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Supporting Information

The Ketosynthase Domain Controls Chain Length in Mushroom Oligocyclic Polyketide Synthases

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Additional Experimental Procedures

Construction of chimeric PKS genes: Chimera I is a variant of CoPKS1 whose amino acids (aa) 1308-1417 were replaced by the equivalent portion of CoPKS4 (aa 1308-1415). Chimera II was the inverse PKS (CoPKS4, but aa 1308-1415 were replaced by aa 1308-1417 of CoPKS1). To create chimera I, the respective *copks1* fragments were amplified from pNAL020^[1] with primer pair oNAL098/145 (condition I, Table S3) and oNAL099/146 (condition II) respectively, while the *copks4* portion was amplified from pNAL006^[1] using the primer pair oNAL147/148 (condition II). The three amplicons were assembled to create pNAL030 (see cloning scheme; Table S2A, bottom) in a one-step cloning process via the NEBuilder HiFi DNA Assembly Master Mix, using *Nsi*I-linearized pSMX2-URA as vector. Plasmid pNAL031 was generated similarly (see cloning scheme; Table S2A, bottom), using primers oNAL149/150 (condition I) and oNAL153/154 (condition II) to amplify the *copks4* fragments from pNAL006 and primers oNAL151/152 (condition II) to amplify the *copks1* fragment from pNAL020.

Chimera III is a variant of CoPKS1 whose amino acids aa 1-401 were replaced by the equivalent portion of CoPKS4 (aa 1-401; representing the N-terminal KS domain), while chimera IV is the inverse construct (CoPKS4, aa 1-401 replaced by aa 1-401 of CoPKS1). To create chimera III, the *copks4* fragment encoding the N-terminal KS domain was amplified from pNAL006 with primer pair oNAL218/219 (condition III), while the following *copks1* downstream portion was amplified from pNAL020 using the primer pair oNAL220/221 (condition I). The two amplicons were assembled to pNAL039 via the NEBuilder HiFi DNA Assembly Master Mix, using *Nsi*I and *Nco*I opened pSMX2-URA as vector (see cloning scheme; Table S2A, bottom). Plasmid pNAL037, encoding chimera IV, was generated likewise, using primers oNAL214/215 (condition III) to amplify the upstream *copks1* fragment from pNAL020 and primers oNAL216/217 (condition I) to amplify the downstream *copks4* fragment from pNAL006 (see cloning scheme; Table S2A, bottom). Sequence data for native CoPKSs1 and 4 and for chimeras I – IV are given in Figure S1.

Construction of expression plasmids for ascomycete PKS genes: The intron-disrupted genes encoding MdpG and MdpF^[2] were amplified from genomic DNA of *A. nidulans* FGSC A4 with oNAL249/251 (condition IV, Table S3) and oNAL259/260 (condition III) respectively. The resulting PCR amplicons were electrophoretically purified in agarose gels and assembled to plasmids pNAL048 (to produce MdpG) and pNAL050 (MdpF) using the NEBuilder HiFi DNA Assembly Master Mix (NEB). The constructs to produce ACAS^[3] (pNAL055) and ACTE (pNAL057) were generated analogously. Both genes were amplified from genomic DNA of *A. terreus* SBUG402 with oNAL268/269 (for ACAS; condition IV) and oNAL272/273 (for ACTE; condition III), respectively. *Nco*I-linearized pSM_StrepTag_X_URA was used as vector backbone for pNAL048 and pNAL055, while *Nco*I-linearized pSM_StrepTag_X_PABA served as vector to yield pNAL050 and pNAL057.

Genetic analysis of transformants: Full-length integration of the respective expression cassettes (*terA* promoter/gene of interest/*trpC* terminator) was then confirmed by diagnostic PCR using oligonucleotides oMG370/oNAL156 (condition IV, Table S3). Three PCR-confirmed transformants were used as biological replicates for metabolite analyses. To compare the predicted with the actual splicing pattern, the cDNAs resulting from the expression of gDNA-based genes for ACAS and ACTE in *A. niger* tNAL059 and MdpG and MdpF in *A. niger* tNAL052 were analyzed. The mycelia were harvested and ground under liquid nitrogen. Total RNA was isolated from the respective expression strains using the SV Total RNA Isolation Kit (Promega) with additional digestion of residual genomic DNA by Baseline-ZERO DNase (Biozym). Reverse transcription was performed with anchored oligo-(dVT)₁₈ primers and RevertAid Reverse Transcriptase (ThermoFisher) at 42 °C for 60 min. The coding sequences were PCR amplified from the first strand reaction, using oligonucleotides oNAL295/306 (Table S2B) for *acas* (condition IV; Table S3), oNAL302/303 for *acte* (condition III), oNAL298/299 for *mdpG* (condition IV), and oNAL304/305 for *mdpF* (condition III). The gel-purified amplicons were ligated to cloning vector pJET1.2, to yield plasmids pNAL103 (*mdpG*), pNAL104 (*mdpF*), pNAL105 (*acas*) and pNAL106

(*acte*). Their inserts were sequenced to analyze the exon/intron-junction pattern. During this work, a previously unrecognized intron of the *mdpG* gene was identified and consequently a revision (Figure S9) of the published MdpG^[2] protein sequence is proposed.

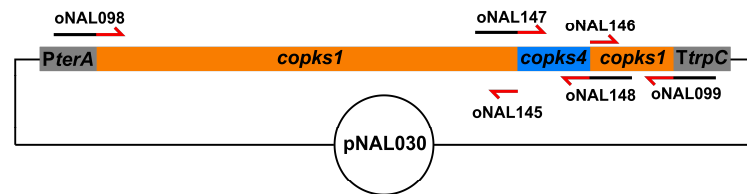
Table S1. Fungal strains and their genotypes.

Strain	Genotype	Reference
<i>Aspergillus niger</i> ATNT16 Δ pyrGx24	TetOn:terR_ble; Δ pyrG::ptrA	[4]
<i>Aspergillus niger</i> ATNT16_2_No. 17.1	TetOn:terR_ble; Δ pyrG::ptrA; Δ pabA	unpublished
<i>Aspergillus niger</i> tNAL000	TetOn:terR_ble; Δ pyrG::ptrA; PterA:His ₆ _pyrG	[1]
<i>Aspergillus niger</i> tNAL002	TetOn:terR_ble; Δ pyrG::ptrA; PterA:copks4_pyrG	[1]
<i>Aspergillus niger</i> tNAL024	TetOn:terR_ble; Δ pyrG::ptrA; PterA:copks1_pyrG	[1]
<i>Aspergillus niger</i> tNAL032	TetOn:terR_ble; Δ pyrG::ptrA; PterA:chimeraI_pyrG	This study
<i>Aspergillus niger</i> tNAL033	TetOn:terR_ble; Δ pyrG::ptrA; PterA:chimeraII_pyrG	This study
<i>Aspergillus niger</i> tNAL039	TetOn:terR_ble; Δ pyrG::ptrA; PterA:chimeraIV_pyrG	This study
<i>Aspergillus niger</i> tNAL041	TetOn:terR_ble; Δ pyrG::ptrA; PterA:chimeraIII_pyrG	This study
<i>Aspergillus niger</i> tNAL052	TetOn:terR_ble; Δ pyrG::ptrA; Δ pabA; PterA:mdpG_pyrG; PterA:mdpF_pabA	This study
<i>Aspergillus niger</i> tNAL059	TetOn:terR_ble; Δ pyrG::ptrA; Δ pabA; PterA:acas_pyrG; PterA:acte_pabA	This study

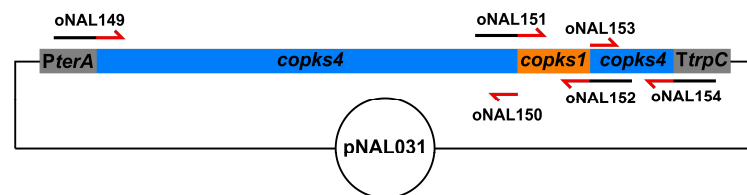
Table S2A. Oligonucleotides used to generate chimeric genes encoding CoPKSs. The positions of the oligonucleotide primers within the respective genes are shown in the graph below. Red: bases that pair with template sequences, black: bases required for overlap during Gibson assembly.

Name	Sequence (5' → 3')	Target	Purpose
oNAL098	TAACAACTTCTCATCACAGCACC ATGCCACCAAACTGCTAAC	<i>copks1</i>	Construction of pNAL030
oNAL099	CGGTTCAGATTGAAATCACTGCTG TCATCCCGCCTTGTTCAACCAACC	<i>copks1</i>	Construction of pNAL030
oNAL145	AGGACGTAATATCTGTAATAGCG	<i>copks1</i>	Construction of pNAL030
oNAL146	CCAGGCCGTACACCCCTTC	<i>copks1</i>	Construction of pNAL030
oNAL147	GGCAGCGCTATTACAGATATTACGTCCT ACACCTTTACCCAAATCGCTCC	<i>copks4</i>	Construction of pNAL030
oNAL148	TGGATAAGCAGAAGGGGTGTACGGCCTGG TGCGTATTGTATAACCTCTGG	<i>copks4</i>	Construction of pNAL030
oNAL149	ATCATTTAACAACTTCTCATCACAGCACC ATGCCACCAAACTACTGTTAAC	<i>copks4</i>	Construction of pNAL031
oNAL150	AGGACGTAGCAACTGTAATAGC	<i>copks4</i>	Construction of pNAL031
oNAL151	AACTTGACATCGCTATTACAGTTGCTACGTCCT GCGCCCTCACCCAAATCG	<i>copks1</i>	Construction of pNAL031
oNAL152	TCAGCAGAAGGGGTGTACGGCCTGGTGC GTGTTGTACAACCTCTGGATTAG	<i>copks1</i>	Construction of pNAL031
oNAL153	GCACCAAGGCCGTACACC	<i>copks4</i>	Construction of pNAL031
oNAL154	ACGGTTCAGATTGAAATCACTGCTGTTATC TCACCCCATCTTGTTCAACCGG	<i>copks4</i>	Construction of pNAL031
oNAL214	ATTAAACAACTTCTCATCACAGCACCATGCAT CCACCAAACACTGCTAACAAAGC	<i>copks1</i>	Construction of pNAL037
oNAL215	TCAAAGAAGGGTTGAAGAGTTGGATTCAATCTGGATGG ATGGAAATCGGCCTGAGGA	<i>copks1</i>	Construction of pNAL037
oNAL216	ACGCCGTGCGATACCTCCTCAGGCCGATTTCAT CCATCCAGATTGAATCCAATC	<i>copks4</i>	Construction of pNAL037
oNAL217	ATACGGTTCAGATTGAAATCACTGCTGTTATCCATG TCACCCCATCTTGTTCAACC	<i>copks4</i>	Construction of pNAL037
oNAL218	CATTTAACAACTTCTCATCACAGCACCATGCAT CCACCAAATACTGTTAACAAAGC	<i>copks4</i>	Construction of pNAL039
oNAL219	AAGAAGGGTTTGAGCGTTGGATTCAATCGGGATGG ATGAAATCGGCCTGAGGAG	<i>copks4</i>	Construction of pNAL039
oNAL220	AACGCCGTGCGATACCTCCTCAGGCCGATTTCAT CCATCCCGATTGAATCCAAC	<i>copks1</i>	Construction of pNAL039
oNAL221	ATACGGTTCAGATTGAAATCACTGCTGTTATCCATG TCATCCCGCCTTGTTCAACC	<i>copks1</i>	Construction of pNAL039

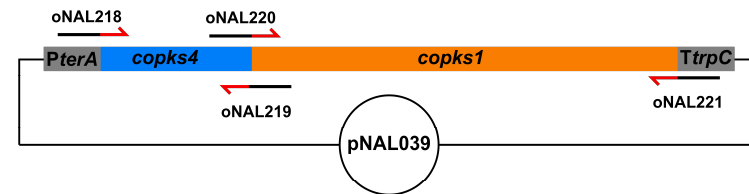
chimera I



chimera II



chimera III



chimera IV

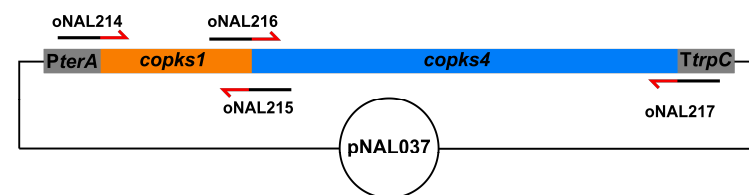


Table S2B. Other oligonucleotides used in this study. Red: bases that pair with template sequences, black (optional): bases required for overlap during Gibson assembly.

Name	Sequence (5' → 3')	Target	Purpose
oMG370	GATCCTCTCTCTGATATTGTCG	<i>PterA</i>	Proof of transgene integration
oNAL156	GTGAGGGTTGAGTACGAGATT	<i>TtrpC</i>	Proof of transgene integration
oNAL249	CACCATGCATTGGTCCCACCCCAGTTCGAGAAGCCCGTATATACTCCTCAATCAGG	<i>mdpG</i>	Construction of pNAL048
oNAL251	ACGGTTCAGATTGAAATCACTGCTGTTACCATGGATTATAGTACTCTAATAGCCAGC	<i>mdpG</i>	Construction of pNAL048
oNAL252	GTGTATGGCTCGTGTGACC	<i>mdpG</i>	Sequencing of pNAL103
oNAL259	CATTGGTCCCACCCCAGTTCGAGAAGCCATGGGCTCAGCCGAGCAGCATAAAGG	<i>mdpF</i>	Construction of pNAL050
oNAL260	AAACTATACGGTTCAGATTGAAATCACTGCTGTTAAAGCACTCCTACTCCAAACC	<i>mdpF</i>	Construction of pNAL050
oNAL268	TCACAGCACCATGCATTGGTCCCACCCCAGTTCGAGAAGGATTTACCCCGTCGACAGG	<i>acas</i>	Construction of pNAL055
oNAL269	TATACGGTTCAGATTGAAATCACTGCTGTTACCATGGCTGTAAATATTCTAGAAGCCAGC	<i>acas</i>	Construction of pNAL055
oNAL272	TGCATTGGTCCCACCCCAGTTCGAGAAGCCATGGAGCGGGGAGGCTACCGTCAAATC	<i>acte</i>	Construction of pNAL057
oNAL273	CCGAAACTATACGGTTCAGATTGAAATCACTGCTGTTATTACGGGCTGGATGTGACATC	<i>acte</i>	Construction of pNAL057
oNAL295	TTACCATGGGCTGTAATATTCTAG	<i>acas</i>	Construction of pNAL105
oNAL298	CCCGTATATACTCCTCAATCAG	<i>mdpG</i>	Construction of pNAL103
oNAL299	TTACCATGGATTATAGTACTCTAATAGC	<i>mdpG</i>	Construction of pNAL103
oNAL302	CCATGGAAGCGGGGAGGCTAC	<i>acte</i>	Construction of pNAL106
oNAL303	TTACGGGCTGGATGTGACATC	<i>acte</i>	Construction of pNAL106
oNAL304	CCCCAGTTCGAGAAGCCATGG	<i>mdpF</i>	Construction of pNAL104
oNAL305	TAAAGCACTCCTACTCCAAACC	<i>mdpF</i>	Construction of pNAL104
oNAL306	GATTTACCCCGTCGACAGG	<i>acas</i>	Construction of pNAL105

Table S3. PCR parameters. All reactions were initiated by a 30 second denaturation step at 98 °C.

Condition	Thermal cycling	Final elongation
I	35 cycles of 98 °C for 10 s, 60 °C for 15 s, 72 °C for 2:40 min	72 °C for 5 min
II	35 cycles of 98 °C for 10 s, 60 °C for 15 s, 72 °C for 45 s	72 °C for 5 min
III	35 cycles of 98 °C for 10 s, 60 °C for 15 s, 72 °C for 60 s	72 °C for 5 min
IV	35 cycles of 98 °C for 10 s, 58 °C for 15 s, 72 °C for 3:30 min	72 °C for 7 min

Table S4. Plasmids used in this study.

Plasmid name	Vector backbone	Gene	Reference
phis_SM-Xpress	pUC19	-	[4,5]
pSM_StrepTag_X_URA	pUC19	-	[1]
pSM_StrepTag_X_PABA	pUC19	-	unpublished
pNAL006	phis_SM-Xpress	<i>copks4</i> (gDNA)	[1]
pNAL020	phis_SM-Xpress	<i>copks1</i> (gDNA)	[1]
pNAL030	phis_SM-Xpress	<i>chimera I</i> (gDNA)	This study
pNAL031	phis_SM-Xpress	<i>chimera II</i> (gDNA)	This study
pNAL037	phis_SM-Xpress	<i>chimera IV</i> (gDNA)	This study
pNAL039	phis_SM-Xpress	<i>chimera III</i> (gDNA)	This study
pNAL048	pSM_StrepTag_X_URA	<i>mdpG</i> (gDNA)	This study
pNAL050	pSM_StrepTag_X_PABA	<i>mdpF</i> (gDNA)	This study
pNAL055	pSM_StrepTag_X_URA	<i>acas</i> (gDNA)	This study
pNAL057	pSM_StrepTag_X_PABA	<i>acte</i> (gDNA)	This study
pNAL103	pJET	<i>mdpG</i> (cDNA)	This study
pNAL104	pJET	<i>mdpF</i> (cDNA)	This study
pNAL105	pJET	<i>acas</i> (cDNA)	This study
pNAL106	pJET	<i>acte</i> (cDNA)	This study

Table S5. HPLC parameters. Eluents in gradient I were: 0.1 % formic acid in water (eluent A) and acetonitrile (eluent B). In case of gradient II, eluent B was 0.1 % formic acid in acetonitrile.

Gradient	Flow [mL min ⁻¹]	Time [min]	Eluent B [%]	Column
I	1	0.00	5	Zorbax Eclipse Plus C18 column (2.1 × 50 mm, 1.8 µm particle size)
		1.00	5	
		1.50	25	
		3.50	25	
	1.1	6.00	30	
		6.50	60	
	1	7.50	100	
II	0.2	0.00	5	Accucore C18 column (100 × 2.1 mm, 2.6 µm particle size)
		7	98	
		10	98	



MPPNTANKAAEIPPFEPPIAIIIGIGIRGPGGINSLTTLWDTLVERKSHCFPLAKDPRFHRRFNPDDFKALFDGIPDSENVLHSN
 LFDETPGLDRTYFSLSREAAAGMDVQKQLLLHVAHEALEDAGYSGVEDGSAFDPSTFGVYVATATDEVIQDPRDYDTDIYHLV
 RTERAFLSGQICFHFGLRGPSSSVDTVCSGSITAINDACRALATGDCRAAIAGGVHVVTVPVSGPISFYSIKRAGFLDRTGQCK
 PFLHNGTGYSRSEGCGLVVMKRLSDAIREGDRIHGVIIRGLCIRSMASPKFISQPNGVFQSVALAQAQVKISGVDPAAISFVEAH
 GPGTAKGDLAEVSSLCVLAQHRDNDNPLTVGSLKGNVGHAEAAASGTHSLAKVIAMFQRRRIPPQADFHP SRLNPTLKPFDFK
 HPRIIENEEDWNASHRIAIVGNFGASGNAGFMVVEEGSTFQPLEGRDVPKVSSPLPFVISAKDQATLVKLIHLYIDWLKQPS
 TFLTPLSDISYATTARRFTHPCMISVQADSHLDLAKKLQERPPMIDSSANRTSRQVAFCFSGQGGGERVDPNRNSTLYNFSASFT
 DAVDMCFRIVESESLIAEEDIAMLELFALEFGLMEMMWSWGINPVALAGHSFGEYVALVCAGVLTVRDALKLLGIRAAALIRAR
 CLDVPGKMAAVRLSVSDVNKCLEQQRSTHVELACVNSDNSVTLAGTPEDLESFRQELLKSYPAAGWHLLNNMTAAAFHSRQVQ
 ILADFTNACNEVKVHPSEMVLVSLGGLGKMCPVGDAVLQQHDYLVHRCHRETNRFGTATISDYQRRNVESETPQPDWIEIGSHSRI
 ISFISLASDQLKFPSHGKTAADGWTTALDTLMRLYSAGHVDFKDLHNDINPSAHHTDLPLYPFQFEPHVYPARREAKAISTT
 LASANEVELCPRPVSTELAPLLLNVHMAGYTLCPATTHVALLLAAAASSASSSQHEGRLAYKLFKLKVIAGFTNTTDGWLQVR
 RHLATSELEIIISNDDNKIHIITARAEVCKEQDMLESLSLYKPFILPFKSFKSLPTTDVLRKELAYSLEFNHTVNYGAHQVLDLV
 WIAEDGHQAWGYSTYPGGARTAEGVPTVLRDFSPMLIESTCQLIGFLMNTSMERKDGAEAFVTDELGECSIALSKLYETKYVEL
 YASFKMVDGGTAGLGNVFAFDDKGQLISAFRDVRMAKMKIYVLKRLIDRGRKPEQVVNTQHVAPEEVEEDNDANNDLEEKVIS
 VLKAALRLSEIPRDKTLGELGLDLSLTALIDVGVQLERLVPHRRDHLTIDPEGNLAALLQILRPAPSPKSLPTTSSTKEIKLTNG
 DLEVKAISTPPSLPITANGIPTANGVATTNVVPAVNGVTVNGVPKANGVPKANGVYTAKMPTEPATALIANLSPEMMEAIISSN
 PEVVQHAPGRTPLLLHDGGGTSFAYYSGLNLDRTVIGIHAPGLQEGKGMVNILHATNEYANARQYLYKHCPGHKSKLLIGGW
 SLGGTISITMAAMFPDLVAGVVTIDTTPPGVGLTAAEAESVLLHPWSRSDGIHGLVRKQLEQNTRALFANPEYKTTIRNTTV
 NVPVYVICAKDPFRPPESLHLQDSSSQWLIDFKEPQVAEVMWKSMLGERLLGVQIIPGNHWTMFTPANAKTTTEALRRGLDVI
 EGWLNKAG

Figure S1A: Amino acid sequence information for native CoPKS1 (1668 aa).



MPPNTVNKAAEIPPFEPPIAIVGIGIRGPGGINSLTTLWDTLVERKSHCFPLAKDPRFQRRFNPDDFKALFDGIPDSENVLHAN
 LFDETPGLDRTYFSLSREAAAGMDVQKQLLLHVAHEALEDAGYSGAEDGSAFDPSTFGVYVATATDEVIQDPRDYDTDIYHLV
 RTERAFLSGQICFHFGLRGPSSSVDTVCSGSITAINDACRALATGDCRAAIAGGVHVVTVPVSGPISFYCIKRAGFLDRTGQCK
 PFLQNGTGYSRSEGCGLVVMKRLSDAIREGDRIHGVIIRGLCIRSMASPKFISQPNGAFQSVALAQAQVKISGVDPAAISFVEAH
 GPGTAKGDLAEVSSLCVLAQHRAVDNPLTVGSLKGNVGHAEAAASGTHSLAKVIAMFQRRRIPPQADFHP SRLNPTLQPFDFK
 HPRIIENEEDWNASHRIAIVGNFGASGNAGFMVIEEGSTFQPLEGRDVPKVSSPLPFVISAKDQATLVKLIHLYIDWLKQPS
 TFLTPLSDISYAMTARRFIHPFMISVQADSHMDLVQKLQERPPMINGSANRTPREVAFCFSGQGGGERVDPNRDSTLYNFSASFT
 DAVDMCFRVAESENVAEEDVAILELFALEFGLVEMMWSWGKIPVALAGHSFGEYVALVCAGVLTVRDALKLLGIRAAALIRAM
 CLDVPGKMAAVRLPLSDVNKCLEQQKSTRVELACVNSDNSVTVAGTPEDLESFRQELLKWYPAASWHLLNNMTAAAFHSRQVQ
 ILADFTNACNEVKVHPSEMVLVSLGGLGKMCPVGDAVLQQHDYLVHRCHRETNRFGTATISDYQRRNVERETPQPDWLEIGSHSRI
 ISFITPASDQLKLPSHGKSAGEGWM TALDALMRLYSAGHVDFKDVHYDVNPSAHHTDLPLYPFQLEPHFYPARREVKASSTM
 LASTNEVQFCPRVPSTELAPLLLNVHMAGYTLCPATTHVALMMAAASTFASSSQEGRLAYKLSKLKVIAGFTNTTDGWLQVR
 RQSSTSDLEIIISNDDNKIHIITARAEVCKEQDLLESLSLYASFILPFKSFKFLPSTDVLRKELAYSLEFNHTVNYGPHGQVLDLV
 WIAEDGYQAWGYSTYPGGASTAEGVPSALRDFSPMLIESVCQLIGFLMNTSTNRKDGAEAFVTDELGDCSIASKLYETKYVEI
 YASYKMVDGGTAGLGNVFAFDDKGQLISAFRDVRMAKMKIYVLKRLIDRGRKPEQVVNTQHVASTEVEEDNVANDIDDKVIS
 VLKAALRLSEIPLDKTLGELGLDLSLTALIDVGVQLERLVPHRRDHLTIDPEGNLSLLQLLRPTPLPKSLPITTSSTKESKVEV
 DLATSPSLPIMPNGVPTTVNGVVPKPNALPTVNLHKNPANGAPANGVPTANGVPTADGTSTEPSQTLVANISPEMLQAIISSNPE
 VIQYAPGRTPLLLHDGGGTSFAYYSGLNLDRTVIGIHCPGLQEGKGIESVHHAANEYANARQYLYKQCPGHKSKVLIGGWSL
 GGTISIMMAALFPDLVAGVVTIDTTPPGVGLTAQQAESVLLHPWSRSDGIHGLVRRQLQLNTRASFAHPEYKTIIRVTAVNV
 PVYVLCALIDPFRPSESLDLPKETQWLLSFKQSDVAEVTWKELIGERLLGVQSVPGNHWTMFTPANVKATTEALKQGLDVIEA
 RLNKMKG

Figure S1B: Amino acid sequence information for native CoPKS4 (1666 aa).



MPPNTANKAAEIPPFEPPIAIIIGIGIRGPGGINSLTTLWDTLVERKSHCFPLAKDPRFHRRFNPDDFKALFDGIPDSENVLHSN
 LFDETPGLDRTYFSLSERAAGMDVQKLLHVAHEALEDAGYSGVEDGSAFDPSTFGVYVATATDEVIQDPRDYDTDIYHLV
 RTERAFLSGQICFHFGLRGPSSSVDTVCSGSITAINDACRALATGDCRAAIAGGVHVVPVSGPISFYSIKRAAGFLDRTGQCK
 PFLHNGTGYSRSEGCGLVVMKRLSDAIREGDRIHGVIIRGLCIRSMASPKFISQPNGVFQSVALAQAQVKISGVDPAAISFVEAH
 GPGTAKGDLAEVSSLCVLAQHRDNDPLTVGSLKGNVGHAEASGTHSLAKVIAMFQRRRIPPQADFHP SRLNPTLKPFDFK
 HPIRIIENEEDWNASHRIAIVGNFGASGNAGFMVVEEGSTFQPLEGRDVPKVSSPLPFVISAKDQATLVKLIHLYIDWLKQPS
 TFLTPLSDISYATTARRFTHPCMISVQADSHLDLAKKLQERPPMIDSSANRTSRQVAFCFSGQGGGERVDPNRNSTLYNFSASFT
 DAVDMCFRIVESESLIAEEDIAMLELFALEFGLMEMWKSNGINPVALAGHSFGEYVALVCAGVLTVRDALKLLGIRAAALIRAR
 CLDVPGKMAAVRLSVSDVNKCLEQQRSTHVELACVNSDNSVTLAGTPEDLESFRQELLKSYPAAGWHLNNMTAAAFHSRQVQ
 ILADFTNACNEVKVHPSEMVLVSGLLGKMCPVGDAVLQQHDYLVHRCHRETNRFGTATISDYQRNVESETPQPDWIEIGSHSRI
 ISFISLASDQLKFP SHGKTAADGWTTALDTLMRLYSAGHVDFKDLHNDINPSAHHTDLPLYPFQFEPHVYPARREAKAISTT
 LASANEVELCPRPVSTELAPLLLNVHMAGYTLCPATTHVALLAAAASSASSSQHEGRLAYKLFKLKVIAGFTNTTDGWLQVR
 RHLATSELEII SNDDNKI HITARAEVCKEQDMLESLSLYKPFILPFKSFKSLPTTDVLRKELAYS LFNHTVNYGAHQVLDLV
 WIAEDGHQAWGYSTYPGGARTAEGVPTVLRDFSPMLIESTCQLIGFLMNTSMERKDGAEFVTDDELGEC SIALSKLYETKYVEL
 YASFVMVDGGTAGLGNVFAFDDKGQLISAFRDVRMAKMKIYVLKRLIDRGRKPEQVVNTQHVAPEEVEEDNDANNDLEEKVIS
 VLKAALRLSEI PRDKTLGELGLDLSLT AIDVGVLRLVPHRRDHLTIDPEGNLAALLQILRP¹³⁰⁸**TPLPKSLPITSSTKESKVE**
TVDLATSPSLPIMPNGVPTTVNGVVPKPNALPTVNLGHKPNGAPANGVPTANGVPTADGTSTEPSQTLVANISPEMLQAISSN
PEVIQY¹⁴¹⁵APGRTPLLLIHDGGGTSFAYYSLGNLDRVTIGIHAPGLQEGKGMVNILHATNEYANIRQYLKQHC PGHSGKLLIG
 GWSLGGTISITMAAMF PDLVAGVVTIDTTPPGVGGTLAEAEASVLLHPWSRSDGIHGLVRKQLEQNTRALFANPEYKTTIRNT
 TVNVVPVYICAKDPFRPPESLHLQDSSSQWLIDFKEPQVAEVMWKS LMGERRLLGVQIIPGNHWTMFTPANAKTTTEALRRGLD
 VIEGWLNKAG

Figure S1C: Amino acid sequence information for chimera I (1666 aa). The swapped portion is shown in blue.



MPPNTVNKAAEIPPFEPPIAIVGIGIRGPGGINSLTTLWDTLVERKSHCFPLAKDPRFQRRFNPDDFKALFDGIPDSENVLHAN
 LFDETPGLDRTYFSLSERAAGMDVQKLLHVAHEALEDAGYSGAEDGSAFDPSTFGVYVATATDEVIQDPRDYDTDIYHLV
 RTERAFLSGQICFHFGLRGPSSSVDTVCSGSITAINDACRALATGDCRAAIAGGVHVVPVSGPISFYCIKRAAGFLDRTGQCK
 PFLQNGTGYSRSEGCGLVVMKRLSDAIREGDRIHGVIIRGLCIRSMASPKFISQPNGAFQSVALAQAQVKISGVDPAAISFVEAH
 GPGTAKGDLAEVSSLCVLAQHRAVDNPLTVGSLKGNVGHAEASGTHSLAKVIAMFQRRRIPPQADFHP SRLNPTLQPFDFK
 HPIRIIENEEDWNASHRIAIVGNFGASGNAGFMVIEEGSTFQPLEGRDVPKVSSPLPFVISAKDQATLVKLIHLYIDWLKQPS
 TFLTPLSDISYAMTARRFIHPFMISVQADSHMDLVQKLQERPPMINGSANRTPREVAFCFSGQGGGERVDPNRNSTLYNFSASFT
 DAVDMCFRVAESENVAEEDVAILELFALEFGLVEMWKSNGIKPVALAGHSFGEYVALVCAGVLTVRDALKLLGIRAAALIRAM
 CLDVPGKMAAVRLPLSDVNKCLEQQKSTRVELACVNSDNSVTVAGTPEDLESFRQELLKWYPAASWHLNNMTAAAFHSRQVQ
 ILADFTNACNEVKVHPSEMVLVSGLLGKMCPVGDAVLQQHDYLVHRCHRETNRFGTATISDYQRQNVRETPQPDWLEIGSHSRI
 ISFITPASDQLKLPSHGKSAGEGWM TALDALMRLYSAGHVDFKDVHYDVNPSAHHTDLPLYPFQLEPHFYPARREVKASSTM
 LASTNEVQFCPRVPSTELAPLLLNVHMAGYTLCPATTHVALMMAAASTFASSSQQEGRLAYKLSKLKVIAGFTNTTDGWLQVR
 RQSSTSDLEII SNDDNKI HITARAEVCKEQDLLESLSLYASFILPFKSFKFLPSTDVLRKELAYS LFNHTVNYGPHGQVLDLV
 WIAEDGYQAWGYSTYPGGASTAEGVPSALRDFSPMLIESVCQLIGFLMNTSTNRKDGAEFVTDDELGDCSIAISKLYETKYVEI
 YASYKMVDGGTAGLGNVFAFDDKGQLISAFRDVRMAKMKIYVLKRLIDRGRKPEQVVNTQHVASTEVEEDNVAHNDIDDKVIS
 VLKAALRLSEI PLDKTLGELGLDLSLT AIDVGVLRLVPHRRDHLTIDPEGNLTSLLQLLRP¹³⁰⁸**APSPKSLPTTSSTKEIKLT**
NGDLEVKAI STPPSLPITANGIPTANGVATTNVVPAVNGVTVNGVVPKANGVVPKANGVYTAKMPT EPATALIANLSPEMMEAIS
SNPEVVQH¹⁴¹⁷APGRTPLLLIHDGGGTSFAYYSLGNLDRVTIGIHCPGLQEGKGIESVHHAANEYANIRQYLKQCCPGHSGKVL
 IGGWSLGGTISIMMAALF PDLVAGVVTIDTTPPGVVGTLAQQAESVLLHPWSRSDGIHGLVRRQLQLNTRASFAHPEYKTTIR
 VTAVNVVPVYVLC AIDPFRPSES LDKPKETYQWLLSFKQSDVAEVTWKELIGERLLGVQSVPGNHWTMFTPANVKATTEALKQG
 LDVIEARLNKMG

Figure S1D: Amino acid sequence information for chimera II (1668 aa). The swapped portion is shown in orange.



MHPNNTVNKAAEIPPFEPPIAIVGIGIRGPGGINSLTTLWDTLVERKSHCFPLAKDPRFQRRFNPDDFKALFDGIPDSENVLHANLFDETPGLDRITYFSLSERAAGMDVQQKLLLHVAHEALEDAGYSGAEDGSAFDPSTFGVYVATATDEVIQDPRDYDTDIYHLVRTERAFLSGQICFHFGLRGPSSSVDTVCSGSITAINDACRALATGDCRAAIAGGVHVVTVPVSGPISFYCIKRAGFLDRTGQCKPFLQNGTGYSRSEGCGLVVMKRLSDAIREGDRIHGVIIRGLCIRSMASPKFISQPNGAFQSVLAQAQVKISGVDPAAISFVEAHGPGTAKGDLAEVSSSLCSVLAQHRAVDNPLTVGSLKGNVGHAEAAASGTHSLAKVIAMFQRRRIIPQADFH⁴⁰²PSRLNPTLKPF
FDKHPIRIIENEEDWNASHRIAIVGNFGASGNAGFMVVEEGSTFQPLEGRDVPKVSSPLPFVISAKDQATLVKLIHLYIDWLKQPSTFLTPLSDISYATTARRFTHPCMISVQADSHLDLAKKLQERPPMIDSSANRTSRQVAFCFSGQGGERVDPNRNSTLYNFSA
SFTDAVDMCFRIVESESLIAEEDIAMLELFALEFGLMEMWKSWSGPNVALAGHSFGEYVALVCAGVLTVRDALKLLGIRAALIRARCLDVP
GKMAAVRLSVSDVNKCLEQQRSTHVELACVNSDNSVTLAGTPEDLESFRQELLKSYPAAGWHLNNMTAAFHSRFVQPILADFTNACNEV
KVHPSEMVLVSGLLGKMCPVGDAVLQQHDYLVHRHCRETNRFGTASDYQRRNVESETPQPDWIEIGSHSRIISFISLASDQLKFPSHGKTAADG
WTTALDTLMRLYSAGHVVDKDLHNDINPSAHHTDLPLYPFQFEPHVYPARREAKAISTTLASANEVELCPRVPSTELAPLLLNVHMAGY
TLCPATTHVALLLAAAASSASSSQHEGRLAYKLFKLKVIAGFTNTTDGWLQVRRHLATSELEIISNDDNKIHITARAEVCKEQDMLESLSLYK
PFILPFKSFKSLPTTDVLRKELAYSLEFNHTVNYGAHQVQLDRVWIAEDGHQAWGYSTYPGGARTAEVPTVLRDFSPMLIESTCQLIGFLMNTS
MERKDGEAFVTDDELGECIALSKLYETKYVELYASFKMVDGGTAGLGNVFAFDDKGQLISAFRDVRMAKMKIYVLKRLIDRGRKPEQVVNTQHV
APEEVEEDNDANNDLEEKVISVLKAALRLSEIPRDKTLGELGLDSLTAIDVGQLERLVPHRRDHLTIDPEGNLAALLQILRPAPSPKSLPTTS
SSTKEIKLTNGDLEVKAISTPPSLPITANGIPTANGVATTNVVPAVNGVTVNGVPAKANGVPKANGVYTAKMPTEPATIALNSPEMMEAI
SSNPEVVQHAPGRTPLLLIDHGGGTSFAYYSLGNLDRVTIGIHAPGLQEGKGMVNILHATNEYANIRQYLKQHCPGHSKLLI
GGWSLGGTISITMAAMFDFLVAGVVTIDTTPPGVGGLTAAEAESVLLHPWSRSDGIHGLVRKQLEQNTRALFANPEYKTTIRN
TTVNVVPYVICAKDPFRPPESLHLQDSSSQWLIDFKEPQVAEVMWKSMLGERLLGVQIIPGNHWTMFTPANAKTTTEALRRGL
DVIEGWLKAG

Figure S1E: Amino acid sequence information for chimera III (1669 aa). The swapped portion is shown in blue.



MHPNNTANKAAEIPPFEPPIAIIIGIGIRGPGGINSLTTLWDTLVERKSHCFPLAKDPRFHRRFNPDDFKALFDGIPDSENVLHNSNLD
ETPGLDRITYFSLSERAAGMDVQQKLLLHVAHEALEDAGYSGVEDGSAFDPSTFGVYVATATDEVIQDPRDYDTDIYHLVRTERAFLSGQICFHFGLRGPSSSVDTVCSGSITAINDACRALATGDCRAAIAGGVHVVTVPVSGPISFYCIKRAGFLDRTGQCKPFLHNGTGYSRSEGCGLVVMKRLSDAIREGDRIHGVIIRGLCIRSMASPKFISQPNGVVFQSVLAQAQVKISGVDPAAISFVEAHGPGTAKGDLAEVSSSLCTVLAQHRVDNPLTVGSLKGNVGHAEAAASGTHSLAKVIAMFQRRRIIPQADFH⁴⁰²PSRLNPTLQPF
FDKHPIRIIENEEDWNASHRIAIVGNFGASGNAGFMVIEEGSTFQPLEGRDVPKVSSPLPFVISAKDQATLVKLIHLYIDWLKQPSTFLTPLSDISYAMTARRFIH
PFMISVQADSHMDLVQKLQERPPMINGSANRTPREVAFCFSGQGGERVDPDRDSTLYNFSA
SFTDAVDMCFRVAESENVAEEDVAILELFALEFGLVEMWKSWSGIKPVVALAGHSFGEYVALVCAGVLTVRDALKLLGIRAALIRAMCLDVP
GKMAAVRLPLSDVNKCLEQQKSTRVELACVNSDNSVTVAGTPEDLESFRQELLKWYPAASWHLNNMTAAFHSRFVQPILADFTNACNEV
KVHPSEMVLVSGLLGKMCPVGDAVLQQHDYLVHRHCRETNRFGTASDYQRQNVRETPQPDWLEIGSHSRIISFITPASDQLKLPSHGKSAGEG
WMTALDALMRLYSAGHVVDKDVHYDVNPSAHHTDLPLYPFQLEPHFYPARREVKASSTMLASTNEVQFCPRVPSTELAPLLLNVHMAGY
TLCPATTHVALMMAAASTFASSSQQEGRLAYKLSKLKVIAGFTNTTDGWLQVRRQSSTSLEIISNDDNKIHITARAEVCKEQDLLESLSLYASFI
LPFKSFKFLPSTDVLRKELAYSLEFNHTVNYGPHGQVQLDRVWIAEDGYQAWGYSTYPGGASTAEGVPSALRDFSPMLIESVCQLIGFLMNTSTNRKDGEAFVTDDELGDCSIAISKLYETKY
VEIYASYKMVDGGTAGLGNVFAFDDKGQLISAFRDVRMAKMKIYVLKRLIDRGRKPEQVVTTQHVASTEVEEDNVAHNDIDDKVISVLKAALRLSEIPLDKTLGELGLDSLTAIDVGQLERLVPHRRDHLTIDPEGNLTSLQLLRPTPLPKSLPITSSSTKESKVETVDLATSPSLPIMPNGVPTTVNGVPKPNALPTVNLHKKPNGAPANGVPTANGVPTADGTSTEPSQTLVANISPEMLQAISSNPEVIQYAPGRTPLLLIDHGGGTSFAYYSLGNLDRVTIGIHCPGLQEGKGIESVHHAANEYANIRQYLKQCCPHGSKVLIGGWSLGGTISIMMAALFPDLVAGVVTIDTTPPGVVGLTAQQAESVLLHPWSRSDGIHGLVRRQLQLNTRASFAPHEYKTIIRVTVNVVPYVLCALDPFRPSESLDLPKETYQWLLSFKQSDVAEVTWKELIGERLLGVQSVPGNHWTMFTPANVKATTEALKQGLDVIEARLNKMG

Figure S1F: Amino acid sequence information for chimera IV (1667 aa). The swapped portion is shown in orange.

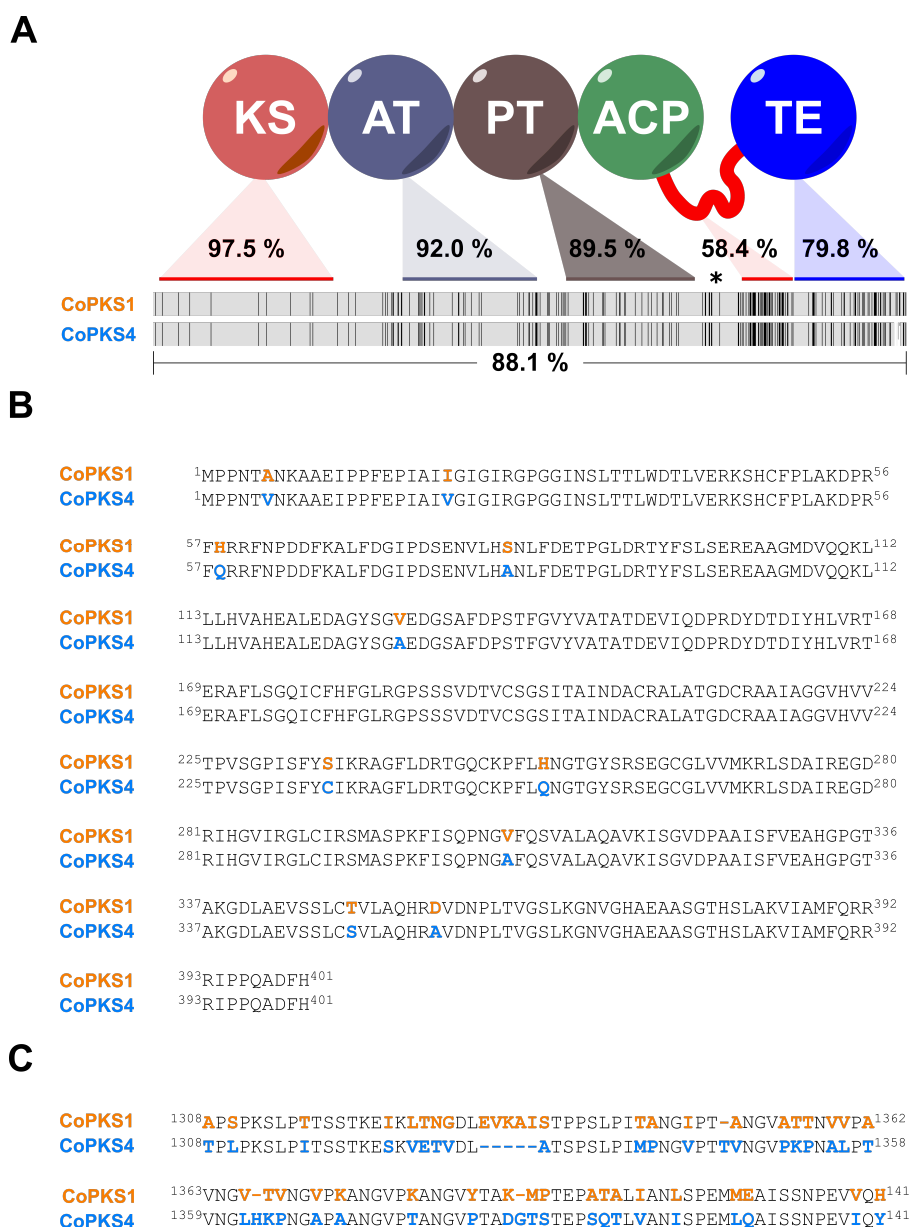


Figure S2: Sequence comparison of CoPKS1 and CoPKS4. A) Overall sequence similarities. Domain acronyms: KS – β -ketoacyl synthase; AT – acyltransferase; PT – product template; ACP – acyl carrier protein; TE – thioesterase. Black ticks in the identity matrix denote differences between CoPKS1 and CoPKS4. The asterisk (*) represents the 4'-phosphopantetheine group, which is bound to the active site of the ACP domain. B) Alignment of β -ketoacyl synthase (KS) domains that were swapped. C) Alignment of proline-rich linker regions that were swapped. A more detailed sequence comparison was published previously.^[1]

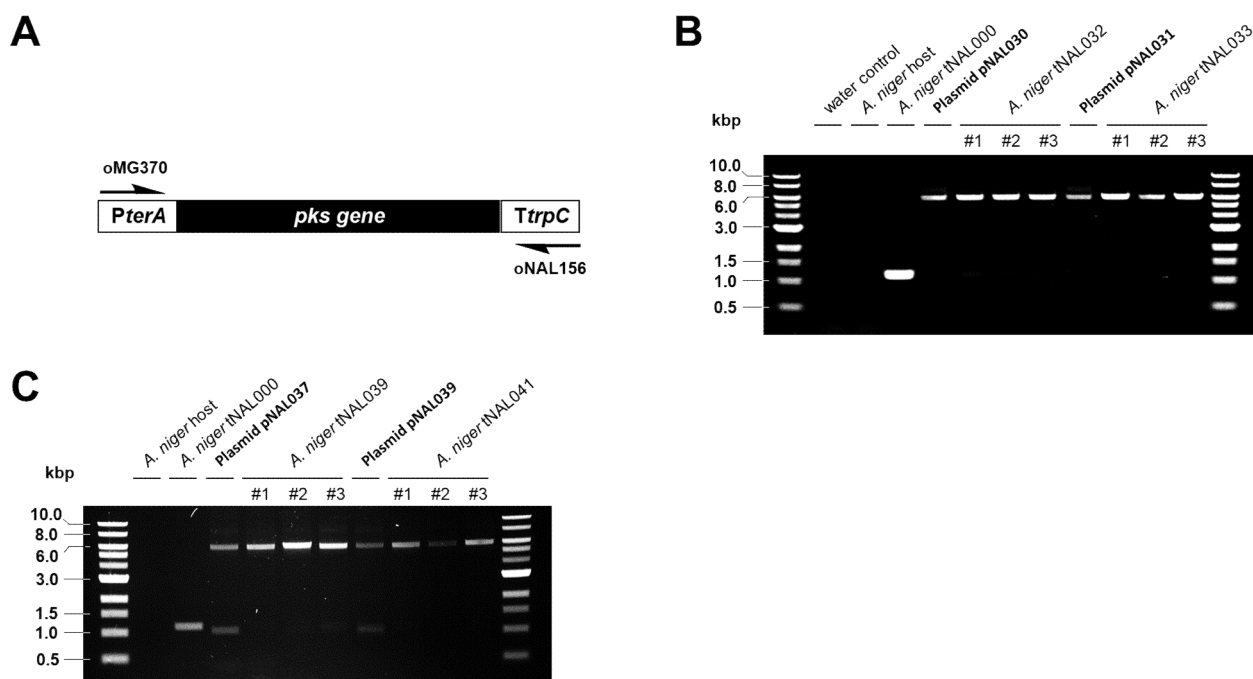


Figure S3A. Agarose gel electrophoresis to confirm the correct integration of chimeric *C. odorifer* PKS genes in the genome of *A. niger* ATNT16 Δ pyrGx24.

A) Oligonucleotide positions relative to the gene. Following this strategy, the entire cassette of *terA* promoter (*PterA*)/PKS gene/*trpC* terminator (*TtrpC*) was amplified. Expected amplicon lengths in case of a full-length accurate genomic integration are about 6.2 kbp.

B) tNAL032 and tNAL033: *A. niger* harboring the genes for chimera I and chimera II, respectively. Sizes of the DNA marker bands in kbp are indicated. Water control: water instead of template DNA was added to the reaction; *A. niger* host: DNA of the untransformed *A. niger* host ATNT16 Δ pyrGx24 was added for negative control; *A. niger* tNAL000: DNA of the *A. niger* host, transformed with insert-less expression vector phis_SM-Xpress was added for negative control.^[1] For positive control, the PCR product obtained with the respective pNAL expression plasmid as template DNA is shown. Three independent transformants are shown per transformed PKS gene

C) tNAL041 and tNAL039: *A. niger* harboring the genes for chimera III and IV, respectively. Three independent transformants are shown per transformed PKS gene.

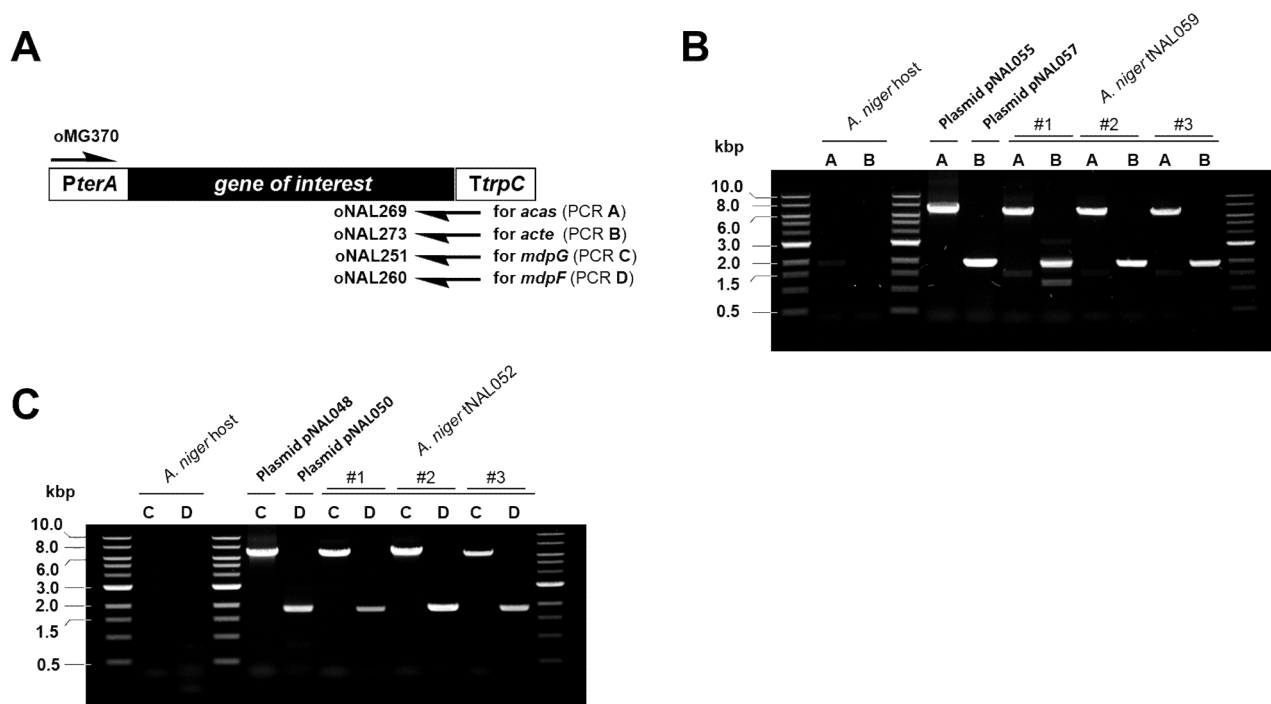


Figure S3B. Agarose gel electrophoresis to verify the integration of ascomycete PKS genes in the genome of *A. niger* ATNT16_2_No. 17.1.

A) Oligonucleotide positions relative to the gene. Due to the double integration of the expression cassette (*terA* promoter (*PterA*)/ gene of interest/*trpC* terminator (*TtrpC*)) into the genome of the double-auxotrophy strain (*A. niger* ATNT16_2_No. 17.1) a different strategy was applied. In addition to oMG370, the specific Gibson primers (Table S2B) at the junction of the respective genes of interest and the *trpC* terminator were used. Expected amplicon lengths in case of an accurate genomic integration are: 6.4 kbp (*acas*; PCR A), 1.9 kbp (*acte*; PCR B), 6.4 kbp (*mdpG*; PCR C) and 1.8 kbp (*mdpF*; PCR D).

B) tNAL059: *A. niger* harboring the genes for ACAS and ACTE. Sizes of the DNA marker bands in kbp are indicated. *A. niger* host: DNA of the untransformed *A. niger* host (ATNT16_2_No. 17.1) was added for negative control. For positive control, the PCR product obtained with the respective expression plasmid (pNAL055 + pNAL057) as template DNA is shown.

C) tNAL052: *A. niger* harboring the genes for MdpG and MdpF. *A. niger* host: DNA of the untransformed *A. niger* host (ATNT16_2_No. 17.1) was added for negative control. For positive control, the PCR product obtained with the respective expression plasmids (pNAL048 + pNAL050) as template DNA is shown.

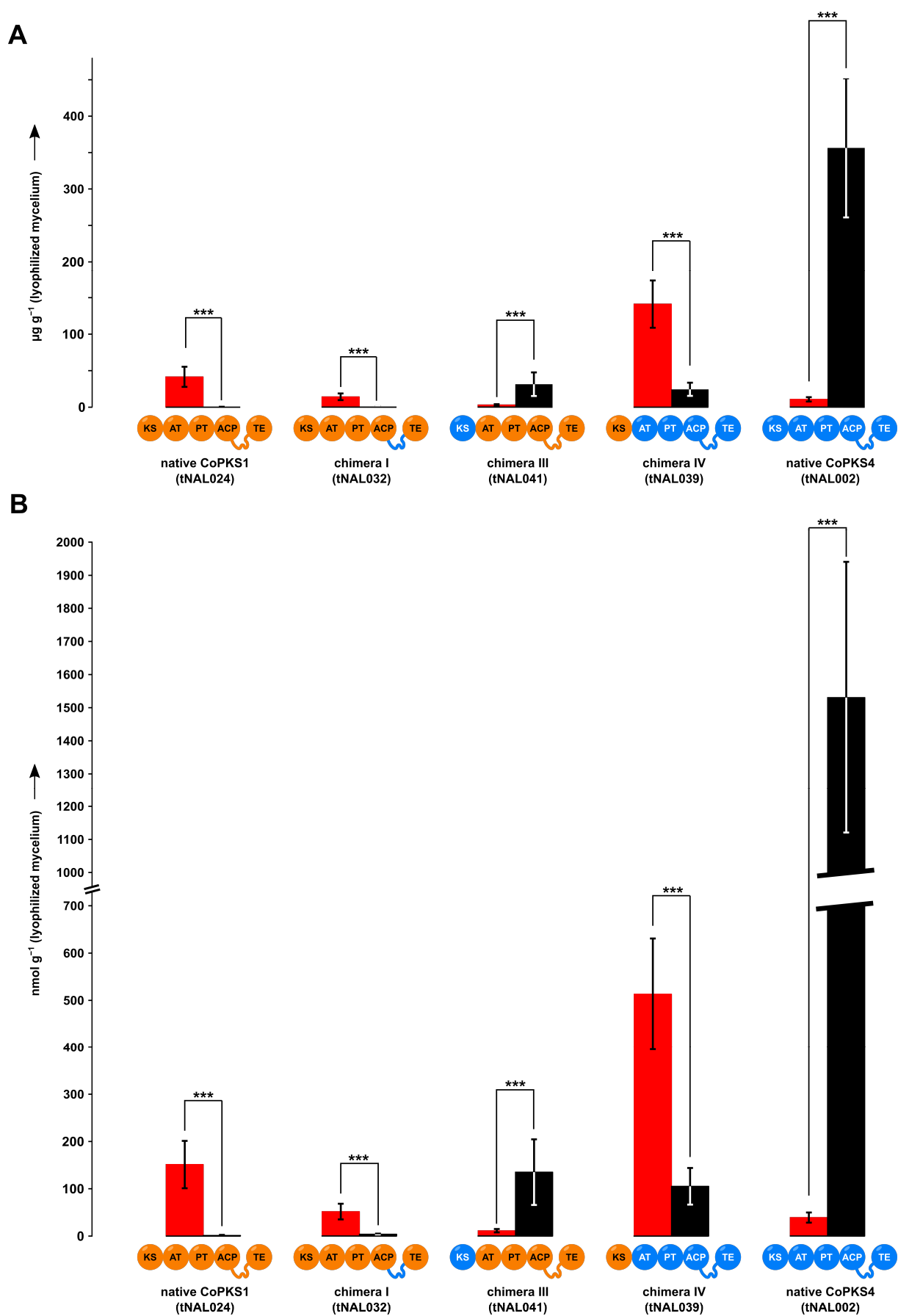


Figure S4. Quantification of the *in vivo* activity of chimeric *Cortinarius* PKSs. A) Concentrations of **1** (red bars) and **2** (black bars) in µg g⁻¹ lyophilized mycelium. B) Concentrations are given in nmol g⁻¹ lyophilized mycelium. Error bars represent three technical and three biological replicates, respectively.

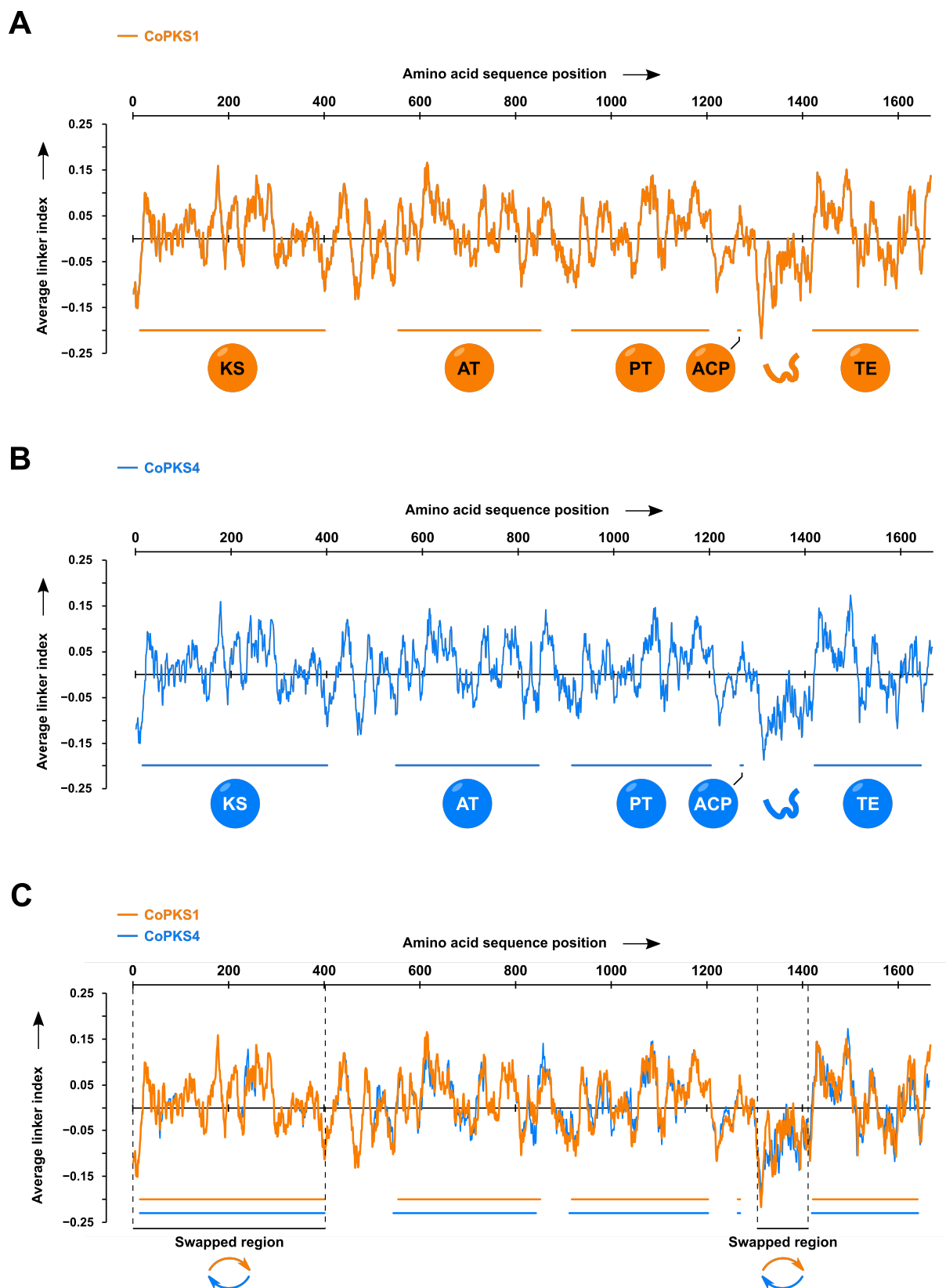


Figure S5. *In silico* domain and linker prediction analyses for CoPKSs 1 (panel A) and CoPKS4 (panel B) with DomCUT.^[6] C) Overlaid profiles from A and B. Domain acronyms: KS – β -ketoacyl synthase; AT – acyltransferase; PT – product template; ACP – acyl carrier protein; TE – thioesterase.

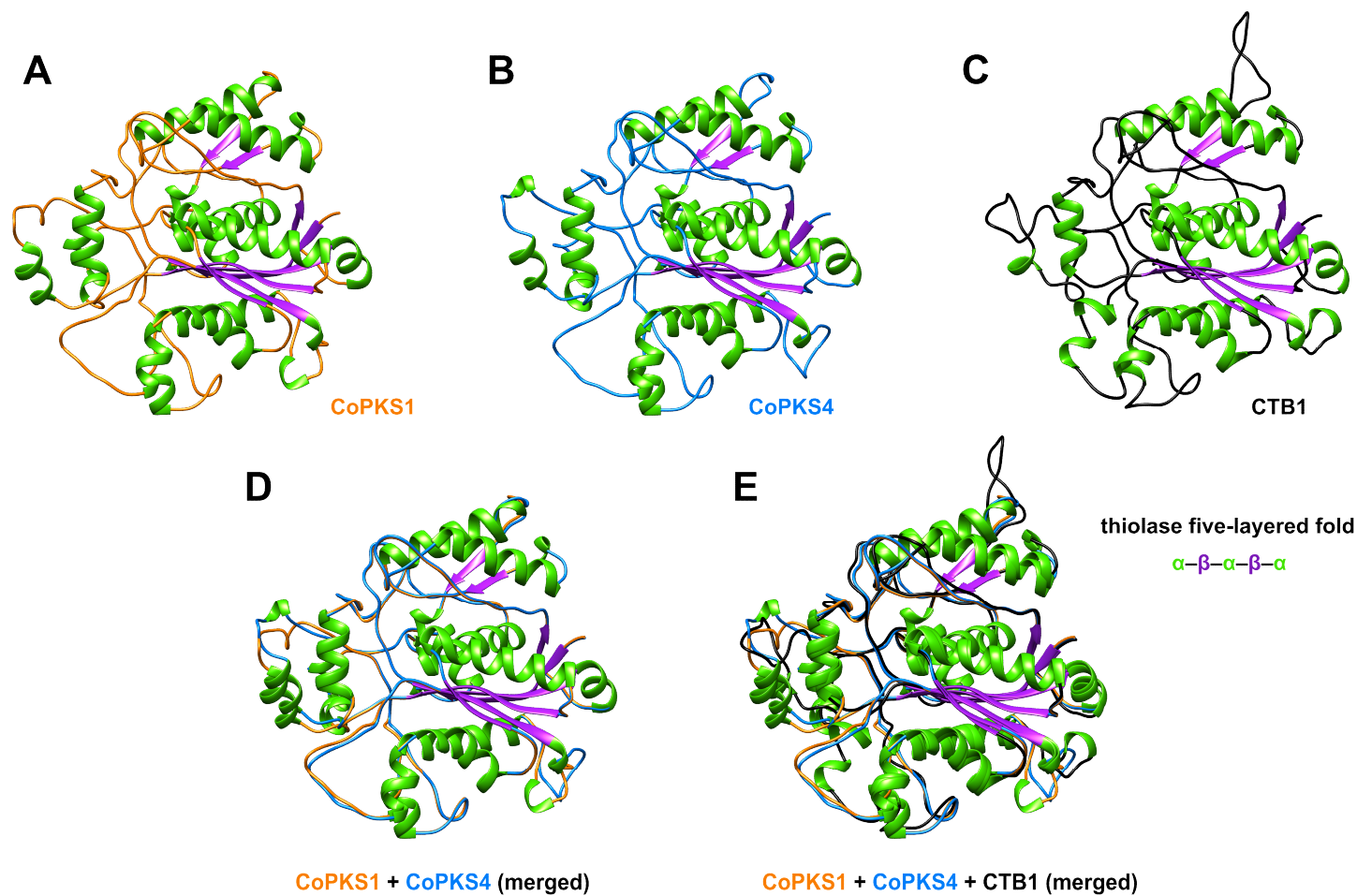


Figure S6. Structural models of KS domains. The 3D models of the KS domain of CoPKS1 (orange) and CoPKS4 (blue) were generated using AlphaFold.^[7] For comparison, the KS domain of CTB1 (black) from the *nor*-toralactone pathway of *Cercospora nicotianae* was used (crystal structure data; PDB: 6FIJ).^[8] The KS domain of CTB1 shares 32.2% and 32.5% sequence identity with those of CoPKS1 and CoPKS4, respectively. All KS domains adopt the canonical thiolase fold^[9] with a α - β - α - β - α five-layered structure (α -helices are highlighted in green, β -sheets in purple). The structural models were superimposed, analyzed and visualized using UCSF Chimera (version 1.13.1).^[10]

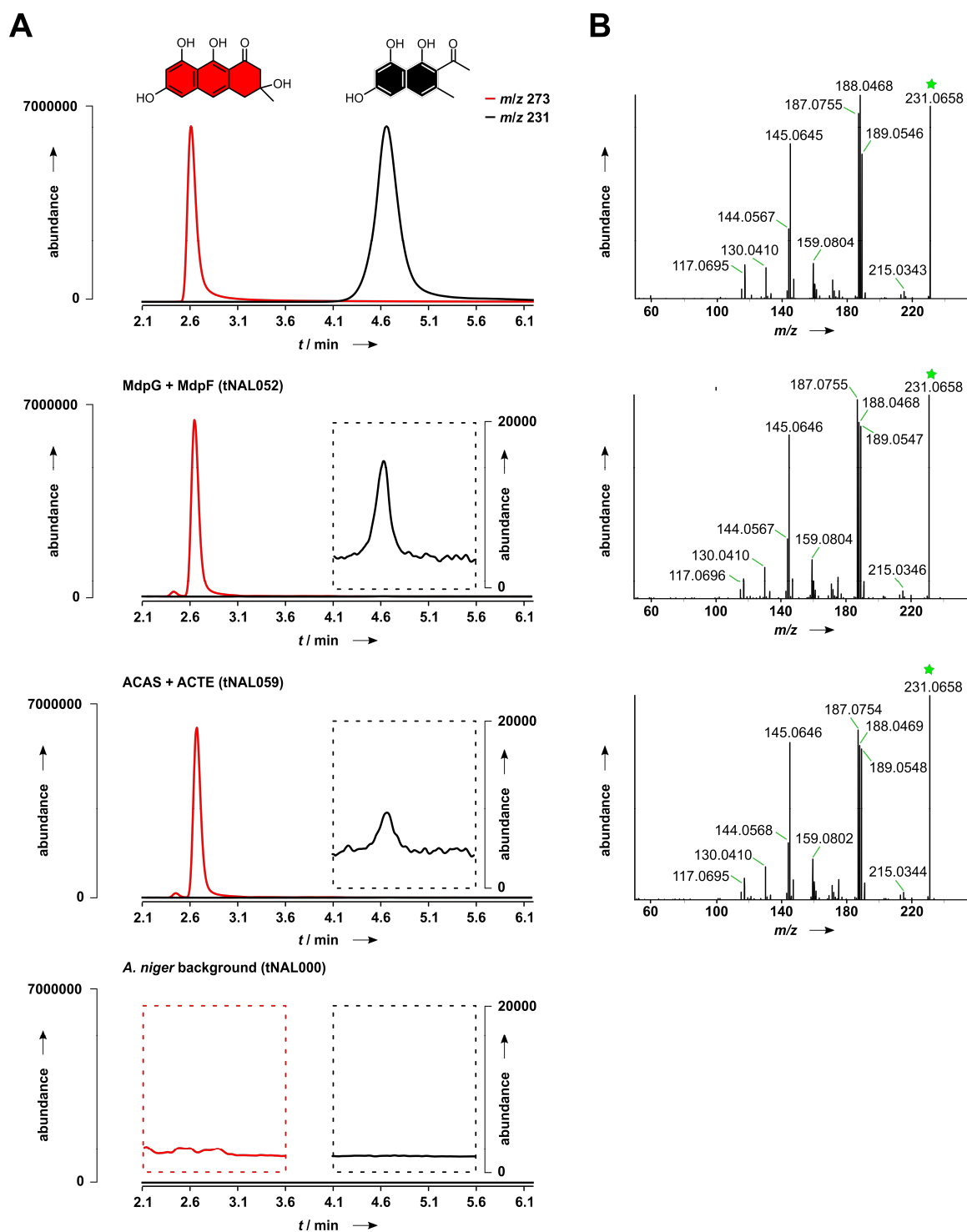


Figure S7. LC-MS analysis of *in vivo* assays with MdpG/MdpF and ACAS/ACTE. A) Single ion monitoring (SIMs) of ethyl acetate extracts are shown for m/z 273 [$M-H$]⁻ (red) to detect the octaketide atrochrysone (**1**) and m/z 231 [$M-H$]⁻ (black) for the heptaketide 6-hydroxymusizin (**2**). Top traces represent an overlay of individual SIMs for standards of **1** and **2**. The *A. niger* strains tNAL052 (producing MdpG and MdpF) and tNAL059 (producing ACAS and ACTE) were analyzed for their capacity to synthesize **2**. In the close-up views (4.1 to 5.6 min), traces of **2** are shown for both samples, which was further confirmed by LC-MS/MS analyses. Neither **1** nor **2** were detected in the *A. niger* host background (tNAL000). B) LC-MS/MS spectra of **2**. The spectra were recorded in negative ([$M-H$]⁻) ionization mode. The upper window displays the spectrum of a **2** standard (calculated mass: m/z 231.0663). The green asterisk denotes the parental ion.

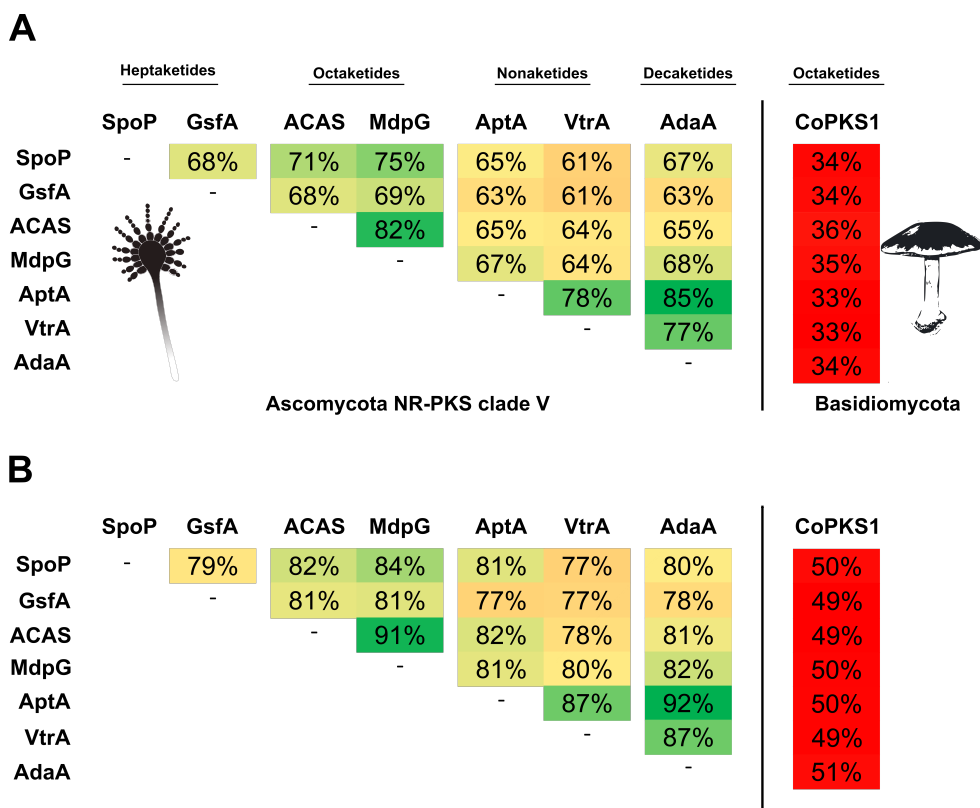


Figure S8. Sequence similarities of ascomycete PKSs and CoPKS1. Comparisons are based on pairwise alignments of the most conserved core regions (around 300 amino acids) of the respective β -ketoacyl synthase (KS) domains. A) Pairwise % sequence identity. B) Pairwise % positive using the BLSM62 substitution-scoring matrix.^[11] Polyketide synthases are: SpoP^[12] (*Chrysosporium merdarium*), GsfA^[13] (*Penicillium aethiopicum*), ACAS^[3] (*Aspergillus terreus*), MdpG^[2] (*A. nidulans*), AptA^[14,15] (*A. nidulans*), VtrA^[13] (*P. aethiopicum*), AdaA^[15] (*A. niger*), CoPKS1^[1] (*Cortinarius odorifer*).

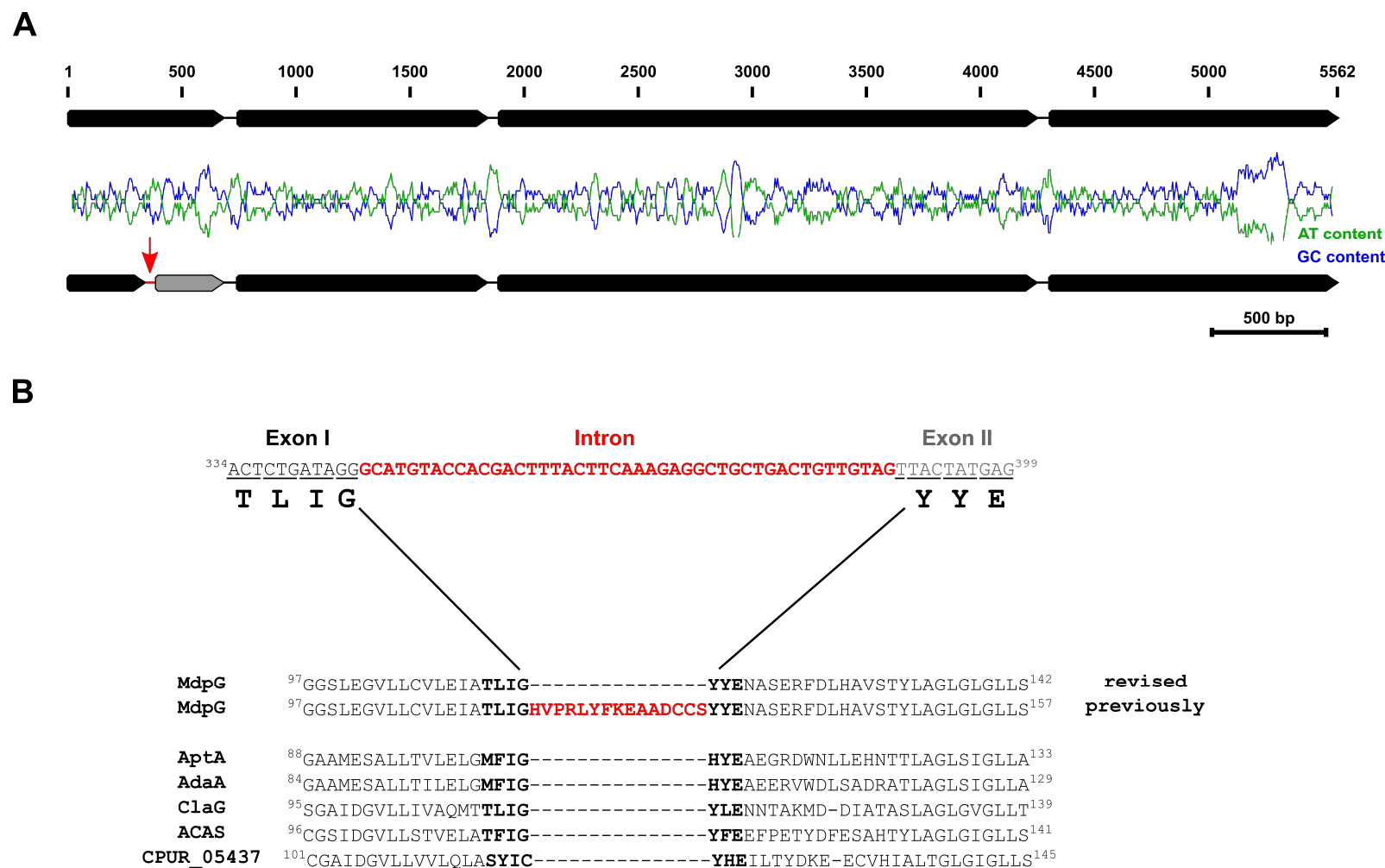


Figure S9. Transcript and cDNA analysis of the *mdpG* gene. A) Intron/exon structure of the *mdpG* gene based on the previously published protein sequence^[6] (top) and with the newly identified intron (bottom; red arrow). B) Sequence alignments of the previously published and revised MdpG protein sequence with related NR-PKSs of clade V. Polyketide synthases are: MdpG^[2] (*Aspergillus nidulans*), AptA^[14,15] (*A. nidulans*), AdaA^[15] (*A. niger*), ClaG^[16] (*Cladosporium fulvum*), ACAS^[3] (*A. terreus*), CPUR_05437^[17] (*Claviceps purpurea*). The revised sequence leads to a gapless alignment of MdpG with its relatives in NR-PKS clade V

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