Short Communication

Efficient heterologous production of atrochrysone carboxylic acid-related polyketides in an *Aspergillus oryzae* host with enhanced malonyl-coenzyme A supply

(Received June 10, 2019; Accepted July 1, 2019; J-STAGE Advance publication date: November 27, 2019)

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We recently developed an *Aspergillus oryzae* strain in which malonyl-coenzyme A (CoA) supply is strengthened by the deletion of *snfA* and *SCAP* as an efficient host to produce a plant polyketide, curcumin. Here, we examined the effectiveness of this strain in producing another polyketide, atrochrysone carboxylic acid (ACA), which is synthesized from eight molecules of malonyl-CoA using an iterative type I polyketide synthase, ACA synthase (ACAS), and atrochrysone carboxyl ACP thioesterase (ACTE) in *Aspergillus terreus*. When ACAS and ACTE were introduced, the *A. oryzae* $\Delta snfA \Delta SCAP$ strain produced approximately four times more ACA-related polyketides than did the control strain expressing both genes. This result further demonstrated the availability of the *A. oryzae* $\Delta snfA \Delta SCAP$ strain for heterologous polyketide production.

Key Words: *Aspergillus oryzae*; atrochrysone carboxylic acid; malonyl-CoA supply; polyketide

The filamentous fungus *Aspergillus oryzae* has long been used to produce traditional Japanese fermented foods, such as soy sauce (*shoyu*), rice wine (*sake*), and soybean paste (*miso*). In this decade, *A. oryzae* has also been used as a convenient host for the production of fungal secondary metabolites, such as atrochrysone carboxylic acid (ACA), aphidicolin, paxilline, aspyridone, and KK-1 (Awakawa et al., 2009; Fujii et al., 2011; Tagami et al., 2013; Wasil

et al., 2013; Yoshimi et al., 2018). *A. oryzae* is very suitable for producing heterologous secondary metabolites because most of the secondary metabolite biosynthetic gene clusters in *A. oryzae* are not expressed under normal culture conditions; therefore, there are fewer background peaks during liquid chromatography (LC) and/or mass spectral (MS) analysis. However, some problems remain in the use of *A. oryzae* as a host strain to produce secondary metabolites for industry. One major problem is that the yields of target compounds are not sufficient for industrial purposes, which is probably because of the low precursor supply.

In a recent study, we showed that the plant polyketide curcumin was successfully produced by an *A. oryzae* strain harboring curcuminoid synthase (CUS) when feruloyl-*N*-acetylcysteamine (NAC), which is a feruloyl-coenzyme A (CoA) analog, was fed on an agar plate (Kan et al., 2019). CUS is an unusual type III polyketide synthase (PKS) that synthesizes curcumin from two molecules of feruloyl-CoA and one molecule of malonyl-CoA (Katsuyama et al., 2007). In that study, we successfully increased the yield of curcumin by strengthening the supply of malonyl-CoA through the deletion of *snfA* and *SCAP*. *SnfA* is a homolog of SNF1 (sucrose non-fermenting 1), which inhibits acetyl-CoA carboxylase (ACC) activity by phosphorylation in *Saccharomyces cerevisiae* (Woods et al., 1994). Because ACC catalyzes the production of malonyl-CoA from acetyl-CoA, deletion of *snfA* (AO090701000767) was expected to have a positive effect on malonyl-CoA production (Fig. 1). Meanwhile, *SCAP* (SREBP cleavage-activating protein) is positively related to sterol biosynthesis in combination with SREBP (sterol regulatory element-

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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.

Fig. 1. Malonyl-CoA metabolism in *A. oryzae*.

Acetyl-CoA carboxylase (ACC) produces malonyl-CoA from acetyl-CoA. Sucrose non-fermenting 1 (SnfA) is assumed to inhibit ACC activity by phosphorylation. The line with a short vertical line at the end indicates a negative effect. Sterol regulatory element-binding protein cleavage-activating protein (SCAP) is assumed to be positively involved in the gene regulation for sterol biosynthesis. The dotted arrow indicates a positive effect to the pathway. See the main text for details.

binding protein) in eukaryotes (Espenshade and Hughes, 2007). In response to the low level of cellular sterol, the SREBP-SCAP complex in the endoplasmic reticulum membrane is transported to a Golgi apparatus, where SREBP is processed by two proteases, and the cleaved N-terminal transcriptional activator domain of SREBP is transported into a nucleus to activate the sterol biosynthesis and uptake genes. Because sterol biosynthesis consumes acetyl-CoA and competes with polyketide biosynthesis, deletion of *SCAP* (AO090701000401) was also expected to have a positive effect on malonyl-CoA production (Fig. 1). The *snfA*-*SCAP* double-deletion mutant (*A. oryzae* $\Delta snfA\Delta SCAP$) expressing *CUS* produced more than six times more curcumin than its parent strain harboring *CUS*. However, curcumin production was examined only in the solid culture because feruloyl-NAC should be efficiently added to the strain. Therefore, we spread a small amount of feruloyl-NAC solution on the surface of a cellophane sheet on an agar plate and after drying, *A. oryzae* strains were inoculated on the cellophane sheet. The effectiveness of the *A. oryzae* $\Delta snfA\Delta SCAP$ strain for polyketide production should also be examined in liquid culture because liquid culture is usually used for industrial fermentation. In addition, the productivity of foreign polyketides other than curcumin in *A. oryzae* $\Delta snfA\Delta SCAP$ is worth examining.

Our laboratory previously succeeded in the heterologous production of ACA-related polyketides in *A. oryzae* (Awakawa et al., 2009). ACA is a biosynthesis intermediate of emodin and endocrocin in *Aspergillus terreus*. An iterative type I PKS, ACA synthase (ACAS; ATEG_08451) condenses eight molecules of malonyl-CoA to produce ACA and atrochrysone carboxyl ACP thioesterase (ACTE; ATEG_08450) releases ACA from ACAS (Fig. 2). In the *A. oryzae* strain expressing ACAS and ACTE, ACA was converted nonenzymatically (or by intrinsic enzymes) to several ACA-related polyketides, including emodin and endocrocin, via decarboxylation, dehydration, and/or oxidation (Fig. 2). Endocrocin anthrone homodimer and endocrocin anthrone/emodin anthrone heterodimer were also produced (Fig. 2). In the present study, we examined the productivity of ACA-related polyketides in *A. oryzae* $\Delta snfA\Delta SCAP$.

For this purpose, we replaced *CUS* with *ACAS* and introduced *ACTE* into another locus (*amyC* locus) in the *A. oryzae* PamyCUS $\Delta pyrG$ (control) and PamyCUS $\Delta snfA\Delta SCAP\Delta pyrG$ strains as follows. All primers used for polymerase chain reaction (PCR) amplification (Table 1) and enzymes used for DNA manipulation were purchased from Thermo Fisher Scientific (Waltham, MA, USA) and Toyobo (Osaka, Japan)/Takara Bio (Shiga, Japan), respectively. The *ACAS* gene was amplified with primers 1 and 2 using pTAex3-ACAS (Awakawa et al., 2009) as a template. The vector part containing “removable” *pyrG*, *amyB* promoter (P), and *amyB* terminator (T) was amplified with primers 3 and 4 using pPamyB-CUS-RpyrG (Kan et al., 2019) as a template. These two amplified DNA fragments were connected using the In-Fusion HD Cloning Kit (Clontech Laboratories, Mountain View, CA, USA), resulting in pPamyB-ACAS-RpyrG (Fig. 3A). The *ACTE* gene was amplified with primers 5 and 6 using pPTRTA-ACTE (Awakawa et al., 2009) as a template. The upstream region from *amyC* promoter was amplified with primers 7 and 8 using the genomic DNA of *A. oryzae* as a template. The region containing *pyrG* and *amyB* promoter was amplified with primers 9 and 4 using pPamyB-CUS (Kan et al., 2019) as a template. The vector part containing *amyB* terminator was also amplified with primers 3 and 10 using pPamyB-CUS as a template. These four amplified DNA fragments were connected using the In-Fusion HD Cloning Kit (Clontech Laboratories), resulting in pPamyC-ACTE (Fig. 3A). Note that the promoter and terminator regions of *amyC* are identical with those of *amyB*. To construct *A. oryzae* strains harboring ACAS, pPamyB-ACAS-RpyrG was linearized by HindIII digestion and introduced into *A. oryzae* PamyB-CUS $\Delta pyrG$ (Kan et al., 2019) and *A. oryzae* PamyB-CUS $\Delta snfA\Delta SCAP\Delta pyrG$ (Fig. 3A(i)), which was generated from *A. oryzae* PamyB-CUS $\Delta snfA\Delta SCAP$ (Kan et al., 2019) by removing the “removable” *pyrG* gene through the positive selection using 5-fluoroorotic acid as described previously (Kan et al., 2019). Then, the “removable” *pyrG* gene derived from pPamyB-ACAS-RpyrG was similarly removed from the resulting ACAS-harboring strains to generate *A. oryzae* PamyB-ACAS and *A. oryzae* $\Delta snfA\Delta SCAP$ PamyB-ACAS (Fig. 3A(ii)). Finally, pPamyC-ACTE was linearized by HindIII digestion and introduced into the resulting ACAS-harboring, *pyrG*-deleted strains to generate *A. oryzae* strains harboring ACAS and ACTE, i.e., *A. oryzae* PamyB-ACAS-PamyC-ACTE and *A. oryzae* $\Delta snfA\Delta SCAP$ PamyB-ACAS-PamyC-ACTE (Fig. 3A(iii)). The insertion of ACAS and ACTE in the chromosome of each strain was confirmed by amplifying the corresponding genes using PCR with primer pairs 11+12 and 13+12, respectively. The DNA fragment amplified by PCR with the primer pair 13+12 was digested with BamHI prior to agarose gel electrophoresis (Fig. 3B).

The spores (5.0×10^6 colony-forming units) of *A. oryzae* strains harboring ACAS and ACTE and their control strains were inoculated into 20 mL Czapek-Dox (CD) (Difco Laboratories, Detroit, MI, USA) minimal medium supplemented with 2% maltose in a shaking flask and cultivated at 30°C for 3 days at 150 rpm. The supernatant of each strain was acidified to pH 3 using 6 M HCl followed by

Name	Sequence (5' to 3')*
1	CAATAAACCCCA CAGAAGGCATTTATGGATTTCACCCCGTCGAC
2	GTACCATCATATACTCTCCACCCT CAGCTGTAATATTCTAGAAGCCAG
3	AGGGTGGAGAGTATATGATG
4	AAATGCCTTCTGTGGGGTTTATTG
5	CAATAAACCCCA CAGAAGGCATTTATGAAGCGGGGAGGCTAC
6	GTACCATCATATACTCTCCACCCTTTACGGGCTGGATGTGACATC
7	GCTTCATGCGCTTGATTGGTAGGGTACCGGTCGAG
8	TACACAGGGTACGTCTGTTGTCTCATCCAATCCCGTCCATC
9	ACAACAGACGTACCTGTGATG
10	CGACCTGCAGGCATGCAAGC
11	GGAAATGCACTGGTATCCAGTCGATG
12	CATCAAACCTCAAACACTCACTGTCCAATGCCAG
13	GCCGATTTTCTCTTCAACCGCAC

ACAS or *ACTE* produced no polyketides as shown by absorbance at 360 nm (Fig. 3C). According to the results of the previous study by Awakawa et al. and the observed *m/z* of each peak in our LC-MS analysis, compounds **1**, **3**, **5**, and **6** were identified as atrochryson, emodin, endocrocin, and endocrocin anthrone/emodin anthrone heterodimer, respectively (see Fig. 2 for their structures). Similarly, compounds **7** and **8** were identified as endocrocin anthrone homodimer (see Fig. 2 for their structures). Importantly, the amount of polyketides produced by *A. oryzae* Δ *snfA* Δ *SCAP* PamvB-*ACAS*-PamvC-*ACTE* was much

Fig. 3. Production of ACA-related polyketides in *A. oryzae* expressing *ACAS* and *CTE*.

A. Schematic representation of the construction of *ACAS-CTE*-expressing strains. See main text for details. **B.** Confirmation of the constructed strains by PCR. Small arrows indicate the primer binding sites. The putative *PamyC-CTE* fragments amplified by PCR were digested with *Bam*HI prior to agarose gel electrophoresis. The size of amplified (and digested) DNA fragments shows that expected recombination had occurred in all strains examined; three independent colonies, #1, #2, and #3, were analyzed for each strain. See main text for details. **C.** LC-MS analysis of the metabolites produced by the recombinant *A. oryzae* strains. “*ΔsnfAΔSCAP*” and “Control” mean *A. oryzae ΔsnfAΔSCAP* and its parent strain, respectively. “*ACAS-CTE*” means that the strain harbors both *ACAS* and *CTE*. Compounds 1, 3, 5, 6, 7, and 8 were identified as described in Fig. 2, according to the results by Awakawa et al. (2009) and observed *m/z*; 273 [M-H]⁻, 269 [M-H]⁻, 313 [M-H]⁻, 553 [M-H]⁻, 597 [M-H]⁻, and 597 [M-H]⁻, respectively. Compound X, which showed *m/z* 257 [M-H]⁻, was assumed to be a heptaketide intermediate by Awakawa et al. (2009), but its structure remains to be elucidated. Compounds included in other small peaks are also unknown, but they seem not to be compounds 2 or 4, as shown by their *m/z*. **D.** Quantitative analysis of emodin production. Error bars represent mean ± standard deviation (*n* = 3).

higher than that produced by *A. oryzae* PamyB-ACAS-PamyC-ACTE. To determine the productivity of ACA-related polyketides, we quantified emodin (**3**) from the peak area in the chromatogram at 440 nm using a calibration curve obtained from authentic emodin (Tokyo Chemical Industry, Tokyo, Japan). The yield of emodin in *A. oryzae* $\Delta snfA\Delta SCAP$ PamyB-ACAS-PamyC-ACTE was $843.4 \pm 121.9 \mu\text{g/L}$, which was more than 3.7 times larger than that of the control strain *A. oryzae* PamyB-ACAS-PamyC-ACTE ($227.0 \pm 8.5 \mu\text{g/L}$) (Fig. 3D). According to the peak area in the chromatogram at 440 nm, ACA-related polyketides other than emodin were also produced in approximately four times larger amounts in *A. oryzae* $\Delta snfA\Delta SCAP$ PamyB-ACAS-PamyC-ACTE than in *A. oryzae* PamyB-ACAS-PamyC-ACTE (Fig. 3C). These results demonstrated that the *snfA*-*SCAP* double-deletion mutant was efficient in producing ACA-related polyketides in liquid culture.

In conclusion, enhancement of the malonyl-CoA supply by the deletion of *snfA* and *SCAP* was proved to be efficient for increasing the heterologous production of polyketides in *A. oryzae*, including not only curcumin, but also ACA-related compounds. Thus, we can safely say that the *snfA*-*SCAP* double-deletion mutant is a useful host for the heterologous production of polyketides in laboratory research, such as the analysis of biosynthetic genes. However, further improvement of production yield (up to a few tens of grams per 1-L culture) is required for industrial use. Not only further genetic engineering but also fermentation engineering, including the selection of the best culture conditions, and traditional strain breeding through mutagenesis appear to be promising methods. The *snfA*-

SCAP double-deletion mutant, *A. oryzae* $\Delta snfA\Delta SCAP$, can be used as a starting strain for these strain improvements.

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