

Correlation Analysis of the Taxane Core Functional Group Modification, Enzyme Expression, and Metabolite Accumulation Profiles Under Methyl Jasmonate Treatment

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Metabolomic and transcriptomic profiling data were obtained and integrated to elucidate the crucial network controls on taxol and its precursor biosynthesis during the taxane core functionalization within methyl jasmonate (MJ)-induced Taxus chinensis cells. Twelve metabolites were identified using liquid chromatography-electrospray ionization-mass spectrometry. These metabolites contain taxol (paclitaxel), baccatin III (B-III) and its analogs, a group structurally bearing multiple free hydroxyls (TAX), and another group of multiple acyl taxanes (MAT), including taxuyunnanin C (TC) and its analogs. The metabolomic profile showed a higher increase in TAX than in MAT. Particularly, the ratio of B-III and taxol to the total taxane content increased more significantly in TAX than in MAT. The MAT proportion did not significantly change, although they are predominant components in cell cultures compared with TAX. Quantitative real-time polymerase chain reaction (qRT-PCR) was used to determine the transcription level of 20 genes, among which 11 were reported responsible for taxol biosynthesis and 9 were obtained from our previous transcriptomic data. The total expression levels of hydroxylase after 24 h and 6 days were higher than those of acylase. The principal component analysis (PCA) results validated the metabolomic analysis data, indicating that hydroxylation was more crucial than acylation for controlling the flux toward TAX biosynthesis. Furthermore, the PCA contribution comparison showed that two undefined genes of OHX1 and ACX3 might have good potential in TAX upregulation and MAT downregulation. To the best of our knowledge, this study provides the first experimental evidence on the contribution of total hydroxylation to taxane biosynthesis.

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Introduction

Taxanes represent a group of unusual diterpenoid skeletons. Approximately 400 taxoids have been found in various *Taxus* plant or cell cultures.¹ Taxol has remarkable efficacy in cancer chemotherapy because of its unique mode of action on the microtubular cell system.² Taxol is also applied in synergistic antiangiogenic activity³ and in slowing down progressive synaptic deterioration linked to Alzheimer-type pathogenesis.⁴ However, the very low concentrations in plant and the slow growth rate of the tree make the increasing demand for taxol nearly impossible to meet. Currently, the commercial supply of taxol and its structural analogs is produced through semisynthesis using 10-deacetyl-baccatin

III(DAB) or B-III as starting substrates. These substrates are likewise separated from biotechnological sources, such as *Taxus* cell cultures or sustainable parts of tree clippings and needles. Understanding the detailed biosynthetic and network controls in *Taxus* cells is essential to effectively produce large quantities of taxol or its pharmaceutical precursors through plant cell culture or cultivation using metabolic-engineering and biological synthesis methods.

Taxol biosynthetic pathways are currently well reported by the Croteau laboratory,⁵ Williams laboratory,⁶ and others. Similar to other diterpenes, the pathways of taxol biosynthesis could be referred to as a “two-phase process”⁷ with at least 19 enzymatic steps. In the first phase (the cyclase phase), taxadiene is cyclized by enzymatically controlled molecular catalysis and rearrangements of taxadiene synthase (TXS) from geranylgeranyl diphosphate (GGPP). In the second phase, cytochrome P450-mediated monooxygenations and acyl transferases are involved, resulting in ~400 taxoid numbers, which provides a large array of taxane structural

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diversity. The enzymatic characteristics and gene sequences of TXS were elucidated and documented.⁵ By balancing precursor supply with GGPP and introducing plant TXS gene into *Escherichia coli* (*E. coli*), Ajikumar et al.⁸ achieved a final concentration of 1.02 g L⁻¹ taxadiene, which is the maximum taxadiene yield in taxol biosynthesis research attained thus far. In contrast, little is known regarding the mechanisms and related regulations of the functionalization of the taxane skeleton during the second phase, although five presumptive taxane core oxygenases and five presumptive acyl and aroyl transferases were cloned and characterized primarily by functional expression in yeast.^{5,9,10} The poor substrate specificity among the P450 enzymes and the substrate dependence of acyl or acetyl transferases rather than a single “path” approach in the second phase suggest the complexity of the existing reaction network. In the network, each oxygenase is designed to hydroxylate the taxane core at a specific position, and the hydroxys are further promiscuously acylated (including acetylate).¹¹ Each hydroxylated and subsequently acylated product can function as a substrate of varying efficiencies for each P450 and acyltransferase, resulting in the formation of diverse taxane fluxes, many of which find their way to DAB, B-III, taxol, and TC. DAB and B-III have the same core as taxol and could be used as precursors for taxol semisynthesis. Nevertheless, TC and its derivatives that possess MAT may present reverse multidrug resistance.¹² However, they often turn out to be shunt metabolites because they are not modified further by downstream enzymes. Clearly, MAT presents significant side routes that divert pathway flux away from taxol to decrease the production yield of target drugs.

The greatest challenge in investigating the net regulation of taxane functionalization may lie on the lack of special substrates because of the low yield from biological sources as well as the difficulty in synthesis using a chemical approach.⁹ Metabolomics research was successfully realized using the genome model plant *Arabidopsis thaliana*.¹³ Considering the information obtained from gene expression, plant secondary metabolomics using liquid chromatography-mass spectrometry (LC-MS) provides valuable information on gene-to-metabolite networks, although the real intermediate biosynthetic substrates are often produced in trace or undetectable amounts. Exposito et al.¹⁴ compared taxadiene synthase activity with the total taxoid contents in different cell lines treated with MJ. In their research, the total taxoids were only represented by five target compounds, namely, DAB, B-III, 10-deacetyltaxol (DAT), cephalomannine (CEP), and taxol. Moreover, a lag of 2–3 weeks was observed among the maximum taxadiene synthase gene(*txs*) expression, activity of TXS and the highest taxoid production. Obviously, the observed enzymatic activity is not correlated with taxoid production, suggesting that taxadiene synthase does not control a flux-limiting step of the biosynthetic pathway leading to taxol and related taxoids. Thus, other MJ-activated metabolic steps downstream of the process might also exist. This speculation was supported by previous research¹⁵ that focused on the relationship between related taxoid contents and the expression of genes encoding TXS and three acyltransferase enzymes based on a study of taxoid production in *Taxus baccata* plantlets grown *in vitro* for 1 year. The results of this study imply that the genes controlling the rate-limiting step are not involved in the early steps of TXS, but in the latter steps of the functionalization of the taxane core in taxol biosynthetic networks.

Our research team has recently completed the transcriptional profile of *Taxus chinensis* (*T. chinensis*) cells in response to MJ and generated 58 million reads (200 bp in length) and 46,581 unigenes,¹⁶ including 70 differentially expressed unigenes of P450 monooxygenase and 48 differentially expressed unigenes of O-acyltransferase that respond to MJ-induction. Using some of these patent data, we analyzed here the correlation between the taxane accumulation profiles and a series of gene expressions of the enzymes possibly involving in the modification of the functional groups of the taxane core. Taxoid metabolic and gene profiling expressed from ~20 known and random monooxygenases and O-acyltransferases selected from transcriptional databases were compared, and metabolomics and transcriptional profiles were integrated.

Materials and Methods

Plant cell culture

Cultured suspension cells of *T. chinensis* cell line 48# were established from callus culture-initiated embryos excised from tender stem (young stems) collected in Wuhan (Hubei Province, China) in May 2003. The culture was maintained using modified Gamborg's B5 medium as previously described.¹⁷ All experimental cell suspensions were obtained from the same cell line and were maintained in a rotary shaker at 105 rpm in the dark at 26°C. MJ (Sigma, USA) was added after 7 days of culture in the production medium at a final concentration of 100 μ M, whereas an equal volume of ethanol was added to the control cultures. MJ and ethanol were sterilized by filtration through 0.22- μ m sterile filters.

Sample collection and treatment

For the transcriptome analysis, ~3 g of fresh cells were collected at 0 h, 3 h, 6 h, 16 h, 24 h, 3 days, 6 days, 8 days, 10 days, 12 days, and 15 days from both the induced and control samples. Then, Trizol (Invitrogen, USA) was added (1 mL g⁻¹ fresh cell). Each sample was placed under room temperature for 10 min and then immediately stored at -80°C until total RNA isolation. For metabolite analysis, ~20 g of fresh cells was taken out at the same timeline from the same cells as that of the transcriptome analysis and immediately stored at -80°C for quenching metabolism until extraction.

Extraction and assay of taxanes

The frozen sample (5 g) was placed in a 50-mL beaker, which was then added with 4 mL g⁻¹ (fresh cell) ice-methanol and 0.85 mL g⁻¹ ice-cold deionized water. The mixture was stirred for ~30 s using a homogenizer (Fluko FA25, Germany) to break the cytoderm. Then, 2 mL g⁻¹ ice-cold dichloromethane was added. The mixture was stirred for another 30 s and then shaken using an ultrasonoscope (Kunshan KQ-250E, China) in ice-cold water for 30 min to thoroughly dissolve the taxanes. After centrifugation (Sigma 3K30C, Germany) at 9000 rpm for 20 min at 4°C, the supernatant (methanol-aqueous phase) was removed. Dichloromethane (2 mL g⁻¹) and ice-cold deionized water (2 mL g⁻¹) were added to the sediment. Homogenization was conducted for ~30 s, and centrifugation was performed at 6,000 rpm for 20 min at 4°C. Subsequently, the liquid

was separated into three layers: an upper methanol–aqueous phase, a lower dichloromethane phase, and a middle thin layer of cell debris. The above two methanol–aqueous phases were mixed together as polar phase, and then was dried using N-Evap (Autoscience MTN-2800D, China) for high pressure liquid chromatography (HPLC) analysis. The lower dichloromethane phase as non-polar phase was also dried using N-Evap for HPLC analysis.

The dried samples were respectively dissolved in acetonitrile at a volume of 2 mL. Each solution was filtered through a 0.45 μm Millipore filter unit, and the filtrate samples were injected (20 μL) into a high-performance liquid chromatography-electrospray ionization-mass spectrometry (HPLC-ESI-MS) system for analysis. All samples were managed and stored in glass containers because of the dissolution of dichloromethane into the plastic materials.

An internal standard content of norethindrone solution was added into each sample at a final concentration of 15 $\mu\text{g mL}^{-1}$. The HPLC peak area of this internal standard was considered as the reference for calculation. The relative content of individual taxane (R_c) was calculated based on the equation:

$$R_c = 15 (f_1 + f_2)$$

where f_1 is the ratio of taxane area in polar phase sample to norethindrone area and f_2 is the ratio of taxane area in non-polar phase sample to norethindrone area.

HPLC-MS conditions

Taxanes were identified using HPLC-ESI-MS (Agilent 1100, USA) based on our method.¹⁷ The HPLC conditions were previously optimized and will not be covered in this article. HPLC analyses were performed using a phenyl-C18 (2.5 mm $\times$ 250 mm $\times$ 4.6 μm) column with a mobile phase consisting of a mixture of water (A) and acetonitrile (B) under a timed gradient program T (min)/%B: 0/5, 10/10, 11/50, 20/60, 30/70, 40/90, and 45/100 with a flow rate of 1 mL min^{-1} and a wavelength of 227 nm. The identification criteria included retention time, UV spectra, and mass spectrum with standard and peak homogeneity determined by a photodiode array detector spiked with an authentic standard.

The mass spectrometer was equipped with an ESI source in positive ion mode with an LC-MSD-Trap-XCT instrument. The source voltage was set to 3.5 kV. The tuning parameters adopted for the ESI source were the following: N_2 , 40 psi; auxiliary gas flow, 10 L min^{-1} ; capillary temperature, 325°C; capillary current, 10 nA; target mass, 650 m/z ; initial scan, 100 m/z ; and final scan, 1,000 m/z .

Gene expression assays through quantitative real-time polymerase chain reaction

The following genes in taxol biosynthetic pathways were selected for transcriptome library analysis according to previous reports: six known hydroxylase genes of *T. chinensis* 13- α -hydroxylase gene (T3 α H), *T. chinensis* taxoid 2- α -hydroxylase gene (T2 α H), *T. chinensis* taxadiene 5- α -hydroxylase mRNA (T5 α H), *T. wallichiana* var. *mairei* taxoid 7- β -hydroxylase gene (T7 β H), *T. chinensis* 5- α -taxadienol-10- β -hydroxylase gene (T10 β H), and *Taxus* $\times$ media taxane 14b-hydroxylase mRNA (T14 β H) as well as five known acyl transferase genes of *T. wallichiana* var. *chinensis* voucher SNJ001 10-deacetyl baccatin III-10-

O-acetyl transferase mRNA (DBAT), *Taxus* $\times$ media 3'-N-debenzoyltaxol N-benzoyltransferase mRNA (DBTNBT), *Taxus* $\times$ media taxane 2- α -O-benzoyltransferase gene (DBBT), *T. cuspidata* taxadienol acetyl transferase mRNA (TDAT), and *T. wallichiana* var. *mairei* phenylpropionyltransferase mRNA (BAPT).

Some candidate genes probably involved in other taxane biosynthesis were selected according to our transcriptome data.¹⁶ Candidate hydroxygenase genes were randomly selected from the transcripts designated as CYP725A and CYP716D subfamilies, in which all known taxane hydroxygenases have been found thus far.^{18,19} Conversely, the candidate acetylase genes were all designated as terpene acetylases. According to the homology at 50–95% settings, the sequences of these genes were aligned with BLAST analysis in the NCBI website. The remaining four hydroxylase genes of OHX1 (gene ID: Unigene35752_All, Blastx: 71%), OHX2 (gene ID: Unigene14738_All, Blastx: 69%), OHX3 (gene ID: Unigene41192_All, Blastx: 59%), and OHX4 (gene ID: Unigene46162_All, Blastx: 50%) were screened out from 65 CYP450 hydroxygenases in the transcriptome of *T. chinensis*. The remaining five acylase genes named ACX1 (gene ID: Unigene8833_All, Blastx: 61%), ACX2 (gene ID: Unigene15184_All, Blastx: 59% to 61%), ACX3 (gene ID: Unigene13707_All, Blastx: 64%), ACX4 (gene ID: Unigene7834_All, Blastx: 52%), and ACX5 (gene ID: Unigene2630_All, Blastx: 95%) were also screened out according to the homology at 50–95% settings from the remaining genes which exclude histone GNC.

Total RNA were isolated from the samples treated with and without MJ for 0 h, 3 h, 6 h, 16 h, 24 h, 3 days, 6 days, 8 days, 10 days, 12 days, and 15 days. For each time point, three biological replicates were obtained and analyzed. Then, cDNA was synthesized from total RNAs treated with DNAase-I (Invitrogen, USA) using Superscript II reverse transcriptase (Invitrogen, USA). Specific primers were designed for genes involved in taxol biosynthesis. SYBR premix Ex-Taq Kit (Takara, Kyoto, Japan) and ABI PRISM 7900 DNA Sequence Detection System (Applied Biosystems, USA) were used to perform qRT-PCR. The comparative threshold cycle method was used to calculate the relative gene expression. The internal control used was 18s rDNA. Each real-time PCR was performed three times.

The amplification multiple M was calculated as follows:

$$M = 2^{-[(Ct - Ctr) - (Cto - Ctro)]}$$

where Ct is the expression quantity of the target gene at any collection time, Ctr is the expression quantity of 18s rDNA at the same collection time as Ct , Cto is the expression quantity of the target gene on day 0 (control), and $Ctro$ is the expression quantity of 18s rDNA on day 0 (control).

Data analysis of metabolites and microarray

The quantifications of metabolites were expressed by relative content (R_c) and the proportion of individual taxane. The R_c of individual taxane has been defined in the section of "Extraction and Assay of Taxanes," and the proportion of individual taxane was the ratio of individual taxane R_c to the sum of all identified taxane R_c in the same sample. For example, in the sample of 12 day-control, the proportion of B-III was calculated as 0.8% based on the ratio of 0.34 (R_c of B-III) to 41.8 (the sum of all identified taxane R_c).

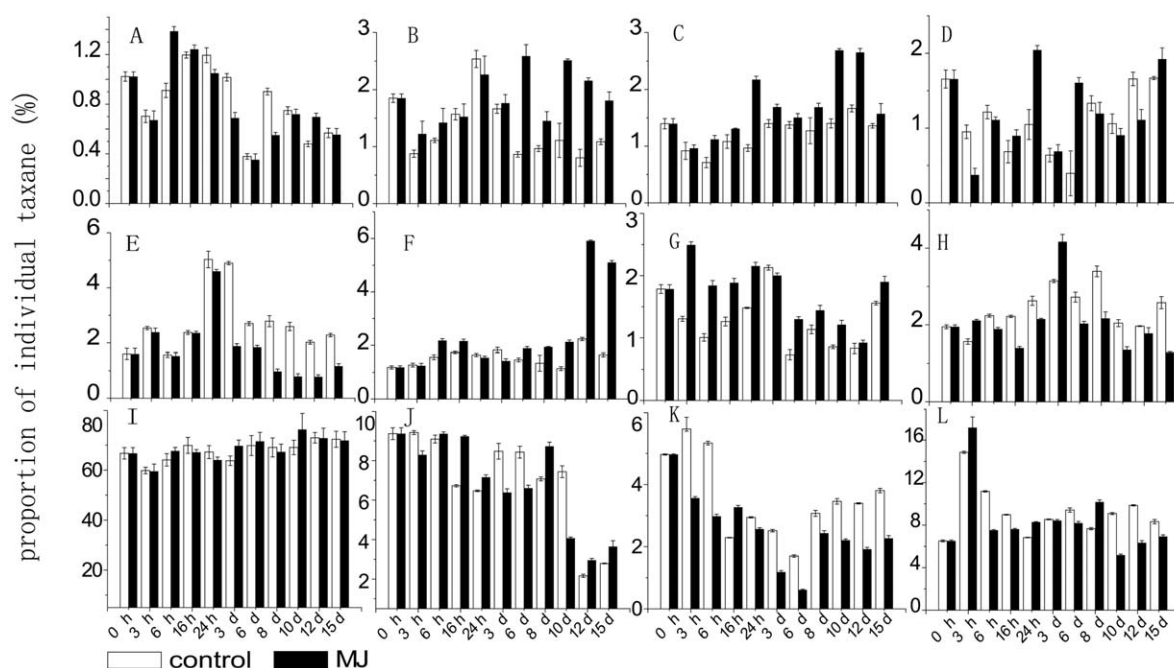


Figure 1. Variations in individual taxane proportion in *T. chinensis* cell culture over 15 days when treated by MJ or without MJ added (control). A–L represent proportional profilings of different taxanes. A: DAB; B: B-III; C: DAT; D: H3T; E: TO; F: Taxol; G: H4T; H: YN; I: TC; J: TPT; K: TIBT; L: TMBT.

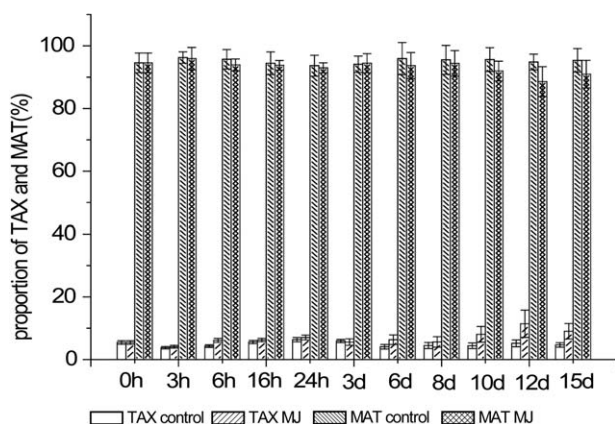


Figure 2. Variations in total taxane proportion in *T. chinensis* during the 15 days cell culture of the MJ-treated and control samples.

The proportion was employed to compare the variation in taxane relative content over different treatments (induced and control) and different times (Figures 1 and 2).

The metabonomic and transcriptomic data were statistically evaluated using principal component analysis (PCA) to reduce the dimensionality of the data to a small number of components. PCA is an unsupervised data analysis method; thus, prior knowledge of the sample is not required. PCA allows interpretation of the results in a fairly intuitive graphical manner. Data from the qRT-PCR results of the 20 selected genes and the liquid chromatography–mass spectrometry (LC-MS) set of all 12 identified taxane metabolites were compared under different induction periods. According to the component matrix, the contribution order of the compounds or genes to the sample can be obtained from their component score coefficients. A higher component score coefficient indicates a higher degree of contribution. Thus,

the contribution of the variations in individual metabolite content and gene expression level could be arranged based on the component score coefficients of the variables. This method was performed using the SPSS 19.0 software.

The results were processed using Origin 8.0. The experiments were repeated three times, and the results shown were the averages of three measurements. Error bars were used to represent the standard errors of the samples from three parallel experiments. The data were subjected to ANOVA, and *P* values consistent within $\pm 5\%$ were considered to indicate statistical significance.

Results and Discussion

Identification and profiling of taxanes

The identification of all possible taxanes (including target taxanes and non-target taxanes) is the first and most important step in metabolic profiling or metabolomics study. A method for analyzing taxanes in *T. chinensis* cell cultures was developed using LC-ESI-MS in our previous study.²⁰ To identify the target taxanes and characterize the molecular ion fragments from MS analysis, a commercially available mixed standard substance containing DAB, B-III, DAT, CEP, 7-epi-10-deacetyl-taxol (EDT), taxol, and 7-epi-taxol (ETOL), and another mixture containing Yunnanxane (YN), TC, and $2\alpha,5\alpha,10\beta$ -triacetoxyl-14 β -(2-methyl)-butyryl oxy-taxa-4(20),11-diene (TMBT) prepared in our laboratory were used for ESI-MS and MS/MS measurements. Approximately 12 specific peaks were observed in the HPLC chromatogram of the *T. chinensis* cell culture samples. The LC-MS data with peak assignments are shown in Table 1.

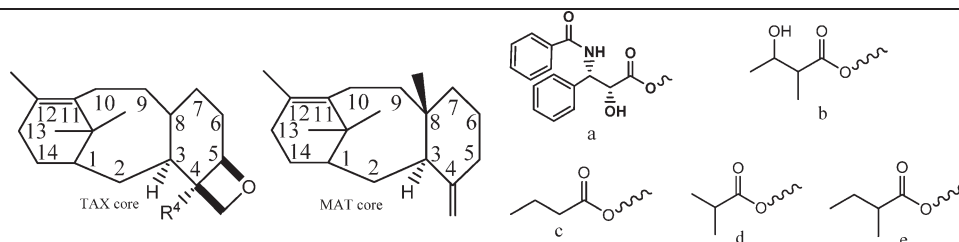
A comparison was performed based on the chromatographic retention time and mass spectrum molecular ion peaks of the standard substance with those of the samples. Peaks 1, 2, 3, and 6 were identified as DAB, B-III, DAT, and taxol, respectively. Peaks 8, 9, and 12 were assigned as

Table 1. LC-MS Data and Identification of Taxanes

No.	t_R (LC/TIC)	MS	MS/MS	MW	Identification
1	15.1/15.6	545	528,509,363,345,327,309	544	DAB
2	16.5/17.2	587	570,509,405,387,327	586	B-III
3	18.1/19.0	812	592,559,527,405,327,309,286	811	DAT
4	18.7/19.7	480; 485	385,361,343,325,283,265,209	462	H3T
5	19.0/19.8	508	490,385,325,283,265,209	508	TO
6	20.4/21.5	854	776,594,569,509,405,387,327,309,286	853	Taxol
7	22.0/22.5	538; 543	461,401,365,341,323,281,221,161	520	H4T
8	23.2/24.0	580; 585	503,461,443,385,343,325,283,265,209	562	YN
9	24.8/26.0	522; 527	385,325,265,209	504	TC
10	26.7/28.0	536; 541	482,422,385,348,265,209,180	518	TPT
11	28.9/29.8	550; 555	445,385,325,265,209,194	532	TIBT
12	29.7/31.4	564; 569	445,385,325,283,265,209,	546	TMBT

Table 2. Structures of Identified Taxanes

No.	TAX Group	R^1	R^2	R^4	R^7	R^9	R^{10}	R^{13}	R^{14}
1	DAB	OH	OBz	OAc	OH	O	OH	OH	H
2	B-III	OH	OBz	OAc	OH	O	OAc	OH	H
3	DAT	OH	OBz	OAc	OH	O	OH	a	H
6	Taxol	OH	OBz	OAc	OH	O	OAc	a	H



No.	MAT group	R^1	R^2	R^5	R^7	R^9	R^{10}	R^{13}	R^{14}
5	TO	H	OAc	OH	OH	OA c	OAc	O	H
8	YN	H	H	OAc	OAc	H	OAc	H	b
9	TC	H	OAc	OAc	H	H	OAc	H	OAc
11	TIBT	H	OAc	OAc	H	H	OAc	H	d
12	TMBT	H	H	OAc	OAc	H	OAc	H	e

No.	MAT Group	No. of Hydroxy	No. of Acetyl
4	H3T	1	3
7	H4T	1	2~4
10	TPT	0	2~4

YN, TC, and TMBT, respectively. The molecular ion peaks of all references in the mixed standard substance were observed as $[M+H]^+$. However, those of YN, TC, and TMBT were considered as $[M+NH_4]^+$ and $[M+Na]^+$ (Table 1), indicating that the adduct ion type in the ESI-MS spectra partly depends on the number of free hydroxyls in the taxane skeleton. For the assignment of nontarget taxanes, a comparison of the corresponding information with the literature¹ and biogenetic backgrounds was used to identify the site and number of functional groups in the taxane core according to our previously established method.²¹

For example, Peak 4 with the molecular-related ion peaks at m/z 485 and m/z 480 (Table 1) was deduced as $[M+Na]^+$ and $[M+NH_4]^+$, and the presumed molecular weight (MW) could be 462 Da. The data of MS/MS for Peak 4 with m/z 283 and m/z 265 indicated a 6/8/6 taxane core containing four substituents.²⁰ Two taxane isomers with a relative MW of 462 can be found in the taxane chemical library,¹ but both of them have one hydroxyl and three acetyls at their taxane molecular cores, indicating that they differ only in the position of the functional groups. In addition, one isomer with MW of 462 was recorded in the literature¹ from cell cultures of *T. yunnanensis*, while another from the twigs of *T. mairli*. Thus, peak 4 was roughly assigned as an isomer

of 2 α -hydroxy-5 α ,10 β ,14 β -triacetoxytaxa-4(20),11-diene (H3T). The remaining undefined taxanes could be similarly assigned (Table 2).

In view of the biosynthetic pathway, the taxanes of DAB, B-III, DAT (Peaks 1, 2, 3, and 6) have the same core in the 6/8/6/4 taxane type with taxol (Table 1). In this report, we defined them as the taxol group (TAX). Other groups include H3T, Taxuspin O(TO), H4T, YN, TC, TPT, 2 α ,5 α ,10 β -triacetoxy-14 β -iso-butyryloxytaxa-4(20),11-diene (TIBT), and TMBT (Peaks 4, 5, 7, 8, 9, 10, 11, and 12, respectively) with the 6/8/6 taxane type. These multiple acyl types were defined as MAT. The molecular structure shows that the TAX group contained at least three free hydroxyls, whereas the MAT group seldom contained free hydroxyls or none at all.

The quantifications of metabolites were expressed by relative content (R_c) and the proportion of individual taxane. R_c of individual taxane (data not shown) has been calculated to its proportion, and the proportions of individual taxanes both in MJ addition and absence during the culture period are shown in Figure 1. The highest proportion of taxane found in the *T. chinensis* cell cultures was 2,5,10,14-Tetracetyl taxanes (TC), which was identified in our previous study²¹ and other research²² that also reported that this compound (TC)

is a predominant taxane produced by *T. chinensis* cells. The highest proportion of TC was found here after MJ-induction of *T. chinensis* cells at 10 days with a value of 76.2% (Figure 1I), followed by TMBT, which exhibited a peak proportion value of 17.2% at 3 h after MJ-induction (Figure 1L). It is noticed that the proportion of Taxol was found to reach its maximum of 5.9% at 12 days after MJ-induction. Other pharmaceutically important precursors of Taxol, such as B-III, DAB, and DAT, accounted for minor proportions, which the peak values were 2.6, 1.4, and 2.7% at 6 days, 6 h, and 10 days, respectively, after MJ-induction.

The addition of MJ to the culture medium changed the proportions of almost all the taxanes, but the variations were different depending on the difference in molecular structures and the time after MJ addition. For example, the proportion of Taxol increased gradually from 2.2% at 6 h, then reduced slightly to 1.4% at 3 days, and then reached a maximum of 5.9% at 12 days. TC did not seem to show a significant response to MJ addition; the proportion fluctuated only in the narrow range from 59.5 to 76.2% in the MJ-induced groups and from 59.8 to 73.0% in the uninduced groups.

Compared with the control, the proportion of some taxanes showed an increase trend after MJ-induction during the culture period. The taxanes contained B-III, DAT and Taxol, whose proportions increased gradually and peaked at 2.5%, 2.7%, 5.9% at 10, 10, and 12 days (Figures 1B,C,F) respectively, while the values were 1.1%, 1.4%, 2.2% in the control at the same time points. The increments that the three taxanes achieved were 2.3-, 1.9-, and 2.7-folds due to MJ-induction. These increases are close to the previous report²³ in which the maximum production of taxane occurred at 12 days after MJ-induction. It is noticed that during the culture period the proportions of some taxanes exhibited more than one peak. For example, DAT displayed another peak of 2.2% at 24 h (Figure 1C), and B-III was observed to exhibit two other peaks of 2.3% and 2.6% at 24 h and 6 days (Figure 1B).

The proportion of some taxanes decreased after MJ-induction. The proportions of TO, TIBT and TMBT decreased from 2.0, 3.4, and 9.8% in the control to 0.8, 1.9, and 6.3%, respectively, corresponding to the culture time of 12, 6, and 10 days (Figures 1E,K,L). There were still some taxanes, such as DAB, H3T, H4T, YN, and TPT, which responded to MJ addition in an unusual way (Figures 1A,D,G,H,J). The variations in the proportions of individual taxanes suggest the complicated nature of taxane functionalization, and that it is difficult to demonstrate the response rules to MJ only using the data pertaining to a single taxane.

To present the change rules of the functionalization, we group these 12 taxanes to TAX and MAT; the calculated total proportions of the two groups are shown in Figure 2. The proportion of MAT in the MJ-induced groups showed a trend to decrease from 94.5% at 1 days to 91.0% at 15 days. While in the control, MAT proportion in the total taxanes showed a slight increase from 94.5 at 1 day to 95% at 15 days. Although the highest proportions were observed at 3 days in both of induced and uninduced groups, being 95.9 and 96.2%, respectively, the variations in the proportions of MAT were not significant. The results indicate that the effect of MJ did not seem to improve the ratio of MAT to the total taxane content.

Compared with MAT, the proportion of TAX showed a trend to increase from 5.4% at 1 day to 9.0% at 15 days in the MJ-induced groups, and a decrease in the control from 5.4% at 1 day to 4.6% at 15 days (Figure 2). The highest

proportion of TAX was observed at 12 days in the induced group with a value of 11.4%; the other peaks of TAX in the induced group were 7.0% at 24 h, while compared with the control at the same time point the values were detected to be 5.1 and 6.3%, indicating that the increase of TAX was much higher than that of MAT ($P < 0.05$). These results indicate that MJ improved the ratio of TAX in the total taxane content.

Concerning biosynthesis, DAB and B-III are important precursors in taxol biosynthesis. The increase in the proportion of the TAX group suggests that MJ-induction could enhance one-step or multiple-step functionalization towards taxol biosynthetic pathways. TC and its derivatives (YN, TPT, TIBT, and TMBT) are unlikely to reside on the route to taxol because of inappropriate acylation patterns on the taxane ring system.²⁴ Thus, the biosynthetic pathway of MAT is an important bypass to taxol.

Variations in the transcriptional profiling of 20 target and nontarget genes

To determine which step between hydroxylation and acylation has a more important role and to identify which gene has the most significant role among the network of oxygenases and acyl transferases in the modification of functional groups at the taxane core, ~20 genes were selected for transcriptome library analysis. These genes contained six known hydroxylase genes of T α H, T2 α H, T5 α H, T7 β H, T10 β H, and T14 β H as well as five known acyl transferase genes of DBAT, DBTNBT, DBBT, TDAT, and BAPT in the taxol biosynthetic pathways (Table 3).

The sequences of the other 9 genes resulted from the screening of our previous transcriptome data¹⁶ containing 65 CYP450 hydroxygenases and 24 acylases in response to MJ-induction. According to the homology at 50–95% settings, the sequences of these genes were aligned with BLAST analysis in the NCBI website. The remaining four hydroxylase genes of OHX1, OHX2, OHX3, and OHX4 were screened out from 65 CYP450 hydroxygenases in *Taxus*. The remaining five acylase genes of ACX1, ACX2, ACX3, ACX4, and ACX5 were also screened out to exclude histone GNC and those unrelated to acetylation genes (Table 3).

Hydroxyl and acylation genes were highly responsive to MJ-induction except for OHX2 (Figure 3). For most selected genes, the response peak appeared at induced times of before 24 h and 6 days. The increase in mRNA expression level of the related taxol biosynthesis within 24 h elicited by MJ is not surprising since this has been observed by other reports for *txs* and *bapt* genes.^{14,23}

Interestingly, we detected a significant increase in gene expression for almost all hydroxylases and acylases at day 6 after MJ-induction (Figure 3). To the best of our knowledge, such results have not been reported by other studies. After the addition of the irritant, some of the genes showed high expression levels, such as T α H, T10 β H, T14 β H, T5 α H, OHX1, and OHX4. While the expression levels of some genes reached peaks only within 24 h, such as T2 α H, T7 β H, and OHX3 (Figure 3). Thus, not only does the metabolite production differ in response to the induction, the expression profilings of some genes will also show disparity.

The expression levels of T α H, T2 α H, T5 α H, T10 β H, T14 β H, DBAT, DBTNBT, TDAT, BAPT, and ACX3

Table 3. Names and Genbank Num/Gene IDs of 20 Selected Genes

Gene Name	Accession Number	Primer
TzH	AY959321	F: GGGAAATCCAACGCCACA R: GCGACGGAGAAGACGAGGT
T2zH	AY518383	F: TAGCATCCCAGCAATCAGG R: GCATTGTGGATGGGCAGA
T5zH	AY741375	F: GTTCTAAACGCCACTCCTCCC R: GGATGCCCAATCAAGGAGGT
T7βH	AY307951	F: CGATAAAGAGCGAAAGCAAC R: TCATAGGAGGCATCCAGCA
T10βH	AY519128	F: CACCTATTCTCGCCATTATTC R: CTACCAAGCTTGTCTCGTTC
T14βH	AY188177	F: GGCAACGACCAAGTGAGTGT R: GCCCGACGGTAAGCAAAT
OHX1	–	F: CATTCTTACCATTGGAGCAG R: GGGTCAACTGGGATGTAGC
OHX2	–	F: TTTATGGACGGCTTATACTACAC R: GGACACCCATTGATAGGAAGA
OHX3	–	F: CCAGCAAGGTTGTCCACG R: AAGGATTTTCATCACGCGATT
OHX4	–	F: TTGGCACTCAACCCAGATA R: GAGGGAATAGGCGTAGTGTTT
DBAT	AF193765	F: AGGCGATTGGGATTGATG R: GGTATTCTTGGGAGGTCGTAT
BAPT	AY082804	F: TTGACAACATGGCAAGAGC R: TTCCAGAAACAGAACACCTT
DBBT	AY864799	F: GGGATCTTGAAGTGGAGTGC R: CTGTGAATACTGAAAGGTCGTTGT
DBTNBT	AY563629	F: CTCAGCCCATCGTTTCATC R: CTCATCCGCCACAGCAA
TDAT	AF190130	F: GGGGAATGCGGTGAGTG R: TCCATCGGGCTTGTCTT
ACX1	–	F: CCCAAATCTCCCAACACTG R: GGGCGGCTCAGAAGTAAG
ACX2	–	F: GCTCCGCCCAATACAC R: GAAACCTACTCCCACAACAAA
ACX3	–	F: CTATGCCCGCCGTCAG R: TGCCGCCACAACCTCG
ACX4	–	F: TGTGGACTTGGGTGGG R: TCCTTTAGGCTTATTCTTCG
ACX5	–	F: GGACGGCTCAGATACAAGAA R: AGATGAAGGTCCACAATGTCTG

(Figure 3) greatly increased. Among these genes, TzH, T2zH, T5zH, T10βH, DBAT, DBTNBT, TDAT, and BAPT are the genes in the taxol biosynthetic pathway. This finding explains the reasons for the extensive growth of the TAX group. At the same time, T14βH (Figure 3F) and ACX3 (Figure 3S) also increased, which is consistent with the increase in the production of related metabolites, such as YN, TC, TIBT, and TMBT. Thus, the gene expression profilings conform to the variations of metabolic product profilings.

Because most of the hydroxyl genes and the acylation genes were highly responsive to MJ-induction, we aggregated all the hydroxyl genes together as well as all the acylation genes to determine which kind of genes were more influenced by MJ. Compared with control, the gene expression level both of total hydroxylase and the acylase fluctuated during MJ-induction. For the hydroxylation gene aggregate, around four peaks appeared at the induction time of 6 h, 16h, 24 h, and 6 days, increasing by 13.5-, 4.2-, 1.9-, and 41-fold, respectively. This was accompanied by 10.4-, 7.7-, 7.3-, and 29.1-fold increases for total acylation genes at the same time points (Figure 4). When compared with total gene expression level, hydroxylation was 2.6-, 1.4-, 2.2-, and 2.5-times higher than acylation at 6 h, 16 h, 24 h, and 6 days, respectively, after MJ-elicitation, suggesting that the hydroxylation fluxes were higher than those of acylation. Thus, the hydroxyl step seems to

influence the functionalization of taxane cores more than acylation.

Multivariate statistical analysis

To elucidate the relationship of the variation between the taxane metabolite profiles and the transcriptome in *T. chinensis* cells, a chemometric approach using PCA was used. PCA, an unsupervised data analysis method, allows interpretation of the results in a fairly intuitive graphical manner. To achieve dimensionality reduction, a linear combination of the original data was carried out. If the variance of linear combination was bigger, we called it PC1 which could represent the most information of the original data, and the smaller was called PC2.²⁵ Data from the qRT-PCR results of 20 selected genes and the LC-MS set of all 12 identified taxane metabolites were compared under different induction periods. The PCA results are presented in Figure 5. The principal components PC1 and PC2 were obtained to differentiate the MJ-induced sample from the control sample. The PC1 of taxane content, hydroxylated gene, and acylated gene expression variance accounted for 61, 64, and 68% (Figures 5B,D,F) of the MJ-induced samples, respectively, and 56, 75, and 69% of the control groups, respectively. The PC1/PC2 loading plots (Figures 5A,B) show that diagonal arrangements of data points were formed and distributed in the right upper of the loading plots. The explanation for the dispersion is shown in Table 4. In the component matrix, the columns represent the order of contribution of the compounds or genes to the whole sample as well as their component score coefficients. A higher component score coefficient indicates higher contribution. Thus, the contribution of the variations in individual metabolite content and gene expression level could be arranged based on the component score coefficients of the variables. In the case of the taxane metabolites, for the control samples the first six variables contributing to PC1 were TC (0.965), DAT (0.930), YN (0.883), H3T (0.831), Taxol (0.772), and TSO (0.739). For the MJ-induced samples, the first six variables contributing to PC1 were Tc (0.986), DAT (0.932), B-III (0.908), axol (0.819), TMBT (0.809), and H3T (0.806). This distinction of metabolite contributors between the control and MJ groups indicated that B-III, TMBT, taxol, and H3T are the dominant variable metabolites because the rank for B-III, taxol, TMBT changed from eleventh to third, from fifth to fourth, and from sixth to fifth, respectively, following MJ-induction. These results were found although TC and DAT are predominant in composition for both the MJ-induced and control samples. Furthermore, the variations in the TAX group are higher than those in the MAT group in terms of MJ-induction response. This rationalization is in accordance with the increase in the related taxane contents based on the relative changes in peak area before and after MJ-induction in this study (Figure 1). Moreover, these results are consistent with those reported on *Taxus × media* cell and its transformed gene *Taxus* strain,¹⁴ *T. baccata* cell,¹⁵ and *T. cuspidata* cell.²⁶

Similarly, in the case of gene expression, the results obtained from PCA analysis show that the predominant contributors to both the MJ-induced and control samples are T5zH and DBTNBT, the first ranks of which remain unchanged in Table 4. The dominant variables were OHX1, T10βH, TzH for the hydroxylated genes, and ACX3 and DBAT for the acylated genes, which contributed to the total variability increase because of MJ-induction. Notably, the rank of T14β declined from third to fourth, suggesting that

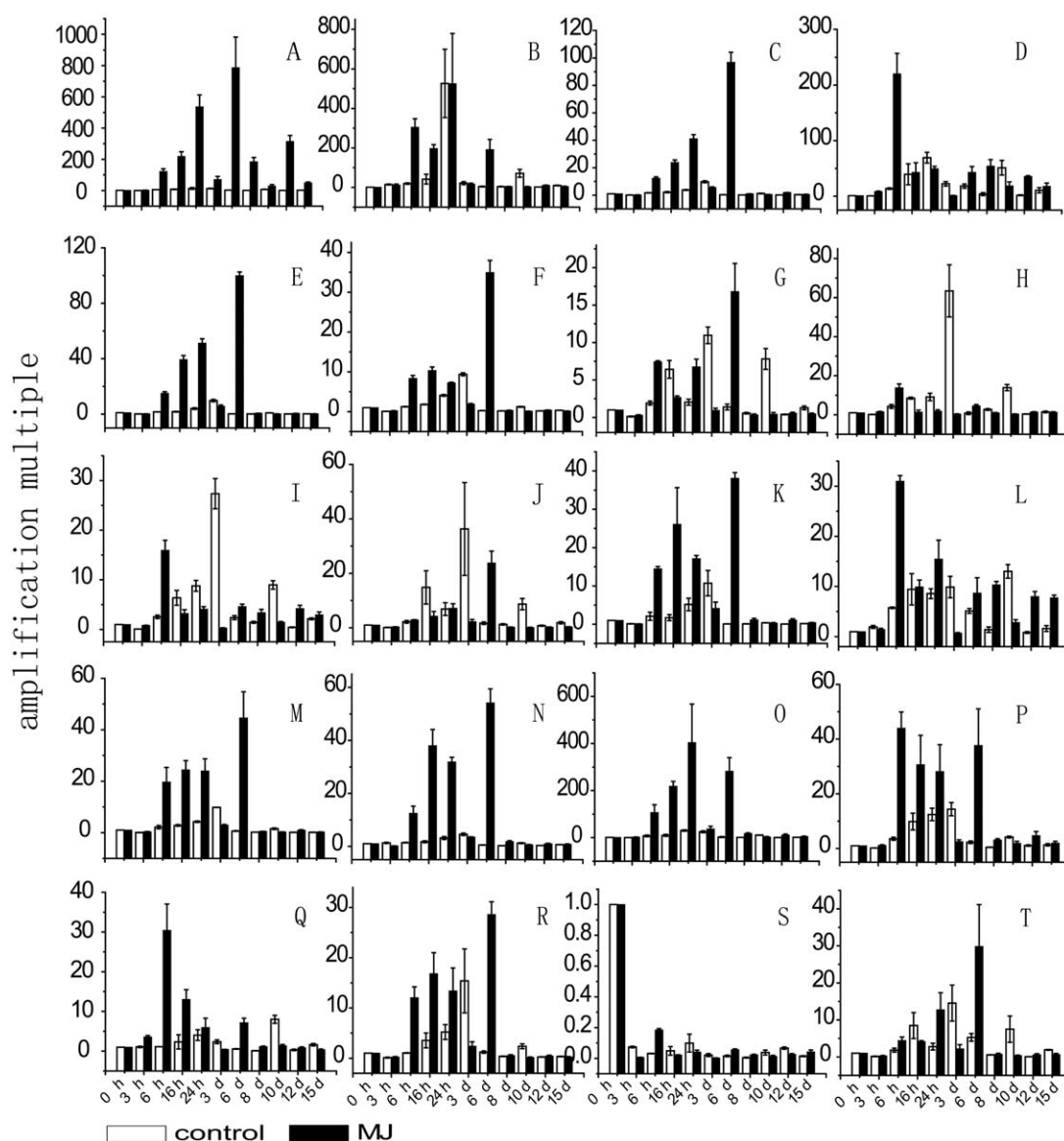


Figure 3. Variations in individual genes in *T. chinensis* during the 15 days cell culture of the MJ-treated and control samples. A-T represent amplification multiple of different genes. A: T α H, B: T2 α H, C: T5 α H, D: T7 β H, E: T10 β H, F: T14 β H, G: OHX1, H: OHX2, I: OHX3, J: OHX4, K: DBAT, L: DBBT, M: DBTNBT, N: TDAT, O: BAPT, P: ACX1, Q: ACX2, R: ACX3, S: ACX3, T: ACX5.

the contribution proportion of gene expression decreased after MJ-induction.

Interpretation of key regulation during the modification of functional groups of the taxane core

All taxanes were derived from taxadiene and were mainly modified by a set of hydroxygenases and acylases, especially CYP450 hydroxygenases and acetylases. The expression levels of these CYP450 hydroxygenases and acetylases directly affect the taxane biosynthesis of *T. chinensis*. However, information on the expression of these CYP450 hydroxygenases and acetylases is lacking at present because of their great diversity and complex biosynthesis processes.

Previous studies defined most of the catalytic genes of the 19 steps in the biosynthetic pathways from GGPP to taxol,²⁷ including the six hydroxylase genes T α H,²⁸ T2 α H,²⁹ T5 α H,³⁰ T7 β H,³¹ T10 β H,³² and T14 β H³³ as well as the five acyl transferase genes DBAT,³⁴ BAPT,³⁵ DBBT,³⁶ DBTNBT,³⁷

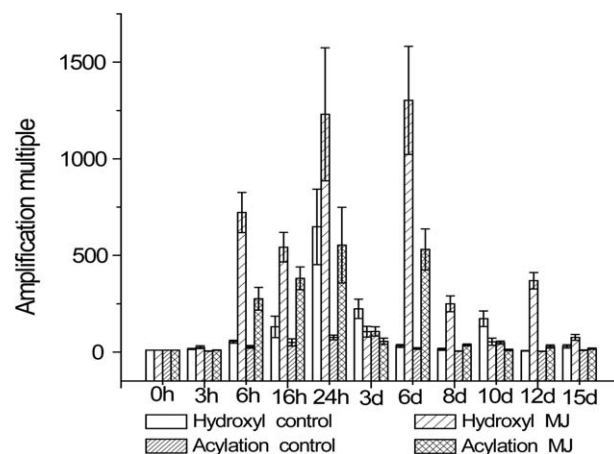


Figure 4. Variations in total hydroxyl genes and total acylation genes in *T. chinensis* during the 15 days cell culture of the MJ-treated and control samples. Error bars were used to represent the standard errors of the genes from three parallel measurements.

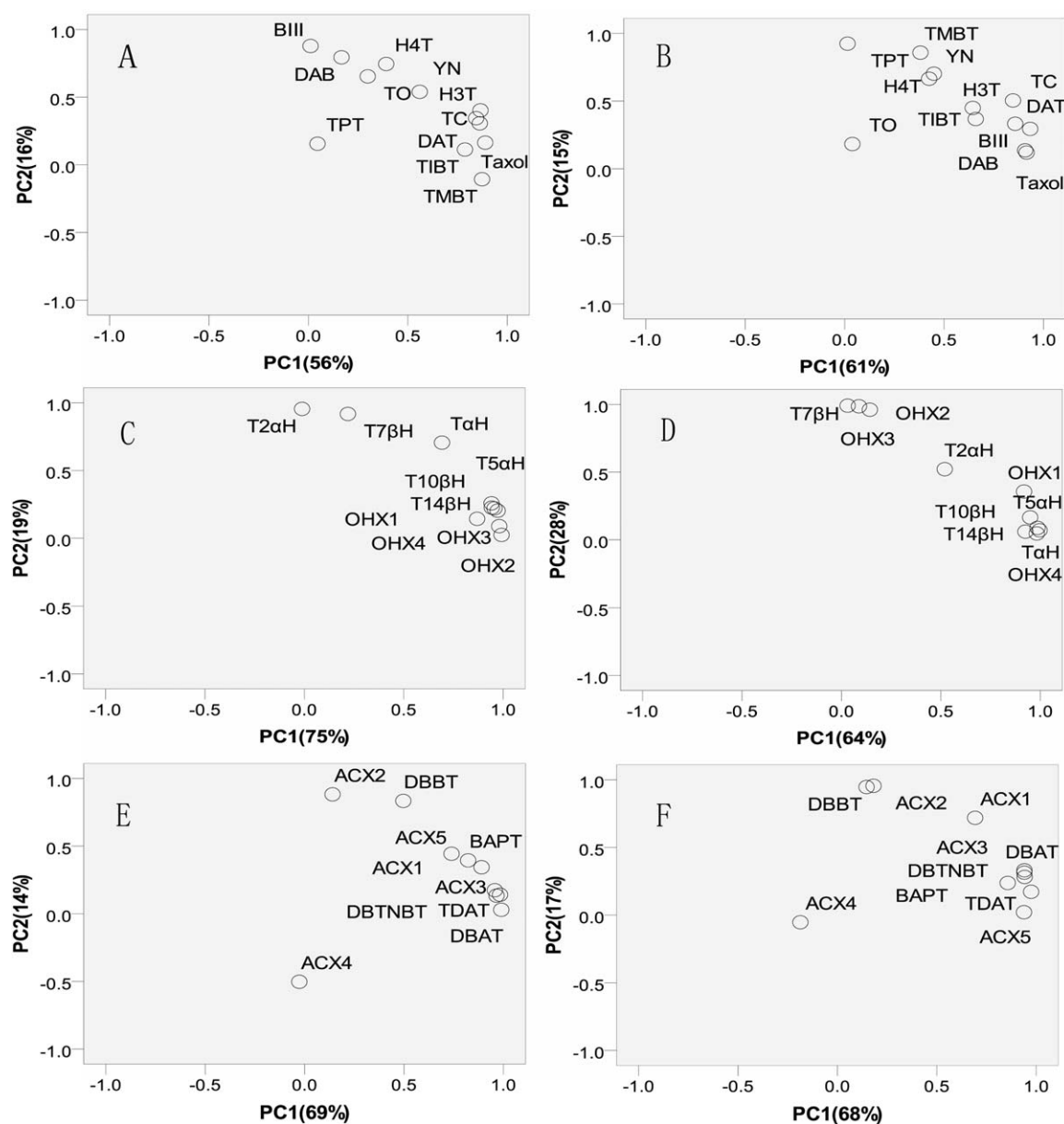


Figure 5. Principal component analysis of the variation in metabolites and gene expression levels. (A) Uninduced metabolites, (B) induced metabolites, (C) uninduced hydroxylation genes, (D) induced hydroxylation genes, (E) uninduced acylation genes, and (F) induced acylation genes.

and TDAT.^{38,39} These hydroxylase genes and acyl transferase genes are respectively responsible for the hydroxylation of the taxol biosynthesis precursors at a specific position in the taxane core (Figure 6) and also for further promiscuous acylation (including acetylation). Prior reports^{28–33} have revealed that all hydroxylation genes belong to the family of CYP450 monooxygenases and have strict specificity for catalytic position and substrate. Meanwhile, all acylases are acetyl-CoA-dependent acyl or acetyl transferases, whose gene functions are not strictly substrate specific, and the sequence homology of the genes between functional similarity is not very high.⁴⁰ However, the pathway of taxol biosynthesis is only one of the pathways in the taxane secondary metabolic network. A large number of genes or key steps still require identification. Unfortunately, the main taxane produced by the *Taxus* species, especially in *T. chinensis* cells, is not TAX but rather MAT (Figures 1 and 2). MAT constitutes significant side routes that divert the

pathway flux away from TAX to decrease the production yields of the target metabolites. Therefore, identification of the key genes or critical steps in such pathway diversions has a significant biotechnological implication, such that elimination of these side-route taxane biosynthetic pathways will increase the yields of TAX. However, at present, little is known regarding this assumption. In general, it is problematic for us and others to distinguish whether total hydroxylation and total acylation is more important for TAX biosynthesis.

The information on gene expression can be combined with the metabolome to provide valuable information on gene-to-metabolite networks, such that the key metabolic steps and critical genes in regulating the fluxes of target metabolites can be identified.⁴¹ Through MJ-induction of *T. chinensis* cells, the relative contents of all detected taxanes and expression levels of the selected genes increased,⁴² indicating that both hydroxylation and acylation are MJ responsive.

MAT comprises the predominant components in cell cultures compared with TAX, indicating that the effect of MJ did not seem to improve the rate of MAT in the total taxane content. The transcriptome analysis results showed that the total gene expression of hydroxylase after 24 h and 6 days was higher than that of acylase. The PCA analysis results also confirm the results of metabolic profiling analysis, indicating that hydroxylation was more crucial than acylation for controlling the flux toward TAX biosynthesis in the metabolic network of *T. chinensis* cells. To the best of our best knowledge, this study is the first experimental evidence on the contribution of total hydroxylation to the biosynthesis of taxol and its structural analogs, and the ratio of total hydroxylation to total acylation may be used to analyze taxol metabolic engineering in the future. Furthermore, the results of the contribution value from PCA showed that T10 β H, T α H, DBAT, and two undefined genes of OHX1 and ACX3 contribute main variables to the samples induced by MJ, suggesting their significant roles in the diverse pathway flux of the taxane secondary metabolic network. In particular, the two unknown genes of OHX1 and ACX3 might have good potential for the up-regulation of TAX and the down-regulation of MAT biosynthesis in *T. chinensis* cells.

Acknowledgments

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Literature Cited

- Baloglu E, Kingston DGI. The taxane diterpenoids. *J Nat Prod*. 1999;62:1448–1472.
- Malika S, Cusidó RM, Mirjalili MH, Moyano E, Palazón J, Bonfill M. Production of the anticancer drug taxol in *Taxus baccata* suspension cultures: a review. *Process Biochem*. 2011;46:23–34.
- Mistry SJ, Bank A, Atweh GF. Synergistic antiangiogenic effects of stathmin inhibition and taxol exposure. *Mol Cancer Res*. 2007;5:773–782.
- Butler D, Bendiske J, Michaelis ML, Karanian DA, Bahr BA. Microtubule-stabilizing agent prevents protein accumulation-induced loss of synaptic markers. *Eur J Pharmacol*. 2007;562:20–27.
- Koksal M, Jin YH, Coates RM, Croteau R, Christianson DW. Taxadiene synthase structure and evolution of modular architecture in terpene biosynthesis. *Nature*. 2011;469:116–122.
- Guerra-Bubb J, Croteau R, Williams RM. The early stages of taxol biosynthesis: an interim report on the synthesis and identification of early pathway metabolites. *Nat Prod Rep*. 2012;29:683–696.
- Liu K, Ding XW, Deng BW, Chen WQ. Isolation and characterization of endophytic taxol-producing fungi from *Taxus chinensis*. *J Ind Microbiol Biot*. 2009;36:1171–1177.
- Ajikumar PK, Xiao WH, Tyo KEJ, Wang Y, Simeon F, Leonard E, Mucha O, Phon TH, Pfeifer B, Stephanopoulos G. Isoprenoid pathway optimization for taxol precursor overproduction in *Escherichia coli*. *Science*. 2010;330:70–74.
- Williams RM. Natural products synthesis: enabling tools to penetrate nature's secrets of biogenesis and biomechanism. *J Org Chem*. 2011;76:4221–4259.
- Chau MD, Jennewein S, Walker K, Croteau RB. Taxol biosynthesis: molecular cloning and characterization of a Cytochrome P450 taxoid 7 β -hydroxylase. *Chem Biol*. 2004;11:663–672.
- Hampel D, Mau CJD, Croteau RB. Taxol biosynthesis: identification and characterization of two acetyl CoA:taxoid-O-acetyl transferases that divert pathway flux away from Taxol production. *Arch Biochem Biophys*. 2009;487:91–97.
- Hosoyama H, Shigemori H, Tomida A, Tsuruo T, Kobayashi J. Modulation of multidrug resistance in tumor cells by taxinine derivatives. *Bioorg Med Chem Lett*. 1999;9:389–394.
- Tohge T, Fernie AR. Web-based resources for mass-spectrometry-based metabolomics: a user's guide. *Phytochemistry*. 2009;70:450–456.
- Exposito O, Syklowska-Baranek K, Moyano E, Onrubia M, Bonfill M, Palazon J, Cusido RM. Metabolic responses of *Taxus media* transformed cell cultures to the addition of methyl jasmonate. *Biotechnol Progr*. 2010;26:1145–1153.
- Onrubia M, Moyano E, Bonfill M, Palazón J, Goossens A, Cusidó RM. The relationship between TXS, DBAT, BAPT and DBTNBT gene expression and taxane production during the development of *Taxus baccata* plantlets. *Plant Sci*. 2011;181:282–287.
- Li ST, Zhang P, Zhang M, Fu CH, Zhao CF, Dong YS, Guo AY, Yu LJ. Transcriptional profile of *Taxus chinensis* cells in response to methyl jasmonate. *BMC Genom*. 2012;13:295–306.
- Zhang CH, Mei XG, Liu L, Yu LJ. Enhanced paclitaxel production induced by the combination of elicitors in cell suspension cultures of *Taxus chinensis*. *Biotechnol Lett*. 2000;22:1561–1564.
- Nelson D, Reichhart DW. A P450-centric view of plant evolution. *Plant J*. 2011;66:194–211.
- Rontein D, Onillon S, Herbette G, Lesot A, Reichhart DW, Sallaud C, Tissier A. CYP725A4 from yew catalyzes complex structural rearrangement of taxa-4(5),11(12)-diene into the cyclic ether 5(12)-oxa-3(11)-cyclotaxane. *J Biol Chem*. 2007;283:6067–6075.
- Zhao CF, Yu LJ. LC-ESI/MS determination of taxol and related taxanes in *Taxus chinensis*(Pilger) Rehd. *Chin J Pharm Anal*. 2006;26:1–6.
- Zhao CF, Yu LJ, Li LQ, Xiang F. Simultaneous identification and determination of major taxoids from extracts of *Taxus chinensis* cell cultures. *Z naturforsch C*. 2006;62:1–10.
- Dong H-D, Zhong JJ. significant improvement of taxane production in suspension cultures of *Taxus chinensis* by combining elicitation with sucrose feed. *Biochem Eng J*. 2001;8:145–150.
- Onrubia M, Moyano E, Bonfill M, Expósito O, Palazón J, Cusidó RM. An approach to the molecular mechanism of methyl jasmonate and vanadyl sulphate elicitation in *Taxus baccata* cell cultures: the role of txs an bap gene expression. *Biochem Eng J*. 2010;53:104–111.
- Ketchum REB, Horiguchi T, Qiu D, Williams RM, Croteau RB. Administering cultured *Taxus* cells with early precursors reveals bifurcations in the taxoid biosynthetic pathway. *Phytochemistry*. 2007;68:335–341.
- Aa JY. Analysis of metabolomic data: principal component analysis. *Chin J Clin Pharmacol Ther*. 2010;15:481–489.
- Nims E, Dubois CP, Roberts SC, Walker EL. Expression profiling of genes involved in paclitaxel biosynthesis for targeted metabolic engineering. *Metab Eng*. 2006;8:385–394.
- Kong JQ, Wang W, Zhu P, Cheng KD. Recent advances in the biosynthesis of taxol. *Acta Pharma Sin*. 2007;42:358–365.
- Jennewein S, Rithner CD, Williams RM, Croteau RB. Taxol biosynthesis: taxane 13 α -hydroxylase is a cytochrome P450-dependent monooxygenase. *Proc Natl Acad Sci USA*. 2001;98:13595–13600.
- Chau MD, Croteau RB. Molecular cloning and characterization of a cytochrome P450 taxoid 2 α -hydroxylase involved in Taxol biosynthesis. *Arch Biochem Biophys*. 2004;427:48–57.
- Jennewein S, Long RM, Williams RM, Croteau RB. Cytochrome P450 taxadiene 5 α -hydroxylase, a mechanistically unusual monooxygenase catalyzing the first oxygenation step of taxol biosynthesis. *Chem Biol*. 2004;11:379–387.
- Chau MD, Jennewein S, Walker K, Croteau RB. Taxol biosynthesis: molecular cloning and characterization of a cytochrome P450 taxoid 7 β -hydroxylase. *Chem Biol*. 2004;11:663–672.
- Schoendorf A, Rithner CD, Williams RM, Croteau RB. Molecular cloning of a cytochrome P450 taxane 10 β -hydroxylase cDNA from *Taxus* and functional expression in yeast. *Proc Natl Acad Sci USA*. 2001;98:1501–1506.

33. Jennewein S, Rithner CD, Williams RM, Croteau RB. Taxoid metabolism: taxoid 14 β -hydroxylase is a cytochrome P450-dependent monooxygenase. *Arch Biochem Biophys.* 2003;413:262–270.
34. Walker K, Croteau RB. Molecular cloning of a 10-deacetylbaicatin III-10-O-acetyl transferase cDNA from *Taxus* and functional expression in *Escherichia coli*. *Proc Natl Acad Sci USA.* 2000;97:583–587.
35. Walker K, Fujisaki S, Long R, Croteau RB. Molecular cloning and heterologous expression of the C-13 phenylpropanoid side chain-CoA acyltransferase that functions in Taxol biosynthesis. *Proc Natl Acad Sci USA.* 2002;99:12715–12720.
36. Walker K, Croteau RB. Taxol biosynthesis: molecular cloning of a benzoyl-CoA:taxane 2 α -O-benzoyltransferase cDNA from *Taxus* and functional expression in *Escherichia coli*. *Proc Natl Acad Sci USA.* 2000;97:13591–13596.
37. Walker K, Long R, Croteau RB. The final acylation step in Taxol biosynthesis: cloning of the taxoid C13-side-chain *N*-benzoyltransferase from *Taxus*. *Proc Natl Acad Sci USA.* 2002;99:9166–9171.
38. Chau MD, Walker K, Long R, Croteau RB. Regioselectivity of taxoid-O-acetyltransferases: heterologous expression and characterization of a new taxadien-5 α -ol-O-acetyltransferase. *Arch Biochem Biophys.* 2004;430:237–246.
39. Walker K, Schoendorf A, Croteau RB. Molecular cloning of a Taxa-4(20),11(12)-dien-5 α -ol-OAcetyl transferase cDNA from *Taxus* and functional expression in *Escherichia coli*. *Arch Biochem Biophys.* 2000;374:371–380.
40. Menhard B, Zenk MH. Purification and characterization of acetyl coenzyme A: 10-hydroxytaxane O-acetyltransferase from cell suspension cultures of *Taxus chinensis*. *Phytochemistry.* 1999;50:763–774.
41. Malitsky S, Blum E, Less H, Venger H, Elbaz M, Morin S, Eshed Y, Aharoni A. The transcript and metabolite networks affected by the two clades of arabidopsis glucosinolate biosynthesis regulators. *Plant Physiol.* 2008;148:2021–2049.
42. Mirjalili N, Linden JC. Methyl jasmonate induced production of taxol in suspension cultures of *Taxus cuspidata*: ethylene interaction and induction models. *Biotechnol Prog.* 1996;12:110–118.

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