Review

Heterologous production of fungal natural products: Reconstitution of biosynthetic gene clusters in model host Aspergillus oryzae

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Abstract: While exploring phytotoxic metabolites from phytopathogenic fungi in the 1970s, we became interested in biosynthetic enzymes that catalyze Diels–Alder reactions involving biosynthesis of several phytotoxins that we isolated. Target enzymes were successfully characterized, and this triggered the identification of various Diels–Alderases in a recent decade. Through our Diels–Alderase project in 1990s, we recognized a highly efficient expression system of various biosynthetic genes with Aspergillus oryzae as a host. With the development of tools such as genomic data and bioinformatics analysis to identify biosynthetic gene clusters for natural products, we developed a highly reliable methodology such as hot spot knock-in to elucidate the biosynthetic pathways of representative fungal metabolites including phytotoxic substances. This methodology allows total biosynthesis of natural products and genome mining using silent biosynthetic gene clusters to obtain novel bioactive metabolites. Further applications of this technology are discussed.

Keywords: heterologous expression, biosynthesis, Aspergillus oryzae, fungal natural products, Diels–Alderase

Introduction

Toxins produced by phytopathogenic microorganisms are one of the important causal factors in the development of many plant diseases.1 In the 1970s, our laboratory isolated a number of phytotoxins from phytopathogenic microorganisms. One of the representative phytotoxins in our research program is coronatine isolated from a causal bacterium of chocolate spot disease Pseudomonas syringae (Fig. 1).2 Recently, this toxin was rediscovered as an agonist of jasmonoyl-L-isoleucine, a genuine bioactive form of plant hormone jasmonic acid, and has played an important role in the recent progress of jasmonic acid research, as in the discovery of the coi1 mutant and the identification of the jasmonic acid receptor.3 Although the actual role of phytotoxins is still difficult to uncover, elucidation of the function of phytotoxins is an important issue for understanding the defense response between plants and phytopathogenic microbes.

During structural elucidations of phytotoxic metabolites produced by phytopathogenic fungi in our laboratory, we frequently encountered Diels–Alder-type adducts, such as betaenones,4 solanapyrones,5 pyrenocines, and pyrenochaetic acids6 (Fig. 1). Our careful search in the literature showed that there are a number of putative natural [4 + 2] adducts in various types of natural products (NPs), including polyketides, terpenoids, phenylpropanoids, and alkaloids.7–9 These observations strongly indicated the involvement of the enzyme responsible for biochemically unusual Diels–Alder reactions, often named “Diels–Alderases (DAases)”. Although considerable efforts have been made to identify the enzymatic Diels–Alder reaction, there has been no report on DAases thus far.

By characterizing two important enzymes in DAase projects, we recognized the importance of a
reliable expression system for studying the biosynthesis of fungal NPs. Such systems can enable us to achieve complete total NP biosynthesis and supply both sufficient amount of intermediates and NPs, and they can also be applied to the genome mining of novel NPs by expression of silent biosynthetic gene clusters (BGCs) on the fungal genome. I will also provide the details of our achievements in this topic.

Discovery of DAases in fungal phytotoxin biosynthesis

The Diels–Alder reaction is synthetically very useful and is one of the most widely used reactions in organic synthesis because it forms a six-membered ring from a 1,3-diene and a dienophile with high regioselectivity and stereo selectivity under mild conditions. In addition, the Diels–Alder reaction is a powerful tool for creating four chiral centers or quaternary stereogenic centers in organic synthesis and has been applied for the synthesis of complex pharmaceutical and biologically active compounds.

The first target of DAase was a cyclization enzyme in the biosynthesis of phytotoxin solanapyrones A and D (Fig. 2A). Initially, we confirmed that the solanapyrone producing fungus Alternaria solani transformed putative achiral linear precursors prosolanapyrones II and III into chiral solnapyrones.10) In the crude enzyme from A. solani, we found enzymatic activity catalyzing the Diels–Alder reaction from prosolanapyrone II to solanapyrone A with excellent enantioselectivity (99% ee) and relatively high exo selectivity (6:1).11) Subsequently, we reported the partial purification and properties of the enzyme, solanapyrone synthase (SPS), which is the first example of a DAase.12) In addition, we observed that the SPS’s cyclase activity is tightly correlated with oxidase activity. In parallel to the purification of the DAase, our genetic study using a heterologous expression of putative PKS genes in Aspergillus oryzae (AO) successfully confirmed the BGC of solanapyrone.13) Our preliminary information led us to express flavin-dependent oxidase Sol5 among several modification enzyme genes in the cluster. Finally, we unveiled that the DAase SPS can oxidize prosolanapyrone II into aldehyde III and cyclized III into (−)-solanapyrones, proposing that the single enzyme catalyzes the oxidation from the alcohol II to the reactive aldehyde III, which is further converted into the adducts A and D by the intramolecular Diels–Alder reaction.13) A similar bifunctional enzyme LovB giving decalin lovastatin was reported in 2000.14) Later, the involvement of PKS LovB as a DAase was firmly established by an in vitro study.15)

For the study of intermolecular DAase, we chose an unusual conversion found in the biosynthesis of macrophomic acid.16) The phytopathogenic fungus, Macrophoma commelinae has the ability to transform 2-pyrole into the corresponding benzoate analogue macrophomate as in the case of pyreno n to pyreno catic acid (Fig. 2B). This complex transformation is catalyzed by a single enzyme, macrophomate synthase (MPS), with oxalacetate as a substrate for the C3-unit precursor. MPS is a Mg2+-dependent enzyme with 339 amino acid residues (MW 36244 Da).16) The catalytic mechanism of the whole pathway was investigated extensively, and it was shown that it proceeds through three separate steps: decarboxylation, two carbon–carbon bond formations, and decarboxylation with concomitant dehydration.16) In the absence of 2-pyrene, MPS simply acts as a decarboxylase with high catalytic efficiency (Fig. 2B). Based on the formation of an
aberrant adduct with pyrone and the observation that dehydration proceeds formally in an anti-sense, it was proposed that the higher-energy \([4 + 2]\) adducts are transformed into either the rearranged product or benzoate analogue. The crystal structure of the MPS complexed with pyruvate and \(\text{Mg}^{2+}\) was determined. In the catalytic cavity, a tight complex was observed between the two oxygen atoms of pyruvate enolate and \(\text{Mg}^{2+}\), which is ligated with two amino acids residues of MPS. The proposed mechanism was further supported by this crystal structure. The rigorous discussion of this proposed mechanism was reviewed.

Other than SPS and MPS, we also studied several other natural \([4 + 2]\) adducts such as PKS catalyzed \([4 + 2]\) adducts betaenones, nonenzymatic DA adducts didymellamide, and macrocyclic DA adduct cytochalasin (Fig. 3). In 2015, several...
groups succeeded in identifying versatile DAases, Fsa2/chg7 that are responsible for the formation of a characteristic decalin ring system in fungal polyketide biosynthesis. In contrast to other DAases, these DAases share high sequence homology, and thus simple bioinformatics analysis such as BLAST could readily identify BGCs having DAase genes. To date, these searches led us to find more than 100 homologous decalin-forming DAases in public databases. Further, extensive studies have characterized more than 10 DAases only from fungi. As described here, our efforts on DAases open the door for DAase research.

**Total biosynthesis of NPs using a versatile fungal host**

Through collaboration with our group, the Fujii and Ebizuka group successfully synthesized core skeletons of alternapyrone, aslanipyrone, and solanapyrones by expression of HR-PKS genes obtained from the genomic DNAs of *Alternaria solani*. These pioneering works on functionally uncharacterized PKS genes that resulted in isolation of novel metabolites encouraged us to develop versatile tools for heterologous expression.

Since 2010, in our group, the use of the AO expression system has been extended for the reconstitution of the biosynthetic machinery for fungal secondary metabolites. At that time, there were a few studies on the synthesis of biologically active NPs by expressing whole genes in the BGCs. For this purpose, multiple vectors are required to introduce all the biosynthetic genes in the clusters. To examine the expression of multiple NP biosynthetic genes, we selected an expression system using the quadruple-auxotrophic host AO NSAR1 (*niaD*, *sC*, *argB*, *adeA*) developed. This host strain can accept multiple vectors such as pTAex3, pUNA, pUSA, and pAdeA, in addition to pPTRI17 with the pyrithiamine-resistant marker. Using this simple modification, we examined the heterologous production of phytotoxins, whose gene clusters we obtained ourselves. We successfully obtained diterpene aphidicolin and polyketide solanapyrone (~100 mg/kg, in solid medium) (Fig. 4). In addition, stepwise introduction of biosynthetic genes provided individual intermediates, thus clarifying each biochemical transformation. By further modification of the gene introduction using tandem transformation, we also succeeded in elucidating the early-stage biosynthesis of fungal indole diterpenes such as paxilline and aflatrem (Fig. 5).
In the next stage, for the rapid reconstitution of the biosynthetic machinery in AO, we used the vectors pUARA2, pUSA2, pUNA2, and pAdeA2 carrying a tandemly arranged promoter/terminator set. Tandem transformation utilizing two plasmids with the same selectable marker is efficient, requiring the use of fewer vectors and allowing a rapid introduction of biosynthetic genes to obtain TFs. By applying this simple method, we completely elucidated the detailed biosynthetic pathway of fungal indole diterpenes such as shearinine,34) lolitrems,35) and especially penitrem 36) whose biosynthesis requires 17 genes, one of the largest number of genes in the fungal metabolite biosynthesis (Fig. 5). Initial diversification of indole terpenes is core construction, such as paspaline, by changing the chain length of the prenyl chain and various cyclization modes of epoxidized oligoprenyl chains. The second diversification is generated by two cytochrome P450 monoxygenase (P450)-catalyzed modifications (P: oxidative elimination of C30-methyl group and C10-oxidation; Q: allylic oxidation(s) at C13 and C7).37) The final diversification is rather specific for indole terpene diprenylation coupled with various oxidative modifications.37)

A similar stepwise reconstitution strategy has been applied to synthesize fungal ribosomally generated peptides,38) antifungal anhydrides,39),40) and fungal polyketides. Furthermore, we recently unveiled the long-standing mystery in the biosynthesis of plant hormone abscisic acid in fungi,41) which is significantly different from that in plants. Recently, fungal gene expression has become popular, especially in pathway elucidation of fungal metabolites. We believe that we have made significant contribution in this research field.

Genome mining of novel NPs from silent BGCs

To date, biologically active NPs have usually been found by suitable bioassay-guided screening. Recently, genome mining has become another attractive approach to discover novel NPs solely using gene sequences. By this approach, we can avoid duplicated isolation of known compounds whose
biosynthetic pathways are already characterized. Even obtaining microorganisms is not necessary when we use synthetic DNAs designed using the sequences from public databases.

Recently, we and others succeeded in identifying a novel type of fungal di-/sesterterpenes, which are biosynthesized by a novel type of cyclopentane forming terpene synthases (CPF-TSs). The preceding study of bifunctional TS (BFTS) with two catalytic domains of prenyltransferase GGPP synthase and diterpene synthase showed that two BFTS PaFS and PaPS generated unique molecular skeletons fusicoxin containing 8-membered ring and methyl phomopsenate multicyclic systems, respectively. Based on these results, we applied a genome mining approach with BFTS genes to find novel diterpenes. When we expressed a BFTS gene AcOS from A. clavatus using the AO expression system, the resultant TF did not give a diterpene but yielded a sesterterpene instead, which is identical to ophiobolin F, suggesting that the PT domain of BFTS can supply either C20- or C25-precursor GGPP/GFPP and the TS domain can produce either C20- or C25-terpenes (Fig. 6A).

Bioactive sesterterpenes are found throughout the tree of life, including in bacteria, plants, animals, and fungi, but remarkably, no sesterterpene-generating enzymes were known. This encouraged us to further explore novel sesterterpene synthases. Phylogenetic analysis of more than 100 BFTSs from the public database allowed us to categorize two clades: I and II. Assuming that the amino acid sequence was strongly correlated with the cyclization mechanism, we employed another genome for mining a clade I-BFTS gene NfSS from Neosartorya fischeri. In the extracts of AO-NfSS, we obtained a novel tetracyclic sesterterpene sesterfisherol (Fig. 6B). Based on the common cyclization mechanism of di-/sesterterpenes putatively derived from type-A and type-B cyclizations, we proposed unified biogenesis for these CPF-TSs. Density functional theory calculations of the sesterfisherol formation revealed two alternative cyclization mechanisms (Paths a and b) that were thermodynamically and kinetically favorable. Actually, two metabolites were found, which were derived via both mechanisms (Fig. 6B).

To explore terpene synthases producing sesterfisherol-related sesterterpenes, we selected four clade I-BFTS genes from Bipolaris maydis and Phoma betae, which showed moderate identities (35%-38%) with NfSS. Applying the same strategy as for functional analysis of NfSS, we obtained four sesterterpenes, Bm1, Bm2, Bm3, and Pb1, whose structures were closely related with the carbocation intermediates in the cyclization giving sesterfisherol (Fig. 6B). The structure of Bm2, including its absolute configuration, is the same as that of the tetracyclic carbocation, whereas the other three products are diastereomers of the bicyclic and tricyclic carbocations. This observation suggested that clade I CPF-TSs can generate various enantiomeric and diastereomeric cation intermediates that give rise to a diverse array of structural derivatives. This genome mining approach attracted several research groups, and nearly 20 CPF-TSs were characterized.

Heterologous expression of genes encoding modification enzymes led to the production of putative NPs. With the exception of the BGCs for fusicoxin (14 genes) and brassicicene (10-15 genes), BGCs of di-/sesterterpenes belonging to this class usually consist of fewer than 5 genes. To date, the function of these terpenoids remains unknown. Although some of them show strong phytotoxicity (ophiobolin) and antiviral/antiangiogenic activity (terpestacin), the remaining terpenes do not exhibit any obvious activities. However, the conidiation-inducing activity of conidigenone suggested that these terpenoids act as a signaling molecule in the fungi.

Heterologous production of NPs from Basidiomycota fungi

Mushroom-forming basidiomycete fungi are known to be prolific producers of structurally diverse, bioactive NPs. However, despite their incredible potential for NP discovery, Basidiomycota fungi are a largely unexplored target for drug discovery compared with Ascomycota fungi. Unfortunately, Basidiomycota have very intron-rich genomes and genes that contain very small and unpredictable exons, and thus currently no reliable tool for automated intron prediction is available. The ability to directly express Basidiomycota biosynthetic genes from genomic DNA in a suitable fungal surrogate host would therefore greatly accelerate the functional characterization of NP pathways.

After successful production of the widely used livestock antibiotic pleuromutilin in AO TF harboring cDNA gene from its producer mushroom, we examined the heterologous expression of genomic DNAs. Preliminary expression of pleuromutilin genes gave the results that two genes ple3/4 for core construction were correctly spliced, and three intron-rich P450 genes ple1/5/6 were mostly spliced in three
Fig. 6. (A) Genome mining of class B di-/sesterterpenes. (B) Genome mining of class S sesterterpenes.
single gene-TFs (2 non-spliced introns remained out of 34 introns). This suggested that we can produce the metabolites in AO using genomic DNA sequence by removing the remaining intron by PCR technique (Fig. 7).

To establish the versatility of basidiomycete heterologous expression using AO, 30 sesquiterpene synthase genes were selected for characterization from two basidiomycete fungi: pleuromutilin producer *Clitopilus pseudo-pinsitus* and *Stereum hirsutum*. In the examination of unpredictable splicing of basidiomycete genes, we used the recently developed technique, a fungal clustered regularly interspaced short palindromic repeat (CRISPR)-Cas9 system specifically optimized for AO, to ensure the expression of target genes.\(^{49),51}\) In addition, the STS genes were introduced (knocked-in) to the hot spot (HS) loci where target genes were integrated in the highly yielding TFs found in our previous studies. Sequence analysis of cDNAs recovered from the TFs revealed that 87% of total introns were correctly spliced, and nonspliced introns were simply skipped with no complicated missplicing. Following the removal of nonspliced introns in amplified cDNA, the fixed cDNAs were expressed by *E. coli*, which successfully yielded terpene products except a single TF.\(^{49),51}\)

To confirm the versatility of the AO expression system, total biosynthesis of a typical Basidiomycota metabolite erinacine Q, a potential drug for Alzheimer’s disease, was conducted. Before heterologous production, we prepared AO-transformants carrying the genomic DNA sequences of *eriA-C*, and *eriH-L* and confirmed their correct splicing (> 91%) in all genes. In this study, we faced the problem of detecting the activities of two P450 EriAC. These enzymes showed oxygenase activity in the coexpression of extra dehydrogenase EriH. In addition, expression of all required genes (*eriEGACIJKLH*) resulted in the production of erinacine Q when we enhanced the supply of UDP-xylene by adding two plant genes *AtUGD1* (UGD: UDP-D-glucose dehydrogenase) and *AtUXS3* (UXS: UDP-D-glucuronic acid decarboxylase) to the TF (Fig. 7). The detailed function of SDR is currently unknown, but this phenomenon is likely mushroom-specific owing to the presence of EriH homologs in other basidiomycete terpene BGCs.\(^{51}\) Overall, the complete elucidation of the erinacine biosynthetic pathway was achieved, and this study showed that heterologous production of mushroom NPs in AO is one of the promising methods.

**Conclusion and perspective**

Frequent occurrence of putative Diels–Alder adducts in phytotoxins from phytopathogenic fungi led us to study biosynthetic enzymes that catalyze Diels–Alder reactions involved in the biosynthesis of several phytotoxins that we isolated. The corresponding enzymes SPS and MPS were successfully characterized by our extensive chemical and biochemical analyses including X-ray crystallographic analysis. Our achievement triggered the identification of various Diels–Alderase in the last 10 years. A highly efficient expression system of various biosynthetic genes with AO as a host played a key role for identification of DAases (in the 1990s). With the advancement of DNA sequencing for accumulation of genomic data and tools for bioinformatics analysis
to identify BGCs for NPs, we developed methods for multiple gene introduction and applied it to elucidation for the biosynthetic pathways of representative fungal NPs including phytotoxic substances. Eventually, we established the highly reliable methodology HS-knock-in to study biosynthesis of NPs from Basidiomycota fungi. Total biosynthesis and genome mining became accessible for genomic data-based heterologous production of biologically active metabolites.

These technologies are now applied to isolate NPs that are produced transiently in a specific infection period by phytopathogenic fungi. Target NPs are usually unable to obtain a large amount and are therefore difficult to isolate because of conditional expression. However, the expression profile of the gene clusters may be sufficient information to fish out the target NPs if we apply the heterologous expression of silent gene clusters. For example, we successfully overexpressed biosynthetic genes to yield conidiogenone, which is a conidiation-inducing factor for the genus *Penicillium*. Conidiation is important for the pathogenicity of phytopathogens, and thus, understanding the cellular mechanism of conidiation is an urgent research topic in plant disease.

Basically, enzymatic synthesis of biologically active NPs solely depends on the information about the DNA sequence of the gene. In the future, automated systems may generate TFs with plasmid-carrying genes derived from synthetic DNA. Once we establish these systems, we can use them to produce novel NPs by the TFs expressing whole genes of unidentified BGCs. Furthermore, the NPs thus obtained can be linked to the timing of individual BGC expressions under specific conditions of life cycle events. This information (where and when) may unveil the exact role of NPs in phytopathogenic fungi.

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Profile

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