Research Article

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Bioproduction of baccatin III, an advanced precursor of paclitaxol, with transgenic *Flammulina velutipes* expressing the 10-deacetylbaccatin III-10-*O*-acetyl transferase gene

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Abstract

BACKGROUND: 10-Deacetylbaccatin III (10-DAB) and baccatin III are intermediates in the biosynthesis of Taxol (an anti-cancer drug) and useful precursors for semi-synthesis of the drug. In this study, a bioconversion system was established for the production of baccatin III, an advanced precursor of paclitaxel, in the transgenic mushroom *Flammulina velutipes* expressing the 10-deacetylbaccatin III-10β-*O*-acetyltransferase gene. The expression vector pgFvs-TcDBAT containing the 10-deacetylbaccatin III-10β-*O*-acetyltransferase (DBAT) gene was constructed and transformed into the cells of *F. velutipes* by polyethylene glycol-mediated protoplast transformation.

RESULTS: Polymerase chain reaction and Southern blotting analysis verified the successful integration of the exogenous *DBAT* gene into the genome of *F. velutipes*. Reverse transcription polymerase chain reaction and enzyme activity analyses confirmed that the *DBAT* gene was expressed in *F. velutipes*, and DBAT is able to convert substrate into baccatin III.

CONCLUSION: The *DBAT* gene from the plant *Taxus chinensis* can be functionally expressed in *F. velutipes*. Transgenic *F. velutipes* expressing the *DBAT* gene is able to produce the target product, baccatin III. This is the first report about the transformation and expression of paclitaxel biosynthetic gene in the edible mushroom *F. velutipes*. This represents a significant step towards bio-production of paclitaxel and its advanced precursor baccatin III in an edible fungus.

Keywords: 10-deacetylbaccatin III-10β-*O*-acetyltransferase; paclitaxel; baccatin III; bioconversion; *Flammulina velutipes*

INTRODUCTION

Taxol (paclitaxol), which has been approved by the US Food and Drug Administration (US FDA) for the treatment of ovarian and breast cancers etc. since 1992,[1] is one of the most effective and widely used anti-neoplastic drugs currently available in extremely low yield from the bark of the *Taxus* species.[2] The diterpenes 10-deacetylbaccatin III (10-DAB) and baccatin III are intermediates in Taxol biosynthesis and useful precursors for the semi-synthesis of the anti-cancer drug. 10-Deacetylbaccatin III-10β-*O*-acetyltransferase (DBAT) catalyses the conversion of 10-DAB to baccatin III in the presence of acetyl-coenzyme A in *Taxus* species.[3,4] The reaction from 10-DAB to baccatin III is a key step, because the supply of Taxol is currently largely sustained by semi-synthetic means in which 10-DAB isolated from yew needles is used to produce Taxol and the closely related analogue Taxotere. Semi-synthetic production of baccatin III involves protection of the 7-hydroxyl of 10-DAB and chemical acetylation of the 10-hydroxyl to give 7-O-protected baccatin III. This method involves many steps and is expensive.[4,5] However, a biosynthetic approach to yield Taxol by using enzyme catalysis could eliminate complicated steps and reduce production costs. In fact, the gene encoding 10-DBAT has been successful cloned.[6,7] If the gene can be strongly expressed in a soluble and active form, the use of a biosynthetic approach to produce baccatin III is feasible.[8,9]

Some basidiomycete fungi possess many advantages over other microorganisms and plants as models for recombinant

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expression of functional and medicinal proteins. They have been utilised for the expression of exogenous genes, such as the green fluorescent protein gene (*gfp*) from the animal *Aequorea victoria* in *Agaricus bisporus*,[10] the antifreeze protein gene (*afp*) from the insect *Choristoneura funiferana* in *Volvariella volvacea*,[11] the *afp* in *Pleurotus nebrodensis*,[12] a multi-functional cellulase gene (*mfc*) from the animal *Ampullaria crossean* in *Coprinopsis cinerea*,[13] a fungal immunomodulatory protein gene (*fip-gsi*) from *Ganoderma sinense* in *C. cinerea*,[14] and *gfp* in *Flammulina velutipes*.[15] However, little effort has been made in the homologous expression of Taxol biosynthetic genes in basidiomycetes.

In our previous study, a full-length cDNA of the *DBAT* gene was isolated from *Taxus chinensis* var. *mairei*, which had a 1323 bp open reading frame encoding a protein of 440 amino acid residues, and alignment analysis showed that the nucleotide sequence and the deduced amino acid sequence of *DBAT* had very high similarity (over 95% identity) to *DBAT* of other *Taxus* species such as *Taxus wallichiana* var. *mairei*, *Taxus x media* and *Taxus baccata*.[6,16] In this present report, the eukaryotic expression vectors pgFvs-TcDBAT containing the endogenous *gpd* promoters from *F. velutipes*, and the *DBAT* gene from the plant *T. chinensis* var. *mairei*, was successfully constructed and transformed into the mycelia of *F. velutipes* by polyethylgene glycol (PEG)-mediated protoplast transformation. Heterologous expression of DBAT in *F. velutipes* demonstrated that DBAT was a functional enzyme, which represents a valuable step toward bioengineering the production of Taxol and will also be useful for further transgenic manipulation and expression of Taxol biosynthetic genes in *F. velutipes* or other basidiomycetes.

MATERIALS AND METHODS

Cultures and materials

F. velutipes was conserved at −70°C in our laboratory and maintained on potato dextrose agar (PDA) medium (200 g L^{-1} extract of boiled potato, 20 g L^{-1} glucose, 3 g L^{-1} KH_2PO_4, 1.5 g L^{-1} $MgSO_4 \cdot 7H_2O$). The plasmid pgFvs-mfc (containing the *mfc* gene from the animal *A. crossean* with the *gpd* promoter from *F. velutipes* (*gpd-Fvs*) and the *trp* terminator (trp1+) from *Aspergillus nidulans*),[13] the plasmid pGEM-TcDBAT (containing the *DBAT* gene from the plant *T. chinensis* var. *mairei*), and the plasmid pBgGI-hph (containing the selectable marker gene *hph* from *Streptomyces hygroscopicus* and the *gpd* promoter from *Ganoderma sinense*) were constructed by our laboratory. The *E. coli* DH5α (GIBCOBRL; Life Technologies, Grand Island, NY, USA) was used for DNA manipulations and grown in Luria broth medium (Sigma, Shanghai, China) at 37°C. Plasmid DNA was isolated from *E. coli* DH5α using the QIAgen MiniPrep kit (QIAgen, Mississauga, Ontario, Canada). 10-DAB and baccatin-III standards were purchased from Sigma, (Munich, Germany).

Construction of the expression vector

Two plasmids, pgFvs-TcDBAT and pgFvs-hph, were constructed based on the backbone of plasmid pgFvs-mfc (Fig. 1).[13] The open reading frame of the *DBAT* gene was amplified from plasmid pGEM-TcDBAT using the primers TcDBAT-F (5′-ACTAGTATGGCAGGCTCAACAGAA-3′) and TcDBAT-R (5′-TGTACATCAAGGTTTAGTTACATA-3′), and *Spe* I and *BsrG* I restriction sites (underlined) were designed for flanking the polymerase chain reaction (PCR) product at the 5′- and 3′-terminus, respectively. The *mfc* gene in the plasmid pgFvs-mfc was replaced with the digested PCR fragment to construct the expression plasmid pgFvs-TcDBAT containing the *gpd* promoter from *F. velutipes* and *DBAT* gene from *T. chinensis* var. *mairei* and the *trp* terminator (trp1+) from *A. nidulans* (Fig. 1B). The *mfc* gene in the plasmid pgFvs-mfc was replaced with the digested plasmid pBgGI-hph fragment (containing the selectable marker gene *hph* from *S. hygroscopicus*) by restriction enzyme *Spe* I and *Bsr*G I (Roche, Penzberg, Germany) to construct the expression plasmid pgFvs-hph containing the *gpd* promoter from *F. velutipes* and *hph* gene from *S. hygroscopicus* and the *trp* terminator (trp1+) from *A. nidulans* (Fig. 1C). All DNA and RNA manipulations followed standard procedures.

Preparation of the protoplast

F. velutpes was grown in PDA liquid medium and incubated at 25°C for 5 days. The mycelia (1 g, wet weight) were collected by centrifugation and washed three times with double-distilled H_2O and 0.6 mol L^{-1} KCl, respectively. Then the mycelia were suspended in 0.6 mol L^{-1} KCl containing 1.5% lywallzyme (Guangdong Institute of Microbiology, China) and incubated at 28°C on a rotary shaker (180 × *g*). After incubation for 3 h, the supernatant was filtered though four-layer glass filters to remove the mycelial debris and was centrifuged at 3000 × *g* for 10 min at 4°C.

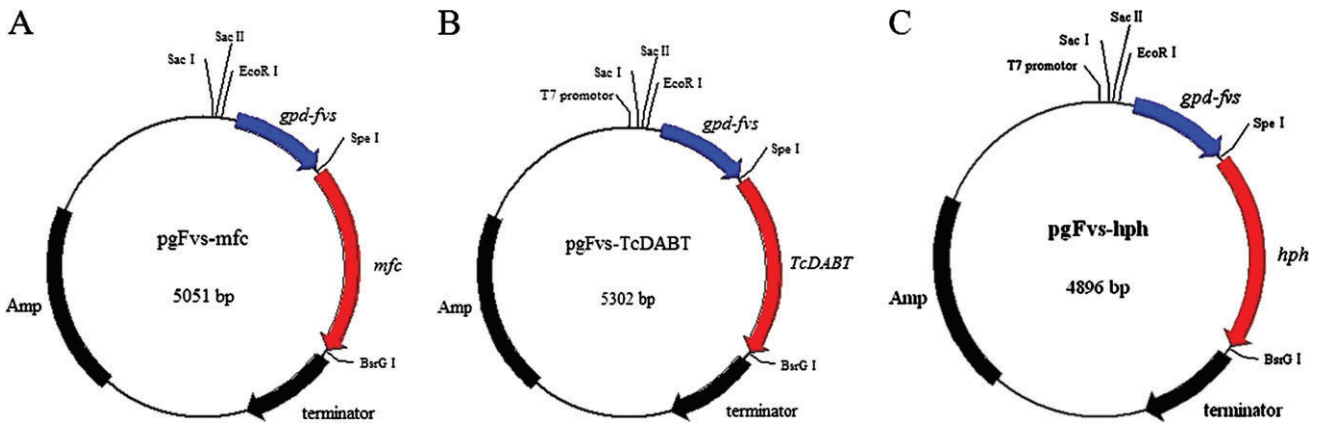

Figure 1. Schematic diagram of the construction of pgFvs-mfc (A), pgFvs-TcDBAT (B) and pgFvs-hph (C). The *DBAT* gene from *Taxus chinensis* and the *hph* gene from *Streptomyces hygroscopicus* replace the *mfc* gene in the plasmid pgFvs-mfc (A) with the *gpd* promoter from *F. velutipes* (gpd-Fvs) and the *trp* terminator (trp1+) from *Aspergillus nidulans*, to produce the expression plasmid pgFvs-TcDBAT (B) and pgFvs-hph (C), respectively.

Then the precipitates were washed twice with 0.6 mol L^{-1} KCl, and suspended in 100 μL of 0.6 mol L^{-1} KCl and density of protoplasts were counted using a haemocytometer.

Co-transformation of *Flammulina velutipes*

In co-transformation experiments, protoplasts (approximately 1×10^8 in 250 μL PDA liquid medium containing 0.6 mol L^{-1} KCl) were gently mixed with pgFvs-TcDBAT (10 μg), pBgFvs-hph (10 μg), heparin (100 μg mL^{-1}), 50 μL PEG buffer (25% PEG-4000, 50 mmol L^{-1} $CaCl_2$, 10 mmol L^{-1} Tris-HCl, pH 7.5, filter sterilised), and 10 μL Tris-EDTA (pH = 8.0), and incubated for 30 min on ice. One millilitre of PEG buffer was then added and the mixture was incubated for an additional 15 min at 28°C. Treated protoplasts were recovered by centrifugation (3000 × *g*, 10 min) and resuspended in 1 mL of PDA liquid medium containing 0.6 mol L^{-1} KCl for 3 days at 25°C before embedding into regeneration medium (PDA medium containing 0.6 mol L^{-1} KCl, 30 μg mL^{-1} hygromycin B and 30 μg mL^{-1} ampicillin (Sigma, St. Louis, MO, USA). Regenerated colonies appeared approximately 7 days after plating and the hygromycin B resistancy test was applied to the regenerated colonies. A control culture were treated the same way but omitting the DNA.[17]

Regeneration of the protoplast

The protoplast solution obtained as described above was diluted to a concentration of 10^5 cells mL^{-1} using PDA liquid medium containing 0.6 mol L^{-1} KCl. Aliquots of the suspension (100 μL) were added to the PDA medium containing 0.6 mol L^{-1} KCl and placed on Petri dishes. Eight concentrations of hygromycin B (0, 10, 20, 25, 30, 35, 40, 45 μg mL^{-1}) were prepared to determine the lowest sensitive concentration of protoplasts to hygromycin B.[15] Protoplasts were incubated at 25°C for 7–15 days and regenerated protoplasts were counted as individual colonies.

Polymerase chain reaction analysis

Genomic DNA was extracted from the control untransformed and transformed hygromycin B resistant regenerated colonies according to the method described previously and was analysed by PCR.[18] Amplification of the *DBAT* gene was carried out using primers TcDBAT-IF (5′-ACATCCATCCTCTGGTGGTTCA-3′) and TcDBAT-IR (5′-CTTCATCAAATCCCAATCGCCT-3′). The PCR amplification protocol consisted of an initial denaturing cycle of 4 min at 95°C, followed by 35 cycles with 60 s denaturation (94°C), 60 s annealing (52°C) and 2 min elongation (72°C). The amplified fragments were further identified with sequencing test by the Beijing Genomics Institute. China.

Southern blotting analysis

Approximately 10 μg of DNA samples from transformed and non-transformed (negative control) strains were prepared and completely digested with restriction enzyme *Hin*d III (Roche, Penzberg, Germany) and separated on 8 g L^{-1} agarose gel. The DNA fragments in the agarose gel were transferred to a Hybond N + nylon membrane (Amersham, Buckinghamshire, UK) using 10× saline–sodium citrate. The digested DNAs were subjected to Southern hybridisation analysis, which was carried out using the digoxigenin (DIG) system (Roche, Mannheim, Germany). The cDNA probe corresponding to the *DBAT* gene was prepared using the random priming procedure.

Reverse transcription polymerase chain reaction analysis and stability of the transformants

For RT-PCR reaction, 10 μg of total RNA was isolated from the mycelia of transformants and genomic DNA was removed from the RNA sample by digestion with RQ1 RNase-Free DNase digestion Kit (Promega, Madison, WI, USA). The RT reaction was conducted using PrimeScript RT-PCR Kit (Takara, Shiga, Japan), according to the manufacturer's instructions (Takara). The *DBAT* gene was amplified by PCR using RT reaction products as the templates with primers TcDBAT-RTF (5′-TTAGAGAGAGTGATGGTGGCT-3′) and TcDBAT-RTR (5′-TTCTTGGGAGGTCGTATGA-3′). The *DBAT* gene stability was assayed by transferring randomly selected transformants to medium without antibiotic selection for weeks to months, followed by the hygromycin B resistance test.

Activity assay of 10-deacetylbaccatin III-10β-*O*-acetyltransferase from *Flammulina velutipes*

For detection of DBAT activity, DBAT from *F. velutipes* was extracted by a modification of previously described methods.[19,20] Transformants were cultured in 10 mL potato dextrose broth. After 7 days of incubation, the mycelia were washed with sterilised water and ground in liquid nitrogen with a mortar and pestle. The resulting powder was extracted with buffer containing 100 mmol L^{-1} Tris-HCl pH 7.8, 10 mmol L^{-1} $Na_2S_2O_5$, 10 mmol L^{-1} sodium ascorbate, 5 mmol L^{-1} dithiothreitol, 10 g L^{-1} (w/v) polyvinylpyrrolidone (relative molecular mass, Mr, 10 000), 10 mL L^{-1} (v/v) glycerol. The homogenate was centrifuged at 15 000 × *g* for 60 min, and the resulting supernatant was desalted by passage through a Sephadex G 25 column which was previously equilibrated with the same buffer (without dithiothreitol) and used as crude extract for the experiments. A 10 mL crude extract was incubated in solution [5 mmol L^{-1} $MgCl_2$, 10-DAB (400 μM), acetyl CoA (400 μM)] for 2 h at 31°C. The reaction mixture was then extracted, evaporated *in vacuo* at 4°C and the residue was re-suspended in 1 mL methanol. The product was assayed by high-performance liquid chromatography (HPLC) with an Agilent 1200 HPLC system (Agilent, Santa Clara, California, USA). The sample was loaded onto an Hypersil ODS C_{18} column (250 mm × 4.6 mm × 5 μm; Thermo, Milford, Massachusetts, USA), eluted at 1.5 mL min^{-1} with a mobile phase that consisted of 10% acetonitrile, 20% methanol and 70% water. The column temperature was ambient and the detection wavelength was 227 nm. The retention times for 10-DAB and baccatin III were 5.15 min and 7.90–8.32 min, respectively.

RESULTS

Isolation and regeneration of the protoplast

F. velutipes protoplasts extrude through ruptures in the cell wall, thus they completely lack a cell wall. Microscopic observation revealed that protoplasts prepared in the present study contained neither cell wall debris nor mycelium. The protoplast concentration obtained varied under different conditions, ranging from 5×10^7 to 8×10^8 protoplasts of *F. velutipes*, and the average yield was $(1.5 \pm 0.1) \times 10^8$. The growth of protoplasts was tested in regeneration medium containing various concentrations of hygromycin B. Colonies did not appear at concentrations exceeding 30 μg mL^{-1}, therefore 30 μg mL^{-1} of hygromycin B was used for screening of transformants. The regeneration frequency of the protoplasts was 2.27 ± 0.07 % on PDA medium containing 0.6 mol L^{-1} KCl, and the regeneration frequency of the protoplasts was 0.003% on PDA medium containing 0.6 mol L^{-1} KCl and 30 μg mL^{-1} hygromycin B.

Co-transformation of *Flammulina velutipes*

Protoplasts treated with plasmids pgFvs-TcDBAT and pBgFvs-hph in the presence of PEG was screened for drug resistance on regenerating plates containing hygromycin B. After incubation at 25°C for 7 days, a number of germinating protoplasts were observed (Fig. 2A and B), whereas no such germination was observed when protoplasts were treated with no plasmid DNA (Fig. 2C). In successive incubations, some of the germinating protoplasts ceased growth when the diameter of the colony reached about 3 mm. The remaining continuously growing colonies were picked up and cultured on the PDA medium in the presence of 30 μg mL^{-1} hygromycin B. These strains were regarded as putative transformants because only colonies containing the *hph* gene could grow on regeneration agar medium with hygromycin B and be taken for further analysis.

Detection and sequencing of the 10-deacetylbaccatin III-10β-*O*-acetyltransferase gene of *Flammulina velutipes* by using polymerase chain reaction

Since these putative transformants might contain plasmid pBgFvs-hph or plasmid pBgFvs-hph and plasmid pgFvs-TcDBAT simultaneously, PCR analysis was performed to detect the *DBAT* gene in putative transformants. Result showed the amplification of a bright band under a 800 bp ladder corresponding to the *DBAT* gene fragment size (699 bp), indicating the presence of exogenous gene in nine of 20 putative pgFvs-TcDBAT transformants (45%). After being sequenced and aligned, the sequence of this band had 99% similarity to the *DBAT* gene of *Taxus wallichiana* var. *mairei* (GenBank accession number: JQ029678.1) published on the GenBank. PCR result also showed that the *DBAT* gene was present in the positive control but absent in the non-transformed negative control.

Southern blotting analysis

Southern blotting analysis with seven randomly selected putative transformants, negative control (*F. velutipes*) and positive control (pgFvs-TcDBAT) was performed with the specific *DBAT* gene probe (Fig. 3). The hybridisation signals showed that one transformant (lane 7) had a single copy and another transformant (lane 4) had three copies, while another four transformants (lanes 5 and 6, lanes 8 and 9) had two copies of the *DBAT* gene in their genomes. The variation in the copy numbers and position of the *DBAT* gene among transformants indicated that the integration of the gene was a random event and might occur by non-homologous recombination. DNA isolated from negative control strain (lane 3) and t20 (lane 10) did not hybridise with the *DBAT* probe. The result indicated that the *DBAT* gene was successfully integrated into the genome of *F. velutipes*.

Reverse transcription polymerase chain reaction analysis

The detection of transcription of the introduced *DBAT* gene in transformants was carried out by using the RT-PCR technique. With the primers specific to the recombinant construct, a strong signal band under the 2000 bp ladder was amplified using the RNA samples prepared from transformants as templates (Fig. 4), which were consistent with the anticipated size of amplification. In contrast, no expected band was observed in the negative control. This result suggested that the introduced *DBAT* gene was expressed predominantly in the early stage of culture of the transformants.

Genetic stability of the transformants

After five generations of sub-cultivation, six transformants (t2, t4, t5, t7, t11 and t12) were able to grow well on PDA plates containing 100 μg mL^{-1} hygromycin B (Fig. 5), and demonstrated that the hygromycin B resistance trait remained stable and hereditable during mitotic cell division for generations. PCR detection of the *DBAT* gene showed that there exists an amplified band equivalent to the expected *DBAT* gene fragment about 1184 bp in size. This further confirmed that the *DBAT* gene had been integrated into the genomes of *F. velutipes* and the exogenous *DBAT* gene possessed hereditary stability in the transformants, which provides a basis for further research.

Activity assay of 10-deacetylbaccatin III-10-*O*-acetyl transferase from *Flammulina velutipes*

HPLC analysis revealed that the acetylation product catalysed by the expressed 10-deacetylbaccatin III-10-*O*-acetyl transferase (DBAT) from *F. velutipes* transformants with the co-substrates 10-DAB and acetyl CoA had the same HPLC chromatogram characteristics (the retention times and spectrum) as did the standard purchased sample of baccatin III (Fig. 6F), which could not be detected in non-transformants with same substrate (Fig. 6E), indicating that the cloned *DBAT* was a functional gene. This DBAT was able to catalyse the acetylation of the C-10 hydroxyl group of the advanced metabolite 10-DAB to yield baccatin III, which is the immediate diterpenoid precursor of Taxol.

DISCUSSION

Due to the shortage of yew trees, the low yield of Taxol in nature and the environmentally destructive prospect of large-scale harvesting of *Taxus* trees,[9,20] alternative less destructive sources and methods of Taxol production, such as chemical synthesis, cell culture and fungal fermentation, have been sought.[21,22] Among these methods, chemical semi-synthesis is one of the most widely adopted approaches for commercial production of Taxol. This method uses 10-DAB, a *Taxus* metabolite, which is much more readily available than Taxol itself, as the starting material.[4,23]

The Taxol biosynthetic pathway starts with the cyclisation step from geranylgeranyldiphosphate to taxadiene, and most of the 19 known enzymatic steps in the biosynthesis are related to hydroxylation and other oxygenation reactions of the taxadiene skeleton.[24,25] These enzymes were isolated and identified from different *Taxus* species, and many genes have been isolated and functionally expressed in *Escherichia coli*,[24] yeast[1,8] or *Spodoptera* cells.[26] The first intermediate, taxadiene, can now be produced in *E. coli*. Co-expression of the taxadiene synthase from *Taxus brevifolia* with a geranylgeranyl diphosphate synthase isolated from *Erwinia herbicola*,[27,28] isopentenyl diphosphate synthase from *Schizosaccharomyces pombe*,[29] and the endogenous deoxyxylulose 5-phosphate synthase from *E. coli* resulted in a production of 1.3 mg taxadiene L^{-1} of cell culture.[30] This proved the principle of genetically engineering *E. coli* for the heterologous production of taxanes by combining enzymatic biosynthetic steps derived from several different organisms.

However, *E. coli* does not have an efficient isoprenoid biosynthetic pathway and is the limited supply of complementary nicotinamide adenine dinucleotide phosphate (reduced form) (NADPH):cytochrome P450 reductase that is also essential for the correct function of reconstituted plant cytochrome P450 enzymes. As the lowest eukaryotic organisms, yeasts usually produce hyper-glycosylation modified expression proteins.[31,32] Insect hosts are

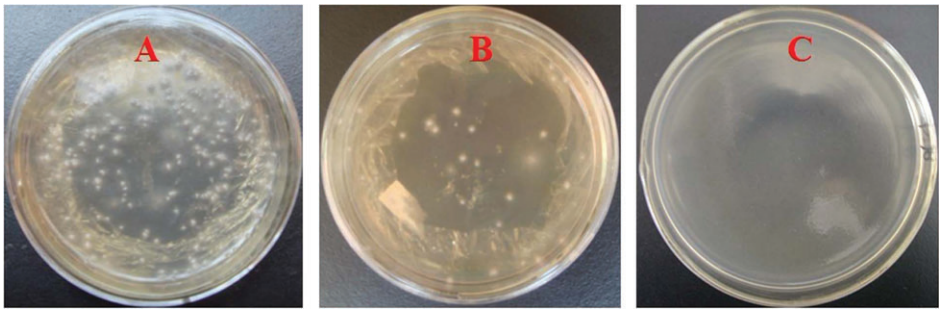

Figure 2. The regeneration colonies of protoplasts of *Flammulina velutipes* growing on the selective medium. (A) Co-transformants with pgFvs-TcDBAT and pBgFvs-hph culturing on the potato dextrose agar (PDA) medium containing 0.6 mol L^{-1} KCl. (B) Co-transformants with pgFvs-TcDBAT and pBgFvs-hph culturing on the PDA medium containing 0.6 mol L^{-1} KCl and 30 μg mL^{-1} hygromycin B. (C) Non-transformants culturing on the PDA medium containing 0.6 mol L^{-1} KCl and 30 μg mL^{-1} hygromycin B.

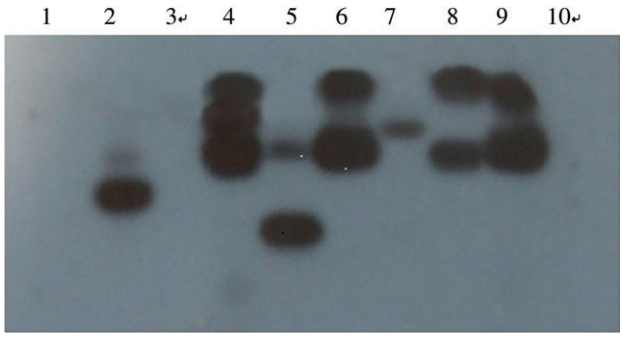

Figure 3. Southern blot hybridisation analyses of DNA isolated from putative *Flammulina velutipes* transformants (PCR positive for *DBAT*). Genomic DNA (approximately 15 μg) was isolated from broth cultures, digested with *Hin*dIII, and probed with the digoxigenin (DIG)-labelled *DBAT* gene sequence. Lane 1: DNA molecular size markers (kilobases); lane 2: DNA isolated from plasmid pgFvs-TcDBAT as positive control; lane 3: negative control of wild-type genomic DNA of *F. velutipes*; lanes 4–10: *Hin*dIII-digested genomic DNA of putative transformants (t2, t4, t5, t7, t11, t12 and t20, respectively) were probed with the DIG-labelled *DBAT* sequence.

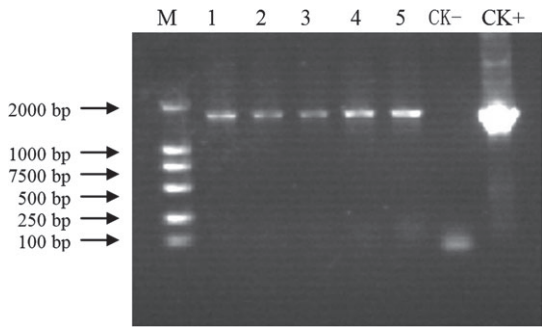

Figure 4. The detection of the *DBAT* transcripts in the transformants by RT-PCR. Total RNA was extracted from mycelium of transformants and then subjected to RT-PCR analysis. Lanes: CK−, RNA isolated from *Flammulin velutipes* as negative control; 1 to 5, RNA isolated from DBAT transformants t2, t4, t5, t7, t11, respectively; CK+, RNA isolated from *Taxus chinensis* as positive control; M, DNA molecular size markers.

higher eukaryotic expression systems and produce recombinant proteins with almost natural characteristics. However, these systems require strict culture conditions and they are difficult to manipulate, accompanied with high production costs and safety risk concerns, and hence are difficult to put into industrial production.[33,34]

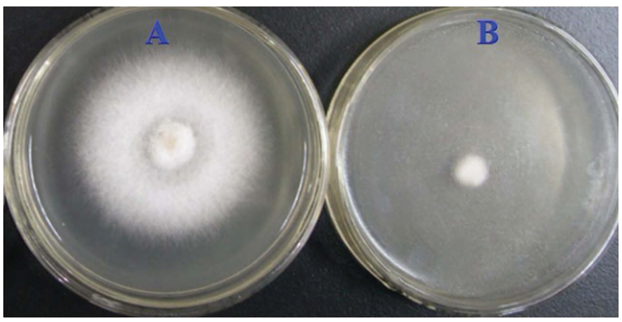

Figure 5. The analysis of transformants t2 on the tolerance test. (A) Transformants t2 mycelia were able to grow well on potato dextrose agar (PDA) plates containing 100 μg mL^{-1} hygromycin B; (B) Non-transformed mycelia stopped growing on PDA plates containing 100 μg mL^{-1} hygromycin B.

In this present study, the lignin-degrading basidiomycete *F. velutipes* was chosen as the host for heterologous expression of the *DBAT* gene from the plant *T. chinensis* var. *mairei* not only because it is genetically amendable and with endogenous microsomal cytochrome P450 enzymes and energy supporting systems, but also because it can be cultivated on low cost solid agro-residual wastes on a large scale.[15]

The successfully heterologous expression of *DBAT* in *F. velutipes* demonstrated that DBAT was a functional enzyme and catalyses the conversion of 10-DAB to baccatin III in the presence of acetyl-coenzyme A *in vitro*, which implies a new promising approach to semi-synthesise baccatin III, and even Taxol, using enzyme catalysis by engineered *F. velutipes* in future, based on successful isolation of related genes involved in Taxol biosynthesis.

Similar to DBAT, the hygromycin B phosphotransferase gene (*hph*) from *E. coli* also has been successfully heterologously expressed in *F. velutipes*, with the homologous gpd promoters.[35] The transformation procedure used in this study could be used in DBAT and other Taxol biosynthetic enzymes, such as geranylgeranyl diphosphate synthase, taxadiene synthase, cytochrome P450 taxadienyl acetate 10-β-hydroxylase, and so on.

CONCLUSION

This is the first report about transformation and expression of the Taxol biosynthetic gene *DBAT* in the edible mushroom *F. velutipes*, which holds significance for further transgenic manipulation and expression of other Taxol biosynthetic genes in *F. velutipes* or other basidiomycetes.

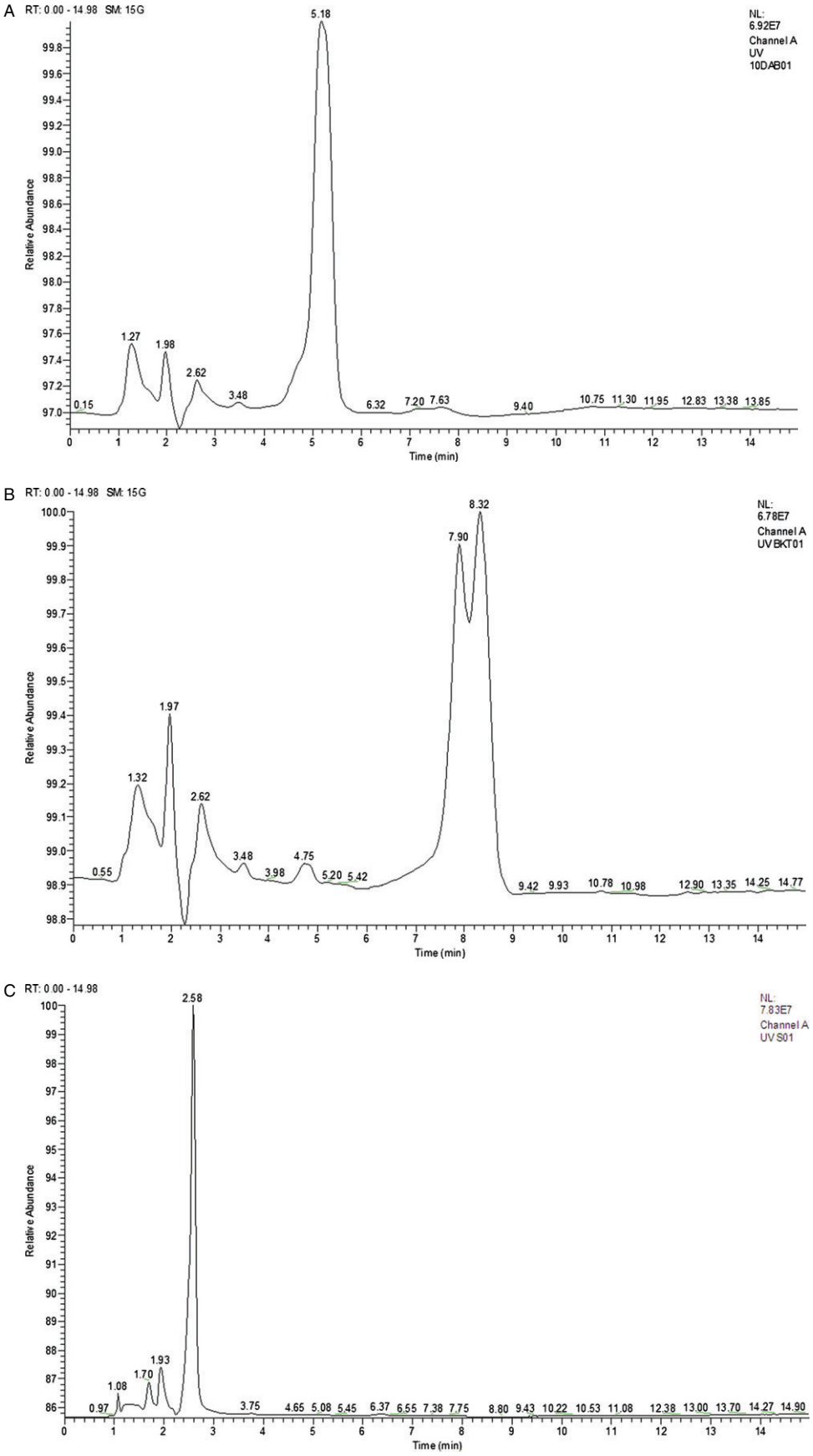

Figure 6. Continued.

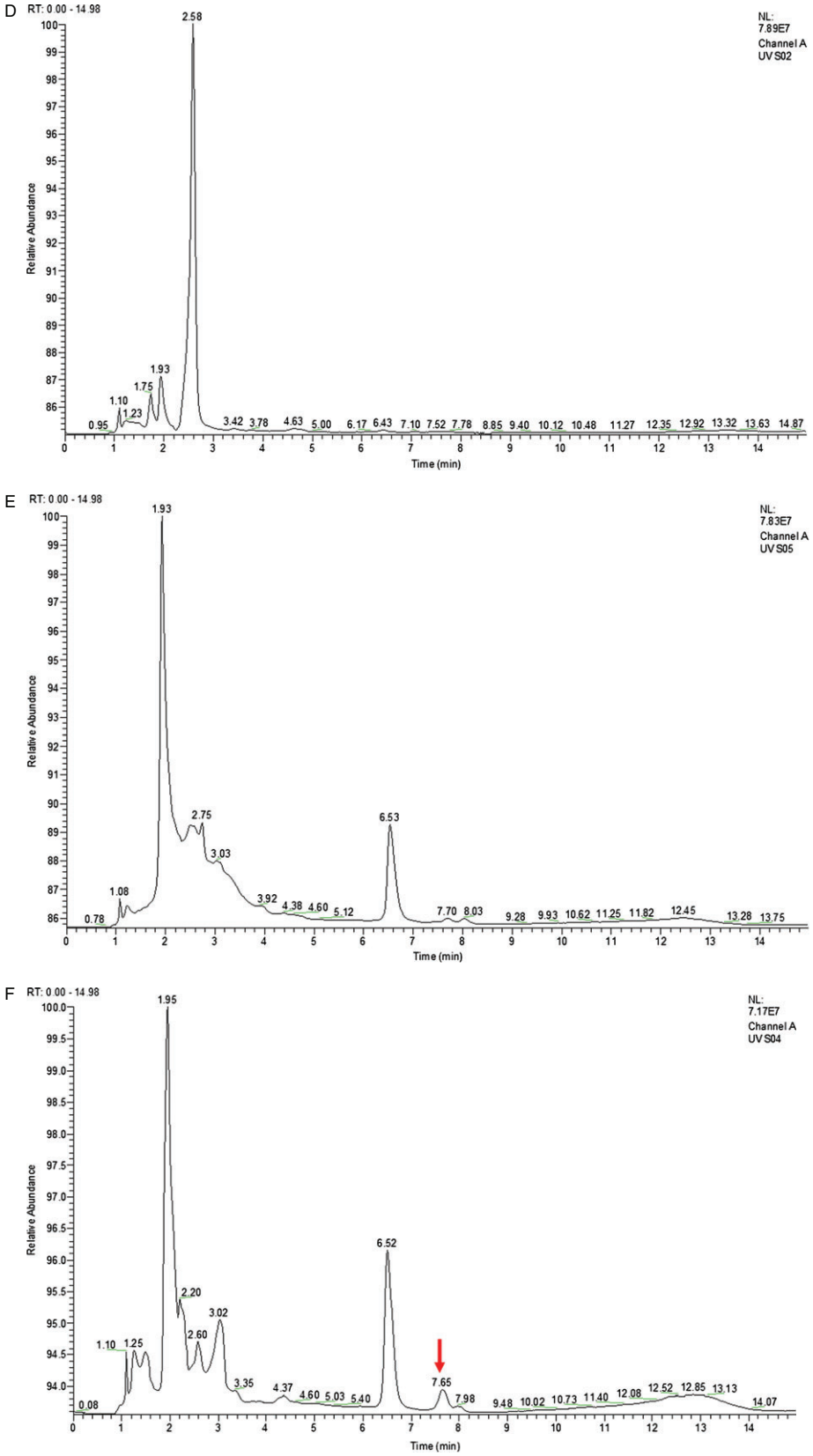

Figure 6. HPLC chromatograms of transformants extraction and standard. (A) Standard 10-DAB; (B) standard bacctin III; (C) non-transformants extraction without added standard 10-DAB; (D) transformants extraction added standard without 10-DAB; (E) non-transformants extraction containing added standard 10-DAB; (F) transformants extraction containing added standard 10-DAB. The retention time for baccatin III which was catalysed by DBAT from *Flammulina velutipes* transformants was 7.65 min.

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