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Metabolic Engineering of *Aspergillus niger* for production of aromatic compounds

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Research aims

A filamentous fungus *Aspergillus niger* is capable of high-yield production of citric acid from molasses and other raw materials containing saccharides, and widely used for industrial production of citric acid, which reaches 1.6 million tons per year in the world[1]. On the other hand, aromatic compounds are important precursors of many useful polymers, pharmaceuticals, and agrochemicals, etc. Presently, the aromatic compounds are mostly prepared from petroleum. However, in near future, production of aromatic compounds from renewable raw materials would be highly desirable from the view point of building a low-carbon society. In this study, we examine the possibility that aromatic compounds can be produced from glucose, a renewable carbon source, in *A. niger*, using fungal type III polyketide synthases.

Methods

Two fungal type III polyketide synthase (PKS) genes, AB206758 (*csyA*)[2] and XM_001390834 (*An-csyA*), were amplified by RT-PCR from *Aspergillus oryzae* RIB40 and *A. niger* NRRL328, respectively. These genes are cloned into the pET-21a(+) vector (Merck, Darmstadt, Germany), and *Escherichia coli* BL21 (DE3) was transformed with the recombinant plasmids. The recombinant PKS proteins were produced by culturing the transformed *E. coli* strains for 16 hours at 16°C. For transformation of *A. niger*, a *pyrG*$^-$ mutant of citric acid-producing *A. niger* strain WU-2223L was co-transformed with each of the PSK genes cloned in the pNAN8142 vector[3] and a fragment of *pyrG* gene by protoplast-PEG method. The standard conditions for citric acid production were essentially the same as described previously[4].

The reaction mixture for the assay of PKS contains 125 mg/l of purified PKS obtained through Ni^{2+}-affinity chromatography, 0.3 mM starter substrate, and 0.7 mM malonyl-CoA in 50 mM K_2HPO_4-KH_2PO_4 buffer (pH 7.0). The reaction was performed at 30°C for 16 hours.

The extracellular metabolites and reaction products were extracted from supernatant by ethylacetate-HCl, dried, resuspened in methanol, and analyzed by HPLC. The ODS-100V column (Tosoh, Tokyo, Japan) and PDA (SPD-M20A, Shimadzu, Kyoto, Japan) or ESI-IT-MS (LCQ-Deca, ThermoFishier, MA, USA) detectors were used for the HPLC analyses.

The intracellular metabolome analysis was performed at Human Metabolome Technology Inc., Tsuruoka, Japan.

Results

In this study, we focused on PKS as a key enzyme for production of aromatic compounds (Fig. 1), and cDNAs of *A. oryzae* PKS gene (*csyA*) and its homologue from *A. niger* (*An-csyA*) were cloned for overexpression of the genes. The deduced amino acid sequence of the *An-csyA* gene product contains the residues and active centers that are conserved among PKSs (Fig. 2). To examine the enzymatic activity of the *An-cysA* gene product, the recombinant An-CsyA protein was incubated with each of fifteen different fatty acyl-CoAs (a starter substrate) and malonyl-CoA (an elongation substrate). The LC-MS/MS analysis of extracted reaction mixture revealed structures of reaction products as shown in Table 1. The reaction products were pyrone-type polyketides, which are made up of a molecule of a starter substrate (C2–C12 fatty acyl-CoA) and 2–5 molecules of the elongation substrate (malonyl-CoA). Therefore, An-CsyA is a novel type III PKS capable of using a smaller starter substrate, a property that is suitable for the aromatic compounds synthesis[5].

The genes, *An-csyA* and *csyA*, were introduced into *A. niger* WU-2223L and placed under the control of a promoter for high level expression. The transcripts of *An-csyA*

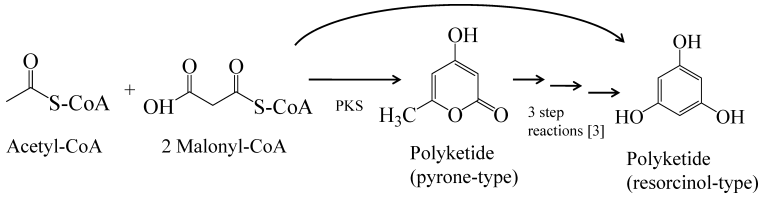


Fig. 1 Synthesis of aromatic compounds from renewable glucose by *A. niger*.

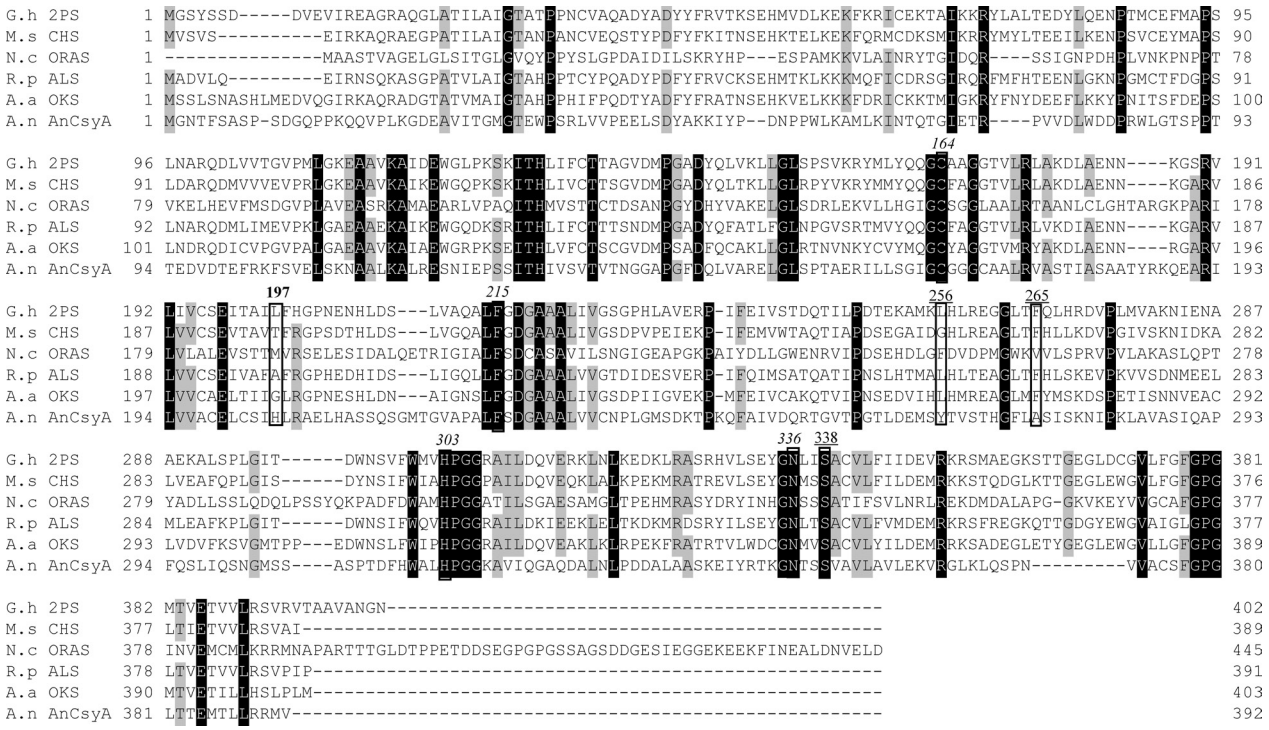
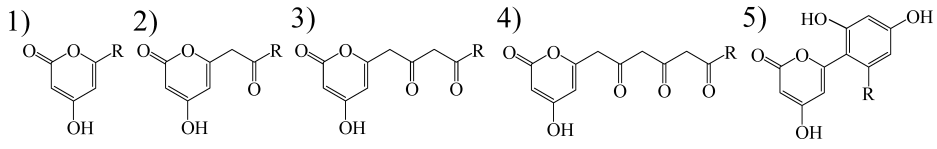


Fig. 2 Comparison of the deduced amino acid sequences of *A. niger* An-CysA and other type III PKSs. G.h 2PS, *G. hybrida* 2PS; M.s CHS, *M. sativa* CHS; N.c ORAS, *N. crassa* ORAS; R.p ALS, *R. palmatum* ALS; A.a OKS, *A. arborescens* OKS; A.n AncsyA, *A. niger* An-CsyA. The critical active-site residue 197 is marked in bold number, the active center (Cys164, Phe215, His303, Asn336) are marked in italicized numbers, and the residues lining the active site (Gly256, Phe265, Ser338) are marked in underlined numbers.

Table 1. Polyketides synthesized by reactions with An-CsyA

Starter substrate	Side chain	Triketide pyrone[1)]	Tetraketide pyrone[2)]	Pentaketide pyrone[3)]	Hexaketide pyrone
Acetyl-CoA	R=CH_3	+	+	−	−
Acetoacetyl-CoA	R=CH_3	+	+	−	−
Malonyl-CoA	R=CH_3	+	+	−	−
Methylmalonyl-CoA	R=$CH(CH_3)COOH$	+	−	−	−
Isobutyryl-CoA	R=$CH(CH_3)_2$	+	+	−	−
Benzoyl-CoA	R=C_6H_5	+	+	−	−
Butyryl-CoA	R=$(CH_2)_2CH_3$	+	−	−	−
Hexanoyl-CoA	R=$(CH_2)_4CH_3$	+	+	−	−
Octanoyl-CoA	R=$(CH_2)_6CH_3$	+	+	−	−
Decanoyl-CoA	R=$(CH_2)_8CH_3$	+	+	+	+[4)]
Lauroyl-CoA	R=$(CH_2)_{10}CH_3$	+	+	+	+[4),5)]
Myristoyl-CoA	R=$(CH_2)_{12}CH_3$	−	−	−	−



or *cysA* were detected in transformants by RT-PCR analysis. The transformants were then cultivated under the conditions for citric acid production for 7 days, and the extracellular metabolites were extracted and analyzed. However, we detected no remarkable accumulation of polyketides in the cells (data not shown).

The metabolome analysis of *A. niger* WU-2223L cells after 7 days cultivation under the conditions for citric acid production revealed the intracellular accumulation of acetyl-CoA (0.3 nmol/g-wet cell). But malonyl-CoA, which can serves as an elongation substrate in An-CysA-mediated reaction, was not detected in the cells.

Conclusion

In this study, we examined the properties of a novel type III PKS from *A. niger* (An-CysA). The An-CysA exhibited activities towards many different fatty acyl-CoAs and produced pyrone-type polyketides made up of a molecule of C2–C12 fatty acyl-CoA and 2–5 molecules of malonyl-CoA. The simple overexpression of these PKS genes in *A. niger* did not resulted in the production of polyketides under the conditions described previously for citric acid production.

The pyrone-type polyketides are precursors of resolcinol-type polyketides, which are aromatic compounds. At pres-

ent, we are attempting to modify the metabolic pathways in *A. niger* to increase intracellular levels of malonyl-CoA for production of polyketides.

References

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