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Elimination of the mycotoxin citrinin production in the industrial important strain *Monascus purpureus* SM001

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ABSTRACT

The application of the high-producing pigments industrial strain *Monascus purpureus* SM001 has been greatly limited by the synchronous production of mycotoxin citrinin. Here we have tried both traditional mutagenesis and metabolic engineering methods to eliminate the production of citrinin. Traditional chemical and physical mutagens were applied to induce mutagenesis, and a bio-screening method based on the antibacterial activity of citrinin against *Bacillus subtilis* was designed to select mutants. Among the resulting four citrinin-free mutants, only mutant MU2411 can maintain the similar pigments yield. A binary vector system was constructed and successfully disrupted the polyketide synthase gene *pksCT* in *M. purpureus* SM001 through the *Agrobacterium tumefaciens*-mediated transformation. The resulting citrinin-free Δ*pksCT* mutants maintained the same level of pigments yield. The established *Monascus* genetic system was comprehensively evaluated and showed high efficiency and specificity, which provides us a potential approach to manipulate and improve industrial *Monascus* strains.

1. Introduction

The filamentous fungus *Monascus purpureus* has been used to produce fermented products such as fermentation starters, food colorants and medicinal agents in Asian countries for centuries (Lin et al., 2008). It can synthesize many secondary metabolites including red and yellow pigments, monacolins and gama-aminobutyric acid, which are industrially and medicinally important compounds (Blanc et al., 1994; Ma et al., 2000; Narahara, 1994). However, *M. purpureus* is also a toxigenic strain that can produce the nephrotoxic and hepatotoxic mycotoxin citrinin; this greatly limits the wide application of the *Monascus*-related products (Blanc et al., 1995). For example, *M. purpureus* SM001 is one of the best *Monascus* pigment producers isolated in China, but it is also a good producer of citrinin. Thus, elimination of citrinin production in *M. purpureus* is essential to increase the safety of *Monascus*-related products and extend their application.

A traditional way of purposefully improving microorganisms or screen high-throughput strains is via chemical or physical mutagens-induced mutagenesis, which is normally time-consuming to select target mutants and is difficult to continue the consecutive improvement due to the incomprehension of the random mutation sites. Comparably, genetic mutagenesis based on the correlative biosynthetic mechanism is an increasingly powerful tool to manipulate microorganisms, which has been widely applied in microbiologic studies. Unlike traditional methods, the genetic approach is directed by the understanding of biosynthetic machinery and is aimed at targeted genes, which is more specific and effective. And the obtained mutants can be further modified through certain manipulation to gain desired characteristics.

However, the genetic mutation for *Monascus* species is mainly executed in the lab level for fundamental researches, and no industrial application has been reported so far. Recently, a polyketide synthase gene *pksCT* involved in citrinin biosynthesis has been successfully cloned from *M. purpureus* (Shimizu et al., 2005), which provides us a basis to modify the industrial strain *M. purpureus* SM001 through this potential method. On the other hand, *Agrobacterium tumefaciens*-mediated transformation (ATMT) has been widely applied to engineer the metabolic pathway in plant (Jadhav et al., 2005; Morris et al., 2006; Muoz-Bertomeu et al., 2008) and it represents a powerful tool to carry out our genetic design. In this work, we have tried both traditional and genetic mutagenesis methods to eliminate the citrinin production in *M. purpureus* SM001. The comparison results have indicated that the ATMT is a very efficient method to achieve this purpose and it has great potential for further development. This study not only is the first example characterizing gene replacement and patterns of T-DNA integration in the industrial strain of

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genus *Monascus* but also sets up the base for future genetic manipulation of industrial *Monascus* strains.

2. Materials and methods

2.1. General materials and methods

All highly pure chemicals were purchased from Sigma-Aldrich (Milwaukee, WI). Restriction enzymes for DNA digestion were purchased from TaKaRa Bio Inc. (Shiga, Japan). Buffers and chemicals for gel electrophoresis were purchased from Bio-Rad (Hercules, CA). The vector pDH2857 was kindly offered by Dr. S.U. Kim (Seoul National University, Seoul, Korea). It contains *M. anka gpd1* promoter (P*mgpd*, GPD: D-glyceraldehyde-3-phosphate), hygromycin B-resistant gene (*hph*), and *Aspergillus nidulans trpC* terminator (TRP: tryptophan) (Kim et al., 2003). The binary vector pCAMBIA0380 was obtained from Mr. Leon M.A. Smith (Center for the Application for Molecular Biology to International Agriculture, Canberra, Australia). General DNA isolation and molecular manipulation were performed according to standard protocols (Sambrook and Russell, 2001). All inserted fragments in constructed plasmids were sequenced including the cloning sites. Oligonucleotides synthesis and nucleotide sequencing were carried out by GenoTech Corp. (Taejon, Korea). PCRs were performed in an iCycler thermal cycler (Biorad, Richmond, USA) by using BSplus Taq DNA polymerase (Biosesang, Sungnam, Korea).

2.2. Strains and media

Escherichia coli DH5α (Life Technologies Inc., Gaithersberg, USA) was cultivated in Luria-Bertani (LB) medium containing 100 μg/ml ampicillin or kanamycin at 37 °C for plasmid propagation. *B. subtilis* was purchased from the Korean Federation of Culture Collections (KFCC, Seoul, Korea). It was inoculated in potato dextrose broth (PDB) at 30 °C for 24 h; then an aliquot (200 μl) of the culture was spread on a PDA plate for bioassay of *M. purpureus* strains. *A. tumefaciens* strain AGL1 was kindly provided by Dr. Tang Li (Korea Research Institute of Bioscience & Biotechnology, Teajon, Korea). This strain (with carbenicillin resistance) was grown on YEP (yeast extract 1%, peptone 1%, NaCl 0.5%) medium containing 100 μg/ml carbenicillin, or induction medium (IM, composed of 10 mM K_2HPO_4, 10 mM KH_2PO_4, 2.5 mM NaCl, 2 mM $MgSO_4$, 0.7 mM $CaCl_2$, 9 μM $FeSO_4$, 4 mM $(NH_4)_2SO_4$, 10 mM glucose, 40 mM 2-(N-morpholino) ethanesulfonic acid, pH 5.3, 0.5% glycerol (w/v), and 200 μM acetosyringone) (de-Groot et al., 1998). *M. purpureus* SM001, a wild-type high producer of red pigments and citrinin, is stocked on Potato Dextrose Agar (PDA). For sporulation, the fungus was grown at 28°C in the dark on modified complete medium for 12 d (Lakrod et al., 2003). For genomic DNA preparation, 10^6 spore suspensions were inoculated in 100 ml starch mineral medium and grown at 30°C with agitation at 200 rpm for 36 h (Kim et al., 2003). For ATMT, transformants were screened on SM1 agar plate (PDA supplemented with 150 μg/ml hygromycin B and 200 μg/ml cefotaxime). Monoconidial isolates were obtained by harvesting conidia from SM1 plates and then by plating diluted conidia suspension on SM2 agar (PDA plate containing 150 μg/ml hygromycin B). To determine the growth inhibition of *Monascus* strains by hygromycin B, precultures of the parent strain and transformants were grown on PDA at 30 °C for 5 days. The resulting precultures with size of 6 mm diameter were transferred to PDA plates containing different concentrations of hygromycin B. Ten replicas at each concentration were incubated at 30 °C in the dark, and the growth of fungus was recorded after 7 days. For analysis of citrinin and pigment production, liquid cultivation of the strains was done as described by Shimizu et al. (2005).

2.3. Traditional fungal mutagenesis

The parental strain *M. purpureus* SM001 was cultured at 30 °C for 7 days on PDA plate for collecting the fungal spore. Three different procedures were applied to induce the fungal mutagenesis.

UV irradiation: 4 ml spores suspension (10^6 spores/ml) of *M. purpureus* SM001 was evenly spread on a Petri dish, which was then placed under an ultraviolet lamp (15 W) with a distance of 45 cm and irradiated for different times. The handled spores were harvested and washed three times with sterile distilled water, and then inoculated in fresh PDB medium. After 6–8 h growth at 30 °C in dark, the cells were plated on PDA agar plates and incubated at 30 °C for 72 h. The formed colonies were used for subsequent bioassay screening.

Diethyl sulfate (DES) treatment: 2% of DES (in 0.1 M phosphate buffer, pH7) was added to 1 ml spores suspension (10^6 spores/ml) of *M. purpureus* SM001. After incubation at 30 °C for different time intervals ranging from 1 to 90 min, the mixture was diluted 1000 times with sterile distilled water immediately. The viable cell (death rate 50%) was harvested by centrifugation and washed three times with sterile distilled water, and then was suspended in fresh PDB medium. After 6–8 h growth at 30 °C in dark, the cells were plated on PDA agar plates and incubated at 30 °C for 72 h. The formed colonies were used for subsequent bioassay screening.

LiCl treatment: Different concentrations of LiCl were added to PDA plates. In total, 4 ml spores suspension (10^6 spores/ml) of *M. purpureus* SM001 was evenly spread on PDA agar plates and was incubated at 30 °C for 72 h. The formed colonies were used for subsequent bioassay screening.

2.4. Bioassay screening for citrinin-free mutants

B. subtilis was grown in PDB culture at 30 °C for 24 h. After that, an aliquot (200 μl) of such culture was spread on a PDA plate. The mutant strains (precultures with size of 6 mm diameter) were placed on the PDA plate for 3 days to observe the size of inhibition zone. The strains with small or no inhibition zone were selected and further analyzed for citrinin production in the solid-state fermentation and submerged fermentation.

2.5. Plasmid construction

(i) *pDBJ051*: The 0.6-kb downstream DNA region (CTR) of the *pksCT* gene was amplified by PCR from *M. purpureus* SM001 genomic DNA using primers CTRF (5′-AAAA*GTCGAC*ATGGTAGCCTCTCGTGGTGT-3′) and CTRR (5′-AAAA*AGCTTG*GGAGTTCCAATAGCCTGAA-3′). The resulting PCR fragment was cloned into pDH2857 (Kim et al., 2003) through *Sal*I and *Hind*III sites to generate pDBJ051.

(ii) *pDBJ052*: The 1.4 kb upstream DNA region (CTL) of the *pksCT* gene was amplified by PCR from *M. purpureus* SM001 genomic DNA using primers CTLF (5′-AAAA*GAGCTC*AACAGTGAGAGCCACCAACA-3′) and CTLR (5′-CGG*GGTACC*GAGCGGGGAAAGAAGCAC-3′). The resulting PCR fragment was cloned into pDBJ051 through *Sac*I and *Kpn*I sites to generate pDBJ052.

(iii) *Binary vector pDBJ053*: pDBJ052 was digested with *Pvu*II and *Bgl*II to give a 5.0 kb fragment that contains the CTL-P*mgpd*-*hph*-T*trp*-CTR cassette. This fragment was then