Efficient agrobacterium-mediated transformation of *Shiraia bambusicola* and activation of a specific transcription factor for hypocrellin production

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**ABSTRACT**

*Shiraia bambusicola*, as a well-known medicinal and edible macro-fungus, is generally located on the branches of bamboo and plays an important role in herbal treatment. Furthermore, the natural products from its fruiting bodies, hypocrellins, are attracting increasing amounts of attention due to their notable bioactivities. In this report, we focused on constructing a fast and universal transgenic system for *S. bambusicola*, which could easily regulate the biosynthesis of hypocrellins by altering gene expression. By the consideration of the broadened application of the mycelial transformation, it was determined to implement the *Agrobacterium tumefaciens*-mediated transformation (ATMT) technique, and a transcription factor (*SbTF1*) was employed to activate the downstream genes in different producing strains. After real-time polymerase chain reaction analysis, gene *SbTF1* was verified to be overexpressed in all of the transgenic strains, and the yield of hypocrellins was significantly improved in strain CNU103846. To confirm the regulated function, the transcript levels of the downstream genes and the corresponding products at different stages were screened. In this study, we developed a stable and easily available assay for genetic transformation of *S. bambusicola* and employed the method to demonstrate that one transcription factor *SbTF1* could effectively increase the yield of the final products.

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**Introduction**

*Shiraia bambusicola* P. Henn. is hosted on the branches of bamboos and is widespread over the southern region of China. As a traditional herbal medicine, fruiting bodies of *S. bambusicola* have been used to treat rheumatic arthritis, pertussis, tracheitis and skin diseases for several centuries. Hypocrellins, including hypocrellin A (HA), hypocrellin B (HB), hypocrellin C (HC) and hypocrellin D (HD), are the dominant secondary metabolites in *S. bambusicola* [1, 2]. Currently, these components have emerged as notably potent novel therapeutic medicine, especially in photodynamic therapy (PDT), with antifungal, antibacterial, antiviral and antitumor activities [3–6]. However, the low yield of produced hypocrellins has become a considerable bottleneck for further improvement.

Therefore, when considering hypocrellin production, the construction of an effective transgenic assay and the improvement of the fermentation yield by regulating gene expression are attractive. For the *Shiraia* species, protoplasts derived from conidia have been used for transformation, which yielded transgenic lines with stable inheritance [7]. Unfortunately, most of the industrial strains could not effectively produce the corresponding spores, and furthermore, the higher-yield stains usually had weaker sporulation. Among the generally available methods, the *Agrobacterium tumefaciens*-mediated transformation (ATMT) technique is an easy and flexible transgenic assay and has been previously used in many plants, from the leaves of *Arabidopsis thaliana* to the callus of *Zea mays* (maize). Several species of *Fusarium* and other fungi have also smoothly produced transgenic fragmented mycelia by the same technique [8–10].

In fungi, the functional genes are always clustered into neighboring locations and produce the metabolites in an integrated fashion. In certain instances, a specific transcription factor hidden in the gene cluster can directly regulate the other corresponding genes. *AcstuA* was identified from *Acremonium chrysogenum* as a transcriptional regulatory gene, and its disruption drastically reduced the cephalosporin production and blocked the expression of downstream genes [11]. The transcription factor *GaaR* is needed for the expression
of the genes required for D-galacturonic acid biosynthesis in *Aspergillus niger*, and the overexpression of GaaR resulted in an increased transcription of the D-galacturonic acid transporter [12]. The putative gene cluster for the synthesis of hypocrellins was identified from the *S. bambusicola* genome [13], and one putative transcription factor also appeared among them.

In this study, we aimed to optimize the genetic transformation of fragmented mycelia from *S. bambusicola* using ATMT assay, and furthermore, to make use of one specific transcription factor to increase the yield of hypocrellins.

**Materials and methods**

**Organisms and plasmids**

The original strains of *S. bambusicola* were preserved in the China Forestry Culture Collection Center (CFCC), including zzz816, GZ4-6, CNU103846 and B18. The strains were used as the wild-type recipients in the transformation experiments. Before transformation, these strains were incubated in PDB (potato dextrose broth) for two days. *Agrobacterium tumefaciens* strain AGL-1 (provided by Gang Liu, Institute of Microbiology, Chinese Academy of Sciences) was stored at −80 °C and revitalized in YEB (yeast extract broth) medium containing kanamycin before use. *Escherichia coli* DH5α (Mei5 Biotechnology Co., Ltd) was used in vector construction. A binary vector was constructed on the backbone of pAg1-H3 [14] (provided by Gang Liu, Institute of Microbiology, Chinese Academy of Sciences), which harbors a hygromycin B resistance gene as a selection marker under the control of the fungal promoter *PtrpC* and terminator *TrpC*. A 1.3-kb fragment of the transcription factor *SbTF1* sequence, which was amplified from the genome of *S. bambusicola*, was incorporated into the plasmid driven by the *PgpdA* promoter. pAg1-OE was linearized by endonucleases, and the *SbTF1* sequence was subsequently ligated to the linear vector using a ClonExpress™ II One Step Cloning Kit (Vazyme Biotech Co., Ltd.). The structures of pAg1-OE and pAg1-TF1 are shown in Figure 1, and the primers used in this investigation appear in Table 1.

**Fungal transformation**

*S. bambusicola* (inoculated with a 2-day-old culture) were grown in 150 mL PDB under shake-culture

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**Figure 1.** Construction of the binary vectors pAg1-OE (a) and pAg1-TF1 (b).

**Note:** The plasmids were constructed for the ATMT of *Shiraia bambusicola* with the *SbTF1* gene placed under the strong promoter *PgpdA* from *Aspergillus nidulans* and the terminator *TrpC*.

**Table 1.** Primers used in this investigation.

| Primer          | Sequence(5'-3')                                      |
|-----------------|------------------------------------------------------|
| SbTF1-F         | TTAGACGAGACATCCCCTGGCCACTCAACTCCCTACC               |
| SbTF1-R         | AAGGTAAATGGATCTGCCAATTCCCTG                         |
| PgpdA-F         | AACGACGGCCAGATGAAATCCCTTGATCTCTAC                  |
| PgpdA-R         | TCGAGAGGCTGACTGAGCTGACTG                           |
| TrpC-F          | CGCTTGAGCAGACATCGACACTCCCTGCCAC                     |
| TrpC-R          | TTAACGTAGTACCTTGAAAT                                |
| PgsdAtest-1     | GAAATCCTGGATCATCTTACA                               |
| TrpCtest-R      | GAGATACGCTGACACTGACGACATG                          |
| Hyg500-F        | ATCGGTATCGATTTTATCGCACT                             |
| Hyg500-R        | TGGGCAGCGCTGATTTGG                                  |
| PKS1q-F2        | ACCCTCATTCAAGCCAAAC                                 |
| PKS1q-R2        | ACGACCTTTCCGATGATTGG                                |
| SbTF1q-F1       | TTGACGCCCCATTTCCATGG                               |
| SbTF1q-R1       | TGGAATGGGAGTCGCCGTTC                                |
| Sb5AS1q-F       | GCAATCCGAGAAGACTG                                  |
| Sb5AS2q-R       | ATGGTCCTGAAACGGCTCCTG                              |

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conditions at 26°C and 180 rpm for 4 days. The new culture was filtered through sterilized gauze to gather small mycelial pellets. Next, the mycelial pellets were gently washed with 20 mL of 0.5 mol/L MgSO₄ and 0.05 mol/L maleic acid (pH 5.9) [10]. The pellets were later milled with quartz sand in sorbitol solution, and the small mycelium, suspended on the face of the liquid, was picked up to soak on the filter for the transformation operation. *A. tumefaciens* strain AGL-1 was grown in minimal medium (MM) supplemented with kanamycin (100 µg/mL) at 28°C for 2 days and was diluted to optical density at 600 nm (OD₆₀₀) of 0.2 in induction medium (200 µmol/L acetosyringone) and incubated for 48 h at 26°C. After co-cultivation, the mycelial pellets were washed by cephalosporin at 100 µg/mL, transferred to PDA containing hygromycin (300 µg/mL) and cephalosporin (100 µg/mL), and subsequently incubated at 26°C for 4 days to select the correct mutants. We randomly identified mycelia that were growing on the selective medium by polymerase chain reaction (PCR). Transformants were grown on PDA medium with hygromycin B together with the wild type as the control. In the sensitivity analysis the following were employed in selection media for ATMT: hygromycin, glufosinate and hygromycin B, were applied for the sensitivity analysis of *S. bambusicola* 816. In view of the popularity of different industrial strains, we decided to focus on the fragmented mycelia for transformation, and therefore, the results were dependent on the diameter of the colonies. As shown in Table 2, hygromycin B (300 µg/mL) and nourseothricin (200 µg/mL) displayed high bioactivity against the growth of mycelial strains; the others were incompatible with this species.

### Table 2. Diameter of colonies incubated in increasing concentrations of hygromycin and nourseothricin.

| Concentration (µg/mL) | Diameter of colonies, x±SD (mm) |
|-----------------------|---------------------------------|
| 0                     | Hygromycin | Nourseothricin |
| 0                     | 4.65 ± 0.05 | 4.65 ± 0.05 |
| 100                   | 1.98 ± 0.11 | 1.51 ± 0.04 |
| 150                   | 1.23 ± 0.08 | 0.65 ± 0.03 |
| 200                   | 0.98 ± 0.07 | 0.40 ± 0.04 |
| 250                   | 1.79 ± 0.04 | — |
| 300                   | 0.44 ± 0.01 | — |

*a±SD, mean diameter (mm) with standard deviation.

SbSAS1 was identified as the most stable gene among the candidate reference genes [16], and therefore, it was chosen as the reference gene to normalize the data for the analysis of the relative gene expression. The relative expression level of each gene was calculated using the 2⁻ΔΔCT method.

### High performance liquid chromatography (HPLC)

Extraction of cell-associated hypocrellins from *S. bambusicola* was done as described by Shen et al. [17]. The content of the extracts was analyzed by an Agilent 1200 Series HPLC system equipped with a Kromasil 100-5 C18 (250 × 4.6 mm). The content of elsinochrome was also detected by the same technique.

### Data analysis

Data are presented as mean values with standard deviation (±SD) from three independent measurements.

### Results and discussion

#### Sensitivity analysis for the chosen selection agents and concentrations

Four common selection agents, benomyl, nourseothricin, glufosinate and hygromycin B, were applied for the sensitivity analysis of *S. bambusicola* 816. In view of the popularity of different industrial strains, we decided to focus on the fragmented mycelia for transformation, and therefore, the results were dependent on the diameter of the colonies. As shown in Table 2, hygromycin B (300 µg/mL) and nourseothricin (200 µg/mL) displayed high bioactivity against the growth of mycelial strains; the others were incompatible with this species.

#### Mycelial transformation by Agrobacterium tumefaciens and protoplasts

With the rapid improvement in the hypocrellins industry, more and more attention has been paid to...
molecular modification of the relevant strains. There have been studies for improvement of the genetic transformation techniques in recent years, mainly depending on protoplast transformation from spores [18–21]. It is common for filamentous fungi to make use of spores as methods of reproduction, but this technique is not ideal to produce diverse strains for producing hypocrellins, especially since the higher yield strains have weaker sporulation. To remove this obstacle, we focused on the transformation of fragmented mycelia and even abandoned the optimized protoplast system by polyethylene glycol-mediation. The more convenient and robust ATMT tool was selected for further study, and this transformation system was able to guide the genes into the diverged strains. Breaking away from the limit of weak sporulation, almost all of the culturable strains from S. bambusicola successfully accepted the molecular modification, and similar species could also be modified with this reliable tool for genetic engineering.

Genomic DNA of both transformants and the wild type were extracted using the CTAB method. The hph gene was identified by PCR using primers Hyg500F/Hyg500R, which amplified a 500-kb fragment of the internal region of the hph gene.

Cloning and characterization of the putative transcription factor SbTF1 in S. bambusicola

The specific transcript factor SbTF1 was identified through homology searches with the BLASTP algorithm and it was located on the putative gene cluster for the hypocrellins synthesis pathway. The factor had a close relationship with the transcriptional activator of the sterigmatocystin biosynthesis gene cluster in Emericella nidulans and the cercosporin biosynthesis gene cluster in Cercospora nicotianae. RT-PCR analysis confirmed that the expression of SbTF1 was up-regulated during the fermentation stage but not very strongly or for long.

Analysis of different transformants using SbTF1 activation

Through the ATMT assay, the full length of the SbTF1 gene was added into plasmid vector pAg1-OE for high-efficiency expression and was transformed into different strains of S. bambusicola including CNU103846, zzz816, B18 and gangzhu4-6. Under routine testing, these transformations were finished successfully, and the transcript levels of SbTF1 were improved considerably. By the analysis of phenotype and HPLC results, the yield of hypocrellins was clearly boosted in CNU103846 (Figure 2), but not in the other species (data not shown).

Figure 2. Amount of hypocrellins (mg/L) and elsinochrome (mg/L) produced by the wild-type strain, and the overexpression mutants.
Note: Each sample was detected after 96 h and 144 h of incubation.
Regulation of SbTF1 in hypocrellin and elsinochrome production

The relevant functional genes, covering, *mono* and *hyd*, have been employed to improve the yield of hypocrellins [19], and some amylase genes could also assist to produce the natural products [20, 21]. The cluster-specific transcription factor, which usually appears in the biosynthetic gene cluster for natural products and could directly regulate the expression of the adjacent genes, was not involved [22]. Among the similar perylenequinoid compounds, *EfPKS1* and *CTB1* have been respectively found in the gene cluster for elsinochrome and cercosporin, and gene knockout demonstrated their regulatory effects [23–25]. However, none of them was reported to increase the yield of the relevant pigments.

In this study, we explored the putative transcription factor SbTF1 for hypocrellins and uniquely employed it to enhance perylenequinone production for the first time. By a trial of various strains with different productivity, we observed that the modified strain CNU103846 had made great progress in the synthesis of hypocrellins, considerably higher than the former strains, either the original CNU103846 or the high yield zzz816 strains. The yield of elsinochrome also displayed a similar variation trend but not so clearly. Even among the present studies with diverse modified strains, such as *S. bambusicola* zzz816 and *Shiraia* sp. Slf14, the strain CNU103846 was still at the top of the list [17, 26]. It was noticeable that the premature overexpression of SbTF1 hindered the normal growth of mycelia; therefore, the final mass was significantly lower than in the primary strain. In the next step, we plan to improve the induction of the promoter and further re-optimize the process of submerged fermentation.

In the transgenic strain CNU103846, the period of hypocrellin production was shortened, which was consistent with the alteration of SbTF1. At a prior stage of submerged fermentation, the output per unit of hypocrellins had reached its peak, and it remained steady during the process. Although the early overexpression of SbTF1 played a negative role in mycelial growth, it is still worthy to note that the final yield of hypocrellins increased to 210.65% of the original strain and even exceeded that of the high yield strain zzz816 (Figure 2).

Moreover, the key downstream gene SbPKS1 was also analyzed by RTFQ-PCR, and its abnormal overexpression demonstrated that SbTF1 had up-regulated the putative gene cluster for hypocrellins synthesis. On the basis of the elevated production efficiency (Figure 3), the results from Figure 2 also supported the close association of the gene cluster with production of hypocrellins.

During submerged fermentation, the industrial strains were not always able to maintain the high yield of hypocrellins, where the culture conditions and media components both might interfere with the process directly. In the general, the PDA medium is adapted to the *S. bambusicola* strains, but some important strains only produce hypocrellins in addition to Triton X-100 [27, 28]. The employment of other media would receive more restrictions and would often eliminate the red pigments, especially including the natural components [17, 29, 30]. The wave length and intensity of light could also determine the biosynthetic efficiency, so the increase of the production has to be limited to the update of equipment [31–33]. These requirements are very likely to increase the cost of large-scale production and hamper the further development of the corresponding industries. In our study, the overexpression of SbTF1 was able to activate the relevant gene cluster and ensure the stability of hypocrellin production under the more compatible conditions. Thereof, the edited strains in this study

![Figure 3. Relative expression levels of SbTF1 and SbPKSI by real-time PCR.](image)
were anticipated to have the great adaptability of various cultural conditions, and we would greatly advance the production of hypocrellins through the broader range of medium components and the more permissive way for fermentation.

Conclusions
In this study, the ATMT technique was employed to build a stable and easily available technique for genetic transformation of S. bambusicola, which could be used in the mycelial transformation for industrial strains. By this method, the cluster-specific transcription factor SbTF1 was overexpressed, and it improved the yield of the hypocrellins, up to 210.65% of the original strain. The stable performance of the fermentation process indicated that the modified strain would have an extensive application in the corresponding industry.

Disclosure statement
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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