Taxomyces andreanae: A Presumed Paclitaxel Producer Demystified?

Authors **Agata Staniek, Herman J. Woerdenbag, Oliver Kayser**

Affiliation Pharmaceutical Biology Department, Groningen Research Institute of Pharmacy, University of Groningen, Groningen, The Netherlands

Key words
- paclitaxel
- *Taxomyces andreanae*
- hyphomyces
- endophyte
- taxadiene synthase gene
- phenylpropanoyl transferase gene
- PCR

Abstract

The 1990s brought an abundance of reports on paclitaxel-producing endophytes, initially heralded as a discovery having tremendous implications for cancer therapy. As the vision of large-scale fermentation tanks producing vast quantities of relatively inexpensive paclitaxel and novel taxanes has faded and has been replaced by controversial silence, we carried out an in-depth investigation of *Taxomyces andreanae* – the very first presumed endophytic synthesizer of the diterpenoid. On one hand, metabolic profiling by means of chromatographic, spectroscopic and immunoenzymatic techniques predominant in literature was taken up. On the other, the experimental procedure was brought to an alternative, previously unattempted level aiming at revealing the genetic background of paclitaxel biosynthesis in the endophyte. The profound PCR-based screening for *taxadiene synthase* (*txs*) – a gene unique to the formation of the primary taxane-skeleton, as well as *phenylpropanoyl transferase* (*bapt*) encoding for the catalyst of the final acylation of the core structure rendering the ultimate efficacy of the drug, confirmed the molecular blueprint for paclitaxel biosynthesis to be an inherent genetic trait of the endophyte. However, as the thorough metabolic analysis of *Taxomyces andreanae* commercial isolate brought no confirmation of endophytic paclitaxel production even after considerable up-scaling endeavors, we postulate that proclaiming the strain "a fungus factory for Taxol" might have been premature.

Introduction

Since its discovery in the 1960s, through structure elucidation completed in 1971 and FDA approval in 1992, paclitaxel – a highly functionalized diterpenoid, has evolved to become a blockbuster drug with commercial sales of well over US $3 billion in 2004 [1]. Since it was primarily obtained from the inner bark of *Taxus brevifolia*, a relatively rare and slow-growing tree, the supply of this potent antineoplastic agent was soon to become the issue of scarcity. Even early estimations indicated that the demand for paclitaxel might exceed 300 kg per year, which would amount to 750 000 yew trees [2]. The supply crisis, as well as the ecological implications resulting in the plant-endangered status, prompted the search for paclitaxel-producing microorganisms among the endophytic fungi of *Taxus*. This approach yielded the discovery of *Taxomyces andreanae* reported in 1993 [3].

Initially dubbed "a hot commodity" [4], the fungus was to become an inexhaustible supplier of the valuable diterpene. After nearly two decades, however, no conclusive follow-up data on the endophytic metabolite profile or the genetic background of the paclitaxel biosynthetic pathway are available.

While the original study [3] relied solely on metabolic means to confirm the endophytic taxane production, applying chromatographic, spectroscopic and immunoenzymatic detection methods shortly after removing the fungal symbiont from its plant host, our research concerned a commercially available, pure *Taxomyces andreanae* strain (CBS 27992) and was brought to an alternative, more advanced level. Namely, in parallel to utilizing the aforementioned techniques to confirm taxane presence in fungal cultures, we also addressed the question of the molecular blueprint for paclitaxel production being an inherent genetic trait of the endophyte.

The presumed route leading to paclitaxel *in planta*, encompassing genes encoding for the consecutive biosynthetic enzymes, has been extensively investigated and well documented by Croteau and coworkers [5]. The paclitaxel pathway in

received June 25, 2009
revised August 6, 2009
accepted Sept. 1, 2009

Bibliography
DOI 10.1055/s-0029-1186181
Published online October 6, 2009
Planta Med 2009; 75: 1561–1566 © Georg Thieme Verlag KG Stuttgart · New York · ISSN 0032-0943

Correspondence
Prof. Dr. Oliver Kayser
Pharmaceutical Biology Department
University of Groningen
Antonius Deusinglaan 1
9713 AV Groningen
The Netherlands
Phone: + 31 5 03 63 32 99
Fax: + 31 5 03 63 30 00
o.kayser@rug.nl

Taxus is considered to require 19 enzymatic steps from the ubiquitous diterpenoid precursor geranylgeranyl diphosphate (GGPP) which is subsequently cyclized to taxa-4(5),11(12)-diene. Following stages involve a series of cytochrome P450-mediated oxygenations, an array of acylations, oxetane ring formation and several steps of side chain assembly, resulting ultimately in the synthesis of paclitaxel (**Fig. 1**).

Taxadiene synthase (TXS, EC 4.2.3.17) is considered to be an enzyme catalyzing the commited step of paclitaxel biosynthesis [6], i.e., the slow bioconversion of the parent olefin (GGPP) to taxadiene, whereas phenylpropanoyl transferase (BAPT, EC 2.3.1.-) provides for the final attachment of the side chain – a structural requirement for anticancer efficacy [7]. For this reason, the in-depth study of *txs* and *bapt* as target genes, bearing clear implications for paclitaxel biosynthetic pathway elucidation in *Taxomyces andreanae*, was taken up. It was further anticipated that determining the intraspecific homology by alignment studies might shed some light on the widely debated hypothesis of horizontal gene transfer [8,9], thus explaining the distant distribution of the secondary metabolites in question between taxonomically unrelated species.

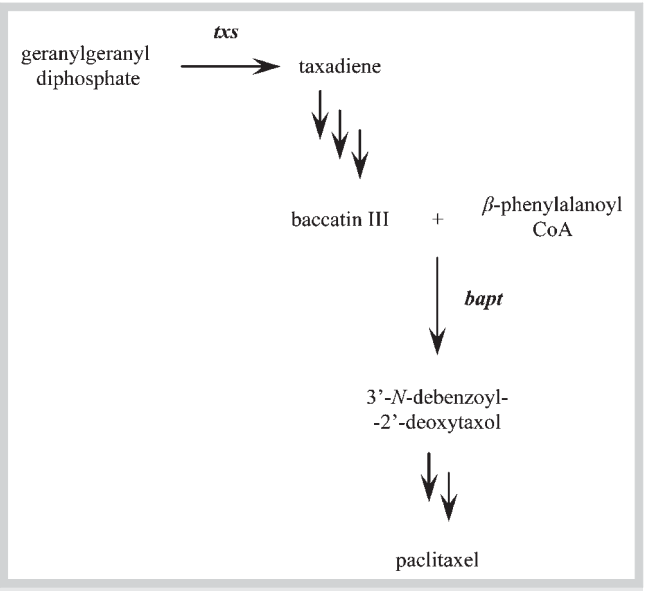

Fig. 1 A simplified outline of paclitaxel biosynthesis indicating steps crucial for our investigation.

Materials and Methods

Chemicals

Paclitaxel (≥ 95%, HPLC) and baccatin III (≥ 95%, HPLC) reference compounds as well as benzoic acid, thiamine, biotin and pyridoxal were purchased from Sigma-Aldrich. Glucose, fructose, sucrose, peptone and yeast extract were supplied by Duchefa. All other chemicals and organic solvents, including the deuterated methanol and chloroform, were obtained from Merck.

Plant and fungal material

Taxus baccata L. was purchased at a local market in Groningen and authenticated at the Botanical Garden "De Kruidhof", Buitenpost, the Netherlands. A voucher specimen (Gro-ASOK-01) is deposited in our department.

Taxomyces andreanae (CBS 27992) was obtained from Centraalbureau voor Schimmelcultures (CBS), Institute of the Royal Netherlands Academy of Arts and Sciences, Utrecht, the Netherlands.

Fungal cultivation

Initial cultivation was performed in Sabouraud-Dextrose medium (peptone 10 g/L, glucose 20 g/L, pH 5.6). A slightly modified S7 medium [3] comprising: glucose 1 g/L, fructose 3 g/L, sucrose 6 g/L, peptone 1 g/L, sodium acetate 1 g/L, yeast extract 250 mg/L, thiamine 1 mg/L, biotin 1 mg/L, pyridoxal 1 mg/L, $Ca(NO_3)_2$ 6.5 mg/L, phenylalanine 5 mg/L, $MgSO_4$ 3.6 mg/L, $CuSO_4$ 1 mg/L, $ZnSO_4$ 2.5 mg/L, $MnCl_2$ 5 mg/L, $FeCl_3$ 2 mg/L, benzoic acid 100 mg/L, 1 M KH_2PO_4 buffer (pH 6.8) 1 mL/L was the experimental medium.

Growth incubation was conducted at room temperature with moderate rotation speed (100 rpm) under a day/night regime (16/8 h; 1200 lux). 1, 5 and 10 L cultivations were attempted. Subculturing was performed by medium refreshment of a one-week-old fungal suspension in a 1 : 100 ratio.

Extract preparation

Crude and purified extracts of dried *Taxus baccata* needles were prepared as described previously [10]. After Büchner-filtration, the fungal mycelium was macerated and extracted with CH_2Cl_2:MeOH (1 : 1), while the filtrate was extracted with CH_2Cl_2. The solvent was removed from the organic extracts by rotary evaporation under reduced pressure at ~ 40 °C [11].

TLC

TLC analysis was conducted on silica gel plates Merck F_{254} (5 × 10 cm) with CH_2Cl_2:ACN (7 : 3, v/v) as the solvent system. Taxane detection was carried out by UV light absorption (254 nm) and spraying with vanillin/H_2SO_4 [11].

MPLC

The Büchi MPLC system comprised the following units: C-615 pump manager, two C-605 pump modules, C-660 fraction collector, C-630 UV monitor and a PC computer. A Büchi C-690 series borosilicate glass column (230 × 15 mm), packed with reversed phase silica gel RP_{18} was applied to perform the separation. Water (solvent A) and methanol (solvent B) were the mobile phase. The solvent flow was set at 10 mL/min and the UV detection wavelength at 220 nm. Dry fungal extracts were dissolved in 2 mL of methanol and injected to the system. The fractions were collected by signal or by volume (max. 15 mL/tube). The analysis was commenced at 20% B, followed by linear gradient with an endpoint at 40 min, 100% B and finalized by 15 min of isocratic 100% B elution. The separation procedure was monitored by TLC.

HPLC

The HPLC system comprised an LC-10AT pump, a SCL-10Avp controller, a FCV-10AL low-pressure gradient mixer and an SPD-M10A diode array detector (Shimadzu CLASS-VP), operated with CLASS-VP software version 6.12SP4. Low-pressure gradient chromatography was performed using an Eclipse XDB C_{18} column (250 × 4.6 mm with 5 µm packing). The injection volume was 50 µL and the flow rate was set at 1.5 mL/min at room temperature. The mobile phase system consisted of A: H_2O : ACN (80 : 15.44, w/w) with 10 mL 0.1 M phosphoric acid/L, and B: H_2O : ACN (20 : 62.56, w/w) with 10 mL 0.1 M phosphoric acid/L. The separation was commenced at 45% solvent B for 5 min, fol-

lowed by a linear gradient with given time endpoints: 24 min, 100% B; 26 min, 100% B; 28 min, 45% B; 30 min, 45% B (adopted from Ballero et al. [12]). The detection limit for paclitaxel as reference was established as the amount of analyte that provides a signal-to-noise ratio of 3, and amounted to 400 ng/mL.

HPLC-MS

A Perkin-Elmer SCIEX API-3000 apparatus with atmospheric pressure electrospray ionization and triple-quadrupole mass spectrometer operating in the positive mode was used for the HPLC-MS analysis. The instrument was equipped with a Perkin-Elmer series 200 HPLC micro-gradient pump and an autosampler. The mass spectrometer was interfaced to an Alltima C_{18} column (150 × 2.1 mm, 5 µm particle size). The mobile phase comprised: 100% H_2O (solvent A) and 100% ACN (solvent B) both containing 0.1% formic acid. The separation was commenced at 45% solvent B for 1 min, followed by a linear gradient with given time endpoints: 20 min, 80% B; 22 min, 80% B; 24 min, 45% B; 25 min, 45% B. The injection volume was 30 µL and the flow rate 1 mL/min at room temperature. The positive ion mode was employed and spectra were obtained with a spray voltage of 5.2 kV. The source temperature was 450 °C. The scan rate was 2 sec/scan, a full ion scan was applied in the range of 100–1100 amu with a step size of 1 amu and 10 V entrance potential. Nitrogen was used both as the nebulizer and curtain gas at a pressure of 0.7 Torr and a flow rate of 13 mL/min. Data processing was performed using the Analyst version 1.4 software (MDS Sciex).

NMR

^{1}H- (500 MHz) and ^{13}C- (125.69 MHz) one- and two-dimensional NMR spectra were recorded on a Varian INOVA-500 spectrometer.

Immunodetection

CIEIA – a competitive inhibition enzyme immunoassay system for the quantitative detection of taxanes in biological matrices sensitive to paclitaxel at a concentration of 0.5 ng/mL [13] was performed using a commercially available kit (Hawaii Biotech, Inc.).

DNA isolation

Efficient extraction of plant and fungal DNA was accomplished using DNeasy® Plant Mini Kit (Qiagen, Inc.) according to the manufacturer's protocol. Plasmid DNA purifications were performed using QIAprep® Spin Miniprep Kit (Qiagen, Inc.). PCR products were purified with QIAquick® PCR Purification Kit & QIAquick® Gel Extraction Kit (Qiagen, Inc.).

PCR

Temperature gradient PCR reactions were conducted with 2 alternative enzymes: *Taq* DNA Polymerase (5 U/µL) (Fermentas GmbH) and *Phusion*™ DNA Polymerase (2 U/µL) (Finnzyme OY), in the Eppendorf Mastercycler gradient thermocycler. *Thermal conditions: Taq*: 95 °C for 3 min (initial denaturation), 30 × [95 °C for 30 sec (denaturation), 40 sec (annealing) in temperature gradient, 72 °C (extension) for the period of time dependent on the length of the amplified gene fragment and enzyme efficiency], 72 °C for 5 min (final extension). *Phusion*™: 98 °C for 1 min (initial denaturation), 30 or 35 × [98 °C for 10 sec (denaturation), 20 sec (annealing) in temperature gradient, 72 °C (extension) for the period of time dependent on the length of the amplified gene fragment and enzyme efficiency], 72 °C for 10 min (final extension).

Primers: taxadiene synthase gene specific primer set for the amplification of *txs* sequence encoding the full-length protein [14]:
- txs_fwd (5′-atggctcagctctcatttaatg-3′)
- txs_rev (5′-tgccaatacaataataagtc-3′)

Alternative specific forward starter for the amplification of a truncated *txs* sequence encoding the mature enzymatic entity (without the plastid targeting signal peptide):
- txs_fwd_noPTS (5′-atgagcagtagcactggcactagca-3′)

Specific primer set for the amplification of highly conserved core DNA fragment of *txs* [15]:
- ctxs_fwd (5′-caaacccatgtcgaattgagaag-3′)
- ctxs_rev (5′-caagtttgcatacactctggaatct-3′)

Degenerate PCR primers:
- LHQ_fwd (5′-ytrcaycargargarytraargarytr-3′)
- LHQ_rev (5′-yarytcyttyarytcytcytgrtgyar-3′)
- DDXXD_fwd (5′-gtnathgaygayacntaygaygaytay-3′)
- DDXXD_rev (5′-rtartcrtcrtangtrtcrtcdatnac-3′)
- DDXXDspec_rev (5′-aaagatgtcagccatatcatcaaaaag-3′)

Phenylpropanoyl transferase gene [16]:
- bapt_fwd (5′-cctctctccgccattgacaa-3′)
- bapt_rev (5′-tcgccatctctgccatactt-3′)

The primers were purchased from Invitrogen™/Illumina® laboratory and Operon Biotechnologies.

Molecular cloning and sequence analysis

Taq and A-tailed *Phusion* amplification products were ligated into pGEM®-T and pGEM®-T Easy Vector Systems (Promega Corporation), whereas cloning experiments on the blunt-ended PCR products synthesized with *Phusion* polymerase were conducted by means of Zero Blunt® TOPO® PCR Cloning Kit (Invitrogen). The obtained plasmid vectors were transformed into NovaBlue Singles™ chemically competent *E. coli* cells (Novagen, Inc.), or XL1-blue competent *E. coli* strain (Stratagene). Endonuclease digestions of DNA inserts were performed using restriction enzymes from New England BioLabs, Inc. Sequence analysis was performed by ServiceXS B.V. and Macrogen Ltd.

Results and Discussion

In order to gain the first insight into the presence of taxane compounds in the extracts of *Taxomyces andreanae* prepared from 1-, 5- and 10-L cultures, thin layer chromatography was employed. As R_f values of analyzed mixtures' components corresponded to the one of paclitaxel reference (0.30–0.34) yielding the same intense blue color reaction with the vanillin-sulphuric acid spray as the authentic compound, the initial indication of endophytic paclitaxel production seemed to have been provided for the organic extracts of fungal culture medium filtrates. High-performance liquid chromatography was applied subsequently. The chromatograms of culture broth filtrate extracts revealed a peak eluting around the retention time of 10.96 min corresponding, more or less, to that of the reference compound (10.91 min).

While both baccatin III and paclitaxel were successfully detected in the purified extract of *Taxus baccata* needles, providing for a positive control of taxane presence verification by means of

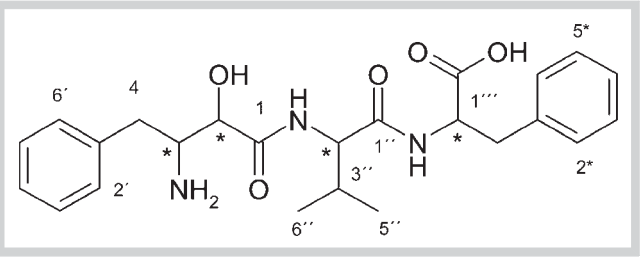

Fig. 2 3-Amino-2-hydroxy-4-phenylbutanoic acid – Val-Phe.

HPLC-MS, no such reaffirmation concerning endophytic taxane production was obtained.

As current chromatographic methods for the determination of taxanes in biological matrices are considered to be relatively insensitive, an immunoenzymatic system (CIEIA), with the reactivity towards paclitaxel at concentrations 800 times lower than those detectable by HPLC, was employed. The average taxane concentration values in crude and purified extracts of *Taxus baccata* needles amounted to 270 µg/mL and 20 µg/mL, respectively, which constitutes 0.05% of dry weight and corresponds to taxane concentration in *Taxus* needles as previously reported [10]. Despite numerous trials, results obtained for the samples of fungal origin remained inconsistent and led to the conclusion that the diterpenoids of interest were absent from the endophytic extracts.

Medium-pressure liquid chromatography was applied as a preparative method for the isolation of individual natural products from the complex fungal extracts, with a major effort put on the identification of the intriguing paclitaxel-mimicking constituent. The separation procedure was monitored by TLC and reaction with vanillin/H_2SO_4 spraying reagent. The signal representing the compound in question was registered at a retention time of 14.10 min while 6 consecutive fractions of the eluate showed intense blue staining on the silica gel plates. The pure fungal metabolite was further subjected to HPLC analysis and eluted at 5.66 min. While present in the original chromatograms of endophytic extracts, this peak did not correspond to the one of paclitaxel registered at 10.91 min.

The endophytic compound (**1**) obtained was an amorphous white solid (7 mg). The TLC and HPLC pure metabolite was subjected to mass spectrometry as well as ^{1}H- and ^{13}C 1D/2D NMR. The spectral data were in good agreement with an unusual tripeptide 3-amino-2-hydroxy-4-phenylbutanoic acid – Val-Phe (**Fig. 2**). According to LC-MS/MS the molecular mass was determined at 441.53 (calcd. for $C_{24}H_{31}N_3O_5$). The fragmentation pattern showed three major fragments at *m/z* (%) = 257.2 (100), 239.2 (23), 216.2 (20), and 198.2 (48). The assignment of proton resonances gave the following correlation: PBA (3-amino-2-hydroxy-4-phenylbutanoic acid) 2 (3.83 ppm, d, 10 Hz), PBA3 (3.5 ppm, dd, 10 Hz, 8 Hz), PBA4 (2.72 and 2.85 ppm, dd, 8 Hz); PBA2′-6′ (7.25–7.37 ppm, m), Val2″ (6.42 ppm, d, 15 Hz), Val3″ (2.18 ppm, m), Val5″ (0.96 ppm, t, 19 Hz), Val6″ (0.89 ppm, t, 19 Hz), Phe2‴ (3.98 ppm, d, 21 Hz), Phe3‴ (3.16 ppm, dd, 3.32, dd, 21 Hz), Phe2*-6* (7.25–7.37 ppm, m). A ^{13}C NMR showed 24 resonances as documented in **Table 1**.

Table 1 ^{13}C NMR data of compound **1**.

C	1
PBA1	171.3
PBA2	77.2
PBA3	54.8
PBA4	39.9
PBA1′	140.8
PBA2′	131.5
PBA3′	127.8
PBA4′	126.3
PBA5′	127.8
PBA6′	131.5
Val1′′	168.3
Val2′′	61.8
Val3′′	30.1
Val4′′	20.0
Val5′′	18.1
Val6′′	19.7
Phe1‴	54.6
Phe2‴	38.2
Phe1*	134.9
Phe2*	127.9
Phe3*	128.4
Phe4*	127.4
Phe5*	128.4
Phe6*	127.8

* Values in ppm were recorded at 125.69 MHz (in $CDCl_3$)

The basic tool of research on the genomic level was the polymerase chain reaction. The initial PCR trials were carried out using specific primers designed after very highly conserved sequences in the *txs* cDNAs from *Taxus brevifolia* and *Taxus chinensis*, as reported in literature [14], and allowing the amplification of the full-length *Taxus baccata* gene. As this original transcript encompasses a long *N*-terminal targeting sequence for localization to and processing in the plastids, consecutive reactions were performed using an alternative forward oligonucleotide for the amplification of a truncated *txs* sequence encoding the mature enzymatic entity.

Further investigation required designing suitable degenerate primer pairs providing for amplification of the target gene. The ultimate design was preceded by an extensive study concerning the nature of prenyltransferases as a group of enzymes encompassing taxadiene synthase, e.g., [17–19], and completed by means of the NCBI Conserved Domain Search for *txs* [20] revealing the presence of highly conserved amino acid sequences characteristic of terpene cyclases of plant as well as microbial origin [21]. Although the evolutionary relationship of plant and microbial prenyltransferases is unclear, all of these enzymes contain the aspartate-rich DDXXD motif involved in coordination of divalent metal ions for substrate binding [22]. This region was, therefore, an obvious choice for primer design basis. An alternative primer set (LHQ) was constructed with reference to an up-stream highly conserved amino acid sequence of unknown function, as identified by the NCBI Search tool.

All the aforementioned oligonucleotides were employed in various combinations, in a wide range annealing temperature gradient, with and without DMSO included in the reaction mixture, and with two alternative DNA polymerases as reaction catalysts. This extensive PCR-based screen yielded a number of potentially interesting amplification products. These were either directly sequenced or, if applicable, further purified and employed as templates for nested PCR experiments.

Primary alignment studies at the nucleotide and amino acid levels did not reveal any significant similarities between the sequences of the analyzed DNA fragments and those of *txs*, nor

any other known gene of the prenyltransferase family, be it of plant or microbial origin.

Final experimental steps addressing the genetic capacity of *Taxomyces andreanae* to synthesize the tricyclic taxane core were prompted by a recent publication postulating successful PCR-based screening for *txs* presence in several endophytic entities. Thus, the genomic DNA of fungal origin served as a template yet again, in hope for efficient annealing with the specific starters proposed by Zhou et al. [15]. As no amplification products could be observed while applying a standard quantity of 30 PCR cycles, the cycle-number was increased to 35 finally yielding the desired amplicon (**Fig. 3a**). Further successful semi-degenerate/nested (ctxs_fwd and DDXXD_rev and ctxs_fwd and DDXXDspec_rev) chain reactions, sequencing and alignment studies followed, ultimately confirming *txs* presence in the genome of *Taxomyces andreanae* (97% sequence similarity with *txs* of *Taxus* sp. within the conserved region amplified). Interestingly, the desired sequence could not be retrieved while utilizing the forward starter annealing to the plant *txs* fragment immediately following the plastid targeting sequence (txs_fwd_noPTS) in combination with the core reverse oligonucleotide (ctxs_rev), suggesting that the functional fungal gene is further cropped on its *N*-terminus.

While crucial for the taxane biosynthetic pathway elucidation, the detection of *taxadiene synthase* affords no definitive proof for endophytic paclitaxel production. Thus, in order to gain one, *phenylpropanoyl transferase* was selected as an alternative molecular marker [16]. The core fragment of the gene diagnostic for one of the very last steps of paclitaxel biosynthesis was successfully amplified (**Fig. 3b**), cloned and sequenced, showing high homology with its plant counterparts (96% sequence identity). *Bapt* detection, in turn, allows speculating about the origins of the *β*-phenylalanoyl-paclitaxel side chain. Namely, the fact that phenylalanine constitutes one of the building blocks of the tripeptide identified in the course of our research deems it plausible that, what we initially considered to be merely a paclitaxel counterfeit, is actually its crucial progenitor.

To sum up, the absence of taxane metabolites from endophytic extracts derived from 1-, 5- and 10-L cultures proves *Taxomyces andreanae* incapable of independent (*ex planta*) paclitaxel production under axenic culture conditions. Thus, while initially unable to detect the fungal *taxadiene synthase*, we primarily ascribed the biosynthesis of paclitaxel *ab initio* to be a genuine feature of the yew host rather than its microbial inhabitant in question [23]. Interestingly, the presence of the *N*-terminal plastid targeting sequence within *taxadiene synthase* transcript of plant origin, further supported by ample evidence of the antineoplastic diterpene production by sterile cell suspension cultures of *Taxus* [24] and the successful isolation of paclitaxel biosynthetic genes therefrom [5], seemed indeed to indicate that this gene was plant-derived rather than a fungal product.

However, the ultimate success of our profound screen for the microbial *taxadiene synthase*, accompanied by the detection of *bapt*, encoding for the enzyme affording the efficacious antineoplastic natural product, provides crucial evidence for the molecular blueprint of paclitaxel production being an inherent genetic trait of *Taxomyces andreanae*. Consequently, the results thus obtained seem to give a new insight to the controversial hypothesis of horizontal gene transfer (HGT). One might assume that the biosynthesis of paclitaxel has been repeatedly invented during evolution. However, the fact that approximately 20 genes are involved in the formation of this highly functionalized and unique diterpenoid [5] makes it rather unlikely. Therefore, it seems possible that

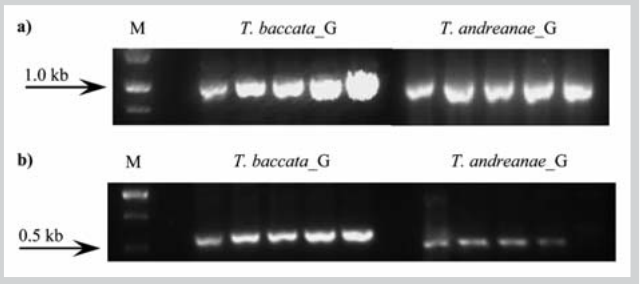

Fig. 3 PCR analysis for the presence of **a** *taxadiene synthase* (~ 1.0 kb) and **b** *phenylpropanoyl transferase* (~ 0.5 kb) in *Taxomyces andreanae. Taxus baccata* needles were a source of plant DNA samples, providing for a positive control of *txs* & *bapt* presence verification (M – GeneRuler™ 1 kb DNA Ladder, Fermentas; G – annealing temperature gradient: 45–65 °C).

a lateral transfer of genetic information shaped the evolutionary trajectory of taxonomically unrelated, yet coexisting, species. Moreover, postulating HGT to be a driving force in the evolution of fungal gene clusters – a phenomenon now considered a hallmark characteristic of secondary metabolic biosynthetic pathways [9,25], raises an intriguing question as to whether the genes responsible for paclitaxel formation in *Taxomyces andreanae* are indeed grouped in a contiguous cluster. Therefore, although deemed largely anecdotal thus far [8,9], the ultimate plausibility of the horizontal gene transfer hypothesis should be revisited and further investigated.

All in all, our results suggest that specific plant environment may be required for the induction of paclitaxel biosynthetic genes in the fungal symbiont. Identifying the regulatory mechanisms will be of considerable future interest and will provide further insight into the true nature of the fine-tuned equilibrium of plant-microbe interactions [23,26].

Regardless of the aforementioned fundamental speculations, practical aspects should be decisively invoked. As the highly desirable search for sustainable and economically feasible paclitaxel sources has tempted various authors to draw premature conclusions proclaiming endophytes to be true taxane bio-factories, e.g., [3,27,28], and reviewers still seem to dwell on the potential of the microbial taxane synthesizers to revolutionize the pharmaceutical arena [29]. We contend that such reports should be viewed as extraordinary claims that demand ultimate justification, namely: unambiguous evidence for the molecular blueprint underlying the postulated microbial paclitaxel biosynthesis, suggesting a means for its activation and manipulation.

Acknowledgements

Thanks are due to Jan Willem Zwart, Botanical Garden "De Kruidhof", Buitenpost, for providing plant material, Wim Kruizinga, Synthetic Organic Chemistry Department, University of Groningen, for performing NMR analysis, as well as Annie van Dam, Analytical Chemistry Departrment, University of Groningen, for performing HPLC-MS/MS analysis.

References

1 *Croteau R.* Taxol biosynthesis and molecular genetics. Wageningen, the Netherlands: Terpnet; 2005
2 *Stierle A, Strobel GA, Stierle DB, Grothaus P, Bignami G.* The search for a taxol-producing microorganism among the endophytic fungi of the Pacific yew, *Taxus brevifolia*. J Nat Prod 1995; 58: 1315–1324

3 *Stierle A, Strobel GA, Stierle DB.* Taxol and taxane production by *Taxomyces andreanae*, an endophytic fungus of Pacific yew. Science 1993; 260: 214–216
4 *Stone R.* Surprise! A fungus factory for taxol? Science 1993; 260: 154–155
5 *Walker K, Croteau R.* Taxol biosynthetic genes. Phytochemistry 2001; 58: 1–7
6 *Wildung MR, Croteau R.* A cDNA clone for taxadiene synthase, the diterpene cyclase that catalyzes the committed step of taxol biosynthesis. J Biol Chem 1996; 271: 9201–9204
7 *Walker K, Long R, Croteau R.* The final acylation step in taxol biosynthesis: cloning of the taxoid C13-side chain *N*-benzoyltransferase from *Taxus*. PNAS 2002; 99: 9166–9171
8 *Kurland CG, Canback B, Berg OG.* Horizontal gene transfer: a critical view. PNAS 2003; 100: 9658–9662
9 *Rosewich UL, Kistler HC.* Role of horizontal gene transfer in the evolution of fungi. Annu Rev Phytopathol 2000; 38: 325–363
10 *Grothaus PG, Bignami GS, O'Malley S, Harada KE, Byrnes JB, Waller DF, Raybould TJ, McGuire MT, Alvarado B.* Taxane-specific monoclonal antibodies: measurement of taxol, baccatin III, and "total taxanes" in *Taxus brevifolia* extracts by enzyme immunoassay. J Nat Prod 1995; 58: 1003–1014
11 *Strobel GA, Stierle A, Stierle DB.* Taxol production by *Taxomyces andreanae*. US Patent 5322779; 1994
12 *Ballero M, Loi MC, van Rozendaal EL, van Beek TA, van de Haar C, Poli F, Appendino G.* Analysis of pharmaceutically relevant taxoids in wild yew trees from Sardinia. Fitoterapia 2003; 74: 34–39
13 *Grothaus PG, Raybould TJ, Bignami GS, Lazo CB, Byrnes JB.* An enzyme immunoassay for the determination of taxol and taxanes in *Taxus* sp. tissues and human plasma. J Immunol Methods 1993; 158: 5–15
14 *Besumbes O, Sauret-Güeto S, Phillips MA, Imperial S, Rodriguez-Concepcion M, Boronat A.* Metabolic engineering of isoprenoid biosynthesis in *Arabidopsis* for the production of taxadiene, the first committed precursor of taxol. Biotechnol Bioeng 2004; 88: 168–175
15 *Zhou X, Wang Z, Jiang K, Wei Y, Lin J, Sun X, Tang K.* Screening of taxol-producing endophytic fungi from *Taxus chinensis* var. *mairei*. Prikl Biokhim Mikrobiol 2007; 43: 490–494
16 *Zhang P, Zhou P, Jiang C, Yu H, Yu LJ.* Screening of taxol-producing fungi based on PCR amplification from *Taxus*. Biotechnol Lett 2008; 30: 2119–2123
17 *Bohlmann J, Meyer-Gauen G, Croteau R.* Plant terpenoid synthases: molecular biology and phylogenetic analysis. PNAS 1998; 95: 4126–4133
18 *Lesburg CA, Zhai G, Cane DE, Christianson DW.* Crystal structure of pentalenene synthase: mechanistic insights on terpenoid cyclization reactions in biology. Science 1997; 277: 1820–1824
19 *Trapp SC, Croteau R.* Genomic organization of plant terpene synthases and molecular evolutionary implications. Genetics 2001; 158: 811–832
20 *NCBI.* Available at http://www.ncbi.nlm.nih.gov/BLAST. Accessed May 3, 2007
21 *NCBI.* Available at http://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi?uid=cd00868& version=v2.06. Accessed May 17, 2007
22 *Tarshis LC, Yan M, Poulter CD, Sacchettini JC.* Crystal structure of recombinant farnesyl diphosphate synthase at 2.6-A resolution. Biochemistry 1994; 33: 10871–10877
23 *Staniek A, Woerdenbag HJ, Kayser O.* Endophytes: exploiting biodiversity for the improvement of natural product-based drug discovery. J Plant Interact 2008; 3: 75–93
24 *Ketchum RE, Gibson DM.* Paclitaxel production in suspension cell cultures of *Taxus*. Plant Cell Tiss Organ Cult 1996; 46: 9–16
25 *Lawrence JG, Roth JR.* Selfish operons: horizontal transfer may drive the evolution of gene clusters. Genetics 1996; 143: 1843–1860
26 *Hines PJ, Zahn LM.* What's bugging plants? Introduction to special issue. Science 2009; 324: 741
27 *Chakravarthi BVSK, Das P, Surendranath K, Karande AA, Jayabaskaran C.* Production of paclitaxel by *Fusarium solani* isolated from *Taxus celebica*. J Biosci 2008; 33: 259–267
28 *Strobel GA, Hess WM, Ford E, Sidhu RS, Yang X.* Taxol from fungal endophytes and the issue of biodiversity. J Indian Microbiol Biotechnol 1996; 17: 417–423
29 *Miller K, Neilan B, Sze DMY.* Development of taxol and other endophyte produced anti-cancer agents. Recent Pat Anticancer Drug Discov 2008; 3: 14–19