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Changes in gene transcription and taxane production in elicited cell cultures of *Taxus* × *media* and *Taxus globosa*

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ABSTRACT

The response of two *Taxus* cell systems to the action of cyclodextrin (CD) and coronatine (CORO), supplied to the culture medium either separately or together, was studied. Two-stage *Taxus globosa* and *Taxus media* cell cultures were established and the elicitors were added at the beginning of the second stage. Growth, taxane production, and the expression of known taxol biosynthetic genes, including the recently characterized *CoA ligase* gene, were studied. Although CORO reduced the growth capacity of both cell lines, CD apparently counteracted this negative effect. Taxane production was significantly enhanced by the simultaneous addition of CD and CORO to the medium. The total taxane production in the *T. media* cell line was more than double that of *T. globosa*, but in the latter more than 90% of the taxanes produced were excreted to the medium. Individual taxane patterns also differed: at the height of production, the main taxanes in *T. globosa* cultures were cephalomannine and 10-deacetyltaxol, and in *T. media*, taxol and baccatin III. The low transcript levels of taxane biosynthetic genes found in *T. globosa* cells mirrored the lower taxane production in these cultures, while a high expression was strongly correlated with a high taxane production in *T. media*.

1. Introduction

Plant *in vitro* cultures are regarded as an attractive and cost-effective alternative for plant secondary metabolite (PSM) production. Among their several advantages, these "green cell factories" constitute the only sustainable and eco-friendly system for the production of complex chemical structures biosynthesized by rare or endangered plant species that resist domestication (Georgiev et al., 2009). Plant *in vitro* cultures also serve as a useful experimental platform for gaining new insights into PSM biosynthesis and regulation (Cusido et al., 2014). Notable among PSM with important biological activities is taxol and its derivatives, currently being used as anticancer agents.

Taxol, known by the generic name of paclitaxel, is one of the most effective anticancer drugs ever developed (Abal et al., 2003). The natural source of this complex diterpene alkaloid is the bark of several *Taxus* species, but the cost of its extraction is prohibitively high, since it accumulates in a very low concentration (about 0.02% of dry weight), and harvesting entails the destruction of yew trees (Vidensek et al., 1990). Consequently, the constantly growing demand for taxol and its derivatives cannot be met by isolation from their natural source, and alternative ways of producing this drug are actively being sought (reviewed in Expósito et al., 2009).

The biotechnological production of taxol and related taxanes in *Taxus* spp. cell cultures (TCC) has been empirically optimized, from a small scale to bioreactor level (Cusidó et al., 2002; Bentebibel et al., 2005). Metabolic engineering techniques have also been employed to obtain cell cultures overexpressing genes of the taxol biosynthetic pathway (Exposito et al., 2010; Zhang et al., 2011). With the aim of exploring how the different taxane production-boosting factors also affect gene expression and metabolic profiles in TCC, several rational studies have focused on the molecular bioprocesses in the producer cells (Croteau et al., 2006; Nims et al., 2006; Onrubia et al., 2010, 2011; Sabater-Jara et al., 2014).

All these studies have shown that the addition of elicitors is crucial for obtaining a high taxane accumulation in *Taxus* spp. cell cultures. Recently, coronatine (CORO), a toxin produced by *Pseudomonas syringae* (Bender et al., 1999) with a potential role as a plant growth regulator, was demonstrated to be a potent

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new elicitor (Katsir et al., 2008; Tamogami and Kodama, 2000; Haider et al., 2000; Onrubia et al., 2013b). Also of interest for PSM-production systems is cyclodextrin (CD), which, due to its chemical structure, can form inclusion complexes with poorly hydrosoluble apolar compounds, thus facilitating their excretion from cells and their isolation from the culture medium (Cai et al., 2012). CD has also attracted attention for its capacity to induce defense responses in plant cell cultures, thereby acting as a true elicitor (Bru et al., 2006; Lijavetzky et al., 2008). The action of CD, alone or together with methyl jasmonate (MeJA), has already been studied in *Taxus media* cell cultures (Sabater-Jara et al., 2014). In this case, the presence of CD in cell cultures elicited with MeJA dramatically enhanced the taxol production, which was 31-fold higher than in the non-elicited cultures, whereas the addition of MeJA alone increased production approximately 4-fold. The effectiveness of CORO as an elicitor was also shown, as it enhanced taxol production up to 10-fold (Onrubia et al., 2013b). Further studies were then required to explore the combined effect of the potent elicitor CORO together with the elicitor/excretion activator CD in *Taxus* cell suspensions.

Despite their biological and economic importance, taxane biosynthetic pathway and its regulation remains incompletely described. As a diterpene, taxol is formed from geranylgeranyl diphosphate (GGPP), and subsequent to the formation of GGPP, 19 enzymes are known to be involved (Croteau et al., 2006; Vongpaseuth and Roberts, 2007). After the cyclization of GGPP to taxadiene by taxadiene synthase (TXS), the path toward taxol requires the action of eight hydroxylases, five acyl/aroyl transferases, and one aminomutase, and also includes one epoxidation, one oxidation, two CoA esterifications and an N-benzoylation (Fig. 1). While most of the taxane biosynthetic pathway has been elucidated, and the genes encoding the respective enzymes cloned, six steps are still without an assigned gene (Onrubia et al., 2011).

In order to gain new insights into these unknown steps, the transcript profiling in a MeJA-elicited *Taxus baccata* cell line has been studied by cDNA-amplified fragment length polymorphism (AFLP) and correlated with taxane production (Onrubia et al., 2014). The transcript tags generated by this methodology have proved very useful to identify genes potentially involved in the taxol biosynthetic pathway. When cDNA-AFLP analysis was combined with *in silico* studies, several candidate genes were identified for the different uncharacterized enzymes, including a CoA transferase. In recent functional studies, we have confirmed that this enzyme is a new β-phenylalanine CoA ligase involved in the formation of the taxol side chain (Ramirez-Estrada et al., 2015).

Several *Taxus* plant species have been used for biotechnological taxane production, including *T. baccata* (Bentebibel et al., 2005), *T. media* (Liao et al., 2005; Cusidó et al., 2002), *Taxus cuspidata* (Nims et al., 2006) and *Taxus chinensis* (Hu et al., 2006). Recently, plant cell cultures of *Taxus globosa* (Mexican yew) have also been proposed as a potential new source of taxanes, despite achieving a lower production than other *Taxus* species prior to optimization (Tapia et al., 2013).

The main aim of this work was to improve taxane production in a new biotechnological platform based on *T. globosa* cell cultures by optimizing the elicitor treatment. To achieve this goal, two

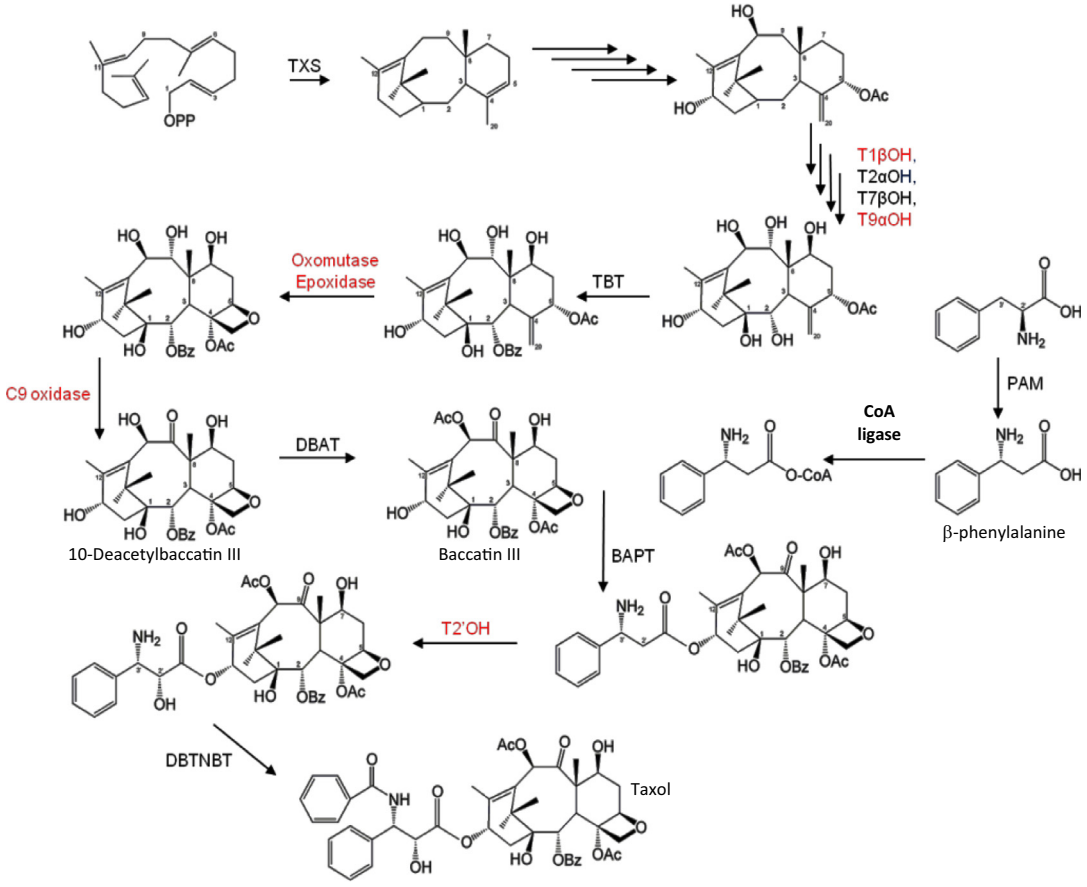

Fig. 1. A summarized scheme of taxol biosynthesis. TXS, taxadiene synthase, T1βOH, taxane 1β-hydroxylase, T2αOH, taxane 2α-hydroxylase, T7αOH, taxane 7α-hydroxylase, T9αOH, taxane 2α-hydroxylase, TBT, taxane-2α-O-benzoyl transferase, DBTA, 10-deacetylbaccatin III-10-O-acetyltransferase, BAPT, baccatin III-3-amino, 13-phenylpropanoyl-CoA transferase, T2′OH, taxane 2′-hydroxylase, DBTNBT, debenzoyltaxol N-benzoyl transferase. Known genes in red. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

potent new elicitors were tested for the first time in this plant species: CORO and CD, either separately or in a combined treatment. The individual effectiveness of both elicitors, as mentioned above, has been demonstrated in studies with other *Taxus* species but until now never in combination. Additionally, we compared the results in *T. globosa* with those obtained in *T. media* cell cultures, a system better characterized at the molecular level. Our ultimate objective was to study how this treatment affects the expression of key genes in taxane biosynthesis, including the recently characterized β-*penylanine CoA ligase* gene, and to gain molecular knowledge of this metabolic pathway and its regulation in cell cultures of *Taxus* spp.

2. Results and discussion

2.1. Effect of the elicitor treatment on the growth capacity of the cell cultures

T. media and *T. globosa* cell lines were cultured in a two-stage system (Cusidó et al., 2002; Tapia et al., 2013). Cell suspensions of both lines were grown for 12 days in culture media optimized for high biomass formation (i.e. until the end of the exponential growth phase), and then transferred to the optimized production medium (PM) for 20 days. The elicitors CORO (1 μM), CD (50 mM), or both (1 μM CORO + 50 mM CD) were added to the medium at the beginning of the second stage.

The *T. media* and *T. globosa* cell cultures were established using an optimal inoculum density of 20 g wet weight/L and 5 g wet weight/L, respectively, based on previous results (Sabater-Jara et al., 2014; Tapia et al., 2013). All results were compared with unelicited cultures of the two cell lines.

Biomass of *T. globosa* cell cultures grown in PM in control conditions, measured as DW, increased (up to 2-fold) until day 16, thereafter decreasing slightly until the end of the culture at day 20 (Fig. 2a). A similar growth pattern was observed in *T. media* but with a lower increase in cell biomass (1.4-fold at day 16; Fig. 2b). The biomass peaked in both cultures at 16 days of growth in the optimized PM.

The addition of CORO (1 μM) to the liquid media decreased the cell growth capacity, especially at the end of the culture period ($p < 0.05$). This effect was particularly observed in *T. globosa* cells, whose growth was reduced by 53% compared with 20% in *T. media* cells. The elicitors CORO and MeJA are known to have a detrimental effect on the growth capacity of cells (Sabater-Jara et al., 2014; Onrubia et al., 2013b).

In contrast, CD (100 mM) increased the biomass 1.2-fold, measured as DW, in both cell lines. It has been shown that CD does not significantly affect growth capacity in *Vitis vinifera* and *T. media* cell cultures (Belchí-Navarro et al., 2012; Sabater-Jara et al., 2014, respectively). Finally, in combined treatments, the addition of CD apparently counteracted the negative effects of CORO on cell growth in both cell lines (Fig. 2), with growth in *T. globosa* and *T. media* being reduced by 22% and 14%, respectively, after 20 days, in relation to the control.

2.2. Taxane production in the elicited cell cultures

As shown in Fig. 3a, the highest total taxane production in *T. globosa* cell cultures was achieved at the end of the experiment, after 16–20 days of elicitation. Most of the taxanes were released to the medium, the cell-associated taxanes never representing more than 10% of the total production, under any conditions (Fig. 3b). Studies carried out with different cell lines of diverse *Taxus* species have found a considerable variation in the capacity of producer cells to excrete taxol to the medium, ranging from 10% to 90% (Naill et al., 2012). The mechanism of excretion is known to require metabolic energy, but has not been resolved to date.

The most effective elicitor treatment for enhancing taxane production in *T. globosa* proved to be the combined addition of CORO and CD (Fig. 3a), the peak yield being over 40-fold higher than in the control. In comparison, the application of CORO and CD separately induced a total taxane production only 5.3- and 1.6-fold higher, respectively. This pattern was observed throughout the experiment, although production was lower in the other samples harvested.

In *T. media* cell cultures, the most effective treatment was also CD + CORO, although in this case the highest total taxane production was achieved after 12–16 days of elicitation (Fig. 3a). At day 16, the total taxane production obtained in the cell suspensions treated with CD + CORO, CORO, or CD, was 17.6-, 2.6- and 1.5-fold higher, respectively, than in the control. When applying the elicitors separately, contrary to the pattern observed in *T. globosa*, production in *T. media* cell cultures at the end of the experiment was more induced by CORO than by CD. The variable response of cell cultures to elicitation depends not only on factors related to the elicitor itself (type, concentration, duration of elicitation, etc.), but also on the species, cell line and state of development of the culture (Onrubia et al., 2010).

Notably, throughout the experiment, the total taxane production of CD + CORO-treated cell cultures was higher than the

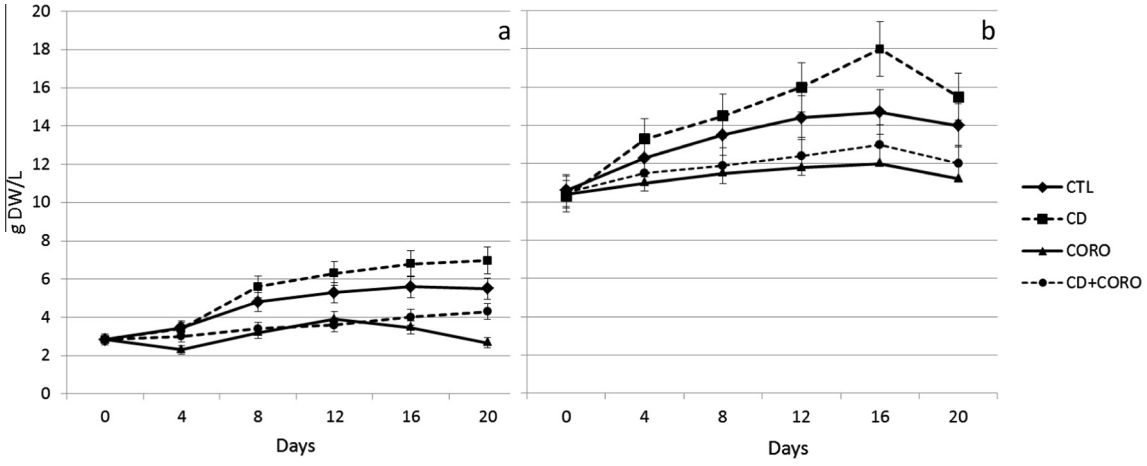

Fig. 2. Time course of growth measured as g DW/L of the *T. globosa* (a) and *T. media* (b) cell lines in a culture period of 20 days in optimized production media. Each value is the average of 3 biological replicates ± SE.

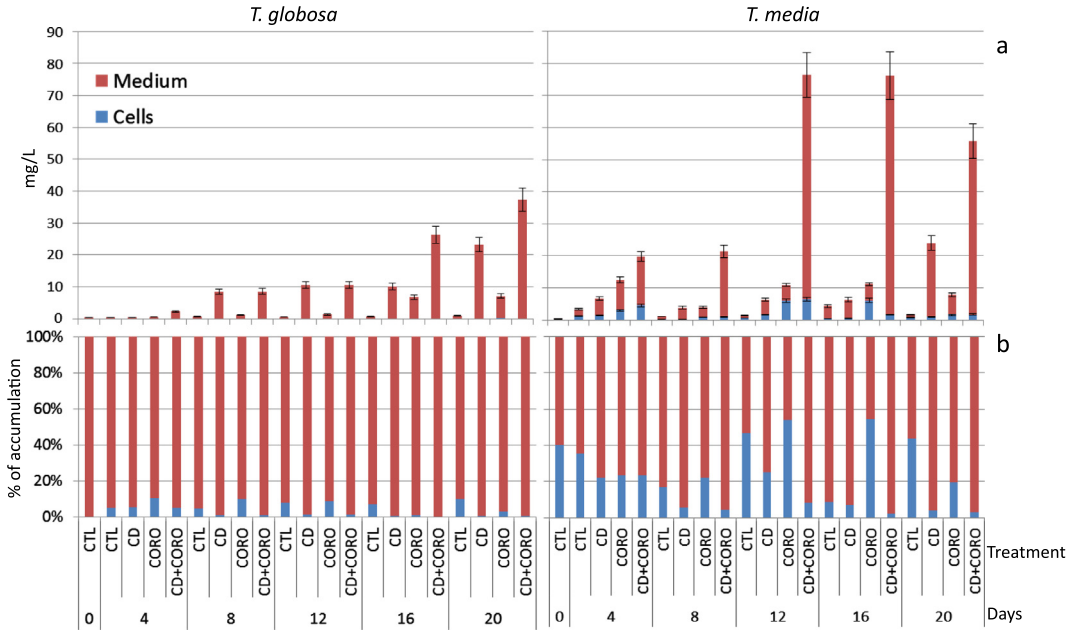

Fig. 3. Total taxane contents (cell-associated and extracellular) in *T. globosa* and *T. media* cell suspensions grown for 20 days in control conditions (CTL) and with the addition of CD (cyclodextrin), CORO (coronatine), or both elicitors together. (a) Total taxane contents expressed as mg/L. (b) Proportion of the taxanes determined, expressed as a percentage of the total taxane content. Data are the mean of three independent replicates ± SD.

combined yield of the two treatments applied separately. After 12 and 16 days of elicitation, in the CD + CORO-treated cell cultures levels were more than 4-fold higher than the sum of taxanes accumulated under the two separate treatments. These results suggest a possible synergistic effect of the two elicitors on taxane production, as previously observed with CD and MeJA in *T. media* cell cultures (Sabater-Jara et al., 2014).

When considering the cell capacity to excrete taxanes to the medium (Fig. 3b), treatment with CD, either alone or, to a lesser extent, in combination with CORO, induced a high release, especially when the taxane production was high (from 12 to 20 days of culture). In contrast, control and CORO-treated cell cultures retained up to 50% of the taxanes produced inside the cells. Cyclodextrins have recently attracted considerable attention not only for their elicitation capacity in plant cell cultures, the result of inducing defense responses (Bru et al., 2006; Lijavetzky et al., 2008), but also for their ability to facilitate the excretion of metabolites with low hydrosolubility from cells (Cai et al., 2012).

The individual taxanes studied, both cell-associated and extracellular, were taxol (T), cephalomannine (CF), 10-deacetyltaxol (DT), baccatin III (B) and 10-deacetylbaccatin III (DB). Their contents in *T. globosa* cell suspensions are shown in Fig. 4a and b, and *T. media* in Fig. 5a and b. The main taxane found in the *T. globosa* cells was DT, although its maximum level never surpassed 2 μg/L, regardless of the conditions; very low amounts of T and CF were also found. The effect of the elicitors on the production of the individual taxanes could not be ascertained due to the scarce quantities found inside the producer cells (Fig. 4a).

The main taxanes accumulated in the medium were those bearing a side chain (CF, DT and T), especially under the conditions most conducive to taxane production (Fig. 4b). At day 16 of elicitation with CD, CORO or CD + CORO, the sum of T, CF and DT was 11-, 5.5-, and 15.2-fold higher, respectively, than of taxanes without a side chain (B and DB). Similar results were obtained at day 20, when the sum of T, CF and DT under the three elicitor treatments was 10.4-, 2.7- and 4.2-fold higher, respectively, than the sum of taxanes without the side chain. The lower ratio between taxanes with and without the lateral chain observed in the CD + CORO-treated cultures at the end of the experiment was mainly due to the high accumulation of B in the medium throughout the culture.

In contrast, in unelicited cultures, the production of B and DB was always higher than the sum of side chain-bearing taxanes (although the quantities were very low). Also noteworthy is that at the beginning of the experiment (days 0 and 4), the main taxane found in the control was B. In *Taxus* cell cultures with a low overall taxane production, either because elicitors have not been added, or have not yet taken effect, the predominance of DB and B has been repeatedly observed (Moon et al., 1998; Nims et al., 2006; Onrubia et al., 2010, 2013a). DB and B, known intermediates in the biosynthesis of taxol and other side chain-bearing taxanes, are formed at an early stage of culture, whereas the attachment of the side chain to B takes place after several days and mainly in the presence of elicitors.

In *T. media* cell cultures (Fig. 5a and b), under all conditions studied, B and taxol were the main cell-associated taxanes, particularly the former; the only exception was at the start of elicitation (day 4), when the main taxane was CF. As mentioned earlier, the excretion capacity of these cells was high, since maximum taxane accumulation inside cells (in CD + CORO-treated cultures 12 days after elicitation, Fig. 5a) represented less than 10% of total taxane production.

The highest taxane levels in the culture medium were obtained with combined CD + CORO elicitation. In cell cultures treated with CORO or CD separately, or unelicited, the maximum taxol in the medium (day 16) represented only 2%, 1.3% and 1.2%, respectively, of the yield obtained after dual elicitation. Similarly, the accumulation of B under these conditions represented only 6%, 9.8% and 7.8%, respectively, of the level achieved with CD + CORO.

In contrast with *T. globosa, T. media* cell cultures showed no significant differences between levels of taxanes with or without a side chain, especially under the most inductive conditions. In fact, the predominant taxane in *T. media* cell cultures was B, except under dual elicitation. Very low amounts of DB, CF and DT were found in the medium throughout the experiment. In previous studies with *T. media* cell cultures, after elicitation with CORO or MeJA, the main taxane obtained has generally been B.

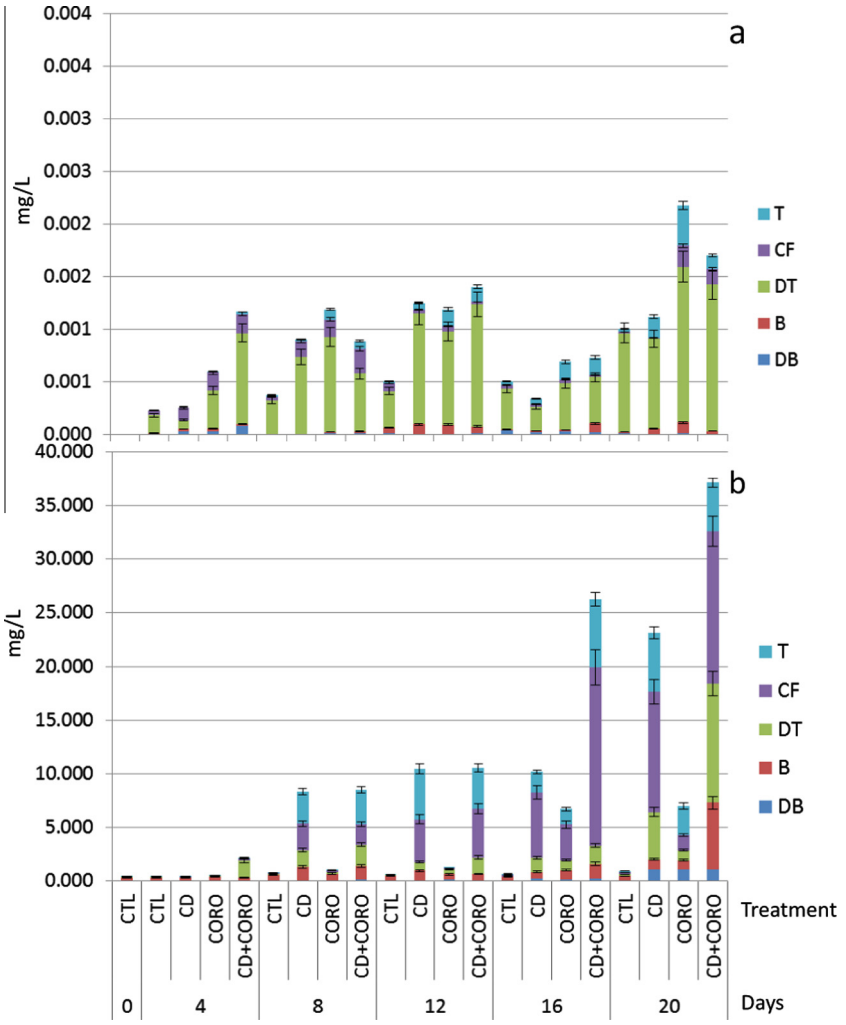

Fig. 4. Individual taxane contents (cell-associated and extracellular) in *T. globosa* cell suspensions grown for 20 days in control conditions (CTL) and with the addition of CD (cyclodextrin), CORO (coronatine), or both elicitors together. (a) Cell-associated taxanes expressed as mg/L. (b) Extracellular taxanes expressed as mg/L. Data are the mean of three independent replicates ± SD.

2.3. Gene expression profiling

To analyze the relationship between the transcript profiles and total and individual taxane production in the *Taxus* cell lines during elicitation, changes in expression levels of *TXS*, *T7βOH*, *DBAT*, *BAPT*, *CoA ligase* and *DBTNBT* genes, which encode enzymes involved in taxol biosynthesis (Fig. 1), were determined by qRT-PCR. Their transcript accumulation was quantified at different time points during 48 h of elicitation (Figs. 6 and 7). Most of these genes have been previously studied in different *Taxus* systems under the action of diverse elicitors, but the expression level of the *CoA ligase* gene has never been determined under the treatments described here, since its involvement in the activation of β-phenylanine to β-phenylanine CoA to form the C13 side chain of taxol has only been reported very recently (Ramirez-Estrada et al., 2015).

In *T. globosa* cell cultures (Fig. 6), the transcript accumulation for these genes was generally very low throughout the 48 h. After two days of elicitation with CD + CORO, the *TXS* gene expression increased to more than 20 times the reference value. Although at very low levels, transcripts corresponding to the *T7OH*, *DBAT* and *CoA ligase* genes were found after 4 h of dual elicitation. Negligible amounts of *BAPT* and *DBTNBT* mRNA were also observed.

It could be inferred that the very low expression of these genes was responsible for the generally low taxane production in the *T. globosa* cell cultures. However, the total taxane production in the cell line after CD + CORO elicitation was higher than 35 mg/L, which is not reflected in the gene expression. Patil et al. (2012), working with MeJA-elicited *T. cuspidata* cell cultures of different cell aggregate size, reported that differences in expression of several taxol biosynthetic genes were minor compared with differences in taxane accumulation. Consequently, there must be other factors controlling taxane biosynthesis besides the expression of the studied genes.

Expression of the *TXS* gene, which controls the first committed step of the taxol biosynthetic pathway (Hezari et al., 1995), was highest in *T. media* cell cultures elicited with CORO, or CD + CORO. Under CORO, *TXS* transcript levels peaked at 12 h after elicitation, decreasing thereafter, although remaining high until 48 h. Maximum *TXS* transcript levels in the CORO-elicited cultures were 18.6-fold greater than in the control, also at 12 h (Fig. 7). Although adding CD to the culture also increased *TXS* gene expression, the effect was not very notable (4-fold higher than the control, at the peak of expression). Dual elicitation also enhanced the accumulation of *TXS* transcript levels (up to almost 12.5-fold that of the control at 12 h), but in this case the peak of expression was achieved after 48 h of the elicitor treatment. The strong expression of the *TXS* gene in the *T. media* cell line in comparison with the untransformed *T. globosa* cultures was not attributed to

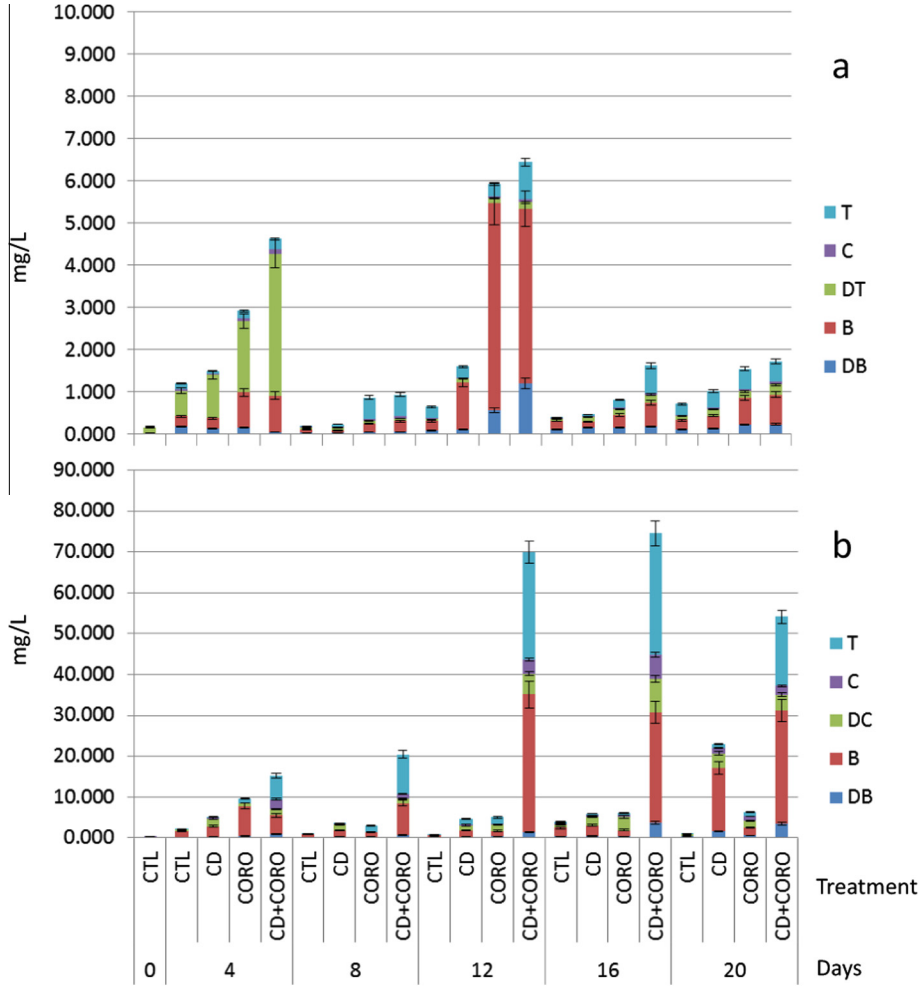

Fig. 5. Individual taxane contents (cell-associated and extracellular) in *T. media* cell suspensions grown for 20 days in control conditions (CTL) and with the addition of CD (cyclodextrin), CORO (coronatine), or both elicitors together. (a) Cell-associated taxanes expressed as mg/L. (b) Extracellular taxanes expressed as mg/L. Data are the mean of three independent replicates ± SD.

its transgenic nature, considering that in previously studied wild-type cell lines of *T. baccata* and *T. chinensis*, the *TXS* transcript levels after 12–16 h increased 60-fold and 12-fold, respectively (Onrubia et al., 2011; Li et al., 2012), in comparison with a mere 5-fold increase in *T. globosa* in the current work. Moreover, after 24 h of elicitor treatment in *T. baccata* cell lines, *TXS* expression increased more than 120-fold (Onrubia et al., 2011) compared to 20-fold in *T. globosa* (Fig. 6). These results confirm that the expression profile of taxane biosynthetic genes depends more on the plant species than the transgenic nature of the studied cell line.

Taxane 7-hydroxylase catalyzes the hydroxylation at the 7C position of the taxane skeleton in one of the intermediate steps of taxol biosynthesis (Chau et al., 2004). The expression of the *T7OH* gene was clearly induced by the addition of CORO or CD + CORO, with little difference between the two treatments, at its peak (at 12 h) being approximately 2 and 3 times higher than in CD-treated and untreated cultures, respectively (Fig. 7).

The same transcription pattern was observed for the *DBAT* gene, which encodes the enzyme transforming DB into B (Walker and Croteau, 2000). The highest *DBAT* gene expression was obtained after 12 h of elicitation with CORO or CD + CORO, when it increased by 268% and 295%, respectively, compared with the control, and 100% and 117.4% compared with CD-treated cultures (Fig. 7).

The three genes, *TXS*, *T7OH* and *DBAT*, participate in metabolic steps leading to B formation (Fig. 1), so their high expression level in the CD + CORO-elicited *T. media* cell cultures could explain the high production levels of B.

As previously indicated, the CoA ligase gene encodes the enzyme that activates β-phenylalanine to β-phenylalanine CoA, before its attachment to B (Ramirez-Estrada et al., 2015). Its expression peaked at 4 h of CD + CORO elicitation, when transcript levels were 3.2-, 8.7- and 6-fold higher than in the cell cultures treated with CORO or CD separately, or without elicitation, respectively. Although the expression of this gene decreased thereafter, the highest transcript levels were always found after dual elicitation (Fig. 7). In previous studies with *T. baccata* cell cultures, we found that MeJA also increased the expression of the *CoA ligase* gene, which peaked at 8 to 24 h of elicitation; after 8 h, the mRNA accumulation was 4-fold that of the control (Ramirez-Estrada et al., 2015). Although the expression level of the *CoA ligase* gene was enhanced by elicitation, it was lower compared to other genes in *T. media* cell cultures, such as several hydroxylases or the *TXS* gene (Onrubia et al., 2013b). We can infer from these results that the *CoA ligase* gene may control a limiting step in the taxol biosynthetic pathway, although further studies are required on this recently elucidated enzymatic activity.

In the final steps of the metabolic pathway. The enzyme baccatin III 13-O-(3-amino-3-phenylpropanoyl) transferase (BAPT) (Walker et al., 2002) is responsible for binding the side chain to B, leading to taxanes with a β-phenylalanine side chain (Fig. 1).

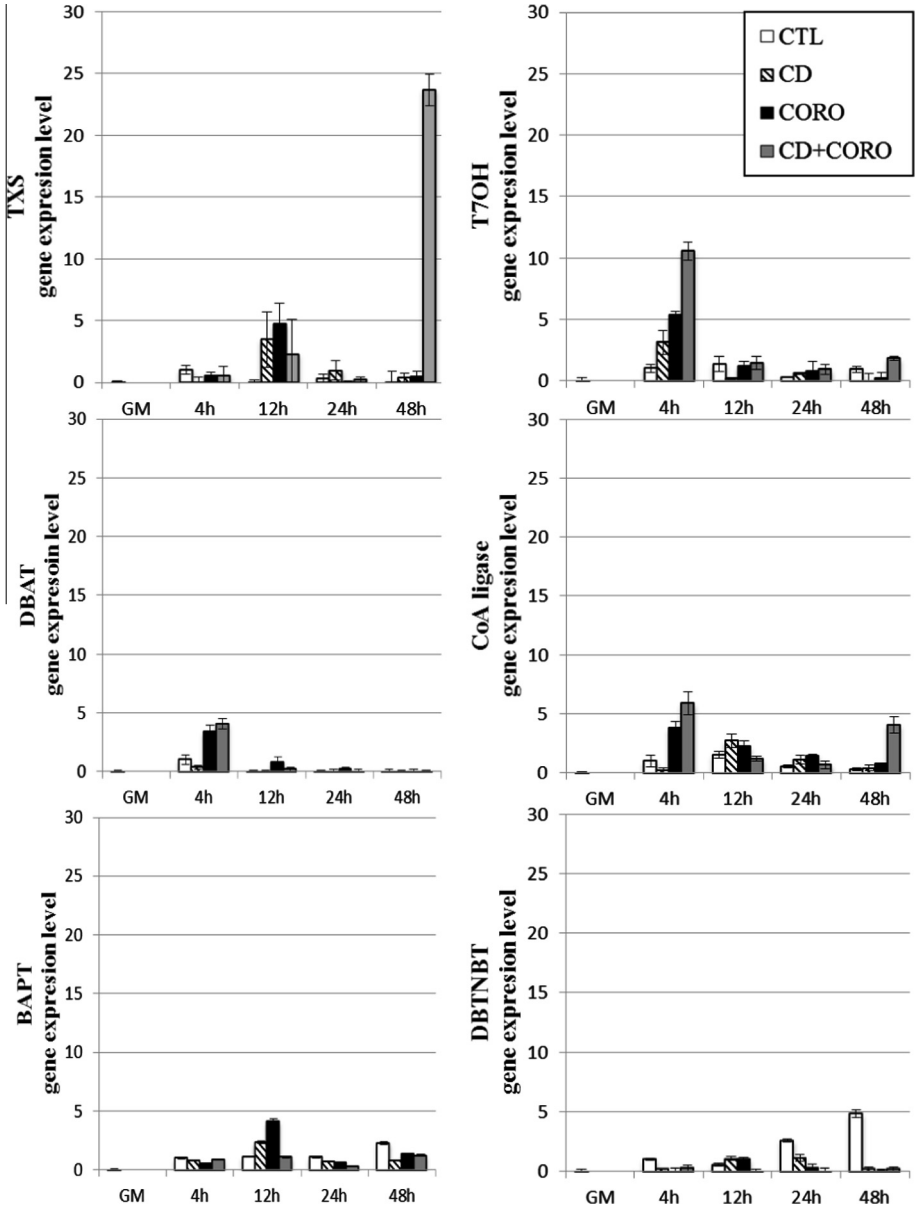

Fig. 6. Gene expression in the *T. globosa* cell line during the first 48 h in the production media supplemented or not with CD, CORO or both elicitors together. *Y*-axis: gene expression relative to that of the expression level in cultures maintained for 14 days in the growth medium, *X*-axis: time of culture. TXS, taxadiene synthase, T7OH, taxadiene 13-β-hydroxylase, DBAT, 10-deacetylbaccatin III-10-O-acetyltransferase, CoA ligase, β-phenylalanine CoA ligase, BAPT, baccatin III-3-amino,13-phenylpropanoyltransferase, DBTNBT, debenzoyltaxol N-benzoyl transferase. Data are the mean of three independent replicates ± SD.

The *BAPT* gene was more induced by CORO alone, achieving the highest expression after 12 h, when its transcript accumulation was 2- and 11.2-fold higher than after elicitation with CD + CORO or only CD, respectively, and 23 times higher than the control (Fig. 7). The increase in *BAPT* expression induced by CORO in this transgenic cell line was notably higher than in the *T. globosa* cultures, where it was less than 5-fold. As mentioned previously, the different behavior of the two studied plants cannot be attributed to the transgenic nature of the *T. media* cell line. In this case, wild-type cell lines of *T. baccata* have shown an increase in *BAPT* gene expression up to 40-fold in elicited conditions (Onrubia et al., 2011), which is higher than the levels achieved in the transgenic *T. media* cell line. On the other hand, the CORO-enhanced expression of *BAPT* did not bring about the total conversion of B into T (Fig. 5), suggesting a limited activity of the BAPT enzyme even under elicitation, or that taxol formation is restricted in subsequent steps. Further studies are required to explore these hypotheses.

The last gene studied was *DBTNBT* (Long et al., 2008), which is involved in the benzoylation of the side chain, the final metabolic step in taxol biosynthesis (Fig. 1). When comparing the elicitor treatments, the highest expression level almost throughout the study was observed under CORO. In this case, the highest transcript accumulation was obtained 4 h after elicitation, being 3-, 18.7- and 17.4-fold higher than the maximum under CD + CORO, CD and the control, respectively (Fig. 7).

Although the expression of the two genes encoding enzymes directly responsible for the formation of taxol and taxanes bearing the β-phenylalanine side chain (*BAPT* and *DBTNBT*) was higher in the cell cultures treated with only CORO, the highest production of these taxanes was obtained under CD + CORO. Consequently, no clear relationship was observed between *BAPT* and *DBTNBT* gene

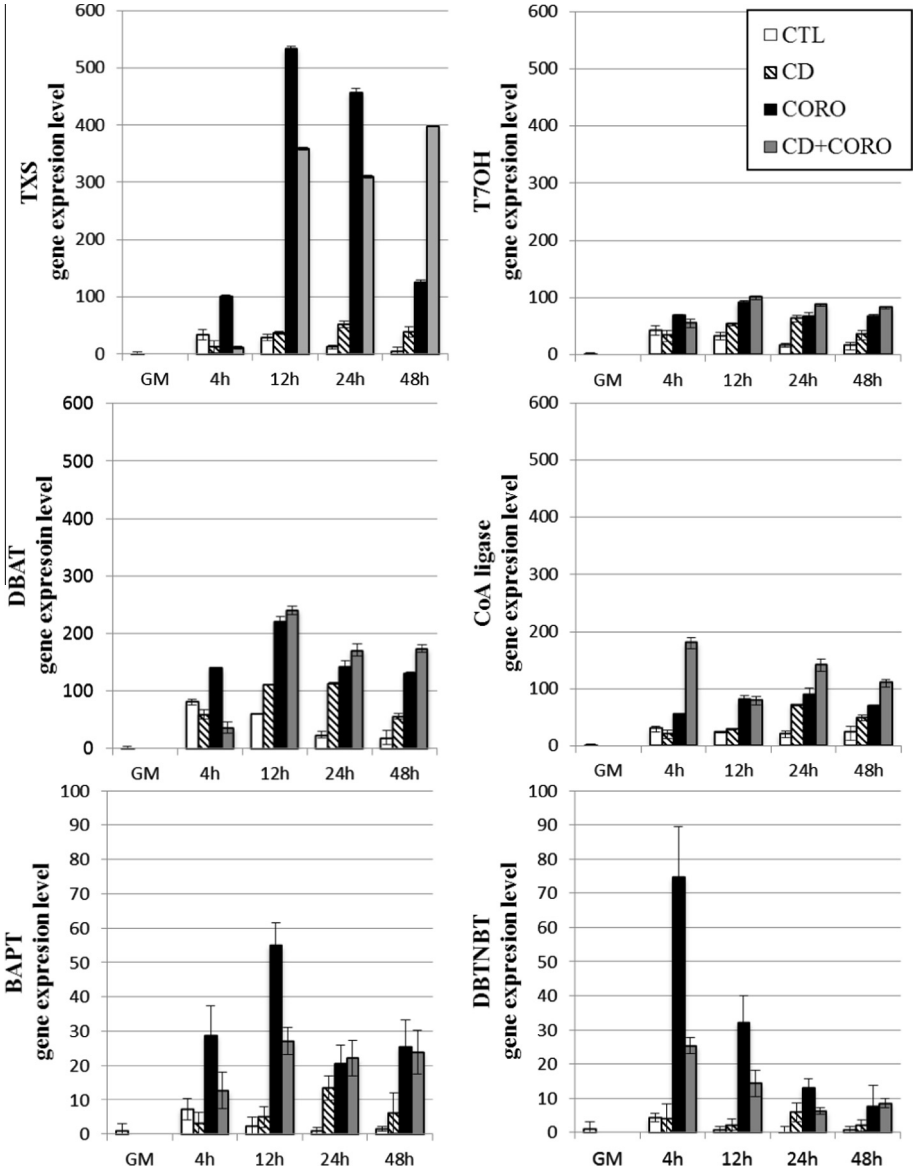

Fig. 7. Gene expression in the *T. media* cell line during the first 48 h in the production media supplemented or not with CD, CORO or both elicitors together. *Y*-axis: gene expression relative to that of the expression level in cultures maintained for 14 days in the growth medium, *X*-axis: time of culture. TXS, taxadiene synthase, T7OH, taxadiene 13-β-hydroxylase, DBAT, 10-deacetylbaccatin III-10-O-acetyltransferase, CoA ligase, β-phenylalanine CoA ligase, BAPT, baccatin III-3-amino,13-phenylpropanoyltransferase, DBTNBT, debenzoyltaxol N-benzoyl transferase. Data are the mean of three independent replicates ± SD.

expression and the production of side chain-bearing taxanes. Patil et al. (2012) observed that although taxane production is related with the expression of genes involved in the biosynthetic process, other factors may be involved, such as post-transcriptional and post-traductional regulation. However, since the other studied genes showed their highest expression after CD + CORO elicitation, or at least matching the levels in CORO-treated cultures, it seems likely that more taxol intermediates were produced by the dual treatment. In this case, the quantity and/or activity of BAPT and DBTNBT would have been sufficient for the formation of high levels of taxol and other side chain-bearing taxanes in the *T. media* cell cultures.

3. Conclusions

Growth, taxane production and transcript profiling of two different cell cultures were studied: *T. globosa* of Mexico and the more widely distributed *T. media*. The metabolism of *T. media* has been extensively studied, but not that of *T. globosa*, and the response of cell cultures of both species to the addition of elicitors has not been compared before.

Although the growth capacity of the two cell lines in the optimum production medium was good, *T. globosa* presented a shorter average doubling time than *T. media*. In both cell lines the addition of the elicitors CD and CORO, especially when applied together, proved a very efficient strategy for improving taxane biosynthesis and accumulation. Total taxane production was clearly higher in *T. media* than in *T. globosa* cell cultures, although the latter presented a higher excretion capacity. These results were corroborated by the transcript profile of taxol biosynthetic genes, since in *T. globosa* their transcript levels were very low, even under elicitation. The known variability of taxane biosynthesis among *Taxus* species and cell lines was borne out by the differing individual taxane patterns observed in the two cell lines. Whereas *T. media* elicited with CD + CORO produced high amounts of taxol and its hemisynthetic precursor baccatin III, *T globosa* cells

produced mainly DT and CF, little taxol and scarce levels of B. The production of taxanes in *T. globosa* can potentially be improved as the culture conditions for this relatively unstudied species have not yet been optimized.

Previous studies with other *Taxus* cell cultures and elicitors have found a similar expression pattern to that of the genes studied here, and that the bottlenecks in taxol biosynthesis in elicited conditions are generally the last steps of the pathway (Nims et al., 2006; Onrubia et al., 2013b; Sabater-Jara et al., 2014). In a recent transcriptomic study of *T. chinensis*, Li et al. (2012) found that 16 h after MeJA elicitation the most expressed genes were *TXS* and *T7bOH*, whereas levels of *DBAT*, *DBTNBT* and *BAPT* were low. The *CoA ligase* gene could also be considered as another limiting gene of taxol biosynthesis, since its response to elicitation in *T. media* cell cultures was not very remarkable, similar to our previous observations in MeJA-treated *T. baccata* cell cultures (Ramirez-Estrada et al., 2015).

Our study showed once again the feasibility of taxol and related taxane production in a sustainable *Taxus* cell culture system, and the possibility of increasing taxane biosynthesis and excretion from the producer cells to the medium by the addition of different elicitors. Cell cultures of *T. globosa* were established, showing the potential of using this new *Taxus* species for taxane production, although the process still requires optimization. Finally, new insights have been gained into the limiting steps of taxane biosynthesis, essential for determining suitable metabolic engineering targets to obtain highly productive cell cultures.

4. Experimental

4.1. Plant material

The transgenic *Taxus* × *media* TXS calli obtained by Exposito et al. (2010) were grown in solid Gamborg's medium (Gamborg et al., 1968) supplemented with vitamins, sugars and hormones, as described previously (Exposito et al., 2010).

The *T. globosa* cell line was established as previously described (Tapia et al., 2013), it was grown and maintained in solid WPM (McCown and Lloyd, 1981) supplemented with sucrose (1%), fructose (1%), and the growth regulators picloram (PIC) (2 mg/L), kinetin (KN) (0.1 mg/L) and gibberellic acid (GA3) (0.5 mg/L). Both cultures were grown in the corresponding growth media (GM) at 25 °C in darkness, and subcultured every two weeks to obtain enough friable calli to establish cell suspension cultures.

4.2. Cell suspension elicitation

Taxus × *media* and *T. globosa* cell suspensions were established using calli as the inoculum and the corresponding liquid growth media described in the previous section. A 20% inoculum from each species was placed in a rotary shaker (100 rpm) in darkness at 25 °C and subcultured every 14 days to obtain enough biomass for the elicitation experiments.

A two-stage culture was established as previously reported (Cusidó et al., 2002; Tapia et al., 2013). The cells were cultured first in GM and then transferred to production media (PM), which consisted of Gamborg's B5 medium (Gamborg et al., 1968) supplemented with 3% sucrose and 2,4-dichlorophenoxiacetic acid (2,4-D) (2 mg/ml), bencilaminopurine BAP (0.1 mg/ml), and GA3 (0.5 mg/ml). The amount of B5 vitamins was doubled for *T. globosa*.

After 15 days in GM, 2 g of *T. media* cells and 1 g of *T. globosa* cells were transferred to a 200 ml flask containing 10 or 20 ml of the corresponding PM. The cell suspensions were elicited with randomly methylated-β-cyclodextrin (M-β-CD) 50 mM, alone or in combination with CORO (1 μM). Both elicitors were added at the beginning of the second phase of culture.

Samples for taxane determination were taken every 4 days for 20 days, for early gene expression analysis, samples were taken at 0 h (GM), 4 h, 24 h, 12 h and 48 h in PM.

4.3. Biomass accumulation and viability assay

Fresh weight was determined by filtering the cells with Miracloth filters (Calbiochem, San Diego CA, USA). The cells were then freeze-dried to obtain the dry weight and perform the taxane extraction. Cell viability was evaluated by the fluorescein diacetate staining technique, as previously described by Exposito et al. (2010). The percentage of fluorescent cells in relation to the total was determined by observing the samples with a fluorescent microscope at 520 nm.

4.4. Taxane determination

Taxanes were extracted from the culture media and lyophilized cells, as previously described by Onrubia et al. (2013b). Samples with M-β-CD were resuspended in 1 ml of methanol and the rest in 500 μl of the same solvent. Before the analysis, the samples were filtered with 0.22 μm PVDF filters (Milipore, Billerica, MA, USA). Taxanes were quantified by ultraperformance liquid chromatography (UPLC) based on the HPLC method described by Richheimer et al. (1992). UPLC analyses were performed with a Waters Acquity Ultra Performance LC system (Waters, Milford, MA, USA) and taxanes were separated in a SUPELCOSIL LC-F column 25 cm × 4.6 mm (SUPELCO, Bellefonte, PA, USA) using a mixture of water (A) and acetonitrile (B) as the mobile phase, with the following gradient program: time (min)/%B, 0/25, 38/60, 40/100, 43/100, 45/25, and 55/25 with a flow rate of 1 ml/min. The criteria used for identification were retention time, UV spectra and co-chromatography, with the standard peak homogeneity determined by a photodiode array detector when spiked with an authentic standard. Taxanes were quantified by integrating the peaks corresponding to each studied taxane from the samples and comparing them with an external standard calibration curve. Taxol and related taxanes were provided by Chromadex (Irvine, CA, USA).

4.5. Quantitative real-time PCR (qRT-PCR)

For gene expression analysis, total RNA was isolated from 100 mg of frozen cells using the REAL ARNzol SPIN Kit (REAL, Valencia, España.) according with the manufacturer's instructions. The concentration of each sample was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Wilmington, DE, USA). Only the samples with a 260:280 ratio between 1.9 and 2.0 were used for the analysis. The RNA integrity was evaluated by agarose gel electrophoresis. 1 μg of total RNA from each sample was used in cDNA synthesis using the MMLV RT (Invitrogen, Carlsdab, CA, USA) according to the manufacturer's instructions. qRT-PCR was performed with iTAqTM Universal SYBR Green Supermix (BioRad, Hercules, CA, USA) in a 384-well platform system (ABI Prism® 7900HT sequence detection system, Applied Biosystems, Foster, CA, USA) using gene specific primers designed with Primer3 software version 0.4.0 (Table 1). Reaction conditions and primer amplification efficiency were performed as previously described by Sabater-Jara et al. (2014). As the reference gene, we used *TBC41*, which is a gene fragment showing homology with a 3,5-epimerase-4-reductase, due to the high stability according to its GeNoem M-value (Sabater-Jara et al., 2014). For each gene, the relative expression levels were normalized with respect to the same transgenic cell line growing for 14 days in GM (Growth Media) without elicitors (reference value = 1).

Table 1
Sequences of the primers used to amplify the genes under study.

Gene	Primer sequence	Amplicon size (bp)
TBC41	Forward: 5′-CAA GAA GAA AGA GTC AGC AAA TGG-3′ Reverse: 5′-GGA ACG ACA TGA CAT TAT GAA TAG C-3′	91
TXS	Forward: 5′-TTCG CAC GCA CGG ATA CG-3′ Reverse: 5′-TTC ACC ACG CTT CTC AAT TCG-3′	115
T7βOH	Forward: 5′-GGT CCG CCC AAA TTG CCA GAA-3′ Reverse:5′-CCC TGC AGA GCC CAA AAA ACC T-3′	110
DBAT	Forward: 5′-AGT TGG ATT TGG TGA TCG AA-3′ Reverse: 5′-ATC CAT GTT GCA CGA GAC TT-3′	92
CoA ligase	Forward: 5′-AGC AGA CAC TAT GGA ACA-3′ Reverse: 5′-GCC ACA ACT CTC CTC TAT-3′	109
BAPT	Forward: 5′-TAA GCA CTC TAC AAC AAC AAT GG-3′ Reverse: 5′-GCA TGA ACA TTA GTA TCT TGA TTC C-3′	111
DBTNBT	Forward: 5′-CGG GGG GTT TGT TGT GGG ATT A-3′ Reverse: 5′-TTA GCC TCT CCC CTC GCC ATC T-3′	105

4.6. Statistics

Statistical analysis was performed with Excel software. All data are average of three measurements + SD. The multifactorial ANOVA analysis followed by the turkey multiple comparison test were used for statistical comparisons. A p-value of <0.05 was assumed for significant differences.

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