

Table of Contents

Figure S1. Phylogenetic tree of basidiomycete sesquiterpene synthases	2
Figure S2. Gene structure of <i>cubA</i> and alignment of CubA	3
Figure S3. SDS polyacrylamid gel electrophoresis of purified His ₆ -tagged CubA	4
Figure S4. Temperature optimum for CubA	5
Figure S5. pH dependence of CubA	6
Figure S6. CubA-catalyzed sesquiterpene formation <i>in vivo</i>	7
Figure S7. PCR analysis of <i>A. niger</i> transformants.....	8
Scheme S1. Biosynthetic scheme of CubA-produced sesquiterpenes/-terpenoids	9
Table S1. Sesquiterpene products detected <i>in vitro</i>	10
Table S2. Sesquiterpene products detected in <i>A. niger</i> tES02	11
Table S3. Oligonucleotides for PCR	12
Table S4. Oligonucleotides for qRT-PCR.....	12
Table S5. Amino acid sequences of terpene synthases used for phylogenetic analyses	13
References	14

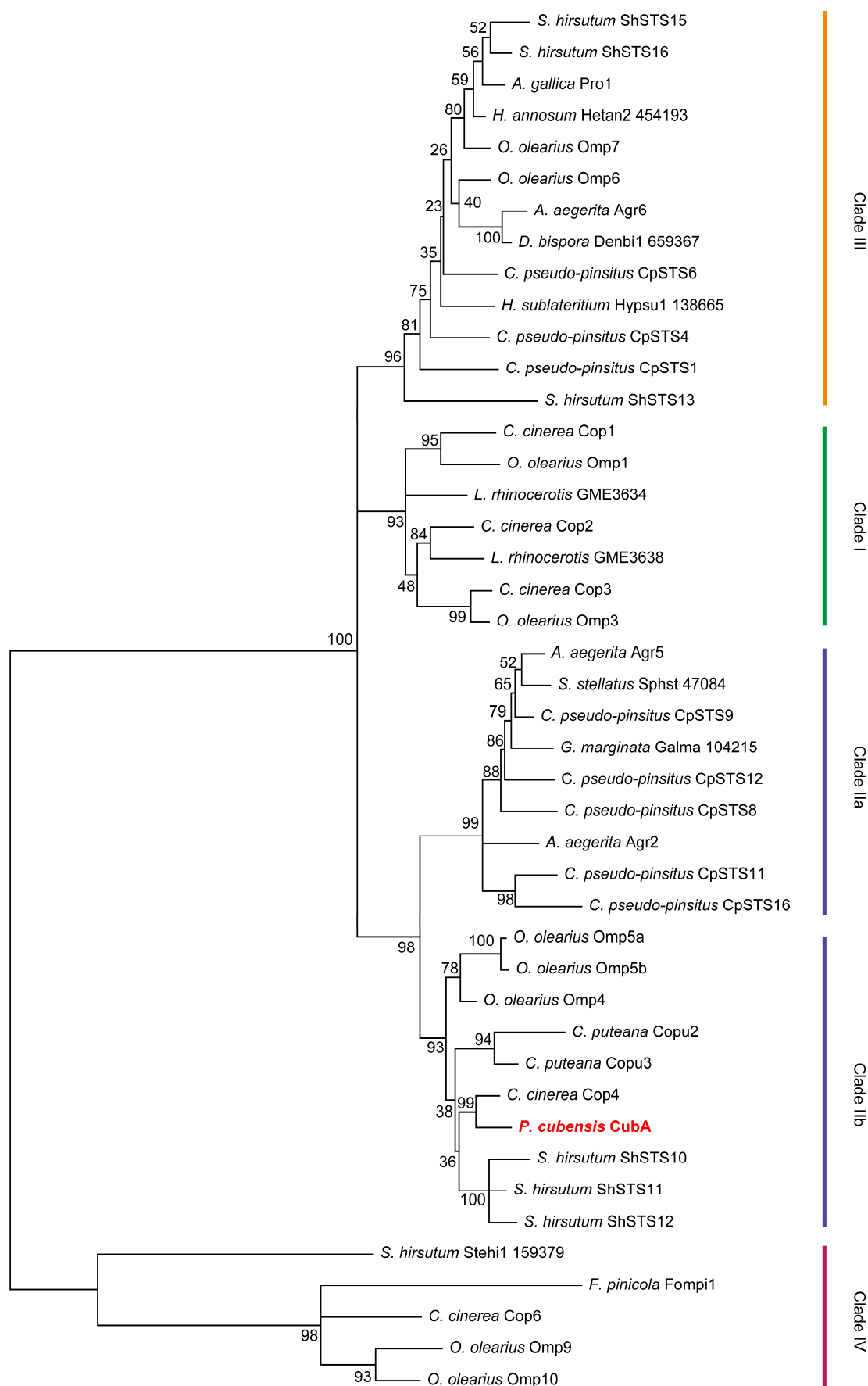
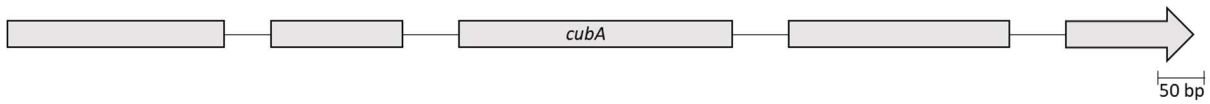


Figure S1. Phylogenetic tree of basidiomycete sesquiterpene synthases and placement of *P. cubensis* CubA. The tree largely follows phylogenies published by Nagamine et al. and Zhang et al.^[12c, 12e] Details of enzymes used to construct this tree are provided in Table S5.

A



B

<i>P. cubensis</i>	CubA	001MSTEQFVLPDLLESCPLKDATNPYYKEAAAESRAWINGYDIFTDRKRAEFIQQQNELLCSHVYWYAGR ⁰⁶⁸
<i>P. cyanescens</i>	TS10	001MSTSTQQFRIPDLFASCPLKDATNPYYKEAAAESRAWINSYDIFTDRKRAEFVQGANELLCSHVYFYAGR ⁰⁷⁰
<i>P. mexicana</i>	TS12	001MSPSPKQFRIPDLLESCPLKDGTPNPYYKEAAGSRAWINSYDIFTDRKRAEFVQGANELLCSHVYCFAGR ⁰⁷⁰
<i>P. serbica</i>	TS3	001MSTSTEQFRIIPDLFESCPLKDATNPYHKEATAESRAWINSYVFTDRKRAEFVQGANELLCSHVYFYAGR ⁰⁷⁰
<i>C. cinerea</i>	Cop4	001MRPTARQFTLPDLFSICPLQDATNPWYKQAAAESRAWINSYNIIFTDRKRAFFIQGSNELLCSHVYAYAGY ⁰⁷⁰
DEXXD		
<i>P. cubensis</i>	CubA	069EQLRRTCDFVN-LLFVV DEVSD EQNGKGARETGQVFFKAMKYPDWDDGSILAKVTKEFMARFTRLAGPRN ¹³⁷
<i>P. cyanescens</i>	TS10	071EQLRRTCDFVN-LLFVV DEVSD EQNGMDARETGQVFFKAMKYPEWDDGSILAKITKEFRARLMRLAGPRN ¹³⁹
<i>P. mexicana</i>	TS12	071EQLRRTCDFVSKPLFVV DEVSD EQNGLDARATGQIFLKAMKYANWDDGSILAKITKEFRARFLRIAGPNN ¹⁴⁰
<i>P. serbica</i>	TS3	071EQLRRTCDFVN-LLFVV DEVSD EQNGLDARATGQIFFKAMKYPEWDDGSILAKITKEFRARLMRLAGPRN ¹³⁹
<i>C. cinerea</i>	Cop4	071EQFRTECCDFVN-LLFVV DEISD DQNGQDARATGRI FVNAMRDAHWDGGSILAKITHEFRERFVRLAGPKT ¹³⁹
<i>P. cubensis</i>	CubA	138TKRFIDLCESYTACVGEAEELRERSELDDLASYIPLRRQNSAVLLCFALVEYILGIDLDEVEYEDEMFMK ²⁰⁷
<i>P. cyanescens</i>	TS10	140AKRFIDLCGSYTDVCGQEAELRERAEELDDLASYTPLRRQNSAVLLCFALVEYILGIDLSDVEYEDENFMK ²⁰⁹
<i>P. mexicana</i>	TS12	141ARRFIELCESYTECVGREAEELRERGELDDLASYMPLRRQNSAVLLCFALNEYNLGLIDLDDVEYQNETFMR ²¹⁰
<i>P. serbica</i>	TS3	140AKRFVDLCESYTECVGQEAELRERRELLDLASYTPLRRQNSAVLLCFALVECVLGLIDLSDVEYEDETFMK ²⁰⁹
<i>C. cinerea</i>	Cop4	140VRRFADLCESYTDVAREAEELRERNQVLGLNDFIALRRQNSAVLLCYSLVEYILGIDLDDVEYEDPTFAK ²⁰⁹
NDXXSXXXE		
<i>P. cubensis</i>	CubA	208AYWAACDQVCW TNDIYSYDME QSKGLAGNNIVSILMNENGTNLQETADYIGERCGEFVSDYMSAKSQISP ²⁷⁷
<i>P. cyanescens</i>	TS10	210AYWAACDHVCW ANDVYSYDME QSKGLAGNNIVSILMNENGTSLQETSDFIGARCSEFVTDYLSAKRELSP ²⁷⁹
<i>P. mexicana</i>	TS12	211AYWAACDHVCW ANDVYSYDME QSKGLAGNNIVTILMNENGTTLQETS DYIGVRCKEFCRRLSVGKSQSP ²⁸⁰
<i>P. serbica</i>	TS3	210AYWAACDHVCW ANDVYSYDME QSKGLAGNNIVSILMNENGTSLQETADYIGVRCSEFTADYLSAKSQSP ²⁷⁹
<i>C. cinerea</i>	Cop4	210AYWAACDFVCW ANDVYSYDME QAKGHTGNVVTVLMKEKDLSLQEASDYIGRECEKQMRDYLEAKSQLLQ ²⁷⁹
<i>P. cubensis</i>	CubA	278S--LGPEALQFIDFVGWYWMIGNIEWCFETPRYFGSRHLEIKETRVVHLRPKEVPEGLSSEDCIESDDE ³⁴³
<i>P. cyanescens</i>	TS10	280S--LGPEAAHFIESIGSWTIGNVAWSFETVRYFGPRHLEVKETRVVYLKPKVPEGLSSEDCIESDDE ³⁴⁵
<i>P. mexicana</i>	TS12	281S--LGPEAARFIEAIGSWMIGNIAWSFETVRYFGSRHLEVKETRVVHLRPREC--QLSEDSSESDEE ³⁴⁴
<i>P. serbica</i>	TS3	280S--LGPEAVRFIESIGSWMIGNIAWSFETVRYFGSRHLEVKETRVVYLRPKVPEPDLSEGCPESDGE ³⁴⁵
<i>C. cinerea</i>	Cop4	280STDLPQEAVRYIEALGYWVGNLWVSFESQRYFGAQHERVKATHVVHLRPSVLEASCDSDS-DSDC ³⁴⁵

Figure S2. The *P. cubensis* sesquiterpene synthase CubA. A) Gene structure of *cubA*. Lines represent introns. B) Alignment of the CubA amino acid sequence with *Coprinopsis cinerea* Cop4 and other *Psilocybe* clade IIb sesquiterpene synthases. The portions highlighted in green represent conserved motifs for Mg²⁺ cation coordination at the active site entrance.

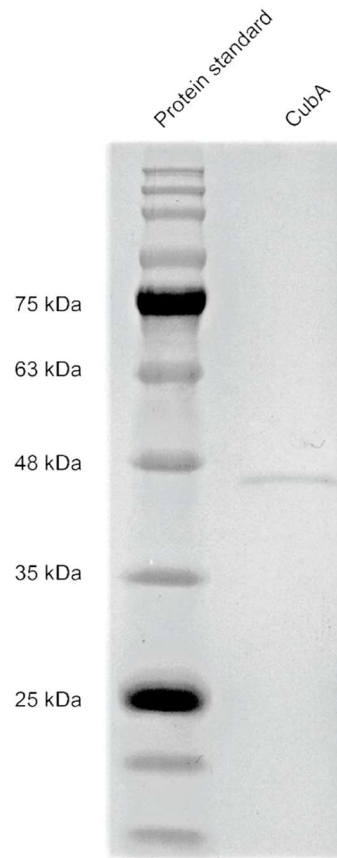


Figure S3. SDS polyacrylamide gel electrophoresis of purified His₆-tagged *Psilocybe cubensis* CubA. The calculated protein mass for CubA is 41.3 kDa. As protein standard, the Blue Eye prestained marker (Jena Bioscience) was loaded.

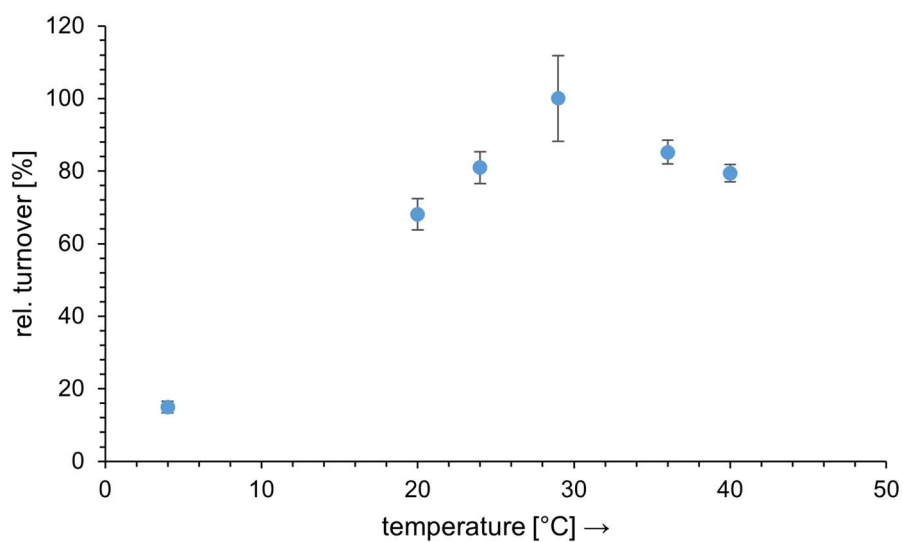


Figure S4. Temperature optimum of *P. cubensis* CubA. The activity was assessed *in vitro* by chromatographically measuring total product formation, i.e., areas under the curve of all identified sesquiterpene products in the GC-chromatogram. Error bars indicate the standard error (n = 3).

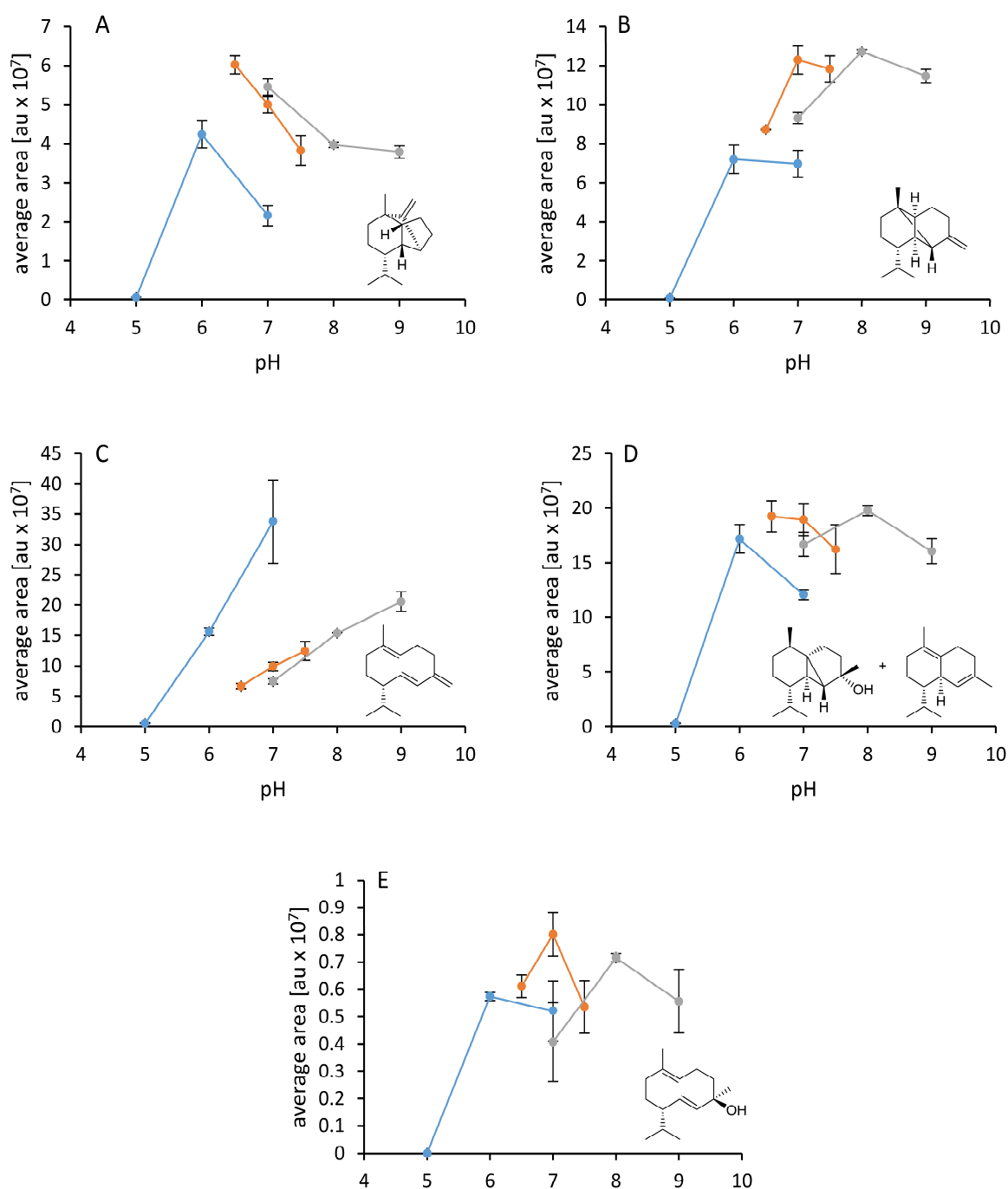


Figure S5. pH dependence of *P. cubensis* CubA product formation. The activities were assessed individually for all products > 3% of the cumulated peak area of the *in vitro* reaction. Panel A shows sativene formation, B: β -copaene, C: germacrene D, D: cubebol/ δ -cadinene, E: germacrene D-4-ol. Chemical structure in the respective panels represent the relative configuration. Relative abundance refers to the area under curve in arbitrary units for the signals of the respective compound. Error bars indicate the standard error (n = 3). Color code: blue – citrate buffer, orange – MOPS buffer, grey – TRIS buffer.

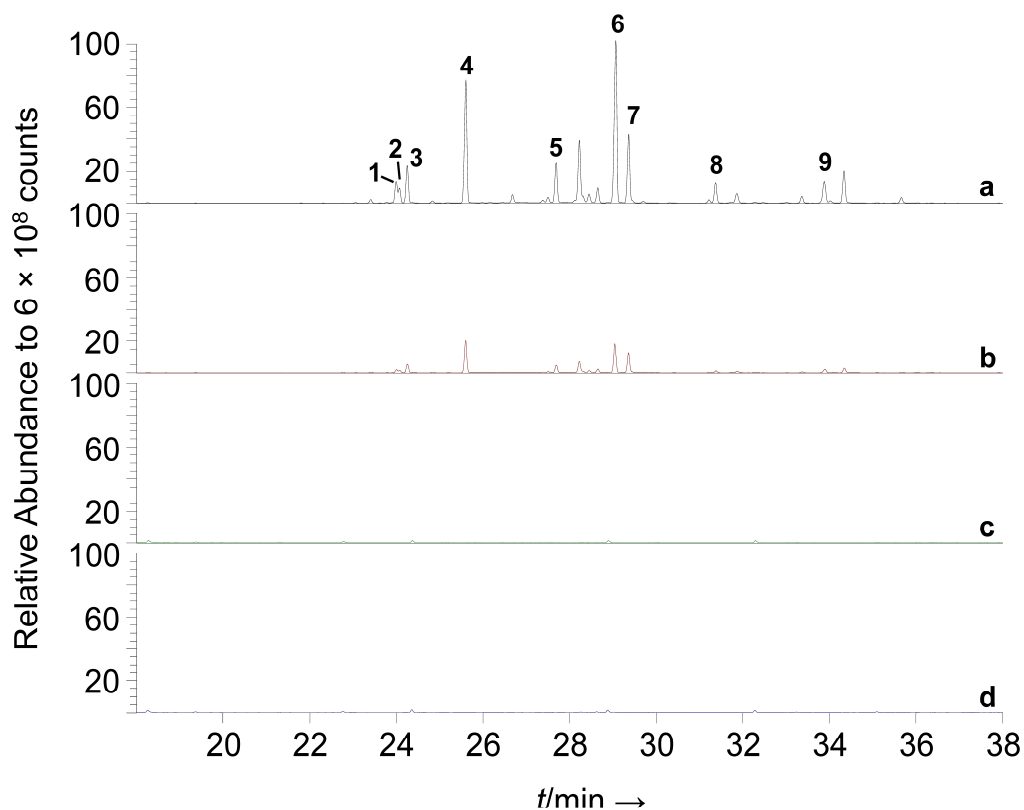


Figure S6. Gas chromatographic analysis of CubA-catalyzed sesquiterpene formation in *Aspergillus niger*. Shown are ethyl acetate extracts of a doxycycline-induced (a) and non-induced culture (b) of *A. niger* tES02. Chromatograms c and d represent extracts of induced and non-induced cultures of the empty vector control strain, *A. niger* tNAL000. Compound numbers are assigned as follows: β -cubebene (1), β -elemene (2), sativene (3), β -copaene (4), germacrene D (5), cubebol (6), δ -cadinene (7), germacrene D-4-ol (8), and τ -muurolol (9). Structures are shown in Scheme S1.

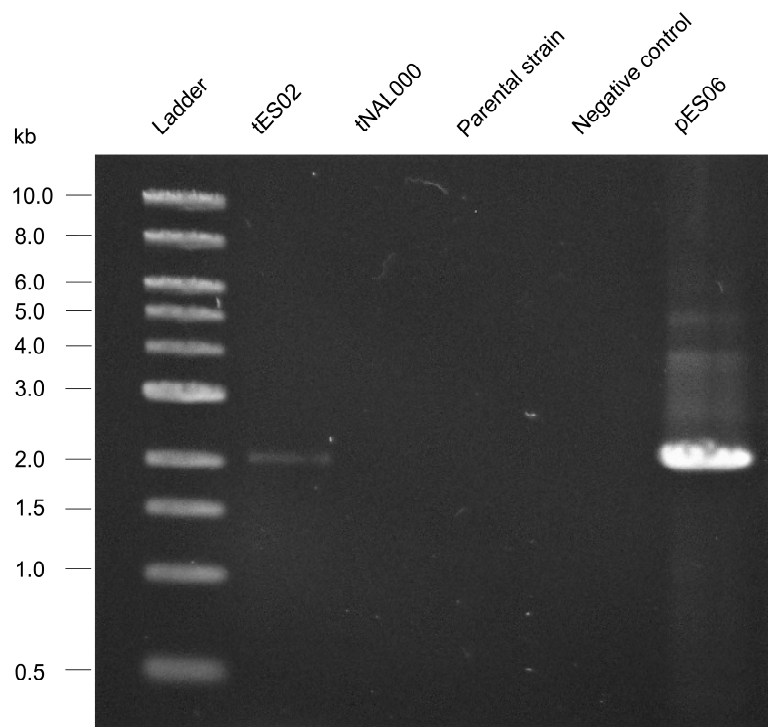
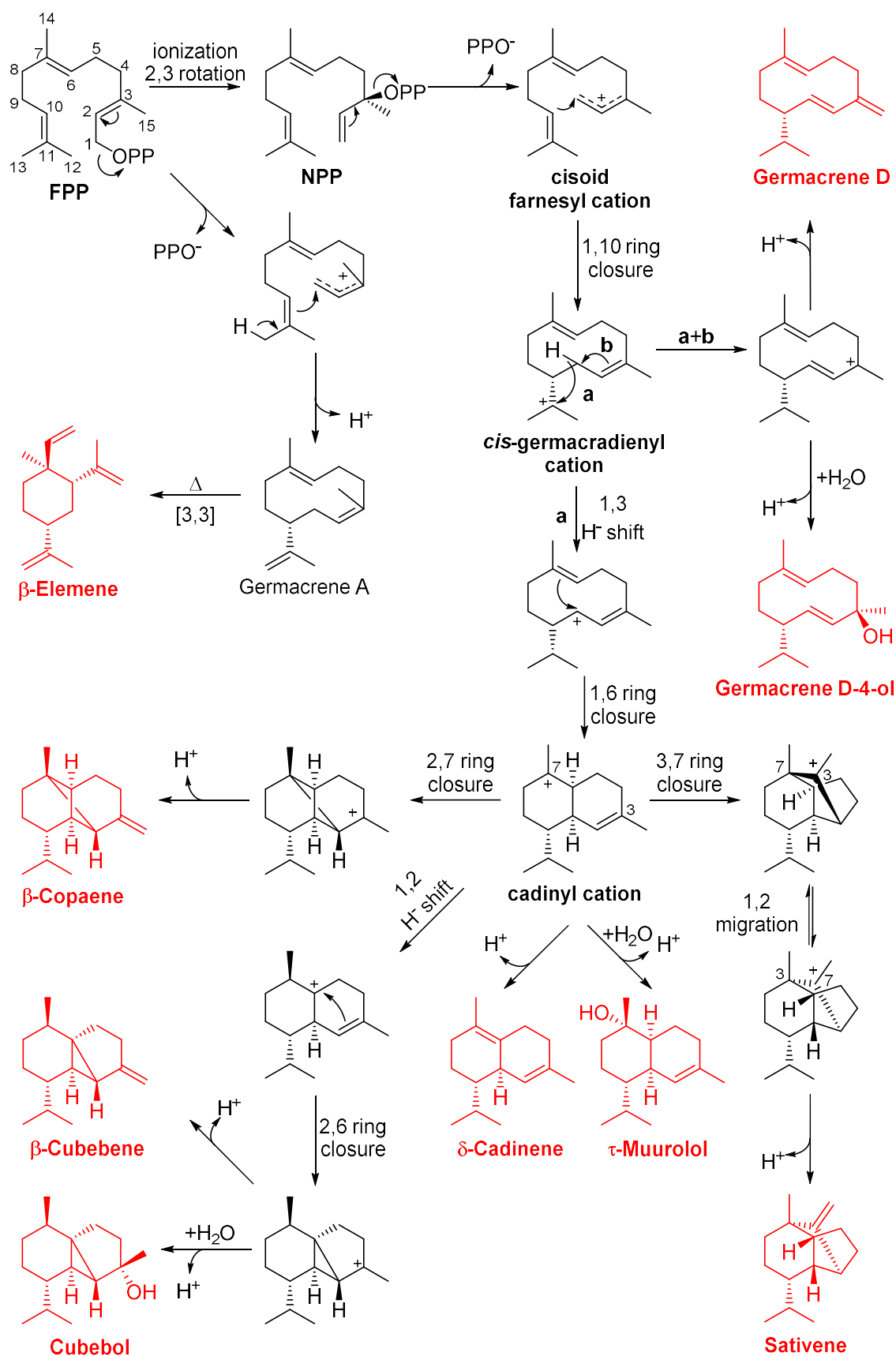


Figure S7. Agarose gel electrophoresis of PCR amplicons to verify the full-length integration of the *cubA* expression construct pES06 in the *Aspergillus niger* genome.



Scheme S1. Proposed scheme of the biosynthetic relationship of sesquiterpenoid products resulting from the terpene synthase activity of *Psilocybe cubensis* CubA. Confirmed products (relative stereochemistry shown) are highlighted in red. The scheme was compiled following various publications.^[17,18,35]

Table S1. Sesquiterpenes and -terpenoids identified after *in vitro* product formation assays with *Psilocybe cubensis* CubA. Percentages are referenced to total integral of the selected sesquiterpenoids.

Compound	t_R (min)	Integral	Integral (% of total area)	Retention Index (observed)	Retention Index (Adams) ^[30a]	Retention Index (NIST) ^{[30c]*}	Present in <i>Piper cubeba</i> oil? ^[20a,20b]	Present in <i>Angelica archangelica</i> oil? ^[20c]
β -Cubebene (1)	23.97	6.94 x 10 ⁶	0.3	1391	1387	1389	yes	no
β -Elemene (2)	24.05	2.57 x 10 ⁷	1.3	1393	1389	1391	yes	yes
Sativene (3)	24.23	1.06 x 10 ⁸	5.2	1398	1390	1396	no	no
β -Copaene (4)	25.57	3.02 x 10 ⁸	15.0	1431	1430	1432	no	yes
Germacrene D (5)	27.67	3.38 x 10 ⁸	16.7	1483	1484	1481	yes	yes
Cubebol (6)	29.03	9.02 x 10 ⁸	44.6	1517	1514	1515	yes	no
δ -Cadinene (7)	29.33	7.05 x 10 ⁷	3.5	1525	1522	1524	yes	yes
Germacrene D-4-ol (8)	31.35	2.67 x 10 ⁸	13.2	1577	1574	1574	yes	no
τ -Muurolol (9)	33.86	3.96 x 10 ⁶	0.2	1643	1640	1642	yes	no

*NIST: National Institute for Standards and Technology.

Table S2. Sesquiterpenes and -terpenoids identified after heterologous production of CubA in *Aspergillus niger* tES02. The host was induced with 30 $\mu\text{g mL}^{-1}$ doxycycline. Percentages are referenced to the total integral of the selected compounds.

Compound	t_R (min)	Integral	Integral (% of total area)	Retention Index (observed)	Retention Index (Adams) ^[30a]	Retention Index (NIST) ^{[30c]*}	Present in <i>Piper cubeba</i> oil? ^[20a,20b]	Present in <i>Angelica archangelica</i> oil? ^[20c]
β -Cubebene (1)	24.00	2.92 x 10 ⁸	3.8	1391	1387	1389	yes	no
β -Elemene (2)	24.07	1.69 x 10 ⁸	2.2	1392	1389	1391	yes	yes
Sativene (3)	24.25	5.21 x 10 ⁸	7.2	1397	1390	1396	no	no
β -Copaene (4)	25.61	1.87 x 10 ⁹	23.9	1430	1430	1432	no	yes
Germacrene D (5)	27.69	5.81 x 10 ⁸	7.5	1482	1484	1481	yes	yes
Cubebol (6)	29.07	2.57 x 10 ⁹	32.7	1516	1514	1515	yes	no
δ -Cadinene (7)	29.36	1.03 x 10 ⁹	13.3	1524	1522	1524	yes	yes
Germacrene D-4-ol (8)	31.37	3.06 x 10 ⁸	3.8	1576	1574	1574	yes	no
τ -Muurolol (9)	33.93	4.67 x 10 ⁸	6.1	1643	1644	1642	yes	no

*NIST: National Institute for Standards and Technology.

Table S3. Oligonucleotides used for PCR.

Name	Sequence in 5'-3' direction	Target
oES03	GGTGCCGCGCGGCAGCCATATGTCTACTGAACAATTCGTCC	<i>cubA</i> (cDNA)
oES04	CTCGAGTGCGGCCGCAAGCTTCTACTCATCGTCGGACTCAATGC	
oES11	TCTCATCACAGCACCATGCATATGTCTACTGAACAATTCGTCC	<i>cubA</i> (gDNA)
oES12	AATCACTGCTGTTATCCATGGTCACTCATCGTCGGACTCAATGC	
oMG370	GATCCTCTCTCTGATATTGTCG	<i>terA</i> promoter in pSMXpress 2

Table S4. Oligonucleotides used for qRT-PCR in this study. Primer efficiency, linear correlation coefficient (R^2), and expected amplicon size are indicated for the primer pairs used for qRT-PCR analysis.

Name	Sequence in 5'-3' direction	Target	Efficiency (R^2)	Amplicon size (bp)	
				gDNA	cDNA
oMG388	GTGTCAACAACAACATCATTCC	<i>gpdA</i>	95% (0.99984)	208	133
oMG389	AGATCAACGACAGAGACATCG				
oPS532	GATCAAGTCTGCTGGACCAA	<i>cubA</i>	98% (0.99991)	175	113
oPS533	TGCAGGTTTCGTTCCATTTTCG				

Table S5. Amino acid sequences of terpene synthases used for phylogenetic analyses.

Clade	Organism	Name	Accession number
I	<i>C. cinerea</i>	Cop1	EAU89322.2
	<i>C. cinerea</i>	Cop2	EAU85264.1
	<i>C. cinerea</i>	Cop3	EAU88892.1
	<i>L. rhinocerotis</i>	GME3634	KX281943.1
	<i>L. rhinocerotis</i>	GME3638	KX281944.1
	<i>O. olearius</i>	Omp1	jgi Ompol1 1311 MUSStwsD_GLEAN_10001317
	<i>O. olearius</i>	Omp3	jgi Ompol1 4636 MUSStwsD_GLEAN_10003938
IIa	<i>A. aegerita</i>	Agr2	A0A5Q0QNJ2
	<i>A. aegerita</i>	Agr5	A0A5Q0QSI8
	<i>C. pseudo-pinsitus</i>	CpSTS8	LC436352.1
	<i>C. pseudo-pinsitus</i>	CpSTS9	LC436353.1
	<i>C. pseudo-pinsitus</i>	CpSTS11	LC436355.1
	<i>C. pseudo-pinsitus</i>	CpSTS12	LC436356.1
	<i>C. pseudo-pinsitus</i>	CpSTS16	LC436360.1
	<i>G. marginata</i>	Galma_104215	jgi Galma1 104215 estExt_Genewise1Plus.C_370136
	<i>S. stellatus</i>	Sphst_47084	jgi Sphst1 47084 fgenesh1_pg.39_#_2
IIb	<i>C. cinerea</i>	Cop4	A0A5Q0QNJ2
	<i>C. puteana</i>	Copu2	A0A5Q0QSI8
	<i>C. puteana</i>	Copu3	LC436352.1
	<i>O. olearius</i>	Omp4	LC436353.1
	<i>O. olearius</i>	Omp5a	LC436355.1
	<i>O. olearius</i>	Omp5b	LC436356.1
	<i>S. hirsutum</i>	ShSTS10	LC436360.1
	<i>S. hirsutum</i>	ShSTS11	jgi Galma1 104215 estExt_Genewise1Plus.C_370136
	<i>S. hirsutum</i>	ShSTS12	jgi Sphst1 47084 fgenesh1_pg.39_#_2
III	<i>A. aegerita</i>	Agr6	MN146029.1
	<i>A. gallica</i>	Pro1	AGR34199.1
	<i>C. pseudo-pinsitus</i>	CpSTS1	LC436345.1
	<i>C. pseudo-pinsitus</i>	CpSTS4	LC436348.1
	<i>C. pseudo-pinsitus</i>	CpSTS6	LC436350.1
	<i>D. bispora</i>	Denbi1_659367	jgi Denbi1 659367 e_gw1.88.48.1
	<i>H. annosum</i>	Hetan2_454193	jgi Hetan2 454193 Genemark.7021_g
	<i>H. sublateralitium</i>	Hypsu1_138665	jgi Hypsu1 138665 e_gw1.30.115.1
	<i>O. olearius</i>	Omp6	jgi Ompol1 4774 MUSStwsD_GLEAN_10003820
	<i>O. olearius</i>	Omp7	jgi Ompol1 2271 MUSStwsD_GLEAN_10000831
	<i>S. hirsutum</i>	ShSTS13	jgi Stehi1 50042 e_gw1.2.358.1

	<i>S. hirsutum</i>	ShSTS15	jgi Stehi1 64702 e_gw1.15.166.1
	<i>S. hirsutum</i>	ShSTS16	jgi Stehi1 73029 estExt_Genewise1.C_2_t30189
IV	<i>C. cinerea</i>	Cop6	EAU89298.1
	<i>F. pinicola</i>	Fompi1	jgi Fompi3 84944 Fompi1.gm1.3035_g
	<i>O. olearius</i>	Omp9	jgi Ompol1 3258 MUSStwsD_GLEAN_10000543
	<i>O. olearius</i>	Omp10	jgi Ompol1 3981 MUSStwsD_GLEAN_10000292
	<i>S. hirsutum</i>	Stehi1_159379	jgi Stehi1 159379 gm1.8309_g

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