Development of transformation system in *Monascus purpureus* using an autonomous replication vector with aureobasidin A resistance gene

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Abstract

To enhance the variety of genetic tools and thus to promote molecular genetic study, aureobasidin A and its resistance gene were adopted as a new marker system together with the incorporation of the Gateway system to facilitate the introduction of long heterologous DNA fragments into *Monascus purpureus*. The minimum inhibitory concentration of aureobasidin A against *Monascus* was 0.05 μg/ml and a transformation efficiency of 17 colonies/μg DNA was obtained by the protoplast-PEG method with the vector pAUR316, containing the aureobasidin A resistance gene. Southern analysis of the transformants confirmed that pAUR316 exists as an independent vector, demonstrating that the AMA1 sequence acts as the autonomous replication sequence in *M. purpureus*. Through the use of the Gateway system, a polyketide synthase gene (7.8 kbp) responsible for citrinin biosynthesis was introduced. As a result, the transformants showed 1.5-fold higher production of citrinin than the wild-type strain.

Introduction

In fungal transformations, the autonomous-replication vectors are generally more effective than the genome-integration vectors for introducing an intact gene to complement a gene disruptant or for expressing a heterologous gene to analyze its function, because side effects arising from vector integration into the genome can be avoided and the autonomous-replication vectors show a high transformation efficiency (Ruiz-Diez 2002). In filamentous fungi, the AMA1 sequence derived from *Aspergillus nidulans* is reported to work as a replication origin and the plasmid containing the sequence gives 10–30 copies per genome as an autonomous replication vector (Gems et al. 1991). In addition, the development of an easy way to introduce a large DNA fragment would enhance the utility of replication vectors and expand their use in fungi.

Antibiotic resistance has a great advantage over auxotrophic complementation because it can be applied to any strain that is sensitive to the antibiotic. Furthermore, it can avoid laborious experiments of constructing an auxotrophic mutant as the host and cloning a complementing gene. Aureobasidin A (AbA), from *Aureobasidium pullulans* R106, is a cyclic depsipeptide antibiotic with a molecular weight of ca. 1100 Da (Takesako et al. 1993). AbA inhibits inositol-phosphorylceramide synthase, which catalyzes a key reaction of fungal sphingolipid biosynthesis (Zhong et al. 2000), and shows high toxicity against fungi and yeast. Recently, an AbA resistance gene was isolated from *Aspergillus* and applied to several fungi as a selection system (Kuroda et al. 1999).

Fungi belonging to the genus *Monascus* have been used for centuries in Asian countries to produce fermented food. Secondary metabolites
produced by Monascus species, such as red pigments (Hajjaj et al. 2000), monacolin K, and γ-aminobutyric acid (GABA), are compounds of medicinal importance. Some studies have focused on the transformation in Monascus with the protoplast-PEG method or Agrobacterium tumefaciens-mediated method (Campoy et al. 2003, Kim et al. 2003), but only genome-integration vectors have been used. For further study on Monascus, a wider variety of genetic tools is urgently needed.

In this study, the efficiency of the autonomous-replication vector and the aurebasidin A resistance system was evaluated to increase the variety of genetic tools in Monascus. Integration of the Gateway system into this vector system was demonstrated to be successful for introducing large DNA fragments, such as those of polyketide synthase genes.

**Materials and methods**

**Strain, growth conditions, and transformation**

Monascus purpureus IFO30873, wild-type strain, was cultivated for 7–10 days at 28 °C on a plate (2% agar) of Monascus cultivation (MC) medium consisting of 50 g glucose/l, 7.5 g polypeptdon/l, 2.0 g NH₄H₂PO₄/l, 0.5 g MgSO₄·7H₂O/l, 0.1 g CaCl₂·2H₂O/l, and 2.0 g KNO₃/l. For liquid cultivation, a mycelia mat (square of about 1 cm²) was taken from an agar plate, inoculated into 100 ml of MC medium in a 500-ml baffled Erlenmeyer flask, and incubated for 5 days at 28 °C with shaking at 120 strokes per minute (spm).

**Transformation protocol**

Transformation was done as described by Shimizu et al. (2005) with the following modifications. The vector pAUR316 (Takara Bio, Inc., Otsu, Japan), containing the AMA1 sequence and an AbA resistance gene derived from Aspergillus nidulans, was used for transformation. After mixing with the protoplast, the mixture was spread on MC medium containing 0.1 µg AbA/ml (TaKaRa). The transformants were maintained on MC plates with 0.1 µg AbA/ml.

**Southern and Northern blot analyses**

Southern and Northern blot analyses were conducted essentially by standard methods (Sambrook et al. 1989). For Southern blot analysis, genomic DNA (20 µg) was digested with restriction enzyme overnight, separated on 1% agarose gel, and transferred to a Hybond N+ membrane (Amerham Biosciences). For Northern blot analysis, total RNA (8 µg) was separated on 1% agarose–formaldehyde gel. The probes were amplified by PCR from ampicillin resistance gene in pAUR316 and pksCT gene with the following primer sets; amp-F (5’-GTAGATAACTACGATAACGGG-3’) and amp-R (5’-TATGTTGCAG CGGTATTATCC-3’), and pksCT-F (5’-GGAA TTCTGCAGAAGAGTAATGTCCTTA-3’) and pksCT-R (5’GG AATTCTGCAGAAGAGTAATGTCCTTTA-3’). The position of the pksCT probe is shown in Figure 2a below. The probe was labeled with [α-32P]dCTP using the Random Primer DNA Labeling Kit Ver. 2 (Takara Bio Inc., Otsu, Japan). The signal intensity of the appeared bands was quantified by measuring the radioactivity of the corresponding area on the hybridized membrane with a Beckman Coulter LS6500.

**Construction of pAG-CT vector**

The vector pAG was constructed by inserting Reading Frame Cassette C1 (att-Cm-cciDB-att cassette) (Invitrogen) into the blunt-ended BamHI site in pAUR316, resulting in pAG (Figure 2b). Three separate fragments [6.8-kbp EcoRI–SalI, 1.6-kbp SalI, and 2.5-kbp EcoRI (Figure 2a)] were sequentially introduced into the corresponding sites of pENTR11 (Invitrogen), resulting in pENT-CT which contains whole pksCT gene (DDBJ accession no. AB167465) and its promoter region (Figure 2b). pAG-CT was obtained through the recombination reaction between pAG and pENT-CT by the Gateway system.

**Analysis of citrinin production**

The amount of citrinin was measured in the mycelia and in the filtrate after separation of the mycelia. The filtrate (10 ml) was directly loaded onto a C18 Sep-Pak Plus cartridge (2 ml). After