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A new enzyme involved in the control of the stereochemistry in the decalin formation during equisetin biosynthesis

Naoki Kato [a], Toshihiko Nogawa [a], Hiroshi Hirota [a, b], Jae-Hyuk Jang [c], Shunji Takahashi [a, b], Jong Seog Ahn [c], Hiroyuki Osada [a, *]

[a] Chemical Biology Research Group, RIKEN Center for Sustainable Resource Science, Wako, Saitama 351-0198, Japan
[b] RIKEN-KRIBB Joint Research Unit, Global Research Cluster, RIKEN, Wako, Saitama 351-0198, Japan
[c] Chemical Biology Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), 30 Yeongudanji-ro, Ochang, Cheongwon, Chungbuk 363-883, Republic of Korea

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ABSTRACT

Tetramic acid containing a decalin ring such as equisetin and phomasetin is one of the characteristic scaffolds found in fungal bioactive secondary metabolites. Polyketide (PKS)-nonribosomal peptide synthetase (NRPS) hybrid enzyme is responsible for the synthesis of the polyketide scaffold conjugated with an amino acid. PKS-NRPS hybrid complex programs to create structural diversity in the polyketide backbone have begun to be investigated, yet mechanism of control of the stereochemistry in a decalin formation via a Diels-Alder cycloaddition remains uncertain. Here, we demonstrate that *fsa2*, which showed no homology to genes encoding proteins of known function, in the *fsa* cluster responsible for equisetin and fusarisetin A biosynthesis in *Fusarium* sp. FN080326, is involved in the control of stereochemistry in decalin formation via a Diels-Alder reaction in the equisetin biosynthetic pathway.

1. Introduction

Fungal metabolites derived from polyketide synthase (PKS)-nonribosomal peptide synthetase (NRPS) hybrid enzymes such as fusarin C, tenellin, and equisetin are structurally complex and show a variety of biological activities [1,2]. The fungal PKS-NRPS hybrids are of particular interest because of their capability to synthesize diverse backbone structures [1,2]. Highly reducing PKS module synthesizes linear polyketide backbones, which are linked to an amino acid by the action of the NRPS module. Released products from the megasynthases are further converted to metabolites with more complex structures by tailoring enzymes, which are encoded by genes clustered with the megasynthase genes.

The Diels-Alder reaction, which is a [4+2] cycloaddition reaction to form a cyclohexene ring by the conjugation of a 1,3-diene to a dienophile [3], has been proposed as a key transformation process in some fungal PKS-NRPS hybrid pathways such as the equisetin, chaetoglobosin, and diaporthichalasin/phomopsichalasin pathways [4–6]. The involvement of Diels-Alderases in the formation of a decalin ring derived from similar highly-reducing PKS pathways has been demonstrated [7,8]. Lovastatin nonaketide synthase, LovB, catalyzes the Diels-Alder cycloaddition during the polyketide chain elongation to generate the proper stereochemistry for dihydromonacolin L [7]. It has been proposed that the enzyme-bound hexaketide intermediate is the actual substrate of LovB [9]. Solana-pyrone synthase (Sol5), a flavin-dependent oxidase, catalyzes the oxidation of prosolanapyrone II, which is a post-PKS product released from the prosolanapyrone synthase, Sol1, and the subsequent *exo*-specific cycloaddition reaction to yield solanapyrone A [8]. However, genes involved in a Diels-Alder reaction to form a decalin structure of equisetin (**1**) are yet to be identified.

Fusarisetin A (**2**), which was isolated as an acinar morphogenesis inhibitor, possesses a unique pentacyclic ring system [10] and has been proposed to be converted from **1**, since **2** contains the basic skeleton of **1** [11]. To establish the biosynthetic pathway for **2**, we conducted the genome mining of *Fusarium* sp. FN080326, a

Abbreviations: ATMT, *Agrobacterium tumefaciens*-mediated transformation; C6, $Zn(II)_2Cys_6$-type; ER, enoyl reductase; EtOAc, ethyl acetate; LC/ESI-MS, liquid chromatography/electrospray ionization mass spectrometry; MPLC, medium pressure liquid chromatography; MRM, multiple reaction monitoring; NRPS, nonribosomal peptide synthetase; PDB, potato dextrose broth; PKS, polyketide synthase; WT, wild type.

* Corresponding author. Chemical Biology Research Group, RIKEN Center for Sustainable Resource Science, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan. Fax: +81 48 462 4669.
E-mail address: hisyo@riken.jp (H. Osada).

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producer strain of **2**. In this study, we report that the *fsa* gene cluster is responsible for the biosynthesis of **1** and **2** in *Fusarium* sp. FN080326. A feeding experiment with the *fsa* gene deletion mutant showed that **1** is a biosynthetic intermediate of **2**. Furthermore, we identified a new gene, existing in some fungal PKS-NRPS hybrid gene clusters, that is involved in the stereocontrolled decalin formation of **1**.

2. Material and methods

2.1. Microbial strains

Fusarium sp. FN080326 was previously isolated [10]. *Rhizobium radiobacter* (*Agrobacterium tumefaciens*) C58 and the binary vector pBI121 were used for *A. tumefaciens*-mediated transformation (ATMT) of the fungus. *Escherichia coli* strain Stellar (Clontech, Mountain View, CA, USA) was used for plasmid construction and amplification.

2.2. Genome sequencing and gene prediction

The fungal genomic DNA of the strain FN080326 was extracted with the DNeasy Plant Mini kit (Qiagen, Hilden, Germany) and Genomic tip 20/G (Qiagen), and sequenced using Illumina HiSeq2000 (Illumina, San Diego, CA, USA) at the Genome Network Analysis Support Facility (GeNAS), RIKEN CLST, Yokohama, Japan. Sequence assembly was performed with CLC Genomics Workbench (CLC Bio, Aarhus, Denmark) to yield 820 contigs covering 40.2 Mb (Table S1). Gene annotation and prediction of secondary metabolite gene clusters were performed with the 2ndFind program (http://biosyn.nih.go.jp/2ndfind/).

2.3. Targeted gene inactivation

To construct the gene deletion plasmids, 2-kb DNA fragments upstream of the start codon and downstream of the stop codon of the target genes were amplified by PCR using chromosomal DNA of *Fusarium* sp. FN080326 as the template. The primer pairs, fsa-UF and -UR, and fsa-DF and -DR, were used for amplification of the upstream and downstream regions, respectively. The hygromycin B-resistant cassette (*hph*) was used as a selection marker [12]. These DNA fragments were combined in the original orientation in pBI121 in the following order: the upstream regions, *hph*, followed by the downstream regions (Fig. S1A). In-Fusion cloning system (Clontech) was used for the plasmid construction. The strain FN080326 was transformed with the resultant plasmids using the ATMT method as described previously [13]. Hygromycin B-resistant transformants (Δ*fsa*::*hph*) that resulted from a double-crossover between the deleted *fsa* sequence and the intact chromosomal *fsa* sequence were selected by culture in 50 μg/mL hygromycin B. Correct disruption was checked by PCR (Fig. S1B, C). All DNA fragments amplified by PCR were verified by sequencing. The oligonucleotides that were used for plasmid construction and genotyping are summarized in Tables S2 and S3.

2.4. Characterization of the fsa gene deletion mutants

For the metabolite production analysis, freshly harvested spore suspensions were inoculated in potato dextrose broth (PDB) medium. The fungal strain was cultured at 28 °C and extracted with ethyl acetate (EtOAc). The dried extracts were dissolved in methanol and analyzed by liquid chromatography/electrospray ionization mass spectrometry (LC/ESI-MS). The conditions for LC/ESI-MS and multiple reaction monitoring (MRM) analysis are described in the Supplementary methods.

For expression analysis, total RNAs were isolated from the mycelia of the *fsa* gene deletion mutants and the wild-type strain by using the RNeasy Plant Mini kit (Qiagen). One microgram of total RNA treated with DNase I was used for oligo(dT) primed cDNA synthesis using SuperScript III First Strand Synthesis System for RT-PCR (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocols. Bio-Rad CFX96 Real-Time PCR Detection System was used for qRT-PCR, and SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA, USA) was used for cDNA amplification and detection. The primer pairs used for qRT-PCR are listed in Table S4.

2.5. Isolation of **1** and (3S,6R)-diastereomer of **1** (**3**)

Fusarium sp. FN080326 was cultured at 28 °C for 7 days in PDB medium. The fungal cultures were filtered and extracted with EtOAc. From the dried extracts, **1** was purified by silica gel chromatography, followed by preparative HPLC. Compound **2** was also isolated as described previously [10]. Structures of **1** and **2** were identified as equisetin [14] and fusarisetin A [10] by comparison of their physicochemical properties and NMR data, including 2D NMR spectra.

For the isolation of **3**, the Δ*fsa2* strain derived from FN080326 was cultured at 28 °C for 2 weeks in PDB medium. The whole culture broth (14 L) was extracted 3 times with a half volume of EtOAc. It was evaporated to yield 1.02 g of crude extract. The EtOAc extract was separated into 8 fractions by SiO_2 medium pressure liquid chromatography (MPLC), with a stepwise gradient of $CHCl_3$/MeOH. The first fraction eluted with 100% of $CHCl_3$ was further separated by SiO_2 MPLC with a linear gradient of hexane/EtOAc to afford 5 fractions. The 2nd fraction, showing 2 peaks with the same *m*/*z* value of 374 $[M+H]^+$ in LC/MS analysis, was again separated by SiO_2 MPLC with a linear gradient of hexane/EtOAc to give crude fractions of compounds **3** and **1** as the 2nd and 4th fraction, respectively. Each fraction was purified by C_{18}- HPLC with isocratic elution of MeOH/0.05% aqueous formic acid (8:2) to afford **3** (10.2 mg) and **1** (5.3 mg) as colorless amorphous solids. The physicochemical properties and ^1H and ^{13}C NMR spectra of **3** are summarized in the Supplementary Information (Tables S5 and S6, and Fig. S2–S8). The physicochemical properties of **1** were identical to the data obtained from the compound extracted from the wild-type culture described above.

3. Results

3.1. Identification of the biosynthesis gene cluster for **1** and **2** in Fusarium sp. FN080326

To explore secondary metabolite biosynthetic gene clusters of the producer strain of **2**, the genome of the strain FN080326 was sequenced using Illumina 100-bp paired end sequencing. Sequence analysis showed that the genome harbored a number of PKS and NRPS genes like most filamentous fungi, but fortunately, only one PKS-NRPS hybrid gene, *fsa1*, was found in the genome (Table S1). Enzymatic genes that were likely to be involved in the biosynthesis of **1**, such as those encoding *trans*-acting enoyl reductase (ER) domain protein and methyltransferase as well as $Zn(II)_2Cys_6$ (C6)-type transcription factor genes, were adjacent to *fsa1* (Fig. 1). Features of the gene products in the *fsa* cluster and its neighbors are summarized in Table 1 (GenBank: LC025956). The biosynthetic gene (*eqx*) cluster of **1** in *Fusarium heterosporum* ATCC 74349, which has been corrected recently [15], was very similar to the *fsa* cluster (Table 1).

Knockout experiments were carried out to demonstrate that the *fsa* gene cluster is involved in the biosynthesis of **1** in the fungal strain FN080326. Gene deletion mutants were generated by replacing the entire coding region of the *fsa* gene by the hygromycin B-resistance gene cassette (Fig. S1). Metabolite

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