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Expression of a synthetic *Artemesia annua* amorphadiene synthase in *Aspergillus nidulans* yields altered product distribution

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Abstract A gene encoding a plant terpene cyclase, *Artemisia annua* amorpha-4,11-diene synthase (ADS), was expressed in *Aspergillus nidulans* under control of a strong constitutive promoter, (*p*)*gpdA*. The transformants produced only small amounts of amorphadiene, but much larger amounts of similar sesquiterpenes normally produced as minor by-products *in planta*. In contrast, expression of *ADS* in *Escherichia coli* produced almost exclusively amorpha-4,11-diene. These results indicate that the host environment can greatly impact the terpenes produced from terpene synthases.

Keywords *Aspergillus nidulans* · Amorphadiene · Artemisinin · Sesquiterpene

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Introduction

Aspergillus species have long been utilized in the production of fermented food products, and more recently for organic acids and other small molecule metabolites, such as polyketide drugs, e.g., lovastatin, and proteins, chiefly hydrolytic enzymes. Since the advent of recombinant DNA technology and transformation vectors for fungi, there has been much interest in developing *Aspergillus* as an expression host for heterologous genes. To date, genetically modified *Aspergillus* has been used mainly for overproduction of native proteins or expression of genes from less tractable fungal species; while a number of mammalian proteins have been produced, yields are generally much lower than from fungal genes. There has also been considerable work towards cloning of *Aspergillus* and other fungal genes and their expression in other organisms, including plants; for example fungal phytases have been expressed in crop plants in order to improve yields by enhancing phosphorous uptake from soil, while increasing the nutritive value for animals and reducing water eutrophication from agricultural runoff [1–4]. While most of the biologically active metabolites developed as pharmaceuticals are derived from plants, as yet there has been little work on expressing plant genes in *Aspergillus*; the major examples to date being the sweetening proteins thaumatin and curculin (neoculin) [5, 6]. Recombinantly-produced human proteins and antibody products are emerging as an important new sector in therapeutics, however historically most drugs have been derived from small molecules, such as secondary metabolites. Both plants and fungi are richly endowed with a wide diversity of these metabolites; however they are normally produced only in small amounts, and thus present an obvious target for heterologous expression in a high-yielding microbial host.

A majority of the drugs extracted from plants are alkaloids, but other classes of bioactive compounds are now making larger incursions into pharmaceutical territory. Prominent among these are the terpenoids, a large and diverse class of secondary metabolites, first famous as flavors and fragrances, now known to be a promising source of new drugs, e.g., Taxol®. The biosynthetic precursor to these compounds, isopentenyl pyrophosphate (IPP), is also the precursor to sterols, essential in cell membrane structure, and the biosynthetic pathways involved are ubiquitous in nature; IPP is derived from the mevalonate pathway in higher organisms and some bacteria, and from the 2-methylerythritol 4-phosphate pathway in most prokaryotes and in plant chloroplasts [7]. This class of compounds is alternately known as isoprenoids, since the carbon backbones are formally oligomers of isoprene (isopentadiene or 2-methyl-buta-1,3-diene). The committing step to isoprenoid production is the condensation of IPP with its isomer dimethylallyl pyrophosphate (DMAPP) to form geranyl pyrophosphate (GPP). Sequential addition of IPP can follow to form farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) (Fig. 1).

Isomerization of GPP, FPP, or GGPP followed by release of the pyrophosphate group leads to a variety of backbone structures with a reactive carbocation center, from which are derived the monoterpene (C_{10}), sesquiterpene (C_{15}), and diterpene (C_{20}) families of compounds, respectively. Quenching of the carbocation with water produces an alcohol, and deprotonation an olefin, the simplest of which is isoprene. More complex terpenes are formed via directed cyclization of the reactive intermediate by specialized enzymes known as terpene cyclases, which guide the folding of the backbone and location of the reactive center, and thus the bonds formed. Further functionalization of the released terpene product, as well as combination of the reactive terpene intermediates with each other and with other compounds, leads to the wide diversity of isoprenoids observed in nature; nearly 50,000 known compounds [8], many of these first isolated from plants.

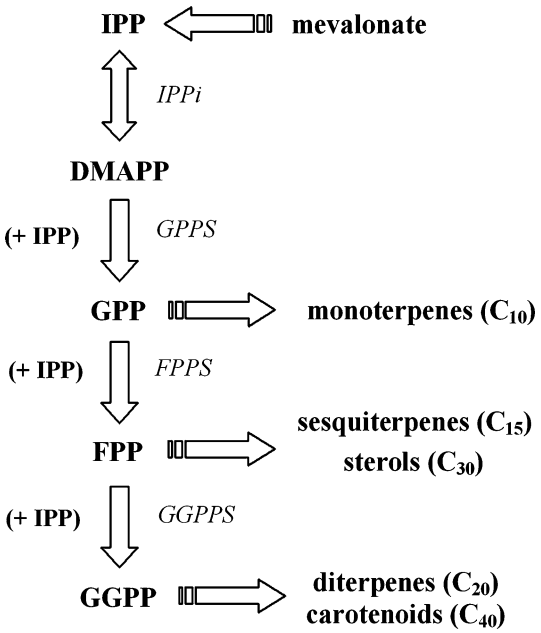

Fig. 1 Isoprenoid biosynthetic pathway intermediates and enzymes. *IPPI* IPP isomerase, *GPPS* GPP synthase, *FPPS* FPP synthase, *GGPPS* GGPP synthase. See text for details. The gene encoding the *A. nidulans* GGPP synthase was recently cloned and expressed in *E. coli*; in-vivo assays showed that the synthase accepts *DMAPP*, *GPP* and *FPP* as allylic substrates to synthesize *GPP*, *FPP*, and *GGPP*, respectively, with *FPP* as the preferred substrate (Wang and Keasling, unpublished results)

A number of fungal isoprenoids have also been isolated, including bioactive sesquiterpenes formed from FPP or its isomers **1–3**; e.g., developmental regulators such as sporogen-AO **4** in *Aspergillus oryzae*, and toxins such as PR-toxin **5** in the blue cheese mold *P. roqueforti* (Fig. 2). Toxins that are specific in their mode of action have been explored as therapeutic drug candidates, e.g., the fungal immunosuppressant ovalicin **6** [9], and the closely related fumagillin **7**, with derivatives in clinical trials as broad spectrum antitumor compounds [10].

Fumagillin and ovalicin biosynthesis begins with oxidation of the sesquiterpene precursor *β*-bergamotene **8** [11, 12]; sporogen-AO, PR toxin and other mycotoxins are similarly produced from aristolochene **9** in *Aspergillus* and *Penicillium* species [13]. The toxicity of these compounds likely derives from their common epoxide moiety; epoxy or peroxy groups are also found in cytotoxic plant isoprenoid metabolites, such as the important anti-malarial drug artemisinin **10** from Chinese wormwood (*Artemisia annua*), also produced via oxidation of a sesquiterpene precursor, amorpha-4,11-diene **11** (AD) [14].

Artemisinin is extracted commercially from *A. annua*, which produces only small amounts (0.01–0.8% dcw) and like other crop plants is subject to the vagaries of weather and limits in the infrastructure of the producing nations. While the price of a course of treatment is modest by Western standards at US $2–3, this remains far too expensive for the millions suffering from malaria in developing nations. Chemical synthesis of artemisinin is a laborious multi-step process, and thus the economics of production, coupled with the demand for inexpensive anti-malarials, make it a highly attractive target for heterologous biosynthesis. A considerable amount of work has been done to produce a precursor to artemisinin in microbial hosts, starting with the construction of a synthetic version of amorphadiene synthase (ADS) and its expression in *E. coli*, followed by engineering of the isoprenoid pathway for increased precursor production [15]. In our search for a suitable high-level production host for artemisinin, it was hoped that as many

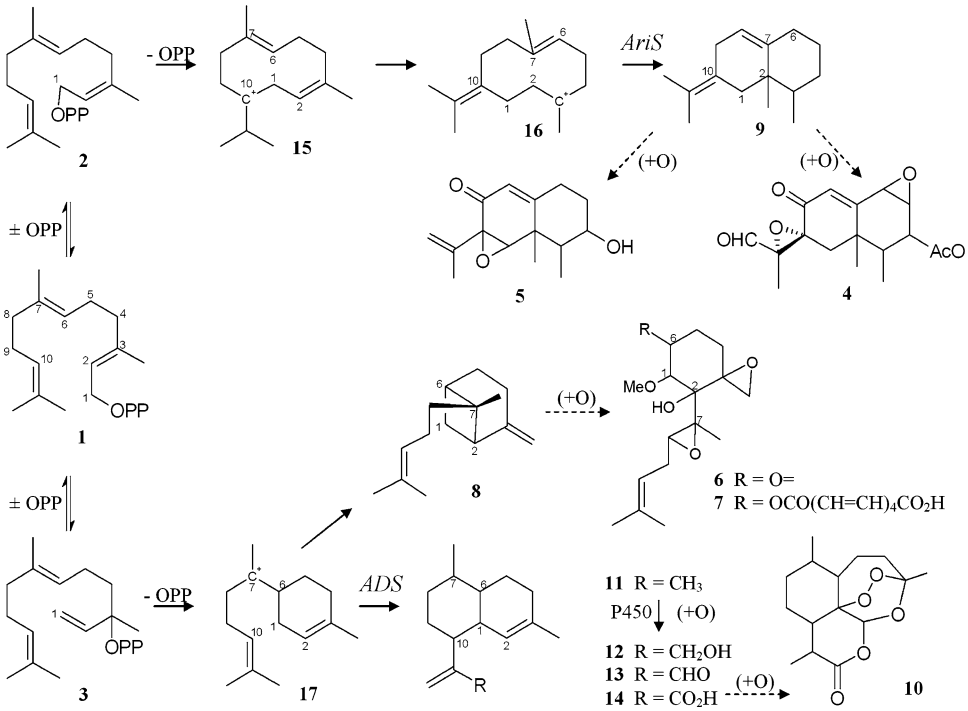

Fig. 2 Structures of selected sesquiterpenoid pathway members. Farnesyl pyrophosphate (FPP) isomers *2E*, *6E*– FPP (**1**), *2Z*, *6E*– FPP (**2**) and nerolidyl pyrophosphate (**3**); sesquiterpenes *β*-bergamotene (**8**), (+) aristolochene (**9**), and amorpha-4,11-diene (AD, **11**); derived isoprenoid metabolites ovalicin (**6**), fumagillin (**7**), sporogen AO (**4**), PR toxin (**5**), artemisinic alcohol (**12**), artemisinic aldehyde (**13**), artemisinic acid (**14**) and artemisinin (**10**) and reactive intermediate species: germacrenyl (**15**), eudesmanyl (**16**) and bisabolyl (**17**) cations. Aristolochene results from 10-1 and 7-2 closures; bergamotene from 6-1 and 7-2 closures; and amorphadiene from 6-1 and 10-1 closures. *AriS* aristolochene synthase, *ADS* amorphadiene synthase

oxidation steps downstream of amorphadiene as possible could be performed in vivo to reduce later chemosynthetic steps to artemisinin and keep process costs low. A key breakthrough came recently with the cloning of the *A. annua* cytochrome P450 monooxygenase responsible for the first oxidation steps and its successful expression in yeast [16].

As *Aspergillus* is known to possess a sesquiterpenoid biosynthetic pathway similar in some respects to that of *Artemisia annua*, it was deemed a worthwhile host to explore. In this study, *A. nidulans* was transformed with the synthetic ADS gene previously expressed in *E. coli*, after verifying that the codon usage was compatible [17]. The *argB* selection marker and the strong constitutive *Aspergillus* glyceraldehye-3-phospate dehydrogenase promoter (*p*)*gpdA* were used, as this marker-promoter combination had shown the highest expression levels of those tested previously [18].

Materials and methods

Strains, media, and culture conditions

Plasmids were propagated in *E. coli* strains DH5α, DH10b, and DM1 (Life Technologies), and XL1-Blue (Stratagene). PCR products for sequencing or ligation were cloned into the pCR4-TOPO vector (Invitrogen) and maintained in *E. coli* TOP10 (Invitrogen). Transformed *E. coli* hosts were cultivated at 37 °C on Luria–Bertani (LB) medium (Sigma) containing ampicillin (100 μg/mL) or kanamycin (50 μg/mL) from Fisher Scientific.

Saccharomyces cerevisiae strain BY4742 was cultured on synthetic defined (SD) medium (Qbiogene); transformation was performed by the standard lithium acetate method. For induction of genes expressed from the *GAL1* promoter, *S. cerevisiae* strains were grown in 2% galactose as the sole carbon source.

Aspergillus nidulans strain A89 (*biA1 argB2*) was obtained from the Fungal Genetics Stock Center (FGSC), University of Kansas. *Aspergillus* stocks for spore and protoplast production were grown on glucose/nitrate *Aspergillus* minimal medium (AMM) [19] plus 4 mM arginine and 0.2 μg/L biotin. *Aspergillus* transformation was performed as described by Dawe [20], with the exception of replacing Novozym® 234 and *β*-glucuronidase with Glucanex® (Sigma L1412; at 10 mg/mL) and Driselase® (Interspex 0456; at 2 mg/mL); additionally the washed protoplast suspension was filtered through a 60 μM nylon mesh (Small Parts, Inc) to remove undigested mycelia. Ten micrograms of transforming DNA was added to ~10^5 protoplasts in 150 μL for each transformation. Transformed protoplasts were initially selected on AMM plus 1 M sucrose and vitamins, and propagated on AMM plus vitamins. Individual clones were streaked to purity three times on selective medium.

DNA manipulations and reagents

General recombinant DNA techniques were performed essentially as described in Sambrook [21]. *E. coli* plasmid

DNA was extracted using the Bio101 Mini-Prep Express kit (for colony screening), Qiagen mini-spin kit (for sequencing and ligation), and the Qiagen Midi kit (for *Aspergillus* transformation). *Aspergillus* genomic DNA was extracted from colonies on agar, including spores, by a "quick prep" boiling lysis method [22] for screening PCR, and from broth-cultured pellets by lyophilization, grinding and phenol–chloroform extraction [23] for sequencing. Restriction enzymes and T4 DNA ligase were from Roche and New England Biolabs, and were used with the manufacturers' buffers. Gibco DNA *Taq* polymerase was used for PCR screening of transformants, and Stratagene *Pfu* Turbo polymerase for PCR products to be sequenced.

Plasmids pDL12 [18] and pUCADS [15] were previously constructed in our lab. Plasmid pRPS1 consists of a 1.6-kb *Nco*I–*Xma*I fragment from pUCADS containing the synthetic *ADS* gene, cloned into a 6.5-kb *Bsp*EI–*Nco*I digest of pDL12, containing the *A. nidulans* glyceraldehyde-3-phosphate dehydrogenase promoter ((*p*)*gpdA*) and the 800-bp terminating region of the *Aspergillus trpC* gene ((*t*)*trpC*).

Molecular genetic analysis of *Aspergillus* transformants

PCR screening of transformants for integration of *ADS* utilized primers 3 and 4, amplifying a 1.7-kb fragment including the 5′ and 3′ terminal fusions to (*p*)*gpdA* and (*t*)*trpC*, along with primers 5 and 6, amplifying an 850-bp fragment starting in the 5′ untranslated region of *argB* as a positive control for genomic template. DNA sequencing was done at the UC Berkeley DNA Sequencing Facility. Oligonucleotide synthesis services were by Operon Biotechnologies, Huntsville, AL (Table 1).

Gas chromatography–mass spectroscopy analysis of terpenoids

Aspergillus, yeast, and *E. coli* transformants were cultured at 30 °C for 72 h, 30 °C for 48 h and 37 °C for 24 h, respectively, in broth overlaid with *n*-dodecane (5% vol/vol, Sigma Aldrich) as described in Newman et al. [24] and the organic phase, suitably diluted in ethyl acetate, was subjected to analysis by gas chromatography–electron impact mass spectrometry (GC–MS) using a Hewlett Packard HP6890 gas chromatograph and HP5973 mass detector. Splitless 1-μL injections onto a capillary column, Agilent DB-5 (30 m × 250 μm ID × 0.25 μm film thickness; Agilent Life Sciences and Chemical Analysis, San Jose, CA), were separated using an oven temperature program of 80 ºC for 2 min, followed by a variable two-step ramp up to 300 °C. The carrier gas was helium at a flow rate of 1 mL/min. Injector and MS quadrupole detector temperatures were 250 and 150 °C, respectively. MS parameters were: electron energy, 70 eV; scan rate, 20 Hz; and scanned-mass range (m/z), 40–240. Identification of unknown peaks was with MSD Chemstation software (Agilent Technologies) using a proprietary terpene mass spectral library (Finnigan MAT). Amorphadiene amounts are reported as caryophyllene equivalents, as described previously [25].

Table 1 PCR primers used in this study

Primers	Sequence 5′→3′
1	GGATCCAACAATGCCGTCCGCTATTGACACCG
2	GCACTAGTCTACGCGCTAGCGGTTGCC
3	CCGCTTGAGCAGACATCACC
4	GAACACCATTTGTCTCAACTCCGG
5	TCAGACGGCGAATCGGG
6	AAAGCATTGATGACTGGAACCG

Results and discussion

Molecular genetic analysis of transformants

Aspergillus nidulans A89 was transformed with the ADS expression plasmid pRPS1. Genomic DNA extracted from the transformants was screened by PCR for integration of *ADS*, using primers 3 and 4, amplifying the full gene and the 5′ fusion region. Since a single amino acid change can radically alter the product distribution of a terpene cyclase [26], sequencing of the PCR products ensured that the ADS expression cassette was intact and free of mutations. ADS-positive transformants were analyzed for terpene content by gas chromatography–mass spectrometry.

Analysis of terpene production in transformants

Organic extracts of ADS-transformed *A. nidulans* cultures showed evidence of much increased terpene production over the wild type. Initial GC–MS analysis parameters were based on methods for detection of sesquiterpenes described in Martin et. al. [25] and further optimized for better separation of unknown sample components. The chromatograms of the ADS-transformed *A. nidulans* cultures had large peaks at the same retention times as the caryophyllene and amorphadiene (AD) standards, and numerous other peaks not present in the wild-type extracts (Fig. 3). Spectrometric analysis of the novel peaks showed a number of ions characteristic of sesquiterpenes, but the spectra of the largest two were not readily identifiable in the compiled library of terpene mass spectra. Using a slower ramp, these peaks were better separated from the standards; peaks **I**–**IV** in the transformant chromatogram were identified as Z-β-farnesene, α-.-bergamotene, E,E-α-farnesene and β-bisabolene,

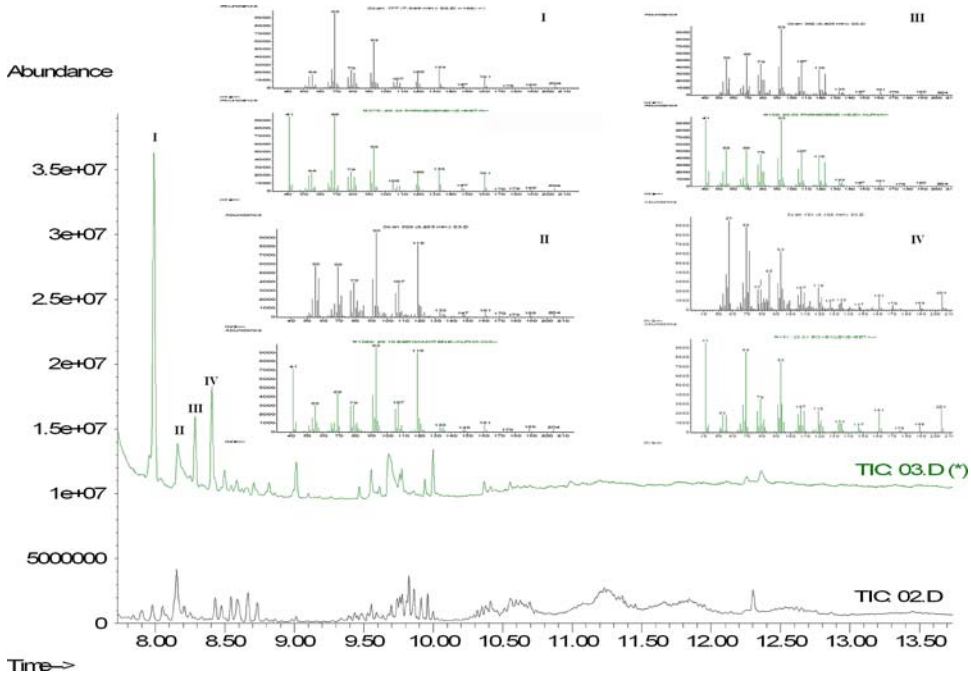

Fig. 3 Production of terpenes by the ADS-engineered *A. nidulans*. Total ion chromatograms of *A. nidulans* organic extracts. Samples: 02, *A. nidulans* A89; 03, *A. nidulans* ADS transformant. *Inset* Mass spectra of peaks I–IV compared with terpene library reference spectra: *I* Z-*β*-farnesene, *II* α-*cis*-bergamotene, *III* E,E-α-farnesene; and *IV* *β*-bisabolene

respectively, along with several other minor terpene components (see Supplementary Data for a complete list of identified terpenes and mass spectra). None of the spectra of the peaks in the chromatogram of the extract from wild-type *A. nidulans*, grown under the same conditions, matched any library spectra for terpenes; most matches showed similarity to C_8–C_{10} alcohols, likely partial oxidation products of dodecane, possibly aided by enzymatic activity in *Aspergillus*.

Although other terpenes dominated, amorphadiene was produced by the engineered *A. nidulans*. While in the total ion chromatogram (TIC) of the ADS transformant, no peaks were visible above the basal noise level at the retention time of AD (7.40 min), ion chromatograms for the characteristic ions for AD, m/z 189 and 204, extracted from the TIC data each showed small peaks in the transformant that were absent in the wild-type (Fig. 4). The mass spectrum of the peak centered at 7.40 min matches the reference spectrum of AD nearly exactly. Ion abundance of this peak is ~3,000 counts, equivalent to ~5 ng/L of AD; nearly five orders of magnitude less than the largest peaks I and IV, but still well within the theoretical limit of detection of terpenes with this instrument (0.1 ng/L [25], or ~50–100 ion counts on this chromatogram).

It appears that the ADS transformants are producing significant quantities of α- and *β*-farnesene, α-bergamotene and α- and *β*-bisabolene, yet only very small amounts of amorphadiene. As with many terpene-producing plants, in *Artemesia annua* amorphadiene is found along with a number of related terpene products, including the sesquiterpenes *β*-farnesene, germacrene A and D, selinadiene and *β*-caryophyllene, as well as monoterpenes camphor, α-pinene, pinocarvone and others [14, 27, 28]. While in *E. coli*, the synthetic ADS produces no other sesquiterpenes in detectable quantities [24], it is conceivable that the cellular environment might influence the product distribution of ADS in vivo. Other terpenic plants are known to contain several cyclases, but a single cyclase can also form a variety of similar products [29]. It has been hypothesized that many cyclases have evolved from promiscuous catalysts to more specific ones [30, 31]. Thus, a "broken" specifically evolved cyclase might be expected to produce a wider variety of products, perhaps including simpler, non-cyclized sesquiterpenes, such as farnesene. Incubation of FPP with crude lysate from the transformants showed no ADS activity in vitro (data not shown), which, given the well-known high protease activity of *Aspergillus* species, is not surprising, even though protease inhibitors were added. Since ADS was apparently active in *A. nidulans*, we hypothesized that the low levels of AD might be due to either precursor limitation or rapid bioconversion, or an altered product distribution that occurred as a result of alteration of enzyme activity in vivo, perhaps due to proteolysis or improper folding.

It is possible that native *Aspergillus* terpene cyclases could have produced these sesquiterpenes. *β*-Bergamotene has been found in *A. fumigatus* and *Pseudeurotium ovalis* [11, 12] and was identified as the precursor to fumagillin, although neither compound has been reported in *A. nidulans*. A BLAST search of the *A. nidulans* genome sequence revealed an ORF (AN3277.3, [32]) with high homology to the published sequences of the *A. terreus* and *P. roquefortii*

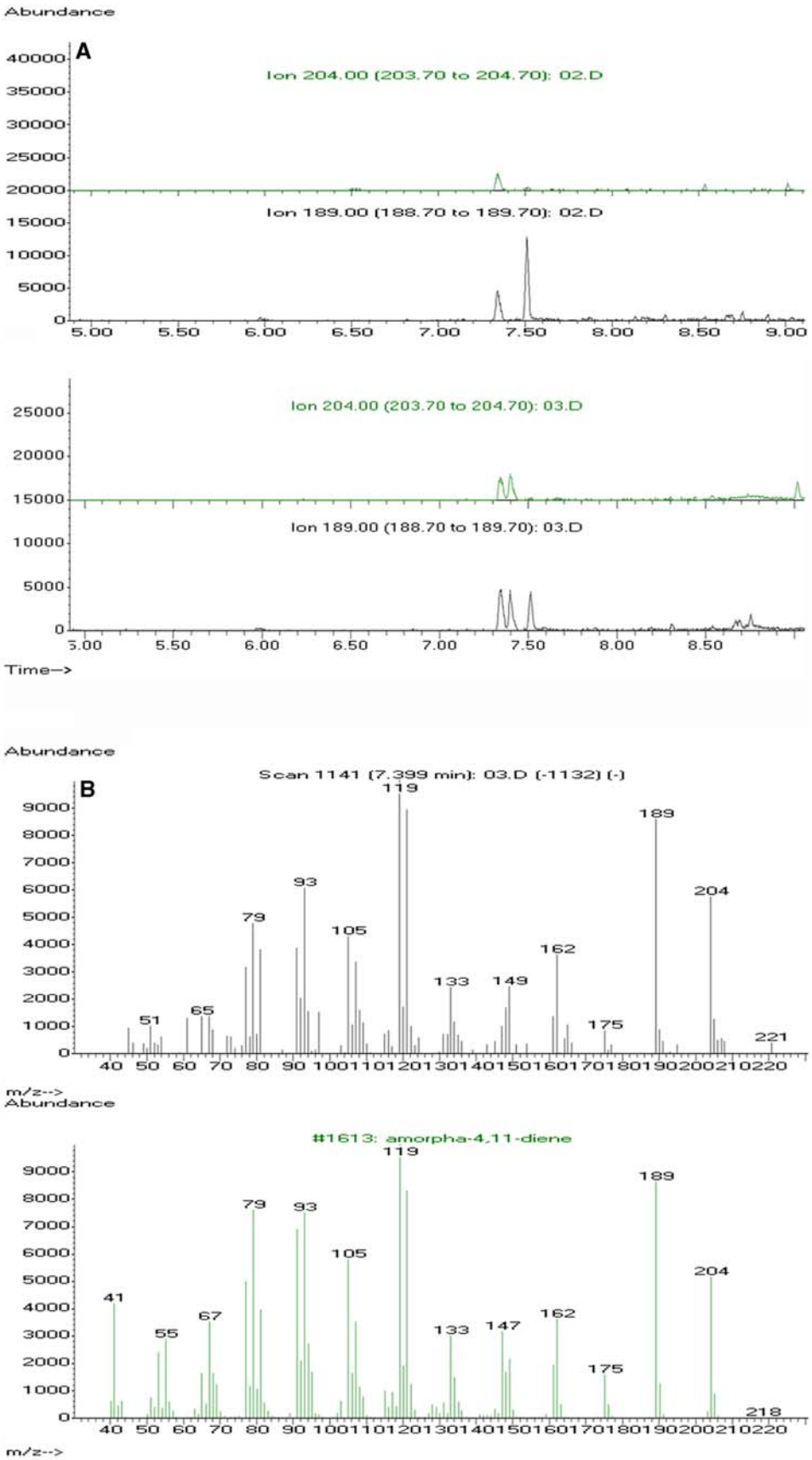

Fig. 4 Amorphadiene production by ADS-engineered *A. nidulans*. **a** Chromatogram traces of AD characteristic ions (m/z 189 and 204) extracted from the TIC data of Fig. 3. *Top A. nidulans* A89, *bottom* ADS transformant. **b** Mass spectrum of transformant peak at 7.40 compared with library reference spectrum of AD

aristolochene synthase (*ariS*) genes; expression of AN3277.3 cDNA in *Saccharomyces* confirmed its identity as an aristolochene synthase (Supplementary Data). Aristolochene was not detected in organic extracts of *A. nidulans*, suggesting that it is either produced only in small amounts under these conditions, or is rapidly converted to other metabolites. The wild type was also negative for all of the sesquiterpenes detected in the ADS transformants, indicating that the introduced amorphadiene cyclase is responsible for their formation, rather than AriS or an unknown native cyclase.

Another hypothesis is that the ADS transformants could be producing relatively larger quantities of amorphadiene, which is then converted to other metabolites in *Aspergillus*. Since sporogen-AO and PR toxin are known to be produced from aristolochene by oxidation, the structurally similar AD might also be oxidized in *Aspergillus*, perhaps to form artemisinic alcohol **12**, aldehyde **13** or acid **14**, intermediates identified in *A. annua* [14]. However, these intermediates were not detected in the transformants (data not shown).

Summary

Many terpene cyclases are known to produce a distribution of similar products rather than a single one; not surprising, given that these terpenes are isomers formed via a guided folding of an alkene with a reactive carbocation, rather than catalyzing the formation or cleavage of a particular chemical bond. The altered product distribution of ADS in *A. nidulans* is likely due to the premature release of reactive intermediates, which to some extent explains the distributions found in native terpene biosyntheses. For example, aristolochene is produced from *Z,E*-FPP via consecutive 10-1 and 7-2 ring closures, yielding the germacrenyl **15** and eudesmanyl **16** cationic intermediates, respectively [33]. Thus it would be expected to find germacrene and eudesmane as co-products of AriS; expression of AN3277.3 cDNA in *Saccharomyces* did indeed yield these sesquiterpenes along with aristolochene (Supplementary Data). Likewise AD and bergamotene are synthesized via the bisabolyl cation **17** [12, 34, 35], hence bisabolene and bergamotene are not unlikely co-products; the farnesenes are simply uncyclized FPP.

While it is known that a point mutation of the cyclase can alter the product distribution, it remains an open question how differing conditions in vivo might change it. Further study on over-expression of heterologous and native cyclases in *Aspergillus* may help to answer this question; clearly the expression of terpene cyclases in *Aspergillus* is not as straightforward an affair as in bacteria or yeast. While cDNA for the few fungal cyclases that have been cloned to date has been well expressed in these hosts, there are as yet not enough to make generalizations on their suitability. For the converse, no other attempts at expressing a plant terpene cyclase in *Aspergillus* were found in the literature; given its potential as a host for both native and heterologous metabolites, hopefully this study will be the first of many in the near future.

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References

1. Peng RH et al (2006) Codon-modifications and an endoplasmic reticulum-targeting sequence additively enhance expression of an *Aspergillus* phytase gene in transgenic canola. Plant Cell Rep 25(2):124–132. doi:10.1007/s00299-005-0036-y
2. George TS et al (2005) Expression of a fungal phytase gene in *Nicotiana tabacum* improves phosphorus nutrition of plants grown in amended soils. Plant Biotechnol J 3(1):129–140. doi:10.1111/j.1467-7652.2004.00116.x
3. Drakakaki G et al (2005) Endosperm-specific co-expression of recombinant soybean ferritin and *Aspergillus* phytase in maize results in significant increases in the levels of bioavailable iron. Plant Mol Biol 59(6):869–880. doi:10.1007/s11103-005-1537-3
4. Brinch-Pedersen H et al (2000) Generation of transgenic wheat (*Triticum aestivum* L.) for constitutive accumulation of an *Aspergillus* phytase. Mol Breed 6(2):195–206. doi:10.1023/A:1009690730620
5. Faus I et al (1998) Secretion of the sweet-tasting protein thaumatin by recombinant strains of *Aspergillus niger* var. awamori. Appl Microbiol Biotechnol 49(4):393–398. doi:10.1007/s002530051188
6. Nakajima K et al (2006) Extracellular production of neoculin, a sweet-tasting heterodimeric protein with taste-modifying activity, by *Aspergillus oryzae*. Appl Environ Microbiol 72(5):3716–3723. doi:10.1128/AEM.72.5.3716-3723.2006
7. Withers ST, Keasling JD (2007) Biosynthesis and engineering of isoprenoid small molecules. Appl Microbiol Biotechnol 73(5):980–990. doi:10.1007/s00253-006-0593-1
8. Mann J (1987) Secondary metabolism. In: Atkins PW, Holker JSE, Holliday AK (eds) Oxford Chemistry Series, vol 33, 2nd edn. Clarendon Press, Oxford
9. Stahelin HF (1996) The history of cyclosporin A [Sandimmune(R)] revisited: another point of view. Experientia 52(1):5–13. doi:10.1007/BF01922409
10. Satchi-Fainaro R et al (2004) Targeting angiogenesis with a conjugate of HPMA copolymer and TNP-470. Nat Med 10(3):255–261. doi:10.1038/nm1002
11. Nozoe S, Kobayashi H, Morisaki N (1976) Isolation of beta-*trans*-bergamotene from *Aspergillus fumigatus*, a fumagillin producing fungi. Tetrahedron Lett (50):4625–4626. doi:10.1016/S0040-4039(00)93948-0
12. Cane DE, McIlwaine DB (1987) The biosynthesis of ovalicin from beta-*trans*-bergamotene. Tetrahedron Lett 28(52):6545–6548. doi:10.1016/S0040-4039(00)96909-0
13. Cane DE, Kang I (2000) Aristolochene synthase: purification, molecular cloning, high-level expression in *Escherichia coli*, and characterization of the *Aspergillus terreus* cyclase. Arch Biochem Biophys 376(2):354–364. doi:10.1006/abbi.2000.1734
14. Bertea CM et al (2005) Identification of intermediates and enzymes involved in the early steps of artemisinin biosynthesis in *Artemisia annua*. Planta Med 71(1):40–47. doi:10.1055/s-2005-837749

15. Martin VJJ et al (2003) Engineering a mevalonate pathway in *Escherichia coli* for production of terpenoids. Nat Biotechnol 21(7):796–802. doi:10.1038/nbt833
16. Ro DK et al (2006) Production of the antimalarial drug precursor artemisinic acid in engineered yeast. Nature 440(7086):940–943. doi:10.1038/nature04640
17. Kazusa DNA Research Institute (2007) Codon Usage Database. http://www.kazusa.or.jp/codon
18. Lubertozzi D, Keasling J (2006) Marker and promoter effects on heterologous expression in *Aspergillus nidulans*. Appl Microbiol Biotechnol 72(5):1014–1023. doi:10.1007/s00253-006-0368-8
19. Barratt RW, Johnson GB, Ogata WN (1965) Wild-type and mutant stocks of *Aspergillus Nidulans*. Genetics 52(1):233–246
20. Dawe AL, Willins DA, Morris NR (2000) Increased transformation efficiency of *Aspergillus nidulans* protoplasts in the presence of dithiothreitol. Anal Biochem 283(1):111–112. doi:10.1006/abio.2000.4658
21. Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual, 3rd edn. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, NY
22. Chiou CH et al (2004) Distribution and sub-cellular localization of the aflatoxin enzyme versicolorin B synthase in time-fractionated colonies of *Aspergillus parasiticus*. Arch Microbiol 182(1):67–79. doi:10.1007/s00203-004-0700-6
23. Chow TYK, Kafer E (1993) A rapid method for isolation of total nucleic acids from *Aspergillus nidulans*. Fungal Genet Newsl 40:25–27
24. Newman JD et al (2006) High-level production of amorpha-4, 11-diene in a two-phase partitioning bioreactor of metabolically engineered *Escherichia coli*. Biotechnol Bioeng 95(4):684–691. doi:10.1002/bit.21017
25. Martin VJJ, Yoshikuni Y, Keasling JD (2001) The in vivo synthesis of plant sesquiterpenes by *Escherichia coli*. Biotechnol Bioeng 75(5):497–503. doi:10.1002/bit.10037
26. Deligeorgopoulou A, Allemann RK (2003) Evidence for differential folding of farnesyl pyrophosphate in the active site of aristolochene synthase: a single-point mutation converts aristolochene synthase into an (E)-beta-farnesene synthase. Biochemistry 42(25):7741–7747. doi:10.1021/bi034410m
27. Woerdenbag HJ et al (1991) Analysis of artemisinin and related sesquiterpenoids from *Artemisia annua* L. by combined gas chromatography/mass spectrometry. Phytochem Anal 2(5):215–219. doi:10.1002/pca.2800020507
28. Tellez MR et al (1999) Differential accumulation of isoprenoids in glanded and glandless *Artemisia annua* L. Phytochemistry 52(6):1035–1040. doi:10.1016/S0031-9422(99)00308-8
29. Deguerry F et al (2006) The diverse sesquiterpene profile of patchouli, *Pogostemon cablin*, is correlated with a limited number of sesquiterpene synthases. Arch Biochem Biophys 454(2):123–136. doi:10.1016/j.abb.2006.08.006
30. Yoshikuni Y, Ferrin TE, Keasling JD (2006) Designed divergent evolution of enzyme function. Nature 440(7087):1078–1082. doi:10.1038/nature04607
31. Yoshikuni Y et al (2006) Engineering cotton (+)-delta-cadinene synthase to an altered function: Germacrene D-4-ol synthase. Chem Biol 13(1):91–98. doi:10.1016/j.chembiol.2005.10.016
32. Broad Institute of MIT and Harvard (2003) *Aspergillus nidulans* database. http://www.broad.mit.edu/annotation/genome/aspergillus_nidulans/Home.html
33. Shishova EY et al (2007) X-ray crystal structure of aristolochene synthase from *Aspergillus terreus* and evolution of templates for the cyclization of farnesyl diphosphate. Biochemistry 46(7):1941–1951. doi:10.1021/bi0622524
34. Kim S-H et al (2006) Cyclization mechanism of amorpha-4, 11-diene synthase, a key enzyme in artemisinin biosynthesis. J Nat Prod 69(5):758–762. doi:10.1021/np050356u
35. Picaud S et al (2006) Amorpha-4, 11-diene synthase: mechanism and stereochemistry of the enzymatic cyclization of farnesyl diphosphate. Arch Biochem Biophys 448(1–2):150–155. doi:10.1016/j.abb.2005.07.015