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Strain Improvement by Overexpression of the *laeA* Gene in *Monascus pilosus* for the Production of *Monascus*-Fermented Rice

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Monascus species have been used to produce fermented rice called *Monascus*-fermented rice (MFR). To improve a *Monascus* strain *via* activation of secondary metabolite (SM) gene clusters for use in the production of MFR, we overexpressed an ortholog of the *laeA* gene, which encodes a global positive regulator of secondary metabolism under the control of the strong heterologous *Aspergillus nidulans alcA* promoter in *Monascus pilosus*. The *OE::laeA* transformant produced more SMs, including those not detected under uninduced conditions. MFR produced using the *M. pilosus OE::laeA* strain contained 4 times more monacolin K, a cholesterol-lowering agent, than MFR produced using the wild-type strain. In addition, pigment production was remarkably increased, and the antioxidant activity was increased as well. The results from this study suggest that *Monascus* species, which are important industrial fermentative fungi in Asia, can be improved for the production of functional foods by overexpressing the *laeA* gene.

Keywords: *Monascus pilosus*, secondary metabolite, *Monascus*-fermented rice, *laeA*, monacolin K

Introduction

Monascus species are used to produce *Monascus*-fermented rice (MFR) (also called red mold rice, red yeast rice, and *red-koji*). MFR has been used for thousands of years in East Asian countries to produce fermented foods such as red-rice wine and fermented soybean curd [12]. MFR has also been used as a folk medicine to improve blood circulation, and spleen and stomach health, and its pharmaceutical properties have been described in the ancient Chinese pharmacopeia, *Ben Cao Gang Mu* by Shi-Zhen Li (1518-1593 AD). Such health-promoting effects of MFR have been supported by findings of various bioactive compounds such as angiotensin-converting enzyme (ACE)-inhibitory peptides, γ-aminobutyric acid (GABA), monacolins, pigments, and dimerumic acid (reviewed in [12, 17]). In addition to its traditional use, MFR is being developed as a functional food or food supplement with hypolipidemic effects, because it contains monacolin K (also known as mevinolin or lovastatin), which is known to reduce blood cholesterol levels [7].

Recently, substrates besides rice, including garlic, dioscorea, red ginseng, and soybean, have been used to prepare *Monascus*-fermented products (reviewed in [13]). For example, monacolin K-enriched mugwort is developed by fermentation with *Monascus pilosus* [15]. Because *Monascus* species produce a wide range of bioactive compounds, *Monascus*-fermented products may contain other bioactive compounds in addition to the intrinsic ones. During the fermentation of some substances, *Monascus* species provide an additional advantage of transforming intrinsic bioactive substances to ones with improved functionality. *Monascus*-fermented red ginseng contains high levels of monacolin K and deglycosylated ginsenosides, and it could offer improved health benefits owing to the additional bioactive compounds and readily absorbable deglycosylated ginsenosides produced by fermentation with *M. pilosus* [9].

Most of the health-promoting compounds in *Monascus*-fermented products are fungal secondary metabolites (SMs). Recent genomic studies in several fungi have revealed the presence of additional SM gene clusters than expected in a given fungus, suggesting that many SM gene clusters are

not fully expressed under standard laboratory or industrial culture conditions [5, 6, 29]. Therefore, it would be possible to improve the fungal production of bioactive compounds by activating SM gene clusters. LaeA would be a good target for improving SM production, because it controls SM production in several filamentous fungi [3]. In *Aspergillus nidulans*, overexpression of the *laeA* gene activated the terrequinone A biosynthetic gene cluster, leading to the discovery of terrequinone A, which had previously not been identified in this fungus [2]. Perrin *et al*. [20] reported that LaeA positively regulates 20−40% of SM gene clusters in *Aspergillus fumigatus*. Overexpression of *laeA* also increased penicillin production by 25% in *Penicillium chrysogenum* [11]. Therefore, we hypothesized that the overexpression of a *laeA* ortholog in *Monascus* species would lead to the activation and/or elevation of SM production, thereby making *Monascus* strains more valuable in functional food-processing applications. In this study, we constructed a *laeA*-overexpressing *M. pilosus* strain and evaluated its applicability in MFR production.

Materials and Methods

Chemicals and Media

Monacolin K and cyclopentanone were purchased from Sigma Aldrich Chemical Co. (USA) and Merck Schuchardt OHG (Germany), respectively. All HPLC-grade solvents were from Mallinckrodt Baker (USA). Rice was purchased from a local market in Seoul City (Korea). Potato dextrose agar (PDA) and potato dextrose broth (PDB) were from Acumedia (USA). A lactose minimal medium (LMM) was used for both uninduced and induced cultures, and cyclopentanone was added to a final concentration of 10 mM in induced cultures only. LMM has the same composition as GMM [23], except that glucose is replaced with 2% lactose in LMM. GMM containing cyclopentanone was used for repressed cultures.

Fungal Strains and Culture Conditions

Monascus pilosus IFO4480 was purchased from the Korean Culture Center for Microorganisms (KCCM, Seoul, Korea). It was routinely grown on PDA at 30°C. *Aspergillus nidulans* H109 was obtained from Prof. Nancy P. Keller (University of Wisconsin-Madison, WI, USA).

Spores of *M. pilosus* were harvested using a 0.8% saline buffer after culturing the strains on PDA plates for 5 days at 30°C. Spores were inoculated into liquid medium at 1.0×10^7 spores/ml. All broth cultures were incubated at 30°C with shaking (150 rpm).

Molecular Genetic Manipulation and Southern Analysis

DNA manipulations and Southern analysis were performed according to standard procedures [22]. Fungal DNA was isolated as described previously [23]. The PCR primers used are listed in Table 1. For probe labeling and detection in Southern analysis, a digoxigenin (DIG)-DNA labeling and detection kit was used according to the manufacturer's instructions (Roche, Basel, Switzerland).

Cloning and Overexpression of the *M. pilosus laeA* Gene

When this study was initiated, no *laeA* gene sequence was available for *Monascus* spp. We had previously cloned and sequenced the *Monascus ruber laeA* gene (Kim and Lee, unpublished data). Based on the *M. ruber laeA* gene sequence, we designed PCR primers (laeAF and laeAR) to amplify 3.8 kb fragments including the *laeA* open reading frame (ORF) and 5′- and 3′-flanking sequences (1.2 and 1.4 kb, respectively), The PCR fragments were sequenced by Macrogen (Korea). The *alcA* promoter was amplified from genomic DNA of *A. nidulans* H109 by using the primers OEF and OER. The *laeA* promoter was replaced by the *alcA* promoter by single-joint

Table 1. Primers used in this study.

Primer	Sequence (5'−3')	Use
laeAF	GAA AGA TTT TCT CTT GCT CA	Amplification of the *laeA* gene
laeAR	GGA CGC GAG TGG ATG AAT TC	
OEF	GTT CCG TTC TGC TTA GGG TA	Amplification of the *alcA* promoter
OER	TTT GAG GCG AGG TGA TAG GA	
AnlaeAF	TCC AAT CCT ATC ACC TCG CCT CAA AAT GTT TGG ACA ACA ACA GCA	Joining of the *A. nidulans alcA* promoter and the *M. pilosus laeA* gene to generate *OE::laeA*
PolylaeAR	GAC CTA CCT ACC TAT TCA GG	
AnnestF	TCC TAT CAC CTC GCC TCA	Amplification of the *OE::laeA* construct
OEnestR	CCT ACC TAT TCA GGG AAA AG	
OE-R	GGC TCC TGG AGA AGA ATG AG	Screening of *OE::laeA* transformants
RTlaeAF	GAT CGC CTG GAC ATC TTC CA	Amplification of the *laeA* gene by real-time RT-PCR
RTlaeAR	GGC TGT TCA GAG GCT GTA TG	
ACT-F	TGA CAA TGT TAC CGT AGA GAT CC	Amplification of the actin gene by real-time RT-PCR
ACT-R	GGA GAA GAT CTG GCA TCA CA	

PCR using the primers AnalcAF and polylaeAR [30]. The *OE::laeA* construct was amplified by nested PCR using the primers AnnestF and OEnestR and cloned into pTop V2 using a pTop V2 blunt kit to yield pLSS1 (Enzynomics, Korea). The *Bam*HI/*Not*I fragments of pLSS1 containing *OE::laeA* were transferred to the equivalent restriction enzyme sites in pUCH2-8, which harbors the selectable marker hygromycin B phosphotransferase [1], to generate pLSS2. The pLSS2 was used to transform *M. pilosus* IFO4480, as described elsewhere [16]. Transformants were screened for the presence of *OE::laeA* by PCR using the primers OEF and OE-R. The copy number and insertion position of *OE::laeA* were confirmed by Southern analysis of the PCR-screened candidates by using a DIG-dUTP-labeled probe, which covers the *laeA* ORF.

RNA Isolation and Real-Time RT-PCR Analysis

Real-time RT-PCR was used to detect the relative expression levels of the *laeA* gene in *M. pilosus* as described previously [16], with a few modifications. Wild-type and *OE::laeA* strains were grown in LMM for uninduced and induced conditions, and GMM for repressed conditions, at 30°C with shaking (200 rpm). After 36–48 h, cyclopentanone (final concentration, 10 mM) was added to the induced and repressed cultures. Mycelia were harvested after 5 days of culture. Total RNA was extracted from lyophilized mycelia using RiboEx total RNA isolation solution (GeneAll Inc., Korea). DNaseI-treated RNA (1 μg) from each strain was used as a template for cDNA synthesis using the RevertAid first-strand cDNA synthesis kit (Fermentas, Lithuania). Real-time RT-PCR was performed in triplicate using Sofast EvaGreen Supermix (Bio-Rad, USA). RTlaeAF and RTlaeAR, and ACTF and ACTR were used as primers for the *laeA* and actin genes, respectively, and the My CFX96 real-time PCR detection system (Bio-Rad) was used for the relative expression analysis. Temperature gradients were analyzed for all primers and melt curve analysis was performed using the same product to identify nonspecific products and primer dimers. For more accurate results, real-time efficiencies of the *laeA* and actin genes were examined with a standard curve using the Ct (threshold cycle) value of each real-time PCR reaction mixture, including the 1:1, 1:2, 1:4, 1:8, and 1:16 dilution series of cDNA. Relative expression levels were calculated with the Bio-Rad CFX Manager software (version 1.6) using the 2^{-Ct} method [18]. All values were normalized using the expression ratio of the actin gene as a reference and the wild-type *laeA* expression values as a calibrator.

Production of MFR

Rice purchased from a local market (Seoul, Korea) was soaked in water for 8 h. After removal of excess water, a 30 g aliquot was transferred to a 250 ml Erlenmeyer flask and sterilized by autoclaving for 20 min at 121°C. Spores (5×10^5) were inoculated into sterilized rice and incubated in a 30°C chamber with 90% RH (EYELA, Japan) for 10 days. After completion of fermentation, MFR was immediately frozen in a −70°C deep freezer (Sanyo, Japan) and then lyophilized prior to analysis using a lyophilizer (Hanil Science Industrial Co., Korea). Steamed and autoclaved rice (without fungal inoculation) were used as controls to examine the functional properties of the MFR.

SM Analysis by TLC

SM production was assessed by thin-layer chromatography (TLC), as described elsewhere [4]. For TLC of broth culture, 50 μl of 1×10^3 spores/μl was inoculated into 50 ml of LMM (for uninduced and induced conditions) or GMM (for repressed conditions) at 30°C with shaking (200 rpm) for 7 days. Cyclopentanone (final concentration, 10 mM) was added to the induced and repressed cultures after 36–48 h to minimize growth inhibition by cyclopentanone. Culture broths were extracted with 50 ml of chloroform. The chloroform layer was separated from the aqueous layer by centrifugation at 2,000 ×*g* for 10 min and concentrated using a rotary evaporator (EYELA, Japan). For TLC of MFR, 2 g of MFR powder was extracted with 15 ml of 80% ethanol. Undissolved solids were removed by centrifugation at 15,000 ×*g* and the ethanol layer was concentrated using a rotary evaporator (EYELA). Extracts (10 μl/sample) were loaded onto Silica 60 F254 plates (25 DC-Alufollien Kieselgel 60 F254; Merck, Germany) and metabolites were separated in the developing solvent of 20:1 chloroform:methanol (v/v, %). Photographs were taken following exposure to UV radiation at wavelengths of 254 and 366 nm.

Measurement of Monacolin K, Pigments, and Antioxidative Activity

Monacolin K was quantified by HPLC as described previously [10, 15]. Briefly, 2 g of freeze-dried MFR was extracted with 10 ml of 30% acetonitrile, 30% methanol, and 40% water (0.1% phosphoric acid) in a 45 ml centrifuge tube by stirring at 150 rpm in a water bath (30°C) for 60 min. After centrifugation at 1,750 ×*g* for 10 min, the extracted supernatant was filtered using a PTFE filter (0.45 μm, Target; National Scientific Company, USA), and used for HPLC analysis. The HPLC analysis was using an Agilent HPLC (Agilent Technologies, USA) equipped with a Hypersil Gold column (4.6 mm × 150 mm, 5 μm; Thermo, USA). Acetonitrile/0.1% phosphoric acid water (65:35 (v/v)) was used as the mobile phase. The eluent was pumped at a flow rate of 0.5 ml/min. UV detection was set at 238 nm.

Total pigments were determined using a procedure reported elsewhere [21, 28]. Briefly, 1 g of freeze-dried MFR was resuspended in 10 ml of 80% ethanol and extracted for 2 h at room temperature. After centrifugation at 1,750 ×*g* for 10 min, 1 ml of the supernatant was transferred to a cuvette. Absorbance was measured at 510, 487, and 410 nm for red, orange, and yellow pigments, respectively, using a spectrophotometer (GE Healthcare Life Sciences, Sweden).

Antioxidative activity was measured using a method reported elsewhere [15]. Briefly, 1 g of freeze-dried MFR was extracted with 100 ml of ethanol for 24 h at room temperature. The extract was filtered through a Whatman No. 42 filter paper (Whatman PLC, UK) and concentrated in a rotary evaporator (EYELA). Antioxidative activity against the 1,1-diphenyl-picrylhydrazyl

(DPPH) radical was measured using an *in vitro* assay system [8]. A 200 µl aliquot of the ethanol extract was mixed with 800 µl of 0.15 mM DPPH. The DPPH free-radical-scavenging activity was determined by reading the absorbance at 517 nm after a 30 min reaction.

Statistical Analysis

Data are expressed as the mean ± SEM. Statistical analysis was performed using the Student's *t*-test following ANOVA. A *p*-value <0.05 was considered significant.

The *laeA* sequence has been deposited at GenBank under Accession No. KC907393.

Results and Discussion

Cloning and Overexpression of the *M. pilosus laeA* Gene

Before the *M. pilosus laeA* gene sequence was published, we had cloned and sequenced the *M. ruber laeA* gene (Kim and Lee, unpublished data). Based on the *M. ruber laeA* gene, we designed PCR primers to amplify 3.8 kb fragments containing the *laeA* ORF, and 1.2 and 1.4 kb of the 5′- and 3′-flanking sequences, respectively. Of the 3,795 nucleotides sequenced, 2,413 nucleotides overlapped with the published sequence of the *laeA* gene of *M. pilosus* IFO4520 [31]. Two sequences were almost identical, except for a 12 bp deletion (GAGATGGGTGCC) and a single nucleotide change (A→T) at 867 and 678 bp upstream from the start codon in IFO4480, respectively, which may not affect *laeA* gene expression. The ORF consists of 367 amino acids, coding for a 42 kDa polypeptide (pI = 5.81), which exhibits 70% sequence identity with LaeAs of *A. nidulans* and *Aspergillus oryzae*, respectively [3, 19]. Like these LaeAs, the *M. pilosus* LaeA has an *S*-adenosylmethionine-dependent methyltransferase binding site. LaeA has been proposed to be a methyltransferase.

A *laeA*-overexpressing strain was constructed by transforming *M. pilosus* with pLSS2, in which the *laeA* promoter was replaced with the *alcA* promoter of *A. nidulans*. Several transformants were prescreened by PCR, and putative *OE::laeA* transformants were analyzed by Southern hybridization to confirm the presence of the overexpression cassette in the genome (Fig. 1). Two transformants exhibited bands corresponding to the overexpression cassette (4.6, 2.4, 1.8, and 2.2 kb for *Sal*I, *Eco*RI, *Bam*HI/*Sph*I, and *Eco*RI/*Sac*I digests, respectively), in addition to bands of the expected sizes for the endogenous *laeA* gene (2.4, ≥3.0, 2.2, and 2.9 kb for *Sal*I, *Eco*RI, *Bam*HI/*Sph*I, and *Eco*RI/*Sac*I digests, respectively). This band pattern suggests that the overexpression vector is inserted into the 3′-region of the *laeA* gene.

One of the 2 *OE::laeA* transformants was chosen for further analysis and real-time RT-PCR was performed to examine the level of *laeA* expression. As shown in Fig. 2A, *laeA* expression levels were up to 3-fold higher in the *OE::laeA* transformant than in the parental strain, even under uninduced conditions. The *laeA* expression was further increased upon induction by the addition of cyclopentanone, and decreased under repressed conditions. The higher expression in *OE::laeA* under uninduced conditions is likely due to the presence of 2 copies of the *laeA* gene.

Effects of *M. pilosus laeA* Gene Overexpression on SM Production

The effect of *laeA* overexpression on global SM production was examined by TLC analysis (Fig. 2B). The production of several SMs was increased in *OE::laeA* and further increased under induced conditions. Moreover, some novel spots were detected in the *OE::laeA* extracts. These spots may be the result of increased SM production or the expression of silent SM gene clusters.

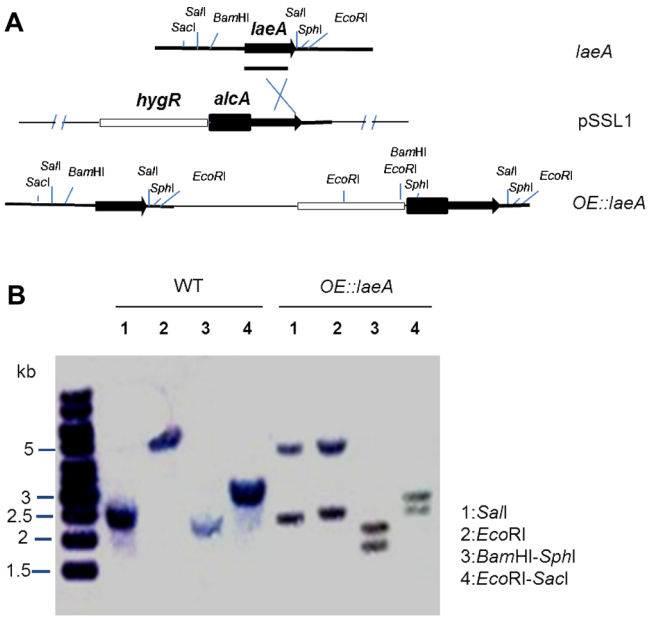

Fig. 1. Overexpression of the *laeA* gene in *M. pilosus*.
(**A**) Schematic diagram showing the *laeA* open reading frame, the *OE::laeA* construct, and its insertion to yield a *laeA*-overexpressing strain. (**B**) Southern analysis of wild-type (WT) and *laeA*-overexpressing strains. Genomic DNA was digested with restriction enzymes and separated by electrophoresis on a 0.7% agarose gel, blotted, and hybridized with a probe that covers the *laeA* ORF. The band pattern for the *OE::laeA* transformant indicates that the *OE::laeA* construct is inserted into the 3′-end of the *laeA* gene.

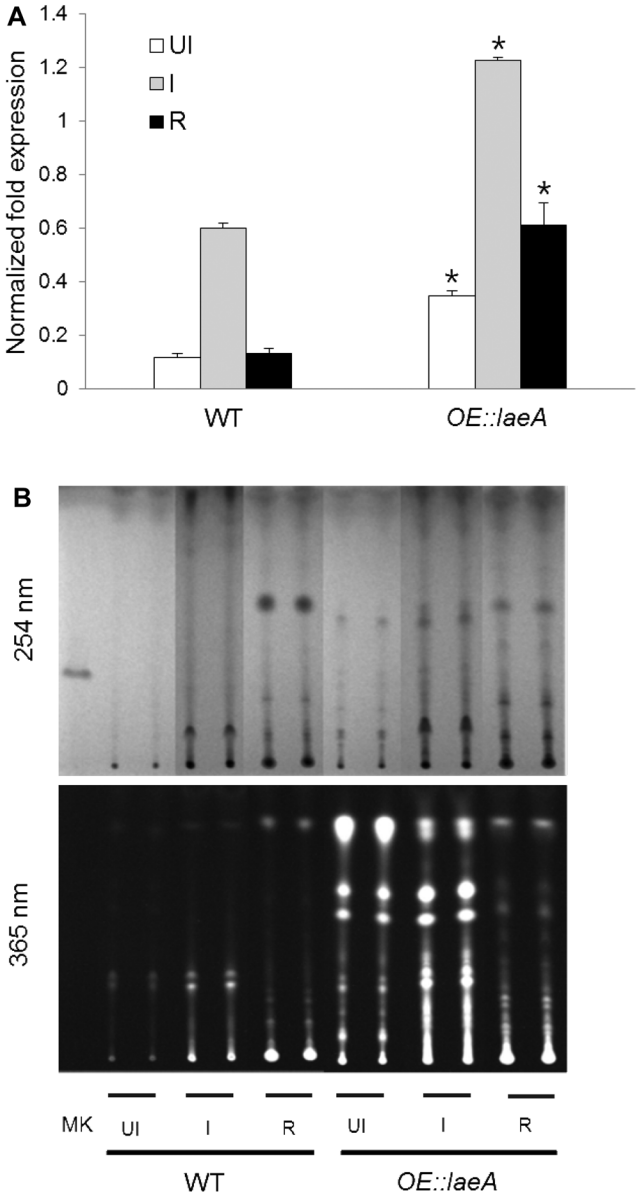

Fig. 2. Expression of the *laeA* gene and SM production by WT and *OE::laeA* in LMM under uninduced (UI), induced (I), and repressed (R) conditions.

(**A**) Real-time RT-PCR analysis of the *laeA* expression level. Data are expressed as the mean ± SEM of 3 reactions. *$p < 0.05$ compared with WT. (**B**) TLC analysis of SM production. Chromatograms were imaged at 254 and 365 nm.

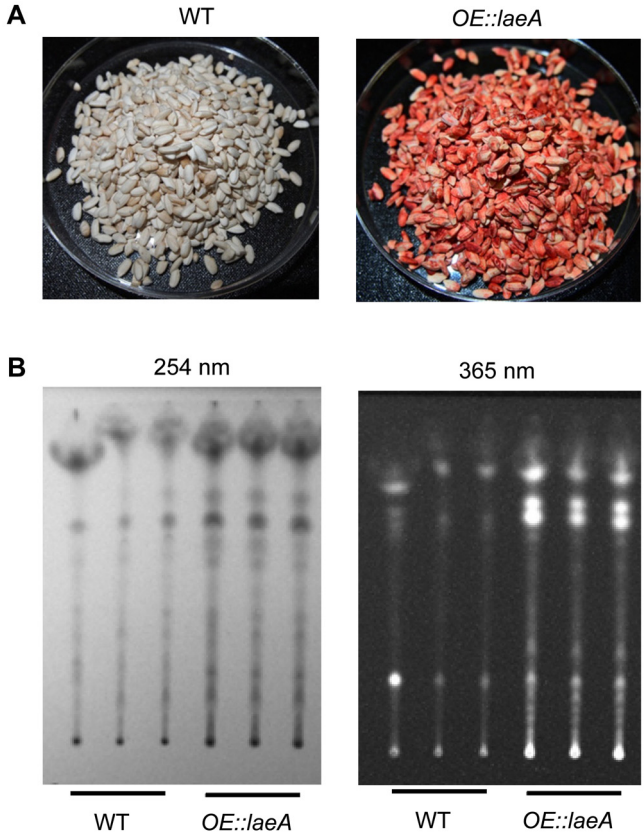

Fig. 3. MFR produced by fermentation with WT and *OE::laeA* strains.

(**A**) Photographs of MFR. (**B**) TLC analysis of SM production in MFR. Chromatograms were imaged at 254 and 365 nm.

MFR Production by Fermentation with *OE::laeA*

To evaluate the potential industrial application of *OE::laeA* in the production of improved functional foods, MFR was produced using *OE::laeA*, and its functional characteristics were examined. MFR produced using the wild-type strain (MFR-W) appeared as dried steamed rice, whereas that produced using *OE::laeA* (MFR-OE) was red and resembled typical commercial MFR, even under uninduced conditions (Fig. 3A). In addition, more SMs were detected in MFR-OE than in MFR-W in LMM broth culture (Fig. 3B). New spots may represent SMs resulting from the activation of silent SM gene clusters. The identities and functions of these newly detected SMs remain to be elucidated; however, MFR-OE may have additional functionality derived from these new SMs. The level of monacolin K (increasing from 2.45 to 15.59 mg/kg) was increased over 4-fold in MFR-OE compared with MFR-W. Consistent with the color of MFR-OE, pigment production was more than 1.5-fold higher in MFR-OE than in MFR-W. Antioxidant activities were also over 4-fold higher in MFR-OE than in MFR-W. Thus, MFR-OE would have improved functionality compared with MFR-W owing to increased and/or newly produced SMs.

Several approaches to increase the production of bioactive compounds, including monacolin K, in *Monascus*-fermented

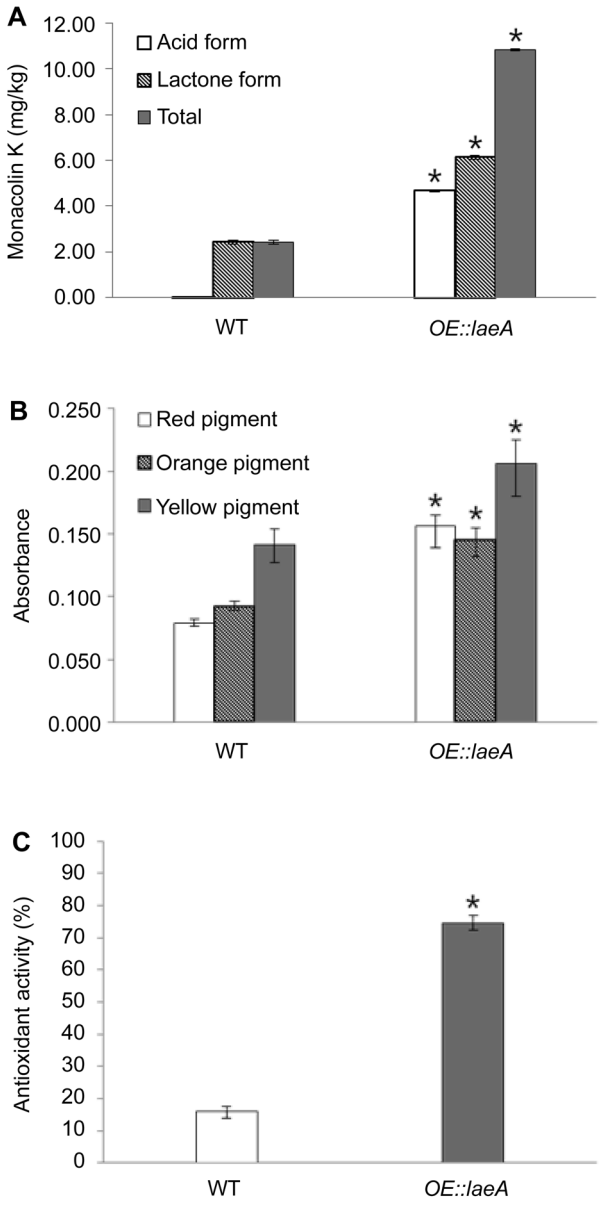

Fig. 4. Levels of monacolin K (**A**) and pigments (**B**), and antioxidant activities (**C**) in MFR produced by fermentation with WT and *OE::laeA* strains.

Data are expressed as the mean ± SEM of 3 experiments. *$p < 0.05$ compared with WT.

products have been reported [14, 24–27]. Most approaches depend on the screening of appropriate strains from natural sources for a given substrate or the optimization of culture conditions. However, a few molecular genetic approaches for strain improvement have been addressed. This study demonstrated that *laeA* overexpression is a useful strategy to improve *Monascus* species for the better production of functional foods. Until now, overexpression of the *laeA* gene has been mainly considered as a means to discover novel compounds *via* activation of silent SM gene clusters.

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