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Coronatine, a more powerful elicitor for inducing taxane biosynthesis in *Taxus media* cell cultures than methyl jasmonate

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ABSTRACT

Coronatine is a toxin produced by the pathogen *Pseudomonas syringae*. This compound has received much attention recently for its potential to act as a plant growth regulator and elicitor of plant secondary metabolism. To gain more insight into the mechanism by which elicitors can affect the biosynthesis of paclitaxel (Px) and related taxanes, the effect of coronatine (Cor) and methyl jasmonate (MeJA) on *Taxus media* cell cultures has been studied. For this study, a two-stage cell culture was established, in which cells were first cultured for 14 days in a medium optimised for growth, after which the cells were transferred to medium optimised for secondary metabolite production. The two elicitors were added to the medium at the beginning of the second stage. Total taxane production in the cell suspension was significantly enhanced by both elicitors, increasing from a maximum level of 8.14 mg/L in control conditions to 21.48 mg/L (day 12) with MeJA and 77.46 mg/L (day 16) with Cor. Expression analysis indicated that the txs, *t13oh*, *t2oh*, *t7oh*, *dbat*, *pam*, *bata* and *dbtnbt* genes were variably induced by the presence of the elicitors. Genes encoding enzymes involved in the formation of the polihydroxylated hypothetical intermediate (TXS, T13OH, T2OH, T7OH) and the phenylalanoil CoA chain (PAM) were stronger induced than those encoding enzymes catalysing the last steps of the Px biosynthetic pathway (DBAT, BAPT and DBTNBT). Notably, although taxane accumulation differed qualitatively and quantitatively following MeJA- or Cor-elicitation, gene expression induction patterns were similar, inferring that both elicitors may involve distinct but yet uncharacterised regulatory mechanisms.

Introduction

A biotechnological approach to the production of the anticancer drug Px and other taxanes by means of *Taxus* spp. cell cultures has proven to be a good alternative to the use of whole plants, and several companies are currently obtaining taxanes on an industrial scale using cell cultures of different *Taxus* species. Nonetheless the generally low Px production in plant cell cultures requires the use of elicitors, notably methyl jasmonate (MeJA). As several studies have shown, MeJA dramatically enhances not only the production of taxanes in cell cultures of different *Taxus* species (Onrubia et al., 2010) but also of other secondary metabolites of interest, such as triterpene glycosides, lignans and alkaloids (Ionkova, 2009; Shohael et al., 2007; Goossens et al., 2003).

Jasmonates (JAs) play many important roles in wound response and secondary metabolite production in plants. Coronatine (Cor) is a phytotoxin produced by several pathovars of the plant bacteria *Pseudomonas syringae* (Bender et al., 1999), and acts as a molecular mimic of the isoleucine-conjugated form of jasmonic acid (JA–Ile) (Katsir et al., 2008). Several studies have reported that Cor exerts its virulence effects by activating the host's jasmonate signalling pathway (Zhao et al., 2003) and plants insensitive to Cor, like the *Arabidopsis* (*Arabidopsis thaliana*) *coi1* mutant, exhibit resistance to Cor-producing strains like *P. syringae* (Zhao et al., 2003). The actions of Cor include the induction of JA biosynthesis, impact on phytohormonal signalling responses in tomato (Uppalapati et al., 2005), and a wide range of biological functions, such as tendril coiling, inhibition of root elongation, hypertrophy, chlorosis, secondary metabolite production, ethylene emission, accumulation of proteinase inhibitors and apoptotic cell death (Tamogami and Kodama, 2000; Yao et al., 2002; reviewed in Uppalapati et al., 2005).

Cor seems to be a structural and functional analogue of jasmonic acid (JA) and related signalling compounds such as MeJA and 12-oxo-phytodienoic acid (12-OPDA), the C_{18} precursor of JA and MeJA.

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It consists of the polyketide coronafacic acid (CFA), which is a product of polyketide biosynthesis, and coronamic acid (CMA), a cyclised derivative of isoleucine. It has been shown that CFA mimics MeJA, and that CMA or other aminoacids enhance toxicity. CFA acid has been found conjugated to other amino acids such as isoleucine, serine and threonine, and these conjugates also possess phytotoxic activity (Lauchli and Boland, 2003). JA and 12-OPDA control an astonishingly large number of plant functions, notably the activation of secondary metabolism, by acting as signalling compounds in plant defensive responses against several stress situations. Svoboda and Boland (2010) have reported that the defense responses of plants to different external plant invaders are controlled by a suite of phytohormones among which JA and JA–Ile play a major role. As Cor resembles the JA–Ile conjugate their mode of action could be similar. Importantly however, the chemical structure of Cor is more stable due to the rigid *cis*-orientation in its bi-cyclic skeleton, which does not permit transformation to a less active stereoisomer or catabolism, and consequently may not be susceptible to signal attenuation to avoid an exaggerated response, as do the natural compounds involved in the JA pathway formation (Heitz et al., 2012; Koo et al., 2011). This may explain the higher levels of induction of secondary metabolism observed in plants/cell cultures treated by Cor as compared to those treated with 'natural' JAs.

Whereas the effect of exogenous natural and synthetic JAs (Hu et al., 2006; Qian et al., 2005) on secondary metabolite biosynthesis has been widely studied, there are relatively few reports on the action of Cor on secondary metabolite production. Tamogami and Kodama (2000) showed induced accumulation of some flavonoid phytoalexins when rice leaves were treated with different concentrations (0.05–0.4 mM) of Cor. The effect of Cor on flavonoid production was greater than that of JA or 12-oxo-PDA (all at 0.5 mM concentrations). Haider et al. (2000) showed the positive action of Cor and some structural analogues on benzo[c]phenanthridine alkaloid production in *Eschscholzia californica* cell cultures, although in these studies Cor had a lower elicitor effect than MeJA and some analogues. The accumulation of glyceollins, the phytoalexins of soybean (*Glycine max* L.), in soybean cell suspension cultures has been studied after the addition of several elicitors related with the JA biosynthetic pathway. JA and MeJA showed weak phytoalexin-inducing activity when compared to an early jasmonate biosynthetic precursor, 12-oxo-phytodienoic acid (OPDA), or the bacterial phytotoxin Cor and certain 6-substituted indanoyl-L-isoleucine methyl esters, which were all highly active (Fliegmann et al., 2003; Lauchli et al., 2002).

No information about the effect of Cor elicitation on Px and related taxane production and the Px biosynthetic pathway in *Taxus* cell cultures is currently available. In this work, a selected *Taxus media* cell line was elicited with 1 μM Cor or 100 μM MeJA at the beginning of the second culture stage, to compare the effects of both elicitors on Px production. The expression levels of several genes related with the biosynthesis of Px and related taxanes were studied to shed light on the molecular changes that take place in elicited *T.* × *media* producer cells.

Materials and methods

Plant material

The *Taxus media* TXS cell line (Expósito et al., 2010) was grown in a two-stage culture as described previously (Cusidó et al., 2002a,b). Elicitors (MeJA and Cor; Sigma–Aldrich, St. Louis, MO, USA) were added to the production medium prior to inoculation. All compounds were filter-sterilised (0.22 μm sterile PES filters, Millipore, Billerica, MA, USA) and added to give the final concentrations of 100 μM MeJA and 1 μM Cor. For analysis, three flasks were harvested for each treatment at different time points: 0 h, 30 min, 1 h, 2 h, 4 h, 8 h, 12 h, 1 day, 2 days, 4 days, 8 days, 12 days, 16 days, 20 days and 24 days after elicitor treatment.

Cell growth and viability were determined as previously described (Expósito et al., 2010).

Taxane determination

Taxanes were extracted from the culture media as described (Bonfill et al., 2007) with some modifications. 20 mL of media was mixed and vortexed during 2 min with 5 mL of dichloromethane (DCM), followed by 1 h sonication at 25 °C. Once the organic phase was recovered, it was evaporated. Taxanes were extracted from freeze-dried cells with a microwave-assisted extraction protocol adapted from Talebi et al. (2004). 2 mL of methanol:water (9:1, v/v) was added to 50 mg lyophilised material, heated for 8 min in the microwave at 80 W, and filtered through nylon (0.50 μm filter, Maissa, Spain). The process was repeated twice, and both methanolic extracts were combined. After adding 4 mL of hexane, the samples were centrifuged at 2500 × *g* for 20 min at room temperature. The aqueous phase was recovered, mixed with 3 mL DCM:water (2:1, v/v) and vortexed until an emulsion of both phases was obtained. After recovering the organic phase, the aqueous phase was vortexed again with 3 mL DCM:water (2:1, v/v). Finally, both organic extracts were combined and evaporated. All samples were resuspended in 500 μL methanol and filtered prior to analysis (0.22 μm PVDF filters, Millipore, Billerica, MA, USA).

Quantification of Px and related taxanes was performed by high performance liquid chromatography (HPLC) as described (Richheimer et al., 1992). Criteria for identification included retention time, UV spectra, co-chromatography with standards and peak homogeneity by photo-diode detection when spiked with authentic standards. The peak areas corresponding to the studied taxanes from the samples, with the same retention time as authentic taxanes, were integrated by comparison with an external standard calibration curve. Px and related taxanes were provided by Hauser Chemicals, Boulder (USA).

Quantitative real-time PCR (qRT-PCR)

RNA was isolated using the "RNeasy Mini Protocol for isolation of total RNA from Plant cells and tissues and filamentous fungi" (Qiagen, Germany). cDNA was prepared from 1 μg of RNA with SuperscriptII reverse transcriptase (Invitrogen, CA, USA) and qRT-PCR was performed with SYBR Green PCR Mastermix (Roche, USA) in a 384-well platform system (LightCycler® 480 Instrument, Roche, USA). Gene specific primers were designed with Primer3 software version 0.4.0 (Table 1) and the amplification efficiency of each primer pair was determined empirically by 10-fold serial dilutions of cDNA and calculated as described by Qiagen. Only those primer pairs with an efficiency of over 0.8 were used. Expression levels were normalised to the levels of the *18S* from *Taxus baccata*.

For each gene, expression levels were indicated relative to those at day 14 of culture in the growth medium (reference value = 1).

Statistics

Statistical analysis was performed with Statgraphics (Centurion XV) and Excel software. All the data are the indicated as the mean of 3 measurements ± SD. The multifactorial ANOVA analysis followed by the Tukey multiple comparison tests were used for statistical comparisons. A p-value of <0.05 was assumed for significant differences.

Table 1
Sequences of the primers used to amplify the genes by quantitative real-time PCR.

Gene		Primer sequence	Amplicon size	Reference – Acc. Number
18S	Sense Reverse	5′-GTGCACAAAATCCCGACTCT-3′ 5′-GCGATCCGTCGAGTTATCAT-3′	102	Onrubia et al. (2010)
txs	Sense Reverse	5′-TTCGCACGCACGGATACG-3′ 5′-TTCACCACGCTTCTCAATTCG-3′	115	Onrubia et al. (2010)
t13oh	Sense Reverse	5′-GCCCTTAAGCAATTGGAAGT-3′ 5′-CAGAGGAATGGCGTTTAGAG-3′	100	AY866412 (*T.* × *media*)
t2oh	Sense Reverse	5′-CGTGCCATTTGGAGGAGGGAGA-3′ 5′-CGTGAGGGTCGATTGGCGTGTA-3′	122	AY518383 (*T. canadensis*)
t7oh	Sense Reverse	5′-GGTCCGCCCAAATTGCCAGAA-3′ 5′-CCCTGCAGAGCCCAAAAAACCT-3′	110	AY307951 (*T. cuspidata*)
dbat	Sense Reverse	5′-AGTTGGATTTGGTGATCGAA-3′ 5′-ATCCATGTTGCACGAGACTT-3′	92	Onrubia (2012)
pam	Sense Reverse	5′-CCCGGAGGCATGACGTGAAG-3′ 5′-CGCCGTCTTCCGCCTTGC-3′	99	AY866411 (*T.* × *media*)
bapt	Sense Reverse	5′-TAAGCACTCTACAACAACAATGG-3′ 5′-GCATGAACATTAGTATCTTGATTCC-3′	111	Onrubia et al. (2010)
dbtnbt	Sense Reverse	5′-CGGGGGGTTTGTTGTGGGATTA-3′ 5′-TTAGCCTCTCCCCTCGCCATCT-3′	104	Onrubia (2012)

Results

Cell biomass and viability

Two-stage suspension cultures of the TXS cell line were established (Cusidó et al., 2002a,b). In the second stage the cells were maintained for 24 days in the optimum medium for production and elicited or not with MeJA or Cor. MeJA was applied at 100 μM, the concentration previously found to be best for our culture system (Bonfill et al., 2007). Cor was applied at 1 μM, which earlier studies established as the optimum concentration for promoting taxane production in our TXS cell line with the least effect on biomass formation (Onrubia, 2012). Samples were taken just before and during this second stage of culture. Despite being in production media, growth was still observed in all three conditions (Fig. 1).

The capacity for biomass formation of Cor-elicited and unelicited cells was similar, except at the end of the experiment when the former exhibited a reduced growth capacity whereas the latter continued to grow actively. The growth observed in MeJA-elicited cultures was lower than in the control and Cor-treated cells throughout the experiment, especially after 12 days, when highly significant differences were observed ($p < 0.05$). The growth index (biomass obtained at the end of the experiment/inoculum biomass) achieved by the control cultures and those treated with MeJA or Cor was 3.5, 2.1 and 3.0, respectively. The dry weight values obtained during the time-course of the experiment corroborated the results indicated by the fresh weight (data not shown).

Viability, determined as the percentage of living cells in relation to the total cells, was also high, being on average 77%, 68% and 80% for the control, MeJA- and Cor-treated cultures, respectively. The fact that the fresh and dry weight results followed the same pattern suggests that the lower fresh weight of MeJA-treated cell cultures was not due to osmotic changes but to a decrease in living cells, which was also supported by the viability study.

Taxane production

Total taxane production (measured as the sum of 10-deacetylbaccatin III (DAB III), 10-deacetyltaxol (DAT), baccatin III, cephalomannine and Px) increased significantly when cells were transferred from the growth medium to the production medium. The highest taxane levels in the control cultures were observed in the second part of the second culture stage (8.8 mg/L at day 24) (Fig. 2). At the end of the culture, the production obtained was almost 6 times higher than at the beginning.

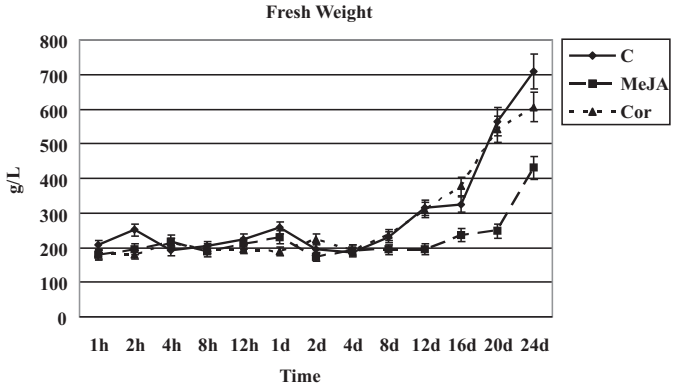

Fig. 1. Time courses of biomass production (expressed as g fresh weight/L) of the TXS cell line cultured for 24 days in the production medium without elicitors (C, control) or with the addition of 100 μM methyl jasmonate (MeJA) or 1 μM coronatine (Cor). In all cases, the inoculum consisted of 200 g/L of cells. Data represent the mean of three independent experiments ± SD.

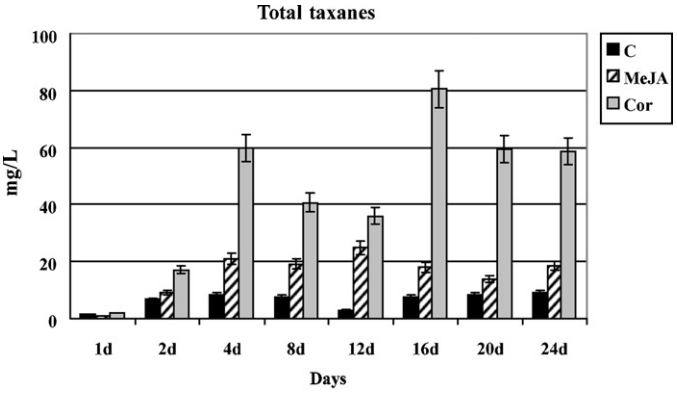

Fig. 2. Total taxane content (cell associated + extracellular) in the TXS cell line growing for 24 days in the production medium without (C) and with the addition of 100 μM methyl jasmonate (MeJA) or 1 μM coronatine (Cor). Data are the mean of three independent replicates ± SD.

The time course of taxane production was similar in the MeJA- and Cor-treated cells, but production in the latter was much higher throughout the culture period (Fig. 2). In both cases, taxane contents reached an early peak at day 4, but the overall maximum production in the Cor-treated cells occurred at day 16 (80.6 mg/L), 4 days later than in MeJA-treated cells (24.7 mg/L). The productivity achieved by the cultures under Cor elicitation corresponded to 5 mg L^{-1} day^{-1}. The total taxane profile obtained in this study for the control and treated cells agrees with the one observed by our group in *Taxus baccata* cell cultures, also grown in a two-stage system, with or without the addition of MeJA (Palazón et al., 2003). The increase of taxane production after MeJA elicitation is well-documented (Bonfill et al., 2007; Cusidó et al., 2002a,b; Ketchum et al., 1999; Yukimune et al., 1996).

The cell line studied showed a high capacity for taxane excretion from the producer cells to the medium, independent of elicitor treatment. The percentage of taxane excretion, which equals the total taxanes in the medium divided by the total taxanes in the medium plus cells, was extremely high: in control conditions it was over 91% until day 16, and subsequently over 50%. Under MeJA and Cor treatment, more than 80% of total taxanes were excreted, with maximum values reaching over 98% during the first days of culture. The release of accumulated taxanes from the cells to the medium is desirable, since it avoids toxic effects in the cells, enhances biosynthesis (Navia-Osorio et al., 2002), and also facilitates downstream processing.

Regarding the qualitative accumulation of the determined taxanes (Fig. 3), the major one in the control and MeJA-treated cell cultures during the first 8 days of culture was DABIII, which then decreased until the end of the experiment. In this period, DABIII represented on average 78% and 52% of the total taxane contents in the control and MeJA-elicited cell cultures, respectively. This was an expected result, since during the first days of culture, when the level of total taxanes is at its lowest, the most abundant taxanes are those without a lateral chain, especially DABIII, which is the precursor of baccatin III. This corresponded with the observation of Nims et al. (2006), who found DABIII to be the main taxane during the first 7 days of culturing *Taxus cuspidata* cells, independently of elicitation with 100 μM MeJA. In contrast, in Cor-treated cultures, although the absolute levels of DABIII were not much lower than in those under control and MeJA conditions, its content was very low relative to other taxanes (on average 7% of the total taxanes considered).

Baccatin III was the main taxane in the elicited cultures, especially when the total production was at its highest. Cell cultures treated with MeJA achieved a baccatin III production of 10.7 mg/L at day 12 (Fig. 3), which corresponded to 43% of the total taxanes produced at that point. In cell cultures treated with Cor, baccatin III peaked at day 16, achieving a level of 51 mg/L (Fig. 3), which represented more than 63% of the total taxanes produced. In control cultures, baccatin III was found in low or very low amounts throughout the experiment.

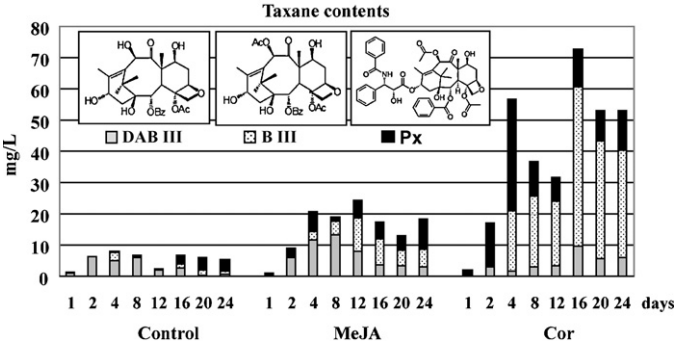

Fig. 3. Taxane content (expressed as mg L^{-1}) in the TXS cell line when maintained for 24 days in the optimum medium for production complemented or not with elicitors. MeJA: 100 μM methyl jasmonate; Cor: 1 μM coronatine; DABIII: deacetyl baccatin III; BIII: baccatin III.

Table 2
Production of the minority taxanes (DAT: 10-deacetyltaxol; Ceph: cephalomannine), expressed as mg/L in the TXS cell line growing for 24 days in the production medium without (C) and with the addition of 100 μM methyl jasmonate (MeJA) or 1 μM coronatine (Cor). Data are the mean of three independent replicates ± SD.

Treatments	Days	DAT	Ceph
Control	1	Traces	–
	2	Traces	Traces
	4	0.32 ± 0.02	Traces
	8	0.68 ± 0.08	Traces
	12	0.38 ± 0.05	Traces
	16	0.34 ± 0.05	0.50 ± 0.08
	20	0.91 ± 0.08	1.22 ± 0.20
	24	1.01 ± 0.09	2.61 ± 0.31
MeJA	1	–	–
	2	Traces	–
	4	Traces	–
	8	Traces	–
	12	–	–
	16	0.75 ± 0.08	–
	20	–	0.65 ± 0.07
	24	Traces	Traces
Coronatine	1	–	Traces
	2	Traces	Traces
	4	1.65 ± 0.33	1.59 ± 0.33
	8	1.42 ± 0.20	2.58 ± 0.41
	12	1.12 ± 0.09	3.15 ± 0.43
	16	2.14 ± 0.31	5.91 ± 0.59
	20	1.72 ± 0.23	4.74 ± 0.60
	24	1.73 ± 0.24	3.85 ± 0.45

Px in the unelicited cultures increased with time, peaking at day 20, when production was almost 4 mg/L (Fig. 3). The highest Px contents in the MeJA-treated cultures, almost 10 mg/L, were found at day 24. Px production in the Cor-treated cultures followed a different pattern, since two peaks were observed during the experiment. The first peak occurred very early, when at day 4 the Px content reached 36 mg/L, and the second peak was at the end, when almost 13 mg/L Px was obtained (Fig. 3).

The content of DAT (Table 2) was not detectable or very low, both in the control and elicited cell cultures. Since no information on the biosynthesis of DAT is available, it is difficult to interpret this result. Cephalomannine (Table 2) was found in significant levels at day 24 under control conditions and at day 16 under Cor elicitation. It is known that cephalomannine differs from Px in carrying a tigloylation instead of a benzoylation at position C3 of the lateral chain, while both have baccatin III as a precursor. Although the main taxane formed from baccatin III in Cor-treated cultures was Px, a small amount of the very high levels of this precursor could have been directed to the production of cephalomannine. Nevertheless, once again it is difficult to provide a reliable explanation since the regulation and characteristics of cephalomannine biosynthesis are not yet understood. Nonetheless, DAT and cephalomannine are both important taxanes since they can be used for the semisynthesis of Px analogues.

When comparing the maximum levels of taxane production in the three culture conditions, the content of baccatin III in the Cor-treated cultures was 21.6- and 4.8-fold higher than in the control and MeJA-treated cultures, respectively, whereas MeJA-elicitation increased baccatin III production 4.5-fold compared to the control. Px production under Cor treatment was 3.6- and 9.0-fold greater than in MeJA-treated and control cultures, respectively.

Also worth emphasising is the high baccatin III level accumulated in the Cor-treated cultures (52 mg/L) at day 16, when

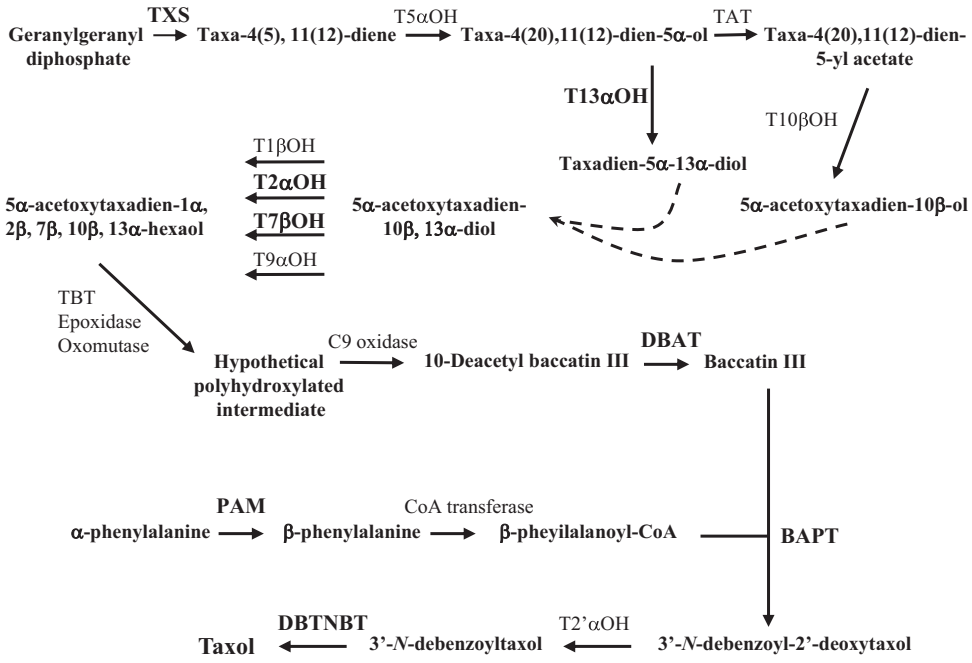

Fig. 4. The taxol biosynthetic pathway. In bold: the enzymes encoded by the studied genes.

the productivity rate reached 3.3 mg L^{-1} day^{-1}. This result is very promising since new Px-related compounds with improved efficacy and less toxicity are currently being sought, and most of them are being obtained semisynthetically from the natural precursor baccatin III (Expósito et al., 2009).

Gene expression profiles

To investigate the mode of action of the elicitors in taxane biosynthesis and the relationship between gene expression and the pattern of taxane production, the expression of genes encoding enzymes involved in Px biosynthesis was profiled by qRT-PCR. The studied genes were: *txs* (encoding taxadiene synthase) and *t13αoh* (encoding taxadiene 13α-hydroxylase), both involved in early synthetic steps; *t7βoh* (encoding taxane 7*β*-hydroxylase), *t2αoh* (encoding taxane 2α-hydroxylase) and *dbat* (encoding 10-deacetylbaccatin III-10β-*O*-acetyltransferase), which control intermediate synthetic steps; and *pam* (encoding phenylalanine aminomutase), *bapt* (encoding baccatin III-3-amino-13-phenylpropanoyltransferase) and *dbtnbt* (encoding 3′*N*-benzoyl transferase), which are involved in the last synthetic steps (Fig. 4).

Gene expression was determined from 1 h to 4 days after elicitation. Subsequent transcript accumulation is not shown in the figures because we observed that the highest expression and induction thereof occurs in this early period (Expósito et al., 2010; Onrubia et al., 2010). Similar results have been observed by other research groups (Hu et al., 2006; Nims et al., 2006).

The expression of the *TXS* gene, which controls the first committed step of the Px biosynthetic pathway (Hezari et al., 1995), was greatly enhanced by the presence of elicitors in the culture medium (Fig. 5). The accumulation of *txs* transcripts under MeJA-treatment was detectable 1 h after elicitation, and reached a maximum at days 1–4, decreasing thereafter (data not shown). At day 4, *txs* transcript levels were 5.2 times higher in the MeJA-treated cultures than in the control. Under Cor, *txs* transcript levels clearly increased after 1 h and peaked at 24 h after elicitation, decreasing thereafter, although remaining high until day 4. Maximum *txs* transcript levels in the Cor-elicited cultures were 4.8 times greater than in the control, also at 24 h (Fig. 5). Although values were similar under both elicitors (140 and 130 times higher than the reference value) ($p < 0.05$), it is notable that *txs* transcript levels peaked 3 days earlier in Cor-treated cultures. We have previously shown that the *txs* gene is highly induced in MeJA-elicited cell suspension cultures (Onrubia et al., 2010).

Taxadiene-13α-hydroxylase (T13αOH) is the enzyme that hydroxylates taxa-4(20),11(12)-dien-5α-ol to taxa-4(20),11(12)-dien-5α-13α-diol (Jennewein et al., 2001). As shown in Fig. 5, transcript accumulation of this gene was significantly higher in Cor-treated cultures than in those supplemented with MeJA ($p > 0.05$). At the same time, both cultures presented significantly higher *t13oh* mRNA levels than unelicited cultures ($p > 0.05$). Expression under MeJA began 1 h after elicitation, increasing until day 4, and under Cor it increased from 1 h to day 2, decreasing thereafter. Results obtained by Nims et al. (2006), indicate that the expression of this gene increases during the first hours after MeJA treatment. In our case, although RNAm accumulation of the *t13oh* gene was evident after 1 h of elicitation, the highest level was achieved after 4 days. The difference is probably due to the use of different cell lines and culture systems.

It is known that there is a branch point after the biosynthesis of taxa-4(20),11(12)-dien-5α-ol, which can be the substrate either for an acetylation at C5OH and a subsequent hydroxylation at C10, or for an hydroxylation at the C13 of the taxane skeleton, giving the taxa-4(20),11(12)-dien-5α-acetoxy-10β-ol or taxa-4(20),11(12)-dien-5α-13α-diol, respectively (Fig. 4). Although the expression of genes involved in the metabolic branch leading to taxa-4(20),11(12)-dien-5α-acetoxy-10β-ol (*TAT* and *T10βOH* genes) has not been studied here, the high levels of the *t13oh* gene observed in our TXS cell line could indicate that, after elicitation with MeJA and Cor, Px biosynthesis proceeds mainly through the step catalysed by the T13αOH enzyme. Nims et al. (2006) also suggested a preference for the T13αOH-side of the branch pathway in elicited *T. cuspidata* cell cultures, which confirms metabolic profiling data (Ketchum et al., 2003) showing that precursor flux leading to Px is via the 5α, 13α-diol through T13αOH, rather than the 5α-yl-acetate derived from the alternative branch controlled by the TAT enzyme.

In the intermediate steps of Px biosynthesis, the enzymes taxane-2α-hydroxylase (T2αOH) and taxane-7β-hydroxylase

(T7βOH) catalyse the hydroxylation of the 2C and 7C positions of the taxane skeleton, respectively (Chau and Croteau, 2004; Chau et al., 2004) (Fig. 4). The two corresponding genes showed similar expression patterns, which were comparable to that of the *t13oh* gene.

The expression of the *dbat* gene, corresponding to the enzyme for the transformation of 10-deacetylbaccatin III into baccatin III (Walker and Croteau, 2000) (Fig. 4), peaked at day 1 in the unelicited cultures (3.6 times higher than the reference value). Elicitation with MeJA resulted in a higher induction of the gene than with Cor, respectively 17.4 and 10.3 times the reference value. Levels of *dbat* transcripts in MeJA-treated cell cultures were already very high at 12 h, and continued increasing until day 1, decreasing again thereafter to values similar to those observed at 12 h. A similar trend was observed after treatment with Cor but in this case the highest value was observed at day 2. The highest values observed in MeJA- and Cor-supplemented cultures were 5 and 3 times higher, respectively, than in the control (Fig. 5).

The *pam* gene, encoding the enzyme responsible for the formation of β-phenylalanine from its isomer α-phenylalanine (Walker

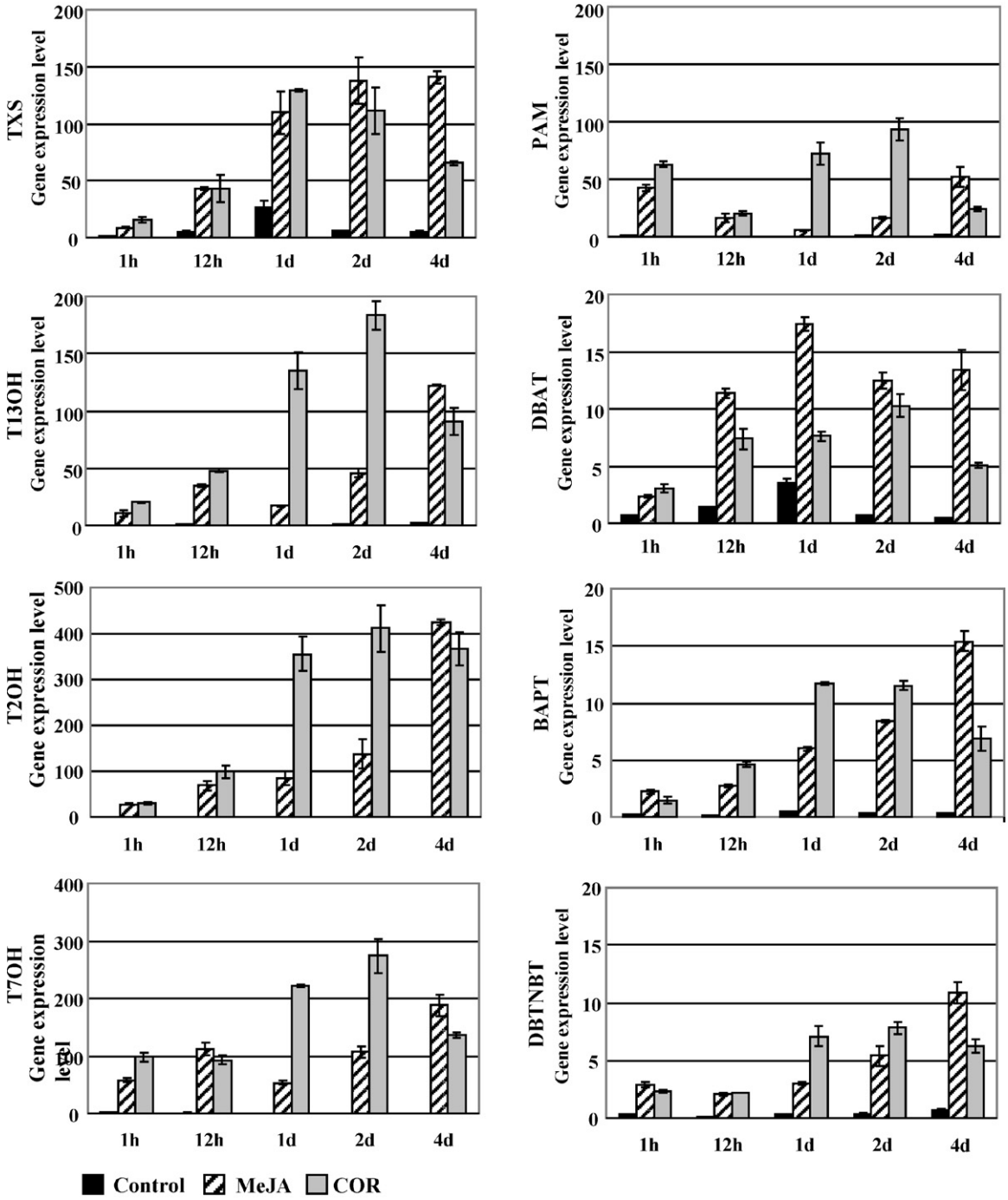

Fig. 5. Gene expression in the TXS cell line during the first 4 days in the production media complemented or not with the elicitors methyl jasmonate (MeJA, 100 mM) or coronatine (Cor, 1 mM). *Y*-axis: gene expression relative to that of the expression level in cultures maintained for 14 days in the growth medium; *X*-axis: time of culture, 14 days in growth medium, posterior time points in production medium with or without treatment. *TXS*, taxadiene synthase; *T13αOH*, taxadiene 13α-hydroxylase; *T2αOH*, taxane 2α-hydroxylase; *T7βOH*, taxane 7β-hydroxylase; *DBAT*, 10-deacetylbaccatin III-10-*O*-acetyltransferase; *PAM*, phenylalanine aminomutase; *BAPT*, baccatin III-3-amino, 13-phenylpropanoyltransferase; *DBTNBT*, debenzoyltaxol *N*-benzoyl transferase.

et al., 2004) (Fig. 4), is considered to be the first gene involved in the biosynthesis of the lateral chain of Px. The expression of this gene, in control cells, remained very low throughout the 4 days considered. In the elicited cultures (Fig. 5) two expression peaks were observed. The first occurred already 1 h after elicitation, with *pam* transcript levels in the MeJA- and Cor-treated cultures being 43 and 63 times higher, respectively, than the reference value. The second peak, which was the maximum *pam* transcript level achieved, was observed at day 4 under MeJA and day 2 under Cor treatment, being 52 and 93 times higher, respectively, than the reference value, and 29 and 53 times higher than in the control. It is notable that Cor was almost twice as effective as MeJA for the induction of the *pam* gene.

In the final steps of the metabolic pathway (Fig. 4), the enzyme baccatin III 13-*O*-(3-amino-3-phenylpropanoyl) transferase (BAPT) (Walker et al., 2002) is responsible for binding the lateral chain to baccatin III, leading to taxanes with β-phenylalanine as a lateral chain. The enzyme *N*-benzoyl transferase (DBTNBT) (Long et al., 2008) is involved in the last metabolic step leading to Px. Control cultures grown in the production medium presented only a slight increase in the expression of the two corresponding genes over the 4 sampled days (Fig. 5). When comparing the elicitor treatments, the highest expression level was observed for both genes under MeJA, being 1.3 times higher than the maximum under Cor. However, Cor caused an earlier activation, as shown in Fig. 5 (days 1–2 and 4, respectively).

Discussion

The highest expression levels of the studied genes were observed during the first hours/days after elicitation, however the highest taxane production was generally achieved later. Similar results have been found for other pathways (Pauwels et al., 2009). Nims et al. (2006) reported that a few days after their expression, the transcripts of several genes are no longer found in the cells, indicating that enzyme activity may persist long after their cognate mRNAs are absent from the cell. Nevertheless, our MeJA- and Cor-treated TXS cell cultures showed a significant increase in Px and baccatin III production only 4 days after elicitation, although the highest level was obtained at days 12 and 16, respectively.

Although the differences in the expression level of the studied genes in the MeJA- and Cor-treated cell cultures were not very remarkable, the total taxane production in these cultures differed considerably, suggesting that the elicitors may involve distinct but as yet uncharacterised regulatory mechanisms. Patil et al. (2012), working with MeJA-elicited *T. cuspidata* cell cultures of different cell aggregate size, reported that differences in expression of several genes involved in paclitaxel biosynthesis were minor compared with differences in taxane accumulation, The authors concluded that additional factors need to be uncovered before Px productivity can be fully optimised.

In our case, a possible explanation for the increased production of taxanes in the Cor-treated cultures is that this elicitor is less toxic than MeJA, since the cell viability study showed a higher percentage of living cells under Cor, especially towards the end of the culture period. A greater number of Px-producing cells in the Cor-treated cultures would thus be responsible for the increased taxane production. Alternatively, Uppalapati et al. (2005) showed that Cor induces an accumulation of proteinase inhibitors and consequently, enzymes involved in the Px biosynthetic pathway would have a more persistent activity in cell cultures treated with this elicitor, resulting in higher taxane contents than in the control or MeJA-treated cultures.

As indicated above, the expression of the *txs* gene in MeJA- or Cor-treated cell cultures was much higher than in control conditions. We have previously shown (Expósito et al., 2010) that although TXS controls the first committed step in the Px biosynthetic pathway, it is not a limiting enzyme involved in taxane production, as taxadiene is not a limiting substrate in Px formation. Hezari et al. (1997) suggested that taxadiene did not accumulate for longer than 2 days to any appreciable level in *Taxus* cell cultures, indicating a rapid conversion of this metabolite by downstream reactions.

The studied hydroxylases participate at different levels in the formation of the hypothetical polyhydroxylated intermediate (Croteau et al., 2006), which is the substrate for the formation of DABIII and baccatin III. In our study, the three genes encoding these enzymes were the most strongly activated by the elicitors. It is also worth noting that the highest transcript levels occurred two days earlier under Cor than MeJA. This might suggest that the taxane substrates were available earlier for the downstream metabolic steps in the Cor-treated cells and explain the higher taxane accumulation in these cultures.

Of the three genes, *pam*, *bapt* and *dbtnbt*, involved only in the biosynthesis of taxanes with a phenylisoserine lateral chain attached to the C13 of baccatin III, the *PAM* gene was the most induced by the addition of Cor to the culture medium, probably resulting in a high amount of β-phenylalanine, which would not limit the lateral chain formation and consequently the biosynthesis of Px in the Cor-treated cultures. However, when total taxane production was at its peak (day 16), the average baccatin III accumulation was 2.3 times higher than the average sum of lateral chain-bearing taxanes during the same period. This result indicates a limited transformation of baccatin III into Px or lateral chain-bearing taxanes, which might be due to the relatively low induction of the *bapt* and *dbtnbt* genes by Cor (in terms of fold induction relative to the reference value). It is also notable that although total taxane production was higher in the Cor-treated cells, the proportion of Px was higher in MeJA-treated cultures. This could reflect the aforementioned enhanced levels of *bapt* and *dbtnbt* in the MeJA cultures, particularly at the end of the experiment.

Our results have shown that all the studied genes were induced to a variable extent by the presence of the elicitors MeJA (100 μM) and Cor (1 μM). The elicitors enhanced the expression of genes involved in the formation of both precursor parts of the Px molecule, the polyhydroxylated precursor of Px and the phenylalanoil CoA chain, but had a more limited effect in the last two steps of the Px biosynthesis pathway (Figs. 4 and 5). Clearly, the elicited cultures showed different taxane patterns and gene expression profiles, suggesting differing action mechanisms, even though Cor is structurally similar to the active jasmonate JA–Ile. Previous studies carried out by Weiler et al. (1994) report that 1 μM Cor conditioned more accumulation of indole alkaloids in *Rauwolfia serpentina* cells than 100 μM MeJA. However, the same authors observed that the content of benzophenacetridine alkaloids as a function of Cor concentration and the time course of response was about 50–100-fold lower than for MeJA in *E. californica* cell cultures. Katsir et al. (2008) have shown that Cor is more efficient than MeJA and JA–Ile in stimulating the JA pathways. Uppalapati et al. (2005) demonstrated that a 10,000-fold higher concentration of MeJA was necessary to have the same effect as Cor on anthocyanin content in tomato.

Although it has been shown that both JA–Ile and Cor have the COI1 protein as a receptor (Katsir et al., 2008), the different response in MeJA and Cor-treated cell cultures might be explained by the different signal transduction steps downstream of the perception machinery. This could explain the different taxane-accumulation behaviour in Cor- and MeJA-elicited cultures. Several authors studying the action mechanism of Cor, MeJA and other chemical analogues (Svoboda and Boland, 2010; Tamogami and Kodama, 2000; Uppalapati et al., 2005) have reported that the recognition process of these elicitors is very complex, so while

they might be recognised by several members of the same family of proteins, the differential recognition may trigger a variety of defensive responses. Alternatively, the absence of downstream catabolic steps to attenuate Cor effects, which exist for JA–Ile or other 'natural' JAs (Heitz et al., 2012; Koo et al., 2011), may also be an explanation for the distinct effects observed in the Cor-treated *T.* × *media* cell cultures.

Conclusion

The metabolic studies carried out have shown that the presence of Cor in the culture medium dramatically enhanced taxane production, particularly of baccatin III and to a lower extent Px, the highest levels of both taxanes being 4.8- and 3.6-fold greater than in the MeJA-elicited cultures.

Taken as a whole, our results show that both MeJA and Cor induce a dramatic reprogramming of gene expression in *Taxus* cell cultures, which likely accounts for the enhanced production of Px and related taxanes. Although the studied taxane biosynthetic genes did not show a significantly higher induction by Cor than MeJA, peak expression levels were generally observed earlier under Cor elicitation. The resulting earlier availability of precursors could be responsible for the higher total taxanes achieved in cultures elicited by Cor rather than MeJA (3.3-fold higher, on average). At the same time, a clear relationship was observed between the expression profile of the studied genes and the taxane production and pattern in the TXS cell line. Further enzymatic studies are warranted, however, to confirm the dependence of the taxane accumulation pattern on the expression of genes involved in the Px biosynthetic pathway.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jplph.2012.09.004.

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