Synergistic effect of cyclodextrins and methyl jasmonate on taxane production in *Taxus x media* cell cultures

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Summary

Methyl jasmonate and cyclodextrins are proven effective inducers of secondary metabolism in plant cell cultures. Cyclodextrins, which are cyclic oligosaccharides, can form inclusion complexes with nonhydrophilic secondary products, thus increasing their excretion from the producer cells to the culture medium. In the present work, using a selected Taxus x media cell line cultured in a two-stage system, the relationship between taxane production and the transcript profiles of several genes involved in taxol metabolism was studied to gain more insight into the mechanism by which these two elicitors regulate the biosynthesis and excretion of taxol and related taxanes. Gene expression was not clearly enhanced by the presence of cyclodextrins in the culture medium and variably induced by methyl jasmonate, but when the culture was supplemented with both elicitors, a synergistic effect on transcript accumulation was observed. The BAPT and DBTNBT genes, which encode the last two transferases involved in the taxol pathway, appeared to control limiting biosynthetic steps. In the cell cultures treated with both elicitors, the produced taxanes were found mainly in the culture medium, which limited retroinhibition processes and taxane toxicity for the producer cells. The expression level of a putative ABC gene was found to have increased, suggesting it played a role in the taxane excretion. Taxol biosynthesis was clearly increased by the joint action of methyl jasmonate and cyclodextrins, reaching production levels 55 times higher than in nonelicited cultures.

Keywords: cell cultures, cyclodextrins, gene expression, methyl jasmonate, taxane production.

Introduction

Taxol is a complex diterpene alkaloid with an intense antitumor activity, widely used for the treatment of several types of cancer. Its natural source is the inner bark of several *Taxus* species, although it accumulates in very low quantities (0.01% referred to dry weight). The effectiveness of taxol, and the achievement of structural analogues with enhanced activity by semisynthetic processes from the precursors, baccatin III and deacetylbaccatin III (DAB III), together with the growing world demand for these compounds, has prompted a search for alternative sources to the original plant.

The biotechnological production of taxol and related taxanes has been studied since the early 1990s when it was demonstrated that calli and cell suspensions were able to produce this compound and its precursors at least to the same extent as the plant (Fett-Neto *et al.*, 1992; Wickremesinhe and Arteca, 1993). To increase taxane production, various strategies have been examined, including optimization of culture conditions, selection of highly producer cell lines and the use of elicitors and precursors (reviewed by: Expósito *et al.*, 2009; Frense, 2007; Jennewein and Croteau, 2001; Malik *et al.*, 2011; Onrubia *et al.*, 2013a; Sabater-Jara *et al.*, 2010). In fact, the addition of methyl jasmonate (MeJ) to cell cultures is currently one of the most effective strategies for increasing taxane yields, because its exogenous application enhances secondary metabolite production in a variety of plant species, including *Taxus* sp. (Bentebibel *et al.*, 2005; Ketchum *et al.*, 1999; Yukimune *et al.*, 1996).

Similarly, in recent years, cyclodextrins (CDs) have also attracted considerable attention as agents capable of inducing defence responses in plant cell cultures and therefore acting as true elicitors (Bru *et al.*, 2006; Lijavetzky *et al.*, 2008; Zamboni *et al.*, 2009). In *Vitis vinifera*, CDs have been shown to trigger a signal transduction cascade that activates different families of transcription factors regulating the expression of genes related to the *trans*-resveratrol biosynthetic pathway. Moreover, the chemical structure of these compounds also allows them to form inclusion complexes with apolar compounds with low hydrosolubility, facilitating their excretion from cells and their isolation from the culture medium (Cai *et al.*, 2012). Thus, Belchí-Navarro *et al.* (2011) have demonstrated that CDs stimulate the biosynthesis and extracellular accumulation of silymarin (a pharmacologically active flavolignan) in *Silybum marianum* cell cultures.

The biosynthetic pathway leading to taxol is a very complex metabolic process that is still not fully understood (last reviews by Croteau *et al.*, 2006; Onrubia *et al.*, 2013a; Vongpaseuth and

Please cite this article as: Sabater-Jara, A.-B., Onrubia, M., Moyano, E., Bonfill, M., Palazón, J., Pedreño, M.A. and Cusidó, R.M. (2014) Synergistic effect of cyclodextrins and methyl jasmonate on taxane production in *Taxus x media* cell cultures. *Plant Biotechnol. J.*, doi: 10.1111/pbi.12214

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Roberts, 2007). Several authors have shown a positive correlation between the transcript abundance of genes involved in taxol biosynthesis and the amount of taxane accumulation (Bruňáková and Košuth, 2009; Bruňáková *et al.*, 2010; Onrubia *et al.*, 2010), but the genes that control the bottleneck steps in the taxol pathway are still unknown. It has been postulated that 19 enzymes are involved in taxol biosynthesis after the formation of geranylgeranyl diphosphate (GGPP). Once GGPP undergoes cyclization into taxadiene, taxol is obtained after eight oxidative steps, the addition of five acyl/aroyl groups, one epoxidation, the action of one aminomutase, two CoA esterifications and one N-benzoylation step (Figure 1).

The aim of this work was to determine the effect of CDs, alone or together with MeJ, on taxane production in cell suspensions of a selected *Taxus x media* cell line. The expression levels of several genes involved in the biosynthesis of taxol and related taxanes were also studied to shed light on the molecular changes that take place in elicited *T. x media* cell cultures and the possible bottlenecks that control taxane yield. To our knowledge, no information about the effect of CDs on the production of taxanes and their biosynthetic pathway in *Taxus* cell cultures is currently available.

Results and discussion

Effect of CDs on taxane production in *Taxus x media* cell cultures

Two-stage suspension cultures of a selected T. x media cell line were established as described by Cusidó et al. (2002). In the second stage, cell cultures were treated with three different CDs: randomly methylated- β -CDs (M- β -CD), hydroxypropylated- β -CDs (H- β -CD) or γ -CDs, either alone or combined with 100 μ M MeJ, with the aim of increasing taxane production and excretion to the culture medium. Cell growth capacity was not significantly affected by the presence of CDs, as has also been observed in Vitis vinifera cell cultures (Belchí-Navarro et al., 2012). In contrast. the addition of MeJ alone to the cell cultures resulted in a reduced growth (20-30%) compared with the control cells, as it was reported by other authors in various cell cultures (Belchí-Navarro et al., 2012; Pauwels et al., 2008) including those of Taxus (Furmanowa et al., 2000; Onrubia et al., 2013b). However, the addition of MeJ to Taxus cell cultures, which have been previously treated with CDs, reduces this negative effect of MeJ as the cell biomass (g dry weight/L) at the end of the experiment was almost



Figure 1 Taxol biosynthetic pathway. The enzymes encoded by the studied genes are encircled in black. TXS, taxadiene synthase; T7βOH, taxane 7β-hydroxylase; DBAT, 10-deacetylbaccatin III-10-O-acetyltransferase; BAPT, C-13-phenylpropanoyl-CoA transferase; DBTNBT, debenzoyl taxol *N*-benzoyl transferase. Adapted from Onrubia *et al.* (2013a).

identical to that of the control cells and cell viability was slightly affected (data not shown).

Total taxane production (cell-associated + extracellular), measured as the total content of DAB III, 10-deacetyltaxol (DAT), baccatin III, cephalomannine and taxol, in control cell cultures was very low, reaching a maximum level at the end of the experiment (5.24 mg/L at day 16) (Figure 2). As expected, the addition of MeJ to the medium after 7 days of culture increased taxane production, which peaked at day 16 (17 mg/L). Although the kinetics of production in MeJ-elicited cell cultures coincided with those observed in other *Taxus* cell lines (Bonfill *et al.*, 2003, 2006; Ketchum *et al.*, 2003; Palazón *et al.*, 2003), the cell response to this elicitor depends on a variety of factors, particularly the biosynthetic capacity of the cell line investigated.

The addition of CDs (M- β -CD, HP- β -CD or γ -CD) at the beginning of the experiment also increased the total taxane production (7.7, 9.0 and 15.1 mg/L, respectively, at day 16). However, in cultures treated with both MeJ and CDs, taxane production increased dramatically, reaching levels 6.1, 3.6 and 4.8 times higher, respectively, than the maximum content obtained by the same cell line treated only with MeJ (Figure 2). The respective productivities (6.5, 3.9 and 5.2 mg/L/day) obtained in these cultures were also very high, especially when M-β-CD was used, being higher than when the same cell line was treated with 1 µm coronatine (5 mg/L/day), which is a powerful elicitor of taxane production (Onrubia et al., 2013b). This enhancement of taxane productivity observed using CDs is due to the physicochemical properties of CDs because they have a hydrophilic external surface and a hydrophobic central cavity that trap apolar compounds, including taxol and related taxanes, forming complexes with CDs and obtaining highly water-soluble taxanes. In fact, the complexation of taxol and related taxanes with natural or chemically modified CDs has been widely studied to increase their water solubility for pharmaceutical formulations (Hamada et al., 2006; Kagkadis, 2007; Loftsson and Duchene, 2007). Indeed, Hamada et al. (2006) showed that chemically modified β-CDs (i.e. methyl-B-CD and HP-B-CD) were more effective in solubilizing taxol than nonmodified CDs such as γ -CD, and complexes of taxol maintained their bioactivity on in vitro tubulin assay. Moreover, Dordunoo and Burt (1996) showed that the solubility of taxol increased in presence of HP-β-CD, and its stability was pH dependent, being more stable in the 3-5 pH range. They also observed less than 1% decomposition of taxol in CD solutions stored for 1 month. These studies agree with the high levels of taxol and related taxanes found in the extracellular medium of T. x media cell cultures elicited with M-B-CD and HP- β -CD (which have a pH around 3.5) because they allow a stable accumulation of taxanes in the extracellular medium over time during which the experiment was carried out.

On the other hand, the total content of taxanes in cell cultures simultaneously elicited with M- β -CD, HP- β -CD or γ -CD, and MeJ was higher than the sum of total taxanes achieved by the separate application of CDs and MeJ. These results suggest that both elicitors could act synergistically in the production and accumulation of taxanes. Figure 2 also shows the capacity of *Taxus* cells for taxane excretion. In control and MeJ-elicited cultures, the average taxane excretion throughout the experiment represented about 45% and 65% of total taxanes respectively. However in cell cultures elicited with M- β -CD, HP- β -CD or γ -CD together with MeJ, the release of taxanes to the medium was on average 86, 72 and 67% respectively.

We can conclude from the results that CDs, especially M- β -CD, were able to increase taxane production and their secretion to the culture medium. A clear decline in retroinhibition processes and toxicity of taxanes could explain the high taxane production found in these cultures.

Gene expression profiles and taxane production

To analyse the relationship between the transcript profiles and individual taxane production in the *T. x media* cell line during elicitation, the changes in expression levels of *TXS*, *TTβOH*, *DBAT*, *BAPT* and *DBTNBT* genes (Figure 1), which encode enzymes involved in taxol biosynthesis, were determined by qRT-PCR. Thus, the transcript accumulation of these genes was quantified at different time points in cell cultures elicited with M-β-CD and MeJ, separately or combined. M-β-CD was added to the production medium at the beginning of the experiment, and MeJ 7 days later, taxane production was analysed during a 23-day period (8 days longer than in the first experiment) to check whether the production of taxanes was maintained along the time (Figure 3).

Total taxane production (extracellular + cell-associated), measured as the total content of DAB III, DAT, baccatin III, cephalomannine and taxol, in a nonelicited production medium (control cultures) (Figure 3) was virtually nil in the first days, thereafter increasing slightly until day 21 (7 mg/L). As before, separate elicitation with M- β -CD or MeJ had a marked and variable impact on taxane production (Figure 3). Compared with the control, MeJ-treated cells achieved significantly (P < 0.05) higher levels of total taxanes almost throughout the experimental period, reaching a maximum of 56 mg/L at day 23 (which corresponded with day 16 of elicitation with MeJ). At this point, the production was 32.5 times higher than in control conditions. Taxane levels obtained in M- β -CD-treated cell cultures were also

Figure 2 Total taxane production in *T. x media* cell cultures elicited with 50 mM methyl-β-cyclodextrin (M-β-CD), hydroxypropylated-β-cyclodextrin (HP-β-CD) or γ-cyclodextrin (γ-CD) alone or in combination with 100 μm methyl jasmonate (MeJ) for 16 days of culture. CDs were added at the beginning of the experiment and MeJ 7 days later. Black bars: extracellular taxane content; white bars: cell-associated taxanes. Data are the mean of three independent replicates ± SD.





Figure 3 Total taxane production in *T. x media* cell cultures elicited with 50 mM methyl- β -cyclodextrin (M- β -CD) alone or in combination with 100 μ M methyl jasmonate (MeJ) for 23 days of culture. CDs were added at the beginning of the experiment and MeJ 7 days later. Data are the mean of three independent replicates \pm SD.

higher than in the control, especially at the end of the experiment.

As in the preliminary experiment previously described, the joint action of MeJ and M- β -CD clearly enhanced the total taxane production, which reached a maximum level (around 140 mg/L) at day 23. At this point, the total taxane content was 8.8, 2.5 and 83.2 times higher than in cell cultures treated separately with M- β -CD or MeJ, or nonelicited, respectively. Notably, at the end of the experiment, the total taxanes produced by cell cultures treated with both M- β -CD and MeJ was more than double the combined yield of the two treatments applied separately (Figure 3). These results corroborate the aforementioned synergistic effect of both elicitors on taxane production.

All the analysed genes were considerably induced by the presence of MeJ, alone or in combination with M- β -CD, whereas M- β -CD-treated cells did not significantly enhance transcript levels compared with the control (Figures 4 to 8). In these



Figure 4 Relative expression level of *TXS* gene in *T. x media* cell cultures elicited with 100 μ M methyl jasmonate (MeJ) and/or 50 mM methyl- β -cyclodextrin (M- β -CD). The relative gene expression levels were normalized with respect to the same transgenic cell line growing for 14 days in the growth medium (Gm) without elicitors (reference value = 1). Data are the mean of three independent replicates \pm SD.

figures, the relative expression levels of each gene were normalized with respect to the same transgenic cell line growing for 14 days in the growth medium (Gm) without elicitors (reference value is taken as 1) in order to ascertain differences among treatments.

The expression of the first gene involved in the taxol biosynthetic pathway, TXS, was strongly enhanced in MeJ-treated cells, increasing significantly after 4 h of treatment (7 days-4 h in Figure 4), peaking 72 h (10 days-72 h), when levels were about 31 times higher than in control cells. These results are in accordance with those obtained by Vongpaseuth and Roberts (2007), who showed that the expression of genes encoding enzymes involved in the first steps of taxol biosynthesis (GGPPS and TXS) increased during the first 6 h of MeJ elicitation, peaking 1-2 days later. The expression of the TXS gene was also enhanced when cells were treated simultaneously with both elicitors, reaching the highest level 1-2 days after the addition of MeJ, when it was almost 16 times higher than the reference value. Expósito et al. (2010) showed that although TXS controls the first committed step in the taxol biosynthetic pathway (Figure 1), it is not a limiting enzyme in taxane production, as taxadiene is not a limiting substrate in taxol formation. In fact, taxadiene does not accumulate at any appreciable level in Taxus cell cultures for longer than 2 days (Hezari et al., 1997), indicating a rapid conversion of this metabolite into intermediates involved in the consecutive reactions downstream. Likewise, Nims et al. (2006) reported that TXS transcripts and also other MeJ responding genes in T. cuspidata cell cultures did not persist for more than 30 h after elicitation, suggesting that the very similar response of the genes involved in the taxol production to the presence of MeJ could be due to the fact that these genes may be under the control of a single regulatory regime. As the time of highest expression varied very little among the genes and cell lines studied and in almost all the genes a rapid up-regulation was guickly followed by a down-regulation, it is likely that MeJ elicitation induced a positive regulation for the first hours/days, and a negative regulation thereafter, as suggested by Nims et al. (2006).

The enzyme T7BOH catalyses the hydroxylation of C7 in the taxane skeleton in the intermediate steps of the taxol biosynthetic pathway (Figure 1). As shown in Figure 5, the transcript accumulation of the $TT\beta OH$ gene was significantly higher in cells treated with MeJ, either alone or in combination with M-B-CD. than in nonelicited cell cultures or in cells treated only with M-β-CD. Under MeJ treatment, *TTβOH* expression increased immediately after elicitation (7 days-1 h, Figure 5), reaching a maximum level at 4 h, when mRNA accumulation was found to be 150 times higher than the reference value. It then decreased (8 days-24 h), and later its expression increased and maintained until the end of the experiment. In contrast, in M-B-CD-treated cells, TTBOH transcript levels remained low throughout the experiment, at most being only 6.7 times higher than the reference value. However, the joint action of both elicitors caused a considerable increase in transcript accumulation, with two peaks at 4 and 72 h after elicitation, reaching levels more than 400 times higher than the reference value. At these two peaks, the expression of $T7\beta OH$ was higher than that achieved by the sum of M-β-CD and MeJ applied separately, reflecting a synergistic effect of the two elicitors on $T7\beta OH$ expression (Figure 5). In previous studies with the same T. x media cell line, we also observed that the expression level of different genes encoding other hydroxylases, which are involved in taxane



Figure 5 Relative expression of *TTβOH* gene in *T. x media* cell cultures elicited with 100 µm methyl jasmonate (MeJ) and/or 50 mm methyl-β-cyclodextrin (M-β-CD). The relative gene expression levels were normalized with respect to the same transgenic cell line growing for 14 days in the growth medium (Gm) without elicitors (reference value = 1). Data are the mean of three independent replicates \pm SD.

biosynthesis (*T13OH* and *T2OH*), was strongly enhanced under elicitation with coronatine or MeJ (Onrubia *et al.*, 2013b).

The transformation of DAB III into baccatin III is catalysed by 10deacetylbaccatin III-10 β -O-acetyltransferase (DBAT) (Figure 1). As shown in Figure 6a, *DBAT* expression was increased by the presence of MeJ, both alone and in combination with M- β -CD. There was also an initial increase at 4 h in the control cells, and at 24 h under M- β -CD elicitation, decreasing thereafter. In MeJtreated cells, *DBAT* expression peaked at 72 h (10 days–72 h, Figure 6a), being 2.4 and 13 times higher than the reference value (Gm) and control, respectively. Again, the combined action of both elicitors induced the greatest accumulation of transcripts, and this gene expression was maintained throughout the experiment. However, in comparison with the *TXS* and *T7\betaOH* genes, *DBAT* response to the presence of these two elicitors was very limited, with mRNA levels never exceeding the reference value more than sevenfold.

As shown in Figure 1, the expression of the TXS and T7BOH genes, together with various other genes not studied in this work, may regulate the production of DAB III (Figure 6b), the precursor of baccatin III. In control cells and those elicited separately with M-B-CD or MeJ, DAB III levels were variable, remaining low (<5 mg/L) throughout the experiment, whereas the presence of both elicitors increased DAB III production, especially at the end of experiment (total contents: 14.03 mg/L at day 21). In contrast, baccatin III levels were significantly enhanced by elicitation with MeJ alone or in combination with M- β -CD (32.5 mg/L at day 23 and 22 mg/L at day 21, respectively) (Figure 6b). These results suggest that in this T. x media cell line, DAB III was actively transformed into baccatin III. The greater accumulation of baccatin III compared with DAB III suggests that, although the DBAT gene was not highly expressed (Figure 6a), the amount of active enzyme was enough not to limit the biosynthetic pathway leading to taxol. Therefore, the DBAT enzyme does not seem to control a limiting step of this biosynthetic route.

The last two analysed genes, *BAPT* and *DBTNBT*, encode two transferases: BAPT, responsible for the attachment of the side

chain to baccatin III, leading to the formation of taxanes containing β -phenylalanine as a side chain, and DBTNBT, involved in the benzoylation of the side chain, which constitutes the final metabolic step in the biosynthesis of taxol (Figure 1). As shown in Figure 7a,b, in control cultures and those elicited only with M-β-CD, transcript accumulation of these genes was lower than the reference value (Gm). The expression of BAPT (Figure 7a) began 1 h after MeJ elicitation, peaking 96 h later (11 days-96 h), showing levels five times higher than the reference value, while the maximum expression of DBTNBT (Figure 7b) peaked at 72 h after elicitation (10 days-72 h). As with other genes, the two elicitors exerted a synergistic effect on the expression of BAPT at 7 days (7 days-1 h and 7 days-4 h) and DBTNBT at 7 (7 days-1 h) and 10 days (10 days-72 h), being their levels higher than the sum of expression levels in cells treated separately with MeJ and M-β-CD. Notably, the presence of M-β-CD in the dual elicitation resulted in a much earlier activation of these genes than treatment with MeJ alone, as shown in Figure 7.

The taxol content in cell cultures grown in control conditions was very low or nonexistent, probably due to the very low BAPT and DBTNBT gene expression also observed in these cultures. Although taxol production in the T. x media cell cultures (Figure 7c) was slightly enhanced when M- β -CD or MeJ was supplied separately (the highest level being 5.9 and 13.9 mg/L, respectively), the synergistic effect of joint elicitation gave far better results, resulting in taxol levels as high as 65.01 mg/L. Surprisingly, the expression of the BAPT and particularly DBTNBT gene, which was on average 42.6 and 7.4 times higher under joint elicitation than in the control (Figure 7a,b), did not correlate with the 60-fold increase in taxol (Figure 7c), suggesting that their limited transcript accumulation was not responsible for the outstanding taxol production obtained. However, as the BAPT and DBTNBT genes were expressed earlier, a consequent earlier availability of precursors could be partially responsible for the higher taxol levels achieved in cultures elicited by both MeJ and M-β-CD. It should be emphasized that, although the expression levels of all studied genes increased significantly in the presence of both elicitors, those encoding the transferases DBAT, BAPT and DBTNBT showed much lower transcript accumulation than the genes encoding TXS and T7_BOH.

Notably, the highest expression level of genes related to taxol biosynthesis was found from a few hours (mainly at 7 days–4 h) to 3 days (10 days–72 h) after MeJ was added, whereas taxane contents did not peak until at least 14 (21 days) to 16 (23 days) days later (Figures 4 to 7).

Our results are in agreement with Onrubia *et al.* (2010) as the maximum *TXS* and *BAPT* gene expression took place 24 h and 6 h, respectively, after the addition of MeJ to *T. baccata* cell cultures. However, the *T. media* cell line also treated with MeJ presented the highest *TXS* and *BAPT* expression levels at 2 and 4 days, respectively. In this same cell line, when the elicitor added was coronatine (a structural analogue of Ile-MeJ), the highest expression level of both genes took place 1 day after elicitation (Onrubia *et al.*, 2013b). Additionally, Li *et al.* (2012) found the higher levels of gene expression of *TXS and T7βOH*, whereas *DBAT*, *DBTNBT* and *BAPT* were slightly expressed in a transcriptome study of *T. chinensis* at 16 h after MeJ elicitation.

In previous studies, we have shown that genes encoding master regulators such as taximin (Onrubia *et al.*, 2014) are overexpressed after supplementing the culture medium with MeJ, probably contributing to the enhancement of secondary compound production. In fact, De Geyter *et al.* (2012) have



Figure 6 (a) Relative expression of *DBAT* gene in *T. x media* cell cultures elicited with 100 μM methyl jasmonate (MeJ) and/or 50 mM methyl-β-cyclodextrin (M-β-CD). The relative gene expression levels were normalized with respect to the same transgenic cell line growing for 14 days in the growth medium (Gm) without elicitors (reference value = 1). (b) Total content of 10-deacetylbaccatin III and baccatin III in *T. x media* cell cultures elicited with 50 mM M-β-CD and 100 μM MeJ separately or combined for 23 days. Black part of the bars: extracellular taxanes; grey part of the bars: cell-associated taxanes. Data are the mean of three independent replicates \pm SD.

suggested that the transcription factors that drive the expression of secondary metabolic pathway genes are encoded by the jasmonate-responsive genes themselves, as well as many other regulatory proteins. In *Taxus*, two transcription factors have been functionally characterized, both of them being picked up in a yeast-one hybrid screen. The first, TcWRKY1 from *T. chinensis*, interacted with a W-box element in the promoter of the DBAT-encoding gene, and it was shown to be a positive regulator of this gene (Li *et al.*, 2012). The function of the second, TcAP2, has not yet been studied in *Taxus*. The scarcity of these results shows that more studies are needed to obtain a deeper insight into the genetic regulation of the paclitaxel biosynthetic pathway. On the other hand, the other taxanes quantified, DAT and cephalomannine, were either undetectable or found in very low levels (\leq 5 mg/L) in both control cultures and those elicited with M- β -CD or MeJ separately (Figure S1). In contrast, the joint use of elicitors enhanced the total content of both DAT and cephalomannine, which peaked at 16–18 days (16.6 mg/L) and 23 days (33.9 mg/L), respectively. DAT biosynthesis probably requires baccatin III and β -phenylalanoyl-CoA, as well as the action of the BAPT enzyme, but it is unclear whether it is formed as a degradation product of taxol or through a side reaction of the taxol biosynthetic pathway. The BAPT enzyme is also involved in the formation of cephalomannine, which in turn, only differs from taxol in the presence either a N-tigloyl or N-benzoyl group at



DBTNBT (b) genes in *T. x media* cell cultures elicited with 100 μM methyl jasmonate (MeJ) and/ or 50 mM methyl-β-cyclodextrin (M-β-CD). The relative gene expression levels were normalized with respect to the same transgenic cell line growing for 14 days in the growth medium (Gm) without elicitors (reference value = 1). (c) Total content of taxol in *T. x media* cell cultures elicited with 50 mM M-β-CD and 100 μM MeJ separately or combined for 23 days. Black part of the bars: extracellular taxanes; grey part of the bars: cellassociated taxanes. Data are the mean of three independent replicates \pm SD.

Figure 7 Relative expression of BAPT (a) and

C3' of the side chain (Bonfill *et al.*, 2003). The lower levels of cephalomannine (compared with taxol) could be due to less efficient metabolic steps leading to its synthesis.

Moreover, as the addition of M- β -CD to cell cultures increased not only the production of taxanes, but also their excretion to the culture medium, the expression of a gene that encodes an ABC transporter was determined. It is well known that ABC transporters play an important role in the removal of secondary metabolites from the active cytoplasm to be accumulated in places where their toxicity does not interfere with cell viability (Fornalé *et al.*, 2002; Sun *et al.*, 2013). As shown Figure 8, the accumulation of transcripts for the gene encoding the ABC transporter (*ABC* gene) in MeJ-treated cultures peaked at 4 h (7 days–4 h) after elicitation, reaching values more than 12 times the reference value. However, under simultaneous elicitation with MeJ and M- β -CD, the maximum expression was achieved after

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only 1 h (7 days–1 h), and the peak level was more than double that in cells elicited only with MeJ and 23 times greater than the reference value. The two elicitors therefore also seem to act synergistically in enhancing the expression of the *ABC* gene.

These results suggest that the high and very early *ABC* gene expression observed by the joint action of both elicitors might be another decisive factor contributing to the remarkable production of taxol depicted in Figure 7C. In addition, this constant release of taxol could explain the active biosynthesis and extracellular accumulation observed, because it would clearly diminish the usual feedback inhibition processes and/or toxicity resulting from the presence of taxol in the cytoplasm.

Conclusion

The low expression of the studied genes in control conditions could explain the low content of taxol and related taxanes in the *T. x media* cell line. Therefore, the use of elicitation to activate genes involved in taxane metabolic pathways is a good strategy to increase the production of these compounds. Our results show that the joint action of MeJ and CDs induces an important reprogramming of gene expression in *T. x media* cell cultures, which likely accounts for the enhanced production of taxol and related taxanes observed in this study, although the molecular mechanism for the possible synergy between the two elicitors remains unknown.

The fact that taxol and other taxanes form inclusion complexes with CDs, besides decreasing cellular toxicity, also prevents possible intracellular and especially extracellular enzymatic degradation of these compounds. The ability of cell suspensions to excrete target products to the culture medium is crucial in a biotechnological production system at a bioreactor level because it allows continuous production without destroying the cell biomass and makes the extraction and purifying processes simpler, more bio-sustainable and economically more viable.



Figure 8 Relative expression of the *ABC* gene in *T. x media* cell cultures elicited with 100 μ M methyl jasmonate (MeJ) and/or 50 mM methyl- β -cyclodextrin (M- β -CD). The relative gene expression levels were normalized with respect to the same transgenic cell line growing for 14 days in the growth medium (Gm) without elicitors (reference value = 1). Data are the mean of three independent replicates \pm SD.

Experimental procedures

Plant material

A transgenic T. x media cell line carrying the gene encoding taxadiene synthase (TXS) of *T. baccata* under the control of p35S was obtained as described by Expósito et al. (2010). Calli was maintained in solid Gamborg's B5 medium (Gamborg et al., 1968) supplemented with vitamins, sugars and hormones [B5 growth medium (Gm)] as described by Expósito et al. (2010), at 25 °C in the darkness and subcultured every 3 weeks. Cell suspension cultures were established using calli as the inoculum. For this, 10 g of fresh weight of callus pieces was inoculated into 250-mL flasks containing 50 mL liquid growth B5 medium (Sigma, St Louis, MO) and placed in a rotary shaker (100 rpm) at 25 °C in darkness. After 14 days, 4 g of cells was transferred to the B5 production media supplemented with vitamins, sugars and hormones as described by Cusidó et al. (2002) and maintained in the same conditions as described above. Taxane production was increased by adding elicitors to this production media.

Elicitation experiments on *Taxus x media* suspension cultured cells

Taxus x media cells cultivated in production medium were elicited with 50 mM of three types of CDs, randomly methylated- β -CDs (M- β -CD), hydroxypropylated- β -CDs (HP- β -CD) and γ -CD, alone or in combination with 100 μ M of MeJ. Samples were taken every 4 days for 16 days.

Elicitors were added to the production medium at two different times of culture: CDs were added to the liquid medium when the cells were transferred from Gm to the optimum medium for production of taxol and related taxanes, whereas MeJ was added to the production medium 7 days afterwards, in both the production medium (control medium) and the production medium supplemented with CDs.

Taxane determination

Taxanes were extracted from the culture media and freeze-dried cells as described by Onrubia et al. (2013b). All samples were resuspended in 500 µL methanol and filtered prior to analysis (0.22 µm PVDF filters, Millipore, Billerica, MA). Taxane quantification was achieved by high-performance liquid chromatography (HPLC) as described by Richheimer et al. (1992). The chromatographic analyses were performed in an HPLC Agilent 1100 series, and taxane separation was carried out in a SUPELCOSIL LC-F column 25 cm \times 4.6 mm (Supelco, Bellefonte) using a mobile phase consisting of a mixture of water (A) and acetonitrile (B) using the timed gradient program: time (min)/%B: 0/25, 38/60, 40/60, 50/25 and 55/25 with a flow rate of 1 mL/min. Criteria for identification included retention time, UV spectra and cochromatography with standards and peak homogeneity determined by a photodiode array detector when spiked with authentic standard. Quantification was carried out from the calibration curve of each standard: DAB III, baccatin III, cephalomannine, DAT and taxol (Chromadex, LGC Standards, Barcelona, Spain).

Quantitative real-time PCR

For gene expression analysis, *T. x media* cell cultures were elicited with 50 mM of M- β -CD and 100 μ M of MeJ, separately or in combination. Samples were harvested at different time points: 0 h (Gm), 4 h, 24 h, 72 h and 96 h in both control and CD-treated

cells, and 7 (7 days–1 h, 7 days–4 h), 8 (8 days–24 h), 10 (10 days–72 h) and 11 days (11 days–96 h) in MeJ-treated cells.

Total RNA was isolated from frozen cells (0.1 g fresh weight) using the REAL ARNzol SPIN KIT (REAL) according to the manufacturer's instructions. The concentration of each RNA sample was measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Only the RNA samples with a 260 : 280 ratio between 1.9 and 2.1 were used for the analysis. The integrity of RNA samples was also assessed by agarose gel electrophoresis. 1 µg of total RNA from each sample was reverse-transcribed by first-strand cDNA synthesis using MMLV RT (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. gRT-PCR was performed using SYBR Green PCR Mastermix (Roche, Basel, Switzerland) in a 384-well platform system (LightCycler[®] 480 Instrument, Roche). Gene-specific primers were designed with Primer3 software version 0.4.0 (SimGene.com) (Table 1), and the amplification efficiency of each primer pair was determined empirically by serial dilutions of cDNA and calculated as described by Qiagen (Hilden, Germany). Data were analysed with the LightCycler[®] 480 Software release 1.5.0 SP3 (Roche). Only those primer pairs with an efficiency of over 0.8 were used. Three candidate reference genes were selected in this experiment: TBC41, which is a gene fragment showing homology with a 3, 5-epimerase-4-reductase (involved in the metabolism of some cell wall components), maturase K and 18S rRNA. To compare the stability of the reference genes, two programs of Microsoft Excel Visual Basic for Applications (VBA) were used: GeNorm vs. 3.4 (Vandesompele et al., 2002) and NormFinder (Andersen et al., 2004). The stability study allowed us to select TBC41 as the reference gene according to its GeNorm M-value (Table S1). Furthermore, the amplicons were sequenced to confirm its specific identity (it was consistent with the expected fragment size). For this, we extracted DNA from the transgenic line and checked by PCR with specific primers, as previously described by Expósito et al. (2010). Bands were obtained and the resulting amplicons were sequenced with the same primers used for amplification and confirmed its identity by performing BLAST (Table S2). Likewise, for each gene, the relative expression levels were normalized with respect to the same transgenic cell line growing for 14 days in Gm without elicitors (reference value = 1).

 Table 1 Sequences of the primers used to amplify the genes by quantitative real-time PCR. Amplicon sequences are included as Table S2

Gene	Primer sequence	Amplicon size
TBC 41	Forward: 5'-CAA GAA GAA AGA GTC AGC AAA TGG-3'	91
	Reverse: 5'-GGA ACG ACA TGA CAT TAT GAA TAG C-3'	
TXS	Forward: 5'-TTC GCA CGC ACG GAT ACG-3'	115
	Reverse: 5'-TTC ACC ACG CTT CTC AAT TCG-3'	
DBAT	Forward: 5'-AGT TGG ATT TGG TGA TGC AA-3'	92
	Reverse: 5'-ATC CAT GTT GCA CGA GAC TT-3'	
BAPT	Forward: 5'-TAA GCA CTC TAC AAC AAC AGG-3'	111
	Reverse: 5'-GCA TGA ACA TTA GTA TCT TGA TTC C-3'	
DBTNBT	Forward: 5'-CGG GGG GTT TGT TGT GGG ATT A-3'	105
	Reverse: 5'-TTA GCC TCT CCC CTC GCC ATC T-3'	
Т7ВОН	Forward: 5'-GGT CCG CCC AAA TTG CCA GAA-3'	110
	Reverse: 5'-CCC TGC AGA GCC CAA AAA ACC-3'	
ABC	Forward: 5'-AGC CTA TGC ATC CCT AGT GCA A-3'	119
	Reverse: 5'-GTT GCC TGC CAG TGT TAT TT-3'	

Statistics

Statistical analysis was performed with Excel software. All data are the average of three determinations \pm SD. The multifactorial ANOVA analysis followed by the Tukey's multiple comparison tests was used for statistical comparisons. A *P*-value of <0.05 was assumed for significant differences.

Acknowledgements

Sabater-Jara AB held grants from the Ministerio de Ciencia e Innovación. This study was supported by MICINN-FEDER (BIO2011-29856-C02), Fundación Lavoisier (LAVOISIER-1/2013) and Catalan Government (2009SGR1217). All authors gratefully acknowledge the support of Cost Action FA1006-Plant Engine.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 Total content of 10-deacetyltaxol and cephalomannine in *T. x media* cell cultures elicited with 100 μ M methyl jasmonate (MeJ) and/or 50 mM methyl- β -cyclodextrin (M- β -CD) for 23 days. **Table S1** Stability comparison of candidate reference genes according to GeNorm and NormFinder Visual Basic for Applications (VBA) for Microsoft Excel.

Table S2 Amplicon sequences of the studied genes obtained byqRT-PCR.