp deletion: 1226 bp of the ORF
△*brm1*			2542-bp

		brm1 GGTGTAGAAATATTTCGG********** △brm1 GGTGTAGAAATATT---------- brm1 *****TCATGTTTGAGAAGAACGAATTGAAGCCTACGTTCGAGGGTTA********** △brm1 ---------- brm1 *****ACAGCACTTCATCGGCGGTACACGGTGGG**********CAGGCGTTGTAG △brm1 ---------- brm1 **********GATCTATCGACCATGTCATTCAACAACAGCTACGGC △brm1 ----------TATCGACCATGTCATTCAACAACAGCTACGGC	deletion: 279 bp of the 5' UTR, 654 bp of the ORF and 1609 bp of the 3' UTR
△*brm2*		brm2 GTTTGCAGGCTAAACCTT********** △brm2 GTTTGCAGGCTAAAC---------- brm2 *****ATGGCATCAATCGAGCAGACATGGAGCCTTGCCGGCAAGGTC********** △brm2 ---------- brm2 *****CTACAATACTAACTATCACCCAGGCCGT**********GCTTGCATGTAA △brm2 ----------TAACTATCACCCAGGCCGT**********GCTTGCATGTAA	925-bp deletion: 98 bp of the ORF and 827 bp of the 5' UTR
△*brm3*		brm3 AGCTTCGGGCGCGAAGGTGTAGAGCCGCAGGTCCCTGAAGCAGCTATTTTCGGT △brm3 AGCTTCGGGCGCGAAGGTGTAGAGCCGCAGGTCCCTGAAGCAGCTATTTTCGGT brm3 GTTCGTTTCTTCGGATCCCCGCCCGCGGCCGCCTACCGTGAAGACCATCGTTTGA △brm3 GTTCGTTTCTTCGG---------- brm3 *****CGGCCAAACCGCGTACAAGCACATTGAGGACAACG*********CGTCGCAGTAA △brm3 ----------ATTGAGGACAACG*********CGTCGCAGTAA	623-bp deletion: 411 bp of the ORF and 212 bp of the 5' UTR
△*brm3* (△*brm2/brm3 double mutant*)		brm3 AGCTTCGGGCGCGAAGGTGTAGAGCCGCAGGTCCCTGAAGCAGCTATTTTCGGT △brm3 AGCTTCGGGCGCGAAGGTGTAGAGC---------- brm3 *****CGGCCAAACCGCGTACAAGCACATTGAGGACAACG*********CGTCGCAGTAA △brm3 ----------ATTGAGGACAACG*********CGTCGCAGTAA	666-bp deletion:

			411 bp of the ORF and 255 bp of the 5’ UTR
△*lccB*		*lccB* ATGGTTG******CCCAGAGTTCCTGAGGTTCCGTGGAAAGAAACCGGTCTATGGAG △*lccB* ATGGTTG******CCCAGAGTTCCTGAGGTTCCGTGGAAAGA---------- *lccB* ******TTTCTTGAGCGCTCGTCTGAGATTAAGGCCAAGGATGGAT******GGGCTATAG △*lccB* ----------TGAGATTAAGGCCAAGGATGGAT******GGGCTATAG	1864-bp deletion: 1864 bp of the ORF
△*lccC*		*lccC* ATGTTGG******ACTTTCCTTCGTATGAACAGATTGTCAAGAGGCAAGACGGTGCTT △*lccC* ATGTTGG******ACTTTCCTTCGTATGAACAGATTGTC---------- *lccC* ******TACTCAAGAATGGATGCGACGAGTGGGACTCTTACT*********CTGGTATCTAA △*lccC* ----------CGAGTGGGACTCTTACT*********CTGGTATCTAA	1657-bp deletion: 1657 bp of the ORF
△*lccD*		*lccD* ATGCTCT******CACTTCGGATCTACGACCCAGAGCAGCCTGTGACGGAAACACTG △*lccD* ATGCTCT******CACTTCGGATCTACGACCCAGAGCAGC---------- *lccD* ******GACGAGTCATACCGAAACGGTCGGTATTGAGCAGGATGACTCCGGTGTTTAG △*lccD* ----------GGTCGGTATTGAGCAGGATGACTCCGGTGTTTAG	1741-bp deletion: 1741 bp of the ORF
△*lccF*		*lccF* ATGTTGG******TGTAAGTACTCTTATTCCTAGTCACGACCAAAGGCGATAGGCCAC △*lccF* ATGTTGG******TGTAAGTACTCTTATTCCTAGTCA---------- *lccF* ******GCACTTTGCGATGCAGGAAAGAAGAAGTCGAGCGGTAAC******ATTGAGTAG △*lccF* ----------CGAGCGGTAAC******ATTGAGTAG	1596-bp deletion: 1596 bp of the ORF
△*cmrA*			3275-bp deleti

	 cmrA ACATCTACAGCCAGGCTCTCTCCAGGACTACACGTCCAATCCGTGATTCAAAATGGTC △cmrA ACATCTACAGCCAGGCTC---------- cmrA *****GTGTCGCCACCAGGTTTTCCGATGGCATTCGTAGTAAACTC********GCGCGGTTAA △cmrA ----------GTAGTAAACTC********GCGCGGTTAA	on: 3275 bp of the ORF
△*lccF* (△*lccB/lccF double mutant*)	 lccF ATGTTGG*******TGTAAGTACTCTTATTCCTAGTCACGACCAAAGGCGATAGGCCAC △lccF ATGTTGG*******TGTAAGTACTCTTATTCCTAGTCACGA---------- lccF ******GCACTTTGCGATGCAGGAAAGAAGAAGTCGAGCGGTAAC*******ATTGAGTAG △lccF ----------CGAGCGGTAAC*******ATTGAGTAG	1593-bp deletion: 1593 bp of the ORF
△*lccD* (△*lccC/lccD double mutant*)	 lccD ATGCTCT******CACTTCGGATCTACGACCCAGAGCAGCCTGTGACGGAAACACTG △lccD ATGCTCT******CACTTCGGATCTACGACCCAGAGCAGCCTG---------- lccD ******GACGAGTCATACCGAAACGGTCGGTATTGAGCAGGATGACTCCGGTGTTTAG △lccD ----------GGTCGGTATTGAGCAGGATGACTCCGGTGTTTAG	1738-bp deletion: 1738 bp of the ORF

Annotation: The protospacers are underlined; the start codon (red); the stop codon (blue); 5' Un-translated Region (UTR) (gray); 3' UTR (gray); C: negative control; M: 1 kb gene ruler; WT: wild type.

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