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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.