

Full Paper

Heterologous production of ascofuranone and ilicicolin A in *Aspergillus sojae*

(Received May 12, 2021; Accepted August 13, 2021; J-STAGE Advance publication date: April 13, 2022)

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Ascofuranone and its precursor, ilicicolin A, are secondary metabolites with various pharmacological activities that are produced by *Acremonium egyptiacum*. In particular, ascofuranone strongly inhibits trypanosome alternative oxidase and represents a potential drug candidate against African trypanosomiasis. However, difficulties associated with industrial production of ascofuranone by *A. egyptiacum*, specifically the co-production of ascochlorin, which inhibits mammalian respiratory chain complex III at low concentrations, has precluded its widespread application. Therefore, in this study, ascofuranone biosynthetic genes (*ascA-E* and *H-J*) were heterologously expressed in *Aspergillus sojae*, which produced very low-levels of endogenous secondary metabolites under conventional culture conditions. As a result, although we obtained transformants producing both ilicicolin A and ascofuranone, they were produced only when an adequate concentration of chloride ions was added to the medium. In addition, we succeeded in increasing the production of ilicicolin A, by enhancing the expression of the rate-determining enzyme AscD, using a multi-copy integration system. The heterologous expression approach described here afforded the production of both ascofuranone and ilicicolin A, allowing for their development as therapeutics.

Key Words: ascofuranone; *Aspergillus sojae*; filamentous fungi; heterologous expression; ilicicolin A; multi-copy integration; secondary metabolites

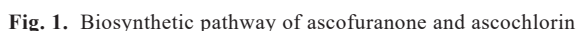
Introduction

Ascofuranone (**1**) is a polyketide-terpene hybrid metabolite (meroterpenoid) originally isolated from the filamentous fungus *Acremonium egyptiacum* (synonym: *Acremonium sclerotigenum*), which is also known as *Ascochyta viciae* (Hijikawa et al., 2017; Sasaki et al., 1972; Sasaki et al., 1973; Summerbell et al., 2018). **1** has diverse physiological activities, including antibiotic, hypolipidemic, anti-inflammatory, and anti-echinococcus (Enkai et al., 2017; Park et al., 2017; Sasaki et al., 1972; Sasaki et al., 1973). In particular, **1** is a strong inhibitor of cyanide-insensitive alternative oxidases (Minagawa et al., 1997; Shiba et al., 2013), and is a potential drug candidate for the treatment of African sleeping sickness caused by the protozoan parasite *Trypanosoma brucei*. (Nihei et al., 2002). *T. brucei* depends exclusively on glycolysis as an energy source in the mammalian bloodstream and relies on trypanosome alternative oxidase (TAO) for the re-oxidation of NADH to continue glycolysis (Nihei et al., 2002). Mammalian hosts lack this protein, and thus TAO is considered to be a unique target for anti-trypanosomal drugs (Nihei et al., 2002). In addition, recent reports indicated that **1** and its precursor, ilicicolin A (**2**) have inhibitory activity against human dihydroorotate dehydrogenase (DHODH), and are thus expected to have applications in the treatment of cancer (Miyazaki et al., 2018).

Recently, the complete set of genes were identified and the biosynthetic pathway of **1** synthesis in *A. egyptiacum* was elucidated (Araki et al., 2019) (Fig. 1). First, the polyketide-terpenoid hybrid molecule, ilicicolinic acid B (**4**) is synthesized by polyketide synthase (PKS) AscC and prenyltransferase AscA, followed by its conversion to **2** by the nonribosomal peptide synthetase (NRPS)-like

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None of the authors of this manuscript has any financial or personal relationship with other people or organizations that could inappropriately influence their work.



reductase AscB and halogenase AscD. Subsequently, **2** is oxidized sequentially by two P450 monooxygenases, namely AscE and AscH, before cyclization by the terpene cyclase AscI. Finally, **1** is obtained through further modifications of **2** by dehydrogenase AscJ. Furthermore, *A. egyptiacum* also produces ascochlorin (**3**), which is similar in structure to **1**, and is synthesized via the common precursor, ilicicolin A epoxide (**6**). However, **3** can be harmful to humans, because it inhibits animal mitochondrial respiratory chain complex III, at low concentrations (Berry et al., 2010). Therefore, the use of *A. egyptiacum* as an industrial host for **1** production is unfavorable.

In filamentous fungi, *Aspergillus nidulans* and *Aspergillus oryzae* are often used for heterologous production of fungal secondary metabolites (Anyagwu and Mortensen, 2015). For industrial use, *A. oryzae* has the advantage of producing low levels of endogenous secondary metabolites under conventional culture conditions; thus, the target compound is expected to be purified easily. In addition, *A. oryzae* is considered to be safe, as it has a long history of use in food fermentation processes for products such as soy sauce, sake, and miso in Japan. In fact, it has been shown that *A. oryzae* produces few mycotoxins, and that these compounds are only produced by a few strains (Frisvad et al., 2018). Also, *Aspergillus sojae*, which has been used in soy sauce manufacturing, does not produce aflatoxins and cyclopiazonic acid, and is suitable as a host for heterologous expression in terms of safety (Chang et al., 2007, Heerikhuisen et al. 2005, Sato et al., 2011).

In this study, we aimed to produce **1** and **2** using a heterologous expression approach in *A. sojae*. In addition, for the purpose of increasing the production of **2**, we constructed strains capable of highly expressing the rate-determining enzyme, AscD, using a multi-copy integration technology.

Strains. The strains and primers used in this study are listed in Table 1 and Table 2, respectively. The DNA frag-

ments for obtaining *A. sojae* transformants were constructed using an In-Fusion HD Cloning Kit (Clontech Laboratories, Inc., Mountain View, CA, USA). Transformation of *A. sojae* was performed as previously reported (Takahashi et al., 2004). For transformants, the correct recombination events were verified by direct PCR using KOD FX Neo (Toyobo Co., Ltd., Osaka, Japan).

A. sojae KP-del (Araki et al., 2020) was used as a host strain for heterologous expression of each *asc* gene (DDBJ/ENA/GenBank accession LC406756 and LC406757). All expression cassettes of *asc* genes were composed of a *tef* promoter (*Ptef*) and an *alp* terminator (*Talp*), derived from *A. sojae* (Araki et al., 2020), and codon-optimized synthetic *asc* genes, without introns. An expression cassette of *cprA* was composed of a *gpd* promoter (*Pgpd*), a *cprA* homolog, and an *amy* terminator (*Tamy*), and amplified from the *A. sojae* NBRC4239 genome DNA using the following primers: P-gpd-F,R for *Pgpd*, AscprA-F, R for *cprA* homolog, and T-amy-F, R for *Tamy*. The DNA sequences (5'- and 3'-arm) required for integration of each *asc* gene into a targeted region by homologous recombination were the same as in the case of *egtA* integration (Hara and Ichikawa, 2019). *A. sojae* NBRC 4239 has at least 8 regions for *asc* gene integration at different chromosomal loci, which contain almost the same sequences, with >99% identity to each other. The loop-out region for *pyrG* marker recycling was used, similar to the case of *egtA* integration (Hara and Ichikawa, 2019). The DNA fragment for the integration of each *ascA-E*, *I* and *J* gene was composed of a 5'-arm, *Ptef*, the *asc* gene, *Talp*, a loop-out region, a *pyrG* marker (Araki et al., 2020), and a 3'-arm, in that linear order. The DNA fragment for co-integration of *ascH* and *cprA* was composed of a 5'-arm, *Ptef*, *ascH*, *Talp*, a *pyrG* marker, *Pgpd*, *cprA*, *Tamy*, and a 3'-arm, in that linear order. Each DNA fragment for *asc* gene expression was introduced to the KP-del strain in the sequential order of the biosynthetic pathway, one by one, by repeating *pyrG* marker recycling using 2 mg/mL 5-fluoroorotic acid (5FOA) and 20 mM uridine and uracil (Ito et al., 2013),

Table 1. *A. sojae* strains used in this study

Strain name	Genotype	Reference
NBRC4239	Wild-type	Sato et al., 2011
KP-del	$\Delta pyrG \Delta ku70$	Araki et al., 2020
P6-1	$niaD^- \Delta pyrG::niaD$	Araki et al., 2020
As-CAB	$\Delta pyrG \Delta ku70$ Ptef::ascC::Talp Ptef::ascA::Talp Ptef::ascB::Talp::pyrG	This study
As-CAD	$\Delta pyrG \Delta ku70$ Ptef::ascC::Talp Ptef::ascA::Talp Ptef::ascD::Talp::pyrG	This study
As-CABD	$\Delta pyrG \Delta ku70$ Ptef::ascC::Talp Ptef::ascA::Talp Ptef::ascB::Talp Ptef::ascD::Talp::pyrG	This study
As-CAB_mcD	$\Delta pyrG \Delta ku70$ Ptef::ascC::Talp Ptef::ascA::Talp Ptef::ascB::Talp::pyrG pUC19-pG56-ascD	This study
As-mcCABD	$niaD^- \Delta pyrG::niaD$ pUC19-pG56-ascC+A pUC19-pG56-ascB+D	This study
As-CABDE	$\Delta pyrG \Delta ku70$ Ptef::ascC::Talp Ptef::ascA::Talp Ptef::ascB::Talp Ptef::ascD::Talp Ptef::ascE::Talp::pyrG	This study
As-CABDEH	$\Delta pyrG \Delta ku70$ Ptef::ascC::Talp Ptef::ascA::Talp Ptef::ascB::Talp Ptef::ascD::Talp Ptef::ascE::Talp Ptef::ascH::Talp::pyrG::PgpD::AscprA::Tamy	This study
As-CABDEHI	$\Delta pyrG \Delta ku70$ Ptef::ascC::Talp Ptef::ascA::Talp Ptef::ascB::Talp Ptef::ascD::Talp Ptef::ascE::Talp Ptef::ascI::Talp Ptef::ascH::Talp::pyrG::PgpD::AscprA::Tamy	This study
As-CABDEHIJ	$\Delta pyrG \Delta ku70$ Ptef::ascC::Talp Ptef::ascA::Talp Ptef::ascB::Talp Ptef::ascD::Talp Ptef::ascE::Talp Ptef::ascI::Talp Ptef::ascJ::Talp Ptef::ascH::Talp::pyrG::PgpD::AscprA::Tamy	This study

Table 2. Oligonucleotide primers used in this study

Primer name	Oligonucleotide sequence (5' to 3')
P-gpd-F	agggtcgggTTCACCGTATCTTAATAGAGAA
P-gpd-R	TGTTTGATGTGTCTGCTGGTGTG
AscprA-F	agacacatccaaacaATGGCGCAACTCGACACTCTCGATT
AscprA-R	tatactctccacctTCATGACCAAACATCCTCCTGGTAG
T-amy-F	AGGGTGGAGAGTATATGATGGTAC
T-amy-R	ACCATTGTAGTTCGTccatgagatactatgatatactaa
sqPCR-ascA-F	ACATACGTCGGCGTGGGTACAA
sqPCR-ascA-R	CCGGTCAGGAGATTAAGGATCG
sqPCR-ascB-F	GCAGGACTATGGCGGTCAACTT
sqPCR-ascB-R	ATCTGCGTAAGCCTTCACGGTC
sqPCR-ascC-F	TGTCTCTCTCTGAAATCCGCCG
sqPCR-ascC-R	CCAGTTCACCCAAGAGGAAGTC
sqPCR-ascD-F	GCAGATCAGGCACCAAGAGGAA
sqPCR-ascD-R	CCCTCAGGTTGGTGATGTCCAT
sqPCR-actA-F	TTCCAGCCTAGCGTTTTGGGTC
sqPCR-actA-R	CGGAGTATTACGCTCAGGAGG
qPCR-ascB-F	TGCACGAATTGGACTTGCAC
qPCR-ascB-R	TCACAAACCAGCTTGCCATC
qPCR-ascD-F	TGGCGGCCCAACAATTATG
qPCR-ascD-R	ATTTACGCTGACTCCATCG
qPCR-H2A-F3	AGGAGTTGAACAAGCTGCTG
qPCR-H2A-R3	TCTTGGGGAGAAGGTCTAGTTC

Lowercase letters indicate the sequence necessary for In-Fusion reaction.

although co-integration of *ascH* and *cprA* was lastly performed to obtain the As-CABDEHI and As-CABDEHIJ strains.

The multi-copy integrative vector containing the attenuated *pyrG* marker (*pyrG56*) was used to obtain As-CAB_mcD and As-mcCABD (Araki et al., 2020). For construction of As-CAB_mcD, a *pyrG* disruptant of As-CAB was transformed using the pUC19-pG56-ascD vector, containing the *ascD* expression cassette. For construction of As-mcCABD, P6-1 (Araki et al., 2020) was simultaneously transformed using two vectors, namely pUC19-pG56-ascC+A and pUC19-pG56-ascB+D, containing expression cassettes of *ascC* and *ascA*, or *ascB* and *ascD*.

HPLC analysis of each product. *A. sojae* strains were cultivated at 30 °C for 4 days in GPY medium, consisting of 2% glucose, 1% Hipolypepton (Nihon Pharmaceutical Co., Ltd., Tokyo, Japan), 0.5% yeast extract (Becton, Dickinson and Co., Franklin Lakes, NJ, USA), 0.5% KH₂PO₄, and 0.05% MgSO₄·7H₂O with 0–10% (w/v) NaCl, for production of ascofuranone or its precursors. Hydrophilic products of *A. sojae* transformants were extracted by acetone from their mycelia with the same weight, after drying on paper towel, and dissolved in methanol after exsiccation. The filtered aliquots were analyzed with a 1220 Infinity LC system (Agilent Technologies, Inc.), using a solvent system of 0.1% formic acid in water (solvent A) and ace-

tonitrile containing 0.1% formic acid (solvent B) at a column temperature of 40 °C using a TSKgel ODS-100V column (4.6 i.d. × 150 mm, 3 µm, Tosoh Corp., Tokyo, Japan). For analysis of ilicicolin A, separation was performed with a linear gradient from a solvent B:solvent A ratio of 80:20 to 95:5, over 15 min, with detection at 220 nm. For analysis of ascofuranone, separation was performed with a linear gradient from a solvent B:solvent A ratio of 40:60 to 100:0, over 50 min, with detection at 295 nm.

Mass Spectrometry analysis. Samples for LC/MS analysis (Figs. 4b and S6) were analyzed using an Agilent 1100 series HPLC-QSTAR Elite mass spectrometer (Applied Biosystems, Foster City, CA, USA), using electrospray ionization with an L-column2 ODS column (3 µm, 2.1 i.d. × 100 mm, Chemicals Evaluation and Research Institute, Tokyo, Japan). The negative mode ESI-MS/MS spectrum of ascofuranone (m/z 419) was obtained with 45 eV collision energies. Samples for LC/MS analysis (Figs. S1 and S4b) were analyzed with a Micromass Quattro Micro API mass spectrometer (Waters).

Semi-quantitative RT-PCR. Total RNA was isolated using ISOGEN (Nippon Gene, Tokyo, Japan) from the As-CABD strain cultured at 30 °C for 2 days in GPY medium without or with 5% (w/v) NaCl. To eliminate DNA contamination, total RNA (40 µg) was treated with 40 units of DNase I (Nippon Gene, Tokyo, Japan) and subsequently purified using the RNeasy Plant Mini Kit (QIAGEN). cDNA was prepared from the same amount of total RNA using oligo dT and random hexamer primers with the PrimeScript RT Master Mix (Takara, Kyoto, Japan). PCR amplification was performed for 20–26 cycles with the KOD Plus Neo DNA polymerase (Toyobo, Osaka, Japan) using the primers listed in Table 2, and the cDNA generated during reverse transcription was directly used as the template. The actin gene (*actA*: homolog of AO090701000065) was used as an internal control.

Quantitative PCR. Genomic DNA was extracted from the cells with a DNeasy Plant Mini Kit (QIAGEN) following the manufacturer's instructions. The primers, qPCR-ascB-F,R and qPCR-ascD-F,R were used for the amplification of *ascB* and *ascD* genes, while qPCR-H2A-F3 and R3 were used for amplification of the histone H2A gene for normalization, using SYBR Premix Ex Taq II (Takara, Kyoto, Japan). Copy numbers of *ascB* and *ascD* in As-CAB_{mcD} and As-mcCABD were calculated by comparison with As-CABD, which was thought to have one copy of *ascCABD*, using an MX3005P instrument (Agilent Technologies, Inc.).

Results and Discussion

Increasing ilicicolin A production in the *ascCABD*-expressing strain by the addition of chloride ions

First, in order to produce **2** in *A. sojae*, we constructed a strain expressing *ascCABD*, derived from *A. egyptiacum*. For the expression of *asc* genes, codon-modified exon sequences were used. The host strain of *A. sojae* did not produce any detectable secondary metabolites under this study conditions (data not shown). As in the case of

expressing *ascCABD* in *A. oryzae* (Araki et al., 2019), the *ascCABD*-expressing strain of *A. sojae* (As-CABD) accumulated significant levels of **5**, instead of **2** (Fig. 2). Therefore, NaCl was added to the medium to provide chloride ions, which serve as a cosubstrate of halogenase AscD. The addition of NaCl above 0.1%, resulted in detectable production of **2**, and the level of production was maximized (8 mg/L) when NaCl was added at 5% (Fig. 2). Similar results were obtained with the addition of other chlorine sources at 5%, for example KCl and NH₄Cl (data not shown).

However, the addition of 10% NaCl decreased the production of **2**, in comparison to supplementation with 5% NaCl, and compound **7**, exhibiting a molecular ion [M-H][−] at m/z 405, was found to accumulate under these conditions (Figs. 2 and S1). A previous report showed that ilicicolinic acid A (5-chloro grifolic acid) is synthesized from ilicicolinic acid B (**4**, grifolic acid) by an AscD homolog derived from *Fusarium* sp. (Okada et al., 2017). The retention time and m/z value of **7** were the same as those of the compound that specifically accumulated in the As-CAD strain, suggesting that **7** was ilicicolinic acid A (Fig. 2).

From the above findings, it was confirmed that the As-CABD strain produced **2**, when the medium was supplemented with chloride ions, in *A. sojae*. However, even when 5% NaCl was added, a large amount of **5** accumulated in the As-CABD strain, in which *ascE* was expressed at a similar level as the other *asc* genes (Fig. S2). Therefore, it was suggested that the step involving AscD was rate-determining. In the case of 10% NaCl addition, the accumulation of **5** was reduced, in comparison to that at 5% NaCl supplementation, and **7**, which was thought to be ilicicolinic acid A, accumulated at significant levels. Furthermore, when 10% NaCl was added, more of compound **4** was found to accumulate (data not shown). These results might indicate that excessive supplementation of chloride ions leads to a decrease in AscB activity and the accumulation of **4**, resulting in higher production of **7**, due to the preferential reaction of AscD with **4**, rather than **5**, as the substrate. Therefore, it was necessary to determine an alternative method to chloride ion addition to increase AscD reactivity.

Improvement of ilicicolin A production by over-expression of *ascD*

Subsequently, we examined whether the *in vivo* reactivity of AscD, which was determined to be a rate-determining reaction in the production of **2**, could be improved by increasing the expression of *ascD*. Previously, we developed an efficient system for high-level heterologous protein production by multi-copy gene introduction using an attenuated selectable marker in *A. sojae* (Araki et al., 2020). Utilizing this system, an *ascD* expression cassette was introduced into the *pyrG*-defective As-CAB strain. Of the 16 transformants, 8 strains were confirmed to produce **2**, even in the absence of NaCl supplementation (data not shown). Among the As-CAB_{mcD} strains obtained, which had the highest productivity, 26 mg/L of **2** was produced without NaCl supplementation. By quantitative PCR, it was found that the As-CAB_{mcD} strain had 46 copies of the *ascD* gene. In addition, we obtained the As-mcCABD

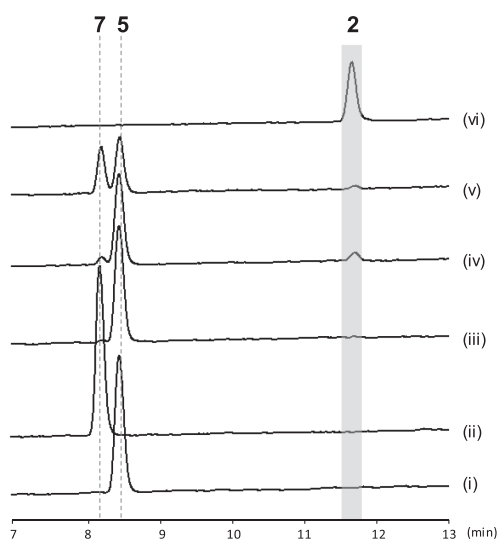


Fig. 2. Heterologous expression of *ascA-D* in *A. sojae*

HPLC profiles of mycelium extracts of *A. sojae* transformants As-CAB (i), As-CAD (ii), and As-CABD (iii)(iv)(v), as well as the profile of authentic ilicicolin A (vi). 5% NaCl was added into the growth media of (ii) and (iv). 10% NaCl was added in (v), and NaCl was not added in (i) and (iii).

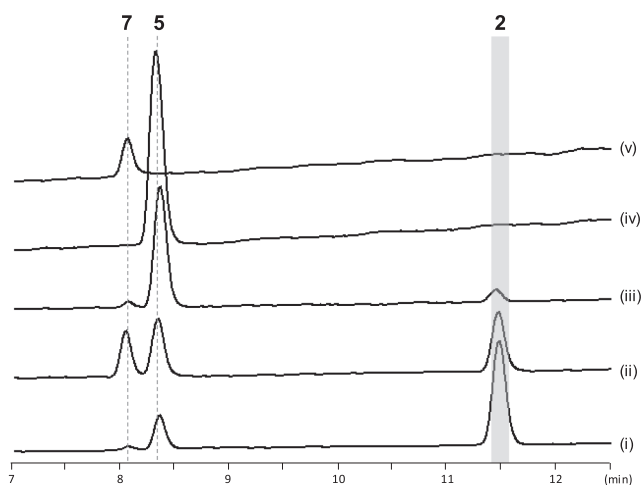


Fig. 3. Improvement of ilicicolin A production by multi-copy introduction of *ascD*

HPLC profiles of mycelium extracts of *A. sojae* transformants As-mcCABD (i), As-CAB_{mcD} (ii), As-CABD (iii), As-CAB (iv), and As-CAD (v). 5% NaCl was added to the growth media of (iii) and (v). NaCl was not added in (i), (ii) and (iv).

strain, comprising 18 copies of *ascB* and 22 copies of *ascD*, using a vector comprising both expression cassettes. In the As-mcCABD strain, the production of **7** was very low, and the accumulation of **2** was further improved, in comparison with the As-CAB_{mcD} strain (Fig. 3).

Next, the positive effect of NaCl supplementation was verified in the As-CAB_{mcD} and As-mcCABD strains. When these strains were cultured in media supplemented with 5% NaCl, the production of **2** per cell was reduced to approximately half (Fig. S3). In contrast, in the As-mcCABD strain, when 0.5% NaCl was added to the medium, the production of **2** improved slightly (85 ± 11 mg/L $\rightarrow$ 110 ± 27 mg/L), but no significant difference was observed

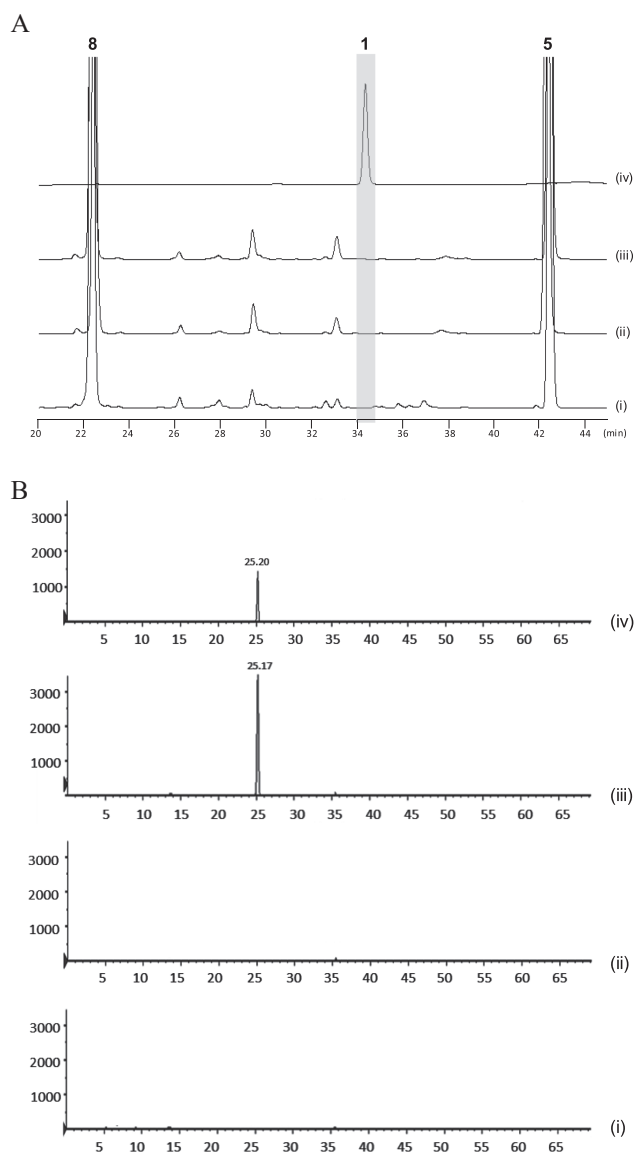


Fig. 4. Heterologous expression of all biosynthetic genes of ascofuranone in *A. sojae*

HPLC profiles (A) and MS spectra of the $[M-H]^-$ ion at m/z 419 (B) from mycelium extracts of *A. sojae* transformants As-CABDEH (i), As-CABDEHI (ii), and As-CABDEHIJ (iii), as well as the profile of authentic ascofuranone (iv).

in comparison to media without NaCl supplementation.

Collectively, the productivity of **2** was significantly improved by introducing the *ascD* gene in multiple copies. In particular, it was found that the As-mcCABD strain, having multi-copy of both *ascB* and *ascD*, increased the production of **2** by more than 10 fold, in comparison with the As-CABD strain, and was able to produce about 100 mg/L of **2**. Meanwhile, the production of **2** on a per cell basis in the As-CAB_{mcD} strain was about half that of the As-mcCABD strain (Fig. S3). This result may suggest that AscD competed with AscB for the substrate (**4**) in the As-CAB_{mcD} strain, owing to overexpression of AscD. In the As-mcCABD strain, we hypothesize that **4** was preferentially used as a substrate for AscB, and as a result, the production of **2** was higher.

Heterologous ascofuranone production by introduction of all ascofuranone biosynthetic genes

Finally, in order to produce ascofuranone in *A. sojae*, we constructed a strain expressing all ascofuranone biosynthetic genes, *ascCABDEHIJ*. Since marker recycling is difficult in both the As-CAB_{mcD} strain and the As-mcCABD strain, expression cassettes of *ascEHIJ* were introduced into the *pryG*-defective As-CABD strain as a host. In expressing AscH, the cytochrome P450 enzyme, a cytochrome P450 reductase (Cpr) ortholog derived from *A. sojae* having 90% identity with CprA from *Aspergillus niger* (van den Brink et al., 1995) was co-expressed, as Cpr is required as an electron donor for the reaction of the P450 enzyme. In addition, to improve the *in vivo* reactivity of AscD, each transformant was cultured in a medium containing 5% NaCl.

The As-CABDE strain produced a new metabolite **8**, not observed in the As-CABD strain (Fig. S4a). Considering the biosynthetic pathway shown in Fig. 1, it was predicted that ilicicolin A epoxide (**6**, *m/z* 405, [M-H][−]) would accumulate in the As-CABDE strain. However, **8** presented a molecular ion [M-H][−] at *m/z* 389 (Fig. S4b). Previous reports have shown that ilicicolin B epoxide (*m/z* 371, [M-H][−]) can be synthesized from **5** via the reaction of an AscE homolog derived from *Fusarium* sp. (Quan et al., 2019). Also, we have previously indicated that **6** is hydrolyzed by an endogenous hydrolase in *A. sojae* (Araki et al., 2019). Considering the above information, **8** was predicted to be a shunt product formed by hydrolyzation of ilicicolin B epoxide (Fig. S4b).

In the As-CABDEH strain, any metabolites were not newly produced, in comparison to the As-CABDE strain (Fig. S5). In addition, all mycelium extracts of the As-CABDEH, As-CABDEHI, and As-CABDEHIJ strains revealed almost identical HPLC profiles, and all HPLC profiles of their strains lacked a peak at the known retention time of **1** (Fig. 4A). On the other hand, LC-MS analysis showed that only the As-CABDEHIJ strain produced a metabolite with an *m/z* of 419 ([M-H][−]), which was detected at the same retention time as **1** (Fig. 4B), and the MS/MS fragmentation pattern coincided with that of **1** (Fig. S6).

From the above, it was confirmed that **1** could be biosynthesized in *A. sojae*, which heterologously expressed all genes successfully (*ascCABDEHIJ*). However, the level of production was very low (about 0.3 mg / L). This may be due to low *in vivo* reactivity of AscH in *A. sojae*. In this study, a Cpr homolog derived from *A. sojae* was co-expressed; however, co-expression of Cpr derived from *A. egyptiacum* may promote the activity of AscH more efficiently. In addition, the reactivity of AscH might be improved by co-expressing not only Cpr, but another electron donor such as cytochrome b5. In fact, in the production of artemisinin in *Saccharomyces cerevisiae*, the production volume was improved by optimizing the combination and the balance of expression levels of P450, Cpr, and cytochrome b5 (Paddon et al., 2013). Furthermore, disruption of the endogenous hydrolase in *A. sojae*, which can hydrolyze **6**, may be an efficient method for improving the production levels of **1**.

Although the As-CABD strain was used as a host for the biosynthesis of **1** in this study, we hypothesize that the

production of **1** can be further increased using a strain producing a high-level of **2**, such as the As-mcCABD strain. However, since the *pyrG* marker could not be recycled in the As-mcCABD strain, it was necessary to use another marker for the insertion of the remaining *asc* genes into this strain. Recently, in *A. oryzae*, an efficient CRISPR/Cas9 system using the drug resistance marker *ptrA*, has been reported to allow for repeated genome editing (Katayama et al., 2019). In the future, the application of a similar CRISPR/Cas9 system to the As-mcCABD strain may provide a strain capable of producing higher levels of **1** in *A. sojae*.

ACKNOWLEDGEMENTS

We are deeply grateful to Dr. Motomichi Matsuzaki and Dr. Takayoshi Awakawa for their insightful comments and suggestions. We thank Ms. Nahoko Endo for experimental assistance.

References

- Anyagou, DC. and Mortensen, UH. (2015) Heterologous production of fungal secondary metabolites in *Aspergilli*. *Front. Microbiol.*, **6**, 77.
- Araki, Y., Awakawa, T., Matsuzaki, M., Cho, R., Matsuda, Y. et al. (2019) Complete biosynthetic pathways of ascofuranone and ascochlorin in *Acremonium egyptiacum*. *Proc. Natl. Acad. Sci. U. S. A.*, **116**, 8269–8274.
- Araki, Y., Yuzuki, M., Masakari, Y., Sato, A., Gomi, K. et al. (2020) High-level heterologous protein production using an attenuated selection marker in *Aspergillus sojae*. *J. Gen. Appl. Microbiol.*, 10.2323/jgam.2020.05.002
- Berry, EA., Huang, LS., Lee, DW., Daldal, F., Nagai, K. et al. (2010) Ascochlorin is a novel, specific inhibitor of the mitochondrial cytochrome bc1 complex. *Biochim. Biophys. Acta.*, **1797**, 360–370.
- Chang, PK., Matsushima, K., Takahashi, T., Yu, J., Abe, K. et al. (2007) Understanding nonaflatoxigenicity of *Aspergillus sojae*: a windfall of aflatoxin biosynthesis research. *Appl. Microbiol. Biotechnol.*, **76**, 977–984.
- Enkai, S., Sakamoto, K., Kaneko, M., Kouguchi, H., Irie, T. et al. (2017) Medical Treatment of *Echinococcus multilocularis* and New Horizons for Drug Discovery: Characterization of Mitochondrial Complex II as a Potential Drug Target. *Echinococcosis*, ed. by Inceboz, T., IntechOpen, 10.5772/intechopen.68565.
- Frisvad, JC., Møller, LLH., Larsen, TO., Kumar, R., and Arnau, J. (2018) Safety of the fungal workhorses of industrial biotechnology: update on the mycotoxin and secondary metabolite potential of *Aspergillus niger*, *Aspergillus oryzae*, and *Trichoderma reesei*. *Appl. Microbiol. Biotechnol.*, **102**, 9481–9515.
- Hara, S. and Ichikawa, K. (2019) Selenoneine production method. Unexamined Patent Application Publication WO2019/240243A.
- Heerikhuisen, M., van den Hondel, C., and Punt, P. (2005) Protein production in *Aspergillus sojae*. In *Production of recombinant proteins. Novel microbial and eukaryotic expression systems*. ed. by Gellissen G., Wiley, Weinheim, pp 191–214.
- Hijikawa, Y., Matsuzaki, M., Suzuki, S., Inaoka, DK., Tatsumi, R. et al. (2017) Re-identification of the ascofuranone-producing fungus *Ascochyta viciae* as *Acremonium sclerotigenum*. *J. Antibiot. (Tokyo)*, **70**, 304–307.
- Ito, K., Koyama, Y. and Hanya, Y. (2013) Identification of the Glutaminase Genes of *Aspergillus sojae* Involved in Glutamate Production during Soy Sauce Fermentation. *Biosci. Biotechnol. Biochem.*, **77**, 1832–1840.
- Katayama, T., Nakamura, H., Zhang, Y., Pascal, A., Fujii, W. et al. (2019) Forced Recycling of an AMA1-Based Genome-Editing Plasmid Allows for Efficient Multiple Gene Deletion/Integration in the Industrial Filamentous Fungus *Aspergillus oryzae*. *Appl. Environ. Microbiol.*, **85**, e01896–18.
- Minagawa, N., Yabu, Y., Kita, K., Nagai, K., Ohta, N. et al. (1997) An antibiotic, ascofuranone, specifically inhibits respiration and *in vitro* growth of long slender bloodstream forms of *Trypanosoma brucei*

- brucei*. *Mol. Biochem. Parasitol.*, **84**, 271-280.
- Miyazaki, Y., Inaoka, DK., Shiba, T., Saimoto, H., Sakura, T. et al. (2018) Selective Cytotoxicity of Dihydroorotate Dehydrogenase Inhibitors to Human Cancer Cells Under Hypoxia and Nutrient-Deprived Conditions. *Front. Pharmacol.*, **9**, 997.
- Nihei, C., Fukai, Y., and Kita, K. (2002) Trypanosome alternative oxidase as a target of chemotherapy. *Biochim. Biophys. Acta.*, **1587**, 234-239.
- Okada, M., Saito, K., Wong, CP., Li, C., Wang, D. et al. (2017) Combinatorial Biosynthesis of (+)-Daurichromenic Acid and Its Halogenated Analogue. *Org. Lett.*, **19**, 3183-3186.
- Paddon, CJ., Westfall, PJ., Pitera, DJ., Benjamin, K., Fisher, K. et al. (2013) High-level semi-synthetic production of the potent antimalarial artemisinin. *Nature*, **496**, 528-532.
- Park, JY., Chung, TW., Jeong, YJ., Kwak, CH., Ha, SH. et al. (2017) Ascofuranone inhibits lipopolysaccharide-induced inflammatory response via NF-kappaB and AP-1, p-ERK, TNF- α , IL-6 and IL-1 β in RAW 264.7 macrophages. *PLoS One*, **12**, e0171322.
- Quan, Z., Awakawa, T., Wang, D., Hu, Y., and Abe, I. (2019) Multidomain P450 Epoxidase and a Terpene Cyclase from the Ascochlorin Biosynthetic Pathway in *Fusarium* sp. *Org. Lett.*, **21**, 2330-2334.
- Sasaki, H., Okutomi, T., Hosokawa, T., Nawata, Y., and Ando, K. (1972) Ascofuranone, a new antibiotic from *Ascochyta viciae*. *Tetrahedron Lett.*, **13**, 2541-2544.
- Sasaki, H., Hosokawa, T., Sawada, M., and Ando, K. (1973) Isolation and structure of ascofuranone and ascofranol, antibiotics with hypolipidemic activity. *J. Antibiot. (Tokyo)*, **26**, 676-680.
- Sato, A., Oshima, K., Noguchi, H., Ogawa, M., Takahashi, T. et al. (2011) Draft genome sequencing and comparative analysis of *Aspergillus sojae* NBRC4239. *DNA Res.*, **18**, 165-176.
- Shiba, T., Kido, Y., Sakamoto, K., Inaoka, DK., Tsuge, C. et al. (2013) Structure of the trypanosome cyanide-insensitive alternative oxidase. *Proc. Natl. Acad. Sci. U. S. A.*, **110**, 4580-4585.
- Summerbell, RC., Gueidan, C., Guarro, J., Eskalen, A., Crous, PW. et al. (2018) The Protean *Acremonium*. *A. sclerotigenum/egyptiacum*: Revision, Food Contaminant, and Human Disease. *Microorganisms*, **6**, 88.
- Takahashi, T., Hatamoto, O., Koyama, Y., and Abe, K. (2004) Efficient gene disruption in the koji-mold *Aspergillus sojae* using a novel variation of the positive-negative method. *Mol. Genet. Genomics*, **272**, 344-52.
- van den Brink, HJ., van Zeijl, CM., Brons, JF., van den Hondel, CA., and van Gorcom, RF. (1995) Cloning and characterization of the NADPH cytochrome P450 oxidoreductase gene from the filamentous fungus *Aspergillus niger*. *DNA Cell Biol.*, **14**, 719-729.