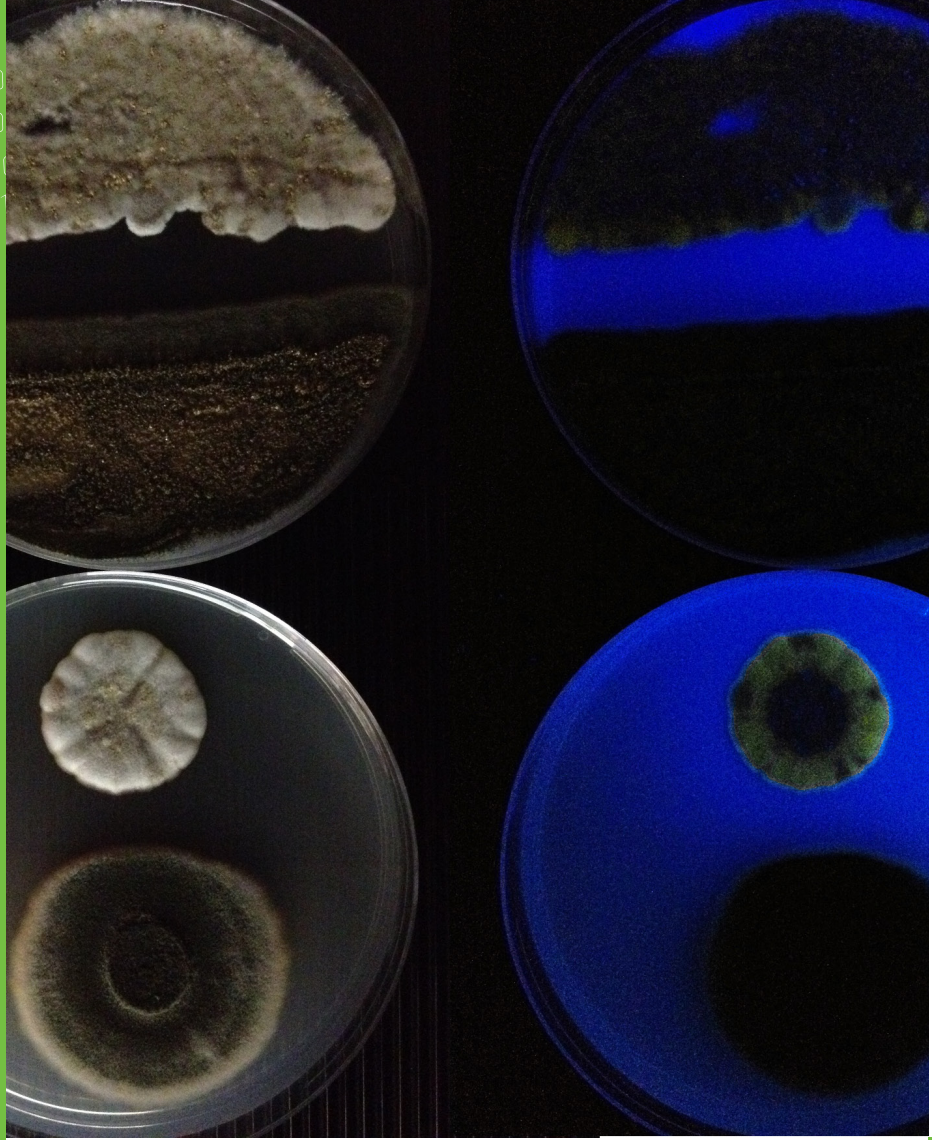

SCIENCE • TECHNOLOGY • RESEARCH HIGHLIGHTS • VISIONS

Dissertation
126

Production of terpenes in *Aspergillus nidulans*

Engineering of the secondary metabolite gene cluster

Kirsi Bromann

VTT

VTT SCIENCE 126

Production of terpenes in *Aspergillus nidulans*

Engineering of the secondary metabolite gene cluster

Kirsi Bromann

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Abstract

Secondary metabolites, or natural products, are produced by a variety of microorganisms and plants but are particularly abundant in filamentous fungi. Secondary metabolites can be toxic or have pharmaceutical applications as antibiotics, anticancer, antiparasitic and anti-fungal agents. Terpenes are a large group of secondary metabolites with wide array of bioactivities.

Filamentous fungus, *Aspergillus nidulans,* is a fungal model organism and a close relative to other industrially relevant *Aspergillus* species. *A. nidulans* is a known producer of many bioactive metabolites, including polyketides, nonribosomal peptides and sesquiterpenes. Sequencing of *A. nidulans* genome has revealed numerous secondary metabolite gene clusters, yet products of many of these biosynthetic pathways are unknown since the expression of the clustered genes usually remains silent in laboratory conditions. Finding ways to induce the gene expression of silent biosynthetic clusters can lead to discovery of novel metabolites with unknown bioactivities. Furthermore, identifying key biosynthetic genes in interesting metabolite pathways is a prerequisite for genetic engineering of heterologous production systems.

In this study, we wanted to explore the terpene producing ability of *A. nidulans*. We discovered a novel diterpene, *ent*-pimara-8(14),15-diene (PD), in *A. nidulans* by predicting a biosynthetic gene cluster with genomic mining. We identified a $Zn(II)_2Cys_6$–type transcription factor, PbcR, and demonstrated its role as a positive regulator for the predicted gene cluster. The transcriptional profile of the *pbcR* overexpression strain (oe:*PbcR*) was analyzed by Real-Time Quantitative Reverse Transcription PCR (qRT-PCR) and DNA microarray. Overexpression of *pbcR* led to upregulation of the predicted biosynthetic cluster genes and subsequent PD production in *A. nidulans*. In addition to PD cluster genes, transcriptional changes were observed for a number of secondary metabolite genes in oe:*PbcR*. Microscopy of the plate cultures revealed changes in the oe:*PbcR* morphology.

To study the bioactivity of PD, we developed and optimized a PD extraction method for the engineered *A. nidulans* production strain. An accelerated solvent extraction (ASE) with a combined High-Performance-Liquid-Chromatography (HPLC) purification enabled us to collect highly pure PD for the Nuclear Magnetic Resonance (NMR) analysis and bioactivity testing. While PD did not exhibit inhibitory function against *Staphylococcus aureus* or *Leishmania donovani*, we observed an anti-oxidant activity for PD in a radical scavenging assay.

We also characterized *A. nidulans* PD synthase (PbcA) with overexpression and deletion assays and showed that it is indispensable for PD production in *A. nidulans*. Furthermore, we extended our genetic engineering to study the potential of *A. nidulans* as a heterologous host for mono- and diterpene production. Overexpression of *Fusarium fujikuro*i *ent*-kaurene synthase (cps/ks) and *Citrus unshiu* gamma-terpinene synthase resulted in *ent*-kaurene and gamma-terpinene produc-

tion, respectively. The results reported in this thesis highlight the advantage of using genomic mining in the search for novel biosynthetic pathways. Additionally, this thesis presents a proof-of-concept study for using *A. nidulans* as heterologous terpene production host.

Keywords *Aspergillus nidulans*, gene cluster, genome mining, secondary metabolism, natural products, terpenoids, diterpenes, *ent*-pimara-8(14),15-diene, *ent*-kaurene, gamma-terpinene

Tiivistelmä

Useat mikro-organismit ja kasvit tuottavat sekundaarimetaboliitteja, jotka ovat toiselta nimeltään luonnonaineita. Nämä yhdisteet ovat erityisen yleisiä filamenttihomeissa. Sekundaarimetaboliitit voivat olla myrkyllisiä, tai niillä voi olla farmaseuttisia sovelluksia antibiootteina, syöpälääkkeinä tai loisten ja sienten vastaisina yhdisteinä. Terpeenit ovat suuri bioaktiivisten sekundaarimetaboliittien ryhmä.

Filamenttihome, *Aspergillus nidulans*, on sienten malliorganismi ja läheisesti sukua muille teollisesti tärkeille *Aspergillus*-lajeille. *A. nidulans* tunnetaan monien bioaktiivisten metaboliittien, kuten polyketidien, nonribosomaalisten peptidien, sekä seskviterpeenien, tuottajana. *A. nidulans*in genomin sekvensointi on paljastanut useita sekundaarimetaboliageeniklustereita. Suurin osa näistä biosynteettisten ketjujen tuotteista on kuitenkin tunnistamatta, sillä klustereiden geenien ilmentymistä ei yleensä tapahdu laboratorio-olosuhteissa. Biosynteettisten klustereiden geenien ekspression indusointi voi johtaa uusien bioaktiivisten yhdisteiden löytymiseen. Tämän lisäksi heterologisten tuottosysteemien geneettisen muokkaamisen perustana on identifioida mielenkiintoisten yhdisteiden metaboliareittien tärkeimmät biosynteettiset geenit.

Tässä työssä halusimme tutkia *A. nidulans*in terpeenien tuottokykyä. Löysimme uuden diterpeenin, *ent*-pimara-8(14),15-dieenin (PD), paikallistamalla kyseisen yhdisteen biosynteettisen geeniklusterin genomin haravoinnilla. Identifioimme $Zn(II)_2Cys_6$-tyypin ilmentymistekijän, PbcR:n, ja osoitimme sen roolin oletetun geeniklusterin positiivisena säätelijänä. *PbcR*:n ylekspressiokannan (oe:*PbcR*) ilmentymisprofiili analysoitiin reaaliaikaisella kvantitatiivisella käänteistranskriptiopolymeraasiketjureaktiolla (Real-Time Quantitative Reverse Transcription PCR, qRT-PCR) ja DNA-mikroarraylla (DNA microarray). *PbcR*:n yliekspressio *A. nidulans*issa johti ennakoitujen klusterigeenien yliekspressioon ja PD:n tuottoon. PD-klusterin geenien lisäksi oe:*PbcR*-kannassa havaittiin transkriptionaalisia muutoksia useiden sekundaarimetaboliittigeenien ilmentymisessä. Maljakasvatusten mikroskopointi paljasti rakenteellisia muutoksia oe:*PbcR*-kannassa.

Kehitimme ja optimoimme PD:n uuton muokatulle *A. nidulans* -kannalle PD:n bioaktiivisuuden tutkimiseksi. Nopeutettu liotinuutto (ASE) yhdistettynä korkean erotuskyvyn nestekromatografiaan (HPLC) mahdollisti erittäin puhtaan PD:n keräämisen ydinmagneettiseen resonanssispektrometria-analyysiin sekä bioaktiivisuustestaukseen. Vaikka PD ei inhiboinut *Staphylococcus aureus*ta tai *Leishmania donovani*a, vapaiden radikaalien pyydystämiskokeessa PD:lle havaittiin antioksidatiivinen aktiivisuus.

Lisäksi karakterisoimme PD-syntaasigeenin (PbcA) yliekspressio- ja deleetiokokeilla ja osoitimme sen olevan korvaamaton *A. nidulans*in PD-tuotannossa. Lisäksi tutkimme *A. nidulans*in potentiaalia heterologisena isäntänä mono- ja diterpeenien tuotossa. *Fusarium fujikuroi*n *ent*-kaureenisyntaasin (*cps/ks*) ja *Citrus unshiu*n gamma-terpineenisyntaasin yliekspressio johti *ent*-kaureenin ja gamma-

terpineenin tuottumiseen. Tässä väitöskirjassa esitetyt tulokset korostavat genomikartoituksen hyödyllisyyttä uusien biosynteesireittien etsinnässä. Tämän lisäksi väitöskirjatyö toimii “proof-of-concept”-tutkimuksena *A. nidulans*in käytöstä heterologisena tuottoisäntänä terpeeneille.

Preface

This study was carried out at VTT Technical Research Centre of Finland Ltd in the Metabolic Engineering team. Financial support provided by the Academy of Finland (SA-Biodive, 213084) and by the Finnish Bioeconomy Cluster (Future Biorefinery 1 & 2), as well as the travel grants provided by the BIOREGS Graduate School for Biomass Refining and the University of Helsinki are greatly appreciated.

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Kirsi

Academic dissertation

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List of publications

This thesis is based on the following original publications which are referred to in the text as I–III. The publications are reproduced with kind permission from the publishers.

I **Bromann K**, Toivari M, Viljanen K, Vuoristo A, Ruohonen L, Nakari-Setälä T (2012). Identification and characterization of a novel diterpene gene cluster in *Aspergillus nidulans*. *PLoS ONE* 7(4):e35450. DOI: 10.1371/journal.pone.0035450

II **Bromann K**, Viljanen K, Moreira VM, Yli-Kauhaluoma J, Ruohonen L, Nakari-Setälä T (2014). Isolation and purification of *ent*-pimara-8(14),15-diene from engineered *Aspergillus nidulans* by accelerated solvent extraction combined with HPLC. *Analytical Methods* **6**, 1227-1234. DOI: 10.1039/C3AY41640B

III **Bromann K**, Toivari M, Viljanen K, Ruohonen L, Nakari-Setälä T (2016). Engineering *Aspergillus nidulans* for *ent*-kaurene and gamma-terpinene production. Accepted to *Applied Microbiology and Biotechnology*.

Author's contributions

Publication I
Kirsi Bromann designed the experimental work and carried out the laboratory work, including genome mining, strain constructions, cultivations, qRT-PCR, DNA array analysis, microscopy, and sample preparation for GC. SPME-GC/MS and GC/MS analyses were done by Anu Vuoristo and Kaarina Viljanen, respectively. Kirsi Bromann contributed to analysis and interpretation of the results; and, wrote the article. Kirsi Bromann is the corresponding author of the article.

Publication II
Kirsi Bromann designed the experimental work and carried out most of the laboratory work, except for the NMR analysis. HPLC and TLC purification were done together with Kaarina Viljanen. Kirsi Bromann contributed to analysis and interpretation of the results and wrote the article. Kirsi Bromann is the corresponding author of the article.

Publication III
Kirsi Bromann contributed to the design of the experimental work and carried out most of the laboratory work, excluding the yeast experiments and the use of GC/MS equipment. Kirsi Bromann analyzed and interpreted the results and drafted the article. Kirsi Bromann is the corresponding author of the article.

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Abstract
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List of abbreviations

qRT-PCR	Real-Time Quantitative Reverse Transcription PCR
GC/MS	Gas Chromatography Mass Spectrometry
SPME	Solid Phase Micro Extraction
PD	*ent*-pimara-8(14),15-diene
PbcA	*Aspergillus nidulans ent*-pimara-8(14),15-diene synthase
Cps/Ks	*Fusarium fujikuroi ent*-kaurene synthase
PbcR	Pimaradiene Biosynthetic Cluster Regulator
Zn2(II)Cys6	Zinc binuclear cluster
Acetyl-CoA	Acetyl coenzyme A
HMG-CoA	Hydroxymethyl glutaryl coenzyme A
GGPP	Geranylgeranyl diphosphate
GGPPS	Geranygeranyl diphosphate synthase
GPP	Geranyl diphosphate
DMAPP	Dimethyl allyl diphosphate
IPP	Isopentenyl diphosphate
ASE	Accelerated Solvent Extraction
HPLC	High Performance Liquid Chromatography
TLC	Thin Layer Chromatography
Hex	Hexane
EtOAc	Ethyl acetate
ACN	Acetonitrile
NMR	Nuclear Magnetic Resonance

CYP	Cytochrome P450
NAD/NADP	Nicotinamide adenine dinucleotide/phosphate
TEF-g	Transcription elongation factor gamma
SDR	Short-chain dehydrogenase/reductase
PKS	Polyketide synthase
NRPS	Nonribosomal peptide synthase
DMATS	Dimethyl allyl tryptophan synthase
PET	Polyethylene terephtalate

1. Introduction

1.1 Framework of this study

In this thesis, we studied secondary metabolism of the filamentous fungus, *Aspergillus nidulans*. The framework for our study stemmed from the idea of exploiting the metabolic diversity of filamentous fungi in the production of commecially valuable compounds. The project was started as a part of the Finnish Biotechnology cluster (FIBIC) Joint Research 2, which focused on finding novel uses for the compounds produced by the Finnish forest industry. Our hypothesis was that filamentous fungi could be used as biological transformation tools to add value to the under-utilized rosin acid, stilbene, and terpenoid sidestreams of the Finnish paper and pulp industry. A variety of compounds in these sidestreams have diterpenoid structures. To find enzymes that could be useful in modification of terpenoids, we focused on studying the diterpene metabolism of *A. nidulans*.

A feasibility study conducted at VTT had suggested that monoterpenes could function as precursors in the chemical synthesis of terephtalic acid. Terephtalic acid is the main component used in the manufacturing process of polyethylene terephthalate, or PET. The physicochemical properties of PET make it an ideal material to be used in containers for carbonated drinks. The global market for PET is expected to reach 48.4 billion US dollars in 2016 (Research 2012). Production of PET precursors in biological hosts, such as filamentous fungi, yeasts, or plants is seen as a favorable biobased alternative to using crude oil as a feedstock. For many large companies, such as Coca-Cola, Pepsi, Danone, and Walmart, the sustainability aspect of the biobased plastics is starting to be a necessity to attract consumers and protect market share in certain regions (Komula 2011). For this reason, we extended our study to exploring if *A. nidulans* could produce terpenoid precursors heterologously.

In addition to the aforementioned applications, fungal metabolites are implemented in a multitude of different commercial applications. Increased understanding of the metabolic cascades of *A. nidulans* is valuable in finding novel compounds, which could possess bioactivities beneficial to humankind.

1.2 Fungi

The different biomes in the world include flora, fauna, fungi, and microorganisms. Fungi is one of the largest and most diverse kingdoms of eukaryotes with about 100,000 species described so far (Hibbett, Binder et al. 2007). Fungal habitats include soil, water, and extreme environments. Fungi are the primary decomposers in all terrestrial ecosystems and play a role in the global carbon cycle and in nutrient recycling. Mutualistic fungi are essential to the survival of their associate organisms, whereas many fungal species are important for their pathogenicity for humans, plants, or animals (Baker, Kennedy et al. 2009). Because of the wide distribution in all habitats and ecosystems on Earth, fungal biodiversity shows remarkable metabolic features. Fungi are of significant potential for agriculture, biotechnology and biological production, and they are well-developed genetic model systems for molecular biologists (Baker, Kennedy et al. 2009). A variety of fungal compounds have commercial potential as pharmaceuticals, biocatalysts, nutritional supplements, cosmetics, agrichemicals, biomaterials, and enzymes (Chambergo and Valencia 2016).

1.3 Filamentous fungi

Fungi can be divided into three major groups by the basis of their life cycles, the presence or structure of their fruiting body and the arrangement of and type of spores they produce. The fungal groups are: multicellular filamentous moulds, macroscopic fungi that are commonly referred to as mushrooms, and yeasts, which are single-celled organisms. While the morphological similarities between moulds and actinomycetes (filamentous bacteria) suggest that the two groups have undergone parallel evolution and both of these microbe groups often inhabit the same ecosystems, all fungi are eucaryotes whereas actinomycetes are clearly prokaryotes (Cole 1996). Filamentous fungi produce branching filaments called hyphae that can fragment into conidia, or spores, for reproduction. Some filamentous fungi, such as *Aspergillus nidulans*, can also undergo sexual reproduction.

1.4 *Aspergillus nidulans*

Aspergillus nidulans belongs to the phylum Ascomycota, and its sexual form, or teleomorph, is referred to as *Emericella nidulans*. It has been used for decades to study eukaryotic cell biology and represents the model organism for filamentous fungi. In addition to its role as an academic study organism, *A. nidulans* is a prolific producer of secondary metabolites. This has led to increased interest in *A. nidulans* as a target for biotechnological engineering.

The genome of *A. nidulans* has been annotated (Galagan, Calvo et al. 2005) and many genetic tools for its engineering have been developed. *A. nidulans* can

be fermented using fairly inexpensive carbon sources, and as a close relative to industrially used *Aspergilli,* the methods developed for *A. nidulans* could potentially be applied to industrially relevant species.

1.5 Primary metabolism

Fungal metabolism can be divided into primary and secondary metabolism. Primary metabolism sustains the growth, reproduction, and development of the organism. In fungi, primary metabolism affects the phenotypical traits including reacting to extracellular stimuli, producing precursor molecules required for cell division and morphological changes, as well as providing monomer building blocks for production of secondary metabolites and extracellular enzymes . Primary metabolism is involved in processes related to cellular morphology, the life cycle and cell cycle of the cells, but also other traits associated with growth and development in general (Andersen 2014).

1.6 Secondary metabolism

Secondary metabolites are derived from the primary metabolic pathways. Secondary metabolites, also known as natural products, are low molecular weight compounds produced primarily by fungi, plants and bacteria. The main classes of secondary metabolites include terpenes, polyketides, non-ribosomal peptides and alkaloids. These compounds, seemingly dispensable for the survival and growth, have been proposed to function as communication cues and defense signals in the producing organism's ecological niche (Fox and Howlett 2008).

A multitude of biological roles have been suggested for secondary metabolites. One example of this communication is the complex signaling network in marine sponges and corals to distinguish between beneficial and hostile microorganisms (Hardoim and Costa 2014). For sponges and corals, the secretion and recognition of the correct secondary metabolite seems to be essential for thriving in the marine environment. Natural product guided defense mechanisms have been reported in plants, which produce metabolites against herbivores, pests and pathogens (Du Fall and Solomon 2011); as well as in fungi, which protect themselves against fungivory by producing insect repelling secondary metabolites (Rohlfs, Albert et al. 2007). In addition to being defense signals, secondary metabolites can act as attractants for pollinators and as cues for soil organisms to establish beneficial symbiotic partnerships. Examples of the secondary metabolite mediated interactions include the pollinator attraction by floral sesquiterpenes of kiwifruit (Nieuwenhuizen, Green et al. 2010) and the function of the secreted strigolactone plant hormones as the branching factor of arbuscular mycorrhizal fungi (Zwanenburg, Pospisil et al. 2016).

In Aspergilli, studies have found that a number of genetic regulators controlling the formation of developmental structures, such as the sexual cleistothecia and asexual conidia formation, also govern the production of secondary metabolites.

Also, the link between secondary metabolism and sclerotia formation has been established. Sclerotia are structures that allow certain fungi to survive for long periods of time under adverse environmental conditions. The secondary metabolites in these structures protect sclerotia from predators, competitors and other environmental stressor (Calvo and Cary 2015).

1.7 Applications of natural products

Structurally, natural products are very diverse and many of them have numerous bioactive properties. Historically, natural products from plants and animals have been the source for medicinal preparations; more recently, natural products have continued to provide leads for compounds entering clinical trials, particularly as anticancer and antimicrobial agents (Harvey, Edrada-Ebel et al. 2015). In addition to pharmaceutical potential, some natural products also exhibit toxic bioactivities. Toxic metabolite producing organisms can be detrimental in agriculture by causing adverse health effects to humans and livestock feeding on contaminated crops (Medeiros, Barbosa et al. 2003, Gerhards, Neubauer et al. 2014). On the other hand, toxic bioactivities of natural products can be useful in applications such as insecticides and fungicides (Abbruscato, Tosi et al. 2014, Ncube and Van Staden 2015). Secondary metabolites are responsible for aromatic qualities of essential oils, flavors of many spices and herbs as well as fragrances and colors of flowers. These qualities have been extensively applied in cosmetic and personal care products, and many natural compounds are used in functional nutraceuticals providing health benefits to consumers. Even more so, the technical applications for natural products can extend to jet fuel components and UV-protective agents. The commercial impact of secondary metabolites in a plethora of applications has led to increasing interest in the biosynthesis and production of these compounds.

As the knowledge of the secondary metabolites' biological roles in nature increases, so does the potential of finding novel drug candidates and bioactivities that can benefit humankind. The remarkable complexity and variety of secondary metabolites make them truly intriguing targets of study.

1.8 Terpenes and terpenoids

Terpenes are one of the largest natural product groups of over 55,000 known compounds. Terpenes with rearrangements or additions in their hydrocarbon skeleton are referred to as terpenoids. Many terpenoids are bioactive metabolites with pharmaceutical or toxic properties. Examples of pharmaceutical terpenoids include cancer drug taxol and anti-malaria drug artemisinin. Total chemical synthesis of many commercially interesting plant-derived terpenoids is difficult and expensive due to the structural complexity of these compounds and collection of plants from their natural habitat for high-value terpenoid purification can cause environmental stress. The need for alternative production methods has been driving research on heterologous natural product production systems. Heterologous

organisms, such as yeast, bacteria and filamentous fungi, could provide an economical and environmentally friendly way for commercial high-value terpenoid production.

Terpenes and terpenoids consist of the simple five-carbon isoprenoid precursors, dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP). In fungi, these molecules are produced from acetyl-CoA via the mevalonate pathway, whereas in bacteria and plants, the DMAPP and IPP precursors can also be produced via 1-deoxy-D-xylulose 5-phosphate (DOXP) pathway (Wanke, Skorupinska-Tudek et al. 2001). In terpene biosynthesis, isoprenoid precursors are linked in head-to-tail fashion to create different length hydrocarbons (Miziorko 2011). Terpene synthases catalyze the cyclization of linear hydrocarbons to produce the terpene backbone (Christianson 2006). Finally, the terpene hydrocarbon can be further modified by tailoring, or decorating, enzymes to produce the final terpenoid product. Figure 1 illustrates the fungal terpenoid biosynthetic pathway and depicts the chemical structures of some of the terpenoids belonging to sesqui-, di- and triterpenoid groups.

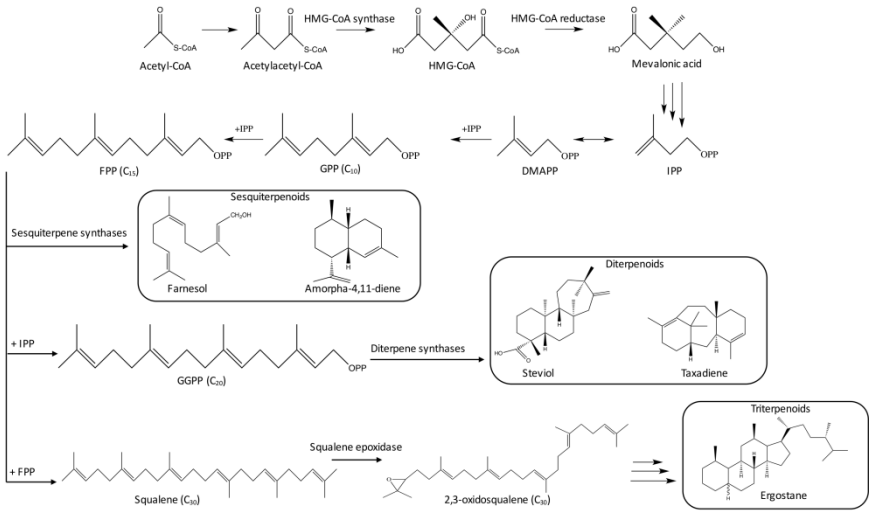

Figure 1. Terpenoid biosynthesis. In fungi, the precursors IPP and DMAPP are synthesized via mevalonate pathway. The chemical structures of some of the commercially interesting and/or representative sesqui-, di- and triterpenoids are depicted.

Terpenes are classified by the number of carbon atoms in their backbone, ranging from five-carbon containing hemiterpenoids to over 40-carbon consisting polyterpenes. Sesqui-, di- and triterpenoids are the main classes of terpenoids found in fungi (Quin, Flynn et al. 2014). Table 1 shows the classification and examples for representative terpenes from each group.

Table 1. Classification of terpenes

Class	Number of carbon atoms	Representative terpene or terpenoid
Hemiterpene	5	isoprene, prenol, isovaleric acid
Monoterpene	10	geraniol, menthol, limonene, terpinene
Sesquiterpene	15	farnesol, amorphadiene, zingiberene
Diterpene	20	taxadiene, steviol, kahweol
Sesterterpene	25	geranylfarnesol, ceroplastol, haslenes
Triterpene	30	cholestane, ergostane, squalene,
Tetraterpene	40	phytoene, carotenoids, xanthophylls
Polyterpene	over 40	gutta-percha, natural rubber, resins

Pharmaceutical terpenoids include the sesquiterpenoid artemisin and the diterpenoid taxol, which are used in treating malaria and cancer, respectively. Other commercially relevant terpenoids include monoterpenoids menthol, carvone and limonene, which are used in flavoring agents, cosmetics, and personal health products (Lange 2015). Because of terpenoid structural complexity, many terpenes are difficult to produce by using total chemical synthesis. There is an increasing demand for inexpensive production methods for commercially valuable terpenoids. Heterologous microbial production hosts that are easily cultured in the laboratory, can provide cost-efficient way to produce commercially interesting terpenoids in large quantities.

1.9 Transcriptional regulation of fungal secondary metabolism

Transcriptional regulation of fungal biosynthetic genes is carried out by narrow and broad domain transcription factors (Yin and Keller 2011). AreA, CreA and PacC are well-characterized broad domain regulators in *Aspergillus nidulans*. They regulate the production of secondary metabolites in response to changes in the environmental nitrogen, carbon and pH, respectively (Fox and Howlett 2008). A global regulator of secondary metabolite gene clusters, LaeA (Loss of *AflR* Expression), was identified in a screen for *A. nidulans* mutants unable to produce sterigmatocystin (Yin and Keller 2011). *AflR,* the positive regulator of aflatoxin and sterigmatocystin biosynthesis, is not expressed in *LaeA* mutants. Expression of also other genes in the sterigmatocystin and penicillin gene clusters is downregulated in the *LaeA* deletion strain (Bok, Hoffmeister et al. 2006). The overexpression of *LaeA* instead activates multiple putative secondary metabolite clusters.

Narrow domain transcriptional regulators are also called cluster-specific or pathway-specific transcription factors. These include zinc binuclear cluster ($Zn(II)_2Cys_6$) proteins, a group of transcription factors found only in fungi (MacPherson, Larochelle et al. 2006). In fungi, Zn(II)2Cys6-type transcription factors have been shown to positively regulate the biosynthetic clusters they reside in. AflR, the regulator necessary for aflatoxin and sterigmatocystin biosynthetic gene activation, is a characteristic $Zn(II)_2Cys_6$ protein (Fernandes, Keller et al. 1998). Pathway-specific transcription factors up-regulate the expression of genes encoding biosynthetic proteins for a particular metabolite.

1.10 Secondary metabolites in *A. nidulans*

A. nidulans produces a number of metabolites with complex chemical structures including polyketides and non-ribosomal peptides (Yaegashi, Oakley et al. 2014). In fungi, the genes encoding consecutive steps in a biosynthetic pathway of secondary metabolites are often clustered together on the chromosomes. The clustering of such genes, along with multiple genome sequencing projects (Keller and Hohn 1997, Quin, Flynn et al. 2014), has facilitated the prediction of new biosynthetic pathways by using bioinformatics. Since secondary metabolites are not crucial for the survival of the organism, their production usually remains silent in normal laboratory conditions (Brakhage and Schroeckh 2011, Lange 2015). As a result, for most cases in which pathways are discovered through bioinformatic analysis, the products remain undetected (Keller and Hohn 1997, Brakhage and Schroeckh 2011, Quin, Flynn et al. 2014, Yaegashi, Oakley et al. 2014, Lange 2015).

1.11 Genome mining

A. nidulans genome mining indicates a high number of putative secondary metabolite gene clusters that exceed the number of known *A. nidulans* metabolites (Bok, Hoffmeister et al. 2006, Andersen, Nielsen et al. 2013, Wiemann and Keller 2014). Andersen et al. reported 58 putative secondary metabolite clusters in *Aspergillus nidulans*, whereas Keller et al. only reported 17 compounds directly linked to predicted biosynthetic gene clusters. By culturing *A. nidulans* on different solid media, Andersen et al. were able detect increased expression of 58 different clusters, and identify 42 metabolites. However, they were able to connect only about half of the dereplicated metabolites to the predicted SM clusters: 12 polyketides from 24 predicted PKS clusters, 7 nonribosomal peptides out of 24 predicted clusters, and 3 compounds linked to dimethylallyl tryptophan synthases from 7 predicted clusters. The three putative terpene clusters predicted in this study were included in the final number. Table 2 shows the number of predicted secondary metabolite clusters in selected fungal species.

Table 2. The number of predicted secondary metabolite clusters in selected fungi.

Species	PKS	PKS-like	NRPS	NRPS-like	Hybrid	DMATS	Total
Aspergillus nidulans	24	2	21	3	1	7	58
Alternaria alternata	10	n.d.	n.d.	n.d.	n.d.	n.d.	10
Cladosporium fulvum	10	n.d.	10	n.d.	2	1	23
Cercospora zea-maydis	11	2	7	8	1	1	30
Phaeosphaeria nodorum	12	9	9	5	1	1	38
Botrytis cinerea	16	6	6	8	0	1	37
Magnaporthe grisea	12	3	5	6	3	3	32
Magnaporthe oryzae	23	2	8	6	5	3	47
Fusarium graminearum	12	2	10	10	0	0	34
Cochiobolus heterostrophus	22	3	9	7	0	3	44
Aspergillus flavus	25	3	18	14	2	8	70
Aspergillus niger	15	1	12	2	5	0	35

n.d.: not detected, PKS: polyketide synthase, NRPS: Nonribosomal peptide synthase, DMATS: Dimethyl allyl tryptophan synthase

Genomic mining for gene clusters usually begins with identifying a core biosynthetic enzyme, such as polyketide synthase, an NRP synthetase or a terpene synthase. In addition to the core synthase gene, secondary metabolite gene clusters contain genes for so-called decorative enzymes. These include cytochrome P450 monooxygenases (CYPs), oxidoreductases, and transferases, which further modify metabolites produced by the core synthases. Many times, natural product gene clusters also contain transcription factors. Because zinc binuclear cluster regulators specifically regulate genes in their close proximity, the existence of these transcription factors can be indicative of a putative secondary metabolite biosynthetic gene cluster.

Various bioinformatics tools for gene prediction can be used. InterPro (Hunter, Apweiler et al. 2009) website [http://www.ebi.ac.uk/interpro/] predicts protein domain according to similarities to known protein domain structures. Tools like BLAST [http://blast.ncbi.nlm.nih.gov/Blast.cgi/] (Altschul, Gish et al. 1990) and ClustalW2 [http://www.ebi.ac.uk/Tools/msa/clustalw2/] also predict unknown gene functions and protein domains based on the sequence homology. Multiple websites, e.g. *Aspergillus* Genome Database (AspGD, http://www.aspergillusgenome.org/GBrowseContents.shtml) and Joint Genome Institute's Integrated Microbial Genomes (IMG, https://img.jgi.doe.gov/cgi-bin/m/main.cgi), provide genome browser tools for different fungal species. In addition to publicly available databases, which can be used in manual genome mining, research groups interested in the metabolite dereplication have developed their own prediction algorithms. One example is the method developed by Andersen *et al.*, that combines biological evidence with the existing sequence data to accurately predict secondary metabolite gene clusters in *A. nidulans* (Andersen, Nielsen et al. 2013). Reports like this underline the interest in the secondary metabolite profiling of this filamentous fungus.

1.12 Aims of this study

Although *Aspergillus nidulans* is widely studied model organism and a producer of various secondary metabolites, diterpenoids have not been reported as its natural products. The aim of this study was to examine the diterpene producing ability of *A. nidulans*, and to investigate the potential of *A. nidulans* as a heterologous terpenoid production host. The experimental part of the thesis consists of three publications. The aim of the Publication I was to mine *A. nidulans* genome for putative diterpenoid biosynthetic gene clusters, activate the cluster gene expression with positive transcriptional regulators and identify diterpene products of the clustered biosynthetic genes. The aim of the Publication II was to develop efficient purification method for the diterpene produced by *A. nidulans*, identify the product further with NMR, and subject the pure compound for bioactivity testing to evaluate its commercial potential. Publication III concentrates on additional characterization of the diterpene synthase gene in *A. nidulans* by using deletion and overexpression models, and assesses the potential of *A. nidulans* as a heterologous host for mono- and diterpene production.

2. Materials and methods

2.1 Fungal strains

The strains used in this work are generally well-characterized and their genome sequences are publicly available. *Aspergillus nidulans* parent strains were acquired from Fungal Genetic Stock Center, Kansas City, MO, USA. Detailed strain information can be found on the strain database at www.fgsc.net. *Saccharomyces cerevisiae* strain was obtained from Dr. P. Kötter (Frankfurt, Germany). The strains used in this study are listed in Table 3.

Table 3. Methods used in Publications I–III.

Method	**Used in**
Genome mining	I
Plasmid construction	I, III
qRT-PCR and transcription analysis	I, III
Expression array and transcriptome profiling	I
Construction of fungal overexpression strains	I, III
Construction of targeted mutation strains	III
Shake flask cultivations	I–III
HPLC and TLC	II
NMR	II
GC/MS	I, II
SPME-GC/MS	I–III
DPPH antioxidant assay	II
Bioactivity testing	II

Ultrasonic extraction for fungal cultures	I, II
ASE extraction for fungal cultures	II
Microscopy and imaging	I–III
Gamma-terpinene synthase enzyme assay	III

3. Results

3.1 Genomic mining of *A. nidulans* reveals two putative diterpene gene clusters

To identify putative diterpene gene clusters in *A. nidulans,* an Inter Pro protein signature database (Hunter, Apweiler et al. 2009) search of the *A. nidulans* genome was conducted. The total *A. nidulans* genome of 10,687 ORFs, had 26 genes with protein domain identifiers 'terpenoid synthase' or 'terpenoid cyclase' were identified in *A. nidulans* FGSC A4 genome. These 26 genes were analyzed by using BLAST, and four genes showed similarity to known diterpene synthases. To analyze if any of these diterpene synthase-like genes could be part of a biosynthetic cluster, about 20 kb area around the ORFs (AN1594, AN3252, AN6810 and AN9314) was again subjected to InterPro domain search using terms 'Fungal transcriptional regulatory protein', Cytochrome P450, E-class, group IV' and 'Cytochrome P450'. These protein identifiers were used to identify putative pathway specific positive transcriptional regulators as well as to mine for hallmark genes for secondary metabolite biosynthesis. Two putative diterpene gene clusters were located, one in chromosome VI and one in chromosome VII.

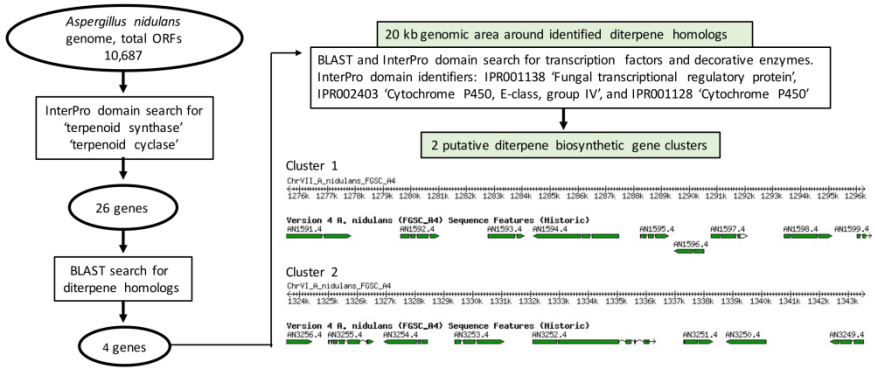

Figure 2. Schematic figure of the genomic mining of *A. nidulans* for putative diterpene gene clusters.

3.2 AN1599 encodes a positive transcriptional regulator

Genomic sequence of the transcriptional regulator at chromosome VII gene locus AN1599 was cloned into expression vector with *A. nidulans gpdA* promoter and transformed into *A. nidulans* FGSC A4 wild type strain by random integration. Expression of the transcriptional regulator (AN1599), and two target genes (AN1594; terpene synthase, and AN1592; cytochrome P450) from the predicted cluster was analyzed by using Real-Time Quantitative Reverse Transcription PCR (qRT-PCR). qRT-PCR results showed that transcription of AN1599 was elevated in all three analyzed transformant strains along with the two other cluster genes compared to wild type control (Publication I, Figure 1A).

The putative transcriptional regulator gene at chromosome VI locus AN3250 was also cloned and overexpressed in FGSC A4. Two isolated transformant strains were analyzed by using qRT-PCR. Although an increase in expression of the putative transcription factor was observed compared to the wild type strain, we could not detect significant upregulation of the two target genes for this cluster (Figure 1B). In sum, these results suggested that AN1599 encodes a positive regulator for the predicted diterpene metabolite cluster at chromosome VII; and, in contrast to AN1599, AN3250 overexpression does not activate its putative terpene cluster. The strain with the highest transcription of AN1599, oe:AN1599_42 was selected for further analysis.

3.3 The predicted diterpene cluster consists of eight adjacent genes on chromosome VII

To define the extent of the genes under regulation of AN1599, expression of 13 adjacent genes in the predicted cluster area was analyzed by using qRT-PCR. The expression of the genes in the wild type strain was very low, whereas significant upregulation of seven adjacent genes from AN1592 to AN1598 was seen in the AN1599 overexpressing strain (Publication I, Figure 2). The highly upregulated genes in the strain were homologous to geranylgeranyl diphosphate synthase (GGPPS; AN1592), hydroxymethyl glutaryl coenzyme A (HMG-CoA) reductase (AN1593), diterpene synthase (AN1594), translation elongation factor (AN1595), short-chain dehydrogenase/reductase (SDR; AN1596), methyltransferase (AN1597), cytochrome P450 (CYP; AN1598) and Zn(II)2Cys6 –type transcription factor (AN1599)

DNA array analysis was used to confirm the changes in the transcriptome of AN1599 overexpressing strain. The eight terpene cluster genes identified by qRT-PCR displayed similar enhanced expression when assessed by using DNA array analysis. In fact, when compared with the wild type strain, the most abundant transcripts were the seven target genes of the predicted terpene cluster. The DNA array data also confirmed the overexpression of AN1599. Figure 2 in Publication I shows the expression pattern for the predicted cluster area genes analyzed by using both qRT-PCR and DNA array. These data suggest that the predicted diter-

pene cluster consists of eight adjacent genes, AN1592 to AN1599, on *A. nidulans* chromosome VII.

3.4 Activation of the terpene cluster results in *ent*-pimara-8(14),15-diene (PD) biosynthesis in *A. nidulans*

To identify potential diterpene compounds produced by the predicted biosynthetic gene cluster activation, cultures of *A. nidulans* oe:AN1599_42 strain were analyzed by using solid phase microextraction gas chromatography mass spectrometry (SPME-GC/MS). SPME-GC/MS was chosen as the analytical method, since it allows identification of volatile and semi-volatile terpenoids (Hamm, Bleton et al. 2005). Analysis of the transformant strain liquid cultures showed an accumulation of a product peak, with a mass spectrum that matched a spectral library compound *ent*-pimara-8(14),15-diene (Publication I, Figure 4). To determine if also non-volatile compounds were produced by oe:AN1599_42 strain, both the cells and the media from fungal cultures were extracted with hexane:ethyl acetate (1:1) and polar phase extracts subjected to GC/MS-analysis. The data from the cell extracts were consistent with the SPME-GC/MS analysis (Publication I, Figure 4). Taken together, these data demonstrate that overexpression of AN1599 upregulates pathway genes for PD biosynthesis. The gene at locus AN1599 was named PbcR, for Pimaradiene Biosynthetic Cluster Regulator, and the strain oe:AN1599_42 was named oe:PbcR.

3.5 Overexpression of pbcR leads to changes in the transcriptome and morphology of *A. nidulans*

In addition to the pimaradiene cluster genes, DNA array revealed changes in expression of a number of secondary metabolite synthase genes in oe:PbcR. A penicillin gene cluster and two other putative polyketide clusters were downregulated in oe:PbcR (Publication I, Figure 3 A–C, Table S2). Additionally, one putative nonribosomal peptide cluster with two NRP synthases was also downregulated in oe:PbcR (Publication I, Figure 3D).

Morphological changes were also observed in oe:PbcR when compared with FGSC A4. The liquid cultures of oe:PbcR typically grew slower than the wild-type strain and the predominant structure in the plate cultures were sexual fruiting bodies (cleistothecia). Microscopic analysis and conidia quantification of the oe:PbcR plate cultures showed that fruiting body size was increased and conidia number decreased in oe:PbcR compared to FGSC A4 (Publication I, Figure 6). Although the precise mechanism is unclear, these data demonstrate that overexpression of *pbcR* is associated with changes in the transcriptome and morphology of *A. nidulans*.

3.6 Extraction of PD from engineered *A. nidulans*

Overexpression of *pbcR* caused accumulation of PD in *A. nidulans*. To verify the structure of a given compound with GC/MS, one needs to have a pure standard compound for retention time comparison. Since we were not able to obtain pure PD from commercial sources, or from other researchers, a purification method was developed. First, we used sonication extraction for manually ground oe:PbcR mycelia. This method consisted of collecting oe:PbcR mycelia at the exponential growth phase, manually grinding it in liquid nitrogen with mortar and pestle, and then extracting the polar components in a sonicating water bath. The manual steps of the protocol were time-consuming and caused some loss of material. Hence, automated extraction protocol was developed using ASE 200 Accelerated Solvent Extraction System (Dionex, Thermo Scientific). Mycelia were lyophilized and ground in a Retsch homogenizer before extraction. Weighing of the freeze-dried sample made it possible to evaluate the extraction yield and method efficiency. ASE extraction was optimized using different solvent compositions and parameters for pressure and temperature. The developed method for ASE 200 proved superior in the recovery of PD when compared to the ultrasonic method.

3.7 Purification of PD with TLC and HPLC

A TLC method was developed in order to isolate PD from crude extracts of oe:PbcR. The extracts were separated on silica plates using different solvent compositions. Since PD was not detectable directly by using UV fluorescence, developed silica plates were visualized by using rhodamine staining (Publication II, Figure 3A). PD was then extracted from the silica and the purity of the compound was analyzed by using GC-MS. TLC purification excluded fatty acids and other interfering compounds from the crude extract. However, the TLC-purified compound still contained some non-polar alkanes that interfered with identification by NMR analysis (Publication II, Figure 3B).

An analytical scale reversed phase HPLC purification method for PD was developed. In HPLC, both the interactions between the purified compound and stationary phase as well as the solvent play a role in the separation process. The purification method was optimized by using different eluent compositions. Because the PD hydrocarbon does not have any functional groups or bonds with UV absorbance, and its absorption maximum was unknown, we used multiple wavelengths to monitor PD fractionation. We collected eluent fractions in six 10 minute intervals to identify the PD compound peak. The UV absorption maximum for the peak corresponding to PD was 222.1 nm (Publication II, Figure 4A) and the monitoring of the following purifications was done at 225 nm. PD was undetectable at wavelengths 250 nm and greater. In order to accommodate larger injection volumes also preparative scale HPLC was performed. Isolated PD fractions were combined, evaporated to dryness, and dissolved in EtOAc prior to GC-MS analysis. Comparison of the GC-MS peak areas for crude extract and for HPLC purified

PD indicated that there were no impurities in the collected fraction and the recovery of the diterpene was consistently over 90% (Publication II, Figure 4C). We collected 25 mg of pure PD after multiple rounds of preparative scale HPLC.

3.8 NMR analysis and bioactivity testing of PD

To verify the structure of HPLC-purified PD, infra-red, MS, and 1D and 2D NMR experiments were performed (Publication II, Table 1). NMR analysis confirmed the structure and stereochemistry of PD isolated from *A. nidulans* strain oe:PbcR. The verified structure was consistent with our earlier report. The structure and mass spectrum of pure PD is shown in Publication II, Figure 6C. In sum, the purification and structural analysis verified the earlier identification of PD as a product of *A. nidulans* and provided us with an authentic standard for future identification of the compound.

With its isolation and purification, we could now test PD in a number of bioactivity assays. The anti-oxidant activity of pure PD in a DPPH radical scavenging assay was tested. The activity was compared to a known tetraterpene antioxidant, beta-carotene. The purified PD had significantly higher DPPH radical scavenging activity than the beta-carotene in all test conditions (Publication II, Figure 5). We also tested the activity of PD for the growth of *Staphylococcus aureus* and axenic amastigote of *Leishmania donovani*. However, even with fairly high PD concentration of 50 μM, PD did not exhibit antimicrobial or anti-leismanial activity in either of these tests.

3.9 Genetic engineering of *A. nidulans* PD cluster

Since we had shown that *A. nidulans* is capable of producing PD diterpene, we tested if *A. nidulans* could be a heterologous host for terpenes. We hypothesized that exchanging the terpene synthase gene in PD cluster to a heterologous synthase gene would result in heterologous terpene production. Our aim was to exchange the PD synthase gene with another diterpene or monoterpene synthase.

3.9.1 The genetic locus AN1594 encodes *ent*-pimara-8(14),15-diene synthase (PbcA) in *A. nidulans*

Along with the cluster gene identification, our sequence analysis suggested that the gene AN1594 codes for diterpene synthase. The bioinformatic analysis demonstrated that AN1594 is related to fungal bifunctional diterpene synthases (Publication, FIgure 7), and lead us to suggest a putative biosynthetic pathway for PD (Publication I, Figure 8). To further identify this putative terpene synthase gene, we created AN1594 deletion and overexpression *A. nidulans* strains.

The terpene synthase deletion strain was created by replacing the AN1594 ORF with *T. reesei ura3* selection marker and *pbcR* was overexpressed by adding

a constitutively active promoter to its 5' region in the biosynthetic cluster. This strain was analyzed by using RT-qRT-PCR and GC/MS. The transcript analysis with RT-qPRC affirmed the overexpression of *pbcR* and two other cluster genes, whereas the expression of AN1594 was undetectable. Production of PD in this strain was undetectable by using GC/MS. These data demonstrate that although the PD gene cluster was activated, the deletion of the putative diterpene synthase AN1594 abolished PD production (Publication III, Figure 1D) in *A. nidulans*.

Overexpression of the putative terpene synthase was achieved by placing AN1594 under the control of constitutively active *A. nidulans gpdA* promoter. The diterpene synthase gene expression was significantly increased in this strain, while expression of three other cluster genes was undetectable (Publication III, Figure 1E). GC/MS analysis of the strain overexpressing AN1594 revealed production of PD (Publication III, Figure 1F).

These data confirmed that AN1594 is both necessary and sufficient for PD production in *A. nidulans* and demonstrate that AN1594 encodes a bona fide PD synthase. Therefore, AN1594 was named PbcA for the PD synthase in PbcR-regulated biosynthetic gene cluster in *Aspergillus nidulans*.

3.10 Genetic engineering of *A. nidulans* for heterologous diterpene production

The identified PD cluster has a gene homolog for GGPP synthase (AN1592). Our hypothesis was that this gene is part of the PD precursor biosynthesis. However, overexpression of *pbcA* alone was sufficient for PD production without PD cluster activation. This indicated that intracellular GGPP levels in *A. nidulans* could support also heterologous diterpene production. Another fungal diterpene, *ent*-kaurene, is a precursor in gibberellin (GA) biosynthesis (Tudzynski 1999). GAs are phytohormones and first identified as secondary products of the rice pathogenic fungus causing overgrowth symptoms in rice (Tudzynski 2005). In addition to GAs, *ent*-kaurene is also a precursor for steviol (Richman, Gijzen et al. 1999). Steviol glycosides are used as sweeteners and steviol derivatives can be used in pharmaceutical applications (Yadav and Guleria 2012).

3.10.1 Overexpression of *Fusarium fujikuroi* kaurene synthase results in *ent*-kaurene production in *A. nidulans*

To test our hypothesis on heterologous diterpene production, we overexpressed *F. fujikuroi* kaurene synthase (cps/ks) in *A. nidulans*. Two strains were used for heterologous expression: *A. nidulans* strain with no PD cluster activation and oe:PbcR that has the PD biosynthesis activated. The expression levels of cps/ks as well as PD cluster genes were analyzed by using qRT-PCR and the metabolite profiling was done with GC/MS. Both strains produced *ent*-kaurene. In addition to *ent*-kaurene, the heterologous oe:PbcR host strain also produced PD (Publication

III, Figure 2B,D). Interestingly, the data revealed 2.4 times higher *ent*-kaurene production in the strain overexpressing both *cps/ks* and *pbcR* (Publication III, FIgure 2D). To our knowledge, this is the first report of *ent*-kaurene production in *A. nidulans*.

3.10.2 PD cluster engineering results in diterpene producing *A. nidulans* strains

To ensure that any given heterologous diterpene synthase has access to the maximal precursor pool, one must presumably delete the competing endogenous synthases. Since we observed higher *ent*-kaurene accumulation in the *A. nidulans* strain overexpressing both *cps/ks* and *pbcR*, we attempted to exchange *pbcA* with *cps/ks* in order to guide GGPP flux towards kaurene production. An exchange construct containing *F. fujikuroi cps/ks* and *T. reesei ura3* flanked by 5' promoter and 3' terminator regions of *pbcA* was constructed and transformed to oe:PbcR. This particular *A. nidulans* strain is capable of non-homologous end-joining, leading to high frequency of randomly inserted genetic fragments in transformations. After analysing a number of transformants, we could not detect insertion at the right locus. Instead, multiple strains with random insertions were identified. Interestingly, when some of these strains were grown and analyzed with GC/MS, we noticed accumulation of both PD and ent-kaurene (Publication III, Figure 2F). Because the exchange construct had integrated randomly, the overexpression of *cps/ks* was not expected. The mechanism of increased *cps/ks* expression is not known but could involve PbcR-mediated activation of transcription (Publication III, Figure 3i) or insertion of the exchange construct into transcriptionally active chromosomal loci (Publication III, Figure 3ii). Regardless of the precise mechanism, the data show that *A. nidulans* oe:PbcR is suitable for heterologous diterpene production.

3.11 Genetic engineering of *A. nidulans* for heterologous monoterpene production

3.11.1 Overexpression of *Citrus unshiu* gamma-terpinene synthase results in gamma-terpinene production in *S. cerevisiae* and in *A. nidulans*

Having shown that *A. nidulans* is suitable for heterologous diterpene production, we further explored the possibility of producing heterologous monoterpenes in *A. nidulans*. Given the improved genetic tractability and relative lack of protease activity in yeast (compared to *Aspergilli)*, the activity of *Citrus unshiu* gamma-terpinene synthase (gTerpS) was first tested in the yeast *Saccharomyces cerevisiae*. The plastid targeting sequence was removed from the gamma-terpinene construct to provide a fully active form of the enzyme and an *Aspergillus* codon-

optimized construct was overexpressed in *S. cerevisiae*. Production of gamma-terpinene was clearly detected with GC/MS (Publication III, Figure 5A).

An overexpression construct for *A. nidulans* was generated by placing gTerpS under control of the constitutively active *gpdA* promoter. Expression of the gamma-terpinene synthase was analyzed by using qRT-PCR and a product peak corresponding to gamma-terpinene was identified with GC/MS (Publication III, Figure 5). The data demonstrate production of gamma-terpinene in *A. nidulans*. To our knowledge, this is the first report of heterologous monoterpene production in *A. nidulans*.

3.12 Activation of the PD gene cluster leads to heterocyclic compound production in *A. nidulans*

While overexpression of *pbcA* alone was sufficient for PD production in *A. nidulans*, we wanted to identify putative roles for the other cluster genes. Overexpression of *pbcR* positively regulated PD cluster genes. Within the *A. nidulans* PD cluster, genes AN1595–AN1598 encode putative decorative enzymes. To study the role of these genes, GC/MS analysis was carried out to compare the metabolite profiles of oe:PbcR strain (that has the entire PD cluster activated) to the strain overexpressing *pbcA* alone. Both strains show PD accumulation, yet additional peaks were noticed in the oe:PbcR strain (Publication III, Figure 6A). These peaks were identified by matching extracted spectra to known mass spectral library compounds with a match percentage of 80 or higher. The matching library compounds were mainly three-ring heterocyclic compounds with a molecular mass of 270 (Publication III, Figure 6B). Many of these matching structures are closely related to known fluorescent compounds and their presence may result in the fluorescence seen in oe:PbcR (Publication III, Figure 6C). Although PD exhibits antioxidant properties, its role in *A. nidulans* is obscure. The compounds seen in oe:PbcR could function in communication or defense of *A. nidulans* in biological situations where *pbcR* transcriptional regulation is triggered. Until these conditions are revealed though, the role of PD and the other metabolites produced in oe:PbcR remains unknown.

4. Discussion

Aspergillus nidulans is a well-characterized fungal model organism and a producer of many secondary metabolites. The significance of fungal natural products as novel high-value chemicals or pharmaceutical leads, along with the sequencing and annotation of fungal genomes, has guided screening efforts for secondary metabolite gene clusters. Genome mining of *A. nidulans* has revealed numerous putative secondary metabolite gene clusters and many of these clusters are linked to already identified metabolites (Andersen, Nielsen et al. 2013, Inglis, Binkley et al. 2013). The predicted number of *A. nidulans* metabolite clusters, however, exceeds the number of known metabolites identified for the fungus. This phenomenon is characteristic for many natural product producing organisms, since the biosynthesis of the compounds remains silent in laboratory conditions.

4.1 Gene cluster analysis

For genetic engineering purposes, it is important to identify the genes involved in production of a given compound. Secondary metabolite clusters usually have one or more backbone gene(s), including: polyketide synthases, nonribosomal peptide synthetases, dimethylallyl tryptophan synthases, and terpene cyclases. Additional hallmark genes include different modifying enzymes, such as oxidoreductases, oxygenases, dehydrogenases, and transferases (Lim, Sanchez et al. 2012). Also, transcriptional regulators are often found in biosynthetic gene clusters.

In this study, we applied genome mining to predict a previously unidentified diterpene gene cluster in *A. nidulans*. We characterized a zinc binuclear protein, PbcR (Pimaradiene Biosynthetic Cluster Regulator), as the positive transcriptional regulator of this normally silent secondary metabolite gene cluster. Overexpression of *pbcR* results in elevated transcription of seven cluster genes and in *ent*-pimara-8(14),15-diene (PD) production. The metabolite profile comparison of *pbcR* overexpressing *A. nidulans* (oe:PbcR) and the wild-type fungus (FGSC A4) showed distinct oe:PbcR product peaks, which were not present in the FGSC A4. The analysis was done to the fungal strains in their exponential growth phase. No product peaks were observed for the wild type fungus with either direct SPME-GC/MS of the liquid cultures or the GC/MS of the solvent extracts. The experimental conditions were picked purposely to avoid observing possible interfering

secondary metabolites that might arise in the late culture phase. The late culture phase, or the idiophase, refers to the growth phase in which the fungus has stopped actively dividing and the secondary metabolite production starts.

To activate the biosynthesis of PD in *A. nidulans*, we used the same approach as has been conducted for polyketide asperfuranone, and PKS-NRPS hybrid metabolites, aspyridone A and B. The biosynthesis of asperfuranone and aspyridones was activated with the overexpression of their pathway-specific transcription factors (Bergmann, Schumann et al. 2007) (Chiang, Szewczyk et al. 2009). Although a few fungal pimaradiene compounds have been identified (Dockerill and Hanson 1977, Kenmoku, Tanaka et al. 2004), this study is the first report of PD as a natural product in *Aspergillus nidulans*.

4.1.1 Transcription factor PbcR (AN1599) and the PD synthase PbcA (AN1594)

Two genes, the cluster-specific transcription factor *pbcR* and the PD synthase *pbcA,* from the PD gene cluster were functionally characterized and named in this study. Other genes in the cluster were identified with expression analysis and their possible role in PD biosynthesis was suggested by using bioinformatics.

Because the fungal and plant terpene synthases share fairly low primary sequence homology, the identification is based on the presence of the catalytic domains. The pimaradiene synthase, PbcA, is homologous to known bi-functional diterpene synthases that catalyze two sequential cyclization steps from GGPP to the final diterpene hydrocarbon via an *ent*-copalyl diphosphate (*ent*-CDP) intermediate. The primary sequence homology of type I terpene cyclases is usually fairly low (Schrepfer, Buettner et al. 2016). PbcA shares 40% homology to *Phomopsis amygdali* phyllocladan-16-ol synthase, 38% identities to *Fusarium fujikuroi ent*-kaurene synthase, 37% homology with *Phaeosphaeria* sp. L487 *ent*-kaurene synthase and *Phoma betae* aphidicolan-16-ol synthase. The similarity between PbcA and plant diterpene synthases is even lower than with the fungal orthologs. For example, PbcA shares only 27% sequence homology with *Oryza sativa* PD synthase and 25% homology with bifunctional *Abies grandis* abietadiene synthase. Nevertheless, PbcA contains the three conserved motifs necessary for the cyclization steps performed by its orthologs, suggesting the ability to perform two cyclization reactions (Publication I, Figure 7).

PbcA was further characterized in this study by deletion and overexpression analysis, which showed that AN1594 truly encodes PD specific synthase. In addition, to study the mechanism of PbcA, we constructed codon-optimized GST-fusion proteins and expressed them in *E. coli*. Site-directed mutagenesis was applied to the cyclization motifs to investigate bi-functionality of PbcA. Despite our efforts, we were not able to detect PD formation from GGPP in our *in vitro* assay by using GC/MS. Possible reasons for undetected terpene may be the poor activity and stability of fungal terpene synthases in *E. coli*. In addition, the protein levels of expressed GST-fusion proteins in the tested cell lysates were fairly low. More

optimization could be done in the future to verify the cyclization domains and the precise mechanism of PbcA. While we lack the experimental data, based on the sequence similarity to other known bi-functional diterpene synthases, we hypothesize that PbcA performs two cyclization steps in PD biosynthesis.

Since the secondary metabolite production is not necessary for the survival of the organisms, we did not see any deleterious affects in deleting the PD synthase gene in *A. nidulans*. Two other diterpene synthase homologs were identified with our genomic mining, AN3252 and AN9314. It is possible that these two diterpene synthase homologs could compensate for the loss of PD synthase in biological conditions where the PD production is needed. In our study, the activation of PD production is artificially triggered by the overexpression of *pbcR* instead of finding the biological stressors for *A. nidulans* to instigate PD production. Since the functional role of PD for *A. nidulans* is unclear, it is hard to know if there is redundancy between the PbcA and the other diterpene synthase homologs.

4.1.2 Precursor pathway enzymes: HMG-CoA reductase (AN1593) and GGPPS (AN1592)

PD cluster genes, AN1592 and AN1593, code for geranylgeranyl diphosphate synthase (GGPPS) and hydroxymethyl glutaryl coenzyme A (HMG-CoA) reductase, respectively. HMG-CoA reductase is a rate-limiting enzyme in the isoprenoid-producing mevalonate (MEV) pathway, whereas GGPPS links the isoprenoid precursors isopentenyl diphosphate (IPP) and dimethyl allyl diphosphate (DMAPP) to form the diterpene-specific building block, GGPP (Chen, Kroon et al. 1994). Given their known role in terpenoid biosynthesis, it was not surprising to find GGPPS and HMG-CoA reductase homologs as part of the PD cluster genes.

4.1.3 Putative decorative enzymes: redox partners cytochrome P450 (AN1598) and short-chain Dehydrogenase/reductase (AN1596)

The precise role of the four additional genes in PD cluster remains unclear. These adjacent genes from locus AN1595 to AN1598 encode putative decorative enzymes. AN1598 encodes a cytochrome P450 (CYP), classified as CYP503B1 by Kelly *et al*. (Kelly, Krasevec et al. 2009). CYPs are found in many secondary metabolite clusters and their role is to catalyze a broad range of decorative steps in metabolite biosyntheses (Renault, Bassard et al. 2014). CYPs generally coordinate oxidation steps, which require an oxidoreductase as a proton donor. *A. nidulans* PD cluster gene AN1596 is homologous with short-chain dehydrogenase/reductases (SDR). SDRs function as NAD- or NADP-dependent oxidoreductases and are therefore potential protein donor partners in CYP guided oxidation. Although oxidation steps are not required for the biosynthesis of the PD hydrocarbon itself, PD cluster CYP503B1 and SDR could be needed for the production of the heterocyclic compounds seen in the metabolite analysis of the oe:PbcR (Pub-

lication III, Figure 6). While PD is the predominant metabolite produced in oe:PbcR, it is possible, that these heterocyclic compounds exhibit beneficial properties for *A. nidulans* in biological conditions where PD cluster activation is triggered. Suggested roles for the discussed PD cluster genes are depicted in Figure 3.

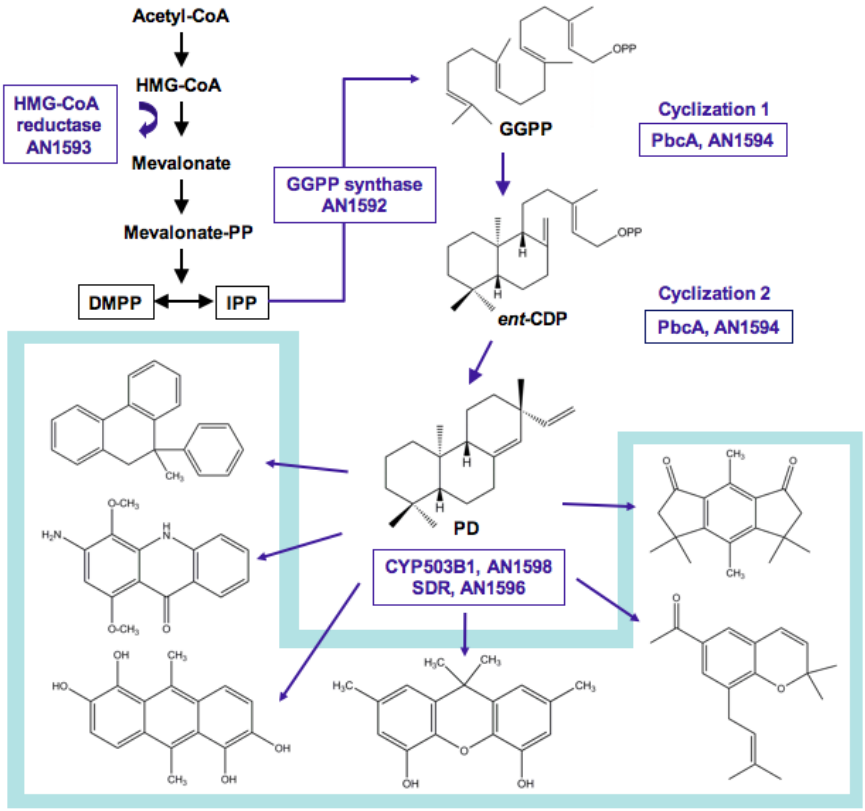

Figure 3. Suggested biosynthetic pathway for PD in *A. nidulans*. HMG-CoA reductase (AN1593) functions as the rate-limiting step on the MEV pathway. GGPPS (AN1592) forms the diterpene specific precursor, GGPP. PbcA catalyzes two cyclization steps from GGPP to PD via *ent*-CDP intermediate. The CYP53B1 (AN1598) and SDR (AN1596) may further modify PD to heterocyclic derivatives (structures in the panel with light green outline).

4.1.4 Hypothetical roles for AN1595 and AN1597 in chromatin modification and translation

Bok et al. (2006) demonstrated upregulation of multiple secondary metabolite clusters, including some PD cluster genes, in *laeA*-overexpressing *A. nidulans* (OE::*laeA*). LaeA is a global regulator for secondary metabolism. While the precise

mechanism of LaeA is unknown, it is thought to act through its S-adenosyl-l-methionine (SAM) binding domain (Strauss and Reyes-Dominguez 2011). SAM domains are identifying features of methyltransferases that modify proteins and small molecules. SAM domains are also present in DNA and RNA-modifying histone methyltransferases. LaeA is suggested to be involved in the heterochromatin histone methylation that opens the chromatin conformation for transcription (Strauss and Reyes-Dominguez 2011). Interestingly, one of the PD cluster genes, AN1597, is homologous to methyltransferase. This gene, along with *pbcR* and AN1595 that encodes a putative translation elongation factor gamma (TEF-g), did not show elevated expression in OE::*laeA*. Significant upregulation of CYP503B1 (AN1598) and SDR (AN1596) expression was seen, while the expression of *pbcA* (AN1594), HMG-CoA reductase (AN1593) and GGPP synthase (AN1592) was only slightly elevated in OE::*laeA*. In contrast, eight adjacent PD cluster genes have significantly elevated expression levels in oe:PbcR.

In the report by Bok *et al.*, the PD cluster in OE::*laeA* was characterized as a cryptic hybrid metabolite cluster (Bok, Hoffmeister et al. 2006) and no PD production was reported. This suggests that the activation of the diterpene cluster genes can be differentially regulated by both PbcR and also LaeA. Given the role of methyltransferases in histone modification, it is possible that PD cluster methyltransferase AN1597 is involved in local chromatin modification when PD cluster genes are activated by PbcR. While LaeA exerts its regulation to multiple secondary metabolite clusters, AN1597 may be needed more specifically in the regulation of PD production. The decorative catalytic activities of PD cluster CYP503B1 and SDR in OE::*laeA* are most likely needed in the modification of metabolites other than PD. A schematic figure of the suggested function of *pbcR*, methyltransferase (AN1597) and TEF-g (AN1595) is shown in Figure 4.

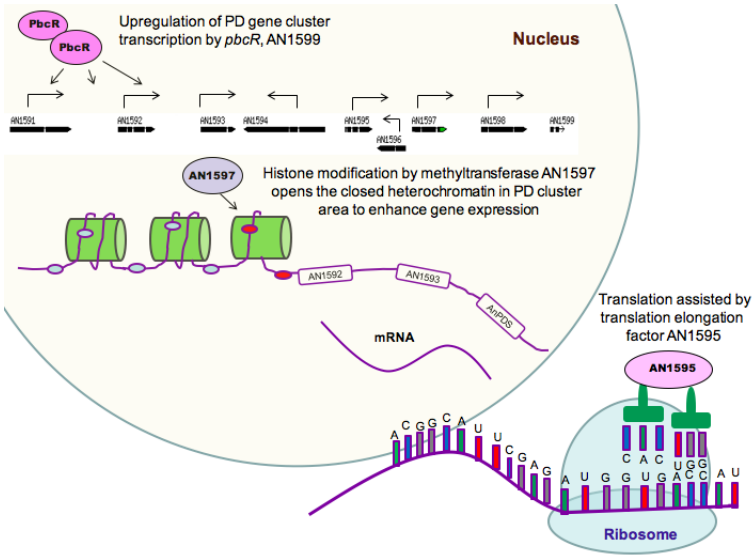

Figure 4. Model for function of PD gene cluster genes pbcR (AN1599), methyltransferase (AN1597), and TEF-g (AN1595). Transcription of PD cluster genes in the nucleus is elevated with the overexpression of pbcR. The methyltransferase may function as a local histone modifier removing heterochromatin marks. Removal of the heterochromatin marks opens up the conformation of chromatin, which allows for transcription to take place. Transcribed mRNA is transported to the cytosol where TEF-g participates in translation.

4.1.5 Hypothetical roles for AN1595 and AN1597 as detoxifying enzymes

Many fungal secondary metabolites are antifungal agents. A recent report shows that endophytic fungus, *Xylaria* sp., produces volatile components that inhibit the growth of agricultural pathogenic fungi *Alternaria solani* and *Fusarium oxysporum* (Sanchez-Ortiz, Sanchez-Fernandez et al. 2016). In the report by Sánchez-Ortiz *et al*., one of the main compounds exhibiting antifungal activity was a sesquiterpene thujopsene. Endophytic fungi are microorganisms that colonize the tissue of their host plant without apparently causing symptoms of illness in the host. Fungal endophytes are known for their capacity to produce compounds that have growth-inhibitory activities toward plant pathogens and herbivores (Hardoim, van Overbeek et al. 2015).

For fungal secondary metabolites that are antifungal agents, the producing fungi must be resistant to their own metabolites (Keller 2015). The glutathione-S-transferases (GSTs) are stress-related detoxification enzymes (Hardoim, van Overbeek et al. 2015). The BLAST sequence analysis of AN1595 reveals the presence of two conserved GST-domains (Figure 5), of which the C-terminal alpha-helical glutathione domains are found in cytosolic dimeric proteins involved in

cellular detoxification. The function of AN1595 in the PD cluster could have protective of detoxifying function for *A. nidulans*.

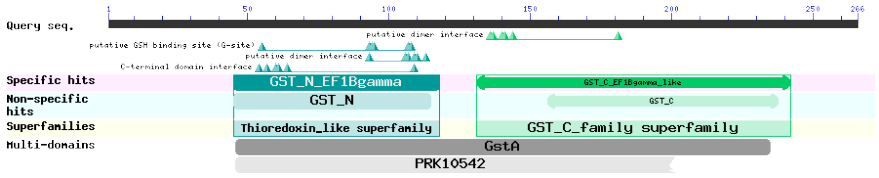

Figure 5. BLAST sequence homology search of AN1595 reveals the conserved GST-protein domains.

The putative roles for the PD cluster genes, exluding *pbcR* and *pbcA*, were predicted with sequence analysis. Mutational analysis of the remaining genes is needed to verify their function in the PD biosynthesis. Since generating targeted mutations in *A. nidulans* can be challenging, implementing the RNAi approach could be more effective way to further study the functionality and necessity of the PD cluster genes in *A. nidulans* PD production.

4.2 Transcriptomic changes in oe:PbcR

According to DNA array analysis 66 genes were significantly upregulated and 75 genes downregulated in oe:PbcR compared to wild type strain (Publication I, Tables S1 and S2). The downregulation of other biosynthetic gene clusters, such as the penicillin cluster, in the PD producing strain could be a way for *A. nidulans* to ensure sufficient primary metabolites for cell growth or for specific production of PD. A schematic figure of genes with altered transcript levels in oe:PbcR compared to the FGSC A4 is shown in Figure 6.

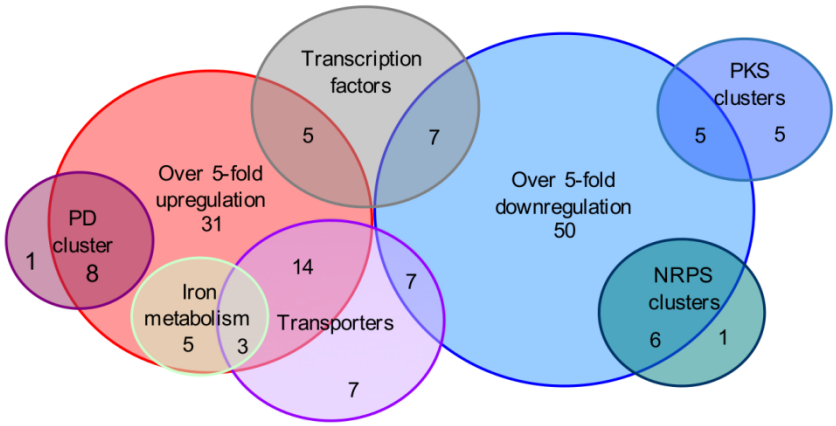

Figure 6. A schematic figure of the genes with altered transcription in oe:PbcR compared to the control FGSC A4 strain. *A. nidulans* strains were cultured to their exponential growth phase, mycelia were collected and freeze-dried and the extracted RNA was provided to Roche Nimblegen for the micro-array processing.

A large number of transporter genes were upregulated in the strain producing PD. Three transporter genes along with 5 other genes with over 5-fold increase in the transcript levels were part of the siderophore iron metabolism that has been linked to *A. nidulans* sexual development. Interestingly, an increase in fruiting body (cleistothecia) formation and a reduction in the number of conidia were observed in the *pbcR* transformants. In addition to the hypothesized detoxifying enzymes within the PD cluster, transporter membrane proteins are perhaps the best-known way that fungi rid themselves of toxic materials. The upregulation of 14 transporter genes in the PD producing strain could very well function in conferring self-resistance to the produced secondary metabolite(s). A number of transcription factors also showed altered expression patterns in oe:PbcR compared to the wild type control. These widespread transcriptomic changes seen with the DNA array could affect the sexual morphology as well as increase the stamina of *A. nidulans* in occasions where PD production is triggered.

4.3 Biotransformation of terpenes and stilbenes with *A. nidulans*

To test our hypothesis that filamentous fungi could be used as a biological transformation tool for the terpenoid or stilbene sidestreams of the paper and pulp industry, we performed biotransformation experiments with dihydroabietic acid

(DHA) and pinosylvin. The acid form of tricyclic diterpenoid dihydroabietadiene was used in the bioconversion experiments for its stability. Pinosylvin is a stilbene with structural resemblance to resveratrol that has been shown to have many pharmaceutical as well as nutraceutical applications (Malhotra, Bath et al. 2015, Rabassa, Zamora-Ros et al. 2015). To study the enzymatic activities in *A. nidulans*, we fed both DHA and pinosylvin to *A. nidulans* wild-type and the oe:PbcR strains and studied the forming compounds with GC/MS. The biotransformation of DHA and pinosylvin resulted in minor amounts of bioconversion products but the amounts were too minute to enable accurate identification with GC/MS. The GC/MS data showed that the compounds were taken into the *A. nidulans* cells within the first 48-hours of incubation and that the converted compounds had carboxyl and/or methyl group additions on the existing DHA and pinosylvin structures. The data of the bioconversion is unpublished.

4.4 The bioactivity of PD

As with many secondary metabolites, the biological function of PD in *A. nidulans* is not known. We tested the antifungal activity of PD against *Aspergiilus nidulans*, *Trichoderma reesei*, *Aspergillus niger*, *Kluyveromyces lactis*, *Saccharomyces cerevisiae*, and *Pichia kudriavzevii* by culturing the different fungi on agar plates with PD containing Hex:EtOAc extracts. The fungal growth was visually compared to the cultures grown on plates without PD. With this method, we were not able to see any antifungal effect of PD.

Since some secondary metabolites have been shown to function as insect repellents, we tested the effect of PD in the *Drosophila melanogaster* larval migration assay. In this assay, both PD producing fungus and the FGSC A4 wild-type were co-cultured on a plate and the first instar larvae of *D. melanogaster* were planted in the middle of the plate in between the two different fungal mycelial mats. The hypothesis was that if PD functions as an insecticide, the larvae will migrate toward the less toxic environment, in this case, toward the wild type fungus. Unforunately, the results from this assay were not conclusive and remain unpublished. Our antifungal and insecticidal assays relied on using the PD containing extracts or the PD producing fungus, respectively. We did not use the pure compound for these experiments and therefore cannot rule out the possibility of PD exhibiting antifungal or insecticidal properties. In the future, these bioactivities could be retested with the pure compound to see if it changes the outcome.

In many plants and fungi, the production of secondary metabolites has been linked to environmental stress such as pathogen invasion, osmotic or oxidative stress. For example, lovastatin biosynthesis and aflatoxin biosynthesis in *A. terreus* and *A. flavus*, respectively, is activated by accumulation of reactive oxygen species (Hong 2013, Miranda, Gomez-Quiroz et al. 2013). Because oxidative stress has also been shown to activate secondary metabolite biosynthesis in *A. nidulans*, we tested the antioxidant activity of PD. Although the biological function of PD in *A. nidulans* is unknown, the antioxidant activity presented here suggest

that one potential role of PD may be to function as an antioxidant. To further strengthen this hypothesis, we tested if PD production is triggered in *A. nidulans* exposed to oxidative stress by culturing FGSC A4 strain in the presence of hydrogen peroxide. Unfortunately, the results of this experiment were never analyzed due to internal changes in the personnel at the time. The aforementioned experiments and additional testing such as co-culturing *A. nidulans* with potentially competing organisms, could be re-done with some modifications to shed more light on the biological function of PD in *A. nidulans*. The purification method developed in this study can be up-scaled to accommodate larger quantities of PD and systematic bioactivity screening of the compound could be done in the future to further assess the putative biological roles or applications of PD. The isolation method developed in our study is relevant not only for PD, but also for purification of other diterpenes with similar properties.

4.5 Engineering of *A. nidulans* for heterologous terpene production

While our data indicated that PbcR functions as a positive regulator for PD biosynthesis in *A. nidulans*, overexpression of the PD synthase gene, *pbcA,* alone, also caused PD accumulation. Since GGPP is a necessary precursor for diterpene production, intracellular GGPP pool must exist in *A. nidulans* to support PD production in conditions where PD cluster *GGPPS* (AN1592) is not expressed. We concluded that *A. nidulans* intracellular GGPP levels may be sufficient to support heterologous diterpene production.

E. coli and *S. cerevisiae* are widely-used host organisms for production of heterologous mono- and diterpenes. However, low intracellular levels of isoprenoid precursors in these organisms can limit the production yields (Martin, Pitera et al. 2003, Chang, Eachus et al. 2007). To test if *A. nidulans* is suitable for heterologous terpene production, we overexpressed *F. fujikuroi* kaurene synthase (*cps/ks*) in *A. nidulans*. This is the first report of *ent*-kaurene production in *A. nidulans*, and affirmed that *A. nidulans* can produce heterologous diterpenes. In addition to its role in gibberellin biosynthesis, *ent*-kaurene is a precursor for steviol (Richman, Gijzen et al. 1999). Steviol glycosides are used as sweeteners, and steviol derivatives have pharmaceutical applications (Yadav and Guleria 2012); and, as a steviol precursor, *ent*-kaurene could be commercially valuable diterpene.

To study the effect of activated diterpene biosynthesis on the heterologous production, we expressed *cps/ks* in the oe:PbcR strain and observed 2.4 fold increase in the *ent*-kaurene production compared to a strain overexpressing *cps/ks* alone. This could be due to activated PD cluster genes and subsequent increase in GGPP precursor pool. Also, the suppression of other secondary metabolite clusters observed in the strain overexpressing *pbcR* could lead to increased intracellular GGPP levels. To guide the flux of intracellular GGPP specifically for *ent*-kaurene biosynthesis, we tried to exchange the *pbcA* to *cps/ks*. Although we were unable to generate the appropriate strain, we observed *ent*-kaurene production in

the strains with randomly inserted DNA fragments. These fragments had *cps/ks* linked to the *pbcA* promoter, and we hypothesized that *pbcR* recognizes the *pbcA* promoter area to upregulate *cps/ks* transcription. Taken together, these data confirm that *A. nidulans* can produce heterologous diterpenes.

The monoterpene, gamma-terpinene, can be used as a precursor molecule in the production of bio-based terephtalic acid (Asikainen, Jauhiainen et al. 2013), but has not been produced in *A. nidulans*. No monoterpenes or monoterpene biosynthetic genes have been reported to *A. nidulans* (Wortman, Gilsenan et al. 2009, Arnaud, Cerqueira et al. 2012, Andersen, Nielsen et al. 2013, Inglis, Binkley et al. 2013). Nonetheless, we tested the monoterpene producing ability of *A. nidulans* and overexpressed N-terminally truncated *Citrus unshiu* HA-tagged gamma-terpinene synthase in *A. nidulans*. The activity of the construct was first assessed in *S. cerevisiae* and clear catalytic activity was observed in an *in vitro* assay using GPP as a substrate. Gamma-terpinene was the predominant product in the assay. When the same construct was expressed in *A. nidulans*, gamma-terpinene was likewise observed by using GC/MS. However, the gamma-terpinene levels were much lower than what was observed for PD or *ent*-kaurene in similar conditions. Low gamma-terpinene yield could be due to toxicity or low intracellular GPP precursor pool. Regardless of the low yield, our data suggest that with further strain modification and optimization of the production conditions, *A. nidulans* could be a potential target host for also monoterpene production

5. Conclusions and future prospects

Terpenes and terpenoids are valuable chemicals used in many applications ranging from jet-fuel components to pharmaceuticals. In this study, we report the first diterpene biosynthetic gene cluster in filamentous fungus *Aspergillus nidulans*. Our results affirm the terpene producing ability of *A. nidulans*, and serve as a proof-of-concept for using this filamentous fungus as a heterologous host for terpene production. The results reported in this thesis emphasize the advantage of using genomic mining with genetic engineering in the search for novel biosynthetic pathways and metabolites.

The positive transcriptional regulator responsible for diterpene biosynthetic gene cluster activation in *A. nidulans* was functionally characterized and named PbcR for Pimaradiene Biosynthetic Cluster Regulator. Also, the first diterpene synthase gene in *A. nidulans* was characterized and named PbcA for PD synthase in PbcR-regulated biosynthetic gene cluster in *A. nidulans*. The remaining genes in the biosynthetic PD cluster were predicted with expression analysis. These results allowed us to suggest a biosynthetic pathway for PD production in *A. nidulans*.

This study describes optimized extraction and purification methods, as well as the NMR characterization of PD. The methods presented here are applicable to compounds with similar properties and will prove useful when intending to produce pure diterpenes for commercial applications or for bioactivity screening. The antioxidant activity of PD and the heterocyclic compounds seen in the PD producing *A. nidulans* could be part of the biological responses of this fungus. Hence, our study indicates a potential biological role for a previously unidentified metabolite in *A. nidulans*.

The potential of using *A. nidulans* as a heterologous host for terpene production was demonstrated by genetic engineering. The expression of heterologous di- and monoterpene syntheses in *A. nidulans* resulted in production of *ent*-kaurene and gamma-terpinene. These data show that *A. nidulans* could be used as a host organism for heterologous mono- and diterpene production.

While we were able to hypothesize the function of PD cluster genes by using bioinformatics, the stepwise deletions of the cluster genes would be needed to verify their role in PD biosynthesis. Also, more systematic bioactivity screening of PD could bring more insight into its role in *A. nidulans*. As this thesis work demonstrates the prospect of using *A. nidulans* in heterologous terpene production, addi-

tional engineering should be applied to make this organism a viable option as an industrial host. Expression of other heterologous terpene synthases in *A. nidulans* would further clarify its potential role as a producer of high-value compounds.

This study displays a previously unidentified natural product compound class for *A. nidulans* and opens up possibilities for developing *A. nidulans* as a producer of commercially valuable secondary metabolites. Furthermore, the results reported in this thesis extend the knowledge of *A. nidulans* secondary metabolism and its regulation.

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Publication I

Identification and characterization of a novel diterpene gene cluster in *Aspergillus nidulans*

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Identification and Characterization of a Novel Diterpene Gene Cluster in *Aspergillus nidulans*

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Abstract

Fungal secondary metabolites are a rich source of medically useful compounds due to their pharmaceutical and toxic properties. Sequencing of fungal genomes has revealed numerous secondary metabolite gene clusters, yet products of many of these biosynthetic pathways are unknown since the expression of the clustered genes usually remains silent in normal laboratory conditions. Therefore, to discover new metabolites, it is important to find ways to induce the expression of genes in these otherwise silent biosynthetic clusters. We discovered a novel secondary metabolite in *Aspergillus nidulans* by predicting a biosynthetic gene cluster with genomic mining. A $Zn(II)_2Cys_6$–type transcription factor, PbcR, was identified, and its role as a pathway-specific activator for the predicted gene cluster was demonstrated. Overexpression of *pbcR* upregulated the transcription of seven genes in the identified cluster and led to the production of a diterpene compound, which was characterized with GC/MS as *ent*-pimara-8(14),15-diene. A change in morphology was also observed in the strains overexpressing *pbcR*. The activation of a cryptic gene cluster by overexpression of its putative $Zn(II)_2Cys_6$–type transcription factor led to discovery of a novel secondary metabolite in *Aspergillus nidulans*. Quantitative real-time PCR and DNA array analysis allowed us to predict the borders of the biosynthetic gene cluster. Furthermore, we identified a novel fungal pimaradiene cyclase gene as well as genes encoding 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase and a geranylgeranyl pyrophosphate (GGPP) synthase. None of these genes have been previously implicated in the biosynthesis of terpenes in *Aspergillus nidulans*. These results identify the first *Aspergillus nidulans* diterpene gene cluster and suggest a biosynthetic pathway for *ent*-pimara-8(14),15-diene.

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Introduction

Filamentous fungi produce various bioactive compounds as secondary metabolites [1,2]. The genes encoding consecutive steps in a biosynthetic pathway of secondary metabolites are often clustered together on the chromosomes [3]. The clustering of such genes, along with multiple genome sequencing projects [4,5], has facilitated the prediction of new biosynthetic pathways using bioinformatics. Since secondary metabolites are not crucial for the survival of the organism, their production usually remains silent in normal laboratory conditions [1,6]. As a result, for most cases in which pathways are discovered through bioinformatic analysis, the products remain undetected [1,4–7]. The role of secondary metabolites for the producing organism is often unclear. They are most likely used as chemical signals in communication and defense to enhance the survival of the organism in its ecological niche [8]. For example, activated secondary metabolite production in *Aspergillus nidulans* has been shown to protect the fungus from fungivory [9]. In addition, for many pathogenic fungi, the virulence that has been hypothesized to protect the fungus in an environment with a diverse array of competing organisms [10] is often mediated by secondary metabolites. Nevertheless, in many cases the biological importance of secondary metabolites for fungi is elusive, and hence the conditions triggering the metabolic biosynthesis are unknown [8].

A variety of methods have been used to uncover the products of silent secondary metabolite clusters in filamentous fungi [3,11]. One approach has been to manipulate the transcriptional control of the genes involved. Transcriptional regulation of fungal biosynthetic genes for many secondary metabolites is carried out by narrow and broad domain transcription factors [12]. AreA, CreA and PacC are well-characterized broad domain regulators in *Aspergillus nidulans*, where they regulate the production of secondary metabolites in response to changes in the environmental nitrogen, carbon and pH, respectively [8]. Another global regulator of

secondary metabolite gene clusters, LaeA (Loss of *AflR* Expression), was identified in a screen for *Aspergillus nidulans* mutants unable to produce sterigmatocystin [12]. *AflR,* the positive regulator of aflatoxin and sterigmatocystin biosynthesis, is not expressed in *laeA* mutants. Expression of also other genes in the sterigmatocystin and penicillin gene clusters is downregulated in the *laeA* deletion strain [13]. The overexpression of *laeA* instead activates multiple putative secondary metabolite clusters. Activation of a biosynthetic gene cluster of previously unknown product in *Aspergillus nidulans*, terrequinone A, was identified in a strain overexpressing *laeA* [13,14]. LaeA has been implicated to function in chromatin remodeling in the subtelomeric regions of fungal chromosomes, where many secondary metabolite clusters are located [15].

In addition to broad domain transcription factors, narrow pathway-specific regulators also take part in the activation of cryptic secondary metabolite gene clusters [16]. Binuclear zinc cluster ($Zn(II)_2Cys_6$) proteins are a group of pathway-specific transcription factors found only in fungi [17]. AflR, the regulator necessary for aflatoxin and sterigmatocystin biosynthetic gene activation, is a characteristic binuclear zinc cluster protein [18]. AflR is encoded within the sterigmatocystin gene cluster, and it binds to 5′-TCG(N5)GCA motifs found in most promoters of sterigmatocystin/aflatoxin biosynthetic genes [12,18]. Structurally, the $Zn(II)_2Cys_6$–type transcription factors have a well-conserved cysteine rich domain that binds two zinc atoms. This DNA binding domain recognizes CGG triplets in varying orientations within the promoter region of the target genes [17]. $Zn(II)_2Cys_6$–type proteins are typically encoded within the biosynthetic gene cluster for which they positively regulate expression, as is the case for *Aspergillus nidulans* polyketide asperfuranone and PKS-NRPS hybrid metabolites aspyridone A and B [19,20]. Likewise in *Fusarium verticillioides*, the overexpression of the $Zn(II)_2Cys_6$–type transcription factor residing in the fumonisin gene cluster is able to activate fumonisin production [21].

In *Aspergillus nidulans,* the discovery of unknown products by using transcriptional upregulation of cryptic gene clusters has been shown to be a potential method for finding novel bioactive metabolites [13]. One such class of compounds, the terpenes, is of particular interest because of their many bioactive and pharmaceutical properties [22,23]. Many pharmaceutical terpenoids have been isolated from plants used in traditional medicine [24,25], but there is increasing interest toward terpenoids produced by fungi [26,27].

The objective of the present study was to determine the terpene producing capability of *Aspergillus nidulans*. Earlier reports suggest the existence of at least one terpene gene cluster in *Aspergillus nidulans* [13]. Here we describe the identification and activation of a novel gene cluster that produces the diterpene *ent*-pimara-8(14),15-diene. We show that genomic mining in the prediction of novel secondary metabolite clusters, and the subsequent transcriptional activation of the clusters, serve as a tool for discovering new metabolites and biosynthetic pathways.

Results

Genomic Mining Reveals Two Putative Diterpene Clusters in *Aspergillus Nidulans*

Despite earlier published work suggesting that the *Aspergillus nidulans* genome has only one terpene cluster [13], our analysis instead revealed multiple terpene synthase genes potentially located in biosynthetic clusters. The genes with 'terpenoid synthase' or 'terpenoid cyclase' InterPro [28] domains were searched from the genome of *Aspergillus nidulans* FGSC A4 [4]. We found 26 such genes, and this group of genes was analyzed by using BLASTp [29] homology search to find putative diterpene synthase homologs. Three ORFs, encoded by locus AN1594, AN3252 and AN9314, showed significant homology to known *ent*-kaurene synthases, whereas the AN6810 sequence shared sequence homology with fungal fusicoccadiene synthase [30]. The genomic neighborhood of these four diterpene synthase homologs was screened for zinc binuclear cluster ($Zn(II)_2Cys_6$) proteins, because these are known to positively regulate the genes within the cluster that encodes them [17–21]. We also searched for genes encoding putative cytochrome P450 monooxygenases, since these enzymes are many times involved in terpenoid biosynthesis [31]. We found two gene clusters containing all three genes encoding putative terpene synthase, cytochrome P450, and $Zn(II)_2Cys_6$ protein. Both clusters were selected for further analysis. The selected clusters also contained other putative secondary metabolism pathway genes [6], including dehydrogenases, oxidoreductases, and terpene precursor synthase genes.

Overexpression of *pbcR* Enhances the Transcription of Seven Diterpene Cluster Genes in *Aspergillus Nidulans*

We discovered a transcriptional regulator, which we named Pimaradiene Biosynthetic Cluster Regulator (PbcR). *PbcR* is encoded by the *Aspergillus nidulans* chromosome VII locus AN1599.4 (GenBank accession number: CBF85190.1), and was cloned as a genomic construct with *Aspergillus nidulans gpdA* promoter and transformed into FGSC A4 wild type strain by random integration. Three independent transformant strains oe:*AN1599*_9, oe:*AN1599*_42 and oe:*AN1599*-_45 were obtained from two different transformations. The presence of the overexpression construct was verified by PCR (Figure S1). FGSC A4 and the transformant strains were grown in YES medium, and the expression levels of *pbcR* (AN1599.4), putative terpene synthase (AN1594.4) and cytochrome P450 (AN1598.4) genes were analyzed by using quantitative real-time PCR (qPCR). As expected, the transcription of *pbcR* was clearly elevated in all three transformant strains compared to FGSC A4; with qPCR analysis demonstrating an 86-fold, 109-fold and 79-fold increase in expression in oe:*AN1599*_9, oe:*AN1599*_42 and oe:*AN1599*-_45, respectively (Figure 1A). The different strain-specific levels of *pbcR* overexpression may have been due to a number of factors including the possibility of overexpression constructs integrating into different portions of the genome or varying *pbcR* copy numbers. For putative terpene synthase gene we observed a 9,000 to 11,000-fold increase in expression in the three transformant strains. For putative cytochrome P450 we observed a 2,400 to 4,500-fold increase in expression (Figure 1A). These results suggest that PbcR is a positive regulator for the diterpene metabolite cluster genes. The strain with the highest transcription of *pbcR*, oe:*AN1599*_42 was selected for further analysis and named oe:*PbcR*.

The putative transcriptional regulator gene at chromosome VI locus AN3250.4 (GenBank accession number: CBF83099.1) was also cloned and overexpressed in FGSC A4. Two isolated transformant strains oe:*AN3250*_9 and oe:*AN3250*_11 were analyzed by using qPCR. Although we detected a 431-fold and 2680-fold increase in expression of the putative transcription factor in oe:*AN3250*_9 and oe:*AN3250*_11, respectively, no significant upregulation of the two target genes for this cluster was observed (Figure 1B). In sum, these results suggest that in contrast to *pbcR*, AN3250.4 overexpression alone does not activate its own putative terpene cluster. However, regulation of AN3250.4 activity at the post-translational level cannot be ruled out.

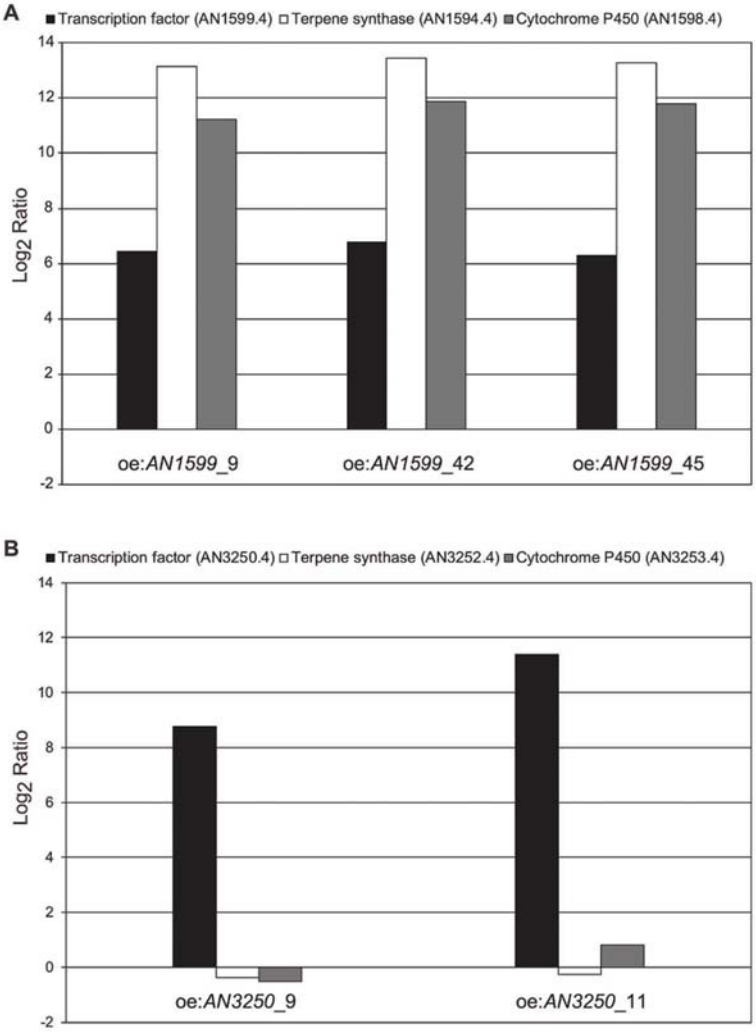

Figure 1. Identification of putative diterpene cluster transcription factor in *Aspergillus nidulans*. Two putative diterpene clusters in *Aspergillus nidulans* were identified by using genomic mining of public databases. Putative transcription factors for the identified clusters were cloned as genomic constructs and overexpressed in *Aspergillus nidulans* FGSC A4. The expression levels of the transcription factors as well as two predicted target genes from each cluster were analyzed by using qPCR. **A)** Overexpression of *pbcR* (AN1599.4) activates transcription of terpene synthase (AN1594.4) and cytochrome P450 (AN1598.4) in three *Aspergillus nidulans* transformant strains (oe:*AN1599_9*, oe:*AN1599*_42 and oe:*AN1599*_45). **B)** Overexpression of the putative transcription factor AN3250.4 fails to alter the transcription of putative terpene synthase (AN3252.4) or cytochrome P450 (AN3253.4) in two *Aspergillus nidulans* transformant strains (oe:*AN3250*_9 and oe:*AN3250*_11).
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To define the borders of the biosynthetic cluster, expression of 13 putative cluster genes was analyzed by using qPCR in oe:*PbcR* and FGSC A4. The expression of these genes in the wild type strain was very low, whereas a massive upregulation of seven adjacent genes was seen in oe:*PbcR* (Figure 2). The highly upregulated genes were homologous to GGPP synthase; AN1592.4 (307,000-fold), HMG-CoA reductase; AN1593.4 (12,000-fold), diterpene synthase; AN1594.4 (21,700-fold), translation elongation factor γ; AN1595.4 (19,000-fold), short-chain dehydrogenase; AN1596.4 (420-fold), hypothetical protein with some similarity to methyltransferase; AN1597.4 (310-fold), cytochrome P450; AN1598.4 (8,400-fold) and $Zn(II)_2Cys_6$ –type transcription factor; AN1599.4 (50-fold). Expression levels of five other putative cluster genes were not as significantly altered by the overexpression of *pbcR* (Figure 2). Taken together, these data suggest that the predicted diterpene cluster consists of eight adjacent genes on *Aspergillus nidulans* chromosome VII in the region AN1592.4 to AN1599.4.

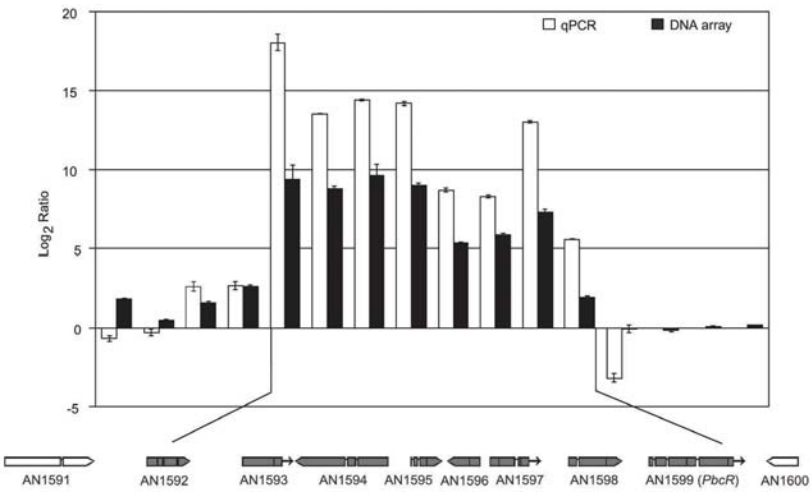

Figure 2. Expression analysis defines the borders of the PbcR activated diterpene cluster in *Aspergillus nidulans*. Transcription factor *pbcR* was overexpressed in *Aspergillus nidulans* strain FGSC A4 (oe:*PbcR*). FGSC A4 and oe:*Pbc*R were grown to their early exponential growth phase in YES-medium. In both strains, the expression levels of 13 genes in the predicted cluster area were measured with qPCR. The fold-change in expression was calculated (white bars). Error bars represent standard error of the mean (SEM, n = 9) for three individual samples with three technical replicates each. The transcriptome of the *pbcR* overexpression strain and the FGSC A4 wild type strain was analyzed by using DNA array and fold differences in expression calculated (black bars). Error bars represent SEM (n = 12) for two cultures with three replicates each; and, each array included duplicate probes. DNA array data represent the comparison of the mean values using confidentiality level 99% with p-values $\leq$ 0.01 in student's t-test. Both qPCR as well as DNA array analysis show that overexpression of *pbcR* (AN1599.4) leads to significant upregulation of seven genes in the predicted diterpene cluster area. Predicted genes in the cluster are GGPP-synthase (AN1592.4), HMG-CoA reductase (AN1593.4), diterpene synthase (AN1594.4), elongation factor 1-gamma (AN1595.4), short-chain dehydrogenase (AN1596.4), conserved hypothetical protein (AN1597.4), cytochrome P450 (AN1598.4), and $Zn(II)_2Cys_6$–type transcriptional regulator *pbcR* (AN1599.4). Chromosomal area has been adapted from Aspergillus Genome Database [19] showing 27 kb from *Aspergillus nidulans* FGSC A4 chromosome VII positions 1275000 to 1302000 (upregulated genes highlighted in gray).
doi:10.1371/journal.pone.0035450.g002

Overexpression of *pbcR* Leads to Widespread Changes in the Transcriptome of *Aspergillus Nidulans*

DNA array analysis was used to analyze the transcriptome of both oe:*PbcR* and FGSC A4. Since most secondary metabolites are produced after the fungus has completed its initial growth phase [32], samples for the expression analysis were taken at the early exponential growth phase when secondary metabolite production for the wild type fungus was expected to be very low. Both strains were grown in YES medium. The eight terpene cluster genes identified by qPCR also displayed similar enhanced expression when assessed by using DNA array analysis. In fact, when compared with FGSC A4, the most abundant transcripts in oe:*PbcR* were the seven PbcR target genes of the predicted terpene cluster, including: AN1592.4 (673-fold), AN1593.4 (439-fold), AN1594.4 (785-fold), AN1595.4 (513-fold), AN1596.4 (41-fold), AN1597.4 (58-fold) and AN1598.4 (156-fold). The DNA array data also confirmed the overexpression of *pbcR* (4-fold) compared to FGSC A4 (Figure 2, Table S1).

In addition to the predicted target cluster genes, the expression of a number of secondary metabolite synthase genes was altered in oe:*PbcR*. The polyketide synthase participating in penicillin biosynthesis, *acvA* (AN2621.4), was 5.7-fold downregulated in oe:*PbcR* (Figure 3A, Table S2). Additionally three other putative polyketide synthase genes; AN0523.4 (17.8-fold) (Figure 3B), AN2032.4 (3.3-fold) and AN2035.4 (2.5-fold) (Figure 3C) were downregulated in oe:*PbcR*. Interestingly, many genes adjacent to these synthases were also downregulated, suggesting that the penicillin and two putative polyketide gene clusters are downregulated in the strain overexpressing *pbcR* (Figure 3 A–C). One putative nonribosomal peptide cluster was also dowregulated in oe:*PbcR* (Figure 3D). A significant decrease in expression was seen for the two nonribosomal peptide synthases (NRPS) of this cluster; AN3495.4 (285-fold) and AN3496.4 (167-fold) (Figure 3D). These data demonstrate that activation of the terpene cluster in oe:*PbcR* is associated with changes in the transcriptome of *Aspergillus nidulans* including downregulation of four secondary metabolite clusters. Chromosomal locations of the synthases from this study are shown in Figure S2.

Activation of the Terpene Cluster Results in *ent*-pimara-8(14),15-diene Biosynthesis in *Aspergillus Nidulans*

To identify potential compounds produced in oe:*PbcR*, we analyzed the strains by using solid phase microextraction gas chromatography mass spectrometry (SPME-GC/MS). This analytical method allows identification of volatile and semi-volatile terpenoids [33]. Oe:*PbcR* and FGSC A4 were grown in complete medium for 44 hours and subjected to SPME-GC/MS analysis without further manipulation. An accumulation of an oe:*PbcR*-specific product was observed (Figure 4A). The mass spectrum for the product peak matched the spectral library compound *ent*-pimara-8(14),15-diene (Figure 4B). Also, the calculated retention index for identified *ent*-pimara-8(14),15-diene was 1943, which is in accordance with previous literature values of 1939–1963 [34–37]. To determine if non-volatile compounds were produced by oe:*PbcR*, both the cells and the media from fungal cultures were extracted with hexane:ethyl acetate (1:1) and polar phase extracts subjected to GC/MS-analysis. The data from the cell extracts were consistent with the SPME-GC/MS analysis (Figure 4A, C)

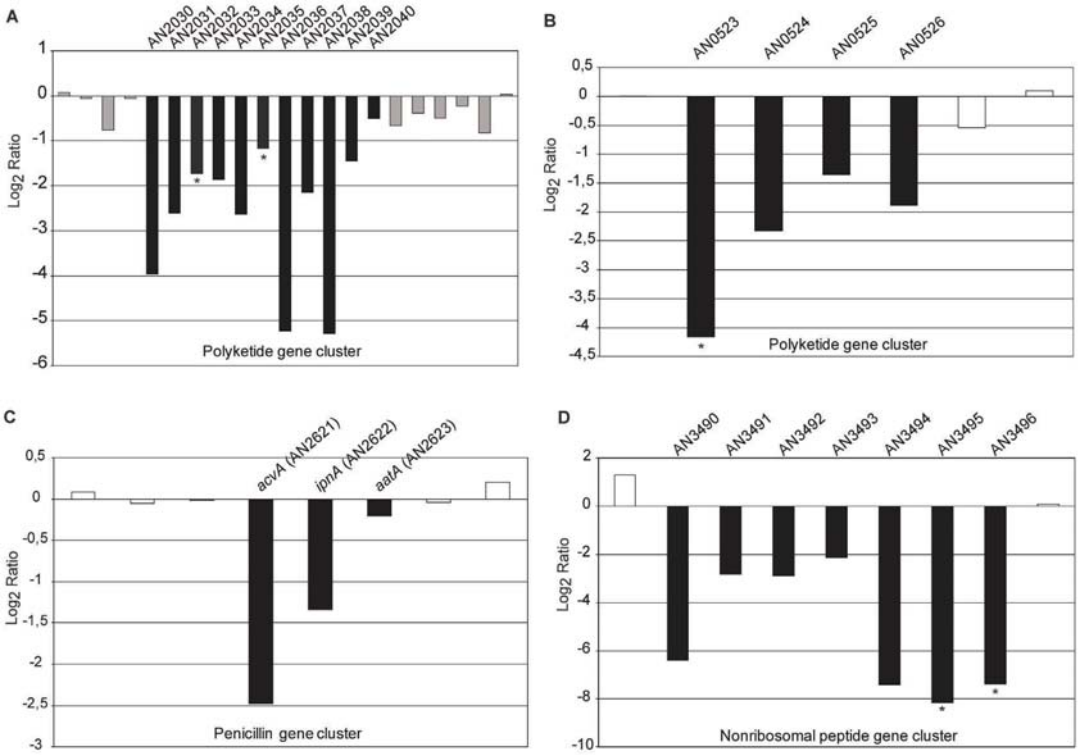

Figure 3. Overexpression of *pbcR* leads to downregulation of four secondary metabolite clusters in *Aspergillus nidulans*. Diterpene cluster transcription factor *pbcR* was overexpressed in *Aspergillus nidulans* strain FGSC A4 (oe:*PbcR*). FGSC A4 and oe:*Pbc*R were grown to their early exponential growth phase in YES-medium. The transcriptome of the oe:*PbcR* and FGSC A4 was analyzed by using DNA array and fold differences in expression calculated. DNA array data represent the comparison of the mean values using confidentiality level 99% with p-values ≤ 0.01 in student's t-test. Downregulation of four putative gene clusters was seen in oe:*PbcR*. **A)** Shown are expression ratios (oe:*PbcR* to FGSC A4) for genes on chromosome VII in the region AN2030.4 to AN2040.4 (black bars) encoding a putative polyketide gene cluster with polyketide synthase genes AN2032.4 and AN2035.4 (asterisks). **B)** Shown are expression ratios (oe:*PbcR* to FGSC A4) for genes on chromosome VIII in the region AN0523.4 to AN0527.4 (black bars) encoding a putative polyketide gene cluster with polyketide synthase gene AN0523.4 (asterisk). **C)** Shown are expression ratios (oe:*PbcR* to FGSC A4) for genes on chromosome VI in the region AN2621.4 to AN2623.4 (black bars) encoding a penicillin gene cluster with genes *acvA* (AN2621.4), *ipnA* (AN2622.4) and *aatA* (AN2623.4). **D)** Shown are expression ratios (oe:*PbcR* to FGSC A4) for genes on chromosome II in the region AN3490.4 to AN3496.4 (black bars) encoding a putative nonribosomal peptide cluster with two nonribosomal peptide synthases AN3495.4 and AN3496.4 (asterisks).
doi:10.1371/journal.pone.0035450.g003

and the oe:*PbcR*-specific product was identified as *ent*-pimara-8(14),15-diene according to its mass spectrum (data not shown). No significant product peaks were detected in the extracts from growth medium (data not shown) suggesting that the *ent*-pimara-8,14(15)-diene is not secreted. Taken together this data demonstrates that an activation of a biosynthetic pathway for a diterpene compound occurs in oe:*PbcR*.

Reduced Conidia and Increased Fruiting Body Formation in Oe:*PbcR*

Morphological changes were observed in oe:*PbcR* compared with FGSC A4. Specifically, oe:*PbcR* cultures typically grew slower than the wild-type strain (data not shown); and, when grown on plates, oe:*PbcR* appeared yellow whereas FGSC A4 were green (Figure 5A). To characterize the morphological phenotype further, microscopic analysis of the plate cultures was performed, and the conidia were quantified. Sexual fruiting body (cleistothecium) was the predominant structure in oe:*PbcR* and the fruiting bodies were also larger (Figure 5). Hülle cell formation was unaffected in oe:*PbcR*, and the sterigmata of the conidiophores in oe:*PbcR* appeared darker compared to FGSC A4 (Figure 5B). Also, the number of asexual spores (conidia) was reduced 6-fold in oe:*PbcR* compared to FGSC A4 (Figure 6).

The Sequence Analysis of Terpene Synthase Orthologs Suggests a Bifunctional Role for AN1594.4

The putative terpene synthase gene orthologs were identified using BLASTp search of public sequence databases. AN1594.4 (accession XP_659198.1) showed sequence homology to known bifunctional terpene synthases. Although overall sequence homology was relatively low (Table 1), as is typical for terpene synthase genes generally [38], we identified conserved motifs required for

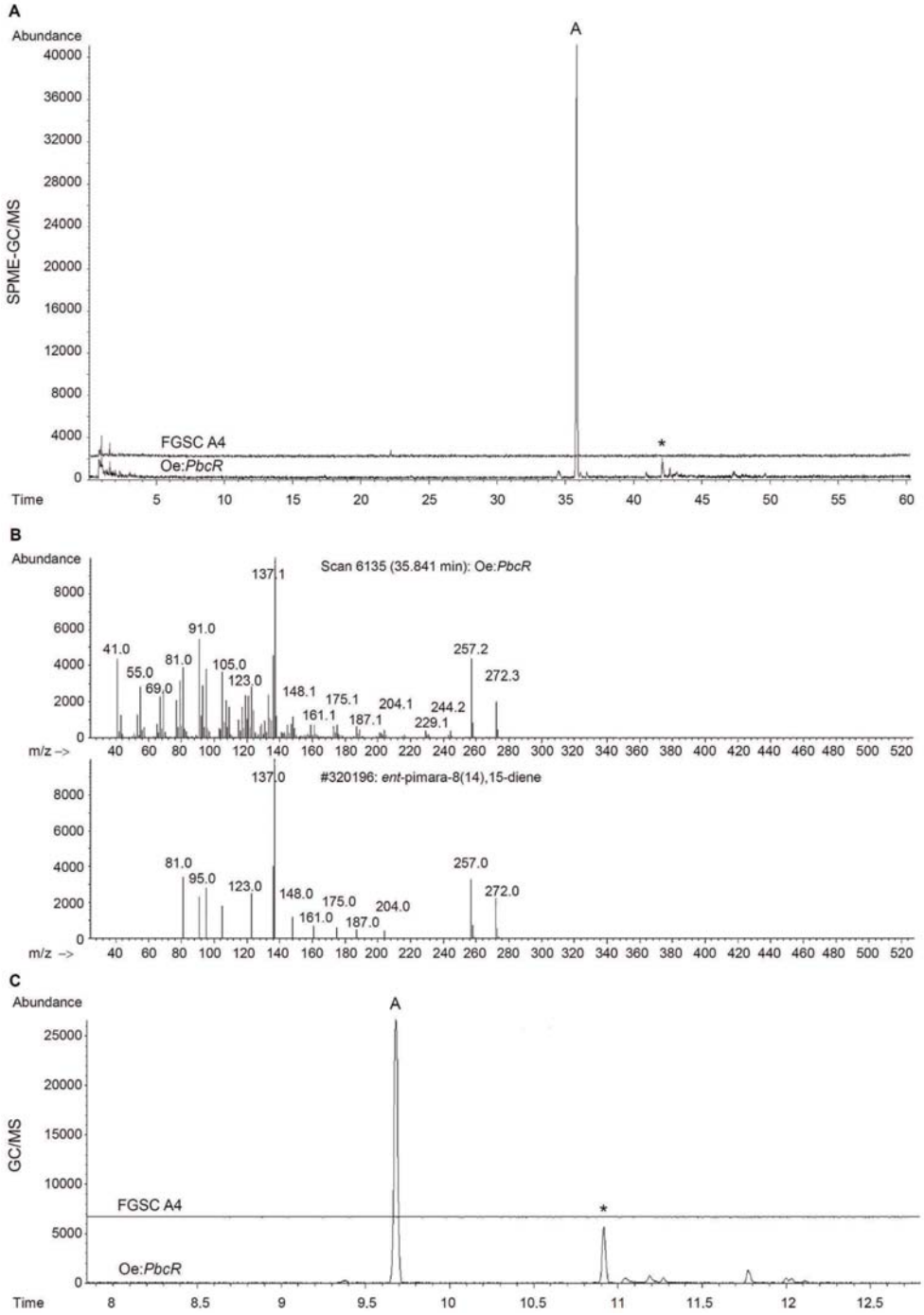

Figure 4. Overexpression of *pbcR* leads to production of *ent*-pimara-8(14),15-diene in *Aspergillus nidulans*. Diterpene cluster transcription factor *pbcR* was overexpressed in *Aspergillus nidulans* FGSC A4 (oe:*PbcR*). The product composition of the oe:*PbcR* and wild type strain

(FGSC A4) was analyzed by using gas chromatography mass spectrometry (GC/MS). Fungal cultures were grown to their exponential growth phase in YES medium. **A)** Cultures were analyzed for the production of diterpene compounds using solid phase micro extraction (SPME)-GC/MS analysis as described in Materials and Methods. One prominent peak (labeled A) was observed in the chromatogram of oe:*PbcR*, but no products were detected in FGSC A4. **B)** Product peak A was identified as *ent*-pimara-8(14),15-diene by comparison of its mass spectrum to Palisade Complete 600K Mass spectral library compounds. **C)** Cells from oe:*PbcR* and FGSC A4 were extracted with hexane:ethyl acetate (1:1) and extracts subjected to GC/MS. As with SPME-GC/MS, one prominent peak (A) was detected. Product peak A was again identified as *ent*-pimara-8(14),15-diene (data not shown).
doi:10.1371/journal.pone.0035450.g004

the two cyclization steps carried out by known bifunctional terpene synthases [39] (Figure 7A). A-type cyclization motif, VYDTAW, was identified at position 34–39 and B-type cyclization motif, DEFME, at position 664–668. In addition, the position 328–331 of AN1594.4 encodes a DADD motif, which is conserved among diterpene synthases [38]. A phylogenetic tree was constructed using ClustalW2 multiple alignment analysis of AN1594.4 and the orthologous genes from other fungi, as well as known pimaradiene synthase genes from *Oryza sativa* [40]. The analysis demonstrates that AN1594.4 is related to fungal bifunctional diterpene synthases. Although there are no annotated fungal pimaradiene synthases, AN1594.4 is nonetheless distantly related to known pimaradiene synthases from plants (Figure 7B). The data from the sequence analysis supports the conclusion that AN1594.4 encodes a bifunctional diterpene synthase.

Discussion

Here we show that the $Zn(II)_2Cys_6$-type transcriptional regulator PbcR (Pimaradiene Biosynthetic Cluster Regulator) activates a normally silent secondary metabolite gene cluster in *Aspergillus nidulans*. Upregulation of eight genes in the biosynthetic gene cluster results in *ent*-pimara-8(14),15-diene production in a strain overexpressing *pbcR* (oe:*PbcR*). To our knowledge, *ent*-pimara-8(14),15-diene has not been reported as a natural product in *Aspergillus nidulans*. Previously unknown genes coding for fungal *ent*-pimara-8(14),15-diene synthase, HMG-CoA reductase, and GGPP-synthase are also present in the cluster we describe. We observed morphological changes in the *pbcR* overexpression strains: the number of asexual spores (conidia) is reduced, and the formation and size of sexual fruiting bodies (cleistothecia) is elevated.

The approach we used has been used previously to identify products of other silent metabolite clusters in *Aspergillus nidulans*. For example, the biosynthesis of polyketide asperfuranone and PKS-NRPS hybrid metabolites aspyridone A and B was activated with the overexpression of their pathway-specific transcription factors [19,20]. The pimaradiene gene cluster upregulated in oe:*PbcR* was previously shown to be one of the putative secondary metabolite clusters upregulated in a *laeA*-overexpressing *Aspergillus nidulans* (OE::*laeA*) [13]. Bok *et al.* [13] demonstrated the upregulation of putative short-chain dehydrogenase (AN1596), cytochrome P450 (AN1598), GGPP-synthase (AN1592), HMG-CoA reductase (AN1593) and terpene synthase (AN1594) in OE::*laeA*. The expression levels of the genes in the pimaradiene gene cluster are different in OE::*laeA* compared to what we observe for oe:*PbcR*. For example, in OE::*laeA*, the expression levels of GGPP-synthase, HMG-CoA reductase and terpene synthase genes were fairly low; and, three cluster genes showed no increase in expression [13]. In contrast, highly elevated expression of eight cluster genes was seen in oe:*PbcR*, and the expression of *laeA* itself was not changed. This suggests that the activation of the diterpene cluster we identified can be differentially regulated by both PbcR and also LaeA. Since multiple secondary metabolite clusters are activated in the *laeA*-overexpressing strain [13], the upregulation of the putative short-chain dehydrogenase and cytochrome P450 genes of the pimaradiene cluster might be needed for modification of secondary metabolites, rather than *ent*-pimara-8(14),15-diene production *per se*. It would be interesting to investigate if *ent*-pimara-8(14),15-diene is produced in the *laeA*-overexpressing strain.

We identified putative genes for HMG-CoA reductase and GGPP-synthase (AN1593.4; GenBank accession number CBF85179.1 and AN1592.4; GenBank accession number CBF85177.1). These genes have not been previously implicated in isoprenoid precursor biosynthesis in *Aspergillus nidulans*. Other studies have identified HMG-CoA reductase (AN3817.2) and GGPP-synthase (AN0654.2, AN2407.2 and AN8143.2) homologs that have been linked to terpenoid biosynthesis in *Aspergillus nidulans* [41–43]. However, HMG-CoA reductase and GGPP-synthase identified in this study may specifically provide precursors for the production of *ent*-pimara-8(14),15-diene. The same phenomenon has been suggested for gibberellin biosynthesis in *Gibberella fujikuroi* [44], where a number of precursor synthase genes function separately in different secondary metabolite pathways. The clustering of the HMG-CoA reductase (AN1593.4) and GGPP-synthase (AN1592.4) with the pimaradiene synthase (AN1594.4) may indicate a need for high precursor production required for the biosynthesis of this particular compound.

Although there are some reports of fungal pimaradiene compounds [45,46], no specific pimaradiene synthases have been identified in fungi. The diterpene synthase identified in our study (AN1594.4; GenBank accession number CBF85181.1) showed similarity to the known fungal *ent*-kaurene synthases GfCPS/KS and PfCPS/KS from *Gibberella fujikuroi* and *Phaeosphaeria* sp., respectively. These terpene synthases catalyze two sequential cyclization steps from GGPP to *ent*-kaurene via *ent*-copalyl diphosphate intermediate [47,48]. *Phomopsis amygdali* phyllocladan-16α-ol synthase PaDC1 is also a bifunctional terpene synthase having three conserved amino acid domains responsible for the different cyclisation reactions [39]. The diterpene synthase AN1594.4 contains all of these three conserved sequences suggesting the ability to perform two cyclization reactions.

Based on the data presented here, we suggest a model for *ent*-pimara-8(14),15-diene biosynthesis in *Aspergillus nidulans*. Specifically, HMG-CoA reductase (AN1593.4) functions in the mevalonate pathway, which produces isoprenoid precursors. GGPP synthase (AN1592.4) is needed in the formation of GGPP, the precursor for diterpenes. Lastly, the two cyclization steps needed to convert GGPP to *ent*-pimara-8(14),15-diene is carried out by pimaradiene synthase (AN1594.4) (Figure 8).

Our analysis revealed four additional genes upregulated in the *Aspergillus nidulans* strain producing pimaradiene. These putative genes encode translation elongation factor 1 gamma (AN1595.4; GenBank accession number CBF85182.1), short-chain dehydrogenase (AN1596.4; GenBank accession number CBF85184.1), hypothetical protein with some similarity to a methyltransferase (AN1597.4; GenBank accession number CBF85186.1), and a cytochrome P450 (AN1598.4; GenBank accession number CBF85188.1). The putative role of these genes in *ent*-pimara-8(14),15-diene biosynthesis is unclear. Cytochrome P450 (AN1598.4), short-chain dehydrogenase (AN1596.4) and methyltransferase (AN1597.4) typically function as decorative enzymes in secondary metabolite biosyntheses [2]. For example, cytochrome

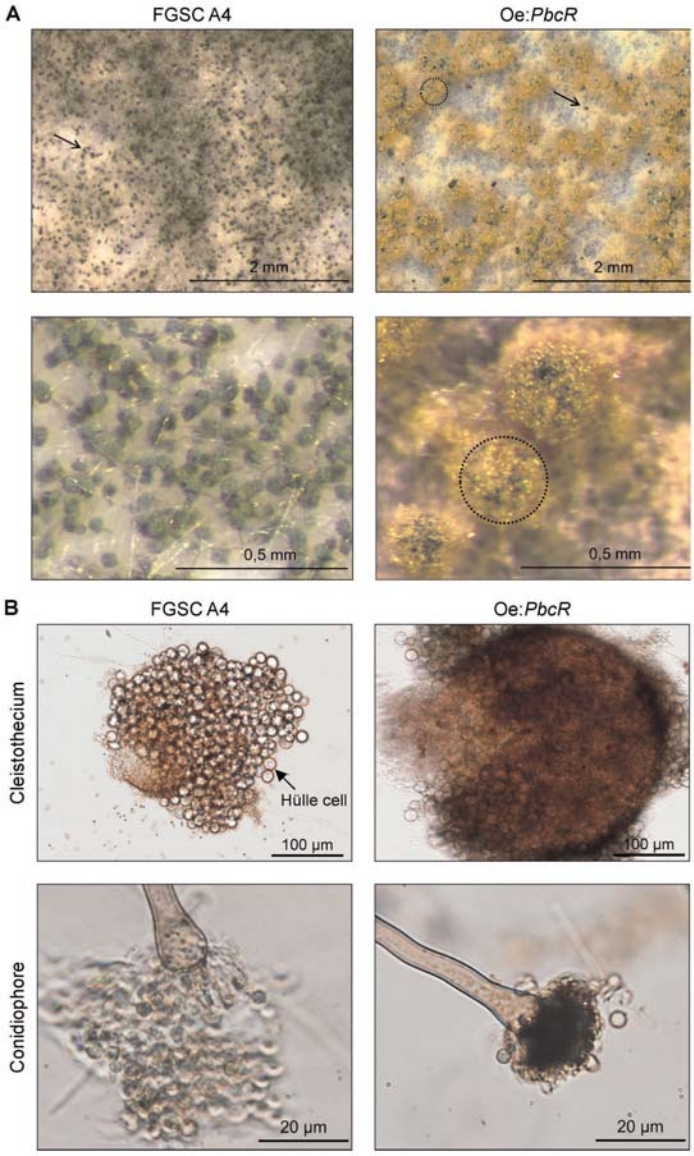

Figure 5. Changes in morphology can be seen in *Aspergillus nidulans* FGSC A4 overexpressing *pbcR* (oe:*PbcR*). Pimaradiene gene cluster regulator *pbcR* was overexpressed in *Aspergillus nidulans* FGSC A4 (oe:*PbcR*). Both wild type FGSC A4 and oe:*PbcR* were grown on potato dextrose plates for 3 days and their morphology studied by microscopy. **A)** Fewer conidiophores (arrows) are seen in oe:*PbcR* compared to FGSC A4. Conidiophore structures in both strains were verified at higher magnification (data not shown). Enhanced sexual fruiting body (cleistothecium, dotted circle) formation can be seen in oe:*PbcR* three-day plate cultures. **B)** The size of cleistothecia in oe:*PbcR* is increased compared to FGSC A4 (upper panels), whereas Hülle cell formation around the fruiting body is similar in both strains. The sterigmata of conidiophores in oe:*PbcR* are darker, and less spores are formed at the tips of the conidiophores than in wild type *Aspergillus nidulans* (lower panels).
doi:10.1371/journal.pone.0035450.g005

P450s add oxygen to the basic terpenoid backbone. This enables chemical modifications of the created hydroxyl group, allowing the formation of a variety of different compounds from the same precursor molecule [31]. Despite the fact that we could not detect the oxidized form of *ent*-pimara-8(14),15-diene in our assay, it is possible that in biological conditions the compound is oxidized to *ent*-pimara-8(14),15-dien-19-oic acid, which is a bioactive diterpene compound predominant in many plant extracts [49,50].

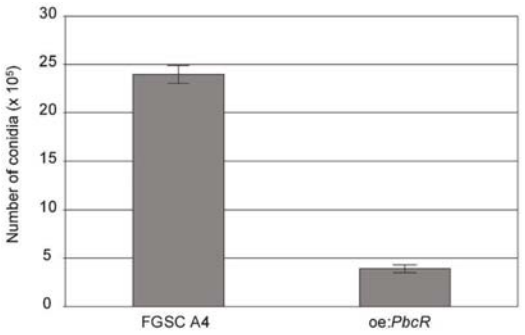

Figure 6. Conidiation is reduced in oe:*PbcR*. FGSC A4 and oe:*PbcR* were grown on potato dextrose plates for three days at 37°C. Spores were quantified from three agar plugs isolated from the PD-plates (average area 85 mm^2). The number of conidia is 6-fold lower in oe:*PbcR* (average number of conidia 3.9×10^5) compared to FGSC A4 (average number of conidia 23.9×10^5).
doi:10.1371/journal.pone.0035450.g006

The expression of two genes (AN1590.4 and AN1591.4) were slightly upregulated in oe:*PbcR*. The expression of these genes was much lower compared to the other putative cluster genes. We identified orthologs of all eight genes included in our cluster in *Neosartorya fischeri*. There, the genes are also adjacent to each other in a putative cluster (Figure S3). But, orthologs of AN1590.4 and AN1591.4 are not present or near this cluster region in *N. fischeri*. Thus, we did not include these genes in our putative cluster. However, the possibility that AN1590.4 and AN1591.4 would be under the regulation of PbcR cannot be ruled out.

An increase in fruiting body formation and a reduction in the number of conidia were observed in all *pbcR* transformants. As the integrations were random in nature, it is possible that the phenotype in the transformants is due to insertional mutagenesis. The velvet family of regulators (*veA*, *velB*, *vosA*, and *velC*) participates in sexual fruiting body formation in *Aspergillus nidulans* [51,52], whereas other genes (e.g., *brlA*, *abaA*, *wetA*, *flbA*, *fluG*, and *fadA*) [53,54] are implicated in asexual conidiation. All of these genes were similarly expressed in both FGSC A4 and oe:*PbcR*, suggesting they are not regulated by PbcR (data not shown). Siderophore iron metabolism has also been linked to *Aspergillus nidulans* sexual development [55]. Eisendle *et al.* [55] showed that the absence of intracellular siderophore impairs both sexual and asexual reproduction in *Aspergillus nidulans*. The same has been reported for ascomycetes *Cochliobolus heterostrophus* and *Gibberella zeae*, where intracellular siderophores are essential for sexual development [56]. The expression levels of siderophore transporter genes *mirA* and *mirB* [57] were upregulated in oe:*PbcR*. In addition, orthologs of genes implicated in SreA-regulated iron metabolism in *Aspergillus fumigatus* [58] were upregulated in oe:*PbcR* (Table S3). It is tempting to speculate that the sexual phenotype seen in oe:*PbcR* is, at least in part, due to altered regulation of siderophore metabolism genes. Identifying specific genes involved in the altered morphogenesis is beyond the scope of this work given that *Aspergillus nidulans* could conceivably possess as many as 2000 genes that function in some aspect of morphogenesis and development [59].

Overexpression of *pbcR* led to the activation of a pimaradiene gene cluster in *Aspergillus nidulans* FGSC A4. There may be as many as 49 putative secondary metabolite clusters in *Aspergillus nidulans* [13], and we detected downregulation of four of them (penicillin gene cluster, two putative polyketide clusters, and one putative nonribosomal peptide cluster) in oe:*PbcR*. The downregulation of other clusters in the pimaradiene-producing strain might be a way for *Aspergillus nidulans* to ensure sufficient primary metabolites for cell growth, or facilitate the specific production of *ent*-pimara-8(14),15-diene. However, the mechanism for the downregulation of these clusters in oe:*PbcR* is not clear.

We report the first diterpene biosynthetic gene cluster in *Aspergillus nidulans*. Our results affirm the terpene producing ability of *Aspergillus nidulans*, and serve as a proof of principle in finding novel metabolites even in a microbe so widely studied. The results reported here highlight the advantage of using genomic mining in the search for novel biosynthetic pathways.

Materials and Methods

Bioinformatic Methods

Putative terpene synthase genes were identified by using InterPro [28] web portal search using domain identifiers IPR008949 'Terpenoid synthase' and IPR008930 'Terpenoid cyclase'. To find the potential terpene biosynthetic gene clusters with a positive regulator and characteristic genes for secondary metabolism, InterPro domains IPR001138 'Fungal transcriptional regulatory protein', IPR002403 'Cytochrome P450, E-class, group IV', and IPR001128 'Cytochrome P450' were searched for in 20 kb genomic area around terpene synthase genes.

Aspergillus Nidulans Strains and Growth

Aspergillus nidulans strain FGSC A4 (wild type, *veA*+) [60] from Fungal Genetics Stock Center was used in all transformations and experiments as wild type control. Overexpression strains oe:*AN1599*_9, oe:*AN1599*_42 (oe:*PbcR*), oe:*AN1599*_45, oe:*AN3250*_9 and oe:*AN3250*_11 were constructed as described below. Strains were grown in liquid YES-media (2% yeast extract, 4% sucrose) supplemented with 3% gelatin. Transformants were selected on Aspergillus minimal medium (MM) [61] with 200 μg/mL of glufosinate ammonium.

Construction of Plasmids

Genomic DNA was isolated from FGSC A4 mycelia disrupted with glass beads using standard phenol extraction and ethanol precipitation protocol [62]. DNA was further purified with Qiagen MiniPrep kit. Genomic sequences of AN1599.4 (GenBank: BN001307) and AN3250.4 (GenBank: BN001306) were cloned into the pCR2.1 TOPO (Invitrogen) with SpeI and SpeI and ApaI sites, respectively. Overexpression vector, pKB1, was constructed by adding glufosinate ammonium resistance gene *bar* from pTJK1 [63] into NotI site of modified pAN52-1NotI-vector [64]. *Bar* gene in pKB1 is fused with *Aspergillus nidulans trpC* promoter. AN1599.4 and AN3250.4 genomic sequences were cloned into their respective restriction sites in pKB1 fused with *Aspergillus nidulans gpdA* promoter. All constructs were analyzed by sequencing before transformations. Primers used in PCR are listed in Table S4.

Transformation

Protoplasting of *Aspergillus nidulans* FGSC A4 was carried out at 30°C in citrate buffer (0.8 M KCl, 0.05 M Na-citrate, pH 5.8) supplemented with 1mM DTT and 1% w/v Hydrolyzing enzymes from *Trichoderma harzianum* (Sigma). Protoplasts were collected by filtration and suspended in 180 μL of cold GTC buffer (1 M glucose, 50 mM $CaCl_2$, 10 mM Tris-HCl, pH 5.8). 20 μg of linearized expression plasmid DNA was added and volume adjusted to 200 uL. 50 μL of PEG-solution (25% PEG6000, 50 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.5) was

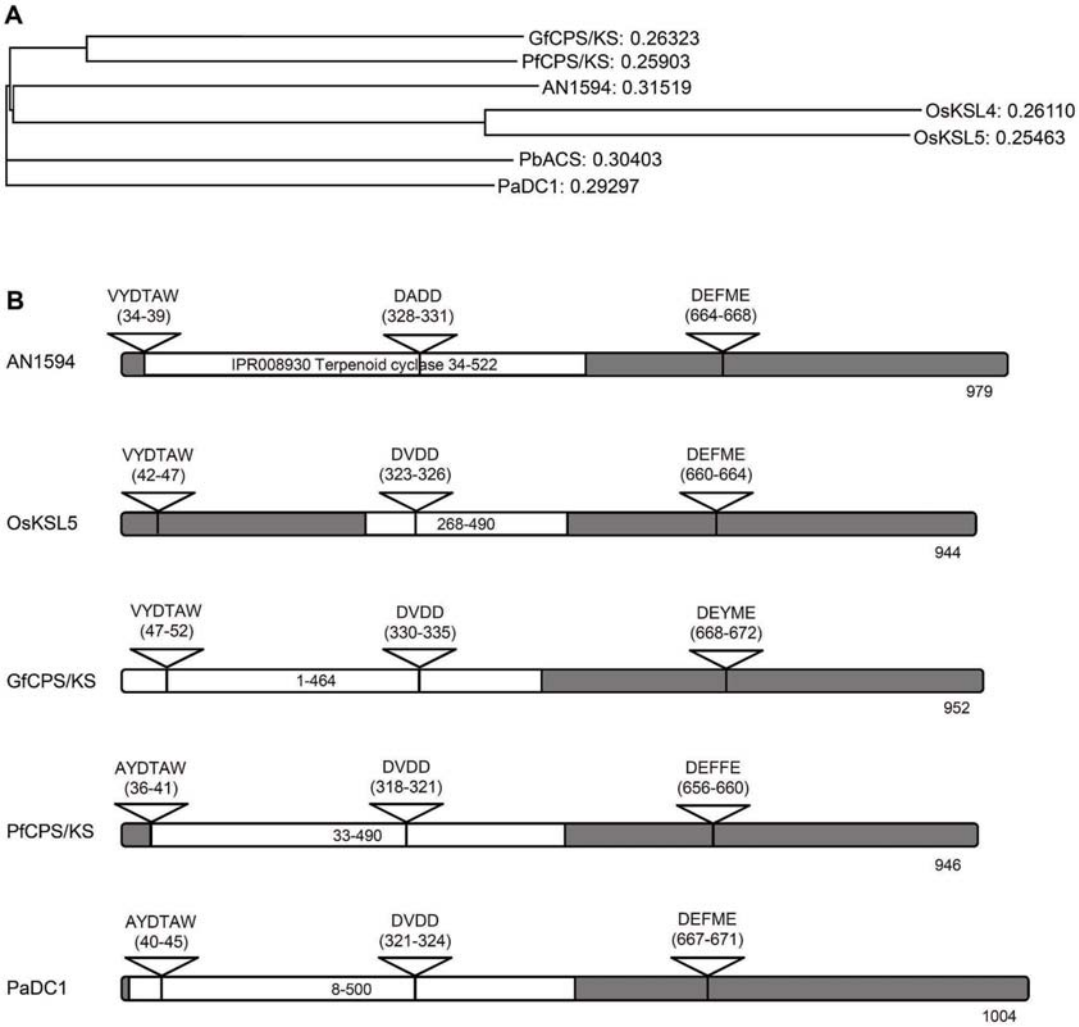

Figure 7. Phylogenetic and primary structure analysis suggests a bifunctional role for *Aspergillus nidulans* pimaradiene synthase (AN1594.4). A) A phylogenetic tree of diterpene synthases was generated by using ClustalW2. Alignment of *Aspergillus nidulans* pimaradiene synthase AN1594 (XP_659198.1) with *Gibberella fujikuroi ent*-kaurene synthase; GfCPS/KS (Q9UVY5.1), *Phaeosphaeria* sp. L487 *ent*-kaurene synthase; PfCPS/KS (O13284.1), *Phoma betae* aphidicolan-16β-ol synthase; PbACS (BAB62102.1), *Phomopsis amygdali* phylloclad-16α-ol synthase; PaDC1 (BAG_30961.1), *Oryza sativa ent*-pimara-8(14),15-diene synthase; OsKSL5 (NP_001047190.1), and *Oryza sativa syn*-pimara-7,15-diene synthase; OsKSL4 (NP_001052175.1). Phylogenetic tree indicates the similarity of AN1594 to fungal bifunctional diterpene synthases GfCPS/KS, PfCPS/KS, PbACS and PaDC1. AN1594 is also distantly related to known plant pimaradiene synthases OsKSL5 and OsKSL4. The phylogenetic distances are indicated next to the gene names. B) The primary structures of AN1594.4, PbACS, GfCPS/KS, PfCPS/KS and PaDC1 are shown. The inverted triangles indicate conserved motifs in fungal diterpene synthases. The AYDTAW motif is conserved among diterpene cyclases from plants and fungi. The DxDD and DExxE motifs are responsible for the type B cyclization (GGPP to copalyl diphosphate) and type A cyclization (copalyl diphosphate to diterpene), respectively. The InterPro domain IPR008930 "Terpenoid cyclase" is indicated with white bars. The total amino acid length of the proteins is indicated.
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added and the suspension incubated on ice for 20 minutes. 2 mL of PEG-solution was added, and the suspension incubated at room temperature for 5 minutes. Protoplasts were plated on selective MM plates in top agar and incubated at 30°C until transformed colonies were visible. Colonies were further grown on selective MM plates and positive colonies verified with PCR.

Quantitative Real-time PCR Analysis (qPCR)

FGSC A4 and transformant strains were grown in YES medium at 30°C for 42 hours. Due to different germination and/or growth rate of different strains, the conidia were inoculated in varying densities to achieve comparable growth of the cultures at the time of collection. Three individual

Table 1. Protein BLAST alignment of AN1594 shows similarity to known fungal diterpene synthases.

Terpene synthase	Accession	Score	Identities	Positives	Coverage
Aspergillus nidulans AN1594	XP_659198.1	2042	100%	100%	100%
Phomopsis amygdali phyllocladan-16α-ol synthase, PaDC1	BAG_30961.1	674	39%	55%	94%
Gibberella fujikuroi ent-kaurene synthase, GfCPS/KS	Q9UVY5.1	629	37%	57%	94%
Phoma betae aphidicolan-16β-ol synthase, PbACS	BAB62102.1	598	36%	53%	97%
Phaeosphaeria sp. L487 *ent*-kaurene synthase, PfCPS/KS	O13284.1	547	36%	53%	94%
Oryza sativa ent-pimara-8(14),15-diene synthase, OsKSL5	NP_001047190.1	63.9	28%	45%	25%
Oryza sativa syn-pimara-7,15-diene synthase, OsKSL4	NP_001052175.1	60.8	28%	45%	23%

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100 μL samples from each culture were collected and frozen in liquid nitrogen. Total RNA of the homogenized samples was extracted using Qiagen RNeasy Plant Mini Kit following manufacturer's suggestions for fungal RNA extraction. Extracted RNA was treated with DNaseI digestion (Qiagen) and quantified using Nanodrop (Thermo Scientific). cDNA synthesis was done with Transcriptor First Strand cDNA Synthesis Kit (Roche). DNA was analyzed by qPCR with LightCycler 480 SYBR Green I Master mix (Roche) on a LightCycler 480 (Roche). All samples were tested in three replicates. Expression levels were normalized to the levels of *β-actin* expression in each sample. Efficiencies for each primer set were calculated, and the expression fold ratios of transformant to FGSC A4 were quantified using pfaffl-equation [65]. Expression levels were checked in similar manner multiple times with consistent results. Primers are listed in Table S4.

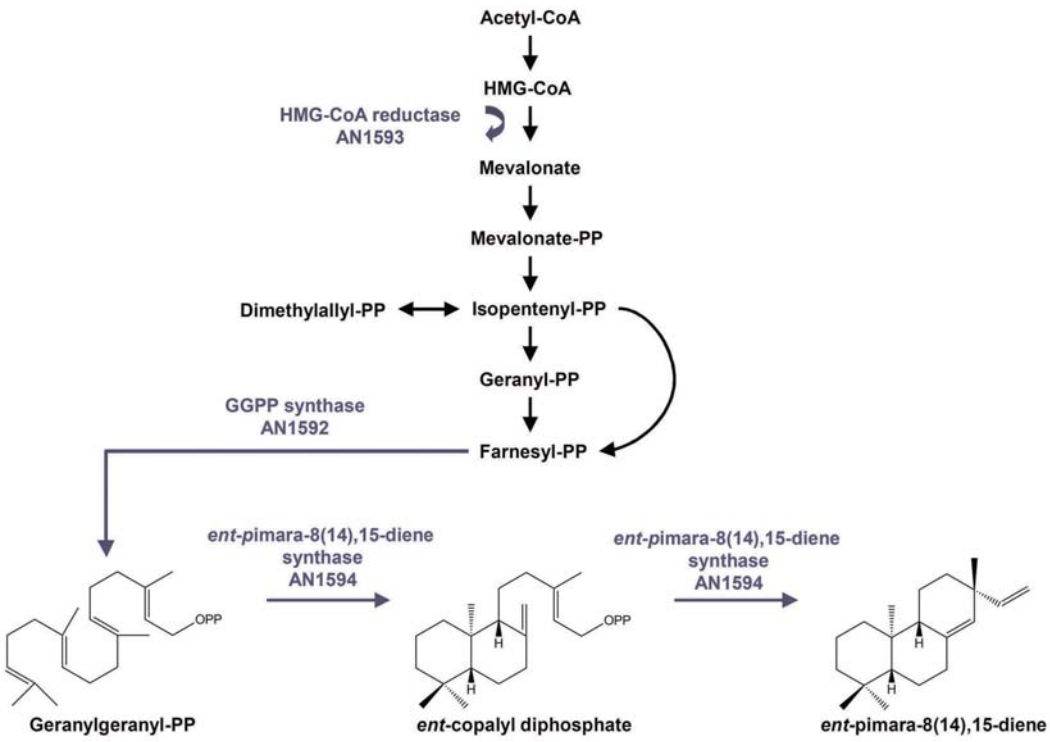

Figure 8. Proposed model of the *ent*-pimara-8(14),15-diene biosynthesis pathway in *Aspergillus nidulans*. PbcR activates key enzymes for pimaradiene biosynthesis. HMG-CoA reductase (AN1593.4) functions as a rate-limiting enzyme in the mevalonate pathway. GGPP-synthase (AN1592.4) provides geranylgeranyl diphosphate precursor for diterpene compounds. Pimaradiene synthase (AN1594.4) is proposed to catalyze two cyclization steps from GGPP to *ent*-pimara-8(14),15-diene via *ent*-copalyl diphosphate intermediate.
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Analysis of Diterpenes by Solid Phase Microextraction Gas Chromatography Mass Spectrometry (SPME-GC/MS) and GC/MS

Conidia of oe:*PbcR* and FGSC A4 were inoculated in varying densities and grown in YES-media at 30°C for 44 hours. SPME-GC/MS was done for cultures with comparable growth. 2 mL of the cultures were transferred into airtight SPME vials. Extraction of volatile and semi-volatile compounds was done at 80°C for 1 hour with preconditioned (250°C, 30 min) 100 µm PDMS fibre (Supelco, USA). Analytes were desorbed during 5 minutes at 250°C in the splitless injector (flow 14.9 mL/min) of the gas chromatograph (Agilent 6890 Series, USA) combined with an MS detector (Agilent 5973 Network MSD, USA) and SPME autosampler (Combipal, Varian Inc., USA). Analytes were separated on BPX5 capillary column of 60 m x 0.25 mm with a phase thickness 1.0 µm (SGE Analytical Science Pty Ltd, Australia). The temperature programme started at 40°C with 1 minute holding, then increased 9°C/min up to 130°C, followed by 2°C/min increase up to 230°C, where the temperature was kept for 1 minute. MSD was operated in electron-impact mode at 70 eV, in the full scan *m/z* 40–550. The ion source temperature was 230°C and the interface was 280°C. Compounds were identified by comparing the mass spectra on Palisade Complete 600 K Mass Spectral Library (Palisade Mass Spectrometry, USA).

For GC/MS analysis, hexane:ethyl acetate (1:1) extracts were prepared. FGSC A4 and oe:*PbcR* cells were homogenized with mortar and pestle in liquid nitrogen. 2 g of homogenized cells and 100 mL of growth media were ultrasonically extracted for 1 hour with 20 mL of hexane:ethyl acetate (1:1) 1µL of solvent phase, concentrated by evaporation, was analyzed by using GC/MS (Agilent 6890 Series, USA combined with Agilent, 5973 Network MSD, USA and Combipal injector, Varian Inc., USA). Analytes were injected on split mode (10:1) and separated on HP-1 capillary column (25 m x 0.2 mm) with a phase thickness 0.33 µm (Agilent, USA). Helium was used as carrier gas, 1.3 mL/min. The temperature program started at 100°C with 0.5 minute holding time, then increased 10°C/min up to 320°C where kept for 25 minutes. MSD was operated in electron-impact mode at 70 eV, in the full scan *m/z* 40–550. The ion source temperature was 230°C and the interface was 280°C. Compounds were indentified with the Palisade Complete 600K Mass spectral library (Palisade Mass Spectrometry, USA). Kovats retention index was determined in relation to a homologous series of n-alkanes (C8–C24) as standards.

DNA Array Expression Analysis

FGSC A4 and oe:*PbcR* were inoculated in different densities in 50 mL of YES medium. The pH values of each culture were monitored during growth and the mycelia harvested from cultures with pH values ranging from 5.76 to 5.94 indicating the early exponential growth phase of the fungal strains. FGSC A4 was grown for 22 hours and oe:*PbcR* for 26 hours at 37°C. Three RNA extractions were made from two separate culture flasks for both WT and oe:*PbcR*. The quality of RNA was assessed with the standard protocol of Agilent 2100 Bioanalyzer (Agilent Technologies). DNA array chip was designed and manufactured by NimbleGen Systems Inc., Madison, WI USA, using Custom Eukaryotic 12×135K Array format. Sequences for the 10597 transcripts in the DNA array design were downloaded from the Central Aspergillus Data Repository, CADRE [66] via FTP server at Ensembl Genomes browser (ftp://ftp.ensemblgenomes.org/pub/fungi/release4/fasta/aspergillus_nidulans/cdna/Aspergillus_nidulans.CADRE2.4.cdna.all.fa.gz). Expression portion was designed by selecting 6 probes per transcript for 10546 out of 10597 transcripts. Each probe had a replicate for a final expression analysis for total of 126,260 probes. cDNA synthesis of total RNA, probe hybridization, scan and preliminary analysis was performed by NimbleGen Systems Inc., Madison, WI USA, following their standard operating protocol. Normalized DNA array data was further analyzed using the ArrayStar (DNASTAR) software. Expression fold changes were calculated with unpaired, two-tailed, equal variance student's t-test with 99% significance level, p-value ≤ 0.01. All data are MIAME compliant and the raw data has been deposited in GEO (Accession # GSE32954).

Conidia Quantification

FGSC A4 and oe:*PbcR* were grown on potato dextrose (PD) plates at 37°C for three days. Three agar plugs were isolated from three PD-plates with a stainless-steel tube with inner diameter of 60 mm (surface area of three plugs is approximately 85 mm^2). Each plug was homogenized in 500 uL ddH20. The conidial suspension was diluted 1:10 and spores counted with hemocytometer. The statistical analysis was done with GraphPad InStat using unpaired student's t-test (p-value <0.0001, $n = 9$).

Microscopy

FGSC A4 and oe:*PbcR* were grown on PD plates for three days at 37°C. Stereomicroscope images of untreated samples were taken using Zeiss SteREO DiscoveryV8 microscope equipped with Olympus Soft Imaging Systems DP-25 camera using 8 X magnification. For higher magnification images, conidia were suspended in 20% glycerol and spread to cover slips. Images were taken using Olympus 1X81 microscope equipped with QImaging Retiga-2000R camera. All image visualizations were performed with Olympus Cell P software.

Supporting Information

Figure S1 PCR analysis shows the presence of overexpression constructs in *pbcR* (AN1599.4) transformants. *Aspergillus nidulans* FGSC A4 was transformed to carry a genomic copy of *pbcR* (AN1599.4) with *Aspergillus nidulans gpdA* promoter. Genomic DNA of FGSC A4 and the overexpression strains (oe:*AN1599*_9, oe:*AN1599*_42 and oe:*AN1599*_45) was purified and the integration of the construct was verified by PCR amplification of a 540 base-pair fragment.
(TIF)

Figure S2 Chromosomal locations of the secondary metabolite synthases from this study. The chromosomal location of *Aspergillus nidulans* pimaradiene synthase (AN1594) is shown in red. The chromosomal locations of nonribosomal peptide synthases (AN3495 and AN3496), polyketide synthases (AN2032, AN2035 and AN0523) and isopenicillin A synthetase (*ipnA*, AN2622) downregulated in oe:*PbcR* are shown in blue. Putative diterpene synthase AN3252 is shown in black.
(TIF)

Figure S3 *Aspergillus nidulans* pimaradiene cluster gene orthologs (AN1592.4 to AN1599.4) are found in *Neosartorya fischeri*. All eight pimaradiene cluster genes in *Aspergillus nidulans* have orthologous genes clustered in *Neosartorya fischeri*. Figure is adapted from Aspergillus Genome Database [19] using ortholog cluster search.
(TIF)

Table S1 Genes with over 5-fold upregulation in oe:*PbcR* compared to FGSC A4 (p-value ≤ 0.01).
(DOCX)

Table S2 Genes with over 5-fold downregulation in oe:*PbcR* compared to FGSC A4 (p-value ≤ 0.01).
(DOCX)

Table S3 Genes implicated in iron metabolism.
(DOCX)

Table S4 Primers used in this study.
(DOCX)

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Author Contributions

Conceived and designed the experiments: KB MT LR TN-S. Performed the experiments: KB KV AV. Analyzed the data: KB KV AV. Contributed reagents/materials/analysis tools: KB KV AV. Wrote the paper: KB MT KV AV. Critically revisited the intellectual content of the article: MT LR TN-S. Final approval of the version to be published: LR TN-S. Supervision of the research group: LR. Acquisition of funding: TN-S.

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Publication II

Isolation and purification of *ent*-pimara-8(14),15-diene from engineered *Aspergillus nidulans* by accelerated solvent extraction combined with HPLC

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Isolation and purification of *ent*-pimara-8(14),15-diene from engineered *Aspergillus nidulans* by accelerated solvent extraction combined with HPLC†

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We recently engineered *Aspergillus nidulans* to produce a pimarane-type diterpene, *ent*-pimara-8(14),15-diene. Here, we describe methods for its isolation and purification from the engineered *A. nidulans* production strain and extend these findings with structural confirmation for the diterpene from this fungal source. The extraction protocol was optimized using accelerated solvent extraction (ASE) with three varying parameters: solvent composition, pressure, and temperature. This ASE method using 100% ethyl acetate at 90 °C with 12.07 MPa was more efficient and faster compared to ultrasonic-assisted extraction. Using both analytical and preparative C18 columns at isocratic elution of acetonitrile developed an HPLC separation; and, the diterpene was well separated within 28 min and 35 min, respectively. Also, TLC of the fungal culture extracts was performed with visualization using rhodamine. The reproduction and efficiency of the purification methods were evaluated by GC-MS; and, the structure of the compound was verified by NMR. The method enabled the isolation of this very lipophilic diterpene in pure form, allowing its testing in a number of bioactivity assays. Notably, we demonstrate for the first time the antioxidant activity for *ent*-pimara-8(14),15-diene using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay.

Introduction

Aspergillus nidulans is a filamentous fungus widely used as a model organism for studies of cell biology and gene regulation. It is a close relative to other *Aspergillus* species with industrial and medical significance – *e.g.*, *A. niger*, *A. oryzae*, *A. flavus*, and *A. fumigatus*. The genus *Aspergillus* produces a variety of natural products, and offers a model system to study secondary metabolism in eukaryotes.[1] Secondary metabolites are microbial organic compounds that are not crucial for the survival of the organism but many times exhibit interesting or useful bioactivities. These are also referred to as natural products. Major groups of secondary metabolites include polyketides, ribosomal and non-ribosomal peptides, and terpenoids. In fungi, the biosynthetic genes of secondary metabolism are organized into clusters.[2] The clustering of the genes along with the sequenced genomes of many *Aspergillus* species has accelerated the discovery of novel natural products.[3] Of the numerous secondary metabolites produced by *Aspergilli*, the most notorious ones include mycotoxins, such as *A. flavus* aflatoxin[4,5] and *A. carbonarius* ochratoxin.[6,7] On the other hand, many of the *Aspergillus* natural products also exhibit beneficial properties and include the antibiotic penicillin[8] and the cholesterol reducing agent lovastatin.[9] Although the functional role of secondary metabolites in the producing organism is often unclear, it has been speculated that secondary metabolites function as chemical signals in communication and defense to enhance the survival of the organism within its ecological environment.[10]

Terpenoids are particularly interesting secondary metabolites because of their many pharmaceutical and toxic bioactivities.[11–13] Specifically, oxygenated derivatives of pimaradiene and kaurene have vasorelaxant, anti-inflammatory, anti-microbial and trypanocidal properties.[14–19] Biosynthesis of fungal natural products is regulated by environmental as well as genetic factors.[8] Recently, we reported that overexpression of a $Zn(II)_2Cys_6$-type transcription factor in *A. nidulans*, called pimaradiene biosynthetic cluster regulator (*pbcR*), led to production of a diterpene hydrocarbon, *ent*-pimara-8(14),15-diene.[20] *ent*-Pimara-8(14),15-diene has previously been identified as a product of the rice (*Oryza sativa* subspecies *japonica*) pimaradiene synthase, OsKSL5j.[21] Many of the diterpene hydrocarbons in rice, for example, *ent*-sandaracopimaradiene, *ent*-cassa-12,15-diene, 9β*H*-pimara-7,15-diene, and stemar-13-ene groups function as pathway intermediates for diterpene

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phytoalexins, which have been suggested to act as antibiotics.[22] Also, *ent*-kaur-16-ene is a precursor of a diterpene phytohormone gibberellic acid that regulates many aspects of development in higher plants. However, the biological activity for *ent*-pimara-8(14),15-diene in rice is still unknown, since OsKSL5 is not involved in the biosynthesis of diterpenoid phytoalexins or gibberellins.[23]

In addition to higher plants, diterpene hydrocarbons have also been found in fungi.[20,24–26] For example, sandaracopimaradiene, isopimara-8,15-diene and pimara-8(14),15-diene have previously been identified among other terpene hydrocarbons in crude extracts of the fungus *Phomopsis amygdali*.[25] Also, many pimarane-type diterpene hydrocarbons are found in the fungus *Phoma betae*, where 8β-pimara-9(11),15-diene has been proposed to be an intermediate in a biosynthetic pathway of aphidicolin, a specific inhibitor of DNA polymerase α.[26] The identification of different diterpene hydrocarbons is many times based on the analysis of terpene synthase products of *in vitro* enzyme assays;[27–29] and, the few reports on the identification of fungal diterpenes do not report bioactivity assay data for these compounds.

Several techniques are used for isolation of oxygenated terpenoids from crude plant and fungal extracts, such as silica gel chromatography, reverse phase chromatography, vacuum liquid chromatography, flash chromatography, preparative thin-layer chromatography, capillary electrophoresis, and recrystallization with methanol.[11,30–32] These methods are most suitable for terpenoids with functional groups, which add hydrophilic properties to the compound and provide UV-absorbent groups. *ent*-Pimara-8(14),15-diene is a very lipophilic compound, and it does not have any functional groups or bonds that have UV absorbance. The compound is not detectable directly by UV fluorescence. In this report we describe the methods for isolation and purification of *ent*-pimara-8(14),15-diene from *A. nidulans* by using ASE combined with reverse phase HPLC. Compared to ultrasonic extraction, ASE was more efficient and was further optimized for *A. nidulans* mycelia. GC-MS was used to analyze the efficiency of the extraction methods and the HPLC separation. We also describe a TLC separation and rhodamine staining detection method, as well as structural confirmation for *A. nidulans ent*-pimara-8(14),15-diene by NMR.

The purification methods enabled us to study the activity of pure *ent*-pimara-8(14),15-diene in a number of bioactivity assays. In DPPH radical scavenging assays, purified *ent*-pimara-8(14),15-diene had higher activity than the known tetraterpene antioxidant, beta-carotene, demonstrating for the first time the antioxidant activity of *ent*-pimara-8(14),15-diene. With our production strain and purification method larger scale production of *ent*-pimara-8(14),15-diene is possible.

Experimental

Aspergillus nidulans strains and culture media

A. nidulans FGSC A4 (Glasgow wild type, veA+)[33] was obtained from the Fungal Genetics Stock Center (Kansas City, Missouri, USA) and used as a wild type control strain. *ent*-Pimara-8(14),15-diene producing strain, oe:*PbcR*, has been described earlier.[20] Briefly, the genomic sequence of the $Zn(II)_2Cys_6$-type transcription factor PbcR (AN1599.4, GenBank:BN001307) was cloned into a fungal expression vector pKB1, where the expression of *pbcR* is under the *A. nidulans gpdA* promoter. 20 mg of linearized expression plasmid DNA was transformed into *A. nidulans* FGSC A4 by protoplasting, and the positive colonies were selected on minimal medium agar plates with 200 mg mL^{-1} of glufosinate ammonium. Strains were grown in liquid YES-media (2% yeast extract, 4% sucrose) supplemented with 3% gelatin at 30 °C with 250 rpm.

Extraction

Extraction conditions of the sonication method have been described earlier.[20] Filtered and freeze-dried *A. nidulans* mycelia were homogenized with a Retsch MM301 Ball Mill at 29 Hz for 30–60 s. The homogenized powder was mixed with diatomaceous earth in ASE sample cells (Dionex) and extracted with ASE 200 (Dionex) using hexane : ethyl acetate (Hex : EtOAc) (1 : 1), EtOAc, or acetonitrile (ACN). Extraction conditions were as follows: pressure, 12.07 MPa or 15.51 MPa; temperature, 70 °C, 80 °C, or 90 °C; preheat time, 1 min; heat time, 5 min; static time, 10 min; flush volume, 30%; and purge time, 90 seconds. When assessing the efficiency of the sonication and ASE methods, one batch of mycelia was extracted three times with EtOAc.

Thin layer chromatography

An aliquot (200 μL) of the Hex : EtOAc (1 : 1) crude extract of FGSC A4 (wild type) and oe:*PbcR* (pimaradiene producing strain) was applied to silica plates (Silica Gel 60, Pre-coated TLC Plates, Merck, Germany) and purified by thin-layer chromatography. The separation was done with EtOAc : heptane : acetic acid (9 : 90 : 1), and the different compound groups were visualized under UV light after spraying the silica plates with 0.01% rhodamine 6B GO (Merck, Germany). The areas with different compounds were marked with a pencil, and silica was scraped and extracted with Hex : EtOAc (1 : 1). The purity of obtained fractions was analyzed by GC-MS.

Gas chromatography-mass spectrometry

GC-MS analysis has been previously described by Bromann *et al.* (2012).[20] The separations were performed using an Agilent 6890 Series GC combined with a 5973 Network MS detector (Agilent, USA) and a Combipal injector (Varian Inc., USA). Samples were dissolved in EtOAc, injected in split mode (10 : 1) and separated on a 25 m × 0.2 mm × 0.33 μm HP-1 capillary column (Agilent, USA) with a helium flow rate of 1.3 mL min^{-1}. The ion source temperature was 230 °C, the interface was 280 °C, and the oven temperature was equilibrated at 100 °C for 30 seconds and then increased to 320 °C at a rate of 10 °C min^{-1} and held at that temperature for 25 minutes. The MS detector was operated in EI mode at 70 eV with a mass range of 40–550. Compounds were identified with the Palisade Complete 600K Mass spectral library (Palisade Mass Spectrometry, USA).

High-performance liquid chromatography

A. nidulans oe:*PbcR* cell extracts were evaporated to dryness and resuspended in HPLC grade ACN. Analytical HPLC was

performed using an XTerra MS C18 column (250 × 4.6 mm; 3.5 μm; Waters) with a Waters 616 pump with a Waters 600 S system controller equipped with a Waters in-line degasser AF, a Waters 717plus autosampler, a Waters 2996 photodiode array detector (200–600 nm), and Waters Empower Pro chromatography software for data processing (Milford, MA). Samples were eluted with a flow rate of 0.5 mL min^{-1}. The injected volume of the crude extract per run was 150 μL and the total analysis time was set to 60 min. Preparative scale purification was done using a Waters XTerra Prep MS C18 column (300 × 10 mm; 10 μm; Waters) with a 2 mL sample loop and a 2.5 mL syringe (Waters). The injected volume was 2 mL of the crude extract and the flow rate was set to 2.5 mL min^{-1}. The total analysis time was set to 60 min. The elution of the different fractions was monitored at 225 nm, and the collected fractions were evaporated to dryness under N_2 flow at 30 °C. Fractions were dissolved in a small volume of EtOAc and the purity was analyzed by GC-MS.

DPPH radical scavenging assay

DPPH radical scavenging assay was performed according to Kähkönen *et al.*[34] with slight modifications. Methanolic 0.1 mM DPPH radical solution was mixed with *ent*-pimara-8(14),15-diene or beta-carotene solution. The concentrations of pure compounds were either 1 mM, 2.5 mM or 5 mM. The absorption at 517 nm was monitored up to 120 min using a UV-1800 UV-Vis spectrophotometer (Shimadzu). The results were calculated as the percentage of radicals scavenged at four different time-points: 20, 60, 90, and 120 min.

IR, NMR, HRMS experimental

The IR spectrum was obtained using a Vertex 70 (Bruker Optics Inc., MA, USA) FTIR instrument. The FTIR measurements were made directly in solids with a horizontal Attenuated Total Reflectance (ATR) accessory (MIRacle, Pike Technology, Inc, WI, USA). The transmittance spectrum was recorded at a 4 cm^{-1} resolution between 4000 and 600 cm^{-1} using the OPUS 5.5 software (Bruker Optics Inc., MA, USA). ESI-MS was performed by direct injection using a Synapt G2 HDMS (Waters, MA, USA) instrument. NMR spectra were obtained using a Varian Mercury Plus 300 spectrometer, in $CDCl_3$ with tetramethylsilane (TMS) as the internal standard. The chemical shifts were reported in parts per million (ppm) and on the δ scale from TMS as an internal standard. The coupling constants J are quoted in hertz (Hz).

***ent*-Pimara-8(14),15-diene (1).** IR (ATR) 3085, 2925, 2895, 1456, 1365, 912, 856 cm^{-1}. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 0.80 (s, 3H, 20-CH_3), 0.85 (s, 3H), 0.88 (s, 3H), 1.01 (m, 1H, 5-H); 1.04 (s, 3H, 17-CH_3), 1.70 (m, 2H), 2.05 (m, 1H, 7β-H), 2.26 (m, 1H, 7α-H), 4.88 (dd, 1H, $J_1 = 1.5$ Hz and $J_2 = 10.6$ Hz, 16-H_b), 4.91 (dd, 1H, $J_1 = 1.5$ Hz and $J_2 = 17.5$ Hz, 16-H_a), 5.21 (s, 1H, 14-H), 5.78 (dd, 1H, $J_1 = 10.6$ Hz and $J_2 = 17.5$ Hz, 15-H). ^{13}C-NMR (75 MHz, $CDCl_3$) δ 14.9 (C20), 18.7, 19, 22 (C19), 22.5, 25.9 (C17), 33.2 (C4), 33.7 (C18), 34.6, 36 (C7), 37.3 (C13), 38.2 (C10), 39.3, 42.1, 50.6 (C9), 54.8 (C5), 109.8 (C16), 128.4 (C14), 137.2 (C8), 149.1 (C15). HMRS calc. for $C_{20}H_{32}$ 273.2582 $[M + 1]^+$, found 273.2578.

Results and discussion

Previously, we reported the identification of the transcription factor, PbcR, in *A. nidulans*. In the strain overexpressing *pbcR* (oe:*PbcR*), transcription of seven genes within a cryptic secondary metabolite gene cluster is activated; and, this results in the production of a pimarane-type diterpene that we identified as *ent*-pimara-8(14),15-diene.[20]

Comparison of extraction methods

In the current study, oe:*PbcR* mycelia were collected at the exponential growth phase and then freeze-dried and homogenized. To compare the previously reported sonication extraction with ASE, we performed three serial extractions of the same batch of mycelia using two methods. In GC-MS analysis of three serial sonication extractions of manually ground mycelia with Hex : EtOAc (1 : 1), 48% of *ent*-pimara-8(14),15-diene was recovered in the first extraction, 19% in the second extraction, and 33% in the third extraction (Fig. 1A). In contrast, ASE efficiently recovered 100% of *ent*-pimara-8(14),15-diene using one extraction (Fig. 1B). Thus, automated homogenization of freeze-dried mycelia and subsequent ASE proved to be superior for the recovery of *ent*-pimara-8(14),15-diene. An additional benefit in

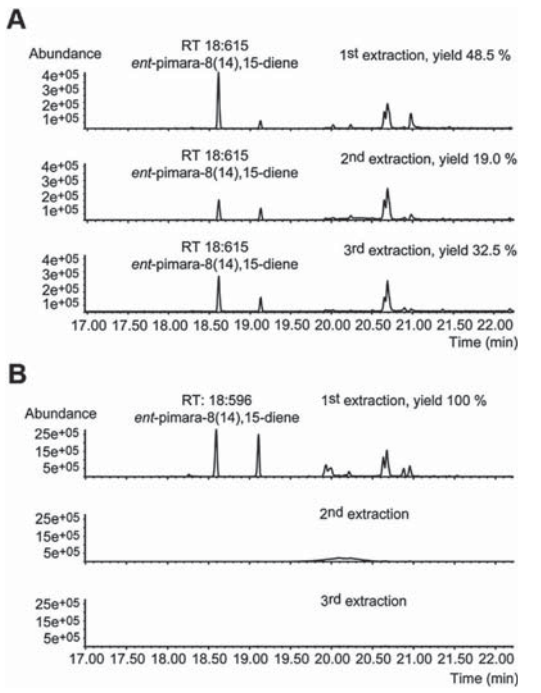

Fig. 1 Comparison of relative yields of *ent*-pimara-8(14),15-diene from two different extraction techniques. The sonication extraction (A) recovers only 48.5% of *ent*-pimara-8(14),15-diene in the first extraction, whereas ASE (B) efficiently recovers 100% of the *ent*-pimara-8(14),15-diene from *A. nidulans* mycelia in the first extraction.

using ASE is that weighing of the freeze-dried sample also makes it possible to quantitate the extraction yield and method efficiency.

The ASE method was optimized for the powdered sample by using different solvent compositions, temperatures, and pressures. The recovery of *ent*-pimara-8(14),15-diene was monitored by analyzing GC-MS peak areas. We tested three different solvents: Hex : EtOAc (1 : 1), 100% EtOAc, and ACN. The highest relative recovery was marked as 100% extraction efficiency and other peak areas were calculated as a percentage of this value. The recovery of *ent*-pimara-8(14),15-diene from the fungal mycelia was highest by using 100% EtOAc (Fig. 2A). Hex : EtOAc was nearly as efficient (92%) in extracting *ent*-pimara-8(14),15-diene as EtOAc, whereas ACN could extract only 52% of the *ent*-pimara-8(14),15-diene compared to EtOAc (Fig. 2A).

The extraction method was further optimized with EtOAc by testing at two different pressures: 12.07 MPa and 15.51 MPa. Recovery of *ent*-pimara-8(14),15-diene was efficient at 12.07 MPa, and increasing pressure slightly lowered the yield (data not shown), suggesting that pressure did not exert a great influence on extraction efficiency. Extraction with EtOAc at three different temperatures (70 °C, 80 °C, and 90 °C), with 12.07 MPa pressure, demonstrated that the highest recovery was achieved at 90 °C (Fig. 2B). In a previous study, Kawamura *et al.*[35] reported that increasing temperature resulted in higher recovery of paclitaxel from bark, although pressure was less important for recovery. In their study, the temperature range used for paclitaxel was 100–150 °C, so it is possible that we could have achieved higher recovery of *ent*-pimara-8(14),15-diene using temperatures over 100 °C. However, to prevent the possible degradation of the compound, we did not test the extraction at temperatures above 90 °C. Taken together, our data suggest optimal extraction conditions for *ent*-pimara-8(14),15-diene from mycelia of oe:*PbcR*.

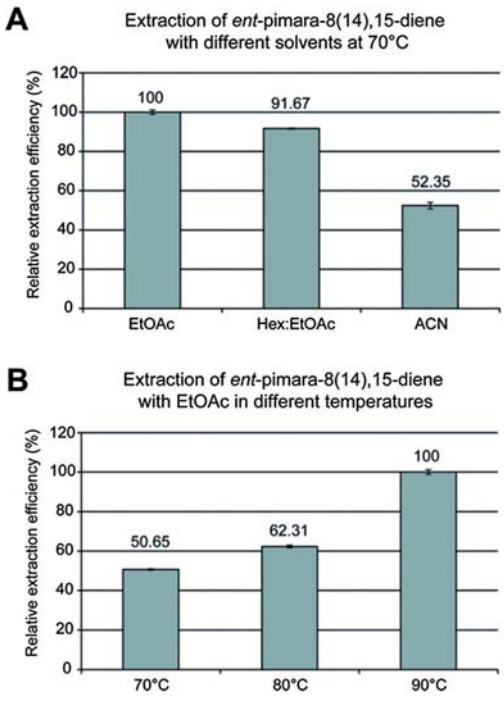

Fig. 2 Influence of different solvents (A) and temperatures (B) on recovery of *ent*-pimara-8(14),15-diene from oe:*PbcR* mycelia. The error bars represent the standard error of the mean (SEM, $n = 3$).

Purification of *ent*-pimara-8(14),15-diene with TLC and HPLC

In order to isolate *ent*-pimara-8(14),15-diene from crude extracts of oe:*PbcR*, a TLC method was developed. Mycelia from wild-type *A. nidulans* (FGSC A4) and oe:*PbcR* were extracted, and the extracts were separated on silica plates. *ent*-Pimara-8(14),15-diene was visualized by rhodamine staining of the developed silica plates (Fig. 3A), since the compound was not detectable directly by UV fluorescence. *ent*-Pimara-8(14),15-diene was separated by using EtOAc : heptane : acetic acid (9 : 90 : 1). Using this eluent composition, the compound moved along the solvent front (Fig. 3A, lane 1). *ent*-Pimara-8(14),15-diene was then extracted from the silica, and the purity of the compound was analyzed by GC-MS (Fig. 3B and C). Many fatty acids and other unknown interfering compounds that were present in the crude extract (Fig. 3B) were not detected in the TLC purified fractions (Fig. 3C). However, the TLC-purified compound still contained some non-polar alkanes that interfered with identification by NMR analysis (Fig. 3C).

To further purify *ent*-pimara-8(14),15-diene, we developed an analytical scale HPLC method. In HPLC, both the interactions between the purified compound and the stationary phase as well as the solvent play a role in the separation process. Specifically, *ent*-pimara-8(14),15-diene was purified from crude extracts of oe:*PbcR* by reversed phase HPLC. The purification method was tested by using three isocratic eluent compositions of 85%, 90%, and 100% ACN; and efficient separation was achieved with 100% ACN in one hour. Low wavelengths (190 nm, 210 nm, 225 nm, and 250 nm) were used to monitor fractionation, because this diterpene hydrocarbon does not have any functional groups or bonds that have UV absorbance; and, the peak corresponding to *ent*-pimara-8(14),15-diene was not detectable at wavelengths 250 nm and greater. To identify the compound peak, eluent fractions were collected in six 10 minute intervals. *ent*-Pimara-8(14),15-diene was detected in the third interval (20–30 minutes), and the individual peaks were then collected and analyzed by GC-MS. The UV absorption maximum for the peak corresponding to *ent*-pimara-8(14),15-diene was 222.1 nm (Fig. 4A), and monitoring of the following purifications was done at 225 nm.

In the analytical scale HPLC, *ent*-pimara-8(14),15-diene was eluted at 25 to 28 minutes after the sample injection (Fig. 4B, upper panel). Preparative scale HPLC was performed in order to accommodate larger injection volumes. The chromatograms of preparative scale and analytical scale HPLC were similar (Fig. 4B), and the total analysis time was kept at 60 minutes. In the preparative scale HPLC, the *ent*-pimara-8(14),15-diene peak

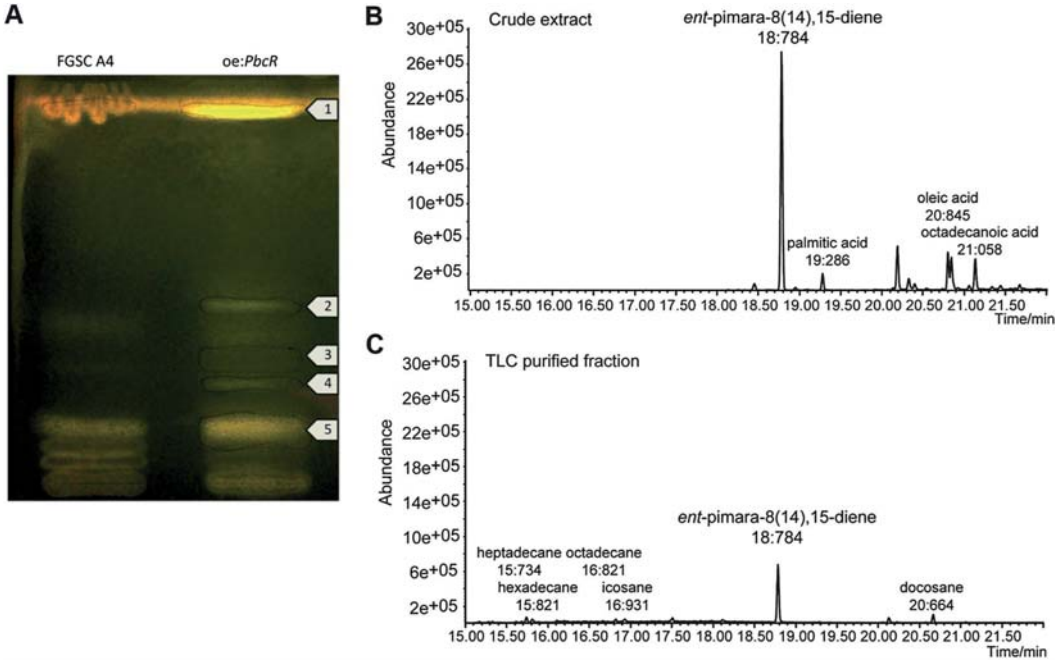

Fig. 3 (A) TLC of crude Hex:EtOAc extracts of *A. nidulans* FGSC A4 (wild-type) and oe:*PbcR*. Separation was done using EtOAc : heptane : acetic acid (9 : 90 : 1), and the developed silica plate was visualized with rhodamine staining. Compounds separated with TLC in oe:*PbcR* were (1) *ent*-pimara-8(14),15-diene fraction, (2) organic acids, fatty acids, (3) organic acids, sugars, (4) aromatic carboxylic acids, fatty acids and alkanes, (5) aromatic carboxylic acids and fatty acids. (B) GC-MS analysis of the oe:*PbcR* crude extract and (C) TLC purified fraction showed that some higher alkanes were still present in the purified fraction. Fatty acids and major impurities can be separated from the crude extracts of oe:*PbcR* with TLC.

was detected at 30 to 35 minutes after injection (Fig. 4B, lower panel). The shoulder seen at the preparative scale HPLC peak was not collected. Isolated *ent*-pimara-8(14),15-diene fractions were combined, evaporated to dryness, and dissolved in EtOAc prior to GC-MS analysis. Comparison of the GC-MS peak areas for the crude extract and for the HPLC purified *ent*-pimara-8(14),15-diene (Fig. 4C) indicated that there were no impurities in the collected fraction; and, the recovery of the diterpene was consistently over 90% (Fig. 4C, small panel). After multiple rounds of preparative scale HPLC, 25 mg of pure compound was collected.

Bioactivity testing of *ent*-pimara-8(14),15-diene

With its isolation and purification, we could now test *ent*-pimara-8(14),15-diene in a number of bioactivity assays. In *Aspergilli*, the production of secondary metabolites has been linked to oxidative stress.[36,37] For example, lovastatin biosynthesis in *A. terreus*, and aflatoxin biosyntheses in *A. parasiticus* and *A. flavus* respond to accumulation of reactive oxygen species (ROS) in these fungi.[38,39] Because oxidative stress has also been shown to activate secondary metabolite biosynthesis in *A. nidulans*, we tested the anti-oxidant activity of pure *ent*-pimara-8(14),15-diene hydrocarbon in a DPPH radical scavenging assay with three different concentrations and four time-points. We compared its activity to a known tetraterpene antioxidant, beta-carotene. Pure *ent*-pimara-8(14),15-diene had significantly higher DPPH radical scavenging activity than beta-carotene in all test conditions (Fig. 5).

Although the function of this compound in *A. nidulans* is not known, the data suggest that one potential role of *ent*-pimara-8(14),15-diene may be to function as an antioxidant. The antioxidant activity of *ent*-pimara-8(14),15-diene could be one way for *A. nidulans* to protect itself against stressful situations where accumulation of free radicals inside the cells occurs. We also tested purified *ent*-pimara-8(14),15-diene for antimicrobial activity against *Staphylococcus aureus*, and for inhibition of axenic amastigotes of *Leishmania donovani*. However, even relatively high concentrations of the compound (50 μM) were ineffective in both of these tests (data not shown). Additional bioactivity and antioxidant assays will give a more complete picture of the potential bioactivity of the compound, and this will be addressed in future studies.

Structural analysis

To verify the structure of HPLC-purified *ent*-pimara-8(14),15-diene, infra-red (IR), MS, and 1D and 2D NMR experiments were performed (Table 1). Inspection of the ^{1}H NMR spectrum

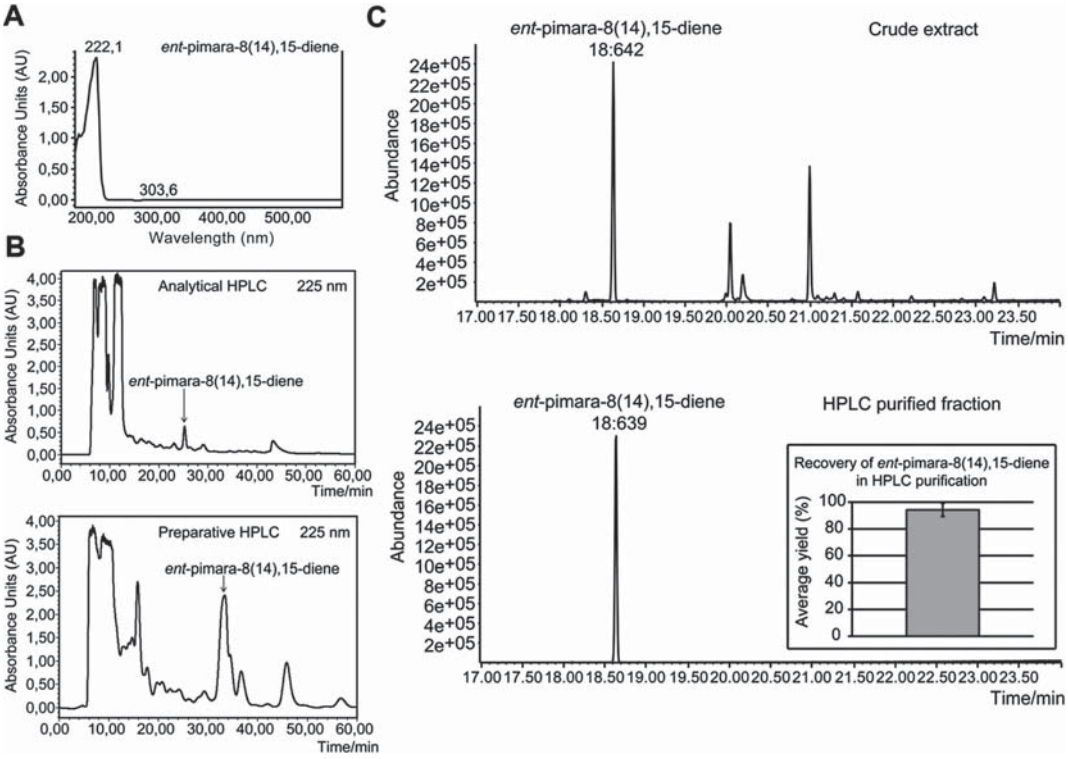

Fig. 4 (A) UV absorption maximum for *ent*-pimara-8(14),15-diene. (B) Chromatograms for analytical (upper panel) and preparative (lower panel) HPLC purification for *ent*-pimara-8(14),15-diene. The peak corresponding to *ent*-pimara-8(14),15-diene eluted at 25 to 28 minutes in analytical scale HPLC and at 30 to 35 minutes in the preparative scale HPLC. Shoulder seen at the preparative HPLC peak was not collected. (C) Gas chromatograms for the crude extract of *A. nidulans* oe:*PbcR* (upper panel) and the HPLC purified *ent*-pimara-8(14),15-diene fraction (lower panel). The yield of HPLC purified *ent*-pimara-8(14),15-diene from the crude extracts was over 90% (lower panel, small figure, error bar represents the SEM, $n = 9$).

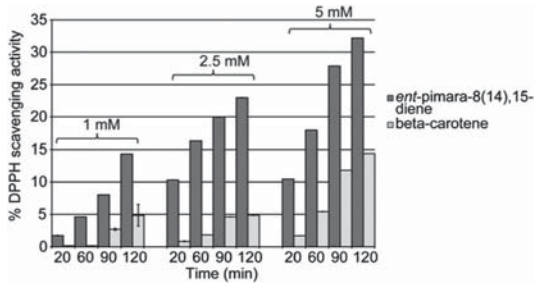

Fig. 5 DPPH radical scavenging activities of *ent*-pimara-8(14),15-diene (dark gray) and beta-carotene (light gray). The concentrations of the pure compounds were 1 mM, 2.5 mM and 5 mM in the DPPH assay with time points 20, 60, 90 and 120 minutes. *ent*-Pimara-8(14),15-diene had higher antioxidant activity than beta-carotene. The error bars represent the SEM ($n = 3$).

Table 1 Selected NMR spectroscopic data for *ent*-pimara-8(14),15-diene

Position	δ_C, type	δ_H (J in Hz)
4	33.2, C	
5	54.8, CH	1.01, m
7α	36, CH_2	2.26, m
7β		2.05, m
8	137.2, C	
9	50.6, CH	1.70, m
10	38.2, C	
13	37.3, C	
14	128.4, CH	5.21, s
15	149.1, CH	5.78, dd (10.6, 17.5)
16a	109.8, CH_2	4.91, dd (1.5, 17.5)
16b		4.88, dd (1.5, 10.6)
17	25.9, CH_3	1.04, s
20	14.9, CH_3	0.80, s

showed the four methyl group signals as singlets at 0.80, 0.85, 0.88, and 1.04 ppm. The downfield double doublet at 5.78 ppm was assigned to 15-H whereas the double doublets at 4.88 and 4.91 ppm were assigned to 16-H_b and 16-H_a, respectively. Thus, the signal at 4.88 ppm displayed the cis vicinal coupling constant of 10.6 Hz between 16-H_b and 15-H and the geminal

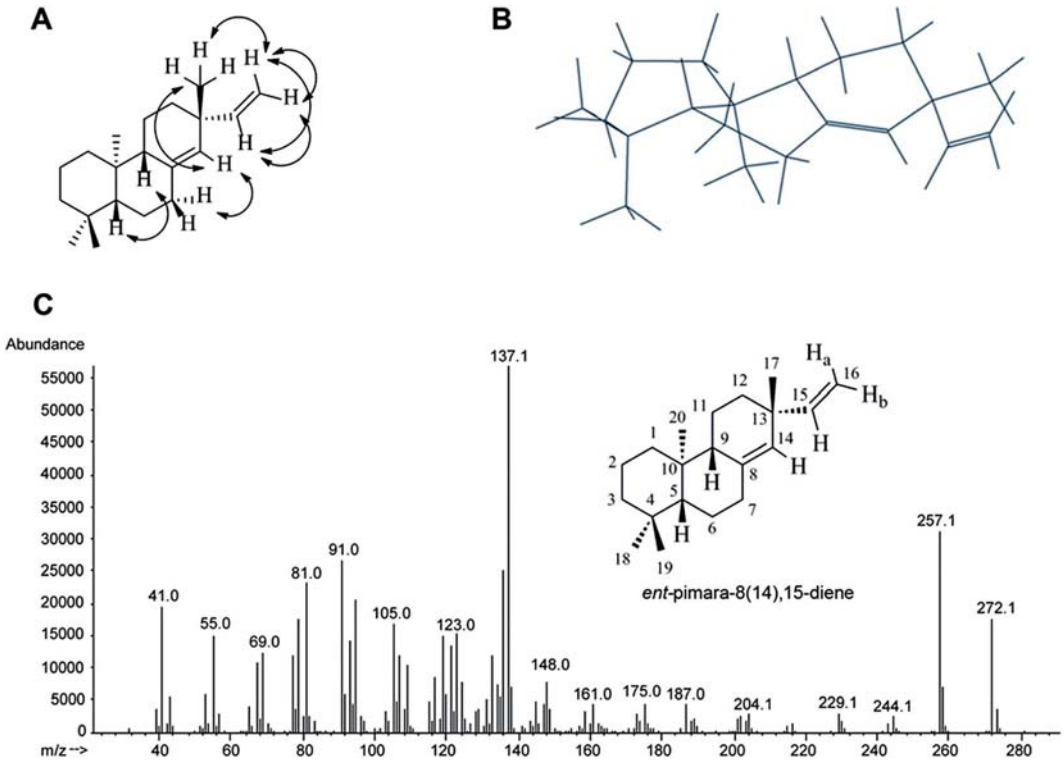

Fig. 6 (A) Selected NOESY correlations and (B) a 3D representation of *ent*-pimara-8(14),15-diene. (C) Structure and the mass spectrum of pure *ent*-pimara-8(14),15-diene.

coupling constant of 1.5 Hz. The *trans* vicinal coupling constant for 16-H_a and 15-H was found to be 17.5 Hz.

The COSY spectrum displayed correlations between the neighboring 15-H, 16-H_a, 16-H_b proton signals. The assignment of C15 (149.1), C8 (137.2), C14 (128.4), and C16 (109.8) was made by observation of their respective correlations on the HSQC experiment. The signal at 1.70 ppm displayed correlation to the signal at 50.6 ppm, belonging to a CH carbon, and was assigned to 9-H.[40] The remaining CH/CH_3 signals to assign correlated to the methyl group protons and to 5-H (C5 at 54.8 ppm). Assignment of the 17-methyl group to the signal at 1.04 ppm was based on the HMBC correlations with the signals of C15, C14, and the quaternary carbon C13 at 37 ppm. The remaining methyl group signals all displayed HMBC correlations to C5. The signal at 0.80 ppm was assigned to the 20-methyl protons based on the HMBC correlations to C9 and quaternary C10 (38.2 ppm). The signals at 0.85 and 0.88 ppm, corresponding to the 19 and 18-methyl protons, both displayed HMBC correlations to quaternary C4 (33.2 ppm). The stereochemistry of *ent*-pimara-8(14),15-diene was verified by the NOESY experiment. Selected NOESY correlations and a 3-D presentation of *ent*-pimara-8(14),15-diene are depicted in Fig. 6A and B, respectively. The axial position of the 13-methyl group and β-orientation for 5-H and 9-H was confirmed by the observed NOESY cross peaks of 14-H/17-CH_3, 14-H/7α-H, and 16a-H/17-CH_3, and is consistent with those found in the literature.[41] Cross-peaks were also observed for the signals of 9-H and 5-H. The assignment of C17 to the signal at 25.9 ppm is also consistent with the axial position of this methyl group, as opposed to the reported values of about 30 ppm for equatorial orientation.[42] These data confirm the structure and stereochemistry of *ent*-pimara-8(14),15-diene isolated from *A. nidulans* strain oe:*PbcR*, and are consistent with our earlier report.[20] The structure and the mass spectrum of pure *ent*-pimara-8(14),15-diene are shown in Fig. 6C. NMR spectra are presented in the ESI (Fig. 1S–6S).†

Conclusions

We developed isolation and purification methods for a lipophilic tricyclic diterpene, *ent*-pimara-8(14),15-diene, produced in fungi. The optimized ASE and HPLC methods developed here were very reproducible and could be scaled up to accommodate larger sample volumes. Biotechnological production of this particular diterpene in *A. nidulans* and the purification methods described here should enable production of large quantities of pure *ent*-pimara-8(14),15-diene. This is necessary for more systematic bioactivity screening of this compound. Finally, the

methods described are relevant for purification of other diterpenes with similar properties.

Conflict of interest

The authors declare no conflict of interest.

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Publication III

Engineering *Aspergillus nidulans* for *ent*-kaurene and gamma-terpinene production

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Title	**Production of terpenes in *Aspergillus nidulans*** Engineering of the secondary metabolite cluster
Author(s)	Kirsi Bromann
Abstract	Secondary metabolites, or natural products, are produced by a variety of microorganisms and plants but are particularly abundant in filamentous fungi. Secondary metabolites can be toxic or have pharmaceutical applications as antibiotics, anticancer, antiparasitic and antifungal agents. Terpenes are a large group of secondary metabolites with wide array of bioactivities. Filamentous fungus, *Aspergillus nidulans*, is a fungal model organism and a close relative to other industrially relevant *Aspergillus* species. *A. nidulans* is a known producer of many bioactive metabolites, including polyketides, nonribosomal peptides and sesquiterpenes. Sequencing of *A. nidulans* genome has revealed numerous secondary metabolite gene clusters, yet products of many of these biosynthetic pathways are unknown since the expression of the clustered genes usually remains silent in laboratory conditions. Finding ways to induce the gene expression of silent biosynthetic clusters can lead to discovery of novel metabolites with unknown bioactivities. Furthermore, identifying key biosynthetic genes in interesting metabolite pathways is a prerequisite for genetic engineering of heterologous production systems. In this study, we wanted to explore the terpene producing ability of *A. nidulans*. We discovered a novel diterpene, *ent*-pimara-8(14),15-diene (PD), in *A. nidulans* by predicting a biosynthetic gene cluster with genomic mining. We identified a Zn(II)2Cys6–type transcription factor, PbcR, and demonstrated its role as a positive regulator for the predicted gene cluster. The transcriptional profile of the pbcR overexpression strain (oe:*PbcR*) was analyzed by Real-Time Quantitative Reverse Transcription PCR (qRT-PCR) and DNA microarray. Overexpression of *pbcR* led to upregulation of the predicted biosynthetic cluster genes and subsequent PD production in *A. nidulans*. In addition to PD cluster genes, transcriptional changes were observed for a number of secondary metabolite genes in oe:*PbcR*. Microscopy of the plate cultures revealed changes in the oe:*PbcR* morphology. To study the bioactivity of PD, we developed and optimized a PD extraction method for the engineered *A. nidulans* production strain. An accelerated solvent extraction (ASE) with a combined High-Performance-Liquid-Chromatography (HPLC) purification enabled us to collect highly pure PD for the Nuclear Magnetic Resonance (NMR) analysis and bioactivity testing. While PD did not exhibit inhibitory function against *Staphylococcus aureus* or *Leishmania donovani*, we observed an antioxidant activity for PD in a radical scavenging assay. We also characterized *A. nidulans* PD synthase (PbcA) with overexpression and deletion assays and showed that it is indispensable for PD production in *A. nidulans*. Furthermore, we extended our genetic engineering to study the potential of *A. nidulans* as a heterologous host for mono- and diterpene production. Overexpression of *Fusarium fujikuroi ent*-kaurene synthase (*cps/ks*) and *Citrus unshiu* gamma-terpinene synthase resulted in *ent*-kaurene and gamma-terpinene production, respectively. The results reported in this thesis highlight the advantage of using genomic mining in the search for novel biosynthetic pathways. Additionally, this thesis presents a proof-of-concept study for using *A. nidulans* as heterologous terpene production host.
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Nimeke	**Diterpeenien tuotto *Aspergillus nidulansissa*** Sekundaarimetaboliageeniklusterin muokkaus
Tekijä(t)	Kirsi Bromann
Tiivistelmä	Useat mikro-organismit ja kasvit tuottavat sekundaarimetaboliitteja, jotka ovat toiselta nimeltään luonnonaineita. Nämä yhdisteet ovat erityisen yleisiä filamenttihomeissa. Sekundaarimetaboliitit voivat olla myrkyllisiä, tai niillä voi olla farmaseuttisia sovelluksia antibiootteina, syöpälääkkeinä tai loisten ja sienten vastaisina yhdisteinä. Terpeenit ovat suuri bioaktiivisten sekundaarimetaboliittien ryhmä. Filamenttihome, *Aspergillus nidulans*, on sienten malliorganismi ja läheisesti sukua muille teollisesti tärkeille *Aspergillus*-lajeille. *A. nidulans* tunnetaan monien bioaktiivisten metaboliittien, kuten polyketidien, nonribosomaalisten peptidien, sekä seskviterpeenien, tuottajana. *A. nidulans*in genomin sekvensointi on paljastanut useita sekundaarimetaboliageeniklustereita. Suurin osa näistä biosynteettisten ketjujen tuotteista on kuitenkin tunnistamatta, sillä klustereiden geenien ilmentymistä ei yleensä tapahdu laboratorio-olosuhteissa. Biosynteettisten klustereiden geenien ekspression indusointi voi johtaa uusien bioaktiivisten yhdisteiden löytymiseen. Tämän lisäksi heterologisten tuottosysteemien geneettisen muokkaamisen perustana on identifioida mielenkiintoisten yhdisteiden metaboliareittien tärkeimmät biosynteettiset geenit. Tässä työssä halusimme tutkia *A. nidulans*in terpeenien tuottokykyä. Löysimme uuden diterpeenin, *ent*-pimara-8(14),15-dieenin (PD), paikallistamalla kyseisen yhdisteen biosynteettisen geeniklusterin genomin haravoinnilla. Identifioimme Zn(II)2Cys6-tyypin ilmentymistekijän, PbcR:n, ja osoitimme sen roolin oletetun geeniklusterin positiivisena säätelijänä. PbcR:n yliekspressiokannan (oe:*PbcR*) ilmentymisprofiili analysoitiin reaaliaikaisella kvantitatiivisella käänteistranskriptiopolymeraasi-ketjureaktiolla (Real-Time Quantitative Reverse Transcription PCR, qRT-PCR) ja DNA-mikroarraylla (DNA microarray). *PbcR*:n yliekspressio *A. nidulans*issa johti ennakoitujen klusterigeenien yliekspressioon ja PD:n tuottoon. PD-klusterin geenien lisäksi oe:*PbcR*-kannassa havaittiin transkriptionaalisia muutoksia useiden sekundaarimetaboliittigeenien ilmentymisessä. Maljakasvatusten mikroskopointi paljasti rakenteellisia muutoksia oe:*PbcR*-kannassa. Kehitimme ja optimoimme PD:n uuton muokatulle *A. nidulans* -kannalle PD:n bioaktiivisuuden tutkimiseksi. Nopeutettu liotinuutto (ASE) yhdistettynä korkean erotuskyvyn nestekromatografiaan (HPLC) mahdollisti erittäin puhtaan PD:n keräämisen ydinmagneettiseen resonanssispektrometria-analyysiin (NMR) sekä bioaktiivisuustestaukseen. Vaikka PD ei inhiboinut *Staphylococcus aureus*ta tai *Leishmania donovani*a, vapaiden radikaalien pyydystämiskokeessa PD:lle havaittiin antioksidatiivinen aktiivisuus. Lisäksi karakterisoimme PD-syntaasigeenin (PbcA) yliekspressio- ja deleetiokokeilla ja osoitimme sen olevan korvaamaton *A. nidulans*in PD-tuotannossa. Lisäksi tutkimme *A. nidulans*in potentiaalia heterologisena isäntänä mono- ja diterpeenien tuotossa. *Fusarium fujikuroi*n *ent*-kaureenisyntaasin (*cps/ks*) ja *Citrus unshiu*n gamma-terpineenisyntaasin yliekspressio johti *ent*-kaureenin ja gamma-terpineenin tuottumiseen. Tässä väitöskirjassa esitetyt tulokset korostavat genomikartoituksen hyödyllisyyttä uusien biosynteesireittien etsinnässä. Tämän lisäksi väitöskirjatyö toimii "proof-of-concept"-tutkimuksena *A. nidulans*in käytöstä heterologisena tuottoisäntänä terpeeneille.
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