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Serial Analysis of Gene Expression in *Monascus aurantiacus* Producing Citrinin[1]

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Objective To construct a tag expression library of *Monascus aurantiacus* that could produce citrinin maximally on the thirteenth (0.966 mg/mL) day in the submerged culture. **Methods** Total RNA was extracted from the mycelium, cDNA was synthesized using the SuperScript choice system, and then, a SAGE library was successfully constructed according to the MicroSAGE method. **Results** Five hundred and ninety eight clones were obtained in SAGE library, and 120 clones were picked out randomly for identification and sequencing purpose. Eighty nine clones had positive inserts, 26 clones had no inserts and the remaining 5 clones had no site of *Nla*III enzyme in inserts. There were seven repeated clones. **Conclusion** With the aid of SAGE2000 software, 901 tags were obtained from 89 clones, representing 686 unique transcripts. Six unique tags of them belong to highly expressed genes (Number of tags≥10) and 143 unique tags to moderately expressed genes (repeat tags≥2).

Key words: *Monascus*; SAGE; Citrinin

INTRODUCTION

The special function and biochemical features of organisms in different growth phases are determined by their different extents of gene expression. However, in many studies were focused on to identify their function, and therefore the complexity of molecule mechanisms might be underestimated[1-2]. Instead of functional analysis of a single gene, high throughput analysis of genes and their functions was developed such as transcriptomics, proteomics and metabomics. The transcriptomics and proteomics have been featured with serial analysis of gene expression (SAGE) and 2-D electrophoresis, respectively[3]. Serial analysis of gene expression (SAGE) is an effective technique to simultaneously analyze the expression profile of a large amount of transcripts by representing them from unique mRNA based on short (9-11bp) nucleotide sequences (tags). These tags were concatenated randomly and subcloned into vectors. The sequence of the concatemers can provide information to comprehend the whole transcriptome and detect the genes of low expression[4,5]. Since the firs report of the SAGE method by Velculescu and his colleagues was published in *Science*, related researches have covered fields including human diseases, especially cancers of all kinds, biometabolism pathway, plant, model organisms and their transcriptome, etc.[6].

Monascus, a typical filamentous fungus, produces an array of diverse secondary metabolites , such as α -amylase, monacolin K and red pigments. Red pigments are used for producing red rice, wine, soybean cheese, fish and red meat in south China, Taiwan, Japan, and Indonesia[7]. Most *Monascus* strains produce red pigments as well as citrinin and the yield of citrinin is regulated by nitrogen and carbon source, culturing conditions and so on[8]. Blanc has investigated the biosynthetic pathway of citrinin in *Monascus Ruber* by C^{13} nuclear magnetic resonance (NMR) and confirmed that red pigments and citrinin arose from the condensation of one acetyl-coenzyme A molecule with three malonyl-coezyme A. After formed tetraketide, the two biosynthetic pathways were uncoupled. The first pathway was that an additional acetyl-coezyme A molecule was added to the tetraketide to form the intermediate of citrinin, while the second one was that an additional malonyl-coezyme A molecule was added to the tetraketide to form pentaketide, the

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intermediate of red pigments[9]. We also confirmed that, although most of *Monascus* strains could produce citrinin, its production could be controlled under different culture media or culture conditions. In this case, the different expressions of genes related to the biosynthesis pathway of citrinin can be investigated using serial analysis of gene expression.

MATERIALS AND METHODS

Monascus aurantiacus Culture and Total RNA Isolation

A suspension of 3×10^5 spores of *Monascus aurantiacus* AS3.4384, derived from the Institute of Microbiology of Chinese Academy of Sciences, was inoculated into a 500 mL flask containing 100 mL yeast extract sucrose (YES) medium and cultured at 28℃. The concentration of citrinin in the broth was determined by high performance liquid chromatography as described previously[10,11]. The mycelium, which produced the citrinin maximally (0.966 mg/mL) on the thirteenth day, was isolated from the medium by filtration with sterile filter paper(10 μm) and washed with 0.01 mol/L PBS (pH 7.2) three times. The mycelium was milled into powder using liquefied nitrogen and the total RNA was extracted as described before[12]. The amount and purity of RNA were determined by reading OD_{260} and $OD_{260/280}$ on a spectrophotometer. The integrity of RNA was detected by 1.5% agarose/ethidium bromide gel electrophoresis.

Linker-ditag-linker Preparation

By using the microSAGE method, Linker-ditag-linker preparation included five steps, such as mRNA separation, cDNA synthesis, cleavage of biotinylated-cDNA with anchoring enzyme, ligating linkers to bound cDNA, release of the cDNA tags using tag enzyme, blunt- ended with klenow fragment, ligation of two sets of linker-tags to form linker-ditag-linker and so on.

mRNA Separation and cDNA Synthesis

According to the polyATtract kit manual (Cat. #Z5482, Promega), 486 μL total RNA (50 μg) was heated in a 65℃ block for 10 min. 2 μL biotinylated-Oligo (dT) probe and 12 μL 20X SSC were added to the RNA. After mixed gently and incubated at room temperature for 30 min, the annealing reaction was added to magnetic beads system for 20 min. After washed with 0.5 X SSC and 0.1 X SSC, the cDNA synthesis was directly performed over the magnetic beads using the SuperScript choice system for cDNA synthesis (Cat. Series 18090, Invitrogen). The bound mRNA over the beads was rinsed three times with 100 μL 1 X first-strand buffer and the first strand cDNA was synthesized for 2 h at 42℃ in a 90 μL reaction volume containing 18 μL 5 X first-strand buffer, 55.5 μL RNA-free water, 9 μL 0.1 mol/L DTT, 4.5 μL dNTP(10 mmol/L each), and 3 μL superscript Ⅱ reverse transcriptase (200 u/μL). The first strand reaction was chilled on ice for 2 min, while the second strand cDNA was synthesized for 2 h at 16℃ in 750 μL reaction volume containing 150 μL 5 X second-strand buffer, 465 μL RNA-free water, 5 μL *E.coli* DNA ligase, 20 μL *E.coli* DNA polymerase, 15 μL dNTP (10 mmol/L each), and 5 μL *E.coli* RNase H. cDNA synthesis reaction was added with 45 μL 0.5 mol/L EDTA and chilled on ice to inhibit the reaction.

Cleavage of Biotinylated-cDNA

The double strand cDNA over the magnetic beads was washed to inactivate the *E.coli* DNA polymerase at 65℃ for 10 min with a wash buffer containing 5 mmol/L Tris (pH7.5), 0.5 mmol/L EDTA, 1M NaCl, 1% SDS, 10 μg/mL mussel glycogen. After the removal of wash buffer, the magnetic beads were washed with 200 μL 1 X NEB buffer 4 (New England Biolabs) and then digested with the anchoring enzyme *Nla*Ⅲfor 2.5 h at 37℃ in a 200 μL reaction volume containing 172 μL water, 2 μL BSA(100 mg/mL), 20 μL 10 X Buffer 4, 6 μL *Nla*Ⅲ (10 u/μL). In the next step, wash buffer was used to inactivate the enzyme at 65℃ for 10 min.

Ligating Linkers to Bound cDNA

The linkerA [5'- TTT GGA TTT GCT GGT GCA GTA CAA CTA GGC TTA ATA GGG ACA TG -3' with the complementary strand (amino mod. C7) 3'-CCT AAA CGA CCA CGT CAT GTT GAT CCG AAT TAT CCC T phosphorylated-5'] and linkerB [5' TTT CTG CTC GAA TTC AAG CTT CTA ACG ATG TAC GGG GAC ATG 3' with the complementary strand (amino mod. C7) 3'-GAC GAG CTT AAG TTC GAA GAT TGC TAC ATG CCC CT phosphorylated-5' (synthesis by Takara)] were annealed at 95℃ for 2 min, at 65℃ for 10 min, at 37℃ for 10 min and at room temperature for 20 min. Annealed products were purified using 12% PAGE. After the removal of the digested unbound cDNA, the 3'-cDNA fragments bound to magnetic beads were divided in half and ligated to linkerA and linkerB at 16℃ for 2 h, respectively. The ligation condition composed of 2 μL linker (25 ng/μL), 14 μL water, 2 μL ligase buffer and 2.5 μL T4 ligase (5 u/μL).

Release of Linker-tags and Formation of Linker-ditag-linker

After the ligation of linker to cDNA, the magnetic beads were washed with 200 μL 1 X NEB buffer 4 (New England Biolabs) and the linker-tags were released by the tag enzyme *Bsmf* Ⅰ, digested for 1 h at 65℃ in a 200 μL reaction volume containing 174 μL water, 4 μL BSA (100 mg/ mL), 20 μL 10 X Buffer 4, 2 μL *Bsmf* Ⅰ (2 u/μL). Linker-tags, consisting of linker and adhering to short cDNA sequence, were released from magnetic beads to the supernatant and then blunt-ended with klenow polymerase at 37℃ for 30 min in 50 μL reaction volume containing 5 μL 10 X klenow buffer, 1 μL BSA(100 mg/mL), 2.5 μL dNTP (10 mmol/L each), 30.5 μL water, 1 μL klenow polymerase (2 u/μL). After the extraction of phenol-chloroform-isoamyl alcohol and ethanol precipitation, the two pellets were dissolved and mixed. The mixture (including linkerA-tags and linkerB-tags) was ligased overnight at 16℃ in a reaction system of 1 μL 10 X ligase buffer, 1 μL T4 ligase (5 u/μL), and 8 μL linker- tags mixture.

PCR Amplification of Linker-ditag-linker

A series of dilutions of the linker-ditag-linker were prepared as template for PCR from 1/5 to 1/800. PCR amplification was carried out as described by Velculescu[4] with some modifications. The reaction system composed of pH 8.8, 2% template, 6.7 μmol/μL Mg^{2+}, 1.5 μmol/μL dNTP, 7 ng/μL primer, 0.05 u/μL Tsg DNA polymerase, 0.02 u/μL pfu DNA polymerase. PCR amplified as the procedure of 2 min at 95℃, 28 cycles of 30 s at 95℃, 1 min at 55℃, 1 min at 70℃ and final extension of 5min at 70℃. One hundred PCR reactions were performed for preparing plentiful linker-ditag-linker.

Generation A Tag Expression Library

Scale-up PCR production was precipitated ethanol and linker-ditag-linker was purified using 10% polyacrylamide gel. Then, ditag digestions were obtained by digesting linker-ditag-linker with *NlaⅢ* as described above. Twenty six bp ditags were observed and recovered from the 12% polyacrylamide gel. Purified ditags were ligated to generate concatemers. The concatemers were cloned into a pZErO®-1 vector and transformed TOP10 electricol *E.coli.* 120 clones were picked out randomly for identification and sequencing purpose. The results of sequencing were analyzed with SAGE2000 software.

RESULTS

Kinetics of Biomass and Citrinin Production During Monascus aurantiacus Growth

The kinetics of biomass (dry weight of mycelium) and citrinin production in submerge culture growth is presented in Fig. 1. From the curve, the trend of citrinin production was basically the same with the growth curve. On the seventh day, *Monascus aurantiacus* (AS3.4384) excreted citrinin to culture broth and produced citrinin maximally on the thirteenth (0.966 mg/mL) day in broth. The mycelium was collected to extract total RNA on the thirteenth day.

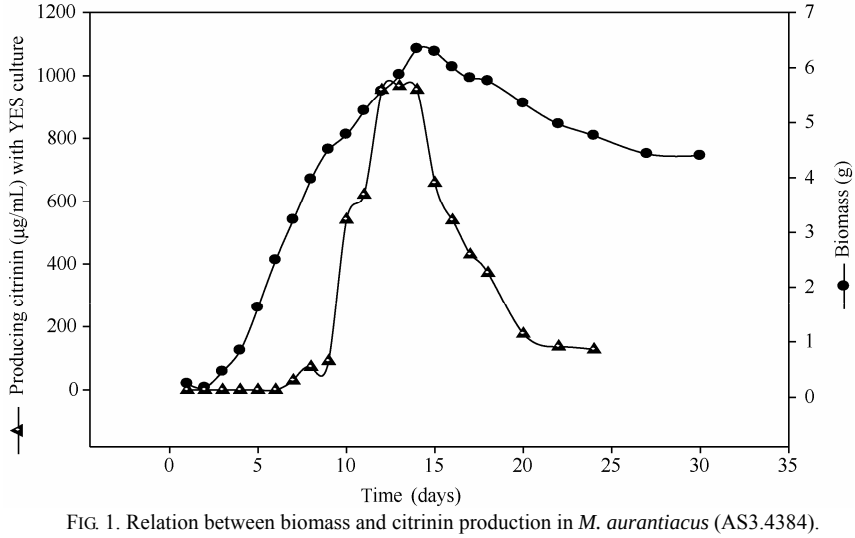

FIG. 1. Relation between biomass and citrinin production in *M. aurantiacus* (AS3.4384).

Total RNA Preparation

2.688 mg total RNA was isolated from 3 g mycelium. The value of $OD_{260/230}$ and $OD_{260/280}$ (2.2 and 2.0) demonstrated that the total RNA had no contamination of protein and guanidine thiocyanate. The intensity of 28 SrRNA band was approximately twice the intensity of 18 SrRNA band (Fig. 2).

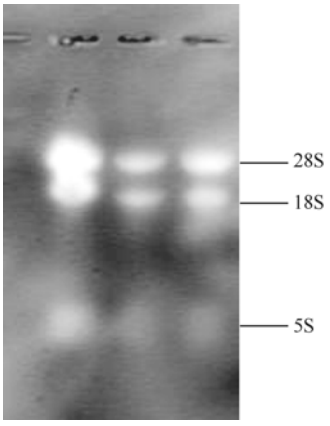

FIG. 2. 1% agarose gel electrophoresis of *M.aurantiacus* (AS3.4384) total RNAlane 1: 20 μg total RNA, lane 2: 10 μg total RNA; lane 3: 5 μg total RNA.

PCR Amplification of the Linker-ditag-linker

PCR amplification was performed according to the standard SAGE protocol with some modifications. In order to improve the yield of linker-ditag-linker, Tsg DNA polymerase plus pfu DNA polymerase was used instead of the Platinum® Taq (Invitrogen), a kind of hot-start Tag DNA polymerase to increase the amplification of proper band. The intensity of 100 bp bands by using Tsg DNA polymerase plus Pfu DNA polymerase was stronger than Platinum® Taq (Figs. 3 and 4). A 1:100 dilution of linker-ditag- linker was selected to scale-up PCR. After 100 PCR reactions were performed, the 100 bp linker-ditag- linker products were digested with *NlaIII* to release 40 bp bands (linkers) and a 26 bp ditags (Fig. 5).

Tag Expression Library Generation

Ditags purified from 12% polyacrylamide gel were ligated to generate concatemers. Concatemers analyzed by 1.5% agarose gel are presented in Fig. 6. The fragments appeared smear bands in range of 100 bp to 2500 bp in lane 2. The concatemers were cloned into the pZErO®-1 vector (Cat. No.2500-01, Invitrogen) and transformed into TOP10 bacteria. A total of 598 clones were obtained.

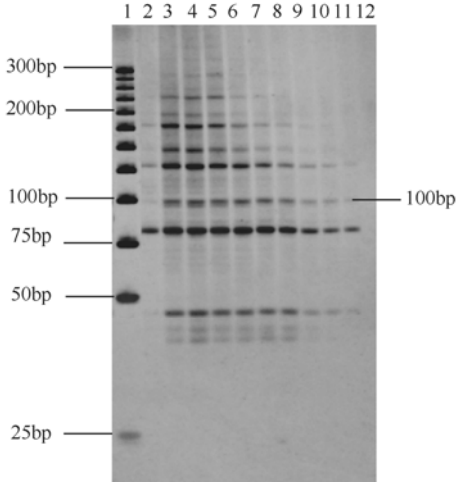

FIG. 3. Optimum template dilution for PCR with Platinum® Taq lane 1: 25 bp ladder; lane 2: control (no template); lanes 3-12: template dilution 1: 5; 1: 10; 1: 20; 1: 40; 1: 60; 1: 80; 1: 100; 1: 200; 1: 400; 1: 800.

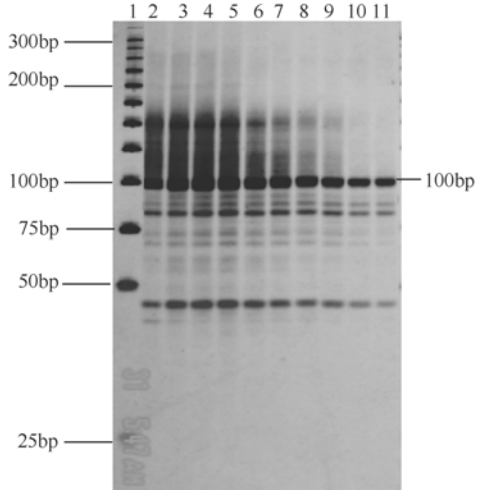

FIG. 4. Optimum template dilution for PCR with Tsg DNA Taq and Pfu DNA Taq. lane 1: 25 bp ladder; lanes 2-11: template dilution 1: 5; 1: 10; 1: 20; 1: 40; 1: 60; 1: 80; 1: 100; 1: 200; 1: 400; 1: 800.

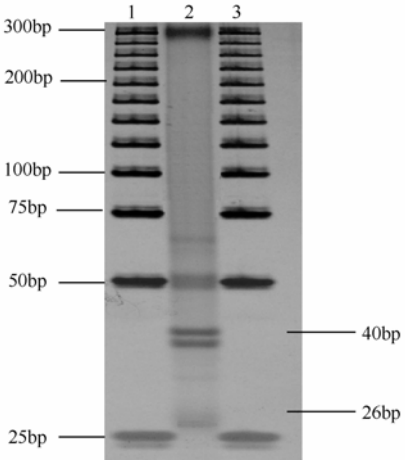

FIG. 5. *NlaⅢ* enzyme digestion of 100 bp linker-ditag-linker Lanes 1, 3: 25 bp ladder; lane 2: the digestion of linker-ditag-linker with *Nla Ⅲ* enzyme.

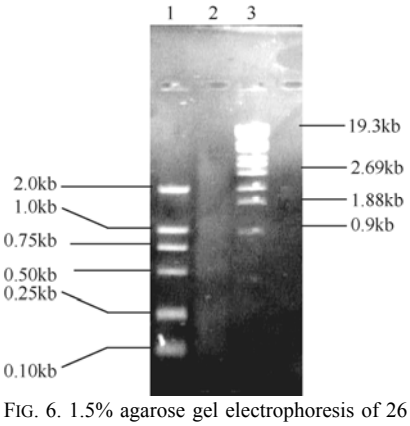

FIG. 6. 1.5% agarose gel electrophoresis of 26 bp ditags concatemers. Lane 1: DL2000 Marker; lane 2：concatemers; lane 3: λ-EcoT14Ⅰ Fragment Marker.

Tag Expression Library Analysis

A total of 120 clones were picked out randomly for identificatioin and sequencing purpose. Eighty nine clones had positive inserts, and, 26 clones had no inserts and the remaining 5 clones had no site of *NlaⅢ* enzyme in inserts. There were seven repeated clones. In the valid clones, the inserts contained eleven tag sequences on average. With the analysis of SAGE2000 software, 901 tags were obtained, representing 686 unique transcripts, among which 6 unique tags belonged to highly expressed genes (Copies of tag≥10); 4 unique tags had five copies, 5 unique tags had four copies, 13 unique tags had three copies, 121 unique tags had two copies and others belonged to a single copy.

Using CGAP UniGene of SAGE2000 software, 26 Tags that expressed more than 3 copies per cell were matched with a unigene cluster of Genbank (Table 1). Among them, 7 tags were matched with a unigene cluster, 12 tags were not matched with Genbank and 9 tags were matched with more than one unigene cluster.

TABLE 1

26 Tags With Expression of More Than 3 Copies Per Cell

Tag Sequence	Copies	UG No.*	Gene Description
CTGTCTTGTA	45	No match	
TCTCCCTGCT	18	HS.98894	Homo Sapiens Transcribed Sequences
CATATACGCG	14	HS.422118	EEF1A1 Eukaryotic Translation Elongation Factor 1 alpha 1
CTACGACCTA	14	No match	
TACCCTGTCT	12	HS.198273	NDUFB8 NADH Dehydrogenase
AGGTTGCTCC	10	No match	
CTATACCCTA	5	No match	
GTTTTATGAG	5	No match	
AAGTATTGTT	4	No match	
TGTATGGGCT	4	No match	
AAGTATTGTT	4	HS.129887	CDH19 Cadherin 19, Type 2
ACGGTAGTAG	3	No match	
CAACGGAATA	3	No match	
GAAACTGTAT	3	No match	
GCGATGGGGT	3	No match	
TACAACCTTT	3	No match	
ATTTGTATGA	3	HS.356624	NID Nidogen (enactin)
CACTCTTATT	3	HS.250619	APOBEC3G Apolipoprotein B mRNA Editing Enzyme, Catalytic Polypeptide- like 3G
GAAGTATTTT	3	HS.1274	BMP1 Bone Morphogenetic Protein 1

DISCUSSION

A SAGE library of *Monascus aurantiacus* AS3.4384 was successfully constructed using SAGE approach. High quality of total RNA was required for the synthesis of large fragment cDNA, which provided enough tag sequences to guarantee the integrity of information reflected by the transcripts in the established SAGE library. In this paper, the total RNA extract was precipitated twice with 1.5 volumes of 4 mol/L NaAC and then assayed with a spectrophotometer and formaldehyde denaturing gel electrophoresis. The results indicated that the total RNA was free of guanidine thiocyanate pollution and 28S band was approximately twice the intensity of the 18S band, and the bands showed no smear and dispersion phenomena, demonstrating that the extracted total RNA was genetically intact.

Amplification with high fidelity of a large amount of the ditags was included as one of the key procedures in the construction of SAGE library. In this study, the amplification effect of Platinum® Taq, a heat starting DNA polymerase recommended by microSAGE systems, and Tsg DNA polymerase plus Pfu DNA polymerase were compared. The results indicated that Tsg+Pfu DNA polymerase was much better, as Pfu DNA polymerase possesses a 3′→5′ endonuclease activity and can amplify the entire transcripts with high fidelity. The purity of linkers A, B is also very important for the obtainment of ditags in the construction of SAGE library, so the PAGE purification process was used to effectively remove the residual single chain linkers brought about by the excess of single chains after the annealing of these synthesized linkers, and therefore avoid the interference of these single chains in the preparation of ditags.

Based on abundant information of related genetic background, serial analysis of gene expression is a kind of high throughput analysis method in determining the gene expression profile of a certain species by sequencing the tags. However, presently there are rather few articles about molecular biology research of *monascus*, and by far the related known genetic background is so little that in this study there were as many as 12 tag sequences that matched no information in Genbank when a total of 28 tags that copied more than 3 times achieved in this paper were analyzed by the UniGene search function of SAGE2000 software CGAP. Nevertheless, through the analysis of transcripts of *monascus*, this study has provided a certain theoretical basis for screening housekeeping genes from the highly expressed genes and also enriched our knowledge about the transcription of primary eukaryotes such as *monuscus*. Meanwhile, in the differential expression aspect, if we can construct two related SAGE libraries from a strain of *monscus* that produces citrinin on different culture conditions, the profiles about the cintrin synthesis related genes will be more and more clear, which will further provide important information for the metabolic pathways and regulation mechanism of citrinin and pigments in *monascus*.

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