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Cloning and Sequence Analysis of the Full-length cDNA of a Novel *yp05* Gene Associated With Citrinin Production in *Monascus aurantiacus*[1]

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Objective To obtain the full-length cDNA of a novel gene (named *yp05*) associated with citrinin production-related genes in *Monascus aurantiacus*. **Methods** Total RNA was extracted from mycelium, 3' and 5' cDNA end of *yp05* gene was amplified using smart™ trace cDNA amplification kit, and the full-length cDNA of a novel gene (named *yp05*) was obtained from the electronic assembly of 3'-RACE and 5'- RACE products. **Results** This *yp05* gene was 787 bp including a 597 bp open reading frame (ORF) and encoded a deduced protein with 199 amino acid residues, and the amino acid sequence of this protein was found similar with the sequences of many fungal manganese-superoxide dismutases in the GenBank with the aid of BLASTp. The transcription of *yp05* gene in *Monascus* strains was analyzed with the aid of Northern blotting. The transcription of *yp05* gene was only detected in *Monascus* strains, provided that citrinin was produced. **Conclusion** The transcription of *yp05* gene belongs to differential expression genes of citrinin yielded from *Monascus* and has no correlation with the biosynthesis pathway of red pigments.

Key words: *Monascus aurantiacus*; Citrinin; RACE; Manganese-superoxide dismutases

INTRODUCTION

Monascus, a typical filamentous fungus, produces diverse physiological active substances during the process of fermentation, such as α-amylase, monacolin K, and red pigments[1]. Red pigments are widely used in traditional food products, such as red rice wine, soybean cheese, fish, and red meat in southern China, Taiwan, Japan, and Indonesia[2]. However, citrinin, a nephrotoxic and hepatotoxic mycotoxin, is also produced by most *Monascus* strains following the yielding of red pigments, and the yield of citrinin is determined by a number of variables, such as *monascus* species, nitrogen, and carbon source, fermentation conditions, *etc.*[3-4]. In investigating the ability of *monascus* species to produce citrinin, Wang *et al.*[5] showed that twenty-three *monascus* type cultures representing eight species can produce citrinin in YES medium. We have constructed two subtractive libraries about genes differential expressed during citrinin production in *Monascus aurantiacus* by SSH analysis. Sequencing of the two libraries produces 299 cDNA EST fragments, among which 8 EST fragments (named *estyp01*, *estyp02*, *estyp03, estyp04*, *estyp05*, *estyp06*, *estyp07*, *estyp08*) are shared by both libraries (data published in Genbank, locus at CB409137, CB409138, CB409139, CB409140, CB409141, CB409142, CB409143, CB409144, respectively). In these 8 cDNA sequences，the sequences of *estyp02*, *estyp04*, *estyp05*, *estyp08* are homologous with the sequences of many fungal manganese- superoxide dismutases by way of BLASTn and BLASTx comparative analysis. In this case, the full-length cDNA of a novel gene (named *yp*05) was obtained by RACE method according to the random sequence (*estyp05*) among the 4 cDNA EST fragments and the relevance of transcription process of *yp*05 gene with regard to citrinin and red pigment production in toxigenic, nontoxigenic *monascus* strains were analyzed by Northern blotting.

MATERIALS AND METHODS

Monascus Strains and Culture Media

Toxigenic strains of *Monascus aurantiacus* AS 3.4384, *Monascus rubber van. Tieghem* IFFI 05032

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and *Monascua anka Nakazawa et. sato* IFFI 05033 were provided by the Institute of Microbiology of Chinese Academy of Sciences and the Institute of Fermentation of Chinese Light Industry Ministry, respectively. Nontoxigenic strains of *Monascus paxii Lingelsheim* AS 3.4453 and *Monascus barkeri Dangerd* AS 3.4452 were provided by the Institute of Microbiology of Chinese Academy of Sciences.

One mL spore (3×10^5 CFU/mL) of the five strains of *Monascus* was inoculated into 100 mL sterile YES broth (containing 4% yeast extract powder and 16% sucrose) and incubated at 28℃ for 5-13 days. In another parallel experiment, one mL spore of *Monascus aurantiacus* AS 3.4384 was inoculated into 100 mL sterile PD broth (containing 20% potato extract and 2% dextrose) and incubated at 35℃ for 3-8 days. Red pigments in the broth were observed subjectively and concentration of citrinin in the broth was determined with the aid of high performance liquid chromatography (HPLC) as described previously[6-7]. Mycelia of strain AS 3.4384, after incubation for 5, 8, 11, 13 days in YES broth and incubation for 3, 5, 7, 8 days in PD broth, were isolated from the culture by filtration with sterile filter paper (10 μm) and washed three times with 0.01 M PBS (pH 7.2). Mycelia of strains AS 3.4452, AS 3.4453, IFFI 05032, and IFFI 05033 were obtained from YES broth, respectively, after incubation for 13 days, and then, mycelium was milled into powder using liquefied nitrogen and total RNA was extracted as described previously[8].

Rapid Amplification of 5' and 3' cDNA End of yp05 Gene

Total RNA from mycelium of strain of AS 3.4384 after incubation for 13 days, was used for rapid amplification of cDNA ends (RACE) according to the manual of the Smart™ trace cDNA amplification kit (Clontech). Preparation of 5'-RACE-Ready cDNA was performed at 70℃ for 2 min and cooled on ice for 2 min in a 5 μL reaction volume containing 2 μL total RNA (3 μg), 1 μL Rnasin(40 U, TakaRa), 1 μL 5'-CDS primer[5'-$(T)_{25}$N-1N–3', (N = A, C, G, or T; N-1 = A, G, or C) 10 μmol/L] and 1 μL Smart II A oligo (5'-AAGCAGTGGTATCAACGCAGAGTACGCGGG–3', 10 μmol/L), and then 2 μL 5X First-Strand buffer, 1 μL DTT (20 mmol/L), 1 μL dNTP Mix (10 mmol/L), 1 μL PowerScript reverse transcriptase were added into the tube and the first strand cDNA synthesis was performed at 42℃ for 1.5 h. 5'-RACE gene specific primer (GSP1: 5'-TACTGCAGGTAATACGCATGCTC-3') was designed according to the *estyp05* sequence (published in Genbank, CB409141). 5'-RACE PCR amplification of the *yp05* cDNA was performed in 25 μL reaction volume containing 2.5 μL 10×PCR buffer, 3 μL 25 mmol $MgCl_2$, 1 μL 5'-RACE first strand product, 1 μL GSP1 (10 μmol), 1 μL nested universal primer A (5'-AAGCAGTGGTATCAACGCAGAGT-3', 10 μmol), 0.5 μL dNTP (10 mmol), 0.2 μL DNA polymerase (5 U/μL) and 15.8 μL PCR-Grade Water. Amplification conditions were as following: 5 cycles of 94℃ for 1 min, 68℃ for 30 s, and 72℃ for 2.5 min; 25 cycles of 94℃ for 1 min, 64℃ for 30 s, and 72℃ for 2.5 min; a final extension for 10 min. Amplified products were run by electrophoresis in 1.2% agarose gel and appropriate bands were purified from the gel and ligated into pGEM-T-vector. The positive clone was sequenced using an automated ABI 310 sequencer.

Preparation of 3'-RACE-Ready cDNA was performed at 70℃ for 2 min and cooled on ice for 2 min in a 5 μL reaction volume containing 2 μL total RNA (3 μg), 1 μL Rnasin (40 U, TakaRa), 1 μL 3'-CDS primer A (5'-AAGCAGTGGTATCAACGCAGAGTAC$(T)_{30}$ N_{-1}N-3') and 1 μL RNA free water, and then 2 μL 5X first-strand buffer，1 μL DTT (20 mmol/L), 1 μL dNTP mix (10 mmol/L) and 1 μL PowerScript reverse transcriptase were added to the tube and the first strand cDNA synthesis was performed at 42 ℃ for 1.5 h. 3'-RACE primer (GSP2: 5'-CAGCAAGCACCACAACACTTAC-3') was designed according to the sequence of 5'-RACE product. 3'-RACE PCR amplification was performed according to the procedure of 5'-RACE PCR. Electrophoresis of the amplified products, purification of the bands, ligation of the vector and sequencing were performed with the methods described above.

Full-length Sequence of yp05 Gene and Computer Program

Full-length cDNA of the *yp05* gene was obtained from the electronic assembly of 5'-RACE, 3'-RACE products and *estyp05* fragment with the aid of DNAStar software and open read frame (ORF) was predicted with the aid of ORFFinder program (http://www.ncbi.nlm.gov/gorf/gorf.html)[9]. The homology of full-length cDNA sequence was determined with the BLASTn and BLASTp program in Genbank (http://www.ncbi.nlm.nih.gov/)[10].

Preparation of DIG-labeled yp05 Gene Probe

Three μg purified 3'-RACE product of the *yp05* gene was dissolved in 16 μL pure water and denaturalized at 100℃ for 10 min, then quickly cooled on ice. Preparation of the *yp05* gene probe was performed at 37℃ for 20 h according to the random primed labeling technique in a 20 μL reaction

volume containing 16 μL denatured template and 4 μL mixed DIG-High Prime. Two μL of 0.2 mol/L EDTA (pH 8.0) was mixed with the sample to stop reaction and the *yp05* gene probe was precipitated at -70℃ for 30 min by adding 2 μL of 4 mol/L LiCl and 65 μL ethanol. The precipitation was dissolved in 50 μL of 0.01 mol/L TE buffer.

Northern Blotting Analysis

Transcription of the *yp05* gene was analyzed with the aid of Northern blotting analysis using the DIG high prime labeling and detection starter kit (Roche). Thirty μg total RNA of the strain AS 3.4384 was prepared from the mycelia grown on YES broth after incubation for 5, 8, 11, and 13 days and on PD broth for 3, 5, 7, and 8 days. Similarly total RNA of strains AS 3.4452, AS 3.4453, IFFI 05032, IFFI 05033 was extracted also from the mycelia grown on YES broth after incubation for 13 days, respectively. All total RNA samples were analyzed with the DIG-labeled yp05 gene probe by Northern blotting as described previously[11].

RESULTS

Characterization of Red Pigment and Citrinin Production During Monascus aurantiacus Growth

To investigate the correlation between transcription of the *yp05* gene with production of citrinin and/or red pigments, 5 representative *monascus* strains producing citrinin or red pigments were selected. The concentration of citrinin in the broth was determined by high performance liquid chromatography and the change of color in mycelium was observed subjectively. As shown in Table 1, toxingenic strains of IFFI 05032 and IFFI 05033 produced citrinin and red pigments in both YES and PD broths. Strain AS 3.4452 and AS 3.4453 produced neither citrinin nor red pigments on YES and PD broths, whereas strain AS 3.4384 synthesized both red pigments and citrinin after incubation for 8 days in YES broth, and produced red pigments instead of citrinin in PD medium.

TABLE 1

Characteristics of *Monascus* Strains Yielding Citrinin and/or Red Pigments in YES or PD Broth

Strain	Yeast Extract Sucrose Broth								Potato Dextrose Broth							
	Citrinin (μg/mL)				Red Pigments				Citrinin (μg/mL)				Red Pigments			
	5 d	8 d	11 d	13 d	5 d	8 d	11d	13 d	3 d	5 d	7 d	8 d	3 d	5 d	7 d	8 d
AS 3.4384	−	57	618	967	−	+	+	+	−	−	−	−	+	+	+	+
AS 3.4452	\	\	\	−	−	−	−	−	−	−	−	−	−	−	−	−
AS 3.4453	\	\	\	−	−	−	−	−	−	−	−	−	−	−	−	−
IFFI 05032	\	\	\	16	−	+	+	+	\	\	\	8	+	+	+	+
IFFI 05033	\	\	\	40	−	+	+	+	\	\	\	15	+	+	+	+

Note. d: days of incubation; −: no citrinin or red pigments; +: excreting red pigments in mycelium; \ : not detected.

Cloning and Sequence Analysis of Full-length cDNA of the yp05 Gene

A 560 base pair (bp) fragment of 5'-RACE product and 656 bp fragment of 3'-RACE product were amplified using the GSP1 and GSP2 primers (Fig. 1). The full-length cDNA (787 bp) was obtained from the electronic assembly of 3'-RACE and 5'-RACE products, containing a 597 bp ORF encoding a protein of 199 deduced amino acid residues (Figs. 2 and 3) with a calculated molecular mass of 22.1 kDa and a *pI* of 8.62[12]. By comparing the sequences of *estyp02*, *estyp04*, *estyp05*, and estyp08 with the full-length sequence of the *yp05* gene, their nucleotide sequences shared identical overlap covering different regions of the full-length sequence, indicating that these EST fragments were portions of the *yp05* gene[13]. The deduced amino acid sequence coded by the *yp05* gene was compared with those published genes by the method of BLASTp with a mode of multiple amino acid sequence alignment. The result showed that the deduced protein had a similarity in their sequences ranged from 44.44% to 47.96% with the superoxide dismutases from other fungi (Fig. 4)[14].

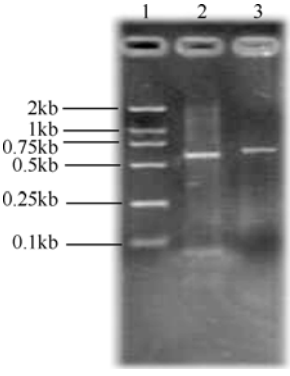

FIG. 1. One percent agarose gel electrophoresis of RACE products. Lane1: DL2000 marker; lane 2: 5'-RACE product; lane 3: 3'-RACE product.

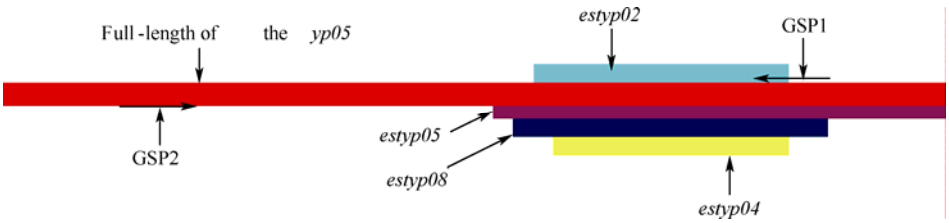

FIG. 2. Scheme for comparing the sequences of *estyp02*, *estyp04*, *estyp05*, and *estyp08* with the full-length sequence of the *yp05* gene.

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5′ -ACGCGGGGGGATCAGTCCAGTCCAGTTATCTTGCTCAAAATGGCCCAGACTTACTCCCTCCCCCC
TCTTCCTTACGAGTAACAATGCTCTTGAACCGGCCATCTCGGCCACCATCATGGAGCTGCACCACAGCA
                    M  L  L  N  R  P  S  R  P  P  S  W  S  C  T  T  A
AGCACCACAACACTTACGTCACAAACCTTATAAGGGCCCTCCACGCCCACGCCGAAGCAACCAAGGTA
 S  T  T  T  L  T  S  Q  T  L  I  R  A  L  H  A  H  A  E  A  T  K  V
ACCGACCTCCCTGCCATTGTGGCCCTGGAGCCCGCCATCAAGTTCAACGCTGGAGGCCACATCAACCAC
 T  D  L  P  A  I  V  A  L  E  P  A  I  K  F  N  A  G  G  H  I  N  H
TCCCTCTTCTGGACCAACCTCACTCCCCAGAAGAGCCCGGAAGCGTCCCCAGACTCCGCCCCGAAGCTG
 S  L  F  W  T  N  L  T  P  Q  K  S  P  E  A  S  P  D  S  A  P  K  L
CACGAGGCCATCCGCCAGCAGTGGGGGGATCGGAAAACCTTCCAGCAGAAGTTCAACGAGCAGCTGCTG
 H  E  A  I  R  Q  Q  W  G  D  R  K  T  F  Q  Q  K  F  N  E  Q  L  L
GGTATCCAGGGTAGTGGATGGGGCTGGCTTGTGCGGCAGGGGACTACCGGCCCGCTGGTTATTGTGACT
 G  I  Q  G  S  G  W  G  W  L  V  R  Q  G  T  T  G  P  L  V  I  V  T
ACCAAGGATCAGGATATCGTGGGGAAGGATCAGGTTCCTATTTTCGGTGTGGATATGTGGGAGCATGCG
 T  K  D  Q  D  I  V  G  K  D  Q  V  P  I  F  G  V  D  M  W  E  H  A
TATTACCTGCAGTATCTGAATGGAAAGGCTGCATATGTCGAGAACATCTGGACGGTCATCAACTGGAAG
 Y  Y  L  Q  Y  L  N  G  K  A  A  Y  V  E  N  I  W  T  V  I  N  W  K
ACGGCCGAGGAACGGTACCTGGGTGGCAATGCAGATGCCTTTAAGGTGCTCAAGGCAGCTATTTGATGA
 T  A  E  E  R  Y  L  G  G  N  A  D  A  F  K  V  L  K  A  A  I  *
ACTACTACATAGCAATGACAGATAGAGTACAGGTAGCTATAGCGTCTGTGAGATTAATCAACCTCGATC
ATGAATGAAAAAAAAAAAAAAAAAAAAAAAAAA-3′
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FIG. 3. Nucleotide and deduced amino acid sequences of *yp05* cDNA.

Northern Hybridization

Yp05 gene probe was hybridized with total RNA of the strain AS 3.4384 on YES broth after incubation for 5, 8, 11, and 13 days and on PD broth after incubation for 3, 5, 7 and 8 days, respectively. Similarly yp05 gene probe was also hybridized with total RNA of strains AS 3.4452, AS 3.4453, IFFI 05032, IFFI 05033 on YES broth after incubation for 13 days, respectively. The results are presented in Fig. 5. Positive hybridization bands were observed in total RNA in the phase of producing citrinin by toxigenic strains AS 3.4384, IFFI 05032 and IFFI 05033. No hybridizing bands were observed in nontoxigenic strains AS 3.4452 and AS 3.4451. No hybridizing bands were detected in total RNA of toxigenic strain AS 3.4384 cultured in PD broth during different growth phases. *Yp05* gene expression was detected from the prophase (5 days) to fastigium (13 days) of biosynthetic citrinin in toxigenic strain AS 3.4384.

DISCUSSION

The biosynthetic pathway biosynthetic pathway of citrinin and red pigments in *Monascus Ruber* has been investigated using C^{13} nuclear magnetic resonance (NMR). Hajjaj *et al.*[15] have confirmed that red pigments and citrinin belong to oxygenated polyketide metabolites and are derived from the polyketide biosynthetic pathway. In brief, acetyl-coezyme A molecules were added to tetraketide to form the intermediate of citrinin, the

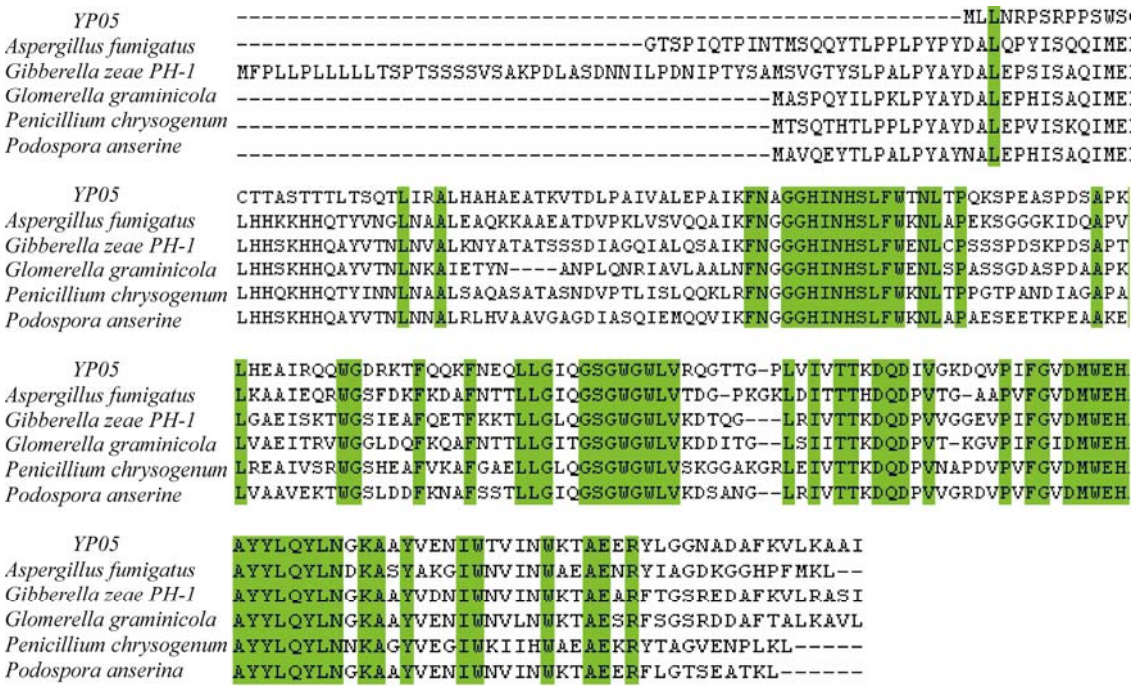

FIG. 4. Multiple alignments of deduced amino acid sequences of *yp05* and other published fungal superoxide dismutases. The dark background shows complete matches of a marker. The similarity between the superoxide dismutases of *Aspergillus fumigatus, Gibberella zeae PH-1, Glomerella graminicola, Penicillium chrysogenum* and *Podospora anserine* and the deduced protein of *YP05* is 44.2%, 45.7%, 50.0%, 45.6%, and 46.7%, respectively.

FIG. 5. Northern blotting analysis of the *yp05* gene transcription in different phases. Northern blotting was analyzed with total RNA from mycelia of strain AS 3.4384 on YES broth after incubation for 13 days (lane 1), 11 days (lane 2), 8 days (lane 3), and 5 days (lane 4), and from mycelia of strain IFFI 05033 (lane 5), IFFI 05032 (lane 6), AS 3.4452 (lane 7), AS 3.4453 (lane 8) on YES broth after incubation for 13 days, and from mycelia of strain AS 3.4384 in PD medium after incubation for 8 days (lane 9), 7 days (lane 10), 5 days (lane 11), and 3 days (lane 12).

subsequent reactions included methylation, condensation, O-alkylation, reduction, oxidation, and dehydration *etc*., leading to the production of citrinin. However, there is experimental evidence that the biosynthetic pathways for secondary metabolites are highly regulated and often modulated by developmental, nutritional, and environmental factors[16]. We know that the metabolism of *Monascus* pigments and citrinin is affected by agitation and aeration in different ways. In case of over-supply of oxygen, a linear relationship is observed between the concentration of oxygen and citrinin yield. In contrast, pigment production is inhibited under aerobic conditions[17]. A similar phenomenon has been observed in aflatoxin biosynthetic process, and oxygen supply coincides with the onset of aflatoxin production which may be regulated by the redox potential of cells[18]. Superoxide dismutase (SOD) which can be found in almost all aerobic organisms is responsible for the principal superoxide dismutase activity during oxidative stress[19]. In *Aspergillus parasiticus*, oxidative stress is a prerequisite for aflatoxin production and the expression of superoxide dismutase gene plays an important role in regulating aflatoxin production through calcineurin-mediated regulation of enzymes involved in aflatoxin biosynthesis[19-20].

The full-length *yp05* gene can be obtained by RACE method according to the *estyp05* sequence from two subtractive libraries about genes differential expressed during citrinin biosynthesis. If the *yp05* gene is citrinin and red pigment production-related genes, the transcript of this gene could be detected in total RNA in the citrinin and red pigment producing phase from toxigenic strains AS 3.4384, IFFI 05032

and IFFI 05033 and no *yp05* transcripts could be detected in total RNA from nontoxigenic strain AS 3.4452, and AS 3.4451. If the *yp05* gene, rather than the red pigment production-related genes, is associated with the differentially expressed gene of citrinin, the *yp05* gene transcripts could not detected in total RNA in the red pigment producing phase but not in the citrinin Producing phase.

In conclusion, the *yp05* gene should be a citrinin production-related genes, but is not related to the synthesis pathway of red pigments in *Monascus* strains. Citrinin production may be regulated by the redox potential of *monascus* cells and the *yp05* gene may be an antioxidant enzyme participating in the redox reaction to regulate the citrinin production in cells.

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