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Expression profiling of genes involved in paclitaxel biosynthesis for targeted metabolic engineering

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Abstract

Taxus plant suspension cell cultures provide a sustainable source of paclitaxel (Taxol®) for the treatment of many cancers. To develop an optimal bioprocess for paclitaxel supply, taxane biosynthetic pathway regulation must be better understood. Here we examine the expression profile of paclitaxel biosynthetic pathway genes by RNA gel blot analysis and RT-PCR in the *Taxus cuspidata* cell line P991 and compare with taxane metabolite levels. Upon methyl jasmonate (MJ) elicitation (100 μM), paclitaxel accumulates to 3.3 mg/L and cephalomannine to 2.2 mg/L 7 days after elicitation but neither are observed before this time. 10-deacetylbaccatin III accumulates to 3.3 mg/L and baccatin III to 1.2 mg/L by day 7 after elicitation. The early pathway enzyme genes *GGPPS*, *TASY*, and *T5αH* are up-regulated by MJ elicitation within 6 h and continue through 24 h before their abundances decrease. This study reveals the preference for one side of the biosynthetic pathway branch in early taxane synthesis, where transcripts coding for TαH are abundant after elicitation with MJ but transcripts encoding the two enzymes for the alternative branch (*TDAT* and *T10βH*) are not highly expressed following elicitation. Transcripts encoding the enzymes DBBT and DBAT are up-regulated upon MJ elicitation. Their products, 10-deacetylbaccatin III and baccatin III, respectively, accumulate within 6 h of the initial increase in transcript abundance. Importantly, the steady-state levels of the two terminal enzyme transcripts (*BAPT* and *DBTNBT*) are much lower than transcripts of early pathway steps. These are potential steps in the pathway for targeted metabolic engineering to increase accumulation of paclitaxel in suspension cell culture.

Keywords: Taxane; Paclitaxel; Taxol; Pathway regulation; Methyl jasmonate; *Taxus*; Cell culture; Plant

1. Introduction

The gymnosperm genus *Taxus* produces the anti-cancer agent paclitaxel (Taxol®, Bristol-Myers Squibb), which is a member of the taxane family of highly functionalized diterpenoids produced by all species of yew. Current FDA-approved uses of the drug include the treatment of ovarian, breast, and lung cancers as well as AIDS-related Kaposi's sarcoma, with over 120 clinical trials underway to test the efficacy of paclitaxel against different cancers and in combination treatments with other chemotherapeutic agents (www.nih.gov). Paclitaxel supply is largely met through a semi-synthetic process whereby a paclitaxel precursor, 10-deacetylbaccatin III, is harvested from yew needles and converted to paclitaxel via chemical synthesis. The plant cell culture process for paclitaxel supply was recently approved by the FDA (www.fda.gov) and is currently being utilized to supply paclitaxel; hence, cell culture is expected to contribute significantly to future paclitaxel supply demands. *Taxus* cell cultures produce paclitaxel and its precursors without forest harvestation and therefore provide a sustainable source of this important secondary metabolite.

Plant cell suspensions are often preferable to callus or organ cultures for commercial applications, as they can be

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easily adapted to existing bioprocess technology and utilized for large-scale production processes. However, low natural yields and selectivity over unwanted by-products complicate bioprocess design. Additionally, cell cultures display a large degree of heterogeneity in secondary metabolite production capabilities. There have been few reports on this variability in plant cell cultures, particularly the long-term stability of cell suspensions to maintain high levels of productivity (Kim et al., 2004). Taxane accumulation in *Taxus* suspensions has been shown to be highly variable between varieties and over time in a single cell line (Ketchum and Gibson, 1996). Recently, work has been undertaken to characterize *Taxus* suspension populations at the single-cell level in terms of growth, taxane accumulation and protein production (Roberts et al., 2003; Naill and Roberts, 2004, 2005a–c) to suggest stabilization strategies. To design an optimal bioprocess for paclitaxel supply, an understanding of taxane biosynthetic pathway regulation is also necessary. Targeted metabolic engineering strategies can be applied once key genes have been identified.

The taxane biosynthetic pathway is not fully characterized; however, multiple steps have been elucidated using directed cloning methods (Croteau et al., 1995) (Fig. 1). Derived from the isoprenoid precursors isoprenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), geranylgeranyl pyrophosphate (GGPP) is synthesized by geranylgeranyl pyrophosphate synthase (GGPPS) (Hefner et al., 1998). GGPP is converted to taxa 4(5), 11(12)-diene by taxadiene synthase (TASY) (Wildung and Croteau, 1996) and then to taxa 4(20), 11(12)-dien-5α-ol by taxadiene 5α hydroxylase (T5αH) (Jennewein et al., 2004a). At this point, a branch in the metabolic pathway occurs. Taxadiene 13α-hydroxylase (TαH) converts taxa 4(20), 11(12)-dien-5α-ol to taxa 4(20), 11(12)-diene 5α, 13α-diol (Jennewein et al., 2001). The alternative branch in the pathway implements taxadiene 5α-ol *O*-acetyltransferase (TDAT) (Walker et al., 1999) to form taxa 4(20), 11(12)-diene-5α-yl acetate from taxa 4(20), 11(12)-dien-5α-ol, which is then further converted to taxa 4(20), 11(12) diene 5α-acetoxy-10*β*-ol by taxane 10*β*-hydroxylase (T10*β*H) (Schoendorf et al., 2001). The steps leading from the acetate or the diol intermediates to functionalized taxanes are unknown. Highly modified taxane production occurs

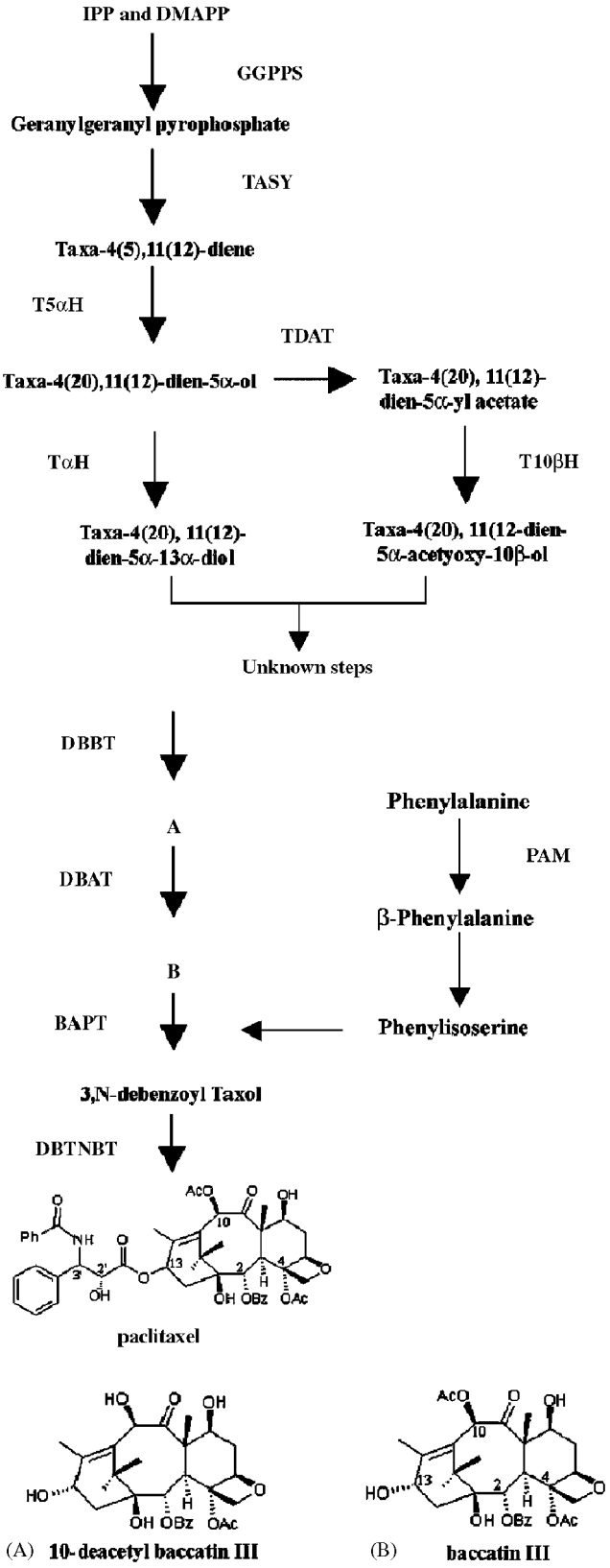

Fig. 1. Paclitaxel biosynthetic pathway. Arrows and abbreviations indicate enzymes: GGPPS: geranylgeranyl pyrophosphate synthase; TASY: taxadiene synthase; T5αH: taxadiene 5α hydroxylase; TDAT: taxadiene 5α-ol O-acetyltransferase; T10*β*H: taxane 10*β*-hydroxylase; TαH taxadiene 13α-hydroxylase; DBBT: taxane 2α-*O*-benzoyltransferase; DBAT: 10-deacetylbaccatin III-10-*O*-acetyltransferase; PAM: phenylalanine aminomutase; BAPT: baccatin III: 3-amino, 3-phenylpropanoyltransferase; DBTNBT: 3′-*N*-debenzoyl-2-deoxytaxol-*N*-benzoyltransferase. Key intermediate taxanes measured by HPLC are shown as **A** (10-deacetyl baccatin III) and **B** (baccatin III). Cephalomannine is similar to paclitaxel except for a substitution at the 2-phe position on the side chain. The many unknown enzymes within the pathway are indicated as 'unknown steps'.

via taxane 2α-*O*-benzoyltransferase (DBBT) (Walker and Croteau, 2000a) to produce 10-deacetylbaccatin III (10-DAB). 10-DAB is then converted to baccatin III by 10-deacetylbaccatin III-10-*O*-acetyltransferase (DBAT) (Walker and Croteau, 2000b). Baccatin III: 3-amino, 3-phenylpropanoyltransferase (BAPT) (Walker et al., 2002b) ligates phenylisoserine (derived from phenylalanine via phenylalanine aminomutase (PAM) (Jennewein et al., 2004b)) to baccatin III to produce 3′-*N*-debenzoyl-2-deoxytaxol. 3′-*N*-debenzoyl-2-deoxytaxol-*N*-benzoyltransferase (DBTNBT) ligates a benzoyl CoA group to 3′-*N*-debenzoyl-2-deoxytaxol to produce 2′-deoxytaxol (Walker et al., 2002a). Finally, benzamidation of 2′-deoxytaxol yields paclitaxel. It is presumed that additional side-chain containing taxanes are synthesized after the ligation; however this has not been confirmed experimentally.

Biotic elicitors are commonly used to stimulate production of secondary metabolites in cell culture systems. In particular, jasmonic acid or its methyl ester have been effective at enhancing paclitaxel accumulation in plant cell culture (Mirjalili and Linden, 1996; Yukimune et al., 1996; Ketchum et al., 1999; Dong and Zhong, 2001). Methyl jasmonate (MJ) specifically induces the up-regulation of secondary metabolic genes involved in stress, wounding and pathogen ingress (Reymond et al., 2004). Conversely, primary metabolic genes such as those involved in photosynthesis, electron transport and cytoskeletal organization are down-regulated or unaffected by MJ elicitation (Hermsmeier et al., 2001). In poppy cell suspension cultures, the response to elicitor treatment has been shown to occur at the level of mRNA within 1 h, with tight correlation to accumulation of the correponding metabolite (Facchini and Park, 2003); however, for *Taxus*, this response to MJ has only been demonstrated at the level of transcript for the first two committed steps in the pathway (Hefner et al., 1998) with no correlation to metabolite accumulation performed.

In this paper, we examine regulation of the taxane biosynthetic pathway using the MJ-responsive, well-characterized P991 *T. cuspidata* cell line. MJ was used to induce taxane synthesis and expression of 11 known pathway genes was examined via RNA gel blot analysis and semi-quantitative RT-PCR. Key nonside-chain and side-chain taxanes were quantified via HPLC. Unelicited cultures produce low levels of nonside-chain taxanes and no paclitaxel or other side-chain taxanes while MJ-elicited cultures accumulate both taxane classes. We demonstrate that regulation of the taxane biosynthetic pathway occurs at the level of mRNA and that there is a tight correlation between steady-state transcript abundance and respective taxane accumulation. We have identified specific pathway bottlenecks to be targeted for metabolic engineering that can potentially increase paclitaxel accumulation. Additionally, we provide new insight into the highly variable nature of taxane accumulation and the lack of paclitaxel accumulation in unelicited cultures. These data can ultimately be used to design superior cell culture processes with enhanced paclitaxel yield.

2. Materials and methods

2.1. Cell culture

The P991 *Taxus cuspidata* cell line was used for all experiments and obtained as a gift from Dr. D. Gibson at the US Plant Soil and Nutrition laboratory, USDA (Ithaca, NY, USA). Suspensions were subcultured every 2 weeks into Gamborg's B5 (Sigma, St. Louis, MO, USA) basal salts (3.2 g/L) with 20 g/L sucrose, supplemented with 2.7 μmol/L naphthalene acetic acid (NAA) and 0.01 μmol/L benzyladenine (BA). Ascorbic acid (156 mg/L), citric acid (156 mg/L) and glutamine (906 mg/L) were filter-sterilized (Millipore Millex 0.2 μm syringe filters) and added post-autoclaving. Cultures were maintained in 125-ml Erlenmeyer flasks capped with Bellco (Vineland, NJ, USA) foam closures at 24 °C and shaking at 125 rpm in the dark.

Cells transfers were accomplished through the addition of 10 ml of 14-day-old suspension cultures into 40 ml of fresh medium. The approximate packed volume of cells transferred was at least 2 ml to maintain optimum culture density. For some experiments, cells were cultured on a larger scale in 500-ml Erlenmeyer flasks with comparable inoculation densities.

2.2. RNA purification

Cells were collected by filtration from the media through Miracloth® (EMD Biosciences, San Diego, CA, USA), and stored at −80 °C in polypropylene tubes. RNA was extracted using the Plant RNA Purification Reagent (Invitrogen, Carlsbad, CA, USA), followed by DNase treatment with the DNA-*free* kit (Ambion, Austin, TX, USA). Quantification of total RNA was performed with the Ribogreen fluorometric assay (Molecular Probes, Eugene, OR, USA).

2.3. RNA gel blot analysis

About 20 μg total RNA was used in each lane in all RNA gel blots shown. Each RNA sample was glyoxalated before nondenaturing agarose gel electrophoresis with 6% glyoxal (Fluka, Ronkonkoma, NY, USA), 50% DMSO (Sigma) and 5 μM sodium phosphate in 20 μl total reaction volume for 30 min at 50 °C. Molecular weight markers (Fermentas, Hanover, MD, USA) were also glyoxalated and loaded onto each gel. Nucleic acids were transferred to Zetaprobe membranes (BioRad, Hercules, CA, USA) via capillary transfer (Sambrook, 1989) using 10× Sodium Chloride/Sodium Citrate (SSC) as transfer buffer. Staining of gels with Ethidium Bromide (EtBr) before and after transfer demonstrated equal loading and complete transfer to the membrane.

Hybridization conditions were as follows: pre-hybridization of the membranes occurred for 6 h in 10% dextran sulfate (average molecular weight of 500,000 kDa; G.E. Health, Piscataway, NJ, USA), 5.5× Sodium Citrate/

Sodium Phosphate (SCP), 0.9% *n*-lauroyl sulfate-sodium salt and 0.5 mg/ml heparin. Probes were labeled with α-^{32}P dCTP (G.E. Health) using DNA polymerase I large (Klenow) fragment (Promega, Madison, WI, USA). Hybridization conditions were 65 °C for 15 h, followed by washing twice with 2 × SCP, 2% sodium dodecyl sulfate (SDS) and then twice using 0.2 × SCP and 0.2% SDS. Probes were stripped off of blots up to three times using 0.1% SDS at 100 °C. Blots were checked for residual hybridization by exposing to film for 24 h before re-hybridizing with a different probe.

2.4. Probe creation

Probes for RNA gel blot experiments were created by amplifying gene fragments from *T. cuspidata* genomic DNA. Primers were designed from previously cloned cDNAs in regions conserved amongst *T. canadensis*, *T. brevifolia*, and *T. cuspidata*, and are listed in Table 1. Amplification of fragments via polymerase chain reaction (PCR) was performed using Expand High Fidelity Polymerase System (Roche, Indianapolis, IN, USA). PCR conditions were 95 °C for 2 min followed by 15 ×: 15 s, 95 °C; 30 s, 63 °C; 5 min, 68 °C. This was then followed by 25 ×: 15 s, 95 °C; 30 s, 63 °C; 5 min + 20 s per cycle, 68 °C.

2.5. RT-PCR

First strand cDNA was synthesized using Superscript™ II Reverse Transcriptase (RT) (Invitrogen) from 5 μg total RNA. For each reaction, 1/50th of the RT reaction was used as template for PCR. PCR conditions were as follows: 95 °C for 2 min followed by 95 °C, 15 s; 63 °C, 30 s, 68 °C, 2.5 min. The numbers of cycles used to amplify each fragment was variable so that each was in the linear range of amplification when analyzed. Linear amplification was determined by testing 20 and 25 cycles then choosing which of these showed the lowest detectable amount of PCR product.

2.6. Taxane extraction from culture samples

Taxanes were extracted from total culture (cells and media) samples. Total culture samples were collected via micropipetting with a cut pipette tip, where a 1000 μl capacity tip was trimmed approximately 0.5 in from the end, to allow for the withdrawal of a 1000 μl sample containing both cells and medium in the same proportion as in the original culture. The use of a cut pipette tip does not influence either the total volume or the dry weight of the sample (data not shown).

After samples were thawed at room temperature, medium was evaporated on a SpeedVac® Plus (Savant, Holbrook, NY, USA) overnight. Pellets were then resuspended in 1 ml of acidified methanol (0.01% glacial acetic acid), and sonicated for 1 h in a VWR Aquasonic® sonication bath. Samples were subsequently centrifuged at 10,000*g* for 25 min using an Eppendorf™ centrifuge (Brinkmann Instruments, Wetbury, NY, USA). Supernatants were recovered and dried down under an air stream

Table 1
Primers used to amplify gene fragments from *T. cuspidata*

Gene	GenBank accession	Primer name	Primer sequence	Expected MW (bp)
GGPPS	AF081514	oTcGGPPS 791–817	GCCATGCCAACTGCCTGTGCAATGGA	688
		oTcGGPPS 1479–1450	CTGAATGCAATGTAATCTGCAAGACCCAAC	
TASY	U48796	oTcTASY 25–48	AATGCAGCGCTGAAGATGAATGCA	608
		oTcTASY 633–610	TTGGCTGTGCCCTGTTTTCCAAAC	
TαH	AY056019	OTcTαH 533–558	GGTAAAAGACCTCGTCTTCTCCGTCG	310
		OTcTαH 843–820	TCTCCTTGTCCGCCAGTGAATTCC	
TDAT	AF190130	oTcTDAT 1100–1124	GGAGCCGATTGGGATTTGATGAAGT	257
		oTcTDAT 1357–1332	AAAATACAAGCCTTCCATCGTTGCAC	
T5αH	AY289209	oTcT5αH 1–25	GGCACCAGGTTTTCTGCTCCTGCTT	218
		oTcT5αH 218–194	AGGGATGCCTAATTTCCCAGGAGGA	
T10βH	AF318211	oTcT10βH 36–61	TTCATCTTGCAGCACATGTACTCCCA	301
		oTcT10βH 337–312	TTGAGGTGTTTCTGATCGGAGTGTCC	
DBBT	AF297618	oTcDBBT 905–930	TGCTATTGGTAATGCATGTGCAATGG	377
		oTcDBBT 1282–1257	TTGAATGGTTTCACCATTGATGCAGG	
DBAT	AF193765	oTcDBAT 1109–1131	CGAAGGCGATTGGGATTTGATGA	302
		oTcDBAT 1411–1386	ATTACTTCAACCACAACCCAGTGCCA	
PAM	AY582743	oTcPAM 1–23	CTCTCATATGGGGTTTGCCGTGG	2190 353 (RT)
		oTcPAM 1837..1857	GGCAGACAACAACGACGCCCT	
		oTcPAM 2190..2165	AGCTACAGTCGCTTCTGCGGAATTTC	
BAPT	AY082804	oTcBAPT 470–494	GGGAGCGAATGTGTATGGTAGTGCA	277
		oTcBAPT 747–723	TTTCTTTGCGTTCTTCCATGATGCG	
DBTNBT	AF466397	oTcDBTNBT 938–965	CCTCTTAAATGGATCTCTTTTGCGTGCT	325
		oTcDBTNBT 1263–1236	TTGTTGGTGGCATACAAGACAGTAGC	
rRNA	AF259290	oTcrRNA 598..580	GGGTGTCCCCGCCCCGGAG	544
		oTcrRNA 54..73	GGGCGTGTTCGGCACGTCCG	

using an Evap-o-rac (Cole Parmer, Vernon Hills, IL, USA). The remaining pellets were resuspended again in 1 ml acidified methanol, sonicated for another hour and centrifuged for 25 min at 10,000*g*. Supernatants were combined, and dried down under an air stream. Dried samples were then resuspended in 100 µl of acidified methanol, sonicated for 30 min, and centrifuged at 10,000*g* for 25 min. Supernatants were filtered through a 0.2 µm PVDF filter into 0.30 ml polypropylene vials (Wheaton, Millville, NJ, USA) capped with PTFE seals liner (Kimble, Vineland, NJ, USA) for HPLC analysis.

2.7. Taxane quantification via HPLC

The HPLC system consisted of a Waters (Milford, MA, USA) Alliance 2690 separation module with 996 photodiode array detector. Data acquisition, processing and equipment control was performed using either Waters Millennium version 3.2 or Empower version 5.0 software. All taxanes were identified and quantified using HPLC in combination with authentic taxane standards (Sigma). Identification of unknown peaks was based upon retention time and UV absorption spectral comparison with known standards. For each separate analysis, fresh standards were prepared and analyzed along with the unknown samples. A 13-mixture taxane standard (InB: Hauser CRO, Boulder CO, USA) was run in parallel to identify other putative taxanes present in the cultures.

Separation was accomplished on a Metachem-Varian Taxsil®, 250 mm × 4.6 mm × 5 µm (Torrance, CA, USA) column equipped with a guard cartridge (Metachem). The mobile phase was acetonitrile and water (52:48) at a flow rate of 1 ml/min. Each sample was analyzed for 25 min. For all analyses, the photodiode array detector scanned from 200 to 400 nm, with peak detection at 228 nm. Paclitaxel, as with most taxanes, has a characteristic absorption peak at 228 nm.

3. Results and discussion

3.1. Gene fragment amplification

Amplification of gene fragments from genomic *T. cuspidata* DNA resulted in amplicons of the predicted molecular weights (no introns were present). Primers used to amplify the probes are listed in Table 1. Fragments were cloned into pTZ19u by blunt-end cloning and sequenced prior to use as probes. Sequences were compared to previously cloned sequences available in GenBank® and show perfect or near perfect homology to previously published *Taxus* sequences: *TASY*: 99%; 606/608 nt to AF326519, *TDAT*: 100%; 257/257 nt to AF190130, *T5αH*: 99%; 216/218 nt to AY289209, *TαH*: 99%; 310/309 nt to AY866412, *T10βH*: 100%; 301/301 nt to AY453403, *DBBT*: 100%; 377/377 nt to AF297618, *DBAT*: 100%; 302/302 nt to AF193765, *BAPT*: 99%; 276/277 nt to AY082804, *DBTNBT*: 98% 321/325 nt to AF466397. The *PAM* gene fragment was not fully sequenced, but showed 98% similarity in the 5′ end; 361/356 nt to AY724736, and 97% similarity to the 3′ end; 268/274 nt of AY724736. No *GGPPS* sequence has previously been reported for *Taxus*. The *GGPPS* clone generated here is most similar to a *GGPPS* from *Abies grandis*: 84%; 197/234 nt to AF513111. The strong nucleotide sequence identity observed between the PCR probes and the published sequences establishes that amplification of the expected fragment was achieved in each case.

3.2. Time course of mRNA accumulation

In preliminary experiments, a subset of probes (*GGPPS*; not shown, *TDAT*; not shown, *DBAT* and *DBAT*) was used to establish a time course for mRNA accumulation of taxane biosynthetic genes. As shown in Fig. 2, mRNA levels of *DBBT* and *DBAT* increased upon MJ elicitation, reaching maximal levels at 12–24 h, declining to undetectable levels by 72–96 h. These initial studies indicate that MJ elicitation results in transient up-regulation of the steady-state levels of mRNA encoding taxane biosynthetic genes during the first 24 h following elicitation. Based on these data, all subsequent studies examined gene expression until 30 h. Because taxane accumulation is first detectable anywhere from 24 to 96 h after MJ elicitation (Mirjalili and Linden, 1996; Yukimune et al., 1996; Ketchum et al., 1999), with maximal levels achieved 2-15D post-elicitation, taxane quantification was performed up to 7 days in culture. Below, we present mRNA expression profiles in conjunction with metabolite data for all 11 known paclitaxel biosynthetic pathway genes in cultures with elevated taxane levels induced by MJ elicitation. Note that we show data from a large-scale experiment (i.e., all genes over a complete time course). Numerous smaller scale studies (e.g., subset of genes, shorter time courses) were conducted and profiles agree with presented results (data not shown).

3.3. Taxane accumulation

As seen in Fig. 3, unelicited cultures accumulate 10-DAB at maximal levels of 0.4–0.8 mg/l, approximately four-fold less than that observed in MJ-elicited cultures. Neither the

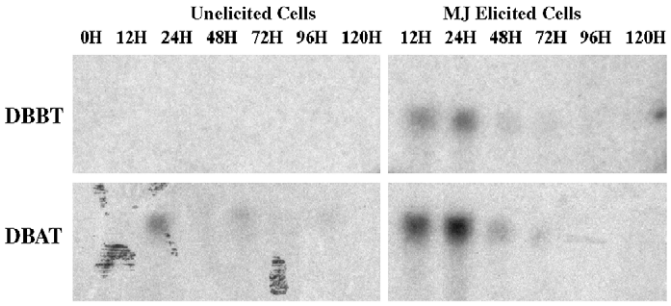

Fig. 2. Time course for mRNA accumulation. RNA gel blot analysis of total RNA extracted from cell cultures at indicated time points. Cultures were elicited with 100 µM methyl jasmonate on day 7 posttransfer and sampled at the time points represented in the figure. Time points after elicitation are indicated above each lane. The probe used for each blot is indicated at the left. About 20 µg RNA were loaded onto each lane.

nonside-chain taxane baccatin III, nor the side-chain taxanes paclitaxel and cephalomannine, could be detected at measurable amounts in unelicited cultures over the entire 7-day time course examined.

In MJ-elicited cultures, both nonside-chain and side-chain taxane accumulation is induced. 10-DAB accumulation increases after 24 h, with a maximum value of 3.30 mg/l measured at 168 h (7 days after elicitation). Baccatin III accumulation is evident at 12 h with a maximum value of 1.24 mg/l achieved at 168 h. Baccatin III levels are 2.7-fold lower than 10-DAB levels. Paclitaxel and cephalomannine are not detected at early time points, but accumulate to 3.34 and 2.18 mg/l, respectively, by 168 h. Further experiments demonstrate that side-chain taxanes first appear in culture as early as 48 h after elicitation (data not shown). Several additional minor taxane peaks were observed via HPLC analysis, including 7-xylosylpaclitaxel and paclitaxel C (data not shown). Through comparison of peak area sizes, 7-xylosylpaclitaxel and paclitaxel C levels followed the typical side-chain taxane pattern, reaching maximum accumulation at 168 h.

3.4. Early pathway regulation

Within the first 6 h after MJ addition, *GGPPS*, *TASY*, and *T5αH* show increases in steady-state transcript abundance (Fig. 4A); however, different temporal patterns of expression after MJ elicitation are observed. *GGPPS* and *TASY* transcripts are most abundant 18 h after elicitation and *T5αH* is most highly expressed at 6 h with a decline in transcript abundance at later time points (Fig. 4A). In unelicited cell cultures, *GGPPS*, *TASY*, and *T5αH* show little (*GGPPS*) or no (*TASY* and *T5αH*) detectable transcript accumulation (Fig. 4A). *GGPPS* is a noncommitted paclitaxel biosynthetic gene, and as such, is expected to be present without elicitation.

To investigate expression further, RT-PCR was performed on RNA from the culture samples that had been elicited for 6 and 18 h. Using this more sensitive technique, steady-state *TASY* transcript levels are greater in MJ-elicited cultures compared to unelicited cultures; however, *TASY* mRNA is present in both elicited and unelicited states (Fig. 4C). Transcripts encoding TASY are not apparent in the unelicited cultures when using RNA gel blot analysis. These data confirm that the three early taxane biosynthetic genes, *GGPPS*, *TASY*, and *T5αH*, are induced by MJ elicitation, but are also present at low levels without MJ elicitation, which explains the low levels of the nonside-chain taxane, 10-DAB, present in unelicited cultures.

3.5. Branch regulation

There is a branch in the pathway in which taxa 4(20), 11(12)-dien-5α-ol can be modified by either TαH or separately by TDAT followed by T10βH. To determine if there was a preference towards one side of the branch upon MJ elicitation, the transcript profiles of three known relevant genes were examined. *TDAT* and *T10βH* are not detected by RNA gel blot analysis in either unelicited or MJ-elicited cultures (Fig. 4B). By contrast, the other side of the branch, *TαH*, shows an increase in transcript abundance by RNA gel blot analysis within 6 h and extending through 18 h (Fig. 4B). RT-PCR analysis of TαH transcripts is in close agreement with RNA gel blot analysis, showing increased PCR product at 6 and 18 h compared to the unelicited cultures (Figs. 4C and D). RT-PCR was used to determine whether gene expression on the TDAT/T10βH side of the pathway occurs at low levels. Expression of *TDAT* is detectable only by RT-PCR in 6 h MJ-elicited cultures (Figs. 4C and D). *T10βH* expression is

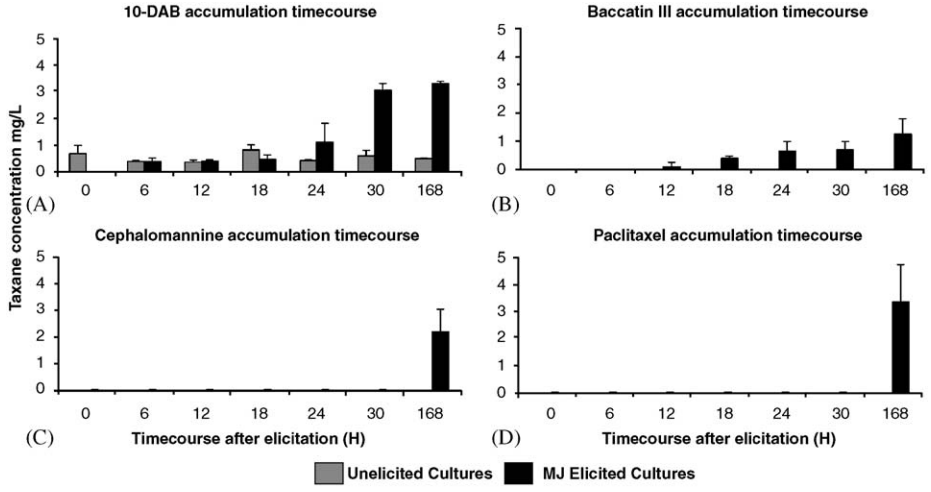

Fig. 3. Taxane profiles in unelicited and methyl jasmonate-elicited *Taxus* cultures. Concentration of taxanes in *Taxus cuspidata* suspension cultures as measured via RP-HPLC, including **A**: 10-deacetylbaccatin III, **B**: baccatin III, **C**: cephalomannine, and **D**: paclitaxel. Cultures were elicited with 100 μM methyl jasmonate on day 7 posttransfer and sampled at the time points represented in the figure. Reported values are the average of three replicate samples and error bars represent one standard deviation.

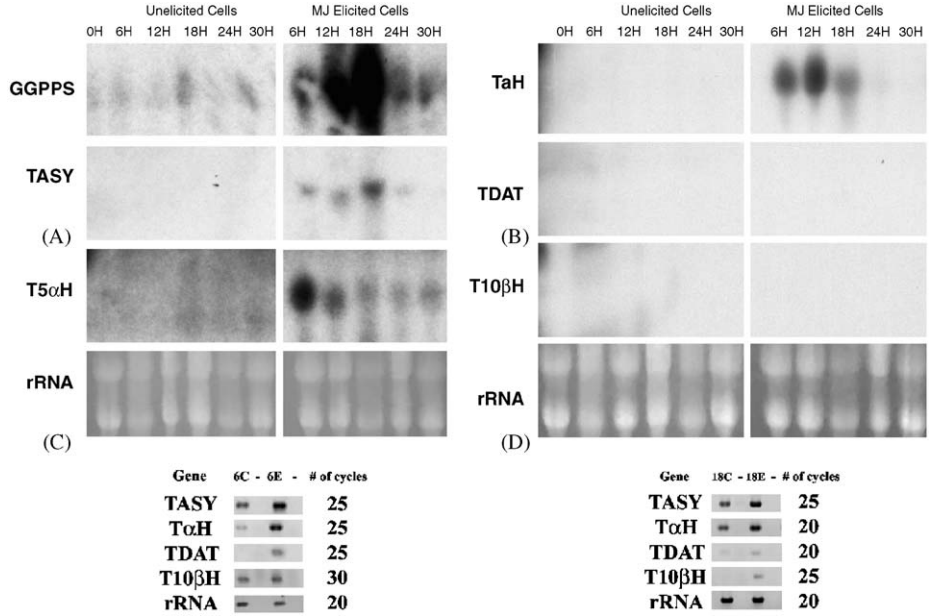

Fig. 4. Early pathway and branch regulation: RNA gel blot analysis and RT-PCR. **A** and **B**: RNA gel blot analysis of total RNA extracted from cell cultures at indicated time points. Cultures were elicited with 100 μM methyl jasmonate on day 7 posttransfer and sampled at the time points represented in the figure. Time points after elicitation are indicated above each lane. **A**: *GGPPS*, *TASY*, and *T5αH* probes. **B**: *TDAT*, *TαH*, and *T10βH* probes. About 20 μg RNA were loaded onto each lane, EtBr stained rRNA to demonstrate equal loading. **C** and **D**: RT-PCR analysis of transcripts at 6 h (**C**) or 18 h (**D**) after MJ elicitation. An *rRNA* fragment was amplified as an internal loading control. The number of PCR cycles used for each RT-PCR reaction is indicated on the right. Lanes labeled as (-) represent the results of amplification of RNA without reverse transcription, as a control for contaminating genomic DNA in RNA preparations.

equally low at 6 h unelicited and MJ-elicited cultures (Fig. 4C). At 18 h, *T10βH* PCR products are only observed in the MJ-elicited cultures, but again, at very low levels (Fig. 4D). The expression profile of these three genes suggests a preference for the TαH-side of the branch pathway in these *Taxus* cell cultures, confirming recent metabolomics data which demonstrate that precursor flux leading to paclitaxel is via the 5α,13α-diol through TαH as compared to the 5α-yl acetate derived from the alternative branch TDAT, which leads to taxine B (Ketchum et al., 2003).

3.6. Late pathway regulation

Using RNA blots, transcripts coding for DBBT and DBAT, enzymes that synthesize 10-DAB and baccatin III, respectively, show increased abundance at 6 h after MJ elicitation, with continued abundance extending through 30 h (Fig. 5A). *DBAT* mRNA is present at low levels in unelicited cultures but transcripts encoding DBBT are not detected in unelicited cultures (Fig. 5A). RT-PCR product for *DBBT* at the 6 h time point confirms that there are transcripts coding for this enzyme in the unelicited cultures, but at low levels compared to MJ-elicited cultures (Fig. 5B). In the unelicited cultures at 18 h, *DBBT* transcripts are not present (Fig. 5C). At 18 h after MJ elicitation, *DBBT* RT-PCR product decreases in abundance compared to its 6 h counterpart (Fig. 5C), which is in excellent agreement with the results from RNA gel blot analysis and supports the observed decrease in steady-state transcript abundance from 6 to 18 h after MJ elicitation. Interestingly, the nonside-chain taxane 10-DAB accumulates in unelicited cell cultures (Fig. 3A), which implies that there is low level of expression of early taxane biosynthetic genes and/or enzyme activity without elicitation. The presence of low levels of *DBBT* (Fig. 5B) transcripts in unelicited cultures explains the presence of 10-DAB in these cultures (Fig. 3A).

The transcript profile of *PAM*, the paclitaxel side-chain-modifying enzyme, indicates that it is under similar regulatory control as taxane biosynthetic pathway genes. *PAM* transcripts accumulate upon elicitation with MJ with high levels of expression at 6 h continuing through 18 h (Fig. 5A). Using RT-PCR, *PAM* transcripts are evident in unelicited cultures at both 6 and 18 h, but with increased abundance upon elicitation (Figs. 5B and C).

The two terminal steps in the pathway involve BAPT and DBTNBT. The abundance of transcripts encoding BAPT and DBTNBT are below the limits of detection for RNA gel blot analysis in both unelicited and MJ-elicited cultures (Fig. 5A). The more sensitive RT-PCR assay, however, detects these rare transcripts (Figs. 5B and C). RT-PCR reveals that *BAPT* expression occurs in unelicited cultures and is increased following MJ elicitation at 6 h (Fig. 5B). *BAPT* transcript abundance is lower at 18 h than at 6 h when detected by RT-PCR (Figs. 5B and C).

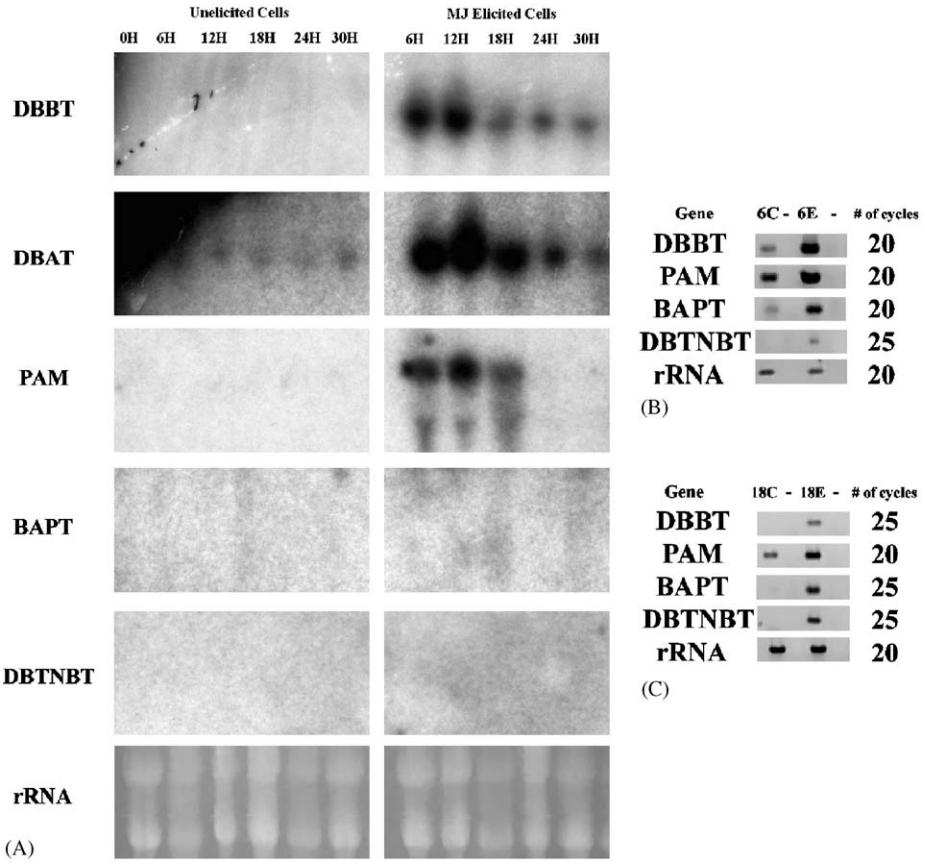

Fig. 5. Late pathway regulation: RNA gel blot analysis and RT-PCR. **A**: RNA gel blot analysis of total RNA extracted from cell cultures at indicated time points. Cultures were elicited with 100 μM methyl jasmonate on day 7 posttransfer and sampled at the time points represented in the figure. Time points after elicitation are indicated above each lane. The probe used for each blot is indicated at the left. About 20 μg RNA were loaded onto each lane, EtBr stained rRNA to demonstrate equal loading. **B** and **C**: RT-PCR analysis of transcripts at 6 h (**B**), or 18 h (**C**) after MJ elicitation. An rRNA fragment was amplified as an internal loading control. The number of PCR cycles used for each RT-PCR reaction is indicated on the right. Lanes labeled as (-) represent the results of amplification of RNA without reverse transcription, as a control for contaminating genomic DNA in RNA preparations.

DBTNBT expression is not observed in unelicited cultures via blot (Fig. 5A) or RT-PCR (Figs. 5B and C), explaining why side-chain taxanes such as paclitaxel and cephalomannine are not detected through metabolite analysis. The amount of *DBTNBT* RT-PCR product increases 6 h after MJ elicitation compared to the unelicited controls (Fig. 5B). At 18 h after MJ elicitation, however, there is a noticeable increase of *DBTNBT* RT-PCR product, suggesting a delayed response for expression of the side-chain-modifying enzyme (Fig. 5C). Because the relevant metabolites, paclitaxel and cephalomannine, were not detected until very late in the time course (168 h), we used RT-PCR to analyze expression of *DBTNBT* and *BAPT* at the 168 h time point. No RT-PCR product was detected for either gene at 168 h after elicitation (data not shown). These data indicate that *DBTNBT* and *BAPT* expression follows a similar time course as the rest of the taxane biosynthesis genes analyzed, with expression peaking within the first 2 days following elicitation. Transcripts from *DBTNBT* and *BAPT* do not persist in 168 h cultures, even though metabolite levels are highest at this time, suggesting that *BAPT* and *DBTNBT* enzyme activity may persist long after their cognate mRNAs are absent from the cell. These data suggest that transcript abundance for these terminal pathway steps is much lower in comparison to earlier steps, making these potential rate-influencing steps in the paclitaxel biosynthetic pathway.

4. Conclusions

Analysis of *Taxus cuspidata* suspension cell cultures upon MJ elicitation via RNA gel blot, RT-PCR and HPLC elucidates the tight correlation of gene expression and taxane accumulation. We provide evidence for a preference towards one side of the taxane biosynthetic pathway branch when cultures are elicited with MJ. Data demonstrate an increase in steady-state transcript abundance for many genes involved in this biosynthetic pathway upon elicitation with MJ, but that the timing and extent of this up-regulation varies for each individual gene. The relative abundance of each mRNA species may reflect the requirements for taxane accumulation *in arbor*, allowing

for accumulation of nonside-chain taxanes, but limiting accumulation of the biologically active side-chain taxanes such as paclitaxel. This study provides direction for future efforts in metabolic engineering of this pathway. Results suggest that targeting the late pathway steps, specifically BAPT and DBTNBT, may be effective in enhancing paclitaxel accumulation in *Taxus* cell suspension cultures. Identification of absolute gene targets for metabolic engineering offers distinct challenges. For example, in some instances, overexpression of a critical regulatory gene is more effective than modifying expression of specific biosynthetic pathway genes. To complement the studies on taxane biosynthesis presented here our laboratory is investigating protein expression and stability as well as developing new strategies to identify key global control genes that may be involved in rate-influencing processes such as transcription, degradation, or transport. The combination of mRNA and protein expression data will provide a more complete picture of taxane biosynthetic pathway regulation. Once a stable transformation method for *Taxus* cell suspension cultures is established (focus of ongoing efforts in our laboratory), targeted metabolic engineering of *Taxus* can be attempted to develop superior strains for use in bioprocesses to synthesize and supply paclitaxel.

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