Architecture and Functional Analysis of Fungal Polyketide Synthases

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Summary

All fungal polyketide synthase (PKS) genes so far cloned code for iterative type I PKSs that are different from bacterial modular type I PKSs and type II PKSs. In order to clarify how fungal PKSs control their reactions to produce specific product compounds, fungal PKSs such as Aspergillus terreus ATX, Aspergillus nidulans WA, Colletotrichum lagenarium PKS1, Aspergillus fumagatus Alb1p and their derivatives, were expressed under β-amylase promoter in heterologous host Aspergillus oryzae. Chemical identification of products confirmed their functions and gave some insights into how their reactions are controlled.

Key words: fungi, polyketide, polyketide synthase, domain architecture, expression

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Polyketides are one of the largest and most important groups of natural products. In spite of their structural diversity, the initial reactions of polyketide biosynthesis follow a common scheme that condensation of acyl primer with malonate extension units to form β-polyketomethylene intermediates and their cyclizations catalyzed by so called polyketide synthases (PKSs)². Although molecular genetic analysis of bacterial polyketide biosynthesis genes have been extensively carried out, only several PKS genes have been cloned from eukaryotic filamentous fungi, which are another rich source of polyketides, especially, aromatic compounds². All fungal PKS genes so far cloned code for iterative multifunctional type I PKS polypeptides. So far, little has been known how fungal PKSs control their reactions, that is, how to regulate the chain-lengths of β-polyketomethylene intermediates and their cyclizations. Thus, expression and functional analysis of fungal iterative type I PKSs were carried out.

Aspergillus terreus ATX³

6-Methyisalicylic acid synthase (MSAS) of Penicillium patulum was the first fungal PKS of which enzyme activity was detected and the gene was cloned⁴. Hopwood et al. indicated the presence of
sequence similarity among streptomycete PKS genes and possible usefulness of the PKS gene as a probe for cloning of new PKS genes. Therefore, MSAS gene was used as a probe for screening of fungal PKS genes. Southern blot analysis of several fungal genomic DNAs with MSAS probe, however, showed a single hybridizing band in (+)-geodin producing strain of Aspergillus terreus. No homologous band was detected in genomic DNA of Penicillium cyclopium which produces orsellinic acid, C-4 hydroxylated derivative of 6-methylsalicylic acid. At the same stringency condition, no homologous bands were detected even in A. terreus when probed with the acyl carrier protein region of MSAS gene. This result indicated the presence of an MSAS like PKS gene in A. terreus, which is not quite identical to that of P. patulum. This A. terreus gene was named atX and cloned from the genomic DNA library. Nucleotide sequence and open reading frame analysis indicated the presence of 5.5 kb-long large open reading frame coding for 1803 amino acid polypeptide of 190 kDa. As was expected, the deduced ATX polypeptide sequence showed a throughout high homology with P. patulum MSAS.

In order to identify its actual function, expression of the atX gene in a heterologous host was carried out using fungal expression vector pTAEex3 with α-amylase promoter of Aspergillus oryzae. The ATX expression plasmid pTA-ATX was constructed and was transformed into Aspergillus nidulans. From the induction culture medium of pTA-ATX transformant with starch, an acidic compound was isolated and identified to be 6-methylsalicylic acid by physicochemical analysis. Thus, the atX gene was identified to code for MSAS of A. terreus. In addition, the A. oryzae transformant with pTA-ATX showed production of 6-methylsalicylic acid more than 0.4 g per liter culture medium, indicating this fungal expression system is quite suitable for expression and functional analysis of fungal PKSs.

**Aspergillus nidulans WA**

The conidiophore formation in A. nidulans has been well studied at genetic level as a model system of multicellular development. The asexual spores, conidia, contain dark green pigments that...
contribute to the strength, rigidity, and impermeability of the conidial cell wall and UV protection. The \( wA \) and \( yA \) genes were proposed to encode enzymes involved in pigment biosynthesis. The product of \( yA \) gene was identified to be a laccase that converts a yellow precursor to the mature green form. The \( wA \) gene product showed significant similarity with prokaryotic and eukaryotic PKSs and was assumed to be a type I multifunctional PKS for yellow spore pigment intermediate\(^8\). In order to identify the product of \( WA \) PKS, we tried to express the \( A. \) nidulans \( wA \) gene by the fungal expression system which was successfully used in ATX expression.

The \( wA \) expression plasmid pTA-\( wA \) was constructed and then introduced into the host fungus \( A. \) oryzae. From the induction culture of \( A. \) oryzae/pTA-\( wA \) transformant, three compounds were isolated. The colorless main compound was identified to be heptaketide citreoisocoumarin by physicochemical analysis and minor compounds were found to be its derivatives. This expression experiment identified that the \( wA \) gene coded for a heptaketide synthase of \( A. \) nidulans\(^9\). However, the expected product of \( WA \) PKS was a yellow pigment which could be polymerized by \( yA \) encoded laccase to green spore pigments. Thus, it was skeptical whether citreoisocoumarin and its derivatives were the true spore pigment intermediates.
In the WA PKS, presence of tandem acyl carrier protein (ACP) motifs was reported. Similar feature is found in the reported fungal PKSs like PKS1 of Colletotrichum lagenarium\textsuperscript{10} and STCA of A. nidulans\textsuperscript{11}. Comparison of active site organization of these tandem ACP type fungal PKSs revealed that the WA PKS alone lacked thioesterase (TE) motif at its C-terminus and had shorter polypeptide length. To confirm this discrepancy, we carried out resequencing of the \textit{wA} gene around the C-terminal region and found an error in the original sequence. The missing one base caused an apparent frame shift in the deduced amino acid sequence. The corrected WA PKS (NWA) has a longer polypeptide length with TE motif at its C-terminus. Thus, the previous expression plasmid pTA-wA lacked a part of WA C-terminus region.

To express the correct full-length WA PKS, a new expression plasmid pTA-nwA was constructed. The \textit{A. oryzae} transformant with pTA-nwA showed yellow pigmentation on agar plates. This observation was quite contrast to the pTA-wA transformant that showed white mycelia. From the induction culture medium of the pTA-nwA transformant, yellow compound was isolated and characterized to be naphthopyrone compound YWA1. Production of citreoisocoumarin derivatives was not observed in \textit{A. oryzae}/pTA-nwA transformant. Since naphthopyrone compounds were isolated from several fungal origins, it is considered that YWA1 is an actual intermediate of conidial spore pigment and the \textit{wA} gene codes for a PKS of heptaketide naphthopyrone YWA1\textsuperscript{12}.

The result that the C-terminus modified WA PKS produced citreoisocoumarin instead of naphthopyorone indicated that the C-terminus region is involved in the cyclization of the second aromatic ring of naphthopyrone. To unravel the actual function of the C-terminus region, we carried out functional analysis of WA PKS mutants by C-terminus deletion and site-directed mutagenesis. Only 32 amino acid deletion from the C-terminus of WA PKS caused product change to heptaketide isocoumarins from heptaketide naphthopyrone YWA1. Further C-terminus deletion up to Ser\textsuperscript{1967}, an active site residue of TE, still produced isocoumarins. Site-directed mutagenesis of amino acid residues in this C-terminus region showed that even a single mutation of S1967A or H2129Q caused production of isocoumarins. These results indicated that C-terminus region of WA PKS is involved in Claisen-type cyclization to form second aromatic ring of naphthopyrone YWA1\textsuperscript{13}.

Mycotoxins

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Fig. 4 Citreoisocoumarin and YWA1
The PKS1 gene was cloned from the phytopathogenic fungus *Colletotrichum lagenarium* which causes anthracnose of cucumber\(^{10}\). During the infection process, the fungus produces melanin which is essential for penetration into the host plants. The PKS1 gene could restore the melanization of albino mutants and its sequence analysis revealed that the gene encodes iterative type I PKS whose architecture is similar to that of the *A. nidulans* WA. Analysis of the blocked mutants showed that its 1,8-dihydroxynaphthalene (DHN) type melanin is biosynthesized via scytalene, 1,3,8-trihydroxynaphthalene, vermelone and DHN. However, no mutant was obtained which accumulated 1,3,6,8-tetrahydroxynaphthalene (T4HN), an assumed initial precursor of DHN melanin. Thus, some ambiguities remained as to whether T4HN is the direct product of PKS1 PKS. The PKS1 was expressed in the heterologous fungal host *A. oryzae* under the starch-inducible \( \beta \)-amylase promoter. The main compound produced by the transformant was isolated and characterized to be T4HN as its tetraacetate. This result unambiguously identified the gene to encode a PKS of pentaketide T4HN\(^{14}\).

In addition, pentaketide \( \beta \)-acetyl orsellinic acid was isolated as a byproduct of the PKS1 PKS reaction. Identification of this monocyclic compound led us to propose the folding pattern of the pentaketide intermediate and two cyclization mechanisms for the second aromatic ring formation of T4HN depending on the starter unit, acetate or malonate. The cell-free extract of *A. oryzae* transformant overexpressing PKS1 showed enzymatic activity of T4HN formation. To identify the starter unit for T4HN synthesis, \(^{14}\)C-labeled acetyl CoA and/or \(^{14}\)C-labeled malonyl CoA were used as substrates for T4HN synthase reaction. Observed was the incorporation of \(^{14}\)C label into T4HN solely from malonyl CoA even in the absence of acetyl CoA and not from acetyl CoA. This in vitro result unambiguously identified that malonyl CoA serves as the starter as well as extender units in the formation of T4HN by the PKS1 PKS\(^{15}\).
Aspergillus fumigatus causes allergy, noninvasive colonization, or life-threatening invasive pulmonary aspergillosis. *A. fumigatus* synthesizes its bluish green conidial pigment through a pentaketide pathway similar to the DHN-melanin pathway. Genetic and biochemical investigations have shown that biosynthesis of the conidial pentaketide melanin in *A. fumigatus* requires a six-gene cluster including the gene *alb1* for PKS which was considered to be a T4HN synthase. However, the *Alb1p* PKS shows higher sequence similarity to a naphthopyrone synthase WA than T4HN synthase PKS1. To clarify the function of Alb1p, the *alb1* gene was expressed in a heterologous host *A. oryzae* under α-amylase promoter. The product compound was identified to be a heptaketide naphthopyrone YWA1 instead of pentaketide T4HN.

To understand how *A. fumigatus* uses a heptaketide synthase to initiate the biosynthesis of a pentaketide melanin, we explored the possible involvement of accessory protein(s) in the biosynthetic pathway. A novel protein, Ayg1p, was shown to be involved in the formation of T4HN by chain-length shortening of a heptaketide precursor in *A. fumigatus*. Phenotypic analyses of double gene disruptants suggested that Ayg1p catalyzes a novel biosynthetic step downstream of Alb1p PKS. Further genetic and biochemical analyses of the reconstituted strain carrying *alb1*, *ayg1*, or *alb1*-*ayg1* indicated that Ayg1p catalyzes the chain-length shortening of the heptaketide product YWA1 of Alb1p to T4HN. Thus, the protein Ayg1p was demonstrated to facilitate the participation of a heptaketide synthase Alb1p in a pentaketide pathway via a novel polyketide-shortening mechanism in *A. fumigatus*.

**Fig. 6 DHN-melanin biosynthesis in Colletotrichum lagenarium**
Concluding remarks

As mentioned above, several fungal iterative type I PKSs were successfully expressed in a heterologous fungal host A. oryzae and their functions were unambiguously identified. Also, expression of mutants led us to identify the role of C-terminus region of WA PKS. Detection of T4HN synthase activity enabled us to identify the starter unit and cyclization mechanism of T4HN synthesis. However, detailed mechanisms were still remained unraveled yet how these iterative type I PKSs regulate choice of starters, condensation cycles, and aldol and Claisen-type cyclizations.

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References


糸状菌ポリケタインド合成酵素の構造と機能解析

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糸状菌の生産するポリケタインド化合物の基本炭素骨格形成に与るポリケタインド合成酵素（PKS）は、アシル基とマロニル伸長単位の総合の繰り返しと生成したポリケートメチレン鎖の閉環などを触媒する。これまで遺伝子がクローニングされ、機能が確認されている糸状菌PKSは数多くはないが、興味深いことにいずれも一つのポリペプチド鎖または閉合反応の活性中心KSやアシル基転移反応の活性中心ATなど各反応の活性部位がリニアに存在するいわゆるタイプI型のPKSタンパクであり、しかも、バクテリアのモジュール型とは異なり、同一の活性中心が総合反応ごとに閲覧して炭素鎖の伸長を触媒する繰り返し型（iterative type）であることが大きな特徴である。我々は、糸状菌PKSが如何に反応を制御し、特異的な炭素鎖長、閉環様式をもつ生成物を与えるのか、その機構解明を目指し糸状菌PKSの遺伝子クローニング、発現、反応解析を行ってきたので紹介する。

キーワード: 糸状菌、ポリケタインド、ポリケタインド合成酵素、ドメイン構造、発現