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**An efficient polyethylene glycol-mediated transformation system of lentiviral
vector in *Shiraia bambusicola***

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Abstract

Shiraia bambusicola can biosynthesize the valuable hypocrellin, which is applied in antibacterial, antitumoral, and antiviral fields. The lack of an effective transformation system limits studies on the hypocrellin metabolism deconstruction. We successfully expressed a lentiviral PgfpHyg vector in the *Shiraia* sp. SUPER-H168 by a polyethylene glycol and CaCl₂ transformation method. The transformant was resistant to 500 µg/mL of hygromycin B and represented by a green fluorescent signal in a fluorescent microscopy. We also overexpressed a multicopper oxidase (MCO) from the gene cluster of hypocrellin biosynthesis. MCO overexpression clearly enhanced hypocrellin production by about five times than the control strain, by improving the transcriptional levels of several hypocrellin metabolic genes. These genes included polyketide synthase, FAD/FMN-dependent oxidoreductase, monooxygenase, and *O*-methyltransferase. The increase of hypocrellin yield can efficiently solve the problem of its shortage from natural extraction. In summary, the polyethylene glycol and CaCl₂ regulation transformation was successfully applied in *S. bambusicola* with a lentiviral vector, which is mainly used in mammal cells. This study will lay a foundation for decoding hypocrellin metabolic pathway by further protein expression, bioconversion, and gene targeting. It will also create a new possibility for gene expression or further gene targeting of other wild filamentous fungi.

Keywords: transformation system, hypocrellin biosynthesis, lentiviral vector, *Shiraia*

bambusicola, relative expression

Introduction

Shiraia bambusicola produces several perylenequinoid compounds, including hypocrellin A and hypocrellin B. These compounds have been widely used in the treatment of skin diseases, rheumatoid arthritis, gastric diseases, and some vascular diseases, because of their light-induced anti-inflammatory, analgesic, antibacterial, antitumoral, and antiviral properties.¹ The hypocrellin was mainly obtained from stromata extraction of the parasitic fungi, such as *Hypocrella bambusae* and *S. bambusicola*^{2,3}; however, yield from this natural extraction was too low to meet its current medical demand. Moreover, studies on hypocrellin mainly focus on fermentation optimization, isolation, and applications.⁴⁻⁶ However, studies on its biosynthesis mechanisms have not yet been conducted because of the deficiency of suitable transformation system for *S. bambusicola*. To date, only transformation systems of fungi, which are similar to *S. bambusicola*, have been established, including those of *Cercospora kikuchii*⁷ and *Colletotrichum acutatum*.⁸

Problems in constructing a transformation system for filamentous fungi can involve three components: exogenous DNA importation into host cells; availability of replication and maintenance of expression vectors; and a selector marker for expression of target proteins.^{9,10} The cell walls of filamentous fungi pose the first challenge for a successful transformation. In order to obtain haploid protoplasts, germinal spores, not hypha, are selected as the original cells and cell walls are eliminated by degeneration enzymes to allow the exogenous gene to enter the cells.

These enzymes include lysing enzyme, driselase, β -glucuronidase, β -D-glucanase, chitinase, and Novozyme 234.¹⁰⁻¹² In addition, many available replication expression vectors for filamentous fungi have been constructed, including pAN7-1 for *Aspergillus nidulans*. Finally, excellent selector markers and screening systems are also essential. Those extensively used fungal markers are proteins that are resistant to chemical drugs, such as Hyg (hygromycin B).¹³ For visualization in blue light wavelengths, green fluorescent protein (GFP) from *Aequorea victoria* is another widely used selector marker in expression vectors.¹⁴ Many fungal expression systems, such as those for *Aspergillus oryzae*¹⁵ and *Neurospora crassa*,¹⁶ use GFP as the reporter gene. Among the fungal transformation methods, polyethylene glycol (PEG) and CaCl_2 transformation protocols have been successfully applied in many filamentous fungi, such as *Fusarium graminearum*,¹⁷ *A. nidulans*,¹⁸ and *N. crassa*.¹⁰

In addition, the genome of *Shiraia* has been sequenced, and the gene cluster for hypocrellin biosynthesis has also been annotated.¹⁹ Recently, Townsend has also inferred the metabolic pathways of cercosporin, a similar perylenequinoid to hypocrellin. On the contrary, he compared the gene clusters between cercosporin and hypocrellin.²⁰ These results inferred that the gene cluster of cercosporin biosynthesis from *Cercospora* was similar to that of *S. bambusicola*, which suggested that cercosporin and hypocrellin have similar biosynthesis pathways.²¹ The genes contained multicopper oxidase (MCO), polyketide synthase (PKS), FAD/FMN-dependent oxidoreductase (*FAD*), monooxygenase (*Mono*), zinc finger

transcription factor (ZFTF), *O*-methyltransferase (*Ome*f), and major facilitator superfamily (*MSF*). Therefore, we proposed the hypocrellin pathway in Fig 1. The hypocrellin was biosynthesized via repetitive decarboxylative claisen condensation of acetyl-CoA and malonyl-CoA by *PKS*, *O*-methylation by *Ome*f, ketone reduction, dimerization by *FAD* or *MCO*, and finally side chain cyclization by *Mono*. Among these genes for hypocrellin biosynthesis, *MCO* acts an essential role in hypocrellin pathway. Bais et al.²² described that *Hyp-I*, a phenol oxidase, was able to convert emodin to hypericin. In addition, *GipI*, an *MCO*, also played an essential role in the biosynthesis of aurofusarin via dimerization of two units of oxidized rubrofusarin.²³

In this study, we aimed to establish a PEG-mediated transformation system for *S. bambusicola* and increase the yield of hypocrellin. This study will guarantee abundant hypocrellin to apply in medical treatment, and confirm the hypocrellin biosynthesis flux by gene targeting in a later study.

Materials and methods

Fungal strains and plasmid

Shiraia sp. SUPER-H168⁵ was cultured in a complete medium (CM, 20-g/L glucose, 1-g/L K₂HPO₄, 0.5-g/L MgSO₄·7H₂O, 0.5-g/L KCl, 0.01-g/L FeSO₄·7H₂O, 3-g/L yeast extract, 10-g/L peptone, 200-g/L potato extract, and 0.6% Triton X-100). *Escherichia coli* DH5α was used as a cloning strain and cultured in Luria–Bertani medium. A lentiviral expression vector, PgfpPuro, obtained from Applied Biological Materials (ABM) Inc. (Richmond, Canada), was chosen as an expression plasmid in *S. bambusicola*. This vector consists of a U6 promoter, GFP, 2A peptide, and puromycin (Fig. 2A). In addition, Ibrahimi described the multicistronic lentiviral vectors with peptide 2A sequences²⁴ in detail. Finally, PgfpHyg and PgfpMCO vectors were constructed in this study (Fig. 2B and 2C).

Plasmid construction

The PgfpHyg expression vector was constructed through fusion polymerase chain reaction (PCR) as described by Szewczyk et al.²⁵ The lentiviral vector was linearized through amplification reaction with PgfpF (5'-ggggccggggtgctctccacg-3') and PgfpR (tccggaaatcaacctctgattac) primers. In order to avoid effects of residual lentiviral plasmid, amplicons were treated with a cloning enhancer (ABM Inc.). The Hyg protein gene was amplified from pUCATPH plasmid²⁶ with primers F (5'-agcaaccccgcccccATGAAAAAGCCTGAACTCACCG-3') and R (5'-gaggttgatttccggCTATTCCTTTGCCCTCGGAC-3'). The amplified Hyg protein

with 15-bp italic bases was reverse-complemented with the linearized lentiviral vector.

On the basis of the 15-bp reverse-complement nucleotides between the linearized lentiviral vector and amplified Hyg gene, the ligation-free cloning kit (ABM Inc., Richmond, Canada) was used to construct PgfpHyg vector by homologous recombination. The PgfpMCO vector was constructed in the same way with primers MCOF (5'-*agcaaccccgcccc*ATGGACGGGCTATATGGCGC-3') and MCOR (5'-*gagggttgatttccgg*TCATTTTGGTTGATCGAATCCGTT-3').

Preparation of protoplasts and fungal transformation

The procedures for fungal protoplast generation and transformation were carried out as described by Chung et al.¹² with several modifications. These modifications included that the spore, not hypha, was selected as **the** original host and the cell wall was eliminated after 4-h degeneration with the enzyme mixture [81.25-U/mL lysing enzyme (Sigma), 1050-U/mL β -glucuronidase (Sigma), **and** 5-mg/mL driselase (Sigma)]. The plasmid transformation was **conducted** as follows. **Plasmid** (5 μ g) was added to 200 μ L ($2-5 \times 10^6$) of protoplasts in STC solution (10 mM Tris-HCl and 10 mM CaCl₂ at pH 7.5), and 100 μ L of cold PTC buffer (50% PEG 3350, 10 mM Tris-HCl, and 10 mM CaCl₂ at pH 7.5) was then added **to the mixture**. The mixture was incubated on ice for 20 min. Then, 800 μ L of PTC was added to the mixture and kept at room temperature for 15 min, followed by centrifugation at 5000 g and 4°C for 5 min. The supernatant was then discarded. The protoplasts were suspended in 3 mL of regeneration medium (CM with 0.7 M sucrose). Then, the medium was

incubated with shaking at 75 rpm and 30°C until the cell walls were degraded. In order to determine the minimum lethal concentration (MLC) of hygromycin B on SUPER-H168, the protoplasts were spread onto the regeneration agar medium with 100, 200, 300, 400, and 500 µg/mL of hygromycin B.

GFP analysis with fluorescence microscopy

The expression of GFP in SUPER-H168 was detected by fluorescence microscopy. The fluorescence signal was visualized using an Axioplan 2 imaging microscope (Zeiss, Oberkochen, Germany) equipped with a filter set matching the excitation and emission spectra for enhanced green fluorescent protein (EGFP) (Ex/Em = 488/509). The images were acquired with AxioVision (V. 4.8) software, setting an exposure time of 900 ms to subtract the background autofluorescent signal of SUPER-H168 or transformants.

Overexpression of MCO and hypocrellin detection

The PgfpMCO transformants were screened according to a red ring-based laccase reaction on 0.004% guaiacol CM, and further confirmed with MCO-F (5'-agcaaccccgccccc-3') and MCO-R (5'-gaggttgattccgg-3') primers²⁷; the PgfpHyg plasmid transformant was chosen as the control strain for analysis of biomass, hypocrellin production, and relative expressions. Hypocrellin was produced, extracted, and detected as described by Cai et al.⁴

The control and *MCO* overexpression strains were further cultured in CM at 200 rpm and 30°C. The cells were harvested from fermentation broth and resuspended in

citrate phosphate buffer (pH 3.0). High-pressure cell press (Ningbo Scientz Biotechnology Co., Ltd, Ningbo, China) was used for cell disruption. The supernatant was obtained after centrifugation at 5000 g and 4°C for 10 min. The MCO activity was further tested as follows. A solution containing 2.4 mL of 0.1 M pH 3.0 citrate phosphate buffer and 0.5 mL of 10 mM 2,6-dimethoxyphenol (DMP) was incubated at 50°C for 10 min, and then 0.1 mL of enzyme solution was added into the reaction mixture. The MCO activity was detected by measuring the rate of increase of absorbance at 469 nm with a spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme that oxidized 1 μ mol of DMP in 1 min. The protein concentration was evaluated by the Bradford method and bovine serum albumin was used as a standard.²⁸

RNA extraction and quantitative real-time PCR analysis

The RNA was isolated from the control strain and *MCO* transformant at 24, 48, 72, and 96 h, using a TaKaRa RNA Extraction Kit (TaKaRa, Otsu, Japan). In order to eliminate genomic DNA, the RNA samples were treated with RNase-free DNase I (TaKaRa). The complementary DNA (cDNA) was obtained using 5 $\times$ All-In-One RT MasterMix (ABM Inc.), and further real-time PCR (RT-qPCR) analysis was tested in 96-well blocks with CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The primers used for RT-qPCR analysis of genes (KM434884.1), including *PKS*, *FAD*, *Omef*, *Mono*, *ZFTF*, *MSF*, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), have a single melting peak, nucleotide length of 100–250

bp, guanine-cytosine (GC) content between 50% and 60%, and no secondary structure and self- and cross-annealing effects. The genes that were used as relative expression included *PKS*, *FAD*, *Omef*, *MCO*, *Mono*, *ZFTF*, and *MSF*. In a previous study,²⁹ we have studied the reference gene selection and relative expression analysis from *Shiraia* sp.SUPER-H168, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was identified as the most stable gene among the candidate reference genes. Therefore, *GAPDH* was also chosen as the reference gene to normalize data for the analysis of the relative gene expression. The homology of primers was detected using NCBI Basic Local Alignment Search Tool (BLAST) analysis on the whole genomic DNA of *S. bambusicola*.

Statistical analysis

All the statistics were performed using SPSS software v. 11.0 (IBM, USA). The production of hypocrellin and **analysis of** relative expression of its biosynthesis genes, between the *MCO* overexpression strain and the control strain, were evaluated by one-way analysis of variance (ANOVA). Statistical significant was set at $p < 0.05$. All experiments were performed in triplicate.

Results

Hygromycin B as selectable marker for SUPER-H168

Hyg was a common genetic marker for research on filamentous fungi. We selected this marker for further molecular studies. In order to determine MLC of

hygromycin B on *Shiraia* sp. SUPER-H168, protoplasts of SUPER-H168 were inoculated onto 0.7 M sucrose CM medium with different concentrations of hygromycin B. When the concentration of hygromycin B was 500 µg/mL, no strain grew on the medium. This indicated that this concentration could be the lowest concentration to select transformant with hygromycin B resistance.

GFP analysis with fluorescence microscopy

Spores of SUPER-H168 germinated (Fig. S1A) in CM under 30°C for 10 h with shaking at 140 rpm. Protoplasts (Fig. S1B) were released after germinal spores were treated with the enzyme mixture solution, including lysing enzyme, β-glucuronidase, and driselase, for 4 h at 30°C. In order to set out a suitable expression vector for SUPER-H168, we constructed a lentiviral vector (PgfpHyg). The plasmid included components of GFP, 2A peptide, and Hyg selective marker by replacing puromycin with the Hyg gene. In order to confirm PgfpHyg expression, transformed SUPER-H168 were grown on a medium containing 500 µg/mL of hygromycin B, and transformants became visible 5 days after incubation on CM with 0.7 M sucrose (data not shown). A single Hyg-resistant clone was continuously cultured in CM until hypha generation. Regular GFP fluorescence was detected around the transformed hyphae, while no fluorescence signal was detected in the control strain. These results indicated that GFP and Hyg protein from PgfpHyg could be expressed in fungal SUPER-H168.

Overexpression of *MCO* and analysis of hypocrellin production

In order to ensure application of biotechnology in protein expressions for *S. bambusicola*, we further overexpressed *MCO* from the gene cluster of hypocrellin biosynthesis. Similar to *Hyp-1* that could transform emodine to hypericin,²² *MCO* is also a phenolic oxidative enzyme and may simulate hypocrellin metabolism. The *MCO* activity (Fig. 3B) from *MCO* strain *in vivo* was developed from 24-h incubation, and reached up to the maximal level with 106.88 U/mg of activity. However, no activity of *MCO in vivo* was detected in control and wild strain (data not shown). Figure 3A shows that hypocrellin production and biomass were gradually increased when both of the control strain and *MCO* transformant were cultured from 24-h incubation ($p < 0.01$). Hypocrellin yield from *MCO* transformant was highly enhanced by comparing the control strain among different culture times ($p < 0.01$). In addition, when the incubation time was 72 h, hypocrellin outputs from *MCO* strain were increased by about five times than the control strain. Then, hypocrellin production in *MCO* transformant represented a reductive tendency together with cell senescence. Moreover, the biomasses between the control and *MCO* overexpression strain exhibited a similar trend.

RT-qPCR analysis of essential genes

In order to analyze the effects of *MCO* overexpression on adjacent genes of hypocrellin biosynthesis, we studied the relative expression levels of the essential genes, such as *FAD*, *Mono*, *ZFTF*, *OmeF*, *MSF*, and *PKS*. Figure 4 depicts that *MCO*

transcription level gradually improved and reached quantities higher than the control strain at 72-h incubation ($p < 0.01$). The *MCO* relative expression in *MCO* transformant increased by about 13.6 times than the control. The relative expressions of genes such as *FAD*, *Mono*, *OmeF*, and *PKS* from *MCO* transformant remained similar changeable patterns. All the relative expressions of these genes were enhanced from 24-h incubation, reached up to the maximal levels at 72 h, and reduced immediately ($p < 0.05$). In addition, expression of *MSF* in *MCO* overexpression strain remained a little higher than the control for the whole process. Moreover, the relative transcription level of *ZFTF* in *MCO* overexpression strain maintained an almost constant and higher level than the control, with $p = 0.005$ at 48 h.

Discussion

The lack of a suitable genetic transformation system for *S. bambusicola* has impeded the deconstruction of the hypocrellin metabolic flux. Among the genetic transformation methods for filamentous fungi, PEG/CaCl₂ is a classical method, because of its easy manipulation and common equipment requirements.³⁰ In this study, we established a lentiviral expression system for the fungus *S. bambusicola* via this method. Protoplast preparation was the first challenge for this system; germinal spore as the original host cell was a critical factor for the preparation of protoplasts in *S. bambusicola*, and the protoplast could avoid the loss of genotype after several generations of heterozygote. These results of protoplast preparation were slightly different from *Colletotrichum*, a similar fungus to *S. bambusicola*,¹² and the digestion

time for *S. bambusicola* was longer, because cell wall compounds of different strains had a little distinction.

Several promoters commonly used for fungi were tested to ensure successful gene expression in *S. bambusicola*. The *GAPDH* promoter¹⁸ is a common fungal constitutive promoter. However, the *GAPDH* promoter of pAN7-1 plasmid¹⁸ from *A. nidulans* did not play a role in activating Hyg protein in our previous study (unpublished). As another familiar promoter for filamentous fungi, the *trpC* promoter of pUCATPH plasmid²⁶ could not yet been activated in *S. bambusicola*. Fortunately, there has been a lentiviral plasmid to widely apply in the fungal expression systems for these years, particularly in mammal cells.²⁴ In addition, on the basis of a ribosome skipping model, 2A peptides of lentiviral vector had the ability to coexpress proteins in many eukaryotic systems, such as plant metabolism engineering and yeast.³¹ Felipe reviewed the process of self-processing polyprotein of the 2A peptide.³² Each constituent protein is generated as a discrete translation product by the processes of a co-translational polyprotein and intraribosomal cleavage. In this study, we coexpressed GFP and Hyg selective markers through these 2A peptides of lentiviral vector, as described by Ibrahimi et al.²⁴ The PgfpHyg transformant was resistant to 500 µg/mL of hygromycin B and represented a green fluorescent signal in fluorescent microscopy, while the wild-type strain could not grow on this hygromycin B medium, and the GFP expression was not detected (Fig. S1). These results indicated that U6 promoter of this lentiviral vector could activate gene expressions of *S. bambusicola*,

and genes could be properly coexpressed by the 2A peptides. These markers were also discussed in *Aspergillus niger* and *Trichoderma reesei*.³³ This U6 promoter has been widely applied in filamentous fungi, such as *Ustilago maydis*³⁴ and *Phytophthora sojae*.³⁵ The successful expression of the lentiviral vector in *S. bambusicola* will create new possibilities for the construction of expression systems of wild filamentous fungi. In other words, we can first select the lentiviral plasmid for exogenous or endogenous gene expression in these wild fungal strains without suitable expression plasmids. This ideal will guarantee the significant gene confirmations in wild strains by further expression or gene targeting.

The *MCO* of hypocrellin biosynthesis was overexpressed to further verify the expression ability of the lentiviral vector. This ensures the role of *S. bambusicola* as a filamentous fungal model strain, such as *A. niger*, *A. oryzae*, and *T. reesei*. It was clearly indicated that hypocrellin **yield** in *MCO* overexpression strain was obviously **increased** (Fig. 3, $p < 0.01$). Bais et al.²² showed that *Hyp-1*, a phenol oxidase, could convert emodin to hypericin, a perylenequinoid compound. Chung et al.²¹ showed that genes such as *PKS*, *FAD*, *Mono*, and *OmeF* played an essential role in cercosporin metabolism through gene coregulation. We inferred that *MCO* was also essential for hypocrellin metabolism by enhancing transcription levels of the biosynthesis genes. In order to verify this assumption, we further tested the relative expressions of the adjacent genes of the *MCO* gene. The *MCO* overexpression led to a rapid increase of transcription levels of genes in the *MCO* overexpression strain than those of the

control strain, including *FAD*, *Mono*, *OmeF*, and *PKS*, after 24-h incubation, and reached the highest expression levels at 72 h (Fig. 4, $p < 0.05$). The production of hypocrellin in *MCO* overexpression strain also maintained similar increase tendencies, and the yield was increased by about five times. These results suggested that *MCO* overexpression promoted transcription levels of several essential adjacent genes. The synergisms of these enhanced gene transcriptional levels undoubtedly stimulated hypocrellin metabolism, resulting in the increase of hypocrellin production. On the contrary, *MCO* overexpression also promoted the relative expression of the *ZFTF*, a transcriptional factor, and enhancement of this transcriptional factor might also strengthen the metabolism of biosynthesis of compounds such as carotenoid.³⁶ Chen et al.²¹ concluded that membrane transporters, such as *CnATR2*, were applied in resistance to the radicals and singlet oxygen production by photoactivating toxin cercosporin. In order to avoid toxicity of radicals and singlet oxygen production by photoactivating perylenequinones,³⁷ transcription-level enhancement of *MSF* maintained a relative constant level, and might ensure the transfer of hypocrellin from *in vivo* to *in vitro* after hypocrellin production reached its maximum level. In summary, enhancement of transcriptional levels of several genes for hypocrellin biosynthesis and stable relative expression of membrane transporters ensured the successive increase of hypocrellin production in *MCO* overexpression strain. The highly effective vector in this study will also ensure the expression of gene target plasmid in *S. bambusicola*, and thus will accelerate confirmation of hypocrellin

metabolic flux by gene knockout.

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Table 1. Primers and relevant information of target genes.

Figure 1. Proposed hypocrellin biosynthesis pathway. Polyketide synthase (*PKS*), FAD/FMN-dependent oxidoreductase (*FAD*), monooxygenase (*Mono*), *O*-methyltransferase (*Omef*). Proposed hypocrellin was biosynthesized via repetitive decarboxylative claisen condensation of acetyl-CoA and malonyl-CoA by *PKS*, *O*-methylation by *Omef*, ketone reduction, dimerization by *FAD* or *MCO*, and finally side chain cyclization by *Mono*.

Figure 2. Construction of expression vectors in this study. (A) PgfpPuro, (B) PgfpHyg, and (C) PgfpMCO. U6, U6 promoter; GFP, green fluorescent protein; 2A, 2A peptide as a coexpression linker; Puro, a protein resistant to puromycin; Hyg, a protein resistant to hygromycin B; MCO, multicopper oxidase.

Figure 3A. Hypocrellin productions and biomasses from the *MCO* overexpression strain and the control strain; 3B. *MCO* activity of *MCO* overexpression strain. □ and ●, the biomasses of the control strain and the *MCO* overexpression strain. □ and ●, the hypocrellin production of the control strain and the *MCO* overexpression strain.

Figure 4. Relative expression of genes, including *PKS*, *FAD*, *Omef*, *Mono*, *ZFTF*, and *MSF* from *MCO* overexpression strain relative to the control strain, using *GAPDH* as the reference gene for data normalization of the relative expression analysis.

Figure S1. The prepared protoplast in optical microscope (40 × 100) and the GFP fluorescence signal in fluorescence microscopy (40 × 100). Bars = 20 μm. (A)

Germinal spores in CM with incubation for 10 h. (B) Protoplasts were liberated from germinal spores with cell wall degradation enzymes, including lysing enzyme, β -glucuronidase, and Driselase for 4 h at low speed. (C and E) The cellular morphology of the *MCO* overexpression strain and the control strain in the optical microscope, respectively. (D and F) Fluorescence images of the *MCO* overexpression strain and the control strain, respectively.

Table 1. Primers and relevant information of target genes.

Gene symbol	Gene name	primer
<i>FAD</i>	FAD/FMN-containing dehydrogenase	F: TGTGACCGCCATCACCTTAC R: TTGTCGTATGGGTGGGAAGC
<i>Mono</i>	Salicylate 1-monooxygenase	F: TCTCGGGGAATTATGGCACG R: ACAACCGTTCTCGCATCAGT
<i>ZFTF</i>	Zinc finger transcription factor	F: GAACACCGTCGCAAGATTCG R: TCATTGGCATCGCTTGGAGT
<i>Omef</i>	<i>O</i> -methyltransferase	F: GAACTACCTGAAGGCACGCT R: GCTCGGAAGGATACTCGCTC
<i>MSF</i>	Major Facilitator Superfamily	F: TCCCGTAGCCTTGCTTTCTG R: CCGGCTTCTTCTTGACGCTA
<i>PKS</i>	Polyketide synthase	F: TGCTGAGGTAGCAGTCAAGC R: TTATGCTACGGTCGTCGCTC
<i>MCO</i>	Multicopper oxidase	F: TATGGCGCTTACGAGTGGAC R: ACTCCCTGGCCGATAACGTA
<i>GADPH</i>	Glyceraldehyde-3-phosphate dehydrogenase	F: TTGACCTGACTGTCCGCATC R: CGAGACGAGCTTGACGAAGT