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Aspergillus nidulans Synthesize Insect Juvenile Hormones upon Expression of a Heterologous Regulatory Protein and in Response to Grazing by Drosophila melanogaster Larvae

Morten Thrane Nielsen1✉a, Marie Louise Klejstrup1✉, Marko Rohlfis2, Diana Chinyere Anyaogu1, Jakob Blæsbjerg Nielsen1, Charlotte Held Gotfredsen3, Mikael Rørdam Andersen1, Bjarne Gram Hansen1✉b, Uffe Hasbro Mortensen1✉, Thomas Ostenfeld Larsen1✉

1 Department of Systems Biology, Technical University of Denmark, Kgs Lyngby, Denmark, 2 J.F. Blumenbach Institute of Zoology and Anthropology, Georg-August-University Göttingen, Göttingen, Germany, 3 Department of Chemistry, Technical University of Denmark, Kgs Lyngby, Denmark

Abstract

Secondary metabolites are known to serve a wide range of specialized functions including communication, developmental control and defense. Genome sequencing of several fungal model species revealed that the majority of predicted secondary metabolite related genes are silent in laboratory strains, indicating that fungal secondary metabolites remain an underexplored resource of bioactive molecules. In this study, we combine heterologous expression of regulatory proteins in Aspergillus nidulans with systematic variation of growth conditions and observe induced synthesis of insect juvenile hormone-III and methyl farnesoate. Both compounds are sesquiterpenes belonging to the juvenile hormone class. Juvenile hormones regulate developmental and metabolic processes in insects and crustaceans, but have not previously been reported as fungal metabolites. We found that feeding by Drosophila melanogaster larvae induced synthesis of juvenile hormone in A. nidulans indicating a possible role of juvenile hormone biosynthesis in affecting fungal-insect antagonisms.

Introduction

Filamentous fungi are capable of synthesizing a wide range of bioactive molecules important for growth and survival in complex and competitive ecological niches [1–3]. A substantial number of these metabolites have been found to have beneficial as well as detrimental impact upon human health. Notable examples of both categories include the pharmaceutically important lovastatin and penicillin [4]; and the mycotoxins fumonisin and aflatoxin that cause health hazards and economical losses when they are present in infected crops [5]. With the release of the full genome sequences of several filamentous fungi it has become apparent that the number of predicted secondary metabolite synthases by far exceeds the number of known metabolites [6,7]. These observations suggest that specific environmental stimuli are required for induction of the majority of secondary metabolites [8]. Despite attempts to identify or mimic these stimuli in order to unravel the secondary metabolism of the model organism Aspergillus nidulans, the product of the majority of predicted syntheses are still not known [9,10]. Genetic approaches have been somewhat successful by manipulating histone methylation [11] or controlled expression of regulatory proteins [12]. As biosynthetic pathways towards secondary metabolites tend to be clustered in the genome [6,7] regulatory proteins likely to be involved in secondary metabolism may be identified by genomic co-localization. However, the number of successful applications of this approach is limited, possibly because far from all predicted gene clusters contain regulatory proteins. We decided to investigate whether induction of secondary metabolites could be achieved through heterologous expression of regulatory genes from other filamentous fungi using the expression of A. niger proteins in A. nidulans as a test case. A selection of putative pathway specific regulators was tested for this purpose by expressing the corresponding genes individually from a defined locus using a constitutive promoter [13]. This genetic approach was combined with a screen of several complex media recently demonstrated to influence A. nidulans
secondary metabolism [14]. This combinatorial approach resulted in the identification of one regulatory protein that strongly induced metabolites not previously reported from *A. nidulans*. Among the induced metabolites were the sesquiterpene hormones methyl farnesoate and insect juvenile hormone-III. Juvenile hormones are required in exact concentrations for correct development of insects and crustaceans [15–17] and therefore hold a strong potential as insecticides [18,19]. To the best of our knowledge, this is the first observation of a fungus with the capacity of synthesizing juvenile hormones in *A. nidulans* was challenged by grazing insects, synthesis of juvenile hormones was induced suggesting that juvenile hormones are part of the fungal defense against invertebrates.

**Results and Discussion**

**Procedure for selection of candidate genes**

Selection of candidate regulatory proteins associated with secondary metabolism was based on genomic co-localization of gene clusters. We utilized a collection of previously published microarray experiments from *A. niger* grown under diverse conditions [20–22] to identify regulatory genes associated with predicted secondary metabolism gene clusters using a recently described co-expression based algorithm [23]. Seven candidate genes associated with predicted gene clusters containing either polyketide synthases or non-ribosomal peptide synthases, were identified (Table 1). All seven putative transcription factors belong to the bincular zinc finger class of proteins, a class often associated with secondary metabolism in fungi [24]. BLAST analysis [25] using the predicted protein sequences against the annotated *A. nidulans* genome (Aspergillus Comparative Database, BROAD institute of Harvard and MIT) revealed that only one candidate (*fgel1* pg C_4000037) had a potential ortholog (ANID_06396, 62% amino acid identity, Table 1). Genes encoding all seven putative regulators were expressed individually in *E. coli* under physiological conditions [20–22] to identify regulatory genes associated with secondary metabolism resulting in increased accumulation of several metabolites not previously reported to be produced by *A. nidulans* (Figure 1A). Hence, we renamed *est_fgel1_pg_C_150220* Secondary Metabolism associated Regulatory protein A (*smra*). The strain that constitutively transcribe *smra* was denoted NID477, see Table 2. Correct integration of *smra* into *Is1*, as well as presence of *smra* mRNA, was confirmed by Southern blot (Figure 2) and quantitative RT-PCR (Figure 3), respectively. Two induced metabolites displaying very similar UV-spectra were isolated from extracts of NID477 and identified by NMR analysis as the sesquiterpenes: methyl (2E,6E)-10,11-dihydroxy-3,7,11-trimethyl-2,6-dodecadienoate (1) [JH-diol] [26] and its formylated analogue (2). The formulation, however, was subsequently demonstrated to occur during the extraction procedure and 2 is therefore an artificial derivative of 1. The sesquiterpene 1 [JH-diol] represents the hydrated form of insect juvenile hormone-III [JH-III]. This observation prompted us to search for *JH-III* and related metabolites using targeted LC-HRMS analysis. Indeed, metabolites with accurate masses corresponding to *JH-III* and the related crustacean hormone methyl farnesoate (MF) [15] were strongly induced in NID477 compared to the reference, NID545 (Figure 1A). Final identification of these metabolites as *JH-III* and MF was established by comparison of retention time and mass spectra with an authentic standard (Figure 1B), or with a reference spectra (Xcalibur software package, Thermo Scientific), respectively. The discovery of *JH-III* and MF as metabolites of *A. nidulans* represents to our knowledge the first report of production of invertebrate juvenile hormones in fungi.

**Chemical analysis of mutant strains identifies juvenile hormones as metabolites of *A. nidulans***

The resulting mutant strains were grown on minimal glucose media as well as four complex media representing diverse physiological conditions. Metabolite profiles of mycelia extracts were analyzed with liquid chromatography-high resolution mass spectroscopy (LC-HRMS) as well as ultra-high pressure liquid chromatography diode array detection (UHPLC-DAD) and compared to a reference strain that constitutively transcribes the *E. coli β*-galactosidase gene (lacZ) from *Is1* (NID545). Of all combinations of candidate genes and growth conditions, only *est_fgel1_pg_C_150220* (annotation from Aspergillus Comparative Database, BROAD institute of Harvard and MIT) propagated under high salt conditions had an immediately appreciable impact on secondary metabolism resulting in increased accumulation of several metabolites not previously reported to be produced by *A. nidulans* (Figure 1A). Hence, we renamed *est_fgel1_pg_C_150220* Secondary Metabolism associated Regulatory protein A (*smra*).

| Strain # | Broad annotation | Transcript ID | Candidate *A. nidulans* homologues | Identity percentage |
|----------|------------------|---------------|-----------------------------------|--------------------|
| NID357  | *fgel1* pg C_4000037 | 38716 | ANID_06396, ANID_03269 | 62%, 27% |
| NID358  | e_gw1_4.316 | 178503 | ANID_07346 | 26% |
| NID360  | e_gw1_11.945 | 188323 | ANID_08894 | 25% |
| NID366  | gw1_10.247 | 123782 | None | – |
| NID367  | *fgel1* pg C_19000192 | 45823 | ANID_11683, ANID_07921 | 43%, 22% |
| NID476  | e_gw1_8.296 | 184613 | ANID_04485 | 30% |
| NID477  | est_fgel1_pg C_150220 | 54836 | None | – |

Candidate genes were selected based on co-localization with predicted gene clusters in *A. niger* containing either a polyketide synthase, a non-ribosomal peptide synthase or both. Transcript ID = Annotation from the DOE Joint Genome Institute (genome.jgi-psf.org), candidate *A. nidulans* homologues = Highest scoring potential homologs in *A. nidulans*, Identity percentage = amino acid identity percentage.

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*Biosynthesis of *JH-III*, JH-diol and MF in *A. nidulans***

Biosynthesis of juvenile hormones is well characterized in insects [27]. Since further elucidation of the potential role of juvenile hormones in fungal-insect antagonisms would benefit substantially from the generation of null mutants, we attempted several homology based strategies for identification of the biosynthetic pathway for juvenile hormones in *A. nidulans*. Initially, BLAST analysis was performed using previously characterized insect enzymes as input, however, no obvious candidates were identified.
We speculate that the long evolutionary distance between insects and *A. nidulans* may have obscured a common origin, but it cannot be excluded that an alternative biosynthetic mechanism has evolved in fungi. We tested whether the mixed PKS/NRPS gene cluster in which *smrA* is located (*A. niger* transcript ID: 192362, 128601, 191998, 44877, 44878, 44880 and 54837) is conserved in *A. nidulans* and could provide an alternative biosynthetic route, however, the cluster is not present in *A. nidulans* as evidenced by BLAST analysis of individual genes (data not shown). Moreover, SmrA does not have any homologs in *A. nidulans* (Table 1). Thus homology based methods seems to be challenging. We expect that microarray based analysis of the

**Figure 1. Induction of metabolites by SmrA.** A) UHPLC-QTOFMS extracted ion chromatogram of m/z 251 (MF, [M+H]⁺), 289 (JH-III, [M+Na]⁺), 307 (JH-diol, [M+H]⁺) and 335 (X2, [M+H]⁺) recorded in positive mode of extracts from the strain constitutively expressing *smrA* (top) and reference (middle) grown under high salt conditions. Chromatograms are normalized by intensity. Chemical structures of JH-diol, compound 2, JH-III and MF are embedded above the corresponding signal peaks. Bottom panel depicts extracted ion chromatogram of m/z 289 (JH-III, [M+Na]⁺) of an authentic JH-III standard (65% pure) purchased from Sigma Aldrich. Note that the standard contains several impurities. Panel B): Corresponding mass spectra of JH-diol, compound 2, JH-III and MF in the mutant strain constitutively expressing *smrA* as well as the authentic JH-III standard. Chemical structure of the corresponding molecule is embedded in each panel. doi:10.1371/journal.pone.0073369.g001
growth condition dependent synthesis of juvenile hormones in NID477 may serve as a more fruitful route for identification of the juvenile hormone synthesis pathway in *A. nidulans*.

**Biological function of Juvenile hormones in *A. nidulans***

Fungal secondary metabolites are known to play an important role in fungal-insect interactions [2,3]. Moreover, the role of juvenile hormones in regulating processes of insect metamorphosis, reproduction and metabolism are well described [16,17]. We therefore hypothesized that the biological function of juvenile hormones in *A. nidulans* is related to interaction with insects. It is known, that timing and dosage of insect exposure to juvenile hormones is crucial for correct development, with fatal consequences of both under- and overexposure [16]. Consequently, synthesis of JH and MF could be employed as a defense mechanism in *A. nidulans* and such a strategy has been demonstrated for the plant *Cyprus iria* [28]. We pursued two experimental lines of evidence in order to test our hypothesis; 1) analysis of the spatial distribution of JH and MF and 2) conducted confrontation experiments between *A.nidulans* and larvae of the saprophagous insect *Drosophila melanogaster*.

**Distribution of JH-III, JH-diol and MF**

The metabolite composition of growth media extracts and collected volatiles of NID477 and the reference, NID545, grown under juvenile hormone stimulating conditions was analyzed by LC-HRMS and gas chromatography mass spectroscopy (GC-MS), respectively. None of the three terpenes were detectable as extracellular metabolites in the growth media. JH-III and JH-diol were also undetectable among the volatiles whereas MF constituted a major metabolite in the volatile fraction of both strains (Figure 4). Taken together with the presence of JH-III and JH-diol in mycelia extracts (see above), we conclude that JH-III and JH-diol are maintained intracellularly in the mycelium. Therefore, insects will ingest juvenile hormones upon foraging on *A. nidulans* which may disturb the careful balance of juvenile hormone dosage.

*D. melanogaster* larvae induce JH-III synthesis upon grazing

*D. melanogaster* was chosen for the confrontation experiments since the versatile role of juvenile hormones in *D. melanogaster* development is well documented [29] and since patterns of interaction between *A. nidulans* and *D. melanogaster* larvae have been described previously [30]. The confrontation experiments were initially performed under the conditions where SmrA stimulated JH-III and MF synthesis. However, the high salt content in the media (5% NaCl) caused severe larval mortality even in mock free controls (data not shown). We therefore decided to perform the experiments under less stressful conditions (standard *Drosophila* medium, [30]). In this experiment, the fitness of grazing *D. melanogaster* larvae was not significantly different between NID545 and NID477 on two of three parameters evaluated (Figure 5). However, flies emerging from the NID477 treatment displayed a significant decreased dry weight, indicating a negative impact of NID477 on *D. melanogaster* fitness compared to NID545. We therefore performed a metabolite analysis of fungal extracts produced from the two strains in the presence or absence of larvae in order to correlate the observed effect with differences in the...
Table 2. Name and description of fungal strains used in this work.

| Strain # | Genotype | Description | Reference |
|----------|----------|-------------|-----------|
| NID74    | argBΔ, pygG89, veA1, nkuAΔ | Parental strain with permanent deletion of nkuA and argB to facilitate gene targeting | This study |
| NID545   | argBΔ, pygG89, veA1, nkuAΔ IS1::PgpdA-lacZ::argB | Reference strain with E.coli lacZ integrated in IS1. | This study |
| NID357   | argBΔ, pygG89, veA1, nkuAΔ, IS1::PgpdA::fge1_pg_C_4000037::argB | Constitutive expression of putative binuclear zinc finger transcription factor fge1_pg_C_4000037 integrated in IS1 | This study |
| NID358   | argBΔ, pygG89, veA1, nkuAΔ, IS1::PgpdA:e_gw1_4.316::argB | Constitutive expression of putative binuclear zinc finger transcription factor e_gw1_4.316 integrated in IS1 | This study |
| NID360   | argBΔ, pygG89, veA1, nkuAΔ, IS1::PgpdA::e_gw1_11.945::argB | Constitutive expression of putative binuclear zinc finger transcription factor e_gw1_11.945 integrated in IS1 | This study |
| NID366   | argBΔ, pygG89, veA1, nkuAΔ, IS1::PgpdA::gwar1_10.247::argB | Constitutive expression of putative binuclear zinc finger transcription factor gw1_10.247 integrated in IS1 | This study |
| NID367   | argBΔ, pygG89, veA1, nkuAΔ, IS1::PgpdA::fge1_pg_C_19000192::argB | Constitutive expression of putative binuclear zinc finger transcription factor fge1_pg_C_19000192 integrated in IS1 | This study |
| NID476   | argBΔ, pygG89, veA1, nkuAΔ, IS1::PgpdA::smrA::argB | Constitutive expression of smrA (est_fge1_pg_C_150220) integrated in IS1 | This study |

Figure 4. Excretion of MF by A. nidulans. Top panel: Total MS chromatogram of the collected volatiles from the smrA expressing strain and the reference. Bottom panel: Mass spectrum of the compound eluting at 26.19 minutes. The compound was identified as MF by comparison to the metabolite library of the Xcalibur software package (Thermo Scientific).

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metabolite profile. When NID545 and NID477 were grown on standard Drosophila medium most of the detectable secondary metabolites (austinol, dehydroaustinol, nidulanin A and sterigmatocystin) did not differ significantly between the two strains (Figure 6A). Importantly, JH-III and the JH-diol were detected in both strains, but was not significantly induced in NID477 on this medium (Figure 6B). These observations are in agreement with the results obtained in the initial screening of the NID77 strain, which revealed that the induction of secondary metabolites was highly condition dependent. Interestingly, comparison of the metabolite profiles obtained from NID77 and NID545 strains grown in the presence or in the absence of grazing D. melanogaster larvae demonstrated that the presence of the insects significantly increased the level of both JH-III and JH-diol irrespective of the strain background (p-values JH-III: 0.0288 and 0.00723 and JH-diol: 0.0006 and 0.02415 for NID477 and NID545, respectively, Figure 6C). Curiously, JH-III accumulated to higher levels in NID545 than in NID477 (p-value <0.025). Perhaps, this reflects that when the natural induction of JH-III takes place, the contribution from the presence of the heterologous transcription factor SmrA is detrimental to JH-III biosynthesis. A simple model could be that the natural A. nidulans transcription factor and SmrA bind in a competitive manner to the promoters of the genes involved in JH-III biosynthesis and that activation is less efficient when SmrA is present. We consider it likely that constitutive expression of the SmrA transcription factor has numerous other effects on A. nidulans that is not reflected in our metabolite analysis, and that collectively these effects cause the observed decrease in D. melanogaster fitness. However, the induction of juvenile hormones upon insect feeding, taken together with the well-established involvement of juvenile hormones in insect development and physiology, strongly suggest that JH-III do impact the relation between insects and A. nidulans.

Perspectives

The findings of this manuscript indicate that juvenile hormones represent previously overlooked compounds in chemical interactions between A. nidulans and insects. In addition, the ability of A. nidulans to synthesize juvenile hormones provides the potential for a bio-based source for juvenile hormone production in cell factories. Juvenile hormones are considered to be among the most potent and promising insecticides due to their high specificity and efficiency [18,19]. Moreover, as A. nidulans releases the juvenile hormone MF to the environment, downstream purification of MF would be simple, as MF could be collected from the volatiles as described previously for other sesquiterpenes [31]. Finally, the findings in this manuscript underline how manipulation of regulatory proteins, systematic variation of physical parameters as well as insect-fungus confrontation systems may be valuable tools for modifying fungal secondary metabolite profiles. The latter approach has the advantage of providing clues to biological function of metabolites. A similar approach simulating bacterial-fungal interactions has previously been successful in identifying novel metabolites in A. nidulans [32] indicating that this more biological approach may constitute a promising route for future studies.

Materials and Methods

Strains and media

Escherichia coli strain DH5α was used to propagate all plasmids. All A. niger genes were amplified from strain ATCC1015. The A. nidulans strain NID74 (argBΔ, veA1, pyrG89, nkuAΔ) was used as background strain for all transformations as it allows gene targeting with the argB marker due to a complete deletion of the A. nidulans argB-open reading frame. NID74 was generated from NID1 (argB2, veA1, pyrG89, nkuAΔ) using the fusion PCR technique essentially as described previously [33]. NID545 (argBΔ, pyrG89, veA1, nkuΔ, IS1::PgdA-lacZ-TtrpC::argB) was used as reference strain for metabolic analysis. Genotypes of all strains are summarized in Table 2. All A. nidulans strains were propagated on solid glucose minimal medium (MM) prepared as described by Cove [34], but with 1% glucose, 10 mM NaNO3 and 2% agar. MM was supplemented with 10 mM uridine (Uri), 10 mM uracil (Ura), where required. Complex media used for chemical analysis.

Figure 5. Influence on D. melanogaster larva-to-adult development. Panel A): Proportion of D. melanogaster larvae that reached the pupal stage as a function of fungal treatment (mold-free control, NID545 or NID477) and time. Panel B): Proportion of flies that emerged from puparia as a function of fungal treatment and time. Panel C): Dry weight of emerged flies as a function of fungal treatment. Different letters indicate statistically significant differences between treatment following a one-way Analysis of Variance (F2,38 = 6.652, p = 0.003) and Holm-Sidak pair-wise comparison. n.s. not significant.

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were prepared as described by Frisvad and Samson [35] and supplemented with 10 mM uridine and 10 mM uracil.

**PCR, USER cloning and A. nidulans strain construction**

USER cloning compatible PCR products were amplified with 30 PCR cycles in 50 μl reaction mixtures using proof-reading PfuX7 polymerase [36]. USER vectors were denoted according to the nomenclature introduced by Hansen et al [13]. Putative A. niger genes were amplified from A. niger genomic DNA, USER cloned into pU1111-IS1, and transformed into A. nidulans as described previously [13]. In order to generate the NID545 reference strain, the E. coli lacZ gene was cloned into a pU1014-IS1 vector generating pU1011-IS1: lacZ which was transformed into a pU1110-IS1-lacZ cassette. All expression plasmids were verified by sequencing. Gene targeting events were verified in all A. nidulans transformants by analytical PCR as described previously [13]. Table 3 summarizes the PCR primers used in this study. In addition, NID477 was confirmed by Southern blotting as described in [37]. For each Southern blot 2 μg genomic DNA was digested with HindIII. Two probes for detecting insertion of the smrA gene into IS1 were generated by PCR. Specifically, primers JBN X66 and JBN X67 were used to generate Probe 1, a 896 bp fragment of smrA using genomic DNA from A. niger as template, and primers JBN X64 and JBN X65 were used to generate Probe 2, a 948 bp fragment at the IS1 locus using genomic DNA from A. nidulans as template, see Figure 2. The probes were labeled with Biotin-11-dUTP using the Biotin DecaLabelTM DNA Labeling kit (Fermentas). Detection was performed with the Biotin Chromogenic detection kit (Thermo scientific).

**RNA isolation and quantitative RT-PCR**

RNA isolation from the A. nidulans strains and quantitative RT-PCR reactions were done as previously described in [13], except that disruption of biomass for RNA isolation was prepared with a Tissue-Lyser LT (Qiagen) by treating samples for 1 min at 45 mHz. The A. nidulans histone 3 encoding gene, hhtA (AN0733) was used as an internal standard for normalization of expression levels. All primers used for quantitative RT-PCR are shown in Table 3.

**Chemical characterization of mutant strains by UHPLC-DAD and LC-HRMS**

All strains were grown as three point inoculations for 7 days at 37°C in the dark on solid glucose minimal, CYAs, RTO and YES media [35]. Extraction of metabolites was performed by the agar plug extraction method [38] using three 6 mm agar plugs/extract. Extracts were analyzed by UHPLC-DAD and LC-HRMS. UHPLC-DAD analysis was performed on a Dionex RSLC Ultimate 3000 (Dionex, Sunnyvale, CA) equipped with a diode-array detector. Separation was performed at 60°C on a 150 mm×2.1 mm ID, 2.6 μm Kinetex C18 column (Phenomenex, Torrence, CA) using a linear water/MeCN (both buffered with 50 ppm tri-fluoroacetic acid (TFA)) gradient starting from 15% MeCN to 100% over 7 min at a flow rate of 0.8 mL min⁻¹. LC-HRMS analysis was performed on a MaXis 3G QTOF (Bruker Daltronics) coupled to a Dionex Ultimate 3000 UHPLC system equipped with a 100×2.0 mm, 2.6 μm, Kinetex C-18 column. The separation column was held at a temperature of 40°C and a gradient system composed of A: 20 mM formic acid in water, and B: 20 mM formic acid in acetonitrile was used. The flow was 0.4 mL/min, 85% A gradually to 100% B in 0–10 min, 100% B 10–13 min, 85% A 13.1–15 min. For calibration, a mass spectrum of sodium formate was recorded at the beginning of each
Table 3. PCR primers used in this study.

| Primer pair (fw/ rv) | Forward primer | Reverse Primer | Description |
|----------------------|----------------|----------------|-------------|
| JBN 2QQ/3QQ          | GCCAAGTGTTGGAATGCCG | gatccccgggaattccatgCAACACTATCGCATACTCTCC | Amplifies 2 kb upstream region from argB (ANID_04409) for fusion PCR |
| JBN 4QQ/5QQ          | aattccagctgaccaccatgCCGAGACACACCTGGATTGTTG | CAGGGCGTGGAGAGATGC | Amplifies 2 kb downstream region from argB (ANID_04409) for fusion PCR |
| JBN 5A/2K            | cagctgaccaccatgCCGAGACACACCTGGATTGTTG | GGAAGAGAGGTTCACACC | Amplifies 5’ A. fumigatus pyrG sequence including 300 bp direct repeat and native promoter for fusion PCR |
| JBN 4Q/2B            | TGATACAGTGTCGGTCC | catgttgtctggaattTGCCAAGCTTAACGCGTACC | Amplifies 3’ A. fumigatus pyrG sequence including 300 bp direct repeat and native terminator for fusion PCR |
|                      |                 |                |             |
| JBN 2QQ/2K           | GCCAAGTGTTGGAATGCCG | GGAAGAGAGGTTCACACC | Amplifies aargB (ANID_04409)::A. fumigatus pyrG upstream gene targeting fragment |
| JBN 4Q/5QQ           | TGATACAGTGTCGGTCC | CAGGGCGTGGAGAGATGC | Amplifies aargB (ANID_04409)::A. fumigatus pyrG downstream gene targeting fragment |
| Motni 165/185        | cgctgcauTGACTATTTTGACACCAGACCA | cagctgcauTAAGCTCCCTAATGCCGAAC | Amplifies the E. coli lacZ ORF for cloning into an AsiSI/Nb.BsmI cassette. Forward primer introduces an upstream AsiSI/Nb.BtsI USER cloning cassette |
| Motni 355/354        | agagcgauTAAGCTCCCTAATGCCGAAC | tctgcgauGCAGTGAGAGCGATCGCAGACACT | Amplifies 0.5 kb of the gpdA promoter for cloning into an AsiSI/Nb.BtsI |
| 287/288             | agagcgauATGATCCTCTGCCGTCGCTCC | tctgcgauTCAGAAATAATTGCTGGAAC | Amplifies the putative ORF of A. niger gene fge1_pg_C_4000037 |
| 289/290             | agagcgauATGAGCCATCGACATCAAAA | tctgcgauTTAATAGAGGCCATGCGC | Amplifies the putative ORF of A. niger gene fge1_pg_C_4000037 |
| 297/298             | agagcgauATGAGCCATCGACATCAAAA | tctgcgauTCAGAAATAATTGCTGGAAC | Amplifies the putative ORF of A. niger gene fge1_pg_C_4000037 |
| 299/300             | agagcgauATGAGCCATCGACATCAAAA | tctgcgauTTAATAGAGGCCATGCGC | Amplifies the putative ORF of A. niger gene fge1_pg_C_4000037 |
| 303/304             | agagcgauATGAGCCATCGACATCAAAA | tctgcgauTTAATAGAGGCCATGCGC | Amplifies the putative ORF of A. niger gene fge1_pg_C_4000037 |
| 305/306             | agagcgauATGAGCCATCGACATCAAAA | tctgcgauTTAATAGAGGCCATGCGC | Amplifies the putative ORF of A. niger gene fge1_pg_C_4000037 |
| 307/308             | agagcgauATGAGCCATCGACATCAAAA | tctgcgauTTAATAGAGGCCATGCGC | Amplifies the putative ORF of A. niger gene fge1_pg_C_4000037 |
| BGHA163/502          | GGTCTACTCCCCCTCGGCTCTAATGCCGAAC | AGGAGGCGTTGTCGCTCTT | Check primers for integration in IS1. Amplifies the junction between A. nidulans chromosome 1 and the gpdA promoter |
| BGHA98/162           | GGTTCCCTGGCTCAATGAGGCAAGAAC | GTTCAGGGTGACCCAGAG | Check primers for integration in IS1. Amplifies the junction between A. nidulans chromosome 1 and the argB marker gene |
| JBN X64/X65          | GAACACGGAGAACGTGCTCT | CTGCCAGGACTAAAGGAG | Detection of smrA by Southern blot |
| JBN X66/X67          | ATGCTGCTCTGCCGTCGCTCC | CGAGAGCGAGGACGGAG | Detection of insertion into IS1 by Southern blot |
| JBN X28/X29          | CACCCCAACAACGTGCTCC | CTGCCAGGACTAAAGGAG | Check primers for transcription of smrA |
| JBN L39/L52          | GAGGCGAGGAGGAAAG | GTGCTAGGTGAGGTAAGAAGC | Check primers for transcription of HHTA (ANID_00733) |

Upper case letters indicate annealing nucleotides, lower case indicate tails for user cloning. doi:10.1371/journal.pone.0073369.t003
down on a freeze drier. The crude extract was separated into three
fractions by partitioning into a heptane phase followed by a dichloromethane (DCM)
phase. The DCM phase was fractionated with a 10 g ISOL Diol column, using 13 steps of stepwise Hexane-dichloromethane-
Dichloromethane-Hexane (10:9:1).

The extract was filtered and the two fractions pooled and dried
under N2(g) to yield 2.0 mg of JH-diol. 1 and 2D NMR experiments could be conducted. Instead, JH-III was
identified based on comparison of accurate mass and retention time with authentic standard. HRMS (m/z): [M+H]+ calcd. for C16H27O12Na 267.1953; found, 267.1957; [M+N+Na]2+ calcd. For C16H27O12Na, 289.1700; found, 289.1774.

NMR studies and structure elucidation

NMR spectra were acquired in DMSO-d6 on a Varian Unity Innova 500 MHz spectrometer for JH-diol and JH-III and on a Bruker Avance 800 MHz spectrometer at the Danish Instrument Center for NMR Spectroscopy of Biological Macromolecules for compound 2 using standard pulse sequences. The spectra were referenced to this solvent with resonances δH ≈ 2.49 and δC ≈ 39.5.

Characterization data of methyl (2E,6E)-10-hydroxy-11-formyl-3,7,11-trimethyl-2,6-dodecadienoate (JH-diol)

NMR data for JH-diol-a: 1H NMR (500 MHz, DMSO-d6): δ 5.65 (s, 1 H), 5.08 (m, 1 H), 4.25 (d, J = 5.6 Hz, 1 H), 4.01 (s, 1 H), 3.57 (s, 3 H), 3.02 (dd, d, J = 10.0, 5.6, 2.5 Hz, 1 H), 2.15 (m, 2 H), 2.13-2.11 (m, 2 H), 2.09 (s, 1 H), 1.87 (m, 2 H), 1.60 (m, 1 H), 1.56 (s, 3 H), 1.15 (m, 1 H), 1.02 (s, 3 H), 0.97 (s, 3 H); 13C NMR (125 MHz): δ 166.1, 159.7, 135.9, 122.2, 114.7, 76.6, 71.5, 50.4, 39.7, 36.2, 29.4, 26.1, 25.1, 24.2, 18.2, 15.7.

NMR data for JH-diol-b: 1H NMR (500 MHz, DMSO-d6): δ 5.65 (s, 1 H), 5.08 (m, 1 H), 3.74 (dd, d, J = 10.0, 3.0 Hz, 1 H), 3.57 (s, 3 H), 2.15 (m, 2 H), 2.13-2.11 (m, 2 H), 2.09 (s, 1 H), 1.96 (m, 1 H), 1.57 (s, 3 H), 1.48 (m, 1 H), 1.41 (m, 1 H), 1.21 (s, 3 H), 1.00 (s, 3 H). 13C NMR (125 MHz): δ 166.1, 159.7, 134.9, 123.0, 114.7, 92.6, 79.4, 50.4, 39.7, 35.7, 29.2, 27.7, 25.1, 22.8, 13.2, 15.6. HRMS (m/z): [M+H]+ calcd. For C16H26O4Na, 285.2060; found, 285.2025; [M+N+Na]2+ calcd. For C16H26O4Na, 307.1883; found, 307.1887; [Na]+ = 0.0 (MeOH).

Characterization data of methyl (2E,6E)-10-hydroxy-11-formyl-3,7,11-trimethyl-2,6-dodecadienoate (JH-diol) (compound 2)

1H NMR (800 MHz, DMSO-d6): δ 8.23 (s, 1 H), 5.65 (q, J = 1.0 Hz, 1 H), 5.05 (t, J = 6.9, 1 H), 4.62 (s, 1 H), 4.52 (d, J = 10.2 Hz, 1 H), 3.57 (s, 3 H), 2.15 (m, 2 H), 2.11 (m, 2 H), 2.09 (d, J = 1 Hz, 3 H), 1.93 (m, 1 H), 1.84 (m, 1 H), 1.75 (m, 1 H), 1.57 (m, 1 H).
1.55 (s, 3 H), 1.46 (m, 1 H), 1.04 (s, 3 H), 1.03 (s, 3 H); 13C NMR (200 MHz): δ 166.2, 162.3, 159.9, 154.6, 123.4, 114.7, 79.7, 70.2, 50.4, 39.6, 35.7, 26.9, 25.2, 25.1, 21.5, 18.3, 15.6; HRMS (m/z): [M+H]+ calcd. for C17H29O5, 313.2010; found, 313.2010.

Characterization data of methyl (2, 6, 10)-3,7,11-trimethyl-2,6-dodecadienoate (MF)

HRMS (m/z): [M+H]+ calcd. for C16H27O2, 251.2006; found, 251.2007.

Confrontation with D. melanogaster larvae

Fungal strains were point-inoculated (1000 conidia in 1 µl Ringer solution) on 3 ml standard Drosophila medium [30] filled in 3.5 cm diameter Petri dishes. Prior to the transfer of ten sterile insects for four days. Subsequently, insects were removed and the plates snap frozen for metabolite profile analysis. Quantitative metabolite profile analyses were performed on groups of five biological replicates. Statistical analysis was performed with pairwise comparisons using the student’s t-test procedure. Evaluation of insect fitness followed the procedure described in Trienens et al. [30]. We confronted the larval stage of the fruit fly Drosophila melanogaster (wild type Oregon R strain) with the reference NID545 or the JH-producer strain NID477. Sterile two-day first-instar larvae were exposed to A. nidulans colonies growing on autoclaved standard Drosophila culture medium in 2 ml micro-tubes. There were N = 20 experimental units per fungal treatment and N = 10 mold-free control units. Insect developmental success was monitored in terms of (1) larva-to-pupa survival and development time, (2) emergence of flies, and (3) fly dry weight. Short development time and high body mass are considered to be positively correlated with fitness in Drosophila [41]. Experimental tubes were checked for pupae and emerged flies at about 2 p.m. each day for a total of 14 days after larval transfer. Emerged flies were removed from the tubes and stored deep-frozen. Subsequently, flies were lyophilized for 24 hours and the dry weight of all flies within each experimental unit was determined as a single value using a micro-balance.

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Author Contributions

Conceived and designed the experiments: MTN MLK MR CHG MRA BGH UMTOL. Performed the experiments: MTN MLK MR CHG MRA BGH UMTOL. Contributed reagents/materials/analysis tools: MTN MRA MR BGH UM TOL. Analyzed the data: MTN MLK MR CHG MRA BGH UMTOL. Performed the experiments: MTN MLK MR CHG. Wrote the paper: MTN, Reviewed the manuscript: MLK.MR DGA JBN CHG MRA BGH UMTOL.

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