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Lower Citrinin Production by Gene Disruption of *ctnB* Involved in Citrinin Biosynthesis in *Monascus aurantiacus* Li AS3.4384

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ABSTRACT: The filamentous fungi *Monascus* spp. have been used in the production of food colorants and health remedies for more than 1000 years in Asia. However, greater attention has been given to the safety of *Monascus* products because they contain citrinin, which is harmful to the hepatic and renal systems. The citrinin biosynthetic gene cluster has been characterized in *Monasucs aurantiacus*. The *ctnB* gene encoding an oxidoreductase is located between *pksCT* and *ctnA*. In this study, a *ctnB* replacement vector (pCTNB-HPH) was constructed to disrupt the *ctnB* gene with a hygromycin resistance gene as the selection marker. The linear vector was transformed into *M. aurantiacus* using the protoplast $CaCl_2$/polyethylene glycol (PEG) method. Three *ctnB*-disrupted strains were obtained by homologous recombination. In comparison to the parental strain, the ΔctnB mutants barely produced citrinin. These data confirmed that the *ctnB* gene is directly involved in citrinin biosynthesis. Moreover, the yields of the pigments of two disruptants were similar to that of the wild-type strain, but the yield of another mutant was slightly higher than that of the latter strain. These results indicate that the production of the mycotoxin citrinin was successfully eliminated through genetic engineering.

KEYWORDS: *Monascus aurantiacus, ctnB gene, gene knockout, transformation, citrinin*

INTRODUCTION

The filamentous fungi *Monascus* spp. have been used in Chinese fermented foods, such as red mold rice wine, red mold rice, and red fermented bean curd, among others, for thousands of years.[1] *Monascus* is known to produce several bioactive metabolites, including monacolin K (lovastatin), red pigments, and γ-aminobutyric acid. Monacolin K can reduce serum cholesterol levels by inhibiting the activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which is the rate-determining enzyme of the cholesterol synthetic pathway.[2] γ-Aminobutyric acid functions to alleviate hypertension,[3] and the red pigments have been used as a natural food colorant by food industries.[4] Monacolin K, pigments, and also a toxic compound citrinin are secondary metabolites synthesized from polyketides by *Monascus* spp., which are catalyzed by polyketide synthase (PKS).

Citrinin is known to have nephrotoxic, hepatotoxic, and carcinogenic activities, which greatly limit the wide application of *Monascus*-related products.[5] In recent years, citrinin, which occurs as a contaminant during the fermentation of *Monascus*, has become a cause for increasing concern. In Japan, the level of citrinin must not exceed 0.2 μg/g of the *Monascus* pigments used in food additives. Citrinin biosynthesis has been reported to begin with the condensation of one acetyl CoA and three malonyl CoAs via a fatty acid synthetase (FAS) and a PKS to form the pentaketide analogue, which is then converted into citrinin through a series of reactions, including methylation, reduction, oxidation, and dehydration.[6]

A citrinin biosynthesis gene cluster has been cloned in *Monascus purpureus*. The gene cluster spanned a 21 kb region containing six genes, including *pksCT*, *ctnA*, and *orf2*.[7,8] *pksCT* encodes a 2593 amino acid protein that contains conservative domains for ketosynthase, acyltransferase, and acyl carrier protein. Gene disruption and nucleotide sequence analyses of *pksCT* have suggested that it encodes a PKS involved in the synthesis of the citrinin polyketide backbone.[7] The *ctnA* gene encodes the $Zn(II)_2Cys_6$ binuclear DNA-binding protein. The disruption of the *ctnA* gene was determined to cause a significant decreases in the transcription of *pksCT*, leading to the reduction of citrinin production to barely detectable levels, indicating that *ctnA* is a major activator of citrinin biosynthesis.[8] The distribution of mycotoxin citrinin biosynthesis genes in 18 *Monascus* strains has also been previously investigated.[9] The production of citrinin in *Monascus* is consistent with the presence of the functional citrinin biosynthesis genes found only in *M. purpureus* and *Monascus kaoliang*. The strains of *Monascus ruber*, *Monascus pilosus*, and *Monascus barkeri* have been found to neither possess the *pksCT*, *ctnA*, and *orf3* genes nor able to produce citrinin. However, our previous results found that *M. pilosus*, *M. barkeri*, and *M. ruber* possessed a highly conserved *pksCT* gene and produced citrinin.[10] The same results were found in other *Monascus* strains, including *M. purpureus*, *Monascus aurantiacus*, and *Monascus anka*. Our results were found different from the results by Chen et al.,[9] and this might be due to the differences in genome plasticity among isolated strains. We also cloned citrinin biosynthesis genes in *M. aurantiacus*. These expected genes have been clustered within a 43 kb DNA region containing 16 open-reading frames, including a polyketide synthase (*pksCT*), a fatty acyl-CoA synthetase (*ctnI*), an oxygenase (*orf3*), short-chain dehydrogenases (*ctnE*, *orf1*, and *ctnH*), oxidoreductases (*ctnD*

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and *orf4*), transcriptional regulator (*ctnA*), membrane transport protein (*orf5*), and WD-repeat protein (*ctnR*).[11] The *orf7* gene is predicted to encode a conserved hypothetical protein, and its function remains unclear. Our previous research has shown that the *orf7* gene disruption leads to higher levels of citrinin than the wild-type strain by approximately 2.5-fold.[12]

In general, *M. purpureus*, *M. ruber*, and *M. anka* are highly useful for their ability to produce red pigments and monacolin K. One of our objectives is to promote the application of *Monascus*-fermented products in the development of health-food products if the citrinin content is reduced to below the allowed level. An understanding of the citrinin biosynthetic pathway may lead to the identification of strategies for overcoming citrinin contamination of *Monascus* fermentation products by gene-knockout technology. The *orf4* and *ctnD* genes, with unknown function in the citrinin cluster, are predicted to encode oxidoreductases. The *orf4* gene is designated as *ctnB*. We attempted to construct this gene disruptant to eliminate citrinin production, and the results confirmed the role of the *ctnB* gene in citrinin synthesis. First, a replacement-disrupted vector was constructed, after which the plasmid DNA was transformed into protoplasts of *M. aurantiacus*. Three *ctnB*-disrupted strains were ultimately analyzed.

MATERIALS AND METHODS

Fungal Strain and Growth Stations. *M. aurantiacus* Li AS3.4384 strain (Institute of Microbiology, Chinese Academy of Sciences, Beijing, China) was used for performing the *ctnB* gene disruption. *Monascus* strains were maintained on 2-(*N*-morpholino)ethanesulfonic acid (MES) medium[10] [6 °Bx wort and 1.2% (w/v) agar] for the propagation of *Monascus* spores.

Construction of *M. aurantiacus cntB* Disruption Vector. The *ctnB* disruption vector was constructed in two steps: First, a 0.8 kb *ctnB* coding region at the 3′ end was amplified by the polymerase chain reaction (PCR) using the following primers: B3, AAAGCATG-CAGCTTCATTGTTCCCACTGAGC; B4, CCCAAGCTTCTGAT-GACTAGGGAGCACCCG (nucleotides 10 414−11 248 in GenBank accession number EU309474). The PCR fragment after digestion with *Sph*I and *Hin*dIII was cloned into the corresponding sites in pUC18-HPH.[11] Second, the 1.2 kb DNA fragment (containing 5′-UTR and the coding region of the *orf3* gene) at the 5′ end of *ctnB* was generated by PCR with the primers B1, TGAGCTCGGTCCTATGATGATG-TACTGG; B2, TTGGTACCCCGGCTCGCTTTTTGGCTCCT (nucleotides 8800−10 015 in EU309474). The fragment digested with *Sac*I and *Kpn*I was cloned into the corresponding sites of the vector obtained from the first step. The disruption vector pCTNB-HPH was linearized by *Sac*I prior to fungal transformation.

Preparation of Protoplasts. In the initial transformation for the disruption of the *ctnB* gene, approximately 10^8 spores harvested from MES agar plates were inoculated into 50 mL of YPD medium [1% yeast extract (Oxoid, Basingstoke, U.K.), 2% tryptone (Oxoid, Basingstoke, U.K.), and 2% glucose] and cultured at 30 °C at 180 rpm for 35 h. The mycelia were harvested and resuspended in 20 mL of a filter-sterilized enzyme solution that contains 120 mg of lysing enzymes (L1412, Sigma, St. Louis, MO), 0.4 mL of cellulase (Sigma, St. Louis, MO), and 100 mg of snailase (Solarbio, Beijing, China) in 0.6 M $MgSO_4$ at pH 6.0. Snailase, extracted from crop and digestive tract of snails, consists of more than 30 enzymes, including cellulase, hemicellulase, β-glucuronidase, etc. The suspension was incubated for 2 h at 30 °C on a rotary shaker at 80 rpm. Protoplasts were collected by filtering through four layers of sterile lens-cleaning tissue and centrifuged at 1000*g*. The protoplasts were washed twice with 1.0 M D-sorbitol solution and finally resuspended in the same solution.

Transformation of *Monascus*. A total of 8 μg of *Sac*I-linearized plasmid DNA was added to 2−5 × 10^6 protoplasts in 200 μL of STC solution (1 M sorbitol, 50 mM Tris−HCl, and 50 mM $CaCl_2$ at pH 8.0) and 50 μL of cold PTC buffer [40% polyethylene glycol (PEG) 4000, 50 mM Tris−HCl, and 50 mM $CaCl_2$ at pH 8.0] and was then added to the reaction, followed by gentle mixing and incubation on ice for 30 min. Next, 900 μL of PTC solution was added, and the mixture was kept at room temperature for 20 min. After TB3 medium (0.3% yeast extract, 0.3% tryptone, and 20% sucrose) was added, the protoplast solution was spread onto a selection regeneration medium (MES broth: 0.6 M sucrose, 100 μg/mL hygromycin, and 1.2% agar). The mixture was incubated at 30 °C in the dark until colonies grew to the desired size for further analysis.

Genomic DNA Isolation. Transformants were selected from the selection medium after incubation for 4−5 generations. For PCR analysis, genomic DNA was extracted according to the glass beads method described by Yu et al.[19] For southern hybridization analysis, DNA was isolated from 1 g of mycelium that had been ground in extraction buffer [100 mM Tris−HCl, 20 mM ethylenediaminetetra-acetic acid (EDTA), 2% (w/v) sodium dodecyl sulfate (SDS), 2% (w/v) Triton X-100, and 1.5 M NaCl] in a mortar with quartz sand and a pestle. The resulting powder was incubated at 65 °C for 1 h and cooled. An equal volume of 5 M KAc was then added, and the mixture was kept at −20 °C for 30 min and centrifuged at 8000*g* for 15 min. The supernatant was extracted with an equal volume of phenol/chloroform (1:1, v/v) and precipitated with 0.6 volume of isopropanol. The DNA pellet was washed with 70% ethanol twice and dissolved in 200 μL of Tris−EDTA (TE) buffer after being air-dried for 15 min.

Confirmation of Gene Disruption by PCR, Dot Analysis, and Southern Blot Analysis. Genomic DNA of hygromycin-resistant transformants was amplified with the primers B5, TCTAGTG-CAGTGGTCGCTTGCT; B6, CGGACAACGAGAGTATCAGGC (nucleotides 10 017 and 10 389 in EU309474), which were used to amplify the deletion fragment of *ctnB* to confirm the disruption of the transformants. The template DNA was amplified using the following thermocycler parameters: 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 51 °C for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 10 min. To confirm the disruption of the *ctnB* gene, PCR of DNA from transformants was performed with the oligonucleotide primer pairs B7, TTTAACTCTCTGCTTCAAGAG (nucleotide 8640 in EU309474) and H3, GCCCGCAAACTTCTCGCCTTAT; H4, ATGGTTGCCTAGTGAATGCTC; and B8, GCGACTAGGGCA-GAGATCAT (nucleotide 11 383 in EU309474). The 3.1 kb *hph* cassette was amplified by PCR using primers for H1 (GAA-GATCTGGCCGTATGTTGTGTGGAAT) and H2 (GAA-GATCTCTCTAAACAAGTGTACCTGTG).

For dot blot analysis, 6 μg of genomic DNA was transferred onto a nitrocellulose membrane. For southern hybridization, 20 μg of genomic DNA was digested with *Bgl*II, electrophoresed on 0.8% agarose gel, and transferred onto a nitrocellulose membrane. Dot blot and sourthern hybridization assays were performed according to the protocols of the manufacturer (Dig High Prime DNA Labeling and Detection Starter Kit I, Roche, Indianapolis, IN).

Pigment Measurement. Rice was used as the substrate for red yeast rice production under solid-state fermentation. It was soaked in distilled water for 8 h. Next, the material was filtered to remove the water and dried in the shade. A total of 20 g of the dry substrate was placed in a 250 mL Erlenmeyer flask and autoclaved for 20 min at 121 °C. The substrate was inoculated with 1 mL of 10^6 spores of AS3.4384 and cultivated at 30 °C for 15 days. The pigments were measured using a spectrophotometer (Ultrospec 4300, GE, Fairfield, CT) according to China National Standards (GB4926-2008, red koji rice). The pigment concentration was estimated at 420 and 505 nm.[15] The results were expressed as optical density (OD) units per gram of dried medium multiplied by a dilution factor.

Citrinin Analysis. To determine citrinin production in *M. aurantiacus* strains, we inoculated 3 × 10^5 spores of AS3.4384 or the transformants into 100 mL of YES (4% yeast extract and 16% sucrose) broth. The culture was incubated at 30 °C for 16 days. The culture supernatant was extracted by methanol and further filtered with a 0.45 μm filter (Millipore, Billerica, MA). The citrinin concentration was determined by high-performance liquid chromatography (HPLC) on a C18 column (5 μm, 4.6 × 250 mm, Waters, Milford, MA). The mobile

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  1 MKGQTGLRSL ALLYISPLYI LERLPLKLSA PDTLVVRGSF IVPTEPLYPS ITMVQTNLEV
 61 VDDTLHLPRI LCLHGGGSNA AIFQAQCRRL IAQLRSEFRF VFAQAPFLSD AEPNVMSVYS
121 QWGPFRRWLR WCPDHPEIRP EDAIRAIDDC LEDVKRQDDA KGATGAWVGL LGFSQGAKMC
181 ASLLYRQQIR QELRGRSFAG SDYRFGVLLA GRAPLVSLDP DLDLNSSLPD VSQITDAKYH
241 GPSQDVLRIP TVHVHGMRDP HVDLHRQLFE EFCAPESRRL VEWDGDHRVP LKYNDVSLVA
301 YQIRELATQT GAP
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Figure 1. Deduced *ctnB* encoding the amino acid sequence. The conserved sequence G−X−S−X−G in common lipases is indicated by the shadowed box, whereas the catalytic triad Ser_{174}−Asp_{259}−His_{288} is underlined.

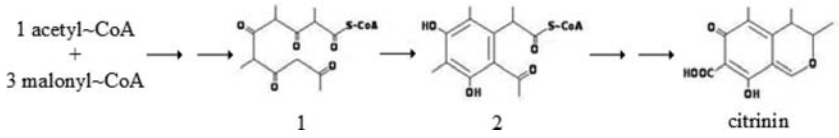

Figure 2. Proposed pathway participated by the CtnB protein.

phase consisted of acetonitrile and water (75:25), the pH level of which was adjusted to 3.0 with H_3PO_4. The flow rate was set at 0.8 mL/min. A 2475 multi-λ fluorescence detector (Waters, Milford, MA) was used at 331 nm of excitation and 500 nm of emission. Commercial citrinin (Sigma, St. Louis, MO) was used as a standard.

HPLC−Electrospray Ionization Mass Spectrometry (ESI−MS) Analysis. The HPLC−MS analyses were performed using a Waters ZQ 4000/2695 HPLC−MS system (Waters, Milford, MA). The ionization conditions were adjusted at 350 °C and 3 kV for capillary temperature and voltage, respectively. The nebulizer pressure was 65 psi, and the nitrogen flow rate was 11 mL/min. The full-scan mass covered the m/z range from 200 to 500 Da.

RESULTS AND DISCUSSION

Disruption of *ctnB* in *M. aurantiacus* Li AS3.4384. Sequence analysis and a database search using the BLASTP program[13] revealed that *ctnB* encoded an oxidoreductase with significant homology to hypothetical proteins found in *Botryotinia fuckeliana* (63% identity), *Aspergillus clavatus* NRRL 1 (57% identity), *Neosartorya fischeri* NRRL 181 (57% identity), and *Aspergillus kawachii* IFO 4308 (55% identity) at the amino acid level. Meanwhile, no significant homologies to known esterases or lipases were observed. The *ctnB* gene-encoded protein has putative conserved domains characteristic of the serine hydrolase or esterase−lipase superfamily. Research has shown that the amino acid sequence GFSQG starting at residue 172 (Figure 1), similar to the Gly−X−Ser−X−Gly motif, is found in most microbial serine hydrolases, such as lipase, esterase, and serine proteinase.[14] The atypical catalytic triad Asp/His/Ser found in most lipases was also present in the deduced amino acid sequence. Its putative location in the sequence is shown as Ser_{174}−Asp_{259}−His_{287} (Figure 1). Generally, the Ser−His−Asp catalytic triad catalyzed deacetylation. Therefore, we deduced that the CtnB protein can catalyze the same reaction and may convert intermediate 1 to intermediate 2 (Figure 2). More experimental data should be collected to support the hypothesis.

The disruption strategy for *ctnB* works such that double homologous recombination of the targeting vector pCTNB-HPH on both sides of *hph* with the *M. aurantiacus* genome would replace a 390 bp internal fragment of the *ctnB* coding region with *hph* and, therefore, disrupt *ctnB* (Figure 3a). The linearized plasmid pCTNB-HPH was transformed into the protoplasts of *M. aurantiacus* Li AS3.4384. In general, the transformants can be divided into four groups: gene replacement mutants through both the up- and downstream homologous regions, insertion type-I mutants through the

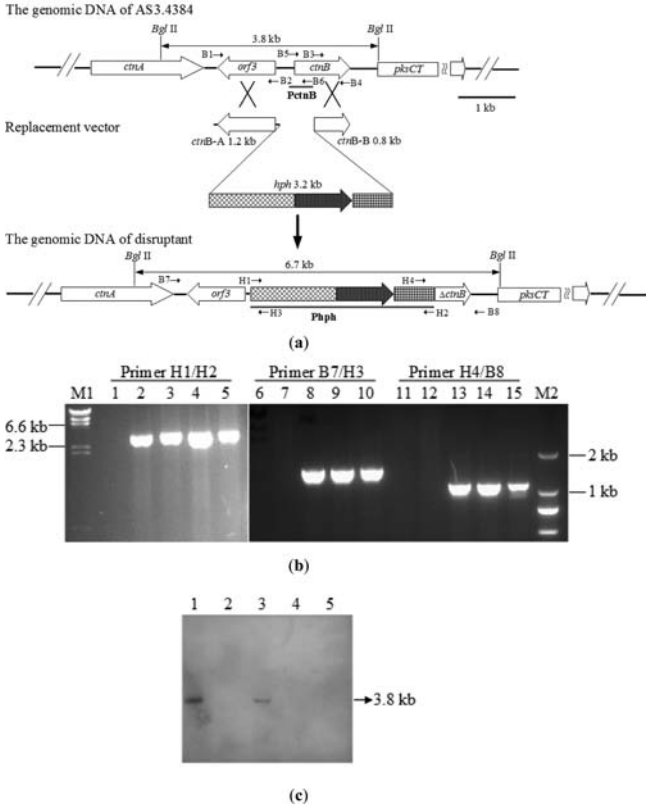

Figure 3. (a) Schematic diagram of gene disruption using the replacement vector pCTNB-HPH. The bar below *ctnB* indicates the position of the probe used in southern blot analysis. Primers for constructing the vector and screening were indicated. (b) PCR confirmation of the *ctnB* gene disruptants. Three primer pairs (primers B7/H3, H1/H2, and H4/B8) were used for confirmation of the double homologous recombination events. Lanes 1, 6, and 11, AS3.4384; lanes 2, 7, and 12, ctnB-0; lanes 3, 8, and 13, ctnB-1; lanes 4, 9, and 14, ctnB-2; and lanes 5, 10, and 15, ctnB-3. (c) Southern blot analysis of putative transformants using the PctnB probe. Lane 1, AS3.4384; lane 2, ctnB-1; lane 3, ctnB-0; lane 4, ctnB-2; and lane 5, ctnB-3.

downstream homologous region, insertion type-II mutants through the upstream homologous region, and random insertion transformants.[15] Moreover, while the target gene was disrupted by a single crossover, the revertant was generated from the disruptant by endogenous homologous recombination between the original gene, with the homologous region derived

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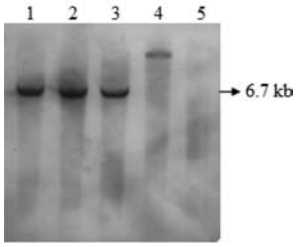

Figure 4. Southern blot analysis of putative transformants using the Phph probe against *Bgl*II-digested genomic DNA. Objective *ctnB* disruptants showed a 6.7 kb band. None was observed in the wild-type strain, whereas a 20 kb band was detected in the non-homologous recombination strain. Lane 1, ctnB-1; lane 2, ctnB-2; lane 3, ctnB-3; lane 4, ctnB-0; and lane 5, AS3.4384.

Table 1. Pigment Production during Solid-State Fermentation by ΔctnB Mutants and AS3.4384

strains	red pigment at 505 nm (units/g)	yellow pigment at 420 nm (units/g)
AS3.4384	195.3 ± 2.1	278.7 ± 1.5
ctnB-1	189.0 ± 2.1	266.3 ± 2.5
ctnB-2	196.0 ± 2.6	279.0 ± 3.1
ctnB-3	223.1 ± 2.6	285.3 ± 2.1

from the vector. After several rounds of cultivation from the spore of the *pksCT* disruptant without hygromycin, a *pksCT* revertant was generated and citrinin production was restored.[7] Because the insertion mutants and random insertion transformants all contain the deletion fragment, while the gene replacement mutants do not contain the deletion fragment, they can be identified by PCR amplifying the deletion fragment DNA. This method may be a simple and rapid selection for the gene replacement mutants from all transformants according to our results. Deletion of *ctnB* was confirmed by PCR with primers specific to the *ctnB* coding region. The primer pair B5/B6 generated a 0.39 kb fragment from the genomic DNA of the parental strain or non-homologous recombination strains that were not produced from the genomic DNA of disruptants (data not shown). Correct insertion of the disrupted gene was checked by PCR with either a forward primer located outside the homologous arm and a reverse primer inside of the *hph* cassette or with primers internal to the marker gene in the disruption cassette. Three primer pairs (primers B7/H3, H1/H2, and H4/B8) were used for confirmation of the double homologous recombination events (Figure 3a). A 1.7 kb fragment with primers B7 and H3, a 1.1 kb fragment with oligos H4 and B8, or a 3.1 kb product with primer pair H1/H2 were amplified from transformants ctnB-1, ctnB-2, and ctnB-3 (Figure 3b). This indicated that the 3.2 kb *hph* cassette had replaced the 0.39 kb region in the resident *ctnB* after double-crossover homologous integration. The putative disruptants were further characterized by dot blot and southern blot analyses. The dot blot analysis was identical with PCR amplification with primer pair B5/B6 (data not shown). As shown in Figure 3c, a probe corresponding to the *ctnB* coding region (PctnB) did not yielded any signal in a dot blot of genomic DNA of *ctnB* deletion mutants compared to the apparent signals in the wild-type strain and random-insertion mutant. A single hybridizing band is detected with the *hph* marker (probe Phph) in the transformants, but none is detected in the wild type, which revealed that the mutants carried a single integrated copy of the *ctnB* targeting construct. The formation of a 6.7 kb hybridizing band confirmed that the 3.2 kb *hph* selection marker was inserted correctly in the *ctnB* transformants (lanes 1−3 in Figure 4). Three transformants

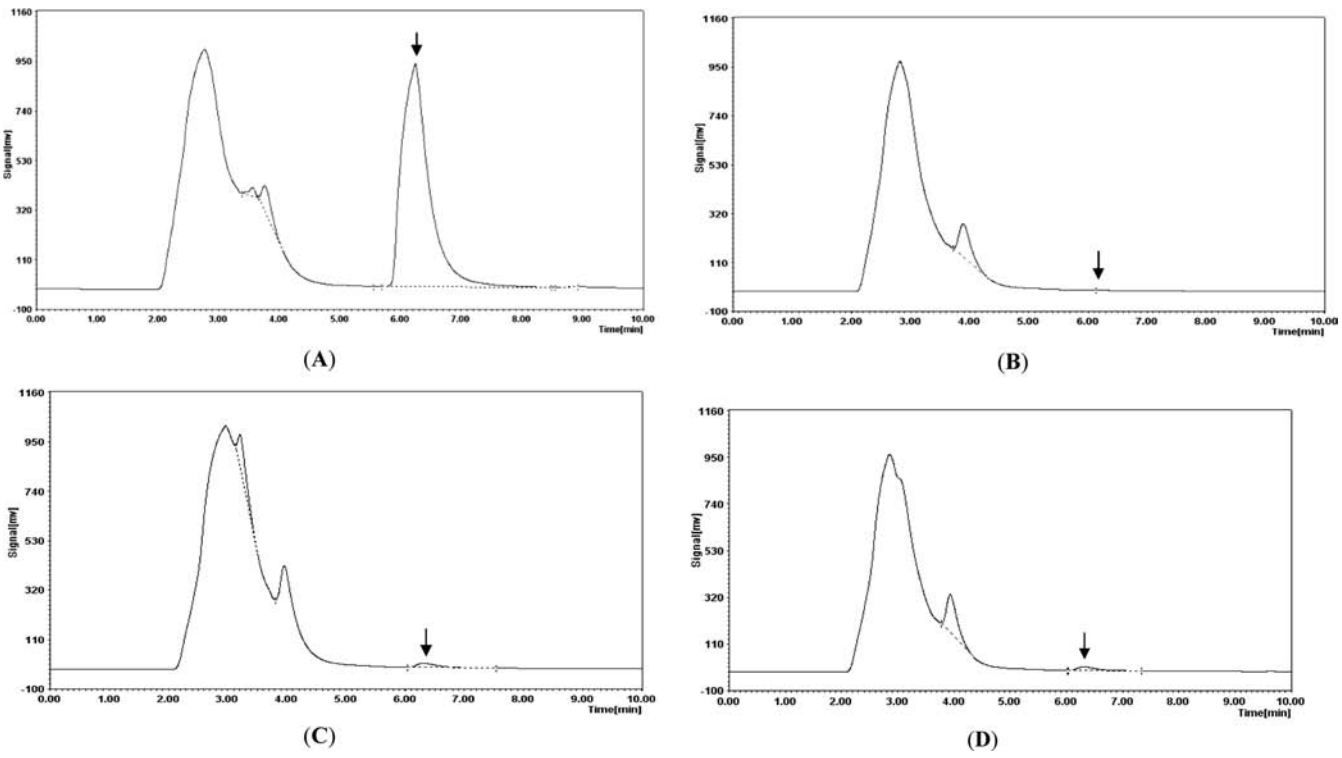

Figure 5. HPLC analysis of citrinin produced by the (A) wild type, (B) ΔctnB mutant ctnB-3, (C) ΔctnB mutant ctnB-2, and (D) ΔctnB mutant ctnB-1. The peaks for citrinin are indicated by arrows.

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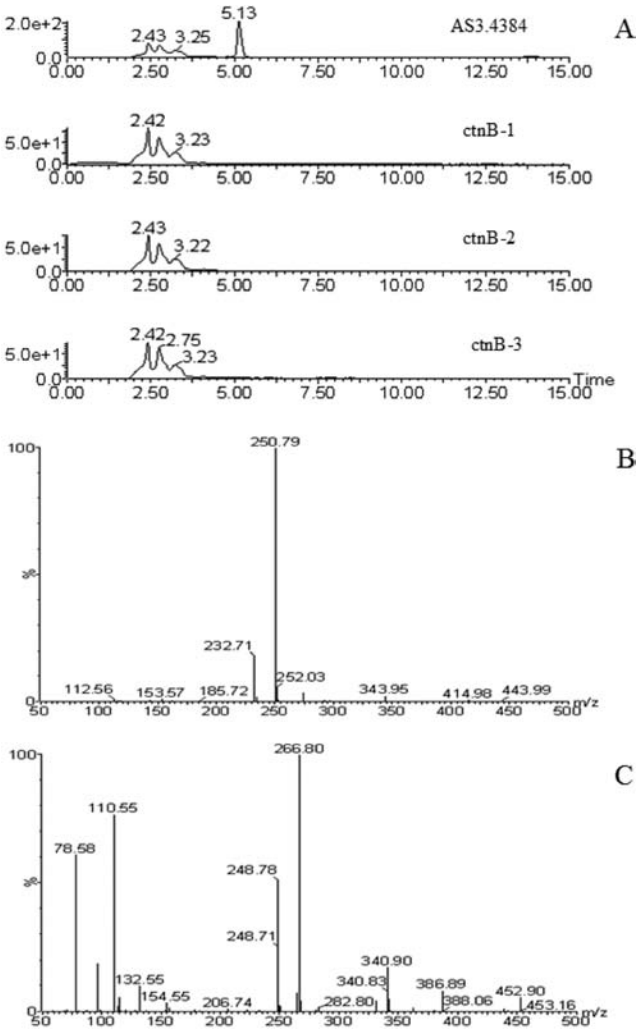

Figure 6. LC−MS for citrinin in a mobile phase of 80% acetonitrile and 20% aqueous 1% formic acid. The molecular weight of citrinin is 250.25. (A) Diode array detection of citrinin produced by the wild type and ΔctnB mutants. The retention time of citrinin was 5.13 min. (B) ESI positive-ion mode of citrinin. (C) ESI negative-ion mode of citrinin.

with a disrupted *ctnB* had been identified among 55 hygromycin-resistant transformants. These disruptants exhibited a similar colony morphology compared to the wild-type strain (data not shown).

Pigment and Citrinin Analysis. The production of pigments in the ΔctnB mutants was detected in solid-state fermentation. OD values representing yellow and red pigment production were determined using a spectrophotometer at 420 nm for the yellow pigment and 505 nm for the red pigment after 70% ethanol extraction. The results showed that the red and yellow pigments produced by ΔctnB mutants ctnB-1 and ctnB-2 were similar to the wild-type strain and that those of ΔctnB mutant ctnB-3 were slightly higher than the wild-type strain (Table 1).

Citrinin from the wild type was used as a control in HPLC analysis. After 10 days of cultivation, the citrinin retention time of the wild-type control was 6.46 min and detected using a fluorescence detector, with a calculated citrinin content of 22.5 μg/mL, and the amount of citrinin produced by the ΔctnB mutant ctnB-3 barely reached detectable levels (detection limit of 0.01 μg/mL), while ctnB-1 and ctnB-2 produced 0.08 and 0.07 μg/mL citrinin, respectively (Figure 5). Moreover, the result of liquid chromatography−mass spectrometry (LC−MS) of citrinin produced by the wild type and ΔctnB mutants (Figure 6) was identical to the result of HPLC. These data revealed that the *ctnB* gene is directly involved in citrinin biosynthesis.

Because of its toxicity, the amount of citrinin in *Monascus* products is strictly regulated in Japan, European Union countries, and the United States according to the new standards. The level allowed in Japan is lower than 0.2 μg/g for *Monascus* color. *Monascus* products should comply with strict limits for citrinin to ensure their safety. However, few *Monascus* products in China can meet this control standard for citrinin. Therefore, identifying ways on how to avoid the inclusion of citrinin warrants immediate attention. Some low-citrinin-producing mutants of *Monascus* have been obtained by genetic engineering.[10,15,16] Our results also revealed that disrupting the citrinin biosynthesis gene by knockout technology is more effective in controlling the production of citrinin than obtaining mutants by ultraviolet (UV)/chemical mutagenesis[17] or optimizing the growth conditions,[18] because it is difficult to eliminate citrinin production through the latter two methods. These results confirmed that the production of mycotoxin citrinin was successfully reduced through genetic engineering to meet the Japanese control standard for citrinin.

A previous study revealed that red pigments and citrinin begin with a common biosynthesis pathway and the same precursors.[6] Citrinin biosynthesis begins with one acetyl-CoA and three malonyl-CoAs condensing to a tetraketide, but pigments are produced from a hexaketide; this suggested the existence of a branch point at the tetraketide level, which could account for differential production of pigments and citrinin during the growth of *Monascus*. The blockage of the citrinin biosynthesis pathway would result in a reduction in citrinin production and an improvement of red pigments.[11,16] However, our results suggested that the biosynthesis of citrinin is independent of that of the pigments because the red pigments did not increase when the *ctnB* gene was disrupted. One could probably speculate that *Monascus* does not share enzymes responsible for citrinin biosynthesis and pigment synthesis, despite the same precursor. None of the enzymes required for citrinin and pigment synthesis has been identified, and few stable intermediates have been purified. The knowledge of genes in the citrinin synthesis cluster needs clarification to understand the biosynthesis pathway. The metabolic network is complicated. It deserves further study to determine whether or not citrinin synthesis gene-encoded enzymes play a role in red pigment production. The fact that the strong decrease in the citrinin content in the ΔctnB mutant does not lead to a strong increase in the red pigment content should be more deeply discussed.

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Notes

The authors declare no competing financial interest.

REFERENCES

(1) Lin, Y. L.; Wang, T. H.; Lee, M. H.; Su, N. W. Biologically active components and nutraceuticals in the *Monascus*-fermented rice: A review. *Appl. Microbiol. Biotechnol.* **2008**, *77* (5), 965−973.

(2) Tobert, J. A. Lovastatin and beyond: The history of the HMG-CoA reductase inhibitors. *Nat. Rev. Drug Discovery* **2003**, *2*, 517−526.

(3) Wu, C. L.; Lee, C. L.; Pan, T. M. Red mold dioscorea has a greater antihypertensive effect than traditional red mold rice in spontaneously hypertensive rats. *J. Agric. Food Chem.* **2009**, *57* (11), 5035−5041.

(4) Wild, D.; Toth, G.; Humpf, H. U. New monascus metabolite isolated from red yeast rice (angkak, red koji). *J. Agric. Food Chem.* **2002**, *50* (14), 3999−4002.

(5) Liu, B. H.; Wu, T. S.; Su, M. C.; Yu, F. Y. Evaluation of citrinin occurrence and cytotoxicity in *Monascus* fermentation products. *J. Agric. Food Chem.* **2005**, *53*, 170−175.

(6) Hajjaj, H.; Klaebe, A.; Loret, M. O.; Goma, G.; Blanc, P. J.; Francois, J. Biosynthetic pathway of citrinin in the filamentous fungus *Monascus ruber* as revealed by ^{13}C nuclear magnetic resonance. *Appl. Environ. Microbiol.* **1999**, *65* (1), 311−314.

(7) Shimizu, T.; Kinoshita, H.; Ishihara, S.; Sakai, K.; Nagai, S.; Nihira, T. Polyketide synthase gene responsible for citrinin biosynthesis in *Monascus purpureus*. *Appl. Environ. Microbiol.* **2005**, *71*, 3453−3457.

(8) Shimizu, T.; Kinoshita, H.; Nihira, T. Identification and *in vivo* functional analysis by gene disruption of *ctnA*, an activator gene involved in citrinin biosynthesis in *Monascus purpureus*. *Appl. Environ. Microbiol.* **2007**, *73*, 5097−5103.

(9) Chen, Y. P.; Tseng, C. P.; Chien, I. L.; Wang, W. Y.; Liaw, L. L.; Yuan, G. F. Exploring the distribution of citrinin biosynthesis related genes among *Monascus* species. *J. Agric. Food Chem.* **2008**, *56* (24), 11767−11772.

(10) Fu, G. M.; Xu, Y.; Li, Y. P.; Tan, W. H. Construction of a replacement vector to disrupt *pksCT* gene for the mycotoxin citrinin biosynthesis in *Monascus aurantiacus* and maintain food red pigment production. *Asia Pac. J. Clin. Nutr.* **2007**, *16* (Supplement 1), 137−142.

(11) Li, Y. P.; Xu, Y.; Huang, Z. B. Isolation and characterization of the citrinin biosynthetic gene cluster from *Monascus aurantiacus*. *Biotechnol. Lett.* **2012**, *34* (1), 131−136.

(12) Zou, L. H.; Li, Y. P.; Huang, Z. B.; Xu, Y. Construction of the *Monascus aurantiacus* AS3.4384 *orf7* gene deletion strains and functional analysis. *China Biotechnol.* **2011**, *31* (7), 79−84 (in Chinese).

(13) Altschul, S. F.; Wootton, J. C.; Gertz, E. M.; Agarwala, R.; Morgulis, A.; Schäffer, A. A.; Yu, Y. K. Protein database searches using compositionally adjusted substitution matrices. *FEBS. J.* **2005**, *272* (20), 5101−5109.

(14) Quevillon-Cheruel, S.; Leulliot, N.; Graille, M.; Hervouet, N.; Coste, F.; Bénédetti, H.; Zelwer, C.; Janin, J.; Van Tilbeurgh, H. Crystal structure of yeast YHR049W/FSH1, a member of the serine hydrolase family. *Protein Sci.* **2005**, *14* (5), 1350−1356.

(15) Jia, X. Q.; Xu, Z. N.; Zhou, L. P.; Sung, C. K. Elimination of the mycotoxin citrinin production in the industrial important strain *Monascus purpureus* SM001. *Metab. Eng.* **2010**, *12* (1), 1−7.

(16) Xu, M. J.; Yang, Z. L.; Liang, Z. Z.; Zhou, S. N. Construction of a *Monascus purpureus* mutant showing lower citrinin and higher pigment. *J. Agric. Food Chem.* **2009**, *57* (20), 9764−9768.

(17) Huang, Z. B.; Li, Y. P.; Wang, Y. H.; Xu, Y. Study on citrinin and pigments in fermented rice of *Monascus aurantiacus* (AS3.4384) and its mutant strains by high performance liquid chromatography. *Chin. J. Anal. Chem.* **2007**, *35* (4), 474−478 (in Chinese).

(18) Wang, J. J.; Lee, C. L.; Pan, T. M. Improvement of monacolin K, γ-aminobutyric acid and citrinin production ratio as a function of environmental conditions of *Monascus purpureus* NTU 601. *J. Ind. Microbiol. Biotechnol.* **2003**, *30*, 669−676.

(19) Yu, J. H.; Hamari, Z.; Han, K. H.; Seo, J. A.; Reyes-Domínguez, Y.; Scazzocchio, C. Double-joint PCR: A PCR-based molecular tool for gene manipulations in filamentous fungi. *Fungal Genet. Biol.* **2004**, *41* (11), 973−981.