

## Transformation of *Monascus purpureus* to hygromycin B resistance with cosmid pMOcosX reduces fertility

**Kamolnan Lakrod<sup>#</sup>**

Department of Microbiology  
Faculty of Science, Kasetsart University  
Bangkhen, Bangkok, 10900 Thailand  
Tel: 66 2 5793769  
Fax: 66 2 5798781  
E-mail: kamolnanl@missioncollege.edu

**Chulee Chairisook**

Department of Microbiology  
Faculty of Science, Kasetsart University  
Bangkhen, Bangkok, 10900 Thailand  
Tel: 66 2 5793769  
Fax: 66 2 5798781  
E-mail: fsciclc@ku.ac.th

**Daniel Z. Skinner<sup>\*</sup>**

USDA-ARS and Department of Crop and Soil Science  
209 Johnson Hall, Washington State University  
Pullman, Washington 99164-6420, USA  
Tel: 509 335 8696  
Fax: 509 335 2553  
E-mail: dzs@wsu.edu

**Financial support:** Thai Research Fund, Graduate School of Kasetsart University.

**Keywords:** biolistic, cosmid, hygromycin, *Monascus purpureus*, transformation.

**Present address:** #Mission College, Muak Lek Campus, 195 Moo 3, Tambon Muak Lek, Muak Lek District, Saraburi Province 18180, Thailand. Tel: 66 36 344777, Fax: 66 36 341629.

**Albino strain KB20M1 of *Monascus purpureus* was genetically transformed to hygromycin B resistance with cosmid pMOcosX, using biolistic bombardment. Conidia and mycelial fragments were used as the recipient material and rupture disk strengths of 6.2 and 9.3 MPa each yielded a transformed isolate from five bombardments. Southern analysis suggested a single copy of the cosmid had integrated into each of the transformants. Both of the independent transformants formed cleistothecia but ascospore formation was greatly reduced or absent, suggesting the integration and expression of the genes carried on pMOcosX interfered with fertilization and/or ascospore formation in this homothallic fungus.**

in meat preservation (Fink-Gremmels et al. 1991). Several studies have focused on optimizing pigment production for use in foods (Chu and Poon, 1993; Berset et al.1995; Teng and Feldheim, 2000; Teng and Feldheim, 2001). The fungus also is of interest from a medical standpoint because it produces monacolin K, an inhibitor of cholesterol biosynthesis (Ma et al.2000). Hence, numerous genes in this fungus are of potential interest from a food and medicine point of view. Studies of genes are greatly facilitated by the availability of a genetic transformation system, but genetic transformation has not been reported in *M. purpureus*. This study was undertaken to develop a simple and robust genetic transformation system applicable to genetic study of *M. purpureus*.

### MATERIALS AND METHODS

#### Microorganism and cultivation

The microorganism used was *Monascus purpureus*. UV-

*Monascus purpureus* is a homothallic fungus found on red rice (Anka or Ang-kak). It has been used as a natural food colorant and traditional medicine in Asia for centuries (Chu and Poon, 1993) and has been used as a substitute for nitrite

\* Corresponding author

induced albino mutant KB20M1 (Yongsmith et al. 2000) was used as the recipient in fungal transformations. Cultures were maintained on modified complete medium (CM) containing 0.5% sucrose, 0.6% yeast extract, and 0.6% casein hydrolysate at 28°C in the dark.

Growth inhibition of KB20M1 by hygromycin B was determined prior to transformation experiments by inoculation of the fungus to CM containing 6.25, 12.5, 25, 50, 100 or 200 µg/ml hygromycin B. Ten replications were incubated at 28°C in the dark and growth rates recorded after 14 d.

### **Biolistic transformation**

Recipient material for biolistic transformation of KB20M1 consisted primarily of conidia. KB20M1 was cultured on CM at 28°C in the dark for 2 weeks. Conidial masses, and some hyphal fragments, were prepared in sterile water by scraping the culture surface with a bent glass rod and pipetting the resulting slurry to a 15-ml sterile conical tube. Conidial masses were concentrated by centrifugation in a Dynac II Centrifuge (Clay Adams, USA) at 2,300 rpm for 20 min and removing the supernatant with a pipette. Approximately 0.5 cm<sup>3</sup> (about 0.2 g) of wet conidial masses were placed in the center of the 9 cm CM plate as the target recipient cells for microprojectile bombardment.

Cosmid pMOcosX (Orbach, 1994) obtained from the Fungal Genetic Stock Center, Kansas City, KS, USA, carrying a hygromycin B resistance gene was used as the exogenous DNA in microprojectile bombardment. The pMOcosX DNA was purified by Aldevron Corp., Fargo, North Dakota, USA.

M5 tungsten microparticles (0.4 µm diameter, Bio-Rad, USA) were surface sterilized in 70% ethanol with vortexing for 3 min, three times. The particles were resuspended at 60 mg/ml in 50% (v/v) glycerol and vortexed at setting 4 on a Fisher Genie 2 Vortex mixer (Fisher Scientific, USA) for 30 min, after which were added 10 µl of pMOcosX DNA at 2 µg/µl, 75 µl of 2.5 M CaCl<sub>2</sub> and 25 µl of 0.1 M Spermidine free base. The suspension was vortexed at the highest speed for 5 min, then incubated on ice for 3 min; this process was repeated three times. The mixture then was incubated on ice for 30 min, centrifuged for 30 sec at 13,000Xg, and the supernatant was removed. The tungsten pellet was gently washed in 300 µl of cold 70% ethanol, and then with 300 µl of cold absolute ethanol. The final cosmid-coated tungsten pellet was briefly vortexed and aliquots of 20 µl containing about 2 µg of DNA and 300 µg of tungsten particles were spread at the center of macrocarriers for a biolistic transformation apparatus (PDS-1000/Helium-driven Biolisticparticle Delivery System, Bio-Rad, USA) and air-dried in a laminar flow hood.

The particle gun was assembled with the distance between the particle launch site and the target cells at 6 cm. A 4.5-cm length of 5 ml pipetter tip was assembled to the macrocarrier's holder to focus the direction of bombardment straight into the target cells (J.P. Fellers, personal communication). The helium gas pressures (rupture disk break pressures) used were 6.2 and 9.3 MPa. The bombardment chamber was evacuated to 85 Kpa prior to bombardment. Five bombardments were conducted at each pressure. Following bombardment, the plates were incubated overnight at room temperature. The bombarded cells were then scraped from the plate surfaces and suspended in 2 ml sterile water. CM agar plates were inoculated with 0.5 ml of the suspension and incubated overnight at room temperature. The bombarded cultures were then overlaid with 12 ml of 0.7% agarose (SeaKem LE) in CM containing 200 µg/ml hygromycin B.

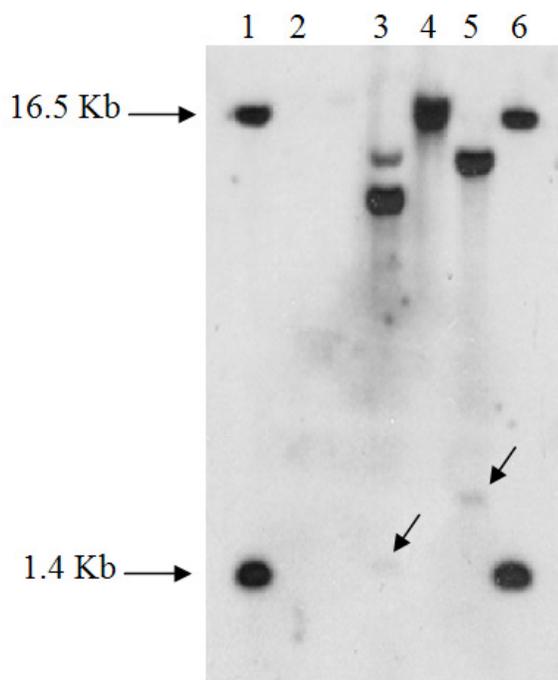
For Southern blot analysis, 15 µg of genomic DNA of putative transformants were isolated and digested singly with *Xho*I, *Xba*I, which linearize pMOcosX, and *Eco*RI. The digested DNA of transformants and undigested DNA of the albino recipient were subjected to gel electrophoresis for 5.5 hrs at 6.25V cm<sup>-1</sup> using 0.8% SeaKem LE agarose in 0.5 X TBE buffer. Random primer labeling was used to incorporate <sup>32</sup>P into 25 ng of pMOcosX used as a probe to detect the integration of this vector in transformants.

### **RESULTS AND DISCUSSION**

Growth of KB20M1 was completely inhibited by 100 µg/ml hygromycin B in CM media. Two hygromycin B-resistant colonies, one from each rupture disk pressure, were found on CM overlaid with 200 µg/ml hygromycin B in 0.7% agarose following biolistic bombardment with pMOcosX, a frequency of one transformant per 10 µg DNA at each pressure. Each transformant resulted from five trials at each pressure, a 20% rate of success. Control plates with KB20M1 with the hygromycin B overlay supported no fungal growth. Single conidia of the hygromycin resistant colonies were isolated and the monoconidial isolates were studied further. There were no significant differences in the growth rates of the transformants and the albino recipient KB20M1 on PDA without hygromycin B (data not shown). Growth of the transformants was inhibited by higher concentrations of hygromycin B, but both transformants continued to grow with hygromycin B concentration in the media up to 1.0 mg/ml, the highest concentration tested ([Table 1](#)).

Southern blot analysis of the transformants indicated two fragments in the DNA digested with *Xho*I, which linearizes the cosmid, and three fragments in the DNA digested with *Eco*RI, which cuts the cosmid at two sites, hybridized to the pMOcosX probe ([Figure 1](#)). In the DNA digested with

*Xba*I, which also linearizes the cosmid, a broad band hybridized to the probe, which probably indicated two fragments of similar size (Figure 1). These results suggested a single copy of the cosmid had integrated into the transformant.

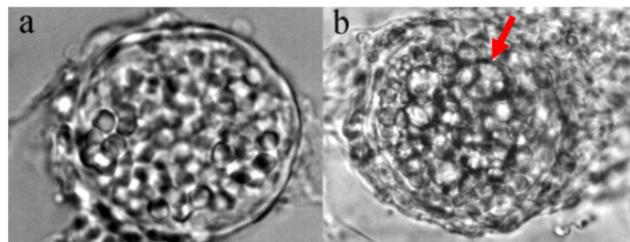


**Figure 1.** Southern blot analysis of a *Monascus purpureus* isolate biologically transformed with cosmid pMOcosX and a particle delivery pressure of 6.2 MPa.

Lanes 1 and 6: size marker, lane 2: uncut DNA from untransformed recipient strain KB20M1, lanes 3-5: transformant DNA digested with *Eco*RI, *Xba*I and *Xho*I, respectively.

To investigate if morphological characteristics of the transformants were affected by the pMOcosX insertion, the transformants were compared to the albino recipient KB20M1 on PDA, SS, CYA, MEA and G25N culture media. Colonial, mycelial and conidial morphological measurements of the transformants and the albino recipient were all statistically indistinguishable (data not shown). However, the transformants failed to produce ascospores, or were extremely delayed in ascospore formation. The albino recipient formed abundant cleistothecia with an abundance of ascospores within 5 days on all media used. Within 5 days of culture initiation, like the albino, the transformants formed apparent cleistothecia, but both transformants either did not produce ascospores at all (after eight months of growth), or infrequently produced a few ascospores after about three months of growth. In the transformants, the cleistothecial walls appeared to be less well-organized than in the recipient strain, and relatively large, variable-sized structures were found instead of ascospores (Figure 2). Dozens of the cleistothecia from the transformants were isolated and crushed over a period of

eight months. In most cases, no ascospores were found and the round structures from within the cleistothecia easily broke into small pieces, suggesting that they were simply oil droplets. This phenomenon was observed in both transformants on the five culture media (above), with and without hygromycin B in the culture media, and therefore is not a direct effect of the hygromycin B.



**Figure 2.** Cleistothecia from (a) recipient *Monascus purpureus* strain KB20M1 and (b) KB20M1 transformed with cosmid pMOcosX.

Both cultures were grown for 10d on PDA. The arrow in (b) points to one of the variable-sized, relatively large structures that were produced instead of ascospores in the transformant. Both (a) and (b) were photographed at 600X.

## CONCLUDING REMARKS

These results showed that hygromycin B selection and cosmid pMOcosX can be used in biolistic transformation of whole conidia and mycelial fragments of *M. purpureus*. Cosmid pMOcosX conferred robust resistance, supporting growth on media containing 1 mg/ml hygromycin B. The two transformants we obtained, both apparently carrying a single copy of the cosmid, did not produce ascospores after eight months growth, compared to abundant ascospore production after 5d growth of the recipient strain. It may be possible to take advantage of this phenomenon with gene expression studies, possibly elucidating the control of ascospore formation in this homothallic fungus. It is not known how frequently this phenomenon occurs in hygromycin B resistant transformants of *M. purpureus*, but occurred in these two independent transformants, which to our knowledge, are the only transformants of *M. purpureus* produced to date.

## ACKNOWLEDGMENTS

To Dr. Busaba Yongsmith, Faculty of Science, Department of Microbiology, Kasetsart University, Bangkok, Thailand, for providing fungal strain KB20M1.

## REFERENCES

BERSET, C.; CLERMONT, H.S. and CHEVAL, S. Natural red colorant effectiveness as influenced by absorptive supports. *Journal of Food Science*, July/August 1995, vol. 60, no.4, p. 858-861.

**Lakrod, K. et al.**

CHU, S.W. and POON, Y.K. Submerged production of *Monascus* pigments. *Mycologia*, March/April 1993, vol. 85, no. 2, p. 214-218.

FINK-GREMMELS, J.; DRESEL, J. and LEISTNER, L. Use of *Monascus* extracts as an alternative to nitrite in meat products. *Fleischwirtsch*, October 1991, vol. 71, p. 1184-1186.

MA, J.; LI, Y.; YE, Q.; LI, J.; HUA, Y.; JU, D.; ZHANG, D.; COOPER, R. and CHANG, M. Constituents of red yeast rice, a traditional Chinese food and medicine. *Journal of Agricultural and Food Chemistry*, November 2000, vol. 48, no. 11, p. 5220-5225.

ORBACH, M.J. A cosmid with a Hyg<sup>R</sup> marker for fungal library construction and screening. *Gene*, January 1994, vol. 150, no.1, p. 159-162.

TENG, S.S. and FELDHEIM, W. The fermentation of rice for anka pigment production. *Journal of Industrial Microbiology and Biotechnology*, September 2000, vol. 25, no. 3, p. 141-146.

TENG, S.S. and FELDHEIM, W. Anka and anka pigment production. *Journal of Industrial Microbiology and Biotechnology*, May 2001, vol. 26, no. 5, p. 280-282.

YONGSMITH B.; KITPRECHAVANICH V.; CHITRADON L.; CHAISRISOOK C. and BUDDA, N. Color mutants from *Monascus* sp. KB9 and their comparative glucoamylases on rice solid culture. *Journal of Molecular Catalysis B: Enzymatic*, October 2000, vol. 10, no. 1-3, p9. 263-272.

## APPENDIX

## Tables

**Table 1. Growth on various concentrations of hygromycin B of *Monascus purpureus* isolates biolistically transformed to hygromycin B resistance.**

Isolate	Colony growth <sup>a</sup> on PDS with hygromycin at µg/ml concentration of									
	0	100	125	200	300	400	500	600	700	1000
KB20M1 <sup>b</sup>	45.5	<sup>c</sup>	-	-	-	-	-	-	-	-
900PSI	46.0	45.1	44.8	44.5	43.6	41.4	39.0	35.4	26.8	12.2
1350PSI	45.1	43.5	43.8	43.4	42.2	40.2	39.8	30.5	20.5	13.5

<sup>a</sup> Colony diameter in mn of 8 day cultures, means of two duplicate cultures.

<sup>b</sup> Non transformed recipient isolate.

<sup>c</sup> No growth.