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Construction of a T-DNA Insertional Library of *Colletotrichum gloeosporioides* ES026 Strain and Cloning of Relevant Gene of Huperzine A Biosynthesis Pathway

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Huperzine A (HupA), an anti-Alzheimer's disease (AD) drug candidate, was isolated from *Huperzia serrata*. Recently, it was found that various endophytic fungi produce Huperzine A. However, it might be due to the innate properties of endophytic symbiosis, that production of HupA in large quantity from endophytes has not yet been put into practice. *Colletotrichum gloeosporioides* ES026 was previously isolated from *Huperzia serrata* and proved to produce HupA. In this study, the strain was successfully transformed using *Agrobacterium tumefaciens* mediated transformation approach. Moreover, the transformated conditions were optimized. The results showed that under the condition of 25<1×10^6 spores /mlμA.tumefaciens OD_{600} = 0.3-0.5<400μM acetosyringone and 48h co-cultivation in the presence of induction medium, a high transformation efficiency (about 95 transformants per10^6 conidia) could be obtained. Under this optimized transformed system, a T-DNA insertional mutant library of 4,000 transformants was constructed. A T-DNA insertional transformant EST010044 was screened, whose yield of Hup A was about 1.83 times of ES026. The border flanking sequence of T-DNA was recovered by TAIL-PCR, and annotated with the highest similarity to a methyltransferase domain. The results provide a foundation for further research of relevant genes of Hup A biosynthesis pathway.

Key words: *Colletotrichum gloeosporioides* ES026, *Agrobacterium tumefaciens* mediated transformation, Huperzine A biosynthesis.

Huperzine A (HupA), a pharmaceutical *Lycopodium* alkaloid, was isolated from the traditional Chinese medicine plant *H. serrat*a. The chemical structure of Hup A (Fig. 1) [1, 2]. Hup A is a promising drug for Alzheimer's disease (AD) being highly specific and reversible inhibitor of acetylcholinesterase (AChE) [3, 4]. Compared to chemically synthesize HupA[5, 6], HupA derived from plant extracts have many characteristics such as significant curative efficiency, short functional cycle, little side-effects, and less drug resistance. Therefore, Hup A on the pharmaceutical market has been predominantly extracted from wild plant *H. serrata*[7, 8], which results in the rapid decline of *H. serrata* population in China due to over-harvesting[9, 10], The original source of Hup A possesses a low content of Hup A (0.007%, w/w) [9, 11]. It belongs to a club moss, has a very limited distribution and grows very slowly [12]. It takes at least 15 years from spore germination through a gametophyte stage to finally reach a mature sporophyte stage [12-14] , thus , the natural production of HupA has been greatly limited. Several studies

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have confirmed that endophytic fungi and the host plant have the same route for synthesis of secondary metabolites and can produce the same or identical physiologically active metabolites [15, 16]. Recently much attention has been paid to find a potential microbial source producing Hup A. A few researches on the isolation of HupA-producing endophytic fungi from different Huperiaceae plants have been reported [11, 12, 17]. In our study, a HupA-producing strain *C. gloeosporioides* ES026 was isolated from a variety of endophytic microbes of *H. serrata* and several factors possibly promoting its yield of HupA through fermentation were researched [12]. However, due to the innate properties of endophytic symbiosis, the total chemical synthesis of HupA in an industrially feasible manner is not available, and the in vitro culture of lycopods has only met limited success [14].Therefore production of HupA in large quantity from endophytes is not practiced yet.

Agrobacterium tumefaciens-mediated transformation (ATMT) becomes a powerful tool for molecular genetic analysis of filamentous fungi and plants due to high transformation efficiency [18, 19]. Using this method ,we obtained extensive mutants , and cloned the silent genes, which has great potential for finding novel genes relevant to metabolism, morphogenesis, and so on [20, 21]. In this study, a T-DNA insertion mutant library of *C. gloeosporioides* ES026 was established, using ATMT. Here, we focused our research on an optimal procedure of the ATMT for efficient isolation of transformants, and screening the transformants relating to the yield of HupA and subsequently cloning the T-DNA junction fragments of high-yielding of HupA transformants using TAIL-PCR.

MATERIALS AND METHODS

Strains, plasmid and culture conditions

The strain *C.gloeosporioides* ES026, which produced the highest HupA in our initial screening, was preserved at the China Center for Type Culture Collection (CCTCC NO: 2011046), Wuhan, China. The strain was cultivated on potato-dextrose-agar (PDA) medium at 25°C and for long term storage maintained at -70°C in 17% glycerol [12]. Spores of *C.gloeosporioides* ES026 were obtained from 7-day-old cultures grown on PDB liquid medium at 25°C, 9h/d, and 150r/min at a shaker. Mycelia and debris were removed filtering through 2 layers of cheesecloth. A concentration of 10^7spores/ml was adjusted with sterile deionized water [22]. The transforming binary vector pTFCM contains a T-DNA harbouring a hygromycinB resistance (*hph*) gene. The vector under the control of the PtrpC promoter was propagated in the hypervirulent strain *A. tumefaciens* EHA105 . The vector and the hypervirulent strain were kindly provided by Professor Guoqing Li (Huazhong Agricultural University, Wuhan, China). The *A. tumefaciens* EHA105 was cultivated on Luria-Bertani (LB) medium with 50µg/ml kanamycin, 50µg/ml rifampicin and 50µg/ml streptomycin sulphate, at 28°C [23].

Agrobacterium-mediated transformation of *C. gloeosporioides* ES026

Agrobacterium-mediated transformation was carried out following the method of Li *et al* and Gong *et al* [24, 25] with some modifications. Bacterial cultures were diluted to an OD_{600} of 0.3 by induction medium (IM) containing 200µM acetosyringone (AS), and were mixed 1:1 with a conidial suspension (10^7spores/ml). Mixture spread over a glass paper on a Co-IM plate containing 400µM of acetosyringone (AS). After co-cultivation at 23°C for 36 h, the membranes were removed, inverted, and placed mycelia-side down onto PDA containing 150µg/ml of cephalosporin to counter-select bacteria, and 200µg/ml of hygromycin B to select *C.gloeosporioides* transformants. After incubation at 25°C for 3-5 days, transformant colonies were transferred to PDA plates containing 150µg/ml of cephalosporin and 200 µg/ml of hygromycinB for a second round of selection.

Optimization of ATMT conditions for efficient isolation of transformants

The effects of co-culture temperature and co-culture time on transformation efficiency were assessed respectively by ranging the time from 12h to 84h and the temperatures from 20 to 30°C. The influence of concentrations of *A. tumefaciens* and *C.gloeosporioides* ES026 during co-cultivation as well as the culture time of conidia of *C.gloeosporioides* ES026 were also assessed. These optimized parameters were then used to investigate the effect of different concentrations of inducer AS on transformation efficiency. To test

for the the stability of the transformants of the integrated hygromycin B resistance cassette, 13 mutants were cultivated on PDA without cephalosporin and hygromycin B. After five generations, transformants were grown on PDA plates containing hygromycinB (200µg/ml) and cephalosporin (150µg/ml), then the specific primers were used to amplify the hph gene fragement, while the wild *C.gloeosporioides* ES026 and the pTFCM acted as control

Evaluation of the transformation efficiency

To examine the genetic stablility of transformants, 13 transformants were randomly selected. After five generations in the absence of hygromycinB [26], transformants were grown on PDA plates containing hygromycinB (200µg/ml) and cephalosporin (150µg/ml), then the specific primers were used to amplify the *hph* gene fragement to identify the transformants' stability.

Quantification of Hup A produced by mutant strains through high performance liquid chromatography (HPLC)

Observations on morphology (color, size and surface smoothness of the mycelium pellets) and Hup A yield of mutant strains were done to find the genes related to Hup A biosynthesis. The extraction method of transformants fermented product was carried out following Zhao *et al* [12] , with some modifications. The mycelia were harvested after fermenting 7 days and grinded into powder after dried at 45°C. 5 ml anhydrous ethanol was added in 1.0 g dried powder for 30 minutes, then centrifuged at 10, 000 r/min for 5 minutes. The supernatant was removed, and the cell-debris pellet was extracted with 0.5% hydrochloric acid (w/v, 15 ml) for 30 minutes, followed by water bath ultrasonication at 35°Cfor 30 minutes. Next, the filtrates were rendered with ammonia solution to pH 9.0. Then the water phase was extracted with $CHCl_3$. After this, the incorporated $CHCl_3$ extracts were evaporated to dryness using the rotary evaporation instrument. The dry residue was dissolved in 1.0 ml methanol and passed through a 0.45µm polytetrafluoroethylene syringe filter into a 1.0 ml brown measuring vial prior to RP-HPLC analysis.

The measurement of HupA content was performed by HPLC using Agilent HPLC 1260 series (Agilent, California, USA) consisting of a WondaSil C18 column (4.6 × 250 mm, 5 µm; Shimadzu, Japan). The temperature of the column compartment was kept at 40°C. The injection volume was set to 20µl. The flow rate was 1.0 ml/min using 80 mM ammonium acetate (pH 6.0)-methanol (7:3, v/v) as mobile phase. The effluent was monitored at 310 nm. Quantification was achieved by using the standard curve generated from the HupA standard in a concentration range of 0.5–8.0µg/ml, where the peak area and height showed linear correlation with the absorbance ($R^2 = 0.997$).

Molecular analysis of transformants

The fungal genomic DNA was extracted with CTAB following Sambrook *et al*[27]. PCR detection of the *hph* gene was done using primers of *hph* gene (Forward primer : 5'-TCGCCCTT CCTCCCTTTATTTC -3', Reverse primer: 5'-GGTCGGCATCTACTCTATTCCTTT-3'), which defined a 1101-bp sequence spanning the *hph* gene.

Transformants' southern blot analyses were carried out according to Li *et al* [24], with slight modifications.Genomic DNA sequences flanking T-DNA insertions were amplified by thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR). The reaction conditions were as described by Liu *et al* [28].

Flanking sequences were recovered by TAIL-PCR and nucleotide sequences were compared with known protein sequences by the BLASTX algorithm of the Basic Local Alignment Search Tool [29] hosted by the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov). Subsequently the results were checked for conserved domains within a protein or coding nucleotide sequence, the Conserved Domain Database of NCBI was accessed (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml).

Statistical analysis

The data of all quantitative assays were analyzed with SPSS statistical analysis software (version19, IBM Company, The United States) , using analysis of variance (ANOVA). When significant treatment effects were found ($P < 0.01$), means were further separated using the test of Duncan's.

RESULTS

Optimization of ATMT condition for fungal transformation

The transformation efficiency from different co-culture time and temperatures was first

investigated. An increased number of fungal transformants was obtained in the range from 12h to 48h, while in the range from 48h to 84h decreased (Fig.2A). The highest number was 60 colonies per 10^7 spores in 48h. The results presented in Fig. 2B showed that the most fungal transformants at elevated temperature (65 colonies /10^7spores at 25°C) However, when the co-culture temperature increased to 30°C(32 colonies /10^7spores), the number of transformants was significantly lower than the number at 25°C. The effects of different concentrations of *A. tumefaciens* and *C.gloeosporioides* ES026 during co-cultivation were measured respectively. The results showed that the transformation efficiency was the highest when the concentration of *A.tumefaciens* was 0.3-0.5 at OD_{600} (68-71 colonies /10^7 spores). However, when the concentration of *A. tumefaciens* was higher than 0.5 or lower than 0.3 the number of transformants was reduced (Fig.2C). As for *C.gloeosporioides* ES026, the highest transformation efficiency was obtained with concentration of spores at 10^6 cfu/ml (Fig.2D). As transformation numbers between 10^6 cfu/ml (74 colonies/per dish) and 10^7 cfu/ml (71 colonies/per dish)were not significantly different, and a higher single T-DNA insertion efficiency was needed, 10^6 cfu/ml was chosen as the optimal concentration of *C.gloeosporioides* ES026 spores.

The culture time of conidia of *C.gloeosporioides* ES026 for transformants was optimized by checking the effect of different culture periods of the 4-11days, each at 1d increment. The results showed that the transformation efficiency on the 7th (78 colonies / per dish) and 8th (76 colonies / per dish) day was significantly higher than other treatment (Fig.2E). The 7th day was chosen as the final culture time, due to the yield of HupA of wild fungal ES026 was significantly higher than that of other treatment on the 6th day and 7th day [12]. At last, we examined whether AS was required for transformation of *C.gloeosporioides* ES026 (Fig.2F). Induction medium (IM) plates with or without AS were used for co-cultivation. The least transformants (40 colonies /10^6 spores) were obtained without AS. The number of transformants was increased with an increasing concentration of AS, reaching a maximum at 600μM (97 colonies / 10^6 spores), and the transformation numbers were not significantly different with 400μM (95 colonies/ per dish). Yet the concentration above 600μM of AS would have resulted in a slight decrease of the transformants' number (63 colonies/10^6 spores). However, to obtain less copy number of T-DNA, we chose 400μM as the optimal AS concentration.

Table 1. The morphological characteristics of different transformants

Transformants	Morphological characteristics of mycelial pellets		
	Color	Diameter (cm)	Smooth or not (+/-)
ES026	black	2	-
EST01005	Black auburn	0.5	+
EST010010	grayish brown	1.5	-
EST010021	yellowish white	1	-
EST010043	grayish brown	1	-
EST010044	brown	1	+

In view of these results, we established optimal conditions for transformation of *C.gloeosporioides* ES026 as follows. The *A.tumefaciens* strain culture was diluted with IM to an optimal density of 0.3-0.5 at 600 nm. Fungal conidia collected from the 7-day-old culture were

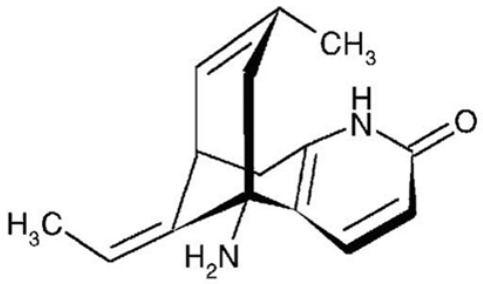
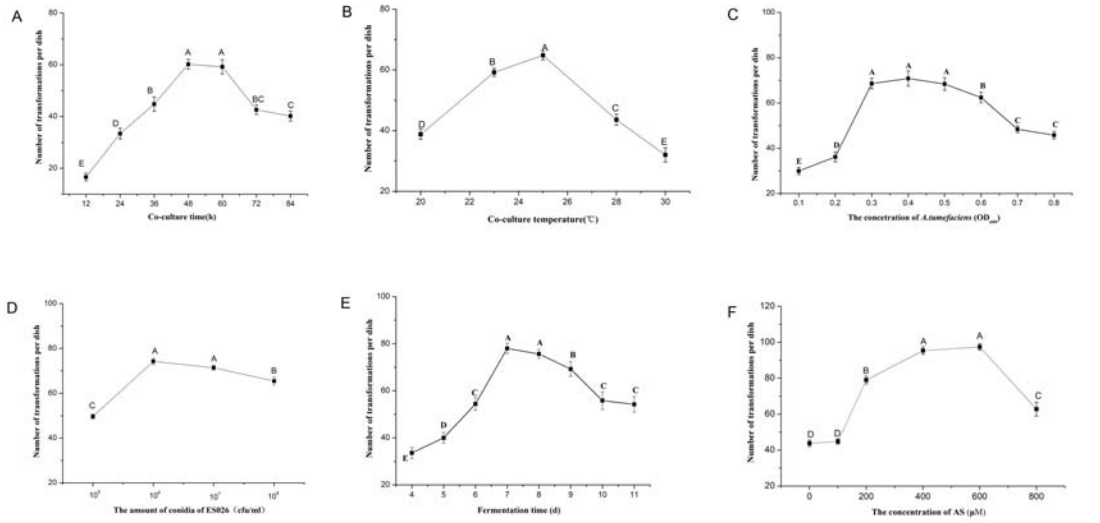


Fig. 1 The chemical structure of Huperzine A

suspended in IM and adjusted to 10^6 conidia/ml. A 100-µl bacterial suspension was mixed with the same volume of conidial suspension and spread over a hardened filter paper on an IM plate containing 400µM of AS. After incubation at 25°C for 48h, the filters were transferred to PDA medium containing hygromycin B 200µg/ml and cefotaxime 150µg/ml. Using the optimized protocol described above, about 4,000 tranformants in total were obtained and approximately 95 transformants in each selection medium.

Evaluation of genetic stability of the *Agrobacterium* mediated *C.gloeosporioides* ES026 mutant library

The integrated T-DNA was mitotically stable, as shown by analyses of 13 randomly chosen transformants: five successive 7-day passages of these transformants on PDA without the selection marker hygromycin did not result in the loss of integrated T-DNA, as indicated by the maintenance of the ability of all 13 transformants to grow on PDA containing hygromycin B (200µg/ml) and cephalosporin (150µg/ml). What's more, DNA fragments with an expected size (1101bp) could be amplified from all tested candidates, and binary vector pTFCM, but not from the original strain *C.gloeosporioides* ES026 (Fig.3). Therefore, stable transformants were obtained within the five generations.



A. Effect of co-culture time on the transformation efficiency. The bacterial suspension (100 µl, OD_{600} = 0.3) was mixed with the same volume of conidial suspension(10^7 conidia/ml) and incubated on each IM plate containing 200µM of acetosyringone at 23°C. **B.** The effect of co-culture temperature on the transformation efficiency. The bacterial suspension (100 µl, OD_{600} = 0.3) was mixed with the same volume of conidial suspension(10^7 conidia/ml) and incubated on each IM plate containing 200µM of acetosyringone for 48h. **C.** Effect of A.tumefaciens concentration on transformation efficiency. The bacterial suspension (100 µl, OD_{600}=0.1-0.8) was mixed with the same volume of conidial suspension (10^7 conidia/ml) and on each IM plate containing 200 µM of acetosyringone for 48h at 25°C. **D.** Effect of the amount of conidia of ES026 on transformation efficiency. The bacterial suspension (100µl, OD_{600}=0.3-0.5) was mixed with the same volume of conidial suspension(10^5-10^8 conidia /ml) and on each IM plate containing 200µM of acetosyringone for 48h at 25°C. **E.** Effect of the culture time of conidia of ES026 on transformation efficiency.The bacterial suspension (100µl, OD_{600}=0.3-0.5) was mixed with the same volume of conidial suspension(10^5-10^8 conidia /ml) and on each IM plate containing 200µM of acetosyringone for 48h at 25°C, in a sustained fenmentation time from 4th day-11th day. **F.** Effect of acetosyringone concentration in inducted medium on transformation efficiency. Acetosyringone was added to induction medium (IM) plates at various concentrations. The bacterial suspension (100µl, OD_{600} = 0.3-0.5) was mixed with the same volume of conidial suspension (fermentation time is 7th day, 10^6 conidia /ml)and incubated on each IM plate at 25°C for 48h. For all panels, each data point was the average of five replicates with error bar representing standard deviation.*Ducan's analysis, p≤0.01.

Fig. 2 Effect of different ATMT conditions on the *C.gloeosporioides* ES026 transformation efficiency

Molecular analysis of fungal transformants

Randomly selected 100 transformants, using the screening criteria (differnet) color, size and surface smoothness of the mycelium pellets, five types of obtained mutants were screened and identified from the T-DNA insertion library of transformants with different morphological characteristics from *C.gloeosporioides* ES026 (Table 1, Fig.4).

To further investigate the difference of the T-DNA insertion mutant on HupA, the yield of HupA of all the mutants with morphology change was analyzed by HPLC. Among these transformants, a transformant EST010044 produced the highest yield of HupA, which was about two times more than the control. The HupA yield of other mutants was altered, but not significantly higher comparing to control (Fig. 5).

All this five mutants were analyzed for T-DNA integration by Southern blot using *hph* gene as a probe. Among several mutants with morphological change the Southern blot analysis showed that, four (EST010005, EST010021, EST010010 and EST010044) contained a single T-DNA copy, while, EST010043 contained two T-DNA copies (Fig.6).

Based on these results, EST010044 was used to amplify the genomic DNA fragment flanking the insertion T-DNA with TAIL-PCR (Fig.7). Subsequently the sequence was further analyzed

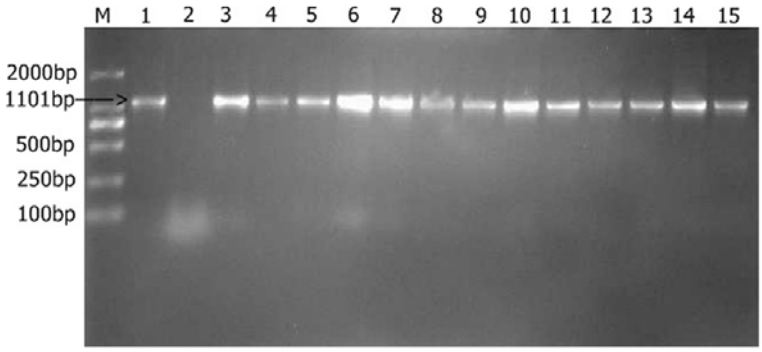


Fig. 3 Identification of transformants' stability by PCR
M: DNA Marker; 1: pTFCM <2-ES026; 3-15 transformants. The specific primers hpha and hphs were used to amplify the *hph* gene fragement.

Fig. 4 The morphological characteristics of mycelium pellets of different transformants.
The fungi were cultured in liquid growth medium consisting of potato 200 g/L (soup extract), and sucrose 20 g/L at 150 r/min shaking at 25°C.The sizes were measured with a vernier caliper. (bar = 1.0 cm).

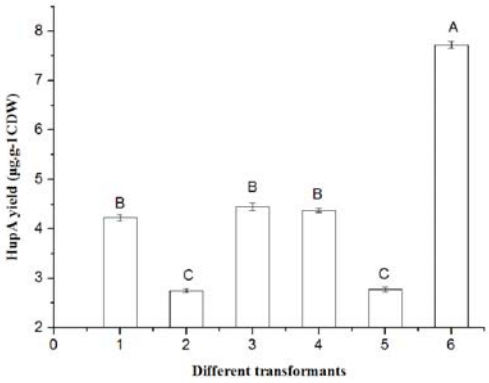
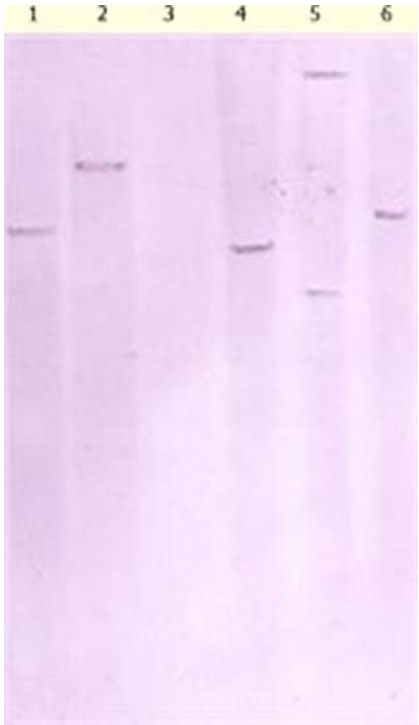


Fig. 5. HupA yield of different transformants
The fungi were cultured in the liquid growth medium consisting of potato 200 g/L (soup extract) and sucrose 20 g/L at 150 r/min shaking at 25°C for 7 days. *Ducan's analysis, p≤0.01. Each data point isthe average of five replicates with error bar representing standard deviation.



Genomic DNA of transformants were digested with SacI. The gel lanes show examples of transformants with morphological changes. Lane1: EST01005, a single T-DNA insertional site; lane 2: EST010010, a single insertional site; lanes 3: Wild type ES026; lanes 4: EST010021, a single insertional site; lanes 5: EST010043, two T-DNA insertional sites; lanes 6: EST010044, a single insertional site

Fig. 6 Southern blot analysis of genomic DNA of mutants of *Colletotrichum gloeosporioides* ES026

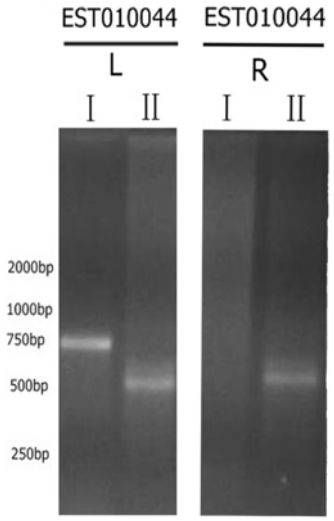


Fig. 7 Identification of T-DNA flanking sequences by TAIL-PCR
Agarose gel analysis of thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR) products generated from mutants EST010044. The'!anda!lanes (from left to right) represent the secondary, and tertiary reactions, respectively of the protocol.

for conserved domains within a protein or coding nucleotide sequence, the Conserved Domain Database of NCBI was accessed. The targeted gene showed the highest sequence similarity to a methyltransferase domain protein gene of *Colletotrichum gloeosporioides*.

DISCUSSION

The endophytic fungi isolated from pharmaceutical plants are simpler in genetics, easier to manipulation and more cost-effective to generate the drugs compared to their host plants [12]. Based on the preponderance, the discovery of HupA-producing endophytic fungi associated with *Huperziaceae* plants is significantly valuable for both basic research and industrial applications [30]. In recent years, HupA-producing strains have been isolated from fungi living in *H.serrat*a, for example, *Cladosporium cladosporioides* LF70 isolated from *H. serrata* by Zhang *et al* [11], *Trichoderma L44* isolated from *H. serrata* by Dong *et al* [31]. *Sharaia sp.* Slf14 isolated from *H.serrata* by Wang *et al*[32]. and so on. However, because maintaining the vitality and stability of the endophytic fungi during passages is a fundamental

obstacle, only scant research on HupA-producing endophytic fungi has been developed by far. Same issue was confronted in our study with C*olletotrichum gloeosporioides* ES026.

To obtain high-yield and stable HupA-producing endophytic fungi and to explore the relevant genes of HupA biosynthesis pathway, a protocol for *Agrobacterium*-mediated transformation technology was established. Up to date, more than 4,000 transformants have been obtained. We selected 200µg/ml hygromycin and 150µg/ml cephalosporin as the optimal concentration for ATMT. The optimal hygromycin concentration of *Colletotrichum gloeosporioides* ES026 was higher than that of some other fungi with the same *A. tumefaciens* and vector , such as *Coniothyrium minitans* (40µg/ml), *Colletotrichum higginsianum* (50µg/ml) [28]. The result was deduced that the resistance to hygromycinB is probably related to fungi specificity, and *Colletotrichum gloeosporioides* ES026's resistance may increase along with a long-term symbiosis with host plant. At the same time, several transformation parameters had been optimized to increase transformation efficiency and minimize the number of integration events per genome. Among the different parameters we studied, co-culture temperature, co-culture time, concentration of *A. tumefaciens* and AS for co-cultivation were already known to affect transformation efficiency in fungi[19, 33]. Our results indicate that the most favorable conditions are: co-cultivation temperature 25°C and co-culture time 48h with the concentration of AS (OD600) at 0.3-0.5. In our study, the addition of AS in IM medium was not essential for ATMT of our fungi, although omission of AS in the IM medium led to a lower transformation frequency, which is contradiction with most previous reports [19, 34]. This further confirmed that addition of AS in IM medium seems not to be an absolute requirement for transformation. The optimum concentration of spores of ES026 for ATMT was 10^6 spores/ml, and out of this range of concentration, few transformants could be obtained. A lower concentration of the spores led less efficient germination of the spores, as well as lower tranformation efficiency. A higher concentration theoretically could obtain a higher transformation efficiency, in fact too many spores bring difficulty in selecting the single transformants producing fewer transformants. Another study investigated the differences in culture time of ES026 spores, stating that lower and a higher culture time of spores resulted in a decreased transformation frequency, probably due to a reduced viability or less efficient germination of spores[20], resulting in less transformation frequency during co-cultivation [35]. Using the optimized protocol described above, approximately 95 transformants (950 colonies/10^6 spores) were consistently obtained in each selection medium. The transformation efficiency of *C.gloeosporioides* ES026 was higher than that of other fungi such as *Colletotrichum lagenarium* (150–300 colonies/10^6 spores) [19], *Fusarium oxysporum* (300-500 colonies/ 10^6 spores) [36], and *Colletotrichum trifolii* (25-35 colonies/10^6 spores) [10]. but it was lower than that of another fungi such as *Cryphonectria parasitica* (more than 1000 colonies/10^6 spores) [37]. Some studies had explained the reason why there are different transformation efficiency, one is the probable biological differences among fungi [18], another is the differences in the binary vector or bacterial strain used [36]. The optimization of these transformation parameters above mentioned provides reference for the construction of other endophytes mutant library.

An additional major advantage of *Agrobacterium* mediated transformation over conventional plasmid transformation is the possibility of recovering T-DNA flanking sequences by PCR-based techniques. Using TAIL-PCR and Inverse-PCR technology, we successfully amplified the LB and RB regions of the transformants. The yield of HupA of EST010044 had been certified two times higher than ES026, and subsequently the targeted gene was analyzed, which encoded a methyltransferase with high sequence similarity to a methyltransferase domain protein gene of *Colletotrichum gloeosporioides*. Several groups had devoted intensive efforts to the chemical synthesis of HupA[14, 38, 39], the total chemical synthesis of HupA in an industrially feasible manner is not available, the relevant genes for HupA synthesis are still not perfect. In our study, a suspected methyltransferase in *C.gloeosporioides* ES026 was destroyed by T-DNA insertion, which consequently improved the yield of HupA. This may be due to a precursor substance in HupA biosynthesis pathway. The precursor

substance was involved in the biosynthesis of two secondary metabolites, which were substance B (an unknown substance) and HupA. However, the methyltransferase was only correlated with substance B. When methyltransferase was inserted by a T-DNA and its biological function was broken, ultimately the biosynthesis pathway of substance B was blocked. Hence, the production of the precursor was accumulated, and the yield of HupA increased. However, whether the lack of the methyltransferase gene may result in the increased yield of HupA or not, as well as the way in which the methyltransferase is paticipating in the synthesis of HupA metabolism, are still not clear. Therefore more detailed studies remain to be done. To certify the function of methyltransferase in the synthesis of HupA, a targeted gene disruption and complementation approach should be used in the following study.

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