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Agrobactrium tumefaciens-Mediated Transformation of *Monascus ruber*

Yang, Yun-Jung and Inhyung Lee*

Department of Foods and Nutrition, Kookmin University, Seoul 132-706, Korea

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***Agrobacterium tumefaciens*-mediated transformation (ATMT) was successfully applied to *Monascus ruber*. The optimum cocultivation time was 84 h with an efficiency of 900 to 1,000 transformants when 1×10^6 spores were used with the same volume of bacteria. The stability of transformants was over 98% after five generations. When *M. ruber* was transformed with *A. tumefaciens* YL-63 containing the green fluorescent protein gene (*egfp*), the green fluorescent signal was observed throughout hyphae, confirming expression of the gene. This efficient transformation and expression system of *M. ruber* by ATMT will facilitate the study of this fungus at a molecular genetic level.**

Keywords: *Monascus ruber*, *Agrobacterium tumefaciens*, fungal transformation

Filamentous fungi of the genus *Monascus* have been applied to produce fermented foods such as *To-Fu-Yo* and *Benikouji* in East Asian countries for a long time, dating back at least to the first century A.D. [8]. Today, they are also used for industrial production of functional dietary supplements because of their capacity to produce various bioactive secondary metabolites. These include monacolin K (also known as lovastatin, mevinolin, and mevacor), an antihypercholesterolemic agent [6, 7], and γ-aminobutyric acid (GABA), an antihypertensive agent [20]. However, *Monascus* also produces the compound citrinin, which has nephrotoxic and hepatotoxic properties [1, 2, 18] and therefore limits the industrial application of *Monascus*. *Monascus ruber* is known to be among the highest producers of citrinin [1], making it an excellent model for the study of citrinin biosynthesis. Unfortunately, this fungus is currently poorly characterized at a molecular genetic level.

Understanding the biosynthesis of secondary metabolites is important for efficient industrial applications. A few studies about the biosynthesis of secondary metabolites in *Monascus* have been reported [9], but molecular genetic studies are often hindered by lack of molecular genetic tools. Recently, genetic transformations including protoplast transformation, polyethylene glycol-mediated protoplast transformation, and electroporation [4, 6, 11, 13] have been successful in certain *Monascus* species [4, 11, 12]. However, these methods often had low transformation efficiency and low mitotic stability. A transformation method with high efficiency and mitotic stability has not previously been reported in *M. ruber*. Recently, application of *Agrobacterium tumefaciens*-mediated transformation (ATMT) has been applied as an alternative transformation method to fungi that were difficult to transform with traditional methods or for which the traditional protocols failed to yield stable DNA integration. The ATMT system has been greatly successful in many filamentous fungi including Ascomycetes, Basidiomycetes, and Zygomycetes [13]. This system was shown to improve transformation frequency by up to 600-fold as compared with conventional techniques in other fungi [5, 16, 17]. In this study, we have established a protocol for optimized *A. tumefaciens*-mediated transformation of *M. ruber* that should greatly facilitate future molecular genetic studies of this fungus and allow us to gain a better understanding of the genetics underlying citrinin biosynthesis.

Materials and Methods

Monascus ruber KCTC6122 was obtained from the Korean Collection for Type Cultures (Daejeon City, Korea). Stock culture, growth, and spore preparation have been described previously [15]. *A. tumefaciens* AGL-1 (pBHt2) and *A. tumefaciens* YL-63 that contains the EGFP gene were obtained from Dr. Y.-H. Lee at Seoul National University [14]. These were grown at 28°C for 24 h in a minimal medium [10] supplemented with kanamycin (75 μg/ml).

For *A. tumefaciens*-mediated transformation, *A. tumefaciens* cells in a 2-ml aliquot were harvested and washed with an induction medium [3]. The cells were subsequently resuspended in 3 ml of an induction medium containing 200 mM acetosyringone and grown for an additional 6 h. The induced cells were mixed with an equal

*Corresponding author
Phone: 82-2-910-4771; Fax: 82-2-910-5249;
E-mail: leei@kookmin.ac.kr

volume of a conidial suspension of *M. ruber*. These mixed cells (100 μl) were plated on nitrocellulose filters (0.45 μm pore size and 45 mm diameter) (Whatman International Ltd, Maidstone, England) lying on a cocultivation medium containing or lacking 200 mM acetosyringone. The cocultivation medium was the same as an induction medium except it contained 5 mM instead of 10 mM glucose. Following cocultivation at 25°C for various times, the fungal and bacterial cells on the filter were transferred to a YM agar plate containing hygromycin B (100 μg/ml) as the selection agent for fungal transformants, and cefotaxime (200 μg/ml) in order to eliminate the *A. tumefaciens* cells [11]. Individual transformant was transferred to a C-medium containing hygromycin B (100 μg/ml) and incubated until conidiation. Conidia of the individual transformant were suspended with sterile water and inoculated into PDB containing hygromycin B (100 μg/ml) for molecular analysis.

To determine the mitotic stability of the transformants, randomly selected putative transformants were successively cultured on a C-medium for five generations in the absence of hygromycin B. After the fifth generation, transformants were tested for resistance to hygromycin B (100 μg/ml).

The introduction of the marker gene in transformants was confirmed by PCR and Southern analysis. The PCR primers were hph1 (5'-GAATTCAGCGAGAGCCTGAC-3') and hph2 (5'-GGATCCGGTCGGCATCTACT-3'). The amplification protocol consisted of an initial denaturation at 95°C for 5 min; followed by 30 cycles of 45 sec denaturation (94°C), 1 min annealing (55°C), and 1 min polymerization (72°C); and a final 5 min polymerization at 72°C.

Southern blots were made essentially as described by Sambrook *et al.* [19]. Genomic DNA (10 μg) isolated from randomly selected hygromycin-resistant transformants was digested with EcoRI, which cuts once in the T-DNA. Digested genomic DNA was separated on a 0.8% agarose gel, and transferred to a Hybond N^+ membrane (Amersham Biosciences, Piscataway, NJ, U.S.A.). The probe, a 0.8 kb *hph* gene fragment, was labeled with digoxigenin-dUTP using the DIG DNA Labeling kit (Roche, Basel, Switzerland). Hybridization and detection of signal were carried out following the manufacturer's instructions.

For evaluation of expression of the transformed gene, *M. ruber* was transformed using *A. tumefaciens* YL-63 that contains the EGFP gene. Conidia of a randomly selected transformant were cultured on 5 ml of YM broth containing hygromycin B (100 μg/ml) at 30°C for 12 h with shaking. The young hyphae were harvested by centrifugation at 12,000 ×*g* for 5 min, and washed with and resuspended in 500 μl of sterile water. Finally, the hyphae were observed using a fluorescence microscope.

Results and Discussion

The main objective of this work was to establish optimized conditions of ATMT for *M. ruber*. According to Michielse *et al.* [13], factors influencing ATMT efficiency in fungi were fungal starting materials, *Agrobacterium* and host strains, acetosyringone concentration, cocultivation conditions such as cocultivation time, conidia concentration (the number of conidia per ml), and *Agrobacterium* cell volume (μl). In this study, the starting material was the most generally used type of conidia, and the *Agrobacterium* strain was the supervirulent derivative, AGL-1.

The pretreatment of bacterial cells with acetosyringone, a compound that induces the expression of virulence genes in *A. tumefaciens* during induction, yielded more transformants than without acetosyringone; however, this difference was not significant (data not shown). By contrast, there were no transformants in the absence of acetosyringone in the cocultivation medium, suggesting that the presence of acetosyringone in the cocultivation medium is imperative for transformation. This result was similar to the study by de Groot *et al.* [5]. Therefore, acetosyringone was always added to the cocultivation medium.

Cocultivation of *Agrobacterium* cells carrying pKHt with conidia of *M. ruber* KCTC6122 in the presence of acetosyringone led to the formation of hygromycin B-resistant colonies approximately 7 days after transferring to YM plates as selection media. The binary vector pKHt contains the *hph* gene under the control of the *Aspergillus nidulans trpC* promoter as a selectable marker for fungal transformants.

We examined the effects of cocultivation time (duration of cocultivation) from 60 to 120 h at 12-h intervals. For this, the *M. ruber* conidia concentration and *Agrobacterium* cell volume were fixed at 10^6 conidia/ml and at 200 μl, which were applied in other studies [5, 17]. In 60-h cocultivation, transformants were abortive, yielding no true transformants. The occurrence of abortive transformants has been previously described in other fungi [16]. True transformants began to appear after 72 h and their numbers were highest at approximately 800 to 1,000 after 84-h cocultivation (Fig. 1A). This optimum cocultivation time was longer than in other fungi such as *M. grisea*, *A. niger*, *F. venenatum*, *T. reesei*, *N. crassa*, and *A. bisporus* [5, 17]. The efficiency of transformation for *M. ruber* was even higher than for *M. purpureus* by approximately 10-fold [4]. The long cocultivation was due to a difference in receptivity [13], which may be the main barrier of successful transformation of *M. ruber*.

With the optimum cocultivation time of 84 h, the transformation efficiency was the highest at 10^6 conidia/ml (Fig. 1B). This was comparable to the transformation efficiencies in other fungi [4, 5]. Too many fungal recipients can result in too much fungal growth during cocultivation, which makes the subsequent isolation of single transformants difficult [13].

Finally, we determined the effect of the bacterial cell volume as compared with conidia suspension volume. With the same volume of the *Agrobacterium* cells (OD_{660}=0.8) as conidia (200 μl), the highest efficiency was observed (Fig. 1C). The use of a greater volume (400 μl) of bacteria yielded many transformants, but many of them were abortive transformants, which caused difficulty in selection of a single transformant. These results corresponded with those

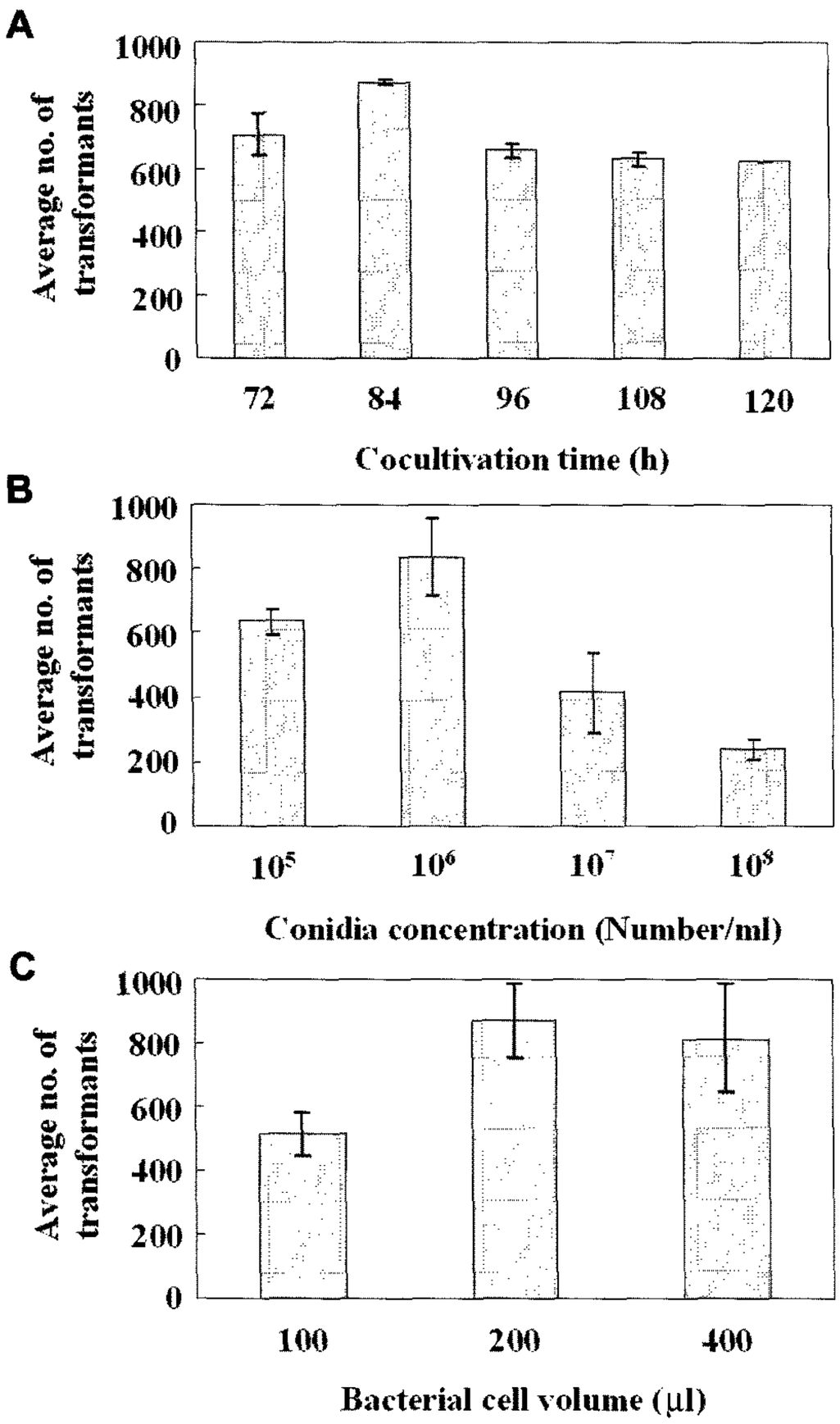

Fig. 1. Optimization of ATMT for *M. ruber*.
A. For optimization of cocultivation time, 10^6 conidia/ml of *M. ruber* was mixed with an equal volume of *A. tumefaciens* (pBHt2) cells and incubated for various times. **B**. For optimization of conidia concentration, various conidia concentrations of *M. ruber* were mixed with an equal volume of *A. tumefaciens* (pBHt2) cells and incubated for 84 h. **C**. For optimization of *A. tumefaciens* cell volume, 10^6 conidia/ml of *M. ruber* was mixed with various volumes of *A. tumefaciens* (pBHt2) cells and incubated for 84 h. The number of transformants is calculated per ml of conidia.

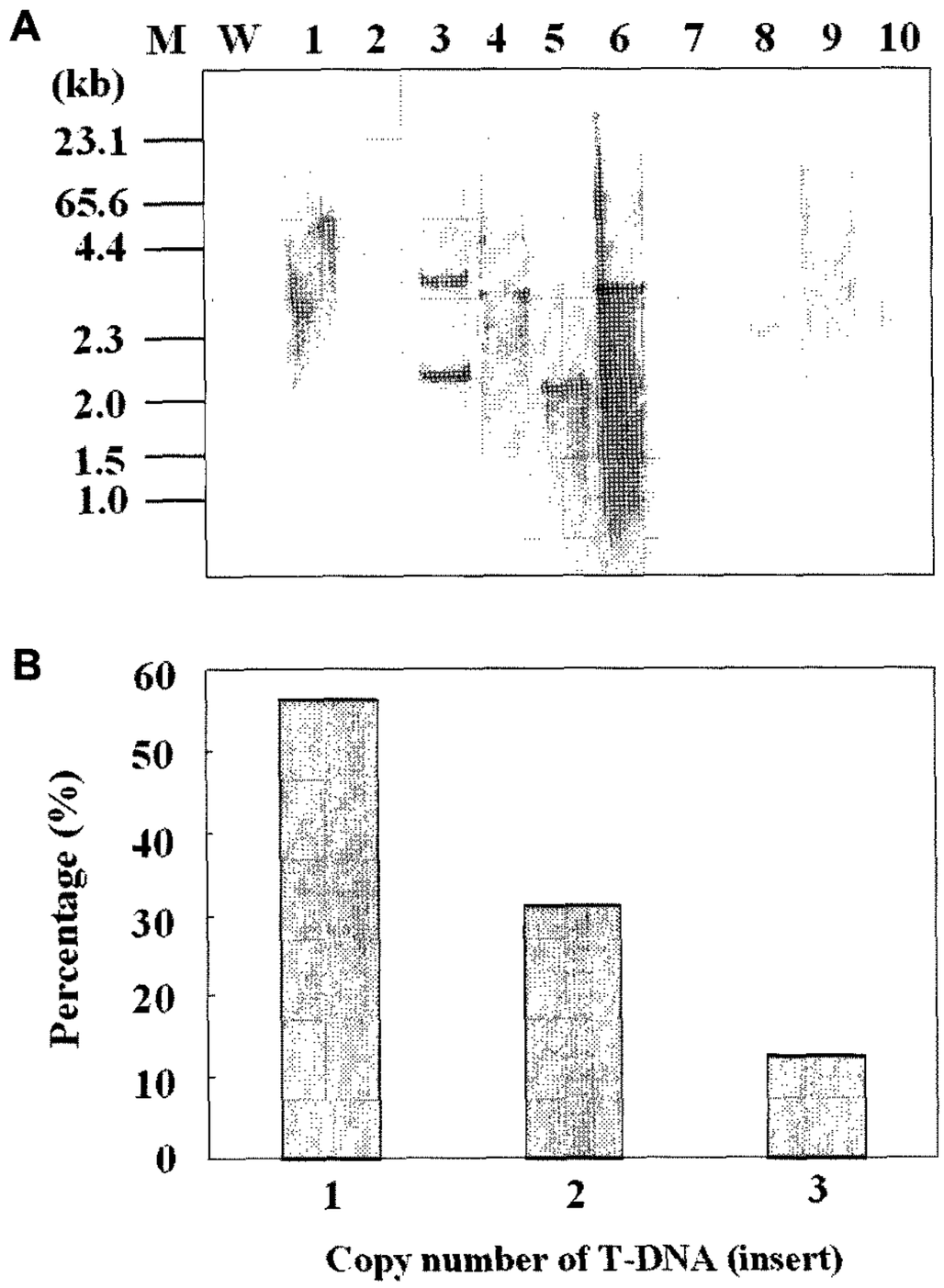

Fig. 2. Southern analysis of transformants.
A. Genomic DNA was digested with EcoRI, which cuts once in the T-DNA. Hybridization was performed using a 0.8 kb *hph* gene fragment. Lanes 1–10, randomly selected hygromycin-resistant transformants; lane W, *M. ruber* wild type; lane M, molecular mass in kb. **B**. Percentage of copy number of T-DNA.

of Michielse *et al.* [13], who found that the addition of too many *A. tumefaciens* cells can result in a decrease in transformation efficiency, probably due to nutritional or space limitations. However, other studies contradict this finding, such as the one performed in *M. grisea*, in which transformation efficiency was found to increase as the *A. tumefaciens* cell number increased [17].

Thirty-nine out of 40 transformants randomly selected from cocultivation time batches (8 per each) maintained their hygromycin B resistance after being cultured for five generations in the absence of hygromycin B. This suggests that the transforming marker is very mitotically stable.

The physical existence of the introduced DNA in transformants was evaluated by PCR and Southern analyses. DNA fragments of the expected 800-bp size were amplified from the *hph* gene, confirming that the introduced *hph* gene rendered hygromycin B resistance to transformants. Southern analysis also confirmed the existence of the *hph* gene in transformants (Fig. 2A). Different sizes of bands were hybridized among genomic DNA isolated from different transformants, indicating that the loci of integration were random among transformants. Among 31 analyzed transformants, 17, 10, and 4 showed single, double, and more than triple hybridizing bands, respectively (Fig. 2B), which would be indicative of multiple integrations. A high percentage of single integrations was observed, which is often advantageous in transformation studies. The addition of acetosyringone in an induction medium and an elongated cocultivation time of more than 48 h are known to increase multiple integrations [17]. Therefore, the relatively long

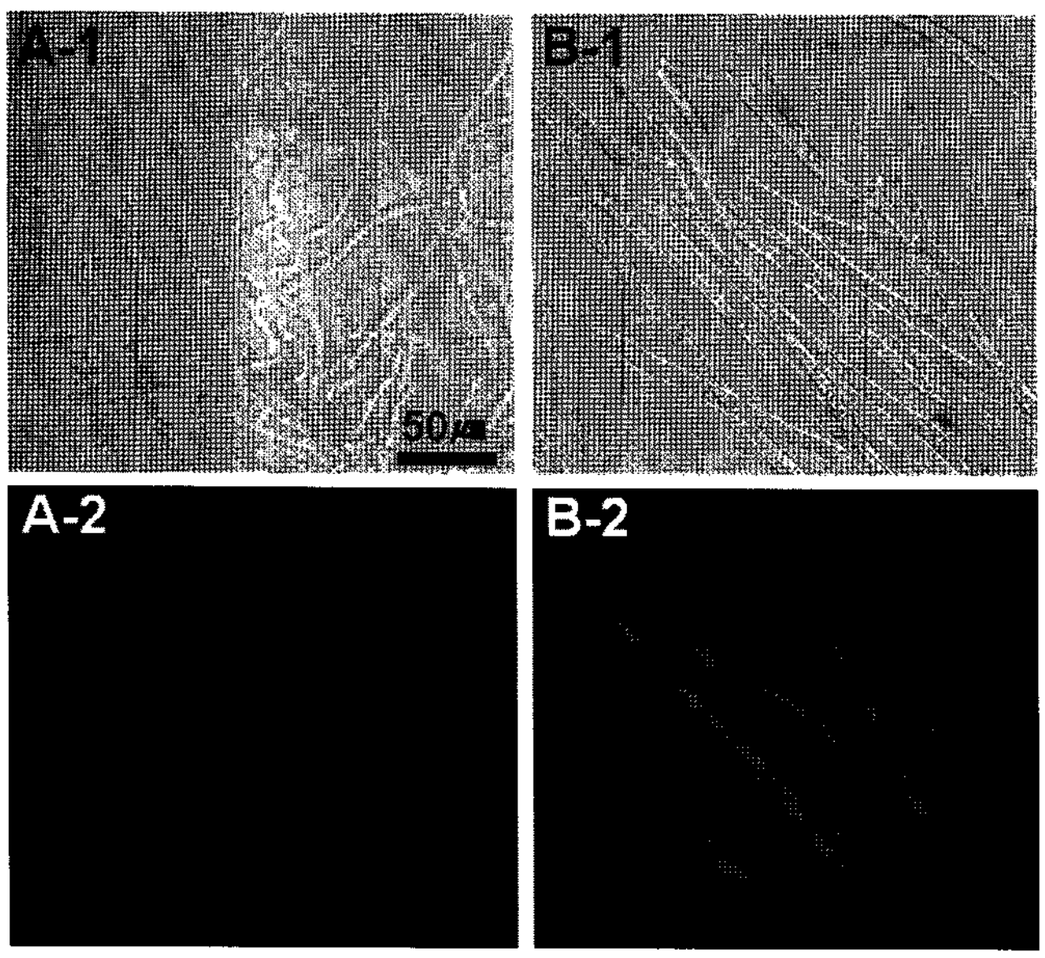

Fig. 3. Fluorescent microscopy of transformants with EGFP.
The transformants with *A. tumefaciens* YL-63 containing the EGFP gene were observed under a microscope. The photographs are of DIC of wild type (A-1) and transformant (B-1) and those of GFP of wild type (A-2) and transformant (B-2). All photographs were taken at 1,000× magnification.

duration of cocultivation used might have affected the copy number of integrated DNA in this study. Increasing the cocultivation time from 72 to 120 h increased the tendency for multiple integrations, but the integration copy number was not increased proportionally.

Expression of the transformed gene was evaluated using the EGFP gene. *M. ruber* was transformed using *Agrobacterium* YL-63 containing the EGFP gene under the optimized conditions. The representative transformants showed bright green signals through whole hyphae under a fluorescence microscope (Fig. 3), suggesting that the transformants not only had the integrated EGFP gene but also expressed it.

In this study, we have established optimized ATMT conditions for *M. ruber*, which we were previously unable to transform by electroporation of protoplasts [15]. Development of efficient transformation and expression systems for this fungus – an important model for citrinin production – will provide useful tools for molecular genetic analysis and gene manipulation, such as targeted gene disruption. Such tools should be instrumental in the improvement of industrial strains of *Monascus*, enabling more efficient production of desirable secondary metabolites.

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