The Journal of Antibiotics (2010) 63, 207–218

www.nature.com/ja

REVIEW ARTICLE

Functional analysis of fungal polyketide biosynthesis genes

Isao Fujii

Fungal polyketides have huge structural diversity from simple aromatics to highly modified complex reduced-type compounds. Despite such diversty, single modular iterative type I polyketide synthases (iPKSs) are responsible for their carbon skeleton construction. Using heterologous expression systems, we have studied on ATX, a 6-methylsalicylic acid synthase from *Aspergillus terreus* as a model iPKS. In addition, iPKS functions involved in fungal spore pigment biosynthesis were analyzed together with polyketide-shortening enzymes that convert products of PKSs to shorter ketides by hydrolytic C–C bond cleavage. In our studies on reducing-type iPKSs, we cloned and expressed PKS genes, *pksN*, *pksF*, *pksK* and *sol1* from *Alternaria solani*. The *sol* gene cluster was found to be involved in solanapyrone biosynthesis and *sol5* was identified to encode solanapyrone synthase, a Diels-Alder enzyme. Our fungal PKS studies were further extended to identify the function of PKS-nonribosomal peptide synthase involved in cyclopiazonic acid biosynthesis.
The Journal of Antibiotics (2010) **63,** 207–218; doi:10.1038/ja.2010.17; published online 5 March 2010

Keywords: biosynthesis; fungi; heterologous expression; polyketide; polyketide synthase

INTRODUCTION

Fungal polyketides have huge structural diversity from simple aromatics to highly modified complex reduced-type compounds. However, single modular iterative type I polyketide synthases (iPKSs) are responsible for their carbon skeleton construction. Historically, fungal metabolite 6-methylsalicylic acid (6-MSA) is the key natural product in the establishment of polyketide biosynthetic pathway. Birch demonstrated acetate incorporation into 6-MSA using *Penicillium griseofulvum* in 1955.[1] Lynen followed to detect 6-MSA synthase (MSAS) activity in the cell-free extract of *P. patulum* in 1961, and then purified the enzyme as the first PKS (1971).[2] Schweizer's group succeeded in cloning of the MSAS gene from *P. patulum* as the first fungal PKS gene (1990),[3] though some bacterial PKS genes from streptomycetes had been cloned at that time. Since then, a number of PKS genes have been cloned and now sequence information on PKSs is rapidly increasing because of the progress of genome projects. According to their architecture, PKSs are classified into three types, type I, II and III.[4] Interestingly, most of fungal PKSs belong to iPKSs with a single modular architecture consisting of ketosynthase (KS), acyltransferase (AT) and acyl carrier protein (ACP) domains as a minimum together with additional catalytic domains such as ketoreductase (KR), dehydratase (DH), enoylreductase (ER), methyltrnasferase (MeT) and thioestase.[5]

Owing to reiterative use of domains by iPKSs, co-linearity rule of modular type I PKSs is not applicable to assume iPKS function.[6] We have been working on expression of fungal iPKSs and product identification for their functional analysis. In this review, I tried to summarize our recent contribution to functional analysis on fungal iPKSs and related enzymes involved in fungal polyketide biosynthesis.

ATX

At the time we started to work on fungal PKS, MSAS was the only fungal PKS whose *in vitro* activity was detectable. After the cloning of *P. patulum* MSAS gene by Schweizer's group,[3] we cloned the *atX* gene from *Aspergillus terreus* by screening the genomic DNA library with the MSAS probe. To identify the function of ATX, we applied the fungal expression system using α-amylase promoter.[7] Although ATX was found to be an MSAS of *Aspergillus terreus* by this heterologous expression experiment, high 6-MSA productivity of this fungal expression system led us to study functions of other fungal PKSs such as *Aspergillus nidulans* WA,[8,9] *Aspergillus fumigatus* Alb1p,[10] *Colletotrichum lagenarium* PKS1,[11,12] etc.

Of the fungal iPKSs whose functions have been identified, MSAS is the smallest in size, consisting of KS, AT, DH, KR and ACP domains on a polypeptide of around 190 kDa. Therefore, we chose ATX as a target iPKS for detail functional analysis. However, the fungal expression system that[13] we have used is not suitable for detail analysis mainly because of difficulty in copy number control in transformants because the vector is a genome integration type. Thus, we chose yeast expression system for further analysis on ATX function. *P. patulum* MSAS was previously expressed in *Saccharomyces cerevisiae* using

Laboratory of Natural Products Chemistry, School of Pharmacy, Iwate Medical University, 2-1-1 Nishitokuta, Yahaba, Iwate, Japan
Correspondence: Professor I Fujii, Laboratory of Natural Products Chemistry, School of Pharmacy, Iwate Medical University, 1/2/2001 Nishitokuta, Yahaba, Iwate 028-3694, Japan.
E-mail: ifujii@iwate-med.ac.jp
Received 28 October 2009; revised 4 February 2010; accepted 5 February 2010; published online 5 March 2010

ADH2 promoter with co-expression of *Bacillus subtilis* Sfp for ACP phosphopantetheinylation.[14] To attain easier induction control, we used the GAL10 promoter for ATX expression and the GPD promoter for constitutive expression of Sfp[15] (Figure 1).

ATX INTER DOMAIN REGION

After confirmation of ATX expression and production of 6-MSA in the yeast transformant, we first carried out deletion mutant analysis.[16] Deletion mutant ATXN1 lacked the *N*-terminus upstream of the intron located just upstream of KS domain. ATXN1 produced 6-MSA, but ATXN2 that had a longer deletion retaining 92% of the KS domain no longer produced 6-MSA. On the other hand, the C-terminal deletion mutant ATXC1 that lacked only nine amino acids and kept the conserved ACP domain was found to be inactive. Prediction of the secondary structure[17] of ATX indicated that helical elements at both N- and C-termini were lost in the inactive deletion mutants. We then tried to analyze subunit–subunit interaction by co-expression of deletion mutants.

First, inactive N-terminal deletion mutant ATXN2 was co-expressed with inactive C-terminal deletion mutant ATXC1, and the co-transformant was found to produce 6-MSA. This result indicated that at least two subunits could interact to reconstitute the catalytic reaction center; that is, the inactive KS of ATXN2 should be complemented with the active KS of ATXC1 and the inactive ACP of ATXC1should be complemented with the active ACP of ATXN2. To confirm domain complementation by counterpart subunits, further deletion mutants were constructed from both N- and C-termini. The productivity of 6-MSA was tested with the series of deletion mutants and found to be getting lower by co-transformants with longer deletions. Even the combination of DHd3-KRd4 co-expression transformant showed a production of 6-MSA, but co-expression of further deletion mutants resulted in non-productivity (Figure 2).

These complementation data indicated that the region overlapped by DHd3 and KRd4 is an inter domain (ID) region required for subunit–subunit interaction to constitute the catalytic reaction center(s). This proposed ID region of 122 amino acids was located between DH domain and KR domain. BLAST search indicated that this ATX ID sequence is conserved in not only MSASs but also some bacterial iPKSs; for example, orsellinic acid synthase CalO5 from *Micromonospora echinospora*.[18] However, no homologous sequence was found in other fungal iPKSs for multi-ring aromatic compounds.

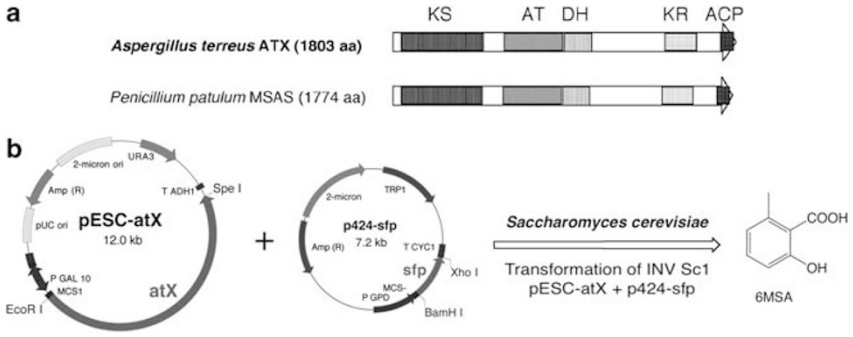


Figure 1 Architecture of ATX and its expression system in yeast. (**a**) Architecture of *Aspergillus terreus* ATX and *P. patulum* MSAS. (**b**) ATX expression plasmid pESC-atX and Sfp expression plasmid p424-sfp were used to express active ATX in *S. cerevisiae*.

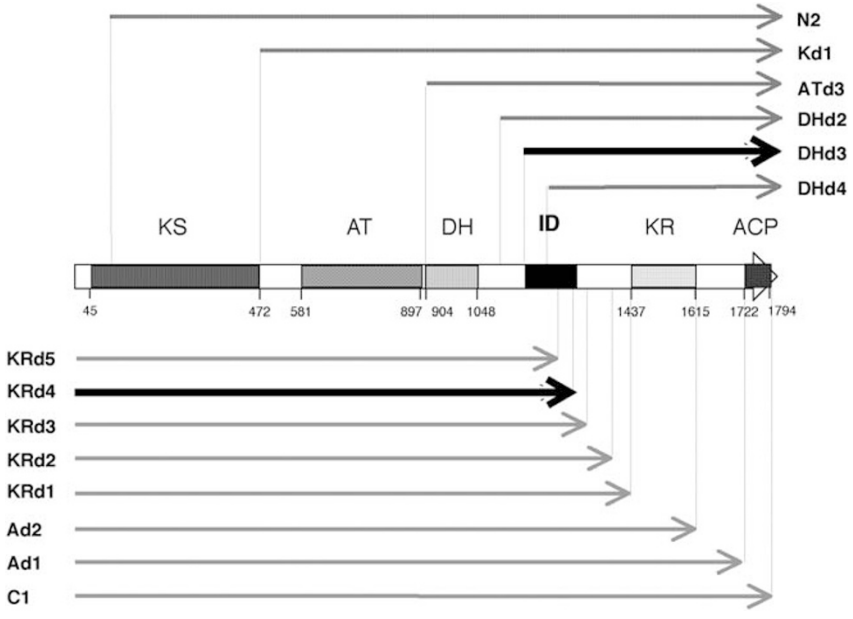


Figure 2 ATX deletion mutants expressed in *S. cerevisiae*. Co-expression of DHd3 and KRd4 gave a production of 6-MSA. Further deletion from either of these abolished 6-MSA production.

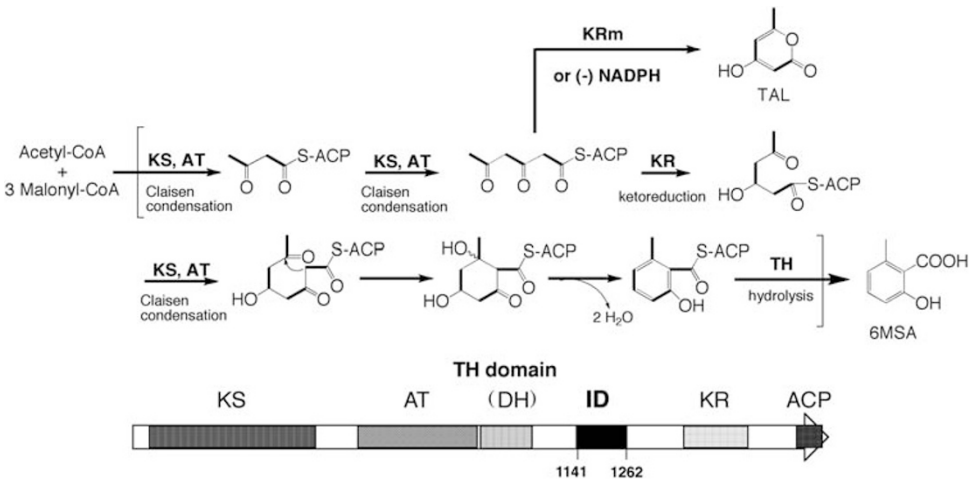


Figure 3 Newly identified thioester hydrolase (TH) domain involved in product release in MSAS reaction.

ATX SUBUNIT–SUBUNIT INTERACTION

For further analysis of domain functions and their interactions in ATX reaction center, we then carried out expression of ATX catalytic domain mutants in yeast.[19] Transformant of each domain mutant was cultured in induction medium. ATX KS domain mutant C216A (KSm) could not produce 6-MSA. Similarly, AT domain mutant S667A (ATm) and ACP domain mutant S1761A (ACPm) lost 6-MSA production ability as expected. Thus, the crucial roles of these catalytic domain residues were confirmed for ATX 6-MSA synthesis. The ATX KR domain mutant (KRm) in which a consensus sequence GxGxxG was mutated to AxPxxA produced triacetic acid lactone (TAL) as reported earlier[20] (Figure 3). The DH domain mutant (DHm), ATX H972A, was then constructed and expressed in yeast. HPLC analysis of DHm induction culture could not give any detectable product compounds, though production of triketide derivative by DHm was expected as dehydration was believed to occur just after the reduction of triketide intermediate in MSAS reaction. This result on DHm indicated that His[972] is crucial for ATX reaction but not just for simple dehydration.

As described above, all catalytic domain mutants of ATX, KSm, ATm, ACPm, KRm and DHm lost 6-MSA production ability. Then, these mutants were used for analysis of the catalytic domain–domain interaction by co-expression in yeast. As a result, the co-transformant expressing KSm and ACPm could produce 6-MSA. Moreover, co-expression of DHm with either KSm or ACPm could produce 6-MSA possibly by reconstitution of the catalytic reaction centers. This result also confirmed that DHm itself was not inactive because of secondary structure change, caused by introduction of mutation, but was active except DH domain. Then, co-expression of all other combinations of ATX domain mutants was analyzed and all of them could reconstitute ATX activity (Table 1).

Mammalian FAS is homodimeric, and has similar domain architecture with iPKS.[21] In FAS mutant complementation analysis, heterodimers comprised of a subunit containing either a KS or AT domain mutant, and a subunit containing mutations in any one of the other five domains, DH, ER, KR, ACP or thioestase, were active in fatty acid synthesis. However, domain mutant heterodimers in either DH, ER, KR, ACP or thioestase were inactive.[22] Therefore, it had been presumed that only KSm and ATm could reconstitute the reaction center by co-expression with other domain mutants in ATX. However, we observed that each domain mutant could be complemented by any other kinds of domain mutants to reconstitute the reaction center for the synthesis of 6-MSA. In addition, when KS domain mutant KSm was co-expressed with the four domains mutant KS-ATm-DHm-KRm-ACPm, the co-transformant could produce 6-MSA. This kind of complementation was observed in all other domain mutants.

Table 1 Results of domain mutant co-expression experiments

	Mutant 1				
	Domain mutation				
Mutant 2	*KS*	*AT*	*DH*	*KR*	*ACP*
Domain mutation					
KS	–	+	+	+	+
AT		–	+	+	+
DH			–	+	+
KR				TAL	+
ACP					–

Abbreviations: ACP, acyl carrier protein; AT, acyltransferase; DH, dehydratase; KR, ketoreductase; KS, ketosynthase; 6-MSA, 6-methylsalicylic acid; MSAS, 6-MSA synthase.
+: 6-MSA was detected in the culture of the co-transformant of mutant 1 and mutant 2.
–: no 6-MSA was detected in the co-transformant culture. All heterologous combinations of inactivated catalytic domain mutants could reconstitute MSAS activity.

The ID region could have a role to interact two subunit polypeptides in head-to-head and tail-to-tail manner as mammalian FAS subunits interact, which further interact in head-to-head and tail-to-tail or head-to-tail manner as MSAS is a homotetramer.[3] Thus, co-expression of KS-ATm-DHm-KRm-ACPm subunit and KSm-AT-DH-KR-ACP subunit, for example, resulted in stochastic formation of active catalytic center in homotetrameric ATX enzyme; that is, N- half (KS-ATm-DHm) interacts with another N- half (KSm-AT-DH) and C- half (KR-ACP). In the ATX catalytic center, five active domains, KS, AT, DH, KR and ACP, even when each on either one of four subunits, could interact with each other with substantial flexibility and carry out 6-MSA synthesis.

ATX THIOESTER HYDROLASE DOMAIN

In the DHm *in vitro* incubation with acetyl-CoA, malonyl-CoA and NADPH, production of neither 6-MSA nor other product was detected. However, DHm produced TAL when NADPH was eliminated from the reaction solution (Figure 3). Thus, it was confirmed

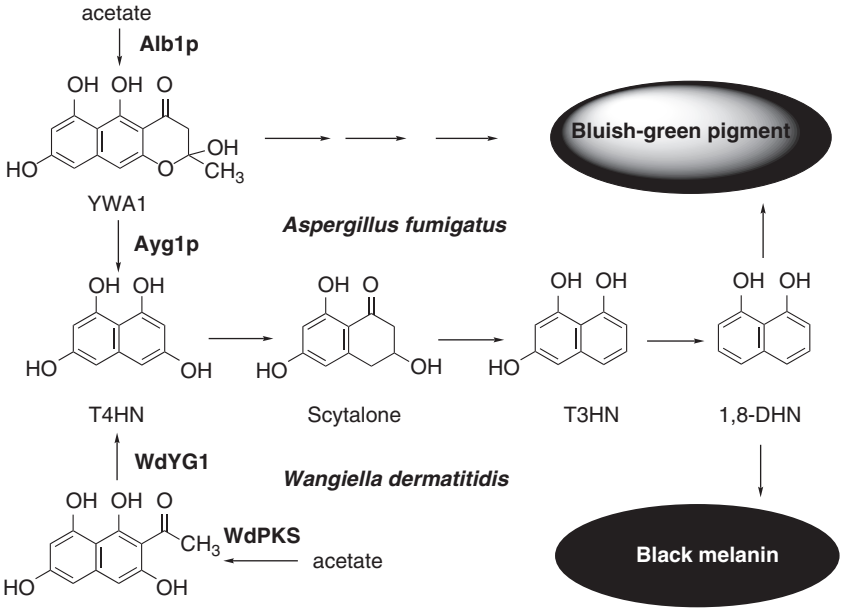


Figure 4 Spore pigment biosynthesis in *Aspergillus fumigatus* and *Wangiella dermatitidis*.

that DHm was active to form triketide intermediate and the mutated domain was not involved in TAL release. These results suggested that DHm could catalyze the formation of reduced intermediate in the presence of NADPH, which could not be released but retained on DHm. In other words, DH-like domain could be involved in product release. Interestingly, DH motif HxxxGxxxxP is also found in the bacterial orsellinic acid synthases, *Streptomyces viridochromogenes* AVIM[23] and *M. echinospora* CalO5[18] although no apparent dehydration is involved in orsellinic acid synthesis reaction.

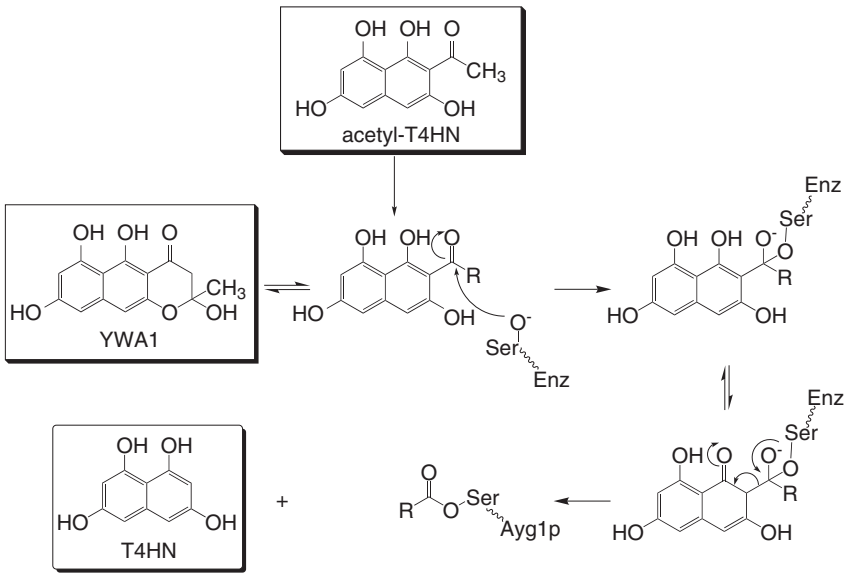
When DHm was incubated with [2-^{14}C]malonyl-CoA and NADPH, ^{14}C-labeling of DHm protein was observed. Then, alkaline hydrolysis was attempted to release the possible thioester intermediate bound on DHm and release of ^{14}C-labeled 6-MSA was detected. This result strongly indicated that 6-MSA formation proceeds in the presence of NADPH without DH domain-catalyzed dehydration and the formed 6-MSA is covalently bound on ACP phosphopantetheine arm as thioester. Then, the formed 6-MSA thioester is hydrolyzed by DH-like domain to release the free acid 6-MSA as ATX product. Therefore, catalytic dehydration of β-hydroxy triketide intermediate by DH domain is not necessary for tetraketide formation and the following aldol cyclization and aromatization. Finally, 6-MSA is released by the function of so far called DH domain. Thus, we renamed the DH domain as thioester hydrolase domain[24] (Figure 3).

PKSs INVOLVED IN SPORE PIGMENT BIOSYNTHESIS IN FUNGI

In fungi, spore pigmentation seems to enhance survival and competitive abilities of fungi in certain environments though not essential for growth and development. We previously identified the function of *Aspergillus nidulans* WA PKS to produce heptaketide napthopyrone YWA1.[8,9] In *Aspergillus nidulans* and related ascomycetes, yellow pigment YWA1 is considered to be a precursor of polymerization by phenol oxidase to form green spore pigments.[25,26] In black fungi such as *Magnaporthe grisea* and *C. lagenarium*, pentaketide 1,3,6,8-tetrahydroxy naphthalene (T4HN) is converted to 1,8-dihydroxynaphthalene (DHN) and then to polymerized DHN-melanin.[11,12,27,28] In these spore pigment biosynthesis, PKSs produce T4HN as a direct product. Recently, we identified the presence of new melanin biosynthetic pathways in human pathogenic fungi, *Aspergillus fumigatus* and *Wangiella dermatitidis*.

In *Aspergillus fumigatus*, a human pathogen causing aspergillosis, conidial pigmentation of characteristic bluish-green color is an important virulence factor.[29] The gene cluster was cloned from *Aspergillus fumigatus* and confirmed to be involved in spore pigment biosynthesis.[30] The *alb1*-coded protein, Alb1p showed a typical iPKS architecture with Claisen cyclase domain.[31] Thus, the Alb1p was first assumed to be a pentaketide T4HN synthase of *Aspergillus fumigatus*. However, the product of Alb1p was identified to be heptaketide YWA1,[10] the same product as that of *Aspergillus nidulans* WA PKS. This intriguing phenomenon that heptaketide naphthopyrone is involved in DHN-melanin biosynthesis was solved by the functional identification of Ayg1p, which surprisingly converts YWA1 to T4HN by hydrolytic side-chain cleavage.[32] YWA1 is considered to be in equilibrium between hemiketal form and side-chain open form in solution. Ayg1p catalyzes hydrolytic cleavage of side chain of its open form by retro-Claisen manner to form T4HN and acetoacetate (Figure 5). Thus, formed T4HN is converted to DHN-melanin by sequential reduction, dehydration and polymerization as in black fungi. Probably, naphthopyrone YWA1 or its dehydrated derivatives serves as substrates of polymerization to form green spore pigments. In the combination of black and green pigmentation, *Aspergillus fumigatus* shows characteristic bluish-green conidial color (Figure 4).

Similar polyketide shortening was found to be involved in *W. dermatitidis* DHN-melanin biosynthesis. The responsible iPKS in *W. dermatitidis* DHN-melanin biosynthesis was cloned and named WdPKS1.[33,34] Heterologous expression of WdPKS1 in *Aspergillus oryzae* under α-amylase promoter resulted in the production of hexaketide acetyl-T4HN.[35] Although this compound was previously isolated as a product of the chimeric iPKS between heptaketie synthae *Aspergillus nidulans* WA and pentaketide *C. lagenarium* PKS1,[36] this was the first time that hexaketide acetyl-T4HN was identified as the direct product of intact iPKS. Then, *WdYG1* gene was cloned from *W. dermatitidis* as an orthlog of *Aspergillus fumigatus ayg1*.[37] *In vitro*



R = -CH_2-CO-CH_3 from YWA1; R = -CH_3 from acetyl-T4HN

Figure 5 Reactions catalyzed byAyg1p and WdYG1 for synthesis of T4HN. Ayg1p recognizes YWA1 and WdYG1 does acetyl-T4HN. R=–CH_2COCH_3 when the substrate is YWA1; R=–CH_3 when the substrate is acetyl-T4HN.

polyketide shortening activity of WdYG1 indicated that hexaketide acetyl-T4HN is converted to T4HN and then to DHN for DHN-melanin formation in *W. dermatitidis* (Fujii *et al.*, in preparation) (Figure 5).

Following the above-mentioned functional analysis of genes involved in melanin biosynthesis in *Aspergillus fumigatus*, we tried to reconstitute the whole biosynthetic pathway in yeast as a model of polyketide biosynthesis system. Thus far, DHN formation was observed in the recombinant yeast by co-expression of genes in the *Aspergillus fumigatus* conidial pigment biosynthetic gene cluster. (Nambu *et al.*, in preparation).

REDUCING-TYPE iPKSs IN *ALTERNARIA SOLANI*

In addition to aromatic polyketides, fungi also produce lots of complex reduced-type polyketides.[38,39] Statins such as lovastain and compactin are famous fungal metabolites whose derivatives are widely used for hypercholesterolemia.[40] These fungal reduced-type compounds are also produced by iPKSs which possess additional reductive domains, KR, DH, ER and MeT domains.[41] Our reducing-type iPKS (RD-iPKS) studies were carried out on *Alternaria solani*, which is a phytopathogenic fungus causing early blight disease in solanaceae plants such as potato and tomato. The fungus produces various structurally unrelated polyketide metabolites, both aromatic and reduced-type compounds. Of these, alternaric acid was indicated to be biosynthesized from two polyketide chains rather than a single chain.[42] *Alternaria solani* also produces other complex reduced-type polyketides, solanapyrones.[43–45] Biosynthetic studies strongly suggested that a biological Diels-Alder reaction is involved in formation of their decalin skeleton.[46–48] Chemical studies on *Alternaria solani* products were carried out by Professors Ichihara and Oikawa group of Hokkaido University including biosynthetic studies. Therefore, *Alternaria solani* RD-iPKS studies have been done as a collaboration work with Professor Oikawa group.

To clone RD-iPKS genes for complex reduce-type polyketides in *Alternaria solani*, we designed degenerate primer pairs for amplification of iPKS fragments from *Alternaria solani*, targeting the highly conserved regions in iPKS functional domains. Using the amplified iPKS fragments as probes, we carried out genomic DNA library screening and/or genome walking method to determine iPKS gene sequences and whole biosynthetic gene clusters. Then their functional analyses were carried out using *Aspergillus oryzae* expression system.

PKSN FOR ALTERNAPYRONE

On the basis of conserved amino acid sequence E-C/A-H-H-G-T-G-T and G-Q-G-A-Q-W located between KS and AT active sites, KSD-F and KSD-R primers were designed.[49] After amplification from the *Alternaria solani* genomic DNA, one of the fragment showed high homology with fungal RD-iPKSs such as LDKS[50] and FUM1p.[51] Using this fragment as a screening probe, the biosynthetic gene cluster containing five genes named *alt1*∼*5* was cloned from from *Alternaria solani*. Homology search indicated that the *alt1*, *2* and *3* genes code for cytochrome P450s and the *alt4* gene for an FAD-dependent oxygenase/oxidase. The *alt5* gene encodes an RD-iPKS, named PKSN, which was found to possess an MeT domain. To identify the PKSN function, the *alt5* gene was introduced into the fungal host *Aspergillus oryzae* under α-amylase promoter. The transformant produced a new polyketide compound, named alternapyrone, whose structure was identified to be 3,5-dimethyl-4-hydroxy-6-(1,3,5,7,11,13-hexamethyl-3,5,11-pentadecatrienyl)-pyran-2-one. Labeling experiments confirmed that alternapyrone is a decaketide with octa-methylation from methionine on every C_2 unit except the third unit.[49]

Several linear α-pyrone containing polyketides have been isolated from fungi, such as citreomontanin,[52] citreoviridin,[53,54] asteltoxin[55] and so forth. These are all α-pyrones substituted with multimethylated alkyl chains. In the biosynthesis of these multi-methylated open chain fungal polyketides, PKSs similar to PKSN could be operative for their initial biosynthetic steps to elaborate their carbon skeletons. PKSN was, to our knowledge, the first PKS to be identified for biosynthesis of this class of compounds (Figure 6).

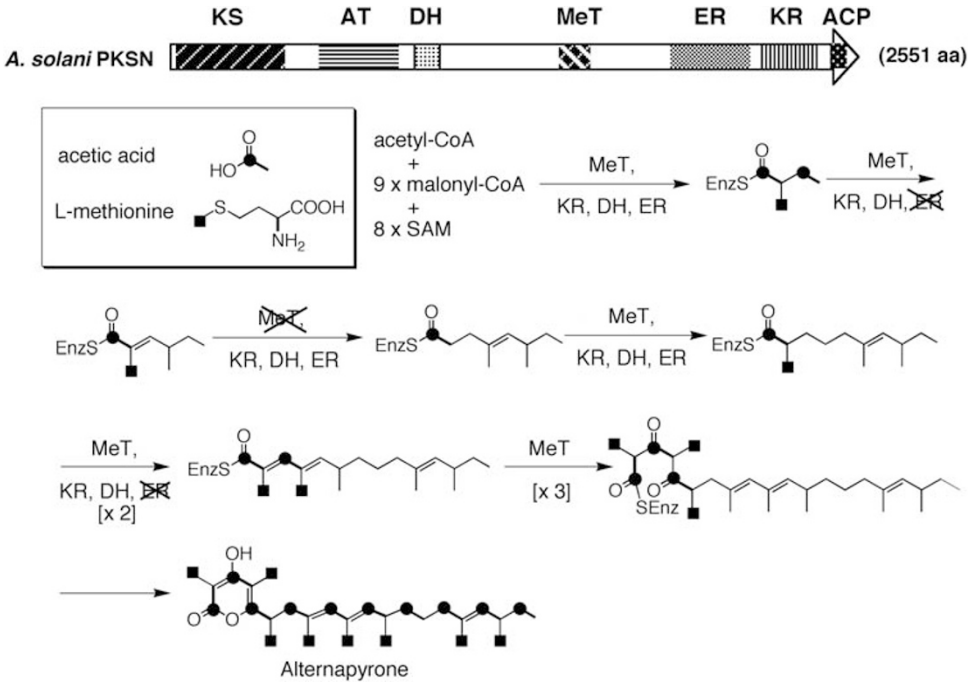


Figure 6 Architecture of PKSN and its reaction to produce alternapyrone.

PKSF FOR ASLANIPYRONE AND ASLANIOL

To investigate other interesting features of polyketide biosynthesis in *Alternaria solani*, particularly the biosynthesis of complex reduced-type compounds, we continued the molecular genetic approach, and cloned additional RD-iPKS genes from the fungus. Amplification with KSD-F and KSD-R primers gave two new PKS gene fragments in addition to that of PKSN. Then, their entire ORF sequences were determined. The full-length PKS gene named *pksF* was 6881 bp long with two putative introns encoding a protein of 2259 amino acids. The other PKS gene was named *pksK*, which was 7600 bp long with seven putative introns encoding a 2533 amino acid protein. Typical RD-iPKS conserved domains, such as KR, DH and ER, were identified in both PKSF and PKSK but no MeT domain as found in either PKSF or PKSK[56] (Figure 7).

Expression of both PKS genes was tried in *Aspergillus oryzae* under α-amylase promoter. Although the *pksK* transformant did not produce any specific PKS product, the *pksF* transformant gave strong yellow pigmentation, which was not observed in the control transformant. The *pksF* transformant's mycelia were extracted and its products were analyzed by LC-TOFMS. The results demonstrated the production of a complex series of polyketides numbering more than 11 metabolites. They varied from C_{18} to C_{26} polyene compounds with absorption maxima around 400 nm. Two main products of *pksF* transformant were isolated by silica gel column chromatography and then MPLC and HPLC. Physicochemical analysis indicated that one of the main compounds was proposed to be the all *trans*-polyene phenol compound, 3-(16-hydroxy-heptadeca-1,3,5,7,9,11,13-heptaenyl)-phenol, which was named aslaniol. The other main compound was deduced to be the all *trans*-polyene pyrone compound, 4-hydroxy-6-(16-hydroxy-heptadeca-1,3,5,7,9,11,13-heptaenyl)-pyran-2-one, named aslanipyrone[56] (Figure 8).

The formation of the major PKSF products, aslanipyrone and aslaniol, can be rationalized as follows. PKSF catalyzes the formation

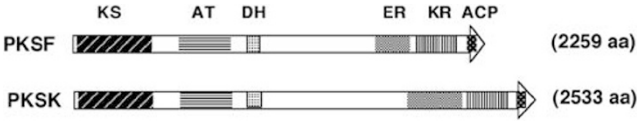


Figure 7 Architecture of PKSF and PKSK.

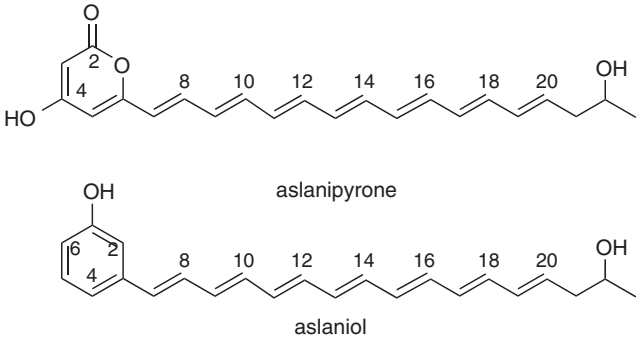


Figure 8 Chemical structures of PKSF main products aslanipyrone and aslaniol.

of a C_{22}-intermediate attached to the ACP. Release through intramolecular hydrolysis by the enolised δ-carbonyl would give the pyrone product aslanipyrone. Alternatively, KR-mediated reduction of the β-carbonyl of the C_{22}-intermediate would form a β-hydroxy thioester intermediate, which could be a substrate for a further KS-mediated condensation of an additional C_2 unit to form a C_{24}-intermediate, which cyclizes through aldol condensation followed by decarboxylation to form aslaniol as shown in Figure 9. The formation of the other minor metabolites can be rationalized by inappropriate release of the

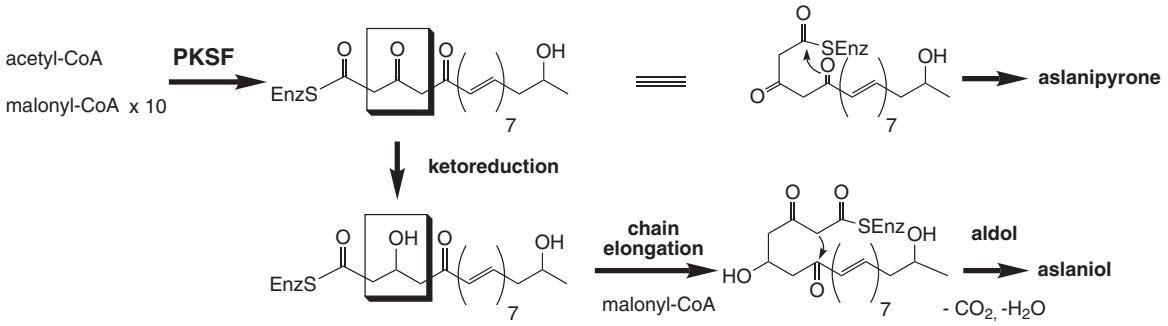


Figure 9 Proposed PKSF reaction for aslanipyrone and aslaniol production.

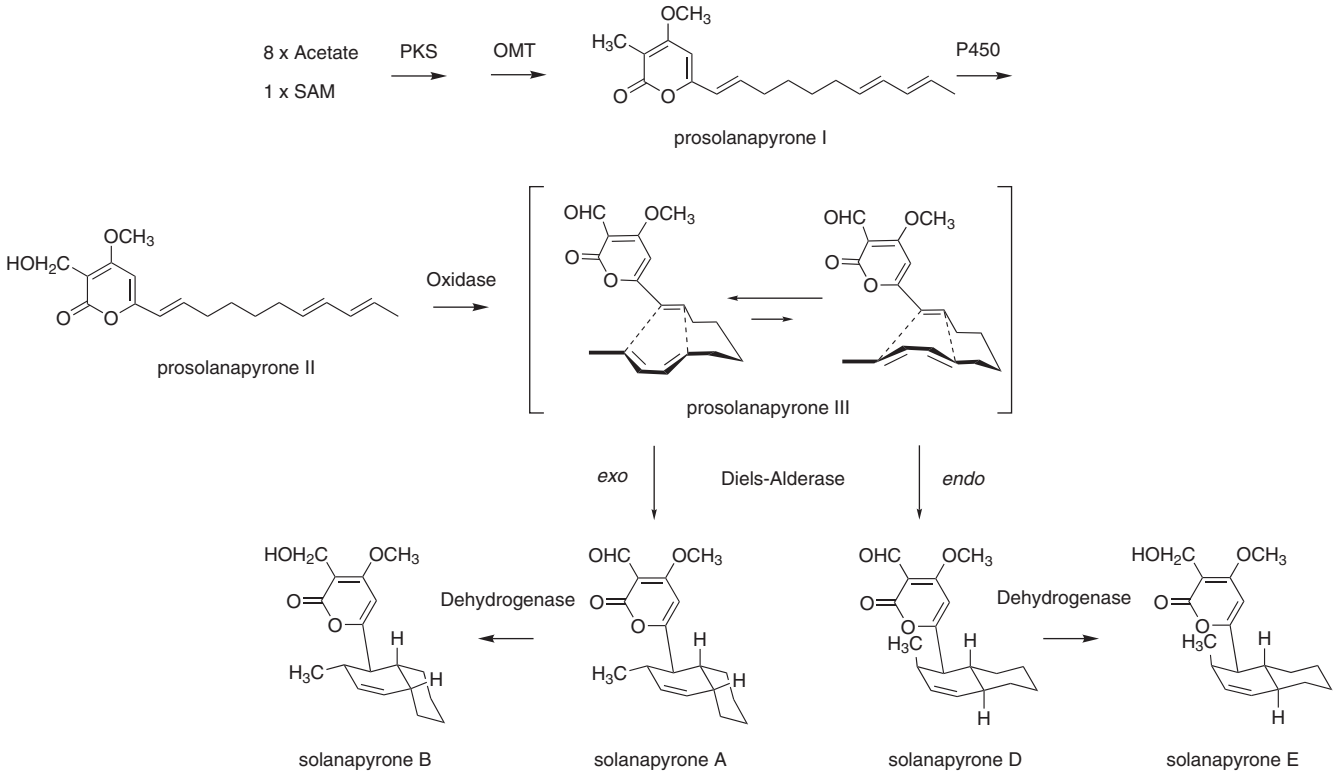


Figure 10 Solanapyrone biosynthesis in *Alternaria solani*.

growing polyketide and/or priming of the polyketide by either butyrate or hexanoate. It has been known that the impaired reduction of β-carbonyl of the triketide intermediate in MSAS reaction inhibited further condensation with C_2 unit and released TAL[20] (Figure 3). Similarly, partial reduction of β-carbonyl of C_{22}-intermediate, probably because of the rate-limiting lower activity of KR on the intermediate, might be the reason for co-production of C_{22} pyrone and C_{23} phenol. The former could be derived from the C_{22}-intermediate without further KR reduction and the latter could be a decarboxylation product of C_{24} aromatic carboxylic acid formed by additional KR reduction and C_2 condensation.

The ER domain in PKSF was found to be much shorter than those of other fungal RD-PKSs, and in its nucleotide-binding domain (G/AxGxxG), the second Gly, was replaced by a Ser residue, suggesting it to be non-functional. This is consistent with the lack of reduction of the double bonds formed by the DH domain in the PKSF products. Similar to PKSF, fusarin synthase[57] and lovastatin nonaketide synthase[50] are fungal RD-iPKSs known to have inactive ER domains. The inactivity of ER in fusarin synthase corresponds with the polyene structure of fusarin C, whereas lovastatin nonaketide synthase requires an accessory protein LovC, an ER enzyme, for nonaketide production.[50] Further sequencing is required to identify presence or absence of the *lovC*-like ER gene in the *pksF* gene cluster.

Although the *pksF* expression in *Alternaria solani* was confirmed by reverse transcription PCR, neither aslanipyrone and aslaniol nor their derivatives have been detected in *Alternaria solani*, probably because of low productivity and/or intensive modification by tailoring enzymes. Aslaniol appears to be the first example of an RD-iPKS product formed through aldol cyclization and aromatization.

SOLANAPYRONE BIOSYNTHESIS GENE CLUSTER

As described above, we cloned three RD-iPKS genes, *pksN*, *pksF*, *pksK* from *Alternaria solani*. However, none of them was involved in solanapyrone biosynthesis (Figure 10). In our previous studies,

primers used were designed from the amino acid sequence E-C/A-H-H-G-T-G-T conserved in KS domain and the amino acid sequence G-Q-G-A-Q-W conserved in AT domain. Owing to accumulation of fungal PKS sequence data by fungal genome projects, minor variations in these conserved amino acid sequences have been found. Among them, E-C-H-G-T-G-T variation was considered to be critical for fungal PKS gene amplification. Thus, we redesigned degenerate primers derived from (V/F)-E-C-H-G-T-G-T sequence for forward primer and T-G-Q-G-A-Q-W sequence for reverse primer, and used other highly conserve regions such as D-T-A-C-S-S in KS domain, G-H-S-S-G-E in AT domain.

In combination use of these primers, four new PKS fragments *AS1*∼*4* were amplified from the *Alternaria solani* genomic DNA. RT–PCR analysis of these genes was carried out with mRNA prepared from *Alternaria solani* mycelia cultured for 15 days at the timing when solanapyrone biosynthesis began. Only *AS2* expression was detected at significant level, indicating that *AS2* was involved in solanapyrone biosynthesis. Then, the full-length *Pks-AS2* gene was determined to be 8118 bp long with three introns encoding a protein of 2641 amino acids. BLAST search indicated that encoded was an RD-iPKS with KS, AT and ACP, and KR, DH and ER. In addition, MeT domain was identified, which is essential for synthesis of prosolanapyrone, a biosynthetic precursor of solanapyrones. The *Pks-AS2* gene was subcloned into a fungal expression plasmid. *Aspergillus oryzae* transformants with *Pks-AS2* gave a specific product in the mycelial extract, which was identified to be a new octaketide, 4-hydroxy-3-methyl-6-undeca-1,7,9-trienyl-pyran-2-one. This compound was a demethylated form of prosolanapyrone I, which was the established biosynthetic precursor of solanapyrones by feeding experiments.[58,59] Thus, the PKS product was named desmethylprosolanapyrone I, and the *Pks-AS2* coding PKS was prosolanapyrone synthase (PSS)[60] (Figure 11).

Identification of the *Pks-AS2* gene to encode PSS prompted us to determine the whole gene cluster for solanapyrone biosynthesis by further genome walking. In the 5′ upstream region of the *Pks-AS2* gene, a possible solanapyrone biosynthetic gene cluster was found consisting of additional five genes for *O*-methyltransferase, dehydrogenase, transcription factor, oxidase and P450. The proposed biosynthetic scheme for solanapyrone could be well explained by the function of these enzymes encoded in the cluster. Thus, the genes in this cluster were named *sol1*∼*6* for PKS, *O*-methyltransferase, dehydrogenase, transcription factor, oxidase and P450, respectively (Figure 12).

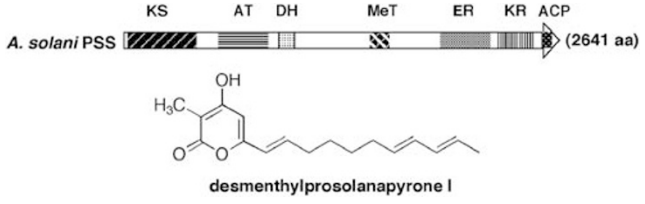


Figure 11 Architecture of PSS coded by *sol1* and its product desmethylprosolanapyrone I.

The *sol5* encoded a 515 amino acid long polypeptide, which was considered to be solanapyrone synthase (SPS), a Diels-Alderase for solanapyrone biosynthesis. Conserved domain search indicated the presence of flavin-binding domain and His^{128} residue for covalent flavinylation. For confirmation, we first expressed the Sol5 oxidase in *Aspergillus oryzae* under the starch inducible α-amylase promoter. The crude enzyme prepared from the induction mycelia of the *sol5* transformant showed enzyme activity to convert prosolanapyorne II to solanpyrone A and D as expected, and thus confirmed was that the *sol5* encodes SPS. However, attempts to purify the recombinant SPS from the *Aspergillus oryzae* transformant resulted in partial purification.

Functional expression of the Sol5 oxidase in *Escherichia coli* was attempted intensively, but no enzyme activity was detected in spite of high-level protein expression. One of the possible reasons was assumed that proper covalent flavinylation of Sol5 could not occur in *E. coli*. To purify Sol5 oxidase, we tried to express it in *Pichia pastoris*. The *sol5* cDNA without secretion signal sequence was placed under *Pichia* α-signal peptide secretion sequence to construct pPIC-sol5. With methanol induction, the induction culture medium of *P. pastoris* transformant showed SPS activity. From induction culture medium of the transformant, SPS was purified by seven steps. After the second Phenyl Sepharose column, the preparation still showed broad diffuse banding on SDS–PAGE, possibly because of glycosidation. In fact, endoglycosidase digestion gave a clear band of SPS of the expected size on SDS–PAGE. After Mono Q ion exchange and Superdex gel filtration purification afforded the homogeneous SPS preparation as judged by SDS–PAGE analysis.

The enzymatically formed *exo*-adduct solanapyrone A by the recombinant SPS from prosolanapyrone II was subjected to circular dichroism analysis. Its observed spectrum was identical with that of natural (−)-solanapyrone A. These results unambiguously confirmed that the SPS encoded by *sol5* catalyzes the oxidation and following *exo*-selective Diels-Alder cyclization to form (−)-solanapyrone A from prosolanapyrone II.[60]

PKS-NONRIBOSOMAL PEPTIDE SYNTHASE HYBRID CPAA

Gene cloning and sequencing were required for functional studies on target genes. In this context, we strived to clone PKS genes from fungi. However, genome projects including fungi dramatically changed this situation. In fungi, genome sequences of three *Aspergillus* species (*Aspergillus nidulans*, *Aspergillus fumigatus* and *Aspergillus oryzae*) were reported in 2005.[61–63] Fungal genome projects revealed the presence of quite large number of secondary metabolism genes. *Aspergillus oryzae* possesses 30 PKS genes, but most of them are not expressed in the culture conditions so far examined. Thus, their functions have been unknown. Hence, we started the project to identify the functions of *Aspergillus oryzae* PKSs using our induction expression system, which is now underway. Out of 30 PKSs genes in *Aspergillus oryzae*, 2 genes were found to be truncated, thus non-functional. We noticed one of them located in chromosome 3 near

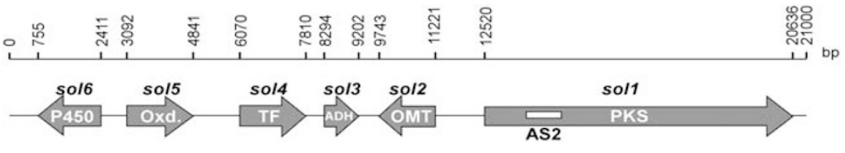


Figure 12 *Sol* gene cluster for solanapyrone biosynthesis cloned from *Alternaria solani*.

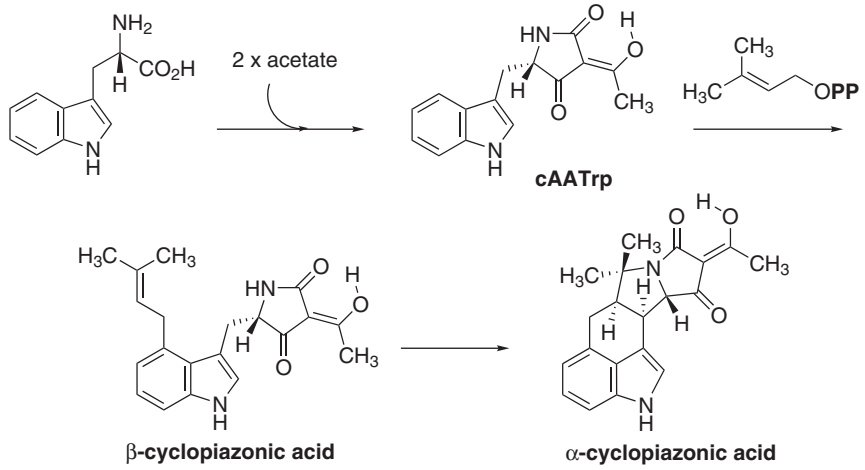


Figure 13 Biosynthesis of α-cyclopiazonic acid.

telomeric end should have been involved in cyclopiazonic acid (CPA) biosynthesis because of gene cluster organization and some other information.[64]

CPA is an indole tetramic acid that was first isolated from *P. cyclopium* as a toxic metabolite in 1968.[65] CPA inhibits sarcoplasmic reticulum Ca^{2+}-ATPases,[66] and it is known that inhibition of Ca^{2+}-ATPases results in cell death through apoptotic pathways within the endoplasmic reticulum and the mitochondria.[67] In addition to *Penicillium* species several other fungi including the *Aspergillus* species have been reported to produce CPA,[68,69] whose contamination of agricultural products, such as maize, corn and peanut, is a serious economic and health problem.[70–72] It is also noteworthy that some strains of *Aspergillus oryzae,* which are used in fermentation industry, are capable of producing CPA.[73]

As *Aspergillus oryzae* RIB40, the genome reference strain, does not produce CPA, we analyzed the corresponding chromosomal regions of *Aspergillus oryzae* NBRC 4177 and its close relative *Aspergillus flavus* NRRL 3357. Both are CPA producing strains. Although *Aspergillus oryzae* RIB40 has only KS and AT domains as the truncated PKS, we identified a full-length PKS-nonribosomal peptide synthase (NRPS) hybrid gene in CPA producing strains. Its gene disruption made NBRC 4177 to be CPA non-producing.[64] Thus, we identified the PKS-NRPS gene, named *cpaA*, involved in CPA biosynthesis in *Aspergillus oryzae* and *Aspergillus flavus*.

From the previous biosynthetic studies, cycloacetoacetyltryptophan (cAATrp) was established to be the first stable intermediate in CPA biosynthesis starting from Trp.[74] Its tetramic acid moiety could be formed from diketide acetoacetate and Trp by a PKS-NRPS hybrid enzyme (Figure 13).

Thus, the product of PKS-NRPS hybrid enzyme CpaA was assumed to be cAATrp, but no direct confirmation has been carried out. As *Aspergillus flavus* genome sequence was available (http://www.aspergillusflavus.org/genomics/) before we carried out *Aspergillus oryzae cpaA* sequencing and its gene disruption, we first set out the heterolgous expression of *Aspergillus flavus* CpaA PKS-NRPS in *Aspergillus oryzae*. Later, it was confirmed that CpaAs from *Aspergillus flavus* NRRL 3357 and *Aspergillus oryzae* NBRC 4177 are quite identical with each other with 92% amino acid sequence identity.

The *cpaA* gene from *Aspergillus flavus* NRRL 3357 is 11 721 bp long and encodes a PKS-NRPS hybrid enzyme of 3906 amino acids with a calculated molecular mass of 431 kDa. Dot matrix comparison of the PKS module in CpaA N-terminal half with LDKS, which is a diketide

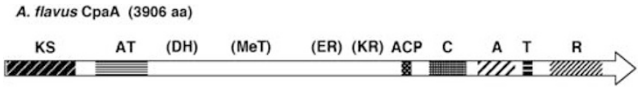


Figure 14 *Aspergillus flavus* CpaA architecture.

synthase involved in lovastatin biosynthesis,[50] indicated that they are similar in amino acid lengths and show fairly high homology from the N-terminus to ACP domain. Detailed comparison showed that CpaA PKS module possesses, in addition to KS, AT and ACP domains, MeT, DH, ER and KR domain regions as LDKS does though these domains in CpaA seem to be non-functional. The C-terminus half of CpaA contains an NRPS module with condensation (C), adenylation (A), thiolation (T or peptidyl carrier protein) and putative releasing (R) domains (Figure 14). To verify whether CpaA is responsible for the formation of cAATrp or any other biosynthetic intermediate, we carried out the heterologous expression of CpaA PKS-NRPS.

The *cpaA* of *Aspergillus flavus* was expressed under α-amylase promoter in *Aspergillus oryzae* M-2-3 and its product was analyzed by HPLC. A single major peak observed was isolated and identified to be cAATrp. Identification of cAATrp as the product of CpaA rationalized the reactions catalyzed by the PKS-NRPS as follows. Essentially, the diketide acetoacetyl-ACP formed from condensation of the acetyl-CoA and malonyl-CoA by the PKS module, and L-Trp activated by A domain of the NRPS module is loaded on the peptidyl carrier protein in T domain, and then condensation is catalyzed by C domain. Thus, the formed intermediate is released from T domain catalyzed by R domain with the formation of tetramic acid moiety (Figure 15).

Presence of PKS-NRPS hybrid in fungi was first reported in *Fusarium moniliforme* and *F. venenatum* involved in fusarin C biosynthesis.[57] Since then, fungal tetramic acids and derivatives, including tenellin,[75] pseurotin A[76] and chaetoglobosin A,[77] have been revealed to be biosynthesized by PKS-NRPSs. Their deduced enzyme architecture is quite similar with each other including CpaA. Our fungal PKS expression system under inducible α-amylase promoter in *Aspergillus oryzae* was applied to fungal PKS-NRPS functional analysis, *Beuveria bassiana* TenS expression, by Cox and co-workers.[78] Considering the relatively simple reactions involved, and specific and high production of cAATrp by CpaA, our result not only adds to successful example of fungal PKS-NRPS expression, but also provides an ideal platform for PKS-NRPS functional analysis.

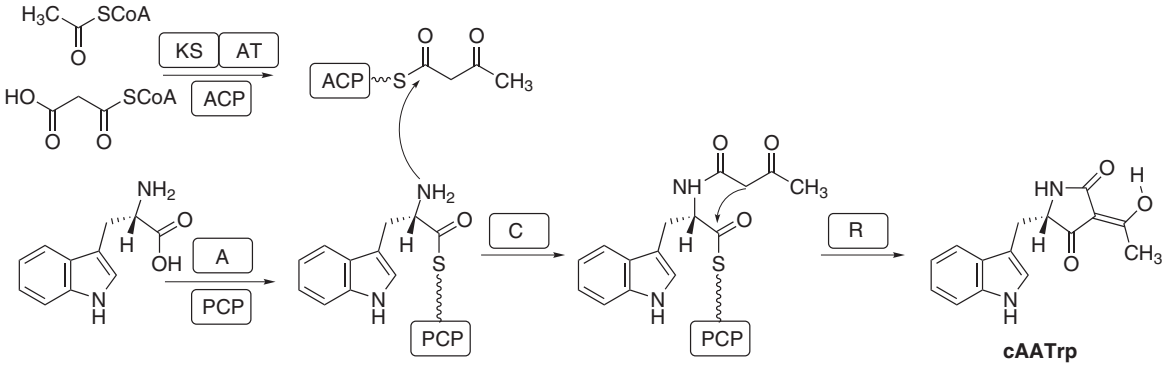


Figure 15 Reaction catalyzed by PKS-NRPS CpaA.

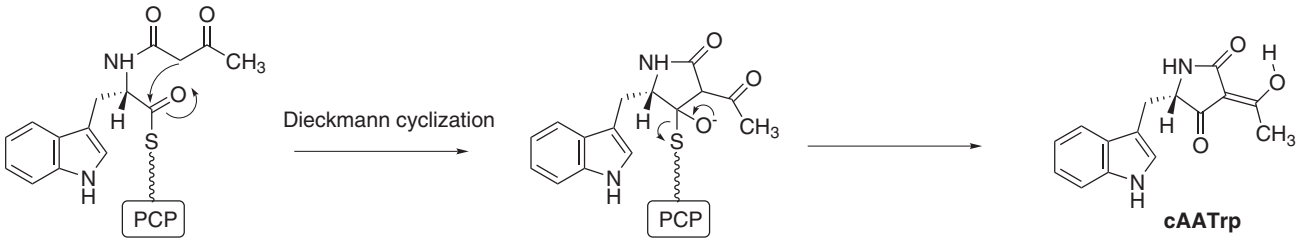


Figure 16 Proposed product release mechanism by CpaA R domain.

The C-terminus of CpaA is a putative releasing (R) domain as are other fungal PKS-NRPSs. It had been believed that fungal PKS-NRPS R domain could act on acyl amide thioester intermediate to release it as an aldehyde by NAD(P)H aided reduction. However, products of TenS expression in *Aspergillus oryzae* were identified to be tetramic acid/hydroxypyrolinone intermediates that are considered to be directly formed by TenS PKS-NRPS. From these results, Cox and co-workers proposed that the putative R domain catalyzes a Dieckmann cyclization of the bound *N*-β-ketoacyl α-aminothioester intermediate and releasing the cyclized product.[78] In our experiments of CpaA expression, cAATrp with tetramic acid structure was identified to be a PKS-NRPS product as was reported for TenS expression. Sims and Schmidt[79] carried out *in vitro* functional analysis of equisetin synthase (EquiS) R domain to determine the enzymatic basis on tetramic acid formation. Their results using synthetic substrate analogs strongly supported that EqiS R domain catalyzes a Dieckmann condensation (Figure 16). This mechanism will further be verified by functional analysis of CpaA R domain using the expression system that we reported here.

PROSPECTS

Compared with the progress in bacterial PKS studies, fungal iPKS studies were much retarded because of intrinsic single modular iterative reaction nature. However, yeast and *E. coli* expression systems[80–82] have recently been shown to be applicable for fungal iPKS studies in addition to the fungal expression system we have used. These successful expressions have led to reconstitution of lovastatin nonaketide synthase system,[82] deconstruction and reconstruction of PksA for aflatoxin biosynthesis,[81] and more recently structural analysis of the product template domain from PksA.[83] Further progress will not only contribute to functional and structural analysis in detail but also provide tools to produce novel compounds based on this class of enzymes. In the post-genomics era, lots of sequence information including sequences of iPKS and their gene clusters are waiting to be elucidated of their functions. Different approaches have been applied to identify gene functions, such as promoter exchange[84,85] and chromatin-level modification.[86] Moreover, expression of global regulators induced the production of compounds.[87–89] In combination, our continuous efforts will contribute to identify gene functions and production of useful compounds based on iPKSs and related enzymes.

ACKNOWLEDGEMENTS

These studies were performed in my current laboratory at Iwate Medical University and my previous laboratory at The University of Tokyo, together with Dr T Moriguchi, Dr K Kasahara, Dr Y Seshime, Mr S Shimomaki, Mr Y Yasuoka, Mr M Naruse, Ms N Yoshida, Ms N Nambu and Professor Emeritus Y Ebizuka. I thank to collaborators, Professor H Oikawa (Hokkaido University), Dr J Kwon-Chung and Dr Tsai (US NIH), Dr M Wheeler (USDA), Professor P Szaniszlo (University of Texas), Professor K Kitamoto (The University of Tokyo), Professor K Gomi (Tohoku University). These works were supported in part by Grant-in-Aids for Scientific Research (B) (No. 19310139) to IF from the Japan Society of the Promotion of Science and by a Grant-in-Aid for Scientific Research on Priority Areas 'Applied Genomics', to IF from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

1 Birch, A. J., Massy-Westropp, R. A. & Moye, C. J. Biosynthesis (VII) 2-hydroxy-6-methyl benzoic acid in *Penicillium griseofulvum*. *Aust. J. Chem.* **8,** 539–544 (1955).
2 Dimroth, P., Walter, H. & Lynen, F. Biosynthese von 6-methylsalicylsäure. *Eur. J. Biochem.* **13,** 98–110 (1970).
3 Beck, J., Ripka, S., Signer, A., Schiltz, E. & Schweizer, E. The multifunctional 6-methylsalicylic acid synthase gene of *Penicillium patulum*. Its gene structure relative to that of other polyketide synthases. *Eur. J. Biochem.* **192,** 487–498 (1990).
4 Shen, B. Polyketide biosynthesis beyond the type I, II and III polyketide synthase paradigms. *Curr. Opin. Chem. Biol.* **7,** 285–295 (2003).
5 Fujii, I., Watanabe, A. & Ebizuka, Y. More functions for multifunctional polyketide synthases. in *Advances in Fungal Biotechnology for Industrial, Agriculture, and Medicine* (eds Tkacz, J.S. & Lange, L.) 97–125 (Kluwer Academic/Plenum Publishers, New York, 2004).

6 Wenzel, S. C. & Müller, R. Formation of novel secondary metabolites by bacterial mutimodular assembly lines: deviatons from textbook biosynthetic logic. *Curr. Opin. Chem. Biol.* **9,** 447–458 (2005).
7 Fujii, I. *et al.* Cloning of the polyketide synthase gene *atX* from *Aspergillus terreus* and its identification as the 6-methylsalicylic acid synthase gene by heterologous expression. *Mol. Gen. Genet.* **253,** 1–10 (1996).
8 Watanabe, A. *et al.* Product identification of polyketide synthase coded by *Aspergillus nidulans wA* gene. *Tetrahedron Lett.* **39,** 7733–7736 (1998).
9 Watanabe, A. *et al.* Re-identification of *Aspergillus nidulans wA* gene to code for a polyketide synthase of naphthopyrone. *Tetrahedron Lett.* **40,** 91–94 (1999).
10 Watanabe, A. *et al. Aspergillus fumigatus alb1* encodes naphthopyrone synthase when expressed in *Aspergillus oryzae*. *FEMS Microbiol. Lett.* **192,** 39–44 (2000).
11 Fujii, I. *et al.* Heterologous expression and product identification of *Colletotrichum lagenarium* polyketide synthase encoded by the PKS1 gene involved in melanin biosynthesis. *Biosci. Biotechnol. Biochem.* **63,** 1445–1452 (1999).
12 Fujii, I. *et al.* Enzymatic synthesis of 1,3,6,8-tetrahydroxynaphthalene solely from malonyl coenzyme A by a fungal iterative Type I polyketide synthase PKS1. *Biochemistry* **39,** 8853–8858 (2000).
13 Fujii, T., Yamaoka, H., Gomi, K., Kitamoto, K. & Kumagai, C. Cloning and nucleotide sequence of the ribonuclease T_1 Gene (*rntA*) from *Aspergillus oryzae* and its expression in *Saccharomyces cerevisiae* and *Aspergillus oryzae*. *Biosci. Biotech. Biochem.* **59,** 1869–1874 (1995).
14 Kealey, J. T., Liu, L., Santi, D. V., Betlach, M. C. & Barr, P. J. Production of a polyketide natural product in nonpolyketide-producing prokaryotic and eukaryotic hosts. *Proc. Natl Acad. Sci. USA* **95,** 505–509 (1998).
15 Nakano, M. M., Gorbell, N., Besson, J. & Zuber, P. Isolation and characterization of *sfp*: a gene that functions in the production of the lipopeptide biosurfactant, surfactin, in *Bacillus subtilis*. *Mol. Gen. Genet.* **232,** 313–321 (1992).
16 Moriguchi, T., Ebizuka, Y. & Fujii, I. Analysis of subunit interactions in the iterative type I polyketide synthase ATX from *Aspergillus terreus*. *Chembiochem* **7,** 1869–1874 (2006).
17 Rost, B., Yachdav, G. & Liu, J. The PredictProtein server. *Nucleic Acid Res.* **32,** W321–W326 (2004).
18 Ahlert, J. *et al.* The calicheamicin gene cluster and its iterative type I enddiyne PKS. *Science* **297,** 1173–1176 (2002).
19 Moriguchi, T., Ebizuka, Y. & Fujii, I. Domain-domain interactions in the iterative type I polyketide synthase ATX from *Aspergillus terreus*. *Chembiochem* **9,** 1207–1212 (2008).
20 Richardson, M. T., Pohl, N. L., Kealy, J. T. & Khosla, C. Tolerance and specificity of recombinant 6-methylsalicylic acid synthase. *Metab. Eng.* **1,** 180–187 (1999).
21 Maier, T., Leibundgut, M. & Ban, N. The crystal structure of a mammalian fatty acid synthase. *Science* **321,** 1315–1322 (2008).
22 Rangan, V. S., Johi, A. K. & Smith, S. Mapping the functional topology of the animal fatty acid synthase by mutant complementation *in vitro*. *Biochemisry* **40,** 10792–10799 (2001).
23 Gaisser, S., Trefzer, A., Stckert, S., Kirshning, A. & Bechthold, A. Cloning of an avilamycin biosynthetic gene cluster from *Streptomyces viridochromogenes* Tü57. *J. Bacteriol.* **179,** 6271–6278 (1997).
24 Moriguch, T., Kezuka, Y., Nonaka, T., Ebizuka, Y. & Fujii, I. Hidden function of catalytic domain in 6-methylsalicylic acid synthase. *J. Biol. Chem.* (in press) (2010).
25 Mayorga, M. E. & Timberlake, W. E. Isolation and molecular characterization of the *Aspergillus nidulans wA* gene. *Genetics* **126,** 73–79 (1990).
26 Mayorga, M. E. & Timberlake, W. E. The developmentally regulated *Aspergillus nidulans wA* gene encodes a polypeptide homologous to polyketide and fatty acid synthases. *Mol. Gen. Genet.* **235,** 205–212 (1992).
27 Takano, Y. *et al.* Structual analysis of *PKS1*, a polyketide synthase gene involved in melanin biosynthesis of *Colletotrichum lagenarium*. *Mol. Gen. Genet.* **249,** 162–167 (1995).
28 Kubo, Y., Nakamoto, H., Kobayashi, K., Okuno, T. & Furusawa, I. Cloning of a melanin biosynthesis gene essential for appressorial penetration of *Colletotrichum lagenarium*. *Mol. Plant Microb. Interact.* **4,** 440–445 (1991).
29 Tsai, H.- F., Washburn, R. G., Chang, Y. C. & Kwon-Chung, K. J. *Aspergillus fumigatus arp1* modulates conidial pigmentation and complement deposition. *Mol. Microbiol.* **26,** 175–183 (1997).
30 Tsai, H.- F., Chang, Y. C., Washburn, R. G., Wheeler, M. H. & Kwon-Chung, K. J. The developmentally regulated *alb1* gene of *Aspergillus fumigatus*: its role in modulation of conidial morphology and virulence. *J. Bacteriol.* **180,** 3031–3038 (1998).
31 Fujii, I., Watanabe, A., Sankawa, U. & Ebizuka, Y. Identificatin of Claisen cyclase domain in fungal polyketide synthase WA, a naphthopyrone synthase of *Aspergillus nidulans*. *Chem. Biol.* **8,** 189–197 (2001).
32 Tsai, H.- F. *et al.* Pentaketide melanin biosynthesis in *Aspergillus fumigatus* requies chain-length shortening of a heptaketide precursor. *J. Biol. Chem.* **276,** 29292–29298 (2001).
33 Feng, B. *et al.* Molecular cloning and characterization of *WdPKS1*, a gene involved in dihydroxynaphthalene melanin biosynthesis and virulence in *Wangiella* (*Exopphiala*) *dermatitidis*. *Infect. Immun.* **69,** 1781–1794 (2001).
34 Wheeler, M. H. & Sipanovic, R. D. Melanin biosynthesis and the metabolisim of flavilin and 2-hydroxyjuglone in *Wangiella dermatitidis*. *Arch. Microbiol*. **142,** 234–241 (1985).
35 Wheeler, M. H. *et al.* New biosynthetic step in the melanin pathway of *Wagiella* (*Exophiala*) *dermatitidis*: evidence for 2-acetyl-1,3,6,8-tetrahydroxynaphthalene as a novel precursor. *Eukaryot. Cell* **7,** 1699–1711 (2008).
36 Watanabe, A. & Ebizuka, Y. A novel hexaketide naphthopyrone synthesized by a chimeric polyketide synthase coomposed of fungal pentaketide and heptaketide synthases. *Tetrahedron Lett.* **43,** 843–846 (2002).
37 Wheeler, M. H., Cheng, Q., Puckhaber, L. S. & Szaniszlo, P. J. Evidence for heptaketeide melanin biosynthesis and chain-shortening of the melanin precursor YWA1 to 1,3,6,8-tetrahydroxynaphthalene in *Wangiella (Exophilla) dermatitidis*. in *105th Gen. Meet. Am. Soc.* Vol. abst. F036 266 (Washington, DC, 2005).
38 Cox, R. J. Polyketides, proteins and genes in fungi: programmed nano-machines begin to reveal their secrets. *Org. Biomol. Chem.* **5,** 2010–2026 (2007).
39 Hoffmeister, D. & Keller, N. P. Natural products of filamentous fungi: enzymes, genes, and their regulation. *Nat. Prod. Rep.* **24,** 393–416 (2007).
40 Davidson, M. H. & Robinson, J. G. Lipid-lowering effects of statins: a comparative reiview. *Expert Opin. Pharmacother.* **7,** 1701–1714 (2006).
41 Hutchinson, C. R. *et al.* Molecular genetics of lovastatin and compactin biosynthesis. in *Handbook of Industrial Mycology* (ed An, Z.) 479–491 (Marcel Dekker, New York, 2005).
42 Ichihara, A. & Oikawa, H. Biosynthesis of phytotoxins from *Alternaria solani*. *Biosci. Biotechnol. Biochem.* **61,** 12–18 (1997).
43 Ichihara, A., Tazaki, H. & Sakamura, S. Solanapyrones A, B and C, phytotoxic metabolites from the fungus *Alternaria solani*. *Tetrahedron Lett.* **24,** 5373–5376 (1983).
44 Ichihara, A., Miki, M. & Sakamura, S. Absolute configuration of (−)-solanapyrone A. *Tetrahedron Lett.* **26,** 2453–2454 (1985).
45 Oikawa, H. *et al.* Biosynthesis of solanapyrone A, a phytotoxin of *Alternaria solani*. *J. Chem. Soc. Chem. Commun.* 1282–1284 (1989).
46 Oikawa, H., Katayama, K., Suzuki, Y. & Ichihara, A. Enzymatic activity catalyzing exo-selective Diels-Alder reaction in solanapyrone biosynthesis. *J. Chem. Soc. Chem. Commun.* 1321–1322 (1995).
47 Oikawa, H., Kobayashi, T., Katayama, K., Suzuki, Y. & Ichihara, A. Total synthesis of (−)-solanapyrone A via enzymatic Diels-Alder reaction of prosolanapyrone. *J. Org. Chem.* **63,** 8748–8756 (1998).
48 Ichihara, A. & Oikawa, H. The Diels-Alder reaction in biosynthesis of polyketide phytotoxins. in *Comprehensive Natural Products Chemistry* Vol. 1 (ed Sankawa, U.) 367–408 (Elsevier, Oxford, 1999).
49 Fujii, I., Yoshida, N., Shimomaki, S., Oikawa, H. & Ebizuka, Y. An iterative type I polyketide synthase PKSN catalyzing synthesis of the decaketide alternapyrone with regio-specific octa-methylation. *Chem. Biol.* **12,** 1301–1309 (2005).
50 Kennedy, J. *et al.* Modulation of polyketide synthase activity by accessory proteins during lovastatin biosynthesis. *Science* **284,** 1368–1372 (1999).
51 Proctor, R. H. & Desjaradins, A. E. A polyketide synthase gene required for biosynthesis of Fusarium mycotoxins in *Gebberella fujikuroi* mating population A. *Fungal Genet. Biol.* **27,** 100–112 (1999).
52 Rebuffat, S., Davoust, D., Molho, L. & Molho, D. Citreomontanin, a new polyene a-pyrone isolated from *Penicillium pedemontanum*. *Phytochemistry* **19,** 427–431 (1980).
53 Sakabe, N., Goto, T. & Hirata, Y. Structure of citreoviridin, a mycotoxin produced by *Penicillium citreo-viride* molded on rice. *Tetrahedron* **33,** 3077–3081 (1977).
54 Niwa, M., Endo, T., Ogiso, S., Furukawa, H. & Yamamura, S. Two new pyrones, metabolites of *Penicillium citreo-viride* Biourge. *Chem. Lett.* **10,** 1285–1288 (1981).
55 Kruger, G. J., Steyn, P. S., Vleffar, R. & Rabie, C. J. X-ray crystal structure of asteltoxin, a novel mycotoxin from *Aspergillus stellatus* Curzi. *J. Chem. Soc. Chem. Commun.* 441–442 (1979).
56 Kasahara, K., Fujii, I., Oikawa, H. & Ebizuka, Y. Expression of *Alternaria solani* PKSF generates a set of complex reduced-type polyketides with different carbon-lengths and cyclization. *Chembiochem* **7,** 920–924 (2006).
57 Song, Z., Cox, R. J., Lazarus, C. M. & Simpson, T. J. Fusarin C biosynthesis in *Fusarium moniliforme* and *Fusarium venenatum*. *Chembiochem* **5,** 1196–1203 (2004).
58 Oikawa, H., Suzuki, Y., Naya, A., Katayama, K. & Ichihara, A. First direct evidence in biological Diels-Alder reaction of incorporation of diene-dienophile precursors in the biosynthesis of solanapyrone. *J. Am. Chem. Soc.* **116,** 3605–3606 (1994).
59 Oikawa, H. *et al.* Involvement of Diels-Alder reaction i biosynthesis of secondary natural products: the late stage of the biosynthesis of the phytotoxins solanapyrones. *J. Chem. Soc. Perkin Trans.* **1,** 1225–1232 (1999).
60 Kasahara, K. *et al.* Solanapyrone synthase, a possible Diels-Aldrase, and iterative type I polyketide synthase encoded in a biosynthetic gene cluster from Alternaria solani. *Chem. Biol.* (in press) (2010).
61 Galagan, J. E. *et al.* Sequencing of *Aspergillus nidulans* and comparative analysis with *A. fumigatus* and *A. oryzae*. *Nature* **438,** 1105–1115 (2005).
62 Nierman, W. C. *et al.* Genomic sequence of the pathogenic ana allergenic filamentous fungus *Aspergillus fumigatus*. *Nature* **438,** 1151–1156 (2005).
63 Machida, M. *et al.* Genome sequencing and analysis of *Aspergillus oryzae*. *Nature* **438,** 1157–1161 (2005).
64 Tokuoka, M. *et al.* Identification of a novel polyketide synthase-nonribosomal peptide synthase (PKS-NRPS) gene required for the biosynthesis of cyclopiazonic acid in *Aspergillus oryzae*. *Fungal Genet. Biol.* **45,** 1608–1615 (2008).
65 Holzapfel, C. W. Isolation and structure of two new indole derivatives from *Penicillium cyclopium*. *Tetrahedron* **24,** 2101–2119 (1968).
66 Seidler, N. W., Jona, I., Vegh, M. & Martonosi, A. Cyclopiazonic acid is a specific inhibitor of the Ca2+-ATPase of sarcoplasmid reticulum. *J. Biol. Chem.* **264,** 17816–17823 (1989).
67 Venkatesh, P. K., Vairamuthu, S., Balachandran, C., Manohar, B. M. & Raj, G. D. Induction of apoptosis by fungal culture materials containing cyclopiazonic acid and T-2 toxin in primary lymphoid organs of broiler chickens. *Mycopathologia* **159,** 393–400 (2005).

68 Dorner, J. W., Cole, R. J. & Diener, U. L. The relationship of *Aspergillus flavus* and *Aspergillus parasiticus* with reference to production of aflatoxin and cyclopiazonic acid. *Mycopathologia* **87,** 13–15 (1984).
69 Vinokurova, N. G., Ivanushkina, N. E., Khme'nitskaya, I. I. & Arinbasarov, M. U. Synthesis of a-cyclopiazonic acid by fungi of the genus *Aspergillus*. *Appl. Biochem. Microbiol.* **43,** 435–438 (2007).
70 Gallgher, R. T., Richard, J. L., Stahr, H. M. & Cole, R. J. Cyclopiazonic acid production by aflatoxigenic and non-aflatoxigenic strains of *Aspergillus flavus*. *Mycopathologia* **66,** 31–36 (1978).
71 Urano, T. *et al*. Co-occurrence of cyclopiazonic acid and aflatoxin in corn and peanuts. *J. AOAC Int.* **75,** 838–841 (1992).
72 Lansden, J. A. & Davidson, J. I. Occurrence of cyclopiazonic acid in peanuts. *Appl. Environ. Microbiol*. **45,** 766–769 (1983).
73 Orth, R. Mycotoxins of *Aspergillus oryzae* strains for use in the food industry as starters and enzymes producing molds. *Ann. Nutr. Aliment* **31,** 617–624 (1977).
74 Holzapfel, C. W. & Wilkins, D. C. Biosynthesis of cyclopiazonic acid. *Phytochemistry* **10,** 351–358 (1971).
75 Eley, K. L. *et al.* Biosynthesis of the 2-pyridone tenellin in the insect pathogenic fungus *Beauveria bassiana*. *Chembiochem* **8,** 289–297 (2007).
76 Maiya, S., Grundmann, A., Li, X., Li, S.- M. & Turner, G. Identification of a hybrid PKS/NRPS required for pseurotin A biosynthesis in the human pathogen *Aspergillus fumigatus*. *Chembiochem* **8,** 1736–1743 (2007).
77 Schümann, J. & Hertweck, C. Molecular basis of cytochalasan biosynthesis in fungi: gene cluster analysis and evidence for the involvement of a PKS-NRPS hybrid synthase by RNA silencing. *J. Am. Chem. Soc.* **129,** 9564–9565 (2007).
78 Halo, L. M. *et al.* Authentic heterologous expression of the tenellin iterative polyketide synthase nonribosomal peptide synthase requires coexpression with an enoyl reductase. *Chembiochem* **9,** 585–594 (2008).
79 Sims, J. W. & Schmidt, E. W. Thioesterase-like role for fungal PKS-NRPS hybrid reductive domains. *J. Am. Chem. Soc.* **130,** 11149–11155 (2008).
80 Ma, S. M. *et al.* Enzymatic synthesis of aromatic polyketides using PKS4 from *Gibberella fujikuroi*. *J. Am. Chem. Soc.* **129,** 10642–10643 (2007).
81 Crawford, J. M. *et al.* Deconstruction of iterative multidomain polyketide synthase function. *Science* **320,** 243–246 (2008).
82 Ma, S. M. *et al.* Complete reconstitution of a highly reducing iterative polyketide synthase. *Science* **326,** 589–592 (2009).
83 Crawford, J. M. *et al.* Structural basis for biosynthetic programming of fungal aromatic polyketide cyclization. *Nature* **461,** 1139–1143 (2009).
84 Zarrin, M., Leeder, A. C. & Turner, G. A rapid method for promoter exchange in *Aspergillus nidulans* using recombinant PCR. *Fungal Genet. Biol.* **42,** 1–8 (2005).
85 Bergmann, S. *et al.* Genomics-driven discovery of PKS-NRPS hybrid metabolites from *Aspergillus nidulans*. *Nat. Chem. Biol.* **3,** 213–217 (2007).
86 Bok, J. W. *et al.* Chromatin-level regulation of biosynthetic gene clusters. *Nat. Chem. Biol.* **5,** 462–464 (2009).
87 Bok, J. W. & Keller, N. P. LaeA, a regulator of secondary metabolism in *Aspergillus* spp. *Eukaryot. Cell* **3,** 527–535 (2004).
88 Bayram, Ö. *et al.* VelB/VeA complex coordinates light signal with fungal development and secondary metabolism. *Science* **320,** 1504–1506 (2008).
89 Bok, J. W. *et al.* Genomic mining for *Aspergillus* natural products. *Chem. Biol.* **13,** 31–37 (2006).