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Product Identification of Polyketide Synthase Coded by *Aspergillus nidulans wA* Gene

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Abstract

The *wA* gene cloned from *A. nidulans* which was assumed to code for polyketide synthase involved in conidial spore pigment biosynthesis was expressed in a heterologous host fungus *A. oryzae* using starch inducible fungal expression plasmid pTAex3. The *A. oryzae* transformant produced starch inducible compounds, whose structures were identified to be citreoisocoumairn and its derivatives. Apparent heptaketide nature of these compounds identified that the *wA* gene codes for a heptaketide synthase of *A. nidulans*.

Keywords: biosynthesis, fungi, isocoumarins, polyketides.

The conidiophore formation in *Aspergillus nidulans* has been well studied at genetic level as a model system of multicellular development [1]. The asexual spores, conidia, contain dark green pigments that contribute to the strength, rigidity, and impermeability of the conidial cell walls and UV protection. A number of genetic loci are required in conidial pigmentation [2]. In addition to regulatory genes, both *wA* and *yA* genes have been proposed to encode enzymes involved in pigment biosynthesis. White spores and yellow spores are formed in *wA* mutants and *yA* mutants, respectively [3]. The product of *yA* gene was identified to be a laccase (E.C. 1.10.3.2) that converts a yellow pigment precursor to the mature green form [4]. Several *yA* mutants were analyzed to identify the yellow pigment structure, but low accumulation and high background production of pigmented compounds prohibited its identification and characterization.

Previous characterization of the *wA* gene indicated the significant sequence similarity with both prokaryotic and eukaryotic polyketide synthases and vertebrate fatty acid synthases [5]. Thus, the coded WA polypeptide was assumed to be a type I multifunctional polyketide synthase to produce a yellow spore pigment intermediate which could be converted to the polymerized green mature spore pigment by *yA* gene coded laccase.

We previously cloned the *atX* gene from *Aspergillus terreus* IMI 16043 and identified it to be a 6-methylsalicylic acid synthase gene by heterologous expression in *A. nidulans* [6]. The expression plasmid pTAex3 contains starch inducible α-amylase promoter and terminator of *Aspergillus oryzae* [7]. This successful expression of the *atX* gene in a heterologous fungus indicated the potential usefulness of this system at least for functional analyses of fungal multifunctional type I polyketide

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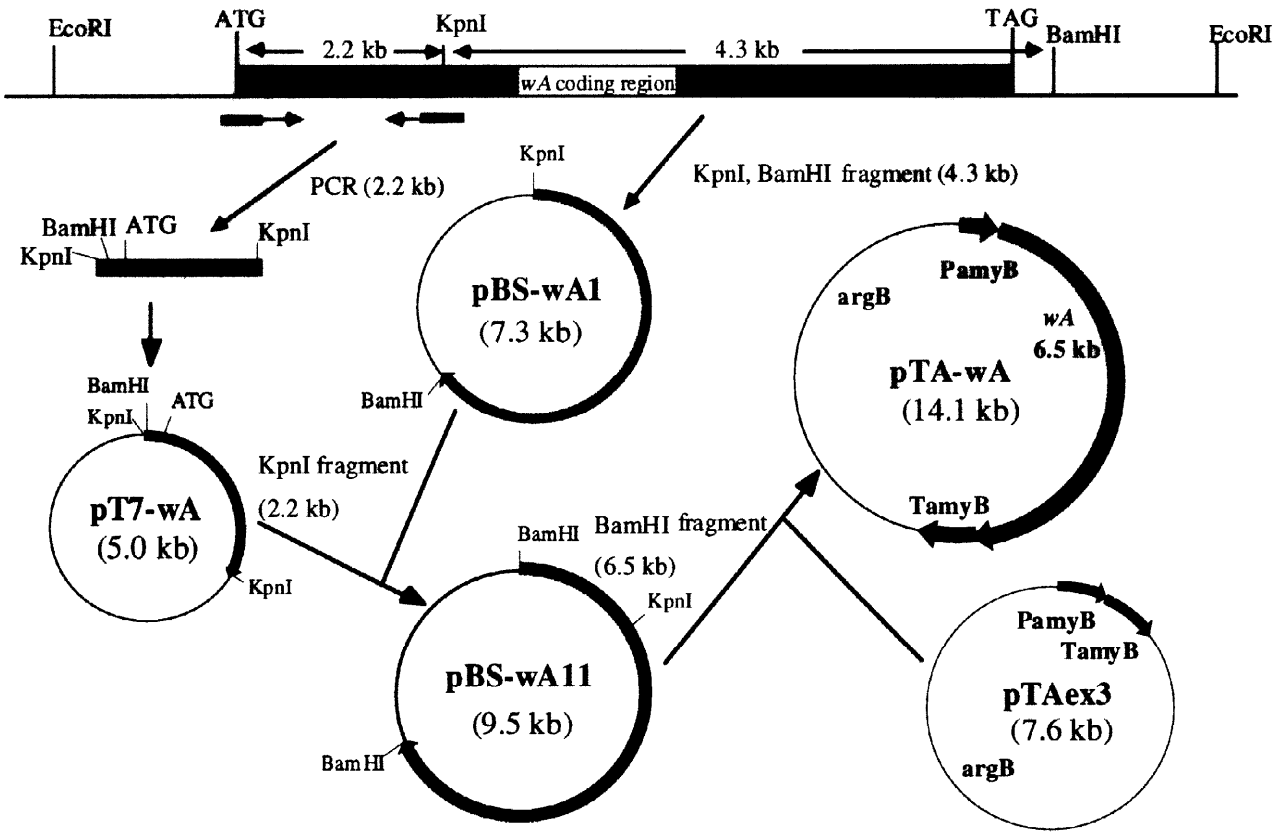


Fig. 1 Construction of expression plasmid pTA-wA

synthase genes. Thus, we tried to express the *A. nidulans wA* gene by this fungal expression system in order to identify the product of WA polyketide synthase.

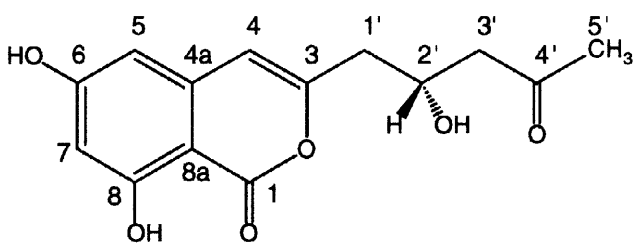
The *wA* expression plasmid pTA-wA was constructed as shown in Fig. 1. The *wA* gene was divided into two parts to introduce a new *Bam*HI site just upstream of the start codon. The N-terminal region was amplified by polymerase chain reaction using the specific primers with the restriction enzyme sites (*Bam*HI and *Kpn*I) attached at the 5' end. After confirming the nucloetide sequence, the *Kpn*I fragment of pT7-wA was used to reconstruct the whole *wA* gene in pBS-wA11, which was then introduced into the cloning site of expression plasmid pTAex3 just downstream of α-amylase promoter (PamyB) to construct pTA-wA. The control plasmid pTA-wAR with the insert of opposite direction was also prepared. These plasmids were introduced by protoplast transformation into the host fungus *A. oryzae* M-2-3 [8] harboring an auxotrophic selection marker *argB* mutation. Transformants appeared on minimal medium plates were then confirmed for the integration of introduced plasmids into host genomes by Southern blot analysis. Thus obtained transformant *A. oryzae* / pTA-wA was first precultured in Czapek-Dox medium containing glucose as carbon source and then transferred into induction medium (Czapek-Dox with starch). After induction culture for three days, the culture medium was extracted with ethyl acetate. TLC analysis indicated the production of compounds which were absent in the control cultures, namely, host fungus, pTAex3 transformant, and pTA-wAR transformant. The crude extract of 740 mg obtained from 4 liter induction culture was separated on oxalic acid impregnated silica gel column with a benzene-acetone solvent system. Two oily fractions I and II with light brown color were obtained. From fraction I, 50 mg of colorless crystals were isolated. This compound (**1**)

showed a molecular ion peak at 278.0744 by HR-EIMS for $C_{14}H_{14}O_6$ (278.0790). A close inspection of the ^1H and ^{13}C NMR spectral data (Table 1) identified (**1**) to be citreoisocoumarin previously isolated from *Penicillium citreo-viride* B [9], though (**1**) showed positive optical rotation ($[\alpha]_D$ +46.4°) suggesting 2' ***R***-configuration opposite to that of (**1**) isolated from *P. citreo-viride* B. HMBC correlation and NOE interactions shown in Fig. 2 and HMQC measurement unambiguously confirmed the assignment of the spectral data shown in Table 1.

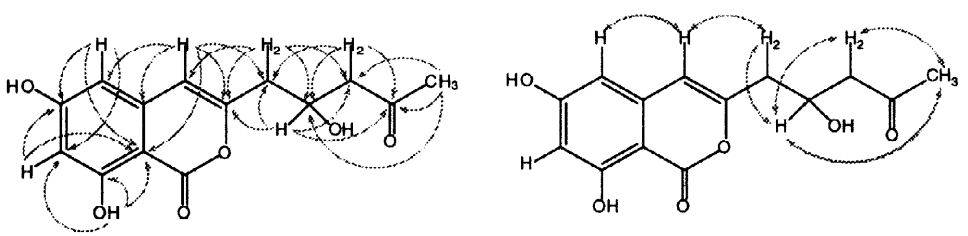
Table 1
^{13}C and ^1H NMR data of compounds produced by *A. oryzae* transformant

	(1) [a]		(2) [b]		(3) [b]	
position	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)
1	167.2		168.1		165.7	
3	155.9		153.3		153.4	
4	106.9	6.53 (1H, s.)	108.9	6.51 (1H, s.)	108.9	6.60 (1H, s.)
4a	141.0		141.5		141.4	
5	103.5	6.49 (1H, d., *J*=2.1)	104.8	6.45 (1H, d., *J*=2.4)	105.0	6.45 (1H, d., *J*=2.1)
6	166.4		165.6		167.6	
7	102.4	6.45 (1H, d., *J*=2.1)	103.8	6.41 (1H, d., *J*=2.4)	103.9	6.42 (1H, d., *J*=2.1)
8	164.7		165.6		167.7	
8a	100.1		100.9		100.9	
1'	42.0	2.72 (1H, d.d., *J*=8.1, 14.5) 2.83 (1H, d.d., *J*=4.8, 14.5)	49.0	3.79 (2H, s.)	45.0	3.63 (2H, s.)
2'	66.2	4.55 (1H, m.)	205.7		192.6	
3'	50.8	2.80 (1H, d.d., *J*=7.8, 16.3) 2.86 (1H, d.d., *J*=4.7, 16.3)	53.2	2.7 (2H, m.)	101.8	5.82 (1H, s.)
4'	207.6		65.5	4.25 (1H, m.)	192.6	
5'	30.8	2.23 (3H, s.)	24.9	1.18 (3H, d., *J*=6.2)	25.1	2.06 (3H, s.)
6-OH		9.70 (1H, br.s.)				
8-OH		11.22 (1H, br.s.)		11.14 (1H, br.s.)		11.06 (1H, br.s.)

Proton ([a] 500.00, [b] 399.79 MHz) and carbon ([a] 125.65, [b] 100.54 MHz) NMR spectra were obtained in acetone-d_6.

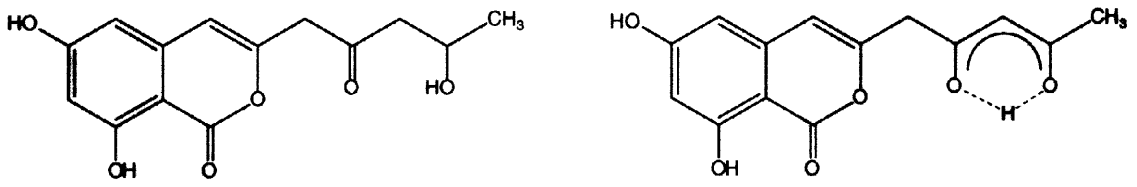


(+)-citreoisocoumarin (**1**)



HMBC correlations NOE interactions

Fig. 2 Structure of citreoisocoumarin (**1**) and its HMBC correlations and NOE interactions



(2) (3)

Fig. 3 Structures of citreoisocoumarin derivatives produced by *A. oryzae* transformant

The remaining portion of fraction I was found to be a mixture of citreoisocoumarin (**1**) and compound (**2**) with one to one ratio by spectroscopic analysis. Compound (**2**) is an isomer of (**1**) with exchanged 2'-keto and 4'-hydroxy groups. Fraction II was also found to be a keto-enol equilibrium mixture of dehydrocitreoisocoumarin (**3**), which gave a molecular ion peak at 277 $(M + H)^+$ by FAB-MS.

These isocoumarin compounds (**1**)~(**3**) were produced by *A. oryzae* transformant harboring the *wA* gene of *A. nidulans* and are typical heptaketide compounds with oxygen atoms located at alternate carbons. Carbonyl carbons at 2' and 4' of compound (**3**) might be reduced by a host fungus reductase to form compounds (**1**) and (**2**). This successful expression experiment identified that the *wA* gene codes for a heptaketide synthase of *A. nidulans*. The expected product of WA polypeptide was a yellow pigment, which could be polymerized by *yA* coded laccase to green spore pigments. However, both fraction I and II showed light brown color and the major product, citreoisocoumarin (**1**) was colorless. Thus, it is still skeptical whether compounds (**1**)~(**3**) are the true spore pigment intermediates. They might be further modified to yellow intermediates by additional reactions although no corresponding genetic locus has been identified, or they might not be normal products of the WA polyketide synthase reaction due to incorrect folding of WA polypeptide expressed in a heterologous host fungus.

A number of heptaketide compounds have been isolated from fungi. Isolation of parasperone A from spores of a laccase deficient strain of *Aspergillus parasiticus* [10] should be noted since its yellow color suggests it to be an intermediate of green spore pigments of mature conidia. Further investigation is underway to identify whether citreoisocoumarin (**1**) is a true spore pigment intermediate of *A. nidulans* or not.

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