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Complete biosynthetic pathways of ascofuranone and ascochlorin in *Acremonium egyptiacum*

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Ascofuranone (AF) and ascochlorin (AC) are meroterpenoids produced by various filamentous fungi, including *Acremonium egyptiacum* (synonym: *Acremonium sclerotigenum*), and exhibit diverse physiological activities. In particular, AF is a promising drug candidate against African trypanosomiasis and a potential anticancer lead compound. These compounds are supposedly biosynthesized through farnesylation of orsellinic acid, but the details have not been established. In this study, we present all of the reactions and responsible genes for AF and AC biosyntheses in *A. egyptiacum*, identified by heterologous expression, in vitro reconstruction, and gene deletion experiments with the aid of a genome-wide differential expression analysis. Both pathways share the common precursor, ilicilicolin A epoxide, which is processed by the membrane-bound terpene cyclase (TPC) AscF in AC biosynthesis. AF biosynthesis branches from the precursor by hydroxylation at C-16 by the P450 monoxygenase AscH, followed by cyclization by a membrane-bound TPC AsCF. All genes required for AC biosynthesis (ascABCDEFG) and a transcriptional factor (ascR) form a functional gene cluster, whereas those involved in the late steps of AF biosynthesis (ascHIJ) are present in another distantly located cluster. AF is therefore a rare example of fungal secondary metabolites requiring multi-locus biosynthetic clusters, which are likely to be controlled by the single regulator, AscR. Finally, we achieved the selective production of AF in *A. egyptiacum* by genetically blocking the AC biosynthetic pathway; further manipulation of the strain will lead to the cost-effective mass production required for the clinical use of AF.

Ascofuranone (AF) and ascochlorin (AC) are fungal natural products with similar chemical structures, originally isolated from *Acremonium egyptiacum*. Both have many useful biological properties; in particular, AF is a promising drug candidate against the tropical disease, African trypanosomiasis. However, the difficulty of the synthetic method and the inaccessibility of bioengineering methods have inhibited industrial production. This study identified all of the genes required for the branched biosynthetic pathways of AF/AC, which are clustered at two separate loci in the genome. In addition, we established the *A. egyptiacum* strain selectively producing AF, by genetically blocking the AC biosynthetic pathway. This study benefits the field of combinatorial biosynthesis through presenting biocatalysts and paves the way to cost-effective AF production with bioengineering.

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Data deposition: The data reported in this paper have been deposited in the DNA Data Bank of Japan (accession nos. DRA008136, E-GEAD282, LC006756, and LC006757).

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Interestingly, these compounds have been isolated from various fungi; for example, 2 from Cylindrocladium ilicicola (15, 16), Cylindrocarpos sp. (17), Fusarium sp. (18), Microcera sp. (19), and Nectria coccinea (20), and 1 from Paecilomyces variotii (21) and Verticillium hemipterigenum (22), indicating the broad distribution of the biosynthetic pathways. Compounds related to 1 and 2 are considered to be synthesized through the prenylation of orsellinic acid (3) and terminal cyclization via epoxidation (17, 18, 23) (Fig. 2A), but the details of their biosyntheses have not been established. Recently, we reported that Stachybotrys bisbyi PYH05-7 encodes the 3-producing polyketide synthase (PKS) StbA, the UbiA-family prenyltransferase StbC, which produces ilicicolic acid B (4) (Fig. 2A), and the nonribosomal peptide synthetase (NRPS)-like reductase StbB for the synthesis of LL-Z1272 (ilicicolin B) (5), a putative precursor of 1 and 2 (24). By analogy to the biosynthetic pathways of other fungal meroterpenoids, such as pyripyrperene, paxilline, and aflatrem (25), the terminal olefin of the prenyl chain of 5 is thought to be epoxidized by a flavin monooxygenase (EMO), and then cyclized by a membrane-bound terpene cyclase (TPC). Considering the different cyclization patterns of 1 and 2, two distinct TPCs were assumed to exist in A. egyptiacum.

Developments in genomic resources and bioinformatics tools have accelerated the elucidation of the biosynthetic genes in various organisms (26). In particular, the biosynthetic genes for fungal secondary metabolites often form functional gene clusters (27), which can be predicted by searching the genome for collocated genes with functional motifs for putative biosynthetic enzymes (26). However, it is difficult to predict the relevance of genes with novel functions and to find biosynthetic clusters separated at multiple loci. For plant metabolites, in contrast, coexpression analyses have been effective in identifying nonclustered biosynthetic genes (28, 29); naturally, such a transcriptome-based approach is also promising in the case of fungal multilocus clusters (30). Since previous studies showed that 1 and 2, as well as other related compounds, suppress respiratory chain activities at multiple targets (31, 32), the fungal production of 1 and 2 is likely to be tightly regulated to avoid inhibiting fungal growth. Therefore, we can reasonably anticipate that determining the gene clusters that are differentially expressed in association with 1 production will lead to the identification of the responsible genes.

In this study, we aimed to find the biosynthetic genes for 1 by using a comparative transcription analysis of A. egyptiacum cultures exhibiting all-or-none production. Taking advantage of heterologous expression systems in Aspergillus spp., in vitro reconstruction assays, and a newly established gene disruption method for A. egyptiacum, we identified the complete biosynthetic enzymes of 2 encoded by the most prominently induced gene cluster. We then discovered another gene cluster at a discrete locus involved in the remaining steps of AF production, by virtue of a combination of motif-based and differential expression approaches. In addition, we analyzed the transcriptional factor regulating the biosynthetic genes of both compounds located in separate chromosomal regions.

Results and Discussion

Discovery of the asc-1 Cluster. First, we performed a genome-wide transcription analysis using the A. egyptiacum strain F-1392, to identify the differentially expressed gene clusters possibly responsible for the biosynthesis of 1 or 2. The production of 1 and 2 in A. egyptiacum varied dependent on the culture medium, and two media, designated as F1 and AF here, showed the virtual all-or-none difference. We thus prepared poly(A) selected RNAs from mycelia grown in F1 and AF media, which yielded 0.96 and 399 mg of 1 per liter culture, and obtained 611,740 and 846,811 RNA-seq reads, respectively. Although the experiment was preliminary, in terms of read numbers and lacking replicates, mapping the differential expression profiles to the 31-Mb draft genome (SI Appendix, Table S1) shed light on a 32-kb region on
Cluster Genes. (SI Appendix, Figs. S4 and S5 and Table S3). Subsequently, the A. oryzae transformant harboring ascCABD (SI Appendix, Figs. S4 and S5 and Table S3) yielded an additional compound (SI Appendix, Fig. S4), which was identified as ilicicolin A (6) (0.04 mg/L) by comparison with the authentic standard (SI Appendix, Fig. S6A). These data showed that AscCABD were responsible for the biosynthesis of 6.

Next, we performed an in vitro reconstruction of the succeeding steps, since the meager production of 6 complicated the analysis of the downstream metabolites in A. oryzae transformants. To prepare the Asc-E-G proteins, we employed an Aspergillus sojae high-copy expression system in which the pyrG selective marker with a truncated promoter enables the transformation of high-copy plasmids (SI Appendix, Fig. S7) (39). Since AscF and AscG are membrane-bound proteins and difficult to purify, a cell-free homogenate was used for the in vitro enzyme reaction. We hypothesized that 6 was epoxidized into ilicicolin A epoxide (7) by AscE or AscG before cyclization by AscF, as in the case of other fungal meroterpenoid biosynthesis in which terpene cyclization follows epoxidation of the prenyl group (25). This is also supported by the previous report of accumulation of 7 in A. egyptiacum mutant strain obtained through random chemical mutagenesis (23). However, the in vitro assay showed that 7 was not detected with either the Asc-E- or Asc-G-containing homogenate; alternatively, a compound (11) with m/z 423 ([M–H]–) was detected only with the Asc-E-containing homogenate (Fig. 3B and SI Appendix, Fig. S8 and Table S7). The m/z suggested that 11 was a diol, hydrolyzed from the epoxide 7 (m/z 405, [M–H]–), so we considered that AscE catalyzes the epoxidation to produce 7. Since 11 is unlikely to participate in the production of 1 or 2 in view of the reaction mechanisms, the diol 11 was presumed to be a shunt compound formed by endogenous hydrolase in A. sojae, as previously observed in the A. oryzae system (36, 40). These assumptions were later confirmed by our deletion experiment (see below). Another reaction product (8) was detected when a mixed homogenate containing AscE and AscF was incubated with 6 (Fig. 3B). Compound 8 was deduced to be ilicicolin C from the high-resolution (HR)-MS data (SI Appendix, Fig. S8 and Table S7), which was supported by the fact that ilicicolin C was isolated from A. egyptiacum (41). Finally, when the protein extracts containing AscE, AscF, and AscG were incubated with 6, the reaction product was identified as ascochlorin (2) (Fig. 3B). Confirmation of the structure was obtained by direct comparison of the MS/MS fragmentation pattern, and by coincidence with the authentic standard.
(SI Appendix, Figs. S6 B and C). These results indicated that the AscF-catalyzed terpene cyclization product 8 was oxidized into 2 by the P450 monooxygenase AscG. The absolute configuration of 8 is thought to be identical to that of (14S,15R,19R)-2, which was established by X-ray structure analysis (42).

To experimentally establish the involvement of 7 in the biosynthesis of 1 and/or 2, we constructed A. egyptiacum gene disruptants, using the kai70-deleted strain that we constructed in this study to enhance homologous recombination (43) (SI Appendix, Fig. S9). The ascF-deleted strain (ΔascF) no longer produced 2 (Fig. 3C), but instead accumulated 7 (1.22 g/L; SI Appendix, Table S8), which was isolated and structurally determined by NMR and HR-MS analyses (SI Appendix, Figs. S8 and S10 and Tables S4 and S7). In contrast, the ascE-deleted strain (ΔascE) only accumulated 6 (2.32 g/L; SI Appendix, Table S8), but neither 1 nor 2 (Fig. 3C). These results supported experimental proof that AscE epoxidized the terminal olefin of 6 to produce 7, and AscF cyclized 7 into 8 in AC biosynthesis. For AF biosynthesis, another TPC other than AscF should be involved, since ΔascF still produced 1 (0.50 g/L; SI Appendix, Table S8) (Fig. 3C). Given the fact that ΔascE no longer produced 1, the epoxide 7 was indicated as the last common precursor for the biosyntheses of 1 and 2.

Considering these results, we concluded that the seven genes, ascA-G, in the asc-1 cluster encode the biosynthetic enzymes for the production of 2. AscE is a P450 monooxygenase that catalyzes stereoselective epoxidation of the terminal double bond of the prenyl group (44). In contrast, in most of the cases, fungal meroterpenoid biosynthesis employs FAD-dependent monooxygenases for the formation of epoxide (25). Notably, AscF is the rare meroterpenoid TPC that produces a monocyclic terpene, and the cyclization reaction is proposed to be initiated by protonation of the terminal epoxide of 7 to generate a monocyclic tertiary cation, which is followed by a series of hydride and methyl shifts with oxygen participation, leading to the formation of the (14S,15R,19R)-trimethylcyclohexane ring structure of 8 (Fig. 2B). AscA-E are also involved in AF biosynthesis, but additional enzymes are required for the later steps, after the common precursor 7, which should be encoded outside of the asc-1 cluster.

Identification of the Ascosfurane Biosynthetic Genes. Once again, the differential expression analysis was exploited to identify the genes responsible for the late stage of AF biosynthesis. Since one additional oxygen atom must be incorporated into the side chain of 7, we focused on P450s, the most abundant oxygenases in fungi. Among the gene clusters induced at least 10-fold in AF medium, only three harbored P450 monooxygenases genes other than the asc-1 cluster (SI Appendix, Table S5). One cluster on scaffold 6 encodes homologs of desmethylcinamin biosynthesis enzymes, DmbA, DmbC, and DmbA (45), and thus was not likely to be involved in AF biosynthesis. Another cluster on scaffold 3 was excluded since it encodes an acetyltransferase and a peptidase, both of which would seem irrelevant to AF biosynthesis. Combining the function prediction (see below), we targeted the other cluster on scaffold 3 (asc-2 cluster; DDBJ/ENA/GenBank accession no. LC406757) located on the different scaffold from asc-1 cluster (SI Appendix, Fig. S1).

The asc-2 cluster is composed of three genes, designated as ascHJ (Fig. 4A). All three genes exhibited higher (log_{10} values ≥ 1.5) expression in AF medium (SI Appendix, Table S2). AscH is a P450 sharing 38% amino acid identity with the hexadecane hydroxylase P450-ALK2-A from Candida tropicalis (46). AscJ is classified into the NAD(P)-dependent, short-chain alcohol dehydrogenase family, sharing 33% identity with the aflatoxin biosynthesis protein AflH (47). Finally, AscJ is an eight-transmembrane protein deduced by Sosui program (48) without any Pfam motif; however, it is similar to the citreoviridin biosynthetic protein CtvD (49) and Aorf in aureoverin biosynthesis (50), although it shares only 27% and 29% identities, respectively. Both enzymes are regiospecific hydroxylases for cyclizing a bisepoxide to give a 3,4-dihydroxy-tertahydrofuran structure, suggesting that AscJ is the TPC for AF biosynthesis.

Characterization of the asc-2 Cluster Genes. To link the candidate genes ascHJ to AF biosynthesis, each candidate gene was disrupted in the A. egyptiacum ΔascF strain accumulating the precursor 7. HPLC analyses of these double disruptants showed that ΔascF/ΔascH and ΔascF/ΔascJ could not produce 1, indicating that AscH and AscJ are essential for AF biosynthesis, although its function is compensated by the endogenous dehydrogenase in A. egyptiacum. These results clearly demonstrated that AscHIJ are responsible for AF biosynthesis.

The double disruptant ΔascF/ΔascJ specifically accumulated a new compound 9 (0.05 g/L, SI Appendix, Table S8) (Fig. 4B), which was isolated from the large-scale culture, and analyzed by HR-MS and NMR. Its molecular formula was established as...
C₂₂H₃₁ClO₃ from the HR-MS data (m/z 421.1799, calc. 421.1787; SI Appendix, Fig. S8 and Table S7), indicating that it contains an additional oxygen compared with 7. The NMR data revealed that 7 and 9 are very similar to each other, but one methylene signal (δH: 2.01/2.05 ppm; 13C: 36.4 ppm) disappeared and one oxymethine signal (δH: 4.20 ppm; 13C: 75.5 ppm) appeared in 9 (SI Appendix, Fig. S12 and Table S4). Based on the association with the spin system of H-17/H-18, we assigned this signal as H-16. Notably, under acidic condition, 9 was non-enzymatically converted into ascofuranol (10), whose planar structure was identified by HR-MS and NMR analyses, and its absolute configuration was determined as (16S,18S)-10 by the Mosher and NOESY analyses (SI Appendix, Figs. S13 and S14 and Table S6). The absolute configurations of 7 and 9 were thus thought to be (16S)-7 and (16S,18S)-9, respectively.

We further conducted in vitro assays to attribute the succeeding reactions to the candidate genes. The microsomal fraction of the yeast expressing asc1 efficiently converted 9 into 10, while that from the yeast harboring a blank vector did not afford any product (Fig. 4C). The Km and Vmax values of Asc1 were 50.4 ± 11.1 μM and 129 ± 13 nmol/min, respectively. In addition, we found that the cell-free homogenate of A. sojae expressing asc1 dehydrogenates 10 into 1 (Fig. 4D). Thus, we demonstrated that 7 is hydroxylated by the P450 monoxygenase AscH, and the resultant 9 is cyclized by Asc1 to 10, which is oxidized into 1 by Asc1 (Fig. 2A).

Next, we investigated the reaction mechanism of the meroterpenoid TPC Asc1, which lacks significant sequence similarity to any known TPCs. Sequence alignment revealed that Asc1 also harbors several conserved acidic residues that are thought to be essential for the catalysis (36) (SI Appendix, Fig. S15). Indeed, the alanine substitutions of Asc1 D61, E103, D296, E353, and D355 resulted in significant loss of activities (SI Appendix, Fig. S16). Notably, D61, D296, and E353 are also conserved in AuvD and CbV (SI Appendix, Fig. S15), suggesting that these residues are thought to be (16S)-7 and (16S,18S)-9, respectively.

The evolutionary origin of fungal meroterpenoid biosynthetic clusters has also been a matter of debate. In the cases of the biosyntheses of aflatoxin and astacin, ancestrally single-locus clusters were presumably divided into two separated loci by chromosomal rearrangement, supported by the presence of the relict copy in one cluster and the functional gene in the other cluster (51, 52). However, phylogeny and synteny analyses of trichothecene biosynthesis genes revealed that ancestrally separated genes could work with the major biosynthetic cluster as a coregulated pathway and, depending on the species, were later merged into the major cluster (53). For AF biosynthesis, there was no trace of past duplication suggesting a split into the asc-1 and -2 clusters. BLAST searches revealed partially syntenic clusters of the asc-1 cluster in several related fungi of the class Sordariomycetes, but there is no asc-2 syntenic cluster. Interestingly, the asc-1 syntenic cluster in Coniella hystericola also contains the ascH ortholog (PSR83571; 50% amino acid identity), whereas the fungus does not encode a plausible asc1 ortholog. Since the orthologs of the ascH and asc-1 cluster genes are widely distributed among Sordariomycetes, ascH is likely to play another role in the biosynthesis of other AC-related compounds. Given that the report of AF-producing fungi (13) and the distribution of ascH orthologs were biased to the class Eurotiomycetes, we assume that asc1 was horizontally transferred and grafted upon the preexisting ascH to make a coregulated asc-2 cluster, although the elucidation will require further detailed investigation including synteny and phylogeny analyses using a wide range of fungal species.
heteronuclear multiple quantum coherence, and COSY. Full experimental procedures are described in SI Appendix.

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