Expression of *dbat* and *dbtnbt* Genes Involved in Paclitaxel Biosynthesis during the Growth Cycle of *Taxus baccata* L. Callus Cultures

Zuzana Katkovčinová, Martina Lázárová, Katarína Bruňáková*, Ján Košuth, and Eva Čellárová

P. J. Šafárik University in Košice, Faculty of Science, Institute of Biology and Ecology, Mánesova 23, 041 54 Košice, Slovakia. Fax: +4 21-55-6 33 73 53.
E-mail: katarina.brunakova@upjs.sk

* Author for correspondence and reprint requests

Z. Naturforsch. **63 c**, 721–730 (2008); received February 25/April 4, 2008

The time-course of expression of *dbat* and *dbtnbt* genes involved in the later steps of paclitaxel biosynthesis and the intracellular taxane accumulation were investigated through a 64-day subculture interval of VI/M1 and VI/M2 *Taxus baccata* callus cultures. HPLC proved traces of baccatin III and an intracellular content of paclitaxel up to 90 μg/g DW. The steady-state of the respective gene transcripts was measured by quantitative real-time RT-PCR. The expression profile of *dbat* and *dbtnbt* genes was slightly different and varied within the subculture. The highest level of *dbat* expression was detected 24 h after inoculation followed by a decrease in both cultures. In contrast with *dbat* no substantially high expression of the *dbtnbt* gene after inoculation was observed. The impact of the conditions during inoculation on gene expression is discussed. Although the increase in transcriptional activity of both genes positively correlated with callus growth, the intracellular accumulation of paclitaxel varied during subculture with the maximum in the stationary (VI/M1) or at the end of the linear (VI/M2) phase. The increase of the steady-state mRNA level of the *dbtnbt* gene was followed by paclitaxel accumulation with a delay of approx. 28 (VI/M1) and 14 days (VI/M2).

Key words: Gene Expression, Real-Time RT-PCR, *Taxus baccata* L.

Introduction

Paclitaxel (Taxol®, Bristol-Myers Squibb Co.), a complex diterpenoid with a unique tumour-suppressing mechanism (Schiff *et al.*, 1979; Cragg *et al.*, 1993) is an effective antineoplastic drug widely used in the treatment of a variety of cancers, including carcinomas of the breast, ovary, lung, head and neck, AIDS-related Kaposi's sarcoma or non-small cell lung cancer either alone or in combination with other chemotherapeutics (Rowinsky *et al.*, 1990; Brown, 2003; Sledge, 2003).

The use of the natural source of paclitaxel – the bark of *Taxus* trees – caused mass devastation of yew populations around the world. The low amount of paclitaxel in the bark of *Taxus* spp. in the range of 0.0001 to 0.076% dry weight (DW) (Videnšek *et al.*, 1990; Kelsey and Vance, 1992; Rao, 1993; Nadeem *et al.*, 2002) and the slow growth of yew trees led to identifying new, more sustainable sources of this important drug. The total chemical synthesis or the isolation and purification of the active compounds from the plantation-grown *Taxus* plants are expensive and complicated processes (Guo *et al.*, 2006). The semisynthesis of paclitaxel from its precursor 10-deacetylbaccatin III (10-DAB III) or baccatin III which can be isolated from the needles, the renewable source, is a more feasible and commercially viable method (Jennewein *et al.*, 2004).

Nevertheless, the most promising way for large-scale production of paclitaxel and related taxanes seems to be the use of various *in vitro* systems – fungal fermentation (Xu *et al.*, 2006), genetically engineered microorganisms (DeJong *et al.*, 2006) or plant cell culture process, which was recently approved by FDA for paclitaxel supply. Currently, Bristol-Myers Squibb reported that Taxol® is now being made solely by plant cell cultures (Tabata, 2006).

Currently, a large-scale culture process was developed with a paclitaxel production in the range of 140–295 mg/L (Tabata, 2006). However, the commercial feasibility of a large-scale bioprocess of *Taxus* cell cultures in the near future will probably depend on detailed understanding of the taxane biosynthetic pathway, the possibilities to influence the production rates of desirable taxanes by overexpression of genes controlling slow steps, by

0939–5075/2008/0900–0721 $ 06.00

suppressing the production of undesired taxanes or by redirection of the pathway for the production of novel taxanes with greater range and potency of anticancer activity and decreased side-effects (Jennewein and Croteau, 2001). Genetic transformation may also be a promising approach for revealing and engineering the complex paclitaxel biosynthetic pathway. Currently a stable transformation of *Taxus* cell suspension cultures was achieved (Ketchum *et al*., 2007).

The paclitaxel biosynthesis is considered to require 19 enzymatic steps from the universal diterpenoid precursor geranylgeranyl diphosphate (Hezari and Croteau, 1997). Except for a few undefined steps the whole biosynthetic pathway was proposed and 13 genes for its enzymes have been cloned and characterized (reviewed by Croteau *et al*., 2006; Guo *et al*., 2006). As indicated by Nims *et al*. (2006) the regulation of the taxane biosynthetic pathway occurs at the level of mRNA with a tight correlation between steady-state transcript abundance and respective taxane accumulation. Although many experiments have been made in order to characterize the *Taxus* cell cultures by growth, taxane accumulation and enzyme production in elicited cultures (Hefner *et al*., 1998; Tabata, 2006), little information is available regarding the growth and taxane accumulation in the relation to the transcriptional activity of the responsible genes in cell cultures without elicitation.

In this report we have focused on the study of the time-course of transcriptional activity of *dbat* and *dbtnbt* genes coding for two enzymes of the later steps of the paclitaxel biosynthetic pathway and accumulation of the respective taxanes, baccatin III and paclitaxel in unelicited *Taxus baccata* callus cultures within a prolonged subculture period. As proposed by Walker and Croteau (2000) and Walker *et al*. (2002) the *dbat* gene codes for 10-deacetylbaccatin-III-10β-*O*-acetyltransferase (DBAT), which converts 10-deacetylbaccatin III (10-DAB III) to baccatin III, and the *dbtnbt* gene codes for 3′-*N*-debenzoyl-2′-deoxytaxol-*N*-benzoyltransferase (DBTNBT), which ligates a benzoyl CoA group to 3′-*N*-debenzoyl-2′-deoxytaxol to produce 2′-deoxytaxol, respectively. This is the first report on the expression profile of genes involved in the paclitaxel biosynthesis during growth of a callus culture applying accurate quantification of gene expression by real-time RT-PCR (qRT-PCR).

Materials and Methods

Plant material

The callus cultures (VI/M1, VI/M2) used in the experiments were initiated from *in vitro* grown seedlings originated from seeds of one *T. baccata* L. tree growing in Botanical Garden of P. J. Šafárik University in Košice, Slovakia, according to Bruňáková *et al*. (2004). For the experiments both cultures were selected after 2 years of cultivation on the basis of growth characteristics like doubling time and biomass accumulation.

The induction of callus formation was performed in Gamborg's B_5 medium (Gamborg *et al*., 1968) supplemented with 1.5% phenolic-binding compound, soluble polyvinylpyrrolidone (PVP), 3 mg/L 2,4-dichlorophenoxy-acetic acid (2,4-D) and 0.5 mg/L kinetin; for long-term culture the content of 2,4-D was reduced to 1.5 mg/L. All media were solidified with 0.6% agar – the pH value was adjusted to 5.6 before autoclaving – and sterilized at 121°C for 15 min. For the routine callus cultivation the above described media were used; for the gene expression studies the inocula were transferred onto *fresh* and also so-called *reused* media, *i.e*., the media which were already used in a previous subculture.

The calli were cultivated at (22 ± 2) °C and 75% relative humidity under dark conditions. The inoculation was routinely performed under light conditions (white daylight, approx. 72 μmol m^{-2} s^{-1}); for a part of gene expression studies the inocula were transferred under dark conditions.

The regular subculture interval for long-term cultivation was 21 (VI/M1) and 28 d (VI/M2), respectively. In our experiment the studied characteristics of both cultures were recorded during a 64-day interval; the samples for the analyses were taken on days 0, 1, 2, 3, 4, 8, 15, 22, 29, 36, 43, 50, 57 and 64.

In order to assign the reproducibility and homogeneity, the samples were prepared as a mixture of calli grown in 9 cultivation containers representing a stock specimen for all analyses on the respective days. The measured values were expressed as the mean ± standard deviation. Growth curves were determined by weighing the fresh and dry cell mass; each data point represents the mean of 9 replicates ± standard deviation.

Analysis of gene expression

The samples for gene expression analysis were homogenized in liquid nitrogen to a fine powder and stored at −80 °C until the RNA isolation.

RNA isolation and reverse transcription

Total RNA was extracted from 100–120 mg of ground callus using the RNeasy Plant Mini kit (Qiagen, Valencia, USA). To eliminate the residual genomic DNA present in the samples, RNA was treated with RNase-Free DNase I (Qiagen, Hilden, Germany). The integrity of the extracted RNA was checked by agarose gel electrophoresis. Total RNA concentration was determined by spectrophotometry at 260 nm and more accurately by fluorescent dye RiboGreen using the Quant-iT™ RiboGreen® RNA Kit (Invitrogen, Carlsbad, USA) according to the manufacturer instructions. RiboGreen dye was diluted 1:1000 (v/v). Fluorescence was measured by a FLUOstar fluorometer (BMG Labtechnologies, Offenburg, Germany) fitted with 485 nm (excitation) and 520 nm (emission) filters.

Equal amounts (1 μg) of total RNA quantified by RiboGreen were reverse transcribed for each sample. Reverse transcription was performed at 37 °C in 20 μL volume using 10 mM anchored oligoT primer and 200 U M-MLV reverse transcriptase (Invitrogen) according to the manufacturer instruction.

Quantification of taxane biosynthetic genes

Quantification of *dbat* and *dbtnbt* genes expression was performed by quantitative real-time RT-PCR (qRT-PCR) in the iCycler iQ Real Time PCR Detection System (BioRad, Hercules, USA). PCR primers specific for *dbat* (dbat-F 5′-CCAAGCCAGCCATCGCCCAAAG-3′, dbat-R 5′-GGCAGAAACTCACCCCCACAACAAA-3′) and *dbtnbt* (dbtnbt-F 5′-TTGCCGTTGGAGTGACTTTGC-3′, dbtnbt-R 5′-AGCATTGGAGGTGGGCATATCG-3′) genes were designed based on the published cDNA sequences coding for DBAT from *T. baccata* (GenBank accession no. AF193765) and DBTNBT from *T. cuspidata* (GenBank accession no. AF466397) using GenTool Lite 1.0 software. qRT-PCR was performed in duplicates in 30 μL reaction volume containing: 1 × iQ™SYBR Green Supermix (0.2 mM dNTP; 3 mM $MgCl_2$) (BioRad), 0.5 μM forward and reverse primer and 50 ng of reverse transcribed RNA. The reaction conditions were as follows: 95 °C for 3 min; 40 cycles at 94 °C for 30 s; 59 °C (*dbat*) or 59.5 °C (*dbtnbt*) for 25 s; 72 °C for 25 s; 74 °C for 4 min; followed by melting curve analysis.

The amplified products (430 bp for *dbat* and 199 bp for *dbtnbt* gene fragments) were separated by electrophoresis in 1.6% agarose gel, stained with ethidium bromide and visualized under UV light. The amplifications of targeted genes were confirmed by sequencing the amplicons. The relative amounts of both gene transcripts were evaluated by the method of standard curves. These were obtained by amplification of a serially diluted mixture of cDNA samples (diluted 2-fold or 4-fold) with 5–6 dilutions, each one in two replicates.

Taxane analysis

Extraction

The lyophilized callus of about 2 g was powdered and ultrasonicated for 30 min in 50 mL methanol. After filtration the methanolic extract was evaporated *in vacuo*. The residue was then dissolved in 12.5 mL methanol and partitioned with 12.5 mL water and hexane (2 times 25 mL). After discarding the hexane extract, the water-methanol layer was partitioned with dichloromethane (2 times 25 mL). The dichloromethane-soluble fractions containing taxanes were combined, filtered over anhydrous sodium sulfate and evaporated to dryness *in vacuo*. The residue was dissolved in 1 mL methanol and analyzed by HPLC.

Analytical HPLC

Analyses were performed on a stainless-steel column (250 × 4.6 mm i. d.) packed with Kromasil 100–7 μm, C18 (EKA Chemicals AB, Bohus, Sweden). A mixture of acetonitrile and water (65:35 v/v) was used as mobile phase, at a flow rate of 0.7 mL/min. The volume of samples injected was 20 μL. The identification of compounds examined in the samples was carried out by retention time and co-chromatography with paclitaxel and baccatin III standards (Sigma, St. Louis, USA). Taxane compounds were detected by UV absorbance at 227 nm. The amount of both taxane compounds was calculated by a comparing of the sample peak area and the standard peak area. Each data point represents the mean of 3 independent extractions of the sample.

Results

In the present study the kinetics of expression of two genes of the later steps of taxane biosynthesis –

dbat and *dbtnbt* – was quantitatively analyzed during a prolonged 64-day subculture interval. The steady-state of gene transcripts of *dbtnbt*, which is to our knowledge the last sequenced gene in the biosynthetic paclitaxel pathway, as well as the accumulation of intracellular paclitaxel were studied. The expression profile of the *dbat* gene, coding for the enzyme-catalyzing conversion of 10-DAB III to baccatin III, the last intermediate without a phenylisoserine side-chain in the biosynthetic pathway, was also determined. The intracellular accumulation of this compound in undifferentiated cell cultures was already proven in our previous study (Bruňáková *et al*., 2005). For quantification of the steady-state mRNA level of *dbat* and *dbtnbt* genes fluorescence-based quantitative real-time RT-PCR (qRT-PCR) with SYBR Green I detection of the product was performed. The concentration of RNA was accurately quantified by the fluorescent dye RiboGreen to assign equal amounts of RNA/cDNA template in qRT-PCR in all compared samples. The accurate quantification of RNA allowed the quantification of *dbat* and *dbtnbt* expression without searching for a suitable housekeeping gene/s and to confirm its/their stable expression in all compared samples.

Growth characteristics

A time-course of FW (fresh weight) and DW (dry weight) accumulation over a 64-day growth period (Fig. 1) showed a relatively slow growth of both callus cultures with the doubling time of approx. 12 days (VI/M1) and 18 days (VI/M2), re-

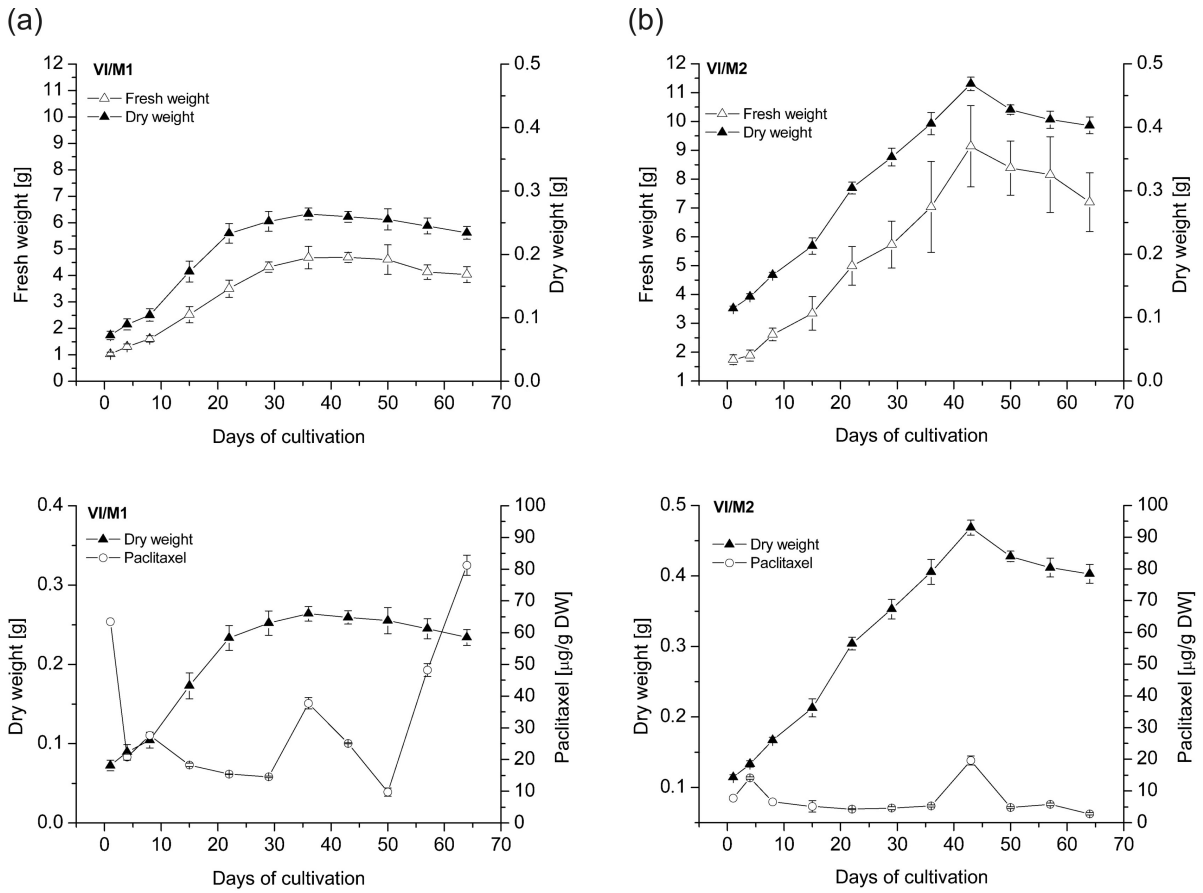

Fig. 1. Time-course of fresh (FW) and dry weight (DW) accumulation and intracellular paclitaxel content over a 64-day growth period of (a) VI/M1 and (b) VI/M2 callus cultures. Each data point represents the mean of calli grown in 9 cultivation containers ± standard deviation.

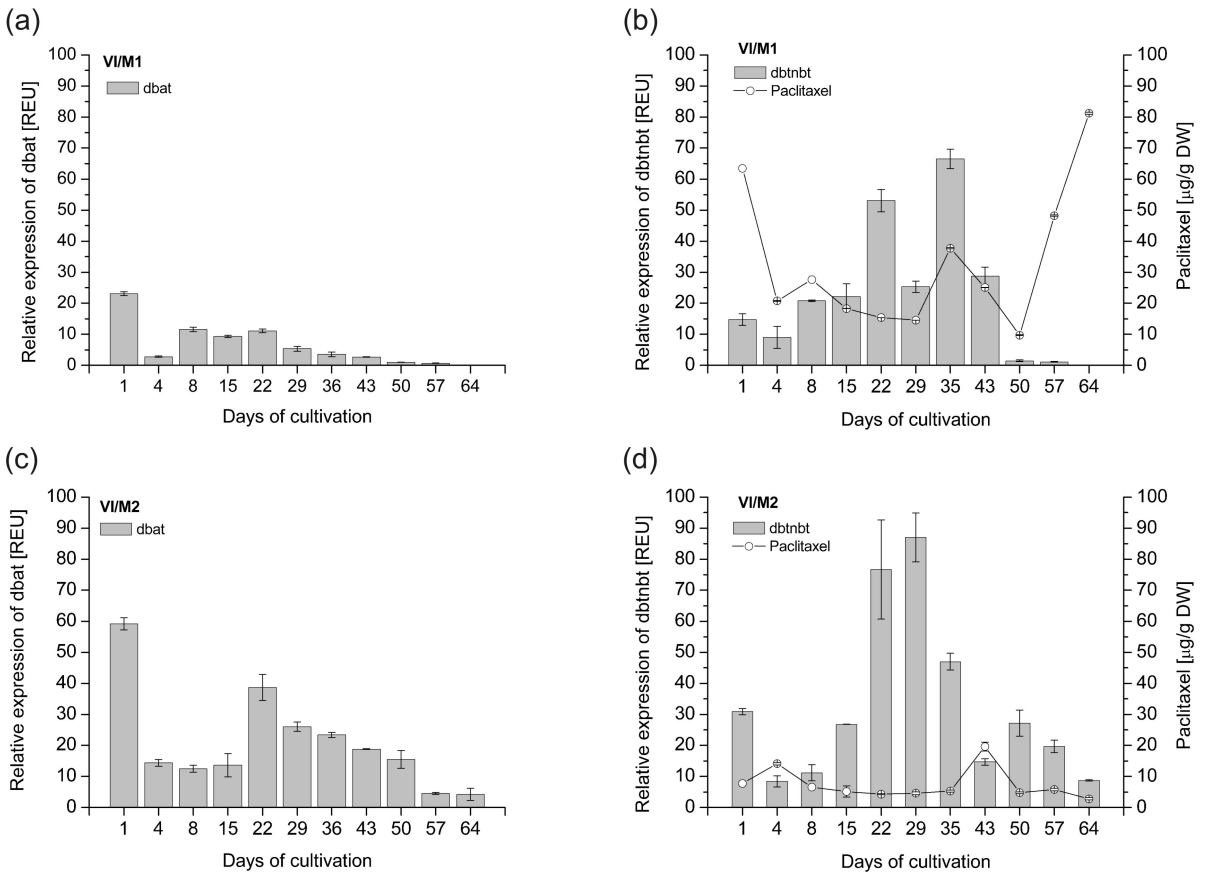

Fig. 2. Course of expression of (a, c) *dbat* and (b, d) *dbtnbt* genes during subculture of VI/M1 (a, b) and VI/M2 (c, d) callus cultures. The expression data represent the mean of 2 replicates ± standard deviation ($n = 9$ cultivation containers sampled for each data point); REU, relative expression units.

spectively. The growth curve of VI/M1 calli showed a lag phase of 8 days followed by an almost linear period of growth between days 8 and 22. The maximum biomass accumulation was reached at day 36 and then it has decreased steadily. The essentially linear growth of VI/M2 during the first 43 days was followed by a gradual decline until day 64. The increase of FW correlated well with the DW in both cultures.

Taxane accumulation

The intracellular paclitaxel content varied during the growth cycle (Fig. 1). In both cultures two peaks of endogenous paclitaxel accumulation occurred; at the beginning (27.6 μg/g DW for VI/M1 and 14.1 μg/g DW for VI/M2) and at the end (37.8 μg/g DW for VI/M1 and 19.6 μg/g DW for VI/M2) of the phase of active growth. Apart from these, in the VI/M1 culture higher paclitaxel levels were observed 24 h after inoculation as well as in the late stationary phase (63.5 μg/g and 81.3 μg/g DW). Although the presence of baccatin III was confirmed in both cultures, it occurred below the limit of quantification.

Expression of dbat and dbtnbt during subculture

The *dbat* transcriptional activity during subculture revealed a similar course in both cultures (Figs. 2a, c). The surprisingly highest level of *dbat* expression detected on day 1, *i. e.* 24 h after inoculation, dramatically decreased on day 4 (8.3-fold for VI/M1 and 4.1-fold for VI/M2). In the VI/M1 culture

the increased *dbat* expression was detected within days 8 to 22, followed by a slight decrease until the end of subculture. Similarly, the *dbat* expression in the VI/M2 culture culminated on day 22 and then gradually decreased.

The expression pattern of the *dbtnbt* gene in both callus cultures was also comparable (Figs. 2b, d). An initial state of expression on day 1 was followed by a decrease on day 4 (1.6-fold for VI/M1 and 3.7-fold for VI/M2). After that the transcriptional activity of *dbtnbt* gradually increased with the maximum level in the middle of subculture on day 36 (VI/M1) and 29 (VI/M2). These values were approx. 7-fold (VI/M1) or 10-fold (VI/M2) higher than those at the beginning of the growth cycle, *i.e.* on day 4. At the end of the culture interval the *dbtnbt* expression was decreasing.

The expression profile of *dbat* and *dbtnbt* revealed surprisingly high expression of *dbat* on day 1 which rapidly decreased on day 4. Because the inocula for this experiment were taken in the time when the expression of the both studied genes was relatively high (Fig. 2), the increased expression levels of the *dbat* gene 24 h after inoculation could reflect the steady-state of the culture at the time of inoculation. To explore the rapid variation in the expression level at the beginning of subculture, the steady-state of mRNA expression of both genes at the time of inoculation (*i.e.* in the inoculum) and within the first four days of culture was studied. For the analysis the VI/M2 culture was used. The preliminary results revealed that the *dbat* gene expression raised 24h after inoculation approx. 4.5-fold in comparison with the expression level in the inoculum. In case of the *dbtnbt* gene the immediate change of expression on day 1 was not that considerable (1.4-fold increase of expression) (data not shown). During the next days, the expression level of both genes significantly decreased (5.5-fold for *dbat* and 5.8-fold for *dbtnbt*) on day 2 and remained at approx. the same level until day 4.

Our results showed that the expression of the studied genes was significantly altered within the first two days after inoculation. In the forthcoming experiment we examined factors which could influence the gene expression at this time, particularly the transfer on nutrient fresh medium and the exposure to light during inoculation. The inocula were transferred under light and dark conditions on the *reused media*, which were already used in the previous subculture, and on the *fresh media* with a nutrient composition as defined in Materials and Methods. As shown in Fig. 3, the expression pattern of both genes was very similar under dark or light conditions during inoculation. The light did not significantly affect the expression pattern of both genes either on fresh or reused medium. In accordance with our preliminary results the level of *dbat* expression (Fig. 3) was increased on the first day after inoculation on fresh medium. On day 2 the expression of *dbat* markedly decreased to the initial level. Afterwards the expression was gradually decreasing. On the other hand, after inoculation on reused medium the variation of the *dbat* expression level was not so rapid. The expression gradually raised until day 2 followed by a slow decrease.

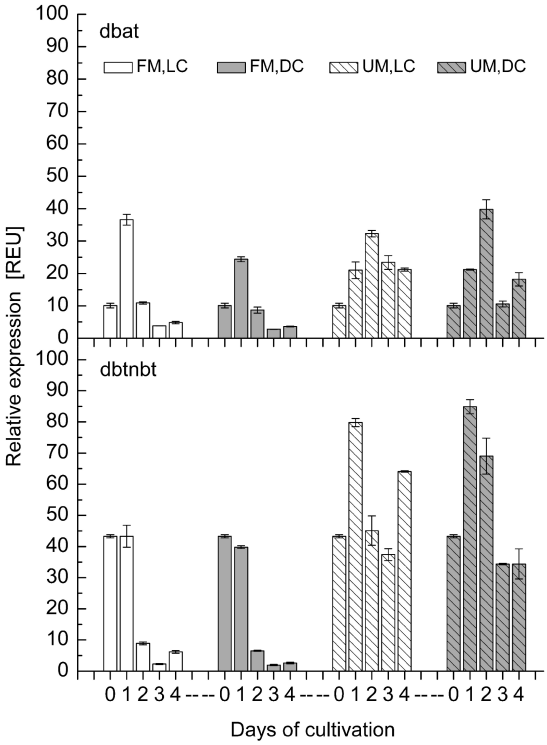

Fig. 3. Influence of different culture conditions on *dbat* and *dbntbt* expression during subculture of a VI/M2 callus culture. Expression data are mean values of 3 replicates ± standard deviation ($n=9$ cultivation containers sampled for each data point). FM, fresh nutrient medium; UM, used nutrient medium; LC, light condition during inoculation; DC, dark condition during inoculation.

In contrast to *dbat,* the expression of *dbtnbt* on day 1 after inoculation on fresh medium remained almost the same level as it was at the time of inoc-

ulation (Fig. 3). A rapid decrease was detected on day 2 and did not change until day 4. In contrast the inoculation on reused medium resulted in an increase in *dbtnbt* transcription on day 1 followed by a subsequent decrease until day 4.

Discussion

During the last several years serious attention has been devoted to the study of the paclitaxel biosynthetic pathway. The expression profile of several genes of the paclitaxel biosynthetic pathway in elicited *Taxus* cell cultures was investigated semiquantitatively via Northern blot analysis or RT-PCR (Tabata, 2006; Nims *et al*., 2006; Kai *et al*., 2005, 2006). Nowadays, there is still only little information about the intracellular accumulation of relevant metabolites in correlation to the expression of relevant genes in *Taxus* callus cultures at quantitative level.

For this purpose we employed qRT-PCR to quantify the gene expression of key enzymes of the paclitaxel biosynthesis. We have surveyed the expression of two genes coding for enzymes involved in later steps of the biosynthetic pathway during the growth of callus cultures. Furthermore, we have assessed the relation between gene expression and paclitaxel intracellular accumulation within a subculture.

Growth of callus in relation to taxane accumulation

During the 2-year period of cultivation both cultures remained unchanged with respect to growth rate, colour and degree of aggregation. Although both seedlings-derived cell cultures originated from the seeds of the same *T. baccata* L. individual, both cultures had different growth characteristics and paclitaxel accumulation profiles.

The kinetic study of biomass production confirmed a relatively slow growth rate of both callus cultures. The doubling time of 12 (VI/M1) and 18 days (VI/M2) was comparable with the other *Taxus* spp. (Wickremesinhe and Arteca, 1993; Fett-Neto and DiCosmo, 1997). The growth curves of both cultures were similar to other *Taxus* cell cultures as reported for *T.* × *media* (Wickremesinhe and Arteca, 1993).

In the paclitaxel accumulation profile a marked difference between both cultures was observed at the beginning and at the end of the growth cycle. Even though both cultures were transferred on fresh medium in the same phase of linear growth when the intracellular paclitaxel content was relatively low, a surprisingly high initial amount of paclitaxel was measured only in the VI/M1 culture. Similarly, a dramatic increase in paclitaxel accumulation observed in the late stationary phase in the VI/M1 culture was in contrast with decreasing paclitaxel content in the same growth period of the VI/M2 culture. The variability of paclitaxel production in *Taxus* spp. cell and organ cultures is well-known and also well documented. Numbers of factors causing the variation were identified including the differences in cell lines and their genetic stability (Ketchum *et al*., 1999; Ketchum and Gibson, 1996), the sensitivity of productivity to culture conditions (Hirasuna *et al*., 1996), the ability of the cells to accumulate the intermediates and the final products, the excretion of metabolites to the medium, *etc.*

An accumulation pattern with two peaks was also reported for suspensions of *T. cuspidata* by Fett-Neto and DiCosmo (1997) and Seki *et al.* (1997). The lack of paclitaxel increase at the end of the 64-day growth period of the VI/M2 culture could be caused by the onset of the progressive deceleration phase with a 21-day delay in comparison with the VI/M1 culture. The highly variable proportion of excreted to endogenously accumulated product depending on the species and cell line should also be taken into account (Fett-Neto and DiCosmo, 1997).

Paclitaxel is not a typical non-growth associated product like many other secondary metabolites; for its accumulation at least two maxima, the first in the phase of active growth and the second during the stationary phase, are typical (Srinivasan *et al*., 1995). According to our observations this can be explained by the fact that the expression level of the relevant *dbtnbt* gene was non-zero during all growth phases, including the phase of active growth.

Expression profile of dbat and dbtnbt genes

Although baccatin III was detected in the samples only in traces, qRT-PCR of *dbat* confirmed the presence of its gene transcripts in all stages but in lower quantities as compared to *dbtnbt* mRNA in the VI/M1 culture (Figs. 2a, b). Similarly, the levels of mRNAs coding for DBAT in unelicited *Taxus cuspidata* suspension cultures detected by RNA blot analysis were low as detected by Nims

et al. (2006) in a comparative study of the expression profile of several paclitaxel biosynthetic pathway genes. The mRNA transcripts for the DBAT enzyme that converts 10-DAB III to baccatin III, the last non-side-chain intermediate in the paclitaxel biosynthetic pathway, showed increased abundance on day 1, which was typical for all experimental conditions – fresh or reused medium, light or dark during inoculation (Fig. 3).

Interestingly, the initial increase in gene expression on day 1 was not so unambiguous for the *dbtnbt* gene, which is involved in one of the terminal steps in the paclitaxel pathway, when the side-chain is modified. The approx. 2-fold increase was observed exclusively after inoculation on the reused medium in both light and dark conditions.

There are several parameters which can affect the gene expression during inoculation: (i) the exposure to light (1–2 h) during the process of inoculation (the callus cultures are ordinarily cultivated in dark conditions); (ii) new nutrients supplied in fresh medium; (iii) presence/absence of metabolites excreted in the medium; (iv) wounding of the inoculum/callus surface during transferring to the fresh medium.

Assuming the gene expression profile within the first four days after inoculation, the expression of the studied genes was not affected by the inoculation process in light conditions. The increased abundance of mRNA transcripts for DBAT and DBTNBT enzymes was observed after inoculation on reused medium, suggesting the potential role of medium composition in the induction of gene expression. In comparison with the fresh medium, the reused medium differs in the nutrient concentration, secreted metabolites, *etc.* Therefore the increased level of gene expression can be the result of a major nutrient decrease, suggesting the diversion of precursors from primary to secondary metabolism (Srinivasan *et al*., 1995). On the other hand, some metabolites, including taxanes may inhibit the paclitaxel production and/or its secretion (Seki *et al*., 1997).

Gene expression and taxane accumulation

In relation to the expression pattern of the *dbtnbt* gene which was similar in both VI/M1 and VI/M2 cultures, the different behaviour of endogenous accumulation of paclitaxel in these cultures is still ambiguously and questioning. The differences in intracellular accumulation of paclitaxel in the cultures could also be related to the proportion of excreted to endogenously accumulated product which is highly variable depending on the *Taxus* species and the cell line (Fett-Neto and DiCosmo, 1997). The ability of *Taxus* cell cultures to release paclitaxel in the culture medium varies in the range of 10–90% (Hirasuna *et al*., 1996; Wickremesinhe and Arteca, 1993). Moreover, *Taxus* cell cultures in the active growth phase are able to release at least 55–60% of paclitaxel by means of active secretion rather than cell lysis which is common for later stages of the growth cycle (Fornale *et al*., 2002). The interconversion of the intermediates and alternative branches of the diterpene pathway in *Taxus* should be also taken into consideration (Laskaris *et al*., 1999).

Although *dbat* mRNA was detected in all stages of callus growth, the relatively low level of transcripts could explain why the relevant metabolite, baccatin III, was present in traces. For unelicited *Taxus* cell cultures the unmeasurable amount of non-side-chain as well as side-chain taxanes like baccatin III and paclitaxel is common (Nims *et al*., 2006).

Surprisingly the intracellular accumulation of paclitaxel did not correlate with transcriptional activity of the *dbtnbt* gene. The *dbtnbt* expression profile indicated that the enhancement of gene expression preceded the increase of the intracellular paclitaxel amount by at least 28 days (VI/M1) and 14 days (VI/M2). Likewise in the experiments provided by Nims *et al*. (2006), the transcripts of the *dbtnbt* gene did not persist in abundance until the late stationary phase, when the paclitaxel level was the highest. This suggests that although *dbtnbt* mRNA transcripts were at their minimum level, the enzyme activity may last much longer. On the other hand the RNA level may not reflect the level of corresponding protein and its activity (Gygi *et al*., 1999). It is interesting that while an inverse relationship is usually referred for growth and taxane accumulation (Fett-Neto and DiCosmo, 1997; Seki *et al*., 1997) the gene activity profile seems to be growth-associated.

Conclusion

In conclusion, (i) the presence of both mRNA transcripts was confirmed by qRT-PCR in all stages of the growth cycle of *T. baccata* callus cultures; (ii) the expression profile of *dbat* and *dbtnbt* was similar with an exception of the first 24 h after inoculation, when a highly elevated level of *dbat*

gene expression occurred; (iii) no evident relation was observed between the intracellular accumulation of paclitaxel and the steady-state of *dbtnbt* mRNA in a unelicited *T. baccata* callus culture.

The intracellular accumulation of paclitaxel is a complex process depending on the transcriptional activity of possibly all genes in the biosynthetic pathway, the availability of substrates, the interconversion of metabolites, the ability of the cells to accumulate the intermediates and the final product, the excretion of metabolites to the medium, *etc.* To improve the production capacity of yew cell cultures all factors should be considered. With respect to gene expression studies, the levels of gene transcript should be correlated with immediate and final product(s) of the respective biosynthetic pathway as well. In addition, more homogeneous experimental systems such as well defined cell lines enabling more exact approach to the determination of the accumulated/excreted amount of the product would bring more understanding to this complex process.

Acknowledgements

This work was supported by grant VEGA 1/1273/04. The authors would like to thank Mrs. Hana Michalčová for her technical assistance in taxane isolation and quantification.

Brown D. T. (2003), Preclinical and clinical studies of the taxanes. In: The Genus *Taxus* (Itokawa H. and Lee H. K., eds.). Taylor & Francis, London, pp. 387–435.

Bruňáková K., Babincová Z., and Čellárová E. (2004), Selection of callus cultures of *Taxus baccata* L. as a potential source of paclitaxel production. Eng. Life Sci. **5**, 465–469.

Bruňáková K., Babincová Z., and Čellárová E. (2005), Production of taxanes in callus and suspension cultures of *Taxus baccata* L. In: Liquid Culture Systems for *in vitro* Plant Propagation (Hvoslef-Eide A. K. and Preil W., eds.). Springer, Dordrecht, The Netherlands, pp. 567–574.

Cragg G. M., Schepartz S. A., Suffness M., and Grever M. R. (1993), The taxol supply crisis – new NCI policies for handling the large scale production of novel natural product anticancer and anti HIV agents. J. Nat. Prod. **56**, 1657–1668.

Croteau R., Ketchum R. E. B., Long R. M., Kaspera R., and Wildung M. R. (2006), Taxol biosynthesis and molecular genetics. Phytochem. Rev. **5**, 75–97.

DeJong J., Liu Y., Bollon A., Long R., Jennewein S., Williams D., and Croteau R. (2006), Genetic engineering of taxol biosynthetic genes in *Saccharomyces cerevisiae*. Biotechnol. Bioeng. **93**, 212–224.

Fett-Neto A. G. and DiCosmo F. (1997), Taxol and taxane production by cell culture. In: Encyclopedia of Molecular Biology and Molecular Medicine, Vol. 6 (Meyers R. A., ed.). VCH, Weinheim, pp. 10–17.

Fornale S., Esposti D. D., Navia-Osorio A., Cusido R. M., Palazon J., Pinol M. T., and Bagni N. (2002), Taxol transport in *Taxus baccata* cell suspension cultures. Plant Physiol. Biochem. **40**, 81–88.

Gamborg O. L., Miller R. A., and Ojima K. (1968), Nutrient requirements of suspension cultures of soybean root cell. Exp. Cell Res. **50**, 151–158.

Guo B. H., Kai G. Y., Jin H. B., and Tang K. X. (2006), Taxol synthesis. Afr. J. Biotechnol. **5**, 015–020.

Gygi S. P., Rochon Y., Franza B. R., and Aebersold R. (1999), Correlation between protein and mRNA abundance in yeast. Mol. Cell Biol. **19**, 1720–1730.

Hefner J., Ketchum R. E. B., and Croteau R. (1998), Cloning and functional expression of a cDNA encoding geranylgeranyl diphosphate synthase from *Taxus canadensis* and assessment of the role of this prenyltransferase in cells induced for taxol production. Arch. Biochem. Biophys. **360**, 62–74.

Hezari M. and Croteau R. (1997), Taxol biosynthesis: an update. Planta Med. **63**, 291–295.

Hirasuna T. J., Pestchanker L. J., Srinivasan V., and Shuler M. L. (1996), Taxol production in suspension cultures of *Taxus baccata*. Plant Cell Tiss. Org. Cult. **44**, 95–102.

Jennewein S. and Croteau R. (2001), Taxol: biosynthesis, molecular genetics, and biotechnological applications. Appl. Microbiol. Biotechnol. **57**, 13–19.

Jennewein S., Wildung M. R., Chau M., Walker K., and Croteau R. (2004), Random sequencing of an induced *Taxus* cell cDNA library for identification of clones involved in taxol biosynthesis. Proc. Natl. Acad. Sci. USA **101**, 9149–9154.

Kai G., Zhao L., Zhang L., Li Z., Guo B., Zhao D., Sun X., Miao Z., and Tang K. (2005), Characterization and expression profile analysis of a new cDNA encoding taxadiene synthase from *Taxus media*. J. Biochem. Mol. Biol. **38**, 668–675.

Kai G., Miao Z., Zhang L., Zhao D., Liao Z., Sun X., Zhao L., and Tang K. (2006), Molecular cloning and expression analyses of a new gene encoding 3-hydroxy-3-methylglutaryl-CoA synthase from *Taxus* × *media*. Biol. Plant. **50**, 359–366.

Kelsey R. G. and Vance N. C. (1992), Taxol and cephalomannine concentrations in the foliage and bark of shade-grown and sun-exposed *Taxus brevifolia* trees. J. Nat. Prod. **55**, 912–917.

Ketchum R. E. B. and Gibson D. M. (1996), Paclitaxel production in suspension cell cultures of *Taxus*. Plant Cell Tiss. Org. Cult. **46**, 9–16.

Ketchum R. E. B., Gibson D. M., Croteau R. B., and Shuler M. L. (1999), The kinetics of taxoid accumulation in cell suspension cultures of *Taxus* following elic-

itation with methyl jasmonate. Biotechnol. Bioeng. **62**, 97–105.

Ketchum R. E. B., Wherland L., and Croteau R. B. (2007), Stable transformation and long-term maintenance of transgenic *Taxus* cell suspension cultures. Plant Cell Rep. **26**, 1025–1033.

Laskaris G., Bounkhay M., Theodoridis G., Van Der Heijden R., Verpoorte R., and Jaziri M. (1999), Induction of geranylgeranyl diphosphate synthase activity and taxane accumulation in *Taxus baccata* cell cultures after elicitation by methyl jasmonate. Plant Sci. **147**, 1–8.

Nadeem M., Rikhari H. C., Kumar A., Palni L. M. S., and Nandi S. K. (2002), Taxol content in the bark of Himalayan yew in relation to tree age and sex. Phytochemistry **60**, 627–631.

Nims E., Dubois C. P., Roberts S. C., and Walker E. L. (2006), Expression profiling of genes involved in paclitaxel biosynthesis for targeted metabolic engineering. Metab. Eng. **8**, 385–394.

Rao K. V. (1993), Taxol and related taxanes. I. Taxanes of *Taxus brevifolia* bark. Pharm. Res. **10**, 521–524.

Rowinsky E. K., Donehower R. C., Spivak J. L., Burke P. J., Griffin C. A., and Jones R. J. (1990), Effects of the differentiating agent hexamethylene bisacetamide on normal and myelodysplastic hematopoietic progenitors. J. Natl. Cancer Inst. **82**, 1926–1931.

Schiff P. B., Fant J., and Horwitz S. B. (1979), Promotion of microtubule assembly *in vitro* by taxol. Nature **277**, 665–667.

Seki M., Ohzora C., and Furusaki S. (1997), Taxol (paclitaxel) production using free and immobilized cells of *Taxus cuspidata*. Biotechnol. Bioeng. **53**, 214–219.

Sledge G. W. (2003), Gemcitabine combined with paclitaxel or paclitaxel/trastuzumab in metastatic breast cancer. Semin. Oncol. **30**, 19–21.

Srinivasan V., Pestchanker L., Moser S., Hirasuna T. J., Taticek R. A., and Shuler M. L. (1995), Taxol production in bioreactors – kinetics of biomass accumulation, nutrient-uptake, and taxol production by cell suspensions of *Taxus baccata*. Biotechnol. Bioeng. **47**, 666–676.

Tabata H. (2006), Production of paclitaxel and the related taxanes by cell suspension cultures of *Taxus* species. Curr. Drug Targets **7**, 453–461.

Vidensek N., Lim P., Campbell A., and Carlson C. (1990), Taxol content in bark, wood, root, leaf, twig and seedling from several *Taxus* species. J. Nat. Prod. **53**, 1609–1610.

Walker K. and Croteau R. (2000), Molecular cloning of a 10-deacetylbaccatin III-10-*O*-acetyl transferase cDNA from *Taxus* and functional expression in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **97**, 583–587.

Walker K., Long R., and Croteau R. (2002), The final acylation step in taxol biosynthesis: Cloning of the taxoid C13-side-chain *N*-benzoyltransferase from *Taxus*. Proc. Natl. Acad. Sci. USA **99**, 9166–9171.

Wickremesinhe E. R. M. and Arteca R. N. (1993), *Taxus* callus cultures. Initiation, growth optimization characterization and taxol production. Plant Cell Tiss. Org. Cult. **35**, 181–193.

Xu F., Tao W. Y., Cheng L., and Guo L. J. (2006), Strain improvement and optimization of the media of taxol producing fungus *Fusarium maire*. Biochem. Eng. J. **31**, 67–73.