Original Article

Construction of a replacement vector to disrupt *pksCT gene* for the mycotoxin citrinin biosynthesis in Monascus aurantiacus and maintain food red pigment production

Guiming Fu DSc, Yang Xu PhD, Yanpin Li PhD and Wenhui Tan MSc

Key Laboratory of Food Science of Ministry of Education, Jiangxi-OAI Joint Research Institute, Nanchang University, Nanchang, China

More and more people pay attention to citrinin produced *by Monascus*, which has nephrotoxic activity in mammals. It was reported that *pksCT* gene is responsible for citrinin biosynthesis in *Monascus purpureus.* In this paper, two DNA fragments in both ends of *pksCT* were amplified by genomic PCR from fourteen *Monascus spp*. strains. The PCR products were gained from all of the strains. It is suggested that *pksCT* gene was highly conserved in different citrinin-producing *Monascus* strains. A *pksCT*-replacement vector (pHD106) was constructed to disrupt *pksCT* with a hygromycin resistance gene as the selection marker, and was transformed into *M. aurantiacus Li AS*3.4384. Three transformants **(***M. aurantiacus* PHDS18, PHDS26, PHDS31**)** were selected from **t**ransformant selective plates. The targeting fragment D was gained by genomic PCR from PHDS18 and PHDS26 except PHDS31. The expressing citrinin capacities of PHDS26 was decreased by about 98%, while PHDS18 was reserved the high capacity of producing citrinin, after 10 days of growth on YM medium. The results indicated that PHDS26 is a *pksCT*-disrupted strain. There are maybe other genes besides *pksCT* responsible for citrinin biosynthesis in *M. aurantiacus*. It is the effective way to solve the problem of citrinin in *M. aurantiacus* products by constructing replacement vectors to disrupt the genes responsible for citrinin biosynthesis to reduce the capacity of expressing citrinin.

Key Words: replacement-disrupted vector, pksCT gene, mycotoxin, citrinin biosynthesis, Monascus aurantiacus, red pigment, food colour

Introduction

Monascus are small filamentous fungi. The applications of *Monascus* in China have been for more than one thousand years.[1,2] A lot of studies showed that *Monascus* could produce many active substances, such as red pigments, Monacolin, γ-aminobutryric acid, acetylcholine, ergosterol and others. Recently, more and more studies have been focused on three kinds of secondary metabolites of *Monascus* spp, i.e., red pigments, Monacolin K and citrinin. Red pigments are widely used in the meat industry (such as sausages or ham) in occidental countries.[3] Monacolin K is also used as a therapeutic agent to reduce blood cholesterol levels.[4] Citrinin, a mycotoxin which has nephrotoxic activity in mammals, was isolated from most cultures of *Monascus* strains in 1993.[5] Kidney is the target organ of citrinin, resulting in not only teratogenicity and carcinogenicity, but also in mutagenicity. Many countries including U.S.A., European Union members and Japan have worked out new standards to strictly limit the content of citrinin in *Monascus* products. It is requested that the content of citrinin must be lower than 200 ng/g in the Japanese standard. Otherwise, products are forbidden to be imported. Few *Monascus* products in China can reach the control standard of citrinin. The problem of citrinin has become a bottleneck to the exports of *Monascus* products. Now, how to decrease the content of citrinin is becoming an urgent problem which should be solved as early as possible.[6]The traditional methods to control the producing of citrinin are the optimization of the conditions of fermentation and selection of the citrinin low-producing strains. But red pigments and citrinin begin with a common synthesis pathway in *Monascu*. [7] In experiments to optimize the conditions of fermentation and mediums, the quantity of red pigments reduced with the decrease in citrinin.[8] It is very important to obtain citrinin low-producing strains for the commercial production of red pigments and Monacolin K.

Although some citrinin low-producing mutants of *Monascus* have been obtained by UV/chemical mutagenesis,[9] the mutants generated revertants easily, and recovered the citrinin expressing capacity. Therefor it is essential to disrupt the gene for citrinin biosynthesis by genetic engineering. In *Monascus* species, there are few studies of gene disruption.

Corresponding Author: Professor Yang Xu, Key Laboratory of Food Science of Ministry of Education, Jiangxi-OAI Joint Research Institute, Nanchang University, 235 East Nanjing Road, Nanchang, Jiangxi, China 330047
Tel: 86 791 8329 479; 86 13320019900
Fax: 86 791 8333 708
Email: xuyang1951@yahoo.com.cn

Only one paper has reported *pksCT*-insert-disruption in *M. purpureu*. A full-length *pksCT* gene of 7,838 bp with a single 56-bp intron was achieved. The *pksCT*- disrupted strain produced little or no citrinin, and maitained a high capacity to produce red pigments. But a *pksCT* revertant which was generated by successive endogenous recombination events in the *pksCT* disruptant restored citrinin producible capability.[10]Results showed that *pksCT* is only correlated with citrinin production.

According to the homologous recombination way between vector and genomic DNA, there were two types of disrupted vectors: insert-disrupted vector and replacement –disrupted vector. The split position of insert-disrupted vector was in homologous sequence. When homologous recombination happened, the target gene and vector were exchanged once, and the whole vector was inserted into the sites of the target gene. The insert-disrupted strains easily generated a second homologous recombination in successive cultivation and the insert-disrupted vector fell off from the chromosome to form revertants. The split positions of replacement –disrupted vector were in both of the two ends of the homologous sequence or outside the homologous sequence, while the target gene was in the homologous sequence. When homologous recombination occurred, the target gene and vector were exchanged twice. The result was only the homologous sequence with the part to replace a target sequence in the chromosome. Sequence of the vector outside the homologous sequence was cut off. The replacement–disrupted strains have few revertants generated by successive cultivation.[11,12]

Monascus aurantiacus is a new strain of *Monascus*, which was found by Professor Zhongqing Li.[13] Since 1996, Professor Yang Xu has conducted a series of research activities in producing citrinin by *M. aurantiaaeus* and in the synthesis pathway **a**nd expression gene of citrinin,[14,15] and it is reported that *M. aurantiacus* is the red-pigment low-producing and the citrinin high-producing strain.[16-18] Our objective in this study was to disrupt *pksCT* gene in *Monascus aurantiacus* using a replacement –disrupted vector and to gain the steady *pksCT* disruptant, and to provide an effective way to avoid the risk of citrinin contamination in *Monascus* products.

Material and methods

Bacterial and fungal strains

Escherichia coli Dh5α was used for the propagation of recombinant plasmid. *Monascus purpureus (*AS3.4451, AS3.4453), *M. aurantiacus Li* (AS3.4384), *M. pilosus Sato (*AS3.976, AS3.4444), *M.barkeri (*AS3.4452), *M. anka* (AS3.2636), *M. anka* (IFFI05012, IFFI05013, IFFI05022, IFFI05031, IFFI05032, IFFI05033), *M.ruber* IFFI05007 (Institute of Microbiology, Chinese Academy of Sciences) are producers of citrinin, and were used for the experiment of homologous character of *pksCT* gene. *M. aurantiacus Li AS*3.4384 was used for disrupting *pksCT*.

Culture conditions

E. coli was grown at 37℃ in LB medium broth or agar supplemented with ampicillin or ampicillin plus X-gal/IPTG as appropriate.[19] *Monascus* strains were maintained on MES medium [6 °Bé wort and 20 g/L agar and pH 7] for the propagate spores of *Monascus*. Regenerate plates [MES agar medium containing the different osmotic stabilizers (0.6 M sucrose, 0.6 M glucose, 0.6 M sorbitol, 0.6 M NaCl, or 0.6 M $MgSO_4$, respectively)] were used in regeneration of protoplasts. Transformant selected plate [MES medium containing 100 mg/L hygromycin B] was used in protoplast transformant experiments of *Monascus*. For liquid cultivation of *Monascus*, fungi were grown in MPPY medium[20], and YM medium.[21]

Genomic PCR

For genomic DNA preparation, the fourteen *Monascus spp*. strains were used. The method of extraction and purification of Genomic DNA from *Monascus* with benzyl chloride was described by Yuan Y.F.[22] Homologous sequence fragments A and B of *pksCT* gene (accession No. AB167465 in GenBank) were amplified by genomic PCR. Fragment A was amplified from the transcriptional start region of *pksCT* with primer K (5'-GGGGATCCCCG AAGGAGATAAACAGTGAGAG-3'), and primer L (5'-GCTCATGAAGGCGTTGATGAGA TGTAG-3') .The underlined letters indicate *Kpn I*, *Xbal I* sites. Primer M (5'-GCTCATGAGCTACTATCCACT TCGCTAC-3') and primer N (5'- AACTGCAGAATCTCTCGTC TTA GTCGTATC-3') used to amplify fragment B were based on the sequences of the stop codon region of *pksCT*. The underlined letters indicate *Xbal I* and *Pst I* sites, respectively.

The amplification conditions were denaturation at 94℃ for 10 min; then 30 cycles, each consisting of denaturation (94℃ for 50 s), annealing (56℃ for 1 min), and extension (72℃ for 1 min); and finally a single extension at 72℃ for 10 min. The PCR products were subcloned into pMD18-T (Takara, Inc.), then sequenced by Shanghai Sangon Biological Engineering Technology & Service Co., Ltd (China).

Construction of a replacement vector

Plasmid DNA extraction from *E. coli*, DNA restriction, ligation, and *E. coli* transformation were carried out using standard methodologies.[19] pSGF957 containing hygromycin B-resistant gene was a gift from Professor Soo-Un Kim.[23] The replacement vector was constructed as follows (Fig 1). Homologous sequence Fragments A and B were ligated to generate fragment C with *Xbal* I site. Then fragment C was digested with *Kpn* I + *Pst* I and ligated to pUC18 (Takara Inc.), to generate pHC6. A 3.1-kb fragment containing hygromycin B-resistant gene from pSGF957 was cloned into *Xbal* I site of pHC6 to generate pHD116.

Protoplast preparation

1×10^8 Spores of *M. aurantiacus Li AS*3.4384 were inoculated on 100 ml MPPY medium, and cultured at 30℃ and 120 rpm for 18-20 h. Young hyphae were collected and suspended in osmotic stabilize buffer (0.6 M $MgSO_4$). To generate protoplasts, young hyphae was treated with the lytic enzyme mixtures(consisting 10 mg/ml snaliase, 3 mg/ml lysing enzyme, 40 mg/ml cellulase, and 0.6 M

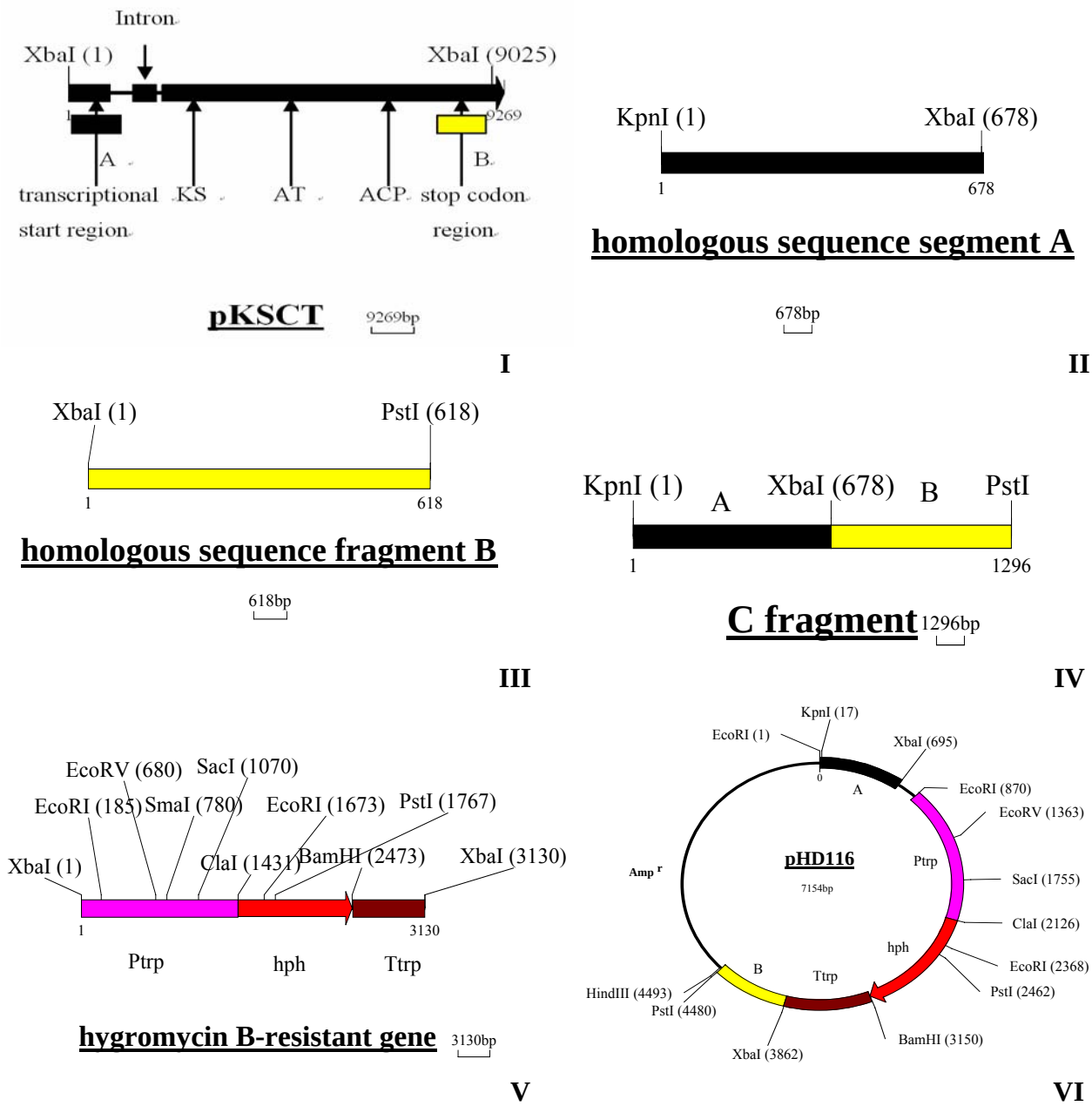

Figure 1. Construction of *pksCT*-replacement vector. Thin black line, pUC18; thick black line (A), homologous sequence A; thick blue line (Ptrp), *Aspergillus nidulans trpC* promoter; thick red line (*hph*), coding sequence of hygromycin B-resistant gene; thick green line (T*trp*), *A. nidulans trpC* terminator; thick yellow line (B), homologous sequence B

$MgSO_4$, pH6.0).[23] Regenerate plates were used to measure the regeneration ratio.

Transformation

Protoplast-PEG described by Campoy was used in transformation, [24] then protoplast suspension (200 μl) was spread on the transformant selection plates and incubated at 28℃ for 4-5 days in darkness.[25]

Analysis of pksCT disruptants

Following transformation, transformants were selected out from the transformant selection plates after incubation for 4-5 days. Genomic DNA was isolated from transformants mycelia, and genomic PCR was used to amplify the targeting fragment D which included homologous sequence fragments and *hph* gene.

To analyze the expression of the red pigments and citrinin, transformants were cultured on 100 ml YM medium [containing 100 mg/L hygromycin B] at 28℃ for 10 to 13 days, while original strains were cultured on 100 ml YM medium. Red and yellow pigment color values were measured by spectrophotometry.[26] Citrinin was analyzed by high-performance liquid chromatography (HPLC) on a Symmetry C_{18} column (5 μm, 250×4.6 mm) (Syknm Inc., Japan) with acetonitrile/water (77/23[v/v], pH2.5) as the mobile phase at a flow rate of 0.8 ml/min, and at the column temperature of 28℃, then detected by fluorescence (λ_{ex}=331 nm, λ_{em}=500 nm). [27] Commercial citrinin (Sigma Ltd., USA) was used as the standard.

Results and discussion

Analysis of homologous character of pksCT

From Figure 2, results showed that the fragments A and B which were two portions in both of the two ends of *pksCT* were gained from fourteen *Monascus spp*. strains. Results of DNA sequencing showed that PCR products exhibited

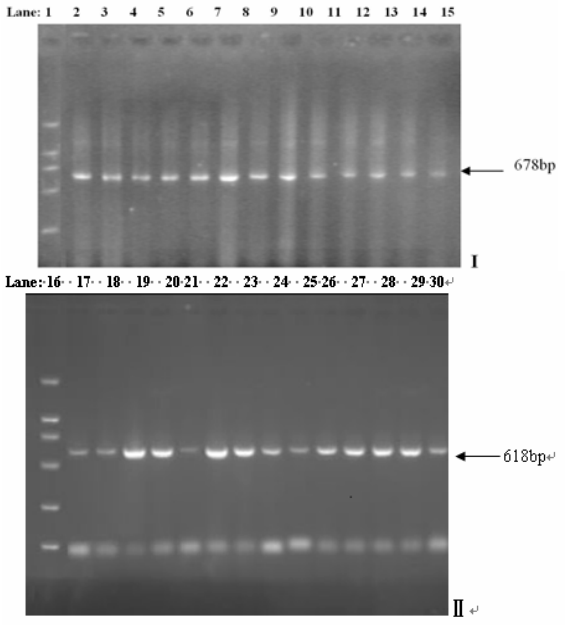

Figure 2. PCR products of *pksCT*. Lanes 1 and 16, DNA marker DL2000; Lanes 2-15, PCR products of A fragment from AS3.4451, AS3.4453, AS3.4384, AS3.976, AS3.4444, AS3.4452, AS3.2636, IFFI05012, IFFI05013, IFFI05022, IFFI05031, IFFI05032,IFFI05033, IFFI05007; Lanes 17-30, PCR products of B fragment from fourteen strains respectively.

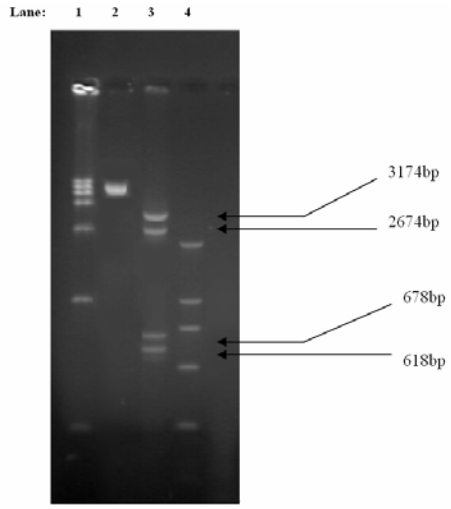

Figure 3. pHD116 and its digested products. Lane 1, DNA marker DL15000; Lane 2, pHD116; Lane 3, digested products of pHD116; Lane 4, DNA marker DL2000.

95%-98% identity with each other. It is suggested that *pksCT* gene was highly conserved in different citrinin-producing *Monascus* strains.

Analysis of the pksCT- replacement vector pHD116

PHD116 was digested with *Xbal* I+*Kpn I* +*Pst* I, and cut into four fragments, which included hygromycin B-resistant gene fragment (3184 bp), pUC18 fragment (2674 bp), homologous sequence fragment A (678 bp) and homologous sequence fragment B (618 bp) in Figure 3. Results showed that pHD116 was the *pksCT*-replacement vector, which contains two homologous sequence fragments of *pksCT* and a hygromycin B-resistant gene as selection marker.

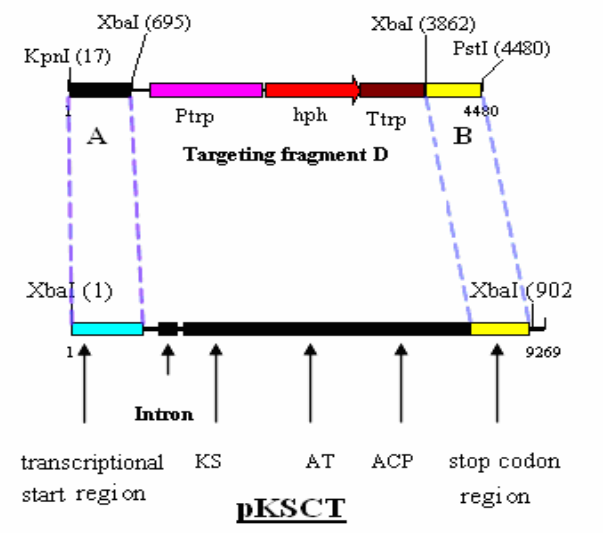

Figure 4. Schematic diagram of representation of gene disruption.

Table 1. Average capacity of expressing citrinin of original strain and transformants (n=3)

strain	citrinin (μg /ml)	Percent of citrinin
original strain	781.5±23	100%±2.9
PHDS 18	121.2±17	15.5%±2.2
PHDS 26	16.16±2	2.1%±0.2
PHDS 31	203.5±11	26.1%±1.4

Protoplast preparation and regeneration

Results showed the lytic enzyme mixture improved the release of protoplasts, and the highest yield of protoplast was about 8×10^7 protoplasts/ml. After being cultivated on the regeneration mediums at 28℃ for 4-5 days in darkness, colony of regeneration was counted. The peak of regeneration ratio was 18%, which was found in regeneration mediums with 0.6 M sucrose.

Analysis of pksCT disruptants

Linearizing pHD116 was transformed into protoplasts by protoplast-PEG method, homologous recombination happened between *pksCT* and vector, and *pksCT* was replaced by the targeting fragment D which included two homologous sequences and hygromycin B-resistant gene in them. The sites of insertion of the targeting fragment D were shown in Figure 4.

Three transformants (*M. aurantiacus* PHDS18, PHDS26 and PHDS31) were seeked out from transformant selection plates. After 10 days of growth on YM medium, the concentrations of citrinin in fermentation liquids were measured by HPLC, and the results were shown in Table 1. The expressions of citrinin of PHDS18, PHDS26 and PHDS31 were 15.5%, 2.1% and 26.1% of the expressions of citrinin of original strain respectively. PHDS18 and PHDS31 were reserved the high capacity of producing citrinin, although their capacity of producing citrinin were lower than that of original strain. It is may

Table 2. Average capacity of expressing pigment of original strain and transformants (n=3)

Strain	red pigment color value (U /ml)	Percent of red pigment color	yellow pigment value (U /ml)	Percent of yellow pigment color
original	34.6±4.3	100%±12.4	46.2±2.2	100%±4.8
PHDS 18	37.8±1.9	109.2%±5.5	50.1±1.7	108.4%±2.7
PHDS 26	51.7±1.6	149.4%±4.6	59.5±2.9	128.8%±6.3
PHDS 31	38.3±2.5	110.7%±7.2	45.4±3.0	98.3%±6.5

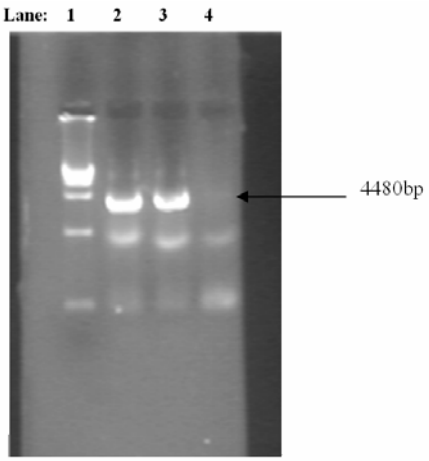

Figure 5. PCR product of targeting fragment D from transformants. Lane 1, DNA marker DL15000; Lane 2-4, PCR products of PHDS18, PHDS26 and PHDS31.

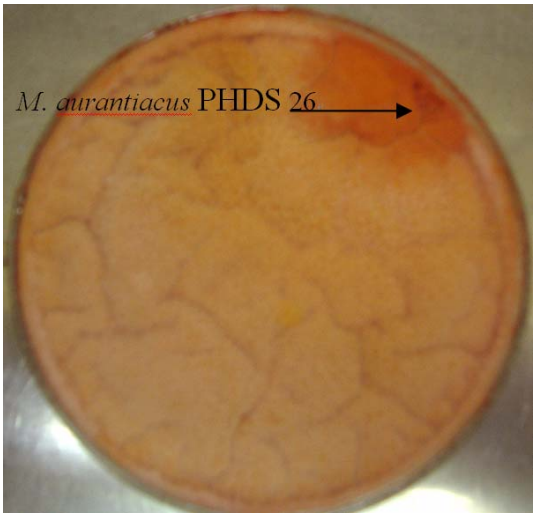

Figure 6. Colonies of transformants growth on the transformant selection plate at 28℃ after 5 days.

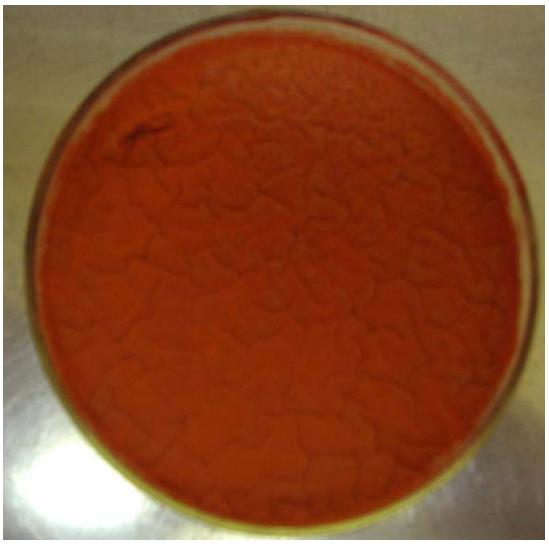

Figure 7. Colonies *of M. aurantiacus* PHDS 26 growth on YM agar medium (contain 100 mg/L hygromycin B) at 28℃after 7days

be that *Monascus* produce lower citrinin in YM medium containing hygromycin B.

The targeting fragment D which was used to replace *pksCT* was amplified by genomic PCR from transformants. PCR product was gained from PHDS18 and PHDS26 except PHDS31 (Fig 5). The results of PCR products and expression of citrinin indicated that PHDS26 is a *pksCT*-disrupted strain, and homologous recombination did not happen between *pksCT* and replacement vector in PHDS31, and PHDS18 is a false positive *pksCT*-disrupted strain for its reservation of the citrinin-high expressing capacity.

Results of the pigment color values in fermentation liquids were shown in Table 2. The red pigment color values of the PHDS18, PHDS26 and PHDS31 were increased by 9.2%, 49.4% and 10.7% respectively, higher than that of the original strain. The yellow pigment values of PHDS18 and PHDS26 were increased by 8.4% and 28.8% compared with the original strain, while the yellow pigment value of PHDS31 was reduced by 1.7%.

PHDS26 was selected from the transformant selection plate in Figure 6, and colonies of PHDS26 was shown in Figure 7, after cultivation on YM mediums (contain 100 mg/L hygromycin B) for 7 days. The color of PHDS26 became deep red, while the capacity of expressing citrinin was decreased.

It was reported that red pigments and citrinin begin with a common biosynthesis pathway in *Monascus*, and have a common precursors, such as acetyl-CoA and malonyl-CoA. [7] Then the synthesis is separated to two pathways. One is to synthesize red pigments, another is to synthesize citrinin. *PksCT* encodes the PKS responsible for the pathway of citrinin biosynthesis.[10] In the *pksCT* disruptant, the capacity of expressing citrinin should be decreased, while the capacity of producing red pigments increased. In this experiment, the *pksCT*-disrupted strain showed its low capacity of producing citrinin, and high capacity of producing red pigments. The results also indicated that there might be other genes besides *pksCT* that are responsible for citrinin biosynthesis in *M. aurantiacus*. *PksCT* is responsible for the main pathway of citrinin biosynthesis in *M. aurantiacus*, and others are responsible for the subordinate synthesis pathway. It is also suggested that it is the effective way to solve the problem of citrinin

content beyond the limit in *Monascus* products by a replacement -disrupted vector to disrupt the genes responsible for citrinin biosynthesis.

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