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Selection of an Effective Red-Pigment Producing *Monascus pilosus* by Efficient Transformation with Aurintricarboxylic Acid

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The filamentous fungus *Monascus pilosus* was genetically transformed with a reporter plasmid, pMS-1.5hp, by aurintricarboxylic acid (ATA) treatment to obtain an efficient red-pigment producing mutant. The transformation efficiency of *Monascus pilosus* was higher with the ATA-treatment than with either a non-restriction-enzyme-mediated integration (REMI) or a REMI method. This valid and convenient random mutagenesis method shows that ATA can be applied in fungi for efficient genetic transformation.

Monascus spp. are filamentous fungi known to produce various secondary metabolites with polyketide structures, such as pigments, monacolins, and citrinin.[1,2] The red pigments produced by the various species of *Monascus* are extensively used as natural food colorants.[3] They have enormous commercial value in terms of increasing the production of pigments. Previous studies have shown that growth of *Monascus* at high temperatures, a high concentration of NaCl, and greater oxygen concentrations can increase the yield of pigments.[4,5] Development of an efficient method of inducing random mutagenesis in *Monascus* should allow for the isolation of a genetic transformant with increased productivity of red pigment. Typically, fungal protoplasts are prepared for the purpose of delivering the transforming DNA into the filamentous fungus. During this process, the protoplasts are mixed with a combination of $CaCl_2$ and polyethylene glycol (PEG), which promotes the movement of the transforming DNA into the protoplasts. In mammalian systems, aurintricarboxylic acid (ATA) is used to inhibit the activity of DNaseI, S1 nuclease, exonuclease III, and RNAse A.[6] This has been found to be useful in protecting plasmid DNA from nuclease degradation and increasing the transfer efficiency of DNA in the respiratory tissues and the skin of mice, pigs, and macaques.[7–11] Nevertheless, there have been few studies in which ATA was used in fungal transformation. In this study, the use of ATA was investigated for its effect on the genetic transformation of *M. pilosus*. Furthermore, the efficiency of genetic transformation was analyzed and compared between ATA-treatment and restriction-enzyme-mediated integration (REMI), which employed a combination of $CaCl_2$ and PEG. Additionally, we were able to obtain a high red pigment-producing *M. pilosus* by pretreating the fungal protoplasts with ATA.

To screen the *M. pilosus* transformants for high productivity of red pigment, the pMS-1.5hp plasmid, which includes a fusion of the hygromycin B resistance gene (HPH) with enhanced green fluorescent protein (EGFP), was constructed. Based on a bacterial artificial chromosome (BAC) library constructed from *M. pilosus* BCRC38072,[12] a primer set (Mps9259F: AGTGGCA-GCCAACCCTCACC and Mps7782R: CGGGCTGA-TAGAGCAGATAGATAGATG) was designed to amplify the promoter of the *hsp*90 gene (heat shock protein) (GenBank accession no. DQ983312). Since Hsp90 is the most abundant protein and occupies 1–2% of all cellular proteins in the cytosols of eukaryotic cells,[13] it can strengthen the production of hygromycin-resistance protein using the *hsp90* promoter from *M. pilosus*. The human cytomegalovirus (CMV) promoter sequence in the pHygEGFP plasmid (BD Biosciences Clontech, Palo Alto, CA) was replaced with the *hsp*90 gene promoter from *M. pilosus* BCRC38072 to
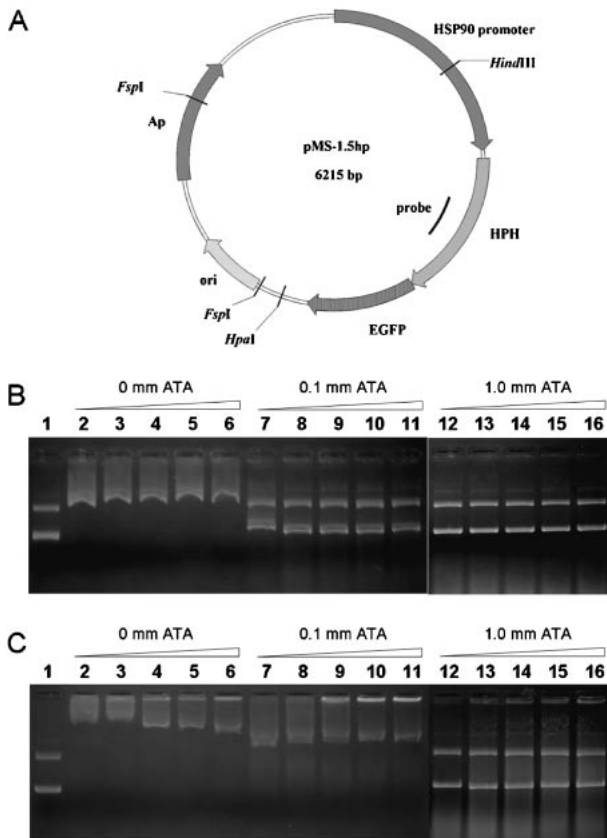
Fig. 1.　Map and Stability of pMS-1.5hp Plasmid.

A, Map of pMS-1.5hp plasmid used to overexpress a fusion protein of the hygromycin B resistance gene (HPH) with enhanced green fluorescent protein (EGFP). The pMS-1.5hp plasmid was modified from the pHygEGFP plasmid. The small black bar represents the probe for analyzing integrations by Southern hybridization. A time course of the stability of the pMS-1.5hp plasmid in the *M. pilosus* BCRC38072 lysate was analyzed by including total protein of $1 \, mg \, ml^{-1}$ (B) or $3.5 \, mg \, ml^{-1}$ (C). Five µg of the pMS-1.5hp plasmid was incubated at $37 \, °C$ with the lysates for 5, 15, 30, 45, and 60 min, containing 0, 0.1, and 1.0 mm ATA. The plasmid DNA was separated on 1.0% agarose gel by electrophoresis. Lane 1, untreated control plasmid without lysate; lanes 2–6, plasmid DNA with lysate protein containing 0 mm ATA incubated for 5, 15, 30, 45, and 60 min; lanes 7–11, plasmid DNA with lysate protein containing 0.1 mm ATA incubated for 5, 15, 30, 45, and 60 min; lanes 12–16, plasmid DNA with lysate protein containing 1.0 mm ATA incubated for 5, 15, 30, 45, and 60 min.

yield the pMS-1.5hp plasmid (Fig. 1A). This vector was used to achieve overexpression of the dual-function marker for genetic transformation in *M. pilosus* BCRC38072. Furthermore, a time course of pMS-1.5hp plasmid stability was analyzed in lysates from *M. pilosus* pretreated with 0, 0.1 and 1.0 mm ATA. We observed that 0.1 mm ATA protected the plasmid in $1 \, mg \, ml^{-1}$ of protein lysate, whereas the stability of the plasmid decreased in $3.5 \, mg \, ml^{-1}$ of protein lysate (Fig. 1B, C). These results indicate that the presence of higher ATA concentrations (1.0 mm) can prolong the stability of plasmid DNA.

The REMI method was established to improve the frequency of transformation substantially and has been adopted for use in various filamentous fungi.[14–16] The method is based on the addition of a restriction enzyme into the protoplasts and transforming DNA mix, such that the specific restriction enzyme generates a site for inserting DNA into the genomic locus.[14] The REMI method increases the efficiency of transformation, but different efficiencies of transformation are achieved by adding different restriction enzymes at different enzyme concentrations.[14,17] To understand the efficiency of genetic transformation with ATA treatment (1.0 mm), three procedures, including non-REMI, REMI, and ATA-treatment, were carried out by the PEG-based protoplasts method.[18] The conidia from 1 week culture of *M. pilosus* were incubated in 100 ml of Vogel medium for 16–18 h at $30 \, °C$. The mycelia were harvested on miracloth (Millipore, Billerica, MA) and washed in MA digestion solution (0.1 m maleic acid, pH 5.5, and 1.2 m $(NH_4)_2SO_4$). A mixture of 100 mg of Yatalase, 100 mg of lysing enzyme, and 100 µl of β-glucuronidase was sufficient to convert hyphae into protoplasts when digested for 4–5 h in 50 ml of digestion solution, after which the protoplasts were used in genetic transformation. The protoplast yield for *M. pilosus* was $1.0–3.0 \times 10^7 \, ml^{-1}$. The non-REMI method using *Fsp*I-linearized pMS-1.5hp had the lowest efficiency of genetic transformation (17 ± 2 transformants per 5 µg of linear plasmid), while the *Fsp*I-REMI had an efficiency of genetic transformation (48 ± 4 transformants per 5 µg of linear plasmid) 2.8-times greater than non-REMI treatment. The *Hpa*I-REMI had a 7-times higher efficiency of genetic transformation (119 ± 11 transformants per 5 µg of linear plasmid) than the non-REMI treatment. ATA-treatment yielded a 9.4-times higher efficiency of genetic transformation (159 ± 24 transformants per 5 µg of linear plasmid) than the non-REMI transformation, and a 3.3- and 1.3-times higher efficiency of genetic transformation than *Fsp*I-REMI and *Hpa*I-REMI respectively. These results suggest that adding ATA to the protoplasts increases the efficiency of genetic transformation in *M. pilosus* BCRC38072. This result is consistent with results reported for the transformation of the respiratory tissues and skin of mice, pigs, and macaques.[7–11] The ATA effect on genetic transformation is probably a result of ATA's ability significantly to reduce nuclease activity in cells, which prevents the transforming DNA from being degraded.[8] The results of this study are also consistent with the findings of Cantone and Vandenberg, who indicated that adding ATA results in a high efficiency of genetic transformation in the pathogen *Paecilomyces fumosoroseus*.[14]

Thirty transformants with increased red pigment production were screened from ATA-treatment, and that which had the highest production of red pigment was characterized. These results indicate that random mutagenesis by an efficient genetic transformation method using ATA can produce a transformant with a high red-pigment production level. To determine the
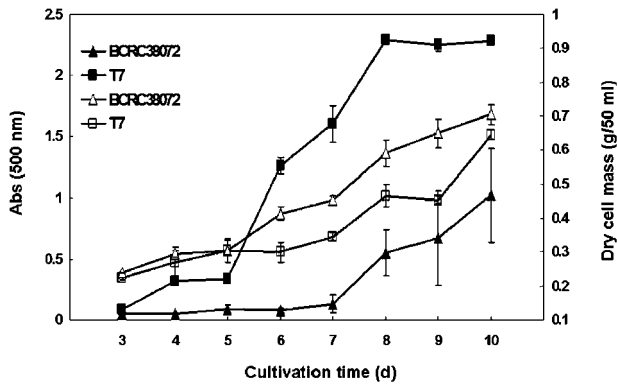
Fig. 2. Submerged Cultures of Wild-Type BCRC38072 and the T7 Transformant Incubated on a 200-rpm Rotary Shaker for 10 d at 25 °C.

△, dry cell mass of the wild type; □, dry cell mass of the transformant; ▲, red pigment production of the wild type; ■, red pigment production of the transformant. The red pigment level was detected by measuring the absorbance at 500 nm using a spectrophotometer.

level of production of red pigment, the wild-type and the most effective pigment-producing transformant were harvested from liquid medium (7% glycerol, 3% glucose, 3% monosodium glutamate, 1.2% polypetone, 0.2% $NaNO_3$ and 0.1% $MgSO_4 \cdot 7H_2O$) after incubation for 3 to 10 d at 25 °C with constant agitation. Aliquots from the wild-type and the transformant culture were cleared of cells and analyzed at 500 nm using a spectrophotometer. The T7 transformant was compared with the wild type during the submerged fermentation process, which lasted for 10 d. The red-pigment production of the T7 transformant was 4- to 5-fold higher than the corresponding values for the wild type, while the cell mass from the culture revealed no significant difference (Fig. 2). After 5 d of cultivation, red-pigment production increased in the T7 transformant, and the maximum red pigment level was reached after 8 d of cultivation. Red pigment was also produced by the wild-type, and reached maximum level during the 10th d of cultivation.

The most effective red pigment-producing transformant and seven other transformants of *M. pilosus* BCRC38072 were selected for Southern hybridization and fluorescence microscopy analysis. Their identities were also verified by transferring the stably grown colonies onto the new plates with hygromycin. The eight transformants were identified by Southern hybridization using *Hin*dIII and *Pvu*II restriction enzymes (Fig. 3A, B). *Hin*dIII cuts at one site in the pMS-1.5hp plasmid, while *Pvu*II does not cut within the plasmid. The *hph* gene probe was DIG-labeled by PCR amplification using the PCR DIG probe synthesis kit (Roche Diagnostics, Mannheim, Germany). The primer set for the *hph* gene was as follows: pMS-f, GAACTGCCCGCTGTTCTG-CA, and pMS-r, ACATCGCCTCGCTCCAGTCA. The results of Southern hybridization showed that the T5 transformant had a single copy integration, while T6 and T8 revealed two integration events (two copies). Using
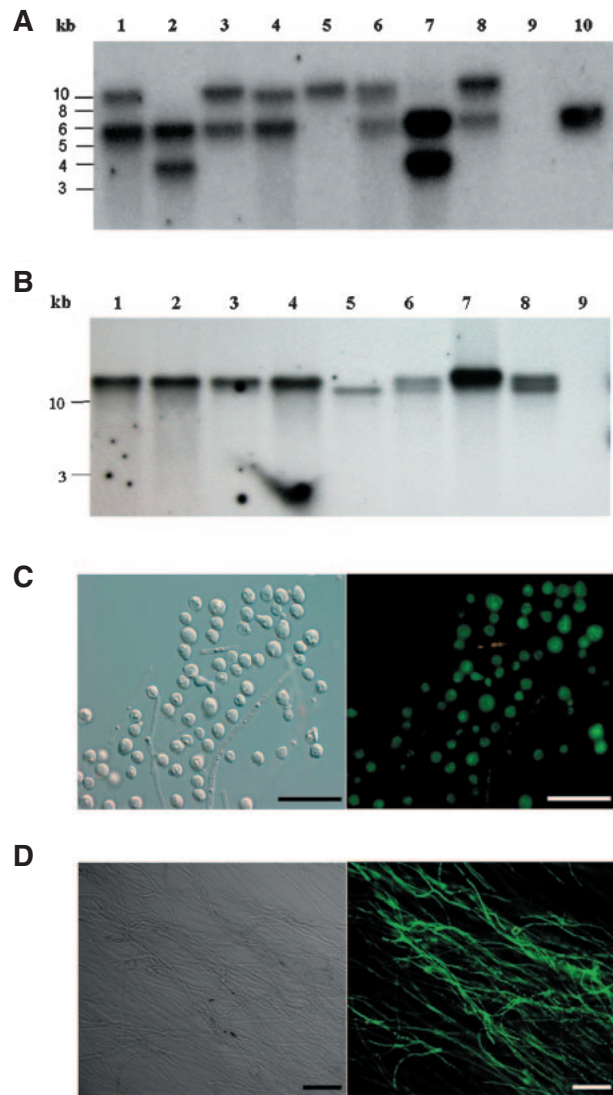

Fig. 3. Analyses of Southern Hybridization and Fluorescence of *M. pilosus* Transformants.

Southern hybridization analyses of the genomes of eight transformants digested with *Hin*dIII (A) or *Pvu*II (B) and hybridized with an *hph* probe. Lanes 1–8 show DNA from the transformants (T1–T8); lane 9 shows DNA from wild-type *M. pilosus* BCRC38072; lane 10 shows the plasmid, pMS-1.5hp. Southern hybridization was performed using a DIG labeling system. Enhanced green fluorescent protein (EGFP) was expressed in the transformed *M. pilosus*. The conidia (C) and hyphae (D) of the transformant were analyzed by fluorescence (right panel) and bright field microscopy (left panel). The small bars represent distances of 50 μm and 100 μm in the conidia (C) and hyphae (D) respectively. The photograph was taken at a magnification of 1000× with an exposure time of 1 to 2 s.

the *Hin*dIII restriction enzyme, 6-kb and 10-kb fragments were observed in the transformants labeled T1, T3, and T4, while 4-kb and 6-kb fragments corresponded to the transformants that were labeled T2 and T7 (Fig. 3A). However, only one fragment (>10 kb), corresponding to transformants T1, T2, T3, T4, and T7, was detected when *Pvu*II was used as the restriction enzyme (Fig. 3B). These results suggest that the plasmid integrated as a tandem repeat in trans-

formants T1, T2, T3, T4, and T7. This was attributed to rearrangement of the integrated plasmid. Further studies of the loci of gene disruption and the mechanism of gene regulation should clarify the molecular mechanisms by which the biosynthesis of red pigment occurs. To confirm that the transformants expressed EGFP from the integrated DNA, expression of EGFP was detected (Fig. 3C, D) by fluorescence microscopy (Leica RXA, Mannheim, Germany). Fluorescence photomicrography of the specimens was conducted, and fluorescence was clearly observed in the transformants when the samples were excited with a red filter. The filter (Chroma, Rockingham, VT), with a peak transmission of excitation at 450 to 490 nm and a peak transmission of suppression at 525 to 575 nm, was used to detect EGFP. These results indicate that the EGFP protein was extensively expressed in the mycelia and spores. Moreover, the most effective red-pigment producing mutation was stably inherited through the next generation after several rounds of cultivation.

In conclusion, this study indicates that the nuclease inhibitor aurintricarboxylic acid (ATA) can be adopted to transform *M. pilosus* stably. The addition of ATA increased the efficiency of genetic transformation in *M. pilosus*. A major advantage of ATA is that different restriction enzymes need not be tested. Due to the fact that the transformant that produces high amounts of red pigment can be isolated by random mutagenesis with efficient genetic transformation using ATA, this valid and convenient method can be used to screen transformants with high amounts of monacolin K, as well as other highly economic polyketides.

Acknowledgments

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