Supplementary information

Transportation of citrinin is regulated by the *CtnC* gene in the medicinal fungus *Monascus purpureus*

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Materials and methods

Fungal strains and culture medium

The *Monascus purpureus* CCTCC CF 2008719 strain was gifted from the Institute of Fungal Resources of Guizhou University (Guizhou Province, China), which was utilized to generate the *CtnC* or *Cas9* over-expression strains. The *Cas9* chassis strain was used for in vitro sgRNAs transformation to generate the *CtnD* mutant strain. Sporulation medium (SPO) was composed of 100 g sucrose, 1 g KH_2PO_4, 0.5 g $MgSO_4·7H_2O$, 2 g $NaNO_3$, 0.5 g KCl, 0.01 g $FeSO_4·7H_2O$, 3 g yeast extract, 5 g tyrosine, 20 g agar, and dH_2O to 1000 mL. Induction medium (IM) was composed of 80 μL K-buffer (pH 5.7), 2.0 mL M-N buffer (3.0 g $MgSO_4·7H_2O$, 1.5 g NaCl, per liter), 100 μL 1% $CaCl_2·2H_2O$, 100 μL 0.1% $FeSO_4·7H_2O$, 250 μL 20% NH_4NO_3, 1.0 mL 50% glycerol, 1.0 mL 20% glucose, 4.0 mL MES (pH 5.5), 200 μL 100 mmol/L acetosyringone (Sigma-Aldrich, USA), 2 g agar, and dH_2O to 100 mL. CM regeneration medium was composed of 210.2 g sucrose, 6 g yeast leach powder, 6 g tyrosine, 20 g agar, and dH_2O to 1000 mL. For phenotypic observation, the Sabouraud dextrose agar (SDA) medium was used and supplemented with appropriate antibiotics. For the chemical analysis, DNA and RNA extraction, *M. purpureus* and the genetic transformants were fermented in Sabouraud dextrose broth (SDB) with antibiotics. For ATMT, the IM and co-cultivating medium (Co-IM, IM medium with halved glucose) were used. For protoplast regeneration, the CM regeneration medium was used. The Luria-Bertani (LB) medium supplemented with antibiotics was used to cultivate *Agrobacterium tumefaciens.*

Transformation of *CtnC* and *Cas9*

The expression vectors of pCAMBIA1303-CtnC (containing the PgpdA-GUS-CtnC-Tnos expression cassette) and pDHt/sk-PC (containing the Ppdc-toCas9-Tpdc expression cassette; Addgene No: 92125) were respectively transformed into *A. tumefaciens* EHA 105 by the freeze-thaw method. The bacteria were screened on LB plates containing 50 mg/L kanamycin (kan), and the resistant colonies were tested by PCR and GUS staining. The resulting positive strain was cultured in a liquid LB medium containing 50 mg/L kan, which had an OD_{600} value of 0.6. The *Monascus* spores were diluted to $1×10^6$/mL with the positive transformed *A. tumefaciens* solution*,* and 200 μL was spread on a Co-IM plate covered with a 0.45 μm nitrocellulose membrane. The culture was incubated in the dark at 25 ℃ for 3 d, and the *Monascus* colonies and the nitrocellulose membrane were transferred together to a new plate containing 20 mg/L hygromycin and 300 mg/L cephalosporins for screening. Three days later, the nitrocellulose membrane was removed. Resistant single colonies were selected for PCR testing using *Hyg*, *CtnC*, and *Cas9* primers (Table S1), and GUS staining was carried out on mycelium.

Table S1 PCR primers and sgRNA sequences

Target genes	Primer/sgRNA	Sequences (5'-3')	Targets length (bp)	Descriptions
Hyg	Hyg-F Hyg-R	atgtctgctatccctcctgc tcacttgtaagtgtagattg	383	Used to amplify the *Hyg*
GUS	GUS-F GUS-R	taccgacgaaaacggcaaga tccagttgcaaccacctgtt	235	Used to amplify the *GUS*
pyrG	pyrG-sgr	ggcttgaagttcctgcgttg<u>TGG</u>	/	Target sequence of *pyrG* sgRNA (PAM underlined)
	pyrG-F pyrG-R	gttgaaacgaaccccagcac ccacatcgacatcctctccg	380	Used to amplify the near region of *pyrG* sgRNA
CtnC	CtnC-sgr	tgcctatgaactttccccta<u>TGG</u>	/	Target sequence of *CtnC* sgRNA (PAM underlined)
	CtnCgr-F CtnCgr-R	ccgtaccaccaccatcgaaa ggttcccaacgtcatgacct	338	Used to amplify the near region of *CtnC* sgRNA
	CtnC-1 CtnC-2	atgtctgctatccctcctgc tcacttgtaagtgtagattg	316	Used to amplify the *CtnC*
	CtnC-F CtnC-R	cattggtaaatggggtctgg tatgtctcgggaagggtgag	157	Used for RT-qPCR of *CtnC*
Actin	actin-F actin-R	ttcgagaccttcaacgccc accctcgtagatgggaacga	135	Used for RT-qPCR of *Actin*
CtnA	CtnA-F CtnA-R	aaatgccgcgaagaagaca ggaattgcgcggaaagg	105	Used for RT-qPCR of *CtnA*
CtnD	CtnD-F CtnD-R	ctactccatcgtcgtcagca ctatgccagcagcaaaaca	143	Used for RT-qPCR of *CtnD*
CtnE	CtnE-F CtnE-R	gacaccaaggcaaagttggt tacatcccagttggcactca	117	Used for RT-qPCR of *CtnE*
CtnF	CtnF-F CtnF-R	ccaggcaatccacaatctct gaggagacgaccaattccac	135	Used for RT-qPCR of *CtnF*
pksCT	pksCT-F pksCT-R	gatttagtcaaggcgcgaaga cgtagttcctggcggatttg	105	Used for RT-qPCR of *pksCT*
MpPKS5	MpPKS5-F MpPKS5-R	tgtccgacgagtttctgcaa tatcaacgctgcttgggcat	134	Used for RT-qPCR of *MpPKS5*

Preparation of protoplast

After culturing the *Cas9*-expressing *Monascus* chassis strain on SPO plates containing 20 mg/L hygromycin for 2 weeks, the spores were washed with sterile water to make spore suspension with a concentration of 1×10^8 mL^{-1}. The spore suspension was spread on the SDA plate covered with cellophane, and the hyphae were scraped after culturing for 40 h. Approximately 0.2 g of mycelium were collected, washed with 1.0 mol/L $MgSO_4$, resuspended in 15 mL of enzyme solution (1.0 mol/L $MgSO_4$ buffer with 0.3% lyase enzyme, 0.1% cellulase, and 1% snailase), and incubated at 60 r/min and 30 °C for 2.5 h. The mixture was then filtered and centrifuged at 4000 r/min and 4 ℃ for 10 min, and the pellet was resuspended in a 1.0 mol/L precooled sorbitol solution (1 mol/L sorbitol, 0.1 mol/L Tris-HCl pH 7.5) and centrifuged again. The pellet was resuspended in STC buffer (1 mol/L sorbitol, 50 mmol/L Tris-HCl pH 8.0, and 50 mmol/L $CaCl_2$), and the concentration of protoplasts was adjusted to 1×10^7 cells/mL.

Transformation of protoplasts with sgRNAs

The *pyrG* (orotidine-5′-decarboxylase gene, GenBank: GU723506.1) and *CtnC* were compared with the genome (GenBank: GCA_011319195.1) of *M. purpureus*, the consensus sequence was submitted to http://crispor.tefor.net/, for the online design of sgRNA and detection primers. The sgRNA was synthesized using an in vitro transcription kit (Inovogen, China) and detected by electrophoresis. Approximately 5 µL of CtnC-sgRNA or pyrG-sgRNA were added to 100 µL protoplast suspension and kept on ice for 30 min. The suspension was added to 125 µL of PTC buffer (40% PEG4000, 50 mmol/L Tris-HCl pH 8.0, and 50 mmol/L $CaCl_2$) and stayed at room temperature for 5 min, followed by the repeated addition of PTC seven times. The protoplast mixture was spread on CM regeneration medium containing 20 mg/L hygromycin, 20 g/L uridines,

and 1.5 mg/mL 5-FOA (5-fluoroorotic acid; Solarbio, China) and cultivated at 28 °C for 3–7 d. The resistant strain was amplified by PCR using CtnCgr-F/R primers, and the product was digested by T7E1 (Beijing Weishanglide, China) and analyzed by sequencing.

Analysis of red pigment

The spores of wild and mutant strains were inoculated in a liquid fermentation medium at 28 °C. After 14 days of fermentation, the mycelium was filtered, dried at 45 °C, and crushed. The analysis of red pigment yield was based on the method of Li et al. (2015) with a slight modification. A total of 0.100 g mycelium powder was accurately weighed in a 10 mL stopper tube, and 10 mL of 70% ethanol was added to the extract at 60 °C for 1 h and filtered by the filter paper. The absorbance values of the filter liquor at 505 nm were detected using a spectrophotometer (the pigment content=OD_{505}×Dilution ratio×volume of extraction solvent/sample weight).

Analysis of citrinin

The citrinin detection method was partly referred to the Chinese standard of GB 5009.222-2016, weighing 0.500 g of dry mycelium powder into a 15 mL centrifuge tube, and adding 10 mL of extract solution (methanol: water=7:3). After vortex mixing for 30 min and filtering, 1.0 mL of the filtrate was taken and mixed with 39 mL of PBS buffer and then filtered with glass fiber filter paper. Then 1 mL of filtrate and 19 mL of PBS buffer passed through the immunoaffinity column (Qingdao Puruibang, China) at a flow rate of 1–2 drops/s. The column was then rinsed with 10 mL of 0.1% Tween 20-PBS solution at a flow rate of 1–2 drops/s, and the flow-through solution was discarded. Then, 1.0 mL of eluent (methanol: 0.1% phosphoric acid=7:3) was added at a flow rate of 1–2 drops/s. The citrinin content was detected by HPLC on a C_{18} column (5 μm, 4.6×250 mm; Waters, USA) at λ_{ex}=331 nm and λ_{em}=500 nm with a fluorescence detector. The citrinin was detected at a flow rate of 1.0 mL/min, an injection volume of 20 μL, and a column temperature of 30 °C. The column was equilibrated and eluted with the mobile phase (acetonitrile: 0.1% phosphoric acid=75:25).

RT-qPCR for gene expression analysis

Specific primers used for expression analysis of genes of *CtnA* (GenBank: AB243687), *CtnC*, *CtnD* (GenBank: EU309474), *CtnE* (GenBank: EU309474), *CtnF* (GenBank: EU309474), *pksCT* (GenBank: AY954027) and *MpPKS5* (GenBank: KC148521) are listed in Table 1, and each production size was 100–250 bp to ensure the accuracy of quantitative real-time PCR (RT-qPCR) results. RT-qPCR reactions were performed with the ABI StepOne Detection System (Applied Biosystems, USA). The qPCR reaction consisted of 2 μL templates, 7.5 μL 2×SYBR Premix, 300 nmol/L of each primer, and 0.3 μL ROX (TaKaRa, Japan). Expression data were analyzed using StepOne software (ABI, Applied Biosystems, USA) and transcript levels were calculated relative to the reference gene *Actin* (GenBank: AJ417880). Each gene in each sample was analyzed for at least three technical replicates.

Reference

Li YP, Tang X, Wu W, et al., 2015. The *CtnG* gene encodes carbonic anhydrase involved in mycotoxin citrinin biosynthesis from *Monascus aurantiacus*. *Food Addit Contam: Part A*, 32(4):577-583. https://doi.org/10.1080/19440049.2014.990993