Alles Wissen und alles Vermehren unseres Wissens endet nicht mit einem Schlußpunkt, sondern mit einem Fragezeichen.

Hermann Hesse (1877-1962)
Produktion und Identifikation phyto- und cytotoxischer Metabolite des opportunistisch humanpathogenen Pilzes *Aspergillus terreus*

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1. Einleitung

1.1 *Aspergillus terreus* als opportunistisch humanpathogener Pilz


Die Diagnosemöglichkeiten und die Behandlungsmethoden bei Infektion mit *A. terreus* sind momentan unzureichend, da (i) die Diagnose meist erst spät erfolgt (Paholcsek *et al*., 2014) und (ii) der Pilz eine erhöhte Resistenz gegenüber gängigen Antimykotika wie Amphotericin B aufweist (Lass-Flörl *et al*., 2005; Walsh *et al*., 2008). Ein Infektionsmodell für *A. terreus* wurde in Zusammenarbeit

Abbildung 1. Differenzierung in Aspergillus terreus. Der Stamm SBUG844 wurde für 48 Stunden in Apfelsaft angezogen, um die Differenzierung von verschiedenen Zellmorphologien zu induzieren. (A) Substratmycel; (B) Konidiophor mit endständigen phialidischen Konidien; (C) Konidiophor mit phialidischen Konidien am Substratmycel; (D) akzessorische Konidien (Aleuriokonidien); (E) Sporensuspension aus phialidischen Konidien (schwarze Pfeile) und akzessorischen Konidien (weiße Pfeile). Der Skalierungs balken entspricht 10 µm.

1.2 Aspergillus terreus als Sekundärmetabolit-Produzent


Das sequenzierte Genom von Aspergillus terreus Stamm NIH2624 (FGSC A1156) codiert für mindestens 64 anotierte Sekundärmetabolit-Gencluster (Tabelle 1 im Anhang). Dabei scheint das metabolische Potential weit über die bisher bekannten Metabolite von A. terreus hinauszugehen. Zu Beginn dieser Arbeit konnten lediglich drei der Gencluster eindeutig einem SM zugeordnet werden (Gencluster für Methylsalicylsäure, Lovastatin, Atrochrysin) (Fujii et al., 1996; Hendrickson et al., 1999; Awakawa et al.; 2009). Im Verlauf der Arbeit konnten weitere Biosynthesen von SM aufgeklärt

### 1.3 Die Biosynthese von Sekundär metaboliten


#### 1.3.1 Polyketide

Polyketide werden aus Acetat-Einheiten durch Polyketidsynthesen (PKS) – ähnlich wie in den Fettsäuresynthasen (FAS) in höheren Eukaryonten – aufgebaut. Fungale PKS des Typ I sind iterativ arbeitende Multidomänenenzyme, d.h. alle für die Biosynthese eines Polyketids notwendigen enzymatischen Funktionen befinden sich auf einem Polypeptid. Während der Biosynthese wird das Polyketid durch Malonyl-CoA unter Decarboxylierung um je eine C2-Einheit verlängert, wobei die enzymatischen Domänen wiederholt (iterativ) genutzt werden.

Ein minimales PKS-Modul besteht aus drei Domänen: Die Acyltransferase-Domäne (AT) lädt neue Malonyl-Einheiten von Malonyl-CoA auf das Protein (Abb. 2A). Anschließend werden durch die β-Ketosynthase-Domäne (KS) die C2-Einheiten (unter Freisetzung von CO₂) auf die nascierende Polyketidkette am Acylcarrierprotein (ACP) durch Thio-Claisen-Kondensation übertragen. Die Abspaltung des finalen Produktes vom Enzym erfolgt entweder über Hydrolyse durch eine Thioesterase-domäne (TE) in nicht-reduzierenden PKS I (NR-PKS-I) oder über durch Reduktion an einer Reduktionsdomäne (R) in NR-PKS-II. Beiden Typen von NR-PKS sind weiterhin zwei Domänen gemeinsam: Die Starter-Acyl-Transferase (SAT, am N-Terminus) bestimmt die Art der Acyl-Einheit, auf dem die nascierende Polyketidkette aufgebaut wird (Crawford *et al.*, 2006), während die Produkt-Template-Domäne (PT, mittig im Protein) für die Anzahl der Iterationen und die Faltung (Cyclisierung)
1. Einleitung


1.3.2 Nicht-ribosomale Peptide

Abbildung 2. Schematische Darstellung der Domänenstruktur von SM-Schlüsselenzymen.

Die Polyketidsynthasen (PKS, grün; A), nicht-ribosomalen Peptidsynthetasen (NRPS, blau; B) und PKS-NRPS-Hybride (grün und blau; C) sind mit charakteristischen Primärprodukten dargestellt. NR, nicht-reduzierend; PR,
partiell reduzierend; HR; hochreduzierend. SAT, Startereinheit-Acyl-Transferase; KS, β-Ketoacylsynthase; AT, Acyltransferase; PT, Produkttemplate-Domäne; ACP, Acylcarrierprotein; TE, Thioesterase; MT, Methyltransferase; R, Reduktionsdomäne; TH, interne Thioesterhydrolase; KR, β-Ketoreduktase; DH, Dehydratase; ER, Enoylreduktase; A, Aktivierungsdomäne; T, Thiolierungsdomäne (Peptidylcarrierprotein); C, Kondensationsdomäne; D, Dieckmann-Kondensationsdomäne. ER<sub>0</sub>, ER nicht aktiv.

* Die PKS13 nutzt ein reduziertes Polyketid als Startereinheit.

### 1.3.3 Polyketid-Aminosäure-Hybride


1.4 Beispiele fungaler Sekundärmetaboliten mit diskreter Funktion


1.4.1 Siderophore und ihre Rolle für den Eisenstoffwechsel


Die SB wurde vor allem in Aspergillus nidulans und dem opportunistisch humanpathogenen Pilz Aspergillus fumigatus sehr gut untersucht (Haas, 2014; Abb. 3). In A. fumigatus kann zwischen extrazellulären (Triacetyl-fusarinin C, TAF; Fusarinin C), Hyphen- (Ferricrocin, FC) und Konidien-Siderophoren (hydroxyliertes Ferricrocin, HFC) unterschieden werden. Während A. nidulans ebenfalls TAF als extrazelluläres Siderophor produziert (Eisendle et al., 2003), sind die Hydroxamat-Siderophore Coprogen B bzw. Coprogen und Ferrichrysin für Aspergillus niger und A. terreus beschrieben (Franken et al., 2014; Bertrand et al., 2010). Der Biosynthese von TAF und FC geht eine
1. Einleitung

Hydroxylierung von L-Ornithin an \( N^\delta \) durch die L-Ornithin-\( N^\delta \)-Monooxygenase SidA voraus, um die Hydroxamat-Grundstruktur auszubilden (Eisendle et al., 2003; Hissen et al., 2005). Anschließend wird das entstandene \( N^\delta \)-Hydroxy-L-ornithin im Falle der TAF-C-Biosynthese mit Anhydromevalonyl-CoA durch die Transacetylase SidF acyliert (Schrettl et al., 2007). Anhydromevalonyl-CoA entstammt dem Mevalonatstoffwechsel der Peroxisomen und zeigt somit eine Verknüpfung der Isoprenoid- und der Siderophorbiosynthese (Yasmin et al., 2012; Haas et al., 2014). Die NRPS SidD verbindet drei Moleküle \( N^\delta \)-cis-Anhydromevalonyl-\( N^\delta \)-hydroxy-L-ornithin über Esterbrücken zu Fusarinin C, welches anschließend durch SidG zum finalen Chelator TAF-C transacyliert wird und an die Umgebung abgegeben wird (Schrettl et al., 2007). Fusarinin C kann seine Funktion als 6-zähniger Chelator bereits erfüllen, sodass eine Deletion von sidG das Wachstum von A. fumigatus nicht beeinträchtigt (Schrettl et al., 2007). Extrazelluläres TAF-C oder alternative Siderophore können anschließend Eisen(III)-Ionen binden und in ihrer Ferri-Form über Siderophortransporter (SIT) wie MirB wieder in die Zelle transportieren (Raymond-Bouchard et al., 2012). Eisen(III)-Ionen werden anschließend durch Reduktion zu Eisen(II)-Ionen oder Abbau des Siderophors über Esterasen wie EstB vom Chelator wieder freigesetzt (Kragl et al., 2007). Intrazellulär können Eisen(III)-Ionen einerseits in Vakuolen andererseits auch durch Siderophore wie FC gespeichert werden (Eisendle et al., 2006; Wallner et al., 2009).

Für die Biosynthese von FC ist ebenfalls die Bildung von \( N^\delta \)-Hydroxy-L-ornithin durch SidA essentiell. \( N^\delta \)-Hydroxy-L-ornithin wird anschließend acetyliert (u.a. durch die N-Acetyltransferase SidL) (Blatter et al., 2011). Drei Moleküle \( N^\delta \)-acetyl-\( N^\delta \)-hydroxy-L-ornithin, zwei Moleküle L-Glycin und ein Molekül L-Serin werden über Peptidbindungen durch die NRPS SidC zu FC verknüpft, welches Eisen(III)-Ionen im Cytosol nicht nur speichert, sondern auch intrazellulär transportieren kann (Schrettl et al., 2007; Wallner et al., 2009). In Sporen findet man zur Speicherung eine hydroxilierte Variante von FC in A. fumigatus, aber nicht in A. nidulans (Schrettl et al., 2007; Haas et al., 2008).

Eine Deletion von sidA verhindert somit sowohl die extra- als auch intrazelluläre SB: Eine \( \Delta sidA \)-Mutante kann weder unter Eisenmangel auskeimen noch eine invasive Aspergillose in immunsupprimierten Mäusen (Cortisonacetat-Modell) auslösen (Hissen et al., 2005). Dies lässt vermuten, dass (i) unter Infektionsbedingungen Eisenmangel herrscht und (ii) die SB essentiell für die Etablierung einer Infektion ist. Siderophore sind nicht nur bei Pilzinfektionen, sondern auch bei bakteriellen Infektionen notwendig für die vollständige Virulenz (Takase et al., 2000). Die Siderophor-Aufnahme stellt damit einen wichtigen Angriffspunkt für Antibiotika dar: Siderophor-gekoppelte Antibiotika werden leichter durch Pathogene wie Pseudomonas spec. aufgenommen und erlauben daher einen gerichteten Transport des Antibiotikums zum Wirkungsort (Möllmann et al., 2009).

Obwohl die SB in *A. terreus* noch nicht im Detail untersucht ist, codiert das Genom viele orthologe Proteine der SB. Das Fehlen eines *sidG*-Orthologs weist darauf hin, dass *A. terreus* kein TAFC als extrazelluläres Siderophor produziert. Stattdessen konnte aus dem Überstand von *A. terreus* Ferrichrysin, Coprogen und – in geringeren Mengen – dessen Vorläufer Dimeruminsäure isoliert werden (Zähner et al., 1963; Bertrand et al., 2010). Das Genom von *A. niger* codiert ebenso kein *sidG*-Ortholog – auch hier lässt sich nicht TAFC, sondern Coprogen B als extrazelluläres und Ferrichrom als intra- und extrazelluläres Siderophor nachweisen (Franken et al., 2014). Das Genom von *A. terreus* weist außerdem eine Vielzahl von putativen SITs auf, was darauf hindeutet, dass *A. terreus* ein funktionales Siderophoraufnahmesystem besitzt.


*A. fumigatus* kann neben dem Siderophor-vermittelten Eisentransport auch Eisen(III)-Ionen durch ein hoch-affines, reductives Eisenassimilationssystem (reductive iron assimilation, RIA) aufnehmen: Dabei werden Eisen(III)-Ionen zunächst durch die membrandgebundene Eisen(III)-Reduktase FreB zu Eisen(II)-Ionen extrazellulär reduziert (Blatzer et al., 2011). Anschließend werden die Eisen(II)-Ionen über einen membrangebundenen Proteinkomplex (aus der Eisen(II)-Oxidase FetC und der Eisen-Permease FtrA) wieder oxidiert und dabei in die Zelle transportiert (Schrettl et al., 2004) (Abb.3). Orthologe RIA-Gene lassen sich im Genom von *A. terreus* ebenfalls nachweisen. Die
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Siderophor-vermittelte Eisenaufnahme dominiert gegenüber dem reduktiven Aufnahmesystem: Defekte im RIA werden erst im Hintergrund einer sida-Deletion phänotypisch sichtbar (Schrettl et al., 2004; Blatzer et al., 2011).

1.4.2 Naphthopyron-basierende Melanine und ihr Einfluss auf die Virulenz

Auffälligste Eigenschaft phialidischer Konidien von A. fumigatus ist die Pigmentierung, die ein wichtiges morphologisches Unterscheidungskriterium innerhalb der Aspergillen darstellt: Während A. fumigatus rauchgraue Konidien ausbildet, sind Konidien von A. nidulans grün und von A. niger schwarz gefärbt. Die farbgebenden Melanine wurden auch für viele weitere Pilze beschrieben. Sie dienen vor allem der Resistenz der Sporen gegen oxidativen oder UV-Stress (Pihet et al., 2009; Henson et al., 1999). Darüber hinaus sind Melanine in vielen Pflanzen-Pathogenen integraler Bestandteil der Appressorien und damit an der effizienten Penetration ins Pflanzengewebe beteiligt (Howard and Ferrari, 1990; Ludwig et al., 2014). Melanine sind makromolekulare Pigmente, die ein vermutlich ungeordnetes Polymer aus kleinen Vorläufermolekülen darstellen. In A. fumigatus und A. nidulans werden die Vorläufermoleküle durch die beiden NR-PKS PksP/Alb1 bzw. WA synthetisiert (Fujii et al., 2001; Tsai et al., 2001; Fujii et al., 2004). In A. fumigatus wird das Primärprodukt, das Naphthopyron YWA1, durch die Hydrolyase Ayg1 zu 1,3,6,8-Tetrahydroxynaphthalen (THN) verkürzt (Fujii et al., 2004). THN wird anschließend durch verschiedene „Tailoring Enzymes“ (Arp2, Arp1) zu 1,8-Dihydroxynaphthalen (DHN) umgesetzt, welches vermutlich durch die Laccase Abr2 oxidativ zu DHN-Melanin polymerisiert wird (Heinekamp et al., 2012).

1. Einleitung


1.5 Induktion und Regulation der Sekundärmetabolit-Produktion
in filamentösen Pilzen

Die Regulation der SM-Biosynthese in filamentösen Pilzen ist sehr komplex und oft hierarchisch geordnet, da die energieaufwendige Synthese nur bei Bedarf stattfinden soll. Daher werden auch viele SM-Gene unter Standard-Laborbedingungen nicht exprimiert (silent gene clusters) (Tominaga et al., 2006; Bergmann et al., 2007). Es bedarf diskreter Bedingungen, um die Genexpression zu induzieren. Dazu gehören:

1. Änderung der Kultivierungsbedingungen wie die Verfügbarkeit von Stickstoffquellen (Ehrlich et al., 2002) und Kohlenstoffquellen (Bracarense et al., 2014) sowie die Änderung von pH-Wert (Bizukojc et al., 2012), Temperatur (Medina et al., 2014), Kultivierungsduauer (Kang et al., 2013), Sauerstoffeintrag (Vödisch et al., 2011) und der Wechsel zwischen Submers- oder Standkultur (Jørgensen et al., 2011)

2. Co-Kultivierung mit anderen Mikroorganismen (Schroeckh et al., 2009; König et al., 2013; Ross et al., 2014)

3. Zugabe von Agenzien, die die Chromatin-Struktur verändern (Williams et al., 2008; Bok et al., 2009; Nützmann et al., 2011)
4. Überproduktion eines spezifischen Transkriptionsfaktors eines Genclusters (Bergmann et al., 2007; Ahuja et al., 2012)

5. Überproduktion oder Deletion eines globalen Transkriptionsregulators oder -repressors (Mihlan et al., 2003; Bok et al., 2004)

1.5.1 Änderung der Kultivierungsbedingungen

Das Anschalten der SM-Gene ist der Schlüssel zur erfolgreichen Produktion von SM. Die systematische Variation der Kultivierungsbedingungen (OSMAC-Ansatz, one strain many compounds) wird auch heute noch zum einfachen und kostengünstigen Screenen nach induzierenden Bedingungen verwendet (Wei et al., 2010; Hewage et al., 2014). Allerdings lassen sich im Labor nicht alle natürlichen Kultivierungsbedingungen nachstellen. „Stille Gencluster“ bedürfen komplexerer Induktionsbedingungen und lassen sich meist nicht über den OSMAC-Ansatz aktivieren.

1.5.2 Co-Kultivierung mit anderen Mikroorganismen


1.5.3 Epigenetische Modifikatoren

Die Induktion der Orsellinsäure-Biosynthese in A. nidulans erfolgt über verstärkte Acetylierung an Histon H3 und der damit verbundenen Auflockerung der Chromatinstruktur am Gencluster (Nützmann et al., 2011). In Übereinstimmung dazu führte auch die Deletion der Histonomethyltransferase (HMT) CcIA zu einer Genclusteraktivierung (Bok et al., 2009). Die Fütterung mit epigenetischen Modifikatoren wie Inhibitoren der HMT, Histon-Acetyltransferasen (HAT), Histondeacetylasen (HDAC) und DNA-Methyltransferasen (DNMT) stellt damit eine weitere Möglichkeit der Genclusteraktivierung dar (Shwab et al., 2007; Williams et al., 2008).
1.5.4 Überproduktion eines gencluster-spezifischen Transkriptionsfaktors


1.5.5 Überproduktion oder Deletion eines globalen Transkriptionsregulators


Unter Berücksichtigung all dieser Faktoren zeichnet sich ein äußerst komplexes Bild in der Regulation der SM ab. Es bedarf verschiedener teils kombinierter molekularbiologischer Ansätze, um fungale SM-Gencluster zu induzieren.

1.6 Heterologe Expressionssysteme in filamentösen Pilzen

oder NRPS, die in *E. coli* überproduziert werden, müssen *in vitro* aufgereinigt und mit Phosphopantethenylintransferasen (PPTasen) aktiviert werden (Owen *et al.*, 2012). Da Aspergillen eigene PKS-spezifische PPTasen besitzen, können „neue“ Metabolite direkt aus den Kulturen extrahiert werden. Damit sind Aspergillen das Mittel der Wahl zur heterologen Expression von SM-Genen.


Konzentrationen an DES (1 µM) toleriert (Mitterbauer et al., 2003), ist das Wachstum in Anwesenheit
von 10 nM DES in Aspergillus spec. beeinträchtigt, was auch die Produktionsraten reduziert
(Pachlinger et al., 2005). Des Weiteren ist der Promotor durch Zearalenon induzierbar, einem von
Fusarium spec. gebildeten Mycotoxin. Die Überproduktion von derartigen SM-Proteinen ist also
erschwert, weil SM-Endprodukte mit dem Expressionssystem interferieren können. Eine Alternative
ist das Tetracyclin-induzierbare Promotorsystem (Tet-on-System), in dem ein reverser, tetracyclin-
abhängiger Transaktivator durch Zugabe von Doxycyclin zur Genexpression führt (Vogt et al., 2005;
Meyer et al., 2011). Die Expressionslevel sind dabei mit dem gpdA-Promotor vergleichbar (Meyer
et al., 2011). Da gängige Expressionssysteme Vor- und Nachteile aufweisen, ist hier ein Bedarf zur
weiteren Optimierung angezeigt. Eine Möglichkeit könnte hierbei die gezielte Nutzung regulierbarer
Elemente eines SM-Clusters sein.
2. Zielsetzung


Im Genom von *A. terreus* lässt sich nur ein einziges vollständiges PKS-NRPS-Hybrid nachweisen. Da bisher noch keine Produkte solcher Hybride aus *A. terreus* isoliert wurden, aber alle bekannten fungalen PKS-NRPS-Produkte potente Mycotoxine darstellen, sollte dieser Gencluster vornehmlich analysiert werden.

3. Übersicht zu den Manuskripten

Gressler M, Zaehle C, Scherlach K, Hertweck C, Brock, M.

**Multifactorial Induction of an Orphan PKS-NRPS Gene Cluster in *Aspergillus terreus***


**Status zum Zeitpunkt der Fertigstellung der Dissertationsschrift:**

veröffentlicht in „Chemistry and Biology“


**Eigenanteil:** 40%

Slesiona S, Gressler M, Mihlan M, Zaehle C, Schaller M, Barz D, Hube B, Jacobsen ID, Brock M.

**Persistence versus Escape: Aspergillus terreus and Aspergillus fumigatus Employ Different Strategies during Interactions with Macrophages**


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**Status zum Zeitpunkt der Fertigstellung der Dissertationsschrift:**

veröffentlicht in „PLoS One“

**Inhalt:**

Die Arbeit beschreibt die Überlebensstrategie von *A. terreus* bei Interaktion mit Makrophagen. Im Gegensatz zu *Aspergillus fumigatus* kann *A. terreus* die Ansäuerung des Phagolysosomes nicht verhindern, da *A. terreus* keine Naphtopyron-Synthesen zur Pigmentierung der Konidien besitzt. Die heterologe Expression der Naphthopyron-Synthase WA aus *A. nidulans* ermöglicht *A. terreus* die Inhibition der Phagolysosom-Ansäuerung mit entsprechend gesteigerter Zytotoxizität für die Makrophagen.

**Eigenanteil:**

10%

Zaehle C, Gressler M, Shelest E, Geib E, Hertweck C, Brock M.

Terrein Biosynthesis in *Aspergillus terreus* and Its Impact on Phytotoxicity


**Status zum Zeitpunkt der Fertigstellung der Dissertationsschrift:**

veröffentlicht in „Chemistry and Biology“

**Inhalt:**


**Eigenanteil:** 35%

Gressler M, Hortschansky P, Geib E, Brock M.

A new high-performance heterologous fungal expression system based on regulatory elements from the *Aspergillus terreus* terrein gene cluster


**Status zum Zeitpunkt der Fertigstellung der Dissertationsschrift:**

veröffentlicht in „Frontiers in Microbiology“

**Inhalt:**

**Eigenanteil:** 35%

Der Eigenanteil an dieser Arbeit besteht in der Erstellung der Transkriptionsfaktor- und Polyketidsynthase-Überproduktionsstämmen sowie der Reporterstämmen. Außerdem wurden die Expressionsanalysen sowie die Analyse der Rohextrakte von mir durchgeführt.
Gressler M, Meyer F, Heine D, Hortschansky P, Hertweck C, Brock M.

Global transcription factors mediate phytotoxin production in
Aspergillus terreus by independent sensing of environmental signals

Status zum Zeitpunkt der Fertigstellung der Dissertationsschrift:
ingereicht (in Begutachtung bei „PLOS Genetics“)


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Gressler M, Zaehle C, Scherlach K, Hertweck C, Brock, M.

Multifactorial Induction of an Orphan
PKS-NRPS Gene Cluster in *Aspergillus terreus*


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SUMMARY

Mining the genome of the pathogenic fungus Aspergillus terreus revealed the presence of an orphan polyketide-nonribosomal-peptide synthetase (PKS-NRPS) gene cluster. Induced expression of the transcriptional activator gene adjacent to the PKS-NRPS gene was not sufficient for the activation of the silent pathway. Monitoring gene expression, metabolic profiling, and using a lacZ reporter strain allowed for the systematic investigation of physiological conditions that eventually led to the discovery of iso-flavipucine and dihydroisoflavipucine. Phytotoxin formation is only activated in the presence of certain amino acids, stimulated at alkaline pH, but strictly repressed in the presence of glucose. Global carbon catabolite repression by CreA cannot be abolished by positive-acting factors such as PacC and overrides the pathway activator. Gene inactivation and stable isotope labeling experiments unveiled the molecular basis for flavipucine/fruit rot toxin biosynthesis.

INTRODUCTION

Fungi are known as prolific reservoirs of natural products. Not only are these compounds important leads for the development of therapeutics, like penicillin or lovastatin (Hoffmeister and Keller, 2007), many secondary metabolites represent infamous toxmins (Möbius and Hertweck, 2009). In recent years, with the advent of whole genome-sequencing projects, it has become increasingly obvious that the biosynthetic potential of fungi is much higher than what can be observed under standardized fermentation conditions (Brakhage and Schroeckh, 2011; Chiang et al., 2009a; Hertweck, 2009). In various studied Aspergillus the majority of genes coding for polyketide synthases (PKSs), nonribosomal peptide synthetases (NRPs), and hybrids thereof (PKS-NRPs) remain dormant in the absence of particular stimuli (Brakhage et al., 2008). It seems plausible that particular environmental conditions are required to activate the expression of these otherwise cryptic or silent gene loci. Present challenges in mycological research, also from a biotechnological point of view, are to understand the molecular basis of gene regulation and to find ways to stimulate the activation of otherwise downregulated or silent biosynthesis gene clusters (Scherlach and Hertweck, 2009). Because it is often not feasible to transfer and express multiple pathway genes in a heterologous host (Schümann and Hertweck, 2006), several new avenues have been established to harness the biosynthetic potential of fungi. For example, variation of cultivation conditions, addition of epigenetic modifiers or epigenetics (Bok et al., 2009; Cichewicz, 2010), and even cocultivation with trigger organisms (Schoeckh et al., 2009) in many cases resulted in the remodeling of the secondary metabolome. However, a more targeted approach involves the ectopic expression of cluster-specific activator genes (Bergmann et al., 2007). Although this appears to be a generally applicable approach, care must be taken not to oversimplify the regulatory complexity behind fungal gene expression.

In the present study we shed light on the hidden metabolic potential of Aspergillus terreus, a ubiquitously distributed saprobic mold fungus, which has also been implicated in various diseases (Laham and Carpenter, 1982; Lass-Flörl et al., 2005). Here, we demonstrate that the induction of a cryptic PKS-NRPS gene locus in A. terreus in fact requires fine-tuned physiological conditions, and identify the encoded metabolite as a heavily rearranged hybrid molecule that belongs to an underexplored mycotoxin family.

RESULTS

Analysis of the Orphan PKS-NRPS Gene Cluster in A. terreus

Inspection of the recently sequenced genome of A. terreus (http://www.broadinstitute.org/annotation/genome/aspergillus_group/MultiHome.html) revealed a gene locus around ATEG00316-ATEG00331 by a SMURF analysis (www.jcvi.org/smurf/index.php) harboring the gene ATEG00325 that could code for a PKS-NRPS hybrid (Figure 1A). However, searches for the closest relative for each predicted gene product imply that the cluster may only comprise genes spanning the region from ATEG00325 to 00331 because other genes seem to code for proteins involved in stress response, protein production, and stability (see Table S1 available online). A more detailed analysis of the deduced gene product of the PKS-NRPS hybrid...
from ATEG00325 shows typical motifs for the minimal set of catalytic domains for the PKS, namely ketosynthase (KS), acyltransferase (AT), acyl carrier protein (ACP), as well as signature NRPS domains for adenylation (A), condensation (C), a peptidyl carrier protein (PCP), and a C-terminal domain for reductive off-loading. The PKS fragment shows a remarkably long stretch between AT and ACP with weak homology to \( \beta \)-keto processing domains (Figure S1). Yet, a closer analysis indicated that these are devoid of motifs for essential catalytic sites and, thus, likely inactive (Figure S1). Furthermore, bioinformatic analysis by the program NRPS-PKS (Ansari et al., 2004) suggested that the A domain would have a preference for amino acids with an aliphatic substituent, such as leucine or isoleucine. Analysis of the flanking genes did not provide any additional clue about potential enzymatic-tailoring reactions. Taken together, the PKS-NRPS could produce a hybrid metabolite composed of a straight-chain, nonreduced (or only partially reduced) polyketide fused to an aliphatic amino acid. However, no such metabolites have been reported for \( \text{A. terreus} \).

Under Standard Cultivation Conditions, \( \text{A. terreus} \) Does Not Produce PKS-NRPS Hybrid Molecules

Initially, we tested whether the orphan PKS-NRPS gene is expressed when culturing \( \text{A. terreus} \) under standard fermentation conditions. By semiquantitative RT-PCR, only a faint signal could be observed after prolonged incubation (Figure 1B). To verify whether this weak basal expression could result in metabolite formation, we compared the metabolic profiles of \( \text{A. terreus} \) wild-type variants and a mutant lacking an intact PKS-NRPS gene denoted SBUG844/\( \Delta \text{kub}/\Delta 00325 \) (Figure S2). Metabolite profiles from strain SBUG844, strain SBUG844/\( \Delta \text{kub} \), and the partial deletion mutant SBUG844/\( \Delta 00325 \) were initially analyzed from solid media containing 50 mM glucose minimal medium, yeast peptone dextrose (YPD), or Czapek yeast autolysate (CYA) medium, and strains were grown for 7 days at 30°C on agar plates. Metabolites were extracted from agar plugs (Smedsgaard, 1997) and analyzed by LC-MS analysis. Although carbon source-dependent differences in the metabolite profiles were obtained, no marked differences among the three strains were observed (data not shown). Because secondary metabolites that are only produced in minute amounts might have been missed by the agar plug extraction, all three strains were additionally cultivated in liquid media, either as shake flask cultures or without agitation, and cultures were harvested after 65–137 hr. Although the number of metabolites detected by this method increased, no differences among the three strains were observed. Apparently, the basal expression of the PKS-NRPS gene is not sufficient for hybrid metabolite production.

gpdA Promoter-Controlled Overexpression of ATEG00326 Does Not Induce Metabolite Production

Adjacent to ATEG00325, two putative transcriptional regulator genes (ATEG00326 and ATEG00328) were detected. Semiquantitative RT-PCR showed no expression of ATEG00328, but a faint band for ATEG00326, which was similar to that observed for the
structural gene ATEG00325 (Figure 1B). Previous studies have shown that overproduction of transcriptional activators can lead to the activation of "silent" gene clusters (Bergmann et al., 2007; Chiang et al., 2009b), which tempted us to produce ATEG00326 under the control of the A. terreus gpdA promoter. This promoter is highly active in the presence of glucose and only induced to a minor extent on gluconeogenic carbon sources (data not shown).

The promoter was fused with ATEG00326, and the construct was used to transform the wild-type SBUG844. Southern analyses showed that strains with ectopic single, double, and triple integrations were obtained. Semiquantitative RT-PCR from mycelia grown on glucose minimal media (gpdA-inducing) revealed that the expression level of ATEG00326 increased with the number of integrations. In agreement with an activating effect of ATEG00326 on ATEG00325, the transcript levels for ATEG00325 increased in dependence of the ATEG00326 copy number. However, despite induction of ATEG00325, transcript levels remained at a rather low level (Figure 1C). No differences in the metabolic profiles of ATEG00326 overexpressing strains, wild-type, and the ATEG00325 deletion strain were observed. Thus, we concluded that, despite an apparent involvement of ATEG00326 in regulation of PKS-NRPS gene expression, activation is not sufficient to yield a significant proportion of the product. This indicates that specific physiological conditions might be required for product formation. Thus, to enable a rapid screening for such inducing physiological conditions, we generated a β-galactosidase producing reporter strain as a fusion of the ATEG00325 promoter with the lacZ gene from E. coli.

**A lacZ Reporter Strain Reveals Physiological Conditions for Metabolite Production**

β-Galactosidase reporter strains have been used to investigate gene expression in penicillin biosynthesis (Bergh et al., 1996). Here, we applied this tool to screen for ATEG00325 inducing conditions by fusing the ATEG00325 promoter with the E. coli lacZ gene. A transformant with a single ectopic integration denoted SBUG844/P00325::lacZ was selected for screening different cultivation conditions.

Besides the above mentioned media (glucose minimal medium,YPD, and CYA), we used potato dextrose broth (PDB), potato broth (PB), Sabouraud (Sab) medium, and minimal media supplemented with 0.5% olive oil, 1% casamino acids (CAs), or 1% peptone to screen for ATEG00325 induction. After 41 and 65 hr incubation, specific β-galactosidase activity was observed (Figure 2A), and no induction was detected on all sugar-rich media (glucose minimal medium, CYA, YPD, PDB, and Sab). In addition, β-galactosidase activity was not induced in the absence of glucose when olive oil served as sole carbon source. In contrast a sharp increase in β-galactosidase activity was observed when amino acid-containing media lacking glucose were used for cultivation, and activity was higher on peptone and CA than on PB medium (containing starch). Thus, we assumed that glucose might act as a repressor for ATEG00325 expression.

In agreement with the extremely low β-galactosidase activity in the presence of sugars, the metabolite profiles of SBUG844/ΔakuB and the partial deletion mutant, SBUG844/ΔakuB::Δ00325, did not show any difference. In contrast the HPLC...
profiles of extracts from strain SBUG844/P00325 cultured in media containing only peptone or CAs showed at least one minor peak (11.8 min, m/z = 237) and two major peaks (12.0 and 12.2 min, both m/z = 239), which were completely absent from the metabolite profile of the partial ATEG00325 deletion strain (Figure 2B). Notably, according to the nitrogen detector, all of these metabolites contain nitrogen, which was expected for metabolites produced by a functional PKS-NRPS hybrid. Yet, in order to establish stable production conditions for these metabolites, more information on the regulation of gene expression and on optimal physiological conditions were required.

**Various Sugars Inhibit ATEG00325 Expression**

First, we investigated the specific inhibiting activity of glucose on ATEG00325 expression and tested the influence of different glucose and CA concentrations on lacZ expression from the reporter strain. The β-galactosidase activity was determined after 28 hr of growth. When 50 mM glucose was supplemented with 0.1%, 0.5%, or 1% CAs, induction of ATEG00325 expression, although even in the presence of 5 mM (which was completely consumed after 28 hr), the β-galactosidase activity was only 60% of that obtained in the complete absence of glucose. Thus, it can be concluded that even trace amounts of glucose inhibit ATEG00325 expression.

To test the time-dependent effect of glucose addition on ATEG00325 expression, 1% CA media were supplemented at different time points with glucose and observed over a total incubation period of 38 hr. As shown in Figure 3B, within the first 12 hr of incubation, addition of glucose strongly suppressed ATEG00325 expression. When added after 24 hr, significant amounts of β-galactosidase activity were obtained, but the specific activity remained 50% below the value without glucose. This indicates that glucose addition terminates ATEG00325 expression.

We then utilized different sugars (glucose, fructose, mannosse, and ribose) to test their effect on ATEG00325 expression. None of the sugars was able to induce ATEG00325 expression and tested meditate a strong negative effect on ATEG00325 expression.

**CreA Represses ATEG00325 Expression**

CreA is a carbon catabolite repressor of filamentous fungi, repressing transcription of genes required for alternative carbon source utilization in the presence of glucose (Felenbok et al., 1998). CreA-mediated gene expression has not been studied for A. terreus, but glucose repression tempted us to search for a CreA homolog in the A. terreus genome and revealed a protein with 66% sequence identity to A. nidulans CreA (protein accession XP_001208483). Despite the relatively low overall identity due to a truncated C-terminal region (96 bp missing), the two DNA-binding zinc finger domains (Jacobs, 1992) are highly conserved in the A. terreus protein, with 95% and 96% identity and 100% similarity (Dowzer and Kelly, 1991). Thus, the high conservation of the zinc finger domains implies that the consensus sequence identified for A. nidulans CreA might also apply to CreA from A. terreus. Indeed, four putative CreA-binding sites were detected in the promoter of ATEG00325 (Figure 4). Although further analyses need to confirm the functionality of these binding sites in glucose-mediated repression of...
ATEG00325, these data are highly suggestive for an involvement of CreA.

**Gene Expression Levels Depend on the Environmental pH**

CAs revealed the strongest induction of ATEG00325, and during growth the pH increased to $\geq 9.0$. To determine an effect of environmental pH on ATEG00325 expression, the pH was adjusted in the range between 5.0 and 8.0 by buffering media with sodium phosphate. SBUG844/P00325: lacZ was cultivated in media composed of glucose, glucose with CA (only pH 7.0 and 8.0), or only CA. After 48 hr of cultivation, the pH was retested, confirming that the initial pH had remained nearly constant. Activity determination showed that glucose persisted as a potent inhibitor of gene expression. However, on CA the specific $\beta$-galactosidase activity steadily increased with increasing pH of the medium (Figure 3).

PacC is the main transcriptional regulator responding to alkaline pH in filamentous fungi (Denison, 2000). An A. terreus PacC homolog was detected by BLAST analysis at locus tag ATEG04740, but the A. terreus protein appeared C-terminally extended, probably due to an incorrect intron prediction overwriting a putative stop codon. However, PacC activation occurs by C-terminal truncations, and the N-terminal sequence containing three zinc finger domains is of major importance.

The two DNA-binding fingers are identical in the A. terreus protein, and it is likely that the A. nidulans consensus sequence 5’-GCCARG-3’ (Tilburn et al., 1995) is also recognized. Three putative PacC-binding sites were detected in the promoter of ATEG00325 (Figure 4). Thus, increased expression of ATEG00325 at alkaline pH seems PacC dependent, but glucose repression dominates the effect of alkalization.

**Distinct Amino Acids Are Sufficient but Not Essential to Activate ATEG00325 Expression**

CAs were the best-suited inducers for ATEG00325 expression, and we were interested in elucidating the role of distinct amino acids on gene expression. A prescreening was performed with strains SBUG844/ΔakuB and SBUG844/ΔakuB/Δ00325 on agar plates, each containing one of the 20 proteinogenic L-amino acids as sole nutrient source and X-Gal to monitor $\beta$-galactosidase activity. Only Ala, Arg, Asn, Asp, Glu, Gln, and Pro showed different degrees of X-Gal conversion. In liquid cultures the strongest induction was observed by Asn, followed by Gln and Pro (Figure 5A), whereas Ala, Arg, Asp, and Glu remained at low activity levels. Addition of Arg to Asn as well as the addition of ammonium chloride or nitrate neither stimulated nor inhibited ATEG00325 gene expression (data not shown). Because the noninducing Asp is a precursor of Asn, we tested the combination of Asp with different nitrogen-containing compounds for a detailed explanation, refer to the text. Error bars denote the standard deviation calculated from three biological replicates each measured in technical triplicates. See also Figures S7 and S8.

**Figure 4. Schematic Presentation of Putative CreA, PacC, and AreA Binding Sites within the First 1250 bp of the ATEG00325 and ATEG00326 Promoter Regions**

Arrows denote the orientation of the consensus sequence. Numbers denote the 5’ position of the consensus in respect to the translational start point. (A) The PKS-NRPS promoter (P00325) contains four AreA and CreA, and three PacC sites. (B) The promoter of the transcription factor (P00326) contains three CreA and six AreA-binding sites, but no consensus sequence for PacC binding. See also Figures S7 and S8.

**Figure 5. Amino Acid and Nitrogen Source-Dependent Expression of ATEG00325**

Gene expression was monitored by determination of $\beta$-galactosidase activities from the reporter strain SBUG844/P00325: lacZ. Amino acids are denoted in the three-letter code.

(A) Single amino acids served as sole nutrient sources, and $\beta$-galactosidase activity was determined after 72 hr.

(B) The noninducing amino acid aspartate (50 mM) served as basal nutrient source, and media were supplemented with different nitrogen sources (10 mM). Activity was determined after 62 hr of cultivation. Cit, citrulline; Orn, ornithine.
Figures S4 and S8

Errors denote the standard deviation from three independent biological replicates measured in technical triplicates. ND, not determined. See also the induced near its maximum level. In contrast, on glucose neither of the parental strain, indicating that ATEG00325 is already

Enables PKS-NRPS Induction

Arginase Promoter-Controlled ATEG00326 Expression

Table 1. Arginase and \(\beta\)-Galactosidase Activities from Reporter Strains Cultivated on Different Nutrient Sources

<table>
<thead>
<tr>
<th>Nutrient Source</th>
<th>Arginase (mU/mg)</th>
<th>(\beta)-Galactosidase (mU/mg)</th>
<th>(\beta)-Galactosidase (mU/mg)</th>
<th>(\beta)-Galactosidase (mU/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PagaA: lacZ(^a)</td>
<td>PagaA: lacZ(^b)</td>
<td>P00325: lacZ(^b)</td>
<td>P00325: lacZ PagaA:00326(^c)</td>
</tr>
<tr>
<td>1% CAs</td>
<td>ND</td>
<td>168 ± 79</td>
<td>1419 ± 225</td>
<td>1760 ± 166</td>
</tr>
<tr>
<td>50 mM glucose</td>
<td>50 ± 8</td>
<td>32 ± 11</td>
<td>7 ± 0</td>
<td>7 ± 2</td>
</tr>
<tr>
<td>50 mM arginine</td>
<td>2510 ± 129</td>
<td>733 ± 65</td>
<td>3 ± 0</td>
<td>1202 ± 415</td>
</tr>
<tr>
<td>50 mM arginine/50 mM glucose</td>
<td>1620 ± 288</td>
<td>429 ± 78</td>
<td>12 ± 1</td>
<td>51 ± 7</td>
</tr>
</tbody>
</table>

Errors denote the standard deviation from three independent biological replicates measured in technical triplicates. ND, not determined. See also Figures S4 and S8.

\(^a\) Arginase and \(\beta\)-galactosidase activity were determined from strain SBUG844/PagaA: lacZ containing a fusion of the agaA promoter with the E. coli lacZ gene.

\(^b\) \(\beta\)-Galactosidase activity was determined from strain SBUG844/P00325:lacZ containing a fusion of the ATEG00325 promoter with the E. coli lacZ gene.

\(^c\) \(\beta\)-Galactosidase activity was determined from strain SBUG844/P00325: lacZ additionally containing a fusion of the agaA promoter with the transcription factor ATEG00326.

From these results we conclude that: (i) ATEG00326 acts as a transcriptional activator for ATEG00325 expression; (ii) ATEG00326 expression is sufficient to stimulate all genes required for product formation; but (iii) only in the absence of glucose, which mediates a dominant negative effect on ATEG00325 expression; and (iv) as assumed above, the cluster seems to span mainly the region starting at ATEG00325 and ending at ATEG00331.

A. terreus is Capable of Producing Members of the Flavipuce Family of Mycotoxins

Expression and LC-MS analysis revealed that the expression of the PKS-NRPS gene correlates with metabolite formation, and production is dramatically increased in the presence of CAs at alkaline pH in the strict absence of glucose. To obtain larger metabolite quantities for a full structure elucidation, the fermentation of wild-type strain SBUG844 was upscaled (2 × 4 liters). Samples taken at 24, 36, 48, and 72 hr showed maximum production levels toward the end of fermentation.

After chromatographic purification of the nitrogen-containing metabolites 1 and 2, their structures were fully elucidated (Figure 6A) by 1D and 2D nuclear magnetic resonance (NMR) spectroscopy and MS. The \(^1^H\) and \(^13^C\) NMR data obtained for 1 and 2 appeared to be highly similar, indicating that these compounds are related. From high-resolution electrospray ionization mass spectrometry (HRESI-MS), we deduced the molecular formula of C_{12}H_{13}O_{12}N for 1 and C_{12}H_{17}O_{3}N for 2.

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Heteronuclear multiple bond correlation (HMBC) experiments revealed the common pyridone substructures for \( 1 \) and \( 2 \). The unusual chemical shift of C-7 (Table S2) for a CH group with \( \delta \) 113.4 ppm for compound \( 2 \) and \( \delta \) 106.1 ppm in compound \( 1 \) indicates a dioxygen substitution. The structure of the branched aliphatic side chain of compounds \( 1 \) and \( 2 \) was deduced from correlation spectroscopy (H,H-COSY) data. \( ^{13}C \) NMR data for compound \( 1 \) showed a chemical shift of C-8 (\( \delta \) 202.0 ppm) that

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**Figure 6. Metabolites Produced by ATEG00325 and Proposed Biosynthesis**

(A) Structures of isoflavipucine \( 1 \) and dihydroisoflavipucine \( 2 \). (B) The domain architecture of ATEG00325 implies the formation of a hybrid molecule. Modules in brackets seem not to contribute to biosynthesis. After formation of linear PKS-NRPS product \( 5 \) by ATEG00325, the first cyclization could take place \( 6 \). Starting from the following oxidation step \( 7 \) hydrolysis involving a trans-amidation step might lead to the formation of \( 9 \), which is further oxidized to flavipucine. Formation of isoflavipucine from flavipucine requires an unusual rearrangement. Alternative rearrangement reactions could build up rubrobramide, representing a branching of flavipucine biosynthesis. See also Figure S5 and Table S2.
indicates the presence of a keto group, which is not present in compound 2 (Figure S5). From these analyses we concluded that metabolites 1 and 2 represent isoflavipucine and dihydroiso-
flavipucine, respectively.

Isoflavipucine (1), a constitutional isomer of the antibiotic flavipucine, is a known metabolite that has previously been isolated from Aspergillus flavipes (Casinovi et al., 1968; Findlay et al., 1977). Dihydroiso-
flavipucine (2) has been described as a semi-synthetic product obtained through chemical reduction of isofla-
vipucine (Findlay et al., 1977), but it has not yet been isolated as a natural product. We succeeded in separating diastereomers of dihydroiso-
flavipucine by an achiral stationary HPLC phase (2a
and 2b in Figure 2B), and using a chiral stationary phase allowed the separation of all four stereoisomers of 2 (Figure S6). The occurrence of all four possible stereoisomers implies that there is no stereochemical control over the formation of these two asymmetric centers during biosynthesis.

**Isoflavipucine Is a PKS-NRPS Hybrid Metabolite Derived from Leucine**

Although the formation of compounds 1 and 2 strictly correlates with the presence of the PKS-NRPS hybrid, their structures do not immediately provide an insight into the biosynthetic pathway. Former isotope-labeling experiments using [1-13C]-, [2-13C]-, and [1,2-13C2]-acetate indicated that the pyridone moiety of flavipucine is derived from acetate (Grandolini et al., 1987). However, no labeling of the isobutyl side chain of the nitrogen heterocycle was observed, and its biogenetic origin has remained enigmatic. A plausible biosynthetic precursor for this side chain would be leucine. To support this hypothesis we performed labeling experiments with [1-13C]- and [5,2-13C]-leucine. For this purpose we composed a synthetic medium containing l-asparagine and l-arginine, supporting both sufficient growth rates and high PKS-NRPS activity and supplemented the medium with unlabeled, deuterated, or

13C-labeled l-leucine (2.5 mM), respectively. Samples were analyzed by LC-MS to compare the mass peaks for labeled and native l-leucine. The labeled sample resulting from the incorporation of [1-13C]-leucine showed an M+1 mass shift, whereas the sample resulting from the incorporation of [5,2-13C]-leucine showed an M+3 mass shift. To identify the position of 13C labeling in 1, the metabolite was purified by preparative LC-MS and analyzed by NMR. Comparison of carbon NMR data showed that the signal of C-7 in the 13C NMR spectrum of isoflavipucine was enriched (calculated incorporation rate 92%), Detailed analysis of the 13C NMR data of isoflavipucine (1) resulting from the incorporation of [1-13C]-leucine showed a doublet for C-8 at 202.1 and 201.8 ppm (J = 46.8 Hz), respectively (Figure S4). Consequently, both isotope-label experiments showed labeling of the aliphatic chain, and the carbonyl (C-1) of l-leucine is detected in metabolite 1, indicating that the entire l-leucine backbone was incorporated, albeit disrupted.

**DISCUSSION**

In this study we have identified a cryptic PKS-NRPS gene cluster in *A. terreus* and unveiled its function using a multidisciplinary approach. Our strategy involved the generation of gene deletion mutants, the construction of reporter strains, screening for gene expression and metabolite production under a variety of culture conditions, and the overproduction of a transcription factor under the control of different inducible promoters.

Gene-activation strategies involving the overproduction of a pathway-specific transcription factor have proven helpful to identify new products from secondary metabolite gene clusters, which appear not inducible under standard laboratory conditions. An example for a successful application of this strategy is the identification of the PKS-NRPS derived metabolite aspyridone from *A. nidulans* (Bergmann et al., 2007). However, here, we show that such a simple approach might be insufficient. Our analyses revealed that glucose acts as a dominant repressing factor, even when the transcription factor was expressed under the control of the glucose-induced gpdA promoter. This negative effect of glucose, which is most likely mediated by the global carbon catabolite repressor CreA, cannot be abolished by other positive-acting factors such as PacC. The processed form of PacC (PacC<sup>C</sup>) can directly act as an inducer of genes required for growth at alkaline pH and additionally acts as a repressor of genes required under acidic conditions (Penalva et al., 2008). In terms of secondary metabolite produc-
tion, PacC was shown to stimulate penicillin production at alkaline pH (Brakhage et al., 2004). Similarly, the production of iso- and dihydroiso-
flavipucine in *A. terreus* is induced at alkaline pH but remains negatively dominated by glucose. Furthermore, the available nitrogen sources, especially amino acids, are important for gene activation, as indicated by our expression analyses and several AAreA-binding sites in the promoters of ATEG00325 and ATEG00326. Thus, despite the ability of the transcription factor ATEG00326 to induce PKS-NRPS expres-
sion, regulators of primary metabolism, e.g., CreA, PacC, and AAreA, possess a major impact.

In sum these results imply that the activation of downregulated or silent genes through the expression of activator genes and/or promoter exchange may not be sufficient for metabolite produc-
tion. Thus, the use of a reporter system is strongly recommended to screen for nutritional and other physiological conditions, which might negatively affect gene expression or may be crucial for pathway induction.

Through our expression analyses and a targeted gene inacti-
vation, we finally succeeded in the functional analysis of the, to our knowledge, only complete PKS-NRPS hybrid from *A. terreus* and showed that this multifunctional enzyme is involved in the biosynthesis of flavipucine-type molecules. A full characterization of the metabolites revealed the structures of isoflavipucine and dihydroiso-
flavipucine. Although isoflavipucine and flavipu-
ce, the biosynthetic progenitors of dihydroiso-
flavipucine, have been isolated from *Aspergillus flavipes* (Casinovi et al., 1968; Findlay et al., 1977; Findlay and Radics, 1972; Grandolini et al., 1987), to our knowledge, they have not been reported as metabolites of *A. terreus*. Due to the close phylogenetic relation between the *Terrei* and *Flavipes* sections (Varga et al., 2005), a conservation of secondary metabolite clusters among these species is well conceivable. Aside from that, also ascomycetes such as *Macrophoma* species or *Cladobotryum rubrobrunnescens* produce flavipucine derivatives or closely related compounds such as fruit rot toxin A (*Sassa and Onuma, 1983*) or rubrobramide (Wagner et al., 1998), respectively, but the responsible gene clusters have remained unknown. Due to
In this respect the identification of the PKS-NRPS and the requisite metabolites, in conjunction with our isotope-labeling experiments, provide new insights into the biosynthesis of these unusual compounds. Inspection of the architecture of the multifunctional hybrid synthetase and the observed incorporation of labeled [1-13C]-leucine and [5-2H2]-leucine allows us to propose a model for isoflavipucine (1) biosynthesis (Figure 6B).

First, the PKS part of the PKS-NRPS could assemble a triketide from an acetyl starter and two malonyl-CoA extender units. The poly-β-keto intermediate would then be fused to the leucine unit by the NRPS part. The resulting amide would be liberated from the NRPS part. The resulting amide would be liberated from the NRPS part. The resulting amide would be liberated from the NRPS part. The resulting amide would be liberated from the NRPS part. The resulting amide would be liberated from the NRPS part. The resulting amide would be liberated from the NRPS part. The resulting amide would be liberated from the NRPS part. The resulting amide would be liberated from the NRPS part. The resulting amide would be liberated from the NRPS part. The resulting amide would be liberated from the NRPS part. The resulting amide would be liberated from the NRPS part. The resulting amide would be liberated from the NRPS part. The resulting amide would be liberated from the NRPS part. 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Strains and Genome Information
The genome of the A. terreus strain AT1156 (NHK624) has been sequenced, and all locus tag and sequence information refers to this strain. Sequence information is available from the BROAD Institute website (http://www.broadinstitute.org/annotation/genome/aspergillus_group/MultiHome.html). Strain AT1156 was obtained from the Fungal Genetics Stock Center and was used in some experiments as reference strain. Most work was performed on A. terreus strain SBUG844, available from an in-house culture collection. Details on the relationship between SBUG844 and AT1156 from sequence analyses can be found in Figure S7. From both strains, ΔαkB derivatives with defect in the nonhomologous end-joining repair mechanism (Kück and Hoff, 2010) were generated (AT1156/ΔαkB and SBUG844/ΔαkB), which facilitated the targeted gene deletion by homologous recombinant (data not shown). For plasmid propagation, the E. coli strain DH5α (Invitrogen) was used.

Media and Cultivation Conditions
Aspergillus minimal media (AMM, http://www.fsgs.net/methods/animed.html) with nitrate as nitrogen source and adjusted to pH 6.5 generally served as basal medium. Solid media were prepared by adding 1.5%–2% agar prior to autoclaving. The following nutrient sources were added at concentrations and combinations as indicated in the respective experiments: 5–100 mM D-glucose; 50 mM D-fructose, D-mannose, or D-ribose; 0.1%–1% CAs; 1% peptone; 2.5–100 mM of proterogenic L-amino acids; 0.5% olive oil. In the presence of amino acids, nitrate was generally omitted from the medium. In experiments requiring a fixed pH value, the medium was additionally buffered by supplementation with 150 mM sodium phosphate adjusted to the respective pH. As complex media, PDB, YPD (10 g/l yeast extract, 20 g/l glucose, 20 g/l peptone), PB, Sab, and CYA (Pit, 1979) were used. All complex media except YPD were adjusted to pH 6.5. β-Galactosidase activity from solid media was determined by addition of 50 μg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) after autoclaving. Liquid media were inoculated with conidia suspensions at a final concentration of 1 × 10^6–2 × 10^7 conidia/ml and incubated on a rotary shaker at 30°C and 200 rpm, if not indicated otherwise.

For preparation of conidia suspensions, conidia were harvested in distilled water from AMM/glucose solid media incubated for 3 days at 37°C, and suspensions were filtered over 40 μm cell strainers. Conidia suspensions were stored at 4°C for a maximum of 2 weeks without significant loss of viability. Large-scale fermentations were described in the Supplemental Experimental Procedures.

Molecular Biological Techniques
All oligonucleotides used in this study have been consecutively numbered (P1, P2, etc.), and detailed information is shown in Table S4. Details on kits and reagents used for molecular biological procedures are provided in the Supplemental Experimental Procedures. Chemically competent E. coli DH5α cells were transformed by standard techniques (Maniatis et al., 1982). A. terreus strains were transformed from protoplasts as described for Aspergillus fumigatus (Brock et al., 2008) except that 1.2 M sorbitol instead of 0.6 M KCl was used as osmotic stabilizer. As selection marker, either 180 μg/ml hygromycin B (Roche) or 0.1 μg/ml pyrithiamine (Sigma-Aldrich) was used.

Semiquantitative PCR
For comparative quantification of transcript levels, cDNA was used as a template, and amplification was performed in a SpeedCycler (Analytik Jena) using the GoTaq Polymerase (Promega). To adjust template concentrations, actin transcript levels (locus ATEG06973) were used as a reference.

Strain Constructions
Details on the cloning procedures for plasmid construction to obtain fungal transformants are given in the Supplemental Experimental Procedures. In brief, ATEG00325 deletion mutants were generated by replacing the first 1594 bp of the ATEG00325 coding region and 22 bp upstream the ATG start codon with the pyrithiamine resistance cassette (ptra) from plasmid pJET1 (Fleck and Brock, 2010) (Figures S8A–S8B). β-Galactosidase reporter strains were generated by using the lacZ gene from E. coli fused with the arginase promoter (Pajaxa, locus ATEG04679) or the ATEG00325 promoter (P00325), respectively. As selection marker, either the ptra or the hygromycin B resistance cassette (pH) (Fleck and Brock [2010] was used (Figures S8F and S8B). For generation of transcription factor overexpression strains, the A. terreus gpdA (Pgfpda, locus ATEG10199) and agaA promoters were fused with the putative transcription factor ATEG00326. The ptra or pH resistance cassette was used for transformation of strain SBUG844 and strain SBUG844/P00325/Δc, which facilitated the targeted gene deletion by homologous recombinant (data not shown). For plasmid propagation, the E. coli strain DH5α (Invitrogen) was used.

Cell-Free Extracts and Enzyme Assays
For preparation of cell-free extracts, mycelia were harvested from liquid cultures, washed with sterile tap water, pressed dry, frozen in liquid nitrogen, ground to a fine powder, and resuspended in 50 mM Tris/HCl buffer (pH 8.0). Debris was removed by centrifugation at 14,800 × g, and the cell-free extract was used for activity determination on a Lambda 25 UV/VIS double-beam spectrophotometer (Perkin Elmer). Protein concentrations were determined by the Bio-Rad Protein assay (Bio-Rad) as described in the manufacturer’s protocol with bovine serum albumin as standard.

β-Galactosidase activity was determined by a continuous assay as described previously (Ebel et al., 2006). For determination of arginase activity, a coupled enzymatic assay with urease and L-glutamate dehydrogenase as helping enzymes was developed. Details are provided in the Supplemental Experimental Procedures. Activities were generally determined from three biological replicates measured in three technical replications. Error bars in figures represent the standard deviation obtained from all data points.

Analysis, Isolation, Purification, and Structure Elucidation of Isoflavipucine and Dihydroisoflavipucine
For metabolite extraction from liquid cultures, fluid-fluid extraction of culture broth with ethylacetate was performed. When mycelia were analyzed, cells were disrupted in ethylacetate by using an IKA-UltraTurrax T25 (Janke & Kunkel GmbH). The organic phase was collected and concentrated in vacuo. Standard analysis of the extracts from mutants and wild-type strains was performed on an Agilent 1100 series HPLC-DAD system coupled with a MSD trap (Agilent) operating in alternating ionization mode and an Antek 8060 HPLC-CLN-detector (Antek Instruments GmbH). As stationary phase, a C8 column (Zorbax Eclipse XDB C8, 4.6 × 150 mm; 5 μm) with a binary solvent system consisting of methanol (solvent B) and water containing 0.1% formic acid (solvent A) was used. The following gradient with a flow rate of 1 ml/min was applied: 0.5 min, 10% B; 0.5–15 min, 10%–90% B; 15–17 min, 90% B; 17.5–22 min, 100% B; and 22–23 min, 100%–10% B. For separation of stereoisomers a chiral stationary phase column (Phenomenex Lux Cellulose 2, 250 × 4.6 mm; 5 μm) with a solvent system consisting of heptane (55%) and methanol/ethanol 1:1 (45%) under isocratic conditions with a flow rate of 0.5 ml/min at 40°C was used. The crude ethylacetate extract solved in methanol was applied to silica gel chromatography (30 × 120 mm) for initial purification using a stepwise gradient: chloroform, chloroform/methanol (8:2, 95:5, 90:10, 50:50), and 100% methanol. For further purification of metabolites from silica gel chromatography, respective fractions were applied to preparative LC-MS using a Waters autopurification system (Waters GmbH) consisting of a 2525 binary gradient module, a 515 HPLC pump, a column fluidics organizer, a 2996 PDA, a ZQ 2000 mass detector, and a 2767 sample manager. The Waters X-Terra prep MS C18 column (19 × 50 mm; 5 μM) was equilibrated with 90% solvent A (water/0.1% formic acid) and 10% solvent B (methanol), with a flow rate of 20 ml/min. The following gradient was applied: 1 min, 10% B; 1–4 min, 10%–30% B; 4–20 min, 30% B; 20–23 min, 30%–100% B; 23–25 min, 100% B; and 25–27 min, 100%–100% B. The HPLC-MS was controlled with an AccQ-ESI UPLC-system (Thermo Scientific) combined with an Exactive Max Mass Spectrometer (Thermo Scientific) operating in positive ionization mode. Separation was carried out on a Betasil C18 column (2.1 × 150 mm, 3 μm; Thermo Scientific) using water (solvent A) and acetonitrile (solvent B), each containing 0.1% formic acid, as binary solvent system. A flow
rate of 250 µl/min and the following gradient was used: 0–1 min, 5% B; 1–16 min, 5%–98% B; 16–19 min, 98% B; and 19–20 min, 98%–5% B.
NMR spectra were recorded on a Bruker Avance III 500 and a Bruker Avance III 600 spectrometer (Bruker BioSpin GmbH) equipped with a cryoprobe head using DMSO-d6 as solvent and internal standard.

Feeding Studies
Feeding studies of SBUG844 were carried out in 100 ml AMM containing 25 mM arginine and asparagine, and supplemented at start of fermentation with 2.5 mM of [13C6]-leucine, [5-13C]-leucine (both Cambridge Isotope Laboratories), or unlabeled -leucine, respectively. Cultures were inoculated with 2 x 10^6 conidia/ml and incubated on a rotary shaker for a total of 72 hr at 30°C and 200 rpm. Addition of leucine was repeated after 48 hr of cultivation. Culture supernatants were extracted with ethylacetate. Extracts were applied to LC/MS and NMR analysis as described above. 13C contents were calculated based on the natural 13C content of unlabeled carbons. A description for the calculation is provided in the Supplemental Experimental Procedures.

ACCESSION NUMBERS
The nucleotide sequences of the region spanning ITS1 and ITS2 from A. terreus strains A1196 and SBUG844 as well as that of the S. untranslated region of ATGC00352 from strain SBUG844 have been deposited in the DDBJ/EMBL/GenBank nucleotide sequence database and assigned the accession numbers HQ380176, HQ380177, and HQ380178, respectively.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures, eight figures, and four tables and can be found with this article online at doi:10.1016/j.chembiol.2010.12.011.

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Chemistry & Biology

Cellular Physiology Controls Metabolite Production


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**Persistence versus Escape: Aspergillus terreus and Aspergillus fumigatus Employ Different Strategies during Interactions with Macrophages**


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Persistence versus Escape: *Aspergillus terreus* and *Aspergillus fumigatus* Employ Different Strategies during Interactions with Macrophages

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**Abstract**

Invasive bronchopulmonary aspergillosis (IBPA) is a life-threatening disease in immunocompromised patients. Although *Aspergillus terreus* is frequently found in the environment, *A. fumigatus* is by far the main cause of IBPA. However, once *A. terreus* establishes infection in the host, disease is as fatal as *A. fumigatus* infections. Thus, we hypothesized that the initial steps of disease establishment might be fundamentally different between these two species. Since alveolar macrophages represent one of the first phagocytes facing inhaled conidia, we compared the interaction of *A. terreus* and *A. fumigatus* conidia with alveolar macrophages. *A. terreus* conidia were phagocyted more rapidly than *A. fumigatus* conidia, possibly due to higher exposure of β-1,3-glucan and galactomannan on the surface. In agreement, blocking of dectin-1 and mannose receptors significantly reduced phagocytosis of *A. terreus*, but had only a moderate effect on phagocytosis of *A. fumigatus*. Once phagocyted, and in contrast to *A. fumigatus*, *A. terreus* did not inhibit acidification of phagolysosomes, but remained viable without signs of germination both in vitro and in immunocompetent mice. The inability of *A. terreus* to germinate and pierce macrophages resulted in significantly lower cytotoxicity compared to *A. fumigatus*. Blocking phagolysosome acidification by the v-ATPase inhibitor bafilomycin increased *A. terreus* germination rates and cytotoxicity. Recombinant expression of the A. nidulans w4 naphthopyrone synthase, a homologue of *A. fumigatus* PksP, inhibited phagolysosome acidification and resulted in increased germination, macrophage damage and virulence in corticosteroid-treated mice. In summary, we show that *A. terreus* and *A. fumigatus* have evolved significantly different strategies to survive the attack of host immune cells. While *A. fumigatus* prevents phagocytosis and phagolysosome acidification and escapes from macrophages by germination, *A. terreus* is rapidly phagocyted, but conidia show long-term persistence in macrophages even in immunocompetent hosts.

**Introduction**

Invasive bronchopulmonary aspergillosis (IBPA) is a life-threatening disease mainly caused by *Aspergillus fumigatus* [1]. Additionally, several other *Aspergillus* species, including *A. terreus*, have been identified as causative agents [2]. Approximately 3–12.5% of IBPA cases are caused by *A. terreus* [3,4,5] with dramatic mortality rates of up to 98% [6,7]. Therapy of IBPA caused by *A. terreus* is complicated by its intrinsic resistance to Amphotericin B [8,9] and prophylactic Amphotericin B monotherapy in high risk patients results in an increased likelihood to acquire IBPA caused by *A. terreus* [10]. In addition, invasive aspergillosis due to *A. terreus* is accompanied by a high rate of dissemination to secondary organs further complicating treatment of affected patients [5,11]. *Aspergillus conidia* are ubiquitous and, due to their small size, reach the lower airways upon inhalation. In the immunocompetent host, these spores are cleared by phagocytic immune cells [12]. If the phagocytic immune defense is impaired, e.g. by prolonged corticosteroid therapy or chemotherapy, conidia are able to germinate and form hyphae which invade the lung tissue and establish an infection [13,14]. Alveolar macrophages (AM) are the first professional phagocytic cells encountering pathogens in the lung. In addition to eliminating pathogens by phagocytosis, AM participate in orchestrating the immune response [15]. Therefore, the initial step of successful infection depends on the pathogen’s ability to prevent or survive recognition and phagocytosis by macrophages. Consequently, pathogens have developed diverse escape and survival strategies [16,17,18,19]. The interaction of *A. fumigatus* with macrophages has been well studied: In immunocompetent mice, AM phagocytose *A. fumigatus* conidia and control fungal burden unless overwhelmed by a large number of conidia [20,21],
Aspergilli Differ in Their Macrophage Interactions

A. terreus is phagocytosed more rapidly than A. fumigatus

Upon inhalation, alveolar macrophages are the first professional phagocytic cells facing fungal conidia in the lower respiratory tract. We proposed that the different efficiency of phagocytosis and intracellular killing of A. fumigatus or A. terreus conidia by macrophages might contribute to the different epidemiology of these two fungi. While phagocytosis of A. fumigatus conidia by macrophages has been extensively studied [28,29,30], little is known about the phagocytosis of A. terreus conidia. Therefore, we compared phagocytosis of both Aspergillus species using alveolar and peritoneal macrophage cell lines (MH-S and J774.A1, respectively).

Resting A. terreus conidia were phagocytosed significantly faster than resting A. fumigatus conidia by both MH-S (Fig. 1A) and J774.A1 cells (data not shown). While phagocytosis of resting A. terreus conidia was completed after three hours (96% phagocytosed), the ratio of phagocytosed resting A. fumigatus conidia continued to increase for at least eight hours (Fig. 1A).

Since resting Aspergillus conidia swell in the cell culture media, we additionally determined the phagocytosis of pre-swollen conidia. Preliminary experiments revealed that A. terreus conidia require a prolonged time for germination and possess a lower growth speed than A. fumigatus. To account for these differences, the swelling and germination speed of both fungi was evaluated in different cell culture media (Fig. S1). Swelling was judged by microscopic and flow cytometry analysis using the (i) increase of conidial diameter, (ii) opacity of conidia and (iii) the loss of spore coloration as markers. Homogenous populations containing >95% swollen conidia but no germings were obtained for A. fumigatus by 3.5 h pre-incubation in RPMI and 6 h pre-incubation in DMEM medium, respectively. Due to slower swelling of A. terreus conidia, pre-incubation for 7 h in RPMI and 10 h in DMEM medium were required to obtain homogenous populations of swollen conidia (Fig. S1). Since phagocytosis can be influenced by particle size, we additionally determined the size of resting and pre-swollen conidia using FACS. The diameter of both resting and swollen A. terreus conidia was smaller than that of A. fumigatus conidia (Fig. S2).

Pre-swelling led to a significant increase in the phagocytosis of A. fumigatus conidia. In contrast, phagocytosis of A. terreus conidia was not affected by pre-swelling (Fig. 1A). Using pre-swollen conidia, no difference between A. terreus and A. fumigatus was observed for initial (30 min) and late phagocytosis (8 h). However, we observed significant differences between 1 h to 3 h. Therefore, we analyzed phagocytosis of pre-swollen conidia on a more detailed time scale (Fig. 1B). Significant differences in the proportion of phagocytosed pre-swollen conidia were observed between 1 h and 6 h.

β-1,3-glucan and galactomannan significantly contribute to pathogen recognition in the phagocytosis of A. terreus

It has been shown that phagocytosis of A. fumigatus conidia depends on the exposure of pathogen associated molecular patterns (PAMPs) such as β-1,3-glucan and galactomannan [31]. PAMPs are recognized by pathogen recognition receptors (PRRs) located on the surface of macrophages. Known PRRs involved in recognition of fungi include dectin-1 recognizing β-1,3-glucan [32], mannose receptors recognizing galactomannan [33,34], and the toll like receptors TLR2 and TLR4 recognizing yet unknown factors [35]. We hypothesized that the observed differences in phagocytosis rates could result from differences in PAMP exposure and PRR recognition.

Therefore, we quantified β-1,3-glucan and galactomannan exposure on the surface of resting and swollen conidia by flow cytometry using specific antibodies (kindly provided by A. Cassone and F. Ebel). Resting conidia of A. terreus displayed significantly higher exposure of β-1,3-glucan (as recently shown by Deak et al. [36]) and galactomannan than resting A. fumigatus conidia. As expected, swelling of conidia increased the exposure of galactomannan in both fungi as indicated by an increase in specific fluorescence (Fig. 1C). The pre-swelling conditions applied to both fungi did not significantly increase the exposure of β-1,3-glucan determined by mean fluorescence levels (Fig. 1D). This confirmed that no germ tubes were formed, which have been shown to display high β-1,3-glucan levels [37]. Importantly, swollen conidia of A. terreus showed higher exposure of both β-1,3-glucan and galactomannan than A. fumigatus. However, within the A. terreus population, β-1,3-glucan exposure was distributed inhomogeneously.

To investigate the role of different PAMPs, we determined phagocytosis of pre-swollen conidia 3 h and 8 h after blocking the receptors of MH-S cells by specific substrates or specific antibodies. Pre-swollen conidia were chosen for these and subsequent in vitro experiments because differences in phagocytosis rates between A. fumigatus and A. terreus were less pronounced for pre-swollen compared to resting conidia. Blocking of dectin-1 by laminarin and blocking of mannose receptors by mannann significantly decreased the phagocytosis of A. terreus conidia within the first three hours by 50% and 30%, respectively. The overall phagocytosis rate of A. terreus did not further increase after prolonged co-incubation (Fig. 2A and 2B). In contrast, blocking of either dectin-1 or mannose receptors did not influence phagocytosis of A. fumigatus (Fig. 2A and 2B). These data suggested that
Figure 1. Phagocytosis rates and surface exposure of galactomannan and β-1,3-glucan on resting and swollen *A. terreus* and *A. fumigatus* conidia. Results are shown for *A. terreus* SBUG844 and *A. fumigatus* CBS14489. (A, B) Phagocytosis of conidia by alveolar macrophages (MH-S cell line). Phagocytosis rates are shown as mean±SD from three independent experiments. Statistical analysis was performed using 1-way ANOVA. * P<0.05, ** P<0.01, *** P<0.001. (A) Phagocytosis of resting and pre-swollen conidia after 0.5, 3 and 8 h of co-incubation. (B) Phagocytosis of pre-swollen conidia over 12 h. (C) FACS analysis of galactomannan and (D) β-1,3-glucan exposure. Histograms (one representative result from three independent experiments is shown) and scatter plots of median fluorescence intensity are shown (mean±SD, three independent
recognition by dectin-1 and mannose receptors played a major role in the early recognition of \(A.\) terreus, but not \(A.\) fumigatus. Blocking of either TLR2 or TLR4 led to a moderate but significant reduction in phagocytosis, but these differences were only observed after 0 h (Fig. 2C and 2D). The influence of TLR2 and TLR4 on the long-term phagocytosis of pre-swollen \(A.\) fumigatus conidia coincided with the occurrence of germings (Fig. S1), suggesting that the ligands for TLR2/4 are either exposed on germings or that the recruitment of PRRs to the phagosome and the subsequent antigen presentation affects long-term phagocytosis [15]. It appeared possible that blocking of a single receptor can be bypassed by other receptors. Thus, we performed simultaneous blocking of mannose receptors and dectin-1 as well as simultaneous blocking of all four tested receptors. Simultaneous blocking of mannose receptors and dectin-1 nearly completely abolished phagocytosis of \(A.\) terreus, but had only a moderate effect on \(A.\) fumigatus (>60% phagocytosed, Fig. 2E). Blocking of all receptors did not further reduce phagocytosis of \(A.\) terreus (Fig. 2F), but further decreased phagocytosis of \(A.\) fumigatus conidia (50% phagocytosed, Fig. 2F). These data confirm that, at least in MH-S cells, dectin-1 and mannose receptors are the main PRRs recognizing \(A.\) terreus conidia, whereas different and probably yet unknown receptors are involved in phagocytosis of \(A.\) fumigatus conidia.

Viable \(A.\) terreus conidia persist in macrophages after phagocytosis

Phagocytosis is one of the first steps in the inactivation of many microbial pathogens. However, some microbes are able to escape killing after phagocytosis [37,38,39]. Thus, we investigated which proportion of phagocytosed pre-swollen conidia were inactivated.

In a first set of experiments, we discriminated dead and viable conidia on a single-conidia level by using a fluorescent dye, which indicates metabolic activity of cells. The ratio of inactivated \(A.\) fumigatus conidia steadily increased over time after phagocytosis by MH-S cells (Fig. 3A). Furthermore, in addition to the metabolic inactivation indicated by color changes of the dye, \(A.\) fumigatus conidia appeared deformed, suggesting that conidia were not only inactivated, but also partially degraded. In striking contrast, no inactivation of \(A.\) terreus conidia was observed (Fig. 3A), indicating an increased resistance of \(A.\) terreus within macrophages. Similar results were obtained using \(J774.1\) cells (data not shown). In addition, we determined survival by counting colony forming units (CFU) 3 h and 10 h after infection. \(A.\) fumigatus showed the expected time dependent inactivation, while \(A.\) terreus CFUs remained stable (Fig. 3B). Increased survival of \(A.\) terreus was likewise observed upon interaction with primary mouse macrophages (Fig. 3C). To confirm that differences in survival rates were not strain but species specific, two additional strains from each species were investigated for their survival within MH-S cells. No significant differences between species specific strains were observed, confirming that our findings are not limited to a specific strain (Fig. S3).

\(A.\) fumigatus conidia which are not inactivated after phagocytosis tend to form germ tubes that pierce macrophages and eventually lead to lysis of phagocytic cells [40]. To investigate whether the increased persistence of \(A.\) terreus conidia in macrophages was likewise associated with host cell damage, we determined the LDH release from macrophages as a marker for macrophage damage. We used pre-swollen conidia to minimize effects caused by the overall slower germination (Fig. S1) and growth speed of \(A.\) terreus compared to \(A.\) fumigatus, which could affect damage of macrophages by \(A.\) terreus in co-culture experiments [41]. Furthermore, we analyzed fungal germination and growth in cell culture medium in the absence of macrophages. No gross differences between \(A.\) fumigatus and \(A.\) terreus were observed in these experiments (Fig. S4A). In co-incubation with macrophages, only mild cell damage was observed with either of the two fungi within the first 12 h. However, cell damage caused by \(A.\) fumigatus strongly increased 24 h post infection, whereas \(A.\) terreus caused significantly less damage. This difference was significant after 24 h in MH-S cells (Fig. 3D), independent of the \(A.\) terreus and \(A.\) fumigatus strain used (Fig. S4B). The difference in cytotoxicity caused by \(A.\) terreus and \(A.\) fumigatus, respectively, was even more pronounced in human primary macrophages (Fig. S4C).

Microscopy revealed that phagocytosed \(A.\) fumigatus conidia developed hyphae, which pierced macrophages within the first 12 h (Fig. 3F). In contrast, germination of phagocytosed \(A.\) terreus conidia was not observed after 12 h and rarely after 24 h (Fig. 3F). Non-phagocytosed conidia (approximately 5% of both \(A.\) fumigatus and \(A.\) terreus total conidia) of both species formed filaments to the same extent (Fig. S4A). Thus, we propose that, despite the high survival rate of \(A.\) terreus conidia, the slow germination of phagocytosed \(A.\) terreus conidia compared to \(A.\) fumigatus led to low macrophage damage.

Phagolysosomes containing \(A.\) terreus conidia mature and acidify

Upon phagocytosis, phagosomes mature via a series of fusion events to mature, acidified phagolysosomes. The fusion events are characterized by the recruitment of several marker proteins, including LAMP-1 (lysosome-associated membrane protein) and Cathepsin D (lysosomal aspartyl protease) [42]. In fully matured phagolysosomes, v-ATPase within the membrane builds up a proton gradient resulting in a pH decrease. The low pH provides an optimal environment for phagolysosomal enzymes and is essential for successful killing of most pathogens [43] including \(A.\) fumigatus [24,25]. Viable \(A.\) fumigatus conidia inhibit this acidification [24,25], allowing the fungus to germinate within the phagolysosome and to escape by piercing through the macrophage membrane as shown previously [44] and in our experiments described above (Fig. 3). To determine whether the observed differences in fungal inactivation of \(A.\) terreus and \(A.\) fumigatus by macrophages and the observed macrophage damage might be related to differences in phagosome maturation, we analyzed phagosome maturation using fluorescence microscopy.

Phagosomes of MH-S cells containing pre-swollen conidia of either \(A.\) fumigatus or \(A.\) terreus successfully underwent fusion with lysosomes 3 h (Fig. 4A) and 8 h (data not shown) after infection as indicated by the presence of LAMP-1 and Cathepsin D, characterizing a mature phagolysosome [45]. However, by using the acidic probe Lysotracker DND99 we observed that only phagolysosomes containing \(A.\) terreus conidia were invariably (>99%) acidified at both time points, whereas few \(A.\) fumigatus conidia were found in acidified phagolysosomes (10–15% after 3 h; 25–30% after 8 h) (Fig. 4A). All \(A.\) fumigatus conidia in acidified phagosomes looked deformed. Similar results were obtained using primary murine alveolar macrophages and primary human monocyte-derived macrophages (Fig. S5). Inhibition of acidification by \(A.\) fumigatus and acidification of phagolysosomes

experiments). Statistical analysis was performed using paired t-test. * \(P<0.05\), ** \(P<0.01\), *** \(P<0.001\). Dashed and dotted lines in the histogram show the respective isotope controls while the full lines represent specific fluorescence for \(A.\) fumigatus (thin) and \(A.\) terreus (thick).

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containing A. terreus was likewise observed with two additional strains of each A. fumigatus and A. terreus (Fig. S6). Thus, in combination with our observation that phagocytosed A. terreus conidia remain viable, these findings suggest that A. terreus does not block acidification but survives in acidified phagolysosomes. The persistence of intact A. terreus conidia within phagolysosomes was additionally confirmed by transmission electron microscopy 8 h and 24 h after infection (Fig. 4B).

Figure 2. Role of PRRs in phagocytosis of pre-swollen A. terreus and A. fumigatus conidia by MH-S cells. Results are shown for A. terreus SBUG844 and A. fumigatus CBS14489. Phagocytosis rates are shown as mean±SD from three independent experiments. Statistical analysis was performed using 1-way ANOVA. * P<0.05, ** P<0.01, *** P<0.001. (A) Blocking of dectin-1 by laminarin. (B) Blocking of mannose receptors (MR) by mannan. (C) Blocking of TLR2 or (D) of TLR4 by specific antibodies. (E) Simultaneous blocking of dectin-1 and mannose receptors. (F) Simultaneous blocking of dectin-1, mannose receptors, TLR2 and TLR4.

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A. terreus is trapped in the non-germinated stage but resistant to acidic pH

Since A. terreus conidia were not inactivated after phagocytosis, we hypothesized that resistance to acidic pH may enable A. terreus to survive in the phagolysosome. Thus, we determined the in vitro survival of conidia from both species in phosphate buffer at different pH values without addition of nutrients, a condition which may closely resemble that in phagolysosomes. In this nutrient-limited environment, no germination of either Aspergillus species was observed. However, A. terreus survived incubation at...
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whether persistence in macrophages also occurs
survive for a prolonged time within macrophages. To determine
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Phagocytosed

Figure 4. Maturation of phagolysosomes containing A. fumigatus or A. terreus conidia. Results are shown for A. terreus SBUG844 and A. fumigatus CBS144.89 with MH-S cells. (A) FITC labeled conidia were used. Yellow signal in the merged pictures indicate co-localization of conidia with the specific phagolysosome stain. All lanes show representative fluorescence microscopy pictures. Blue: DAPI (nucleus); green: FITC labeled conidia; red: specific antibody for the phagolysosomal marker indicated on the left. Bars represent a size of 10 μm. (B) Transmission electron micrograph of phagocytosed A. terreus conidia 8 h and 24 h after infection. Bars represent 0.5 μm. Phagocytosed conidia are surrounded by a phospholipid bilayer at both time points.

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pH 3 and 4 to a higher extent than A. fumigatus (Fig. 5A). The addition of nutrients showed that both species germinated at pH values > 5, whereas swelling and germination of conidia was suppressed at lower pH (Fig. 5B). Thus, we assume that while A. terreus conidia survived within the acidified phagolysosome, they were unable to germinate, did not pierce the macrophage membranes and caused less cell damage. In contrast, the ability of A. fumigatus to inhibit phagolysosome acidification allowed rapid germination of conidia at ambient pH, which caused the high cytotoxicity observed after 24 h (Fig. 3).

To determine the effect of phagolysosome acidification on conidial germination, we investigated macrophage damage in the presence of bafilomycin. Bafilomycin is a potent v-ATPase blocker, which inhibits the acidification of phagolysosomes [46]. Treatment with bafilomycin significantly increased cell damage caused by A. terreus (Fig. 5C) to the level observed in macrophages incubated with A. fumigatus conidia in the absence of bafilomycin. In contrast, only a slight increase in cell damage by A. fumigatus was observed after bafilomycin treatment (Fig. 5C). In agreement with increased macrophage damage, A. terreus showed faster germination and piercing of macrophages in bafilomycin-treated macrophages (Fig. 5D). These differences were also observed in other A. fumigatus and A. terreus strains tested (Fig. S7A). In the absence of macrophages, bafilomycin had no influence on fungal germination and growth (Fig. S7B and data not shown).

These results support the hypothesis that inhibition of germination is due to acidification of phagolysosomes, which prevents escape and the accompanied macrophage damage by A. terreus.

Phagocytosed A. terreus conidia survive in the lungs of immunocompetent mice

Our in vivo data suggested that A. terreus conidia are able to survive for a prolonged time within macrophages. To determine whether persistence in macrophages also occurs in vivo, we infected immunocompetent mice intranasally with 1 x 10⁵ A. terreus conidia. All animals remained clinically healthy and displayed no macroscopic alterations at necropsy 24 h, 3 days and 5 days after infection. Semiquantitative isolation of A. terreus from the lungs of these animals revealed persistently high fungal burdens of 5 x 10⁷ CFU and higher without differences between the time points. Histologically, un-swollen but intact conidia within macrophages could be observed in the lungs at all time points (Fig. 6A). No germings or hyphae were found. The lung tissue itself appeared unaltered 24 h and 3 days after infection; however, in all infected mice analyzed on day 5, small, single foci of mononuclear cell infiltrates were found in the lungs (Fig. 6A). The levels of myeloperoxidase (as neutrophil marker), TNFα, INFγ, GM-CSF, IL-1β and IL-6 transiently increased in lungs after infection (Fig. 6B), while levels of IL-10, IL-17A and IL-12 were indistinguishable from PBS controls. Thus, A. terreus conidia persist in vivo for several days within macrophages in the lung despite a detectable immune response.

Recombinant expression of the A. nidulans waA gene allows A. terreus to prevent phagolysosome acidification

The ability of A. fumigatus to inhibit phagolysosome acidification is linked to the grey-greenish spore coloration derived from DHN-
melanin [25,47]. Deletion of the polyketide synthase gene pksP in A. fumigatus inhibits melanin synthesis and the pksP mutant is not able to block phagolysosome acidification, resulting in increased susceptibility to killing by macrophages [25]. A homologous polyketide synthase responsible for conidial coloration is also present in other aspergilli, like A. nidulans or Aspergillus niger [48,49]. In A. nidulans, the green spore color derives from the yellow melanin precursor naphthopyrrole and is produced by the waA gene product [50,51]. Interestingly, although PksP produces DHN-melanin in A. fumigatus, the product formed after recombinant expression of pksP in Aspergillus oryzae is naphthopyrrole, which indicates that the initial products formed by A. nidulans waA and A. fumigatus pksP are identical [52].

Despite the brownish coloration of A. terreus conidia and several polyketide synthase genes present in its genome, A. terreus seems to lack a homologue of pksP and waA (Fig. S8). Consequently, A. terreus produces neither DHN-melanin nor naphthopyrrole (Fig. S9C). While DHN-melanin seems essential for the inhibition of phagolysosome acidification by A. fumigatus, it has not yet been investigated whether naphthopyrrole is also able to prevent acidification. Therefore, we tested the hypothesis that the lack of a pksP-homologue is responsible for the inability of A. terreus to inhibit phagolysosome acidification by introducing the waA gene from A. nidulans into the genome of A. terreus. The mutant with a single integration of the waA gene, denoted as A. terreus waA, showed a yellowish phenotype of conidia comparable to the color of an A. nidulans waA mutant, which lacks a p-diphenol oxidase polymerizing naphthopyrrole [53] (Fig. S9A and S9B). HPLC-MS analysis confirmed that A. terreus waA produced the naphthopyrrole derivatives YWA1 and YWA2 [50] in conidia (Fig. S9D).

Although initial phagocytosis of A. terreus waA conidia was slightly delayed in comparison to A. terreus wild type, phagocytosis rates after 2 h largely resembled the wild type (Fig. 7A). Thus, expression of waA in A. terreus did not seem to have a major impact on phagocytosis. Compared to the wild-type strain, A. terreus waA caused increased damage to MH-S cells, although damage was still significantly less than that caused by A. fumigatus (Fig. 7B). This increase in cytotoxicity was even more pronounced in human monocyte-derived macrophages (Fig. 7C). Consistent with the inhibitory role on phagolysosome acidification proposed for PksP in A. fumigatus, phagolysosomes containing viable A. terreus waA conidia did not acidify in MH-S cells (Fig. 7D), primary murine alveolar macrophages and human monocyte-derived macrophages (Fig. S10). Furthermore, in accordance with our observation that blocking of acidification by bafilomycin allowed faster germination of A. terreus, A. terreus waA germinated and formed filaments within the first 12 h of incubation within macrophages (Fig. 7E). Since the phagocytosis rate of A. terreus waA was comparable to A. terreus wild type, the increased cell damage was unlikely to be mediated by increased numbers of non-phagocytosed A. terreus waA conidia germinating extracellularly. However, cell damage and germination of A. terreus waA was further enhanced by addition of bafilomycin (Fig. 7F and G).

Thus, the observed difference in cytotoxicity of A. terreus waA compared to A. fumigatus could be due to a lower efficiency of

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naphthopyrone in blocking of phagolysosome acidification. In summary, these data demonstrate that the expression of \( W A \) and the production of naphthopyrone was sufficient to significantly alter the interaction of \( A. \) terreus with macrophages, resulting in a phenotype displaying some characteristics of \( A. \) fumigatus interaction with macrophages.

Figure 5. Influence of environmental pH on in vitro survival, germination and effect of bafilomycin on macrophage lysis. Results are shown for \( A. \) terreus SBUG844 and \( A. \) fumigatus CBS144.89. Data shown represent mean±SD from three independent experiments. Statistical analysis was performed using paired t-test (A, C; * \( P < 0.05 \)) or display representative pictures (B, D) from three independent experiments. (A) Relative survival of \( A. \) terreus and \( A. \) fumigatus conidia after incubation in nutrient-free, pH-stabilized buffer. Survival at pH 7 is set to 100%. Left panel: survival after two days, right panel: survival after five days. (B) Germination of \( A. \) terreus and \( A. \) fumigatus conidia in minimal medium with defined pH. All pictures were acquired after 8 h incubation at 37 °C. (C) Relative cytotoxicity (LDH release) mediated by \( A. \) terreus and \( A. \) fumigatus conidia on alveolar macrophages treated with the v-ATPase blocker bafilomycin or without bafilomycin treatment. (D) Brightfield microscopy of \( A. \) terreus in co-incubation with macrophages (MH-S) without (upper lane) and with bafilomycin (lower lane) 12 h. Bafilomycin treatment allows \( A. \) terreus to germinate and escape from macrophages.

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Figure 6. Persistence of *A. terreus* conidia in the lungs of immunocompetent mice. Infection was performed with *A. terreus* SBUG844. (A) Histological sections, Periodic-Acid-Schiff stain. 24 h, day 3, day 5: time after infection. Upper row: overview 4× magnified; the arrow indicates immune cell infiltrate. Lower row: 100× magnified; arrows indicate *A. terreus* conidia stained in pink. Scale bars represent 100 μm (overview) and 10 μm (100×). (B) Immune response in the lungs determined by myeloperoxidase and cytokine levels. White bars: PBS-mock infected mice; grey bars: *A. terreus* infected mice. Data shown as mean±SD from 5 animals per group. Statistical analysis was performed per time point by unpaired, two-tailed t-test. *P<0.05; **P<0.01; ***P<0.001.

doi:10.1371/journal.pone.0031223.g006
A. terreus wA displays enhanced virulence in a mouse model of aspergillosis

Given that wild type A. terreus conidia persist within alveolar macrophages of immunocompetent mice in vivo, we postulated that the accelerated escape of A. terreus wA conidia from macrophages observed in vitro could influence virulence in vivo. To test this hypothesis, we infected mice rendered either leukopenic by cyclophosphamide, or immunosuppressed by cortisone acetate, intranasally with wild type A. terreus or A. terreus wA, respectively. Survival curves of leukemic mice infected with A. terreus wA were indistinguishable from animals infected with wild type A. terreus (Fig. 8A). Histologically, invasive fungal growth and tissue necrosis were observed in the lungs of moribund mice in both groups (Fig. S11). In contrast, cortisone acetate treated mice infected with A. terreus wA succumbed faster and at a significantly higher proportion than mice infected with A. terreus wild type (Fig. 3B). Lung pathology was characterized by fungal mycelium surrounded by extensive immune cell infiltrates dominated by neutrophils in moribund animals of either group (Fig. S11). However, immune cell infiltrates on day 3 after infection were more prominent in mice infected with A. terreus wA (Fig. 8C). Furthermore, A. terreus wild type was isolated from lungs of all surviving animals 14 days after infection and conidia residing within macrophages were found in all lungs at this late time point (Fig. S11). Non-germinated conidia were likewise observed in the lungs of moribund mice infected with A. terreus wild type in lung regions not showing cellular infiltrates. Myeloperoxidase, a marker for neutrophils, was >250-fold increased in the lungs of all moribund animals compared to PBS controls (Fig. S11). However, myeloperoxidase in surviving animals infected with A. terreus wild type was 10-fold lower than in moribund mice (Fig. S11), consistent with histological analysis, which showed little or no inflammatory cell infiltrates (Fig. S11). These results suggest that either inflammation was less pronounced in surviving animals compared to moribund animals, or that these animals might have survived severe acute inflammation which resolved over time.

Discussion

Several studies have focused on the interaction of A. fumigatus conidia with alveolar macrophages, elucidating mechanisms involved in the uptake of conidia, the fate of conidia within phagolysosomes and the outbreak of hyphae from macrophages [24,44]. However, it has not been addressed whether mechanisms used by A. fumigatus represent common strategies used by all Aspergillus spp. able to cause invasive aspergillosis. Although infections with A. terreus are less frequently observed than those with A. fumigatus, the prognosis for patients is poor [54]. We propose that the outcome of the initial confrontation of inhaled conidia with alveolar macrophages plays a key role in either fungal clearance, persistence, or initiation of lung infections. Furthermore, different survival strategies used by different Aspergillus species may lead to different pathological patterns.

Previous studies have shown that the uptake of A. fumigatus conidia by alveolar macrophages is a dynamic process and that the number of phagocytosed conidia increases over time [55,56]. We likewise observed a time dependent increase of A. terreus phagocytosis. However, uptake of A. terreus conidia was significantly faster than phagocytosis of A. fumigatus conidia, especially if resting conidia were used. Rapid complete uptake of A. terreus conidia was likewise observed by Green et al. [57] in vivo. Phagocytosis kinetics are influenced by two factors: (i) form and size of the particle and (ii) interaction with specific cell surface receptors, e.g. PAMPs and PRRs [58]. A. terreus conidia are comparatively smaller than A. fumigatus conidia, which might impact phagocytosis. However, we showed that the size difference of resting and swollen A. terreus conidia did not affect phagocytosis rates (Fig. S2 and S3). Thus, the difference in the size of conidia is unlikely to fully explain the different phagocytosis rates of A. fumigatus and A. terreus. Importantly, we observed higher exposure of β-1,3-glucan and galactomannan on the surface of pre-swollen resting and swollen conidia in agreement with the microscopical analysis of β-1,3-glucan exposure performed by Deak et al. [59]. This exposure is likely to contribute to or mediate the faster phagocytosis rates of A. terreus conidia. However, it should be noted that prolonged pre-incubation of A. fumigatus conidia leads to a strong increase of β-1,3-glucan exposure on the surface of early germlings that impacts recognition and uptake by macrophages [22,23].

Recognition of β-1,3-glucan by macrophages presented on A. fumigatus germlings is mediated by dectin-1 [22,23] while recognition of mannans is more complex and involves a variety of pathogen recognition receptors, including the mannose receptors, TLR-2 and TLR-4 [34,60]. Our blocking experiments demonstrate that dectin-1 and mannose receptors are the main PRRs mediating phagocytosis of A. terreus conidia. In contrast, even the combined blocking of dectin-1 and mannose receptors and simultaneous blocking of the four receptors dectin-1, mannose receptors, TLR-2 and TLR-4 only moderately reduced phagocytosis of A. fumigatus conidia. Thus, additional PRRs, for example DC-SIGN (dendritic cell-specific ICAM-3-grabbing nonintegrin) [61], may be required for phagocytosis of A. fumigatus, but not A. terreus conidia.

As mentioned above, the pre-swelling time used significantly influences PAMP exposure on A. fumigatus conidia and longer pre-swelling exposes β-1,3-glucan facilitating interaction with dectin-1 [22]. In resting A. fumigatus conidia, immunogenic molecules are masked by the rodlet layer [12]. However, the immunogenic molecules become exposed during germination, thus explaining enhanced phagocytosis of pre-swollen A. fumigatus conidia. The
higher exposure of β-1,3-glucan and galactomannan on the surface of resting *A. terreus* conidia and the high phagocytosis rate raises the question to which extend a similar masking rodlet layer is present on *A. terreus*.

One function of alveolar macrophages is the elimination of inhaled pathogens by intracellular killing after phagocytosis. We determined killing of *A. fumigatus* and *A. terreus* conidia by two independent methods: CFU plating and analysis on the single-conidia level using a metabolic dye [62]. Results obtained with either method showed significantly higher survival rates of *A. terreus* compared to *A. fumigatus*. This effect was independent from the fungal strain and type of macrophage used. In contrast, Perkhofer et al. [63] did not observe these striking differences. In their study, survival was analyzed after a short two hour co-incubation using resting instead of pre-swollen conidia. Since the initial swelling process is essential for conidia inactivation [64], this experimental setup might have masked the differences between both species.

Besides an impact of macrophage NADPH oxidase in killing of *A. fumigatus* conidia [64], microbial inactivation by macrophages has been shown to be associated with acidification of the phagolysosome [24,25]. Similar to other pathogenic fungi [16], *A. fumigatus* is able to inhibit phagolysosome acidification at least to

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**Figure 8. Outcome of infection with *A. terreus* and *A. terreus wA* in immunocompromised mice.** (A, B) Kaplan-Meyer survival curves analyzed by log rank test. (A) Leukopenic mice immunosuppressed with cyclophosphamide. (B) Mice immunosuppressed with cortisone acetate. (C) Histology 24 h, day 3 and day 5 post infection (p. inf.). Periodic Acid Shiff stain, fungal elements stain pink. Magnifications are indicated below the columns; scale bars represent 100 μm (10×) and 10 μm (40× and 100×). Upper row: *A. terreus* wild type. Lower row: *A. terreus wA*.

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a certain extent [25]. Surprisingly, despite the high survival rates of phagocytosed conidia, phagolysosomes containing A. terreus acidified frequently, independent of the macrophage type and A. terreus strain used, suggesting that (i) A. terreus lacks the ability to block acidification and (ii) A. terreus is not inactivated within mature acidic phagolysosomes. Supporting the later, we found that low pH does not impact survival of A. terreus conidia under nutrient limited conditions, although acidic pH inhibits germination. In contrast, survival of A. fumigatus conidia was significantly impaired at low environmental pH, highlighting the need of A. fumigatus to inhibit phagolysosome acidification. However, it should be noted that survival within acidified phagolysosomes does not only depend on pH resistance, but also on the resistance against microbicidal enzymes and compounds, which display highest activity at low pH.

Since conidia are more resistant to damage by adverse environmental conditions than hyphae [63], the delayed germination of A. terreus at low pH might enhance persistence in phagolysosomes. In contrast, rapid germination due to inhibition of acidification allows A. fumigatus to escape from macrophages, but also renders the fungal cells more vulnerable to antifungal host molecules. This might explain the observed inactivation of a significant proportion of A. fumigatus conidia during the time course of co-incubation.

Alveolar macrophages have been shown to efficiently clear A. fumigatus conidia in immunocompetent mice [64], resulting in steadily decreasing fungal burdens [66,67]. In contrast, we found A. terreus conidia to persist in stable numbers in the lungs of immunocompetent mice. Histologically, we observed conidia within alveolar macrophages, suggesting that persistence occurs within these immune cells. Although several cytokines and myeloperoxidase significantly increased in immunocompetent mice infected with A. terreus, the absolute increase was lower than described for A. fumigatus by others [66,67]. Furthermore, immunocompetent mice infected with A. fumigatus rapidly recruit immune cells to the site of infection, peaking two days after infection and resolving towards day 4 [67]. In comparison, immune cell recruitment after A. terreus infection was delayed. These results suggest that the differences in macrophage interaction observed for the two fungal species in vivo have significant consequences for fungal survival and inflammation in vivo. It appears that divergent strategies have evolved within pathogenic Aspergillus species: While A. terreus survives within acidified phagolysosomes of macrophages, A. fumigatus interferes with phagolysosome maturation, germinates and eventually escapes from macrophages. This hints to different strategies to evade the recognition by the immune system. Similarly, different strategies for phagolysosomal survival have also been described for other pathogenic fungi [16].

It has been shown that the ability of A. fumigatus to prevent phagolysosome maturation depends on the presence of a functional pksP gene. PksP is essential for the synthesis of DHN-melanin and deletion of pksP results in white colored conidia [72]. In agreement with the proposed role of DHN-melanin in preventing acidification [73], an A. fumigatus pksP deletion mutant cannot inhibit acidification of phagolysosomes and shows altered conidia morphology [47] with significantly increased β-1,3-glucan exposure [23]. Thus, this mutant is killed to a higher rate by macrophages [25]. A. terreus lacks this specific pksP gene and, although the origin of its pigment is unknown, its conidia color appears not to be DHN-melanin related. Expression of the pksP homologue wA from A. nidulans in A. terreus, which leads to the production of the heptaketide naphthopyrone YWA1 [52], was sufficient to prevent acidification of phagolysosomes for the majority of intracellular conidia. This result emphasizes the role of naphthopyrone derivatives in the interaction of Aspergillus species with macrophages. However, the reduced acidification by A. terreus wA conidia was not sufficient to increase fungal cytotoxicity to the level of A. fumigatus and addition of fluconazole still enhanced cytotoxicity of A. terreus wA mutants. This suggests that (i) naphthopyrone might be less efficient than DHN-melanin in interfering with phagolysosome acidification, (ii) that the expression level of wA in A. terreus wA does not reach the level of DHN-melanin in A. fumigatus or (iii) that additional, yet unidentified, A. fumigatus factors absent in A. terreus interfere with phagolysosome maturation. Furthermore, it should be noted that the lack of naphthopyrone production alone does not sufficiently explain the difference between A. terreus and A. fumigatus in the interaction with macrophages. For example the mechanisms leading to the high survival rates of A. terreus in acidified phagolysosomes remain to be determined.

It is tempting to speculate that the lack of a pksP homologue in A. terreus contributes to the lower incidence of A. terreus infections compared to A. fumigatus, as the impact of pksP on infection in vivo has been demonstrated for A. fumigatus [72]. However, the distribution of pksP homologues within aspergilli does not directly correlate with virulence in mouse models: Both, A. nidulans and A. niger possess pksP homologues and show a green or black spore coloration. Nonetheless, only A. niger is highly virulent in mice [74] while A. nidulans [75,76] shows a more attenuated phenotype. Therefore, we investigated the virulence of the wA expressing A. terreus mutant in leucopenic and cortisone acetate treated mice.

For A. fumigatus it has been shown that the outcome of infection in leucopenic animals is mainly determined by fungal growth [77], due to the absence of the recruited cellular immune response [78]. At the inoculum used, resident macrophages are not able to prevent myelica formation. In agreement, no differences in virulence were observed between A. terreus wild type and A. terreus wA in this model, suggesting that A. terreus wA does not have a general growth advantage within the lung.

Corticosteroid treatment does not affect phagocytosis of A. fumigatus by alveolar macrophages but impairs the ability of macrophages to kill ingested conidia [64,79]. Thus, due to the low frequency of germination of A. terreus wild-type conidia after phagocytosis by macrophages and the accelerated escape of A. terreus wA conidia, we expected significant differences in the outcome of infection in the corticosteroid-based model. In agreement, animals infected with A. terreus wA succumbed to infection faster and to a significantly higher rate. As persisting, non-germinated conidia were found in all lungs of mice surviving A. terreus wild type infection, the difference in virulence could be explained by a comparatively lower induction of inflammation in surviving mice, due to a lower percentage of conidia which germinate. The assumption of low germination rates and persistence is also in agreement with the isolation of fungal burdens from immunocompetent mice. After infection with 1×10⁷ A. terreus wild type conidia, at least 5×10⁴ conidia were isolated five days after infection of immunocompetent mice [67]. Thus, fast escape from macrophages (A. terreus wA and A. fumigatus) appears to cause increased virulence in corticosteroid treated animals, whereas a “sit and wait” strategy (A. terreus) allows long-term persistence, especially in immunocompetent hosts. However, we cannot exclude that expression of wA in A. terreus affects...
general metabolism or expression of virulence-associated factors and, therefore, might indirectly affect virulence. It should also be noted that, in general, the virulence potential of aspergilli is the result of a multitude of factors which contribute to survival and growth within the host. Thus, it is likely that in addition to pigment production other factors influence the virulence potential of different Aspergillus species.

Interestingly, A. terreus infections are accompanied by high dissemination rates [5,11]. We speculate that dissemination might be due to persistence in macrophages. By hiding from immune responses, A. terreus might use macrophages as a vehicle to reach secondary organs, establishing disease under severe immunosuppression. Macrophages as vehicle for dissemination have been described for other microbes [80,81,82].

It should be noted that A. terreus also produces accessory conidia, which differ from the phialidic conidia used in this study in surface structure, cell wall composition and germination kinetics [59]. Furthermore, accessory conidia have been shown to expose higher levels of surface β-glucan, associated with a stronger immune response compared to phialidic conidia in vivo and in vitro [36]. It thus appears likely that the interaction of accessory conidia with macrophages might differ significantly from phialidic conidia; however, phialidic conidia are the commonly produced distributable spores and thus represent the primary infectious structures of A. terreus.

In summary, A. fumigatus and A. terreus demonstrate striking differences in the interaction with macrophages. A. fumigatus prevents phagolysosome acidification due to the presence of DHN-melanin, germinates and escapes from macrophages, leading to establishment of invasive disease in immunocompromised hosts. In contrast, A. terreus is rapidly recognized and phagocytosed by macrophages and persists without germination in acidified phagolysosomes. It appears possible that A. terreus conidia inhaled by patients prior to severe immunosuppression persist in a resting, but viable state. Thus, development of disease by A. terreus might progress more slowly and require a stronger immunosuppression regimen than A. fumigatus but does not necessarily require a de novo infection due to long-term persistence in macrophages.

Materials and Methods

Ethics statement

The use of human primary cells in this study was conducted according to the principles expressed in the Declaration of Helsinki. All protocols used in this study were approved by the local ethics committee of the University Hospital Jena (Permit Number: 2207-01/08; studies on the interaction of human pathogenic fungi with blood and blood components). Written informed consent was provided by all study participants.

All animals used in this study were housed in groups of five in individually ventilated cages and were cared for in strict accordance to the principles outlined in the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (http://conventions.coe.int/Treaty/en/Treaties/Html/123.htm). All animal experiments and protocols were in compliance with the German Animal Welfare Act. All protocols were approved by the responsible Federal State authority (Thüringer Landesamtes für Lebensmittelsicherheit und Verbraucherschutz) and its ethics committee (Permit Number: 03-012/10). All efforts were made to minimize suffering. Animals were clinically monitored at least twice daily by a veterinarian and humanely sacrificed if moribund (defined by lethargy, dyspnoea, hypothermia and weight loss).

Fungal strains and growth conditions

All experiments were performed with A. terreus isolate SBUG844 (HKI strain collection, Jena, Germany, [83]), and A. fumigatus isolate CBS 144.89 (CBS, Utrecht, Netherlands). For selected experiments, A. terreus A1156 (NIH 2624, Fungal genetics stock center, Kansas; USA) and the clinical isolate T9 (kindly provided by Cornelia Lass-Flór, Innsbruck, Austria), as well as A. fumigatus ATCC 1293 (ATCC, Manassas, USA) and ATCC 46645 (ATCC, Manassas, USA) were used. Strains were grown on malt extract agar slants for 7 days at room temperature. Conidia were harvested in 5 ml sterile phosphate buffered saline (PBS, PAA Laboratories, Coelbe, Germany) and filtered at least once through a 40 μm cell-strainer (BD, Heidelberg, Germany). If not indicated otherwise, pre-swollen conidia were obtained after 7 h (A. terreus) or 3.5 h (A. fumigatus) incubation in sterile RPMI 1640 (PAA Laboratories) supplemented with 10% heat-inactivated fetal bovine serum (FBS, PAA Laboratories) at 37°C and 20 rpm shaking [64]. Heat inactivation was facilitated by exposing the FBS 20 min to 56°C. After incubation, cells were centrifuged, washed three times with sterile PBS and again filtered through a 40 μm cell-strainer.

For phagocytosis and killing assays, conidia were pre-labeled with FITC after swelling as described elsewhere [84]. Conidia were counted with a Neubauer counting chamber and diluted in sterile PBS or RPMI with 10% FBS to give the desired concentration. Absence of clumping was confirmed in all conidia preparations by microscopy immediately prior to use.

Macrophase cell lines

The alveolar macrophase cell line MH-S, derived from the lung of a Balb/C mouse and immortalized by simian virus SV40 transfection (ATCC, Manassas, USA), was routinely kept in RPMI 1640 supplemented with 10% heat-inactivated FBS. These alveolar macrophages display the morphologic features and functions of normal, mature alveolar macrophages which were derived from bronchioalveolar lavages [85]. The peritoneal macrophase-like cell line J774.A1 (DSMZ, Braunschweig, Germany) derived from an ascites of a Balb/C mouse and was kept in Dulbecco’s modified Eagle’s medium (DMEM, PAA Laboratories) with 10% heat-inactivated FBS. Both cell lines were maintained and propagated at 37°C and 5% CO₂ atmosphere. These cells show the morphologic, cytotoxic and adherence properties of primary peritoneal macrophages [86].

For interaction experiments, macrophages were used between passages 10 and 20 and seeded at a concentration of 2×10⁵ per well in 8 well chamber slides (Nunc, Roskilde, Denmark) 24 h prior to co-incubation with conidia, leading to a confluent monolayer at the time of experimentation.

Primary macrophages

Human peripheral blood mononuclear cells (PBMCs) were isolated with Histopaque-1077 (Sigma-Aldrich) density centrifugation fromuffy coats donated by healthy volunteers. To differentiate PBMC into monocyte-derived macrophages (MDMs), 2×10⁵ PBMCs were plated in RPMI 1640 media (with L-glutamine and 25 mM HEPES, PAA Laboratories, Inc.) containing 10% heat-inactivated FBS (Gibco, Invitrogen) in cell culture dishes. Ten ng/ml human M-CSF (ImmunoTools, Friesoythe, Germany) was added to the cultures to induce differentiation into macrophages. After 5 days at 5% CO₂ and 37°C, non-adherent cells were removed and purity of MDMs was determined by staining with an anti-CD14 antibody and flow cytometry. The percentage of CD14-positive cells was ≥80%. All experiments
were performed with cells isolated from at least three different donors.

For infection experiments, adherent MDMs were detached with 50 mM EDTA and plated in flat-bottom 96 or 24 well plates containing RPMI 1640 with 10% FBS to give a final concentration of approximately 5 x 10^5 cells/well or 2 x 10^5 cells/well, respectively. For microscopic analyses cells were allowed to adhere over night to 8-well chamber slides prior to infection.

Murine alveolar macrophages and bone marrow-derived macrophages were isolated from healthy, specific pathogen free eight to ten weeks old CD-1 mice. In brief, mice were euthanized, and the trachea was prepared in situ. 1 ml of sterile PBS was instilled via the trachea into the lung and the procedure was repeated twice. The bronchoalveolar lavage fluid of ten mice was pooled and centrifuged for 10 min at 300 x g. The pellet was resuspended in RPMI 1640 supplemented with 10% FBS and 1% Penicillin/Streptomycin (PAA Laboratories). Cells were plated in chamber slides at a density of 2 x 10^5 cells/well and used one day after inoculation. For bone marrow-derived macrophages, the femurs were aseptically opened and the bone marrow was removed by washing with 2 ml of sterile, ice-cold PBS. After washing, the cell suspension was vortexed vigorously. The cells were pelleted by centrifugation for 10 min at 300 x g, resuspended in RPMI 1640 supplemented with 10% FBS and filtered through a 40 μm cell strainer (BD). Cells were seeded at 1 x 10^6 cells/well in 6-well polystyrene plates containing RPMI 1640 supplemented with 10% FBS, 1% Penicillin/Streptomycin and 10 ng/ml murine M-CSF (ImmunoTools, Friesoythe, Germany). After five days of incubation at 37 °C and 5% CO2, non-adherent cells were removed by washing three times with sterile PBS. Fresh RPMI 1640 supplemented with 10% FBS was added prior to challenge with conidia.

**Phagocytosis assay**

Phagocytosis assays were performed as described previously [24] with some modifications. Macrophage cell lines were plated in 8-well chamber slides in RPMI or DMEM, depending on the cell line, supplemented with 10% heat-inactivated FBS. Cells were infected with an MOI of 0.1 (1 conidium per 10 macrophages). After brief centrifugation, slides were further incubated for up to 12 h. At the end of each incubation period the assay medium was removed and cells were fixed directly with Histofix® (Roth, Karlsruhe, Germany) for 10 min at room temperature (RT). After washing with 0.05 M NH4Cl, cells were incubated for 10 min in 0.05 M NH4Cl dissolved in PBS.

Prior to addition of antibodies, cells were blocked for 30 min at RT with 1% bovine serum albumin (BSA; Sigma). After blocking, slides were incubated for 30 min at RT with a specific rabbit anti-conidia antiserum at a dilution of 1:50 (kindly provided by Frank Ebel). For fluorescence detection, slides were incubated with a specific anti-rabbit Texas Red conjugated antibody (Invitrogen, Groningen, Germany) diluted 1:1000 in blocking solution for 30 min at room temperature in the dark.

Slides were washed three times with PBS and analyzed by microscopy. Phagocytosed conidia only showed a bright green fluorescence (deriving from FITC-labeling) while extracellular conidia additionally showed a red signal. Phagocytosis rate in percent was determined as the number of ingested conidia per 1000 conidia counted. Three individual wells were evaluated per slide, strain and time point and experiments were performed in biological triplicates.

**Receptor blocking assay**

For dectin-1 blocking, macrophages were incubated for 10 min prior to infection on ice in culture medium containing 10% heat-inactivated FBS with 2 mg/ml laminarin from Laminaria digitata (Sigma). Mannose receptors were blocked by addition of 2 mg/ml mannan from Saccharomyces cerevisiae (Sigma) to the culture medium 15 min prior to infection [87]. Blocking of TLR2 and TLR4 was performed over night at 37 °C before infection by adding 15 μg/ml of specific antibodies (anti-TLR2: affinity purified anti-mouse Toll-like receptor 2 (CD282, Thermo, USA); clone 6C2, eBioscience, Frankfurt, Germany; anti-TLR4: affinity purified anti-mouse Toll-like receptor 4 (TLR4/MD-2, clone MTS510, eBioscience) [88].

Prior to infection, the medium containing blocking reagents was removed, the cells were washed three times with sterile PBS (PAA) and then infected with conidia in medium containing 10% heat-inactivated FBS. After infection with pre-swollen conidia the standard phagocytosis assay was performed as described above.

**Flow cytometric analysis of conidia**

Flow cytometry was used to quantify the β-1,3-glucan and galactomannan exposure of *A. terreus* and *A. fumigatus* on resting and pre-swollen conidia by using specific antibodies (mouse IgG2b for β-1,3-Glucan [89]; mouse IgM anti-galactomannan [90]; isotype controls: mouse IgG2b/IgM; eBioscience). Resting and pre-swollen conidia were investigated in parallel. Resting and pre-swollen conidia were incubated for 1 h in Histofix® (Roth) at RT, then for 1 h in NH4Cl (0.05 M) and finally washed three times with PBS. To inhibit unspecific binding of primary antibodies, conidia were incubated in 1% BSA for 1 h at RT. After washing with PBS, conidia were incubated at 4 °C overnight on a vertical rotator with primary antibodies diluted in blocking solution. Following three times washing with PBS a FITC-labeled goat anti-mouse IgG/IgM antibody (Invitrogen) was applied, and conidia were incubated for 3 h at RT in the dark. After additional five washings with PBS, measurement was performed in flow cytometry buffer (BD) on a Becton Dickinson LSRII. 20,000 events were counted. At least three biological replicates were analyzed. Data were further analyzed with the FlowJo software (Tree Star, Ashland, USA).

**Killing assay**

To quantify killing of conidia by macrophages, co-incubations of macrophages and pre-swollen conidia [performed as described for phagocytosis assay] were stained with the FUN-1 dye (Invitrogen) [62] according to the manufacturers guidelines, resulting in distinct fluorescence patterns. Living conidia showed green to yellowish cytoplasm with a bright red vacuole, while dead conidia showed a yellow cytoplasm (Axio Imager M1, Filter Set 01; BP 365/12, FT 395, LP 397, Zeiss, Jena, Germany). Viability of conidia without macrophages was assessed in each experiment. Both *Aspergillus* species were analyzed in parallel. Killing rate was determined from the number of inactivated/dead conidia per number of conidia investigated. In each experiment 1000 conidia per strain were evaluated. The experiments were performed in three biological replications each containing three technical replicates. Alternatively, killing was quantified as CFUs. Here, either primary murine BMDMs or the alveolar macrophage cell line MH-S were plated at a density of 1 x 10^6 cells/well in 6 well polystyrene plates one night prior to infection. Conidia of the indicated strains were added at an MOI of 0.1 and incubated for 5 h and 10 h. After incubation, macrophages were lysed with ice-cold sterile deionized water for 15 min. After lysis the cells were scraped from the bottom of the well with a rubber policeman and the suspension was diluted for plating. Serial dilutions were plated on malt extract agar plates and incubated for at least 36 h at 37 °C. The percentage of survival was calculated with reference to control conidia incubated in the absence of cells.

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**Aspergilli Differ in Their Macrophage Interactions**

[Reference Text]

[62] This reference is not provided in the text, but it seems to be a key component of the study. Further reading would be necessary to complete the connection.**
Macrophage monolayer damage assay
Damage to macrophages after co-incubation with pre-swollen conidia (performed as described for phagocytosis assay) was determined as the release of lactate dehydrogenase (LDH) into the medium using a Cytotoxicity Kit-LDH (Roche, Mannheim, Germany). The assays were performed according to the manufacturer’s instructions and the measurements from three biological replications containing technical triplicates were evaluated. To measure cell damage, the following calculation was used: 100 × LDH release (infected macrophages−uninfected macrophages−conidia only)/(100% lysed macrophages−uninfected macrophages) = relative cytotoxicity (%). Uninfected macrophages, conidia only and 100% macrophage lysis (achieved by adding 0.2% Triton X-100, Applichem) served as controls and were determined individually for each treatment and incubation period.

Transmission electron microscopy
For TEM, alveolar macrophages were grown in 24 well plates with a polystyrene bottom. A. terreus was added at a MOI of 1. Eight and 24 h post infection, samples were washed 3 times with PBS to remove non-adherent cells. Samples were then fixed with Karnovsky fixative (3% paraformaldehyde, 3.6% glutaraldehyde, pH 7.2) for 24 h. Post-fixation was based on 1.0% osmium tetroxide containing 1.5% K-ferricyanide in 0.1 M cacodylate buffer for 2 h. Following standard methods, blocks were embedded in glycid ether and cut using an ultra microtome (Ultracut, Reichert, Vienna, Austria). Ultra-thin sections (30 nm) were mounted on copper grids and analyzed using a Zeiss LIBRA 120 transmission electron microscope (Carl Zeiss, Oberkochen, Germany) operating at 120 kV.

pH resistance of conidia
1 × 10⁶ pre-swollen conidia were incubated for up to seven days at 37°C and 220 rpm in 1 ml 0.1 M sodium phosphate buffer supplemented with 0.3 M potassium chloride and adjusted to the desired pH. From different time points, suspensions were analyzed microscopically and serial dilutions were plated in triplicate on malt extract agar plates (Applichem). Additionally, the pH of the buffer was measured to confirm that the suspension maintained at the desired pH. CFU were counted after 24 h incubation at 37°C. Experiments were performed in triplicates. Survival at pH 7 was set as 100%, and the relative survival compared to pH 7 was determined. For microscopical analysis of the germination speed at different pH values, resting conidia were incubated for 8 h at 37°C and 5% CO₂ in Aspergillus minimal medium [80] with the pH adjusted to the desired value.

Inhibition of macrophage v-ATPase
To inhibit phagolysosome acidification, bafilomycin A1 (Sigma) was added to macrophage monolayers to a final concentration of 25 nM 10 min prior to infection. After 24 h the relative cytotoxicity was determined by LDH release as described above. Cells treated with bafilomycin in the absence of conidia served as controls.

Fluorescence microscopy of phagolysosome maturation
Phagolysosome maturation was determined from co-incubations with pre-swollen conidia in 8-well chamber slides. For immunofluorescence analysis, cells were fixed after 3 h and 8 h of co-incubation, permeabilized for 15 min with 0.1% Triton X-100 (Applichem) and blocked for 30 min with Image IT FX signal enhancer (Invitrogen). After incubation, samples were washed with PBS and either incubated for 1 h at RT with the primary antibody against Lamp-1 (rat anti-mouse Lamp-1 clone 1D4B Alexa Fluor 647; Santa Cruz Biotechnology, Heidelberg, Germany; 1:100 diluted in 1% BSA); or with a Cathepsin D specific antibody (rabbit anti-mouse Cathepsin D antibody clone EPR3057Y; Epitomics Inc., Burlingame, United States; 1:1000 diluted in 1% BSA). For immunostaining against Cathepsin D an additional 1 h incubation step at RT with a monoclonal goat anti-rabbit DyLight 549 conjugated antibody (Rockland Immunochemicals, Gilbertsville, United States) diluted 1:5000 in blocking solution was performed. For investigating the acidification of the phagolysosome, cells were pre-incubated 2 h prior to infection at 37°C with Lysotracker DND99 (Invitrogen) diluted 1:5000 in RPMI supplemented with 10% FBS. After co-incubation for 3 h and 8 h with conidia, cells were fixed as described above, and investigated under a Zeiss LSM 710 microscope (Carl Zeiss, Jena, Germany).

Murine infection models
Female specific-pathogen-free inbred Balb/C mice (6–8 weeks old, 18 to 20 g) were used in the studies for the leucopenic model, while female specific-pathogen-free outbred CD-1 (6–8 weeks old, 18 to 22 g) were used in the corticosteroid model (Charles River, Germany).

For the leucopenic infection model, animals were immunosuppressed by intraperitoneal (i.p.) injections of cyclophosphamide (140 mg/kg; Sigma, Germany) on days −4, −1, 2, 5, 8, 11 and an additional dose of cortisone acetate (200 mg/kg, Sigma, Germany) subcutaneously (s.c.) on day −1. For the corticosteroid based model, i.p. injections of cortisone acetate (25 mg/mouse) were applied on days −3 and 0 prior to infection.

The effect of the administered drugs was assessed by differential blood cell counts before immunosuppression, before infection and on the day of post mortem analysis.

Studies in immunocompetent mice were carried out by using specific-pathogen-free CD-1 mice untreated prior to infection.

Mice were challenged under general anaesthesia on day 0 intranasally with either 5 × 10⁶ (cyclophosphamide) or 1 × 10⁷ (cortisone acetate/immunocompetent) conidia in 20 μl sterile PBS. Control animals received 20 μl of sterile PBS. For general anaesthesia a mixture of Medetomidin (0.5 mg/kg, Fort Dodge Veterinär GmbH, Würselen, Germany), Midazolam (5 mg/kg, Roche, Mannheim, Germany) and Fentanyl (0.05 mg/kg, Janssen-Cilag, Neuss, Germany) was used and directly antagonized after infection with Antipamezol (2.5 mg/kg, Pfizer GmbH, Berlin, Germany), Flumazenil (0.5 mg/kg, Roche, Mannheim, Germany) and Naloxon (1.2 mg/kg, Hameln Pharma Plus GmbH, Hameln, Germany).

In time course experiments five mice per group were sacrificed 24 h, 72 h and on day 5 after infection. Gross pathological changes were recorded during post mortem analysis. All animals were further analyzed for histological alterations, MPO content in the target organs and cytokines.

Histology
The right lung of every animal used in this study was fixed in buffered formalin, flatly embedded in paraffin and 5 μm sections were stained with periodic-acid Schiff (PAS) stain using standard protocols. Two distinct sections from every lung were examined qualitatively by light microscopy.

Generation of A. terreus strains heterologously expressing the A. nidulans WaA gene
In order to generate an A. terreus strain capable of producing the A. nidulans conidial pigment naphtopyrone, the responsible
polyketide synthase gene \( \text{acl} \) from \( \text{A. nidulans} \) (gene accession X65866) with its native promoter and terminator (\text{‘}\text{acl-P}+\text{T}’\text{‘}); 8131 bp) was amplified from the genomic DNA of the \( \text{A. nidulans} \) reference strain FGSC A4. The Takara PrimeSTAR HS DNA polymerase (MoBiTec, Göttingen, Germany) with oligonucleotides NotI_AN_8209_f1 (5’-GGGCCGCCG TTCATTTGCCAAAGCCAC-3’) and NdeI_AN_8209_r13 (5’-CATATGGTGCCGACACTCTATGACG-3’) was used with the following program: initial denaturation for 4 min at 95°C; 31 cycles with denaturation for 30 s at 95°C, annealing for 30 s at 55°C, elongation for 9 min at 72°C; final elongation for 20 min at 72°C. The gel purified fragment was ligated into pJET1.2/blunt (Fermentas, St. Leon-Rot, Germany) and further subcloned via NotI restriction (Fermentas) and the pyrithiamine resistance \( \text{ptrA} \) cassette from plasmid pJET-pUC19 [91] was inserted. Seven µg of plasmid DNA were used for protoplast transformation of \( \text{A. terreus} \) SBU1844 as described previously [83] using 0.1 µg/ml pyrithiamine hydrobromide (Sigma Aldrich, Hamburg, Germany) as selection marker. A digoxigenin-labeled probe (Roche, Mannheim, Germany) directed against the \( \text{acl} \) gene was generated by PCR amplification with oligonucleotides AN_8209_f9 (5’-CTACAGCCGACTTTCC-3’) and AN_8209_r11 (5’-GGAAGGAGCAATGTGCGAGGATC-3’). This probe was used for Southern blot analysis on genomic DNA of transformants to determine the number of genomic integrations. Naphthopyrone production from \( \text{A. terreus} \) was quantified by HPLC (Betasil C18 column (2.1 mm × 150 mm); 3 µm, Thermo Scientific) combined with an Exactive mass spectrometer (Thermo Scientific) operating in positive ionisation mode. Separation was carried out on a Betasil C18 column (2.1 mm × 150 mm); 3 µm, Thermo Scientific) using water (solvent A) and acetonitrile (solvent B), each containing 0.1% formic acid, as binary solvent system. A flow rate of 250 µL/min and the following gradient was used: 0–1 min = 5% B; 1–16 min = 5%–98% B; 16–19 min = 98% B; 19–20 min = 98%–5% B.

Detection of naphthopyrone from conidia of \( \text{A. terreus} \) \( \text{wA} \)

To detect naphthopyrone from conidia of strain \( \text{A. terreus wA} \), conidia were harvested in water from malt extract agar plates. The suspension was mixed with an equal volume of ethyl acetate and stirred for 10 min using an Ultra Turrax at 20 000 rpm. The ethylacetate phase was removed and extraction was repeated two additional times. Ethyl acetate was evaporated and the resulting dried extract was solved in methanol. The same procedure was repeated for the \( \text{A. terreus} \) wild-type strain. High resolution electrospray ionisation mass spectrometry (HR-ESI MS) was carried out on an Accela UPLC-system (Thermo Scientific) combined with an Exactive mass spectrometer (Thermo Scientific) operating in positive ionisation mode.

Myeloperoxidase and cytokine quantification

Organs were homogenized in tissue lysis buffer (200 mM NaCl, 5 mM EDTA; 10 mM Tris, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 µg/ml leupeptin and 28 µg/ml aprotinin). Following two centrifugation steps (15000g, 10 min 4°C) the supernatants were stored at −80°C until use.

Myeloperoxidase and cytokine levels were determined using commercially available murine enzyme-linked immunosorbent assays (ELISA) kits (MPO, Hycult Biotechnology, Uden, Netherlands; cytokine ELISA, eBioscience, Hatfield) according to the manufacturers recommendations.

Statistical analysis

Statistical analysis was performed using the GraphPad Prism Software (GraphPad Software Inc., La Jolla, United States). Differences were analyzed using the student’s paired or unpaired t-test (two-tailed \( P \) value) or ONE-WAY ANOVA. \( P \)-values<0.05 were considered as significant (*), and <0.01 as highly significant (**).
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Terrein Biosynthesis in *Aspergillus terreus* and Its Impact on Phytotoxicity


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Terrein Biosynthesis in *Aspergillus terreus* and Its Impact on Phytotoxicity

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SUMMARY

Terrein is a fungal metabolite with ecological, antimicrobial, antiproliferative, and antioxidative activities. Although it is produced by various microbial, antiproliferative, and antioxidative activities, among others, it is produced by *Aspergillus terreus* as one of its major secondary metabolites, not much is known about its biosynthetic pathway. Here, we describe an unexpected discovery of the terrein biosynthesis gene locus made while we were looking for a PKS gene involved in production of conidia coloration pigments common for Aspergilli. The gene, ATEG_00145, here named *terA*, is essential for terrein biosynthesis and heterologous production of *TerA* in *Aspergillus niger* revealed an unusual plasticity in the products formed, yielding a mixture of 4-hydroxy-6-methylpyranone, orsellinic acid, and 6,7-dihydroxymellein. Biochemical and molecular genetic analyses indicate a low extension cycle specificity of *TerA*. Furthermore, 6-hydroxymellein was identified as a key intermediate in terrein biosynthesis. We find that terrein production is highly induced on plant-derived media, that terrein has phytotoxic activity on plant growth, and induces lesions on fruit surfaces.

INTRODUCTION

*Aspergillus terreus* is a ubiquitous filamentous fungus frequently isolated from soil rhizospheres (He et al., 2004; Wijeratne et al., 2003), decaying organic matter (Reddy and Singh, 2002), and marine environments (Damare et al., 2006). *A. terreus* is able to produce potent lipases (Yadav et al., 1998) and cellulases (Narra et al., 2012) and secretes itaconic acid (Kuenz et al., 2012), which makes this fungus attractive for white biotechnology. In addition, *A. terreus* produces a variety of natural products, most notably the medically important HMG-CoA reductase inhibitor lovastatin (Alberts et al., 1980; Tobert 2003). Additionally, a range of metabolites has been extracted from *A. terreus* cultures, among them territrems, citreoveridin, and butyroloactones (Samson et al., 2011). Several gene clusters in *A. terreus* responsible for metabolite production have been analyzed, including lovastatin (Hendrickson et al., 1999), methylsalicylic acid (Fuji et al., 1996), atrochryson (Awakawa et al., 2009), acetylaranotin (Guo et al., 2013), and terretonin (Guo et al., 2012), as well as the gene cluster for the PKS-NRPS hybrid metabolite isoflavipucine (Gressler et al., 2011). The latter belongs to the family of fruit rot toxins, implying that *A. terreus* could have specifically adapted to the decay of plant organic matter.

However, the origin of the conidia pigment in *A. terreus* remains to be elucidated. All Aspergilli analyzed so far employ a polyketide synthase to produce naphthopyrone precursors. *A. terreus* neither produces naphthropyrones (Slesiona et al., 2012) nor contains the highly conserved naphthopyrone synthase (Ahuja et al., 2012). This points to a separation of *A. terreus* from other Aspergillus species in terms of secondary metabolite production.

This is also highlighted by the fact that *A. terreus* is the only *Aspergillus* species producing terrein as one of its major secondary metabolites (Yin et al., 2013). Terrein was first described approximately 80 years ago (Raistrick and Smith, 1935), and its structure was elucidated in the 1950s (Barton and Miller 1955; Grove 1954). However, a series of recent studies revealed the ecological importance of terrein as an inhibitor of plant growth (Phattanawasin et al., 2007). Furthermore, a number of important biological activities have been attributed to terrein, such as antimicrobial, antiproliferative, and antioxidative activities, among others (Arakawa et al., 2008; Demasi et al., 2010; Lee et al., 2010; Liao et al., 2012; Park et al., 2004).

Considering the great interest in terrein functions, it is striking that so little is known about its biosynthesis. Classical stable-isotope-labeling experiments revealed that terrein biosynthesis involves the condensation of five acetate units, whereas the molecular formula of C_{21}H_{23}O_{7} implied that it is composed of four acetate units only (Birch et al., 1965). It was proposed that the cyclopentanone ring derived from an oxidative decarboxylation with ring contraction of a dihydroisocoumarin (Hill et al., 1981). However, experimental support for this model has been lacking, and to date, the molecular basis for terrein biosynthesis has remained elusive.
Here, we report the serendipitous discovery of the terrein biosynthesis gene locus while searching for polyketide synthases that might be responsible for the pigmentation of *A. terreus* conidia. Through targeted mutations, heterologous expression of the terrein PKS, and analyses of pathway intermediates, we not only discovered the hydroxylated dihydroisocoumarin, which was predicted as a terrein precursor, but also found that the terrein PKS produces a variety of products. Finally, we describe that terrein and congeners exert a phytotoxic effect on radish seeds and fruits.

**RESULTS**

**The PKS ATEG_00145 Is Uniquely Positioned in Aspergillus PKS Phylogeny**

The color of asexual conidia of *Aspergillus* derives from polyketide des that generally originate from the precursor naphthopyrone (*Jørgensen et al., 2011; Langfelder et al., 1998; Mayorga and Timberlake, 1992; Watanabe et al., 1999*). All these PKSs share the following in common: (1) they are nonreducing (NR), and (2) their domain structure shows tandem ACP domains (*Fujii et al., 2001*). Interestingly, in *A. terreus* no PKS with close homology to naphthopyrone synthases was identified (*Slesiona et al., 2012*). However, BLAST analyses revealed two PKSs with the same modular organization, namely, ATEG_00145 and ATEG_07500. To analyze the phylogenetic relation of these *A. terreus* PKSs with other nonreducing fungal PKSs, a phylogenetic tree based on the ketosynthase (KS) domains was constructed. This analysis included naphthopyrone synthases, putative melanin, and orsellinic acid synthases. NRPS–PKS hybrids appeared as an outgroup (Figure 1). Whereas ATEG_07500 appeared at least very distantly related to naphthopyrone synthases, we found that ATEG_00145 does not belong to any of the selected groups of PKSs and seems unique for *A. terreus*. However, because neither ATEG_00145 nor ATEG_07500 closely clustered with naphthopyrone synthases, a contribution in conidia pigmentation remained in question.

**ATEG_00145 and ATEG_07500 Do Not Contribute to Conidia Coloration**

Deletion mutants of ATEG_00145 and ATEG_07500 were generated in *A. terreus* SBUG844ΔakuB (*Gressler et al., 2011*) to investigate their contribution to the development of the conidia color. When the resulting transformants, ΔATEG_00145 and ΔATEG_07500, were tested on various media for conidia pigmentation, none of the mutants showed a difference in comparison to the wild-type (as exemplified in Figure 2A). However, when grown on phosphate-buffered Sabouraud agar plates, the mutant ΔATEG_00145 completely lacked a typical secreted red pigment observed by all other strains (Figure 2B). Additionally, cultivation in liquid potato dextrose broth (PDB) resulted in a red culture broth with the wild-type and ΔATEG_00145 mutant, but not with ΔATEG_00145.

**The Metabolic Profile of ATEG_00145 Deletion Mutant Lacks Terrein**

To investigate the impact of ATEG_00145 on metabolite production, we cultivated the parental strain and ΔATEG_00145 on various media, such as glucose containing *Aspergillus* minimal medium (AMM), YPD, Sabouraud (Sab), and PDB. Culture supernatants were extracted with ethylacetate and were analyzed by liquid chromatography-tandem mass spectrometry (LC–MS/MS) (Figures 2C–2F). On AMM, no significant differences were observed. In contrast, YPD and PDB wild-type extracts contained a highly complex mixture of metabolites that largely lacked in the mutant. Furthermore, although the metabolic profile from Sabouraud medium showed a lower complexity than those from YPD and PDB, a major metabolite at 7.5 min (1) with an apparent molecular mass of 154 Da was completely absent from the mutant. This indicates that the deleted polyketide synthase ATEG_00145 is responsible for the production of various metabolites. However, because (1) was detected in significant amounts from all complex media, we isolated and purified the metabolite by crystallization. High-resolution mass determination revealed a molecular formula of **C₇H₄O₃** indicative for the metabolite terrein. Comparing nuclear magnetic resonance (NMR) data with reported spectroscopic data (*Hill et al., 1981*) unequivocally confirmed this assumption. Therefore, we denoted the polyketide synthase encoded at locus ATEG_00145 as *TerA*.

**ORFs terA–terJ Constitute the Terrein Biosynthesis Gene Cluster**

To confirm expression of *terA* in complex media and to analyze the genes coregulated with *terA*, we monitored expression of 25 genes (ATEG_00126–ATEG_00150) by semiquantitative RT-PCR analyses. Transcripts were analyzed after 36 and 48 hr from AMM (as negative control), YPD, Sab, and PDB media (Figure 3A). Expression of ATEG_00126 to ATEG_00134 and ATEG_00146 to ATEG_00150 did not correlate with expression of *terA* (ATEG_00145). Thus, these genes were excluded as constituents of a putative terrein biosynthesis gene cluster. In contrast, all genes spanning a region from ATEG_00135 to ATEG_00144 were coexpressed with ATEG_00145 and seemed to constitute the terrein biosynthesis gene locus (Figure 3B). The corresponding genes were denoted as *terA–J*, and a putative transcriptional regulator was termed *terH*.

To verify their specific contribution, all genes, except those of the MFS transporters (*terG* and *terI*), were deleted in strain SBUG844ΔakuB (*Gressler et al., 2011*). Gene deletions were confirmed by Southern blot analyses (Figure S1 available online), and mutants were cultivated on PDB medium to monitor medium coloration, metabolite profiles, and terrein production level (Figure 4). As expected, the *terA* mutant did not produce any detectable levels of terrein, and most of the prominent metabolites found in the wild-type extract were absent. A similar profile was detected for the *ΔterR* mutant, implying that transcription of the gene cluster strictly depends on this putative transcription factor. Additionally, mutants *ΔterB, ΔterC, ΔterD, ΔterE, and ΔterF* did not produce terrein. However, several metabolites accumulated in culture broth of mutants, some of which could also be detected in wild-type extract (1–5 in Figure 4B). Interestingly, although unable to produce terrein, the mutants *ΔterD and ΔterE* especially showed an enhanced coloration of the medium, implying that the colored substance does not depend on terrein production but seems to derive from TerA-derived intermediates. Although *terH* and *terI* were clearly coexpressed with *terA* (Figure 3A), both mutants still produced terrein, though the amount in relation to the biomass was reduced. In conclusion, *TerA* to *TerF* directly contribute to terrein synthesis with
Figure 1. Phylogenetic Analysis of Fungal KS Domain Sequences
PKSs belonging to pigment biosynthesis pathways and the orsellinic acid clade were used for analysis. KS domains from fungal NRPS/PKS hybrids served as an outgroup. *A. terreus* ATEG_00145 and ATEG_07500 neither group with pigment biosynthesis KS domains nor with orsellinic acid synthases. See also Figure S5.

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Terrein Biosynthesis in *A. terreus*

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TerR as an essential regulator, whereas TerH and TerI are dispensable. Therefore, the “minimal” gene cluster required for the synthesis of terrein spans the region from terR-terA and most likely uses one of the two MFS transporters (terG or terJ) for efficient secretion of metabolites.

6-hydroxymelleine Is the Key Biosynthetic Precursor of Terrein

The accumulation of several metabolites in the mutants ΔterB to ΔterF implied the accumulation of putative terrein precursor molecules. Indeed, when culture broth extracts from ΔterC, ΔterD, ΔterE, or ΔterF were added to the culture medium of the ΔterA mutant, terrein (1) biosynthesis was restored (Figure 5A). However, ΔterB extracts did not restore terrein formation. Thus, metabolites (3) and (4) (Figure 4B), which are also produced by ΔterB, are not pathway intermediates, but are rather shunt or side products. To identify a true terrein precursor, we fractionated the ΔterC extract. We collected eight fractions by preparative high-performance liquid chromatography (HPLC) that baseline separated the major peaks (Figure 5B), including the major metabolite (2), and added these fractions to the ΔterA mutant. Interestingly, only fraction VII consisting of metabolite (2) restored terrein synthesis. Its characterization by high-resolution mass spectrometry (HR-MS) and NMR revealed a molecular mass of 194 Da and the molecular formula C_{13}H_{13}O_4. Structure elucidation by one-dimensional and two-dimensional NMR revealed the identity of 2 with 6-hydroxymelleine (6-HM), a postulated intermediate en route to terrein (Hill et al., 1981).

To unequivocally prove that 6-HM (2) is an immediate terrein precursor, we added [1-13C]-glucose to the ΔterC culture medium (2 x 100 ml cultures) and isolated 4 mg of labeled 6-HM (2). 13C-NMR analysis revealed the expected 13C-labeling pattern of an isocoumarin derive with polyketide origin (Figure 5B). Next, we added labeled 6-hydroxymelleine to the ΔterA mutant and succeeded in isolating 13C-labeled terrein (Figure 5B). NMR experiments revealed two adjacent nonlabeled carbon atoms in the cyclopentanone ring of terrein (Figure S6), which is completely in line with the model involving the loss of carbon atoms during ring contraction of 6-HM (2) (Hill et al., 1981; Zamir and Chin, 1982).

Recombinant TerA Produces Polyketides of Different Chain Length

Because TerA is a NR-PKS, we assumed that 6-hydroxy-2,3-dehydromelleine was the product of TerA. Therefore, we heterologously expressed terA in Aspergillus oryzae FGSC A1144. To test for suitable promoters, β-galactosidase fusions (Gressler et al., 2011) with Aspergillus nidulans glyceraldehyde-3-phosphate dehydrogenase (PgpdA) promoter and the Aspergillus oryzae α-amylase promoter (PamyB) were analyzed. Although PgpdA was constitutively active under all conditions, the PamyB allowed inducible expression on AMM and YM medium but was not induced on casamino acids (Figure S3). Thus, PamyB was selected to construct a promoter fusion with terA for heterologous expression in A. niger.

Transformants with different integration numbers were screened for metabolite profiles on casamino acids and AMM. Unexpectedly, instead of a single metabolite, three new metabolites that were absent from the parental A. niger strain were

Figure 2. Phenotypic Characterization of ATEG_00145 and 07500 Deletion Mutants and Metabolic Profiling of Δ00145 in Comparison to the Parental Strain SBUG844ΔakuB

(A and B) Colony morphology of wild-type and deletion mutants on solid AMM and Sabouraud medium in (A) top and (B) bottom views. The Δ00145 mutant lacks the typical red pigmentation on Sabouraud medium.
(C–F) HPLC profiles (UV 254 nm) of culture extracts from strain ΔakuB (upper profiles) and Δ00145 mutant (lower profiles) cultivated on (C) AMM, (D) YPD, (E) Sab, and (F) PDB. “1” denotes the major metabolite terrein, which is lacking in the deletion mutant.

See also Figure S1.
metabolite, has a molecular mass of 168 Da (C\textsubscript{9}H\textsubscript{16}O\textsubscript{4}). HR-MS analyses revealed that compound (G100), YPD, Sab, and PDB medium. Genes from ATEG\textsubscript{00135–00145} (3 and 4) showed the same retention time and UV spectra as metabolites (3 and 4) of the ΔterB mutant (Figure 4A), which did not restore terrein production (Figure 5A). Purification of the three metabolites with subsequent NMR-based structure elucidation revealed the structures of orsellinic acid (OA) for (3) and 4-HMP (5) for the minor metabolite 5. OA (4) and 4-HMP (5) represent typical products of an NR-PKS. In contrast, 6,7-DHM (3) lacks the expected double bond of 6-hydroxy-2,3-dehydromelalein and has a hydroxyl-group on position C7, although there is no oxygenase domain within TerA.

Although 6,7-DHM (3) is closely related to 6-HM (2) and had been identified from different cluster mutants (ΔterB–ΔterA; Figure 4A), feeding of complete culture extracts from A. niger transformants or their purified metabolites did not restore terrein production in the ΔterA mutant strain (data not shown). Thus, we assumed that the unexpected hydroxylation at C7 was nonspecifically introduced by the host, thus hampering uptake of the compounds and/or its further conversion to terrein.

To test this hypothesis, we supplemented AMM media with 6-HM (2) and inoculated them with either A. terreus or A. niger wild-type strains. Subsequent analyses of culture extracts revealed metabolites corresponding to hydroxylated forms of 6-HM, among them 6,7-DHM (Figure 5A). Thus, this particular hydroxylation takes place in the absence of terrein biosynthesis enzymes. Consequently, recombinant expression of terA in A. niger yields 6-HM, which is subsequently converted into 6,7-DHM by unspecific enzymes in the heterologous host.

**TerA Is Exclusively Primed with Acyl-CoA Starter Units**

TerA is an unusual NR-PKS that produces polyketides of different chain length. This may be rationalized by the loading of starter units of different chain lengths or by a different number of elongation cycles. The first scenario would require a "relaxed" specificity of the starter acyl transferase (SAT) domain that may not only load acetyl-CoA but may also load other short-chain acyl-CoA starter units. In SAT domains, a catalytic diad formed by a conserved cysteine and histidine residue initiates the loading process (Crawford et al., 2006). However, inspection of the annotated protein sequence of TerA revealed no conserved cysteine in the N-terminal region. Further analysis of the proposed open reading frame (ORF) revealed an unusual large intron sequence of 223 bp. To rule out an incorrect intron prediction, we generated cDNA from A. terreus and sequenced the 5' region. Indeed, our analysis revealed that the intron only spanned a region of 49 bp (accession KF647874). The resulting corrected SAT domain contained the expected cysteine residue (Figure 5S). This indicates that an acyl-CoA unit is loaded, but it gave no direct clue on the kind of acyl-CoA ester utilized. Because OA (4) was one of the products of TerA, we investigated a possible increase in product specificity by exchanging the TerA SAT domain with that of the orsellinic acid synthase OrsA from A. nidulans (Sanchez et al., 2010; Schroeckh et al., 2009). Yet A. niger expressing the SAT\textsubscript{orsA–terA} construct completely lacked synthesis of both 6,7-DHM (3) and OA (4) (data not shown). This finding suggests that the OrsA SAT domain and TerA are not compatible, although both PKS produce at least one common polyketide.

**Figure 3. Expression Analysis of the Putative Terrein Synthesis Locus and Gene Annotations**

(A) Semiquantitative PCR analysis of ATEG\textsubscript{00135–00145} from actin-normalized cDNA generated from mycelium grown for 36 and 48 hr on AMM (G100), YPD, Sab, and PDB medium. Genes from ATEG\textsubscript{00135–00145} (terJ–terA) show the same expression pattern and form a putative terrein synthesis cluster.

(B) Genome map of the terrein gene cluster (filled arrows). Putative functional domains of the encoded proteins are listed.

detected in the transformants on glucose minimal medium (Figures 5C and 5D), but not on casamino acids (data not shown). HR-MS analyses revealed that compound 4, the most abundant metabolite, has a molecular mass of 168 Da (C\textsubscript{9}H\textsubscript{16}O\textsubscript{4}). For congeners 3 and 5, masses of 210 Da (C\textsubscript{10}H\textsubscript{18}O\textsubscript{5}) and 126 Da (C\textsubscript{9}H\textsubscript{16}O\textsubscript{4}) were inferred. Interestingly, the two major metabolites

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Figure 4. Metabolic Profiling and Terrein Quantification from Terrein Cluster Deletion Mutants

(A) Photographs of cultures were taken after 72 hr of growth in PDB medium at 30°C (column 2). Metabolite profiles of culture supernatants from control, wild-type, and mutants recorded at 254 nm (column 3). Specific terrein (1) concentrations per gram of mycelial dry weight were determined from three independent cultures (column 4). The metabolite peaks annotated with # (156 Da; molecular formula C_7H_7O_4) and * (154 Da; molecular formula C_7H_6O_4) are present in all mutants at varying extent and not related to terrein biosynthesis. Error bars denoted ± SD.

(B) Structures of metabolites representing the numbered peaks in the HPLC profile: terrein (1), 6-hydroxymellein (2), 6,7-dihydroxymellein (3), orsellinic acid (4), and 4-hydroxy-6-methylpyrone (5).

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We next tested alternative PKS priming. First, we added [1-13C]-acetate to the culture broth of \textit{A. niger A1144_PamyB:terA} and analyzed the labeling pattern of the metabolites. Analysis confirmed the condensation of three acetyl units in 4-HMP (5), four acetyl-units in OA (4), and five acetyl-units in 6,7-DHM (3). Although the incorporation rate in 6,7-DHM (3) was rather weak, the labeling pattern was already confirmed by addition of labeled [1-13C]-glucose to the \textit{A. terreus terC} mutant (Figure 5B). Analysis by 13C-NMR revealed 6,7-DHM with an equal distribution of 13C on all carbon atoms, indicating that 3-hydroxybutyrate was cleaved into acetyl units prior to chain elongation and thus did not specifically serve as a starter unit (data not shown). We additionally tested for a relaxed specificity of the SAT domain by adding the N-acetyl-cysteamine-esters of butyrate, 3-hydroxybutyrate, 3-hydroxypentanoate, and 3-hydroxyhexanoate. LC-MS/AMS analysis neither revealed metabolites with altered or extended carbon skeleton nor an altered relative quantity of one of the three metabolites (data not shown). From these results, we conclude that TerA only utilizes acetyl-CoA starter and malonyl-CoA extender units, and the metabolites of different chain length seem to derive from low extension cycle specificity.

Interestingly, in all experiments, we exclusively detected the reduced 6,7-DHM (3) rather than a 6,7-dihydroxy-2,3-dehydro-mellein. This implies that, independent from the gene cluster, \textit{A. niger} enzymes reduce 6,7-dihydroxy-2,3-dehydro-mellein to 6,7-DHM (3). Similarly, this reduction also seems to occur nonspecifically in \textit{A. terreus}, because all cluster mutants, except
the ΔterA and the ΔterR strain, accumulated 6,7-DHM (3) or 6-HM (2). However, the cluster also contains a putative ketoreductase (TerB) that may be essential for the correct timing of this reduction step during terrein production, but coexpression of terB together with terA in A. niger resulted in the same product pattern as observed for the strain solely expressing terA (data not shown).

**The TerA Thioesterase Domain Is Exchangeable without Alteration of Product Formation**

Bioinformatics and labeling studies imply that different numbers of elongation cycles are responsible for the different chain lengths of the TerA products. Thioesterase (TE) domains have been shown to contribute to chain length specificity and product release (Watanabe and Ebizuka, 2004). In the A. nidulans naphthopyrone synthase WA, the TE domain acts as a Claisen cyclase involved in closure of the “B-ring” in the heptaketide naphthopyrone that is also the sole product of this PKS (Fujii et al., 2001). When deleted, ring closure occurs via nonenzymatic lactonization, resulting in an isocoumarin heptaketide. In contrast, PKS1 from Colletotrichum lagenarium produces the tetraketide OA (10%), the pentaketides α-acetylorsellinic acid (25%), tetrahydronaphthalene (50%), and the hexaketide 2-acetyl-1,3,6,8-tetrahydronaphthalene (15%) (Watanabe and Ebizuka, 2004). Removal of the TE domain resulted in >95% production of an isocoumarin hexaketide. Thus, it was concluded that PKS1 generally produces a hexaketide but that the Claisen cyclase domain interferes with chain growth (Watanabe and Ebizuka, 2004), which could also be true for TerA. Thus, two different constructs were generated: (1) a truncated version of TerA lacking the TE domain (called terAΔTE) and (2) a construct in which the C-terminal part next to the phosphopantetheine binding site was replaced by the TE domain from A. nidulans WA (called terA:TE

**DISCUSSION**

In this study, the molecular basis for terrein biosynthesis in *A. terreus* was elucidated. The discovery of this gene cluster was unexpected, because we were initially searching for secondary metabolites responsible for coloration of asexual conidia. In all related Aspergillus species, the conidia color is naphthopyrone derived (Langfelder et al., 1998; Watanabe et al., 1999) and essential to inhibit phagolysosome acidification and allows for escape from macrophages (Slesiona et al., 2012; Thywissen et al., 2011). As putative candidates for conidal pigment synthesis in *A. terreus*, we selected two nonreducing polyketide synthases that revealed the same domain structure (SAT-KS-AT-PT-ACP-ACP-TE) as naphthopyrone synthases. When deleted, both mutants showed normal coloration of conidia, and phylogenetic analyses revealed that, despite the same domain structure, none of the two PKSs clustered with PKSs for which products had been described. Especially the ketoreductase domain of ATEG_00145 (subsequently denoted as TerA) formed a distinct phylogenetic branch. This observation was in agreement with a recent study, in which aromatic PKSs from Aspergillus were phylogenetically investigated, and ATEG_00145 (TerA) could not be explicitly assigned to any particular clade (Ahuja et al., 2012).

We showed that the terA gene encodes for a functional polyketide synthase. When terA was expressed in *A. niger*, it produced the triketide 4-hydroxy-6-methylpyranone (5), the tetraketide orsellinic acid (4), and a pentaketide, which is 6,7-dihydroxymellein (3). Although these products were also identified from *A. terreus* wild-type and some of the cluster mutants, the production of 6,7-DHM (3) especially did not fit to the domain structure of a NR-PKS. Subsequent analyses revealed that the
were expressed in an intrinsic secondary metabolite gene clusters. Thus, although as shown by feeding studies with terA produced by TerA, 6-HM restored terrein production in the hydroxylation at position 7 of 6,7-DHM (3) occurs independent from TerA, implying that a derivative of 6-HM (6 or 2) rather than of 6,7-DHM (3) is the pentaketide produced by TerA. In this respect, one would have expected that 2,3-dehydro-6-HM (6) is produced because of a lacking ketoreductase domain in TerA. Indeed, this product was identified by an independent investigation (Ahuja et al., 2012), in which different NR-PKS were expressed in an A. nidulans strain deleted for several intrinsic secondary metabolite gene clusters. Thus, although 2,3-dehydro-6-HM (6), rather than 6-HM (2), is the pentaketide produced by TerA, 6-HM restored terrein production in the terA and terB mutants and acts as a direct precursor for terrein as shown by feeding studies with 13C-labeled 6-HM (2). In contrast, triketide (5) and tetraketide (4) did not serve as precursors for terrein in any of the mutants. Thus, we were interested in the reason for the low chain-length specificity of TerA, especially because we discovered that the recombinant PKS produced the tetraketide (4), rather than the pentaketide (3), as major product.

For other PKSs, such as the norsolorinic acid synthase, it was shown that the SAT domain accepts various starter units (Crawford et al., 2006), resulting in products of different chain length, whereby under natural conditions the main substrate is provided by a coregulated fatty acid synthase (Brown et al., 1996; Watanabe et al., 1996). In contrast, our feeding studies showed that in TerA the low specificity for a product with a specific chain length is independent from the utilization of alternative starter units. A respective SAT-domain-independent mechanism has previously been described for PKS1 from C. lagenarium (Crawford et al., 2006), in which the TE domain causes low product specificity. In C. lagenarium, the deletion of the TE domain strongly altered the product spectrum in the direction of the product with the highest chain length (Watanabe and Ebizuka, 2004). This is unusual, because in other polyketide synthases, such as the naphthopyrone synthase WA from A. nidulans, the TE domain is important for product chemistry but does not direct chain-length specificity (Fuji et al., 2001). In the case of TerA, neither deletion nor exchange of the TE domain altered the product spectrum, but a TE domain was essentially required for high product yields. This implies that the TE domain is not directly involved in chain-length determination but is essential for efficient product release and thus overall activity of the PKS.

These data suggest that the low specificity is a trait inherent to the KS and likely the product template (PT) domain. This PT domain limits the pocket size and thus the possible substrate chain length and substrate orientation prior to the cyclization step (Crawford et al., 2009). It is remarkable that the KS domain of TerA did not fall into any KS clades in the phylogenetic tree. Thus, this part of the PKS may indeed cause the low product specificity. In this context, it should also be noted that fungal HR-PKS have been shown to produce polyketides of different chain lengths when taken out of the biosynthetic context (Kennedy et al., 1999). It is very conceivable that accessory enzyme contribute to the fidelity of the PKS.
In this study, we showed that TerA generates 2,3-dehydro-6-HM (6) as a precursor of terrein (Figure 7). The timing of hydroxylation at position C7 and hydrogenation between C2 and C3 appears important. When both reactions are performed on 2,3-dehydro-6-HM (6), 6,7-DHM (3) is formed, which is not converted into terrein in any of the mutants. However, the hydrogenation of 2,3-dehydro-6-HM (6) to 6-HM (2) yields an appropriate pathway intermediate for the δterA and δterB strains. Thus, we conclude that the specific reduction of 2,3-dehydro-6-HM (6) to 6-HM (2) without hydroxylation at position C7 is performed by TerB, which is in agreement with an N-terminal PKS dehydratase domain that could open and close the lactone ring to enable the C-terminal ketoreductase domain the hydrogenation at positions C2 and C3. However, because 6,7-DHM (3) is also found in the δterB strain, unspecific ketoreductases may also perform this reduction on a substrate hydroxylated at position C7.

Although our analyses revealed that terC, terD, terE, and terF are essential for terrein production, it remains difficult to assign a specific function to each of the enzymes. One can assume that at least two additional monooxygenase reactions are required. One reaction could involve the hydroxylation at position C9 (Zamir and Chin, 1982) to set the stage for the subsequent decarboxylation at the lactone ring. Another hydroxylation would be required at position C7 or C5 (Hill et al., 1981) to allow for the ring contraction. Because terC, terD, and terE encode either FAD-dependent monooxygenases or multicopper oxidases, all three enzymes are putative candidates for these reactions. Despite many attempts, potential downstream products evaded isolation and structure elucidation because of their instability and/or reactivity.

(2) inhibits pollen development in Arabidopsis thaliana (Shimada et al., 2002). We also found that terrein, but none of the other compounds produced, causes lesions on fruit surfaces. Thus, in its natural habitat terrein may be specifically required during the interaction with plants and may trigger release of nutrients from the interaction partners. However, various intermediates of terrein synthesis display different beneficial or phytotoxic effects on plant cells, which may explain the high diversity of products released during terrein synthesis.

**SIGNIFICANCE**

Aspergillus terreus is an important fungus that plays a pivotal role in biotechnology, not only for the production of enzymes and fine chemicals but also for pharmaceutically relevant compounds. Moreover, A. terreus has been implicated in various diseases and is regarded as an emerging fungal pathogen. Thus, detailed knowledge about its biosynthetic potential is essential to improve metabolite production and to identify and study potential risk factors. Terrein is a famous, characteristic A. terreus metabolite that is endowed with a variety of biological activities, such as antimicrobial and antiproliferative activities. Yet little is known about terrein biosynthesis, and the genes involved remain fully unknown. In this study we unveil the molecular basis for the biosynthesis of this important natural product and gain insights into regulation and mechanisms of the terrein pathway. The identified terrein PKS is unusual as it yields three compounds of different sizes and shapes. Functional and mutational analyses contribute to a better understanding of terrein
assembly. Most notably, we identify a key intermediate of the terrein pathway and unequivocally show that it is transformed into the rearranged polyketide metabolite. Beyond biosynthetic studies, we also report an additional role — we have not explored thus far — for gene x, in which it forms lesions on fruit surfaces. This finding is particularly noteworthy as terrein biosynthesis is upregulated in organic media that may mimic the natural habitat. Taken together, our studies not only shed light on the biosynthesis of an important fungal metabolite but may also unveil an additional role for the terrein pathway in environmental competition and phytotoxin production.

EXPERIMENTAL PROCEDURES

Reconstruction of the Phylogenetic Tree
Protein sequences of known and predicted PKSs belonging to pigment biosynthesis pathways and the orsellinic acid clade were collected from the National Center for Biotechnology Information. KS domains from fungal biosynthetic studies, we also report an additional role — we have not explored thus far — for gene x, in which it forms lesions on fruit surfaces. This finding is particularly noteworthy as terrein biosynthesis is upregulated in organic media that may mimic the natural habitat. Taken together, our studies not only shed light on the biosynthesis of an important fungal metabolite but may also unveil an additional role for the terrein pathway in environmental competition and phytotoxin production.

EXPERIMENTAL PROCEDURES

Reconstruction of the Phylogenetic Tree
Protein sequences of known and predicted PKSs belonging to pigment biosynthesis pathways and the orsellinic acid clade were collected from the National Center for Biotechnology Information. KS domains from fungal NRPS/PKS hybrids were used as an outgroup. The KS domains were extracted and aligned with Muscle, and the tree was inferred with the neighbor-joining (NJ) method using the Phylip package, with the number of bootstrap trials set to 1,000. Numbers at the nodes indicate the bootstrap support for each clade.

Strains, Cultivation Conditions, and Metabolite Extraction
All strains and mutants used and generated in this study are listed in the Supplemental Experimental Procedures. Minimal media are based on Aspergillus minimal medium (AMM; http://www.tgc.net/methods/atidmed.htm). The following media were used: AMM (containing 100 mM glucose as standard medium), AMM-CA1% (nitrate and glucose replaced by 1% casamino acids), YPD (20 g/l peptone, 20 g/l glucose, and 10 g/l yeast extract), Sabouraud (Sigma Aldrich), buffered in solid media with sodium phosphate buffer (pH 6.5) to a final concentration of 150 mM, potato dextrose broth (PDB; Sigma Aldrich), and yeast extract/malt extract medium (YM; 5 g/l peptone, 3 g/l malt extract, and 3 g/l yeast extract). Liquid cultures were inoculated with a final concentration of 1 x 10^5 conidia/ml and incubated at 30°C and 200 rpm. When required, plates were supplemented with 140 μg/ml hygromycin B (Roche) or 0.1 μg/ml pyrithiamine (Sigma Aldrich). Conidia were harvested from solid media in water and filtered over 40 μm cell strainers (VWR). Culture broth was extracted by adding 50 ml ethylacetate to 50 ml broth, and the extraction was repeated once. Evaporated residues were solved in 1 ml methanol and filtered. Standard extract analyses were performed on an Agilent 1100 series HPLC-DAD system coupled with a MSD trap (Agilent Technologies) operating in alternating ionization mode (Gressler et al., 2011).

Generation of Deletion Mutants in A. terreus
For generation of deletion mutants, A. terreus SBUG844/lakub (Gressler et al., 2011) was used as the parental strain. Briefly, between 0.5 and 1.3 kbp 5'- and 3'-flanking regions were amplified from genomic DNA and fused with the pyrithiamine resistance cassette ptrA (Fleck and Brock, 2010). A. terreus was transformed by protoplast fusion and regenerated on osmotically balanced medium with pyrithiamine as a selection marker. Transformants deriving from single-haploid conidia were analyzed by Southern hybridization to confirm the complete integration of the constructs. Transformants were checked by PCR and Southern hybridization to confirm the complete integration of the constructs (Figure S2). For details concerning oligonucleotides used for fragment amplification, cloning strategies, and generation of labeled probes, refer to the Supplemental Experimental Procedures.

Semiquantitative PCR on Terrein Cluster Genes
RNA from A. terreus SBUG844 grown for 36 and 48 hr on AMM, Sabouraud, YPD, and PDB media was isolated using TRIzol (Bioline). After DNase treatment, cDNA was generated using anchored oligo-dT primers, and cDNA amounts were normalized against the actin gene (Gressler et al., 2011). Oligonucleotides P61–F114 (Supplemental Experimental Procedures) were used for semiquantitative PCR analyses on AMEG 00126–00150. Genomic DNA from SBUG844 was used as a positive control during PCR amplifications.

β-galactosidase Assay
As described above, β-galactosidase assays were performed on cell-free extracts from A. niger transformants harboring lacZ fusion constructs. Mycelia were harvested from cultures grown for 48 hr in AMM, YM, or AMM-CA1% media at 30°C. Mycelia were ground to a fine powder under liquid nitrogen and resuspended in 50 mM MOPS buffer (pH 7.5) with 2 mM MgCl2 and 10 mM β-mercaptoethanol. After centrifugation at 21,000 × g, the supernatant was used for β-galactosidase assays as described (Gressler et al., 2011).

Isolation and Structure Elucidation of Metabolites
Purified terrein was obtained by recrystallization from crude extracts with ethylacetate (Demasi et al., 2010). Details for the isolation of 6-hydroxymellein, 6,7-dihydroxymellein, orsellinic acid, and 4-hydroxy-6-methylpyrone by solid-phase extraction and preparative HPLC are provided in the Supplemental Experimental Procedures. High-resolution electrospray ionization mass spectrometry (HR-ESIMS) was carried out on an Accela UPLC-system combined with an Exact Mass spectrometer (Thermo Scientific) operating in positive ionization mode. Separation was carried out on a Betasil C18 column (2.1 x 150 mm; 3 μm; Thermo Scientific) using water (solvent A) and acetonitrile (solvent B), each containing 0.1% formic acid, as binary solvent system. A flow rate of 250 μl/min and the following gradient was used: 0–1 min = 5% B, 1–16 min = 5%–98% B, 16–19 min = 98% B, and 19–20 min = 98%–5% B. NMR spectra were recorded on a Bruker Avance III 500 and a Bruker Avance III 600 spectrometer (Bruker BioSpin GmbH) equipped with a cryoprobe head using DMSO-d6 and methanol-d4 as solvents and internal standards.

Quantification of Terrein
Extraction of 50 ml cultures with ethylacetate was performed as described above, and 80 ml of extracts were evaporated under reduced pressure, solved in 1 ml methanol, and diluted in a ratio of 1:20. Each sample was applied: 0.5 min = 10% B, 0.5–20 min = 70%–98% B, 20–25 min = 70%–100% B, 25–28 min = 100% B, and 28–33 min = 100%–10% B. Quantification was performed from a calibration curve of known terrein concentrations. For dry-weight correlation the mycelia from the cultures were dried for 48 hr at 37°C and balanced. From these values, the terrein concentrations per gram of mycelium were calculated.
of dried mycelium were calculated. All quantifications were carried out in biological triplicates and technical duplicates.

**Metabolite Supplementation Experiments**

A. niger strains were cultivated for 48 hr on AAM, and A. terreus wild-type and mutant strains were cultivated for 72 hr on PDB medium. In precursor feeding experiments, media were supplemented with unlabeled SNAC derivatives of 3-hydroxy butyric acid, 3-hydroxy pentaenoic acid, 3-hydroxy hexanoic, and labeled [1,2,3,4-13C] 3-hydroxy butyric acid (Cortecnet). Cultures of A. niger A1144, ParryB:terA were supplemented at 0 hr and after 24 hr with 2.5 mM of the respective molecules (solved in DMSO). For labeling of terrein, A. terreus SBUG844 was cultivated for 72 hr at 30°C in a 4 l Biostat B-DCU II fermenter (Sartorius Stedim Biotech) in PDB medium supplemented with 5 mM [1-13C] sodium acetate (Cortecnet). The culture was constantly stirred at 500 rpm, and air was sparged at 2 l/min. Labeled molecules were isolated from A. terreus strain SBUG844:akuB:terE cultivated in 100 ml PDB medium supplemented with 1 g [1-13C]-o-glucose (Cortecnet). In cross-feedings, 100 μl culture extracts from the deletion mutants ΔterB, ΔterC, ΔterD, ΔterE, and ΔterF were added to cultures of SBUG844:akuB:terA. After 72 hr, culture supernatants were extracted and applied to LC-MS/MS, preparative HPLC, and NMR analysis as described.

**Root Growth Inhibition and Fruit Surface Spot Dilution Assays**

Details on root growth inhibition are provided in the Supplemental Experimental Procedures. Briefly, single sanitized radish seeds were transferred to 15 ml tubes with 7 ml of modified solid Hoagland medium and overlaid with organic bananas (type Bio) or pears were cleaned with water, and 5
dilution methods were added to sterile media in final concentrations of 100, 50, 25, 10, or 2 and 7 days of incubation at room temperature in the dark.

**Statistical Analysis**

Error bars in figures represent SD (±SD). Statistical analyses were performed by applying the two-tailed Student’s t test. Data were denoted as significantly different with p values ≤ 0.01 (*) or ≤ 0.001 (**).

**ACCESS NUMBERS**

The DDBJ/EMBL/GenBank nucleotide sequence database accession number of the 5’ coding region spanning intron 1 of the terA gene (locus ATEG_00145) reported in this paper is KF647874.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2014.03.010.

**AUTHOR CONTRIBUTIONS**

C.Z. isolated and characterized metabolites, analyzed data, and contributed in writing the manuscript; M.G. constructed mutants, performed biological activity tests, analyzed transcription profiles, and contributed in writing the manuscript; E.S. performed phylogenetic analyses; E.G. contributed in metabolite isolation and structure elucidation; and C.H. and M.B. designed the study, interpreted data, and wrote the manuscript.

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A New High-Performance Heterologous Fungal Expression System based on Regulatory Elements from the *Aspergillus terreus* Terrein Gene Cluster


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A new high-performance heterologous fungal expression system based on regulatory elements from the Aspergillus terreus terrein gene cluster

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Recently, the Aspergillus terreus terrein gene cluster was identified and selected for development of a new heterologous expression system. The cluster encodes the specific transcription factor TerR that is indispensable for terrein cluster induction. To identify TerR binding sites, different recombinant versions of the TerR DNA-binding domain were analyzed for specific motif recognition. The high affinity consensus motif TCGGHHWYHCGGH was identified from genes required for terrein production and binding site mutations confirmed their essential contribution to gene expression in A. terreus. A combination of TerR with its terA target promoter was tested as recombinant expression system in the heterologous host Aspergillus niger. TerR mediated target promoter activation was directly dependent on its transcription level. Therefore, terR was expressed under control of the regulatable amylase promoter PamyB and the resulting activation of the terA target promoter was compared with activation levels obtained from direct expression of reporters from the strong gpdA control promoter. Here, the coupled system outcompeted the direct expression system. When the coupled system was used for heterologous polyketide synthase expression high metabolite levels were produced. Additionally, expression of the Aspergillus nidulans polyketide synthase gene orsA revealed lecanoric acid rather than orsellinic acid as major polyketide synthase product. Domain swapping experiments assigned this depside formation from orsellinic acid to the OrsA thioesterase domain. These experiments confirm the suitability of the expression system especially for high-level metabolite production in heterologous hosts.

Keywords: Aspergillus niger, secondary metabolites, transcription factor, DNA-binding motif, reporter strains, thioesterase domain

Introduction

Aspergillus terreus is a filamentous ascomycete of biotechnological and medical importance, since it produces the primary metabolite itaconic acid (Calam et al., 1939; Klement and Buchs, 2013) and the HMG-CoA reductase inhibitor lovastatin (Alberts et al., 1980; Hutchinson et al., 2000). Besides that, A. terreus can cause life-threatening invasive
aspergillosis in immunocompromised patients (Slesiona et al., 2012b), which makes its use in biotechnological applications limited. In previous analyses we searched for secondary metabolite gene clusters that are involved in pigment formation of  \textit{A. terreus} conidia (Zaehle et al., 2014). This was of interest, since preliminary analyses suggested that the pigment in  \textit{A. terreus} differs from that found in other related  \textit{Aspergillus} species (Slesiona et al., 2012a). Coincidentally, we identified the gene cluster producing the metabolite terrein (Zaehle et al., 2014). Terrein is a metabolite with various biological activities, but its phytotoxic potential appears to be at least one of its natural functions and may increase competitiveness of  \textit{A. terreus} in the environment (Zaehle et al., 2014).

Interestingly, terrein (Figure 1A) is produced in large quantities, since short-term cultivation in simple potato dextrose broth resulted in more than 1 g of terrein per liter (Zaehle et al., 2014) and even higher yields have been described for cultivation under more optimized conditions (Xu et al., 2012; Yin et al., 2012). This implies that genes from the terrein gene cluster may be expressed at very high levels.

The terrein cluster contains seven genes responsible for terrein biosynthesis (terA-F, terR), whereby terA encodes a non-reducing polyketide synthase (PKS) that produces polyketides of different chain length namely 2,3-dehydro-6-hydroxymellein (C10), orsellinic acid (C8), and 4-hydroxy-6-methylpyrone (C6). The subsequent formation of the cyclopentenoic structure of terrein is proposed to derive from an oxidative ring contraction of the isocoumarinic precursor 2,3-dehydro-6-hydroxymellein. While the terrein gene cluster contains further co-regulated genes, these genes might be dispensable (terH, terI), because deletion only reduces the final terrein production level or are involved in metabolite export (terG, terl) (Zaehle et al., 2014). Importantly, the gene cluster encodes its own transcriptional activator at locus tag ATEG_00139, which is called TerR. TerR is a transcriptional activator with a GAL4-type Zn$^{2+}$Cys$_6$ zinc binuclear cluster DNA-binding domain. This type of DNA-binding domain is very common in fungi and is the most common type of DNA-binding domains in transcriptional regulators of secondary metabolite gene clusters from filamentous fungi such as GliZ for gliotoxin biosynthesis from \textit{Aspergillus fumigatus} (Bok et al., 2006), AflR for aflatoxin biosynthesis from \textit{Aspergillus nidulans} and \textit{Aspergillus flavus} (Yu et al., 1996) or ApdR for aspyridone biosynthesis from \textit{A. nidulans} (Bergmann et al., 2007). A genomic deletion of terR resulted in complete loss of terrein production, suggesting that this transcription factor essentially contributes to cluster expression (Zaehle et al., 2014). However, neither the DNA binding sites recognized by TerR, nor the signals leading to TerR activation have been identified so far. However, if TerR is sufficient to drive expression of cluster genes, we assumed that a combination of TerR together with promoters from its cluster could enable the development of a new heterologous expression system.

Several filamentous fungi have been used for the recombinant production of proteins, among them especially fungi of industrial importance with GRAS status (generally regarded as safe) such as \textit{Aspergillus niger}, \textit{Aspergillus oryzae}, \textit{Trichoderma reesei}, \textit{Acremonium chrysogenum}, and \textit{Penicillium chrysogenum}.

![FIGURE 1](image) 

**FIGURE 1** | Analysis of terrein cluster induction in dependence of terR expression. (A) Chemical structure of terrein (1). (B) Semiquantitative RT-PCR (Continued)
A new terrein cluster-based expression system

Strains and Culture Conditions

Strains used in this study are summarized in Table S1. For maintenance and during transformation procedures, all *Aspergillus* strains (A. nidulans wild type FGSC A4, A. terreus SBUG844 and its derivatives and all *A. niger* FGSC A1144 derivatives) were cultivated for 4 days at 37 or 30°C on solid *Aspergillus* minimal media (AMM) containing 2% agar (http://www.fgsc.net/methods/aniidxmed.html). When required, either hygromycin B (140 μg/ml, Carl Roth GmbH; Germany), pyrithiamine hydrobromide (0.1 μg/ml) or phleomycin (80 μg/ml) (both Sigma Aldrich; Germany) were added. Conidia were harvested in sterile water from solid media and filtered over 40 μm cell strainers (VWR; Germany). Liquid cultures were generally used in a 50 ml scale and were inoculated with 1 × 10^6 conidia per ml. Cultures were incubated at 30°C for 48–72 h as specified in the respective experiments. The following liquid media were used: AMM with 100 mM glucose (AMM-G100) or 1% casamino acids (AMM-CA1%), potato dextrose broth (PDB, Sigma Aldrich), and yeast/malt extract medium (YM, 5 g/l peptone, 3 g/l yeast extract, 3 g/l malt extract).

Bacterial Expression and Purification of TerR Polypeptides for SPR Analysis

All oligonucleotides used in this study are listed in Table S2. The gene sequences encoding for TerR residues 1–153 (TerR<sub>35–153</sub>), 43–138 (TerR<sub>43–138</sub>), and 35–138 (TerR<sub>35–138</sub>) were amplified by PCR from cDNA of *A. terreus* wild-type strain SBUG844 that was cultivated for 48 h at 30°C on PDB medium. The following oligonucleotides introducing 5′-Ndel and 3′-HindIII restriction sites were used: P1/2 for TerR<sub>1–153</sub>, P3/4 for TerR<sub>43–138</sub>, and P5/4 for TerR<sub>35–138</sub>. The fragments were cloned into the pet-29a vector (Novagen; Germany). TerR polypeptides were produced by autoinduction in *E. coli* Rosetta2 (DE3) cells grown at 26°C in 1:1 Overnight Express Instant TB Medium (Novagen) in the presence of 1 mM Zn(OAc)<sub>2</sub>. Fifteen to twenty grams wet cells were collected by centrifugation, resuspended in 200 ml lysis buffer (20 mM HEPES, 150 mM NaCl, 10 μM Zn(OAc)<sub>2</sub>, 5 mM β-Mercaptoethanol, 1 mM AEBSP; pH 7.5) and disrupted using an EmulsiFlex C5 high pressure homogenizer (Avestin; Germany). Cleared cellular extracts were loaded on a SP Sepharose HP (GE Healthcare; Germany) column and eluted with a salt gradient up to 1 M NaCl. Pooled fractions containing TerR<sub>1–153</sub>, TerR<sub>43–138</sub>, or TerR<sub>35–138</sub> were adjusted to 150 mM NaCl and applied on a Cellufine Sulfate (Millipore; Germany) column that was equilibrated with 20 mM HEPES, 150 mM NaCl, 10 μM Zn(OAc)<sub>2</sub>, 5 mM β-Mercaptoethanol, pH 7.5, followed by elution with a gradient to 1 M NaCl. Peak fractions were concentrated with an Amicon Ultra-15 10K centrifugal filter device and purified to homogeneity by size exclusion chromatography on a Superdex 75 prep grade column (GE Healthcare) by using 20 mM HEPES, 150 mM NaCl, 10 μM Zn(OAc)<sub>2</sub>, pH 7.5 as running buffer. TerR proteins were stored in 50% v/v glycerol at −20°C. The absolute molecular mass of TerR<sub>35–138</sub> was determined by static light scattering experiments on a miniDawn TRESOS monitor in series with an Optilab T-rEX differential refractometer.

Materials and Methods

Several post-transcriptional bottlenecks have been reported to limit the production of proteins. These may consist of incorrect protein folding and subsequent degradation, low secretion efficiency, extracellular degradation or hyperglycosylation and several attempts have been made to overcome these limitations (Ward, 2012). However, filamentous fungi have not only been used for protein production, but are also used for the production of lignofuels or metabolic intermediates such as citric acid and the production of secondary metabolites such as antibiotics and other therapeutically useful compounds (Lubertozzi and Keasling, 2009). Despite specific limitations in all expression systems including the codon-adaptation of the target gene and RNA stability, the initial high-level expression of a target gene is the first key step for high production rates.

In general, a strong promoter is used to drive gene expression. In this respect, expression systems frequently rely on endogenous promoters from primary metabolism that are either constitutively active or can be regulated by applying specific inducing or repressing conditions. Examples are alcohol and aldehyde dehydrogenase (*alaA; aldA*), glucoamylase (*glaA*), (Taka) amylace (*amyA; amyB*), glyceraldehyde-3-phosphate dehydrogenase (*gpdA*), sucrase (*sucA*), amylase (*amsl*), endoxylanase (*exlA*), superoxide dismutase (*sodA*), or cellobiohydrolase I (chbI) (Sharma et al., 2009; Fleissner and Der-sch, 2010). Additionally, multiple integrations of an expression construct frequently increases the overall transcription of the target gene, but a linear increase is mainly limited to the first 5–6 copies (Verdoes et al., 1993). Due to these limitations, an inducible system with a promoter that is strongly activated and produces high transcript levels already in single-copy is generally favored.

Interestingly, although high secondary metabolite production rates have been described for several fungal species, information on the use of the involved regulatory elements to drive recombinant gene expression is limited. To analyze, whether elements from the *A. terreus* terrein cluster might be suitable for such a new recombinant expression system, we first analyzed, whether TerR is sufficient to drive terrein cluster expression and identified the respective DNA binding sites. With this knowledge, elements from the cluster were tested by reporter gene expression for their performance in the heterologous host *A. niger*. Finally, the expression system was used to heterologously produce secondary metabolites from *A. terreus* and *A. nidulans* in *A. niger*.

FIGURE 1 | Continued

on terrein cluster genes (terA–terF) normalized against actA transcript levels. Under naturally inducing conditions (PDB) all genes from the cluster are expressed in *A. terreus* SBUG844 and S.BU.8 (parental strain for gene deletions), but not in a ΔterF mutant. The wild-type SBUG844 shows no cluster expression under non-inducing conditions (G100), whereas strains constitutively expressing terR under the *A. nidulans* gpdA promoter (AnP<sub>gpdA;terR</sub> No. 1 and 2) induce the cluster. HPLC analysis of G100 culture extracts from wild type (wt) and terR overexpressing strains AnP<sub>gpdA;terR</sub> No. 1 and 2. The metabolite peak for terrein is denoted by “1.”

(Sharma et al., 2009).
and used for transformation of the bidirectional A1144, or

The following promoters were used:

accession KP100262). The gene coding for a red fluorescent protein was selected (gene

gene was required and a synthetic codon-optimized plasmids containing either the

promoter. The promoter or the terR promoter a second reporter construction was either fused to the terA or orsA gene, whereas the respective natural terminator sequences were maintained. The fusion constructs were ligated into an lhp containing vector and used for transformation of the A. niger P2 strain. To ease heterologous expression of polypeptide synthases in the A. niger P2 strain the expression plasmid SM-Xpress was constructed. A plasmid containing the phleomycin (ble) resistance cassette (AnPgpdAbletrpcT) was linearized with EcoRI. A 786 bp fragment of the A. terreus terA promoter was amplified with oligonucleotides P6/7 and fused with a 363 bp trpC terminator (trpcT) from A. terreus (P8/9) and cloned into the EcorV site of the linearized vector by in vitro recombination using the InFusion HD Cloning Kit (Clontech laboratories; Germany). Thus, the resulting plasmid “SM-Xpress” contains a phleomycin resistance cassette as selection marker and the fusion of PterA and trpCT, which are separated by a Ncol site. This vector was used for domain swapping experiments of the A. nidulans orsA gene, in which the TE domain of OrsA was replaced by the TE domain from TerA. The orsA gene except its TE domain and the TE domain from terA were PCR amplified and fused with the Ncol restricted plasmid SM-Xpress via in vitro recombination. Plasmids were used for transformation of FGSC A1144_PamyBterR (P2).

Metabolite Extraction

To analyze cultures for secondary metabolite production, culture broth was extracted with an equal volume of ethylacetate and the procedure was repeated once. Both fractions were combined and evaporated under reduced pressure. Evaporated residues were solved in 1 ml methanol and filtered. Standard metabolite analyses were performed on an Agilent 1100 series HPLC-DAD system coupled with a MSD trap (Agilent Technologies; Germany) operating in alternating ionization mode as previously described (Gressler et al., 2011).

Expression of terA and orsA in A. niger P2 Strain

Expression of the A. terreus terA gene and the A. nidulans orsA gene in the A. niger P2 strain (containing the PamyBterR construct), the terA promoter was either fused to the terA or orsA gene, whereas the respective natural terminator sequences were maintained. The fusion constructs were ligated into an lhp containing vector and used for transformation of the A. niger P2 strain. To ease heterologous expression of polypeptide synthases in the A. niger P2 strain the expression plasmid SM-Xpress was constructed. A plasmid containing the phleomycin (ble) resistance cassette (AnPgpdAbletrpcT) was linearized with EcoRI. A 786 bp fragment of the A. terreus terA promoter was amplified with oligonucleotides P6/7 and fused with a 363 bp trpC terminator (trpcT) from A. terreus (P8/9) and cloned into the EcorV site of the linearized vector by in vitro recombination using the InFusion HD Cloning Kit (Clontech laboratories; Germany). Thus, the resulting plasmid “SM-Xpress” contains a phleomycin resistance cassette as selection marker and the fusion of PterA and trpCT, which are separated by a Ncol site. This vector was used for domain swapping experiments of the A. nidulans orsA gene, in which the TE domain of OrsA was replaced by the TE domain from TerA. The orsA gene except its TE domain and the TE domain from terA were PCR amplified and fused with the Ncol restricted plasmid SM-Xpress via in vitro recombination. Plasmids were used for transformation of FGSC A1144_PamyBterR (P2).

Quantification of Orsellinic Acid

To quantify metabolite production levels from the direct and the coupled expression system orsellinic acid from the TerA PKS was selected. Metabolites were analyzed on an analytical Shimadzu HPLC system equipped with a DAD type SPD-M20A using a Zorbax Eclipse XDB C8 column (4.5 × 150 mm; 5 μm) with H2O + 0.1% formic acid (buffer A) and methanol (buffer B) as solvents. The following gradient was applied: 0–0.5 min = 10% B; 0.5–12 min from 10 to 90% B; 12–14 min from 90 to 100% B; 14–17 min = 100% B; 17–18 min from 100 to 10% B; 18–21 min = 10% B. A standard curve was generated using defined concentrations of purified orsellinic acid (range from

Surface Plasmon Resonance Measurements

Real-time analyses were performed on a Biacore 2000 system (GE Healthcare) at 25°C. DNA duplexes were produced by annealing complementary 18 bp oligonucleotides using a 5-fold molar excess of the non-biotinylated oligonucleotide. The dsDNA was injected on flow cells of a streptavidin (Sigma Aldrich)-coated CM3 sensor chip at a flow rate of 10 μl/min until the calculated amount of DNA that gives a maximum TerR binding capacity of 50 RU had been bound. TerR proteins were injected in running buffer (10 mM HEPES pH 7.4, containing 150 mM NaCl, 0.005% (v/v) surfactant P20, 5 mM β-Mercaptoethanol and 1 μM ZnCl2) at concentrations from 12.5 to 6400 nM. Sample injection and dissociation times were set to 60 and 120 s at a flow rate of 30 μl/min. Refractive index errors due to bulk solvent effects were corrected with responses from DNA-free flow cell 1 as well as subtracting blank injections. Kinetic raw data were processed and globally fitted with Scrubber 2.0c (BioLogic Software) using a 1:1 interaction model including a mass transport term.

Genetic Manipulation of A. niger and A. terreus

All strains generated in this study are listed in Table S1. The number of genomic integrations of all constructs generated in this study was determined by Southern blot analyses with digoxigenin labeled probes. Blots were developed by chemoluminescence imaging using CDP-star as recommended by the manufacturer (Roche; Germany). For details on specific strain constructions refer to supplementary experimental procedures. In brief, all A. terreus terR expression constructs were amplified from genomic DNA of A. terreus SBUG444. The constructs either contained the native terR promoter or the terR promoter was replaced by the A. nidulans gpdA or the A. oryzae amyB promoter. The terR terminator sequence was maintained in all constructs. Either the pyrithiamine (ptra) or hygromycin B (lhp) resistance cassette was used as a selectable marker in transformations of A. terreus SBUG444, A. niger FGSC A1144, or A. niger FGSC A1144_PterA:lacZ. Transformations were performed as previously described (Zaehle et al., 2014). For β-galactosidase producing reporter strains the lacZ gene from E. coli was fused with different promoters. The following promoters were used: The A. nidulans gpdA promoter, the A. terreus terA promoter in unidirectional (PterA) and bidirectional (PterA/B) orientation, the A. terreus terC promoter and the mutant versions PterCm1 and PterCm2, in which the putative TerR binding site “BS4” was exchanged or mutated. Fusion constructs were cloned into plasmids containing either the ptra or lhp resistance cassette and used for transformation A. terreus SBUG444, A. niger FGSC A1144, or A. niger FGSC A1144_PamyBterR (P2 strain). For analysis of the bidirectional terA/B promoter a second reporter gene was required and a synthetic codon-optimized tdTomato gene coding for a red fluorescent protein was selected (gene accession KP100262). The lacZ and tdTomato genes were fused

in both orientations with PterA/B and cloned in plasmids containing the hph resistance cassette. These reporter plasmids were used for transformation of A. niger FGSC A1144 or A. niger FGSC A1144_PamyBterR (P2 strain).

Metabolite Extraction

To analyze cultures for secondary metabolite production, culture broth was extracted with an equal volume of ethylacetate and the procedure was repeated once. Both fractions were combined and evaporated under reduced pressure. Evaporated residues were solved in 1 ml methanol and filtered. Standard metabolite analyses were performed on an Agilent 1100 series HPLC-DAD system coupled with a MSD trap (Agilent Technologies; Germany) operating in alternating ionization mode as previously described (Gressler et al., 2011).
3.9 to 250 µg/ml). Cultures were cultivated for 48 h at 30°C and 200 rpm in AMM-G100-I-Gln50 liquid medium. Mycelium was collected for dry weight determination and culture supernatants were extracted twice with ethylacetate. Extracts were evaporated, solved in 1 ml methanol and filtered. Different dilutions from each sample in a total volume of 10 µl were loaded to the columns. Peak areas for orsellinic acid at 254 nm were quantified and production levels were calculated against the dry weight biomass.

**RNA Isolation, cDNA Synthesis and Semiquantitative PCR**

*A. terreus* SBUG844 and *A. niger* FGSC A1144 strains were cultivated for 30 or 48 h in PDB, AMM-G100, YM and AMM-CA1% media. Mycelia were ground under liquid nitrogen and RNA was isolated by the RiboPure RNA purification kit (Thermo Scientific; Germany). Residual genomic DNA was removed by the DNA-free kit (Thermo Scientific) and cDNA was synthesized by the Revert Aid Reverse Transcriptase (Thermo Scientific) using anchored oligo dT primers. Semiquantitative PCR was performed as described (Zaehle et al., 2014) previously using oligonucleotides P10–P35. Transcripts were normalized against the actin gene (*actA*, ATEG_06973) for *A. terreus* strains and against the glyceraldehyde-3-phosphate dehydrogenase gene (*gpdA*, est_fge1_pm_C_70216) for *A. niger* strains.

**Determination of β-Galactosidase Activity and tdTomato Fluorescence Intensity**

β-Galactosidase activity was determined as previously described (Gressler et al., 2011). In brief, mycelia from *A. niger* strains containing lacZ fusion constructs were harvested from cultures grown for 48 h at 30°C in PDB, AMM, YM, or AMM-CA1% media. Mycelia were ground under liquid nitrogen and resuspended in 50 mM MOPS buffer (pH 7.5) with 2 mM MgCl₂ and 10 mM β-mercaptoethanol. After centrifugation at 21,000 × g supernatants were used for determination of β-galactosidase activity using o-nitrophenyl-β-galactoside as substrate (Gressler et al., 2011). To detect the fluorescence intensity of *A. niger* transformants containing tdTomato fusion constructs, cell-free extracts were prepared as described above. After determination of protein concentrations by the Bradford assay (BioRad; Germany) protein concentrations were adjusted to 2.0, 1.0, and 0.5 mg/ml. 200 µl of the dilutions were transferred to a black flat-bottom 96 well plate (Nunc; Germany) and analyzed in a microplate reader (FLUOstar Omega, BMG Labtech; Germany). Plates were shaken for 1 min in double orbital mode and emission was detected at 590 nm using an excitation wavelength of 454 nm (50 flashes/well, top reading, gain 2000). The parental *A. niger* strains A1144 and P2 that contained no tdTomato gene served as negative controls and were subtracted from the fluorescence values of reporter strains. Specific activities were expressed as fluorescence units per mg protein. To calculate relative expression levels, fluorescence intensities (FI) of reporter strains were normalized against fluorescence intensities of an *A. niger* strain expressing the tdTomato gene under control of the *A. nidulans* gpdA promoter using the following formula: Relative fluorescence intensity (rFI) [%] = (FIreporter strain – FIp2)/(FIΔgpdA – FIWT). All assays were performed in biological and technical triplicates from at least three individual transformants.

**Accession Numbers**

The DDBJ/EMBL/GenBank nucleotide sequence database accession number for the *Aspergillus* codon-optimized tdTomato gene reported in this paper is KPI00262.

**Results and Discussion**

**The Transcription Factor TerR is Essential for Expression of Terrein Cluster Genes**

Previously, we have shown that *A. terreus* produces large quantities of the metabolite terrein ([Figure 1A](#) and we discovered that the responsible gene cluster contains its own transcription factor TerR, which is encoded at locus tag ATEG_00139 (Zaehle et al., 2014). Gene deletion analysis revealed that TerR is indispensable for terrein production in *A. terreus*, since terR deletion resulted in complete loss of terrein production during cultivation on inducing potato dextrose broth (PDB) (Zaehle et al., 2014). To confirm that TerR is directly involved in the activation of the gene cluster, comparative semiquantitative RT-PCR analyses between *A. terreus* wild type and terR mutant (*AterR*) were performed. Under inducing conditions, all genes from the cluster spanning the region from terA – terF were transcribed in the wild type ([Figure 1B](#)). While all analyzed genes were strictly dependent on TerR for induction, terE, F and H showed some background expression in the ΔterR background. Thus, TerR seems to act as a transcriptional activator with special importance for transcription of the key polyketide synthase TerA, which shares a bi-directional promoter with the terE gene. Interestingly, among the 11 genes spanning the cluster, six of them (terAB, terE/F, and terH/I) share bi-directional promoters, implying that either multiple putative TerR binding sites are found in these promoters or that single binding sites can activate expression in both directions. However, the control of gene expression from bi-directional promoters is frequently found in fungal secondary metabolite gene clusters. As examples, in the allatoxin gene cluster from *Aspergillus parasiticus* 12 out of 25 genes share a bi-directional promoter (Yu et al., 2004) and in the gliotoxin gene cluster from *Aspergillus fumigatus* (Forsyth et al., 2011) 8 out of 12 genes are controlled by shared promoter sequences. Furthermore, similar to our results on TerR dependency for terrein cluster activation, the ZnCySb binuclear transcription factor gliZ from the gliotoxin cluster is essential for activation of cluster genes (Bok et al., 2006).

**TerR is Sufficient for Activation of Terrein Cluster Genes**

In *A. terreus* terrein is only produced under specific environmental conditions, suggesting that transcription and activation of terk is itself under the control of other transcription factors that mediate environmental signals toward terrein production. Thus, to analyze, whether TerR production under non-inducing conditions is sufficient to stimulate terrein production in *A. terreus*, we aimed in the exchange of the native terR promoter against the constitutively active gpdA promoter from *Aspergillus*...
**nidulans** (Punt et al., 1990). The resulting PgpdA:terR fusion construct was used to transform an *A. terreus* wild-type strain. Indeed, when transformants were grown on complete glucose minimal medium, which is a repressive condition for terrein production in *A. terreus*, semiquantitative RT-PCR revealed induction of all cluster genes and high amounts of terrein were produced (Figures 1B,C). Thus, terR expression in *A. terreus* wild type is strictly dependent on specific inducing signals, but once expressed, TerR is sufficient to induce all genes required for terrein production. Similarly, the transcription factors ApdR (Bergmann et al., 2007), AlfR (Liu and Chu, 1998), and GLIZ (Bok et al., 2006) have been shown to induce expression of the respective clusters when overproduced in the homologous host. Therefore, our data indicate that promoter elements of the terrein cluster contain specific DNA binding sites that are recognized by TerR.

**The TerR Zinc Binuclear Cluster DNA-Binding Domain has a Monomeric Structure**

Since TerR was able to induce all genes required for terrein synthesis, we expected that conserved DNA binding sites are present in the promoter regions of genes spanning the terrein cluster. TerR belongs to the family of transcriptional activators with a GAL4-type Zn₂Cy₆ zinc binuclear cluster DNA-binding domain. This type of transcription factor is one of the most abundant transcriptional regulators present in fungi and involved in the regulation of primary and secondary metabolic processes (MacPherson et al., 2006). Their DNA-binding domain is generally located at the N-terminus of the protein and is sufficient to recognize its target sequence (Todd et al., 1998). Additionally, most GAL4-type transcription factors contain a coiled-coil domain near the N-terminus that is involved in dimerization and results in the recognition of consensus repeats (Fitzgerald et al., 2006). Interestingly, no coiled-coil domain is detected in the N-terminus of TerR, instead RADAR (Rapid Automatic Detection and Alignment of Repeats in protein sequences) analysis (Heger and Holm, 2000) detected an unusual amino acid repeat at positions 97–111 and 121–135 with unknown function. Thus, for analysis of the TerR subunit composition and identification of DNA-binding sites we first selected an N-terminal peptide of 153 amino acids (TerR₁₋₁₅₃) that was cloned into an expression vector with or without N-terminal His-tag. Unfortunately, regardless of the peptide version (tagged or untagged) and the purification procedure, we were unable to obtain a monodispersed protein fraction and MALDI-TOF MS/MS analysis showed co-purification of N- and C-terminal degradation products (Figure 2B). A similar phenomenon was observed during purification of the transcriptional activator AlcR of the ethanol utilization pathway in *A. nidulans* (Felenbok et al., 1988; Cabuzac et al., 2001). An AlcR₁₋₁₉₇ fragment enclosing the DNA-binding domain could not be purified to homogeneity (Lenouvel et al., 1997). Therefore, we truncated the TerR domain to a TerR₃₅₋₁₃₈ fragment (Figure 2A). This protein fragment was purified to >98% homogeneity as judged by SDS-gel electrophoresis (Figure 2B) and analytical size exclusion chromatography. Multiangular static light scattering analysis revealed a molar mass of 11.65 kDa (Figure 2C), demonstrating that the purified protein exclusively consisted of monomers in solution (theoretical molar mass of 11.35 kDa). Thus, besides the transcriptional activator AlcR, TerR seems to form another example of GAL4-type transcription factors with monomeric solution structure.

**TerR Recognizes CGG Direct Repeat Consensus Sequences in the Terrein Cluster**

To identify putative TerR binding sites in the terrein gene cluster an *in silico* prediction of candidates constituting regulatory DNA motifs was performed on the intergenic regions of terA—terf (Figure 3A) using the SCOPE motif finder suite (Chakravarty et al., 2007). A SPACER bipartite motif with the direct CGG half-site repeat consensus 5′-CGG ᵃ₋₃₋₃₋₃-CGG-3′ (Figure 3Q) was identified that was present in all promoter regions of the cluster, with the exception of terG, terI, and terH, whereby the latter genes were dispensable for terrein production (Zaehle et al., 2014). Interestingly, the intergenic region that acts as a bidirectional promoter of terA and terB contained three putative binding sites that were annotated as BS1, BS2, and BS3 (Figure 3A). To analyze recognition of these binding sites by the TerR₃₅₋₁₃₈ fragment, protein: DNA real-time surface plasmon resonance (SPR) biosensor interaction analyses were performed. For this purpose, oligonucleotide duplexes with a four-nucleotide overhang at the 5′-CGG half-site and a three-nucleotide overhang at the 3′-CGG half-site were deduced. Biotinylated oligonucleotides were hybridized with complementary unbiotinylated anti-strands and immobilized on streptavidin-coated CM3 sensor chips to give a maximum response (R₉₅) of 50 resonance units (RU) when bound by a single monomeric TerR₃₅₋₁₃₈ domain. Kinetic SPR binding responses of TerR₃₅₋₁₃₈ to BS1–BS3 fitted with K₉₅ values ranging between 0.5 and 0.7 μM. Interestingly, while steady state binding analysis showed that only one monomer binds to the BS1 sequence (Figure 3B, Table 1), BS2 and BS3 revealed that a significant fraction of DNA duplexes were bound by two monomers at these binding sites (Figures 3C,F). To analyze the specificity for the predicted motif, BS2 was selected for mutations in the first and second CGG half-sites. Mutation of the 5′-CGG to CAA led to a predominant loss of BS2-binding (Figure 3D). In contrast, mutation of the 3′-CGG to CAA altered the K₉₅ from 536 to 363 nM, but simultaneously reduced the number of TerR₃₅₋₁₃₈ units that bind to the sequence (Figure 3E). While 1.6 monomers bound to the native sequence, only 0.95 monomers were found to bind at the mutated sequence. A similar phenomenon was observed when the second CGG half-site was mutated in BS7 (Figures 3M,N, Table 1). This implies that the 5′-CGG half-site is required for high affinity binding, whereas the 3′-CGG half-site attracts a second monomer to the motif. To confirm this assumption and to define a consensus sequence, we tested all other binding sites that were predicted by SCOPE using SPR analysis (Figure 3). Indeed, all predicted binding sites were recognized by TerR₃₅₋₁₃₈, whereby K₉₅ values ranged from 26 nM to 1.5 μM with either a tendency to bind only one or two TerR₃₅₋₁₃₈ fragments. In this respect, binding of a second monomer seems to be favored when the last nucleotide of the five base pair spacer is a thymidine (BS2,
BS3, BS5, BS6, and BS9, Figure 3). In contrast, motifs, in which the spacer between the two CGG half-sites was reduced to four instead of five base pairs as well as motifs that harbored inverted CGG half-sites were not recognized by TerR (data not shown). We additionally observed that sequences that contained one or two “T” nucleotides in front of the 5′-CGG half-site had a tendency for high-affinity binding (BS1, BS2, BS4, BS6, BS7, BS9). To analyze the importance of these residues, we mutated the two “T” nucleotides in BS4 into “G” nucleotides (BS4m1; Figure 3H). Indeed, the affinity of the mutated binding site decreased by a factor of 6.5. In contrast, mutation of “C” into “G” directly behind the 5′-CGG half-site only showed a minor effect on affinity (Figure 3I).

A Basic Stretch at the TerR N-terminus Promotes High Affinity DNA Binding

The selected TerR35–138 fragment contained a basic stretch at positions 38–47 that is followed by the predicted DNA-binding domain. In this respect, TerR appears to possess similar features as the regulator of ethanol utilization AlcR from Aspergillus nidulans, which also recognizes DNA-binding sites in monomeric form as mentioned in Section “The TerR Zinc Binuclear Cluster DNA-Binding Domain has a Monomeric Structure.” For AlcR it has been shown that a basic sequence at the N-terminus is important for high affinity DNA binding, since it strengthens the interaction of the monomer with the phosphate backbone (Nikolaev et al., 1999). In order to elucidate the importance of this basic stretch in TerR, we generated a TerR43–138 fragment that lacked four basic amino acids present in the TerR35–138 fragment (Figure 2). When the TerR43–138 fragment was tested by SPR analysis on selected binding sites, a similar order of binding affinity was observed as for the TerR35–138 fragment. However, $K_D$-values increased by a factor of 7–22, confirming that the basic stretch is essential for tight binding to the DNA phosphate backbone (Figure S2). Additionally, while some binding motifs showed the tendency to bind two TerR35–138 fragments, the TerR43–138 fragment exclusively recognized these motifs as a single monomer (Table 2). This implies that recognition of the second CGG half-site is not only dependent on the sequence motif, but also on the general affinity of the TerR fragment toward DNA binding.

Thus, comparison of the different features of the TerR fragments and sequence analyses lead to the following conclusions: (i) A TerR1–153 fragment is unstable in vitro, but the basic stretch at the N-terminus is required for high affinity DNA binding. (ii) The 5′-CGG half-site is of major importance for sequence recognition and binding. (iii) High affinity binding sites are characterized by a “T” before the 5′-CGG half-site. Similarly (iv) when the last nucleotide of the spacer is a “T,” there is a tendency for a second monomer to bind to the 3′-CGG half-site, but only in the presence of the basic amino acid stretch in the TerR domain. (v) The consensus sequence for TerR high affinity binding sites is 5′-TCGGHHWHGWCGG–3′ (Figure 3R).

In Vitro Binding Affinity of TerR35–138 is Reflected by In Vivo Promoter Activity

The in vitro analyses of the TerR35–138 fragment showed that BS4 reflects a high affinity binding site with a $K_D$ of 66 nM, but tendency for single monomer binding (Figure 3G). BS4 is the only confirmed binding site in the intergenic region of terC.
FIGURE 3 | In vitro TerR binding to a consensus motif identified in promoters of the A. terreus terrein biosynthesis gene cluster and in vivo verification of in vitro data. (A) Schematic presentation of the terrein gene cluster. Intergenic positions of the consensus TerR-binding motif identified by the SCOPE motif finder suite (Q) are annotated with BS1–9. (B–I,K–P) Real-time in vitro SPR interaction analysis of TerR<sub>35–138</sub> with DNA containing the predicted natural or mutated (denoted by the letter “m”) binding sites from promoters of terrein cluster genes. Sequences of DNA duplexes used for SPR analysis are shown on top of the sensorgrams. Numbers represent the CGG direct repeat motif positions relative to the start of the open reading frame. CGG half-sites and 5′-flanking thymidines are highlighted in red and blue. Substituted nucleotides relative to the wild-type sequence are underlined and shown in lowercase letters. TerR<sub>35–138</sub> binding responses from triplicate injections of different concentrations (black lines) are overlaid with the best fit derived from a 1:1 interaction model including a mass transport term (red lines). Dissociation constants (K<sub>D</sub>) are plotted inside the sensorgrams. For details on the specific binding motifs refer to the main text. (J) In vivo verification of the PterC binding motif BS4. PterClacZ reporter strains were grown for 48 and 72 h on PDB or for 72 h on AMM and β-galactosidase activity was determined. Highest activity is observed with the native terC promoter (PterC), whereas activity is significantly reduced by replacement of the native binding site (BS4) against the low affinity BS5 from the terD promoter (PterC<sub>m1</sub>) or when the two CGG half-sites of BS4 are replaced by CAA triplets (PterC<sub>m2</sub>). No activity for any reporter is detected on AMM. Statistical significance (p < 0.01) was calculated by the student’s unpaired t-test using data from at least three independent transformants measured in biological triplicates. Error bars represent SEM. *PterC vs. PterC<sub>m1</sub>; †PterC vs. PterC<sub>m2</sub>; ‡PterC<sub>m1</sub> vs. PterC<sub>m2</sub>; all significances for PDB, but not AMM. (R) WebLogo sequence consensus motif of the experimentally mapped high affinity TerR binding sites BS1, BS2, BS4, BS6, BS7, and BS9.
Table 1: Dissociation constants and stoichiometry of analyzed TerR<sub>35−130</sub>:DNA interactions.

<table>
<thead>
<tr>
<th>DNA duplex (Binding site)</th>
<th>Mw duplex (Da)</th>
<th>DNA bound (RU)</th>
<th>R&lt;sub&gt;max&lt;/sub&gt; calculated&lt;sup&gt;*&lt;/sup&gt; (RU)</th>
<th>R&lt;sub&gt;max&lt;/sub&gt; measured (RU)</th>
<th>molar ratio TerR:DNA</th>
<th>K&lt;sub&gt;D&lt;/sub&gt;</th>
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<tbody>
<tr>
<td>BS1</td>
<td>11405</td>
<td>52.4</td>
<td>52.1</td>
<td>58.5 ± 0.5</td>
<td>1.12</td>
<td>527 ± 3 nM</td>
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<tr>
<td>BS2</td>
<td>11401</td>
<td>51.6</td>
<td>51.3</td>
<td>82.9 ± 0.1</td>
<td>1.62</td>
<td>536 ± 3 nM</td>
</tr>
<tr>
<td>BS2m1</td>
<td>11399</td>
<td>54.7</td>
<td>54.5</td>
<td>108.2 ± 0.7</td>
<td>1.93</td>
<td>5.78 ± 0.06 µM</td>
</tr>
<tr>
<td>BS2m2</td>
<td>11339</td>
<td>51.2</td>
<td>51.0</td>
<td>48.4 ± 0.8</td>
<td>0.98</td>
<td>363 ± 2 nM</td>
</tr>
<tr>
<td>BS3</td>
<td>11403</td>
<td>52.0</td>
<td>51.8</td>
<td>90.3 ± 0.1</td>
<td>1.74</td>
<td>676 ± 3 nM</td>
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<tr>
<td>BS4</td>
<td>11403</td>
<td>55.9</td>
<td>55.9</td>
<td>63.6 ± 0.1</td>
<td>1.14</td>
<td>65.6 ± 0.6 nM</td>
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<tr>
<td>BS4m1</td>
<td>11404</td>
<td>53.1</td>
<td>52.8</td>
<td>72.8 ± 0.1</td>
<td>1.38</td>
<td>429 ± 2 nM</td>
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<tr>
<td>BS4m2</td>
<td>11402</td>
<td>52.9</td>
<td>52.7</td>
<td>62.9 ± 0.1</td>
<td>1.19</td>
<td>155 ± 1 nM</td>
</tr>
<tr>
<td>BS5</td>
<td>11401</td>
<td>52.9</td>
<td>52.6</td>
<td>86.6 ± 0.1</td>
<td>1.87</td>
<td>5.32 ± 0.6 µM</td>
</tr>
<tr>
<td>BS6</td>
<td>11402</td>
<td>52.6</td>
<td>52.1</td>
<td>84.6 ± 0.1</td>
<td>1.62</td>
<td>150 ± 1 nM</td>
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<tr>
<td>BS7</td>
<td>11404</td>
<td>50.1</td>
<td>49.9</td>
<td>62.3 ± 0.1</td>
<td>1.25</td>
<td>26.5 ± 0.3 nM</td>
</tr>
<tr>
<td>BS7m</td>
<td>11401</td>
<td>52.4</td>
<td>52.2</td>
<td>48.2 ± 0.1</td>
<td>0.92</td>
<td>25.3 ± 0.2 nM</td>
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<tr>
<td>BS8</td>
<td>11406</td>
<td>52.1</td>
<td>51.8</td>
<td>60.4 ± 0.1</td>
<td>1.17</td>
<td>286 ± 2 nM</td>
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<tr>
<td>BS9</td>
<td>11402</td>
<td>51.7</td>
<td>51.5</td>
<td>77.8 ± 0.2</td>
<td>1.51</td>
<td>41.0 ± 0.4 nM</td>
</tr>
</tbody>
</table>

<sup>*</sup><sup>R<sub>max</sub></sup> calculated = 11350 (Mw TerR<sub>35−130</sub>) / Mw DNA x DNA bound.

Table 2: Dissociation constants and stoichiometry of analyzed TerR<sub>133−138</sub>:DNA interactions.

<table>
<thead>
<tr>
<th>DNA duplex (Binding site)</th>
<th>Mw duplex (Da)</th>
<th>DNA bound (RU)</th>
<th>R&lt;sub&gt;max&lt;/sub&gt; calculated&lt;sup&gt;*&lt;/sup&gt; (RU)</th>
<th>R&lt;sub&gt;max&lt;/sub&gt; measured (RU)</th>
<th>Molar ratio TerR:DNA</th>
<th>K&lt;sub&gt;D&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>BS2</td>
<td>11401</td>
<td>56.3</td>
<td>50.5</td>
<td>52.4 ± 0.2</td>
<td>1.04</td>
<td>3.73 ± 0.03 µM</td>
</tr>
<tr>
<td>BS4</td>
<td>11403</td>
<td>58.5</td>
<td>52.4</td>
<td>45.9 ± 0.1</td>
<td>0.88</td>
<td>1.07 ± 0.06 µM</td>
</tr>
<tr>
<td>BS4m1</td>
<td>11404</td>
<td>57.7</td>
<td>51.7</td>
<td>49.3 ± 0.3</td>
<td>0.95</td>
<td>9.3 ± 1 µM</td>
</tr>
<tr>
<td>BS4m2</td>
<td>11403</td>
<td>58.0</td>
<td>52.0</td>
<td>45.9 ± 0.1</td>
<td>0.88</td>
<td>1.37 ± 0.05 µM</td>
</tr>
<tr>
<td>BS6</td>
<td>11403</td>
<td>57.3</td>
<td>51.3</td>
<td>43.4 ± 0.1</td>
<td>0.85</td>
<td>2.08 ± 0.09 µM</td>
</tr>
<tr>
<td>BS7</td>
<td>11403</td>
<td>60.0</td>
<td>53.8</td>
<td>48.9 ± 0.1</td>
<td>0.91</td>
<td>591 ± 2 nM</td>
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<tr>
<td>BS8</td>
<td>11406</td>
<td>60.9</td>
<td>54.6</td>
<td>58.0 ± 1.0</td>
<td>1.06</td>
<td>17.2 ± 0.5 µM</td>
</tr>
<tr>
<td>BS9</td>
<td>11402</td>
<td>59.6</td>
<td>53.4</td>
<td>49.3 ± 0.1</td>
<td>0.92</td>
<td>820 ± 4 nM</td>
</tr>
</tbody>
</table>

<sup>*</sup><sup>R<sub>max</sub></sup> calculated = 10218 (Mw TerR<sub>133−138</sub>) / Mw DNA x DNA bound.

(ATEG_00143, Figure 3A), which is no longer expressed when terR is deleted (Figure 1B). Thus, we were interested, whether the in vitro binding properties of the predicted binding sites reflect promoter activation in vivo. To address this question, we generated three reporter constructs. The first construct contained the native terC promoter fused with the E. coli lacZ gene (PterClacZ). For the second construct, BS4 from the terC promoter was exchanged with BS5 and also fused with the lacZ gene resulting in PterCm1: lacZ. BS5 is one of two binding sites in the intergenic region of terD (ATEG_00142), which showed a 23 times reduced TerR<sub>35−133</sub> binding affinity in vitro, but a tendency for binding two monomers (Figure 3K). Finally, for the last construct, the two CGG half-sites of BS4 were replaced by CAA in PterC and fused to the lacZ gene resulting in PterCm2: lacZ. These mutations in both CGG half-sites were assumed to lead to a complete loss of binding site recognition. The A. terreus wild-type strain was transformed with the three constructs and several independent transformants with single genomic integrations were selected for analysis of β-galactosidase activity under inducing and non-inducing conditions (Figure 3J). As expected, none of the transformants revealed β-galactosidase activity when cells were grown under non-inducing conditions (AMM). In contrast, activity significantly increased, when cells were grown on PDB medium. Here, β-galactosidase activity was at least 6 times higher from strains containing the native BS4 site compared to strains containing the less affinity BS5 site (PterCm1). Moreover, compared to the native promoter, the conversion of both CGG half-sites to CAA (PterCm2) reduced promoter activity by a factor 17. Thus, the binding sites predicted from in silico and in vitro analyses are indeed important for promoter activation by TerR and in vitro parameters reflect the in vivo situation.

TerR Activates the terA Promoter in the Heterologous Host A. niger, but Requires a Promoter Exchange

In order to generate a heterologous expression system using regulatory elements from the terrein gene cluster, we selected the filamentous fungus A. niger, which is frequently used in biotechnological applications (Sharma et al., 2009). Here, an analysis was required that tested the possibility of TerR mediated induction of terrein cluster promoters in the heterologous host. Thus, to
analyze, whether TerR is sufficient to activate terA in *A. niger*, different β-galactosidase reporter strains were generated (Figure 4 and Figure S1). First, the promoter of terA (PterA), which contained the three DNA binding sites B51-3 was fused with the *E. coli* lacZ gene and transferred to *A. niger*. After cultivation on different media, β-galactosidase activity hardly exceeded the background level, indicating that the terA promoter is not recognized by *A. niger* transcription factors that are present under the applied conditions (Figure 4A and Figure S3). Unfortunately, when terR under its native promoter was additionally introduced into the PterA::lacZ strain, again no β-galactosidase activity was detected (Figure S3). This indicated that either (i) TerR is not able to activate the terA promoter in the heterologous system or that (ii) the native promoter of TerR is not recognized in *A. niger*. To test these assumptions, we analyzed the expression of terR by semiquantitative PCR. Indeed, no terR transcript was detected, which confirms that specific regulatory elements from *A. terreus* are required to drive activation of terR expression (Figure S3). However, to show that TerR can also activate the terA promoter in a heterologous host, terR was fused with the constitutively active glyceraldehyde-3-phosphate dehydrogenase promoter (PgpdA) from *A. nidulans* and the resulting PgpdA::terR construct was introduced into the *A. niger* strain with the PterA::lacZ reporter. Indeed, a strong β-galactosidase activity was detected, which confirmed the specific recognition of terA promoter elements by TerR even in a heterologous system (Figure 4A). Additionally, β-galactosidase activity doubled in a strain that contained two copies of the PgpdA::terR constructs, indicating that TerR levels are the rate limiting step in terA promoter activation (Figure 4A). In conclusion, the native terR promoter is not stimulated and transcribed in *A. niger*, but when TerR is produced under control of an active promoter, this activates the expression of reporter genes (lacZ and tdTomato) presumably in different strength (indicated by arrows).

![Figure 4](https://example.com/figure4.png)

**FIGURE 4** | Analysis of recombinant terR and terA expression in *A. niger* by semiquantitative RT-PCR, LacZ, and tdTomato reporter activity. (A) Semiquantitative RT-PCR (lower panels) and β-galactosidase activity (upper panel) of *A. niger* A1144, PterA::lacZ either without terR or co-expressing the terR gene under control of the *A. nidulans* gpdA promoter (PgpdA::terR, PterA::lacZ; with one (1 x) or two (2 x) copies of PpgpdA::terR) or the *A. oryzae* amyB promoter (P amyB::terR, PterA::lacZ). Expression of terA requires terR co-expression and is dependent on terR expression levels. *A. niger* gpdA was used as reference gene in semiquantitative RT-PCR analyses. Lane 1, control genomic DNA (gDNA) of *A. niger* PterA::lacZ; Lane 2, control gDNA of *A. niger* P amyB::terR, PterA::lacZ. (B) Scheme of the terR dependent expression system using the bi-directional promoter of terA and terB. After sugars induced expression of terR, the TerR protein (blue circles) recognizes the three bindings sites in the bi-directional terA/terB promoter and activates the expression of reporter genes (lacZ and tdTomato) presumably in different strength (indicated by arrows). (C) Semiquantitative RT-PCR analysis of wild type *A. niger* A1144 and A1144_PamyB::terR (P2) expressing terR under control of the amyB promoter. terR is strongly expressed on PamyB inducing media (G100 and YM), but hardly detectable on CA1% medium. (D) Relative promoter activity of PterA and PterB from the reporter system described in (C). The parental strain P2 and the reporter strains P2_PterA::lacZ, PterB::tdTom and P2_PterB::lacZ, PterA::tdTom were cultivated for 48 h on G100, YM and CA1% and β-galactosidase activity and fluorescence intensity from cell-free extracts were determined. All values were normalized against reporter activities from the A. nidulans gpdA promoter (AnPgpdA::lacZ and AnPgpdA::tdTom) that were used as 100% reference values. Each experiment was carried out with three independent mutants in biological and technical triplicates.
transcription factor is sufficient to stimulate expression of terA also in the heterologous system.

**Control of terR Expression Under the Inducible amyB Promoter Allows Regulated Gene Expression from the Bi-Directional terA/B Promoter**

Experiments in Section “TerR Activates the terA Promoter in the Heterologous Host A. niger, but Requires a Promoter Exchange” revealed that control of terR expression under the gpdA promoter leads to constitutive activation of the terA promoter. In order to regulate expression, the amyB promoter was selected, since it was previously shown to be highly active in the presence of maltose or glucose, but only shows low background activity on sugar-free media such as 1% casamino acids (CA medium) (Ward, 2012; Zaehle et al., 2014). Indeed when the A. niger strain carrying the PterA: lacZ reporter was transformed with a PamyB:terR construct, high β-galactosidase activity was obtained when cells were cultivated on maltose containing YM medium, whereas activity after cultivation in CA medium was approximately 20 times lower (Figure 4A). This was also reflected by terR expression levels that were highly abundant on YM medium, but hardly detected on CA medium (Figure 4A). This again confirms that high terR expression levels are crucial for strong activation of target promoters. This also indicates that expression from promoters of the terrein cluster can be regulated by modifying the production level of the TerR regulator.

The selected terrein cluster promoter is assumed to depict a bi-directional promoter, because it separates the reading frames of terA and terB that are transcribed in opposite directions as already described in Section “The Transcription Factor TerR is Essential for Expression of Terrein Cluster Genes.” Interestingly, it has been shown in the bi-directional promoters of penicillin biosynthesis from A. nidulans (acvaAtipnA) (Brakhage, 1997) and cephalosporin biosynthesis from Acremonium chrysogenum (pcbA/pcbC) (Menne et al., 1994) transcription rate is favored in one direction. To test transcriptional activation from the terA/B promoter, the lacZ gene and a codon-optimized tdTomato gene encoding a red fluorescent protein were selected as reporters in both reading directions (Figure 4B). An A. niger strain carrying a single copy of the PamyB:terR construct (P2 strain) that shows the expected expression pattern of terR under inducing and non-inducing conditions (Figure 4C), was used as recipient strain for the different reporter constructs. Four to eight single copy transformants from each construct were analyzed in a pre-screening approach to test for variations in the expression pattern due to positioning effects (Minetoki et al., 1998; Liu et al., 2003; Blumhoff et al., 2013). All transformants revealed a similar tendency of expression levels (±25% from average). Therefore, three independent transformants were selected from each construct and strains were cultivated in biological triplicates for determination of the average promoter activation rate in comparison to the gpdA control promoter. Selected transformants with single copy integration were grown on glucose, YM or CA medium and screened for β-galactosidase activity and fluorescence intensity from cell-free extracts. All measurements were background corrected against extracts from an A. niger strain without reporter integration. Reporter activities were normalized against activities obtained from expression of the respective reporters under control of the A. nidulans gpdA promoter (Figure 4D).

Results from both reporters show that (i) terA/B promoter activity in both directions is strongly induced on glucose and YM medium, whereas activity remained near background values when cells were grown on non-inducing CA medium. (ii) Reporter activity in direction of the terA gene exceeded that of the gpdA promoter by up to three times, whereas activity in terB direction only reached 20–40% of the control value. Thus, transcription in the direction of the polyketide synthase gene terA exceeds that of terB by 8–14-fold. Additionally, the relative accumulation of TdTomato was slightly higher than that of the β-galactosidase, which could be due to increased stability of the codon-optimized TdTomato gene. In conclusion, these results indicate that terA/B promoter activation is directly dependent on the level of the TerR regulator.

Previous approaches to develop high-level expression systems used strongly expressed and, preferentially, inducible promoter elements for direct control of target gene expression. Examples are the thiamine promoter (Pthia) for expression in A. oryzae (Shoji et al., 2005), the xylose induced xy1 promoter in A. chrysogenum (Blatzer et al., 2014), synthetic promoters containing the human estrogen receptor (hERa) response elements in A. nidulans and A. niger (Pachlinger et al., 2005) or the Escherichia coli tetracycline resistance operon (Tet-on system) in A. fumigatus and A. niger (Vogt et al., 2005; Meyer et al., 2011). Here, we identified that the activity of the artificial promoter that controls terR expression is amplified via TerR-dependent activation of the terA promoter. This assumption derives from the following observation: In previous studies we have shown that the gpdA and amyB promoter display similar activation levels on G100 medium (Zaehle et al., 2014). Thus, in case there is no signal amplification, reporter activity from the coupled system should be equivalent to PgpdA-driven activity. However, the analyses performed here show that reporter activity increased by a factor >2 when PamyB controls terR expression and the reporter is under terA control. Therefore, the coupled system of terrein cluster elements leads to an amplification of the strength of the promoter that is used to regulate terR expression. We assume that activity of promoters that are even stronger than PamyB could be amplified in our new coupled system. Although it might be expected that a saturation of the terA promoter may occur at a specific TerR level, this could either be compensated by increasing the number of terR binding sites in the terA promoter or by adding additional copies of the fusion of PterA with the gene of interest. However, a preliminary analysis of multicopy integrants with the PterA: lacZ construct revealed that expression levels run into saturation at approximately five integrations.

**The PamyB:terR/PterA System is Suitable for High-Level Heterologous Secondary Metabolite Production**

Due to the high reporter activities obtained from the terA promoter in combination with the PamyB:terR control element, we were interested in the use of this system for the heterologous
A new terrein cluster-based expression system for production of secondary metabolites. In a previous study, we expressed the terrein synthase gene terA under direct control of the amyB promoter in A. niger (Zaehle et al., 2014). Sufficient quantities of the TerA metabolites orsellinic acid (4), 6,7-dihydroxymellein (3) and 4-hydroxy-6-methylpyrone (2) were produced (for molecular structures refer to **Figure 5E**). However, we assumed that metabolite production in the heterologous system could strongly exceed the quantity of metabolites compared to the previous construct. To confirm this hypothesis, the P2 strain with the single copy of the PamyB:terR integration was transformed with the A. terreus terA gene under control of its native terA promoter. Transformants were cultivated in glucose minimal medium, which was selected since HPLC profiles of the P2 strain showed no significant background metabolite production under this condition (**Figure 5A**).

The metabolic profile of transformants was directly compared with that of a PamyB:terA strain that was cultivated under the same conditions (**Figures 5B,C**). Indeed, although all three metabolites were detected in similar ratios as detected from the PamyB:terA strain, the amount of metabolites produced by the new expression system strongly exceeded that of the former strain. In order to quantify the increase of metabolite production in the coupled system, we selected three PamyB:terA single copy and three PamyB:terA double copy integrants in the A. niger A1144 genetic background (Zaehle et al., 2014) and four single copy and two double copy PterA:terA integrants from the P2 strain. Transformants were cultivated in AMM-G100+Gln50 for 48 h, supernatants were extracted and orsellinic acid concentration was quantified by HPLC and normalized against the mycelial dry weight (**Figure S4**). Although production rates varied among independent transformants, the single and double copy strains with the PamyB:terA construct produced on average 0.37 (±0.19) and 0.43 (±0.13) mg orsellinic acid per gram of dried mycelium. Single copy PterA:terA integrants produced on average 1.16 (±0.64) and double copy integrants 3.20 (±1.69) mg of orsellinic acid per gram of dried mycelium. This results in an approximated 3-fold increase in the coupled expression system for single copy and a 7.5-fold increase for the double copy integrants in comparison to the direct expression system under control of the amyB promoter. Although increases in production rates may be metabolite dependent, these results reflect the increased transcription rate from the coupled expression system as shown in **Figure 4D** and confirms the suitability of the system for heterologous expression.

In another approach, we selected the orsA PKS gene from A. nidulans for heterologous expression in A. niger. Previous analyses have shown that the orsA gene is induced during the cocultivation of A. nidulans with the bacterium

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**FIGURE 5** | Metabolite detection from culture filtrates of recombinant A. niger strains expressing different polyketide synthases under control of the terR/PterA expression system. All strains were grown for 48 h in AMM-G100 medium with 20mM glutamine as nitrogen source. **(A-D)** HPLC profiles from **(A)** A1144_PamyB:terR (P2, parental control strain), **(B)** A1144_PamyB:terA (control strain) with conventional direct terA expression, (Zaehle et al., 2014), **(C)** P2 PterA:terA (coupled system), and **(D)** P2 PterA:orsA (coupled system). A schematic drawing of the integrated constructs is given on the left next to the respective profile. **(E)** Structures of metabolites identified in the extracts are: 4-hydroxy-6-methylpyrone (2), 6,7-dihydroxymellein (3), orsellinic acid (4), and lecanoric acid (5). *Indicate non-reproducible metabolite peaks; # refers to (2) that is only visualized by loading concentrated extracts of the PamyB:terA transformants.
Streptomyces hygroscopicus (Schroeckh et al., 2009). Structure elucidation of the metabolites produced by OrsA required a 14 liter cocultivation of both organisms to obtain sufficient amounts of metabolites. Interestingly, orsellinic acid and lecanoric acid, a depside of two orsellinic acid molecules, were identified. However, it remained unclear, which of the two products is the major metabolite produced by OrsA and whether OrsA is directly performing the transesterification of two orsellinic acid molecules (Schroeckh et al., 2009). Therefore, we generated a PterA:orsA construct that was transferred into the A. niger P2 strain and single copy integrants were tested for product formation in 50 ml culture scale. Ethylacetate extracts revealed one major and two minor metabolites (Figure 5D) that were identified by HRESI-MS as lecanoric acid (5), orsellinic acid (4) and 4-hydroxy-6-methylpyrone (2) and the identity was confirmed by reference against known standards and 1H and 13C NMR spectra of purified lecanoric acid (Figure S5 and supplementary experimental procedures). Since lecanoric acid was by far the most prominent metabolite produced by all independent transformants, it can be assumed that the thioesterase (TE)-domain of OrsA performs the transesterification of the p-hydroxy-group of one orsellinic acid molecule with the carbonyl-group of a second molecule rather than releasing orsellinic acid by simple hydrolysis. A similar mechanism of depside formation from orsellinic acid-derived metabolites might be present in lichen-forming fungi in which lecanoric acid and derivatives thereof constitute major secondary metabolites (Parrot et al., 2015). However, whether the involved polyketide synthases perform depside formations has not yet been characterized in detail (Armaleo et al., 2011).

A New Expression Vector Enables Rapid Cloning and Modification of Secondary Metabolite Genes

In order to ease the use of the heterologous expression system, we constructed a plasmid that allows rapid generation of expression constructs called SM-Xpress. For this purpose, we used a plasmid with a pleomycin resistance cassette in which the ble gene was controlled by the A. nidulans gpdA promoter. Subsequently, the 786 bp terA promoter and a 363 bp fragment of the A. terreus trpC terminator were amplified by PCR and integrated into the plasmid by in vitro recombination. This resulted in plasmid “SM-Xpress” that contained a Ncol restriction site that separated promoter and terminator and allowed insertion of the gene of interest by restriction ligation or in vitro recombination (Figure 6A). Resulting overexpression plasmids are suitable for direct transformation of the A. niger P2 strain that contains the PamyB:terR construct.

To test this system, we went back to the orsA gene from A. nidulans. As stated in Section “The PamyB:terR/PterA System is Suitable for High-Level Heterologous Secondary Metabolite Production,” OrsA predominantly produced lecanoric acid and we hypothesized that the depside formation from two orsellinic acid molecules is mediated by the thioesterase domain (TE) of this enzyme. In general, TE domains are important for product release, but can also perform an interketide esterification (cross-coupling) between the carboxyl-function of the polyketide and an alcohol, whereby in a recent example of melleolide formation in Armillaria mellea the hydroxyl group derives from a sesquiterpene (Lackner et al., 2013). To confirm that the depside formation in lecanoric acid is specific for the OrsA TE domain, we performed an in vitro recombination between the orsA gene lacking its TE domain and the TE domain from A. terreus terA that only releases monomers of its products (Figures 5C,D and Zaehle et al., 2014). In this approach, two different fusions were made: (i) the complete TE domain starting directly 3′ of ACP2 was removed from orsA and fused with the complete TE domain of terA (orsA:TEterA:complete; Figure 6E). (ii) The TE domain from orsA was exchanged at a conserved region of the active site of the TE domain (orsA:TEterA:partial; Figure 6D). The A. niger P2 strain was transformed with the fusion constructs and transformants were analyzed for their metabolic profile (Figures 6B–E). The partial exchange of the active site only resulted in trace amounts of orsellinic acid (Figure 6D), indicating that this exchange led to loss of function of the TE domain. Additionally, it indicates that a functional TE domain is required for product release. This is in agreement with other investigations that showed that TE domains are required for efficient product

![FIGURE 6](Image 314x529 to 541x741)

**FIGURE 6** | Scheme of the new expression vector SM-Xpress and its use for rapid cloning of polyketide synthases in domain swapping experiments. (A) The plasmid SM-Xpress contains the native terA promoter and a trpC terminator and allows cloning of a gene of interest (GOI) by restriction cloning or in vitro recombination into the Ncol site. Due to a pleomycin resistance cassette (AnPgasA:ble:trpC′), the plasmid is suitable for direct transformation of the A. niger P2 strain expressing terA under PamyB control. (B–E) HPLC profiles of culture filtrates from A. niger P2 strains grown for 40 h in AMM-G100 + Gln60 and expressing different versions of the original A. nidulans orsA gene or chimeric versions with exchanged thioesterase (TE) domains as depicted in the schemes on the left. Green parts originate from orsA and pink parts from A. terreus terA. Asterisks indicate the active sites of the TE domains. (B) P2 control strain (PamyB:terR). (C) P2_A1144_PterA:orsA. (D) P2_PterA:orsA:TEterA:partial and (E) P2_PterA:orsA:TEterA:complete. Indicated peaks correspond to 4-hydroxy-6-methylpyrone (2), orsellinic acid (4) and lecanoric acid (5). Note that a partial exchange of the TE domain (D) leads to a chimeric protein that hardly releases products and only trace amounts of orsellinic acid are detected (magnified inset). * indicate non-reproducible metabolite peaks.
release from PKS enzymes, whereby the specific product release mechanism is mainly directed by the TE domain (Du and Lou, 2010; Xu et al., 2013).

In contrast to the chimeric protein with partial exchange of the TE domain at the active site, A. niger transformants expressing the chimeric orsA with the complete terA domain exclusively produced orsellinic acid in high yields and lecanoric acid was no longer detected (Figure 6E). Due to these results we provide the first experimental evidence that the depside formation in lecanoric acid is attributed to the TE domain of orsA. This opens a new avenue for domain swapping experiments, in which the orsA TE domain could be used for fusions with other polyketide synthases to generate new depsides. Furthermore, these results not only confirm the suitability of our expression system for rapid analysis of products formed from secondary metabolite gene clusters, but also shows that the system provides a tool for rapid combinatory domain swapping experiments.

Conclusions and Outlook

In this study we confirmed specificity of the transcriptional activator TerR from the terrein gene cluster for its target promoters. Furthermore, we were able to identify DNA-binding motifs recognized by TerR. Interestingly, TerR predominantly binds as a monomer to a single CGG half-site, but a second CGG motif that is separated by a five nucleotide spacer can be bound by a second monomer. High affinity sites with tendency for binding of two monomers are characterized by the consensus motif TCG-GHHWYHCGGH. At least one site with this high affinity motif is present in all promoters of genes required for terrein synthesis. Interestingly, no high-affinity consensus motifs are detected in terG, terI, and terI that are dispensable for terrein production, but show the same TerR dependent regulation pattern. Thus, single half-site motifs may be present in these promoters, but these are difficult to predict by bioinformatical methods. On the contrary, the terA promoter contains three CGG direct repeat motifs and especially expression of the terA gene is strongly activated by TerR. The terC promoter contains only a single, but high affinity binding motif and its expression level is much lower than that from terA. Therefore, the number and probably also the distance of the motifs relative to the transcriptional start point appear important for high-level transcriptional activation. To confirm this assumption, future studies will replace all binding sites in the terA promoter by motifs from other promoters (such as BS6 and BS7). Furthermore, the number of binding sites will be increased to analyze the effect on the promoter activation potential.

Although there might be space for further optimization of the system, heterologous expression analyses in A. niger clearly showed that a combination of TerR with its terA target promoter is highly efficient and leads to an amplification of the expression level of the promoter that controls terR expression. Thus, additional promoters will be used to control terR expression to analyze the maximum amplification rates that can be obtained. Another important aspect is the transfer of the system to other fungi of industrial importance. While preliminary analyses showed that the system also works in other Aspergillus species, we will study the functionality of the system in yeasts such as Saccharomyces cerevisiae and various Basidiomycetes that are amenable to genetic modifications.

Last but not least, our proof of principle studies for heterologous expression of secondary metabolite genes by the TerR/PterA coupled system demonstrate an easy and rapid method for production of secondary metabolites. The vector SM-Xpress allows fast cloning of genes and eases domain swapping experiments. By this approach, we were able to show that the TE domain of OrsA performs a depside formation and future studies will use this domain for generating chimera with other polyketide synthases.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fmicb.2015.00184/abstract

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Global transcription factors mediate phytotoxin production in *Aspergillus terreus* by independent sensing of environmental signals

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**Running title:** Environmental induction of terrein biosynthesis

**Key words:** Methionine, iron, nitrogen starvation, siderophores, rhizosphere, terrein
Aspergillus terreus is a saprophytic fungus frequently isolated from the rhizosphere. While A. terreus causes invasive aspergillosis in immunocompromised patients, it has recently been identified as a pathogen of potato leaves. In agreement, plant-derived media induce the production of the phytotoxin terrein, but the biological impact of terrein and the inducing signals remained elusive. Knowledge on naturally inducing conditions combined with biological activities allows inference of the impact of secondary metabolite production in natural competition. Therefore, we performed a detailed analysis on environmental signals that induce the terrein biosynthesis gene cluster and drew a picture on the benefit of terrein production in ecological niches of A. terreus. In agreement with terrein production on plant-derived media, this compound induces lesion formation on fruit surfaces and inhibits plant seed germination. We additionally discovered a moderate antifungal activity of terrein and showed for the first time that terrein reduces ferric to ferrous iron, supporting growth of A. terreus under iron starvation in the absence of a functional siderophore system. In accordance with these biological activities, the lack of nitrogen or iron, and also elevated methionine levels induce gene cluster expression, in which each factor alone is sufficient for activation. This indicated that independent signals regulate terrein biosynthesis gene cluster expression. Analysis of transcriptional activators involved in environmental signal transduction revealed that induction under nitrogen starvation and methionine supplementation is dependent on AreA and AtfA, whereas the global iron response regulator HapX induces expression under iron starvation. Nitrogen-poor, methionine-rich and iron-limited conditions resemble signals frequently found in the rhizosphere, in which all species compete for limited nutrient resources. Therefore, in agreement with its broad spectrum of biological activities, induction of terrein biosynthesis provides a prominent example for secondary metabolite production in response to competition in the natural habitat.

Author summary

Secondary metabolite gene clusters frequently contain a specific transcription factor, which activates the structural genes. In turn, induction of these specific transcription factors generally depends on signalling cascades that mediate environmental signals. This allows the adapted production of secondary metabolites in direct response to ecological competition. Aspergillus terreus is a filamentous fungus that can be isolated from the rhizosphere and has recently been described as a pathogen on potato leaves. The major secondary metabolite from A. terreus is terrein, which is produced in high quantities during cultivation in potato dextrose broth and fruit juices. We discovered that signals inducing terrein production result from either nitrogen starvation, methionine or iron limitation. These activating signals perfectly correlate with the biological activities of terrein that are phytotoxic, antifungal and iron reducing. This allows A. terreus to respond to environmental competition and to access new nutrient sources. The signal transfer is mediated by three global transcription factors: AreA and AtfA act in concert in nitrogen source dependent activation, whereas signaling of iron starvation is exclusively mediated by HapX. We assume that independent signal sensing produces a highly resolved environmental image that enables a perfectly adapted response to the ecological situation.

Introduction

Aspergillus terreus is a filamentous ascomycete of biotechnological and medical importance. While this fungus is well known for its ability to produce the cholesterol-lowering drug lovastatin (Hendrickson et al., 1999), it can also cause life-threatening invasive aspergillosis in immunocompromised patients (Baddley et al., 2003; Slesiona et al., 2012b) and has recently described as a pathogen of potato leaves (Louis et al., 2013). Despite its close phylogenetic relation to other Aspergillus species such as A. fumigatus, A. oryzae or A. niger, A. terreus possesses unique features that distinct this fungus from its relatives. A. terreus is the only Aspergillus species identified so far that produces the biotechnologically important itaconic acid (Okabe et al., 2009). In addition, A. terreus displays an expanded spectrum of secondary metabolite biosynthesis gene clusters with several loci unique to A. terreus (Guo et al., 2013; Takeda et al., 2014). On the contrary, gene clusters that are typically conserved in Aspergillus species are lacking. An example is the conidial pigment, that is made from a common polyketide precursor called naphthopyrone (Latgé, 2001; Zaehle et al., 2014). The respective non-reducing polyketide synthase is not present in the genome of A. terreus, indicating that the colour of A. terreus conidia is made from a different molecule (Slesiona et al., 2012a). While searching for a respective candidate for pigment synthesis in A. terreus conidia, we serendipitously identified the gene cluster responsible for terrein production (Zaehle et al., 2014), which is one of the major secondary metabolites formed by A. terreus. Terrein production is highly pronounced on sugar rich plant-derived media such as potato dextrose broth, but the cluster is not expressed in glucose containing minimal media (Zaehle et al., 2014). Since terrein showed phytotoxic activities such as inhibition of seed germination and lesion formation on fruit surfaces (Kamata et al., 1983; Zaehle et al., 2014), it was assumed that specific plant-derived molecules trigger pathway gene expression.

The terrein biosynthesis gene cluster consists of eleven genes (terA-J, terR), whereby terA encodes a non-reducing polyketide synthase called terrein synthase (Zaehle et al., 2014). The cluster contains its own transcriptional activator, TerR, encoded at locus tag ATEG_00139. TerR is a transcriptional activator with a GAL4-type Zn(II)6Cys6 zinc binuclear cluster DNA-binding domain as frequently found in fungal secondary
metabolite gene clusters. A genomic deletion of terR resulted in the complete shutdown of terrein biosynthesis gene expression, indicating that this transcription factor is a key regulator (Zaehle et al., 2014). Recent analyses have shown that all promoters of genes required for terrein production contain a sequence of two CGG half sites with the high affinity binding motif TCGGHHWHCGGH (Gressler et al., submitted manuscript). Interestingly, the 5’ CGG half site is of major importance for monomeric TerR binding and mutation of binding sites leads to a lack of promoter activation under in vivo conditions. Therefore, activation of cluster genes is strictly dependent on the presence of TerR. However, signals that lead to TerR activation under in vivo conditions have not been identified yet, but it has been shown that the terR promoter is not activated in the heterologous host A. niger (Gressler et al., submitted manuscript).

Since terrein is the main secondary metabolite produced by A. terreus, we assumed a benefit from its production in the natural habitat of A. terreus. To elucidate this question, a detailed knowledge on the inducing factors stimulating terrein production and analyses of its biological activities were required. Therefore, we aimed at investigating environmental signals and the signalling factors that lead to terR expression and, eventually, to the production of terrein. From these analyses, the impact of different global transcription factors on cluster induction was studied. Unexpectedly, besides the quality and availability of nitrogen sources that are sensed by two global transcription factors, the iron responsive regulon played a vital role in cluster induction, which tempted us to study the specific contribution of terrein in modulating iron bioavailability.

Results

Fruits are natural inducers of terrein production

While transcription of terrein biosynthesis genes is directly dependent on the transcription of the cluster specific transcription factor TerR, the factors specifically inducing terR expression remained unknown (Zaehle et al., 2014) (Gressler et al., submitted manuscript). In previous studies we showed terrein is produced on plant derived media such as potato dextrose broth (PDB), which is in agreement with its phytophobic biological activity (Zaehle et al., 2014). To address the question on specific conditions that induce TerR, we generated an A. terreus reporter strain that contains a fusion of the terrein synthase promoter PterA with the β-galactosidase gene lacZ from Escherichia coli (PterA:lacZ). Due to the dependence of PterA expression on TerR, this strain served as a direct indicator of terR expression and terrein production.

In agreement with a lack of terrein production, β-galactosidase activity was near the detection limit when the A. terreus PterA:lacZ strain was grown on complete glucose minimal medium. In contrast, when grown on PDB medium a 200 – 500 fold induction was observed (Fig. 1A). This confirmed previous results concerning the stimulatory effect of PDB medium on terrein cluster induction. Similarly, on Sabouraud and YPD medium induction levels reached 10 – 30% compared to PDB, which is in agreement with reduced terrein production titers on these media (Zaehle et al., 2014). However, when cells were grown on potato broth or casamino acids without the addition of glucose, β-galactosidase activity remained at low levels, indicating that the presence of glucose may be required for terrein production. Indeed, when glucose medium was supplemented with 1% casamino acids as nitrogen source, a 20 – 30 fold activation was detected. Furthermore, when non-inducing potato broth (PB) medium (without glucose) was supplemented with varying amounts of glucose (0 to 2% final concentration), promoter activity increased in dependence of the glucose concentration (Fig. S1).

Since terrein can cause lesions on fruit surfaces and inhibits plant seed germination (Kamata et al., 1983;Zaehle et al., 2014), we assumed that sugar-rich fruit and root juices might have a strong stimulatory capacity. Therefore, we cultivated the reporter strain on banana, carrot, peach and apple juice (the latter adjusted to pH 6.5). Mycelia harvested from these media revealed β-galactosidase activities that by far exceeded the activity of the already strong inducing PDB medium (Fig. 1B). At least 5 times increased activity was observed for banana juice compared to PDB medium. Additionally, in culture extracts a strong HPLC signal for terrein was detected (Fig. S2A-D). This led us to infect fresh bananas with the A. terreus wild-type strain, a ΔterR and a ΔterA deletion mutant.

Phenotypically, although difficult to give an exact measure, bananas inoculated with the wild type appeared to loose their structural integrity more rapidly than bananas inoculated with the mutants. Most importantly, an ethyl acetate extraction of wild-type infected bananas revealed high amounts of terrein, whereas no terrein was detected after infection with the ΔterR or ΔterA mutant (Fig. 1C). Similar results were obtained, when fresh apples or nectarines were infected with the three A. terreus strains (Fig. S2 E and F). Furthermore, when banana extracts were used to test lesion formation on the banana peel, strong lesion formation was observed with extracts of wild-type infected bananas, but to a much lower extent with extracts of bananas infected with the mutants (Fig. 1 D).

Taken together these results support our in vitro reporter assays and confirm terrein production in a natural habitat. However, except the requirement of a sugar source, the specific factors leading to terrein production remained unclear. Thus, we performed two additional experiments: First, PDB medium was extracted with ethyl acetate and the extract was added to complete glucose minimal medium. No β-galactosidase activity or terrein production was observed (data not shown). Second, we added a small amount of either PDB medium, banana, apple or...
carrot juice (1 ml) to 50 ml complete glucose minimal medium. Again, neither β-galactosidase activity nor terrein production were detected (data not shown). This implied that (i) either the inducing compound was not extracted by ethyl acetate or (ii) that larger quantities of the inductor are required or (iii) that the compound cannot overwrite repressing conditions from glucose minimal medium.

Methionine supplementation acts as inducing signal in non-inducing media

Although fruits strongly induced expression of the terrein biosynthesis gene cluster the stimulating compound remained unresolved. However, as stated above, casamino acids supplemented with glucose resulted in moderate activation (Fig. 1A). Therefore, we assumed that specific amino acids could act as inducers. To test this assumption, glucose minimal media were prepared that contained defined mixtures of amino acids as sole source of nitrogen. All 20 amino acids were covered in six distinct pools (I- VI). Media were inoculated with the Pter:A: lacZ reporter strain and β-galactosidase activity was determined after 48 and 72 h of cultivation (Fig. 2A).

Pool I with the aliphatic amino acids caused moderate induction and pool VI containing cysteine and methionine strongly induced terrein biosynthesis gene expression especially after 72 h of cultivation.

To analyse, if single amino acids are able to cause the observed induction, we prepared media with non-inducing glutamine (50 mM) as nitrogen source to support high growth rates and added different amounts of the single amino acids from the two activating pools (Fig. 2B). The aliphatic amino acids from pool I only resulted in a weak induction and mainly required high concentrations for this effect, indicating that the presence of glutamine counteracted the activating effect. Similarly, activation with cysteine was low and activity decreased at higher concentrations, which may be due to the accumulation of toxic intermediates from cysteine degradation.

![Figure 1. Terrein production and expression of terA on plant derived media.](image-url)

(A) Promoter activity of strain PterA: lacZ on Aspergillus minimal medium (AMM; supplemented with glucose (Gluc), casamino acids (CA) or glucose + casamino acids (Gluc CA)), Sabouraud (Sab), YPD, potato dextrose broth (PDB) or potato extract (PB) after 48 h and 72 h of cultivation. (B) Promoter activity of strain PterA: lacZ grown in carrot, banana, apple and peach juice after 24, 36 and 48 h of cultivation. (C) HPLC analysis from banana extractions from infection with A. terreus SBUG844 strains ΔakuB, ΔakuBΔterA or ΔakuBΔterR. A mock-infected fruit served as control. 1 – terrein. Terrein is only detectable from ΔakuB infection, but not from ΔterA and ΔterR mutants. (D) Lesion formation on banana surfaces caused by extracts from bananas inoculated with either A. terreus strain SBUG844 wild type (wt), ΔakuB, ΔakuBΔterA and ΔakuBΔterR. Photos were taken after 40 and 60 h. Lesions only occur with extracts from the wild type and the parental strain of the mutants (ΔakuB) strain. Numbers indicates the serial two-fold dilution of the extracts starting from undiluted crude extracts down to 1:256 dilutions.
In contrast, methionine provoked a significant induction even at low concentrations, albeit lower than in the absence of glutamine. To further confirm methionine as an inducing compound, we also analysed the effect of other sulphur-containing compounds in low (5 mM) and high (10 mM) concentrations. However, no induction was observed when homocysteine, cystathionine, glutathione, dimethylsulfoxide, sulfate or sulfide was tested (Fig. 2C). This indicates that methionine itself rather than a sulphur source causes the induction.

When solid media were supplemented with methionine, another phenotype was observed. Methionine caused a strong red colouration of the medium, which we previously reported to be accompanied with terrein production on complex media and which is especially observed in mutants of the cluster accumulating intermediates from terrein synthesis (Zaehle et al., 2014). A strong red colouration of the medium is specifically observed, when A. terreus is grown on PDB medium and is therefore frequently used as a quick diagnostic marker for species identification (Zain et al., 2009; Zaehle et al., 2014). Thus, methionine might represent at least one of the inducing factors that is also present in PDB medium.

**Nitrogen starvation in the presence of sugars acts as a second stimulatory signal**

While methionine might represent the inducing compound in PDB medium, the induction on methionine and PDB was five times lower than on banana juice, in which no red colouration of the medium was observed. This implied that additional or alternative factors could also act as inducing signals.

When the different sets of amino acids were tested for their stimulatory potential, concentrations in the high millimolar range were used to avoid nitrogen limitation. For this reason we also added glutamine when testing the activating effect of single amino acids. However, fruit juices contain a very high sugar content and the C:N ratio in fruits is very high, which could result in severe nitrogen limitation at a later growth state. Therefore, another set of experiments was performed in which...
Figure 3. Terrein biosynthesis gene cluster activation under nitrogen starvation. All tests were performed in biological triplicates and technical duplicates. (A) β-galactosidase activity of SBUG844_PterA: lacZ grown in AMM(-N)G100 supplemented with different concentrations of various nitrogen sources: 70 mM and 10 mM of NaNO₃ or NH₄Cl, 1% or 0.1% of casamino acids (CA) and 50 or 10 mM of the amino acids aspartate (Asp), asparagine (Asn), glutamate (Glu) or glutamine (Gln). (B) β-galactosidase assay and ammonia consumption of SBUG844_PterA: lacZ grown in AMM(-N)G100 with 10 mM NH₄Cl. β-galactosidase activity and ammonia concentrations were determined after 24, 36, 48, 72 and 96 h. (C) β-galactosidase assay of SBUG844_PterA: lacZ in nitrogen shift experiments. After pre-cultivation for 48 h in AMM(-N)G100 + 70 mM NH₄Cl mycelium was washed and transferred to AMM(-N)G100 with (+N) and without 70 mM NH₄Cl (-N). The promoter activity was determined from the pre-culture 15 h after the shift. (D) Determination of β-galactosidase activity of strain SBUG844_PterA: lacZ in dependence of the carbon source under nitrogen starvation. After 48 h pre-cultivation of the reporter strain on AMM-CA1% without sugar addition mycelium was washed and transferred to AMM media without nitrogen supplemented with different carbon sources. Promoter activity was determined from the preculture and 24 h after the shift. (E) β-galactosidase activity from bananas infected with conidia suspension of SBUG844 wild type and PterA: lacZ and a mock-infected control. After 7 days incubation at room temperature a 5 × 5 mm slice from the infected area (highlighted with a red box) was ground to a fine powder and β-galactosidase activity was determined from extracts. From bananas of the mock-control and infected with the wild type, no activity was detected, whereas high activities were obtained from the reporter strain. (F) Quantification of terrein from bananas supplemented with or without ammonium and infected with SBUG844 wild type. Bananas were incubated for 7 days at room temperature. p-values were calculated by the Student’s paired t-test with a two-tailed distribution: ** p < 0.01.

the total concentration of amino acids was limited to either 50 mM (high) or 10 mM (low) and PterA induction from the lacZ reporter strain was determined at different time points (Fig. 3A). While high concentrations of amino acids such as glutamine, glutamate, asparagine or aspartate did not induce the cluster, limited amino acid concentrations (10 mM) resulted in a time-dependent 40–400 fold induction. Since these amino acids support high growth rates and biomass formation, a rapid depletion of the low-abundant nitrogen source can be assumed. Similarly, low concentrations of nitrate, ammonium or casamino acids that were given as sole nitrogen source caused a time-dependent strong PterA induction and β-galactosidase reporter
activity levels exceeded those observed for methionine induction or PDB medium.

To confirm that nitrogen starvation acts as an inducing signal, we supplemented media with 10 mM ammonium chloride and monitored nitrogen consumption and cluster expression. Indeed, as soon as nitrogen levels reached the detection limit, cluster expression was strongly induced (Fig. 3 B). To further confirm that nitrogen starvation acts as an inducing signal, a culture shift experiment was performed. Mycelium was pre-grown in a medium containing 70 mM ammonium as nitrogen source, which inhibited gene cluster activation for at least 40 h. Then, mycelium was harvested, extensively washed and transferred for 15 h to glucose minimal medium with or without a nitrogen source (Fig. 3 C). In the nitrogen starvation cultures the reporter strain showed high β-galactosidase activity accompanied by terrein accumulation in the medium. In contrast, no induction was observed when cultures were shifted back to nitrogen-rich conditions. Similar results were obtained from cultures pre-grown on nitrate or glutamine as nitrogen source (data not shown).

These results confirmed that nitrogen starvation acts as an inducing factor that might be responsible for the high terrein production levels on fruit juices. In agreement, when ammonium chloride was added to banana or apple juice, β-galactosidase activity was strongly reduced (Fig. S3), whereas ammonium addition to PDB medium only had a minor effect (data not shown). Therefore, methionine supplementation and nitrogen limitation resemble independent signals that lead to cluster induction and different plant-derived media may contain either one of these inducing signals.

However, it needs to be taken into account that the presence of high sugar concentrations was always a prerequisite for gene cluster induction. When mycelia were shifted to nitrogen starvation in the presence of different carbon sources, especially hexoses composed of mono- and disaccharide caused strongest induction. This was followed by pentoses and polysaccharides (Fig. 3D). Only very low activation was observed on non-glycolytic carbon sources such as ethanol, acetate or olive oil and carbon starvation did not induce gene cluster expressions.

Nitrogen limitation is a major inducer for terrein synthesis during in vivo fruit infection

While methionine induction plays a major role for terrein production in PDB medium, our in vitro experiments on banana juice indicated a major induction by nitrogen limitation. To test this assumption under conditions mimicking a natural scenario, we infected bananas with A. terreus wild type or the PertA::lacZ reporter strain. Banana peels were cut and supplemented with ammonium chloride prior to infection with the wild-type. After 7 days bananas were homogenised, weighted and extracted with ethyl acetate. As expected, when bananas were supplemented with ammonium chloride only 50% of the terrein concentration compared to bananas without supplementation was observed (Fig. 3F), which is in agreement with results from nitrogen supplementation of fruit juices. This finding clearly indicates that nitrogen limitation is a major inducer for terrein production under natural conditions.

AreA is the main global nitrogen regulator in A. terreus

Nitrogen as well as the presence of methionine marked important signals for terrein cluster induction. To unveil the global regulators that could be involved in signal transduction, the A. terreus genome was analysed for the presence of transcription factors known to play a role in nitrogen sensing, cross-pathway control of amino acid synthesis and stress response. The global nitrogen regulator AreA (ATEG_07264) (Hynes, 1975; Davis et al., 2005), the cross pathway control regulator CpcA (ATEG_03131) (Hoffmann et al., 2001; Krappmann et al., 2004), the stress response bZIP transcription factor AtfA (ATEG_04664) (Balazs et al., 2010; Lara-Rojas et al., 2011) and the nitrogen starvation induced ras-protein RhbA (ATEG_09480) (Panepinto et al., 2002) were selected for gene deletions. Additionally, a double deletion of the areA and atfA genes was generated (ΔareA/ΔatfA). All mutants were tested for their growth properties in the presence of proteinogenic amino acids that were used as sole nitrogen source. Additionally, ornithine, citrulline, urea and casamino acids, the latter with and without glucose, as well as the complex media YPD, PDB and malt extract were analysed (Fig. S4).

The cpcA mutant showed a general reduction in colony diameter on nearly all single nitrogen sources, whereas growth on complex media was similar to that of the wild type. This phenotype is in agreement with phenotypes previously described for cpcA mutants from A. nidulans (Hoffmann et al., 2001). Growth defects of this mutant were most strongly pronounced on nitrate and the amino acids Asp, Arg, Gly, His, Lys, Pro, Ser, Thr, Trp and Orn. Similar to the cpcA mutant, the rhbA mutant showed a general growth reduction compared to the parental strain, and this was not only observed on amino acid containing minimal media, but also on YPD medium. The strongest growth reduction was observed on nitrate, Asp, Gln, His, Ser and casamino acids.

Among all mutants tested, the areA mutant revealed the most severe growth defects. This mutant was only able to grow on ammonium chloride and the nitrogen-rich amino acids Asn,
Arg, Gin and His as well as on urea. As described for other Aspergillus species (Hunter et al., 2014), these results confirm an essential role of AreA in nitrogen sensing and utilisation in A. terreus.

The atfA mutant only showed a growth defect at high aspartate concentrations (50 mM), whereas growth on all other nitrogen sources was unaffected. However, the atfA mutant was unable to form pigmented conidia regardless of the growth medium. This indicated a regulatory role of atfA on secondary metabolite production in A. terreus. Additionally, the typical red colouration of the medium in the presence of methionine was completely lost in the atfA mutant, which was of special interest, since experiments from above indicated that the red colouration in the presence of methionine was accompanied by terrein production. However, no altered phenotype was observed, when the atfA mutant was confronted with different H$_2$O$_2$ concentrations (0.1 - 16 mM) or high osmotic stress (up to 2 M NaCl; data not shown). Thus, AtfA from A. terreus seems to act independent from external oxidative or osmotic stress signals, which are required for AtfA activation in other Aspergillus species (Balazs et al., 2010; Lara-Rojas et al., 2011).

Finally, growth of the double deletion mutant ΔareA/ΔatfA resembled the phenotypes of both single mutants, since it was only able to grow on the media that supported growth of the areA mutant, where it formed white conidia as observed for the ΔatfA strain. In conclusion, the transcription factor AreA is the major regulator of nitrogen metabolism, while the cpcA and rhbA mutants showed only slight growth defects. AtfA is not essentially required for growth on different nitrogen sources, but the white phenotype of conidia and the lack of the red medium colouration on methionine indicate an involvement in regulation of secondary metabolism.

The global transcription factors AreA and AtfA are essential for terrein biosynthesis gene cluster induction during nitrogen starvation

To test the effect of transcription factor mutations on terrein biosynthesis gene cluster activation, all mutants were pre-grown for 40 h on non-inducing glucose medium with 50 mM glutamine. Mycelium was transferred to medium with or without nitrogen and terrein was quantified after 24 h (Fig. 4A). The wild type showed the expected low terrein production in the presence of nitrogen, whereas high terrein levels were detected when nitrogen was omitted. This pattern of terrein production was similar in the ΔcpcA and ΔrhbA mutant, confirming that these regulators are not involved in gene cluster regulation. In contrast, both the ΔareA and the ΔatfA mutant only produced marginal amounts of terrein under nitrogen-limited conditions and production did not significantly increase after prolonged cultivation (Fig. 4A and Fig. S5). This effect was completely cured in the complemented mutants areA' and atfA'. Furthermore, while some traces of terrein were still detectable in the culture extracts of the ΔareA and in the ΔatfA mutants, no terrein was detected in a ΔareA/ΔatfA double mutant. Therefore both, areA and atfA, seem to be important for terrein production under nitrogen limitation. However, due to the additive effect of both mutations, both transcription factors seem to act independently on gene cluster activation. To confirm the assumption that reduced terrein levels are due to the lack of transcriptional activation rather than a lack of the provision of acetyl-CoA units for terrein production, two independent experiments were performed. First, we expressed the cluster specific transcription factor gene terR under control of the gpdA promoter in a ΔareA/ΔatfA mutant background. A constitutive terrein production was observed, indicating that AreA and AtfA regulate terR expression, but are not essential for induction of the structural genes (Fig. 4A). Second, we analysed the effect of areA and atfA deletion by qRT-PCR. For this purpose, the wild type, the ΔareA, ΔatfA, ΔareA/ΔatfA mutants and the complemented strains were pre-grown in glutamine supplemented medium and subsequently shifted for 0 h, 12 h and 24 h to nitrogen starvation. qRT-PCR was performed on the cluster genes terA, terB, the specific activator terR and the global transcription factors areA and atfA (Fig. 4B). Expression levels were normalised against the actin or the enolase gene with similar results.

In the wild type the regulators areA and atfA showed a time-dependent increase in gene expression. In contrast, the gene of the terrein cluster regulator terR was most strongly upregulated 12 h after the shift to nitrogen starvation, but its expression level ceased after prolonged incubation. However, expression of the TerR-controlled terrein biosynthesis genes terA and terB continued to increase after 12 h and reached 13.3 and 3.2 times of the expression level of the actin control gene at 24 h (Fig. 4B). The strong induction of these genes also explains the high terrein production rates in A. terreus culture supernatants that reached concentrations of up to 8 mM under the applied conditions. On the contrary, while complemented mutants behaved like the wild type, the areA and atfA deletions strongly reduced activation of terR and, in turn, the expression of terA and terB. These results are in perfect agreement with the substantially reduced terrein production under nitrogen limitation in these mutants (Fig. 4A). While DNA binding motifs for the basic leucine zipper transcription factor AtfA are difficult to predict, AreA recognises the DNA-binding motif HGATAR and two adjacent binding sites are generally required for transcriptional activation due to dimer formation of AreA monomers (Ravagnani et al., 1997). Two putative AreA HGATAR binding sites were detected within the intergenic region of the terA and terB gene (BS1 and BS2, Fig. S6) and surface plasmon resonance (SPR) interaction analyses with an A. nidulans AreA DNA-binding domain—that is
Figure 4. Terrein quantification and expression of terrein cluster genes in nitrogen regulator mutants. In all experiments biological triplicates with technical duplicates were analysed. Statistical significances in comparison to the parental ∆akuB strain were calculated by the Student’s paired t-test with a two-tailed distribution: * p < 0.05, ** p < 0.01; *** p < 0.001. (A) Terrein quantification from strains SBUG844∆akuB (∆akuB), SBUG844∆akuB∆pcpA (∆pcpA), SBUG844∆akuB∆rhbA (∆rhbA), SBUG844∆akuB∆areA (∆areA), SBUG844∆akuB∆areA/areA C (∆areA C), SBUG844∆akuB∆atfA (∆atfA), SBUG844∆akuB∆atfA/∆atfA C (∆atfA C), SBUG844∆akuB∆areA∆atfA (∆areA∆atfA) and strain SBUG844∆akuB∆areA∆atfA/∆areA∆atfA C::AnPgdA:terR with terR overexpression in the ∆areA∆atfA background. Cultures were pre-grown in nitrogen-rich AMM-G100Gln50. Mycelia were washed and transferred to AMM(-N)G100 with (+N) or without (-N) 50 mM Gln. Terrein was quantified from
supernatants 24 h after the shift. (B) qRT-PCR of strains ΔakuB, ΔareA, areAΔC, ΔatfA, atfAΔC and ΔareA/atfA grown in the presence or absence of a nitrogen source. Strains were pre-cultivated for 40 h in AMM-G100Gln50 medium and mycelia were washed and transferred to AMM-(N)G100 without nitrogen. RNA was isolated before the shift (0 h; ‘+N’) and after 12 and 24 h of starvation. Transcript levels were normalised against the actin gene actA by fold expression = 2^[(C_{T,\text{target}} - C_{T,\text{actA}})] in AMM-G100Gln50Met10 medium. (C) Terrein quantification from strains shown in (B) after 72 h of cultivation in AMM-G100Gln50Met10 medium. (D) qRT-PCR of from RNA of strains shown in (C) isolated after 48 h of growth in AMM-G100Gln50Met10. Transcript levels were normalised against the enolase gene enoA by fold expression = 2^[(C_{T,\text{target}} - C_{T,\text{enoA}})]. (E) Top and bottom view from colonies of A. terreus wild type (ΔakuB) and mutants (ΔterA, ΔatfA, ΔareA) grown for 72 h on AMM-G100Met1Z5 solid plates. The red pigmentation of the wild type (bottom view) is lost in the ΔterA and ΔatfA mutants that show some enhanced growth while unable to produce terrein. No colony formation is observed for the ΔareA strain.

91% identical to the respective A. terreus AreA domain- (Fig. S6) revealed that both binding sites are recognised. This result implies a direct control of these structural genes by AreA. However, since overexpression of terR in the ΔareA/ΔatfA mutant background allowed terrein production (Fig. 4A), AreA regulation at this promoter might be dispensable. In contrast, consistent with the assumed AreA-dependent regulation of terR expression, the terR promoter contains one AreA binding sites that does not fit to the complete HGATAR consensus (BS3) and two putative sites that match the consensus (BS4 and BS5; positions -59 and -72 relative to the translational start point). SPR analysis with the A. nidulans AreA DNA-binding domain revealed that only BS4 and BS5 are recognised with high affinity (Fig. S6). Furthermore, when oligonucleotides comprising BS4+BS5 were analysed, a synergistic effect was observed that indicates cooperative binding of two AreA polypeptides (Fig.S6). This strengthens the model of a direct involvement of AreA in the activation of terR expression. Although direct evidence for AtfA binding to the terR promoter is lacking, AtfA appears of even higher importance for terR expression, because its deletion reduced terrein production and gene cluster expression to a greater extent. Nevertheless, both transcription factors seem to regulate terR expression, but in an independent manner. In agreement, terR, terA and terB expression and terrein production showed the strongest decrease in the ΔareA/ΔatfA double knock-out mutant (Fig. 4).

AreA and AtfA mediate terrein biosynthesis gene cluster induction in the presence of methionine

Besides the importance of atfA and areA on terrein biosynthesis gene cluster induction under nitrogen starvation, we also tested their impact on terrein production in the presence of methionine. As described above, the ΔareA strain is unable to grow on methionine as sole nitrogen source and the ΔatfA mutant culture lacks the methionine-specific red colouration. To avoid a bias from growth limitations, the strains were grown on glucose minimal medium supplemented with 50 mM glutamine and 10 mM methionine and terrein production was quantified after 72 h of incubation (Fig. 4C). While the wild-type strain and complemented mutants showed high terrein production rates, the terrein levels in the ΔareA, the ΔatfA or the ΔareA/ΔatfA double mutant remained near the detection limit, but levels in the areA mutant that were again higher than in the atfA mutant. To confirm that the lack in terrein production under methionine stimulation was again due to a lack of gene expression, qRT-PCR was performed on the different strains.

Both, areA and atfA were expressed in the wild-type under the applied conditions with glutamine and methionine, indicating that a possible repression of transcription by glutamine is overruled by methionine (Fig. 4D). In agreement with low terrein production levels, the deletion of areA reduced terR and, consequently, terA and terB expression. In contrast, no terR transcript was detectable in the ΔatfA mutant and, in agreement, transcription of terA and terB was no longer observed. These results indicated that AreA might be essential for the uptake of methionine prohibiting an internal signalling from methionine towards cluster induction. In contrast, the atfA mutant is able to grow on methionine, indicating that its uptake is not disturbed. However, the complete lack of cluster induction in this mutant in the presence of methionine indicates a methionine-dependent signalling cascade that activates atfA expression. From this assumption we speculated that constitutive expression of atfA could lead to terrein production under non-inducing conditions. To test this hypothesis, we expressed atfA under control of the constitutively active A. nidulans gapD promoter (Figure S7). Indeed, while no terrein was detected in the culture supernatant of the parental SBUG844 wild-type strain grown on non-inducing AMM medium with either nitrate or glutamine as nitrogen source, terrein was produced in two independent SBUG844_ΔanPgapD/ΔatfA strains constitutively expressing the atfA gene. This confirms that AtfA is sufficient to induce terR expression and cluster induction. Thus, atfA-dependent induction acts directly on terR expression, but is not required for the expression of structural genes, since constitutive expression of terR in the ΔareA/ΔatfA background restored terrein production (Fig. 4A).

Iron limitation acts as a third independent signal for terrein cluster induction

Our results clearly indicated that either methionine supplementation or nitrogen starvation act as inducing signals and that AreA and AtfA are essential for mediating the environmental signals on TerR expression. In contrast, starvation for other macroelements such as glucose, sulphur or phosphate
Figure 5. Expression of terA and genes from iron acquisition systems and coprogen isolation under iron supplemented and limited conditions. All analyses were performed from biological triplicates and technical duplicates. Statistical significances were calculated by the Student's paired t-test with a two-tailed distribution. (A) β-galactosidase activity from SBUG844_PterA:lacZ in AMM-(N)G100Gln50 medium with reduced amounts of trace elements (TE). Asterisks indicates p-values vs. 100% TE: ** p < 0.01; *** p < 0.001. (B) β-galactosidase activity from SBUG844_PterA:lacZ in AMM-(N)G100Gln50 medium with 1% trace elements supplemented with 20 µM of specific trace elements: FeSO₄, ZnSO₄, CuSO₄, Na₂MoO₄, CoCl₂, or H₃BO₃. Asterisks indicates p-values vs. activity without supplementation: *** p < 0.001. (C) β-galactosidase activity from SBUG844_PterA:lacZ in AMM-(N)G100Gln50 medium with and without 40 µM FeCl₃. Asterisks indicates p-values vs. 40 µM FeCl₃: *** p < 0.001. (D) qRT-PCR of SBUG844 wild type grown in AMM-G100Gln50 under iron supplemented (40 µM; +Fe) and starved conditions (-Fe). qRT-PCR was performed on genes assumed to be responsible for siderophore biosynthesis, siderophore transport or reductive iron assimilation. Transcript quantities were normalised against enoA by fold expression = 2^(C_T target - C_T eno). Asterisks indicates p-values vs. 0 µM FeCl₃: ** p < 0.01; *** p < 0.001. (E) HPLC profiles of lyophilised culture supernatants of ΔterA after 72 h of cultivation for in AMM-(N)G100Gln50 supplemented with 0, 20 or 200 µM FeCl₃. Peaks for the siderophores ferrichrysin (2) and coprogen (3) are indicated. (F) CAS-assay of coprogen and terrein. Both substances were solved in methanol and serial dilutions (125 – 15.63 µg) were added to punched holes of CAS-plates. Photographs were taken 48 h after incubation at 30°C.

Did not induce terrein production (Figure S8). However, due to the rather undefined composition of plant-derived media, we could not exclude that the presence or absence of trace elements might act as inducing signals. However, since trace elements are highly abundant in synthetic Aspergillus minimal medium supplemented with Hutner’s trace elements solution, we...
excluded the inducing effect from the high abundance of specific trace elements. In contrast, we investigated the effect of trace elements limitations and cultivated the PterA::lacZ reporter strain in glucose minimal medium with 50 mM glutamine as nitrogen source, which was supplemented with 100%, 10%, 1% and 0% of Hutner’s trace elements. β-Galactosidase activity was determined after 48 h of cultivation.

As expected, no induction was observed, when the medium was supplemented with 100% trace elements. Surprisingly, a slight, but significant induction was observed in media supplemented with a ten times reduced amount of trace elements (Fig. 5A). Even more, mycelium grown in the presence of 1% trace elements revealed strong induction of β-galactosidase activities exceeding those obtained from PDB medium (Fig. 5A). To elucidate, whether limitation of a specific trace element could act as trigger for gene cluster activation, we prepared media with 1% trace elements and added 20 µM of the respective trace element (FeSO₄, ZnSO₄, CuSO₄, MnCl₂, Na₂MoO₄, CoCl₂, H₂BO₃). Cluster induction was observed in all cultures except from the medium that was supplemented with 20 µM FeSO₄ (Fig. 5B). To confirm inducing properties of iron limitation, we prepared a trace elements solution that contained all metals with the exception of iron. Indeed, when no iron was added, the cluster was strongly induced, whereas the addition of 40 µM FeCl₃ completely repressed cluster induction (Figure S3). In agreement, HPLC analyses confirmed that terrein was produced under iron-limited conditions (data not shown). Thus, besides methionine and nitrogen limitation, the lack of iron, but no other trace element, induces the terrein biosynthesis gene cluster. Furthermore, induction by iron limitation is independent from the nitrogen source.

Based on results from synthetic media, we supplemented banana juice with additional iron, but β-galactosidase activity hardly changed (Figure S3). This indicates that iron limitation is not the major activator of terrein production in these media. However, when banana juice was supplemented with nitrogen and iron, activity was reduced below the level observed under only nitrogen supplementation, indicating some contribution of iron limitation on induction. When the iron and nitrogen supplemented medium was additionally supplemented with methionine, β-galactosidase activity increased again by approximately seven fold (Figure S3). Taken together, these results indicate that (i) iron limitation is not the major cause of cluster induction in plant-derived media, but (ii) each single factor is on its own sufficient for induction.

Iron starvation induces siderophore-based and reductive iron assimilation pathways

Since terrein production is induced under iron-limited conditions, our next aim was to study the correlation of terrein production and induction of iron acquisition systems. However, while fungal iron acquisition has been well investigated in A. nidulans (Eisendle et al., 2003) and A. fumigatus (Haas, 2014) only limited information was available on the iron acquisition systems in A. terreus. Aspergillus generally possess a siderophore-based and a reductive iron assimilation (RIA) system to counteract iron starvation and the respective genes are assumed to be transcriptionally up-regulated when iron becomes limited (Haas, 2014). Since we expected a quite high conservation of the iron assimilation systems in A. terreus, a BLASTp search for orthologous genes in the genome of A. terreus NIH2624 was performed (Table 1). Indeed, all genes required for a reductive iron uptake system (fetC, fetB, ftrA) were identified. Additionally, genes for siderophore biosynthesis and at least five genes coding for putative siderophore transporters (mirA-D, sitA) were identified. Interestingly, among the siderophore biosynthesis genes a transacylase (sitG) that converts fusarine C to the major siderophore triacetyl fusarine C (TAFC) in A. fumigatus was lacking. However, the lack of a sitG gene was consistent with previous reports in which ferrichrysin and coprogen rather than TAFC are produced by A. terreus cultures (Zähner et al., 1963;Bertrand et al., 2010). To confirm that the predicted orthologous genes from A. terreus are regulated by the availability of iron, we performed qRT-PCR analyses on selected genes (Table 1; Fig. 5D). Indeed, all analysed genes from siderophore biosynthesis and the reductive iron assimilation pathway were strongly induced under iron limitation. The same was true for the transporter genes mirA, mirD and sitA, whereas mirB and mirC were also expressed in the presence of iron and only showed slight induction under iron starvation (Fig. 5D). To confirm siderophore production and the types of siderophores produced by our A. terreus strain under iron limitation, we cultivated the wild type SBUG8444aakuB for 72 h under iron-rich (200 µM FeCl₃) and iron-limited conditions (no or 20 µM FeCl₃). HPLC analyses from lyophilised extracts revealed production of ferrichrysin (2) and coprogen (3) under iron limitation (Fig. 5E). Coprogen is a well-known extracellular siderophore that has previously been isolated from several Aspergillus, Cochliobolus, Penicillium, Neurospora and Paracoccidioides species (Oide et al., 2006;Franken et al., 2014;Silva-Bailao et al., 2014). Although both siderophores were produced under iron limitation, coprogen revealed a higher stability compared to ferrichrysin. Therefore, subsequent analyses on siderophore production based on the quantification of coprogen. Purified coprogen was tested for its potential to chelate iron by applying it to a CAS agar plate assay (Fig. 5F), where a concentration-dependent reddish “halo-zone” was observed. In contrast, when terrein was tested under identical conditions, no iron chelating effect was observed. Thus, although terrein production is induced under iron limitation, it is unlikely that it depicts a siderophore.
Table 1. BLASTp analysis of homologous genes for iron uptake and regulation of iron homeostasis (adapted by Haas, 2012). 
\(^1\)Genes annotated according to Schrettl et al. (2008). \(^2\)Genes annotated according to Blatzer et al. (2011a).

<table>
<thead>
<tr>
<th>Function</th>
<th>A. fumigatus</th>
<th>A. terreus</th>
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<tr>
<td><strong>Reductive iron assimilation (RIA)</strong></td>
<td></td>
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<tr>
<td>FetC</td>
<td>Ferroxidase</td>
<td>AFUA_5G03790 −Fe</td>
</tr>
<tr>
<td>FreB</td>
<td>Ferric reductase</td>
<td>AFUA_1G17270 −Fe</td>
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<tr>
<td>FtrA</td>
<td>Iron permease</td>
<td>AFUA_5G03800 −Fe</td>
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| **Siderophore biosynthesis (SB)** |              |            |
| EstA                       | TAFC esterase | AFUA_3G03660 −Fe | ATEG_04072 | 44%/58% |
| NpgA/PptA                  | Phosphopantetheinyl transferase | AFUA_2G08590 −Fe | ATEG_09695 | 56%/65% |
| SidA                       | Ornithine monooxygenase | AFUA_2G07680 −Fe | ATEG_06879 | 78%/85% |
| SidC                       | FC NRPS       | AFUA_1G17200 −Fe | ATEG_05073 | 60%/76% |
| SidD                       | FSC NRPS      | AFUA_3G03420 −Fe | ATEG_07488 | 43%/59% |
| SidF                       | Transacylase  | AFUA_3G03400 −Fe | ATEG_05075 | 52%/67% |
| SidG                       | Transacetylase | AFUA_3G03650 −Fe | none - | - |
| SidH                       | Mevalonyl hydratase | AFUA_3G03410 −Fe | ATEG_01509 | 53%/67% |
| SidI                       | Mevalonyl ligase | AFUA_1G17190 −Fe | ATEG_05074 | 86%/91% |
| SidL                       | Transacetylase | AFUA_1G04450 −Fe | ATEG_03770 | 64%/76% |

| **Siderophore transporter (SIT)** |              |            |
| MirA                       | Enterobactin transporter | AN7800; - −Fe | ATEG_04071 | 68%/77% |
| MirB                       | TAFC transporter | AN8540; AFUA_3G03640 −Fe | ATEG_02711 | 50%/68% |
| MirC\(^1\)                 | AN7485; AFUA_2G05730 −Fe | ATEG_06762 | 78%/87% |
| MirD\(^2\)                 | Trichotecene efflux pump | AFUA_3G03440 −Fe | ATEG_07487 | 40%/58% |
| SIIA/SIT\(^1\)             | ANS378; AFUA_7G06060 −Fe | ATEG_06329 | 62%/73% |

| **Regulatory proteins** |              |            |
| HapX                      | bZip TF      | AFUA_5G03920 −Fe | ATEG_08014 | 77%/83% (hapX, re-annotated) |
| SreA                      | GATA TF      | AFUA_5G11260 +Fe | ATEG_07741 | 67%/75% |
| SrbA\(^2\)               | HLH TF       | AFUA_2G01260 −Fe | ATEG_08156 | 72%/82% |

**Terrein reduces ferric to ferrous iron by its oxidation to propylene-maleic acid**

While terrein does not seem to contribute to the siderophore-mediated iron assimilation system, some antioxidative properties of terrein had previously been described (Trabolsy et al., 2014). Therefore, we investigated, whether terrein might support the reductive iron assimilation pathway by reducing ferric (Fe\(^{3+}\)) to ferrous (Fe\(^{2+}\)) iron as described for the strong antioxidant ascorbic acid (Elmagirbi et al., 2012). To test this assumption, terrein was diluted in acetic acid buffer at pH 4.0 to give a final concentration in the range of 0.25 - 4 mM when incubated with 0.05 mg/ml FeCl\(_3\). The reduction of Fe\(^{3+}\) was followed by
addition of the ferrous iron chelator 2,4,6-tripyridyl-S-triazine (TPTZ; Fig. 6A). The experimental setup was tested in parallel with ascorbic acid as positive control. Although ascorbic acid showed a much higher reducing potential, terrein was also able to convert ferric to ferrous iron in a concentration dependent manner. Next, we investigated the pH-dependency of terrein-mediated iron reduction by performing the assay with 4 mM terrein in a pH range of 3.0 to 6.0 (Fig. 6B). Ferric iron reduction required an acidic pH with a marked decrease in the reductive potential at pH 6.0. A. terreus generally prefers a neutral to slightly acid pH value during vegetative growth and cultures grown in the presence of iron maintained at a pH of about 7.0. However, under iron limitation we noticed a significant drop of the culture pH to about 3.5, which is the optimum pH for terrein-mediated iron reduction. Thus, since terrein concentrations of 8 mM were easily achieved during growth under iron limitation and accompanied by a drop in pH, terrein could indeed increase iron availability.

Next, we aimed at the structure of the terrein oxidation product after iron reduction. For this purpose, the terrein-mediated iron reduction assay was scaled up (see supporting experimental procedures), incubated with either ferric or ferrous iron and subjected to HPLC analysis. Here, besides a major proportion of terrein that remained in its original structure, a new peak (4) was observed, which was identified as 2-((E)-prop-1-en-1-y)maleic acid (PMA) (Fig. 6C and D). The structure of the oxidation product implies that oxidation took place at the two hydroxylated carbon atoms of terrein, leading to a cleavage of the pentenone ring system as proposed by (Trabolsy et al., 2014). In contrast, conversion of terrein to PMA was not observed during incubation with Fe²⁺, confirming that iron reduction was the cause of PMA formation.

Terrein seems to possess a lower reductive potential than ascorbic acid and we studied the reductive potential on other metal ions. No PMA formation was observed, when copper ions (Cu²⁺ or Cu⁺) were incubated with terrein. In contrast, terrein was oxidised to PMA when incubated together with KMnO₄ but not MnCl₂ (Fig. 6C). This indicates that the electrode potential of copper is too low, while the high electrode potential of permanganate in an acidic solution allows reduction by terrein. In conclusion, terrein production is strongly induced under iron starvation. While it cannot chelate iron to support the siderophore-mediated iron uptake, its reductive potential is sufficient to reduce ferric to ferrous iron, which may increase iron solubility and could ease the direct uptake of iron via the ferrous iron transport system.

**Figure 6. Iron reductive properties of terrein.** (A) Fe(III) reduction by terrein determined by the TPTZ assay. Reduction by ascorbic acid served as control maximum reduction by ascorbic acid was set as 100%. The analyses were performed from duplicates. (B) pH and time dependent Fe(III) reduction of 10 µM FeCl₃ by terrein (4 mM fixed concentration). Acetate buffer in a pH-range from 3.0 to 6.0 was used. The analyses were performed from duplicates. (C) HPLC profiles of terrein after incubation with different oxidised (upper, red lines) or reduced (lower, blue lines) metal ions. Left: Cu(II)SO₄ and Cu(I)Ac; middle: Fe(III)Cl₃ and Fe(II)SO₄, right: Mn(II)SO₄ and KMn(VII)O₄. 1 – terrein; 4 – propylen maleic acid. (D) Scheme of terrein oxidation during iron reduction leading to the formation of propylen maleic acid.
The iron response regulator HapX regulates siderophore and terrein biosynthesis in A. terreus

Induction of terrein synthesis under iron limitation is independent from AreA and AtfA since the \( \text{areA/3atfA} \) double deletion mutant still produced terrein under iron limitation (Fig. S9). Due to the co-regulation with siderophore biosynthesis we assumed that transcriptional regulators involved in iron homeostasis could also regulate terrein biosynthesis. In \( A. \text{nidulans} \) and \( A. \text{fumigatus} \) siderophore biosynthesis is regulated by the interplay of the transcription factors HapX and SreA, whereby HapX acts as a transcriptional inducer under iron limitation and SreA as a repressor in the presence of iron (Haas, 2012). To identify homologous proteins in \( A. \text{terreus} \) a BLASTp analysis was performed on the genome of \( A. \text{terreus} \) NIH2624 (Table 1). While a SreA homologue with 67% identity to \( A. \text{fumigatus} \) SreA was detected at locus tag ATEG_07714, the search for a HapX homologue was more difficult. The most promising candidate derived from ATEG_08014. Unfortunately, the sequence at this locus was incomplete at both ends as indicated by spacer nucleotides in the genome sequence of strain NIH2624. Furthermore, an intron was predicted in the coding region that is lacking in \( \text{hupX} \) genes from any other \( Aspergillus \) species. However, the predicted partial protein sequence of ATEG_08014 revealed 67% identity to \( A. \text{fumigatus} \) HapX, which made ATEG_08014 a very likely HapX candidate in \( A. \text{terreus} \).

To investigate the impact of SreA and HapX in siderophore (coprogen) and terrein synthesis, both genes were deleted. However, only a partial deletion of \( \text{hupX} \) was performed, because sequence information at the borders of the gene were lacking. The \( \text{sreA} \) mutant was complemented with the \( \text{sreA} \) gene from \( A. \text{terreus} \) and the \( \text{hupX} \) partial deletion mutant was complemented with the \( \text{hupX} \) gene from \( A. \text{nidulans} \) FGSC A4 under control of its native promoter. The parental wild-type strain, both mutants and the complemented strains were cultivated for 72 h in iron-supplemented (40 \( \mu \text{M} \) FeCl\(_3\)) and iron-limited media and the terrein and coprogen content was determined from culture supernatants (Fig. 7 A and B). All complemented mutants behaved similar to the wild type, indicating that the \( A. \text{nidulans} \) \( \text{hupX} \) gene was functional in \( A. \text{terreus} \). In these strains only marginal amounts of coprogen and terrein were produced in the presence of iron, whereas production rates strongly increased under iron starvation.

In contrast, the partial \( \text{hupX} \) deletion reduced coprogen production under iron limitation caused by approximately 50% and terrein concentrations ceased by about 90% (Fig. 7 A and B). This indicates that HapX directly activates both, siderophore and terrein biosynthesis in \( A. \text{terreus} \). On the contrary, the \( \text{sreA} \) mutant produced significantly higher amounts of coprogen in the presence of iron, whereas terrein concentrations varied in independent cultures resulting in no significant difference compared to the wild type (Fig. 7 A and B). Furthermore coprogen, but not terrein concentrations significantly increased in the \( \text{sreA} \) mutant under iron limitation. This indicates that SreA acts as a negative feedback regulator in siderophore, but not terrein production, whereas HapX positively controls both synthases.

To confirm these results at the transcriptional level, qRT-PCR was performed on all strains under both growth conditions (Fig. 7C and S10). We found that, \( \text{hupX} \) was up-regulated under iron limitation, whereas \( \text{sreA} \) was up-regulated in the presence of iron. This indicates the inverse regulation of both genes, which might be interconnected in a negative feedback loop as shown for other Aspergilli (Haas, 2012). Furthermore, while \( \text{sreA} \) deletion resulted in a slight, but significant up-regulation of \( \text{hupX} \) expression under iron-supplemented conditions, the lack of \( \text{HapX} \) derepressed \( \text{sreA} \) expression under iron limitation. Even more importantly, the inactivation of \( \text{hupX} \) prohibited \( \text{terR} \) transcription, resulting in an inability to induce the terrein cluster genes \( \text{terA}; \text{terB} \) and \( \text{terC} \). Furthermore, and as expected from product quantification, deletion of \( \text{sreA} \) neither influenced \( \text{terR} \) activation nor expression of other terrein cluster genes in the presence of iron. Therefore, we conclude, that \( \text{hupX} \) is the major regulator for terrein cluster induction during iron starvation.

We also tested the impact of the \( \text{hupX} \) and \( \text{sreA} \) deletion on expression of genes involved in siderophore biosynthesis and siderophore transport (data not shown). Under iron starvation the inactivation of \( \text{hupX} \) significantly reduced expression of \( \text{sidD} \) and \( \text{sitA} \), whereas these genes were up-regulated in the \( \Delta\text{sreA} \) strain in the presence of iron. Here, it should be mentioned that in \( P. \text{brasiliensis} \) species \( \text{sidD} \) and \( \text{sitA} \) are assumed to be directly involved in coprogen biosynthesis and its transport (Silva-Bailao et al., 2014).

The regulatory role of HapX not only on siderophore synthesis, but also on terrein production tempted us to identify the complete coding region of the \( A. \text{terreus} \) \( \text{hupX} \) gene. First, we used degenerate primers to amplify the main proportion of \( \text{hupX} \) from cDNA. Indeed, the \textit{in silico} predicted intron was not confirmed, resulting in an intron-less \( \text{hupX} \) gene as found in other \( Aspergillus \) species. Next, we closed the gaps of the untranscribed borders by long-run PCR. The complete sequence of the \( A. \text{terreus} \) \( \text{hupX} \) gene is found under accession number KP233834. The sequence of the full-length HapX protein matches with that of HapX proteins from other \( Aspergillus \) species such as \( A. \text{nidulans} \) (73% identity), \( A. \text{fumigatus} \) (77%), \( A. \text{niger} \) (81%) and \( A. \text{oryzae} \) (81%).

Terrein supports growth in absence of the siderophore system and shows antifungal properties

Under iron limitation HapX regulated both, the classical iron acquisition systems and terrein production. In general, siderophore-based iron acquisition is highly efficient and assumed to be more important than the reductive iron
assimilation pathway. In *A. fumigatus* growth and virulence defects caused by the interruption of the reductive iron assimilation pathway are only observed, when the siderophore-based system is also inactivated. However, deletion of the siderophore biosynthesis gene *sidA* resulted in intracellular iron accumulation via increased iron reductase activity (Blatzer et al., 2011b).

In order to elucidate a positive effect of terrein on iron acquisition, we deleted the *sidA* gene in *A. terreus* that encodes the ornithine-\(\text{-N}^5\)-monoxygenase which generates the precursor for all hydroxamate siderophores. The wild type, the \(\Delta\text{sidA}\) strain and a complemented \(\text{sidA}^+\) strain were pre-grown with 200 \(\mu\text{M FeCl}_3\) and shifted to medium without iron. Coprogen determination confirmed its production in the wild type and the complemented strain, but not in the \(\text{sidA}\) mutant (Fig. 8A).

Subsequently different mutant strains were tested on solid media for growth phenotypes, whereby all complemented mutants behaved like the wild type (Figure S11A). The \(\Delta\text{sidA}\) mutant,
Figure 8. Siderophore production in \( \Delta \text{sidA} \) mutants and growth-supporting effect of terrein under iron-limitation. (A) Coprogen quantification from \( \Delta \text{akuB} \) wild type, \( \Delta \text{sidA} \) mutant and complemented mutant \( \text{sidA}^\text{C} \). Strains were cultivated for 40 h in AMM(-N)G100Gln50 supplemented with 200 µM FeCl\(_3\). Mycelium was washed and transferred to iron-free AMM(-N)G100Gln50 medium. Coprogen was quantified after 30 h of incubation. Analyses were performed from biological triplicates and technical triplicates. Statistical significance was calculated in comparison to the parental \( \Delta \text{akuB} \) strain by the Student’s paired t-test with a two-tailed distribution: * p < 0.05; *** p < 0.001. (B) Growth of wild type \( \Delta \text{akuB}, \Delta \text{terA}, \Delta \text{hapX} \) and \( \Delta \text{sidA} \) on AMM(-N)G100Gln10 plates containing different iron concentrations. Photos were taken after four and, if required, after 17 days of incubation at 37°C. Iron-free medium was supplemented with 100, 20, 2, or 0 µM FeCl\(_3\). Plates without iron were additionally supplemented with bathophenanthroline disulphonate (BPS; 100 µM) to simulate harsh starvation conditions or with either ascorbic acid (1 mM) or terrein (5 or 10 mM). (C-D) Importance of terrein production in the \( \Delta \text{sidA} \) background. AMM-G100Gln50 plates were supplemented 0, 2, 20 or 100 µM FeCl\(_3\) and either inoculated with (C) \( \text{A. terreus} \) conidia or (D) mycelial pellets from cultures pre-grown for 40 h in AMM-G100Gln50.
with 200 µM FeCl₃. The A. terreus wild-type ΔakuB, the ΔakuBΔsidA strain and the ΔakuBΔterAΔsidA are shown. Mycelia from a 40 h culture in AMM-G100Gln50 with 200 µM FeCl₃ was washed with iron-free medium and three pellets were applied to the plates. Plates were incubated at 37°C for 5 d. (E) HPLC profiles of agar plugs taken from plates shown in panel D. Wild type and ΔsidA mutant produce terrein (1) under iron limited conditions, whereas no terrein was formed by the ΔterAΔsidA mutant.

despite some reduced conidiation, showed a normal growth in presence of high to moderate iron concentrations (100 or 20 µM FeCl₃) (Fig. 8B). However, when the iron concentration was reduced to 2 µM FeCl₃ growth was strongly retarded resulting in colonies only at very prolonged incubation. No growth was observed when iron was omitted (Fig. 8B). These phenotypes were cured, when external coprogen was added to the plates (Fig. S11B). The hapX mutant showed a similar sensitivity towards iron starvation as the sidA mutant, although residual growth was observed on media without iron addition. This is in agreement with reduced coprogen production and reduced expression of the coprogen transporter as stated above (Fig. 7).

Harsh iron limitation, as induced by the addition of the iron chelator bathophenanthroline sulfonate (BPS) completely repressed growth of the hapX mutant. No altered phenotype was observed for the terA mutant in a sidA-positive background (Fig. 8B), indicating that the siderophore-based iron assimilation is the most efficient assimilation pathway. However, we additionally tested the beneficial effect of ascorbic acid or terrein supplementation on the different mutants. While the ΔsidA strain did not grow without iron supplementation even after prolonged incubation, growth was partially restored in the presence of ascorbic acid and also, although to a lesser extent, by addition of terrein (Fig. 8B). This result is in agreement with previous studies, in which a growth-supporting effect of ascorbic acid was also observed for an A. nidulans ΔsidA mutant (Eisenlle et al., 2003). Colony formation also increased with all other strains on ascorbic acid or terrein supplemented media, confirming that iron reductive properties of these compounds increases the availability of trace amounts of iron present in these media. Interestingly, when an A. fumigatus ΔsidA mutant was grown on plates with terrein supplementation, no growth stimulation was observed (data not shown). Subsequent analyses on the parental A. fumigatus wild-type strain revealed antifungal activity of terrein at the concentrations tested (Figure S12A). Additionally, further analyses revealed that growth of the phytopathogen Fusarium graminearum was strongly inhibited, when extracts from A. terreus PDB medium mainly containing terrein and its intermediates were added (Figure S12B). This indicates that terrein and/or its intermediates possess an antifungal potential against competitors in the soil environment.

Since terrein supplementation appeared especially beneficial for the A. terreus ΔsidA mutant, we were interested in the impact of intrinsic terrein production capacity for growth under iron limitation. Therefore, we deleted the sidA gene in the ΔterA background and compared growth of wild type, ΔsidA and ΔterA/ΔsidA under iron-supplemented and limited conditions (Fig. 8C). When AMMG100Gln10 plates were supplemented with 0, 2, 20 or 100 µM FeCl₃ and inoculated with a defined conidia concentration, both mutants (ΔsidA and ΔterA/ΔsidA) were unable to grow on media containing 2 µM or less FeCl₃. Therefore, we conclude that the siderophore system is essential for conidia germination under iron limitation. However, since conidia are unable to produce terrein, a second approach was performed, in which mycelium was generated in the presence of 200 µM FeCl₃, extensively washed and transferred to the same plates as used for inoculation with conidia (Fig. 8D). While growth from mycelia of the ΔakuB strain looked identical to that from conidia, the two mutant strains showed different phenotypes. The ΔsidA mutant started to form small colonies even in the absence of iron, whereas the ΔterA/ΔsidA mutant revealed some weak growth only at 2 µM FeCl₃, but not in the absence of external iron supplementation. To confirm the production of terrein, agar plugs were extracted from these plates. The wild type and the ΔsidA mutant produced substantial amounts of terrein under iron limited conditions, whereas terrein production was fully abrogated in the ΔterA/ΔsidA mutant (Fig. 8E). These results confirm that terrein production supports iron acquisition, although the siderophore system is of major importance for iron acquisition.

Discussion

A. terreus is known to be a human pathogen that causes severe invasive bronchopulmonary and disseminated aspergillosis (Lass-Flörl et al., 2005; Slesiona et al., 2012a; Slesiona et al., 2012b). In addition, a plant pathogenic potential has been described for A. terreus by causing foliar blight of potato leaves (Louis et al., 2013; Louis et al., 2014b; Louis et al., 2014a). On the contrary, A. terreus can inhibit proliferation of other plant pathogens such as Fusarium udum (Upadhyay and Rai, 1987) and acts as a mycoparasite on the plant pathogenic fungus Sclerotinia sclerotiorum (Melo et al., 2006). Therefore, A. terreus may not only cause detrimental effects on plants, but may also act beneficially by out-competing other pathogens.

Production of secondary metabolites is assumed to play a vital role in interaction and competition in the plant and soil environment and terrein appears perfectly suited to support the competitiveness of A. terreus in these interactions. The natural biological activities of terrein includes the inhibition of plant seed germination, the induction of fruit surface lesions (Zaehle et al., 2014) and the newly discovered reduction of ferric to ferrous iron accompanied with some antifungal activity. However, the
production of terrein at the correct timing requires coordinated sensing and transduction of environmental signals.

The structural genes of the terrein biosynthesis gene cluster are directly controlled by the Zn(II)$_2$Cys$_6$ binuclear zinc cluster transcription factor TerR (Gressler et al., submitted manuscript). Therefore, regulation of gene cluster induction is controlled by the activation of terR expression. While sugars are indispensable for high terrein production rates (Zaehle et al., 2014), three major inducing conditions were identified that are either methionine dependent induction, nitrogen or iron limitation. Each single factor, but especially in combination, the plant and rhizosphere environment is unequivocally sensed.

In the rhizosphere the sugar requirement for terrein production may be satisfied by plant root exudates. Plant roots are leaky for carbon compounds that derive from C-fixation during photosynthesis. Although difficult to determine its exact amount, laboratory experiments have shown that up to one third of plant-assimilated carbon may end up in the rhizosphere and especially in the extramatrical mycelium of ectomycorrhiza (Churchland and Grayston, 2014). Here, at least a fraction of carbohydrates is subsequently exuded by ectomycorrhiza, making it available to other soil microorganisms (Sun et al., 1999). Despite sugar availability in the rhizosphere, mycorrhiza fungi mobilise nitrogen to feed their symbiotic plant partner, resulting in nitrogen limitation for surrounding microorganisms. *A. terreus* is frequently isolated from the rhizosphere (Gao et al., 2013; Rajalakshmi and Mahesh, 2014), in which sugar availability and nitrogen limitation stimulate terrein production that might affect existing plant-microbe interactions by its phytotoxic and antifungal activity. Therefore, terrein production can be assumed to increase the competitiveness of *A. terreus* in the soil environment.

Another inducing signal is derived from methionine, which may be of particular importance when *A. terreus* acts as a potato plant pathogen (Louis et al., 2013). Notably, potato dextrose broth induces high terrein production rates (Zaehle et al., 2014), which we assume to be due methionine-dependant induction. Methionine is an extremely important amino acid in plants, because it is required for ethylene production in environmental stress response (Wang et al., 2002) and fruit ripening (Yang and Hoffman, 1984), polyamine production in plant defence reactions and it is a precursor for iron chelating siderophores such as nicotianamine and mugineic acid (Roje, 2006). During infection by fungal pathogens an up-regulation of S-adenosyl-methionine synthase has been described and methionine is an abundant amino acid in plant leaves (Figuereo et al., 2008). Additionally, methionine has been identified from plant root exudates (Dakora and Phillips, 2002). Therefore, besides nitrogen starvation, elevated levels of methionine depict a signal that indicates the presence of a plant environment and stimulates terrein production. However, although further studies are required to elucidate the specific contribution of terrein on plant infection, its ability to induce lesion formation on several fruit surfaces indicates that terrein interacts with plant defence mechanisms (Zaehle et al., 2014).

The third, at first sight unrelated factor inducing terrein production is a limitation of iron. As mentioned above, methionine is required for siderophore production in plants (Shojima et al., 1990) and plants compete with the surrounding microbiota for iron, leading to an iron-limited environment. Although terrein can reduce ferric to ferrous iron, a direct positive growth promoting effect of terrein during iron limitation in *A. terreus* monocultures was only observed in the absence of a functional siderophore system. However, it should be mentioned that most microbial extracellular siderophores show a high affinity for ferric, but not for ferrous iron (Hider and Kong, 2010). Thus, during environmental competition the reduction of ferric iron reduces the efficiency of iron chelation by siderophores that are secreted from competing microorganisms. A similar strategy for iron acquisition has previously been described for the human pathogenic fungus *Histoplasma capsulatum* (Timmerman and Woods, 1999). *H. capsulatum* secretes glutathione accompanied with the γ-glutamyltransferase Ggt1. This enzyme releases the dipeptide cysteinylglycine from glutathione, which reduces extracellular ferric to ferrous iron (Zarnowski et al., 2008). While *H. capsulatum* also secretes siderophores such as coprogen, coprogen B and fusarine (Howard et al., 2000), silencing of ggt1 mRNA reduced virulence in macrophages even in the presence of the siderophore system (Zarnowski et al., 2008). This indicates that extracellular iron reduction is a suitable strategy for iron acquisition in the presence of ferric iron chelating compounds. Thus, together with its antifungal activity, terrein may interfere with competing siderophore systems and increases the competitiveness of *A. terreus* in the environment.

In this study we were able to show that transduction of environmental signals to the promoter of the transcriptional activator TerR requires activation by the global transcription factors AreA, AtfA and HapX. While AreA has previously been described to regulate several secondary metabolite gene clusters in other fungi (Tudzynski et al., 1999), HapX specifically activates iron acquisition systems (Schrett et al., 2010) and AtfA plays a specific role in osmotic and oxidative stress responses (Lara-Rojas et al., 2011).

In aspergilli and maybe all saprophytic ascomycetes the GATA transcription factor AreA plays a substantial role in regulation of nitrogen metabolism and its RNA stability depends on the absence of nitrogen sources (Morozov et al., 2000). Additionally, AreA activity is controlled by its co-repressor NmrA (Andrianopoulos et al., 1998). Under nitrogen starvation AreA is released from NmrA and transported into the nucleus (Todd et al., 2005), where it binds a 5´-HGATAR-3´ sequence in its target promoters (Ravagnani et al., 1997). This leads to transcriptional activation via chromatin remodelling (Muro-
Pastor et al., 1999) as shown for genes encoding nitrate reductase (NiaD) and nitrite reductase (Nid) (Chang et al., 1996) that are upregulated in the absence of preferred nitrogen sources (Johnstone et al., 1990;Punt et al., 1995). In A. parasiticus AreA also binds in the promoter regions of the transcriptional regulators AfIR and AfIU from the aflatoxin biosynthesis cluster (Chang et al., 2000). In contrast to nitrate utilisation, this binding impedes aflatoxin production probably due to inhibition of transcriptional activation of the regulator genes (Yu et al., 2004). Thus, AreA may either act as activator or inhibitor of gene transcription. We have shown that terrein biosynthesis in A. terreus during nitrogen starvation is AreA-dependent and at least two a reA binding sites were found in the terR promoter that were recognised by the AreA DNA-binding domain. Therefore, AreA directly binds to the terR promoter and supports the expression of the terrein biosynthesis genes. Similarly, biosynthesis of mycotoxins such as fumonisins, deoxynivalenol, zearalenone, fusarilcin, fusarin C, and aurofusarin or gibberellins is activated by AreA in the phytopathogenic Fusarium species (Mihlan et al., 2003;Kim and Woloshuk, 2008;Giese et al., 2013). This implies that AreA plays a general role in regulation of secondary metabolite production of plant pathogens and the plant-derived environment may be characterised as habitat with high C:N ratio.

Nitrogen starvation responses are also mediated by the basic leucine zipper transcription factor AtfA in several ascomycetes (Hong et al., 2013). In A. nidulans, nitrogen starvation, oxidative or osmotic stresses induce phosphorylation of several kinases including the downstream MAP kinase SakA (Hog1/SakA signalling pathway) that is transported into the nucleus after phosphorylation (Lara-Rojas et al., 2011). Here, activated SakA interacts with the constitutively expressed transcription factor AtfA and activates transcription of target genes, especially those encoding catalases (Lara-Rojas et al., 2011). Interestingly, in A. terreus AtfA activation appears to act independent from SakA. A deletion of the sakA gene did not reduce terrein production levels (data not shown) and an A. terreus atfA mutant displayed no increased sensitivity to osmotic or oxidative stresses. Since an A. terreus atfA mutant also showed no increased sensitivity to other stressors such as SDS, benzoic acid or UV radiation (data not shown), AtfA is not required for stress adaptation in this fungus. However, in A. terreus AtfA acts as an inducer of secondary metabolite production since its deletion resulted in non-pigmented conidia and in the loss of terrein production during nitrogen starvation or methionine supplementation. This finding contrasts with studies on Botrytis cinerea, in which deletion of the atfI gene resulted in accumulation rather than depletion of the polyketide botcinin A and the sesquiterpene botrydial (Temme et al., 2012). Therefore, AtfI seems to act as an inhibitor of secondary metabolite production in B. cinerea, whereas AtfA acts as an inducer in A. terreus. Interestingly, AreA and AtfA were both required for induction of the terrein biosynthesis gene cluster during nitrogen starvation and methionine supplementation. The deletion of either one of both activators was sufficient to avoid terrein production and both factors seem to act in concert.

To our biggest surprise, activation of the terrein biosynthesis gene cluster was also triggered by the iron response regulator HapX and this activation was completely independent from AreA and AtfA. Iron starvation requires several metabolic (Haas, 2014). To overcome severe iron starvation, microorganisms frequently produce siderophores (Pollack and Neilands, 1970;Eisendle et al., 2003;Kreutzer and Nett, 2012) or utilise siderophores from competing species (xenosiderophores) (Boukhalfa and Crumbliss, 2002). A. fumigatus and A. nidulans produce TAFCS as major extracellular siderophore (Eisendle et al., 2003;Schrettl et al., 2007) and biosynthesis and uptake is regulated by the opposing transcription factors HapX and SreA in dependence of iron availability (Schrettl et al., 2007;Schrettl et al., 2008;Schrettl et al., 2010). Similarly, A. terreus produces the HapX and SreA regulated siderophores ferrichrys and coprogen. In this respect, one might assume that the reducing effect of terrein on ferric iron may counteract the efficacy of siderophore mediated iron uptake due to the reduced affinity of siderophores for ferrous iron. However, in competition with other microorganisms iron reduction could reduce binding of iron to siderophores from competitors that cannot be used by A. terreus. Interestingly, the control of terrein production by HapX depicts an unprecedented example for the activation of a secondary metabolite gene cluster outside the siderophore biosynthesis system. Here, terrein production is only under positive HapX control, but not under negative SreA feedback-control. This is confirmed by two observations: (i) Deletion of hapX strongly reduced the expression of terrein cluster genes under iron limitation, whereas deletion of sreA neither elevated cluster gene expression nor terrein production under iron-rich conditions. (ii) No putative SreA binding sites (Schrettl et al., 2008) were detected in the terR promoter region, whereas a CCAAT box required for binding of the CCAAT-binding complex (CBC) and subsequent CBC interaction with HapX (Hortschansky et al., 2007;Gisler et al., 2014) was present. In this respect, due to the independent induction of terrein biosynthesis in the presence of iron via AreA and AtfA, a negative control by SreA would counteract this induction.

In conclusion, here we show that the terrein biosynthesis gene cluster is activated by at least three independent environmental signals: Nitrogen limitation, methionine and iron starvation. Additionally, the three global transcription factors AreA, AtfA and HapX are essential to transfer these signals to the terrein biosynthesis gene cluster. Although one environmental signal is sufficient to induce the gene cluster, a combination of different signals provides an environmental image of higher resolution and allows a response that is perfectly adapted to the situation in the ecological niche. In case of terrein
particularly its phytotoxic and antifungal potential combined with its iron reductive properties enables *A. terreus* to access new nutrient sources and to acquire nutrients that might otherwise be consumed by competitors. Notably, similar to terrein, the mycotoxin production in *Fusarium* species is generally associated with sensing of nitrogen availability and *AreA* plays an important regulatory function. However, the possible involvement of *AtfA* as regulator and methionine as inducer in these species needs to be tested future experiments. However, we assume that multiple regulation of secondary metabolite gene clusters might depict a general mechanism in fungal gene cluster activation.

**Experimental procedures**

**Media and cultivation conditions**

All genotypes of strain used in this study are summarised in Table S1. *A. terreus* strain SBUG844 and its derivative SBUG844/ΔakuB that shows increased rates of homologous integration served as parental strains (Gressler et al., 2011). If not indicated otherwise all strains were cultivated in *Aspergillus* minimal medium (AMM; http://www.fgsc.net/methods/anidmed.html) supplemented with different carbon and nitrogen sources: AMM with 100 mM glucose (AMM-G100), with 100 mM glucose and 50 mM glutamine (AMM-G100Gln50), with 100 mM glucose, 50 mM glutamine and 10 mM methionine (AMM-G100Gln50Met10), with 1% casamino acids (AMM-CA1%). Additionally, the following complex media were used: Sabouraud (Sab, Sigma Aldrich, Germany), YPD (10g/l yeast extract, 20 g/l peptone, 20 g/l dextrose), potato dextrose broth (PDB, Sigma Aldrich), potato broth (2% potato extract, Sigma Aldrich), apple juice (pH adjusted to 6.0 with NaOH), carrot juice with honey (both Wiesgart, Germany), banana juice or peach juice (both FruchtOase, Kiberg GmbH, Germany). When required, 140 µg/ml hygromycin B (Roht), 0.1 µg/ml pyrithiamine or 80 µg/ml phleomycin (both Sigma Aldrich) were added. For preparation of conidia suspensions all strains were cultivated for 4 days at 37°C on solid 2% AMM-G50Gln10 agar plates. Conidia were harvested by overlaying colonies with water and filtering of the suspension over 40 µm cell strainers (VWR, Germany). Liquid media were generally inoculated at a final concentration of 1 × 10⁶ conidia per ml. For the *ΔareA* strains inoculation densities were generally doubled to compensate for reduced growth rates. For metabolite extraction and quantification liquid cultures in 50 ml scale were used and inoculation densities were generally doubled to compensate for water) were used in final concentrations of 10, 50, 100 or 200 µM. The protein methyltransferase inhibitor BIX01294 trihydrochloride hydrate (BIX, Sigma Aldrich, solved in DMSO) were used in final concentrations of 1, 5, 10 and 20 µM. For nitrogen or iron shift experiments, strains were pre-cultivated for 40 h in 150 ml AMM-G100Gln50 or AMM-G100Gln50 with 200 µM FeCl₃. Mycelia were harvested over sterile miracloth (Merck, Germany) washed two times with AMM lacking a nitrogen (AMM-N) or iron source (AMM-Fe) and aliquots were transferred to fresh 50 ml media with or without 50 mM Gln or with or without 200 µM FeCl₃. Depending on the specific experiments, samples were analysed after 12, 24 or 48 h of cultivation.

**Metabolite extraction, quantification, purification and structure elucidation**

Metabolites were extracted from culture broth as described previously (Gressler et al., 2011). In brief: An equal volume of ethyl acetate was added and collected after defined shaking of the mixture. The procedure was repeated once. After evaporation of the solvent residues were taken up in 1 ml methanol each and filtered. Standard extract analyses were performed on an Agilent 1100 series HPLC-DAD system coupled with a MSD trap (Agilent Technologies, Germany) operating in alternating ionisation mode. Terrein quantification was carried out from 50 ml cultures as described (Zaehle et al., 2014). For quantification of the siderophore coprogen the complete 50 ml culture supernatants were filtered and lyophilised to dryness. The remaining solids were extracted three times with 10 mL of MeOH. The solvent from the combined organic extracts was removed under reduced pressure and residues were re-dissolved in 2 mL of MeOH. The resulting slurries were filtered and the filtrates analysed by HPLC measurements. HPLC analyses were carried out on an Agilent 1260 device equipped with a quaternary Pump and a UV/Vis detector (Agilent Technologies; Column: Zorbax Eclipse XDB-C8, 5 µm, 150 × 4.6 mm; flow rate 1 mL/min; eluent A: H₂O/0.1% HCOOH, eluent B: MeOH. The gradient started with 10% B and reached 30% B after 4 min, increased to 55% B within 10 min and reached 100% B after 2 min, where it was retained for additional 4 min. Quantification of coprogen was performed from a calibration curve of known coprogen concentrations. For correlation of coprogen to the fungal biomass mycelia from the cultures were dried for 48 h at 37°C and balanced and coprogen concentrations per gram dried mycelium were calculated. All quantifications were carried out in biological triplicates and technical duplicates. Isolation of coprogen for generation of the calibration curve was performed by semi-preparative HPLC from culture supernatants of the *ΔakuB* and *ΔakuBΔareA* strains and fractions were collected by automatic fraction collection. Separation was carried out on a Zorbax Eclipse XDB-C8, 5 µm, 250 × 4.6 mm with a flow rate
of 4.0 mL/min using H$_2$O as eluent A and MeOH as eluent B. The gradient started with 10% B, reached 30% B after 6.5 min, increased to 55% B within 16.5 min, reached 100% B after 2 min and retained at 100% B for additional 6 min. For isolation of 2-(E)-prop-1-en-1-yl)maleic acid the crude product from upscaled terrein reduction assays (see below) was subjected to semi-preparative HPLC using a Zorbax Eclipse XDB-C8, 5 µm, upscaled terrein reduction assays (see below) was subjected to semi-preparative HPLC using a Zorbax Eclipse XDB-C8, 5 µm, 250×4.6 mm with a flow rate of 4.0 mL/min, eluent A: H$_2$O/0.1% HCOOH, eluent B: acetonitrile. The gradient started with 5% B and was hold for 14 min, increased to 10% B within 9 min, increased to 100% B within 2 min where it was retained for additional 7 min. Fractions from the new metabolite formed from ferric iron reduction were collected and evaporated resulting in a white solid, which revealed a m/z value of 150.0495 [M+H$^+$] by HRESI-MS that perfectly matched a calculated molecular formula of C$_7$H$_8$O$_3$ containing four double-bond equivalents. $^{13}$C-NMR measurements revealed the presence of two carbonyl groups, one terminal methyl group and four carbons being part of a conjugated system. 2D NMR data and analysis of all proton coupling constants from the $^1$H-NMR spectrum finally confirmed the structure of 2-((E)-prop-1-en-1-yl)maleic acid (Fig. S14 and S15). NMR spectra were recorded on a Bruker Avance III 500 and a Bruker Avance III 600 spectrometer (Bruker BioSpin GmbH, Germany) equipped with a cryoprobe head using DMSO-d$_6$ and methanol-d$_4$ as solvents and internal standards.

### Fruit infection and terrein quantification from fruits

Fruits surfaces were wiped with a soft tissue saturated with 70% ethanol. Apples (ALDI, type: Tenroy Royal/Gala; Germany), bananas (type Bio, Fairverbindet; tegut, Germany) and nectarines (tegut, type: Sweet Lady, class 1, size A; Italy) were cut lengthwise using a sterile scalpel. The resulting groove was infected with 200 µl of a conidia suspension containing $4 \times 10^7$ conidia and fruits were incubated for 7 to 10 days at room temperature in a humid chamber. Cutted, but uninfected fruits served as controls. For cultivation in presence of high levels of nitrogen, 200 µl of a 3.5 M NH$_4$Cl solution were applied prior to infection. For terrein quantification, fruits were homogenised and extracted twice with 100 ml ethyl acetate and the solid residues were collected and evaporated for dry weight determination. Terrein was quantified from extracts as described above and terrein production rates were calculated as [mg terrein / g fruit dry weight].

### Fruit surface spot dilution assay

To determine the induction of lesion formation on banana fruit surfaces organic bananas (type Bio, Fairverbindet; tegut) were softly cleaned with water and air dried. 5 µl of various sequential dilutions (1:2 to 1:512) of metabolite crude extracts (solved in PBS) were added as a single drop to the surface. Fruits were incubated in the dark at room temperature and photographs were taken after 40 h and 60 h.

### Determination of ammonia in culture supernatants

The determination of ammonia was performed as described by Weichselbaum et al. (1969). From each culture 1 ml aliquots of broth was removed and centrifuged for 5 min at 16000 x g to remove residual mycelium. The supernatant was collected and adjusted to pH 7.0 by addition of NaOH. A 200 µl aliquot was sequential diluted in nitrogen-free medium and transferred to a transparent flat-bottom 96 well plate. After addition of 20 µl of SC solution (6.5 g sodium salicylate (C$_9$H$_7$O$_4$Na), 6.5 g trisodium citrate (C$_6$H$_5$O$_7$Na$_3 \times 2$ H$_2$O), 48.5 mg disodium pentacyanonitrosylferrate (Na$_3$Fe(CN)$_5$NO $\times 2$ H$_2$O) in 50 ml water) the reaction was started with 20 µl of DCIC solution (1.6 g NaOH, 100 mg sodium dichloroisocyanurate (C$_5$N$_2$Cl$_2$O$_7$Na) in 50 ml water). Readings were done after 4.5 h of incubation at room temperature in a FLOUstar Omega microplate reader (BMG Labtech). Plates were shaken for 1 min in double orbital direction and absorbance at 655 nm was measured (50 flashes/well, gain 2000). Fresh growth medium served as positive and water as negative controls. A calibration curve was recorded for calculation of ammonia levels from culture broth.

### Iron reduction assay

The ferric iron-reducing assay was performed virtually as described by Benzie and Strain (1996). In brief: Three different working solutions were prepared by mixing 20 parts of reagent A (0.4% sodium acetate, 1.6% acetic acid; pH 4) with 1 part of reagent B (0.4% 2,4,6-tripyridyl-S-triazine (TPTZ; Sigma) in 0.16% HCl) and either 1 part of reagent C1 (88 mM to 5.5 mM sequential dilutions of terrein), reagent C2 (2% ascorbic acid, serving as positive control) or reagent C3 (water, serving as negative control). From each working solution 200 µl were transferred into wells of a 96-well-plate. An iron stock solution (10 mg/ml FeCl$_3$ in 0.2 M H$_2$SO$_4$) was diluted to 0.05 mg/ml and 100 µl were added to the working solution, which started the reaction. Reduction of ferric to ferrous iron was followed by determination of the change in absorbance at 590 nm for up to 10 h using a FLOUstar Omega microplate reader (BMG Labtech). For determination of the pH optimum of the terrein mediated reduction, reagent A was adjusted to pH values of 3.5 to 6.0. Reductive activity was normalised against the activity determined from ascorbic acid, which was set as 100%. All experiments were carried out in triplicates. Upscaling of the iron reduction assays for metabolite extraction was performed by incubating 20 mg (125 µmol) of terrein in a 1:2 molar ratio with either FeSO$_4$, FeCl$_3$, MnCl$_2 \times$ H$_2$O, KMnO$_4$, KMnO$_5$, CH$_3$COOCu or (CH$_3$COO)$_2$Cu in 10 ml of reagent A. Samples were incubated for 48 h at room temperature under continuous vertical rotation (20 rpm). Metabolites were extracted three times.
amplified in Phusion Polymerase (Thermo Scientific). Plasmids were respective ORF with the pyrithiamine resistance cassette ([Gressler et al., 2011] were generally generated by replacing the 23 with ethyl acetate (10 ml each). The solvent was evaporated and PCR (qRT-PCR) RNA isolation, cDNA synthesis and quantitative real-time 48 h at 30°C prior to photography.

2.50, 1.25, 0.625 and 0.3125 mg/ml. Plates were incubated for coprogen, terrein or propylene maleic acid in concentrations of 23, 1.25, 0.625 and 0.3125 mg/ml. Plates were inoculated with 100 µl methanolic solutions of coprogen, terrein or propylene maleic acid in concentrations of 2.50, 1.25, 0.625 and 0.3125 mg/ml. Plates were incubated for 48 h at 30°C prior to photography.

RNA isolation, cDNA synthesis and quantitative real-time PCR (qRT-PCR)
Myceia from specified cultivations were briefly washed with water and ground under liquid nitrogen. RNA was isolated using the RiboPure RNA Purification Kit (Ambion). Residual genomic DNA (gDNA) was removed by the DNA-free kit (Ambion). cDNA was synthesised by the Revert Aid Reverse transcriptase (Thermo Scientific) using anchored oligo_dT primers. qRT-PCR was carried out on a CFX384 Touch Real-Time PCR Detection System (BioRad) using the EvaGreen 5× QPCR (ROX) Mix (Bio & Sell) following the manufacturer’s protocol and using 1:5 and 1:10 dilutions of cDNA samples serving as templates. The actin gene (actA, ATEG_06973) and the enolase gene (enoA, ATEG_02902) were used for normalisation of transcript levels yielding similar results. Normalised transcript levels were calculated as fold expression = 2^ΔΔCT. Primers used for qRT-PCRs showed a primer efficiency of 1.89 to 2.0 and are listed in Table S2.

Cloning strategies
In general, all PCR amplifications were performed using the Phusion Polymerase (Thermo Scientific). Plasmids were amplified in Escherichia coli DH5α. A detailed description of all cloning procedures and oligonucleotides used for plasmid construction are given in the supporting experimental procedures and Table S2. In brief: Deletion mutants in SBUG844∆akuB (Gressler et al., 2011) were generally generated by replacing the respective ORF with the pyrithiamine resistance cassette (ptra) from plasmid ptrA-pJET1 (Fleck and Brock, 2010). For complementation of deletion mutants the ptra cassette was replaced by the ORF of the deleted gene including its terminator sequence that was fused to the phleomycine resistance cassette (ble) (Gressler et al., submitted manuscript). In case of the ΔareA complementation (ATEG_07264), no resistance cassette was required, since complemented strains were selected by growth on nitrate as sole nitrogen source. Only a partial gene deletion was performed for the hapX gene locus (ATEG_08014) due to lack of complete sequence information at beginning of this study. Detailed information on the identification of the complete hapX locus is given in the supporting experimental section (GenBank accession number KP233834). The hapX deletion mutant was complemented with the A. nidulans hapX gene (ANID_08251) including its promoter and terminator sequences that were fused to the ble cassette. The ΔareA∆antA double mutant was generated by replacement of the atfA ORF by the ble cassette in the ΔareA background. The sidA gene has been deleted in SBUG844∆akuB and SBUG844∆akuB∆areA by replacement of the ORF by the ble resistance cassette. For generation of atfA overexpression strains in A. terreus SBUG844 the A. nidulans gpdA promoter (AnPgpdA, locus ANID_08209) was fused with the atfA ORF (ATEG_04664). Here, the hygromycin B resistance cassette (bph) was used as selection marker for transformation. For constitutive expression of terR in the ΔareA∆antA, SBUG844∆akuB∆areA served as parental strain. The fusion of the A. nidulans gpdA promoter with the terR gene was linked with the ble gene and flanked by atfA upstream and downstream regions. Transformation of the ΔareA mutant resulted in replacement of the atfA locus by the terR overexpression construct. The β-galactosidase reporter strains were generated using the lacZ gene from E. coli fused with the terrein synthase promoter (PterA, locus ATEG00145). As selection marker the ptrA resistance cassette was used. Fungal transformation was performed as described previously (Gressler et al., 2011) and transformants were checked by diagnostic PCR and Southern Blot analyses (see Fig. S13).

Bacterial expression and purification of AreA polypeptide for SPR analysis
The coding sequence for the A. nidulans AreA DNA-binding domain (amino acids 663 to 797) was amplified from plasmid pGEX4T1-AreA-ZnF (Muro-Pastor et al., 2004) as BamHI-HindIII fragment using oligonucleotides P80/81 (Table S2). The amplified fragment was cloned into a modified pET-43.1a vector allowing the addition of a N-terminal (His)6-tag and removable by a tobacco etch virus (TEV) protease (Novagen). (His)6-AreA(663–797) was produced by E. coli BL21 (DE3) cells grown at 30 °C in 1 l Overnight Express Instant TB Medium (Novagen, Germany) in the presence of 1 mM Zn(OAc)2. Cells (15 – 20 g wet weight) were collected by centrifugation, resuspended in 200 ml lysis buffer (50 mM NaH2PO4, 300 mM NaCl, 10 % v/v glycerol, 40 mM imidazole, 5 mM β-mercaptoethanol, 1 mM AEBSF, pH 8.0) and disrupted using an EmulsiFlex C5 high pressure homogenizer (Avestin, Germany). Cleared cellular extract was loaded to a 25 ml Ni Sepharose FF (GE Healthcare, Germany) column and (His)6-AreA(663–797) was eluted with 200 mM imidazole. After removal of the (His)6 tag by adding 4 µg TEV protease per mg peptide and overnight incubation at room temperature, samples were adjusted to 150 mM NaCl and applied on a 40 ml CellulofineSulfate (Millipore, Germany) column that was equilibrated with 50 mM NaH2PO4, 150 mM NaCl, 10 % v/v glycerol, 5 mM β-mercaptoethanol, 10
μM Zn(OAc)$_2$, pH 7.5, followed by elution of AreA$_{663-797}$ with a gradient up to 2 M NaCl. Peak fractions were concentrated with an Amicon Ultra-15 10K centrifugal filter device and purified to homogeneity by size exclusion chromatography on a Superdex 75 prep grade column (GE Healthcare) in 20 mM HEPES, 300 mM NaCl, 5 mM β-mercaptoethanol, 10 μM Zn(OAc)$_2$, pH 7.5 as running buffer. The absolute molecular mass of AreA$_{663-797}$ was determined in series on a miniDawn TROEX static light scattering monitor and an Optlab T-REX differential refractometer (Wyatt, Germany). The molar mass was calculated using Astra 6 software (Wyatt). AreA$_{663-797}$ was stored in 50 % v/v glycerol at −20 °C.

Surface plasmon resonance measurements

Real-time analyses were performed on a Biacore 2000 system (GE Healthcare) at 25 °C. DNA duplexes were produced by annealing complementary oligonucleotides using a 5-fold molar excess of the non-biotinylated oligonucleotide. The dsDNA was injected on flow cells of a streptavidin (Sigma)-coated CM3 sensor chip at a flow rate of 10 µl/min until the calculated amount of DNA that gives a maximum Area coated CM3 sensor chip at a flow rate of 10 µl/min until the dsDNA was injected on flow cells of a streptavidin (Sigma)-molar excess of the non-biotinylated oligonucleotide. The system (GE Healthcare) at 25 °C. DNA duplexes were produced from 3.125 to 200 nM. NMR spectra of secondary metabolites. This work was supported by grant BR 22164/1 from the German Science Foundation (DFG) and internal funding from the Leibniz association.

Statistical significance

All analyses in which statistical analyses were required were performed in biological triplicates with at least two technical replications. Significance was calculated by use of the Microsoft Excel 2007 software package using the Student's paired t-test with a two-tailed distribution. p-values were marked as follows: * p < 0.05, ** p < 0.01; *** p < 0.001.

Acknowledgement

We greatly acknowledge Daniela Hildebrandt for assistance in plasmid construction, Carmen Karkowski and Elena Geib for assistance in metabolite quantification, Andrea Perner for HR-MS analyses and Heike Heincke for recording $^1$H- and $^13$C-NMR spectra of secondary metabolites. This work was supported by grant BR 22164/1 from the German Science Foundation (DFG) and internal funding from the Leibniz association.

References


Supporting Information

Supporting Experimental Procedures

Figure S1. Dependency of terA expression on the available glucose concentration.

Figure S2. HPLC analysis of extracts from A. terreus strains after cultivation in fruit juices and from infected nectarines and apples.

Figure S3. β-galactosidase activity of PterA::lacZ grown in banana juice without (control) or with different supplementations.

Figure S4. Analysis of colony formation and growth phenotypes of nitrogen regulator mutants in presence of different nitrogen sources.

Figure S5. Terrein quantification from A. terreus wild type, nitrogen regulator mutants and complemented strains cultivated in the presence of nitrogen starvation.

Figure S6. Biochemical characterisation of the recombinant AreA DNA-binding domain and in vitro binding to HGATAR motifs identified in the terA and terR promoters of the A. terreus terrein biosynthesis gene cluster.

Figure S7. HPLC analyses from culture filtrates of SBUG844 wild type and two independent atfA overexpression mutants (AnP_{gpdA:atfA}; OE 1 and 2).

Figure S8. terA promoter activation from the reporter strain SBUG844 PterA::lacZ cultivated in presence of limited macroelements.

Figure S9. HPLC analyses from culture filtrate extracts grown in the presence and absence of an iron source.

Figure S10. qRT-PCR analysis of A. nidulans hapX expression in the A. terreus wild-type SBUG844ΔakuB, the hapX mutant SBUG844ΔakuBΔhapX and its complemented strain SBUG844ΔakuBΔhapX/ΔhapX*.

Figure S11. Colony formation of A. terreus mutants in dependence of iron availability, ascorbic acid or terrein.

Figure S12. Antifungal activity of terrein and PDB medium from A. terreus wild-type cultivations.

Figure S13. Southern Blot analyses for A. terreus strains generated in this study.

Figure S14. ¹H NMR (500 MHz, MeOD; upper panel) and ¹³C NMR (150 MHz, MeOD; lower panel) of compound 4, 2-(E)-prop-1-en-1-yl)maleic acid.

Figure S15. HSQC NMR (600 MHz, MeOD; upper panel) and HMBC NMR (600 MHz, MeOD; lower panel) of compound 4, 2-(E)-prop-1-en-1-yl)maleic acid.

Table S1 Genotypes of strains used in the study.

Table S2. List of oligo nucleotides used in the study.

Supporting data

Analytical data of coprogen

Analytical data of ferrichrysin

Supporting Experimental Procedures

Generation of PterA::lacZ reporter strains

The promoter of the terA gene (PterA; 1220 bp) was amplified with oligonucleotides P47/48 from gDNA of A. terreus SBUG844 and ligated into the NotI/BamHI digested plasmid lacZTrpC-T-pJET1.2 (Gressler et al., 2011) containing the E. coli lacZ gene and the trpC terminator sequence. The plasmid was linearised by NotI digestion and the NotI-excised ptra-cassette from plasmid ptra-pJET (Fleck and Brock, 2010) was inserted. The resulting plasmid was used for transformation of A. terreus SBUG844 wild type resulting in SBUG844 PterA::lacZ.

Overexpression of the atfA gene

Constitutive strong expression of the atfA gene was performed by using of the strong constitutive gpdA promoter from A. nidulans FGSC A4 (AnP_{gpdA}; 1387 bp; oligonucleotides P49/50) to control expression of A. terreus atfA. The atfA ORF together with its natural terminator was amplified from gDNA of A. terreus SBUG844 (atfA + atfA*, 2466 bp) using oligonucleotides P51/52. Both fragments were fused by in vitro recombination with the SpeI- restricted hph-pCRIV vector using the InFusion HD cloning kit (Clonetech laboratories). The resulting plasmid AnP_{gpdA:atfA:atfA*}:hph-pCRIV was used for transformation of A. terreus SBUG844 wild type resulting in strain SBUG844 AnP_{gpdA:atfA}.

Deletion of the cpcA and rhbA gene

The upstream and downstream fragments of cpcA (ATEG_03131) were amplified from gDNA of A. terreus SBUG844 using oligonucleotides P53/54 (943 bp) and P55/56 (853 bp). Similarly, the upstream and downstream flanks of rhbA (ATEG_09480) were amplified with oligonucleotides P57/58 (473 bp) and P59/60 (418 bp). The respective fragments were fused by in vitro recombination with the ptrA resistance cassette (1950 bp) from ptrA-pJET1 (Fleck and Brock, 2010) into the KpnI-restricted pUC19 vector using the InFusion HD cloning kit (Clonetech laboratories) resulting in cpcAup-ptrA-cpcAdn-pUC19, rhbAup-ptrA-rhbAdn-pUC19 and dnmTup-ptrA-
Deletion and complementation of the \textit{areA} gene

The upstream and downstream flanks of \textit{areA} (ATEG\_04664) were amplified from gDNA of \textit{A. terreus} SBUG844 with oligonucleotides P61/62 (820 bp) and P63/64 (731 bp). Fragments were fused by fusion PCR and ligated into the pJET1.2 cloning vector (Thermo Scientific). The fragment was excised with SmaI and subcloned into pUC19. After restriction with NolI the \textit{ptrA} resistance cassette (1950 bp) from \textit{ptrA}-pJET1 (Fleck and Brock, 2010) was inserted resulting in \textit{areA}-\textit{ptrA}-\textit{areA}DN-pUC19. The deletion cassette was excised by SmaI and used for transformation of SBUG844\textit{ΔakuB}. For complementation, a fragment spanning the entire \textit{areA} ORF including its promoter and terminator sequence was amplified from gDNA of the \textit{A. terreus} type strain FGSC A1156 (NIH2624) using oligonucleotides P65/66 (4181 bp) and ligated into pJET1.2. gDNA from the type strain was used, because its \textit{areA} promoter sequence contained an additional PstI restriction site that allowed discrimination of complemented strains from the parental SBUG844\textit{ΔakuB} strain. The complementation fragment was excised by Xhol restriction from plasmid A1156\textit{areA}\textit{areA}DN-pJET1.2 and directly used for the transformation of SBUG844\textit{ΔakuB}\textit{ΔareA}. Due to the inability of the \textit{ΔareA} mutant to utilise various nitrogen sources, no additional resistance marker was required and transformants were regenerated on media containing nitrate as sole nitrogen source. Complemented strains were checked by Southern Blot analysis and an additional PstI control digest of the PCR-amplified \textit{areA} upstream flank.

Deletion and complementation of the \textit{atfA} gene

The upstream and downstream fragments of \textit{atfA} (ATEG\_04664) were amplified from gDNA of \textit{A. terreus} SBUG844 with oligonucleotides P66/67 (1102 bp) and P68/69 (890 bp) and fused by \textit{in vitro} recombination with the \textit{NolI}-excised \textit{ptrA} resistance cassette (1950 bp) from \textit{ptrA}-pJET1 (Fleck and Brock, 2010) into the \textit{KpnI}-excised pUC19 vector using the InFusion HD cloning kit (Clontech laboratories) resulting in \textit{atfA}-\textit{ptrA}-\textit{atfA}DN-pUC19. The deletion cassette was excised by \textit{KpnI} and used for transformation of SBUG844\textit{ΔakuB}. For deletion of \textit{atfA} in the \textit{ΔareA} background the \textit{ptrA} resistance cassette was replaced by the phleomycin resistance (\textit{ble}) cassette (\textit{NolI} digest from \textit{ble}-pJET1.2). The deletion cassette was excised by \textit{KpnI} restriction and used for transformation of SBUG844\textit{ΔakuB}\textit{ΔareA}. For complementation of the \textit{ΔatfA} phenotype a fragment spanning the entire \textit{atfA} gene including its promoter and terminator region were amplified with oligonucleotides P66/70 (3319 bp) and an additional downstream fragment was amplified with P71/69 (859 bp). The fragments were fused by \textit{in vitro} recombination with the \textit{NolI}-excised \textit{ble} resistance cassette (2073 bp) from \textit{ble}-pJET1.2 into the \textit{KpnI}-restricted pUC19 vector using the InFusion HD cloning kit (Clontech laboratories) resulting in \textit{atfA}-\textit{up:\textit{atfA}}-\textit{atfA}DN-pUC19. The complementation cassette was excised by \textit{KpnI} and used for transformation of SBUG844\textit{ΔakuB}\textit{ΔatfA}.

Overexpression of \textit{terR} in the \textit{ΔareA}/\textit{ΔatfA} background

The \textit{atfA} flanking regions were used to integrate the \textit{terR} gene under control of the \textit{A. nidulans gpdA} promoter in the \textit{atfA} locus, which results in an \textit{atfA} deletion. The upstream and downstream flanks of \textit{atfA} were amplified from gDNA of \textit{A. terreus} SBUG844 using oligonucleotides P72/73 (1125 bp) and P74/75 (913 bp). Subsequently, a fragment already containing the fusion of \textit{AnPgpdA} with the \textit{terR} ORF including its native terminator (4405 bp) was amplified with oligonucleotides P76/77 from plasmid \textit{pah\_AnPgpdA\_terR}\textit{pUC19} (Gressler et al., submitted manuscript). The plasmid \textit{pah\_pCRIV} (Fleck and Brock, 2010) was restricted with EcoRI to remove the \textit{pah} resistance cassette and all three fragments were fused by \textit{in vitro} recombination into the \textit{EcoRI} site using the InFusion HD cloning kit (Clontech laboratories). The resulting plasmid \textit{atfA\_AnPgpdA\_terR}\textit{pUC19} was linearised with \textit{NolI} and the \textit{ble} resistance cassette from \textit{ble}-pJET1.2 was inserted between the \textit{terR} terminator and the \textit{atfA} downstream region. The final plasmid was restricted with EcoRI, and the fragment \textit{atfA\_AnPgpdA\_terR}\textit{DN-pCRIV} was used for transformation of \textit{A. terreus} SBUG844\textit{ΔakuB}\textit{ΔareA} to replace the \textit{atfA} ORF with the \textit{terR} overexpression construct.

Partial deletion of \textit{hapX}, complementation with \textit{AnhapX} and \textit{hapX} sequence verification

Because the complete sequence information of the \textit{hapX} locus was lacking at beginning of this study only a partial deletion of the \textit{A. terreus hapX} gene was performed. Upstream and downstream flanks inside the \textit{hapX} coding region (ATEG\_08014) were amplified with oligonucleotides P82/83 (387 bp) and P84/85 (345 bp) from genomic DNA of SBUG844 and fused by \textit{in vitro} recombination with the \textit{ptrA} resistance cassette (1950 bp) from \textit{ptrA}-pJET1 (Fleck and Brock, 2010) into the \textit{KpnI}-restricted pUC19 vector using the InFusion HD cloning kit (Clontech laboratories) resulting in \textit{hapX}\textit{up:\textit{ptrA}}-\textit{hapX}\textit{DN-pUC19}. The deletion construct was excised by \textit{KpnI} restriction and used for transformation of SBUG844\textit{ΔakuB}. For complementation of the partial \textit{hapX} deletion by the \textit{A. nidulans hapX}, the upstream and downstream flanks of ATEG\_08014 were amplified with oligonucleotides P82/86 (381 bp) and P87/85 (341 bp) and the complete \textit{hapX} ORF (AN8251) including its native promoter and terminator sequence was amplified from

\textit{dnmTdn-pUC19}. The deletion cassettes were excised by \textit{KpnI} restriction and used for transformation of SBUG844\textit{ΔakuB}. The upstream and downstream flanks of \textit{areA} (ATEG\_07264) were amplified from gDNA of \textit{A. terreus} SBUG844 with oligonucleotides P61/62 (341 bp) and P63/64 (890 bp) to generate a partial deletion construct were amplified with oligonucleotides P61/62 (387 bp) and P63/64 (345 bp) from genomic DNA of SBUG844 and fused by \textit{in vitro} recombination with the \textit{ptrA} resistance cassette (1950 bp) from \textit{ptrA}-pJET1 (Fleck and Brock, 2010) into the \textit{KpnI}-restricted pUC19 vector using the InFusion HD cloning kit (Clontech laboratories) resulting in \textit{atfA\_up:\textit{ptrA}}-\textit{atfA}DN-pUC19. The complementation cassette was excised by \textit{KpnI} and used for transformation of SBUG844\textit{ΔakuB}\textit{ΔatfA}.
gDNA of *A. nidulans* FGSC A4 using oligonucleotides P88/89 (2648 bp). All fragments were fused by *in vitro* recombination with the *ble* resistance cassette (2073 bp) from *ble*-pJET1.2 (2073 bp) into the KpnI-restricted pUC19 vector using the InFusion HD cloning kit (Clontech laboratories) resulting in *hapX* up_PAn.*hapX*:An.*hapX*T_ _ble_ _hapXdn-pUC19. The complementation cassette was excised by KpnI and used for transformation of SBUG844∆akuB∆*hapX*. To identify the complete coding region of the *A. terreus* *hapX* gene, long run PCRs from within the *hapX* gene and the last nucleotides of the known 5´- and 3´-borders were performed with oligonucleotides P110/111 (800 bp 5´ fragment) and P112/113 (2500 bp 3´ fragment). Bands were excised from agarose gels and cloned into the pJET1.2 cloning vector. Fragments were sequenced from both strands using the primer walking method (oligonucleotides P114/115 for both fragments and additionally P116, P117 and P78 for the 3´ fragment). Finally, RNA was isolated from iron starvation conditions, transcribed into cDNA and the *hapX* ORF was amplified with gene specific oligonucleotides 113/79 and sequenced with oligonucleotides P114/115. The complete *hapX* locus information was submitted to EMBL and can be found under accession number KP233834.

**Deletion and complementation of the sreA gene**

The upstream and downstream flanking regions of sreA (ATEG_07714) were amplified from gDNA of *A. terreus* SBUG844 with oligonucleotides P90/91 (763 bp) and P92/93 (756 bp) and fused by *in vitro* recombination with the ptrA resistance cassette (1950 bp) from ptrA-pJET1 (Fleck and Brock, 2010) into the KpnI-restricted pUC19 vector using the InFusion HD cloning kit (Clontech laboratories) resulting in sreA up-_ptrA_-sreA dn-pUC19. For transformation of the sreA deletion mutant, the ptrA cassette was excised by NotI restriction and replaced by the *ble* cassette. The deletion cassettes was excised by KpnI restriction and used for transformation of SBUG844∆akuB and SBUG844∆akuB∆*sreA*. For complementation of strain SBUG844∆akuB∆*sidA* the *sidA* ORF including its promoter and terminator sequence was amplified with oligonucleotides P102/103 (2393 bp) and an additional downstream fragment was amplified with P104/105 (919 bp). The fragments were fused by *in vitro* recombination with the *ble* resistance cassette (2073 bp) from *ble*-pJET1.2 into the HindIII-restricted pUC19 vector using the InFusion HD cloning kit (Clontech laboratories) resulting in *sidA* up:_sidA_ _ble_ _sidAdn-pUC19. The complementation cassette was excised by HindIII restriction and used for transformation of SBUG844∆akuB∆*sidA*.

**Deletion and complementation of the sidA gene**

The upstream and downstream fragments of the *sidA* gene (ATEG_06879) were amplified from gDNA of *A. terreus* SBUG844 using oligonucleotides P98/99 (764 bp) and P100/101 (939 bp) and fused by *in vitro* recombination with the ptrA resistance cassette (1950 bp) from ptrA-pJET1 (Fleck and Brock, 2010) into the KpnI-restricted pUC19 vector using the InFusion HD cloning kit (Clontech laboratories) resulting in *sidA* up:_sidA_ _ble_ _sidAdn-pUC19. For transformation of the *terA* deletion mutant, the ptrA cassette was excised by NotI restriction and replaced by the *ble* cassette. The deletion cassettes was excised by KpnI restriction and used for transformation of SBUG844∆akuB∆*sidA*.
Supporting Figures

Figure S1. Dependency of terA expression on the available glucose concentration. β-galactosidase activity was determined from the reporter strain PterA: lacZ after 42 h of growth in 0.4% potato broth (PB) with addition of 0% to 2% of glucose. Commercial PDB (from Sigma) was used as positive control.

Figure S2. HPLC analysis of extracts from A. terreus strains after cultivation in fruit juices and from infected nectarines and apples. (A-D) HPLC analyses of culture extracts from fruit juices after inoculation with SBUG844_PterA: lacZ (blue line) and mock-inoculated juices that served as negative controls (green line). Culture supernatants were extracted 48 h post inoculation. (A) carrot juice, (B) banana juice, (C) apple juice and (D) peach juice. 1 = terrein. (E-F) HPLC analyses of fruit extracts after infection with A. terreus wild type SBUG844_AkuB (blue line) and terrein biosynthesis gene cluster mutants ΔterA (red) and ΔterR (violet). Mock-infected fruits served as negative control (green line). (E) nectarine, (F) apple. 1 = terrein.
Figure S3. β-galactosidase activity of PterA: lacZ grown in banana juice without (control) or with different supplantments. + Fe = 30 µM FeCl₃; + N = 70 mM NH₄Cl; + Met = 10 mM L-methionine. Tests were performed in biological triplicates that were analysed in technical triplicates. Promoter activity of terA is reduced by nitrogen supplementation and is completely repressed in presence of nitrogen and iron in the medium. The addition of methionine partially overwrites this repression. Statistical significance was calculated in comparison to the control condition without supplementation using the Student’s paired t-test with a two-tailed distribution: p values: ** p < 0.01, *** p < 0.001.

Figure S4. Analysis of colony formation and growth phenotypes of nitrogen regulator mutants in presence of different nitrogen sources. As basal medium AMM-G100 without nitrogen was used, which was supplemented with 70 mM inorganic nitrogen (NaNO₃ or NH₄Cl), 50 mM of standard amino acids, ornithine (Orn), citrulline (Cit), urea, or 1% casamino acids (CA). Additionally, the AMM-CA1% (without glucose) and the complex media yeast extract-peptone-dextrose (YPD), potato dextrose agar (PDA), and malt extract agar (MA) were used. Pictures were generally taken after 96 h of incubation at 37°C. Asterisks denote different incubation times: * = 72 h; ** = 48 h. Note the severely reduced colony formation of the Δ areA strain on virtually all single amino acids except for arginine, glutamine and histidine. Additionally, note the loss of conidia colouration in the Δ atfA strain.
Figure S5. Terrein quantification from *A. terreus* wild type, nitrogen regulator mutants and complemented strains cultivated in the presence of nitrogen starvation. Analyses were performed from biological triplicates measured in technical duplicates. Mycelia from pre-cultures of strains SBUG844ΔakuB (ΔakuB), SBUG844ΔakuBΔareA (ΔareA), SBUG844ΔakuBΔareAΔareA (ΔareA²), SBUG844ΔakuBΔatfA (ΔatfA), SBUG844ΔakuBΔatfAΔareA (ΔareAΔatfA), and SBUG844ΔakuBΔareAΔatfA (ΔareA²ΔatfA) cultivated in AMM-G100Gln50 media were washed and transferred to fresh AMM-(N)G100 without a nitrogen source. Terrein was quantified from culture supernatants after 12, 24 and 48 h of cultivation.

Figure S6. Biochemical characterisation of the recombinant AreA DNA-binding domain and *in vitro* binding to HGATAR motifs identified in the *terA* and *terR* promoters of the *A. terreus* terrein biosynthesis gene cluster. (A) Amino acid sequence alignment of the AreA DNA-binding domains from *A. nidulans* and *A. terreus*. Underlined amino acids denote the 65-residue peptide used for solving the solution structure of the AreA:DNA complex (Starich et al., 1998). Cysteine residues of the Cys²-Cys² zinc finger module are highlighted in yellow. (B) SDS-PAGE analysis of purified AreA663-797. (C) In solution oligomeric state analysis of AreA663-797 as determined via size exclusion chromatography and multiangle static light scattering. The light scattering signal (LS) is shown overlaid with the calculated molar mass (Mw) across the elution profile as monitored by the absorbance at 280 nm (UV) and changes of the refractive index (dRI). (D) Schematic presentation of gene loci in the terrein gene biosynthesis cluster. Putative GATA AreA binding sites are annotated with BS1 - 5. (E-J) Real-time *in vitro* SPR interaction analysis of AreA663-797 with DNA containing the identified GATA motifs from *terA* and *terR* promoters of the terrein biosynthesis gene cluster. Sequences of DNA duplexes used for SPR analysis are shown on top of the sensorgrams. Numbers represent the GATA motif positions relative to the start of the open reading frame. HGATAR sites are highlighted in red. (K) Dissociation constants and stoichiometry of analyzed AreA663-797:DNA interactions analysed by SPR.
Figure S7. HPLC analyses from culture filtrates of SBUG844 wild type and two independent atfA overexpression mutants (AnPgdA::atfA; OE 1 and 2). Strains were cultivated under non-inducing conditions for the wild type. (A) AMM-G100 and (B) AMM-G100Gln50. 1 = terrein. Terrein and its side products are detected from the atfA overexpressing strains, but not from the wild type.

Figure S8. terA promoter activation from the reporter strain SBUG844_PterA::lacZ cultivated in presence of limited macroelements. All tests were performed in biological triplicates and β-galactosidase activity was analysed in technical duplicates. The standard control medium contains 100 mM glucose as carbon source (C), 70 mM NH₄Cl as nitrogen source (N), 10 mM K₂HPO₄ as phosphor source (P) and 2 mM MgSO₄ as sulphur source (S). In each set the concentration of one of the macroelements was varied, whereas that of the others was kept constant. Promoter activity was determined after 48 h of cultivation. Only nitrogen starvation induces promoter activity.
Figure S9. HPLC analyses from culture filtrate extracts grown in the presence and absence of an iron source. The wild-type ΔakuB and different nitrogen regulator mutants (ΔareA; ΔatfA; ΔareA/ΔatfA) and their complemented strains (areAC; atfAC) were cultivated in AMM100Gln50 with 20 µM FeCl₃ (+Fe) or without iron supplementation (-Fe). All strains show terrein production under iron starvation, indicating that terrein biosynthesis gene cluster induction under iron limitation is not dependent on the nitrogen sensing regulators AreA and AtfA.

Figure S10. qRT-PCR analysis of A. nidulans hapX expression in the A. terreus wild-type SBUG844ΔakuB, the hapX mutant SBUG844ΔakuBΔhapX and its complemented strain SBUG844ΔakuBΔhapX/AnhapX. All analyses were performed from biological triplicates and in technical duplicates. All strains were grown for 40 h in the presence (+Fe) or absence (-Fe) of 40 µM FeCl₃. Transcript levels of AnhapX were normalised against enoA gene from A. terreus by fold expression = 2^(Cₜarget - Cₜ enoA). The heterologously expressed AnhapX gene behaves similar as the native hapX gene from A. terreus (compare with Fig. 7 from the main manuscript). Statistical significances were calculated by the Student’s paired t-test with a two-tailed distribution: Asterisks indicates p-values vs. 0 µM FeCl₃: *** p < 0.001.
Figure S11. Colony formation of A. terreus mutants in dependence of iron availability, ascorbic acid or terrein. (A) As basic media AMM-(N)G100Gln10(-Fe) agar plates were used that were supplemented either with different iron concentrations (100, 20, 2, or 0 µM FeCl₃), the iron chelator bathophenanthroline disulfonate (BPS; 100 µM), ascorbic acid (Asc, 1 mM) or terrein (10 mM). Plates were incubated for 4 days at 37°C, except for the ∆terA∆sidA mutant that was further incubated for up to 17 days. (B) Effect of purified coprogen on growth of A. terreus mutants and complemented strains under iron starvation. Conidia (1 × 10⁵) of A. terreus SBUG844ΔakuB, SBUG844ΔakuBΔterA, SBUG844ΔakuBΔsidA/sid², SBUG844ΔakuBΔterAsidA or SBUG844ΔakuBΔterAΔsidA were plated on AMM-G100Gln10 plates supplemented with (+Fe) or without 20 µM FeCl₃ (-Fe). To (-Fe) media filter disks soaked with either 20 µl methanol (MeOH, negative control) or 20 µl of 2.5 mg/ml coprogen (in methanol) were applied. Plates were incubated at 37°C for 72 h until photographed.
Figure S12. Antifungal activity of terrein and PDB medium from *A. terreus* wild-type cultivations. (A) AMM-G100Gln10 containing no iron addition (-Fe) or 2 mM FeCl$_3$ were supplemented with 0, 1, 10 or 20 mM terrein and inoculated with conidia of *A. fumigatus* wild type ATCC46645. Plates were incubated for 84 h at 37°C until photographed. Terrein inhibits growth of *A. fumigatus* independent from the available iron concentration. (B) Analysis of the effect of *A. terreus* inoculated PDB culture broth on growth of *A. terreus*, *A. fumigatus* and *Fusarium graminearum*. The basal medium for *A. terreus* and *A. fumigatus* was AMM-G50 medium with nitrate as nitrogen source, whereas the medium for *F. graminearum* was additionally supplemented with 0.2% potato broth (AMM-G50 + 0.2%PB). All plates were supplemented either with 150 µl of PDB ethyl acetate extract from a mock-inoculated culture (PDB extract) or inoculated with *A. terreus* SBUG844 and cultivated for 4 days at 30°C (PDB metabolite extract). Pictures were taken after 4 days of incubation at 30°C.
Figure S13. Southern Blot analyses for A. terreus strains generated in this study. Genomic DNA was isolated and restricted with the respective restriction enzymes indicated in each panel. Digoxigenin-labelled probes were used in all experiments and were amplified by the oligonucleotide couples as indicated. Signals were detected by the CDP-star chemiluminescent substrate. Expected fragment sizes for are also shown.

(A) General scheme for generation of gene deletions using the pyrithiamine (ptrA) resistance cassette (∆GOI) and for complementation of deletion mutants using the phleomycin (ble) resistance cassette (GOI<sup>C</sup>) via homologous recombination.

(B) Generation of the β-galactosidase reporter strain SBUG844_PterA: lacZ in A. terreus SBUG844 wild type.

(C) Overexpression of atfA in SBUG844 under control of the A. nidulans
The native atfA locus (signal at 7008 bp) is still intact in strains that integrated the overexpression construct. Strains with ectopic single copy integration of the construct used for downstream experiments are denoted by "X". (D) Deletion of the opcA locus (ATEG_03131) in SBUG844\(\Delta\)akuB. (E) Deletion of the rhbA locus (ATEG_09480) in SBUG844\(\Delta\)akuB. (F) Deletion of the areA locus (ATEG_07264) in SBUG844\(\Delta\)akuB and complementation with FGSC A1156 areA. (G) PCR and PstI digest of PCR products from the areA upstream fragments amplified from genomic DNA from FGSC A1156, SBUG844 and SBUG844\(\Delta\)areA. The complemented strain shows the identical PstI restriction pattern as the amplified product from FGSC A1156, whereas no PstI restriction site is present in the SBUG844 product, confirming the complementation of the \(\Delta\)areA strain by the areA gene from A1156. (H) Deletion of the atfA locus (ATEG_04664) in SBUG844\(\Delta\)akuB and complementation with SBUG844 atfA. (I) Deletion of the atfA locus (ATEG_04664) in the areA negative background of SBUG844\(\Delta\)akuB\(\Delta\)areA (\(\Delta\)) and replacement of atfA in SBUG844\(\Delta\)akuB\(\Delta\)areA with the terR overexpression construct under control of the A. nidulans gpdA promoter (\(\Delta\):AnP\(gpdA\):terR). (J) Partial deletion of the hapX locus (ATEG_08014) in SBUG844\(\Delta\)akuB and complementation with the A. nidulans FGSC A4 hapX (AN08251). (K) Deletion of the sreA locus (ATEG_07714) in SBUG844\(\Delta\)akuB and complementation with SBUG844 sreA. (L) Deletion of the sidA locus (ATEG_06879) in SBUG844\(\Delta\)akuB and complementation with SBUG844 sidA. (M) Deletion of sidA (ATEG_06879) in the terA negative background of SBUG844\(\Delta\)akuB\(\Delta\)terA.
Figure S14. $^1$H NMR (500 MHz, MeOD; upper panel) and $^{13}$C NMR (150 MHz, MeOD; lower panel) of compound 4, 2-((E)-prop-1-en-1-yl)maleic acid.$^1$H NMR (500 MHz, MeOD): $\delta$ 6.22 (1H, d, $J=15.8$ Hz), 6.15 (1H, dq, $J=15.8$ Hz, $J=6.5$ Hz, 5.73 (1H, s), 1.85 ppm (3H, d, $J=6.5$ Hz); $^{13}$C NMR (600 MHz, MeOH): $\delta$ 172.6, 169.5, 151.7, 136.4, 130.2, 118.5, 18.8 ppm; HRMS: (ESI$^+$): $m/z$ calculated for C$_7$H$_9$O$_4$: 157.0495, found 157.0495 [M+H]$^+$. 
Figure S15. HSQC NMR (600 MHz, MeOD; upper panel) and HMBC NMR (600 MHz, MeOD; lower panel) of compound 4, 2-((E)-prop-1-en-1-yl)maleic acid.
### Supporting Tables

**Table S1. Genotypes of strains used in the study.**

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**Table S2. List of oligonucleotides used in the study.**

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P82 ptrA_hapXup2_r GTAAAGCTTCTCAGGTAAGGC
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P87 ble_hapXdn_f CCCTCACTCGCGGCCGCAGATAGCTCTGGCAGAATG
P88 ble_hapXup_f CCCTCACTCGCGGCCGCAGATAGCTCTGGCAGAATG
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43
Supporting Data

Analytical data of coprogen

\[
\text{H NMR (500 MHz, DMSO-d}_6\text{): } \delta = 9.84 \text{ (s, b, 3H, NOH)}, 8.27 \text{ (d, 1H, } J = 7.3 \text{ Hz, AcNH}), 8.12 \text{ (s, 2H, NH)}, 6.21 \text{ (s, 3H, H-4, H-16, H-26)}, 4.55 \text{ (s, b, 2H, OH)}, 4.15 \text{ (m, 3H, H-19, H-21)}, 3.80 \text{ (m, 2H, H-9, H-11)}, 3.52 \text{ (t, 4H, } J = 6.7 \text{ Hz}, \text{ H-1, H-29)}, 3.49 \text{ (m, 6H, H-6, H-14, H-24)}, 2.38 \text{ (t, 2H, } J = 6.6 \text{ Hz, H-18)}, 2.22 \text{ (t, 4H, } J = 6.6 \text{ Hz, H-2, H-28)}, 2.01 \text{ (s, 9H, H-3', H-17', H-27')}, 1.83 \text{ (s, 3H, H-31)} 1.73-1.47 \text{ (m, 12H, H-7, H-8, H-12, H-13, H-22, H-23)}, \text{ ppm.} \]

\[
\text{C NMR (125 MHz, DMSO-d}_6\text{): } \delta = 172.1 \text{ (C-20), 169.6 (C-30), 167.9 (C-10, C-10'), 166.6 and 166.2 (C-5, C-15, C-25), 150.9 (C3, C-27), 148.6 (C-17), 117.2 (C-16), 116.3 (C-4, C-26), 62.3 (C-19), 59.2 (C-1, C-29), 53.8 and 53.7 (C-9, C-11), 52.0 (C-21), 46.8 and 46.4 (C-6, C-14, C-24), 43.8 (C-2, C-28), 39.0 (C-18), 30.3 (C-23), 28.0 (C-22), 23.1 (C-7, C-13), 22.1 (C-31), 22.1 (C-8, C-12), 18.2 (C-3’, C-27’), 18.0 (C-17’) ppm. MS (ESI\textsuperscript{+}): m/z 769 [M+H\textsuperscript{+}] (100%). HRMS calcd. for C\textsubscript{35}H\textsubscript{57}N\textsubscript{6}O\textsubscript{13}[M+H\textsuperscript{+}]: 769.3978, found 769.3975.
\]

Analytical data of ferrichrysin

\[
\text{MS (ESI): m/z 746 [M-H] (100%). HRMS calcd. for C}_{29}\text{H}_{48}\text{N}_{9}\text{O}_{14}[M-H]^{+}: 746.3342, found 746.3326.}
\]

Supporting references


5. Diskussion

In der vorliegenden Arbeit wurden die kryptischen Sekundärmetabolit-Gencluster für Isoflavipucin/Dihydroisoflavipucin (Gencluster um das PKS-NRPS-Hybrid ATEG_00325) und Terrein (Gencluster um die NR-PKS ATEG_00145, terA) aus A. terreus identifiziert, charakterisiert und die Cluster-induzierenden Bedingungen im Detail untersucht. Die bemerkenswerte Promotoraktivität des Terrein-Clusters und die äußerstwähnlich hohen Terreinproduktionsraten konnten zur Entwicklung eines heterologen Expressionssystems genutzt werden, das auf den regulatorischen Elementen dieses Clusters aufbaut. Die Analysen dieser Arbeit zeigten, dass A. terreus durch von ihm produzierte SM exzellennt an kompetitive Bedingungen im Boden, auf Früchten und insbesondere der Rhizosphäre angepasst ist. Die Hauptpunkte, die zu dieser Schlussfolgerung führen, sind nachfolgend diskutiert.

5.1 Das Isoflavipucin/Dihydroisoflavipucin-Biosynthese-Gencluster

In dieser Arbeit konnte dem einzigen vollständigen PKS-NRPS-Hybrid aus A. terreus das Metabolitenpaar Isoflavipucin/Dihydroisoflavipucin eindeutig zugeordnet werden. Es konnte nicht nur ein putativer Biosyntheseweg formuliert werden, sondern auch gezeigt werden, dass die Metabolite mit Frucht- und Wurzelfäule-Toxinen verwandt sind. Diese könnten den Pilz bei der Gewinnung von Nährstoffen aus den Pflanzen unterstützen, was nachfolgend ebenfalls diskutiert werden soll.

5.1.1 Die Regulation des Dihydroisoflavipucin-Genclusters


Der TF ATEP_00326 induziert die Expression von ATEG_00325, ATEG_00331, ATEG_00329 und ATEG_00330, die für das PKS-NRPS-Hybrid, einen MFS-Exporter sowie zwei kleinen ORFs mit unbekannten Funktionen codieren. Die Regulation von Genclustern durch cluster-codierte TF wurde erstmals für die Aflatoxin-Biosynthese gezeigt, in dem der C6-TF AfIR die meisten der 21 Biosynthese-


Obwohl die Glucose-Repression die dominant-negative Wirkung auf die Clusteraktivität zeigt, kann die Promotoraktivität von ATEG_00325 unter aktivierenden Bedingungen durch ein alkalisches Milieu verstärkt werden, was eine Beteiligung des pH-Regulators PacC vermuten lässt. In A. nidulans wird PacC unter sauren Kultivierungsbedingungen im Endosom durch einen PalA/Vps32-Proteinkomplex inaktiv gehalten. Unter alkalischen Bedingungen wird eine Cystein-Protease, vermutlich PacB, im Endosom aktiv, die den reprimierend wirkenden C-Terminus von PacC entfernt (Hervás-Aguilar et al., 2007). Die prozessierte Form PacC^{27} wird anschließend weiter zu PacC^{27} verkürzt, in das Cytosol entlassen und in den Zellkern transportiert (Peñalvo et al., 2008). PacC^{27}


Zusammengefasst kann der Isoflavipucin-Gencluster durch Überproduktion seines spezifischen TF induziert werden, aber nur wenn nicht-reprimierende Bedingungen vorliegen. Dieser Umstand sollte auch bei der Induktion von anderen stillen Genclustern Beachtung finden.


5.1.2 Die Funktion von Isoflavipucin/Dihydroisoflavipucin

Isoflavipucin und Dihydroisoflavipucin sind die Produkte des PKS-NRPS-Genclusters um Locus ATEG_00325 in A. terreus. Bereits in den sechziger Jahren konnten Flavipucin und Isoflavipucin aus Kulturen von Aspergillus flavipes extrahiert werden (Casinovi et al., 1968). Dihydroisoflavipucin konnte bisher nur durch einen semisynthetischen Ansatz durch 15-minütige Pyrolyse von Flavipucin...

5.1.3 Die Biosynthese von Isoflavipucin/Dihydroisoflavipucin

5. Diskussion

nicht-reduzierten Polyketiden lässt auf nicht-funktionale reduktive Domänen schließen und wurde bisher nur für das PKS-NRPS-Hybrid CpaS in der α-Cyclopiazoninsäure-Biosynthese (Liu und Walsh, 2009) beobachtet. Interessanterweise haben ATEG_00325 und CpaS die Bildung kleiner, unreduzierter Ketide (Tri- bzw. Diketid) im PKS-Teil gemein. Die meisten bekannten PKS-NRPS-Hybride synthetisieren dagegen höher-reduzierte Polyketide von größerer Kettenlänge wie Tetraketide (Xyrolin; Phonghanpot et al., 2012), Hexaketide (Desmethylbassianin; Heneghan et al., 2011), Heptaketide (Fusarin C; Song et al., 2004) oder Octaketide (Equisetin; Sims et al., 2005). Über Fütterungsversuche wurde in dieser Arbeit gezeigt, dass das Triketid anschließend auf Leucin im NRPS-Modul übertragen wird. Kleine Aminosäuren wie Leucin, Serin oder Homoserin sind als Polyketid-Akzeptoren in der fungalen PKS-NRPS-Biosynthese eher selten (Song et al., 2004; Sims et al., 2005); weitaus häufiger werden aromatische Aminosäuren wie Tyrosin (Eley et al., 2007, Bergmann et al., 2007; Heneghan et al., 2011), Phenylalanin (Maiya et al., 2007; Qiao et al., 2011) oder Tryptophan (Schümann et al., 2007; Liu und Walsh, 2009) verwendet. Die Abspaltung des Primärproduktes vom NRPS-Modul erfolgt in ATEG_00325 wahrscheinlich durch einen reduktiven Mechanismus, da sich in der Reduktionsdomäne sowohl eine katalytische Triade aus Ser-Tyr-Lys als auch eine gut konservierte NADPH-Bindestelle (GXXGXXG) für Kurzketten-Dehydrogenasen (SCD) nachweisen lassen. In einer anderen Studie wurde das NRPS-Modul von ATEG_00325 heterolog in E. coli exprimiert und bestätigte, dass (i) Leucin neben anderen kleineren aliphatischen Aminosäuren preferenziell von der Aktivierungsdomäne erkannt wird und dass (ii) die Produktabspaltung reduktiv erfolgt (Qiao et al., 2012). Durch Fütterungsstudien mit deuteriertem [C5-²H3]-Leucin wurde nachgewiesen, dass die primär gebildete Tetramäsäure oxidativ gespalten wird und sich anschließend zu Isoflavipucin umlagert, wobei das Stickstoffatom vom Kohlenstoffrückgrat der Aminosäure getrennt wird. Derartige Umlagersreaktionen sind bisher für fungale PKS-NRPS-Produkte nicht beschrieben. Die finale Reduktion von Isoflavipucin an C-8 zu Dihydroisoflavipucin könnte anschließend durch eine Ketoreduktase erfolgen, die nicht im Cluster codiert ist. Eine mögliche Ketoreduktase (ATEG_00316), findet sich im Abstand von neun Genen zum Cluster (ATEG_00316), wird allerdings unabhängig von TF ATEG_00326 exprimiert (Daten nicht gezeigt).

Die ungewöhnlichen Biosyntheseschritte der Isoflavipucinsynthase und die strikte Glucose-Repression des Clusters zeigen zum einen das breite Spektrum an Metaboliten, die durch Enzyme ähnlicher Domänenstruktur gebildet werden können und zum anderen die ausgeprägte Adaption von A. terreus an seine Umgebung.
5.2 Einfluss von Naphthopyron-Synthase-ähnlichen PKS auf die Konidien-Pigmentierung

Die ausgeprägt bräunliche Konidienpigmentierung von *A. terreus* erleichtert eine morphologische Unterscheidung zu anderen pathogenen *Aspergillus* spec. wie *A. fumigatus* und *A. niger* (Mirhendi et al., 2007). Die NR-PKS PksP in *A. fumigatus* und seine homologen PKS WA in *A. nidulans* und FwnA in *A. niger* sind für die Melanisierung der Konidien verantwortlich (Mayorga et al., 1992; Jahn et al., 1997; Langfelder et al., 1998; Jørgensen et al., 2011). In *A. fumigatus* stellt pksP eine wichtige Virulenzdeterminante dar, da das DHN-Melanin aus *A. fumigatus* die Ansäuerung der Phagolysosomenbildung in Makrophagen verhindert (Thywissen et al., 2011). In Zusammenarbeit mit Silvia Slesion wurde in dieser Arbeit gezeigt, dass *A. terreus* nicht in der Lage ist, die Phagolysosomen-Ansäuerung zu unterbinden. Im Gegensatz zu *A. fumigatus* bricht *A. terreus* nicht aus Makrophagen aus, sondern verbleibt im Phagolysosom, ohne einen Vitalitätverlust der Sporen zu zeigen (Slesion et al., 2012a). Die durch *A. terreus* ausgelöste Aspergillose sind im Gegensatz zu *A. fumigatus* häufiger disseminierend (Hara et al., 1989; Blum et al., 2013; Lewis et al., 2013). Da die Konidien von *A. terreus* in Makrophagen und anderen Immunzellen persistieren, könnten Makrophagen als zelluläres Shuttle im Wirt dienen und so die disseminierte Aspergillose etablieren.

5.3 Das stille Gencluster um ATEG_07500


5.4 Das Terrein-Biosynthese-Cluster um ATEG_00145


5.4.1 Die Biosynthese von Terrein

Das Gencluster um ATEG_00145 (terA) codiert für Proteine, die für die Terrein-Biosynthese verantwortlich sind. Das Gencluster umfasst insgesamt 11 Gene (ATEG_00136 – ATEG_00145; terA-J, terR), wobei durch die Analyse von Deletionsmutanten gezeigt wurde, dass nur sieben der Gene (terA-F und terR) essentiell für die Terrein-Biosynthese sind. Dabei stellt TerR einen C6-TF dar, der für die Genclusterinduktion essentiell ist.

Die NR-PKS TerA weist eine Naphthopyron-Synthase-ähnliche Domänenstruktur (SAT-KS-AT-ACP-ACP-TE) auf (Fujii et al., 2001). Da reduktive Domänen fehlen, sind aromatische Primärprodukte zu erwarten. Die heterologe Expression in A. niger zeigte, dass drei nicht-reduzierte Produkte
unterschiedlicher Kettenlänge gebildet werden: das Triketid 4-Hydroxy-6-methylyron (HMP), das Tetraketid Orsellinsäure (OA) und das Pentaketid 6,7-Dihydroxymellein (DHM) (Abb. 5). Während HMP und OA Primärprodukte von TerA darstellen, ist DHM kein direktes Produkt einer NR-PKS. Das zu erwartende Pentaketid 2,3-Dehydro-6-hydroxymellein wird wahrscheinlich unspezifisch durch Wirtsenzyme an Positionen C2 und C3 zu 6-Hydroxymellein (HM) reduziert und an C7 zu DHM hydroxyliert. Die unspezifische Hydroxylierung von HM zu DHM konnte in A. niger und A. terreus demonstriert werden. Modifikationen durch Wirtsenzyme sind möglich, da auch A. niger Sekundärmetabolit-Gencluster besitzt. Chiang et al. (2013) haben daher einen Expressionsstamm in A. nidulans entwickelt, in dem spezifisch alle größeren Gencluster (für Sterigmatocystin und Emericellamid) deletiert wurden. In der Tat führte die heterologe Expression von terA in diesem Stamm zur Bildung von HMP, OA und 2,3-Dehydro-6-hydroxymellein, aber nicht zu DHM.


5. Diskussion

an der Produktspezifität beteiligt ist. Dies könnte im Fall von TerA über die Produkt-Template-Domäne geschehen (Crawford et al., 2008).

In dieser Arbeit wurde durch Fütterungsstudien mit $^{13}$C-gelabeltem HM zur $\Delta terA$-Mutante bewiesen, dass HM – aber nicht DHM – das erste isolierbare Vorläufermolekül der Terrein-Biosynthese ist (Abb. 5). Hill et al. (1981) haben durch die Zufütterung von verschiedenen synthetischen Mellein-Derivaten zu Kulturen von A. terreus gezeigt, dass HM ein mögliches Vorläufermolekül darstellen könnte, was in dieser Arbeit durch den gewählten molekularbiologischen Ansatz bestätigt werden konnte.


TerA produziert daher drei Produkte unterschiedlicher Kettenlänge, wobei nur 2,3-Dehydro-6-hydroxymellein als Terreinvorläufer dient. Nach anschließender Reduktion zu HM wird der aromatische Ring vermutlich über oxidative Mechanismen geöffnet, die zur Ausbildung der Terreintypischen Cyclopentanon-Struktur führen.

Die PKS TerA produziert drei Polyketide unterschiedlicher Kettenlänge, von denen nur 2,3-Dehydro-6-hydroxymellein als Terreinvorläufer dient. Der Vorläufer wird zunächst durch TerB zu 6-Hydroxymellein reduziert. Nach der Eliminierung wird das Molekül vermutlich mehrfach hydroxyliert, um anschließend über eine Ringkontraktion unter Decarboxylierung das finale Produkt Terrein zu bilden. Die in Klammern angegebenen Strukturen stellen nicht isolierte, postulierte Zwischenstufen dar.
5.4.2 Induktion des Terrein-Clusters durch TerR

In dieser Arbeit wurde gezeigt, dass die Expression der Terrein-Biosynthese strikt von der Aktivierung des im Gencluster codierten C6-TF TerR abhängig ist. Viele SM-Gencluster von Aspergillen benötigen ein im Cluster codiertes Zn\textsubscript{2}Cys\textsubscript{6}-Zinkfinger-Protein zur Clusteraktivierung, wie beispielsweise ApdR (Bergmann et al., 2007), AfIR (Liu et al., 1998) oder GliZ (Bok et al., 2006), die an der Induktion der Aspyridon-7, Aflatoxin- oder Gliotoxinbiosynthese beteiligt sind. TerR ist ein Transkriptionsaktivator mit einem GAL4-ähnlichen Zn\textsubscript{2}Cys\textsubscript{6}-binuklearen Cluster zur DNA-Bindung. Wie bei anderen TF der gleichen Klasse ist die DNA-Bindedomäne (DBD) am N-Terminus lokalisiert und für die Erkennung der Targetsequenzen verantwortlich (Todd et al., 1998). Die meisten bekannten GAL4-Zinkfingerproteine binden als Homo- oder Heterodimere an ihre Zielsequenzen, wie der durch Galactose-induzierte Regulator Gal4p oder der Eisen-Regulator Hap1A von S. cerevisiae (Carey et al., 1989; King et al., 1999). Die Dimerisierung wird durch hydrophobe Wechselwirkung in einer N-terminal gelegenen Region mit parallelen coiled-coil-Helices unterstützt (Fitzgerald et al., 2006). Coiled-coil-Domänen finden sich in vielen GAL4-ähnlichen TF wie Gal4p (Carey et al., 1989; Marmorstein et al., 1992) Leu3p (Fitzgerald et al., 2006), Put3p (Swaminathan et al., 1997), Ppr1p (Marmorstein und Harrison, 1994), und Hap1p (King et al., 1999) von S. cerevisiae. TerR fehlt diese coiled-coil-Domäne am N-Terminus, weshalb auf Basis der Sequenzhomologie von einer monomereren Proteinstruktur ausgegangen werden kann.


TerR weist ähnliche Eigenschaften wie der Meiose-Regulator Ume9p aus S. cerevisiae (Mitchell, 1994) oder der Alkohol-induzierte Regulator AlcR aus A. nidulans auf (Panozzo et al., 1997), die ebenfalls nur Monomere ausbilden. NMR-Analysen bestätigten für Ume6p eine Bindung an 5’-CCGCCG-3’ Targetsequenzen (Anderson et al., 1995). Im Gegensatz zu TerR ist die DBD von Ume6p aber am C-Terminus positioniert und erkennt daher die Targetsequenz ohne Spacer-Nukleotide zwischen den CCG-Triplets. Ein weiteres Beispiel eines monomeren TF stellt AlcR dar, der in Abwesenheit von Glucose und in Gegenwart von Ethanol aktiviert wird (Mathieu et al., 2005). Dieser GAL4-ähnliche TF erkennt 5’CCGW-3’-Motive an seinen Targetpromotoren von alcA und aldA (Lenouvel et al., 1997). AlcR wechselwirkt mit der DNA an den Spitzen seiner Helices I und III (beide


5.4.3 Induktion des Terrein-Clusters durch Umweltfaktoren und globale Regulatoren


5.4.3.1 Induktion unter Stickstoffmangel

interagiert dort mit seinem Co-Repressor NmrA (Andrianopoulos et al., 1998). Unter Stickstoffmangel wird AreA in den Zellkern transportiert (Todd et al., 2005), bindet dort an die 5′-HGATAR-3′-Sequenzen an seinen Zielpromotoren (Ravagnani et al., 1997) und aktiviert so deren Transkription, wahrscheinlich über Chromatin-Neuorganisation/Remodellierung (Muro-Pastor et al., 1999). In A. nidulans finden sich areA-Bindestellen beispielsweise in Genen, die für die Nitrat-Reduktase (NiaD) und Nitrit-Reduktase (NiiD) codieren (Chang et al., 2000), die in Abwesenheit von bevorzugten Stickstoffquellen heraufgereguliert werden (Wong et al., 2008). AreA hat auch entscheidenden Einfluss auf den Sekundärmetabolismus: Im Afloatoxinsynthese-Cluster von A. flavus bindet AreA an Promotorregionen der Transkriptionsregulatoren AfIR und AfIJ (Chang et al., 2000). Umgekehrt zum Nitrat-Regulon, scheint AreA hier die transkriptionelle Aktivierung der Regulatorgene zu unterbinden und verhindert so die Afloatoxinsynthese (Yu et al., 2012).


Die Signaltransduktion der Stickstofflimitation in filamentösen Pilzen ist noch nicht vollständig verstanden und ist speziesabhängig. In A. nidulans induziert Stickstoffmangel sowie oxidativer und osmotischer Stress die Phosphorylierung von Stresskinase-Kasakaden. Im Hog1/SakA-
5. Diskussion


5.4.3.2 Induktion über Methionin

gefunden (Rexen, 1976) und der Terrein-Cluster wird auf Kartoffel-Dextrose-Medium stark exprimiert. Es ist also möglich, dass Methionin einerseits als Induktor der Stressantwort der Wirtspflanzen dient und andererseits in dem Pathogen die Phytotoxinbiosynthese aktiviert.


Methionin ist darüber hinaus auch Vorläufermolekül der Siderophorbiosynthese von Graminaceae: Unter Eisenmangel – einer Bedingung, unter der auch der Terrein-Gencluster induziert wird – geben Wurzelzellen die Eisenchelatoren Nicotianamin und Mugineinsäure im Wurzelexudat ab (Roje, 2006; Mori und Nishzawa, 1987). Auch die Methioninbiosynthese wird unter Eisenlimitation induziert (Zamboni et al., 2012). Durch den großen Konzentrationsgradienten zwischen Cytoplasma der Wurzeln (9,5 mM) und dem Bodenwasser (0,03 mM), kommt es auch zur unfreiwilligen Freisetzung von Aminosäuren wie Methionin (Jones et al., 1994). Der Pool an Methionin in der Rhizosphäre ist entsprechend hoch (Rovira, 1956). Da A. terreus häufig aus der Rhizosphäre isoliert wird (Gao et al., 2013; Wijeratne et al., 2013; Rajalakshmi et al., 2014), könnte Methionin aus den Wurzelexudaten als Sensor für die Terrein-Biosynthese dienen.


### 5.4.3.3 Induktion unter Eisenmangel

Die Terrein-Biosynthese wird unabhängig vom AreA/AtfA-vermittelten Signaltransduktionsweg auch unter Eisenmangel induziert. Eine Regulation der Eisenhomöostase in Pilzen ist notwendig, da Eisen einerseits für viele Stoffwechselenzyme ein essentielles Spurenelement darstellt, aber andererseits ein Überschuss an Eisen toxische Effekte bewirkt (ROS-Bildung *via*...
5. Diskussion


In A. terreus ist die Expression des Terrein-Clusters nicht durch SreA beeinflusst, wohingegen die SB in ΔsreA induziert wird. Dies zeigt, dass SreA zwar ein Repressor der SB in A. terreus darstellt, aber keinen Repressor der Terrein-Biosynthese. Keiner der Promotoren des Terrein-Clusters weist sreA-Bindestellen auf (5'-ATCGGATAA-3'; Haas et al., 2014). Im Gegensatz dazu findet sich im Promotorbereich von terR eine mögliche Bindestelle des CCAAT-bindenden Komplexes (CBC) (5'-CCAAT-3'; Hortschansky et al., 2007) an Position -551 relativ zum Startcodon, die an der hapX-vermittelten Clusterinduktion beteiligt sein könnte.

Zusammengefasst, wird die Terrein-Biosynthese durch HapX unter Eisenmangel induziert und ist damit mit der Siderophorbiosynthese co-reguliert. Eine Rolle in der Eisen-Akquisition ist daher wahrscheinlich (siehe Kapitel 5.4.4.2).

5.4.4 Die biologischen Aktivitäten des Terreins

Terrein zeigt ein weites Spektrum biologischer Aktivitäten: Es wurden bereits antiinflammatorische, anti-proliferative und sogar pro-apoptotische Eigenschaften auf Säugerzellen nachgewiesen (Ghisalberti et al., 1990; Park et al., 2004; Lee et al., 2008; Kim et al., 2008; Lee et al., 2010; Liao et al. 2010; Chen et al., 2014; Mandai et al., 2014). Diese Aktivitäten scheinen aber im natürlichen Lebensraum von A. terreus nur eine untergeordnete Rolle zu spielen. Daher wurde in dieser Arbeit nach biologischen Aktivitäten gesucht, die unter den natürlichen Expressionsbedingungen des Terrein-Clusters in Betracht kommen und die Vorteile in den von A. terreus besetzten ökologischen Nischen bieten können.
5.4.4.1 Terrein als Phytotoxin


5.4.4.2 Terrein als Eisen-Reductans


Spezies ohne eigenes SB-System nutzen die Siderophile anderer Spezies (Xenosiderophore) oder sind – wie für Saccharomyces oder Candida spec. gezeigt – strikt auf ein hoch-affines, reductives Eisenaufnahmesystem (RIA) angewiesen (Dancis et al., 1992; Srivastava et al., 2014). Zusätzlich zur gesteigerten Expression der SB-Gene unter Eisenmangel zeigt A. terreus auch eine verstärkte Expression der Gene für das RIA, wie dies bereits auch für A. fumigatus und A. nidulans gezeigt wurde (Blatzer et al., 2011). Das RIA besteht aus einem membrangebundenen 3-Komponenten-System, bei dem Eisen(III)-Ionen zunächst extrazellulär zu Eisen(II)-Ionen reduziert werden (Eisen(III)-Reduktase) und anschließend unter erneuter Oxidation zu Eisen(III)-Ionen (Eisen(II)-Oxidase) in die Zelle transportiert werden (Eisen-Permease) (Schrettl et al., 2004; Blatzer et al., 2011). Das RIA stellt daher einen konservierten alternativen Eisenaufnahmeweg in Aspergillen dar. In dieser Arbeit wurde gezeigt, dass Terrein unter Eisenmangel produziert wird und Eisen(III)-Ionen zu Eisen(II)-Ionen reduzieren kann (Abb. 7). Somit kann Terrein oxidiertes, nicht zugängliches Eisen in der Umgebung verfügbar machen. Die Reduktionskraft ist aber im Vergleich zu Ascorbinsäure gering (Trabolsy et al., 2014), sodass die Terrein-vermittelte Eisenreduktion nicht mit dem co-regulierten Siderophorsystem
Abbildung 7. Schema über die Regulation der Eisenhomöostase in *A. terreus*.

Unter Eisenlimitation wird der Transkriptionsfaktor HapX verstärkt exprimiert und unterdrückt die Expression von SreA, dem globalen Repressor für die Expression der Gene der Siderophorbiosynthese (SB) und der Siderophortransporter (SIT). HapX induziert unter Eisenmangel die Expression der SB- und SIT-Gene. Coprogen und Ferrichrysin werden als Siderophore über eine NRPS (vermutlich SidD) assembliert und sekretiert. Die Siderophore komplexieren Eisen(III)-Ionen (Fe\(^{3+}\), rote Kreise) und werden über SITs in die Zelle transportiert, wodurch sie den intrazellulären Eisen-Pool auffüllen. Unter Eisen-Limitation wird ebenso die Expression der Gene für die reduktive Eisenaufnahme (RIA) induziert: Die membranständige Eisen(III)-Reduktase FreB reduziert extrazelluläre Eisen(III)-Ionen zu Eisen(II)-Ionen (Fe\(^{2+}\); grüne Kreise), die anschließend über eine membrangebundene Eisenpermease (FtrA) und -oxidase (FetC) von der Zelle aufgenommen und oxidiert werden. Unter Eisen-Limitation wird auch die Terrein-Biosynthese durch HapX stimuliert und führt so zur Biosynthese und Sekretion von Terrein. Das Polyketid kann extrazelluläre Eisen(III)-Ionen reduzieren und bedient damit das RIA-System von *A. terreus*. Terrein wird dabei zu Propenyl-Maleinsäure oxidiert.

5.4.4.3 Terrein als Antibiotikum

Terrein besitzt neben seiner seiner Wirkung als Phytotoxin und als Eisen-Reduktans auch antifungale Aktivität, zumindest gegen *A. fumigatus* und *F. graminearum*. Die notwendigen Konzentrationen sind allerdings vergleichsweise hoch (Wirkung erst ab 1 mM). Der erforderliche Titer an Terrein kann jedoch durch die starke Promotoraktivität von PterA leicht erreicht werden (2-8 mM Terrein nach 72 Stunden in Kartoffel-Extrakt-Medium). *A. terreus* selbst wird bei diesen Konzentrationen nicht im Wachstum beeinträchtigt, was auf einen Detoxifikationsmechanismus schließen lässt. Im Terrein-Gencluster wird ein Protein (Terl) mit großer Ähnlichkeit zum Phleomycin-Resistenz-Protein (Ble) codiert (Gatignol *et al.*, 1987), welches die Resistenz vermitteln könnte. Tatsächlich ist Terl nicht zur Terrein-Biosynthese notwendig, hat aber einen Einfluss auf die Terreinproduktionsrate (Zaehle *et al.*, 2014). Auf welche Weise Terrein antifungal wirkt, ist bisher unklar. Es ist möglich, dass einige Spezies die Aufnahme von Terrein nicht verhindern können und durch die anti-oxidativen Eigenschaften der zellulären Stoffwechsel negativ beeinflusst wird. Da *A. terreus* jedoch zwei cluster-spezifische MFS-Transporter besitzt, könnte darüber Terrein effizient aus der Zelle transportiert werden.

5.5 Ein Terrein-Gencluster-basiertes Expressionssystem in

*Aspergillus niger*

Viele filamentöse Pilze, darunter *A. niger*, *A. oryzae*, *Trichoderma reesei*, *Acremonium chrysogenum* und *P. chrysogenum*, werden zur rekombinannten Überproduktion von Proteinen verwendet (Sharma et al., 2009). Häufig werden die zu exprimierenden Gene (GOI, *gene of interest*) unter Kontrolle starker Promotoren des Primärmetabolismus gestellt wie beispielsweise der Glyceraldehyd-3-phosphat-Dehydrogenase-Promotor (Punt et al., 1991; Punt et al., 2002), der sich aber nur bedingt regulieren lässt. In dieser Arbeit wurde gezeigt, dass der Terreinsynthase-Promotor (PterA) in *A. terreus* sehr stark induziert wird und sogar die Promotorstärke von Actin um den Faktor 10 - 20 übersteigt, was mit dem großen Titer an Terrein übereinstimmt, der aus Kulturüberständen detektiert werden kann (>1 g/L). Andererseits ist aber die Expression von PterA strikt von TerR abhängig, was den Promotor regulierbar und dadurch für Anwendungen zur rekombinannten Proteinproduktion interessant macht.


der Fall, da TerR als spezifischer TF des Terrein-Gencusters wahrscheinlich ausschließlich an Zielsequenzen in PterA bindet und darüber hinaus keine weiteren Gene im heterologen Wirt reguliert. Das terR/PterA-Regulon zeigt also alle notwendigen Eigenschaften eines dichten, aber sehr stark induzierbaren Expressionssystems.


6. Ausblick


6. Ausblick


Insgesamt zeigte diese Arbeit, dass A. terreus sehr gut an die Kompetition mit anderen Organismen angepasst zu sein scheint. Um dies zu bestätigen, wären Kompetitionsuntersuchungen unter Laborbedingungen notwendig, um die aufgestellten Hypothesen zu verifizieren.
7. Zusammenfassung

Diese Arbeit beschäftigte sich mit der Identifikation der spezifischen Induktoren und Signalwege, die die Sekundärmetabolit-Produktion in dem Saprophyten *Aspergillus terreus* induzieren. Es wurde analysiert, wie Sekundärmetabolite das Wachstum und das Überleben des Pilzes in seinem natürlichen Habitat, der Rhizosphäre und abgestorbenenes organisches Material, unterstützen können.

Im Genom von *A. terreus* codiert nur ein einziges Gen für ein PKS-NRPS-Hybrid. Durch Untersuchungen der natürlichen induzierenden Bedingungen und dem darauf basierenden gezielten Austausch des Promoters wurde hier gezeigt, dass die Aktivierung des Clusters durch die Expression eines benachbarten Transkriptionsfaktors möglich ist. Die Bildung der Clusterprodukte, der Isoflavinucose, findet jedoch nur in Anwesenheit von Asparagin statt, wird durch alkalische pH-Werte stimuliert, ist aber in Gegenwart von Glucose strikt reprimiert. Die globale Katabolitpression verhindert somit eine Aktivierung durch den Cluster-spezifischen Transkriptionsfaktor, was bei *Genome Mining*-Experimenten berücksichtigt werden sollte.

In Aspergillen ist eine Naphthopyron-Synthase für die Konidien-Pigmentierung verantwortlich. Das Pigment verhindert die Phagolysosomen-Ansäuerung und erlaubt damit ein Ausbrechen aus Makrophagen. Da *A. terreus* diese konservierte PKS fehlt, säubern die Phagolysosomen an und verhindern die Auskeimung. Die Konidien persistieren jedoch in Phagolysosomen und könnten die Immunzellen als Shuttle zur Disseminierung des Pilzes benutzen. Die unterschiedliche Herkunft der Konidienpigmente wurde durch die Expression der Naphthopyron-Synthase WA aus *Aspergillus nidulans* in *A. terreus* bestätigt, was ein Ausbrechen aus Makrophagen ermöglichte.


8. Abstract

This work aimed at the identification of specific signals and signaling pathways inducing secondary metabolite production in *Aspergillus terreus*. The question is addressed how secondary metabolites can support competitive fitness of the fungus in its natural habitats, the rhizosphere and decaying organic matter.

Mining of the *A. terreus* genome revealed an orphan polyketide-nonribosomal-peptide synthetase gene cluster. Activation of the gene cluster by expression of a neighbouring transcriptional activator required a combined approach of systematic investigation of physiologically inducing conditions followed by an adapted promoter exchange. This led to the identification of the phytotoxic products (dihydro-)isoflavipucine. Phytotoxin formation relied on the presence of asparagine, was stimulated at alkaline pH, but strictly repressed in the presence of glucose. Therefore, global catabolite repression may overrule gene cluster specific transcription factors, which should be kept in mind when performing genome mining experiments.

*Aspergilli* generally utilise a naphthopyrone synthase for conidial pigment production. This pigment prevents phagolysosome acidification and enhances escape from macrophages by germination. *A. terreus* lacks this conserved PKS. Therefore, phagolysosomes acidify and prevent fungal escape. However, *A. terreus* conidia persist in phagolysosomes and may use immune cells as vehicle for dissemination. The different origin of the conidial pigment was confirmed by recombinant expression of the *Aspergillus nidulans* *wa* gene in *A. terreus*, which resulted in naphthopyrone production and enhanced macrophage escape rates.

The search for a naphthopyrone synthase-like PKS in *A. terreus* serendipitously led to the identification of the terrein gene cluster. In agreement to its expression during growth on fruits, phytotoxic activities such as induction of fruit surface lesions and inhibition of seed germination were observed. While nitrogen starvation is a major inducer of terrein production on fruits, methionine - a trigger of plant defence mechanisms – also activated the cluster. Both signals are mediated by the transcriptional activators AtfA and AreA. Unexpectedly, iron starvation also induced the cluster via the iron regulator HapX and coincides with the ability of terrein to reduce ferric to ferrous iron increasing the iron availability for *A. terreus*. All inducing signals mimic conditions in the rhizosphere and combined with its broad spectrum of biological activities, terrein provides an excellent example for adaptation of *A. terreus* to environmental competition in the soil.

Finally, the exceptionally strong promoter activity of the terrein synthase gene led to the development of an inducible heterologous expression system that based on regulatory elements of the cluster. Expression of PKS genes and domain swapping experiments confirmed the suitability of the expression system especially for high-level metabolite production in heterologous hosts.
Literaturverzeichnis


Abkürzungsverzeichnis

Alle nicht in der Liste aufgeführten Abkürzungen entsprechen der IUPAC-Nomenklatur für chemische Summenformeln und der IUBMB-Nomenklatur für Objekte aus dem Bereich der Biochemie.

A
Abkürzungsverzeichnis

ACAS
Aktivierungsdomäne

ACE1
NR-PKS aus A. terreus

ACP
PKS-NRPS aus M. grisea

AcvA
Acylcarrierprotein

AfIR
(L-α-Aminoadipyl)-L-cysteinyl-D-Valin-Synthetase aus A. nidulans

AIDS
C6-Transkriptionsregulator der Aflatoxin-Biosynthese

Alb1
erworbenes Immundefektsyndrom (Acquired Immune Deficiency Syndrome)

AlcA
Naphthopyron-Synthase aus A. fumigatus (auch PksP)

AlcR
Alkohol-Dehydrogenase

AmyB
Regulator des Ethanol-Katabolismus

AmyR
α-Amylase aus A. oryzae

ApdR
Regulator des Amylose-Katabolismus

AptA
C6-Transkriptionsregulator der Aspyridon-Biosynthese

AreA
NR-PKS aus A. nidulans

AT
globaler Transkriptionsfaktor des Stickstoffmetabolismus in Aspergilli

ATA
Acytransferase

ATEG
Aspergillus terreus-Genlokus

AtfA
globaler bZIP-Transkriptionsfaktor bei osmotischem Stress in Aspergilli

bHLH
DNA-Bindedomäne mit basischer Helix-Schleife-Helix-Struktur

Ble
Phleomycin-Resistenz-Protein

bZIP
DNA-Bindedomäne mit basischer Leucin-Reiβverschluss-Struktur

C
Kondensationsdomäne

C6-TF
Transkriptionsfaktor mit Zn2+Cys6-Zinkfinger-DNA-Bindedomäne

CoA
Coenzym A

CpaS
PKS-NRPS der α-Cyclopiazoninsäure-Biosynthese

CreA
globaler Transkriptionsfaktor der Kohlenstoff-Katabolitpression in Aspergilli

D
Dieckmann-Kondensationsdomäne

DH
Dehydratase

DHM
6,7-Dihydroxyxymellein

DHN
1,8-Dihydroxynaphthalen

DMATS
Dimethylallyltryptophan synthasen

DNA
Deoxyribonukleinsäure

DnmA
putative DNA-Methyltransferase in A. nidulans

DNT
DNA-Methyltransferase

DnmT
putative DNA-Methyltransferase in A. terreus

DOPA
L-3,4-Dihydroxyphenylalanin
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<td>SPR</td>
<td>Oberflächenplasmonresonanz (<em>Surface plasmon resonance</em>)</td>
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<td>XylP</td>
<td>Endoxylanase aus <em>P. chrysogenum</em></td>
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Eigenständigkeitserklärung

Die geltende Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität ist mir bekannt.

Ich erkläre ehrenwörtlich, dass ich die vorliegende Dissertation selbst angefertigt habe. Es wurden keine Textabschnitte eines Dritten oder eigener Prüfungsarbeiten ohne Kennzeichnung übernommen.


Die Hilfe eines Promotionsberaters wurde nicht in Anspruch genommen und Dritte haben weder unmittelbar noch mittelbar geldwerte Leistungen von mir für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen.

Die Dissertationsschrift wurde nicht bereits zuvor als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht.

Die gleiche Arbeit, eine in wesentlichen Teilen ähnliche oder eine andere Abhandlung wurde noch nicht bei einer anderen Hochschule als Dissertation eingereicht.

Jena, d. 22.01.2015

______________________________

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Wissenschaftlicher Lebenslauf

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Liste der wissenschaftlichen Veröffentlichungen

Unterstrichen sind jeweils die Erst-Autoren der Veröffentlichungen.

Gressler M, Zaehle C, Scherlach K, Hertzec C, Brock, M.

Multifactorial Induction of an Orphan PKS-NRPS Gene Cluster in Aspergillus terreus

Slesiona S, Gressler M, Mihlan M, Zaehle C, Schaller M, Barz D, Hube B, Jacobsen ID, Brock M.

Persistence versus Escape: Aspergillus terreus and Aspergillus fumigatus Employ Different Strategies during Interactions with Macrophages

Zaehle C, Gressler M, Shelest E, Geib E, Hertweck C, Brock M.

Terrein Biosynthesis in Aspergillus terreus and Its Impact on Phytotoxicity

Gressler M, Hortschansky P, Geib E, Brock M.

A new high-performance heterologous fungal expression system based on regulatory elements from the Aspergillus terreus terrein gene cluster

Gressler M, Meyer F, Heine D, Hortschansky P, Hertweck C, Brock M.

Global transcription factors mediate phytotoxin production in Aspergillus terreus by independent sensing of environmental signals eingereicht (in Begutachtung bei „PLOS Genetics“)
Liste der wissenschaftlichen Veröffentlichungen auf Fachtagungen

Teile dieser Arbeit wurden auf wissenschaftlichen Fachtagungen präsentiert.

Poster

8\textsuperscript{th} Asperfest in Asilomar, CA, USA (14. – 15. März 2011)

\textbf{Effect of primary metabolism on secondary metabolite production in Aspergillus terreus}

Markus Gressler, Christoph Zaehle, Kirstin Scherlach, Christian Hertweck und Matthias Brock

26\textsuperscript{th} Fungal Genetics Conference in Asilomar, CA, USA (15. – 20. März 2011)

\textbf{Effect of primary metabolism on secondary metabolite production in Aspergillus terreus}

Markus Gressler, Christoph Zaehle, Kirstin Scherlach, Christian Hertweck und Matthias Brock

Jahrestagungen der VAAM in Tübingen, Deutschland (18. – 21. März 2012)

\textbf{A naphthopyrone synthase-like PKS from Aspergillus terreus produces phytotoxins}

Markus Gressler, Christoph Zaehle, Sabrina Boldt, Elena Geib, Christian Hertweck und Matthias Brock

11\textsuperscript{th} European Conference on Fungal Genetics in Marburg, Deutschland (30. März – 02. April 2012)

\textbf{A naphthopyrone synthase-like PKS from Aspergillus terreus produces phytotoxins}

Markus Gressler, Christoph Zaehle, Sabrina Boldt, Elena Geib, Christian Hertweck und Matthias Brock

(Posterpreis)
Anhang


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Tabelle 1. Übersicht der Sekundärmetabolit-Gencluster in *A. terreus* (fortgeführt)

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Danksagung

An dieser Stelle möchte ich mich bei all denjenigen bedanken, die mir immer den Rücken gestärkt, mich begleitet und motiviert haben.

An erster Stelle möchte ich mich bei meinem Doktorvater Matthias Brock bedanken für das Ermöglichen dieser Arbeit am Hans-Knöll-Institut, für die hervorragende Betreuung manchmal bis in die tiefste Nacht hinein und dafür, dass seine Bürotür für wissenschaftliche Dispute aber auch komplizierte Fragestellungen immer offen stand.

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