RAÍSSA MESQUITA BRAGA

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Thesis presented to the Programa de Pós-Graduação Interunidades em Biotecnologia USP / Instituto Butantan / IPT to obtain the degree of Doctor in Science

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Concentration area: Biotechnology

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PROF. DR. PAOLO M.A ZANOTTO
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"I think that there is only one way to science - or to philosophy, for that matter: to meet a problem, to see its beauty and fall in love with it"

Karl Popper

RESUMO

BRAGA, R. M. **Identificação de genes possivelmente envolvidos na biossíntese da epicolactona em *Epicoccum nigrum***. 2016. 128 p. Tese (Doutorado em Biotecnologia) - Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2016.

Epicoccum nigrum é um fungo ubíquo conhecido por sua capacidade de produzir vários metabólitos secundários bioativos e pelo seu uso potencial como agente de controle biológico contra vários fitopatógenos. Apesar de muitos compostos com importância biotecnológica produzidos por *E*. *nigrum* terem sidos descritos na literatura, ainda há uma grande lacuna de conhecimento acerca da biossíntese desses compostos e dos genes envolvidos. Entre os compostos produzidos por *E*. *nigrum*, epicolactona é um policetídeo com um esqueleto de carbono incomum que tem atraído grande interesse acerca da sua síntese. O objetivo desta tese foi identificar e caracterizar genes relacionados à biossíntese da epicolactona em *E*. *nigrum*. Três mutantes anteriormente gerados por mutagênese aleatória que tiveram a capacidade de produzir epicolactona reduzida ou ausente foram analisados. O T-DNA estava inserido em regiões intergênicas nesses mutantes, assim, os genes próximos ao sítio de inserção foram analisados. Entretanto, a expressão desses gene não foi significativamente diferente do fungo selvagem, indicando que esses genes provavelmente não estão relacionados à biossíntese da epicolactona. É possível que o T-DNA tenha se inserido em uma região regulatória. Usando ferramentas de bioinformática, genes candidatos codificadores de PKSs foram identificados no genoma de *E*. *nigrum* e seis genes foram selecionados para deleção. A deleção do gene PKSi12 mostrou que os seus produtos estão relacionados à atividade antagonista contra fitopatógenos. A análise química permitiu a identificação putativa dos preditos precursores da epicolactona, epicoccona B e epicoccina, o quais tiveram sua produção afetada no mutante ΔPKSi12. Com base nas evidências fornecidades pelas análises químicas e *in silico*, foi proposta uma possível biossíntese de epicoccona B e epicoccina por *E*. *nigrum*. Entretanto, ainda há muito a se compreender a cerca da biossíntese da epicolactona e são necessários experimentos adicionais para confirmar o papel da PKSi12 na produção de epicoccona B e epicoccina.

Palavras-chave: *Epicoccum nigrum*. Endófito. Epicolactona. Policetídeo. Policetídeo sintase. Biossíntese.

ABSTRACT

BRAGA, R. M. **Identification of candidate genes contributing to epicolactone biosynthesis in *Epicoccum nigrum***. 2016. 128 p. PhD thesis (Biotechnology) - Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2016.

Epicoccum nigrum is a ubiquitous fungus known mainly for its ability to produce numerous bioactive secondary metabolites and for its potential use as a biocontrol agent against various phytopathogens. Although many compounds of biotechnological significance that are produced by *E. nigrum* have been described in the literature, there is still a large gap in the knowledge on their biosynthesis and on the genes involved. Among the bioactive compounds produced by *E. nigrum*, epicolactone is a polyketide with an unusual carbon skeleton; this compound has attracted great interest in terms of its synthesis. The aim of this thesis was to identify and characterize genes related to epicolactone biosynthesis in *E. nigrum*. Three mutants (previously generated by random mutagenesis) that have a reduced or no ability to produce epicolactone were analyzed. The transfer DNA (T-DNA) was inserted in intergenic regions in these mutants; thus, the genes located near the insertion site were analyzed. Their expression was not significantly different from that in the wild type, indicating that they are probably not related to epicolactone biosynthesis. It is possible that the T-DNA was inserted in a regulatory region. Using a genome-mining approach, genes of polyketide synthases (PKSs) were identified in the *E. nigrum* genome, and six candidate genes were selected for targeted gene replacement. Deletion of the *PKSi12* gene showed that its products are related to *E. nigrum's* inhibitory effects on the growth of fungal phytopathogens. Chemical analysis enabled putative identification of the previously proposed epicolactone precursors—epicoccine and epicoccone B—whose production was altered in the ΔPKSi12 mutant. On the basis of the results of the chemical and *in silico* analyses, a possible biosynthetic pathway for epicoccone B and epicoccine in *E. nigrum* was proposed. There is still a lot to learn about epicolactone biosynthesis, and further experiments are necessary to confirm the role of PKSi12 in the biosynthesis of epicoccone B and epicoccine.

Keywords: *Epicoccum nigrum*. Endophyte. Epicolactone. Polyketide. Polyketide synthase. Biosynthesis.

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LIST OF ABBREVIATIONS

A	Adenylation
Acetyl CoA	Acetyl coenzyme A
ACP	Acyl carrier protein
AT	Acyl transferase
ATMT	*Agrobacterium tumefaciens* mediated transformation
C	Condensation
CHS	Chalcone and stilbene synthases
CLC	Claisen like cyclase
CoA	Coenzyme A
DH	Dehydratase
DHMB	4,6-dihydroxy-2-(hydroxymethyl)-3-methylbenzoic acid
DHMP	5,7-dihydroxy-4-methylphthalide
DHN	Dihydroxynaphthalene
DKP	Diketopiperazine
DMATS	Dimethylallyl tryptophan synthase
ER	Enoylreductase
EST	Esterase/lipase-like
H	HxxPF repeated domain
HDAC	Histone deacetylase
HR-PKS	Highly reducing polyketide synthase
HRS	Homologous recombination sequence
KR	Keto reductase
KS	Ketosynthase
LB	Luria Bertani
MES	2-(N-morpholino)ethanesulfonic acid
MFS	Major facilitator superfamily
MT	Methyltransferase
NR-PKS	Non-reducing polyketide synthase
NRPS	Non-ribosomal peptide synthases
ORF	Open reading frame
PCR	Polimerase chain reaction
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
PDYA	Potato Dextrose Yeast Extract Agar
PDYB	Potato Dextrose Yeast Extract Broth
PK	Polyketide
PKS	Polyketide synthase
PR-PKS	Partially reducing polyketide synthase
PT	Product template
R	Thioester reductase

RAL	Resorcylic acid lactone
R-PKSs	reducing PKSs
SAT	ACP transacylase
TAIL-PCR	Thermal Asymmetric Interlaced PCR
T-DNA	Transfer DNA
TE	Thioesterase
THN	Tetrahydroxynaphthalene
TSB	Tryptic soy broth
USER	Uracil Specific Excision Reagent
WT	Wild type

TABLE OF CONTENTS

1 GENERAL INTRODUCTION

Epicoccum nigrum is a dothideomycete fungus widespread in ambient air, in soil, and on decaying vegetation; this fungus can lead an endophytic lifestyle and is commonly isolated from diverse plant tissues. *E. nigrum* is mainly known for its ability to produce many bioactive secondary metabolites and for its potential use as a biocontrol agent against many phytopathogens. Among the bioactive compounds produced by *E*. *nigrum*, epicolactone is a polyketide with an unusual carbon skeleton. This compound has antimicrobial activity and was first isolated from *E*. *nigrum* P16, the strain used in the present study. Because of its unique and complex structure, epicolactone has aroused a lot of interest in relation to its synthesis. Studies on its biomimetic synthesis and actual biosynthesis have attracted considerable attention from the chemical-synthesis community.

The *E*. *nigrum* P16 strain was isolated from sugarcane leaves and shows *in vitro* inhibitory activity against sugarcane phytopathogens. This strain is also capable of increasing the sugarcane root system biomass. The genome of *E*. *nigrum* P16 was sequenced and has been assembled and annotated by our research group; these data allow for studies on genes and possible metabolic pathways related to biosynthesis of secondary metabolites. Although many compounds of biotechnological importance produced by *E*. *nigrum* have been described in the literature, there is still a large gap in the knowledge on their biosynthesis and on the genes involved. In this sense, identification and characterization of genes involved in the biosynthesis of secondary metabolites in *E*. *nigrum* can contribute to the discovery of new enzymatic functions and to genetic engineering studies. This knowledge will help to expand the pool of potential polyketide drugs and to synthesize new non-native compounds with improved characteristics.

On the other hand, the polyketide epicolactone produced by *E*. *nigrum* has an antimicrobial activity and complex structure. Although there are studies describing the biomimetic synthesis of epicolactone and the possible actual biosynthetic pathway, there are no *in vivo* studies on its biosynthesis, and no genes related to its biosynthesis have been identified to date. In addition, the knowledge about the secondary metabolites produced by *E*. *nigrum* can contribute to the understanding of the ecological

relations of this fungus, such as the plant-endophyte relation and its antagonistic activity in the host plant.

Therefore, the aim of the present study was to identify and characterize genes contributing to epicolactone biosynthesis in *E*. *nigrum*. Three *E*. *nigrum* mutants (generated by random mutagenesis) that have a reduced (or no) ability to produce epicolactone were analyzed. Mutants P16-17, P16-47set, and P16-91 were obtained in a previous study by our research group. The genes located near the transfer DNA (T-DNA) insertion site were annotated and analyzed. In addition, gene expression of two polyketide synthase (PKS) genes located in the same gene cluster (and suggested to be possibly related to epicolactone biosynthesis) was also evaluated. These results are described in chapter 3 "Analysis of epicolactone-deficient *Epicoccum nigrum* mutants P16-17, P16-47set, and P16-91 and screening of a mutant library."

Chapter 4, "Genome mining in *Epicoccum nigrum* reveals a gene cluster associated with antagonistic activity and probably involved in epicolactone biosynthesis," describes the diversity of PKS gene clusters in *E*. *nigrum* and generation and analysis of PKS deletion mutants. Bioinformatic tools were used to identify PKS-encoding genes in the *E*. *nigrum* genome and to select six genes that could be related to epicolactone biosynthesis. A protocol for targeted gene replacement in *E*. *nigrum* was successfully established, and PKS deletion mutants were generated and analyzed.

2 THEORETICAL BACKGROUND

2.1 Biotechnological importance of *Epicoccum spp.*

2.1.1 The genus Epicoccum

Epicoccum is a genus of ubiquitous mitosporic fungi of the Dothideomycetes class. They are widespread in ambient air, in soil, and on decaying vegetation, usually acting as a saprophyte or secondary invader of a plant's senescent tissues (PITT; HOCKING, 2012). *Epicoccum* spp. can also lead an endophytic lifestyle and are commonly isolated from various plant tissues (SCHULZ; BOYLE, 2005). More than 70 species have been described in the genus *Epicoccum*, but they were reduced to only one variable species: *E. nigrum*. Another species, *E. andropogonis* (basionym: *Cerebella andropogonis*), was added later (SCHOL-SCHWARZ, 1959). Currently, only these two species are recognized in this genus (AINSWORTH, 2008; SCHOL-SCHWARZ, 1959; STETZENBACH; YATES, 2003).

E. nigrum Link (syn. *E. purpurascens* Ehrenb. ex Schlecht) is known to be a highly variable species (this case is believed to represent only intraspecific variation). On the other hand, a polyphasic analysis of intraspecific diversity in an *E. nigrum* population identified two genotypes with morphological, physiological, and genetic divergence, strongly suggesting that these genotypes correspond to different species (FÁVARO et al., 2011). Besides endophytic and saprophytic lifestyles, *E. nigrum* can lead a fungicolous lifestyle and has been isolated from the fruit body of *Pholiota squarrosa* (KEMAMI WANGUN; HERTWECK, 2007) and *Cordyceps sinensis* (GUO et al., 2009; ZHANG et al., 2007). It has also been frequently found in the marine environment (ABDEL-LATEFF et al., 2003; PENG et al., 2012; SUN et al., 2011; WRIGHT; OSTERHAGE; KÖNIG, 2003; XIA et al., 2012).

E. nigrum P16, an endophytic strain used in the present study, was isolated from sugarcane (*Saccharum officinarum*) leaves (FÁVARO et al., 2011). This strain has shown *in vitro* antagonistic activity against the sugarcane phytopathogens *Fusarium verticillioides*, *Colletotrichum falcatum*, *Ceratocystis paradoxa*, and *Xanthomonas albilineans*, suggesting that *E. nigrum* P16 probably acts as a natural antagonist in sugarcane tissues. Analysis of the endophytic interaction with sugarcane revealed that *E.*

nigrum P16 is capable of increasing the root system biomass and preferentially colonizes the phylloplane environment and, occasionally, the endophytic environment (FÁVARO; SEBASTIANES; ARAÚJO, 2012).

Epicoccum spp. are mainly known for their ability to produce many bioactive compounds and for their potential use as biocontrol agents against many phytopathogens. *E. nigrum* also has other potential biotechnological applications, such as biotransformation of compounds (ANDRADE et al., 2004; SHIMIZU; ONO; MIYAZAWA, 2013; WEBSTER et al., 1998), biosynthesis of silver and gold nanoparticles (QIAN et al., 2013; SHEIKHLOO; SALOUTI; KATIRAEE, 2011), degradation of biogenic amines in wine (CUEVA et al., 2012), and synthesis of lipids for biodiesel production (BAGY et al., 2014; KOUTB; MORSY, 2011).

2.1.2 E. nigrum as a biocontrol agent

E. nigrum can inhibit the growth and conidial germination of many fungal phytopathogens and has been studied as a biocontrol agent against phytopathogens (as an alternative to fungicides). *E. nigrum* has proven to be an effective antagonist of *Botrytis cinerea* in waxflower (BEASLEY et al., 2001), *Claviceps africana* in sorghum (BHUIYAN et al., 2003), *Pythium* spp. in cotton (HASHEM; ALI, 2004), *Rhizoctonia solani* in potato plants (LAHLALI; HIJRI, 2010), and *Sclerotinia sclerotiorum* in sunflower (PIECKENSTAIN et al., 2001), among other similar applications (HUANG et al., 2000; LARENA et al., 2005; LI et al., 2013; MADRIGAL; PASCUAL; MELGAREJO, 1994; MARI et al., 2007; PENG; SUTTON, 1991). Therefore, this fungus holds great promise as a biocontrol agent against several plant pathogens.

Some studies have been carried out to elucidate the mechanisms of inhibition of fungal phytopathogens by *E. nigrum*. Lahlali and Hijri (2010) evaluated the antagonism of *E. nigrum* against *R. solani* in potato plants, and it was shown that *E. nigrum* improves the potato yield significantly and reduces the stem disease severity index. They found that *E. nigrum* limits the development of *R. solani* by growing along its hyphae and by inducing lysis. Another study analyzed the inhibitory effect of *E. nigrum* on *Phytophthora infestans. E. nigrum* showed significant antagonistic activity against *P. infestans in vitro* and *in vivo*. Microscopic analysis revealed that the protoplasm of the mycelium of *P. infestans* suffers severe degradation when it is cultured near *E. nigrum* on slides. Hyphal

malformation and leakage of the cytoplasm were also observed, suggesting that *E. nigrum* likely secretes compounds that affect cell wall synthesis or membrane permeability (LI et al., 2013).

2.1.3 Compounds produced by Epicoccum spp.

Many secondary metabolites produced by *Epicoccum* spp. have been isolated and characterized. Several of these metabolites were found to have diverse biological activities, such as antimicrobial, antioxidant, and anticancer as well as inhibition of viral replication, making the fungi of the genus *Epicoccum* important producers of compounds with potential biotechnological applications (Table 2.1).

Among the metabolites with biotechnological applications, epicocconone is a fluorophore isolated from *E. nigrum* and is used for cell staining and protein detection in electrophoresis gels commercially available as LavaCell-Fluorescent Cell Stain (Active Motif) and LavaPurple-Total Protein Stain for Gels and Blots (Fluorotechnics) (BELL; KARUSO, 2003; CHOI; VEAL; KARUSO, 2006). The telomerase inhibitor D8646-2-6 was also isolated from *E. nigrum* and reported in a patent by the Mitsubishi Pharma Group in 2002 (KANAI et al., 2007; PENG et al., 2012). Furthermore, *E. nigrum* produces taxol, an anticancer compound originally isolated from the plant *Taxus brevifolia*. Taxol is an expensive drug with increasing commercial demand; therefore, there has been some interest in finding new sources of this compound (SOMJAIPENG et al., 2015; WALL, 1995).

Several secondary metabolites of *Epicoccum* spp. are polyketides (Figure 2.1). Flavipin is a well-known fungal polyketide with a wide range of biological activities. This compound was first described in *Aspergillus* spp. (RAISTRICK; RUDMAN, 1956) and later in *Epicoccum* spp. (BAMFORD; NORRIS; WARD, 1961). This molecule has been reported to be an inhibitor of the growth of several fungal phytopathogens (BAMFORD; NORRIS; WARD, 1961; MADRIGAL; TADEO; MELGAREJO, 1991; RAISTRICK; RUDMAN, 1956; XIAO et al., 2013; YE et al., 2013), oomycetes (BROWN; FINLAY; WARD, 1987), the plant-parasitic nematode *Meloidogyne incognita* (NITAO et al., 2002), and the algae *Chlorella pyrenoidosa* (BURGE et al., 1976).

Table 2.1 - Examples of bioactive secondary metabolites produced by *Epicoccum* spp.

Compound	Type	Biological activity	References
4,5-Dimethylresorcinol	Polyketide	Potential therapeutic agent for diarrhea	Da Silva Araújo et al. (2012)
Flavipin	Polyketide	Antifungal, antioomycete, antialgal, and antinematode	Bamford et al. (1961)
Epicoccine	Polyketide	Antioxidant	Lee et al. (2007)
Epicoccone A and B	Polyketide	A: Antioxidant B: Potential anticancer	Abdel-lateff et al. (2003); Kemami et al. (2008)
Mellein	Polyketide	Antibacterial, antifungal, antialgal, and antiworm	Cabras et al. (2006)
Epicoccalone	Polyketide	Serine protease α-chymotrypsin inhibition	Kemami et al. (2008)
Epicolactone	Polyketide	Antibacterial, antifungal and antioomycete	Da Silva Araújo et al. (2012)
Epicocconigrones A and B	Polyketide	A: Potential anticancer	El Amrani et al. (2014)
Epicoccolides A and B	Polyketide	A-B: Antifungal and antioomycete B: Potential anticancer	Talontsi et al. (2013)
Epicocconone	Polyketide	Fluorophore	Bell and Karuso (2003)
Orevactaene	Polyketide	Potential HIV replication inhibitor	Shu et al. (1997)
Epipyridone	Pyridone alkaloid	Gram-positive antibacterial	Kemami Wangun and Hertweck (2007)
Epicoccarines A and B	Tetramic-acid derivative	Gram-positive antibacterial	Kemami Wangun and Hertweck (2007)
Epicoccamides A–D	Tetramic-acid derivative	D: Potential anticancer	Wright et al. (2003)
Epicoccins A–T and ent-epicoccin G	Diketopiperazine	A: Activity against *Bacillus subtilis*; G-H: HIV-1 replication inhibition; E, J, M, S: Anti-inflammatory	Zhang et al. (2007); Guo et al. (2009); Wang et al. (2010)
Diphenylalazines A and B	Diketopiperazine	A: HIV-1 replication inhibition	Guo et al. (2009)
Epicozarines A and B	Diketopiperazine	Antibacterial (more potent against gram-positive bacteria)	Baute et al. (1978); Deffieux et al. (1978a, 1978b)
iso-D8646-2-6 and D8646-2-6	Pyronepolyene C-glucoside	NF-κB inhibition and anti-influenza A viral (H1N1)	Peng et al. (2012)
β-Carotene, γ-Carotene, Rhodoxanthin, and Torularhodin	Carotenoid	Antioxidant	Gribanovski-Sassu and Foppen (1967)
Taxol	Tetracyclic diterpene	Anticancer	Somjaipeng et al., (2015)
Triornicin (1), ferricrocin (2), and coprogen (3)	Siderophore	1: Antitumor	Frederick et al. (1981a); Frederick et al. (1981b)
Epirodin	Undefined structure	Antibacterial and antifungal	Burge et al. (1976). Ikawa et al. (1978)

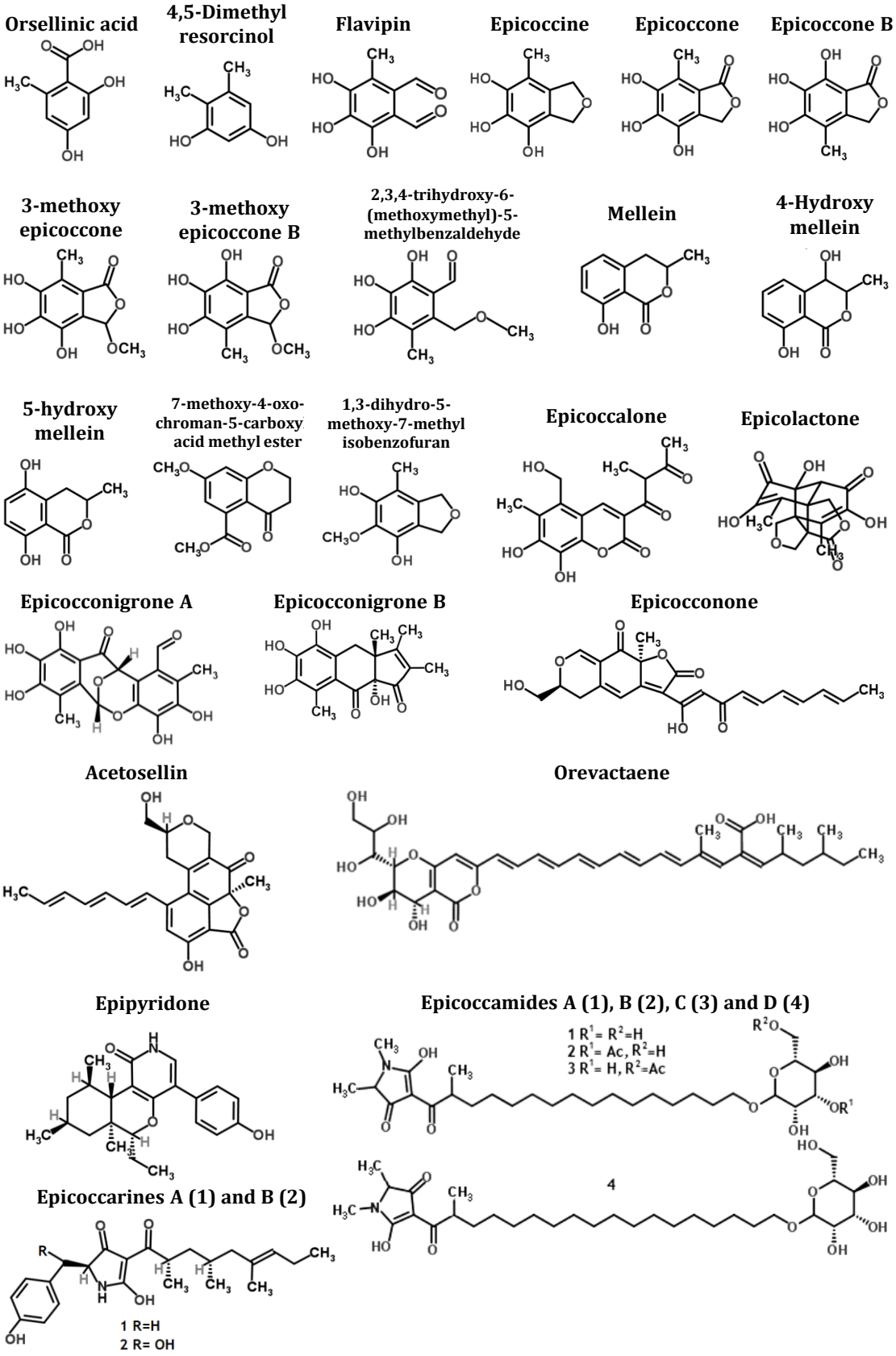

Figure 2.1 – Chemical structure of polyketides and polyketide-nonribosomal peptide hybrids produced by *Epicoccum* spp. The structures were obtained or adapted from the free chemical structure database ChemSpider (Royal Society of Chemistry) available at www.chemspider.com.

Flavipin has been postulated to be a precursor of several compounds produced by *Epicoccum* spp. The antimicrobial compounds epicoccolides A and B are presumably formed via asymmetrical benzoin condensation of two molecules of flavipin. Epicolactone, a compound with antimicrobial activity that has an unusual carbon skeleton, contains the framework of one flavipin molecule and has also been postulated to be derived from flavipin (TALONTSI et al., 2013). More details about the structure and hypothetical biosynthesis of epicolactone will be given in the next chapter.

El Amrani et al. (2014) isolated protein kinase inhibitors and antioxidant compounds from *E. nigrum* that are all probably biosynthetically related to flavipin. Epicoccone A, epicoccone B, and epicoccine, among others, may be derived from one molecule of flavipin by reduction of the aldehyde group followed by ring closure. Furthermore, epicocconigrones A and B may be formed by condensation of two molecules of flavipin. In addition to the potent inhibitory activity against protein kinases related to several types of cancer, epicocconigrone A and epicoccolide B have potent inhibitory effects on total histone deacetylases (HDACs; their inhibitors suppress cancer cell growth) *in vitro*; these properties make these two compounds promising leads for the development of anticancer drugs (EL AMRANI et al., 2014).

Among the polyketides of biotechnological significance produced by *Epicoccum* spp., there is also orevactaene, a potential HIV replication inhibitor. Orevactaene inhibits the interaction between Rev, a transactivator protein of viral gene expression, and Rev response element (RRE) (SHU et al., 1997). Compounds blocking this interaction can inhibit HIV replication (GREEN, 1993).

Some polyketide hybrid metabolites have also been described in *Epicoccum* spp. Epicoccarines A and B and epipyrodine are polyketide-nonribosomal peptide hybrid metabolites that are probably biosynthetically related. These three compounds have shown activity against gram-positive bacteria, especially epicoccarine A has, which exerts a potent antibacterial activity with high selectivity on *Mycobacterium vaccae* (KEMAMI WANGUN; HERTWECK, 2007).

Epicoccamides A–D are unusual polyketide–carbohydrate–nonribosomal peptide hybrid metabolites. The four compounds differ in the substitution pattern and in the length of the central carbon chain. Epicoccamide D, the compound with the longest chain, is cytotoxic to HeLa cells and has antiproliferative effects on mouse fibroblastic and human leukemic cell lines. Furthermore, this compound has been reported to induce

morphogenesis and pigment formation in the phytopathogenic fungus *Phoma destructiva* (WANGUN; DAHSE; HERTWECK, 2007; WRIGHT; OSTERHAGE; KÖNIG, 2003).

Many diketopiperazines (synthesized either by NRPSs or by cyclodipeptide synthases), some with a rare cross-ring sulfur bridge, are also produced by *Epicoccum* spp. These compounds have diverse biological functions, especially epicoccins E, J, M, and S, which have shown potent activities *in vitro* against the release of β-glucuronidase in rat polymorphonuclear leukocytes, when this release is induced by platelet-activating factor (anti-inflammatory activity) (GU et al., 2013; WANG et al., 2010).

Epicoccum spp. are also known for their ability to produce many pigments with hues in red-orange-brown-yellow spectra, mainly polyketides: flavipin (yellow), epicoccones A and B (brown), epicoccalone (yellow), epicocconone (fluorophore, weakly green fluorescent in water and strongly orange-red fluorescent in the presence of proteins), acetosellin (yellow), and orevactaene (orange); and carotenoids: β-carotene (yellow), γ-carotene (orange), rhodoxanthin (red), and torularhodin (violet) (BAMFORD; NORRIS; WARD, 1961; BELL; KARUSO, 2003; FOPPEN et al., 1968; GRIBANOVSKI-SASSU; FOPPEN, 1967; KEMAMI WANGUN; ISHIDA; HERTWECK, 2008; LEE et al., 2007; SHU et al., 1997; TALONTSI et al., 2013).

The pigments produced by *E. nigrum* have been studied for their potential use as colorants in the food industry (MAPARI et al., 2006, 2005; MAPARI; MEYER; THRANE, 2008, 2009; MAPARI; THRANE; MEYER, 2010). Production of pigments from fungi is advantageous because they are readily available sources, and the producers would not depend on the seasonal supply of raw materials (MAPARI; THRANE; MEYER, 2010). Moreover, production of pigments from a likely safe host such as *E. nigrum*, a nonpathogenic and nontoxic fungus, is advantageous in comparison with the traditional use of *Monascus* spp. (mixtures of azaphilone pigments [from *Monascus* spp.] of varying composition are commercially available), which can coproduce the mycotoxin citrinin (MAPARI; THRANE; MEYER, 2010). A yellow mixture of pigments extracted from *E. nigrum* (orevactaene as a major component plus two minor components) has shown enhanced photostability in liquid food model systems in comparison with the commercially available natural colorants turmeric and *Monascus* red, thus adding photostability as another beneficial characteristic of the pigments produced by *E. nigrum* (MAPARI; MEYER; THRANE, 2009).

2.2.4 Genes related to the production of secondary metabolites in Epicoccum spp.

Despite the biotechnological importance of *Epicoccum* spp., there are only a few genetic studies on the biosynthesis of their secondary metabolites. Gribanou et al. (1970) produced mutants of *E*. *nigrum* using ultraviolet irradiation and analyzed formation of carotenoid pigments. They isolated and characterized several carotenoids not present in the wild-type (WT) fungus (putative intermediates of biosynthesis of carotenoids) and confirmed a hypothetical pathway of rhodoxanthin biosynthesis from β-carotene. In another study, mutants of *E*. *nigrum* were produced by chemical mutagenesis, and a mutant strain (with a potential as a source of red pigments) was obtained (BARBU; BAHRIM; SOCACIU, 2006). Amnuaykanjanasin et al. (2009) identified four genes of reducing PKSs in a marine strain of *Epicoccum* sp. along with 145 PKS genes from different tropical fungal species using PCR with PKS-specific degenerate primers and studied their phylogenetic relationship. One of the PKS genes identified in *Epicoccum* sp. constitutes a new reducing clade together with eight other newly cloned PKS genes. Da Silva Araújo et al. (2012) identified three *E*. *nigrum* mutants obtained by random mutagenesis, which show decreased production of the antimicrobial compound epicolactone; however, no biosynthetic gene was characterized. According to the relevant literature, no secondary metabolite has been linked to a gene in *Epicoccum* spp. to date.

2.2 Fungal polyketides: A structurally diverse group of secondary metabolites

2.2.1 Secondary metabolites

Secondary metabolites are natural compounds that do not play an essential role in growth, development, and reproduction of the producing organism. This means that the producers can grow without synthesizing these compounds (KELLER; TURNER; BENNETT, 2005). Nevertheless, these metabolites are often bioactive compounds and can perform important functions in defense, signaling, and ecological interactions (YIN; KELLER, 2011), suggesting that—although these compounds are not essential—they may increase the producer fitness under such environmental conditions.

The major classes of fungal secondary metabolites are polyketides, nonribosomal peptides, terpenes, and indole alkaloids. These classes of secondary metabolites are derivatives of products of the following respective classes of enzymes: polyketide synthases (PKSs), nonribosomal peptide synthases (NRPS), terpene synthase, and dimethylallyl tryptophan synthase (DMATS). These enzymes use simple compounds as substrates, which are rearranged or condensed into more complex molecules. The substrates for PKSs are short-chain carboxylic acids; for NRPSs, the substrates are amino acids, for terpene synthases, isoprene units; and for DMATSs, usually tryptophan and dimethylallyl pyrophosphate. The compounds produced by these enzymes can undergo further modifications such as oxygenation, cyclization, isomerization, and/or condensation, forming metabolites highly variable in chemical structure and biological activity (KELLER; TURNER; BENNETT, 2005).

Many secondary metabolites are bioactive and have important biotechnological applications. Fungi are among the most exploited producers of bioactive secondary metabolites (SURYANARAYANAN et al., 2009). Endophytic fungi are promising sources of bioactive secondary metabolites. Many secondary metabolites with a wide range of potential biotechnological applications have been isolated from endophytic fungi (PRAKASH, 2015; SURYANARAYANAN et al., 2009). Endophytes are organisms that inhabit plants without causing visible disease signs (SCHULZ; BOYLE, 2005). Many of the bioactive secondary metabolites produced by endophytic fungi are probably related to the plant–endophyte interaction and can perform important ecological functions, for example, in the plant development (as growth promoters) and in defense, acting against phytopathogens (SCHULZ; BOYLE, 2005; STROBEL, 2003).

2.2.2 Polyketides

Polyketides are considered an important class of secondary metabolites. Although they are structurally diverse and occur in many organisms, such as plants, bacteria, and fungi, this class is defined by the common biosynthetic origin: polyketides are derived from small carboxylic acids such as acetate (COX, 2007).

In fungi, polyketides are the most abundant class of secondary metabolites, and individual compounds often prove to be highly biologically active (HERTWECK, 2009; KELLER; TURNER; BENNETT, 2005). Some fungal polyketides are known for being

effective therapeutic agents, such as the cholesterol-lowering compound lovastatin, the antifungal agent griseofulvin, and the immunosuppressive drug mycophenolic acid, or for being food-spoiling toxins or virulence factors of pathogens (HERTWECK, 2009).

2.2.3 Polyketide biosynthesis

Polyketides are produced by a sequential set of reactions and are synthesized by PKSs, a large group of multifunctional enzymes. The carbon skeleton is synthesized by decarboxylative condensation of short-chain carboxylic acids, usually acetyl coenzyme A (acetyl-CoA) and malonyl-CoA, in a stepwise fashion. The carbon skeleton can be further modified depending on the PKS (GOKHALE; SANKARANARAYANAN; MOHANTY, 2007).

PKSs are classified by the number of polypeptide chains (single or multiple) in their structure and by the mechanism of synthesis (modular or iterative). Type I and type II PKSs are multienzyme complexes. In type I PKSs (single protein), all the functional domains involved in one cycle are on a single polypeptide chain, whereas in type II PKSs (multiple proteins), these domains are dispersed as individual proteins. Type III PKSs are small homodimeric proteins distinct from the other two types. Type I PKSs have two distinct mechanisms of synthesis: modular or iterative. In the modular mechanism, the synthesis occurs in an assembly line manner, where each active site is used only once. In contrast, in the iterative mechanism, some active sites are used repeatedly (GOKHALE; SANKARANARAYANAN; MOHANTY, 2007).

Fungal polyketides are usually produced by iterative type I PKSs. These enzymes contain a minimum of three domains that are necessary for construction of the polyketide skeleton: β-ketoacyl synthase, acyl carrier protein (ACP) and acyl transferase (Table 2.2). In addition to the three minimal domains, fungal type I PKSs can contain additional domains like the reducing domains—ketoreductase, dehydratase or enoyl reductase; —among others (FRANDSEN, 2010; GOKHALE; SANKARANARAYANAN; MOHANTY, 2007; KEATINGE-CLAY, 2012). Type III PKSs are also found in fungi. These enzymes act iteratively and are independent of the ACP domain (SHEN, 2003).

Table 2.2 - Domains found in fungal iterative type I PKSs and their functions (FRANDSEN, 2010; KEATINGE-CLAY, 2012).

	Domain	Function
Minimal domains	Acyl transferase (AT)	Loads the starter, extender, and intermediate units
	β-ketoacyl synthase (KS)	Catalyzes the condensation reaction between the units
	Acyl carrier protein (ACP)	Holds the growing polyketide chain as a thioester
Reducing domains	Ketoreductase (KR)	Reduces β-ketone groups to hydroxyl groups
	Dehydratase (DH)	Reduces hydroxyl groups to enoyl groups
	Enoyl reductase (ER)	Reduces enoyl groups to alkyl groups
NR-PKS domains	ACP transacylase (SAT)	Loads the starter units
	Product template (PT)	Determines the folding pattern of the unreduced chain
	Thioesterase (TE)	Facilitates the release of the final product from the enzyme
	Thioester reductase (R)	Facilitates the release of the final product from the enzyme
Others	Methyltransferase (MT)	Transfers methyl groups to the growing chain
	Condensation (CON)	Facilitates condensation of the synthesized polyketide with other polyketides

Polyketide biosynthesis (general concept) proceeds by the following steps: 1) β-ketoacyl synthase is acylated; 2) acyl transferase selects an extender unit and loads it onto ACP; 3) the loaded ACP attaches to the acylated β-ketoacyl synthase, which catalyzes decarboxylative condensation to elongate the polyketide chain; 4) ACP transfers the β-ketoacyl intermediate to additional domains (if any) such as methyltransferase, ketoreductase, dehydratase, or enoyl reductase; 5) ACP then transfers the processed polyketide back to β-ketoacyl synthase and repeats the cycle until the chain becomes a substrate for chain-releasing enzymes/domains (Figure 2.2) (KEATINGE-CLAY, 2012). The resulting product may then be subjected to additional modifications by post-PKS modifying enzymes (RIX et al., 2002).

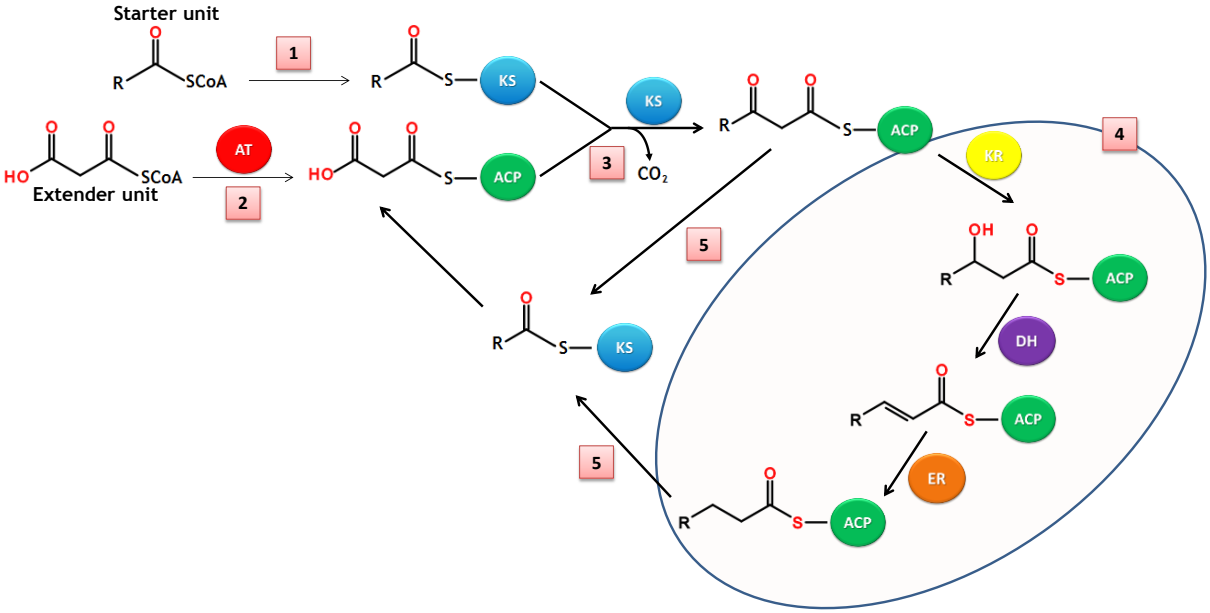

Figure 2.2 – Polyketide biosynthesis (general concept). 1: Loading of a starter unit. 2: Loading of an extender unit. 3: Condensation. 4: Reduction reactions (in reducing PKSs). 5) The cycle is repeated. Abbreviations are defined in Table 2.1. Adapted from Gokhale et al. (2007).

The reducing domains (ketoreductase, dehydratase, and enoyl reductase domains) can catalyze up to three levels of β-keto-reduction: ketoreduction, dehydration, and enoyl reduction. Depending on the absence or presence of these domains, and consequently, on the extent of β-keto-reduction, fungal type I PKSs are classified into nonreducing (NR), partially reducing (PR), or highly reducing (HR) PKSs. NR-PKSs do not contain any reducing domains, PR-PKSs lack only the ER domain, and HR-PKSs contain the three reducing domains. Some fungal PKSs also contain NRPS domains and are classified as PKS-NRPS hybrids (HERTWECK, 2009).

NR-PKSs produce mono- or polycyclic aromatic polyketides by cyclization of the unreduced reactive poly-β-keto chains. HR-PKSs produce a large variety of linear polyketides and cyclic nonaromatic polyketides. PR-PKSs are associated with production of 6-methylsalicylic acid—which requires single reduction of a keto group in a tetraketide intermediate—and with the synthesis of mellein, which requires reduction of a keto group in the diketide and tetraketide intermediates but not in a triketide and pentaketide (Figure 2.3) (CHOOI; TANG, 2012).

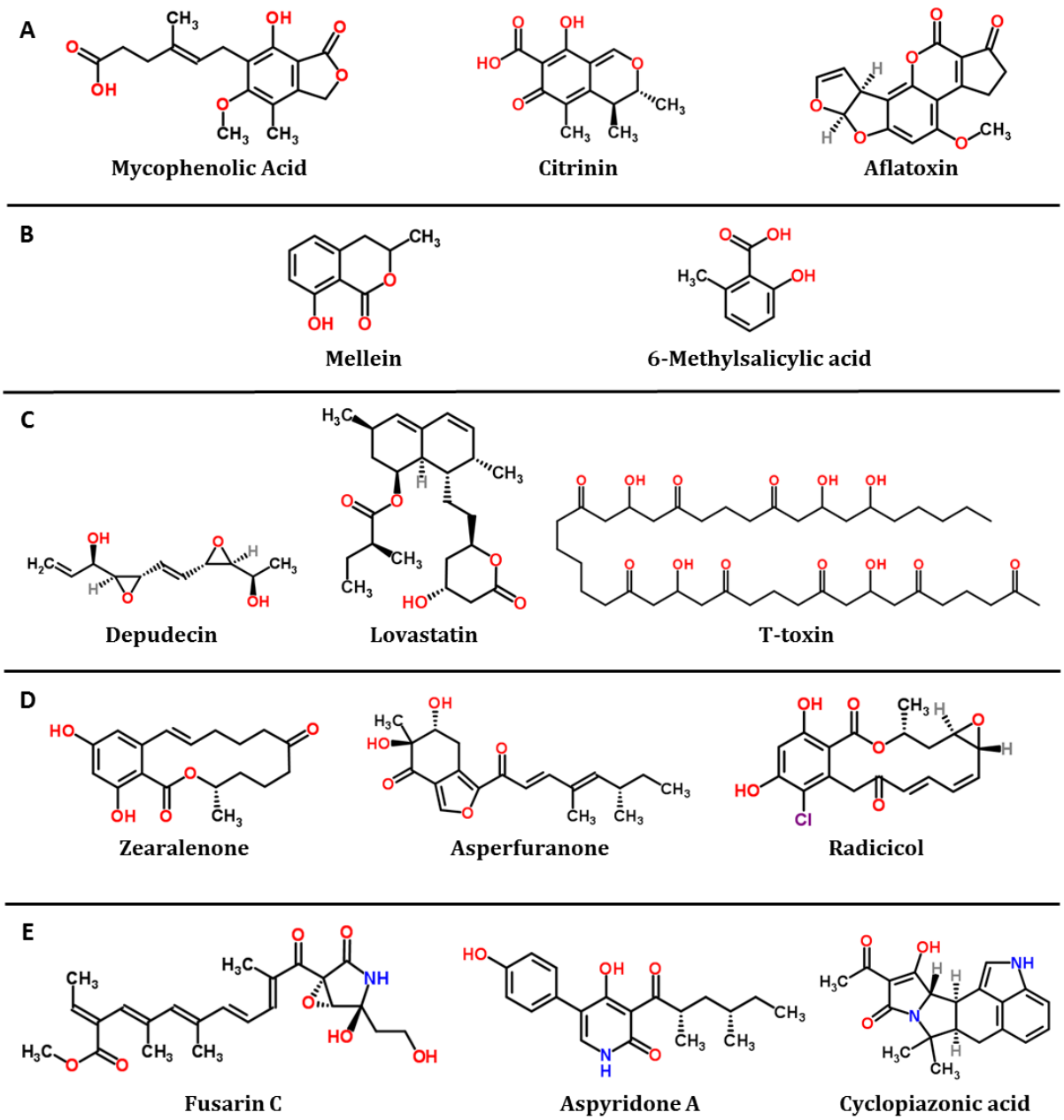

Figure 2.3 – Diverse chemical structures of fungal polyketides that have been linked to a known PKS-encoding gene. Polyketides (final product) produced by NR-PKSs (A), PR-PKSs (B), HR-PKSs (C), dual PKS systems composed of an NR-PKS and an HR-PKS (D), and PKS-NRPS hybrids (E). The structures were obtained or adapted from the free chemical structure database ChemSpider (Royal Society of Chemistry) available at www.chemspider.com.

NR-PKSs have additional domains not present in reducing PKSs: ACP transacylase (SAT), product template, and thioesterase domain. The SAT domain is responsible for loading a starter acyl unit onto the ACP domain, which then primes the β-ketoacyl synthase domain to initiate the first cycle of extension. The folding pattern of the cyclic polyketides synthesized by these enzymes is determined by the product template domain, immediately upstream of the ACP domain (CHOOI; TANG, 2012; CRAWFORD et

al., 2008). Most of NR-PKSs have a thioesterase-releasing domain that usually works as a Claisen cyclase (CLC, facilitates ring formation by means of a Claisen-type cyclization reaction) (FUJII et al., 2001).

HR-PKSs have a higher degree of complexity in their biosynthetic programming. The reducing domains are optionally used in every extension cycle; this situation means that the degree of reduction can vary among extension units. Therefore, reducing PKSs can generate a wide variety of polyketides that vary in the level of reduction, the pattern of reduction, and methylation of the polyketide backbone (HERTWECK, 2009). The mechanism of the programmed keto-reduction by PR-PKSs and HR-PKSs is believed to involve stereospecificity of the ketoreductase domain. This domain can distinguish and selectively reduce polyketide intermediates by substrate specificity (SOEHANO et al., 2014; SUN et al., 2012; ZHOU et al., 2012). Most HR-PKSs lack a releasing domain and require an additional releasing enzyme, often not identified (CHOOI; TANG, 2012).

Some fungal polyketides are biosynthesized by an HR-PKS and an NR-PKS located in the same gene cluster, each responsible for the synthesis of different parts of the molecule. The HR-PKS synthesizes a linear reduced polyketide as an advanced starter unit for the collaborating NR-PKS (CHOOI; TANG, 2012).

The mechanisms that control the chain length of polyketides are not understood completely. The β-ketoacyl synthase domain is believed to participate in the control of the chain length, which is probably limited by cavity size of the β-ketoacyl synthase domain (CHOOI; TANG, 2012; ZABALA et al., 2014). In NR-PKSs, the thioesterase domain can also participate in the control of chain length, intercepting a specific intermediate and releasing it from the PKS (WATANABE; EBIZUKA, 2004). In some HR-PKSs, a thiohydrolase enzyme encoded by a gene present in the PKS gene cluster controls the product chain length (ZABALA et al., 2014).

Fungal PKS-NRPS hybrids can incorporate an amino acid into the polyketide product. These megasynthases are composed of an HR-PKS containing a complete C-terminal NRPS module that includes condensation, adenylation, thiolation [or peptidyl carrier protein (PCP)], and thioester reductase/ Claisen cyclase domains (CHOOI; TANG, 2012).

2.2.4 Organization and regulation of fungal PKS clusters

In fungal genomes, the genes that participate in the production of a particular secondary metabolite are often clustered (KELLER; TURNER; BENNETT, 2005). Secondary-metabolite-related gene clusters are not constitutively expressed and their regulation depends on specific and global transcription factors. These gene clusters usually contain one or more genes encoding transcriptional factors that act specifically in the genes of this cluster. Their expression is also controlled by global transcription factors that are related to the response to environmental conditions (BRAKHAGE, 2013; YIN; KELLER, 2011).

PKS gene clusters also include genes encoding proteins that modify the newly synthesized polyketide, thereby increasing the chemical diversity. These enzymes are called post-PKS modifying enzymes and can be oxygenases, glycosyltransferases, methyltransferases, halogenating enzymes, acyl transferases, cyclases, or aminotransferases. In addition, PKS gene clusters can include genes encoding transporters and factors of self-resistance mechanisms (RIX et al., 2002).

2.2.5 Post-PKS modifying enzymes

Post-PKS modifying enzymes are a diverse group of enzymes that modify a polyketide after it is released from the PKS, thus leading to increased chemical diversity and complexity (Figure 2.4). These enzymes catalyze addition of key functional groups to the polyketide skeleton and therefore are important for the biological activity of polyketides. The most common post-PKS modifying enzymes are oxidoreductases and group transferases (Table 2.3) (OLANO et al., 2010; RIX et al., 2002).

Oxidoreductases are the most common type of post-PKS modifying enzymes. They introduce oxygen-containing functional groups, such as hydroxyl, epoxide, aldehyde, or keto groups, into the polyketide skeleton or modify these functional groups by addition or removal of hydrogen atoms. These modifications can provide a base for additional post-PKS modifications, such as group transfers. Oxidoreductases represent a different type of enzymes: oxygenases, oxidases, peroxidases, reductases, keto-reductases, and dehydrogenases (OLANO et al., 2010; RIX et al., 2002).

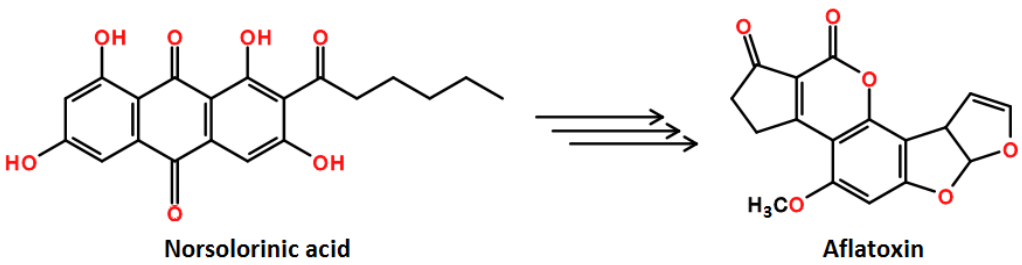

Figure 2.4 – An example of chemical complexity introduced by post-PKS modifying enzymes. Conversion of norsolorinic acid (first product) to aflatoxin B1 (final product, a highly rearranged polyketide) involves 12 steps (YABE; NAKAJIMA, 2004). The structures were obtained or adapted from the free chemical structure database ChemSpider (Royal Society of Chemistry) available at www.chemspider.com.

Table 2.3 – Post-PKS modifying enzymes and their functions (OLANO et al., 2010; RIX et al., 2002).

	Group	Functions
Oxidoreductases	Oxygenase	Inserts one oxygen atom (monooxygenase); Inserts two oxygen atoms (dioxygenase)
	Oxidase	Catalyzes insertion of oxygen or a dehydrogenation reaction (electron acceptor: molecular oxygen)
	Peroxidase	Catalyzes insertion of oxygen or a dehydrogenation reaction (electron acceptor: hydrogen peroxide)
	Reductase	Converts a ketone to a secondary alcohol; removes phenolic hydroxyl groups
	Dehydrogenase	Removes two protons and two electrons from a substrate; catalyzes reduction by addition of two protons and two electrons to a substrate
Group transferases	Glycosyltransferase	Transfers sugar moieties
	Methyltransferase	Transfers methyl groups
	Acyl transferase	Transfers acyl groups
	Aminotransferase	Transfers amino groups
Others types	Halogenating enzyme	Introduces halogen elements (most common is chlorine)
	Cyclase	Catalyzes cyclization

Group transferases catalyze the transfer of functional groups to the polyketide skeleton, introducing novel functional groups, such as a methyl group or a sugar moiety, the most common ones. Methyltransferases can methylate O, N, or C atoms in the polyketide chain and even on a sugar residue. Most of the sugars added by a glycosyltransferase belong to the 6-deoxyhexose family (OLANO et al., 2010; RIX et al., 2002).

Other types of post-PKS modifying enzymes are cyclase (uncommon in type I PKSs), which catalyzes cyclization, and halogenating enzymes, which introduce halogen elements into the carbon skeleton (OLANO et al., 2010; RIX et al., 2002).

2.2.6 PKS engineering

Several drugs on the market are of polyketide origin, for example, immunosuppressant and anticancer agents. Engineering of polyketide biosynthetic pathways with the aim of production of novel metabolites has been attracting great interest because this approach can expand the pool of promising polyketide drugs and allow for production of polyketide drugs with improved characteristics. Nevertheless, there is still a gap in the knowledge about some mechanisms underlying polyketide biosynthesis; thus, PKS engineering is still not established well (BALTZ, 2006; NEWMAN et al., 2014). Therefore, characterization of new proteins and new domains along with optimized tools can help to implement PKS engineering and the production of novel non-native polyketides with improved characteristics (DUNN; KHOSLA, 2013).

Some studies involving fungal type I PKSs have been showing good results in this field. Kakule et al. (2014) were able to produce non-native PKS-NRPS-synthesized hybrid compounds by combinatorialization and fusion of PKS genes responsible for production of chemically diverse polyketides. Another study involved an enzyme deconstruction approach: NR-PKSs were divided into mono- or multidomain fragments and recombined *in vitro*, with reconstitution of enzymatic activity. This approach allows researchers to redirect biosynthesis to non-native products (NEWMAN et al., 2014).

2.3 Epicolactone: a unique compound produced by *Epicoccum* species

2.3.1 Structure and bioactivity of epicolactone

Epicolactone is a polyketide with an antimicrobial activity, is produced by *Epicoccum* species, and has an unusual carbon skeleton (Figure 2.5). This compound was first isolated from *Epicoccum nigrum* P16 (an endophytic fungus of *S. officinarum* [sugarcane]), the strain used in the present study. The culture was cultivated for 60 d in potato broth plus yeast extract, and the molecule was isolated using successive

fractionations selected by means of a bioautography assay against *Staphylococcus aureus* (DA SILVA ARAÚJO et al., 2012). The same molecule was later isolated from *Epicoccum* sp., an endophytic fungus of *Theobroma cacao* (cacao tree), cultivated for 45 d in a rice medium (TALONTSI et al., 2013).

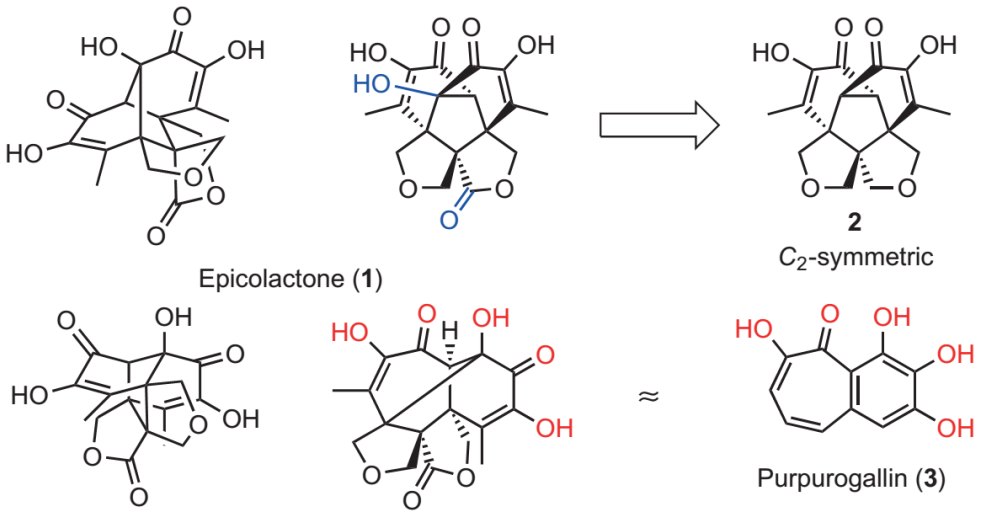

Figure 2.5 – Structure of epicolactone from different perspectives, revealing the C2-symmetric structure and its similarity to the compound purpurogallin. Source: Ellerbrock et al. (2015) (Reprinted by permission from Macmillan Publishers Ltd: Nature Chemistry, ref. Ellerbrock et al. 2015, copyright 2015).

Epicolactone shows an antimicrobial activity against the bacteria *Bacillus subtilis* and *Escherichia coli* although it is not active against *S. aureus*. Epicolactone also inhibits mycelial growth of three phytopathogens, the peronosporomycetes *Pythium ultimum* and *Aphanomyces cochlioides* and the basidiomycetous fungus *R. solani* (TALONTSI et al., 2013). The antimicrobial activity of epicolactone, just as that of other antimicrobial compounds produced by *Epicoccum* spp., may play an important role in protecting the host plant—such as the economically important crops cocoa tree and sugarcane—against phytopathogens.

The structure of epicolactone is composed of five interconnected rings that form a symmetrical carbon skeleton with five contiguous stereocenters, three of which are quaternary, making epicolactone a unique natural molecule (DA SILVA ARAÚJO et al., 2012; ELLERBROCK et al., 2015; ELLERBROCK; ARMANINO; TRAUNER, 2014; TALONTSI et al., 2013). Despite its high structural complexity, epicolactone was isolated

as a racemic mixture in both cases (DA SILVA ARAÚJO et al., 2012; TALONTSI et al., 2013).

2.3.2 Hypothetical biosynthesis of epicolactone and its precursors

The high degree of structural complexity of epicolactone has aroused a lot of interest in its synthesis. Da Silva Araújo et al. (2012) identified and analyzed three *E. nigrum* P16 mutants (obtained by random mutagenesis via *Agrobacterium tumefaciens*-mediated transformation) that lost the ability to inhibit the growth of pathogens: mutants P16-17, P16-47set, and P16-91. Chemical analysis of the fungal extracts showed that production of epicolactone is decreased in the mutants compared to the WT fungus and is absent in the P16-91 mutant. Production of two other compounds is also affected in the mutants: mellein showed increased production, and 4,5-dimethylresorcinol showed decreased production. In addition, the mutants produce a compound not produced by the WT fungus: 5-hydroxymellein. It is likely that regulation of the biosynthetic pathway is altered in the mutants thus activating a monooxygenase responsible for mellein oxidation (DA SILVA ARAÚJO et al., 2012). Mellein has antibacterial, antifungal, antialgal, and antiworm activities (CABRAS et al., 2006; HERZNER et al., 2013; RAMOS et al., 2013; WANG et al., 2014) and is found in fungi (ABOU-MANSOUR et al., 2015; MOORE; DAVIS; DIENER, 1972) and in insects (BLUM; FOOTTIT; FALES, 1992; WEISS; PARZEFALL; HERZNER, 2014). 4,5-Dimethylresorcinol was shown to inhibit rat intestinal Cl^- secretion (OGATA; SHIBATA, 2004).

Flavipin has been postulated to be a precursor of epicolactone because epicolactone contains the framework of one flavipin molecule (TALONTSI et al., 2013). Flavipin was first isolated from *Aspergillus* spp. and has a wide range of biological activities (BAMFORD; NORRIS; WARD, 1961; BURGE et al., 1976; MADRIGAL; TADEO; MELGAREJO, 1991; NITAO et al., 2002; RAISTRICK; RUDMAN, 1956). Pettersson (1965a, 1965b) suggested that flavipin is derived from orsellinic acid and 5-methylorsellinic acid in *A. flavipes*: two compounds isolated from the same culture as flavipin (Figure 2.6). The formyl groups in flavipin are produced by reduction of a carboxyl group and oxidation of a methyl group (PETTERSSON, 1965a, 1965b). Another study showed that methylation in 5-methylorsellinic acid occurs prior to cyclization, indicating that a 5-methylorsellinic

acid synthase (instead of an orsellinic acid synthase) is probably involved in the biosynthesis of flavipin (GATENBECK; ERIKSSON; HANSSON, 1969).

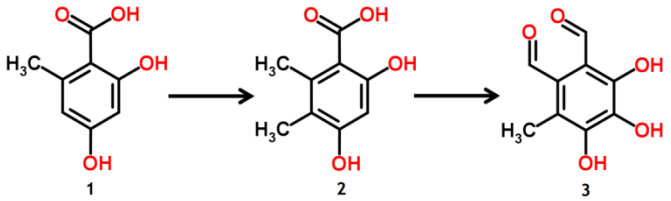

Figure 2.6 – Hypothetical biosynthesis of flavipin. 1: Orsellinic acid, 2: 5-methylorsellinic acid, 3: flavipin. Adapted from Pettersson (1965b).

Ellerbrock et al. (2014) noticed a pattern in epicolactone that could also be recognized in purpurogallin, a much simpler compound with known biosynthesis. By drawing a parallel to the purpurogallin biosynthesis, they suggested that epicolactone is biosynthesized through oxidative dimerization of epicoccone B and epicoccine: two polyketides previously isolated from *Epicoccum* spp. (KEMAMI WANGUN; ISHIDA; HERTWECK, 2008; LEE et al., 2007). Recently, the same research group designed biomimetic synthesis of epicolactone in only eight steps (ELLERBROCK et al., 2015). According to the hypothetical model of biosynthesis of epicolactone, first, epicoccone B undergoes hydrolysis and decarboxylation, and then both epicoccone B and epicoccine undergo oxidation. Their heterodimerization occurs via cycloaddition, and after a few intramolecular steps, the cascade is completed and epicolactone is produced (Figure 2.7) (ELLERBROCK et al., 2015).

Epicoccone B was identified in a strain of *E. nigrum* isolated from the fruit body of the tree fungus *P. squarrosa* (KEMAMI WANGUN; ISHIDA; HERTWECK, 2008). This compound is an isomer of epicoccone, a compound with potent antioxidant activity described in an algicolous marine strain of *Epicoccum* sp. (ABDEL-LATEFF et al., 2003). Epicoccone B has shown protein kinase inhibition (EL AMRANI et al., 2014). Epicoccine was first isolated from *Aspergillus terreus* (ISHIKAWA et al., 1996) and later from a fungicolous strain of *E. nigrum* (LEE et al., 2007). This compound is reported to be an antioxidant and potent lipoxygenase inhibitor (ISHIKAWA et al., 1996).

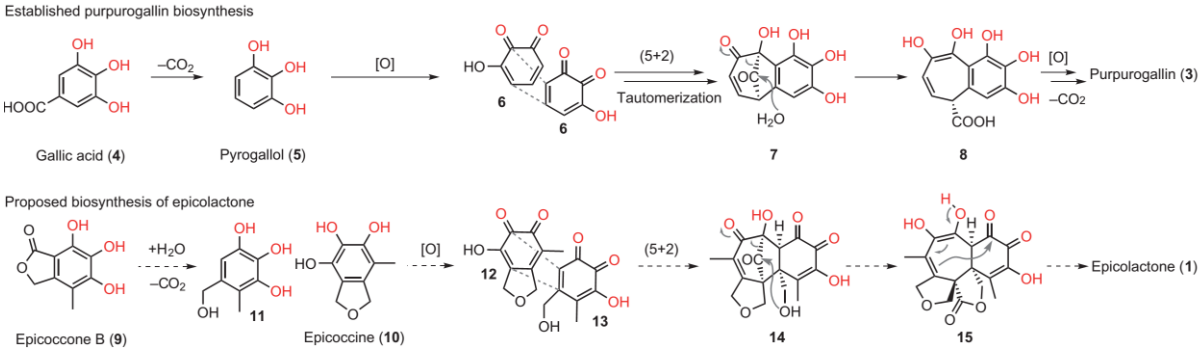

Figure 2.7 – The hypothetical model of biosynthesis of epicolactone and its comparison with the known biosynthetic pathway of purpurogallin. Source: Ellerbrock at al. (2015). (Reprinted by permission from Macmillan Publishers Ltd: Nature Chemistry, ref. Ellerbrock et al. 2015, copyright 2015).

On the basis of epicoccone B and epicoccine structures, Kemami Wangun et al. (2008) proposed that both compounds derive from orsellinic acid (Figure 2.8). The authors also mentioned that epicoccone B structure is reminiscent of mycophenolic acid (KEMAMI WANGUN; ISHIDA; HERTWECK, 2008). El Amrani et al. (2014) and Ellerbrock et al. (2014) suggested that both compounds are derived from flavipin.

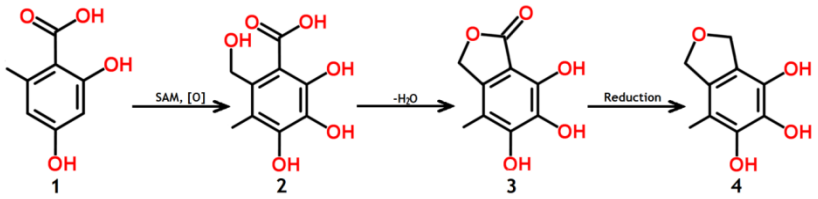

Figure 2.8 – A hypothetical model of biosynthesis of epicoccone B and epicoccine. 1: Orsellinic acid, 2: an intermediary, 3: epicoccone B, 4: epicoccine. SAM: S-adenosylmethionine. Adapted from Kemami Wangun et al. (2008).

Orsellinic acid is considered an archetypal phenolic polyketide because it is the simplest acetate-derived aromatic compound: only three condensation steps are required to obtain the polyacetate chain composed of eight carbons, which cyclizes to orsellinic acid without further modifications (GAUCHER; SHEPHERD, 1968; SCHROECKH et al., 2009). This metabolite was long known to be produced by lichens before it was first isolated from a fungal culture of *Chaetomium cochlioides* (STEYN, 1980). The PKS genes that synthesize orsellinic acid have been identified in *Aspergillus nidulans* (SCHROECKH et al., 2009), *Coprinopsis cinerea* (ISHIUCHI et al., 2012), *Armillaria mellea* (LACKNER et al., 2013), and *Fusarium graminearum* (JØRGENSEN et al., 2014). They

comprise a polyphyletic group with different domain architectures. The orsellinic acid PKSs from *F. graminearum* (PKS14) and *C. cinerea* (CC1G_05377) have one ACP domain, whereas the orsellinic acid PKSs from *A. nidulans* (orsA) and *A. mellea* have two (ISHIUCHI et al., 2012; JØRGENSEN et al., 2014; LACKNER et al., 2013; SCHROECKH et al., 2009).

Although there is a proposed biosynthesis of epicolactone, no *in vivo* study on epicolactone biosynthesis has been reported, and the genes involved have yet to be identified and characterized. Studies on epicolactone biosynthesis and the genes related can lead to the discovery of new gene functions, may facilitate genetic-engineering research and can expand the understanding of the ecological relationships of *Epicoccum* spp., such as the plant–endophyte relation and its antagonistic activity in the host plant.

3 ANALYSIS OF EPICOLACTONE-DEFICIENT *Epicoccum nigrum* MUTANTS P16-17, P16-47SET, AND P16-91 AND SCREENING OF A MUTANT LIBRARY

3.1 Introduction

Epicolactone is a polyketide with an antimicrobial activity, is produced by *Epicoccum* species, and has an unusual carbon skeleton. This compound was first isolated from *Epicoccum nigrum* P16 (an endophytic strain from *Saccharum officinarum* [sugarcane] leaves), using successive fractionations selected by means of a bioautography assay against *Staphylococcus aureus* (DA SILVA ARAÚJO et al., 2012). The same molecule was later isolated from an *Epicoccum* sp. (an endophytic strain from *Theobroma cacao* [cacao tree]) (TALONTSI et al., 2013) and showed an antimicrobial activity against the bacteria *Bacillus subtilis* and *Escherichia coli* and the phytopathogens *Pythium ultimum*, *Aphanomyces cochlioides* and *Rhizoctonia solani* (TALONTSI et al., 2013).

The structure of epicolactone is composed of five interconnected rings that form a symmetrical carbon skeleton with five contiguous stereocenters, three of which are quaternary (DA SILVA ARAÚJO et al., 2012; ELLERBROCK et al., 2015; ELLERBROCK; ARMANINO; TRAUNER, 2014; TALONTSI et al., 2013). Despite its high structural complexity, epicolactone was isolated as a racemic mixture in both cases (DA SILVA ARAÚJO et al., 2012; TALONTSI et al., 2013).

Ellerbrock et al. (2014) suggested that epicolactone is biosynthesized through oxidative dimerization of epicoccone B and epicoccine: two polyketides previously isolated from *Epicoccum* spp. (KEMAMI WANGUN; ISHIDA; HERTWECK, 2008; LEE et al., 2007). Recently, the same research group designed biomimetic synthesis of epicolactone in only eight steps (ELLERBROCK et al., 2015).

Three *E*. *nigrum* P16 mutants (obtained by random mutagenesis) that lost the ability to inhibit the growth of pathogens were previously identified by our research group (DA SILVA ARAÚJO et al., 2012). Chemical analysis of mutants P16-17, P16-47set, and P16-91 showed that production of epicolactone is decreased in the mutants compared to the WT fungus and is absent in the P16-91 mutant. The production of two other compounds is also affected in the mutants: mellein showed increased production, and 4,5-dimethylresorcinol showed decreased production. In addition, the mutants

produce a compound not produced by the WT fungus: 5-hydroxymellein. It is likely that regulation of the biosynthetic pathway is altered in the mutants thus activating a monooxygenase responsible for mellein oxidation (DA SILVA ARAÚJO et al., 2012).

E. *nigrum* P16 was isolated from sugarcane leaves and showed an antagonistic activity *in vitro* against sugarcane pathogens (FÁVARO; SEBASTIANES; ARAÚJO, 2012). The genome of this strain was sequenced, assembled, and annotated by our research group; these data allow for studies on genes possibly involved in epicolactone biosynthesis. Therefore, the aim of this study was to identify the genes that are affected by transfer DNA (T-DNA) insertion in the epicolactone-deficient mutants P16-17, P16-47SET, and P16-91. The expression of the genes located near the T-DNA insertion sites were analyzed by real-time PCR. The expression of two PKS genes (PKSi8 and PKSi9) identified in the sequenced genome that could be involved in epicolactone synthesis was also analyzed. PKSi8 and PKSi9 consist of a highly reducing PKS (HR-PKS) and a nonreducing PKS (NR-PKS), and their mRNA is transcribed divergently from a common region: gene structure similar to the resorcylic acid lactone (RAL) zearalenone cluster in *Fusarium graminearum* (KIM et al., 2005). PKSi8 and PKSi9 genes were considered as candidates because epicolactone has a lactone group, and its complex structure could be the result of action of two PKSs working together, each responsible for the synthesis of a portion of the molecule that could be further modified by tailoring enzymes. Therefore, these two PKSs were included in the bioinformatic and real-time PCR analyses.

3.2 Materials and Methods

The first step was to analyze and expand the mutant library previously constructed by Fávaro (2009) in order to identify more epicolactone-deficient mutants. The antagonistic activity of the mutants from the library was screened in order to identify mutants that had lost the ability to inhibit the growth of microorganisms and could have a deficient production of epicolactone. No mutants with a lack of antagonistic activity were identified. Thus, the experiments were performed on mutants P16-17, P16-47SET, and P16-91 described in the introduction [obtained and identified by Fávaro (2009) and Da Silva Araújo et al., (2012)]. The region of the T-DNA insertion site was then analyzed in order to identify the genes located near the transposon insertion sites (candidate genes to be involved in epicolactone biosynthesis). The gene expression of

the candidate genes was analyzed by real-time PCR. The culture medium from the samples used in the real-time PCR analysis was tested for antibacterial activity against *Bacillus* sp. and *S. aureus* in order to compare the antibacterial activity of the mutants with that of the WT fungus

3.2.1 Strains, the mutant library, and culture conditions

E. nigrum P16 was previously isolated from sugarcane leaves by Fávaro et al. (2011) and maintained in distilled water at 4 °C. The mutant library was previously constructed by Fávaro (2009) by random insertional mutagenesis via *Agrobacterium tumefaciens*-mediated transformation using the binary vector pFAT-GFP (Fitzgerald et al., 2003) and was expanded using the same method, with small modifications (vegetative mycelium was used as starting material instead of conidia). The mutants were maintained on the potato-dextrose-agar (PDA) culture medium supplemented with hygromycin (50 $mg \cdot mL^{-1}$) in distilled water at 4 °C. Mutants P16-17, P16-47SET, and P16-91 were previously reported to have lost the ability to inhibit the growth of microorganisms (FÁVARO, 2009), and their metabolite extracts were chemically analyzed by Da Silva Araújo et al. (2012). The latter analysis revealed that these fungi show decreased or no production of epicolactone. The insertion site of T-DNA was previously identified by TAIL-PCR (Thermal Asymmetric Interlaced PCR) (FÁVARO, 2009). These mutants were found to be stable after 10 successive passages on PDA without hygromycin.

3.2.2 Analysis of the mutant library

The antagonistic activity of the mutants from the library was analyzed in order to identify mutants that had lost the ability to inhibit the growth of microorganisms. To test the antagonistic activity against the bacteria *E. coli*, *S. aureus*, *Bacillus* sp., *Pseudomonas aeruginosa* and the yeast *Candida albicans*, two methods were used: the agar block method and diffusion in semisolid agar. Dual-culture assays were utilized to test the antagonistic activity against the fungal phytopathogens *Fusarium oxysporum*, *Ceratocystis fimbriata*, *Ceratocystis paradoxa*, and *Colletotrichum* sp. (FÁVARO;

SEBASTIANES; ARAÚJO, 2012; ICHIKAWA et al., 1971; MARIANO, 1993; TRILLI et al., 1978). All the assays were performed in triplicate, and WT plates served as controls.

In the agar block method, mutant strains were cultured for 15 d on PDA at 28 °C. The bacterial and yeast cultures were prepared by inoculating isolated colonies into 10 mL of liquid media. The cultures were incubated at 37 °C with shaking at 100 rpm for 18 h. An aliquot of the cultures (50 µL) was spread on the respective solid medium for each bacterium/yeast, and agar plugs with a mutant were then transferred to these plates. The plates were incubated at 4 °C for 6 h (to allow metabolites to diffuse from *E. nigrum* agar plugs to the culture medium containing bacteria or yeast) and then were incubated at 37 °C. After 24 h, the inhibition halo was measured. The control consisted of transference of sterile PDA plugs to the plates containing bacteria or yeast.

In the method of diffusion in semisolid agar, agar plugs with a mutant were inoculated into PDA and incubated at 28 °C for 72 h. The bacterial and yeast cultures were prepared as in the agar block method, and an aliquot was transferred to a semisolid medium. The latter was poured over the mutant cultures, and the plates were incubated at 4 °C for 6 h to allow metabolites to diffuse, and then incubated at 28 °C for 24 h.

Dual-culture assays consisted of direct confrontation of a phytopathogen and a mutant in a solid medium. The mutants and pathogens were cultivated for 7 d on the PDA medium at 28 °C before the confrontation. Mutant agar plugs were transferred to the PDA medium 48 h before inoculation with pathogens. The phytopathogens were inoculated 5 cm away from the mutant colony center. The assays were performed in triplicate, and the plates were incubated at 28 °C.

No mutants with a lack of antagonistic activity were identified by this method. The experiments were performed on mutants P16-17, P16-47SET, and P16-91 [obtained and identified by Fávaro (2009) and Da Silva Araújo et al., (2012)].

3.2.3 Bioinformatic analysis

The region of the T-DNA insertion site in the mutants and the *PKSi8/PKSi9* dual gene cluster were analyzed and annotated using AUGUSTUS (STANKE; MORGENSTERN, 2005). The predicted protein sequences were compared with the UniProtKB/Swiss-Prot database of the National Center for Biotechnology Information

(http://www.ncbi.nlm.nih.gov/) using the BLASTP tool (ALTSCHUL et al., 1997). The protein domains were predicted with CDD (MARCHLER-BAUER et al., 2015). The primers used for real-time PCR analysis were designed in the Primer 3 software (Appendix A) (UNTERGASSER et al., 2012).

The amino acid sequences of 22 characterized fungal NR-PKSs and HR-PKS (that work in partnership and have known products described in the literature) were extracted from the NCBI database (http://www.ncbi.nlm.nih.gov/) (Appendix B). The sequences corresponding to the β-ketoacyl synthase domain of these proteins and of PKSi8 and PKSi9 were aligned using Muscle, and a phylogenetic tree was constructed using the neighbor-joining method with 1000 bootstrap replicates in the Molecular Evolutionary Genetics Analysis software, version 7.0 (MEGA7) (KUMAR; STECHER; TAMURA, 2016). *Caenorhabditis elegans* FAS (fatty acid synthase, a class of enzymes structurally and functionally related to PKSs) served as an external group.

A multiple alignment of the active sites of the C2H2-type zinc-finger domain of the hypothetical protein was implemented with the same type of domain as that in other fungal proteins by means of the MEGA software, version 4 (TAMURA et al., 2007).

3.2.4 Analysis of gene expression by real-time PCR

The WT strain and mutants were grown in potato broth plus 2% yeast extract in triplicate. The mycelia were collected during different growth periods (10 and 30 d), and RNA was extracted with the PureLink® RNA Mini Kit (Life Technologies). The extracted RNA was treated with DNase I (Invitrogen) and quantified using a NanoDrop. The cDNA was synthesized using the SuperScript III First-Strand System Kit for RT-PCR (Invitrogen) and subjected to real-time PCR with the Power SYBR Green PCR Master Mix (Life Technologies) on a StepOnePlus ™ Real-Time PCR System (Life Technologies). The *E*. *nigrum* β-tubulin gene served as an endogenous control. Relative gene expression was calculated as $\log_{10}[2^{-\Delta\Delta Ct}]$. Statistical analysis involved the *t* test in R software at 5% significance to evaluate statistical significance of the difference between the gene expression levels of the WT fungus and mutants.

3.2.5 Extraction and analysis of metabolites

The WT strain and mutants were grown in potato broth plus 2% yeast extract, and the culture filtrates were collected after different growth periods (10, 30, and 60 d) in triplicate. The metabolites were extracted using ethyl acetate as a solvent. The solvent and filtrate were mixed in the ratio 1:3 (v/v), agitated, and separated by means of a phase separation funnel. This step was performed three times. The aqueous phase was discarded, and the organic phase was concentrated in a rota-evaporator. The extracts were diluted to the final concentration of 100 $mg \cdot mL^{-1}$ in dimethylsulfoxide (DMSO) and tested for antimicrobial activity against *Bacillus* sp. and *S. aureus*. Aliquots of 20 µL were pipetted and placed on filter paper discs (6-mm diameter) located in plates with Mueller-Hinton agar inoculated with the bacteria. The controls consisted of DMSO (negative) and hygromycin (positive control). The plates were stored in a refrigerator for 6 h to allow the metabolites to diffuse and then incubated at 37 °C for 18 h. The inhibition zones were measured with a millimeter ruler and subjected to analysis of variance followed by Tukey's test at the significance level of 0.05 in the Sisvar software, version 5.6 (FERREIRA, 2011).

3.3 Results

3.3.1 Genomic context of the T-DNA insertion site in the epicolactone-deficient mutants

The genomic context of the T-DNA insertion site was analyzed in the epicolactone-deficient mutants in order to identify the genes that are potentially affected by the insertion (Figure 3.1). In the P16-47SET mutant, the T-DNA was inserted in an intergenic region between a putative laccase gene and a putative cytochrome P450 oxidoreductase gene. In the P16-17 mutant, the T-DNA was inserted close to a gene of a hypothetical protein that contains a zinc-finger with a C2H2-type domain. In the P16-91 mutant, the T-DNA was inserted downstream of a transcription factor gene. Because the T-DNA was inserted in intergenic regions, it is possible that the genes located near the insertion were affected. Accordingly, the genes located near the T-DNA insertion site were selected as candidates for involvement in epicolactone synthesis. The selected genes to be analyzed are shown in Table 3.1. The predicted proteins (the putative

transcription factor, laccase, and cytochrome P450) contain typical conserved domains and share a similarity with other characterized fungal proteins. The hypothetical protein has a zinc-finger of the C2H2 type with the CxxCx(12)Hx(6)H motif (Figure 3.2).

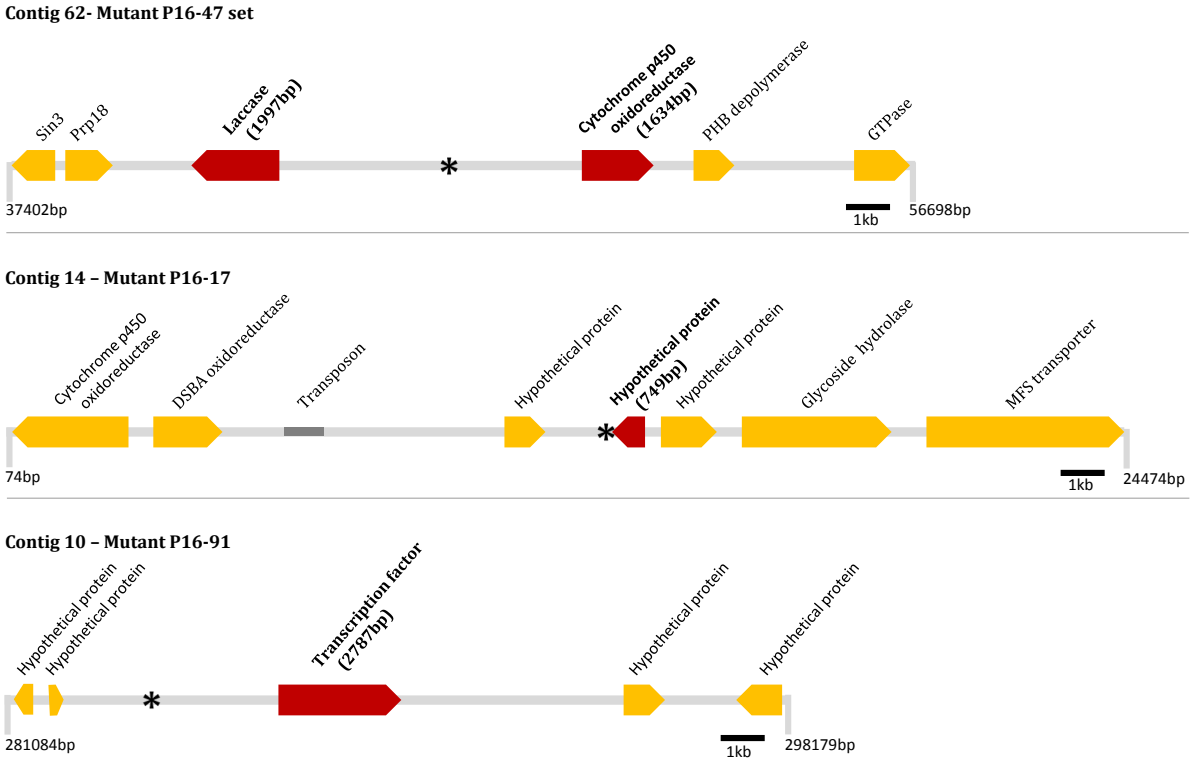

Figure 3.1 – The genomic context of the T-DNA insertion in the epicolactone-deficient mutants. The asterisk represents the T-DNA insertion site.

Table 3.1 - Annotation of the genes near the T-DNA insertion site in mutants P16-47set, P16-17, and P16-91.

Mutant	Predicted protein	ORF length (bp)	Protein length (aa)	Domains	Closest characterized protein homolog (E-value, Identity)
P16-47SET	Cytochrome P450	1634	450	CL12078: Cytochrome P450	Fumitremorgin C synthase: *Neosartorya fischeri* (A1DA63.1), 2e-39, 30%
P16-47SET	Laccase	1827	467	CD13854: The first cupredoxin domain of fungal laccases CL19115: Cupredoxin super family CD13901: The third cupredoxin domain of fungal laccases	Laccase: *Neurospora crassa* (P06811.3), 3e-120, 41%
P16-17	Hypothetical protein	591	196	PFAM12874: Zinc-finger of C2H2 type. Zinc-finger domain with the CxxCx(12)Hx(6)H motif *E-value=0.02	Secreted-in-xylem protein FOXB_02742: *Fusarium oxysporum* (EGU86733.1), 4e-11, 42%
P16-91	Transcription factor	2789	860	CD00202: Zinc-finger DNA-binding domain; binds specifically to a DNA consensus sequence [AT]GATA[AG] PFAM08550: A fungal protein of unknown function (DUF1752) CL20936: Cation channel sperm-associated protein subunit γ	Nitrogen regulatory protein area: *Aspergillus nidulans* (P17429.2), 1e-178, 44%

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Epicoccum nigrum                FNCAICDKGYPRQLDYENHLRSYDHNHR 28
Bipolaris victoriae             FNCAICDKGYPRQLDYENHLRSYDHNHR 28
Pyrenophora tritici-repenti     FKCTICSKDYSRQIEYENHLRSYDHNHR 28
Colletotrichum gloeosporioides  FYCSLCSKGYSRMNDYEAHLSSYDHSH- 27
Beauveria bassian               FYCALCAKGYTRMNDYEAHLGSYDHSH- 27
                                * *::* *.*.*  :** ** ****.*
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Figure 3.2 – Alignment of the zinc-finger C2H2-type domain of the hypothetical protein (*E. nigrum)* with other domains from fungal proteins. The highlighted regions indicate conserved residues of the CxxCx(12)Hx(6)H motif.

The genomic region near the T-DNA insertion site did not contain any PKS-encoding gene (indispensable for polyketide biosynthesis). The complete sequence of the contigs with the T-DNA insertions (contigs 10, 14, and 62) were also screened, but no PKS-encoding genes were identified there.

3.3.2 The PKSi8/PKSi9 *dual cluster*

Two PKS-encoding genes located in contig 52 (*PKSi8* and *PKSi9*), which are transcribed divergently from a common region (the same gene organization is observed in the resorcylic acid lactone [RAL] zearalenone), could be related to epicolactone biosynthesis and were also analyzed (Figure 3.4 and Figure 3.3). The domain architecture analysis of proteins PKSi8 and PKSi9 revealed that both contain the minimal domains necessary for polyketide biosynthesis: β-ketoacyl synthase, acyl carrier protein (ACP), and acyl transferase. In addition to the minimal domains, both PKSs contain a methyltransferase domain, which catalyzes the transfer of methyl groups to the growing polyketide chain. PKSi8 also contains three reducing domains: ketoreductase, dehydratase, and enoyl reductase. PKSi9 does not contain any reducing domain but includes unique domains of the NR-PKSs: ACP transacylase (SAT) and product template as well as a C-terminal thioester reductase domain, which catalyzes the release of the polyketide from a PKS. Thus, PKSi8 is classified as an HR-PKS, and PKSi9 is classified as an NR-PKS.

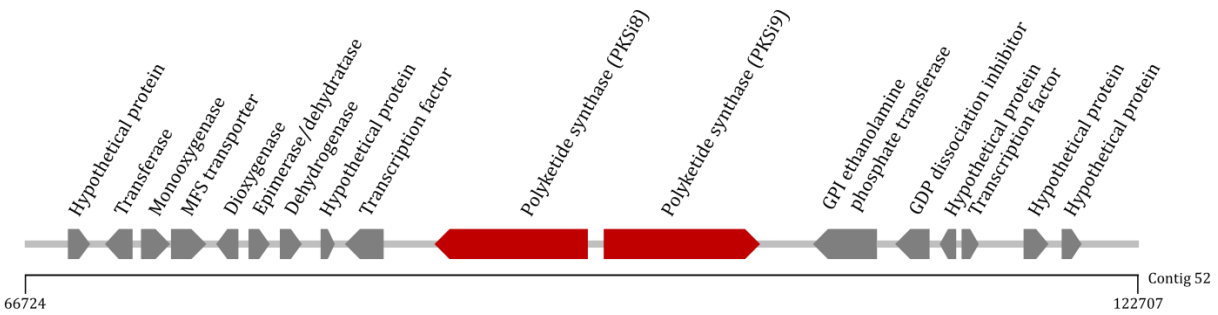

Figure 3.3 – Organization of the predicted PKSi8/PKSi9 gene cluster. Annotation was based on domains and close protein homologs.

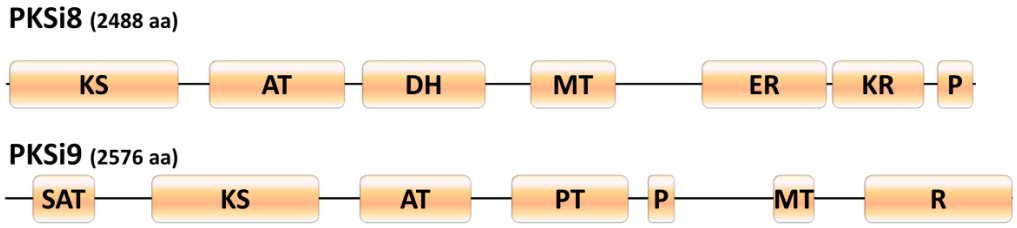

Figure 3.4 – Domain architecture of PKSi8 and PKSi9. KS: β-ketoacyl synthase, AT: acyl transferase, P: acyl carrier protein (ACP), MT: methyltransferase, DH: dehydratase, ER: enoyl reductase, KR: ketoreductase, SAT: ACP transacylase, PT: product template, R: thioester reductase.

Most fungal polyketides are synthesized by a single PKS, but some fungal polyketides are known to require an HR-PKS and an NR-PKS for their biosynthesis, such as RALs, asperfuranone, and azaphilones (CHIANG et al., 2009; GAFFOOR; TRAIL, 2006; ZABALA et al., 2012). Annotation of the gene cluster revealed the presence of a gene encoding typical post-PKS modifying enzymes, such as oxidoreductases and transferases, as well as a major facilitator superfamily (MFS) transporter and transcription factors.

The phylogenetic analysis using the β-ketoacyl synthase domains of the predicted proteins PKSi8 and PKSi9 and 22 characterized fungal PKSs that work in partnership showed that PKSi8 and PKSi9 were grouped with PKSs that synthesize classes of compounds other than RALs, such as azaphilones and asperfuranone (Figure 3.5).

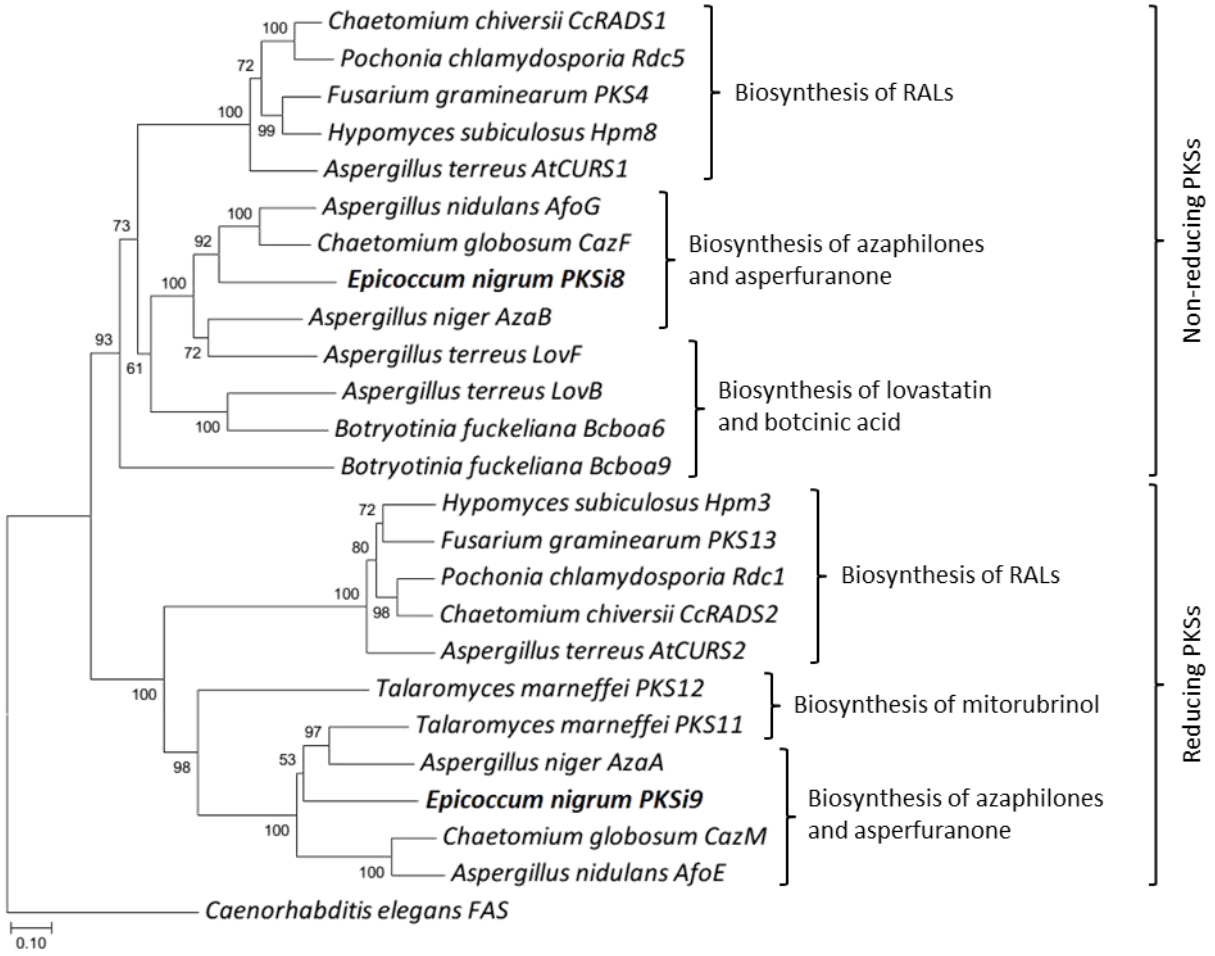

Figure 3.5 – Phylogenetic analysis of amino acid sequences of the β-ketoacyl synthase domain from *E. nigrum* PKSi8 and PKSi9 in comparison with 22 characterized fungal PKSs that work in partnership. Appendix B contains details of gene designations, their corresponding products, and references. Numbers at the nodes indicate bootstrap support based on 1000 replicates. *C. elegans* FAS served as an outgroup.

3.3.3 Gene expression analysis

This analysis by real-time PCR revealed that expression of the selected genes located near the T-DNA insertion site in the epicolactone-deficient mutants was not statistically significantly different from that in the WT fungus, except for the transcription factor, which was downregulated in the P16-17 mutant for a period of 10 d. In contrast, expression of genes *PKSi8* and *PKSi9* was downregulated according to most assays (Figure 3.6).

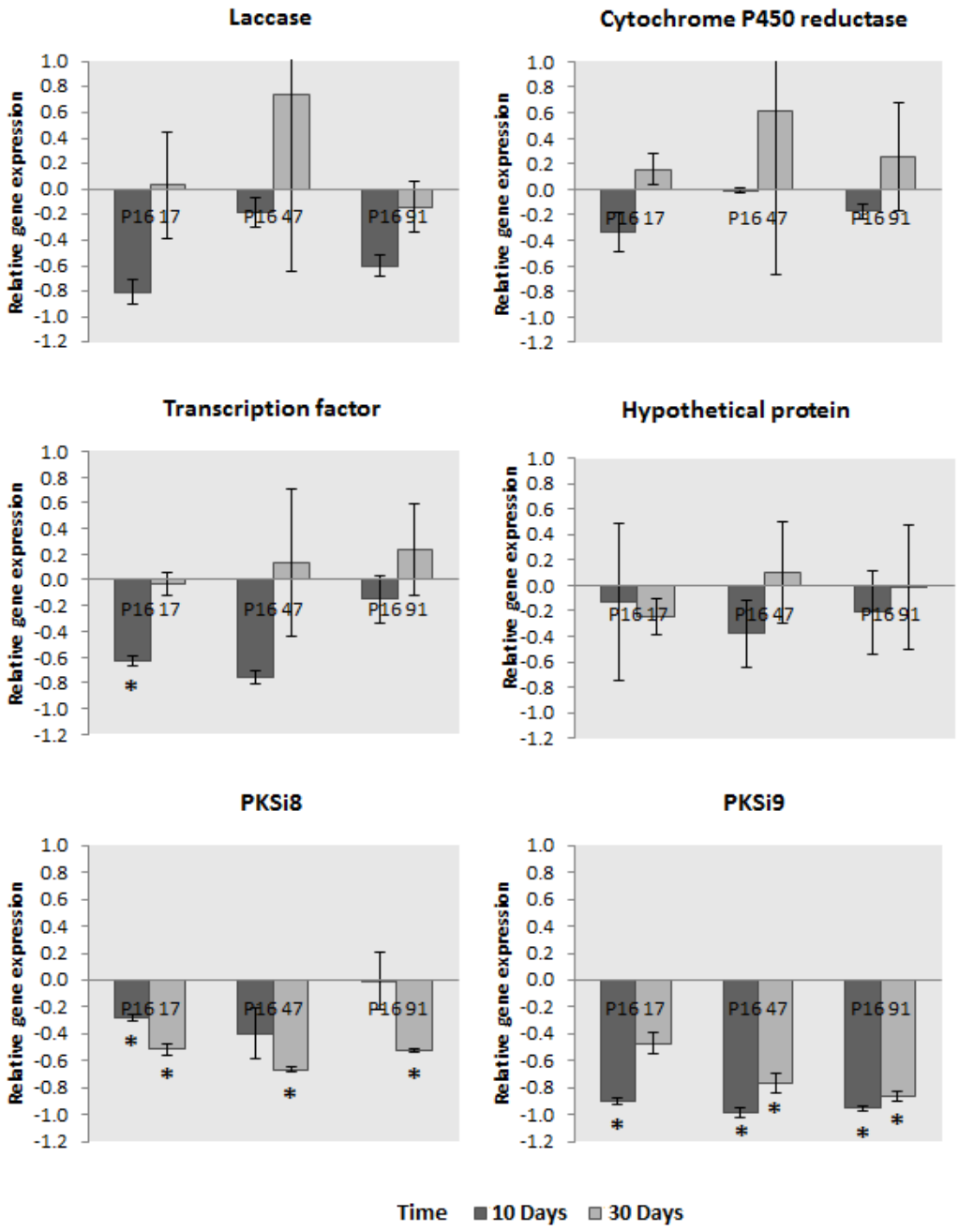

Figure 3.6 – Relative gene expression of the genes located near the T-DNA insertion site in the epicolactone-deficient mutants (laccase, cytochrome P450, a transcription factor, and a hypothetical protein) and the *PKSi8* and *PKSi9* genes. The gene expression was evaluated in two different periods: 10 d and 30 d. Relative gene expression (compared to a endogenous gene: a β-tubulin gene) is expressed as $\log_{10}[2^{-\Delta\Delta Ct}]$. Columns marked with an asterisk are statistically different from the WT ($p < 0.05$, T test).

3.3.4 Analysis of extracted metabolites

In order to collate the antimicrobial activity with gene expression, the culture filtrates from the WT and mutants were subjected to extraction and analyzed in antagonism assays against *Bacillus* sp. and *S. aureus* (Figure 3.7). No statistically significant difference was observed between the mutants and WT, except in the assay against *Bacillus* sp. with the 30-day-old extract, where the mutants yielded a significantly greater inhibition zone than the WT did.

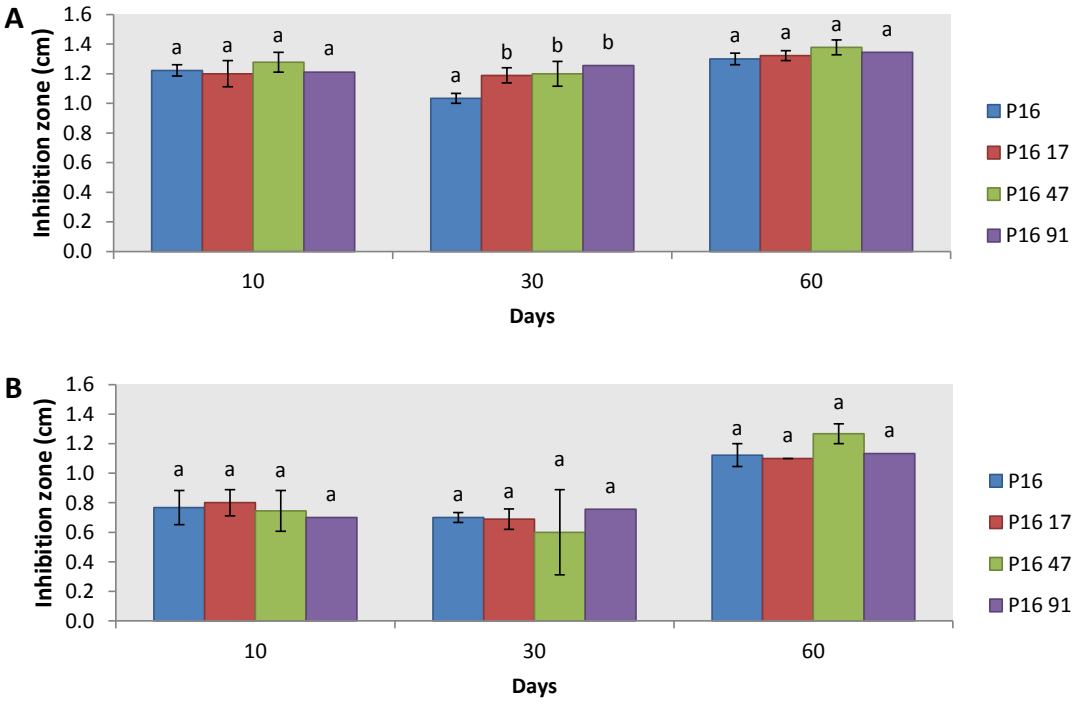

Figure 3.7 – The inhibition zone (cm) produced by ethyl acetate extracts of *E. nigrum* P16 (WT) and mutants P16-17, P16-47set, and P16-91 against *Bacillus* sp. (A) and *S. aureus* (B). In each group, columns marked with the same letter are not statistically significantly different ($p > 0.05$, Tukey's test).

3.4 Discussion

The very complex structure of epicolactone has been attracting great interest in the chemical-synthesis community (ELLERBROCK et al., 2015; ELLERBROCK; ARMANINO; TRAUNER, 2014; MERCER; BURNS, 2015). Although biomimetic synthesis of epicolactone was designed successfully, and a possible biosynthetic pathway was

proposed, the genes involved in the biosynthesis of epicolactone have yet to be discovered (ELLERBROCK et al., 2015).

A common method for analysis of gene function in fungi is preparation of mutant libraries by random insertional mutagenesis. The mutants are screened for a phenotype of interest, and the genomic region carrying the insert is identified by PCR methods, thus possibly leading to the discovery of new genes (SATYANARAYANA; JOHRI; PRAKASH, 2012).

Analysis of the genomic context of the T-DNA insertion in the epicolactone-deficient mutants showed that the insertion occurred in intergenic regions. Thus, the genes located near the insertion site were assumed to be candidates for involvement in epicolactone biosynthesis. It is possible that expression of the genes located near the insertion was affected, leading to decreased production of epicolactone in the mutants.

Laccases and cytochrome P450 oxidoreductases are enzymes usually involved in polyketide synthesis as post-PKS modifying enzymes and act on the polyketide chain after it is released from the PKS, thus causing further modifications and introducing additional chemical diversity into polyketides (RIX et al., 2002). Secondary-metabolite-related gene clusters are not constitutively expressed, and their regulation depends on transcription factors. Thus, transcription factors are also usually encountered in a PKS gene cluster and can control gene expression within this gene cluster. In addition, global transcription factors that are related to the response to environmental conditions are known to control the general gene expression associated with secondary metabolites in fungi (BRAKHAGE, 2013; YIN; KELLER, 2011).

In fungal genomes, the genes that participate in the production of a particular secondary metabolite are often clustered (KELLER; TURNER; BENNETT, 2005). Thus, in the present work, the contigs that contained the T-DNA insertion in the mutants were screened for PKS-encoding genes (a PKS is an essential enzyme in polyketide biosynthesis); however, no PKS-encoding gene was identified.

Real-time PCR analysis showed that in the mutants, the expression of the genes encoding the laccase, cytochrome P450 oxidoreductase, transcription factor, and hypothetical protein located near the T-DNA insertion sites was not significantly different from that in the WT. This result indicates that these genes are probably not involved in epicolactone biosynthesis. It is likely that the T-DNA was inserted in a

regulatory region and caused an alteration in the expression of genes not located near the insertion site.

The real-time PCR analysis revealed that PKSi8 and PKSi9 were downregulated. It is possible that the T-DNA was inserted in a small-RNA-encoding region. The *E*. *nigrum* transcriptome was also sequenced by our research group (FERREIRA, 2016); accordingly, a BLAST search was performed, which showed some small sequences of RNA (maximum of 75 nucleotides) with 100% identity to sequences in the intergenic regions.

The culture medium from the samples used in the real-time PCR analysis was tested for antibacterial activity against *Bacillus* sp. and *S. aureus* in order to compare the antibacterial activity of the mutants with that of the WT fungus; this activity could be due to epicolactone production. No statistically significant difference was observed between the mutants and WT, except in the test against *Bacillus* sp. with the 30-day-old extract. In this assay, the mutants produced a larger inhibition zone than the WT did, which could be due to production of another antimicrobial compound.

As mentioned in the chemical analysis of the mutants by Da Silva Araújo et al. (2012), mutants P16-17, P16-47set, and P16-91 show increased production of mellein. The latter has antibacterial, antifungal, antialgal, and antiworm activities (CABRAS et al., 2006; HERZNER et al., 2013; RAMOS et al., 2013; WANG et al., 2014). Although the mutants show decreased or no production of epicolactone, it is likely that they overexpress mellein, which could have caused the antibacterial action in the analysis of metabolites.

3.5 Conclusions

It is likely that the T-DNA was inserted in a regulatory region in the epicolactone-deficient mutants, such as a small-RNA-encoding region. This regulatory region could be related to the expression of PKSi8 and PKSi9 genes since these genes were downregulated in the mutants. It is possible that genes PKSi8 and PKSi9 participate in epicolactone biosynthesis.

4 GENOME MINING IN *Epicoccum nigrum* REVEALS A GENE CLUSTER ASSOCIATED WITH ANTAGONISTIC ACTIVITY AND PROBABLY INVOLVED IN EPICOLACTONE BIOSYNTHESIS

4.1 Introduction

Epicoccum nigrum is a ubiquitous ascomycete fungus that colonizes various types of soils and plants (PITT; HOCKING, 2012; SCHULZ; BOYLE, 2005). It is mainly known for its potential use as a biocontrol agent and for its ability to produce many bioactive compounds. *E. nigrum* P16, the endophytic strain used in this study, was isolated from sugarcane (*Saccharum officinarum*) leaves (FÁVARO et al., 2011). This strain has shown *in vitro* antagonistic activity against sugarcane phytopathogens, indicating that it probably acts as a natural antagonist in sugarcane tissues. Analysis of the endophytic interaction with sugarcane revealed that *E. nigrum* P16 is capable of increasing the root system biomass (FÁVARO; SEBASTIANES; ARAÚJO, 2012).

E. nigrum can inhibit the growth and conidial germination of many fungi and oomycetes and has been studied regarding biocontrol of phytopathogens such as *Pythium* spp. (HASHEM; ALI, 2004), *Rhizoctonia solani* (LAHLALI; HIJRI, 2010), *Phytophthora infestans* (LI et al., 2013), and *Sclerotinia sclerotiorum* (PIECKENSTAIN et al., 2001). The mechanisms of inhibition of fungal-phytopathogen growth by *E. nigrum* have been investigated, and studies on the biocontrol of *R. solani* and *P. infestans* have shown that *E. nigrum* causes malformation and lysis of phytopathogen hyphae, suggesting that *E. nigrum* likely secretes compounds that affect cell wall synthesis or membrane permeability (LAHLALI; HIJRI, 2010; LI et al., 2013).

Many secondary metabolites produced by *E. nigrum* have been isolated and characterized, and several of these metabolites were found to have diverse biological activities, such as antimicrobial, antioxidant, and anticancer as well as inhibition of viral replication (BAMFORD; NORRIS; WARD, 1961; BROWN; FINLAY; WARD, 1987; BURGE et al., 1976; DA SILVA ARAÚJO et al., 2012; EL AMRANI et al., 2014; ISHIKAWA; ITO; LEE, 1996; KEMAMI WANGUN; ISHIDA; HERTWECK, 2008; LEE et al., 2007; MADRIGAL; TADEO; MELGAREJO, 1991; PENG et al., 2012; SHU et al., 1997; TALONTSI et al., 2013).

Among the bioactive compounds produced by *E. nigrum*, epicolactone is a polyketide with an unusual carbon skeleton (DA SILVA ARAÚJO et al., 2012). Epicolactone has antimicrobial activity against the bacteria *Bacillus subtilis* and

Escherichia coli and against phytopathogens *Pythium ultimum*, *Aphanomyces cochlioides*, and *R. solani* (TALONTSI et al., 2013). The structure of epicolactone is composed of five interconnected rings that form a symmetrical carbon skeleton with five contiguous stereocenters, three of which are quaternary (DA SILVA ARAÚJO et al., 2012; ELLERBROCK et al., 2015; ELLERBROCK; ARMANINO; TRAUNER, 2014; TALONTSI et al., 2013).

Ellerbrock et al. (2014) noticed a pattern in epicolactone that could also be recognized in purpurogallin, a much simpler compound with known biosynthesis. By drawing a parallel to purpurogallin biosynthesis, they proposed that epicolactone is biosynthesized through oxidative dimerization of epicoccone B and epicoccine, two polyketides previously isolated from *Epicoccum* spp. (KEMAMI WANGUN; ISHIDA; HERTWECK, 2008; LEE et al., 2007). Recently, the same research group designed biomimetic synthesis of epicolactone in only eight steps (ELLERBROCK et al., 2015). Nonetheless, there are no *in vivo* studies on epicolactone biosynthesis, and the genes involved have yet to be identified.

Fungal polyketides are usually produced by iterative type I PKSs. These enzymes contain a minimum of three domains that are necessary for construction of the polyketide skeleton: β-ketoacyl synthase, acyl carrier protein (ACP), and acyl transferase. In addition to the three minimal domains, fungal type I PKSs contain other domains, such as the reducing domains ketoreductase, dehydratase, and enoyl reductase (GOKHALE; SANKARANARAYANAN; MOHANTY, 2007; KEATINGE-CLAY, 2012).

On the basis of the domain architecture, fungal type I PKSs are classified as nonreducing (NR), partially reducing (PR), or highly reducing (HR) PKSs. NR-PKSs do not contain any reducing domains, PR-PKSs lack only the enoyl reductase domain, and HR-PKSs contain the three reducing domains (HERTWECK, 2009). NR-PKSs produce mono- or polycyclic aromatic polyketides by cyclization of the unreduced reactive poly-β-keto chains. HR-PKSs produce a large variety of linear polyketides and cyclic nonaromatic polyketides. PR-PKSs are associated with the synthesis of 6-methylsalicylic acid and mellein (CHOOI; TANG, 2012).

NR-PKSs have additional domains not present in reducing PKSs: ACP transacylase (SAT), product template, and thioesterase domain. The SAT domain is responsible for loading a starter acyl unit onto the ACP domain, which then primes the β-ketoacyl synthase domain to initiate the first cycle of extension. The folding pattern of the cyclic

polyketides synthesized by these enzymes is determined by the product template domain (CHOOI; TANG, 2012; CRAWFORD et al., 2008). Most of the NR-PKSs have a thioesterase-releasing domain (FUJII et al., 2001). After the release from the PKS, the polyketide can be further modified by tailoring enzymes, usually oxidoreductases and group transferases, leading to increased chemical diversity and complexity (OLANO et al., 2010; RIX et al., 2002).

The genome of *E*. *nigrum* P16 was sequenced and was assembled and annotated by our research group; these data allow for studies of genes and possible metabolic pathways related to biosynthesis of secondary metabolites. The aim of the present study was to explore genes that may be involved in the biosynthesis of epicolactone by means of a genome-mining approach. Here, the function of five genes encoding NR-PKSs and one gene encoding for an HR-PKS identified in the *E*. *nigrum* P16 genome were studied.

4.2 Materials and Methods

4.2.1 Bioinformatic analysis

PKS gene clusters were identified using antiSMASH 3.0 with default parameters (WEBER et al., 2015). Genes were analyzed and annotated using AUGUSTUS (STANKE; MORGENSTERN, 2005) and FGENESH+ (SOLOVYEV, 2008). The predicted protein sequences were compared with the nonredundant protein database of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) using the BLASTP algorithm (ALTSCHUL et al., 1997). The protein domains were predicted with Pfam 29.0 (FINN et al., 2016) and CDD (MARCHLER-BAUER et al., 2015) at an E-value cutoff of 1e-3.

The primers used for amplification of homologous recombination sequence (HRS) and for verification of the mutants were designed using Vector NTI Advance® 11 (Life technologies) (Appendix C). The amino acid sequences of 62 fungal PKSs with known products described in the literature were extracted from the NCBI database (http://www.ncbi.nlm.nih.gov/) (Appendix D). The sequences corresponding to the β-ketoacyl synthase domain were aligned using Muscle, and a phylogenetic tree was constructed by the neighbor-joining method with 1000 bootstrap replicates in the

Molecular Evolutionary Genetics Analysis software, version 7.0 (MEGA7) (KUMAR; STECHER; TAMURA, 2016).

4.2.2 Strains and culture conditions

E. nigrum P16 was previously isolated from sugarcane leaves by Fávaro et al. (2011) and was maintained in distilled water at 4 °C. *E. coli* DH5α cells were used for cloning and maintenance of the vectors used for transformation. Chemically competent *E. coli* DH5α cells were prepared as described by Frandsen et al. (2012). *Agrobacterium tumefaciens* LBA4404 was used for transformation.

4.2.3 Construction of PKS mutants

4.2.3.1 Construction of the vectors

The vectors for targeted gene replacement by homologous recombination were constructed according to the USER-Brick vector construction system strategy (SØRENSEN et al., 2014) (Appendix E). HRSs (1500 bp upstream and downstream of the target gene, unique for each target gene) and core USER-bricks (vector backbone and selection markers) were amplified using X7 DNA polymerase in 50 µL reactions with the Phusion HF buffer (New England Biolabs) using primers with the respective overhangs necessary for the USER cloning strategy. HRSs were amplified from purified genomic DNA of *E. nigrum* P16, and core USER-bricks were amplified from purified plasmid DNA. The following PCR program was used: 98 °C for 10 min, 35 cycles (98 °C for 20 s, 60 °C for 20 s, and 72 °C for 2 min), and 72 °C for 5 min. The amplicons were gel-purified using the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare).

For USER cloning reactions, 1 µL of each purified USER-brick and 2 µL of the required HRSs were mixed with 1 µL of the USER Enzyme Mix (New England Biolabs) and 1.1 µL of 10× Taq DNA polymerase buffer (Sigma-Aldrich) in a total volume of 11 µL. The reactions were allowed to proceed in a thermal cycler for 25 min at 37 °C, followed by 25 min at 25 °C. The full volume of the reaction was then used for heat shock transformation of 50 µL of a suspension of chemically competent *E. coli* DH5α cells, according to the protocol described by Sambrook and Russel (2001). The resulting *E.*

coli transformants were screened by PCR (using gene-specific primers) and by restriction enzyme digestion. Preparation of electrocompetent *A*. *tumefaciens* cells and electroporation of *A*. *tumefaciens* were performed as described by Frandsen et al. (2012).

4.2.3.2 *A. tumefaciens*-mediated transformation (ATMT)

This procedure was performed on the basis of the protocols described by Frandsen et al. (2012) and Fávaro (2009) with modifications. An isolated colony of *A*. *tumefaciens* LBA4404 pRF-HU2F::ΔPKS*x* was used to inoculate 10 mL of the LB medium (Luria-Bertani broth, Difco™) supplemented with 50 μg·mL^{-1} kanamycin, and the mixture was incubated at 28 °C, with shaking at 200 rpm. After 24 h, aliquots of 100, 200, and 300 μL were used to inoculate 10 mL of the induction medium (10 mM K_2HPO_4, 10 mM KH_2PO_4, 2.5 mM NaCl, 2 mM $MgSO_4$, 0.7 mM $CaCl_2$, 9 μM $FeSO_4$, 4 mM NH_4SO_4, 10 mM glucose, 0.5% glycerol, 40 mM MES, and 500 μM acetosyringone). The diluted cultures were reincubated (28 °C, 100 rpm) and monitored until the optical density at 600 nm reached 0.5–0.7 in one of the cultures. *E*. *nigrum* P16 colonies grown over paper filter circles on PDA plates for 72 h were transferred to a coculture medium (same as the induction medium, but solid) and inoculated with 50 μL of induced *A*. *tumefaciens* over each colony. After 72 h at 25 °C, the paper filter circles containing the colonies were transferred to the PDA medium supplemented with 50 μg·mL^{-1} hygromycin (to select mutants) and 300 μg·mL^{-1} cefoxitin (to eliminate *A*. *tumefaciens*) and incubated for 7 d. Mutants were isolated on PDA supplemented with 50 μg·mL^{-1} hygromycin and screened as described in the next section. Three controls were prepared: a control without *A*. *tumefaciens*, a control without the fungus, and a control without the fungus and *A*. *tumefaciens*.

4.2.3.3 Verification of the mutants

The resulting mutants were analyzed by colony PCR using four primer pairs: 1) primers Hyg588U/Hyg588L to check the presence of the selection marker (hygromycin resistance gene) and confirm that the obtained DNA is suitable for PCR; 2) primers T1/T2 to verify the loss of the target gene (annealing at the center of the coding

sequence); 3) primers T3 in combination with the generic primer RF-1 to verify correctness of the crossover in the left flank; 4) primers T4 in combination with the generic primer RF-2, to verify correctness of the crossover in the right flank.

Southern blot analyzes were performed to ensure that only a single copy of the introduced DNA was present in the genome of the mutants. Genomic DNA (10 μg) was digested with HindIII Fast Digest (Fermentas), and the fragments were separated on an agarose gel and transferred to a nylon membrane (Amersham Hybond-N+, GE Healthcare Life Sciences), according to the manufacturer's instructions. Hybridization was carried out according to the instructions supplied with the Roche DIG High Prime DNA Labeling and Detection Starter Kit I (Roche) with a probe of 588 bp corresponding to a portion of the hygromycin resistance gene.

4.2.3.4 Purification of the mutants

Selected mutants were purified by preparation of protoplasts. Fungi were grown in PDB (potato dextrose broth: infusion of 200 g of potato and 20 g of dextrose per liter) at 28 °C, with agitation (150 rpm) for 3 d. Approximately 300 mg of mycelia was collected and transferred to tubes containing 5 mL of the osmotic stabilizer (1.2 M KCl) supplemented with 15 $mg \cdot mL^{-1}$ Glucanex (Lysing Enzymes from *Trichoderma harzianum*, Novozymes). The hydrolytic preparation was incubated at 30 °C with agitation, and after 4 h, it was filtered through Miracloth. The protoplasts were centrifuged at 710 × *g* for 10 min, resuspended in 5 mL of a stabilizer, centrifuged again, and finally resuspended in 1 mL of the stabilizer. The protoplasts were then regenerated on PDA containing 1.2 M KCl and 50 $mg \cdot mL^{-1}$ hygromycin. The regenerated colonies were isolated, and after culturing of the mutants four consecutive times on PDA in the absence of hygromycin; their genomic DNA was extracted and analyzed again by colony PCR.

4.2.4 Characterization of PKS mutants

4.2.4.1 Mycelial growth on different media

The growth of *E*. *nigrum* (WT and PKS mutants) was analyzed on four media: oatmeal agar (60 g of oatmeal and 12 g of agar per liter), Sabouraud agar (Difco™), sugarcane agar (infusion of 50 g of sugarcane leaves and 15 g of agar per liter) and PDA (Difco™). Agar plugs (7 mm in diameter) from the actively growing edge of a 7-day-old culture on PDA were inoculated into the center of the plates. The assay was performed in triplicate. The diameter of each growing mycelial colony was measured every 4 d in two perpendicular directions until some of the colonies completely covered the plate.

4.2.4.2 Growth-inhibitory action on microorganisms

The growth-inhibitory activity of *E*. *nigrum* (WT and PKS mutants) was analyzed toward the following microorganisms: *R. solani*, *F. verticillioides*, *Colletotrichum falcatum*, *Colletotrichum* sp., *Ceratocystis paradoxa*, *Fusarium oxysporum*, *S. sclerotiorum*, *Phytophthora cinnamomi*, *Candida albicans*, *X. albilineans*, *Bacillus* sp., *E. coli*, and *Staphylococcus aureus*.

Dual-culture assays, which consist of direct confrontation of a pathogen and antagonist in a solid medium, were performed in order to test the antagonistic activity against fungi and oomycetes (MARIANO, 1993). *E*. *nigrum* strains (WT and PKS mutants) and the pathogens were cultivated for 7 d on the PDA medium at 28 °C before the confrontation. *E*. *nigrum* agar plugs (7 mm in diameter) were transferred to the PDA medium 72 h before inoculation with the pathogens. For the assays using *C*. *paradoxa* (a rapidly growing pathogen), *E*. *nigrum* strains were transferred 120 h beforehand. The pathogens were inoculated 5 cm away from the *E*. *nigrum* colony center. The control consisted of the pathogen inoculated without *E*. *nigrum* colonies. The assays were performed in triplicate, and the plates were incubated at 28 °C until the PDA medium in the control was completely covered with the pathogen's mycelia. The inhibition zone and the radial mycelial growth of the pathogen towards *E*. *nigrum* were measured. The percentage of inhibition of a pathogen's growth was calculated in relation to the control.

Growth inhibition in bacteria and yeast was evaluated by the agar block method (ICHIKAWA et al., 1971). *E*. *nigrum* strains were cultured for 15 d on PDA at 28 °C. The bacterial and yeast cultures were prepared by inoculating isolated colonies into 10 mL of PDB for *C*. *albicans*, nutrient broth (Difco™) for *X*. *albilineans*, TSB (tryptic soy broth, Difco™) for *Bacillus sp.*, and LB broth for *S*. *aureus* and *E*. *coli*. The cultures were

incubated at 37 °C or 28 °C with shaking at 100 rpm for 18 or 24 h, in the case of *X. albilineans* (slow growth). An aliquot of the cultures (50 µL) was spread on the respective solid medium for each bacterium/yeast, and *E. nigrum* agar plugs (7 mm in diameter) were then transferred to these plates. The plates were incubated at 4 °C for 6 h to allow the metabolites to diffuse from *E. nigrum* agar plugs to the culture medium containing bacteria/yeast, and then, the plates were incubated at 37 °C or 28 °C. After 24 h, the inhibition halo was measured. The control consisted of transference of the PDA plugs to the plates containing bacteria/yeast. The assays were performed in triplicate.

4.2.4.3 Statistical analyses

Data were expressed as the rate of inhibition (%) of mycelial growth of the pathogen and as inhibition zone size (in centimeters) in the antagonism assays. The inhibition rate was calculated as $[(G_c - G_p)/G_c \times 100]$, where G_p is the growth of a pathogen towards *E. nigrum*, and G_c is the growth of the pathogen on the control plate. In the mycelial-growth assays, the data were expressed as the average diameter (cm) and growth rate ($cm \cdot d^{-1}$). The growth rate was calculated by linear regression. The data were subjected to analysis of variance followed by Tukey's test at a significance level of 0.05 using the Sisvar software, version 5.6 (FERREIRA, 2011).

4.2.4.4 Chemical analysis of the PKS mutants

E. nigrum strains (WT and PKS mutants) were inoculated with three agar plugs on PDA and grown for 7 d. The metabolites were extracted according to the microscale method (SMEDSGAARD, 1997). Three agar plugs (7-mm diameter) from both new and old parts of the culture were transferred to a 2-mL glass tube and mixed with isopropanol:ethyl acetate (1:3, v/v) in 1% formic acid. The metabolites were extracted by sonication for 60 min, and the supernatant was transferred to a new tube and evaporated. After that, 300 µL of methanol was added, and the metabolites were dissolved by sonication for 20 min. The tubes were then centrifuged at 20,000 × *g* for 4 min, and 200 µL of the supernatant was transferred to HPLC vials. The extracts were analyzed in the Department of Systems Biology, Technical University of Denmark, by ultra-high performance liquid chromatography-high resolution mass spectrometry

(UHPLC-HRMS). Samples were analyzed by UHPLC-HRMS as described in detail by Klitgaard et al. (2014). In short, samples were separated on a Dionex Ultimate 3000 UHPLC system (Thermo Scientific, Dionex, Sunnyvale, California, USA) equipped with a Kinetex C18 column (100 × 2.1 mm, 2.6 µm particles)(Phenomenex, Torrance, CA, USA) running an acidic (20 mM formic acid) water-acetonitrile gradient, starting form 10% acetonitrile and increasing to 100 in 10 minutes. This was coupled to Bruker maXis HD quadrupole time-of-flight mass spectrometer system (Bruker Daltonics, Bremen, Germany) equipped with an electrospray source operating in positive polarity, scanning m/z 80-1200.

4.3 Results

4.3.1 The diversity of polyketide synthase genes in E. nigrum

In order to identify genes that could be involved in the synthesis of epicolactone and its hypothetical precursors, the *E. nigrum* P16 genome was screened for genes encoding PKSs. Thirteen PKS-encoding genes were found to be dispersed among 12 gene clusters. Domain architecture analysis revealed 12 type I PKSs: five NR-PKSs, two partially reducing PKSs (PR-PKSs), four HR-PKSs, and a PKS-non ribosomal peptide synthase hybrid (PKS-NRPS). A type III PKS was also present (Figure 4.1).

All the type I PKSs contain the minimal PKS domains: β-ketoacyl synthase, acyl transferase, and ACP. The active sites of the minimal domains are conserved and contain the conserved residues necessary for their catalytic activities (Appendix F). The reducing PKSs also contain the reducing domains: dehydratase, enoyl reductase, and ketoreductase. PKSi1 and PKSi4 do not contain an enoyl reductase domain and were classified as PR-PKSs. The methyltransferase domain in PKSi1 has two substitutions in the glycine-rich region in motif I (ExGxGxG), which is responsible for binding of the substrate (Appendix F) (ANSARI et al., 2008).

The NR-PKSs also contain the unique domains of NR-PKSs: SAT, product template, and a C-terminal domain, typically thioesterase, which catalyzes a release of the polyketide from the PKS and usually works as a Claisen like cyclase (CLC), facilitating ring formation (FUJII et al., 2001).

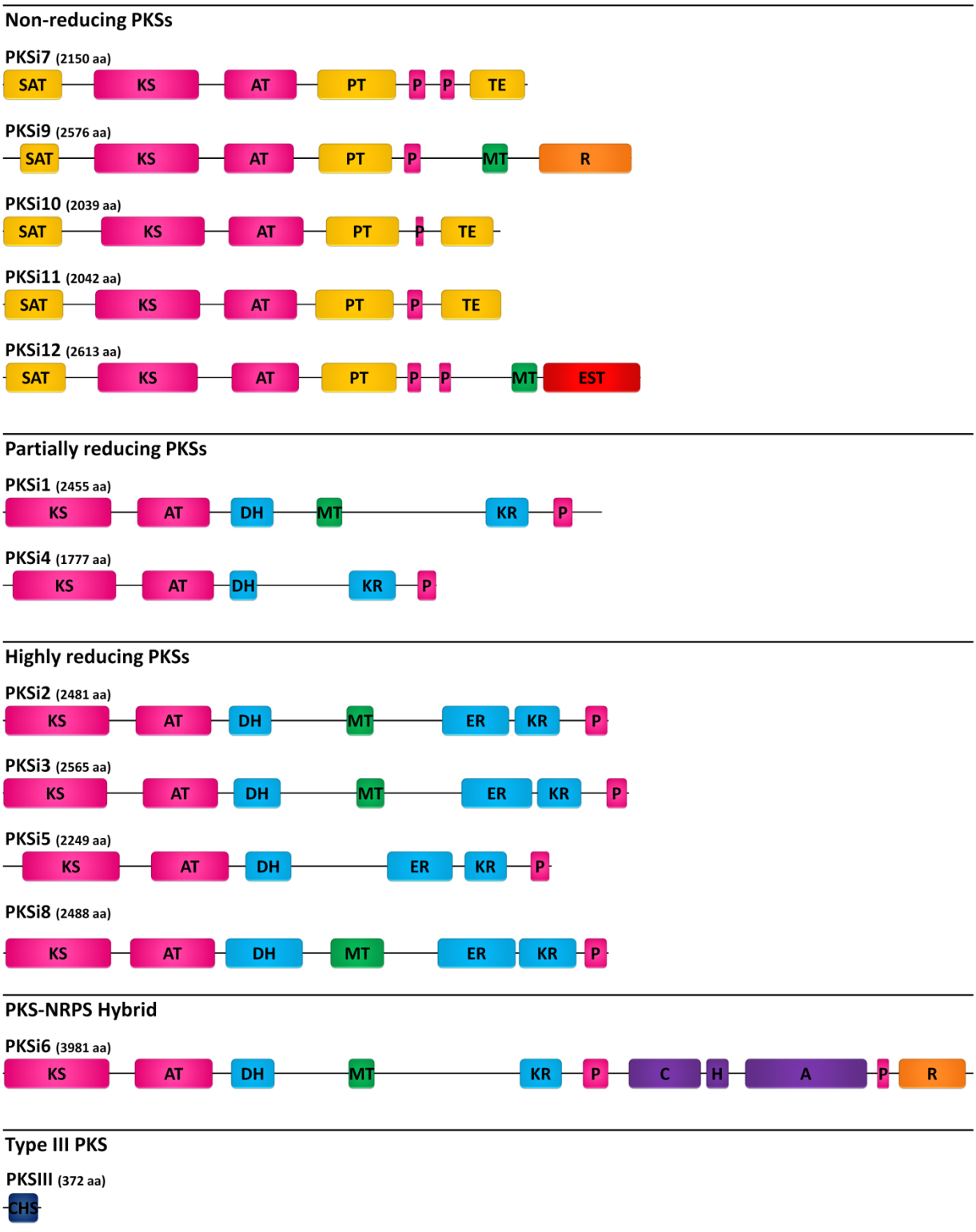

Figure 4.1 – Domain architecture of the PKS and PKS-NRPS hybrid proteins in *E. nigrum*. *Minimal domains (pink).* KS: β-ketoacyl synthase; AT: acyl transferase; P: acyl carrier protein (ACP). *Typical NR-PKS domains (yellow)*. SAT: ACP transacylase; PT: product template, TE: thioesterase. *Reducing domains (blue)*. DH: dehydratase, ER: enoyl reductase, KR: ketoreductase. *NRPS domains (purple)*. C: condensation domain, H: HxxPF repeated domain, A: adenylation domain. *Others:* MT: methyltransferase (green), EST: esterase (red), R: reductase (orange), CHS: chalcone and stilbene synthases (dark blue).

PKSi9 contains a C-terminal thioester reductase domain, which is predicted to catalyze a reductive release and is found in PKSs such as *Talaromyces stipitatus* TropA, which produces 3-methylorcinaldehyde, and *Aspergillus niger* AzaA, which produces a benzaldehyde intermediate in the biosynthesis of azaphilones. Both products are released as an aldehyde (DAVISON et al., 2012; ZABALA et al., 2012).

PKSi12 has a C-terminal esterase/lipase-like domain similar to acetyl esterase/lipase (Aes) of *E. coli* that appears to function as a hydrolase instead of a thioesterase/CLC (CHOOI; TANG, 2012). Similar domains are also found in PKSs shown to produce orsellinic acid derivatives such as 5-methylorsellinic acid (*Penicillium brevicompactum* MpaC, *Chaetomium globosum* CHGG_10128, and *Aspergillus terreus* ATEG_03629), 3,5-dimethylorsellinic acid (*Aspergillus nidulans* AusA, *Aspergillus stellatus* AndM, and *A. terreus* Trt4), and 3-methylorsellinic acid (*A. nidulans* PkbA) (AHUJA et al., 2012; ISHIUCHI et al., 2012; ITOH et al., 2012; MATSUDA et al., 2014; NIELSEN et al., 2011; REGUEIRA et al., 2011; WANG; BEISSNER; ZHAO, 2014).

The PKS-NRPS hybrid (PKSi6) has a PR-PKS region in the N-terminal portion of the enzyme is composed of the essential PKS domains (β-ketoacyl synthase, acyl transferase, and ACP), two reducing domains (dehydratase and ketoreductase), and a methyltransferase domain. The NRPS module is flanked by the ACP domains and is composed of typical NRPS domains: condensation, HxxPF repeated domain (peptidyl carrier protein), and adenylation domains. NRPS modules incorporate amino acids into the polyketide product.

The type III PKS has a chalcone and stilbene synthase (CHS) domain related to chalcone and stilbene synthases: plant enzymes that produce either chalcone or stilbene (SCHRÖDER; SCHRÖDER, 1990). Its closest characterized protein homologs are chain A of PKSIII*Nc* from *Neurospora crassa* (38% sequence identity), which produces resorcinolic lipids (GOYAL et al., 2008), and pyrone synthase CsyA from *Aspergillus oryzae* (36% sequence identity), which produces 3,5-dihydroxybenzoic acid.

To place the *E. nigrum* PKSs in context with other fungal PKSs and to gain some insights into their products, a phylogenetic tree was constructed on the basis of the predicted amino acid sequences corresponding to the β-ketoacyl synthase domains from the 11 type I PKSs, the PKS-NRPS hybrid, and 62 characterized fungal PKSs (Appendix D, Figure 4.2). Table 4.1 presents the closest characterized homologs of the *E. nigrum* PKSs.

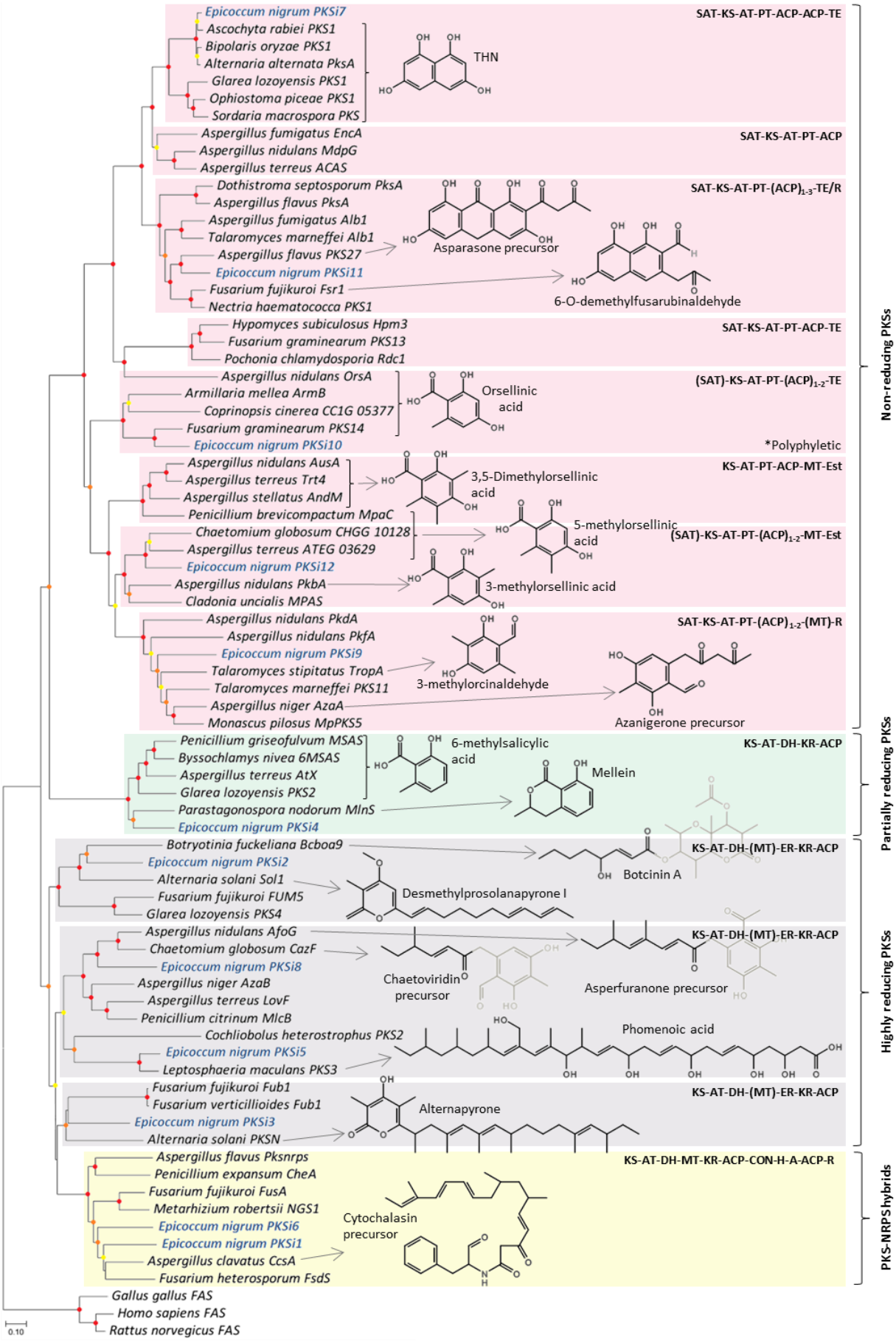

Figure 4.2 – (See legend on next page)

(See figure on previous page)

Figure 4.2 – Phylogenetic analysis of amino acid sequences of the β-ketoacyl synthase (KS) domain from the PKS and PKS-NRPS hybrid proteins of *E. nigrum*. Chemical structures of the products from related characterized fungal PKSs are indicated. The domain architecture of the PKS proteins is indicated for each clade. Appendix D contains details of gene designations and their corresponding products and references. Colored dots indicate bootstrap support based on 1000 replicates: red ≥ 85%, orange ≥ 70%, and yellow ≥ 55%. Animal FASs served as an outgroup.

Table 4.1 – Closest characterized homologs of the *E. nigrum* PKSs

E. nigrum	**Homologs**				
PKS	**Organism**	**PKS name**	**Identity**	**Product(s)**	**Reference**
PKSi1	*Aspergillus clavatus*	CcsA	50%	Cytochalasin	Qiao; Chooi; Tang (2011)
PKSi2	*Fusarium fujikuroi*	FUM5	33%	Fumonisin	Proctor et al. (1999)
PKSi3	*Alternaria solani*	PKSN	41%	Alternapyrone	Fujii et al. (2005)
PKSi4	*Parastagonospora nodorum*	MlnS	52%	Mellein	Chooi et al. (2015)
PKSi5	*Leptosphaeria maculans*	PKS3	58%	Phomenoic acid	Elliott et al. (2013)
PKSi6	*Aspergillus clavatus*	CcsA	55%	Cytochalasin	Qiao; Chooi; Tang (2011)
PKSi7	*Bipolaris oryzae*	PKS1	81%	THN, melanin	Moriwaki et al. (2004)
PKSi8	*Aspergillus niger*	AzaB	45%	Azaphilones	Zabala et al. (2012)
PKSi9	*Talaromyces marneffei*	PKS11	48%	Mitorubrinic acid, mitorubrinol	Woo et al. (2012)
PKSi10	*Fusarium graminearum*	PKS14	55%	Orsellinic acid, orcinol	Jørgensen et al. (2014)
PKSi11	*Aspergillus flavus*	PKS27	59%	Asparasone	Cary et al. (2014)
PKSi12	*Chaetomium globosum*	CHGG_10128	51%	5-methylorsellinic acid	Ishiuchi et al. (2012)

The PKSs were grouped into two major clades, as expected: one composed of NR-PKSs and the other composed of HR-PKSs as well as an intermediate clade composed of PR-PKSs. In this study, the focus was on analysis of the NR-PKSs, which are related to the synthesis of mono- or polycyclic aromatic polyketides, such as the hypothetical precursors of epicolactone.

PKSi7 showed strong identity with tetrahydroxynaphthalene (THN) synthases, and they comprised a clade with 100% bootstrap support. THN is the first intermediate in melanin biosynthesis via the DHN (dihydroxynaphthalene) pathway, the most common pathway for melanin biosynthesis in ascomycetes (BELL; WHEELER, 1986). PKSi11 was present in a clade that also contained THN synthases and other PKSs that synthesize fungal pigments, such as asparasone and fusarubins (AWAKAWA et al., 2012;

CARY et al., 2014; STUDT et al., 2012). The cyclizing pattern (occurrence of first cyclization) of PKSi11 is predicted to be C4-C9 as determined by a phylogenetic analysis of the product template domains (involved in the control of specific aldol cyclization and aromatization) according to the classification of Liu et al. (2015) (Appendix G).

A clade with 100% bootstrap support comprised PKSi10 and orsellinic acid synthases from *C. cinerea* CC1G_05377, *F. graminearum* PKS14, and *Armillaria mellea* ArmB (ISHIUCHI et al., 2012; JØRGENSEN et al., 2014; LACKNER et al., 2013). The orsellinic acid synthase of *A. nidulans* OrsA was present in another clade (SCHROECKH et al., 2009). As observed by Jørgensen et al. (2014) and Lackner et al. (2013), orsellinic acid synthases are polyphyletic and can have variable domain architectures. CC1G_05377 lacks a SAT domain, and ArmB and OrsA have two ACP domains, whereas PKS14 and CC1G_05377 have one.

PKSi9 and PKSi12 were grouped in a clade that comprised PKSs with the unusual C-terminal domains: thioester reductase and esterase/lipase-like. The PKSi9 subclade (100% bootstrap support) comprised PKSs that synthesize orsellinic acid derivatives and PKSs that participate in the synthesis of polyketide pigments that require two PKSs: *A. niger* AzaA (synthesis of azaphilones) and *Talaromyces marneffei* PKS11 (synthesis of mitorubrinol) (AHUJA et al., 2012; DAVISON et al., 2012; WOO et al., 2012; ZABALA et al., 2012). The PKSi12 subclade (91% bootstrap support) also comprised synthases of orsellinic-acid derivatives, and PKSi12 was found to be most closely related to 5-methylorsellinic acid synthases (*C. globosum* CHGG_10128 and *A. terreus* ATEG_03629) (ISHIUCHI et al., 2012; WANG; BEISSNER; ZHAO, 2014). A third subclade (100% bootstrap support) comprised PKSs lacking a SAT domain that also synthesize orsellinic-acid derivatives.

PR-PKSs comprised a clade with 100% bootstrap support. PKSi4 was present in this clade and was most closely related to *Parastagonospora nodorum* MlnS, which synthesizes mellein (CHOOI et al., 2015). It is noteworthy that PKSi1, which does not contain an enoyl reductase domain and was also classified as a PR-PKS, was present in the PKS-NRPS clade together with PKSi6.

HR-PKSs synthesize linear polyketides and cyclic nonaromatic polyketides. The HR-PKSs PKSi2, PKSi3, PKSi5, and PKSi8 were subdivided into three subclades. The PKSi8-and-PKSi5 clade comprised PKSs involved in the synthesis of polyketides that requires two PKSs, such as t-toxin (*Cochliobolus heterostrophus* PKS2), azaphilones (*C.*

globosum CazF), and asperfuranone (*A. nidulans* AfoG) (BAKER et al., 2006; CHIANG et al., 2009; WINTER et al., 2012).

Genes encoding proteins involved in the biosynthesis of a particular fungal secondary metabolite are often clustered in the genome. In order to better understand the roles of the NR-PKS gene clusters in the production of polyketides, these predicted gene clusters were analyzed and annotated (Figure 4.3 and Appendix H).

In addition to the PKS-encoding genes, the clusters included genes encoding transcription factors, transporters, and biosynthetic enzymes (post-PKS modifying enzymes), mainly oxidoreductases (monooxygenases, dioxygenases, oxidases, peroxidases, dehydrogenases, and reductases) and transferases (methyltransferases, acetyltransferases, and prenyltransferases). Some predicted proteins do not have a clear function in PKS biosynthesis and may not belong to the PKS cluster.

The PKS gene clusters usually contained a gene of a transcription factor that could control the gene expression within the cluster. Most of the identified transcription factors contain a Zn(2)-Cys(6) binuclear domain (involved in zinc-dependent binding of DNA) in the N-terminal portion. The second gene of a transcription factor in the predicted PKSi8/PKSi9 dual cluster encoded a smaller protein (267 amino acid residues) with a leucine zipper domain. No transcription factor gene was identified in the predicted PKSi10 gene cluster.

The predicted PKSi7 gene cluster included two genes encoding proteins with high identity to enzymes involved in the biosynthesis of melanin: a reductase with 91% identity to the *Alternaria alternata* THN reductase and a transcription factor with 95% identity to the *Alternaria brassicicola* transcription factor Arm1 (CHO et al., 2012; KIMURA et al., 1993).

PKSi8 and *PKSi9*, genes of an HR-PKS and an NR-PKS located in contig 52, are transcribed divergently from a common region: the same gene organization is observed in the resorcylic acid lactone (RAL) zearalenone gene cluster. The predicted *PKSi8*/*PKSi9* dual cluster contained a gene of a hydroxylase and a gene of a transferase with some similarity to the salicylate hydroxylase (54% identity) and the trichothecene 3-O-acetyltransferase (39% identity), respectively, from *Monascus pilosus*; these two enzymes are involved in the biosynthesis of azaphilones (BALAKRISHNAN et al., 2013).

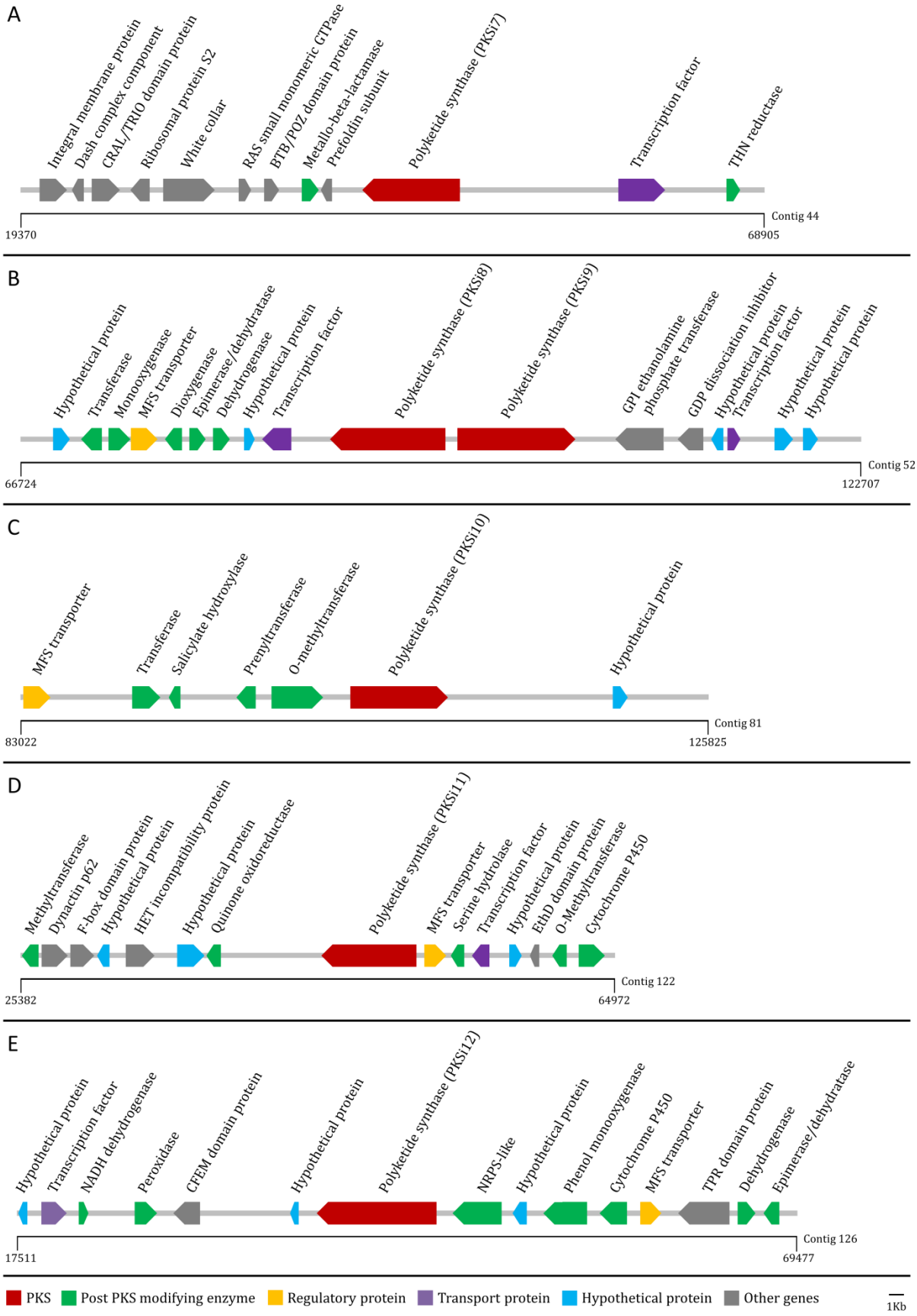

Figure 4.3 – Organization of the NR-PKS and dual HR-PKS/NR-PKS gene clusters in *E. nigrum*. A: PKSi7, B: dual HR-PKS (PKSi8), and NR-PKS (PKSi9) cluster, C: PKSi10, D: PKSi11, and E: PKSi12. Annotation was based on domains and close protein homologs (Appendix H).

The predicted *PKSi10* gene cluster included a gene of a prenyltransferase with an UbiA domain. Prenyltransferase genes are found in PKS gene clusters that synthesize meroterpenoids such as mycophenolic acid, which consists of a polyketide-derived nucleus and a terpene-derived side chain (REGUEIRA et al., 2011).

The open reading frame (ORF) located immediately downstream of the *PKSi12* gene is predicted to encode a 1022-amino acid enzyme that contains three domains: adenylation, ACP, and thioester reductase. Its closest characterized homolog is the NRPS-like enzyme ATEG_03630 of *A. terreus* (46% identity): a novel type of NRPS-like enzyme that activates 5-methylorsellinic acid and reduces it to 2,4-dihydroxy-5,6-dimethyl benzaldehyde (converts an aryl-acid to an aryl-aldehyde). The *A. terreus* ATEG_03630 gene is located immediately downstream of the gene of 5-methylorsellinic acid synthase *A. terreus* ATEG_03629 (WANG; BEISSNER; ZHAO, 2014). A comparison of *E. nigrum PKSi12* cluster and *A. terreus* ATEG_03629 cluster is presented in Figure 4.4.

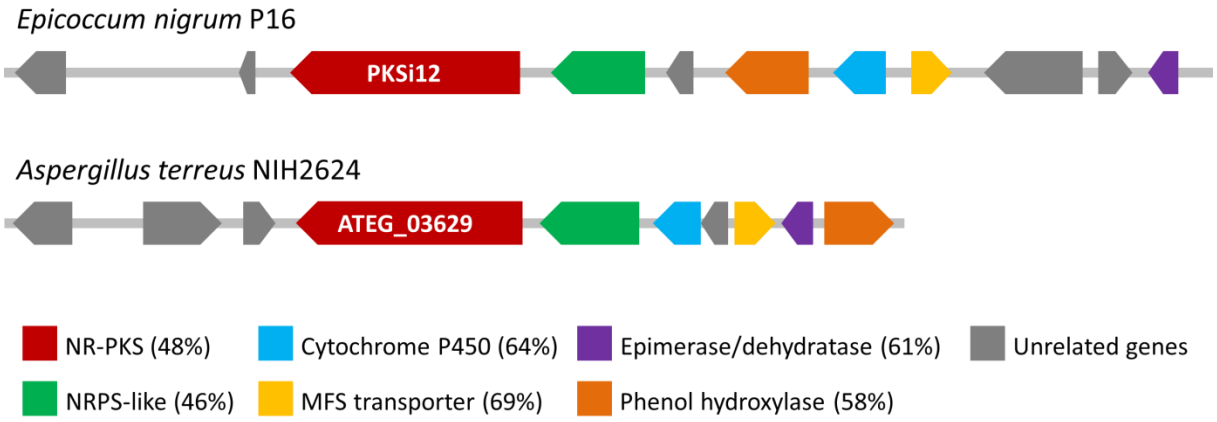

Figure 4.4 – Comparison of the *E. nigrum PKSi12* cluster and *A. terreus ATEG_03629* cluster. Identity of the predicted protein sequences (%) is indicated in the parentheses. The *A. terreus* gene cluster was retrieved from http://aspgd.broadinstitute.org. Predicted proteins encoded by the unrelated genes in the *A. terreus* cluster are a flavin-containing monooxygenase, a hypothetical protein, salicylate hydroxylase, and short-chain dehydrogenase.

The putative cytochrome P450 whose gene is located in the predicted PKSi12 cluster also has similarity to the N-terminal portion of *P. brevicompactum* MpaDE (39% identity). MpaDE is a natural fusion protein of a cytochrome P450 and a hydrolase that catalyzes conversion of 5-methylorsellinic acid to 5,7-dihydroxy-4-methylphthalide (DHMP, structurally similar to epicoccone B except for a hydroxyl group) during the biosynthesis of mycophenolic acid. The N-terminal portion of MpaDE contains a

cytochrome P450 domain that catalyzes hydroxylation of 5-methylorsellinic acid to 4,6-dihydroxy-2-(hydroxymethyl)-3-methylbenzoic acid (DHMB). The hydrolase domain in the C-terminal portion acts as a lactone synthase and catalyzes ring closure (HANSEN et al., 2012; REGUEIRA et al., 2011).

4.3.2 Construction of PKS mutants

The NR-PKSs were selected to be deleted by targeted gene replacement via *A. tumefaciens*. PKSi8 was also selected because the gene of this HR-PKS is in the same gene cluster as PKSi9. In chapter 3, it was shown that both PKSi8 and PKSi9 are downregulated in epicolactone-deficient mutants obtained by random mutagenesis. *E. nigrum* P16 mutants were successfully obtained using the proposed protocol. The isolated mutants were first verified by colony PCR to confirm the loss of the target gene and correct crossover. The percentage of correct mutants with the desired gene replacement showed high variation in each gene deletion experiment (Table 4.2).

Table 4.2 - Results of PCR verification of the constructed PKS deletion mutants.

Gene deletion experiment	Number of tested isolates*	Number of correct mutants**
PKSi7	50	12 (24 %)
PKSi8	45	6 (13 %)
PKSi9	89	9 (10 %)
PKSi10	70	1 (1.4 %)
PKSi11	50	21 (42 %)
PKSi12	50	5 (10 %)

*Hygromycin-resistant isolated mutants;
**Mutants with the desired gene replacement.

The mutants with the desired gene replacement were then analyzed by Southern blot hybridization using a probe corresponding to a part of the hygromycin resistance gene. Results revealed that only a single copy of the T-DNA had integrated into the genome of mutants ΔPKSi7, ΔPKSi8, ΔPKSi9, ΔPKSi11, and ΔPKSi12 (Figure 4.5). Nevertheless, a random ectopic integration represented by a band of approximately 10 kb occurred in combination with the correct gene replacement in the PKSi10 mutant. This mutant was not used in the further experiments because its phenotype could be affected by the additional integration of the T-DNA.

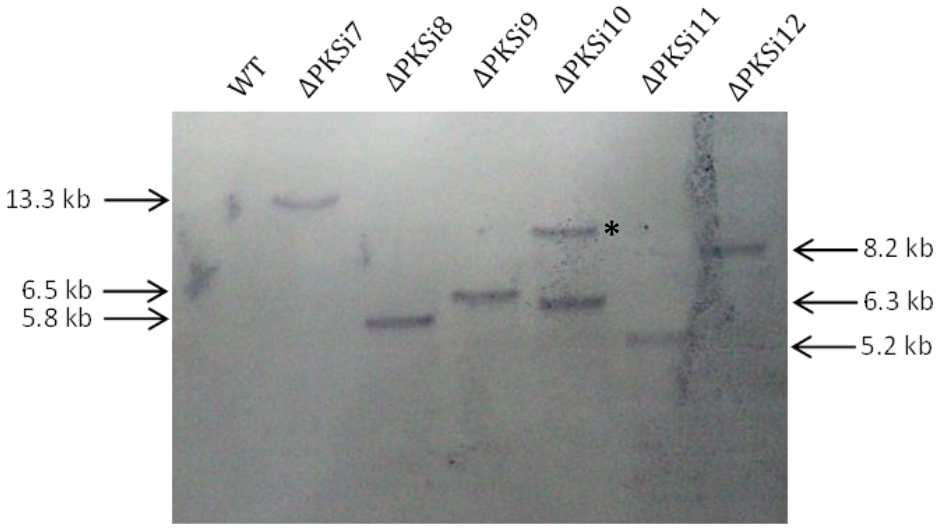

Figure 4.5 – Southern blot analysis of the constructed PKS deletion mutants. Expected band sizes: 13.3, 5.8, 6.5, 6.3, 5.2, and 8.2 kb for mutants ΔPKSi7, ΔPKSi8, ΔPKSi9, ΔPKSi10, ΔPKSi11, and ΔPKSi12, respectively. *Unexpected band.

4.3.3 Characterization of the PKS mutants

4.3.3.1 Mycelial growth on different media

Mycelial growth (diameter of colonies in centimeters) of the strains (WT and mutants) on four media was measured every 4 d until day 12 (Figure 4.6 and Appendix I). On day 16, the mycelia of all strains covered the oatmeal agar and sugarcane leaf extract agar media, except for the WT on the sugarcane medium.

The PDA medium allowed for a clear distinction between the mycelial growth of the different strains: WT had the smallest diameter of a colony, followed by mutants ΔPKSi8, ΔPKSi11, ΔPKSi9, ΔPKSi7, and ΔPKSi12, while on the Sabouraud medium, the strains showed similar growth, except for the ΔPKSi12 mutant. The growth of the strains can also be compared by the average growth rates on the different media for each strain (Table 4.3). The ΔPKSi7 and ΔPKSi12 mutants showed the highest average growth rates, 0.65 and 0.64 $cm \cdot d^{-1}$, respectively, while WT *E. nigrum* showed the lowest average growth rate, 0.48 $cm \cdot d^{-1}$.

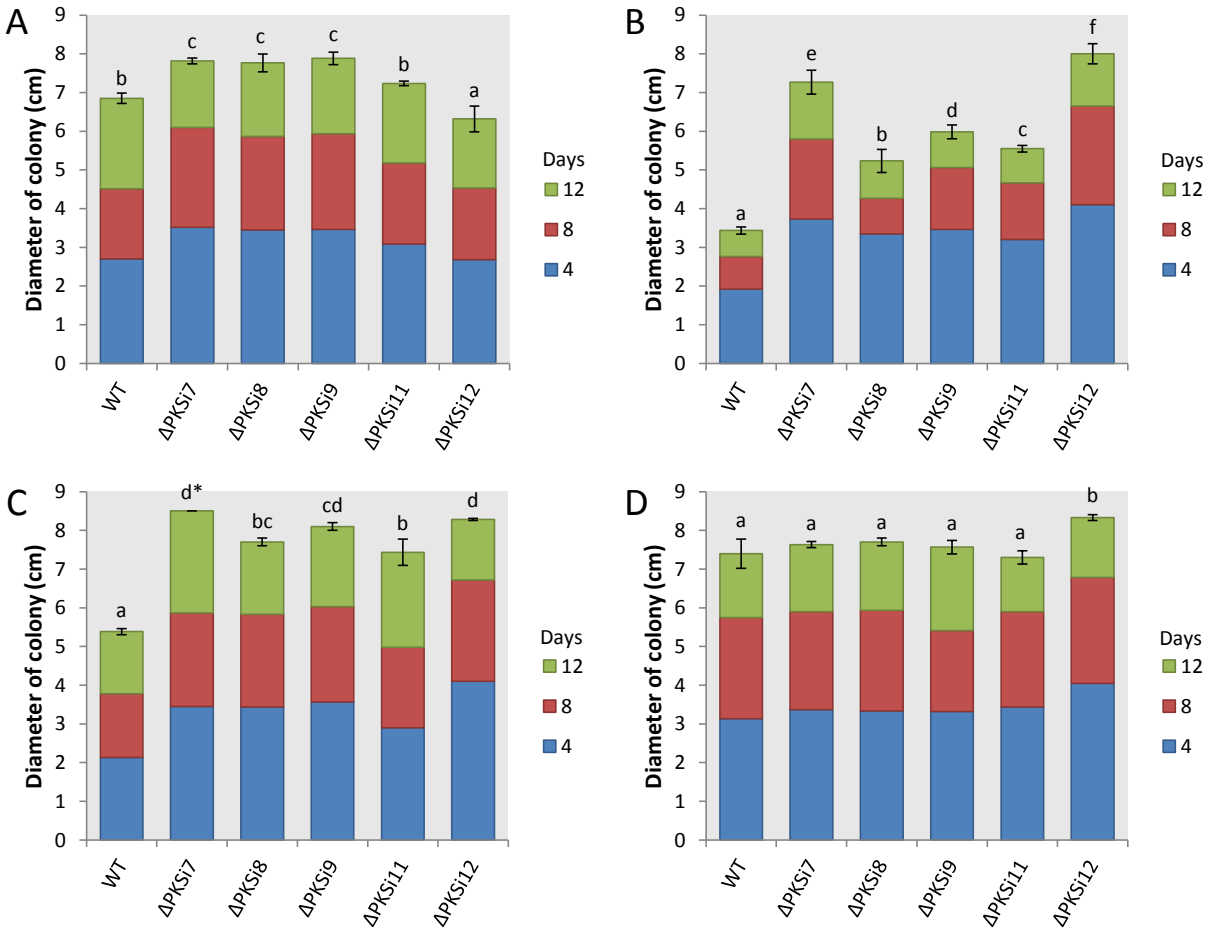

Figure 4.6 – Mycelial growth (diameter of colonies in cm) of *E. nigrum* strains (WT and PKS mutants) on oatmeal agar (A), potato dextrose agar (B), sugarcane leaf extract agar (C), and Sabouraud agar (D) at 28 °C measured after 4, 8, and 12 d of growth. Columns marked with the same letter are not statistically significantly different ($p > 0.05$, Tukey's test). *Strain completely covered the plate on day 12.

Table 4.3 – The growth rate ($cm \cdot d^{-1}$) of *E. nigrum* strains (WT and PKS mutants) on oatmeal agar, potato dextrose agar, sugarcane leaf extract agar, and Sabouraud agar at 28 °C.

Medium / Strain	Oatmeal	Potato dextrose	Sugarcane extract	Sabouraud	Average growth rate
WT	0.56	0.28	0.45	0.62	0.48
ΔPKSi7	0.65	0.60	0.70	0.64	0.65
ΔPKSi8	0.64	0.42	0.64	0.64	0.58
ΔPKSi9	0.65	0.49	0.67	0.62	0.61
ΔPKSi11	0.60	0.45	0.61	0.61	0.57
ΔPKSi12	0.52	0.66	0.69	0.69	0.64

The variation in the colony phenotype depended on the growth medium (Figure 4.7). The growth on oatmeal agar, PDA, and Sabouraud agar media was characterized by

vigorous aerial mycelial growth with high hyphal density, which seemed especially high on PDA. On the PDA medium, colonies had irregular margins and a yellow color (top view) and orange to brown color (reverse view). On the oatmeal agar medium, the colonies had a white color with regular margins, while on the Sabouraud agar medium, the color of colonies varied from white to light yellow-brown with irregular margins. On sugarcane leaf extract agar, the morphology of *E*. *nigrum* strains was different, and colonies showed sparse hyphal growth across the surface of the medium.

The strains produced a yellow compound (or compounds) that diffused in the PDA and Sabouraud media. In the PKSi9 mutant growing on Sabouraud agar, the yellow color in the medium seemed less intense or absent. On sugarcane leaf extract agar, the yellow color was observed only around the oldest part of the WT and the ΔPKSi11 mutant colonies. No diffused colored compounds were observed in the oatmeal agar medium.

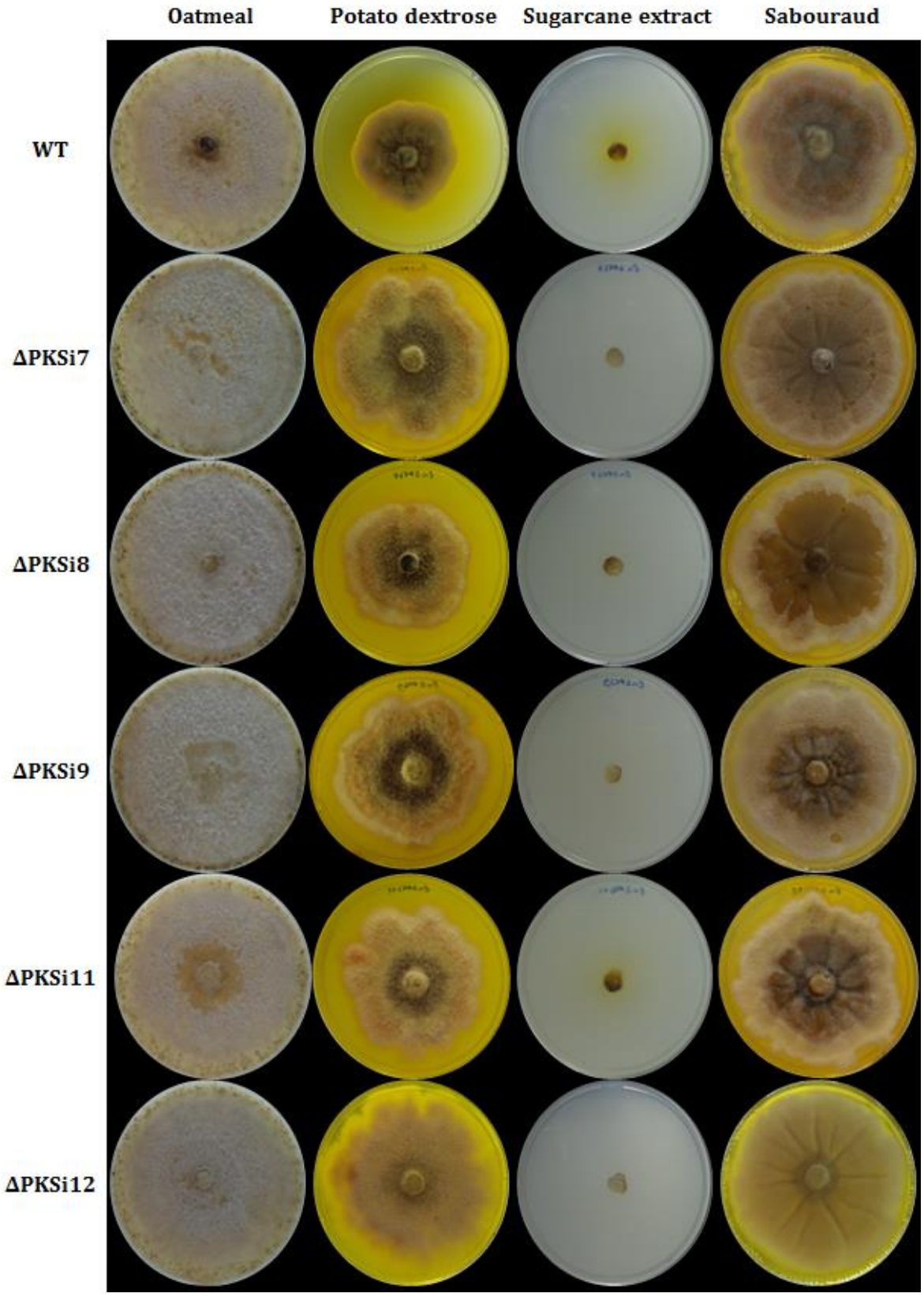

Figure 4.7 – Colony appearance (top view) of the *E. nigrum* strains (WT and PKS mutants) grown on oatmeal agar, potato dextrose agar, sugarcane leaf extract agar, and Sabouraud agar at 28 °C after 16 d.

4.3.3.2 Growth-inhibitory action on microorganisms

None of the *E*. *nigrum* strains exerted growth-inhibitory action on the bacteria *X. albilineans*, *E. coli*, and *S. aureus* according to the agar block method. All the strains showed a growth-inhibitory activity against the bacteria *Bacillus* sp. and the yeast *C. albicans* (Figure 4.8 and Figure 4.9). The inhibition zones produced against *Bacillus* sp. ranged from 0.87 cm (ΔPKSi12 mutant) to 1.23 cm (WT), and only the ΔPKSi12 mutant produced an inhibition zone significantly smaller than that of the WT. Larger inhibition zones, ranging from 2.37 cm (ΔPKSi8 and ΔPKSi12) to 2.47 cm (ΔPKSi11), were produced against *C*. *albicans*. Nonetheless, there was no significant difference among the strains.

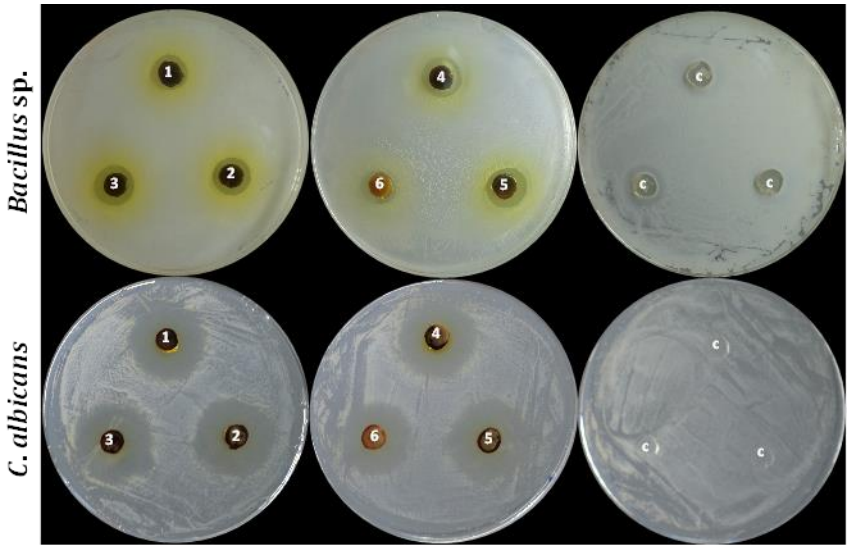

Figure 4.8 – Growth-inhibitory activity of *E. nigrum* strains (WT and PKS mutants) against *Bacillus* sp. and *C. albicans*. 1: WT, 2: ΔPKSi7, 3: ΔPKSi8, 4: ΔPKSi9, 5: ΔPKSi11, 6: ΔPKSi12, C: control (PDA agar plugs were inoculated).

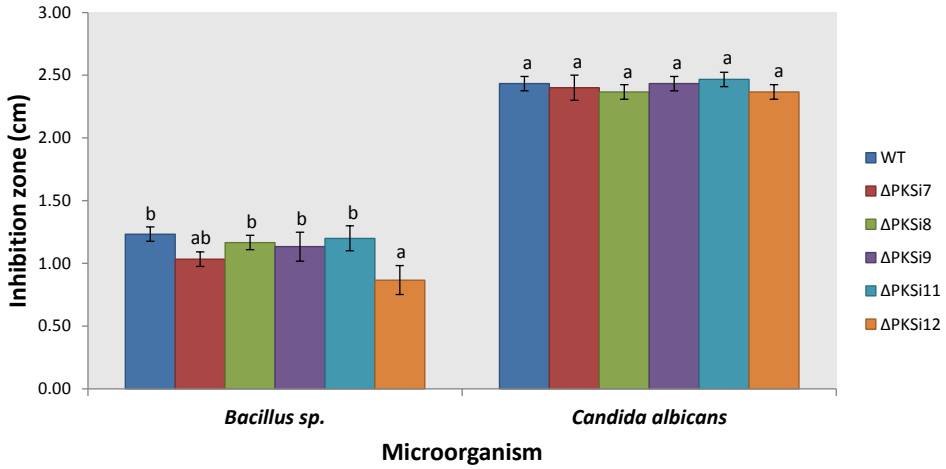

Figure 4.9 – Inhibition zone size (cm) shown by *E. nigrum* strains (WT and PKS mutants) against *Bacillus* sp. and *Candida albicans*. For each group, columns marked with the same letter are not statistically significantly different.

In the dual-culture assays against phytopathogenic fungi and oomycetes, no growth-inhibitory activity was observed against *F. verticillioides* and *P. cinnamomi*. A growth-inhibitory activity against *R. solani*, *C. falcatum*, *F. oxysporum*, *Colletotrichum* sp., *C. paradoxa*, and *S. sclerotiorum* was observed (Figure 4.10, Figure 4.11, and Figure 4.12). On the other hand, mutants ΔPKSi9 and ΔPKSi12 did not show a growth-inhibitory activity against *R. solani*. The ΔPKSi12 mutant also did not show growth-inhibitory activity against *C. falcatum*, *F. oxysporum*, and *C. paradoxa*. In these cases, mycelial contact occurred between the mutant and the phytopathogen. In the dual-culture assay of the ΔPKSi12 mutant against *C. paradoxa,* the phytopathogen mycelium was growing over the ΔPKSi12 mutant colonies.

The inhibition zones produced against *R. solani* by mutants ΔPKSi7, ΔPKSi8, and ΔPKSi11 and by the WT were not significantly different. In contrast, the rates of inhibition of *R. solani* growth shown by the mutants were significantly higher in comparison with the WT. The ΔPKSi8 mutant was the only strain that showed a larger inhibition zone against a phytopathogen than the WT did: against *C. falcatum*. Conversely, the ΔPKSi9 mutant yielded a smaller inhibition zone against *C. falcatum* than the WT did. Nonetheless, this strain showed a higher inhibition rate than the WT did against this phytopathogen's growth. The ΔPKSi9 mutant also showed a higher inhibition rate of *C. paradoxa* growth than the WT did.

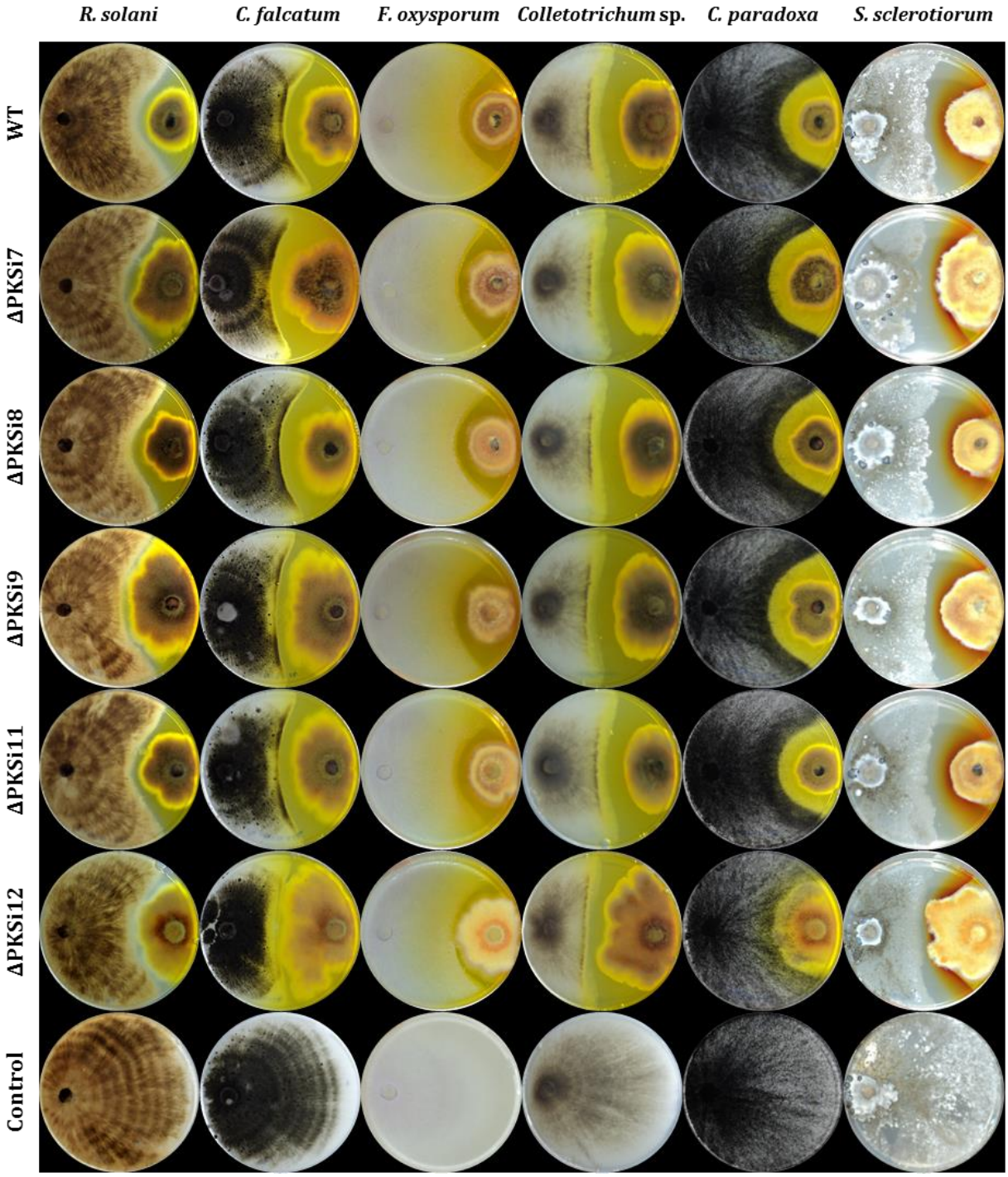

Figure 4.10 – Growth-inhibitory activity of *E. nigrum* strains (WT and PKS mutants) against the phytopathogens *R. solani*, *C. falcatum*, *F. oxysporum*, *Colletotrichum* sp., *C. paradoxa*, and *S. sclerotiorum*.

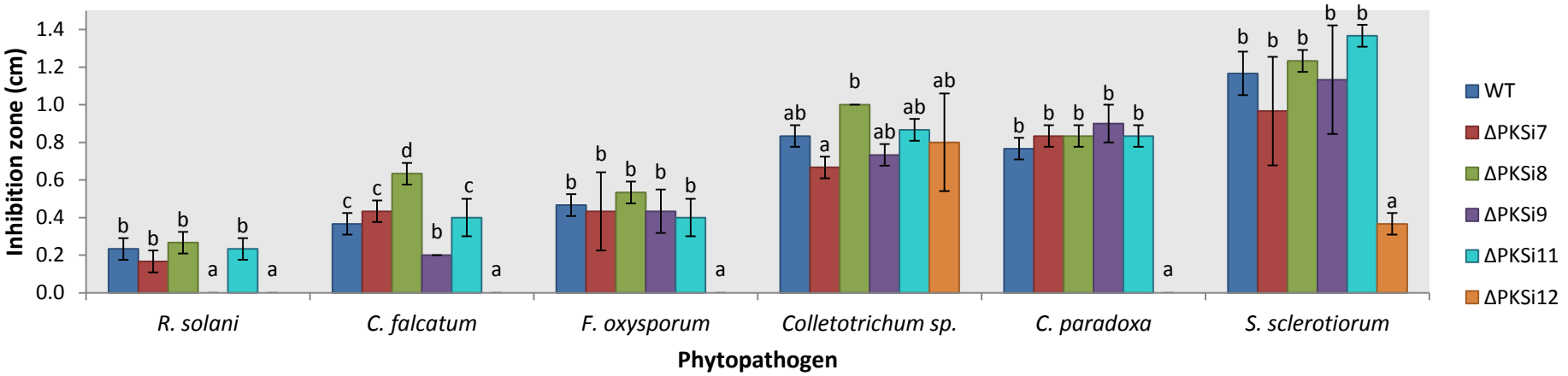

Figure 4.11 – Inhibition zone size (cm) shown by *E. nigrum* strains (WT and PKS mutants) against the phytopathogens *R. solani*, *C. falcatum*, *F. oxysporum*, *Colletotrichum* sp., *C. paradoxa*, and *S. sclerotiorum*. For each group, columns marked with the same letter are not statistically significantly different ($p > 0.05$, Tukey's test).

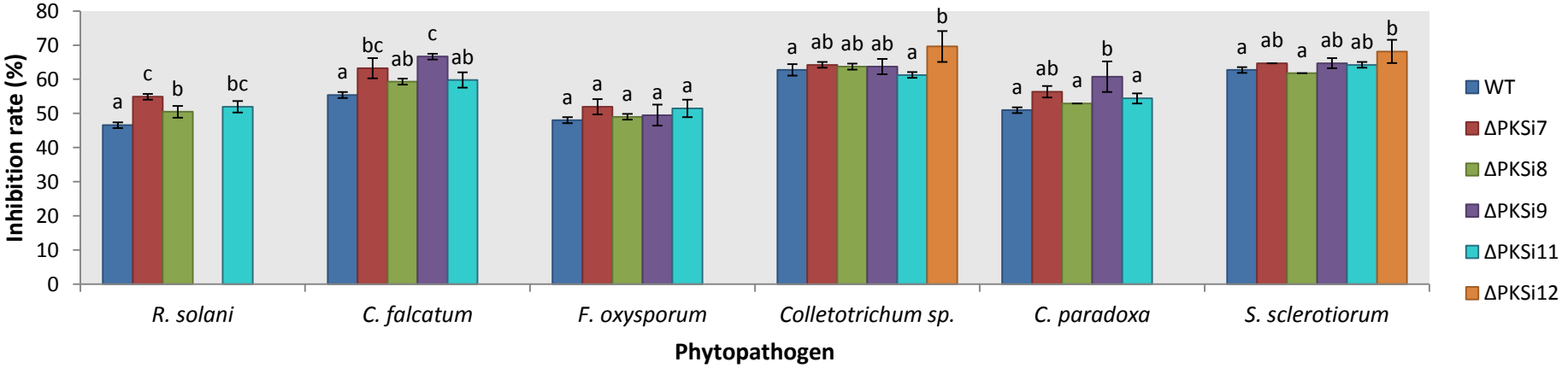

Figure 4.12 – The rate of inhibition (%) toward the mycelial growth of the phytopathogens *R. solani*, *C. falcatum*, *F. oxysporum*, *Colletotrichum* sp., *C. paradoxa*, and *S. sclerotiorum* shown by *E. nigrum* strains (WT and PKS mutants). For each group, columns marked with the same letter are not statistically significantly different ($p > 0.05$, Tukey's test).

I observed the largest inhibition zones against *S. sclerotiorum:* up to 1.37 cm (ΔPKSi11). The mutants did not differ from the WT in this regard, except for the ΔPKSi12 mutant, which yielded a significantly smaller inhibition zone. In contrast, the rate of inhibition of *S. sclerotiorum* growth by the ΔPKSi12 mutant was higher than that of the WT. In addition, the ΔPKSi12 mutant showed a higher rate of inhibition of *Colletotrichum* sp. growth than the WT did.

A red arc formed around the *E. nigrum* colonies in the dual-culture assay against *S. sclerotiorum* (Figure 4.10). After extra 7 d of incubation, *E. nigrum* colonies showed an enhanced red arc, except for the ΔPKSi12 mutant (Figure 4.13). This effect was also described by Alcock et al. (2015) in dual-culture experiments on slides with *E. nigrum* and *Botrytis cinerea*. They attributed this phenomenon to a color change of the yellow compound epirodin, which undergoes a hypsochromic shift under acidic conditions thus turning red. Unfortunately, the chemical structure of epirodin is not known.

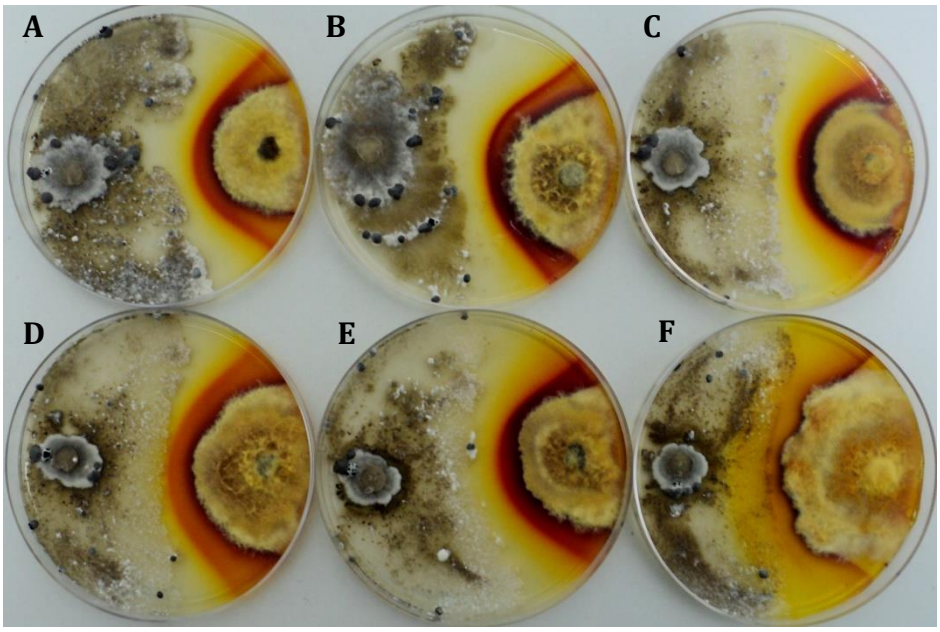

Figure 4.13 – Inhibitory activity of *E. nigrum* strains (WT and PKS mutants) against *S. sclerotiorum* after an additional incubation period of 7 d. A: WT, B: ΔPKSi7, C: ΔPKSi8, D: ΔPKSi9, E: ΔPKSi11, F: ΔPKSi12. An enhanced red arc was observed around the colonies.

4.3.3.3 Chemical analysis of the PKS mutants

Metabolite samples extracted from the WT and mutants ΔPKSi9 and ΔPKSi12 (candidate genes that could be related to the production of orsellinic acid derivatives

according to the bioinformatics analysis) after 7 d of growth on PDA were analyzed chemically. Epicolactone was not detected in any of the samples and probably was not produced under the incubation conditions used. In contrast, epicoccine and epicoccone B were detected by tertiary identification in the WT and in mutant ΔPKSi9. In the ΔPKSi12 mutant, biosynthesis of these two compounds was affected (Figure 4.14). Epicoccine was not produced, and another compound with the same mass represented by a 2-min-earlier peak was produced. In addition, production of epicoccone B was apparently reduced in this mutant.

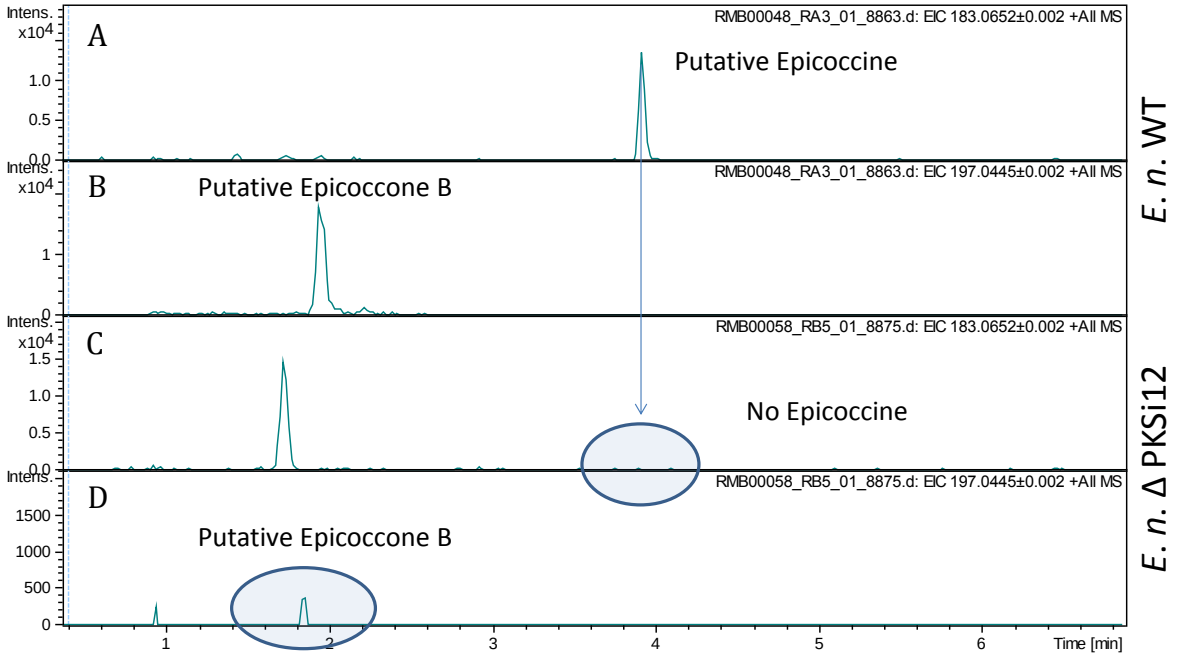

Figure 4.14 – Extracted ion chromatogram of *E. nigrum* strains WT and ΔPKSi12. The two upper chromatograms (A and B) are from the WT and the two lower ones (C and D) are from the ΔPKSi12 mutant. Chromatograms A and C track epicoccine production, whereas B and D track epicoccone B production.

4.4 Discussion

There have been great advances in the research on pathways involved in the production of fungal secondary metabolites. Nonetheless, the basic biology and ecology of the relation between the fungi and their metabolites are poorly understood, especially in endophytic fungi, which are known to produce diverse bioactive metabolites.

Although many compounds of biotechnological significance that are produced by *E. nigrum* have been described in the literature, there is still a large gap in the

knowledge on their biosynthesis. The discovery of pathways and genes involved in the biosynthesis of secondary metabolites in *E*. *nigrum* represents an exciting challenge and can lead to the discovery of new gene functions and may facilitate genetic-engineering research. In addition, these data can expand the understanding of the ecological relationships of this fungus, such as the plant–endophyte relation and its antagonistic activity in the host plant.

Sequenced genomes can yield substantial information on secondary-metabolite pathways and are becoming a powerful tool for discovery of functions of novel genes and gene clusters involved in the biosynthesis of fungal bioactive secondary metabolites. The genome-mining approach has been successfully used, for example, for the discovery of the pneumocandin cluster in *Glarea lozoyensis*, 15-deoxyoxalicine B cluster in *Penicillium canescens*, and viridicatumtoxin cluster in *Aspergillus ustus* (CHEN et al., 2013; PI et al., 2015; YAEGASHI et al., 2015). In the present study, this approach yielded profound insights into the pathways of NR-PKS-driven biosynthesis in *E*. *nigrum*: this is a major advance in exploiting the diversity of secondary-metabolite pathways in this genetically unexplored endophyte.

PKSi7 showed strong identity to THN synthases (>80% identity), which synthesize the first intermediate in the biosynthesis of melanin via the DHN pathway. The predicted cluster also includes a gene of a putative transcription factor and a gene of a putative reductase with strong identity to proteins involved in the biosynthesis of melanin in *Alternaria* spp. (>90% identity) (CHO et al., 2012; KIMURA et al., 1993). However, it seems that the cluster does not contain genes of all the necessary enzymes for melanin production via the DHN pathway as described previously for other ascomycetes, such as *A*. *alternata* (Figure 4.3) (KIMURA et al., 1993). Thus, the function of this cluster is unclear.

Some polyketides are known to require an HR-PKS and an NR-PKS for their biosynthesis, such as RALs, azaphilones, and asperfuranone (CHIANG et al., 2009; GAFFOOR; TRAIL, 2006; ZABALA et al., 2012). The genomic organization of *PKSi8* and *PKSi9*—genes of an NR-PKS and an HR-PKS transcribed divergently from a common region—is similar to the genomic structure of *F. graminearum PKS13* and *PKS4*, which are involved in the biosynthesis of the RAL zearalenone (Figure 4.3) (GAFFOOR; TRAIL, 2006; KIM et al., 2005).

Usually, the HR-PKS/NR-PKS partnerships can occur in two ways: sequentially and convergently. In the sequential mode, an HR-PKS produces a polyketide that is transferred to the NR-PKS for further elongation and processing (GAFFOOR; TRAIL, 2006; KIM et al., 2005; WANG et al., 2008). In the convergent mode, the two PKSs work independently to produce two polyketides that are subsequently condensed by an accessory enzyme, typically an acyl transferase (WINTER et al., 2012; ZABALA et al., 2012).

Phylogenetic analysis showed that PKSi8 and PKSi9 clustered with PKSs involved in the biosynthesis of azaphilones and asperfuranone, instead of RALs, which formed a distinct clade (Figure 4.2). In addition, the predicted *PKSi8/PKSi9* dual cluster contained a gene of a putative transferase that can be responsible for condensation of the two polyketide products and is homologous to *M*. *pilosus* trichothecene 3-O-acetyltransferase involved in the biosynthesis of azaphilones (39% identity) (BALAKRISHNAN et al., 2013).

Judging by the results of the phylogenetic analysis, by the gene cluster organization, and by some reports describing secondary metabolites produced by *E*. *nigrum*, it is likely that the *PKSi8/PKSi9* dual cluster participates in the biosynthesis of the azaphilone epicocconone and of the structurally related compound acetosellin (BELL; KARUSO, 2003; TALONTSI et al., 2013). Epicocconone is a fluorophore produced by *E*. *nigrum* that is commercially available as LavaCell (Active Motif) and LavaPurple (Fluorotechnics) for cell staining and protein detection in electrophoresis gels (BELL; KARUSO, 2003; CHOI; VEAL; KARUSO, 2006).

It is likely that PKSi10 is an orsellinic acid synthase because this PKS was clustered with orsellinic acid synthases in the phylogenetic analysis, and orsellinic acid was described elsewhere as a product of *E*. *nigrum* (MARTIN; RICHARDS; HAIDER, 1967). The presence of a putative prenyltransferase gene in the PKSi10 gene cluster suggests that the final product of this cluster is a meroterpenoid, as observed in the gene cluster of mycophenolic acid (REGUEIRA et al., 2011).

PKSi11 was predicted to produce a compound with the C4-C9 cyclizing pattern, such as asparasone and fusarubins (CARY et al., 2014; STUDT et al., 2012). According to the literature, no polyketides with an apparent C4-C9 cyclizing pattern have ever been described as a product of *E*. *nigrum*.

The phylogenetic analysis suggested that PKSi12 produces an orsellinic acid derivative, probably 5-methylorsellinic acid, because PKSi12 was grouped with the 5-methylorsellinic acid synthases from *A. terreus* and *C. globosum* (91% bootstrap support) (Figure 4.2). The annotation of the predicted PKSi12 cluster uncovered the presence of a putative NRPS-like enzyme homologous to the *A. terreus* NRPS-like enzyme ATEG_03630 (46% identity).

An NRPS-like enzyme with the domain architecture adenylation–ACP–thioester reductase was first described in *Nocardia iowensis*, where it functions as a carboxylic acid reductase, catalyzing the reduction of a wide range of acyl and aromatic carboxylic acids to an aldehyde using ATP (VENKITASUBRAMANIAN; DANIELS; ROSAZZA, 2007). The presence of the gene of this type of NRPS-like enzyme in a PKS gene cluster was recently described by Wang, Beissner, and Zhao (2014). ATEG_03630 catalyzes ATP-dependent activation and NADPH-dependent reduction of 5-methylorsellinic acid in *A. terreus*. A study on substrate specificity of ATEG_03630 revealed that this enzyme has high specificity for 5-methylorsellinic acid compared to various orsellinic acid derivatives and aryl acids (100% relative activity) (WANG; ZHAO, 2014).

The organization is similar in PKSi12 and ATEG_03629 gene clusters: the NRPS-like enzyme gene is located immediately downstream of the PKS gene (WANG; BEISSNER; ZHAO, 2014). These clusters also contain other putative proteins with some sequence similarity: a cytochrome P450, phenol hydroxylase, epimerase/dehydratase, and MFS transporter (Figure 4.4). It should be emphasized that some studies showed that *A. terreus* also produces epicoccine and flavipin (ISHIKAWA; ITO; LEE, 1996; RAISTRICK; RUDMAN, 1956).

Taken together, these bioinformatic results constitute evidence for PKSi12's function, indicating that this PKS probably produces 5-methylorsellinic acid that is then converted to its aldehyde form by an NRPS-like enzyme. The structural similarities also suggest that this compound may be a precursor of epicoccone B and epicoccine.

According to the hypothetical biosynthetic pathway suggested by Ellerbrock et al. (2015), epicolactone is produced via heterodimerization of epicoccone B and epicoccine. The unusually complex structure of epicolactone caught the interest of the chemical-synthesis community (ELLERBROCK et al., 2015; ELLERBROCK; ARMANINO; TRAUNER, 2014; MERCER; BURNS, 2015).

Kemami Wangun et al. (2008) proposed that both epicoccone B and epicoccine derive from orsellinic acid. The authors also stated that epicoccone B's structure is reminiscent of mycophenolic acid. El Amrani et al. (2014) and Ellerbrock et al. (2014) suggested that both compounds are derived from flavipin. In *A*. *flavipes*, flavipin is derived from 5-methylorsellinic acid (GATENBECK; ERIKSSON; HANSSON, 1969; PETTERSSON, 1965a, 1965b).

NR-PKSs, which are responsible for production of aromatic polyketides, were selected for gene deletion experiments in the present study. The HR-PKS PKSi8 was also selected because it is located in the same cluster as the NR-PKS PKSi9 is. An efficient protocol for targeted gene replacement by homologous recombination via ATMT in *E*. *nigrum's* vegetative mycelium was successfully established. To my knowledge, this is the first report of gene replacement in an *Epicoccum* species: a crucial step for identification and manipulation of *Epicoccum* genes related to the synthesis of bioactive secondary metabolites.

The high variation in the percentage of mutants with the desired gene replacement that was observed among the gene deletion experiments may be explained by the location of the genes (Table 4.2). The percentage of correct mutants for the *PKSi10* gene was low (1.4%), and only one mutant with the desired gene replacement was obtained. Unfortunately, a random ectopic integration occurred simultaneously with the correct gene replacement in this mutant. It is possible that the *PKSi10* gene is located in a highly condensed genomic region, and this situation impedes homologous recombination and favors random ectopic integration.

In the mycelial-growth analysis, the mutants usually showed greater growth than the WT did. Moreover, the average growth rate of the WT was the lowest, indicating that the synthesis of polyketides has a metabolic cost (Table 4.3). It is likely that in nature, polyketides give some advantage to *E*. *nigrum* in the competition with other species.

The antagonistic-activity experiments against various microorganisms yielded some insights into the function of polyketides in the antimicrobial activity of *E*. *nigrum*. PKSi9 and PKSi12 are related to this activity, likely producing antimicrobial compounds that inhibit the growth of phytopathogens. PKSi9 is also involved in the production of a pigment because the yellow compound produced by the WT on Sabouraud agar seemed absent in the plates with the ΔPKSi9 mutant. Moreover, during the early growth of the

ΔPKSi9 mutant on PDA plates supplemented with hygromycin, the pigments were absent too (Appendix J).

Chemical analysis allowed for tertiary identification of epicoccone B and epicoccine. In the PKSi12 mutant, putative epicoccine was absent and putative epicoccone B was downregulated. These data suggest that PKSi12 is probably involved in the production of these two compounds. Nonetheless, an analysis involving purified epicoccone B and epicoccine as standards is necessary for accurate identification of the above compounds. The appearance of a new peak with the same mass as epicoccine in the PKSi12 mutant (suggesting that an isomer of epicoccine was produced) could be due to a shift in general regulation of secondary metabolites in *E. nigrum* as a result of deletion of the PKSi12 gene and the absence of its products, leading to production of the compound in question.

The absence of epicolactone in the samples of metabolites from the WT indicates that this polyketide is not being produced under the conditions tested. Further experiments are needed to find the best conditions and duration of incubation for optimal epicolactone production.

According to the evidence provided by the bioinformatic analysis and the chemical-analysis data, a model of biosynthesis of epicoccone B and epicoccine in *E. nigrum* was propose. It is hypothesized that both compounds are derived from 5-methylorsellinic acid within a pathway involving oxidoreductive tailoring steps (Figure 4.15).

In the proposed biosynthetic pathway, PKSi12 synthesizes 5-methylorsellinic acid that is then activated and reduced to 2,4-dihydroxy-5,6-dimethyl benzaldehyde by the NRPS-like enzyme (homologous to *A. terreus* ATEG_03630). The cytochrome P450 (homologous to *P. brevicompactum* MpaD) is predicted to catalyze hydroxylation of the methyl group at C3, and an oxidoreductase (possibly the phenol hydroxylase) is expected to catalyze hydroxylation of C8. After that, a dehydrogenase catalyzes ring closure and produces epicoccone B. Finally, subsequent reduction of epicoccone B yields epicoccine.

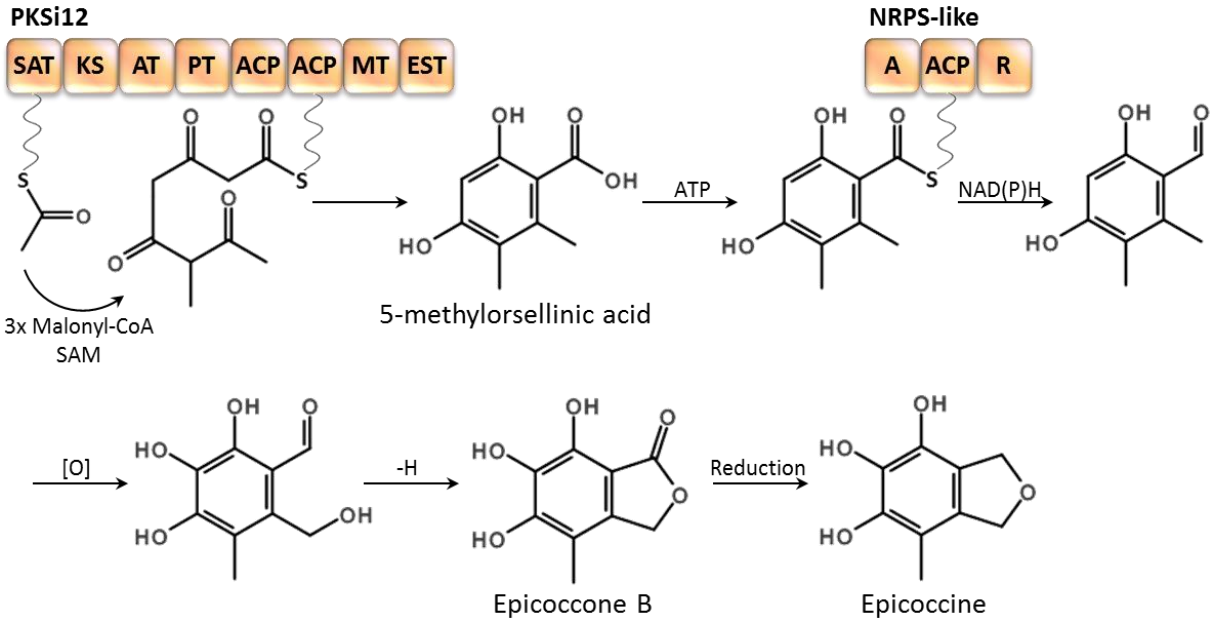

Figure 4.15 – The proposed model of biosynthesis of epicoccone B and epicoccine in *E. nigrum*. The hypothetical biosynthetic pathway was compiled on the basis of the functions of *E. nigrum's* NRPS-like enzyme, PKSi12, and cytochrome P450 homologs identified *in silico;* the last step in the pathway is based on the model of biosynthesis described by Kemami Wangun et al. (2008).

The evidence presented in this study suggests that *E. nigrum* PKSi12 is related to production of epicoccone B and epicoccine: the hypothetical epicolactone precursors as described by Ellerbrock et al. (2014, 2015). The lack of antagonistic action on the phytopathogens *R. solani*, *C. falcatum*, *F. oxysporum*, and *C. paradoxa* by *E. nigrum* ΔPKSi12 indicates that an antimicrobial compound normally produced by the WT is not produced by this mutant, probably epicolactone.

4.5 Conclusions

The phylogenetic analysis, assessment of domain architecture, and evaluation of gene cluster annotation among *E. nigrum* PKSs in this study established a foundation for future studies aimed at elucidating the role of PKS gene clusters in the biosynthesis of various bioactive polyketides in *E. nigrum*.

In addition, an efficient protocol for targeted gene replacement in *E. nigrum* was successfully established here: a crucial step for identification and manipulation of genes related to the production of bioactive secondary metabolites in this genetically unexplored endophyte.

Deletion of the *PKSi12* gene showed that PKSi12's products are related to the growth-inhibitory activity of *E*. *nigrum* toward fungal phytopathogens. The bioinformatic and chemical analyses of this enzyme suggest that it is probably involved in the biosynthesis of epicoccone B and epicoccine, the previously proposed epicolactone precursors. A possible biosynthetic pathway for epicoccone B and epicoccine in *E*. *nigrum* was also proposed here.

OVERALL CONCLUSIONS

Two approaches were used in this study to identify the genes involved in epicolactone biosynthesis: 1) analysis of epicolactone-deficient mutants obtained previously by random mutagenesis and 2) genome mining of candidate genes followed by targeted gene replacement. With the first approach, it was found that the T-DNA got inserted into intergenic regions, and the expression of the genes located near the insertion was not significantly affected in the mutants, suggesting that the T-DNA was probably inserted into a regulatory region. With the second approach, five NR-PKSs that are known to synthesize mono- or polycyclic aromatic compounds were selected for targeted gene replacement. In November 2014, Ellerbrock et al. published a hypothetical biosynthetic pathway for epicolactone; this scheme guided the present study toward identification of an orsellinic acid synthase or a synthase of an orsellinic-acid derivative because the predicted epicolactone precursors (epicoccone B and epicoccine) are believed to derive from orsellinic acid.

Deletion of the *PKSi12* gene showed that PKSi12 is related to antagonistic action of *E*. *nigrum* on fungal phytopathogens. Chemical analysis enabled putative identification of epicoccine and epicoccone B, whose production was altered in the ΔPKSi12 mutant. According to the results of the chemical and *in silico* analyses, a hypothetical biosynthetic pathway for epicoccone B and epicoccine in *E*. *nigrum* was proposed. Nonetheless, there is still a lot to learn about epicolactone biosynthesis, and further research is needed to confirm the function of PKSi12 in the biosynthesis of epicoccone B and epicoccine.

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APPENDIX A - Primers used for real-time PCR analysis

Primer name	Primer sequence (5'-3')	Gene
3P450 F	TGCTGCGAGCTCCGACTACCA	Cytochrome P450 oxidoreductase
3P450 R	CCTGGAATGCCGCTGGTCCG	
2LAC F	AGCGGACTGGGGCGATGAAA	Laccase
2LAC R	GGGCCTTTCGCGTGGAGAGT	
2FT F	AGAGGAGCAGCGCAATAGAG	Transcription factor
2FT R	CATAGTATCGTGCGCCTGTG	
2PKSi_9 F	CATTCTGACACGCTTCCCAG	PKSi9
2PKSi_9 R	AGGCGTCGTGAATGTGAATG	
2PKSi_8 F	TTCAGCAAGCCAGAGACAGA	PKSi8
2PKSi_8 R	CACCGAGCTTTGACTTCGAC	
2PH F	AAGGTACGCGAGAGTCTCTG	Hypothetical protein
2PH R	GTCGACTGAGGAGAAGGAGG	
1Btub F	CAGACCATCTCCGGCGAGCA	β-tubulin
1Btub R	ACCAGCACCAGACTGGCCGAA	

APPENDIX B - Protein sequences included in PKSi8 and PKSi9 phylogenetic analysis

Organism	PKS name	Products	Type	Domain architecture	Accession number	Reference
Aspergillus nidulans	AfoG	Asperfuranone	HR-PKS	KS-AT-DH-MT-ER-KR-ACP	CBF88289.1	(CHIANG et al., 2009)
Aspergillus nidulans	AfoE	Asperfuranone	NR-PKS	SAT-KS-AT-PT-ACP-MT-R	CBF88295.1	(CHIANG et al., 2009)
Aspergillus niger	AzaB	Azaphilones	HR-PKS	KS-AT-DH-MT-ER-KR-ACP	EHA28244.1	(ZABALA et al., 2012)
Aspergillus niger	AzaA	Azaphilones	NR-PKS	SAT-KS-AT-PT-ACP-MT-R	EHA28237.1	(ZABALA et al., 2012)
Aspergillus terreus	AtCURS1	Dehydrocurvularin	HR-PKS	KS-AT-DH-ER-KR-ACP	AGC95324.1	(XU et al., 2013)
Aspergillus terreus	AtCURS2	Dehydrocurvularin	NR-PKS	SAT-KS-AT-PT-ACP-TE	AGC95321.1	(XU et al., 2013)
Aspergillus terreus	LovF	Lovastatin	HR-PKS	KS-AT-DH-MT-ER-KR-ACP	Q9Y7D5.1	(THR et al., 1999)
Aspergillus terreus	LovB	Lovastatin	HR-PKS	KS-AT-DH-MT-KR-ACP-CON-H	Q0C8M3.2	(THR et al., 1999)
Botryotinia fuckeliana	Bcboa9	Botcinic acid	HR-PKS	KS-AT-DH-ER-KR-ACP	CBX87032.1	(DALMAIS et al., 2011)
Botryotinia fuckeliana	Bcboa6	Botcinic acid	PR-PKS	KS-AT-DH-MT-KR-ACP	CAP58786.1	(DALMAIS et al., 2011)
Chaetomium chiversii	CcRADS1	Radicicol	HR-PKS	KS-AT-DH-ER-KR-ACP	ACM42406.1	(WANG et al., 2008)
Chaetomium chiversii	CcRADS2	Radicicol	NR-PKS	SAT-KS-AT-PT-ACP-TE	ACM42403.1	(WANG et al., 2008)
Chaetomium globosum	CazF	Azaphilones	HR-PKS	KS-AT-DH-MT-ER-KR-ACP	EAQ86385.1	(WINTER et al., 2012)
Chaetomium globosum	CazM	Azaphilones	NR-PKS	SAT-KS-AT-PT-ACP-MT-R	AKA40069.1	(WINTER et al., 2012)
Fusarium graminearum	PKS4	Zearalenone	HR-PKS	KS-AT-DH-ER-KR-ACP	ABB90283.1	(KIM et al., 2005)
Fusarium graminearum	PKS13	Zearalenone	NR-PKS	SAT-KS-AT-PT-ACP-TE	ABB90282.1	(KIM et al., 2005)
Hypomyces subiculosus	Hpm8	Hypothemycin	HR-PKS	KS-AT-DH-ER-KR-ACP	ACD39767.1	(REEVES et al., 2008)
Hypomyces subiculosus	Hpm3	Hypothemycin	NR-PKS	SAT-KS-AT-PT-ACP-TE	ACD39762.1	(REEVES et al., 2008)
Pochonia chlamydosporia	Rdc5	Radicicol	HR-PKS	KS-AT-DH-ER-KR-ACP	ACD39774.1	(REEVES et al., 2008)
Pochonia chlamydosporia	Rdc1	Radicicol	NR-PKS	SAT-KS-AT-PT-ACP-TE	ACD39770.1	(REEVES et al., 2008)
Talaromyces marneffei	PKS11	Mitorubrinic acid, mitorubrinol	NR-PKS	SAT-KS-AT-PT-ACP-MT-R	ADH01671.1	(WOO et al., 2012)
Talaromyces marneffei	PKS12	Mitorubrinic acid, mitorubrinol	NR-PKS	SAT-KS-AT-PT-ACP-ACP	ADH01672.1	(WOO et al., 2012)

APPENDIX C - Primers used for amplification of homologous recombination sequences (HRS) and verification of mutants.

Primer name	Primer sequence (5'-3')	Use
PKS7-U1	AGGTCGTATUGGTGTTGCTTGTTTACAGCGTGG	Amplification of HRS of PKSi7 gene
PKS7-U2	AGTATTGCGUGCTATGGCTCTTGTGCACGTAGA	
PKS7-U3	ATGACCTAGUGATTGCGTCTTGGGTTGGAAT	
PKS7-U4	ATTAAACCTUGGTCTACTTCCCGATACTATCGAAATC	
PKS8-U1	AGGTCGTATUAGAAACTCGGTAATTCCTACCCCTT	Amplification of HRSs of PKSi8 gene
PKS8-U2	AGTATTGCGUGAAGCGAACGTTATCTTTGAGTTATT	
PKS8-U3	ATGACCTAGUGCGGACATATTGACTGAAATTATCTTG	
PKS8-U4	ATTAAACCTUGCATGACTAAGTGTGGGGTTATCAGA	
PKS9-U1	AGGTCGTATUTTGGCACCTGACACTACGGACA	Amplification of HRSs of PKSi9 gene
PKS9-U2	AGTATTGCGUGGCTGGCAGTGGGTTTTGA	
PKS9-U3	ATGACCTAGUAGCGCCACCACGATTTTGT	
PKS9-U4	ATTAAACCTUAAATTGCGTCTCGACCTGATATGC	
PKS10-U1	AGGTCGTATUTTCTAAGTATTTACACCTGACTTCTGG	Amplification of HRSs of PKSi10 gene
PKS10-U2	AGTATTGCGUCTCTGAACAGGATGAAGAGATTTGG	
PKS10-U3	ATGACCTAGUTAAAACTGGTACTACCCAACTGCGA	
PKS10-U4	ATTAAACCTUGGGTTGCAAAACCTAGGCTTATAA	
PKS11-U1	AGGTCGTATUCATAATCGGTACGCTCGCTTCC	Amplification of HRSs of PKSi11 gene
PKS11-U2	AGTATTGCGUGAATGGTTGGGTAGAGGCTAGATTG	
PKS11-U3	ATGACCTAGUGGTTGAGTGCAAAAATGAGTGCAAA	
PKS11-U4	ATTAAACCTUAGGAACAAAAAGGCCGCAGAA	
PKS12-U1	AGGTCGTATUACCTTCACGTAACCCTCGAAGAA	Amplification of HRSs of PKSi12 gene
PKS12-U2	AGTATTGCGUAAATAGGGATCGGCTTGTGAAAA	
PKS12-U3	ATGACCTAGUTTTGCAATTGGTTCAAAAGGTCGAGAT	
PKS12-U4	ATTAAACCTUCCAGTATTCGTGGCGTAAGTACATACA	
PKS7-T1	GTAGATAGCTTAGACGGAATCTCAACTGG	Verification of ΔPKSi7 mutants
PKS7-T2	AATAAGCCTCAGATTCCAGTCATGTC	
PKS7-T3	CACGTCGATCAGCATCAACTTCA	
PKS7-T4	TCGTCGATAACGCCAGACCG	
PKS8-T1	AGGATTCTTGAGTGCATATAGACGAAC	Verification of ΔPKSi8 mutants
PKS8-T2	TGTTTCAATCATGAAGCCCGA	
PKS8-T3	AGCTTTCCGTCAGCACCTGTC	
PKS8-T4	GGAGTTTCTTTGTAGTTCTCAGAATGG	
PKS9-T1	TTCGTTCTTACATCACGAAGTTCTGCAAT	Verification of ΔPKSi9 mutants
PKS9-T2	GATCATCTTCATCGTGTCTTCGAGA	
PKS9-T3	GCCCTCAGCCTCTCTATCGAA	
PKS9-T4	CCTGGGCAAGATTAGCGAATTG	
PKS10-T1	GTTGGGTGTTCATCCATTGCG	Verification of ΔPKSi10 mutants
PKS10-T2	CCTCCTGCAATAGCTGCTGTACA	
PKS10-T3	CAGCAACTCGCAAGTAATAGAAGTAGG	
PKS10-T4	TATAAGGTCATCGATGAATCAGGCCTC	
PKS11-T1	CCAGTAATTCTTCAGATCCCACTTG	Verification of ΔPKSi11 mutants
PKS11-T2	ATCGCAAGTTCAGCCAATCCT	
PKS11-T3	TAGTGCTGGGCCCATGCTAA	
PKS11-T4	CGAGGAGCTTCTCTTTGTGCAG	
PKS12-T1	CAATTTCATAACATCGATCGCTGC	Verification of ΔPKSi12 mutants
PKS12-T2	TGAAGTCGCCGATATCCCAATC	
PKS12-T3	CAATACGCGCATCGTTCCTG	
PKS12-T4	TAACTTTCCCTAACCCCTGAGAGG	
RF-1*	TCTCCTTGCATGCACCATTCCTTG	Standard/generic primers for verification of mutants (SØRENSEN et al., 2014)
RF-2*	AAATTTTGTGCTCACCGCCTGGAC	
Hyg588U*	AGCTGCGCCGATGGTTTCTACAA	
Hyg588L*	GCGCGTCTGCTGCTCCATACAA	

The underlined part of the primers represents the overhang necessary for the vector construction strategy. U: 2-deoxyuridine; HRS: homologous recombination sequence. *Reference: SØRENSEN, L. Q. et al. Genetic transformation of *Fusarium avenaceum* by *Agrobacterium tumefaciens* mediated transformation and the development of a USER-Brick vector construction system. BMC molecular biology, v. 15, n. 1, p. 15, 2014.

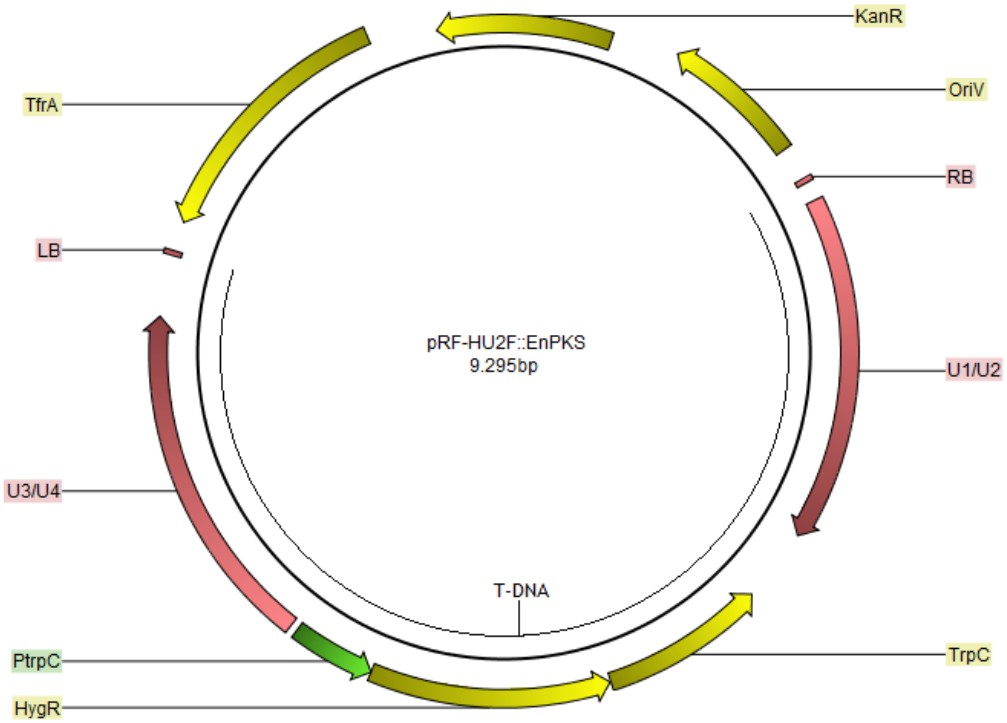
APPENDIX D - Protein sequences included in the phylogenetic analysis and alignments

Organism	PKS name	Products	Type	Domain architecture	Accession number	Reference
Alternaria alternata	PksA	THN	NR-PKS	SAT-KS-AT-PT-ACP-ACP-TE	AFN68292.1	(KIMURA et al., 1993)
Alternaria solani	PKSN	Alternapyrone	HR-PKS	KS-AT-DH-MT-ER-KR-ACP	BAD83684.1	(FUJII et al., 2005)
Alternaria solani	Sol1	Desmethylprosolanapyrone I, Solanapyrone	HR-PKS	KS-AT-DH-MT-ER-KR-ACP	BAJ09789.1	(KASAHARA et al., 2010)
Armillaria mellea	ArmB	Orsellinic acid	NR-PKS	SAT-KS-AT-PT-ACP-ACP-TE	AFL91703	(LACKNER et al., 2013)
Ascochyta rabiei	PKS1	THN	NR-PKS	SAT-KS-AT-?	ACS74449.1	(AKAMATSU et al., 2010)
Aspergillus clavatus	CcsA	Cytochalasin	PKS-NRPS	KS-AT-DH-MT-KR-ACP-CON-H-A-ACP-R	EAW09117.1	(QIAO; CHOOI; TANG, 2011)
Aspergillus flavus	PKS27	Asparasone	NR-PKS	SAT-KS-AT-PT-ACP-TE	EED57518.1	(CARY et al., 2014)
Aspergillus flavus	Pks-nrps	Cyclopiazonic acid	PKS-NRPS	KS-AT-DH-MT-KR-ACP-CON-H-A-ACP-R	XP_002379959.2	(CHANG; HORN; DORNER, 2009)
Aspergillus flavus	PksA	Norsolorinic acid, aflatoxin	NR-PKS	SAT-KS-AT-PT-ACP-TE	AAS90022.1	(EHRLICH et al., 2004)
Aspergillus fumigatus	EncA	Atrochrysone carboxylic acid, Endocrocin	NR-PKS	SAT-KS-AT-PT-ACP	XP_746435.1	(LIM et al., 2012)
Aspergillus fumigatus	Alb1	THN	NR-PKS	SAT-KS-AT-PT-ACP-ACP-TE	AAC39471.1	(TSAI et al., 1998)
Aspergillus nidulans	PkdA	2-ethyl-4,6-dihydroxy-3,5-dimethylbenzaldehyde	NR-PKS	SAT-KS-AT-PT-ACP-R	XP_658127	(AHUJA et al., 2012)
Aspergillus nidulans	AusA	3,5-dimethylorsellinic acid	NR-PKS	KS-AT-PT-ACP-MT-EST	CBF80428.1	(NIELSEN et al., 2011)
Aspergillus nidulans	PkbA	3-methylorsellinic acid	NR-PKS	SAT-KS-AT-PT-ACP-ACP-MT-EST	XP_664052	(AHUJA et al., 2012)
Aspergillus nidulans	MdpG	Anthrone	NR-PKS	SAT-KS-AT-PT-ACP	CBF90097.1	(SCHERLACH et al., 2011)
Aspergillus nidulans	AfoG	Asperfuranone	HR-PKS	KS-AT-DH-MT-ER-KR-ACP	CBF88289.1	(CHIANG et al., 2009)
Aspergillus nidulans	PkfA	Orsellinaldehyde	NR-PKS	SAT-KS-AT-PT-ACP-R	XP_660834	(AHUJA et al., 2012)
Aspergillus nidulans	OrsA	Orsellinic acid	NR-PKS	SAT-KS-AT-PT-ACP-ACP-TE	CBF73505.1	(SCHROECKH et al., 2009)
Aspergillus niger	AzaB	Azaphilones	HR-PKS	KS-AT-DH-MT-ER-KR-ACP	EHA28244.1	(ZABALA et al., 2012)
Aspergillus niger	AzaA	Azaphilones	NR-PKS	SAT-KS-AT-PT-ACP-MT-R	EHA28237.1	(ZABALA et al., 2012)
Aspergillus stellatus	AndM	3,5-dimethylorsellinic acid	NR-PKS	KS-AT-PT-ACP-MT-EST	BAP81867.1	(MATSUDA et al., 2014)
Aspergillus terreus	Trt4	3,5-dimethylorsellinic acid	NR-PKS	KS-AT-PT-ACP-MT-EST	EAU29529.1	(ITOH et al., 2012)
Aspergillus terreus	AtX	6-methylsalicylic acid	PR-PKS	KS-AT-DH-KR-ACP	BAA20102.2	(FUJII et al., 1996)

Organism	PKS name	Products	Type	Domain architecture	Accession number	Reference
Aspergillus terreus	ACAS	Atrochrysone carboxylic acid, Emodin	NR-PKS	SAT-KS-AT-PT-ACP	EAU31624.1	(AWAKAWA et al., 2009)
Aspergillus terreus	LovF	Lovastatin	HR-PKS	KS-AT-DH-MT-ER-KR-ACP	Q9Y7D5.1	(THR et al., 1999)
Aspergillus terreus	ATEG_03629	5-methylorsellinic acid	NR-PKS	SAT-KS-AT-PT-ACP-ACP-MET-EST	EAU35431.1	(WANG; BEISSNER; ZHAO, 2014)
Bipolaris oryzae	PKS1	THN	NR-PKS	SAT-KS-AT-PT-ACP-ACP-TE	BAD22832.1	(MORIWAKI et al., 2004)
Botryotinia fuckeliana	Bcboa9	Botcinic acid	HR-PKS	KS-AT-DH-ER-KR-ACP	CBX87032.1	(DALMAIS et al., 2011)
Byssochlamys nivea	6MSAS	6-methylsalicylic acid	PR-PKS	KS-AT-DH-KR-ACP	AAK48943.1	(PUEL et al., 2007)
Chaetomium globosum	CazF	Azaphilones	HR-PKS	KS-AT-DH-MT-ER-KR-ACP	EAQ86385.1	(WINTER et al., 2012)
Chaetomium globosum	CHGG_10128	5-methylorsellinic acid	NR-PKS	KS-AT-PT-ACP-ACP-MT-EST	EAQ83724.1	(ISHIUCHI et al., 2012)
Cladonia uncialis	MPAS	Methylphloracetophenone, Usnic acid	NR-PKS	SAT-KS-AT-PT-ACP-MT-EST	ALA62323.1	(ABDEL-HAMEED et al., 2015)
Cochliobolus heterostrophus	PKS2	T-toxin	HR-PKS	KS-AT-DH-ER-KR-ACP	AAR90257.1	(BAKER et al., 2006)
Coprinopsis cinerea	CC1G_05377	Orsellinic acid	NR-PKS	KS-AT-PT-ACP-TE	XP_001835415.2	(ISHIUCHI et al., 2012)
Dothistroma septosporum	PksA	Norsolorinic acid, dothistromin	NR-PKS	SAT-KS-AT-PT-ACP-ACP-ACP-TE	AAZ95017.1	(BRADSHAW et al., 2006)
Fusarium fujikuroi	FUM5	Fumonisin	HR-PKS	KS-AT-DH-MT-ER-KR-ACP	AAD43562.2	(PROCTOR et al., 1999)
Fusarium fujikuroi	Fub1	Fusaric acid	HR-PKS	KS-AT-DH-ER-KR-ACP	CCT65184.1	(NIEHAUS et al., 2014)
Fusarium fujikuroi	FusA	Fusarin	PKS-NRPS	KS-AT-DH-MT-KR-ACP-CON-H-A-ACP-R	AFP73394.1	(DÍAZ-SÁNCHEZ; AVALOS; LIMÓN, 2012)
Fusarium fujikuroi	Fsr1	Fusarubins	NR-PKS	SAT-KS-AT-PT-ACP-ACP-R	CCE67070.1	(STUDT et al., 2012)
Fusarium graminearum	PKS14	Orsellinic acid	NR-PKS	SAT-KS-AT-PT-ACP-TE	EYB32182.1	(JØRGENSEN et al., 2014)
Fusarium graminearum	PKS13	Zearalenone	NR-PKS	SAT-KS-AT-PT-ACP-TE	ABB90282.1	(KIM et al., 2005)
Fusarium heterosporum	FsdS	Equisetin	PKS-NRPS	KS-AT-DH-MT-KR-ACP-CON-H-A-ACP-R	AAV66110.2	(SIMS et al., 2005)
Fusarium verticillioides	Fub1	Fusaric acid	HR-PKS	KS-AT-DH-ER-KR-ACP	EWG54266.1	(BROWN et al., 2012)
Glarea lozoyensis	PKS2	6-methylsalicylic acid	PR-PKS	KS-AT-DH-KR-ACP	AAX35547.1	(LU et al., 2005)

Organism	PKS name	Products	Type	Domain architecture	Accession number	Reference
Glarea lozoyensis	PKS4	Pneumocandins	HR-PKS	KS-AT-DH-MT-ER-KR-ACP	EPE34340.1	(CHEN et al., 2013)
Glarea lozoyensis	PKS1	THN	NR-PKS	SAT-KS-AT-PT-ACP-ACP-TE	AAN59953.1	(ZHANG et al., 2003)
Hypomyces subiculosus	Hpm3	Hypothemycin	NR-PKS	SAT-KS-AT-PT-ACP-TE	ACD39762.1	(REEVES et al., 2008)
Leptosphaeria maculans	PKS3	Phomenoic acid	HR-PKS	KS-AT-DH-ER-KR-ACP	AGC79958.1	(ELLIOTT et al., 2013)
Metarhizium robertsii	NGS1	NG-391	PKS-NRPS	KS-AT-DH-MT-KR-ACP-CON-H-A-ACP-R	EXU99208.1	(DONZELLI et al., 2010)
Monascus pilosus	MpPKS5	Azaphilones	NR-PKS	SAT-KS-AT-PT-ACP-ACP-MT-R	AGN71604.1	(BALAKRISHNAN et al., 2013)
Nectria haematococca	PKS1	Fusarubins	NR-PKS	SAT-KS-AT-PT-ACP-ACP-R	XP_003039929	(AWAKAWA et al., 2012)
Ophiostoma piceae	PKS1	THN	NR-PKS	SAT-KS-AT-PT-ACP-ACP-TE	ABD47522.2	(TANGUAY; TANGEN; BREUIL, 2007)
Parastagonospora nodorum	MlnS	Mellein	PR-PKS	KS-AT-DH-KR-ACP	AIW00670.1	(CHOOI et al., 2015)
Penicillium brevicompactum	MpaC	5-methylorsellinic acid, Mycophenolic Acid	NR-PKS	KS-AT-PT-ACP-MT-EST	ADY00130.1	(REGUEIRA et al., 2011)
Penicillium citrinum	MlcB	Compactin	HR-PKS	KS-AT-DH-MT-ER-KR-ACP	BAC20566.1	(ABE et al., 2002)
Penicillium expansum	CheA	Cheatoglobosin	PKS-NRPS	KS-AT-DH-MT-KR-ACP-CON-H-A-ACP-R	CAO91861.1	(SCHUMANN; HERTWECK, 2007)
Penicillium griseofulvum	MSAS	6-methylsalicylic acid	PR-PKS	KS-AT-DH-KR-ACP	P22367.1	(BECK et al., 1990)
Pochonia chlamydosporia	Rdc1	Radicicol	NR-PKS	SAT-KS-AT-ACP-TE	ACD39770.1	(REEVES et al., 2008)
Sordaria macrospora	Pks	THN	NR-PKS	SAT-KS-AT-PT-ACP-ACP-TE	CAM35471.1	(ENGH; NOWROUSIAN; KÜCK, 2007)
Talaromyces marneffei	PKS11	Mitorubrinic acid, mitorubrinol	NR-PKS	SAT-KS-AT-PT-ACP-MT-R	ADH01671.1	(WOO et al., 2012)
Talaromyces marneffei	Alb1	THN	NR-PKS	SAT-KS-AT-PT-ACP-ACP-TE	ADH01664.1	(WOO et al., 2010)
Talaromyces stipitatus	TropA	3-methylorcinaldehyde	NR-PKS	SAT-KS-AT-PT-ACP-ACP-R	DAA64703.1	(DAVISON et al., 2012)

APPENDIX E - Scheme of the vectors used for targeted gene replacement by homologous recombination via *Agrobacterium tumefaciens* mediated transformation



HygR: Hygromycin resistance gene; KanR: Kanamycin resistance gene; LB: Left border; OriV: origin of replication in E. coli; pTrpC: Tryptophan promoter from *Aspergillus nidulans*; RB: Right border; TrfA: replication initiation gene (broad-host-range); T-DNA: Transfer DNA; Trpc: Tryptophan terminator from *A. nidulans*; U1/U2 and U3/U4: homologous recombination sequences.

APPENDIX F - Multiple sequence alignments of domains from *E. nigrum* PKSs and some representative characterized fungal PKSs

β-ketoacyl synthase

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Epicoccum nigrum PKSi1                RVSYFFDWRGPSMTIDTACSSSLVAVHQAVQSLRS-G
Epicoccum nigrum PKSi2                RVNYVFNLKGPSMTIDTACSSTMYALHMACRSLQA-G
Epicoccum nigrum PKSi3                RLSWFFDLRGPSVTIDTACSSSMVALHLGCQSLRT-G
Epicoccum nigrum PKSi4                RISYHLGLQGPSTAVDAACASSLIAIHLARQAIVS-G
Epicoccum nigrum PKSi5                RISHFFDLRGPSYTVDTACSASLVALHNACQSLRT-G
Epicoccum nigrum PKSi6                RLSYFFNWKGPSMTIDTACSSSLIAVHQAVQLLRS-G
Epicoccum nigrum PKSi7                RINYYFKFSGPSYSIDTACSSSLAAIQLACTSLWA-G
Epicoccum nigrum PKSi8                RLSHYFDLRGASMSIDTGCSTTLTALHQACNDLRN-R
Epicoccum nigrum PKSi9                KLSHYFGWLGPSLTLDTACSASAVAIHAACRAILS-G
Epicoccum nigrum PKSi10               RISYFYKLSGPSIVTDTACSSSMVSVYQACRALQN-G
Epicoccum nigrum PKSi11               RQNFYFKFTGPSYSVDTACSSSLAAMHVACNALWR-G
Epicoccum nigrum PKSi12               KISHFFGFDGPSLVFDTACSSSAVAIHTASRAIQT-G
Alternaria solani PKSN                RLSWFYNLRGPSLTVDTACSSSLTAFHLACQSIRT-G
Aspergillus clavatus CcsA             RLSYFFDWHGPSLTIDTACSSSLIAMHHAVQTLRS-G
Aspergillus flavus PKS27              RQNFFYKFSGPSYSVDTACSSGLAALHLACNSLLK-G
Aspergillus niger AzaB                RISHFYDLRGPSMTVDTGCSTSLTALHLACQNLRS-G
Bipolaris oryzae PKS1                 RINYYFRFSGPSYSVDTACSSSLAAIQLACTSLWA-G
Chaetomium globosum CHGG 10128        KISHFFNLSGPSMVFDTACSSSAVAIHTRMPGSQEWG
Fusarium fujikuroi FUM5               RISYEYDLKGPSFTIKAGCSSSLIALHEAVRAIRA-G
Fusarium graminearum PKS14            RISYFYQLSGPSIVTDTACSSSTVSIYQACRAIQN-G
Leptosphaeria maculans PKS3           RISHFFDFRGPSFTVDTACSSSLVAIHTACQSLRN-G
Parastagonospora nodorum MlnS         RISYHLDLMGPSAAVDAACASSMVAVHTGRQAILA-G
Talaromyces marneffei PKS11           KVSHYFGWTGTGLTLDTACSSSLVAVHLACKAILS-G
                                      . .      *..   .:.*::   :.
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Acyl transferase

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Epicoccum nigrum PKSi1                LLVEILRAAGIRFSTVVGHSSGEIAAAYAAGRLSSK
Epicoccum nigrum PKSi2                AMVDLLRSWGVLPAAVVGHSSGEIAAAYCAGHITAE
Epicoccum nigrum PKSi3                ALVNLLREWGVAPQAVVGHSSGEISAAYAAGYVTSE
Epicoccum nigrum PKSi4                GLTALLKSKGVAPQAIIGHSVGEIAAAVASGCLTEQ
Epicoccum nigrum PKSi5                ALVNLLISWGLQPAAVVGHSSGEIGAAYAAGFITFE
Epicoccum nigrum PKSi6                LLTDMLQAVGVSFEAVVGHSSGEIGAAYTCGYLSAW
Epicoccum nigrum PKSi7                ALARMWESWGIKPIAVVGHSLGEYAALHVAGIISAS
Epicoccum nigrum PKSi8                ALVRLLESWGVKPSAVSSHSSGEIASAYAAGVLSLR
Epicoccum nigrum PKSi9                STAKSWIECGVKPVAVVGHSFGELTAMCVSGVLGLE
Epicoccum nigrum PKSi10               GLARLFMSWGIMPDYVMGHSLGEYAALCVSGALSLE
Epicoccum nigrum PKSi11               AMAKLWQSWGIQPVAVVGHSLGEYAALNTAGVLSDA
Epicoccum nigrum PKSi12               SMAMSWLDAGLRVHTIIGHSFGELTALAVAGYLSLE
Alternaria solani PKSN                ALVDLLSGFDVRPVAVLGHSSGEIAAAYAAGFIDQE
Aspergillus clavatus CcsA             MLVDMLREAGIQFSAVVGHSSGEIGAAYAAGCLSAK
Aspergillus flavus PKS27              AMARLWRSWGIEPCAVVGHSLGEYAALNIAGVLSEA
Aspergillus niger AzaB                ALVELLWSWGIHPTAVTGHSSGEIAAAYASKALDMK
Bipolaris oryzae PKS1                 ALARMWGAWGVRPIAVIGHSLGEYAALHVAGVISAS
Chaetomium globosum CHGG_10128        SCAWAWIDAGVQIDAMIGHSFGQLTALCVAGAMSLE
Fusarium fujikuroi FUM5               ALVNLLRSWGITPAAVVGHSSGEMAAAYAAGAISSE
Fusarium graminearum PKS14            ALAKTFISWDIKPHYVMGHSLGEYTALCISGVLTPS
Leptosphaeria maculans PKS3           ALVELLRSWNVRPSAVAGHSSGEIGAAYAADLITFE
Parastagonospora nodorum MlnS         GLIQLLKAKGVHPHAVIGHSVGEIAASVAAGCLTPE
Talaromyces marneffei PKS11           ACGKSWIDSGVQPVAVVGHSFGELTSLCISGVLSLE
                                            .:    : .** *:  :   .  :
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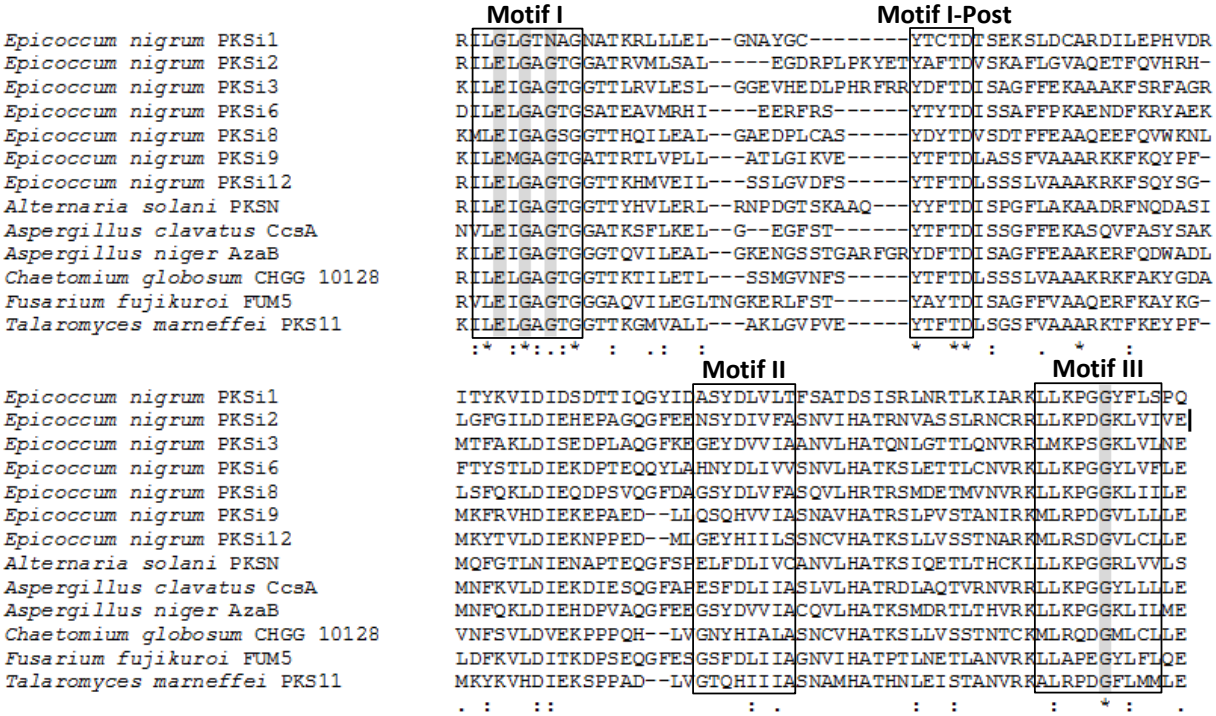
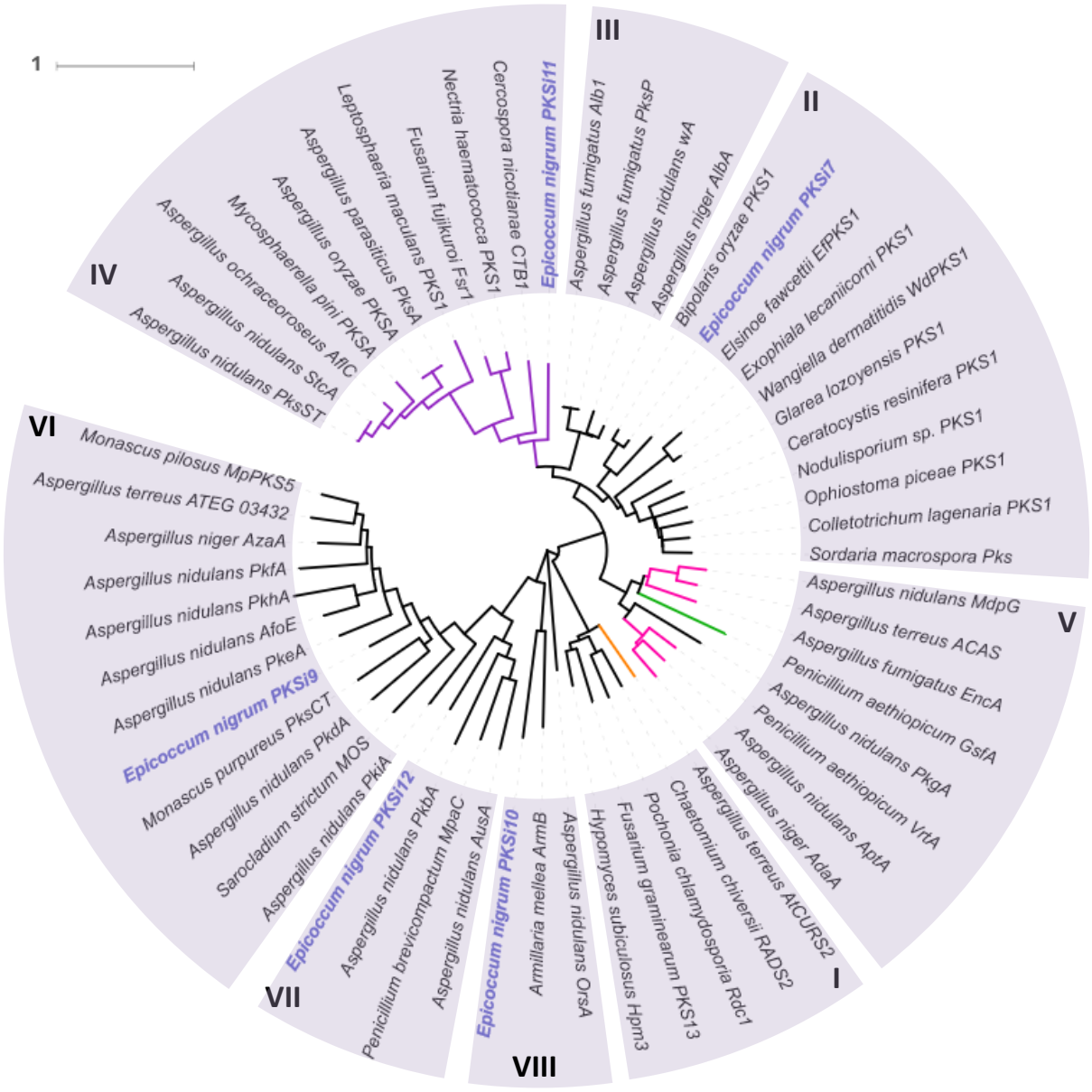
Acyl carrier protein

Methyltransferase domain



APPENDIX G - Phylogenetic analysis of the PT domain amino acid sequences of the NR-PKS proteins in *E. nigrum*.



The PT domains of *E. nigrum* NR-PKSs and 53 characterized fungal PKSs were aligned by Muscle and the phylogenetic tree was constructed using the Neighbor-Joining method in the Molecular Evolutionary Genetics Analysis software version 7.0 (MEGA7) (KUMAR; STECHER; TAMURA, 2016). The PKS sequences from characterized fungal PKSs were retrieved from Liu et al. (2015). The classification of the PT domains follows the description by Liu et al. (2015). The color of the lines indicates the cyclization regioselectivity: black – C2-C7; purple – C4-C9; pink – C6-C11; green – C1-C6; orange – C3-C8.

APPENDIX H - Annotation of the polyketide synthase gene clusters

Annotation of the NR-PKS gene clusters (PKSi7, PKSi10, PKSi11 and PKSi12) and the dual HR-PKS/NR-PKS gene cluster (PKSi8 and PKSi9) in *E. nigrum*. Locations of genes are given according to the respective contig. The predicted function is based on domains and protein homologs obtained by BLASTP. Domains marked with an asterisk are present in two copies.

Gene	Location	ORF length (pb)	Protein length (aa)	Domains/Motifs	Protein homolog obtained by BlastP (Description, Query cover, E-value, Identity)	Predicted function
Cluster PKSi7 (contig 44)						
1	20638-22499	1761	586	CL14616: Eukaryotic cytochrome b(561) (endosomal or secretory vesicle-specific electron transport proteins) PF10355.6: Protein of unknown function (Ytp1)	Integral membrane protein [*Stemphylium lycopersici*] (KNG47669.1), 99%, 0.0, 69%	Integral membrane protein
2	22828-23614	696	231	PF08653.7: DASH complex subunit Dam1 (subunit microtubule-binding complex)	Dash complex component protein [*Stemphylium lycopersici*] (KNG47668.1), 93%, 1e-72, 70%	Dash complex component protein
3	24142-26019	1821	606	PF03765.12: CRAL/TRIO, N-terminal domain PF00650.17: CRAL/TRIO domain (binds small lipophilic molecules)	Cral trio domain-containing protein [*Stemphylium lycopersici*] (KNG47667.1), 76%, 0.0, 71%	CRAL/TRIO domain-containing protein
4	26710-27998	1161	386	PF00318.17: Ribosomal protein S2 (involved in formation of the translation initiation complex)	Ribosomal protein S2 [*Macrophomina phaseolina*] (EKG10462.1), 88%, 1e-135, 60%	Ribosomal protein S2
5	28887-32318	3333	1110	PF13426.4*: PAS domain (signal sensor domain) PF08447.9: PAS fold PF00320.24: GATA zinc finger (binds to DNA)	White collar [*Phaeosphaeria avenaria*] (ACS74817.1), 99%, 0.0, 69%	White collar
6	33903-34747	696	231	PF00071.19: Ras family (class of regulatory GTP hydrolases)	CLG2P [*Curvularia lunata*] (AEI69325.1), 100%, 1e-116, 73%	RAS small monomeric GTPase
7	35609-36598	888	295	SMART00225: Broad-Complex, Tramtrack and Bric a brac; CD07677: The F-BAR (FES-CIP4 Homology and Bin/Amphiphysin/Rvs) domain of FCH and double SH3 domains 2	BTB/POZ domain containing protein [*Metarhizium majus*] (XP_014574675.1), 83%, 4e-20, 31%	BTB/POZ domain containing protein
8	38113-39228	1116	371	CL00446: Metallo-beta-lactamase superfamily	Metallo-hydrolase/oxidoreductase [*Punctularia strigosozonata*] (XP_007380179.1), 75%, 3e-33, 33%	Metallo-beta-lactamase
9	39370-40108	588	195	PF02996.14: Prefoldin subunit (hexameric molecular chaperone complex)	Prefoldin subunit 3 [*Pyrenophora tritici-repentis*] (XP_001933657.1), 95%, 3e-115, 88%	Prefoldin subunit

Gene	Location	ORF length (pb)	Protein length (aa)	Domains/Motifs	Protein homolog obtained by BlastP (Description, Query cover, E-value, Identity)	Predicted function
10	42128-48632	6453	2150	PF16073.2: ACP transacylase (SAT) SMART00825: Beta-ketoacyl synthase (KS) SMART00827: Acyl transferase (AT) TIGR04532: Product template (PT) SMART00823*: Phosphopantetheine attachment site (ACP) SMART00823: Thioesterase (TE)	Polyketide synthase PKS1 [*Bipolaris oryzae*] (BAD22832.1), 100%, 0.0, 81% Biosynthesis of melanin	Polyketide synthase (PKSi7)
11	59186-62299	3045	1014	PF00172.15: Fungal Zn(2)-Cys(6) binuclear cluster domain PF04082.15: Fungal specific transcription factor domain	Transcription Factor Amr1 [*Alternaria brassicicola*] (AEN02471.1), 95%, 0.0, 78%	Transcription Factor
12	66387-67293	804	267	CD05362: Tetrahydroxynaphthalene/ trihydroxynaphthalene reductase-like	1,3,8-trihydroxynaphthalene reductase [*Alternaria alternata*] (BAA36503.1), 100%, 9e-179, 91%	THN reductase
Cluster PKSi8 and PKSi9 (contig 52)						
1	68901-70022	966	321	PF10067.6: Predicted membrane protein	Hypothetical protein [*Bipolaris sorokiniana*] (XP_007702891.1), 96%, 7e-136, 61%	Hypothetical protein
2	70798-72189	1392	463	PF02458.12: Transferase family	Trichothecene 3-O-acetyltransferase [*Monascus pilosus*] (AGN71607.1), 95%, 1e-105 , 39%	Transferase
3	70798-72189	1416	471	PF01494.16: FAD binding domain	Salicylate hydroxylase [*Monascus pilosus*] (AGN71623.1), 90%, 3e-159, 54%	Monooxygenase
4	72646-74107	1482	493	PF07690.13: Major Facilitator Superfamily	Major facilitator superfamily domain, general substrate transporter [*Penicillium italicum*] (KGO65250.1), 100%, 2e-168, 50%	MFS transporter
5	70798-72189	1026	341	PF14226.3: Non-haem dioxygenase in morphine synthesis N-terminal PF03171.17: 2OG-Fe(II) oxygenase superfamily	Non-heme dioxygenase in morphine synthesis N-terminal [*Aspergillus parasiticus*] (KJK63696.1), 94%, 1e-124, 53%	Dioxygenase
6	72646-75950	1026	341	PF01370.18: NAD dependent epimerase/dehydratase family	NAD-dependent epimerase/dehydratase [*Macrophomina phaseolina*] (EKG20229.1), 96%, 6e-115, 54%	NAD dependent epimerase/dehydratase
7	76468-77594	1113	370	PF08240.9: Alcohol dehydrogenase GroES-like domain PF00107.23: Zinc-binding dehydrogenase	Zinc-binding oxidoreductase CipB [*Pyrenophora tritici-repentis*] (XP_001930726.1), 100%, 0.0, 70%	Dehydrogenase
8	78124-79207	711	236	No domains	Hypothetical protein [*Bipolaris victoriae*] (XP_014551926.1), 93%, 4e-137, 85%	Hypothetical protein
9	79741-80853	1662	553	PF00172.15: Fungal Zn(2)-Cys(6) binuclear cluster domain PF04082.15: Fungal specific transcription factor domain	Transcription factor fungi [*Macrophomina phaseolina*] (EKG11416.1), 76%, 6e-177, 60%	Transcription factor

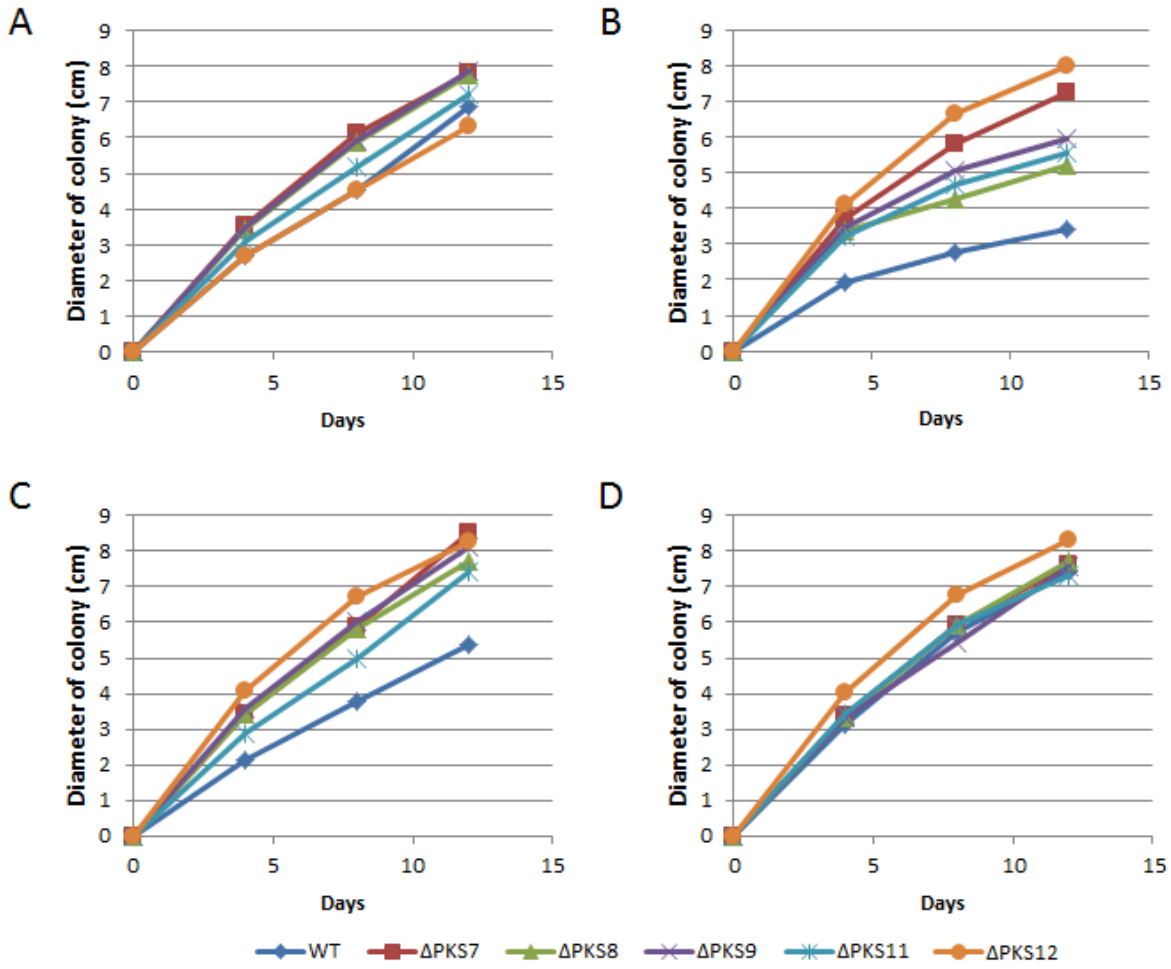
Gene	Location	ORF length (pb)	Protein length (aa)	Domains/Motifs	Protein homolog obtained by BlastP (Description, Query cover, E-value, Identity)	Predicted function
10	81837-82547	7467	2488	SMART00825: Beta-ketoacyl synthase (KS) SMART00827: Acyl transferase (AT) SMART00826: Dehydratase (DH) CD02440: Methyltransferase (MT) SMART00829: Enoyl reductase (ER) PFAM08659: Ketoreductase (KR) SMART00823: Phosphopantetheine attachment site (ACP)	Polyketide synthase AzaB [*Aspergillus niger*] (EHA28244.1), 99%, 0.0, 45% Biosynthesis of azaphilones	Polyketide synthase (PKSi8)
11	83076-85026	7731	2576	PF16073.2: ACP transacylase (SAT) SMART00825: Beta-ketoacyl synthase (KS) SMART00827: Acyl transferase (AT) TIGR04532: Product template (PT) SMART00823: Phosphopantetheine attachment site (ACP) CD02440: Methyltransferase (MT) TIGR01746: Thioester reductase (R)	Polyketide synthase PKS11 [*Talaromyces marneffei*] (ADH01671.1), 99%, 0.0, 48% Biosynthesis of mitorubrinic acid and mitorubrinol	Polyketide synthase (PKSi9)
12	87624-95315	2982	993	PF01663.19: Type I phosphodiesterase / nucleotide pyrophosphatase PF04987.11: Phosphatidylinositolglycan class N (PIG-N)	GPI Ethanolamine phosphate transferase 1 [*Stemphylium lycopersici*] (KNG52028.1), 95%, 0.0, 75%	GPI Ethanolamine phosphate transferase
13	96147-104009	1398	465	PF00996.15: GDP dissociation inhibitor	rab GDP-dissociation inhibitor [*Pyrenophora tritici*-repentis] (XP_001941510.1), 99%, 0.0, 89%	GDP dissociation inhibitor
14	106695-109918	768	255	PF06984.10: Mitochondrial 39-S ribosomal protein L47 (MRP-L47)	Hypothetical protein [*Bipolaris zeicola*] (XP_007715131.1), 95%, 2e-109, 67%	Hypothetical protein
15	110337-112052	804	267	PF07716.12: Basic region leucine zipper	BZIP domain-containing transcription [*Stemphylium lycopersici*] (KNG52029.1) 31%,, 4e-31, 75%	Transcription factor
16	112605-113426	1104	367	No domains	Hypothetical protein [*Setosphaeria turcica*] (XP_008028410.1), 98%, 9e-121, 52%	Hypothetical protein
17	113729-114594	915	304	No domains	Hypothetical protein [*Bipolaris oryzae*] (XP_007689175.1), 87%, 9e-142, 74%	Hypothetical protein
Cluster PKSi10 (contig 81)						
1	83138-84910	1407	468	PF00083.21: Sugar (and other) transporter	Major facilitator superfamily domain, general substrate transporter [*Penicillium expansum*] (KGO40259.1), 84%, 6e-65, 33%	MFS transporter
2	90217-91875	1659	552	PF02458.12*: Transferase family	Transferase [*Colletotrichum graminicola*] (XP_008089247.1) 99%,,0.0, 69%	Transferase
3	92316-93734	1272	423	PRK08163: Salicylate hydroxylase	Salicylate hydroxylase [*Capronia coronata*] (XP_007720118.1), 99%, 4e-81, 35%	Salicylate hydroxylase

Gene	Location	ORF length (pb)	Protein length (aa)	Domains/Motifs	Protein homolog obtained by BlastP (Description, Query cover, E-value, Identity)	Predicted function
4	97329-98396	1068	355	PF01040.15: UbiA prenyltransferase family	4-hydroxybenzoate polyprenyl transferase [*Exophiala mesofila*] (KIV88353.1), 87%, 1e-61, 36%	Prenyltransferase
5	99335-100765	1197	398	PF00891.15: O-methyltransferase	O-methyltransferase [*Trichoderma harzianum*] (KKO99190.1), 95%, 3e-88, 39%	O-methyltransferase
6	102462-109010	6117	2038	PF16073.2: ACP transacylase (SAT) SMART00825: Beta-ketoacyl synthase (KS) SMART00827: Acyl transferase (AT) TIGR04532: Product template (PT) SMART00823: Phosphopantetheine attachment site (ACP) PFAM00975: Thioesterase (TE)	Polyketide synthase PKS14 [*Fusarium graminearum*] (EYB32182.1), 99%, 0.0, 55% Biosynthesis of orsellinic acid	Polyketide synthase (PKSi10)
7	119870-120493	564	187	No domains	Hypothetical protein [*Pyrenophora tritici-repentis*] (XP_001942100.1), 100%, 9e-98, 71%	Hypothetical protein
Cluster PKSi11 (contig 122)						
1	25487-26603	1065	354	PF08241.9: Methyltransferase domain	S-adenosyl-L-methionine-dependent methyltransferase [*Glarea lozoyensis* ATCC 20868] (XP_008080210.1), 96%, 3e-171, 69%	Methyltransferase
2	26855-28591	1737	578	PF05502.10: Dynactin p62 family	Dynactin Arp1 p62 subunit RO2 [*Pyrenophora tritici*-repentis Pt-1C-BFP] (XP_001930463.1), 99%, 0.0, 65%	Dynactin p62
3	28810-30387	1395	464	PFAM00646: F-box domain	F-box domain protein [*Neosartorya fischeri* NRRL 181] (XP_001267622.1), 99%, 2e-28, 25%	F-box domain protein
4	30621-31464	621	206	PF04525.9: LURP-one-related (transmembrane helix)	Hypothetical protein [*Pyrenophora teres* f. teres 0-1] (XP_003295504.1), 95%, 6e-83, 62%	Hypothetical protein
5	32594-34504	1812	603	PF06985.8: Heterokaryon incompatibility protein	Sulfite efflux pump SSU1 [*Phialophora attae*] (KPI39631.1) 94%,, 2e-72, 32%	Heterokaryon incompatibility protein
6	36051-37867	957	318	PF12796.4: Ankyrin repeats (3 copies) (mediate protein-protein interactions)	Hypothetical protein [*Fusarium oxysporum*] (EWZ79009.1), 40%, 3e-05, 33%	Hypothetical protein
7	38040-39002	963	320	CD08241: Quinone oxidoreductase	Quinone oxidoreductase [*Pseudonocardia acaciae*] (WP_028927735.1), 98%, 3e-64, 40%	Quinone oxidoreductase
8	45742-52073	6129	2042	PF16073.2: ACP transacylase (SAT) SMART00825: Beta-ketoacyl synthase (KS) SMART00827: Acyl transferase (AT) TIGR04532: Product template (PT) SMART00823: Phosphopantetheine attachment site (ACP) PFAM00975: Thioesterase (TE)	Polyketide synthase PKS27 [*Aspergillus flavus*] (EED57518.1), 99%, 0.0, 59% Biosynthesis of asparasone	Polyketide synthase (PKSi11)

Gene	Location	ORF length (pb)	Protein length (aa)	Domains/Motifs	Protein homolog obtained by BlastP (Description, Query cover, E-value, Identity)	Predicted function
9	52642-54089	1344	447	PF07690.13: Major Facilitator Superfamily	Monocarboxylate permease-like protein [*Histoplasma capsulatum*] (EEH09155.1), 99%, 1e-157, 54%	MFS transporter
10	54435-55331	819	272	PF03959.10: Serine hydrolase (FSH1)	Phospholipase/carboxylesterase [*Aspergillus niger* CBS 513.88] (XP_001390020.1), 93%, 7e-60, 40%	Serine hydrolase
11	55878-57051	1107	368	PF00172.15: Fungal Zn(2)-Cys(6) binuclear cluster domain	Zinc finger transcription factor [*Aspergillus oryzae*] (EIT81857.1), 96%, 2e-30, 29%	Transcription factor
12	58424-59257	777	258	PF07985.9: SRR1 (signalling proteins)	Hypothetical protein [*Setosphaeria turcica*] (XP_008024370.1), 91%, 4e-67, 54%	Hypothetical protein
13	59837-60452	501	166	PF07110.8: EthD domain (protein involved in the degradation of ethyl tert-butyl ether - ETBE)	Conidial pigment polyketide synthase PksP/Alb1 [*Metarhizium guizhouense*] (KID83384.1), 79%, 7e-35, 45%	EthD domain protein
14	61359-62328	921	306	PF00891.15: O-methyltransferase	Sterigmatocystin 8-O-methyltransferase [*Microsporum gypseum*] (XP_003169142.1), 96%, 2e-41, 32%	O-methyltransferase
15	63165-64902	1503	500	PF00067.19: Cytochrome P450	Pisatin demethylase [*Aspergillus ruber*] (EYE98262.1), 93%, 5e-98, 34%	Cytochrome P450
Cluster PKSi12 (contig 126)						
1	17611-18231	621	206	No domains	Cell wall protein [*Stemphylium lycopersici*] (KNG47560.1), 84%, 3e-40, 44%	Hypothetical protein
2	19254-20849	1545	515	PF00172.15: Fungal Zn(2)-Cys(6) binuclear cluster domain PF04082.15: Fungal specific transcription factor domain	Putative c2h2 finger domain protein [*Botrytis cinerea*] (EMR82773), 91%, 11e-44, 27%	Transcription factor
3	21650-22294	585	194	PF01058.19: NADH ubiquinone oxidoreductase	NADH-ubiquinone oxidoreductase 20 kDa subunit [*Scedosporium apiospermum*] (KEZ38950.1), 85%, 8e-105, 85%	NADH dehydrogenase
4	25372-26867	1290	429	PF01328.14: Peroxidase	Oxidase-like protein [*Stemphylium lycopersici*] (KNG49758.1), 100%, 0.0, 61%	Oxidase
5	27958-29726	1512	503	PF05730.8: CFEM domain (is found in some proteins with proposed roles in fungal pathogenesis)	CFEM domain-containing protein [*Stemphylium lycopersici*] (KNG51755.1), 98%, 0.0, 54%	CFEM domain protein
6	35728-36303	576	191	PF09351.7: Domain of unknown function	Hypothetical protein [*Parastagonospora nodorum*] (XP_001796276.1), 100%, 2e-78, 60%	Hypothetical protein

Gene	Location	ORF length (pb)	Protein length (aa)	Domains/Motifs	Protein homolog obtained by BlastP (Description, Query cover, E-value, Identity)	Predicted function
7	37511-45483	7842	2613	PF16073.2: ACP transacylase (SAT) SMART00825: Beta-ketoacyl synthase (KS) SMART00827: Acyl transferase (AT) TIGR04532: Product template (PT) SMART00823*: Phosphopantetheine attachment site (ACP) CD02440: Methyltransferase (MT) PFAM07859: Alpha/beta hydrolase fold PFAM00326: Prolyl oligopeptidase family	Polyketide synthase CHGG_10128 [*Chaetomium globosum*] (XP_001228055.1), 99%, 0.0, 51% Biosynthesis of 5-methylorsellinic acid	Polyketide synthase (PKSi12)
8	46554-49790	3069	1022	CL1768: Adenylate forming domain (acyl-/aryl-CoA ligases and NRPS) PF00550.22: Phosphopantetheine attachment site TIGR01746: Thioester reductase domain	NRPS-like ATEG_03630 [*Aspergillus terreus*] (XP_001212808.1), 99%, 0.0, 46%	NRPS-like
9	50524-51469	654	217	No domains	Hypothetical protein [*Chaetomium globosum*] (XP_001228057.1),88%, 7e-86, 67%	Hypothetical protein
10	52577-55486	2223	740	PF01494.16: FAD binding domain CD02979: FAD-dependent Phenol hydroxylase	Phenol 2-monooxygenase [*Madurella mycetomatis*](KOP44466.1), 96%, 0.0, 57%	Phenol 2-monooxygenase
11	56329-58156	1563	520	PF00067.19: Cytochrome P450	Pisatin demethylase [*Madurella mycetomatis*] (KOP44342.1), 93%, 0.0, 66%	Cytochrome P450
12	59040-60434	1284	427	PF07690.13: Major Facilitator Superfamily	Putative transporter MCH4 [*Madurella mycetomatis*] (KOP44340.1), 94%, 0.0, 63%	MFS transporter
13	61559-64978	2529	842	PFAM13191: AAA ATPase domain (couple chemical energy from ATP hydrolysis to conformational changes) PF13424.3*, PF13374.3: Tetratricopeptide repeat (protein binding)	TPR domain protein [*Talaromyces marneffei* ATCC 18224] (XP_002150937.1), 92%, 0.0, 44%	TPR domain protein
14	50523-51469	948	315	PF00107.23: Zinc-binding dehydrogenase	Alcohol dehydrogenase superfamily zinc-containing [*Macrophomina phaseolina*] (EKG18346.1), 99%, 8e-89, 47%	Dehydrogenase
15	67240-68290	981	326	PF01370.18: NADH dehydrogenase (ubiquinone)	NAD dependent epimerase/dehydratase family protein [*Pyrenophora tritici*-repentis Pt-1C-BFP] (XP_001931901.1), 99%, 2e-176, 78%	Epimerase/dehydratase

APPENDIX I - Mycelial growth (diameter of colonies in cm) of *E. nigrum* strains (WT and PKS mutants).



A: Oatmeal agar; B: Potato dextrose agar; C: Sugarcane leaves extract agar; D: Sabouraud agar.

APPENDIX J – Comparison of *E. nigrum* WT growing on PDA with *E. nigrum* ΔPKSi9 growing on PDA supplemented with hygromycin (bottom view)