

# Bottleneck Removal of Paclitaxel Biosynthetic Pathway By Overexpression of *DBTNBT* Gene Under Methyl- $\beta$ - Cyclodextrin and Coronatine Elicitation in *Taxus Baccata* L.

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## Research Article

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# Abstract

Paclitaxel is a highly functionalized diterpenoid that is broadly used for the treatment of several cancer types. This valuable specialized metabolite naturally exists in the inner bark of *Taxus* species in low amounts. The limited-scale production of paclitaxel in *Taxus* cell cultures has necessitated the use of several elicitors. Recently, methyl- $\beta$ -cyclodextrin (CD) and coronatine (COR) have been considered to be highly effective elicitors in producing plant specialized metabolites. Given the limited production of paclitaxel due to the rate limiting enzymes' function, bottleneck removal is conducive to the production of more significant amounts of paclitaxel. In the present study, the full length of *DBTNBT* coding sequence (CDS), as one of the paclitaxel pathway bottlenecks, was integrated downstream of the CaMV 35S promoter (pCAMBIA1304-*DBTNBT*) and transiently expressed in *Taxus baccata* leaves via *Agrobacterium tumefaciens* and vacuum infiltration method. Paclitaxel production and the expression level of several involved genes were evaluated through different treatments. The transient overexpression of the *DBTNBT* gene, associated with dual elicitation, resulted in 7.4-fold more paclitaxel production compared with the no-inoculation/no-elicitation control. These ratios were 2.1 and 1.8 in the CD+COR and pCAM treatments, respectively. Among *T13aH*, *T14 $\beta$ H*, *DBAT*, *BAPT*, *DBTNBT*, and *ABC* genes, the most increased expression level belonged to the *DBTNBT* gene, followed by *ABC* and *BAPT* genes. It seems as though in the near future, bottleneck removal could be used on a large scale in *Taxus* metabolic engineering, resulting in the relative removal of some other bottlenecks and an increase in the final paclitaxel production.

## 1. Introduction

Plants are good sources of pharmaceutical compounds, particularly anticancer ones. Certain plant-based anticancer drugs have been introduced, such as paclitaxel (Taxol®), vinblastine, vincristine, camptothecin, podophyllotoxin, and ajmalicine. Among these drugs, paclitaxel and its related compounds have attracted a great deal of attention owing to their high efficiency in treating a wide range of cancers (Cragg and Newman 2005). Paclitaxel has a high anticancer activity because of its unique tumor-suppressing mechanism through which it prevents the mitosis by stabilizing microtubules against depolymerization (Srivastava et al. 2005).

Despite clinical success (Zhu and Chen 2019), the most critical problem associated with paclitaxel production is providing sufficient amounts of this pharmaceutical compound (Sabzehzari and Naghavi 2019; Salehi et al. 2019). Different species of the yew tree (*Taxus* spp.) have been identified as the most important natural resources of taxanes. Roughly 0.0001 to 0.0017% of the *Taxus* bark in dry weight scale is paclitaxel. Due to its scarcity, slow growth rate, low concentration, and the existence of more than 400 similar compounds, paclitaxel extraction and purification procedures are not cost-effective and can destroy these natural reservoirs in the long run (Jaziri et al. 1996; Sabater-Jara et al. 2010).

Currently, chemical synthesis, semi-synthesis, production by endophytes, *Taxus* spp., and *Corylus avellana* cell suspension cultures are proposed for paclitaxel production. Among these methods, cell

suspension culture is one of the most beneficial ones owing to being optimal, renewable, and adaptable, providing the possibility to use elicitors to increase the final product (Jennewein et al. 2001; Pyo et al. 2005).

Since the 1990s, abiotic and biotic elicitors, alone or in combination, have been widely used to improve the production of bioactive metabolites in cell suspension cultures (Chandran et al. 2020; Malik et al. 2011; Onrubia et al. 2013; Satish et al. 2020). Methyl- $\beta$ -cyclodextrin (CD) is a circular oligosaccharide that acts as an elicitation agent for producing specialized metabolites in plant cell suspension cultures (Almagro and Pedreño 2020; Bru et al. 2006; Lijavetzky et al. 2008; Zamboni et al. 2009). It was previously proven that the combined use of methyl jasmonate (MeJA) and CD was more effective than the use of MeJA alone in increasing taxane production (Sabater-Jara et al. 2014). Coronatine (COR) is a bacterial blight phytotoxin produced by several pathovars of *Pseudomonas syringae* (Bender et al. 1999). COR has gained significant attention on account of its potential role in plant growth regulation and as a potent elicitor by triggering the jasmonate signaling pathway (Zhao et al. 2003). This phytotoxin is a molecular mimic of the isoleucine jasmonic acid (JA-Ile), the intracellular switch of the jasmonate pathway (Cusido et al. 2014).

Elicitors are believed to enhance the paclitaxel production up to a certain extent (Exposito et al. 2010; Ho et al. 2005), probably because of the paclitaxel toxicity for producer cells, which leads to the conversion of downstream genes to the bottlenecks in a way that paclitaxel be produced to the extent that it does not harm the cell viability (Kashani et al. 2018). The accumulation of paclitaxel inside the cell causes feedback inhibition, degradation of the final product (Mubeen et al. 2019), and feedback repression effect on the upstream genes involved in the paclitaxel biosynthetic pathway (Cusido et al. 2014); therefore, identifying and removing bottlenecks can be an appropriate approach to increasing the production of paclitaxel.

Paclitaxel is obtained after five steps: 1) providing the precursor of terpenoid compounds from the primary metabolism, 2) cyclizing the geranylgeranyl diphosphate, 3) oxygenation by cytochrome P450-dependent hydroxylases, 4) acylation, 5) conjugation of side-chain, followed by the last steps of paclitaxel biosynthetic pathway. In this pathway, *T13 $\alpha$ H* is a cytochrome P450-dependent hydroxylase that forms taxa-4(20), 11(12)-dien-5 $\alpha$ -13 $\alpha$ -diol. *T14 $\beta$ H* is responsible for the formation of taxa-4(20), 11(12)-dien-5 $\alpha$ -acetoxy-10 $\beta$ -14 $\beta$ -diol. *DBAT* is in charge of baccatin III formation. *BAPT* gene catalyzes the conjugation of  $\beta$ -phenylalanoyl-CoA side-chain to the baccatin III, and the *DBTNBT* gene is responsible for benzoyl-CoA conjugation to 3'-N-debenzoyl-2'-deoxytaxol and yielding the final product (Malik et al. 2011). A putative *ATP-binding cassette (ABC)* was found to play an essential role in taxane excretion (Sabater-Jara et al. 2014) (Fig. 1).

Metabolic engineering may be a robust approach to increasing paclitaxel production in *Taxus* platforms. Previous studies have shown that in the paclitaxel biosynthetic pathways, the genes encoding the downstream enzymes appear to control the limiting biosynthetic steps. The *DBTNBT* gene is the last

transferase in paclitaxel biosynthetic pathway, introduced as a bottleneck gene in *T. baccata* under the dual elicitation of CD and COR (Kashani et al. 2018).

Despite the overexpression of the genes involved in the paclitaxel biosynthetic pathways, elicitation is still required for the maximum paclitaxel production by the producer cells (Exposito et al. 2010; Ho et al. 2005). To the best of our knowledge, no research has ever been conducted on the effects of bottleneck removal via *DBTNBT* gene overexpression in *T. baccata* to enhance the amounts of paclitaxel production. With that in mind, the present study aimed to assess the impacts of *DBTNBT* overexpression on cell-associated and extracellular paclitaxel amounts and the transcriptional profiles of *T13aH*, *T14βH*, *DBAT*, *BAPT*, *DBTNBT*, and *ABC* genes under the elicitation of CD and COR. The results of this study underscore the importance of bottleneck removal regarding paclitaxel biosynthetic pathway; this approach will be applicable in the forthcoming future, particularly in regard to *T. baccata* metabolic engineering.

## 2. Materials And Methods

### 2. 1. Construction of pCAMBIA-DBTNBT vector

A chimeric fragment, including the 5' UTR of *DBTNBT* gene in *T. media* (AY563629.1), the coding region of *DBTNBT* gene (AF466397.1, 1326 bp), and the 3' UTR of the cowpea mosaic virus (GQ497234.1) (Supplementary material- Fig. 1 and Fig. 2) was synthesized by Shinegene company (China). This fragment was integrated between the *EcoRI* and *SphI* restriction sites in the pUC57 vector (pUC57-*DBTNBT*). Plasmid extraction and double digestion reactions of pCAMBIA1304 (AF234300.1) and pUC57-*DBTNBT* vectors were conducted using *SpeI*/*BstEII* restriction enzymes. The desired fragments (the backbone of the pCAMBIA1304 vector (9802 bp) and the target fragment of the *DBTNBT* gene along with its UTRs and designed restriction sites (1584 bp)) were purified and the ligation reaction was carried out with a ratio of 3 (vector) to 1 (insert). The ligation reaction components were 13 ng  $\mu\text{l}^{-1}$  of pCAMBIA1304 backbone, 10 ng  $\mu\text{l}^{-1}$  of insert, T4 buffer (1x), and 0.66 U  $\mu\text{l}^{-1}$  T4 ligase (Fermentas) to a final volume of 30  $\mu\text{l}$ . The reaction tube was incubated at 22°C for 1 h, followed by 4°C for 16-18 h.

Sequencing carried out by South Korean Bioneer Corporation through the use of CA+2 and NOS primers (Supplementary Material- Table 1) and double/ mono digestion reactions were done to confirm the accuracy of the fragment.

Table 1  
Inoculation, elicitation, and perforation conditions of treatments

Treatment Name	Inoculation Condition	Elicitation with CD (50 mM) and COR (1 $\mu$ M)	Leave Perforation with Needle
no-inoculation/no-elicitation control	-	-	-
CD+COR	-	***	***
pCAM	with <i>Agrobacterium</i> harboring pCAMBIA1304 vector (for 2 h using vacuum infiltration method)	-	***
pCAM-DBTNBT/CD+COR	with <i>Agrobacterium</i> harboring pCAMBIA1304-DBTNBT vector (for 2 h using vacuum infiltration method)	***	***
no-inoculation/no-elicitation control; CD+COR, no inoculation- elicitation with methyl- $\beta$ - cyclodextrin (CD) and coronatine (COR); pCAM, inoculation with <i>Agrobacterium</i> harboring pCAMBIA1304 vector- no elicitation; pCAM-DBTNBT/CD+COR, inoculation with <i>Agrobacterium</i> harboring pCAMBIA1304-DBTNBT vector- elicitation with methyl- $\beta$ - cyclodextrin (CD) and coronatine (COR)			

## 2. 2. Bacterial transformation and confirmation

*Escherichia coli* (DH5 $\alpha$ ) competent cells were produced (Green and Rogers 2013) and into which the vectors were transformed (Sambrook and Russell 2001). For bacterial transformation, after transferring the ligation reaction product to the *E. coli* (DH5 $\alpha$ ) competent cells, the tube was placed in an ice container for 30 min followed by incubation at 42°C for 60 s and being put in an ice container for 5 min. 800  $\mu$ l of S.O.C. medium (tryptone 2%, yeast extract 0.5%, NaCl 0.01 M, MgSO<sub>4</sub> 0.01 M, KCl 0.0025 M, MgCl<sub>2</sub> 0.01 M, and glucose 0.02 M) was added to the tubes. The tubes were incubated in a shaker incubator (37°C, 180 rpm) for 80 min. 100  $\mu$ l of tube content was spread on a petri dish containing LB agar medium with 50  $\mu$ g ml<sup>-1</sup> of kanamycin. Petri dishes were incubated overnight at 37°C.

The pCAMBIA-DBTNBT and pCAMBIA1304 vectors were transformed into the *Agrobacterium tumefaciens* (LBA4404) (Rhizobiaceae) competent cells prepared according to the calcium chloride protocol (Sambrook and Russell 2001).

The transformation of *E. coli* and *Agrobacterium* were confirmed using colony PCR, in which Ampliqon Master Mix 1x with 1.5 mM of MgCl<sub>2</sub> (containing *Taq* DNA Polymerase), F (forward) and R (reverse) primers (Supplementary material- Table 1) (each with a final concentration of 266  $\mu$ M), and a colony of bacteria grown in the culture medium containing selective antibiotics were used. The PCR program was as follows: 94°C, 5 min; 35 cycles of 94°C, 30 s; annealing, 50 s; 72°C, 120 s, and 72°C, 10 min

## 2. 3. Plant transformation

The leaves of the perennial yew tree (*T. baccata*), located in the Botanic Garden of Tehran University, were used as the primary plant material for gene transformation. Vacuum infiltration method (using

*Agrobacterium* containing PCAMBIA-DBTNBT and pCAMBIA1304 vectors) was utilized for the transient expression of *DBTNBT* and *GFP-GUS* genes in *T. baccata* leaves.

Overnight culture of *Agrobacterium* and centrifugation (1600 *g*, 15 min, 4°C) were further performed, and the bacterial pellet was re-suspended in an induction medium (pH 5.2) and incubated at 28°C. The final optical density (OD) at 600 nm was 0.6-0.8. The ingredients of this medium were as follows: B5 medium salts except macro-elements (1x), vitamins (2x), sucrose (0.5%), mannitol (0.01 M), glucose (0.1 M), sorbitol (0.1 M), fructose (1 M), KNO<sub>3</sub> (2500 mg l<sup>-1</sup>), myo-inositol (100 mg l<sup>-1</sup>), ascorbic acid (100 mg l<sup>-1</sup>), naphthalene acetic acid (2 mg l<sup>-1</sup>), kinetin (0.1 mg l<sup>-1</sup>), casein hydrolyzate (1000 mg l<sup>-1</sup>), and acetosyringone (0.2 mM). The treatments were no-inoculation/no-elicitation control, CD+COR, pCAM, and pCAM-DBTNBT/CD+COR (Table 1). The leaves were placed in sterilized distilled water containing CD (50 mM) and COR (1 µM) while gently shaken for elicitation. (Singh et al. 2020). The leaves in pCAM and pCAM-DBTNBT/CD+COR treatments were incubated for 16-18 h at 28°C in dark.

## **2. 4. Paclitaxel extraction and determination**

Paclitaxel was extracted from the leaves (Rahpeyma et al. 2015), Afterwards, the freeze-dried leaves were weighted, pulverized, and suspended in 4 ml of HPLC grade methanol, followed by ultrasonication for 30 min and centrifugation at 1340 *g* for 15 min at 22°C. The upper phase was transferred to a vacuum oven to remove the solvent. The extract was resuspended in dichloromethane/water (1:1, v/v) and centrifuged at 1340 *g* for 15 min at 22°C. The lower phase (Dichloromethane) was isolated. Subsequently, vacuum evaporation was carried out. The remaining material was resuspended in 500 µl of HPLC grade acetonitrile and filtered via the 0.22 µm filters (Millipore) prior to high-performance liquid chromatography (HPLC) analysis.

The extracellular paclitaxel was extracted from the media (Onrubia. et al. 2013) with some modifications. 15 ml of the medium was harvested 6, 13, and 17 days after the elicitation time (Kashani et al. 2018), it was mixed with an equal volume of dichloromethane (DCM) and shaken for 2 h, followed by lower-phase separation. The solvent was removed from the organic phase by being transferred to a vacuum oven. The remaining material was resuspended in 0.5 ml of HPLC grade acetonitrile and filtered with the 0.22 µm filters (Millipore) prior to being injected into HPLC instrument.

To determine paclitaxel concentration, an HPLC system (Waters 2695; USA) equipped with an RP C-18 column (KNAUER100-5 C18, 250 × 4.6 mm, Germany) was used and elution was carried out in a gradient system with acetonitrile/water (20: 80- 80: 20 during 60 min) with a flow rate of 1 ml min<sup>-1</sup>. Paclitaxel was detected at 230 nm via a UV detector (PDA Waters 996, USA). The injection was performed using an autosampler injector equipped with a 100 µl loop. The paclitaxel was identified by comparing the retention times with an authentic standard. To draw the calibration curve, the paclitaxel standard was used at 1.5, 3, 6, 12, and 24 ppm. Data acquisition and integration were performed with Millennium 32 software.

## **2. 5. RNA extraction, cDNA synthesis, and Real-Time PCR analysis**

The cDNA was synthesized using 1 µg of total RNA extracted from the frozen leaves (Channuntapipat et al. 2001) and MMLV-RT (Thermo Fisher Scientific). EvaGreen Real-Time PCR master mix, diluted cDNA, and 100 µM of F and R primers were utilized in a 96-well platform of BioRad (USA) instrument. The Real-Time PCR parameters were as follows: 95°C for 15 min and 40 cycles at 95°C for 15 s, 60°C for 20 s, and 72°C for 20 s.

Gene-specific primers were designed employing Oligo 7 software (Supplementary material- Table 2), and the amplification efficiency of each primer pair was calculated using cDNA's serial dilutions (Supplementary material- Table 3). Primer pairs with efficiency ranges of 90-110% were included in the analysis. The obtained data were processed using BioRad CFX Manager software ver. 1.6 (BioRad, USA) and  $(1+Efficiency)^{-\Delta\Delta Ct}$  formula (Livak and Schmittgen 2001). The relative expression levels were normalized with respect to the expression level of *GAPDH* as a reference gene compared to the no-inoculation/no-elicitation control, 4 h after the elicitation time (reference value = 1).

Table 2

The results of relative expression level evaluation, the highest relative expression level, and its related treatment and time

Gene Name	The Highest Relative Expression Level	Treatment of the Highest Relative Expression Level	Time of the Highest Relative Expression Level
T13αH	2.57	pCAM- <i>DBTNBT</i> /CD+COR	4 h after dual elicitation
T14βH	0.66	pCAM	16 h after dual elicitation time
DBAT	5.63	pCAM- <i>DBTNBT</i> /CD+COR	16 h after dual elicitation
BAPT	6.34	pCAM- <i>DBTNBT</i> /CD+COR	16 h after dual elicitation
DBTNBT	13.93	pCAM- <i>DBTNBT</i> /CD+COR	24 h after dual elicitation
ABC	13.04	pCAM- <i>DBTNBT</i> /CD+COR	16 h after dual elicitation
pCAM, inoculation with <i>Agrobacterium</i> harboring pCAMBIA1304 vector- no elicitation; pCAM- <i>DBTNBT</i> /CD+COR, inoculation with <i>Agrobacterium</i> harboring pCAMBIA1304- <i>DBTNBT</i> vector- elicitation with methyl- β- cyclodextrin (CD) and coronatine (COR)			

## 2. 6. Statistical analysis

Statistical analyses were performed using SPSS ver. 16 after ensuring the data normality. All the data were calculated as the average of at least two biological and two technical replicates ± SE. Completely randomized design (CRD) and a factorial experiment in a completely randomized design (CRD) were applied to evaluate paclitaxel contents and gene expression levels. All the analyses were followed by LSD mean comparison tests (P-value < 0.01).

## 3. Results

### 3. 1. Cloning and transformation of overexpression vector

The transformation of pUC57-*DBTNBT* and pCAMBIA1304 into *E. coli* (DH5 $\alpha$ ) (*Enterobacteriaceae*) competent cells was confirmed using colony PCR reaction (Supplementary material- Fig. 3 and Fig. 4). The double digestion reactions with *SpeI*/*BstEII* restriction enzymes confirmed the accuracy of pUC57-*DBTNBT* and pCAMBIA1304 vectors (Supplementary material- Fig. 5 and Fig. 6). The bacterial colonies harboring pCAMBIA1304-*DBTNBT* (pCAMBIA-*DBTNBT*) vector were confirmed using colony PCR reaction (Supplementary material- Fig. 7). Further validations on the vectors extracted from positive colonies were carried out via *SpeI*/*BstEII* (Supplementary material- Fig. 8), *XhoI* (Supplementary material- Fig. 9), and *SalI* (Supplementary material- Fig. 10) enzymes. In addition, the results of pCAMBIA-*DBTNBT* sequencing using CaMV 35S (CA+2) forward and NOS reverse primers verified the identity of the inserted sequence with the *DBTNBT* CDS of *T. canadensis* (AF466397.1). In this CDS, an ORF (1326 bp) was detected, which coded a 441-amino acid protein. Furthermore, the colony PCR confirmed the transformation of pCAMBIA-*DBTNBT* and pCAMBIA1304 vectors into the *Agrobacterium* competent cells (Supplementary material- Fig. 11 and Fig. 12).

### **3. 2. Production of total paclitaxel**

The highest amount of total and cell-associated paclitaxel (140 and 118  $\mu\text{g g}^{-1}$ ) belonged to the treatment of pCAM-*DBTNBT*/CD+COR 17 days after the elicitation (Fig. 2 and Supplementary material- Fig. 13). The transient overexpression of the *DBTNBT* gene, associated with the dual elicitation, culminated in 7.4-fold more paclitaxel production compared to the no-inoculation/no-elicitation control. Among the investigated treatments (pCAM-*DBTNBT*/CD+COR, pCAM, CD+COR, and no-inoculation/no-elicitation control), the highest increase in the total paclitaxel belonged to the pCAM-*DBTNBT*/CD+COR followed by CD+COR and pCAM, respectively. Moreover, the total amounts of cell-associated paclitaxel in all the treatments were higher than those of the extracellular ones. The pCAM-*DBTNBT*/CD+COR and CD+COR treatments resulted in more extracellular paclitaxel amounts on account of the increased secretion of paclitaxel caused by the function of CD (Sabater-Jara et al. 2014) and the prevention of paclitaxel degradation.

### **3. 3. Evaluation of gene expression**

The expression level of *T13aH*, *T14 $\beta$ H*, *DBAT*, *BAPT*, *DBTNBT*, and *ABC* genes were evaluated in different treatments (pCAM-*DBTNBT*/CD+COR, pCAM, CD+COR, and no-inoculation/no-elicitation control). Based on the results, a significant difference was observed among pCAM-*DBTNBT*/CD+COR, pCAM, CD+COR, and no-inoculation/no-elicitation control (Fig. 3 and Table 2).

Concerning the time-course expression profile of *T13aH* (responsible for the formation of taxa-4(20), 11(12)-dien-5 $\alpha$ -13 $\alpha$ -diol), the highest increase in the expression level was observed in the pCAM and pCAM-*DBTNBT*/CD+COR with no significant differences in most of the investigated times and CD+COR ranked second (Fig. 3 and Table 2).

A remarkable feature in the expression level of the *T14 $\beta$ H* gene (in charge of forming taxa-4(20), 11(12)-dien-5 $\alpha$ -acetoxy-10 $\beta$ -14 $\beta$ -diol) was its decreased expression level in all the treatments in comparison with

the no-inoculation/no-elicitation control. The highest reduction in the expression level of *T14βH* belonged to CD+COR treatment, followed by pCAM-*DBTNBT*/CD+COR and pCAM treatments (Fig. 3 and Table 2).

Regarding the expression level of *DBAT* gene (responsible for hydroxylation at the C10 position of the 10-deacetylbaaccatin), pCAM-*DBTNBT*/CD+COR ranked first among the treatments, followed by pCAM and CD+COR (Fig. 3 and Table 2).

Concerning the *BAPT* gene (in charge of catalyzing the conjugation of the β-phenylalanoyl-CoA side-chain to baaccatin III), the highest increase in the expression level among the investigated treatments belonged to the pCAM-*DBTNBT*/CD+COR, followed by pCAM and CD+COR treatments, respectively (Fig. 3 and Table 2).

Compared to the no-inoculation/no-elicitation control, in the pCAM-*DBTNBT*/CD+COR, pCAM, and CD+COR treatments, the expression level of *DBTNBT* gene (responsible for the conjugation of benzoyl-CoA to 3'-N-debenzoyl-2'-deoxytaxol) increased by 14, 3.9, and 3.6 times, respectively (Fig. 3 and Table 2).

As far as *ABC* gene (responsible for paclitaxel secretion) is concerned, pCAM-*DBTNBT*/CD+COR ranked first, followed by pCAM and CD+COR, respectively (Fig. 3 and Table 2).

## 4. Discussion

Cell suspension culture is known as one of the most adaptable methods for taxol production in *Taxus* species, in which the amounts of the produced paclitaxel can be expanded to a limited extent using different elicitors. This constraint is closely related to the function of rate-limiting enzymes (Exposito et al. 2010; Ho et al. 2005). In this study, overexpression of the *DBTNBT* gene together with CD and COR dual elicitation compared to the other treatments (pCAM, CD+COR, and no-inoculation/no-elicitation control) led to some impacts on the expression level of paclitaxel involved genes and taxol production.

The expression profile of *T13aH* indicated that the combined use of *DBTNBT* overexpression and dual elicitation with CD and COR did not significantly affect the *T13aH* expression level, probably because bottleneck removal affects the expression level of genes involved in the downstream region of the paclitaxel biosynthetic pathway.

Comparing the *T13aH* fold-changes in pCAM-*DBTNBT*/CD+COR and pCAM treatments showed that the relative increase in the expression level was probably due to *Agrobacterium* inoculation, not the increased expression level of *DBTNBT* gene. This finding is in accordance with that reported in the research conducted on the overexpression of *TXS* in *Taxus* cells inoculated by *A. rhizogenes* (Exposito et al. 2010).

The relative expression level of *T14βH* showed a decrease, which is considered to be highly favorable because of the essential role of the *T14βH* gene in the paclitaxel biosynthetic pathway towards undesirable taxanes (Li et al. 2011).

The expression profile of the *DBAT* gene indicates that this gene's expression level is affected by the impacts of inoculation with *Agrobacterium* containing pCAMBIA1304 and the *Agrobacterium* containing pCAMBIA-*DBTNBT* vector. The comparative analysis of the treatments showed that the increase in *DBAT* expression level was partly because of *Agrobacterium* inoculation.

According to the results, CD and COR were not highly effective in increasing the expression level of *DBAT* gene. This is consistent with the results of other studies in which the expression level of *DBAT* gene was not significantly affected by the effects of the elicitors (Kashani et al. 2018; Onrubia et al. 2011; Sah et al. 2019). However, the inoculation with *Agrobacterium* harboring pCAMBIA1304 proved to be effective in removing the *DBAT* bottleneck. Comparing the results of this research to those of other studies, using potent elicitors, such as CD and COR, revealed the potential of *Agrobacterium* in the relative removal of the *DBAT* bottleneck. This finding is in line with the results of Exposito et al., who concluded that the T-DNA of the *A. rhizogenes* (wild type) was able to significantly increase the expression level of *TXS* gene along with taxane production in *Taxus* cells (Exposito et al. 2010).

The *BAPT* expression level increased (up to 6.4-fold) in pCAM-*DBTNBT*/CD+COR treatment. Nonetheless, the inoculation by *Agrobacterium* containing the pCAMBIA1304 vector resulted in a 4.9 fold-change in the expression level of the *BAPT* gene. Elicitation by CD and COR showed no remarkable increase in the expression level of this gene; thus, it could be concluded that the *Agrobacterium* transformation may be a powerful alternative (even more potent than CD and COR elicitation) for inducing *BAPT* expression in *T. baccata* leaves. Meanwhile, the higher increase in *BAPT* expression level in *T. baccata* leaves, inoculated with pCAMBIA-*DBTNBT* vector rather than pCAMBIA1304, confirmed that the *DBTNBT* gene is one of the main bottlenecks in the paclitaxel biosynthetic pathway because *DBTNBT* overexpression increases the expression level of other downstream-involved genes, such as *BAPT*. Unquestionably, the increase in paclitaxel amounts caused by *DBTNBT* overexpression in *T. baccata* confirmed this hypothesis.

The results also indicated that the combined use of CD and COR was not highly effective in increasing the expression level of *BAPT* gene. These findings are in accordance with those of previous studies (Kashani et al. 2018; Ramirez-Estrada et al. 2015), in which *BAPT* was recognized as a bottleneck gene in paclitaxel biosynthetic pathway. The results of the current experiment, in line with those of another study (Exposito et al. 2010), revealed that *Agrobacterium* harboring pCAMBIA1304 was much more effective than CD and COR regarding the relative removal of *BAPT* bottleneck.

The results implied that the overexpression of *DBTNBT* gene associated with dual elicitation compared to the *Agrobacterium* inoculation had higher effects on the expression level of *ABC* gene. Therefore, the inoculation of *T. baccata* leaves with *Agrobacterium* could be considered as an incredibly effective approach to increasing the expression level of the *ABC* gene. CD and COR were not able to significantly increase the *ABC* expression level, which is consistent with the results of previous studies (Kashani et al. 2018; Sabater-Jara et al. 2010).

The results of *DBAT*, *BAPT*, and *ABC* expression level indicated that the combined use of transient overexpression and CD and COR dual elicitation was much more effective than that of individual

elicitation. This result is in line with the findings of other researchers (Exposito et al. 2010; Ho et al. 2005).

To sum up, the expression levels of *DBAT*, *BAPT*, and *ABC* genes significantly increased because of the *Taxus* leaves inoculation via *Agrobacterium* harboring no paclitaxel pathway involved gene. This result is probably due to the role of *Agrobacterium* as a pathogen and the function of paclitaxel in *Taxus* spp. defense mechanism (Bentebibel et al. 2005; Collin 2001; Li et al. 2012; M. Cusidó et al. 2002; Onrubia. et al. 2013). Furthermore, the amount of paclitaxel produced in the CD+COR treatment, despite the lower expression level of paclitaxel involved genes compared with the pCAM treatment, ranked second. It was presumably due to the elicitation effects of CD and COR (Kashani et al. 2018; Ramirez-Estrada et al. 2015) in addition to the role of CD in the complex formation with hydrophobic compounds and the secretion of cell-derived paclitaxel (Bru et al. 2006; Ramirez-Estrada et al. 2015; Ramirez-Estrada et al. 2016). The paclitaxel measurement indicated that although the increased relative expression level of the *ABC* gene augmented the extracellular paclitaxel amounts, the increase was not significant and that the elicitation with CD played a vital role in paclitaxel secretion (Fig. 2 and Fig. 3).

Based on the results, the *DBTNBT* overexpression probably leads to related substrate catalysis; this seemingly reduces the feedback repression of this substrate on the *TBT*, *CoA Ligase*, and *BAPT* genes, resulting in the production of more paclitaxel (Cusido et al. 2014). The CD complex formation with paclitaxel and the increased expression level of *ABC* gene culminated in paclitaxel secretion to the extracellular medium, reduced paclitaxel feedback repression on the upstream genes, and the inhibition of extracellular paclitaxel degradation.

## 5. Conclusions

*DBTNBT* overexpression associated with CD and COR elicitation contributed to much more paclitaxel production and the prevention of feedback repression on the upstream bottleneck genes, such as *DBAT* and *BAPT*. The results of this study revealed that *Taxus* metabolic engineering, along with elicitation, is able to produce noticeable amounts of paclitaxel to meet its increasing demand in the foreseeable future.

## Abbreviations

**MeJA:** Methyl jasmonate; **COR:** Coronatine; **CD:** Methyl- $\beta$ -cyclodextrin; **GAPDH:** *Glyceraldehyde-3-phosphate dehydrogenase*; **TXS:** *Taxadiene synthase*; **T13aH:** *Taxane 13 $\alpha$ -hydroxylase*; **T14 $\beta$ H:** *Taxane 14 $\beta$ -hydroxylase*; **TBT:** *Taxane-2 $\alpha$ -O-benzoyl transferase*; **DBAT:** *10-deacetylbaaccatin III-10-O-acetyltransferase*; **BAPT:** *Baccatin III-3-amino 13-phenylpropanoyl- CoA transferase*; **DBTNBT:** *Debenzoyltaxol N-benzoyl transferase*; **PAM:** *Phenylalanine amino mutase*; **ABC:** *ATP-binding cassette*; **ORF:** Open Reading Frame; **GFP:** *Green fluorescent protein*; **GUS:**  *$\beta$ -glucuronidase*; **OD:** Optical Density; **SE:** Standard Error; **CRD:** Completely Randomized Design; **LSD:** Least Significant Difference; **CDS:** Coding Sequence; **CaMV 35S Promoter:** Cauliflower Mosaic Virus Promoter; **NOS Terminator:** Nopaline Synthase Terminator; **cDNA:** Complementary DNA; **HPLC:** High- Performance Liquid Chromatography; **RP:** Reverse Phase; **F:** Forward Primer; **R:** Reverse Primer

# Declarations

## ***Author's contribution statement***

All authors contributed to the study's conception and design. Kimia Kashani performed material preparation, data collection, and analysis. She wrote the first draft of the manuscript and the other authors (Mohammad Sadegh Sabet, Mokhtar Jalali Javaran, and Ahmad Moieni) commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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## ***Conflicts of interest/ Competing interest***

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## ***Ethical approval***

Not applicable.

## ***Availability of data and materials***

Data generated or analyzed during this study was included in this paper.

## ***Code availability***

Not applicable.

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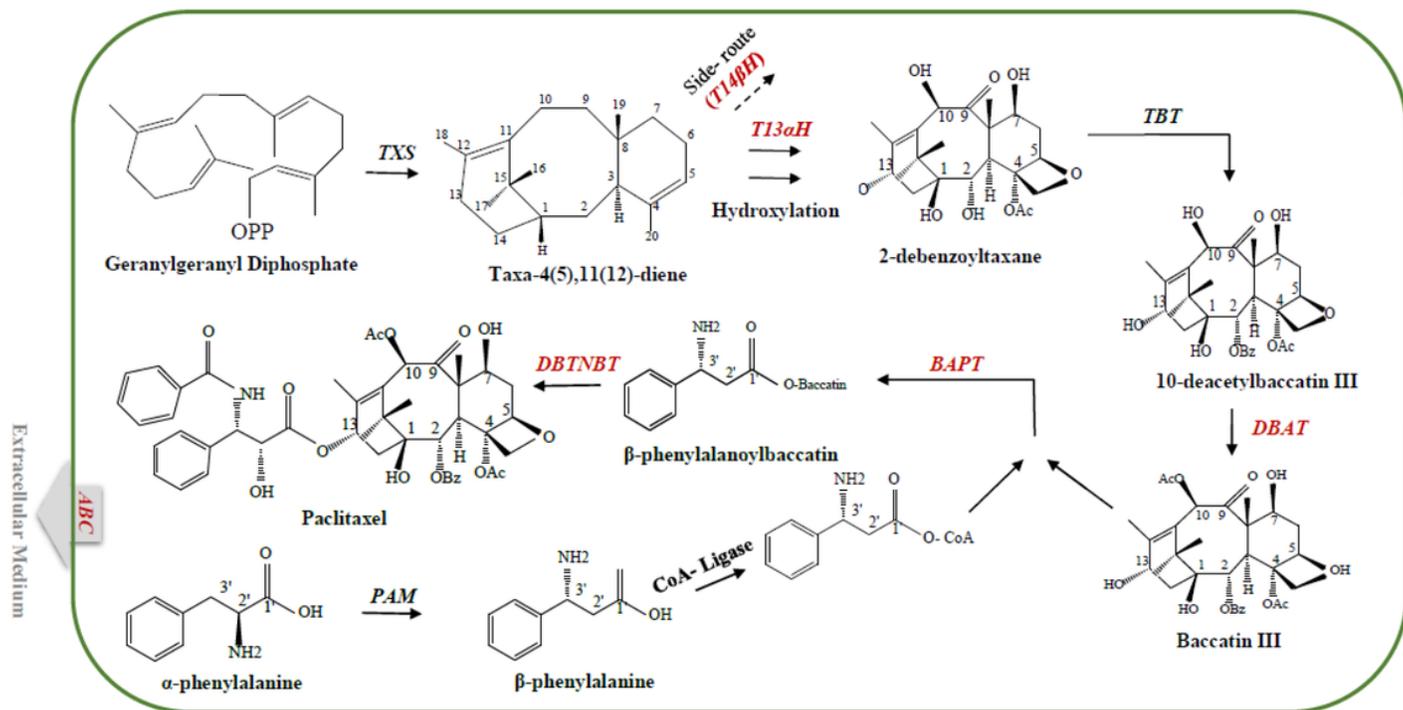
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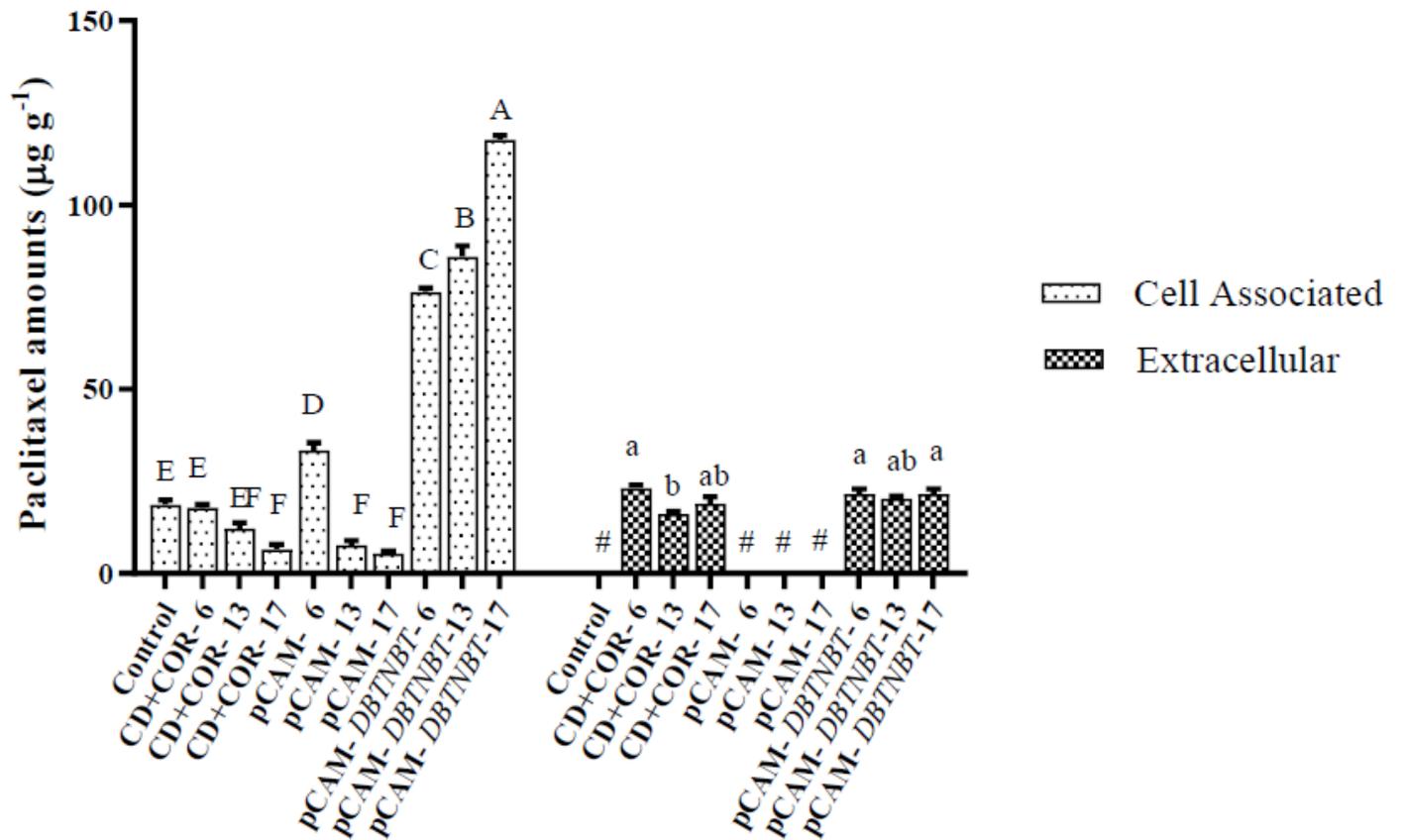
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## Figures



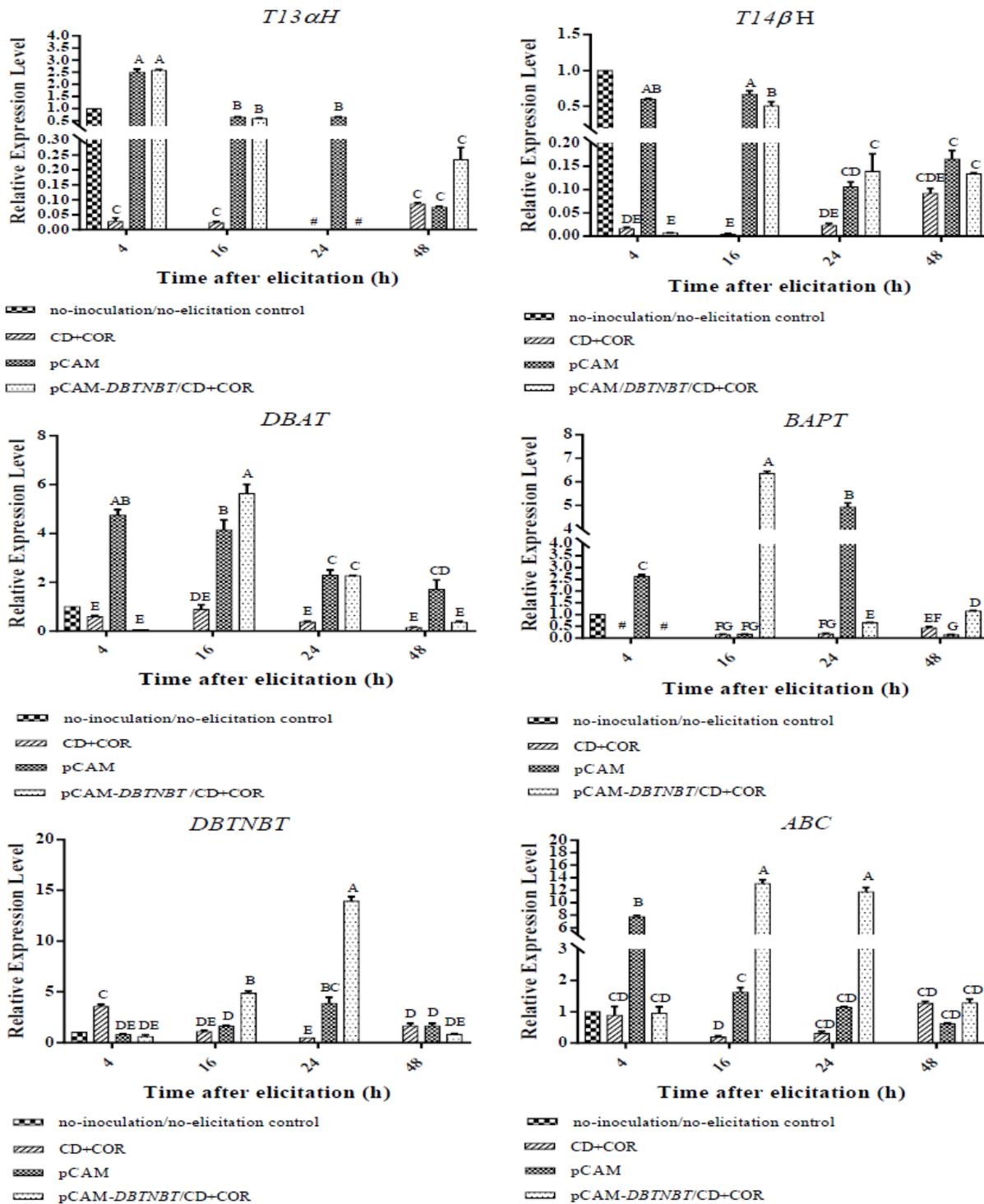
**Figure 1**

A summarized paclitaxel biosynthesis pathway TXS, taxadiene synthase; T13 $\alpha$ H, taxane 13 $\alpha$ -hydroxylase; T14 $\beta$ H, taxane 14 $\beta$ -hydroxylase; TBT, taxane-2 $\alpha$ -O-benzoyl transferase; DBAT, 10-deacetylbaccatin III-10-O-acetyltransferase; BAPT, baccatin III-3-amino 13-phenylpropanoyl- CoA transferase; DBTNBT, debenzoyltaxol N-benzoyl transferase; PAM, phenylalanine amino mutase; CoA Ligase, and ABC, ATP- binding cassette. Colored gene names point out to the genes that have been investigated in this research



**Figure 2**

The comparison of cell-associated and extracellular paclitaxel amounts in *Taxus baccata* leaves. The value of each column represents the average of at least two biological and two technical replications  $\pm$  SE. Upper and lower cases represent the results of LSD mean comparison test ( $P$ -value  $< 0.01$ ) performed on cell-associated and extracellular paclitaxel contents in different treatments, respectively. Means with at least one common letter did not show any significant difference. #) not detected. Different treatments were as follows: Control, no-inoculation/no-elicitation control; CD+COR- 6, 13, and 17, no inoculation-elicitation with methyl-  $\beta$ - cyclodextrin (CD) and coronatine (COR) 6, 13, and 17 days after elicitation; pCAM- 6, 13, and 17, inoculation with *Agrobacterium* harboring pCambia1304 vector- no elicitation (equivalent to 6, 13, and 17 days after elicitation); pCAM-DBTNBT- 6, 13, and 17, inoculation with *Agrobacterium* harboring pCambia1304-DBTNBT vector- elicitation with methyl-  $\beta$ - cyclodextrin (CD) and coronatine (COR) 6, 13, and 17 days after elicitation



**Figure 3**

The relative expression levels of T13αH, T14βH, DBAT, BAPT, DBTNBT and ABC genes in *Taxus baccata* leaves treated differently, including no-inoculation/no-elicitation control, CD+COR (no inoculation-elicitation with methyl- β- cyclodextrin (CD) and coronatine (COR)), pCAM (inoculation with *Agrobacterium* harboring pCambia1304 vector- no elicitation), and pCAM-DBTNBT/CD+COR (inoculation with *Agrobacterium* harboring pCambia1304-DBTNBT vector- elicitation with methyl- β- cyclodextrin (CD))

and coronatine (COR)) Horizontal axis) Times after elicitation by CD and COR. Vertical axis) The expression level of T13αH, T14βH, DBAT, BAPT, DBTNBT, and ABC genes relative to the expression level of GAPDH as a reference gene compared to the no-inoculation/no-elicitation control, 4 h after elicitation time. #) not detected. Each column's value represents the mean of at least two biological and two technical repetitions ± SE. The letters represent the results of LSD mean comparison test (P-value < 0.01) performed on different treatments, and means with at least one common letter did not show any significant difference

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