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ORIGINAL RESEARCH PAPER

Screening of Taxol-producing fungi based on PCR amplification from *Taxus*

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Abstract Genes coding for 10-deacetylbaccatin III-10-*O*-acetyl transferase and C-13 phenylpropanoid side chain-CoA acyltransferase were used as molecular markers for screening of Taxol-producing endophytic fungi. Using PCR, three out of 90 endophytic fungi, isolated from *Taxus x media* and *Taxus yunnanensis,* gave positive results. These 3 strains, when grown in 300 ml potato/dextrose liquid medium at 25°C for 10 days, contained 100–160 μg Taxol/g dry wt of mycelium.

Keywords Taxol · Endophytic fungi · *Taxus* · Anti-tumor

Introduction

Taxol, originally found in *Taxus* spp., has more recently been found in several endophytic fungi isolated from various *Taxus* spp. (Zhou et al. 2007), in particular *Taxomyces andreanae* and *Pestalotiopsis microspora* (Stierle et al. 1993; Strobel et al. 1996). Altogether more than 30 Taxol-producing fungal species have been identified (Ji et al. 2006). The separation of endophytic fungi from plant materials is a comparatively simple process, but the detection of Taxol-producing endophytic fungus is laborious (Zhou et al. 2007). As an alternative method, genes coding for taxol biosynthetic enzymes have been used as molecular markers for screening taxol-producing endophytic fungi.

In this study, we have used the genes coding for 10-deacetylbaccatin III-10-*O*-acetyl transferase (DBAT) and C-13 phenylpropanoid side chain-CoA acyltransferase (BAPT) as molecular markers to screen taxol-producing endophytic fungi. DBAT catalyzes the formation of baccatin III, which is the immediate diterpenoid precursor of taxol (Walker and Croteau 2000). BAPT catalyzes the selective 13-*O*-acylation of baccatin III with *β*-phenylalanoyl-CoA as the acyl donor to form *N*-debenzoyl-2′-deoxytaxol, i.e. it catalyzes the attachment of the biologically important taxol side chain precursor (Walker et al. 2002).

Although Zhou et al. (2007) used a gene coding for taxadiene synthase (TS), which is a rate-limiting enzyme in the taxol biosynthetic pathway (Wildung and Croteau 1996), as a molecular marker to screen for Taxol-producing fungi, we suggest that *dbat* and *bapt* genes are more diagnostic than the *ts* gene because more than ten enzymatic steps after TS are required to reach Baccatin III and taxol itself (Jennewein et al. 2004).

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Using this method, three out of 90 endophytic fungi isolated from *Taxus* × *media* and *Taxus yunnanensis* were selected and found to produce taxol.

Materials and methods

Isolation of endophytic fungi

Samples of *Taxus* × *media* and *Taxus yunnanensis* were collected from the ground of Huazhong University of Science and Technology, in Wuhan, Hubei Province, central China. Samples of bark, 1 × 3 cm, were taken, from the stem of trees about 10 years old, approximately 20 cm from the ground, 3 from *T media* and 4 from *T. yunnanensis.* The bark was cut into pieces, ~0.5 × 0.5 × 0.5 cm, surface-sterilized with 70% (v/v) ethanol, washed with sterilized water and the outer bark removed with a sharp, sterilized blade. Small pieces of the inner bark were placed on the surface of potato/dextrose/agar (PDA) medium supplemented with 50 μg ampicillin/ml in Petri dishes. After several days, fungi were observed growing from the bark fragments. Individual hyphal tips of the various fungal colonies were removed from the agar plates, placed on new PDA medium, and incubated at 25°C for at least 10 days. Each fungal culture was checked for purity and transferred to another PDA plate by the hyphal tip method (Strobel et al. 1996).

Screening of taxol-producing fungi

Samples of fungi isolated as above on Petri dishes were inoculated individually into 150 ml Erlenmeyer flasks containing 20 ml potato/dextrose liquid medium. Cultures were incubated at 120 rpm at 25°C for 3 days and harvested by centrifugation at 12,000*g* for 10 min. 0.5–1 mg of mycelia was ground into powder in liquid N_2. Genomic DNA was extracted using the SDS-CTAB method (Kim et al. 1990).

Based on the conserved sequence of the *dbat* gene (GenBank No. EF028093), primers *dbat*-F (5′-GGGA GGGTGCTCTGTTTG-3′) and *dbat*-R (5′-GTTACC TGAACCACCAGAGG-3′) were designed and synthesized. The primers *bapt*-F (5′-CCTCTCTCCGC CATTGACAA-3′) and *bapt*-R (5′-TCGCCATCTCT GCCATACTT-3′) were designed and synthesized according to Li et al. (2006). PCR amplification was performed in a PTC-100 Peltier Thermal Cycler (Bio-Rad).

The fungal isolates were firstly screened by PCR for the presence of the *dbat* gene. PCR amplification was carried out using the primers *dbat*-F and *dbat*-R in a typical 25 μl reaction mixture containing 2 U *Taq* DNA polymerase (New England Biolabs). The PCR mixture was initially pre-heated at 95°C for 6 min before 35 cycles of amplification, which consisted of incubations at 94°C for 50 s, 50°C for 30 s and 68°C for 50 s, and additionally 68°C for 10 min. The amplified DNA fragments were analyzed by agarose gel electrophoresis and those fungi showing PCR positive for the *dbat* gene were selected for the next screening.

Fungi containing the *dbat* gene were secondly screened by PCR analysis for the gene coding for BAPT. PCR amplification of the *bapt* gene was carried out using the primers *bapt*-F and *bapt*-R in a 25 μl reaction mixture containing 2 U *Taq* DNA polymerase (New England Biolabs) with the following primer extension condition: 6 min at 95°C; 35 cycles of: 94°C (50 s), 55°C (50 s), and 68°C (50 s); after 30 cycles the temperature was held at 68°C for 10 min. The amplified DNA fragments were analyzed by agarose gel electrophoresis. Three fungi showed positive for the *bapt* gene as well as the *dbat* gene, and were selected for the determination of taxol.

Determination of Taxol-producing fungi

The three fungi registering positive for the genes coding for DBAT and BAPT were inoculated into 1 l Erlenmeyer flasks containing 300 ml potato dextrose liquid medium. The flasks were shaken at 180 rpm at 25°C for 10 days, the mycelia were harvested by filtration and dried at 45°C overnight. Dried mycelia were crushed and extracted with 6 ml methanol/chloroform (1:1, v/v) 3 times. The extracts were concentrated under reduced pressure, and dissolved in 1 ml methanol and individual components separated by TLC (Strobel et al. 1996). The band corresponding to Taxol was dissolved in 0.5 ml methanol and its concentration estimated by its absorption at 273 nm: the millimolar absorption coefficient, $\varepsilon = 1.7$.

The extracts of each fungal isolate were examined for the presence of taxol using HPLC-MS. A C18 column (5 × 300 mm, Waters) was used identify taxol by HPLC. 10 μl of the methanol solution of

putative Taxol were injected and elution was done with methanol/H_2O (65:36, v/v). A variable wavelength recorder set at 228 nm was used to detect compounds eluting from the column.

Electrospray mass spectroscopy was done on fungal taxol samples using the electrospray technique with an Agilent 1100 LC/MSD trap. The sample in 100% methanol was injected with a spray flow of 2 µl/min and a spray voltage of 2.2 kV by the loop injection method.

Results and discussion

A total of 90 fungal isolates separated from *T. media* and *T. yunnanesis* were screened for the presence of the *dbat* gene. 15 out of the 90 fungi had about 200 bp fragments of the *dbat* gene (Fig. 1). The *dbat* gene is essential for taxol biosynthesis but is not diagnostic because some fungi containing the *dbat* gene may produce baccatin III, but not produce Taxol. Therefore, the 15 fungi containing the *dbat* gene were also screened for the presence of the *bapt* gene whose enzymic product catalyses the next step for taxol biosynthesis (Walker et al. 2002). Three of the 15 fungi had approximately 530 bp fragments of the *bapt* gene (Fig. 2).

The three possible Taxol-producing fungi were designated MD-2 and MD-3 (from *T. media*) and YN-6 (from *T. yunnannesis*). The extracts of the dried mycelia in methanol all gave a single peak by HPLC, with about the same retention time of 15.4 min as authentic Taxol. Similarly, the extracts yielded an electrospray mass spectrum with an $(M + Na)^+$ peak at 876, which is identical to that of authentic taxol (Fig. 3). These results showed that these three fungi

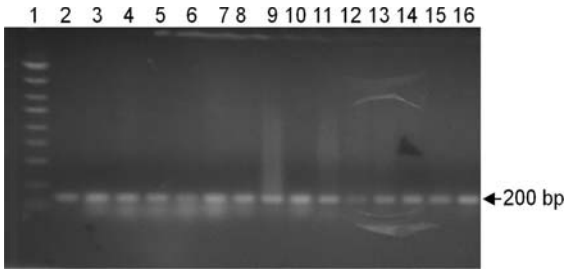

Fig. 1 Representative PCR analysis for the presence of the *dbat* gene in fungi isolates. Lane 1: 250 bp DNA ladder plus; lane 2–16: the PCR product of the *dbat* gene of 15 fungus isolates

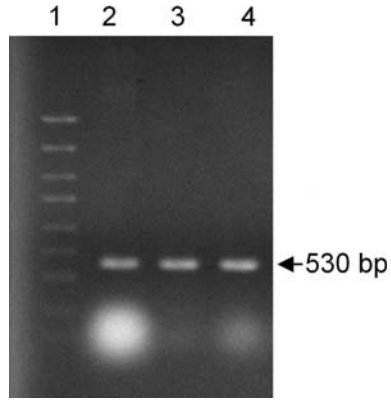

Fig. 2 PCR analysis for the presence of the *bapt* gene in fungi isolates of MD-2, MD-3, and YN-6. Lane 1: 250 bp marker plus; lane 2–4: the PCR product of the *bapt* gene of MD-2, MD-3, and YN-6

can produce taxol. By measuring via the absorption coefficient in the methanol extract, the mean yields of taxol from MD-2, MD-3 and YN-6 were about 160, 112 and 140 µg taxol/g dry wt of mycelium, respectively.

The two pairs of primers used in this study were designed based on the *dbat* and *bapt* genes of *Taxus* because there are no reports of the sequences of these genes from fungi. We do not expect that the sequences of the genes will be precisely the same in all fungi, however, we found that some fungi appeared to have sequences similar to those of the *dbat* and *bapt* genes of *Taxus,* which made it distinctly possible that these species were Taxol-producing ones. Extracts from these species of fungus did contain taxol and this method is therefore feasible.

In addition, the 15 *dbat* positive isolates were tested for Taxol production, and two additional fungi, besides MD-2, MD-3 and YN-6, were found to produce Taxol. One reason for the lack of the PCR analysis for the *bapt* gene in these two isolates may be that they have no *bapt* gene. This also indicates that some Taxol-producing fungi may have a pathway for taxol biosynthesis different from that in *Taxus*. It follows that the inability to find the two enzymes used here may not necessarily mean that a strain does not produce taxol.

We also screened all 90 isolates for the presence of the *bapt* gene and 6 fungi, including MD-2, MD-3 and YN-6, showed positive. The *bapt* genes of these 6 isolates were sequenced and showed high homology to the *bapt* genes from *Taxus* species. The 6 isolates

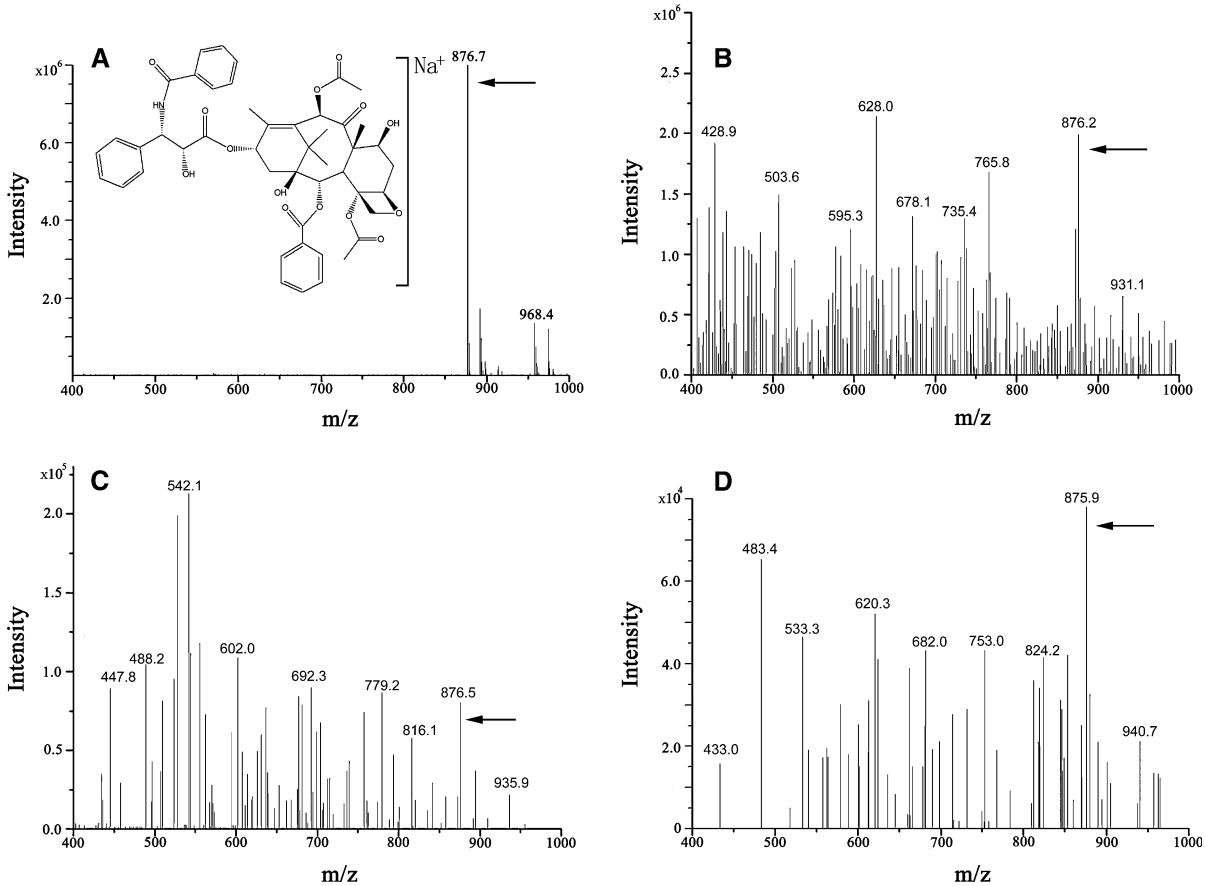

Fig. 3 Electrospray mass spectrum of authentic taxol (**a**) and the fungal taxol of MD-2 (**b**), MD-3 (**c**), and YN-6(**d**). The arrows indicate the identical peak of mass spectroscopy with $(M + Na)^+ = 876$

were examined for taxol production, and only MD-2, MD-3 and YN-6 produced taxol. One reason for the lack of taxol production in the other three *bapt* containing isolates is that the yield of taxol was too low to be detected, another reason may be that the *bapt* genes of these three fungi may not have been expressed.

In this paper, a rapid and economic method using the *dbat* and *bapt* genes as molecular markers has been developed to screen taxol-producing fungi. The finding of the two genes in fungi suggests a means of increasing the yield of taxol in fungi by genetic engineering, which is currently being attempted.

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