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

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Abstract Terpenes are a large and varied group of natural products with a wide array of bioactivities and applications. The chemical production of industrially relevant terpenes can be expensive and time-consuming due to the structural complexity of these compounds. Here, we studied *Aspergillus nidulans* as a heterologous host for monoterpene and diterpene production. Previously, we identified a novel diterpene gene cluster in *A. nidulans* and showed that overexpression of the cluster-specific transcription factor (*pbcR*) led to *ent*-pimara-8(14),15-diene (PD) production. We report further characterization of the *A. nidulans* PD synthase gene (*pbcA*). In *A. nidulans*, overexpression of *pbcA* resulted in PD production, while deletion of *pbcA* abolished PD production. Overexpression of *Fusarium fujikuroi ent*-kaurene synthase (*cps/ks*) and *Citrus unshiu* gamma-terpinene synthase resulted in *ent*-kaurene and gamma-terpinene production, respectively. *A. nidulans* is a fungal model organism and a close relative to other industrially relevant *Aspergillus* species. *A. nidulans* is a known producer of many secondary metabolites, but its ability to produce heterologous monoterpene and diterpene compounds has not been characterized. Here, we show that *A. nidulans* is capable of heterologous terpene production and thus has potential as a production host for industrially relevant compounds. The genetic engineering principles reported here could also be applied to other *Aspergilli*.

Keywords *Aspergillus nidulans* · Terpenoids · *ent*-pimara-8(14),15-diene · *ent*-kaurene · Gamma-terpinene

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Introduction

Fungi produce a large number of secondary metabolites that can be exploited for their beneficial or toxic properties. Terpenoids represent one of the largest groups of bioactive metabolites with more than 55,000 isolated terpenoid compounds to date. Many terpenoids are high-value compounds, including the sesquiterpenoid artemisinin and the diterpenoid taxol, which are used in treating malaria and cancer, respectively. Other commercially relevant terpenoids include monoterpenoid menthol, carvone, and limonene, which are used in flavoring agents, cosmetics, and personal health products (Lange 2015).

Terpenes and terpenoids are structurally diverse natural products consisting of the simple five-carbon isoprenoid precursors, dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP). In fungi, these molecules are produced from acetyl-CoA via the mevalonate pathway and linked in head-to-tail fashion to create different lengths of hydrocarbons (Miziorko 2011). Terpene synthases catalyze the cyclization

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of linear hydrocarbons to produce the terpene backbone (Christianson 2006). Decorative pathway enzymes such as cytochrome P450 monooxygenases (CYPs), oxidoreductases, and transferases modify the terpene backbone to yield the final terpenoid. Sesquiterpenoids, diterpenoids, and triterpenoids are the main classes of terpenoids found in fungi (Quin et al. 2014).

Secondary metabolite biosynthetic genes are clustered in fungi (Keller and Hohn 1997; Brakhage and Schroeckh 2011). *Aspergillus nidulans* is a model organism for filamentous fungus and a well-known producer of secondary metabolites with complex chemical structures, including polyketides and non-ribosomal peptides (Yaegashi et al. 2014). *A. nidulans* genome mining indicates a high number of putative secondary metabolite gene clusters that far exceed the number of known *A. nidulans* metabolites (Bok et al. 2006; Andersen et al. 2013). This phenomenon is also seen in other fungal species (Cacho et al. 2014; Wiemann and Keller 2014). Because many secondary metabolites may have useful bioactivities, it is important to develop methods to study the products of the putative pathway genes. Heterologous expression of cryptic secondary metabolite genes in *Aspergillus* has been used to identify potential beneficial gene products from other fungal species, and a number of recent reports have used *A. nidulans* as a heterologous host by transferring genes or whole gene clusters from other fungi into *A. nidulans* (Anyaogu and Mortensen 2015).

Many of the high-value terpenoids are derived from plants and their abundance in nature is exceeded by usage needs for commercial applications. Because of terpenoid structural complexity, they are difficult to produce using total chemical synthesis. Therefore, it is important to develop alternative production methods. For microorganisms such as *Escherichia coli* and *Saccharomyces cerevisiae*, metabolic engineering has been used in production of high-value terpenes. However, microbial terpenoid production is often limited by the low abundance of hydrocarbon precursors. Thus, these systems rely on extensive engineering of the endogenous pathways or introducing the missing precursor pathways (Reiling et al. 2004; Withers and Keasling 2007). *A. nidulans* can be fermented using fairly inexpensive carbon sources, and as a model organism, the genetic tools for engineering have been developed. While expressing fungal heterologous polyketide synthase genes in *A. nidulans* has been used to create high-value chemicals (Yaegashi et al. 2014); we only know of one report using *A. nidulans* as a heterologous terpene production host (Lubertozzi and Keasling 2008).

A. nidulans has multiple putative isoprenoid pathway genes, including sesquiterpene synthase-like genes (Galagan et al. 2005). For this reason, Lubertozzi and Keasling explored the possibility of using *A. nidulans* as a heterologous host for sesquiterpene amorpha-4,11-diene. Amorpha-4,11-diene is the rate-limiting precursor in artemisinin biosynthesis. Expression of *Artemisia annua* amorphadiene synthase gene in *A. nidulans* resulted in production of multiple different sesquiterpenes, including amorpha-4,11-diene and presented the first example of expressing plant-derived terpene synthase in *A. nidulans* (Lubertozzi and Keasling 2008). Two endogenous sesquiterpenes, which both belong to a class of insect juvenile hormones, have also been identified in *A. nidulans* (Nielsen et al. 2013). Our group previously identified the first *A. nidulans* diterpene gene cluster and demonstrated that *A. nidulans* can also produce diterpenes (Bromann et al. 2012). In the present study, we further characterized the pimaradiene synthase encoded by AN1594 and also investigated the potential of *A. nidulans* to be used in production of heterologous monoterpenes and diterpenes. We expressed the plant-derived monoterpene synthase, *Citrus unshiu* gamma-terpinene synthase, in *A. nidulans*. We also expressed a fungal diterpene synthase, *Fusarium fujikuroi ent*-kaurene synthase (Cps/Ks), in *A. nidulans*. Our data demonstrate that *A. nidulans* can be engineered to produce heterologous gamma-terpinene and *ent*-kaurene.

Materials and methods

A. nidulans and Saccharomyces cerevisiae strains

A. nidulans strains A4 (wild type, veA+) (Pontecorvo et al. 1953), A772 (galD5 pyrG89;AcrA1;chaA1), and A1149 (pyrG89; pyroA4; nkuA::argB) (Nayak et al. 2006) were acquired from the Fungal Genetics Stock Center (Kansas City, Missouri USA). *A. nidulans* strain A4 was used in genomic DNA preparation. *A. nidulans* strain A772 was used in transformations with randomly targeted DNA sequences. *A. nidulans* strain A1149 was used in transformations to create targeted deletion and promoter insertion. All *A. nidulans* liquid cultures were grown in YES-media (2 % yeast extract, 4 % sucrose, 3 % gelatin) and maintained on potato dextrose (PD) plates. *Saccharomyces cerevisiae* strain CEN.PK 113-17 A (H2802; MATα, *ura3–52 HIS3 leu2–3/112 TRP1 MAL2-8cSUC2*) (Entian and Kotter 1998) was obtained from Dr. P. Kötter (Frankfurt, Germany). *S. cerevisiae* strain H2802 was grown in YPD (1 % yeast extract, 2 % peptone, 2 % dextrose). All constructed strains used in this study were deposited in VTT culture collection with the catalog numbers indicated in parentheses after each strain name.

Plasmid and strain construction

Expression vector pHHO5 (Figure S1) contains both the *Trichoderma reesei ura3* (OMPdecase) gene encoding orotidine-5′-phosphate decarboxylase (GenBank: X55880.1) as a fungal selection marker and also a multiple cloning site

(MCS) flanked by *A. nidulans gpdA* promoter and *trpC* terminator.

A772 oe:*PbcR* (D-151,585): Overexpression vector AN1599 in pKB1 has been described earlier (Bromann et al. 2012). PCR-amplified DNA fragments including *bar* selection marker and *pbcR* (AN1599) genomic sequence flanked by *A. nidulans gpdA* promoter *trpC* terminator were used in transformation.

A772 oe:AN1594 (D-151,589): PCR-amplified AN1594 genomic sequence (GenBank: CBF85181.1) was cloned into *Cla*I and *Spe*I sites of pHHO5 MCS to create AN1594 overexpression vector. A772 was transformed with *Pci*I-linearized vector.

A1149 oe:PbcR;ΔAN1594 (D-151,590): AN1594 deletion vector (Figure S2) was constructed by in vivo recombination in *S. cerevisiae* as described by Colot et al. (Colot et al. 2006). Briefly, two 1.4-kb genomic regions flanking the AN1594 open reading frame (ORF) and a fragment containing *T. reesei ura3* were PCR-amplified. The forward primer for the AN1594 5′-untranslated region (5′-UTR) and the reverse primer for AN1594 3′-UTR included *Pml*I restriction sites and short sequences homologous to pRS426 MCS. The 5′-UTR reverse primer and 3′ UTR forward primer included *Not*I, and *Not*I/*SpeI* restriction sites, respectively, as well as short sequences homologous to *T. reesei ura3*. The *Pml*I, *Not*I, and *Spe*I restriction sites were included to facilitate further manipulation of the DNA construct supposing this might be needed in the future studies. DNAs were combined with *Xho*I-linearized pRS426 plasmid and transformed into the *S. cerevisiae* strain SC94721 by using the lithium acetate method (Schiestl and Gietz 1989). Positive *S. cerevisiae* transformants were selected and DNA transformed into *Escherichia coli* strain TOP10 (Life Technologies, USA) to rescue the correct pRS426 plasmid containing the *T. reesei ura3* flanked by AN1594 5- and 3′-untranslated regions (Figure S2). The resulting plasmid was used as a template to generate the PCR amplicon for transformation. Overexpression of *pbcR* in *A. nidulans* A1149 was achieved by introducing *A. nidulans gpdA* promoter into the 5′end of *pbcR* ORF. The plasmid 5′pbcR_AN1599 in pKB1 (Figure S3) was constructed by adding a 1.7-kb *pbcR* 5′-UTR genomic fragment into expression vector AN1599 in pKB1 by using Gibson assembly cloning (Gibson et al. 2009; Gibson 2011). The resulting plasmid (Figure S3) was used as a PCR template to amplify a DNA fragment containing *gpdA* promoter flanked by about 1500 bases of AN1594 5′-UTR and AN1594 ORF. To generate the strain with AN1594 deletion and *pbcR* overexpression, the deletion and the promoter exchange PCR amplicons were cotransformed into *A. nidulans* A1149. All positive transformants were verified by PCR.

A772 oe:*Cps*/*Ks* (D-151,588) and *A772 oe*:*PbcR*; *oe*:*Cps*/*Ks* (D-151,586): Cps/Ks cDNA (GenBank: AB013295.1) originally isolated from *F. fujikuroi* strain m567 (Fungal Culture Collection, Weimar, Germany) was generously provided by Dr. Bettina Tudzynski, Münster, Germany. Cps/Ks cDNA (Tudzynski 2005) was amplified by PCR and cloned into *Apa*I/*Spe*I sites of pHHO5. *Pci*I-linearized vector was transformed into A772 and A772 oe:PbcR.

A772 oe:PbcR; xCps/Ks (D-151,587): To introduce *cps*/*ks* into the AN1594 locus, we created the exchange construct xCps/Ks (Figure S4). Briefly, *F. fujikuroi* Cps/Ks cDNA was *Not*I-digested and subcloned into AN1594 deletion vector (AN1594 deletion, Figure S2). *T. reesei ura3* was then subcloned into the *SpeI* site of the resultant vector (xCps/Ks). PCR amplicons were transformed into A772 oe:PbcR.

A772 oe:gTerpS (D-151,591) and H2802 oe:gTerpS (C-15,954): *C. unshiu* gamma-terpinene synthase (CitMTSL61, GenBank: AB110640.1) codon-optimized for *Aspergillus* (GenBank: KU156821) was obtained from GenScript (Piscataway, NJ, USA). HA-tag and a start codon were introduced to the synthetic gene before the arginine pair at position R56-R57 by using PCR to create gTerpS. For expression in *A. nidulans*, gTerpS was subcloned into the *Bam*HI site of pHHO5. gTerpS was also subcloned into the *Bgl*II site of *S. cerevisiae* expression vector B1181 (Verho et al. 2002). Empty plasmid B1181 was used to generate control *S. cerevisiae* strain H2802 e.v. (C-15,930).

A. nidulans were transformed by using either the PDS-1000/Helium™ system biolistic particle delivery method (Bio-Rad) or by protoplasting as described earlier by Osmani et al. (Osmani et al. 1987). About 5 μg of DNA was used in each transformation. Glufosinate ammonium (400 μg/mL) (PESTANAL, Sigma) was added to *bar* selection MM plates. *S. cerevisiae* were transformed by using lithium acetate method (Schiestl and Gietz 1989), and the transformants were selected on synthetic complete, D-glucose (SCD) without uracil.

Real-time quantitative reverse transcription PCR

A. nidulans strains were grown at 30 °C to culture pH of 5.0–5.5, indicating exponential growth phase (Figure S5). Total RNA was extracted using Qiagen RNeasy Plant Mini Kit following the manufacturer's suggestions for fungal RNA extraction. Extracted RNA was DNaseI (Qiagen) digested and quantified using Nanodrop (Thermo Scientific). cDNA synthesis was done with Transcriptor First Strand cDNA Synthesis Kit (Roche). DNA was analyzed by using real-time quantitative reverse transcription PCR (qRT-PCR) with LightCycler 480 SYBR Green I Master mix (Roche) on a LightCycler 480 (Roche). PD cluster gene transcript levels were normalized to the levels of *β-actin* expression in each sample. Primer efficiencies were calculated, and the expression fold ratios were quantified using the Pfaffl method (Pfaffl

2001). Fold changes in expression are represented as logarithmic value (Log_2-value). Comparisons were made with the corresponding non-modified strain treated equally. For analysis of heterologous gene expression, melting curves were acquired following final amplification cycles by slowly heating to 95 °C with continuous measurement of fluorescence. Temperature curves and fluorescence signal crossing point (C_p) values were generated using a LightCycler 480 (Roche). All samples were tested in three replicates.

Analysis of diterpenes by solid phase microextraction gas chromatography mass spectrometry (SPME-GC/MS)

Five hundred microliters of conidia was grown in a shaker in 2–4 mL YES media with appropriate supplements at 30 °C for 24 h in air-tight SPME vials. Both volatile and semivolatile compounds were extracted at 80 °C for 1 h with preconditioned (250 °C, 30 min) 100 μm PDMS fiber (Sulpelco, USA). Analytes were desorbed during 5 min at 250 °C in the split injector (flow 14.9 mL/min) of the gas chromatograph (Agilent 6890 Series, USA) combined with an MS detector (Agilent 5973 Network MSD, USA) and SPME autosampler (Combipal, Varian Inc., USA). Analytes were separated on BPX5 capillary column of 60 m × 0.25 mm with a phase thickness of 1.0 μm (SGE Analytical Science Pty Ltd., Australia). The temperature program started at 40 °C with 1 min holding, increased 9 °C/min up to 130 °C followed by 2 °C/min increases up to 230 °C, where the temperature was kept for 1 min. MSD was operated in electron-impact mode at 70 eV, in the full scan *m/z* 40–550. The ion source temperature was 230 °C and the interface was 280 °C. Compounds were identified by comparing the mass spectra on Palisade Complete 600K Mass Spectral Library (Palisade Mass Spectrometry, USA). GC/MS data were analyzed with MSD ChemStation (Enhanced ChemStation, E.02.01.1177) software by Agilent Technologies, Inc. All chemical structures were generated with ChemDraw Std 14.0 software.

Semi-quantitative analysis of *ent*-kaurene production

A. nidulans A772 oe:Cps/Ks and A772 oe:PbcR, oe:Cps/KS were grown to exponential phase in YES medium. Mycelia were collected, freeze-dried, and homogenized in Retsch MM301 Ball Mill at 29 Hz for 30 s. For both strains, accelerated solvent extraction (ASE) of 150 mg of homogenized mycelia was performed as described previously (Bromann et al. 2014). Briefly, homogenized powder was mixed with diatomaceous earth in ASE sample cells (Dionex) and extracted with ASE 200 (Dionex) using hexane/ethyl acetate (Hex/EtOAc) (1: 1). The extracts were evaporated to dryness and resuspended in 1 ml of Hex/EtOAc. Gas chromatography mass spectrometry (GC/MS) analysis of resuspended extracts was carried out as described previously (Bromann et al. 2012). For both strains, equal amounts of starting material were processed and analyzed equivalently and in parallel. The relative difference in *ent*-kaurene production was calculated by comparing the GC/MS peak area values.

Gamma-terpinene synthase enzyme assay

S. cerevisiae H2802 e.v. control and oe:gTerpS strains were cultured o/n at +30 °C in 50 mL SCD medium without uracil. Cells were collected, washed, and homogenized with glass beads in lysis buffer (70 mM Tris–HCl pH 7.6, 1 × cOmplete EDTA-Free Protease Inhibitor, Roche). Gamma-terpinene synthase activity was measured as described previously (Shimada et al. 2004). In short, 100 μL of cell extract, 5 μL of phosphatase inhibitor cocktail 2 (Sigma), and 400 μL of reaction buffer (2 mM MgCl2, 2 mM MnSO4, 30 mM NaF, 20 mM Tris–HCl; pH 7.6) were mixed in an SPME vial and 5 μL of 20 mM GPP (Li-salt, no. 76,532, Sigma) was added to start the reaction (final concentration 200 μM). Vials were closed, incubated at +30 °C for 1.5 h, and kept at RT up to 4 h until analysis with SPME-GC/MS.

Fluorescence imaging of *A. nidulans*

Fifty microliters of *A. nidulans* strain A4 oe:PbcR and A4 control strain conidia were plated on PD agar plates. The cultures were grown at +30 °C for 7 days. Digital images were acquired in 366-nm UV-fluorescence as well as in bright field light.

Results

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

We previously reported identification of a biosynthetic gene cluster for *ent*-pimara-8(14),15-diene (PD) production in *A. nidulans.* We demonstrated that this gene cluster was upregulated in a wild-type *A. nidulans* strain overexpressing the cluster-specific transcription factor, *pbcR*, and that upregulation of the cluster genes led to PD production in *A. nidulans* (Bromann et al. 2012, 2014). We hypothesized that AN1594 encoded PD-specific synthase.

To further characterize AN1594, we created AN1594 deletion and overexpression of *A. nidulans* strains. The AN1594 deletion strain (A1149 oe:PbcR;ΔAN1594) was created by replacing the AN1594 ORF with *T. reesei ura3* selection marker in *A. nidulans* strain A1149. In this strain, *A. nidulans gpdA* promoter was integrated into the 5′ region of *pbcR* ORF to overexpress *pbcR* and to activate the PD gene cluster. The AN1594 overexpression strain (A772 oe:AN1594) was created by placing AN1594 under the control of the constitutively active *gpdA* promoter in *A. nidulans* strain A772. A strain

overexpressing PbcR (A772 oe:PbcR) was used as a reference. In all three strains, transcript levels of *pbcR*, AN1594 (putative PD synthase), geranylgeranyl diphosphate synthase (GGPPS, AN1592), and cytochrome P450 (CYP, AN1598) were determined by using real-time quantitative reverse transcription PCR (qRT-PCR). When compared to the parental A772 strain, we detected increased expression of all four cluster genes, including *pbcR* (5.5-fold), AN1594, (putative PD synthase, 11.7-fold), GGPPS (AN1592, 14.2-fold), and CYP (AN1598, 12-fold) in A772 oe:PbcR (Fig. 1a). GC/MS analysis of A772 oe:PbcR confirmed production of PD (Fig. 1b). In A1149 oe:PbcR;ΔAN1594, expression of AN1594 was undetectable by qRT-PCR. When compared to the non-modified A1149 strain, we detected increased expression of *pbcR* (6.8-fold), GGPPS (AN1592, 11-fold), and CYP (AN1598, 8.5-fold) in A1149 oe:PbcR;ΔAN1594 (Fig. 1c). These data confirm that the PD gene cluster was activated in this strain. GC/MS analysis demonstrated that the deletion of AN1594 abolished production of PD (Fig. 1d). In A772 oe:AN1594, only the AN1594 expression was increased (12.6-fold), while expression of other PD cluster genes was not elevated when compared to the non-modified A772 strain (Fig. 1e). Further, overexpression of AN1594 resulted in PD production (Fig. 1f). The retention time of the product peak (11.95 min) matched the retention time of purified PD (Bromann et al. 2014), and the extracted spectra verified the identity and chemical structure of PD (Fig. 1g, h). These data confirm that AN1594 is both necessary and sufficient for PD production in *A. nidulans*. These data demonstrate that AN1594 encodes a bona fide PD synthase in *A. nidulans*, and we name it PbcA.

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

Since *A. nidulans* is capable of producing PD, a diterpene, we tested whether *A. nidulans* could also be used as a production host for other heterologous diterpenes. *F. fujikuroi ent*-kaurene synthase (*cps*/*ks*) was overexpressed by placing Cps/Ks cDNA under the control of the constitutively active *gpdA* promoter in *A. nidulans* strain A772 (A772 oe:Cps/Ks). The transcript levels of PD cluster genes were analyzed by using qRT-PCR. In strain A772 oe:Cps/Ks, PD cluster gene expression was not elevated compared to the A772 strain (Fig. 2a). A melting curve analysis was performed to verify heterologous *cps*/*ks* expression in A772 oe:Cps/ks. The mean crossing point (C_p) value for the fluorescence signal of *cps*/*ks* in A772 oe:Cps/Ks was 19.60 cycles, whereas no *cps*/*ks* fluorescence was observed in the A772 parental strain. The melting point analysis revealed a clear product peak at mean Tm = 84 °C for *cps*/*ks* in the oe:Cps/Ks (Fig. 2b, blue graphs) while no products with Tm values were observed for the parental strain (Fig. 2b, red graphs). Actin was used as an internal control to verify that mRNA preparation was uncompromised for both the control A772 and A772 oe:Cps/Ks. The qRT-PCR amplicon for *cps*/*ks* was also analyzed with agarose gel electrophoresis to further verify that the signal observed with the qRT-PCR is not caused by unspecific amplification products (data not shown). We also introduced the Cps/Ks overexpression construct into the A772 oe:PbcR strain (A772 oe:PbcR; oe:Cps/Ks), and confirmed the increased expression of PD cluster genes, *pbcA* (10-fold), *pbcR* (4.2-fold), GGPPS (9.5-fold), and CYP (10-fold), when compared to non-modified A772 strain (Fig. 2d). The expression for *cps*/*ks* was verified with qRT-PCR. The mean C_p value for the *cps*/*ks* fluorescence signal in A772 oe:PbcR, oe:Cps/Ks was 24.02 (Fig. 2e, blue graphs), whereas C_p values of around 37 cycles were observed for *cps*/*ks* in the parental strain A772. The melting point analysis revealed that the signals observed for the parental strain were due to nonspecific amplification in the qRT-PCR (Fig. 2e, red and green graphs). Actin was used as internal control.

A semiquantitative analysis for the diterpene production in these two strains was carried out. Equal amounts of freeze-dried mycelia were used for both strains, and the extraction and the GC/MS analysis were done in parallel using identical conditions. GC/MS analysis confirmed a 99 % match to *ent*-kaurene for the product peak in A772 oe:Cps/Ks (Fig. 2c); both PD and *ent*-kaurene were produced in oe:PbcR, oe:Cps/Ks (Fig. 2f). Since the size of the GC analysis peaks is proportional to the quantity of the corresponding substances in the analyzed specimen, the *ent*-kaurene peaks in the two equally treated strains were measured from the baseline to the tip of the peak. The peak areas indicating the relative *ent*-kaurene amounts in the two samples were compared, and in oe:Cps/Ks, the numeric value for *ent*-kaurene peak area was 27,881 (Fig. 2b), whereas in A772 oe:PbcR; oe:Cps/Ks, the *ent*-kaurene peak area was 62,975 (Fig. 2d). Interestingly, these data suggest 2.4 times higher *ent*-kaurene production in the strain overexpressing both *cps*/*ks* and *pbcR*. To our knowledge, this is the first report of *ent*-kaurene production in *A. nidulans*.

Genetic engineering of *A. nidulans* to develop diterpene production strains

To ensure that any given heterologous diterpene synthase has access to the maximal precursor pool, one must presumably delete the competing endogenous synthases. Since we observed higher *ent*-kaurene accumulation in an *A. nidulans* A772 oe:PbcR; oe:Cps/Ks strain (that had activated PD biosynthesis genes), we attempted to further increase *ent*-kaurene production by exchanging *pbcA* with *cps*/*ks*. We generated an exchange construct containing *cps*/*ks* and *T. reesei ura3* flanked by approximately 1.5 kb of sequence from the 5′ promoter and 3′ terminator regions of *pbcA*, respectively (Fig. 3).

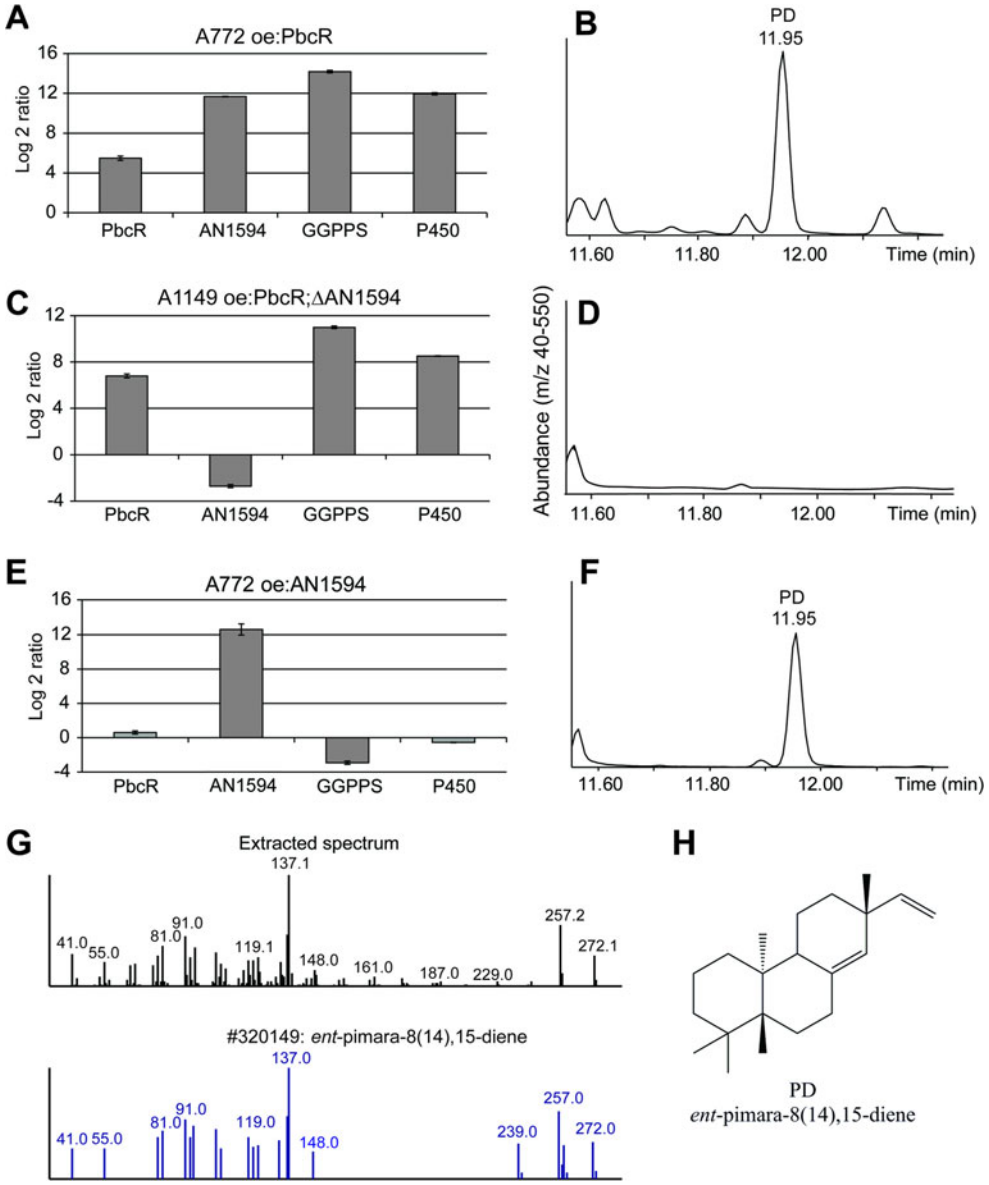

Fig. 1 AN1594 encodes for PD-specific terpene synthase and is required for PD production in *Aspergillus nidulans*. The transcript levels of *pbcR* as well as three PD cluster genes were analyzed by using qRT-PCR (expression fold-changes are presented in $\log_2$ values. *Error bars* represent SEM, $n = 9$). **a** In the A772 oe:PbcR reference strain, overexpression of *pbcR* (5.5-fold) activates transcription of AN1594 (putative PD synthase, 11.7-fold), GGPPS (AN1592, 14.2-fold), and cytochrome P450 (AN1598, 12-fold), when compared to non-modified A772 strain. **b** PD production in A772 oe:PbcR was confirmed by comparing the retention time 11.95 min with the retention time of purified PD using SPME-GC/MS. **c** When compared to non-modified A1149 strain, AN1594 expression is undetectable in AN1594 deletion strain (A1149 oe:PbcR;ΔAN1594), whereas transcript levels of three PD cluster genes are increased (*pbcR*, 6.8-fold; GGPPS, 11-fold; and P450, 8.5-fold). **d** SPME-GC/MS analysis confirms that PD is not produced in A1149 oe:PbcR;ΔAN1594. **e** In A772 oe:AN1594, 12.6-fold overexpression of AN1594 was measured using qRT-PCR, when compared to A772, and **f** PD production was observed by using SPME-GC/MS analysis. **g** The retention time 11.95 min of the product peak matched the retention time of purified PD, and the extracted spectra verified the identity and chemical structure of PD (Fig. 1g, h)

This construct was transformed into A772 oe:PbcR. *A. nidulans* A772 is capable of non-homologous end joining, leading to high frequency of randomly inserted genetic fragments in transformations. After analyzing approximately 60 transformants by diagnostic PCR, we could not detect insertion at the *pbcA* locus. Instead, multiple strains with random insertions were identified (A772 oe:PbcR; xCps/Ks) (data not shown). Interestingly, when some of these strains were grown and analyzed with GC/MS, we noticed accumulation of both PD and *ent*-kaurene (Fig. 2i). The *ent*-kaurene product peak was identified by matching extracted spectrum to known mass spectral library compounds (Fig. 2j). The extracted spectra verified the identity and chemical structure of *ent*-kaurene (Fig. 2k). Because the exchange construct had integrated

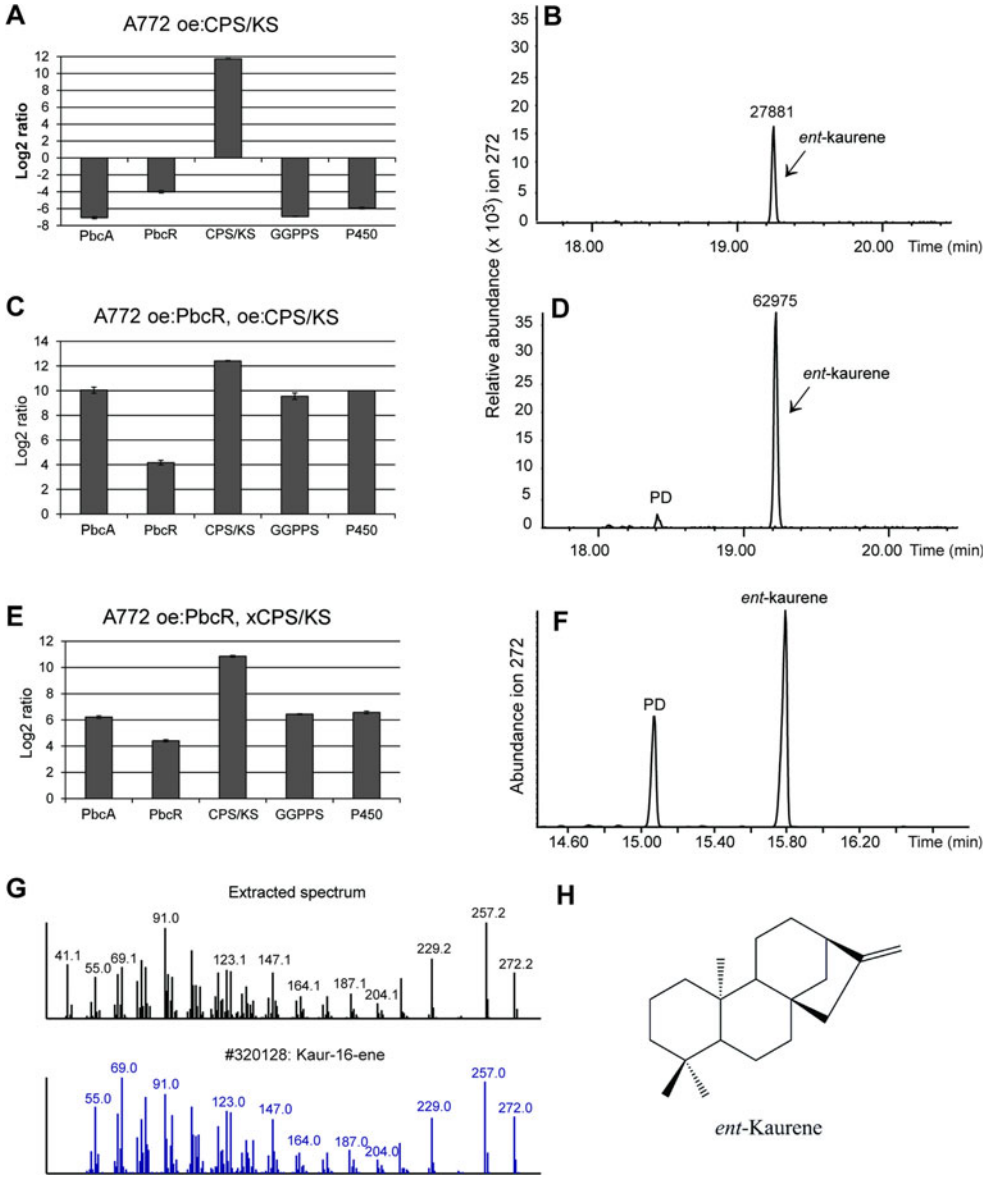

Fig. 2 Kaurene production in *A. nidulans*. Overexpression *cps*/*ks* results in *ent*-kaurene production in *A. nidulans*. The expression of *cps*/*ks* as well as four PD cluster genes was analyzed by using qRT-PCR. Expression ratios for PD cluster genes are presented in $\log_2$ values. *Error bars* represent SEM, $n = 9$. Expression of the heterologous *cps*/*ks* in different strains was verified with melting curve analysis. *Blue graphs* represent the *cps*/*ks* melting curves in heterologous strains. *Red* and *green graphs* represent the *cps*/*ks* melting curves in the A772 control strain. Mean C_p-value is indicated when applicable. **a** qRT-PCR analysis of A772 oe:Cps/Ks shows that PD cluster gene expression is not elevated, while **b** the expression of *cps*/*ks* is observed with the melting curve analysis. **c** *ent*-Kaurene production in A772 oe:Cps/Ks is detected by using SPME-GC/MS analysis (ion 272 chromatogram shown). **d** In A772 oe:PbcR; oe:Cps/Ks, expression of four PD cluster genes is increased (*pbcA*, 10-fold; *pbcR*, 4.2-fold; GGPPS, 9.5-fold; CYP, 10-fold) when compared to A772. **e** The expression of *cps*/*ks* in A772 oe:PbcR; oe:Cps/Ks is observed with the melting curve analysis. **f** SPME-GC/MS analysis confirms that both PD and *ent*-kaurene are produced in A772 oe:PbcR; oe:Cps/Ks. The comparison of the relative *ent*-kaurene levels shows that the amount of *ent*-kaurene in A772 oe:PbcR;oe:Cps/Ks (peak area 62,975) is about 2.4 times higher than in A772 oe:Cps/Ks (peak area 27,881). **g** PD cluster gene expression is elevated (*pbcA*, 6.2-fold; *pbcR*, 4.4-fold, GGPPS, 6.4-fold; and CYP, 6.6-fold) in A772 oe:PbcR; xCps/Ks, which harbors randomly integrated *cps*/*ks* fused to *pbcA* 5′- and 3′-UTR regions, when compared to A772. **h** The expression of *cps*/*ks* is observed with the melting curve analysis. **i** A772 oe:PbcR; xCps/Ks produces both PD and *ent*-kaurene. **j** *ent*-Kaurene product peak was identified with Palisade Spectral library compound match. **k** Chemical structure of *ent*-kaurene

randomly, the overexpression of *cps*/*ks* was not expected. qRT-PCR analysis of these strains confirmed elevated transcript levels for PD cluster genes, *pbcR* (4.7-fold), *pbcA* (6.2-fold), GGPPS (6.5-fold), and CYP (7-fold) (Fig. 2g) when compared to parental strain A772. Also, we noticed a clear expression of *cps*/*ks*. The mean C_p for *cps*/*ks* fluorescence in A772 oe:PbcR, xCps/Ks was 24.10, while the C_p for *cps*/*ks* fluorescence in A772 control was 39. Melting point

analysis showed that the signal observed for the control A772 was due to unspecific amplification in qRT-PCR (Fig. 2h). The mechanism of increased *cps*/*ks* expression is not known but could involve PbcR-mediated activation of transcription (Fig. 3i) or insertion of the exchange construct into transcriptionally active chromosomal loci (Fig. 3ii). Regardless of the precise mechanism, the data show that A772 oe:PbcR can be engineered to also produce heterologous diterpenes.

Overexpression of *Citrus unshiu* gamma-terpinene synthase results in gamma-terpinene production in *A. nidulans*

Having shown that *A. nidulans* is suitable for heterologous diterpene production, we further explored the possibility of producing heterologous monoterpenes in *A. nidulans*. Given the improved genetic tractability and relative lack of protease activity in yeast (compared to *Aspergilli*), the expression and activity of *C. unshiu* gamma-terpinene synthase was first tested in yeast *S. cerevisiae*. Since the removal of the amino-terminal plastid targeting sequence is needed to provide a fully active form of limonene synthase (Williams et al. 1998), we cloned N-terminally truncated and hemagglutinin (HA)-tagged gamma-terpinene synthase, codon-optimized for *Aspergillus* (gTerpS), into a yeast expression vector and transformed the resulting plasmid to *S. cerevisiae* (H2802 oe:gTerpS). GC/MS analysis of cell extracts incubated with geranyl diphosphate (GPP) indicated that the main product peak (retention time 18.825 min) in H2802 oe:gTerpS was gamma-terpinene. No peaks were detected in empty vector transformed cell lysates (H2802 e.v., Fig. 4a). Additional minor peaks were detected in H2802 oe:gTerpS lysates and were identified as monoterpenes with similar structures (Fig. 4b).

The gTerpS was then overexpressed in *A. nidulans* strain A772 by placing gTerpS under control of the constitutively active *gpdA* promoter (A772 oe:gTerpS). The insertion of gTerpS was verified by using PCR (data not shown). The expression of gTerpS in A772 oe:gTerpS was verified by using qRT-PCR. The mean C_p for gTerpS in oe:gTerpS was 19.12, and the melting point analysis revealed a product peak at mean Tm = 85.16 °C (Fig. 5c, blue graphs), while no products with Tm values were observed for the parental strain A772 (Fig. 5c, red graphs). GC/MS analysis detected a peak at retention time 5.014 min in A772 oe:gTerpS, while no peaks were observed in A772 control strain (Fig. 5a). The product peak at retention time 5.014 min was identified by matching extracted spectrum to known mass spectral library compounds (Fig. 5b). The extracted spectra verified the identity and chemical structure of gamma-terpinene with 99 % match (Fig. 5d). The data demonstrate production of gamma-terpinene in *A. nidulans*. To our knowledge, this is the first report of heterologous monoterpene production in *A. nidulans*.

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

GC/MS analysis shows PD accumulation in A772 oe:PbcR and A772 oe:PbcA. However, when comparing GC/MS spectra of these two strains, we noticed additional peaks in the strain A772 oe:PbcR that were absent in A772 oe:PbcA (Fig. 6a). The product peaks had retention times ranging from 11.612 to 14.479 min and were identified by matching extracted spectra to known mass spectral library compounds with match percentage of 80 or higher. The matching library compounds were mainly three-ring heterocyclic compounds containing functional groups and all had molecular mass of 270 (Fig. 6b). Many of these matching structures are closely related to known fluorescent compounds. Interestingly, *A. nidulans* strain A4 overexpressing *pbcR* (oe:PbcR) fluoresces when observed under UV fluorescence, whereas the A4 control strain does not (Fig. 6c).

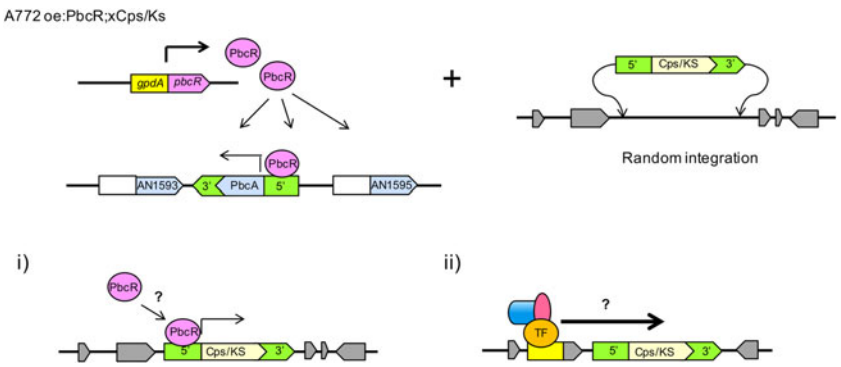

Fig. 3 Schematic diagram of *A. nidulans* genetic modification. *Cps*/*ks* flanked by *pbcA* 5′ promoter and 3′ terminator regions is randomly integrated to A772 oe:PbcR. Two possible models for *cps*/*ks* gene activation in A772 oe:PbcR: *i* PbcR-mediated activation of *cps*/*ks* transcription. PbcR recognizes the *pbcA* promoter and recruits transcriptional machinery to initiate transcription of *cps*/*ks*. *ii* Exchange construct is inserted into a genomic locus with high transcriptional activity that leads to increase in *cps*/*ks* transcription

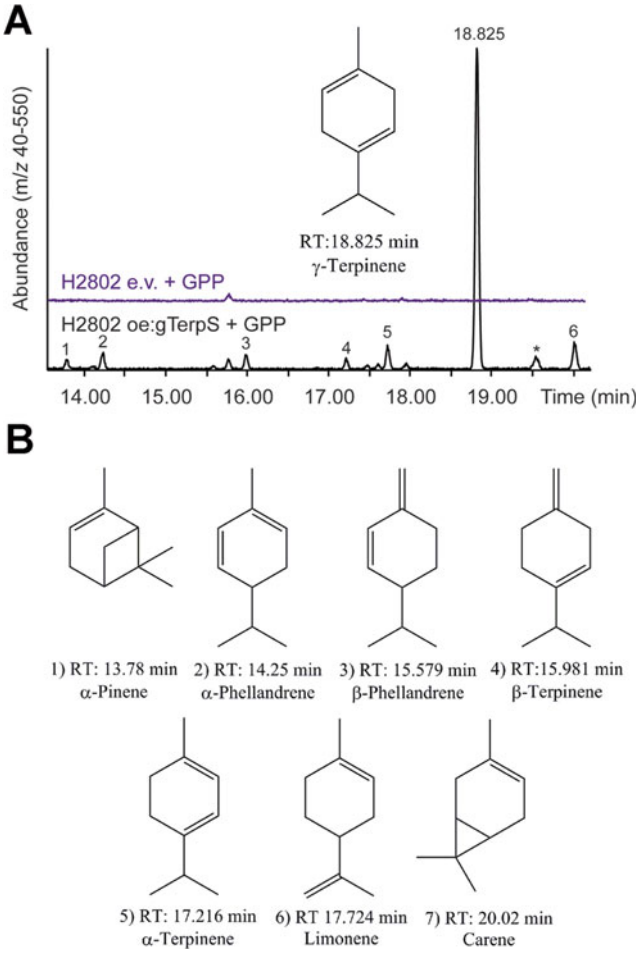

Fig. 4 Gamma-terpinene synthase enzymatic activity in *S. cerevisiae*. Enzymatic activity of *Aspergillus* codon-optimized and HA-tagged *C. unshiu* gamma-terpinene synthase (gTerpS) was measured in *S. cerevisiae* H2802. H2802 oe:gTerpS or H2802 e.v. cell lysates were incubated with GPP and the products analyzed by using SPME-GC/MS. **a** Main product peak in H2802 oe:gTerpS lysates at retention time 18.825 min is gamma-terpinene. This peak is absent in the H2802 e.v. controls. **b** Additional H2802 oe:gTerpS product peak compounds were identified by comparing the mass spectra on Palisade Complete 600K Mass Spectral Library. Chemical structures for the compounds are shown

Discussion

A. nidulans is a model organism and well-characterized filamentous fungus, yet many of its putative secondary metabolites are still unidentified. As many as 58 secondary metabolite gene clusters are predicted for *A. nidulans*, and many are linked to known secondary metabolite biosynthesis (Andersen et al. 2013; Inglis et al. 2013). We previously identified the first diterpene gene cluster in *A. nidulans* (Bromann et al. 2012). The gene cluster borders were predicted using biological data and bioinformatics (Andersen, Nielsen et al. 2013), and both methods agreed on prediction of the cluster borders. The cluster encodes genes AN1592–AN1599, responsible for *ent*-pimara-8(14),15-diene (PD) biosynthesis. *A. nidulans* AN1594 is a putative terpene synthase encoding gene (Bromann et al. 2012). In this report, we analyzed AN1594 in more detail. In an *A. nidulans* strain overexpressing *pbcR*, deletion of AN1594 abolished PD production. Overexpression of AN1594 alone resulted in PD production. These data demonstrate that AN1594 is indispensable for PD production and confirm that AN1594 encodes PD-specific synthase in *A. nidulans*. Therefore, we named this gene PbcA, as the synthase gene in the PbcR-regulated pimaradiene biosynthetic cluster.

The PD gene cluster contains putative genes for hydroxymethyl glutaryl coenzyme A (HMG-CoA) reductase and GGPPS (David et al. 2008; Bromann et al. 2012; Andersen et al. 2013). HMG-CoA reductase is a rate-limiting enzyme in the isoprenoid-producing mevalonate pathway (Luskey and Stevens 1985; Burg and Espenshade 2011). GGPPS links the isoprenoid precursors, isopentenyl diphosphate (IPP) and dimethyl allyl diphosphate (DMAPP), to form the diterpene-specific building block, GGPP (Chen et al. 1994). We previously hypothesized that PD cluster precursor synthase genes were needed for PD production (Bromann et al. 2012). However, the overexpression data presented in this study shows that PD production occurs in *A. nidulans* cells overexpressing *pbcA* alone, even when the expression of PD cluster genes is undetectable. These results suggest that *A. nidulans* contains non-PD cluster genes responsible for GGPP production. In fact, a putative HMG-CoA reductase gene, AN3817, has been annotated in the *A. nidulans* genome (Galagan et al. 2005; David et al. 2008). IMG (Markowitz et al. 2014) BLAST search of AN1592 against *A. nidulans* FGSC A4 genome indicated the existence of four homologous GGPPS genes, including AN0654, AN8143, AN6810, and AN2611 (Mabey et al. 2004; Galagan et al. 2005; Wortman et al. 2009; Arnaud et al. 2012).

Terpenes are a diverse class of secondary metabolites with industrial and medical value. Due to their bioactivities, monoterpenes and diterpenes can be used as specialty chemicals and as pharmaceuticals (Garcia et al. 2007; Hillwig et al. 2011; Koziol et al. 2014). *E. coli* and *S. cerevisiae* are widely-used host organisms for biotechnological production of monoterpenes and diterpenes (Yamano et al. 1994; Newman et al. 2006; Farhi et al. 2011; Misawa 2011). However, genetic modifications are needed to increase the limited intracellular levels of isoprenoid precursors (Martin et al. 2003; Chang et al. 2007; Phelan et al. 2015). In this report, we show that *A. nidulans* can produce PD without PD cluster activation, suggesting that GGPP intracellular levels are sufficient to support heterologous diterpene production. In support of this hypothesis, we were also able to produce *ent*-kaurene in *A. nidulans* by overexpressing *F. fujikuroi* kaurene synthase (another fungal diterpene synthase). *ent*-Kaurene is a precursor in gibberellin (GA) biosynthesis. GAs are phytohormones and first identified as secondary products of the rice pathogenic fungus causing overgrowth symptoms in rice. *ent*-Kaurene is

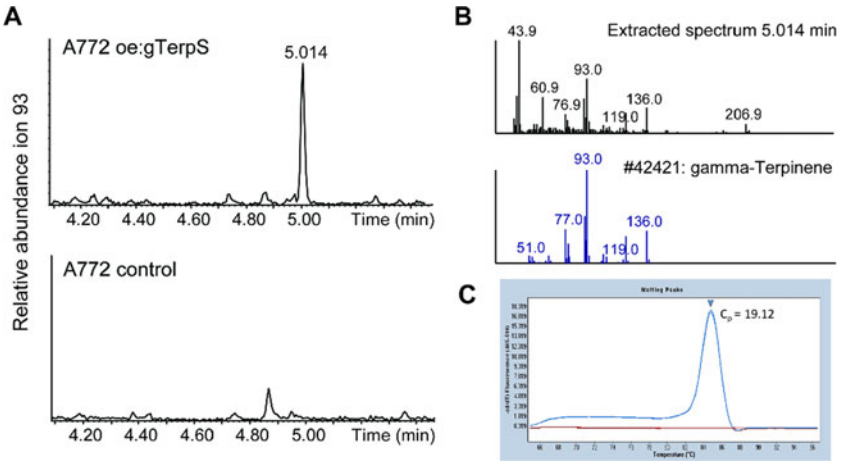

Fig. 5 Gamma-terpinene production in *A. nidulans*. Overexpression of *Aspergillus* codon-optimized and HA-tagged *C. unshiu* gamma-terpinene synthase (gTerpS) results in gamma-terpinene production in *A. nidulans*. gTerpS was overexpressed in *A. nidulans* A772 (oe:gTerpS), and the resultant strain was analyzed by using qRT-PCR and GC/MS. **a** An A772 oe:gTerpS-specific product peak (retention time 5.014 min) is detected by using SPME-GC/MS. The ion 93 chromatogram is shown. **b** Comparison of the extracted spectrum to Palisade Complete 600K Mass Spectral Library compounds identifies this peak as gamma-terpinene. **c** qRT-PCR melting curve analysis verifies gTerpS expression in A772 oe:gTerpS. *Blue graphs* represent gTerpS melting curves in A772 oe:gTerpS. *Red graphs* represent the melting curves for gTerpS in A772. The mean C_p value is indicated where applicable

also a precursor for steviol (Richman et al. 1999). Steviol glycosides are used as sweeteners, and steviol derivatives can be used in pharmaceutical applications (Yadav and Guleria 2012).

Given that intracellular GGPP levels in *A. nidulans* are sufficient to support diterpene production, we anticipated that overexpression of PD cluster genes (specifically, HMG-CoA reductase and GGPPS) would further increase the pool of intracellular GGPP and increase diterpene production. Expression of isoprenoid precursor genes has been used to boost heterologous terpene production in *E. coli* and *S. cerevisiae* (Alonso-Gutierrez, Chan et al. 2013, Yuan and Ching 2014). Also, in *F. fujikuroi*, overexpression of *F. fujikuroi* GGPPS (*ggs2*) together with *cps*/*ks* increases gibberellic acid (GA) formation (Yuan and Ching 2014). However, overexpression of *F. fujikuroi* HMG-CoA reductase (*hmgR*) together with FPP synthase (*fppS*) leads to decreased formation of GA (Albermann et al. 2013). Since the N-terminal part of HmgR is important for sterol-guided protein degradation, overexpression of *hmgR* with *fppS* was suggested to elevate the ergosterol pathway precursor molecules, resulting in negative feedback regulation. However, overexpression of N-terminally truncated *hmgR* was expressed in *F. fujikuroi*; the levels of GAs were drastically elevated (Albermann et al. 2013). When we aligned *A. nidulans* PD cluster HMG-CoA reductase gene, AN1593, *F. fujikuroi hmgR*, and the annotated *A. nidulans* HMG-CoA reductase gene, AN3817, we noticed that the AN1593 is significantly shorter consisting only of the enzymatic domains (Figure S6). This could suggest that AN1593 functions independently of ergosterol feedback regulation. Interestingly, when *cps*/*ks* was expressed in an *A. nidulans* strain with activated PD cluster genes (A772 oe:PbcR; oe:Cps/Ks), we observed increased kaurene production than in the strain overexpressing *cps*/*ks* alone (A772 oe:Cps/Ks) by using our semiquantitative GC/MS analysis. The relative *ent*-kaurene amounts in these two strains suggest that the upregulation of *pbcR* and subsequent activation of the PD biosynthetic cluster is beneficial for heterologous *ent*-kaurene production. The increase in *ent*-kaurene production could be due to activated PD cluster genes and consequently higher intracellular precursor availability for diterpene production. Regardless of the exact mechanism, we demonstrate that genetic modification can be used to enhance diterpene production in *A. nidulans*.

In *A. nidulans*, as in any heterologous production host, modulation of endogenous isoprenoid pathway gene targets is needed to direct carbon fluxes into the production of desired molecules. Given the complex interactions between intracellular carbon fluxes, simple up or down regulation of isoprenoid pathway genes might be insufficient for optimal production of heterologous compounds (Sun et al. 2014). We attempted to exchange *pbcA* with *cps*/*ks* in order to guide GGPP flux toward kaurene production in A772 oe:PbcR. Because strain A772 is capable of functional non-homologous end joining, no insertions at the *pbcA* locus were identified. Instead, the Cps/Ks exchange fragment was inserted randomly in multiple strains. Interestingly, expression of *cps*/*ks* and *ent*-kaurene production was observed in these strains. This was somewhat unexpected given that the exchange construct was under the regulation of the *pbcA* 5′-UTR. A logical mechanism for the activation of *cps*/*ks* expression would be PbcR transcription factor binding to *pbcA* 5′-UTR. Another possible mechanism for increased expression of *cps*/*ks* in A772 oe:PbcR could be the construct insertion into a transcriptional "hot spot," where elevated

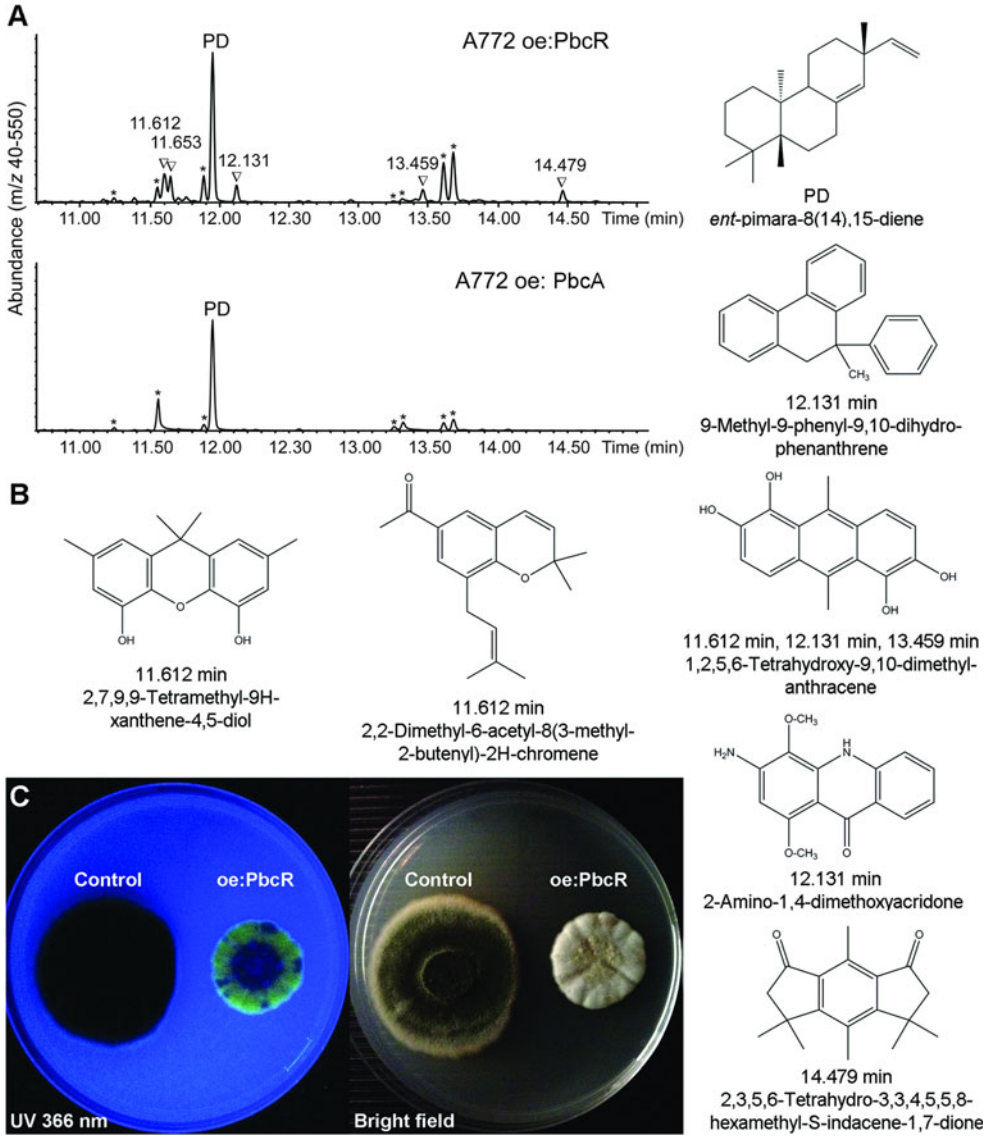

Fig. 6 PD gene cluster activation and heterocyclic compound production in *A. nidulans*. **a** Additional A772 oe:PbcR-specific peaks (marked with *triangles*) are detected by using SPME-GC/MS. These peaks are not present in A772 oe:PbcA spectra. **b** Peak compounds were identified by comparing the mass spectra on Palisade Complete 600K Mass Spectral Library. Chemical structures are shown for the closest matches for the peaks at retention times 11.612, 11.653, 12.131, 13,459, and 14,479 min. Some retention time peaks matched multiple compounds with similar accuracy. **c** Bright field and UV-fluorescence images of A4 and A4 oe:PbcR. *A. nidulans* strain overexpressing *pbcR* (A4 oe:PbcR) is fluorescent under UV light

transcriptional activities of the surrounding genes lead to expression of the inserted construct. For example, this has been observed in yeast (Brem et al. 2002). Analysis of PbcR binding to *pbcA* 5′-UTR (promoter) area would be needed to confirm PbcR-assisted upregulation of expression.

The monoterpene, gamma-terpinene, can be used as a precursor molecule in the production of bio-based terephtalic acid (Asikainen et al. 2013) but has not been produced in *A. nidulans*. Heterologous production of monoterpenes has primarily been studied in *S. cerevisiae* and *E. coli* in which the precursor pathway for GPP has been introduced (Kirby and Keasling 2008; Alonso-Gutierrez et al. 2013; Liu et al. 2013; Formighieri and Melis 2014; Ignea et al. 2014; Zhou et al. 2015). Monoterpene synthases and monoterpenes are rarely identified in fungi. In fact, the only fungal monoterpene synthase identified is 1,8-cineole synthase in *Hypoxylon* sp., *hyp3* (Shaw et al. 2015). No monoterpene biosynthetic genes have been identified by using genomic mining of *A. nidulans* (Wortman et al. 2009; Arnaud et al. 2012; Nielsen et al. 2013; Inglis et al. 2013). However, when we overexpressed N-terminally truncated *C. ushiu* HA-tagged gamma-terpinene synthase in *A. nidulans* A772, we observed gamma-terpinene production. The ability of *A. nidulans* to produce gamma-terpinene indicates the existence of an intracellular GPP pool.

This suggests that *A. nidulans* could harbor still unidentified monoterpene specific synthases. The nearest *hyp3* ortholog in *A. nidulans* is AN3277. BLAST (Altschul et al. 1990) search of AN3277 reveals that it is part of the isoprenoid biosynthesis C1-superfamily and contains a substrate binding pocket, a substrate-Mg^{2+} binding site, and two aspartate rich regions. Other known AN3277 orthologs encode aristolochene synthases in *Sphaerulina musiva*, *Neofusicoccum parvum*, and *Penicillium roqueforti*. Although *hyp3* codes for monoterpene synthase, it is also orthologous to sesquiterpene aristolochene synthases. It would be interesting to further analyze the sesquiterpene-like synthase genes in *A. nidulans*, to test if they in fact encode for monoterpene biosynthetic genes.

Although the production of gamma-terpinene in *A. nidulans* was clearly observed, production levels were lower compared to PD or kaurene production levels (data not shown). Isoprenoid precursors and pathway intermediates are sometimes toxic to microbial hosts (Martin et al. 2003; Sivy et al. 2011). Low gamma-terpinene yields could be due to low and more tolerated intracellular GPP levels in *A. nidulans*. Introducing a heterologous GPP precursor synthase, or identifying and upregulating the putative endogenous GPP precursor synthase(s), could increase the GPP pool and subsequent yield of monoterpenes. Although unlikely, the added HA tag could potentially interfere with the enzyme function. However, we characterized both N-terminally truncated HA-tagged and untagged *C. unshiu* gamma-terpinene synthases in *S. cerevisiae* and observed identical enzyme activities (Fig. 5 and data not shown). The full-length *C. unshiu* gamma-terpinene synthase did not show any activity (data not shown). Although further engineering of *A. nidulans* would be needed for industrial gamma-terpinene production, our results show that *A. nidulans* could be a potential target host for monoterpene production. As a close relative to industrial *Aspergilli*, the methods developed for *A. nidulans* could be useful in these industrial species as well.

The main product of the strain A772 oe:PbcR is PD. PD does not have any oxidation or functional groups attached to the main diterpene backbone. In our earlier report, we identified putative decorative enzymes in the PD gene cluster that are not required for PD biosynthesis in *A. nidulans*. However, here, we have compared the product composition of the *pbcA* overexpressing strain alone with a *pbcR* overexpressing strain; we identified additional compounds in the *pbcR* overexpressing strain. When compared to the strain overexpressing *pbcA* alone, multiple extra product peaks of mass 270 are seen in the *pbcR* overexpressing strain. GC/MS identification to the closest Palisade 600 library matches indicates that the compounds are mainly three ring structures with rearrangement and/or added functional groups in them. These could be derivatives of PD and caused by the action of the other upregulated genes in the PD cluster.

Secondary metabolite clusters usually have one or more backbone gene(s), including polyketide synthases, nonribosomal peptide synthetases, dimethylallyl tryptophan synthases, and terpene cyclases. Other hallmark genes include different modifying enzymes: oxidoreductases, oxygenases, dehydrogenases, reductases, and transferases (Lim et al. 2012). Within the *A. nidulans* PD cluster, *pbcA* is the backbone gene, and AN1595-AN1598 could potentially encode decorative enzymes for PD. AN1598 encodes a putative cytochrome P450 (CYP503B1), which are found in many secondary metabolite clusters (Kelly et al. 2009). These enzymes catalyze a broad range of decorative steps in secondary metabolite biosynthesis (Renault et al. 2014). Usually, CYPs orchestrate oxidation steps and require an oxidoreductase as a proton donor. Within the *A. nidulans* PD cluster, AN1596 encodes a putative short-chain dehydrogenase/oxidoreductase that is a potential partner for CYP503B1 oxidation. In addition, AN1595 and AN1597 encode glutathione S transferase and methyltransferase homologs. These enzymes can add functional groups to the terpene backbone. Stepwise deletions of these PD cluster genes would verify the precise role of these gene products as modifying enzymes.

The *pbcR* overexpressing strain is fluorescent in 366-nm UV light (Fig. 6c), whereas neither wild-type control strain nor the *pbcA* overexpressing strain were fluorescent (Fig. 6c, and data not shown). This suggests that the UV fluorescence is not caused by PD production. The mass spectral library matches of the additional MS/GC peaks seen in the *pbcR* overexpressing strain were structurally similar to derivatives of anthracene, indacene, chromene, xanthene, and phenanthrene. Future NMR verification can be carried out to accurately identify the specific compounds. Since the compounds seen in the *pbcR* overexpressing strain have structural similarity to known chromophores and fluorescent chemicals (Schwarz and Wasik 1976; Wang et al. 2001; Piloto et al. 2006; Xiong et al. 2010; Martinez-Peragon et al. 2014), we speculate that production of these compounds leads to *A. nidulans* fluorescence.

In conclusion, we characterized a novel diterpene-specific synthase, PbcA, in *A. nidulans* by using overexpression and deletion strains. This is also the first report of *ent*-kaurene and gamma-terpinene production in *A. nidulans* and demonstrates that *A. nidulans* has potential as a host organism for heterologous monoterpene and diterpene production.

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Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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