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Gentic overexpression increases production of hypocrellin A in *Shiraia bambusicola* S4201[§]

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Hypocrellin A (HA) is a perylenequinone (PQ) isolated from *Shiraia bambusicola* that shows antiviral and antitumor activities, but its application is limited by the low production from wild fruiting body. A gene overexpressing method was expected to augment the production rate of HA in *S. bambusicola*. However, the application of this molecular biology technology in *S. bambusicola* was impeded by a low genetic transformation efficiency and little genomic information. To enhance the plasmid transformant ratio, the Polyethylene Glycol-mediated transformation system was established and optimized. The following green fluorescent protein (GFP) analysis showed that the gene fusion expression system we constructed with a *GAPDH* promoter Pgpd1 and a rapid 2A peptide was successfully expressed in the *S. bambusicola* S4201 strain. We successfully obtained the HA high-producing strains by overexpressing O-methyltransferase/FAD-dependent monooxygenase gene (*mono*) and the hydroxylase gene (*hyd*), which were the essential genes involved in our putative HA biosynthetic pathway. The overexpression of these two genes increased the production of HA by about 200% and 100%, respectively. In general, this study will provide a basis to identify the genes involved in the hypocrellin A biosynthesis. This improved transformation method can also be used in genetic transformation studies of other fungi.

Keywords: *Shiraia bambusicola*, genetic transformation, hydroxylase gene, O-methyltransferase/FAD-dependent monooxygenase gene

Introduction

Shiraia bambusicola belongs to phylum Ascomycota and has been used as a traditional Chinese medicinal fungus in southern China for several centuries (Kishi *et al*., 1991). Hypocrellin A (HA), which is an extract of *S. bambusicola*, is an effective photosensitive pigment widely uesd in the treatment of vitiligo, psoriasis, and other skin diseases. It also shows great potential in curing of virus infections and cancers due to its antiviral and antitumor properties (Qi *et al*., 2014; Guo *et al*., 2017). HA can damage cellular components, including nucleic acids, proteins and lipids (Cao *et al*., 1992; Choi *et al*., 2016), through attaining an electronically-activated triplet state and generating molecular singlet oxygen (O_2), hydrogen peroxide (H_2O_2), and superoxide radicals (O_2^-) in the presence of oxygen (Zhou *et al*., 2005). However, in previous work, HA application has been limited by the low production in the wild *S. bambusicola* fruiting body. Further, improvements in strain screening and culture condition optimization methods (Yang *et al*., 2009; Cai *et al*., 2010), as well as experimental physical and chemical stimulations of *S. bambusicola* (Pan *et al*., 2012; Liu *et al*., 2016), have been largely insignificant. Thus, molecular biology technologies, including gene overexpression, are expected to improve HA production in *S. bambusicola*. Gene overexpression may stimulate the relevant biosynthetic pathways and improve the yield of the products in filamentous fungi (Wang *et al*., 2015).

Here, manipulating gene overexpression in *S. bambusicola* S4201 has been hindered by the lack of a transformation system with effective promoter and gene fusion expression systems in our study. Transformation systems consist of several components in filamentous fungi, including the materials and methods for the preparation of protoplasts, selection markers, gene fusion expression systems, genetic transformation methods and mediums for the regeneration of transformants. The PEG-$CaCl_2$ transformation method is simple and effective (Sun *et al*., 2015), with hypha as the material commonly used to prepare protoplasts in genetic studies of filamentous fungi (Gao *et al*., 2017). Several degeneration enzymes, such as lysing enzyme, driselase, snailase, yatalase, and cellulase, can be used to eliminate the cell walls of hypha and thereby allow the exogenous gene fragments to enter the protoplasts. Benomyl is an effective fungicide that has been used as a selection marker for many filamentous fungi (Hess and Nakai, 2000). Additionally, the expression of green fluorescent protein (GFP) has been used to determine the effectiveness of gene fusion expression systems in previous studies (Zhou *et al*., 2011).

Recently, HA biosynthetic pathway in *S. bambusicola* has attracted greater research attention aiming to determine the genes involved in HA biosynthesis. The transcriptome sequencing was performed for a wild *S. bambusicola* S4201 strain and mutants were found to not produce HA. Unigenes such as the multicopper oxidase gene (*mco*), major facilitator superfamily gene (*mfs*), polyketide synthase gene (*pks*), O-

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methyltransferase gene (*omef*), O-methyltransferase/FAD-dependent monooxygenase gene (mono), hydroxylase gene (*hyd*), and FAD / FMN-dependent oxidoreductase gene (*fad*) have been found to be down-regulated in the mutants. A comparison of the results of their transcriptome sequencing indicates that they might be involved in the HA biosynthetic pathway (Zhao *et al*., 2016). Cercosporin is another type of perylenequinone with a structure similar to hypocrellin. In the biosynthesis of cercosporin, the O-methyltransferase/FAD-dependent monooxygenase gene (CTB3) gene protein has two functional domains, with O-methyltransferase and FAD-dependent monooxygenase at the N and C termini, respectively (Dekkers *et al*., 2007). CTB3 is involved in polyketide oxidations during ring closure, while mono gene protein in *S. bambusicola* S4201 has the same domains. We infer that mono may be involved in HA biosynthesis and may function similarly to CTB3. Additionally, genes involved in the biosynthesis and modification of many secondary metabolites are often arranged in clusters. The hydroxylase gene (*hyd*) is reportedly located on the HA production gene cluster along with *mono* in *S. bambusicola* (Yang *et al*., 2014) and was down-regulated in the mutants, which indicates that *hyd* performs a role in HA biosynthesis.

In this study, a PEG-mediated transformation system was established and optimized to improve the efficiency of genetic transformation in *S. bambusicola* S4201. The *mono* and *hyd* genes were cloned and overexpressed to obtain the high-producing HA strains. Furthermore, this study will contribute to improving HA production by determining the genes involved in HA biosynthesis and verifying the HA biosynthetic pathway in the second part of the study.

Materials and Methods

Fungal strains and plasmids

The *S. bambusicola* S4201 strain was isolated and screened from the fruiting body of *S. bambusicola* (collected from Anji county, Zhejiang Province, China) and cultured on a potato dextrose agar medium (200 g/L potato extract, 20 g/L glucose, 20 g/L agar, pH 7.0). *Escherichia coli* DH5α (TransGen Biotech) was cultured in Luria-Bertani medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, pH 7.2). Plasmid pDHt-Ben containing a benomyl resistance gene was donated by Dr. Cheng-Shu Wang (Institute of Plant Physiology & Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences). The effectiveness of the promoter we found to co-express the genes was confirmed by GFP analysis with the GFP gene in Plasmid pHAP Ⅱ. A one step cloning kit (Novoprotein) was used for plasmid constructions.

Cloning and sequencing of *mono*, *hyd*

All genomic DNA was extracted by the CTAB method from *S. bambusicola* S4201 (Zhang *et al*., 2008). Then the *mono* and *hyd* genes were amplified from genomic DNA with primers *mono*-t-F/*mono*-t-R, and *hyd* -t-F/*hyd*-t-R, respectively (Table 1), using high-fidelity thermostable DNA polymerase PrimeSTAR HS (Takara). The amplified fragments were connected with the flat end vector pEASY-Blunt Zero Cloning Kit (TransGen Biotech). The consensus primers M13F/M13R were used in the sequencing of these two genes. The primers were designed according to the information from transcriptome sequencing and were synthesized by Sangon Biotech.

Table 1. Primers used in plasmid constructions and transformants detection

Primer name	Nucleotides sequences (5′→3′)
mono-t-F	GTTGAGTGCGTGTCTAAATGG
mono-t-R	TGATCGGTATGTCGTGAATGT
hyd-t-F	CGCTCGATACTCCCTAAACC
hyd-t-R	ATCCAAACCCAAGGCAACAT
Pgpd1-F	AAAACGACGGCCAGTCCAGTCTCAACGTGCCTACT
Pgpd1-R	AGACTTACAATTTCACGCATGATGAAGTGTTTGTTGAGCT
Ben-F	AGCTCAACAAACACTTCATCATGCGTGAAATTGTAAGTCT
Ben-R	CTTGCATGCCTGCAGTTACTCCTCGCCCTCAAGGG
GBen-2A-R	TTTGGCAGCAGCTTCCTTGGCGGCGGCCTCCTTAGCAGCAGCTTCCTCCTCGCCCTCAAGGG
2A-GFP-F	GAAGCTGCTGCTAAGGAGGCCGCCGCCAAGGAAGCTGCTGCCAAAATGGTGAGCAAGGGCGA
GFP-R	CTTGCATGCCTGCAGTTACTTGTACAGCTCGTCCA
2A-*mono*-F	GAAGCTGCTGCTAAGGAGGCCGCCGCCAAGGAAGCTGCTGCCAAAATGGCTTCATCAACCTCTTT
mono-R	CTTGCATGCCTGCAGTCAGTCCGATACAACCGCC
2A-*hyd*-F	GAAGCTGCTGCTAAGGAGGCCGCCGCCAAGGAAGCTGCTGCCAAAATGGCTATCTCCTCTCAAAACGA
hyd-R	CTTGCATGCCTGCAGTTATTCCCTAGATTCAACA
M13F	TGTAAAACGACGGCCAGT
M13R	CAGGAAACAGCTATGACC
D-*mono*-F	CCAGTCTCAACGTGCCTACT
D-*mono*-R	TCAGTCCGATACAACCGCC
D-*hyd*-F	CCAGTCTCAACGTGCCTACT
D-*hyd*-R	TTATTCCCTAGATTCAACA

Note: the underlined parts were overlapping ends used in plasmid constructions.

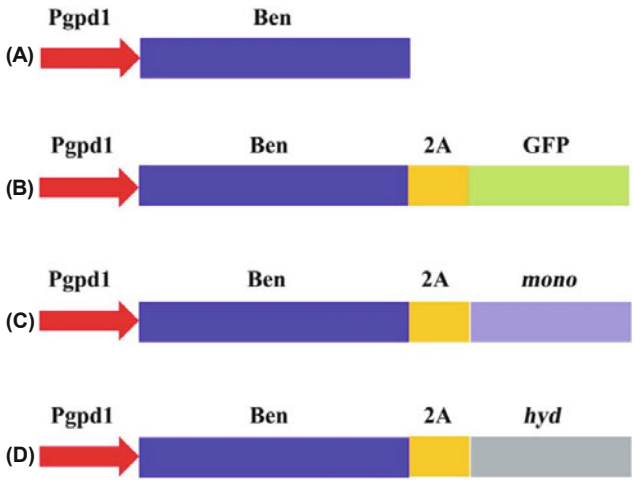

Fig. 1. Construction of expression vectors in this study. (A) Plasmid pGBen (B) Plasmid pGBen-GFP (C) Plasmid pGBen-*mono* (D) Plasmid pGBen-*hyd*. Pgpd1 is a *GAPDH* promoter in *Shiraia bambusicola*. Ben is the benomyl resistance gene. 2A is a rigid 2A peptide with 15 amino acids [$(EAAAK)_3$]. (A) The Plasmid GBen is constructed for the control strains. (B) The pBen-GFP is constructed for GFP analysis. (C, D) The plasmids are constructed for overexpression researches.

Plasmid construction

Primers used for plasmid constructions are listed in Table 1. The primers Pgpd1-F/Pgpd1-R, and Ben-F /Ben-R were used in the amplifications of the *GAPDH* promoter sequence Pgpd1, which we found in the genomic DNA of the S4201 strain and the benomyl resistance protein gene from Plasmid pDHt-Ben, respectively. Then the two sequences were connected with the 15 bp that were overlapping and constructed using the One Step Cloning Kit and the plasmid pGBen. The plasmid pGBen was transformed into *E. coli* DH5α and extracted using the SanPrep column plasmid mini-Preps kit (Sangon Biotech) after 12-h culture in LB medium. The transformants with plasmid pGBen were chosen as the controls for all analyses in this study.

To confirm the efficiency of the gene fusion expression system, plasmid pGBen-GFP was constructed. The Gben sequences were amplified from Plasmid pGBen with the primers Pgpd1-F/GBen-2A-R; the GFP sequences were amplified from plasmid pHAP Ⅱ with primers 2A-GFP-F/GFP-R. There was a rigid 2A peptide with 15 amino acids [$(EAAAK)^3$] between the GBen sequence and GFP sequence (Arai *et al.*, 2001). GFP expression transformants were detected by fluorescence microscopy (Aoix Imager A1). The images were acquired by setting an exposure time of 2000 ms to remove the background autofluorescent signal of the wild strains or transformants. For the following overexpression study, the sequences of *mono* and *hyd* genes were amplified to replace GFP sequence with primers 2A-*mono*-F/*mono*-R, 2A-*hyd*-F/*hyd*-R. Plasmids pGBen-*mono*, pGBen-*hyd*, pGBen and pGBen-GFP were constructed (Fig. 1).

Genetic transformation system and GFP analysis

The high-performance enzyme combination used for *Aspergillus nidulans* by Jiang *et al.* (2014) was implemented here and the effects of spawn age, enzymolysis time, enzymolysis temperature, shaking speed and osmotic stabilizers on the production and regeneration rate of protoplasts made with hypha were tested with an orthogonal experimental design (Table 2). The wild strains cultured in the hypha collection medium (7 g/L peptone, 20 g/L glucose, 0.7 g/L KH_2PO_4, 0.7 g/L $MgSO_4$) were treated with an enzyme mixture [2 mg/ml Yatalase (Takara), 3 mg/ml lysing enzyme (Sigma)] in an osmotic stabilizer to eliminate the cell walls of hypha and to protect the integrity of the protoplasts.

Several genetic transformation methods have been used in genetic studies of filamentous fungi, with the PEG-mediated transformation method is the most common method in the previous studies. The plasmid transformation method was conducted as follows: plasmid (3 μg) was added to 100 μl ($2–3 \times 10^6$) of protoplast stored in 4°C pre-cooled STC buffer (218 g/L sorbitol, 0.47 g/L $CaCl_2$, 10 ml/L 1 M Tris-HCl), and then 100 ml transformation solution (600 g/L PEG 4000, 2.35 g/L $CaCl_2$, 50 ml/L 1 M Tris-HCl) was added to the mixture on ice. The mixture was incubated on ice for 30 min. After that, 800 μl transformation solution was added to the mixture and exposed at room temperature for 20 min. Finally, the mixture was mixed with about 10 ml regeneration medium (CM: 3 g/L yeast extract, 3 g/L casein acid hydrolysate, 10 g/L glucose, 200 g/L sucrose, 8 g/L agar) and benomyl, and spread onto a lower regeneration medium having the same benomyl content (Chi *et al.*, 2009).

Overexpression of *mono* and *hyd* and HPLC analysis of HA production

The plasmid pGBen-*mono* and pGBen-*hyd* were transformed into *S. bambusicola* S4201 with method mentioned above. The *mono* transformants and *hyd* transformants were picked out individually and further detected with the primers D-*mono*-F/D-*mono*-R, and D-*hyd*–F/D-*hyd*–R (Table 1). Meanwhile, in order to analyze the effectiveness of the gene fusion expression system at the protein level, hyd protein was treated with protein Blast. The results indicated that the hyd

Table 2. Factors and levels for orthogonal experimental design of preparing protoplasts with hypha in *Shiraia bambusicola* S4021

Levels	Factors				
	A	B	C	D	E
	Spawn age (h)	Enzymolysis time (h)	Enzymolysis temperature (°C)	Shaking speed (rpm)	Osmotic stabilizers
1	30	2	24	140	0.6 M $MgCl_2$
2	36	2.5	26	160	0.6 M NaCl
3	42	3	28	180	0.6 M Sorbitol
4	48	3.5	30	200	0.6 M Sucrose
5	54	4	32	220	Osmotic Medium (OM)

Table 3. The relevant information of primers used in RT-qPCR analysis

Gene symbol	Gene name		Primer
GADPH	Glyceraldehyde-3-phosphate dehydrogenase	F	ACGATGCGACCAATACGA
		R	AACTCTTCCTCCTACACTTCAG
hyd	Hydroxylase	F	CACTGCGGTCACTTTGCGTAAC
		R	GAGATGCTGGGCGGGCTTG
mono	O-methyltransferase/FAD-dependent monooxygenase	F	CAAACTCTCGCCCAAACTGTATC
		R	GCTTCCTGTGCCGTCTCG
mco	Multicopper oxidase	F	CACGCACCTGGAGAATCG
		R	CACAAACACGGAAACAAAGC
fad	FAD/FMN-containing dehydrogenase	F	CGGGCGATGCGAAGTTGC
		R	AGTAGGTGGCGTTGGTGTGG
pks	Polyketide synthase	F	TCAGACGCACTCACAATAACC
		R	GGACCAAAGCACAACAAAGC
omef	O-methyltransferase	F	GAACTACCTGAAGGCACGCT
		R	GCTCGGAAGGATACTCGCTC
zftf	Zinc finger transcription factor	F	GATACGTCCATTGTTCCA
		R	CAGCAGTCATAGTCTTCTT
mfs	Major Facilitator Superfamily	F	TCCCGTAGCCTTGCTTTCTG
		R	CCGGCTTCTTCTTGACGCTA

protein was one of salicylate hydroxylases. Therefore, the activity of the hyd protein was detected by measuring salicylate hydroxylase activity in *hyd* overexpression strains and control strains. The hypha of overexpression transformants and control strains were dried at 55°C to a constant weight to determine the biomass value. Then, the HA production of overexpression transformants and control strains was extracted with ethyl acetate at room temperature. HA extractive was concentrated by rotary evaporation under reduced pressure at 55°C and re-dissolved by acetonitrile. Then the HA extractive was analyzed by Agilent Technologies HPLC spectrometer (1220 Series, Agilent) at 464 nm (Hu *et al.*, 2012). All samples would run independently in triplicate.

RNA isolation and RT-qPCR analysis

Total RNA of both overexpression transformants and controls on days 1, 2, 3, 4, and 5 were extracted by TRIzol reagent (Invitrogen). To eliminate the effects of genomic DNA, the RNA samples were treated with gDNA wiper (SYBR Premix Ex Taq II, Vazyme). The reverse transcription was performed to synthesize cDNA, and then a real-time PCR analysis was conducted using the StepOne Real-time PCR system (Applied Biosystems) with SYBR Green I fluorescent dye (Takara). The gene-specific primers are listed in Table 3. PCR amplification was performed with an initial step at 95°C for 5 min followed by 40 cycles of 10 sec at 95°C and 30 sec at 60°C. The protocol of the melt curve was as follows: 15 sec at 95°C, 60 sec at 60°C and 15 sec at 95°C. The relative expression levels of target genes were normalized to the level of the reference gene *GAPDH*, and calculated using a $2^{-\Delta\Delta Ct}$ method.

Statistics

Each sample was amplified in triplicate in each experiment. All data is presented as the mean of three biological replicates with standard error (SE). Statistical analyses were conducted with SPSS Statistics 16.0 (SPSS) and one-way analysis of variance (ANOVA) was performed followed by Duncan's multiple-comparison test ($P < 0.05$).

Results

Mono and *hyd* sequencing results and protein predictions

According to the results of the DNA sequencing and transcriptome sequencing, the *mono* gene contains 2,895-bp nucleotides with two exons (2,838 bp) and only one intron (57 bp), encoding 945 amino acids with two domains of O-methyltransferase and FAD-dependent monooxygenase. In contrast, the *hyd* gene contains 1406-bp nucleotides also with two exons (1,314 bp) and one intron (92 bp), encoding 437 amino acids and it was similar to the salicylate hydroxylases in other strains. PSIPRED was used to predict the secondary structure of both *mono* and *hyd* gene proteins (Supplementary data Figs. S1 and S2). The results of the 3D protein prediction performed with the SWISS MODEL (https://www.swissmodel.expasy.org) and are shown in Supplementary data Fig. S3.

An efficient genetic transformation in *S. bambusicola* S4201 and GFP analysis

Previously, the promoter on plasmid pDHt-Ben and other common promoters used in fungi (T7, U6) were unable to express the benomyl resistance gene in the *S. bambusicola* S4201. The cDNA sequence of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) from the results of RNA-sequencing in the *S. bambusicola* S4201 was compared with *Shiraia* sp. slf14 and a sequence in front of the *GAPDH* gene was obtained in the genetics databases. The sequence was supposed to contain a *GAPDH* promoter. Finally, we obtained a potential *GAPDH* promoter from *S. bambusicola* S4201, Pgpd1, which was demonstrated as a constitutive and efficient promoter in filamentous fungi.

The preparation of the protoplasts with hypha was opti-

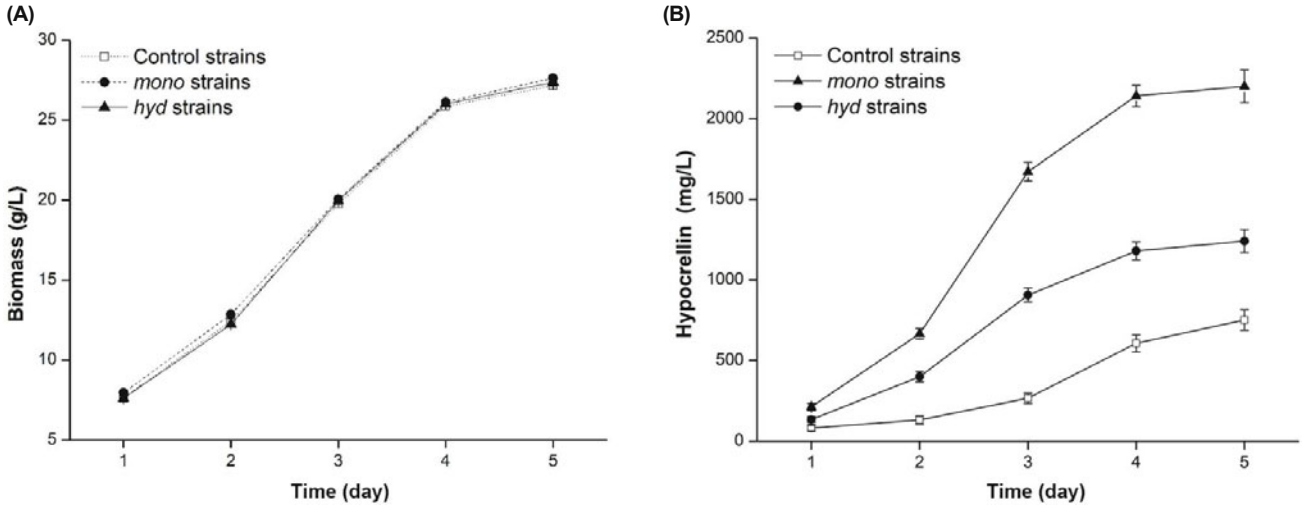

Fig. 2. (A) Biomasses from the overexpression strains and the control strains. (B) Hypocrellin A production from the overexpression strains and the control strains. (□) represents the control strains, (●) represents the *mono* overexpression strains, (▲) represents the *hyd* overexpression strains.

mized through running an orthogonal test. Results showed that the production of protoplasts was highest when the hypha of the *S. bambusicola* S4201 had been cultured at 28°C, spun at 180 rpm for 30 h, treated with enzyme mixture at 32°C, and spun again at 220 rpm for 4 h with Osmotic medium (OM: $MgSO_4$ 1.2 M, NaPB 10 mM, pH 5.8) as osmotic stabilizer. On the other hand, the regeneration rate was highest when the strains had been cultured at 28°C, spun at 180 rpm for 30 h, treated with enzyme mixture at 26°C, and spun again at 180 rpm for 2 h with OM as osmotic stabilizer (Supplementary data Tables S1 and S2).

In GFP analysis, the GFP transformants grown on the regeneration medium with 8 μg/ml benomyl were picked out individually and the single GFP clone strain was continuously cultured until hypha regenerated. No GFP fluorescence signal appeared in the control strains, while a significant fluorescence signal was detected around the hypha of the single GFP clone (Supplementary data Fig. S4). These results confirmed that the *GAPDH* promoter Pgpd1 co-expressed the benomyl resistance gene protein and GFP protein in the *S. bambusicola* S4201.

Overexpression of *mono* and *hyd* and HA production analysis

The *mono* and *hyd* transformants were picked out individually from the medium with 8 μg/ml benomyl and cultured until the hypha regenerated. Next, the single clone strain was cultured in PDA liquid medium with Triton X-100 for 5 days. The results showed that the biomass of the three samples did not differ significantly, with the trend of change similar between overexpression transformants and the control strains at all times ($P < 0.05$). In contrast, significant differences were observed in the production of HA between overexpression transformants and control strains ($P < 0.05$). The HA production was 607 mg/L in the control strains, with HA production in *mono* transformants more than triple that (2,144.1 mg/L) and *hyd* transformants higher-producing than the control strains after the fifth day (1,180.1 mg/L; Fig. 2).

RT-qPCR analysis of *mono*, *hyd* and relevant genes

For confirming the overexpression effects of *mono* and *hyd* genes, RT-qPCR analysis was performed for both transformants and control strains. The relative expression level of the *mono* gene in *mono* transformants peaked at 6 times, while the relative expression level of the *hyd* gene in *hyd* transformants was 11 times higher than in the control strains on the fourth day. The salicylate hydroxylase activity in *hyd* transformants was higher than in the control strains and shared a similar changing trend with the *hyd* transcription level (Fig. 3). These results confirmed that the overexpression plasmids were transformed into the *S. bambusicola* S4201 and successfully expressed. To analyze the effects of *mono* and *hyd* overexpression on other relevant genes, we studied the relative expression levels of *mco*, *pks*, *omef*, *fad*, *mfs*, and zinc fingertrabscription factor (*zftf*). Our results showed that the

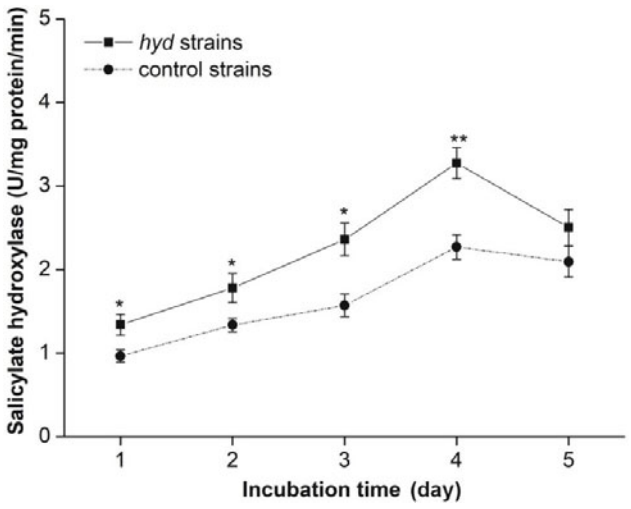

Fig. 3. Salicylate hydroxylase activities in *hyd* overexpression strains and control strains.

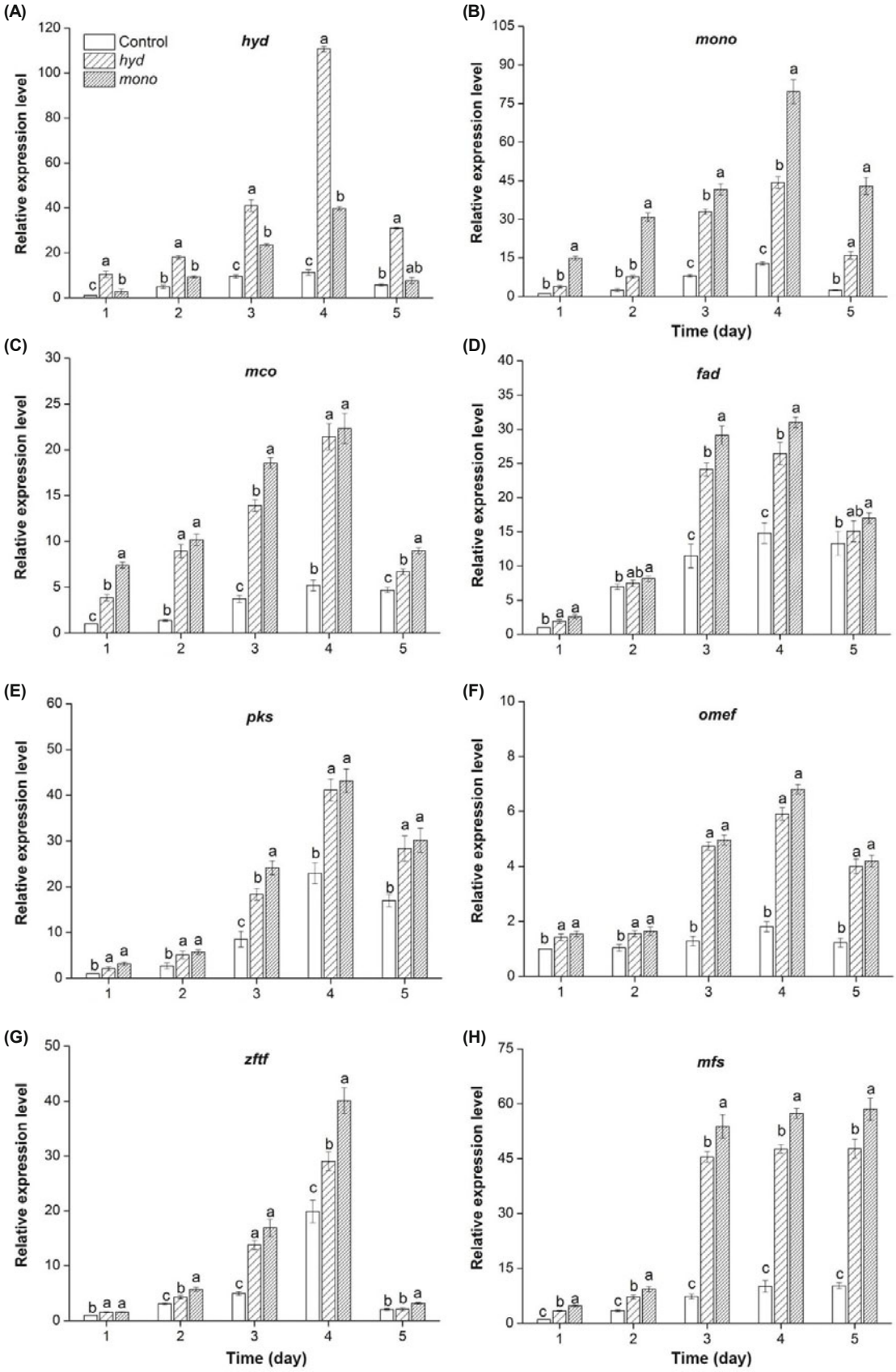

Fig. 4. **Relative expression levels of relevant genes in the HA biosynthesis gene cluster.**

relative expression levels of *mco*, *pks*, *omef*, *fad*, and *zftf* genes gradually increased in the first 1–4 days and declined on the day 5 in both *mono* and *hyd* transformants. The transcription level of *mfs* increased and maintained a high level for the whole process. Moreover, the transcription levels of all six of these genes in *mono* and *hyd* transformants were higher than that in the control strains. Besides, apart from *hyd* gene, the relative expression levels of the other 7 genes in *mono* transformants were higher than that in *hyd* transformants for all times during the culture period (Fig. 4).

Discussion

S. bambusicola is a traditional Chinese medicine used in the treatment of tracheitis, arthritis, pertussis, sciatica and other diseases. However, the application of HA is limited because of its low production in wild *S. bambusicola* strains and its unclearly defined anabolic pathway. To obtain the high-producing HA strains, the genes involved in the HA biosynthetic pathway should be overexpressed in *S. bambusicola* S4201. In our study, research on HA biosynthesis was slowed by the low transformation efficiency of *S. bambusicola* S4201. Meanwhile, hygromycin B, a selection marker widely used in many other filamentous fungi, could not resist the wild *S. bambusicola* S4201 strains even at high concentrations (1 g/ml), though it was previously reported that hygromycin B can be a selection marker for *Shiraia* sp. SUPER-H168 (Deng *et al*., 2016). We introduced a new selection marker for the *S. bambusicola* S4201 and its growth was suppressed on the medium contained 8 μg/ml benomyl. An improved transformation system for *S. bambusicola* S4201 was established by optimizing the protoplast-preparing method with hypha and utilizing the PEG-$CaCl_2$ genetic transformation method. The high protoplast production ensured the improvement of the genetic transformation efficiency. Meanwhile, the changes in osmotic stabilizers, enzyme mixtures and genetic transformation methods also ensured that plasmids could be transformed into the competent cell and expression more effectively (Yu and Chang, 1987). The GFP analysis confirmed that the gene fusion expression system we constructed with the *GAPDH* promoter Pgpd1 was effective in the *S. bambusicola* S4201. Simultaneously, the rigid 2A peptide with 15 amino acids was significant in gene co-expression. Different from other flexible linkers, it can form 3 α-helices and ensure the functional integrity of the two proteins at both ends of the 2A peptide in protein co-expression. These conditions also contribute to the high regeneration rate in overexpression studies. In a word, this efficient genetic transformation system we established will make an important contribution to the application of molecular biology technology in *S. bambusicola*, allowing it to be another model strain for genetic studies of filamentous fungi, in addition to *Aspergillus nidulans* and *Neurospora crassa*. Meanwhile, we plan to perform additional studies to identify the essential genes involved in the HA biosynthetic pathway by editing the genome with this efficient genetic transformation system.

Both hypocrellin and cercosporin are perylenequinones and share an extremely similar structure. Researchers have indicated that their biosynthetic pathways could share a similar process (Zhao *et al*., 2016). The biosynthetic pathway of cercosporin is well understood according to the previous work and CTB3 is an important gene involved in this process (Chen *et al*., 2007). Similarly, two putative functional domains with O-methyltransferase and FAD-dependent monooxygenase at the N and C termini were found in *mono* gene protein of *S. bambusicola*, which has the same domains as CTB3. It could be inferred that the *mono* gene occupies an important position in HA biosynthetic mechanisms and has a similar function to CTB3. It may be involved in the polyketide oxidations, catalysing the addition of one or two methyl groups into the polyketomethylene of the HA backbone formed by the *pks* gene protein (Okubo *et al*., 1975). A later study supported this hypothesis and found a high-producing HA strain with *mono* gene overexpression. The *hyd* gene is another essential gene in our predicted HA biosynthetic pathway and is also located on the HA biosynthetic gene cluster along with *mono* in the *S. bambusicola* S4201. However, the *hyd* is not shown in the predicted HA biosynthetic pathway of *Shiraia* sp. SUPER-H168. Other studies have showed that relatively high expression level of the *hyd* gene was found in a high HA-producing strain stimulated by metal ions such as Ca^{2+} (Liu *et al*., 2018). In later overexpression studies, the production of HA increased about twice as much in *hyd* transformants than control strains. Protein Blast research results in the NCBI database were used to identify the function of the *hyd* gene protein. The conceptually translated *hyd* protein was similar to many salicylate hydroxylases. We infer that some salicylic acid groups can be modified by the *hyd* gene in HA biosynthetic intermediates and that the overexpression of the *hyd* gene stimulates hydroxylation in the HA biosynthetic pathway, thereby promoting HA production.

Meanwhile, the effects of *mono* and *hyd* overexpression on other relevant genes, such as *pks*, *mfs*, *fad*, *omef*, *zftf*, and *mco* in the HA biosynthesis gene cluster, were analyzed. The RT-qPCR method was used on *mono* overexpression strains, *hyd* overexpression strains and the control strains. The results show that both *mono* and *hyd* overexpression promote transcription of other relevant genes in the HA biosynthesis gene cluster. These results suggest that all eight of these genes are involved in the HA biosynthesis system. Furthermore, *zftf* has been confirmed as a transcriptional factor, containing GAL4-like transcription regulators in our study and sharing a similar fluctuating trend with other genes, except *mfs*. We infer that the expression of these eight genes is regulated by *zftf* and co-ordinately induced under HA-producing conditions, like CTB8 in the cercosporin biosynthetic pathway (Chen *et al*., 2007). Further, high transcription of the *mfs* gene was maintained, resulting in the maximum hypocrellin yield. This facilitated the increased transfer of hypocrellin from cell to extracellular space. We speculate that the escape toxicity of the radicals and singlet oxygens produced by hypocrellin during light-induced activation may be partly responsible for the improvement of hypocrellin yield (Daub *et al*., 2005). Considering the results of real-time PCR, HA production in overexpression strains, the cercosporin biosynthetic pathway and a putative HA biosynthetic pathway reported previously, we propose a pathway for HA biosynthesis that resembles fatty acid synthesis. In *mono* overexpression trans-

formants, the slow increase of transcription levels was observed in the first two days, with a sharp significant increase exhibited on the third day in *pks*, *omef*, and *fad* genes, which contrasts the transcription levels of *mco* and *hyd* genes that begin to increase significantly on day one of the culture. The results suggest that there is a feedback regulation mechanism in the HA biosynthetic pathway. We suppose that *mco* and *hyd* genes are located after the *mono* gene in the HA biosynthetic pathway because *mono* overexpression first resulted in the up-regulation of *mco* and *hyd* genes, and the decline of substrates stimulated the up-regulation of *pks*, *omef*, and *fad* genes after two days of culture. For the same reason, the *hyd* gene should be located between the *mono* and *mco* genes in the *S. bambusicola* S4201, based on the results of RT-qPCR in hyd overexpression strains. Taken together, the intermediate products, which have been modified with *pks*, *fad*, *omef*, were first oxidized and hydroxylated by *mono* and *hyd*, generating HA through the oxidation synthesis of *mco* and transporting it to extracellular space by *mfs* at the end. Thus, the whole HA biosynthetic pathway is regulated by *zftf*. To determine the exact locations and functions of mono and hyd in the HA biosynthetic pathway, we will pursue further studies measuring the change in substrate and product using HPLC or Gas chromatography.

In general, the overexpression of *mono* and *hyd* improved the production of HA in *S. bambusicola*. Its production was higher than the chemically or physically-stimulated strains. The overexpression increased the transcription levels of adjacent genes located on the HA biosynthesis gene cluster and they worked collaboratively to increase the production of HA in the *S. bambusicola* S4201. Moreover, the efficient genetic transformation method established in this study will contribute to future gene editing and studies with the CRISPR/Cas 9 system or NHEJ pathway in the *S. bambusicola* S4201, which we hope will confirm whether *mono*, *hyd* or other relevant genes are involved in HA biosynthetic pathway. In the second part of the study, the mono protein and hyd protein will be expressed *in vitro* and we will learn more about their structure and function.

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