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Research review paper

A rational approach to improving the biotechnological production of taxanes in plant cell cultures of *Taxus* spp.

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ABSTRACT

Taxol is a complex diterpene alkaloid scarcely produced in nature and with a high anticancer activity. Biotechnological systems for taxol production based on cell cultures of *Taxus* spp. have been developed, but the growing commercial demand for taxol and its precursors requires the optimization of these procedures. In order to increase the biotechnological production of taxol and related taxanes in *Taxus* spp. cell cultures, it is necessary not only to take an empirical approach that strives to optimize in-put factors (cell line selection, culture conditions, elicitation, up-scaling, etc.) and out-put factors (growth, production, yields, etc.), but also to carry out molecular biological studies. The latter can provide valuable insight into how the enhancement of taxane biosynthesis and accumulation affects metabolic profiles and gene expression in *Taxus* spp. cell cultures.

Several rational approaches have focused on studying the transcriptomic profiles of key genes in the taxol biosynthetic pathway in *Taxus* spp. cell cultures treated with elicitors such as methyl jasmonate, coronatine and cyclodextrins in relation with the taxane pattern, production and excretion to the culture medium. These studies have provided new insights into the taxol biosynthetic pathway and its regulation. Additionally, identifying genes with low levels of expression even in the presence of elicitors, together with metabolomics studies, has shed light on the limiting steps in taxol biosynthesis and could help define suitable metabolic targets for engineering with the main aim of obtaining highly productive *Taxus* cultured cells.

In this review, we have summarized the latest endeavors to enhance the molecular understanding of the action mechanism of elicitors in *Taxus* spp. cell cultures. Developments in the ongoing search for new and more effective elicitation treatments and the application of metabolic engineering to design new transgenic cell lines of *Taxus* with an improved capacity for taxane production are described.

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Introduction

Taxol, also known by the generic name paclitaxel, is one of the most effective anticancer drugs ever developed (Abal et al., 2003; Wani et al., 1971). This complex diterpene alkaloid is characterized by the presence of a taxane ring and an N atom in the phenylisoserine lateral chain (Fig. 1). Due to its unusual mechanism of action, taxol has proved highly effective in the treatment of various types of cancer, and is currently being studied for the treatment of illnesses requiring microtubule stabilization, such as cardiac functional recovery and psoriasis (Ehrlich et al., 2004; Xiao et al., 2012). Consequently, there is a world-wide market for taxol and compounds with similar molecular structures. Since taxol presents some drawbacks in its administration, there is great interest in developing new analogs with improved therapeutic activities (Guo and Huang, 2013; Walji and MacMillan, 2007).

The natural source of taxol is the bark of several *Taxus* species, but the cost of its extraction is prohibitively high, since it accumulates in a very low concentration (about 0.02% of dry weight) and entails the destruction of yew trees. For these reasons, the ever-growing demand for taxol greatly exceeds the supply through isolation from its natural source and alternative ways of producing this drug are actively being sought (reviewed in Exposito et al., 2009a).

Although taxol can be prepared by total synthesis (Holton et al., 1994a, 1994b; Nicolaou et al., 1994), the process is not commercially viable. Taxol and its analogs can also be semisynthetically produced from more abundant taxanes, for example, via the conversion of baccatin III (BIII) extracted from *Taxus* needles (Denis et al., 1988). However, the cost and difficulty of the extraction process of the precursors are also prohibitive. *Taxus* crops continue to be traditionally harvested for taxane extraction, mainly in Yunnan province (China) (Wang et al., 2007), but the most promising approach for a sustainable production of taxol and related taxanes is provided by plant cell cultures at an industrial level. Commercial production of taxol in *Taxus* cell suspension cultures developed by Phyton Biotech has provided Bristol-Myers Squibb with a secure, sustainable and environmentally-friendly source of Taxol® since 1995 (Roberts and Shuler, 1997). In 2004, FDA (Food and Drug Administration, USA) approved the use of plant cell cultures for the supply of taxol and the current main provider worldwide is Phyton Biotech, which is now a subsidiary of DFB Pharmaceuticals Inc. (Leone and Roberts, 2013).

The economic importance of taxanes recently prompted a bibliometric study of global scientific research on *Taxus*, seeking to identify patterns and tendencies in *Taxus*-related articles over the last 20 years (Hao et al., 2012a, 2012b). Among recent "hot topics" identified by a synthetic analysis of article titles, abstracts and keywords are the sustainable production of taxanes by cell culture platforms and semisynthesis of taxane analogs from baccatin III and 10-deacethylbaccatin. The main

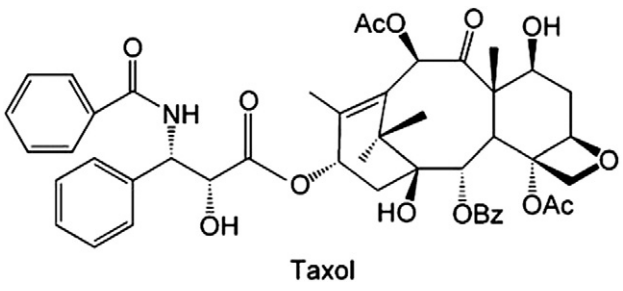

Fig. 1. Chemical structure of the anticancer compound taxol.

foci of ongoing *Taxus*-related research in the 21st century are on the application of taxanes in clinical cardiology, pharmacology and oncology, and the study of *Taxus* metabolism, cytology and microbiology. The bibliometric analysis also revealed that knowledge of taxane genomics, transcriptomics, metabolomics and bioinformatics is still limited, and practically nothing is known about how epigenetic mechanisms regulate taxane biosynthesis. After providing an overview of taxane production in plant cell cultures, this review aims to summarize and highlight the key issues of *Taxus*-related research in the 21st century, with particular emphasis on the use of omics tools and metabolic engineering for improving taxane production using plant cells as biofactories. Special attention is also focused on new taxane sources such as endophytic fungi of *Taxus* and *Corylus avellana* cell cultures, which are promising biological platforms for the biotechnological production of taxanes in the future (Miele et al., 2012).

Production of taxanes in plant cell factories

Plant cell cultures of Taxus, a toolbox for taxane production

When a natural product is scarce in nature, plant biotechnology can provide an alternative system for its production. Plant cell cultures producing phytochemicals have several advantages over whole-plant culture: a) the desired product can be harvested anywhere in the world, maintaining strict production and quality control; b) herbicides and pesticides are not required; c) climate- or ecology-related problems are avoided; and d) growth cycles are measured in weeks rather than years of an intact plant (Ramachandra-Raoa and Ravishankar, 2002). Yet despite all these advantages, there are only few examples of successful bioactive compound production in plant cell factories, which may be due to a lack of knowledge of plant secondary metabolism and its in vitro control. There is therefore an urgent need to develop new strategies to improve our understanding of the metabolic pathways that lead to the formation of bioactive compounds of plant origin. In the case of taxol, although several companies around the world have established plant cell culture platforms for profitable taxane production (Wilson and Roberts, 2012), there is still great scope for improvement to meet the rising market demand for new taxol derivatives.

For some time now, research groups around the world have focused on taxol production, working with cell cultures of *Taxus* spp. from a small scale to bioreactor level. Among the *Taxus* species employed to obtain effective taxane-producing cell cultures are *Taxus baccata* (Malik et al., 2011), *Taxus media* (hybrid of *T. baccata* and *Taxus cuspidata*) (Baebler et al., 2002; Bonfill et al., 2003), *T. cuspidata* (Fett-Neto et al., 1995; Li and Tao, 2009), *Taxus brevifolia* (Collins-Pavao et al., 1996), *Taxus chinensis* (Luo and He, 2004), *Taxus canadensis* (Kim et al., 2006) and more recently *Taxus globosa* (Tapia et al., 2013). However, the capacity of *Taxus* sp. cell lines to produce taxanes depends far more on the culture conditions and the elicitors employed than on the plant species (Exposito et al., 2009).

The first step to obtaining a *Taxus* sp. cell suspension is to establish fast-growing friable callus cultures, such as those of *Taxus* × *media* shown in Fig. 2, which were developed from young stems of 3–4 year old yew trees cultured in optimum conditions. Several basal salt media, including Murashige and Skoog (Murashige and Skoog, 1962; MS), Gamborg (Gamborg et al., 1968; B5) and Woody Plant Medium (Lloyd and McCown, 1980; WPM), and numerous plant growth regulators in different combinations have been used to obtain calli from explants, as comprehensively summarized recently by Onrubia et al.

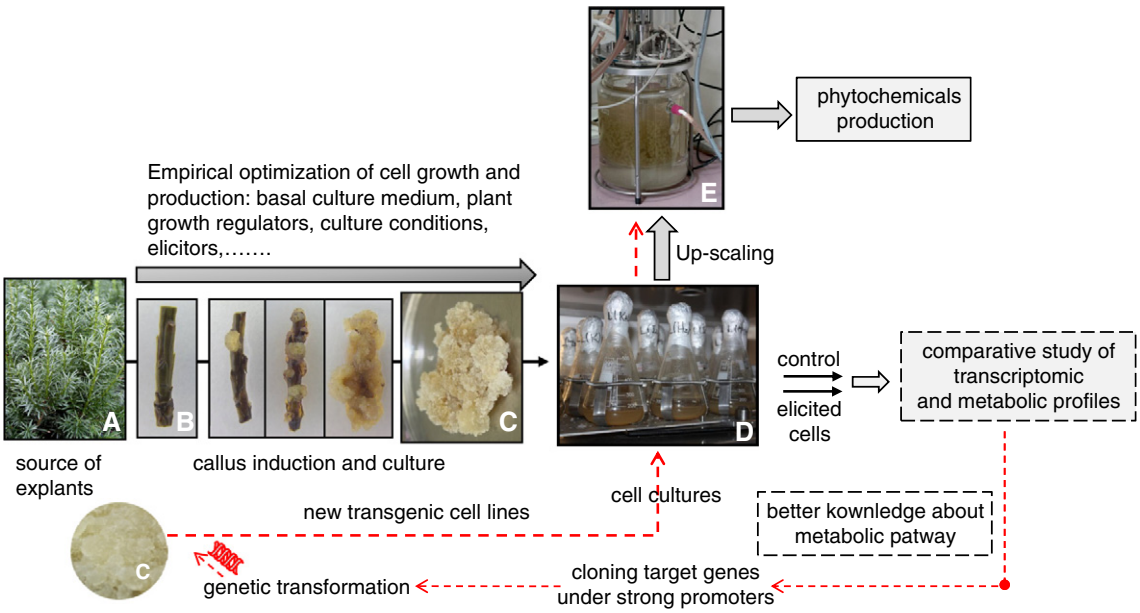

Fig. 2. Proposal for an experimental design based on plant cell cultures to improve the biotechnological production of phytochemicals. (A) 3-year-old *Taxus* plant, (B) explant, (C) *Taxus* calli, (D) *Taxus* cell suspension, (E) *Taxus* cell suspension culture in a 5-L stirred tank reactor.

(2013a). The optimization of conditions for callus induction and growth can be a tedious process, as the response to induction and growth treatment varies according to the type of explant and species of mother plant, thus requiring each process to be optimized separately. The optimization of plant secondary metabolite yield is also a difficult and laborious procedure since in most cases the best conditions for growth are different from those for production of bioactive compounds.

The number of experiments necessary for optimizing the growth and production of a biotechnological system can be reduced by following a fractional factorial design based on an orthogonal array (Dean and Voss, 1999). Working with *T. media* suspension cultured cells for taxane production, Cusido et al. (2002) tested the effects of two basal media [B5 and WPM] and sources of sugar (sucrose and sucrose + fructose), auxin [2,4-dichlorophenoxyacetic acid (2,4-D), 1-naphthaleneacetic acid (NAA), and Picloram] and cytokinin [kinetin, zeatin, 6-benzylaminopurine (BAP), and m-Topolin], reducing the 48 possible culture media combinations to 24. These experiments showed that taxane production is dissociated from cell growth and that it is recommendable to work with a two-stage system in which plant cells are first cultured in a medium optimized for growth and then transferred to a production medium (Cusido et al., 2002).

Elicitation as an empirical strategy for improving taxane production

Elicitation, a process for inducing or enhancing the synthesis of plant secondary metabolites, is one of the most effective established methods to improve secondary metabolite production in plant cell platforms (Patel and Krishnamurthy, 2013) (Fig. 2). Abiotic elicitors include metal ions and inorganic compounds (silver nitrate, copper sulphate, vanadyl sulphate, etc.), while biotic elicitors are organic compounds obtained from living organisms such as fungi, bacteria, viruses, cell wall components and chemicals (arachidonic acid, salicylic acid, jasmonates, etc.) synthesized by the plant at the site of pathogen or herbivore attack (Zhao et al., 2005).

Elicitors for improving taxane production in *Taxus* cell cultures have been extensively used since the 1990s (recent reviews see: Malik et al., 2011; Onrubia et al., 2013a; Sabater-Jara et al., 2010a, 2010b). Cell culture supplementation with abiotic and biotic elicitors such as vanadyl (IV) sulphate, lanthanum salts, arachidonic acid, salicylic acid, jasmonates, and fungal extracts, alone or in combination, has been largely effective in increasing taxane production.

Jasmonic acid (JA) and related compounds are involved in plant stress responses, and can increase plant secondary metabolism by transducing elicitor signals (Wasternack, 2013). It has been shown that exogenous application of JA and methyl jasmonate (MeJA), as well as their conjugated forms, to plant cell cultures stimulates the biosynthesis and accumulation of a wide variety of secondary metabolites, such as terpenoids, alkaloids and polyphenols, many of them with important biological activities yet scarce in nature, like taxol, which makes them putative targets for plant biotechnology (Zhao et al., 2005). MeJA was used for the first time in cell cultures of *T. baccata* in 1996 by Yukimune et al. (1996), who found that it could increase taxane production more than 120-fold. Subsequently, MeJA has been widely applied to increase taxol production in plant cell cultures.

Looking for new elicitors

The search for new and more active elicitors for taxane production is one of the hot research topics for the 21st century. In this context, elicitation with the phytotoxin coronatine (Cor), or MeJA combined with cyclodextrins (CDs), has proved to be more powerful than MeJA alone in boosting taxane production (Onrubia et al., 2013b; Sabater-Jara, 2013).

Coronatine. Coronatine (Cor), a plant pathogenic toxin produced by several strains of *Pseudomonas syringae*, has recently received considerable attention as a potential plant growth regulator and elicitor of plant secondary metabolism. This phytotoxin is known to act as a molecular mimic molecule of the isoleucine-conjugated form of jasmonic acid (JA-Ile), the intracellular switch of the jasmonate pathway (Katsir et al., 2008). Several studies have reported that Cor exerts its virulence effects by activating the host's jasmonate signaling pathway (Zhao et al., 2003) and plants insensitive to Cor, such as the *Arabidopsis* (*Arabidopsis thaliana*) coi1 mutant, exhibit resistance to Cor-producing *P. syringae* strains (Zhao et al., 2003). Cor promotes multiple aspects of bacterial virulence, including reopening of stomata, which facilitates invasion and proliferation in the apoplast and development of disease symptoms. Studies carried out during the last decade suggest that Cor induces JA biosynthesis, affects signaling in tomato via multiple phytohormone pathways (JA, ethylene and auxin pathways) (Uppalapati

et al., 2005) and has a wide range of biological functions, including 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; summed up by Uppalapati et al., 2005).

At a molecular level, it has been shown (Katsir et al., 2008) that Cor promotes virulence by functioning as a potent agonist of JA-Ile, being around 1000-fold more active than JA-Ile in promoting the COI1–JAZ interaction in vitro. Moreover, the formation of the receptor COI1–JAZ1 in *Arabidopsis* and tomato is stimulated by JA-Ile but not by JA, MeJA or the JA precursor 12-oxo-phytodienoic acid (OPDA) (Thines et al., 2007). Thus, JA-Ile, or an analog such as Cor, may be more effective in switching on the JA pathway than a molecule requiring processing, such as MeJA, which only becomes an active jasmonate after undergoing demethylation and conjugation to an amino acid. As Cor is a stable compound, in contrast with JA-Ile, it is able to keep the SCFCOI1 complex active (SCFCOI1 controls genome expression and promotes JA responses; Feng et al., 2003), thereby allowing longer transcription processes. Recently, some interesting biological assays and ultrastructural and gene expression studies have been published about the involvement of Cor in the MeJA signaling pathway and hypersensitive responses, mainly using insensitive mutants (Geng et al., 2012; Kombrink, 2012; Lee et al., 2013).

While 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 impact of Cor on secondary metabolite production. Tamogami and Kodama (2000) described an induced accumulation of some flavonoid phytoalexins when rice leaves were treated with different concentrations (0.05–0.4 mM) of Cor. The effect of this elicitor on flavonoid production was greater than that of JA or OPDA (all at 0.5 mM concentrations). Haider et al. (2000) showed the positive action of Cor and some structural analogs on benzo[c]phenanthridine alkaloid production in *Eschscholzia californica* cell cultures, although in these studies Cor had a lower elicitation effect than MeJA and some analogs. The accumulation of glyceollins, the phytoalexins of soybean (*Glycine max* L.), in soybean cell cultures has been studied after adding several elicitors related with the JA biosynthetic pathway. JA and MeJA showed weak phytoalexin-inducing activity compared to the activity of OPDA or Cor and certain 6-substituted indanoyl-L-isoleucine methyl esters, which were all highly active (Fliegmann et al., 2003; Lauchli et al., 2002). Recently, Onrubia et al. (2013b) reported that Cor very effectively increased the production of taxol and related taxane and the expression of several genes controlling the taxol biosynthetic pathway in *T. media* cell cultures. The taxane levels achieved were significantly higher than those found in the same cell cultures elicited with MeJA, as will be explained in more detail below.

Cyclodextrins. In recent years, great attention has been paid to the use of cyclodextrins as agents capable of inducing secondary metabolism in plant cell cultures by acting as true elicitors (Bru et al., 2006; Lijavetzky et al., 2008; Zamboni et al., 2009). CDs are cyclic oligosaccharides consisting of α-D-glucopiranose residues linked by α (1 → 4) glycosidic bonds and produced from starch by enzymatic conversion (Szejtli, 1997). The most common CDs are α-, β-, and γ-CDs, which contain six, seven, and eight glucose units, respectively (Saenger, 1980). Their action as secondary metabolite inducers in plant cell cultures resides in their chemical structure, which is similar to that of the alkyl-derived oligosaccharides released from the plant cell walls when a fungal infection occurs (Bru et al., 2006). In this way, under CD elicitation, plant cell cultures respond by synthesizing secondary metabolites, which are secreted and accumulated outside the cells in the culture medium, allowing their accumulation at elevated concentrations. Thus, in *Vitis vinifera* cv Monastrell cell cultures elicited with CD, the production of *trans*-resveratrol (phytoalexin from *Vitis* species belonging to the stilbene family) and its accumulation in the culture media were more than 2000 mg L^{-1} (Bru and Pedreño, 2003). Moreover, Zamboni et al. (2009) studied the gene profiles by microarrays in grapevine cell cultures elicited with CD after 2 and 6 h of treatment. They observed early CD-induced defense responses mediated by the activation of protein kinases and/or protein phosphatases, as other authors also described (Belchí-Navarro et al., 2013a). In addition, CD triggered a cascade of signal transduction which in turn, activated different families of transcription factors regulating the expression of genes related to the *trans*-resveratrol biosynthetic pathway (like stilbene synthase or phenylalanine ammonia-lyase), and defense proteins such as pathogenesis-related proteins (Belchí-Navarro et al., 2013b; Zamboni et al., 2009). Therefore, CD elicitation not only increases the production of secondary metabolites, mainly those related to defense, by inducing the expression of genes responsible for their biosynthesis but also allows the accumulation of these compounds in the extracellular medium, thereby reducing feedback inhibition. As this procedure prevents cell death caused by the high amount of metabolites accumulated in the spent medium, it has also been applied successfully to improve the production of other secondary metabolites such as phytosterols in *Daucus carota* (Sabater-Jara and Pedreño, 2013), indole alkaloids in *Catharanthus roseus* (Almagro et al., 2011) and silymarins in *Silybum marianum* (Belchí-Navarro et al., 2013c). In fact, Sabater-Jara and Pedreño (2013) have developed a method based on the use of CD to enhance phytosterol production and their extracellular accumulation by using *D. carota* cell cultures. This effect is mainly based on CD characteristics since they have a hydrophilic external surface and hydrophobic central cavity and so can trap apolar compounds by forming inclusion complexes, thus increasing the solubility of poorly water-soluble compounds. Besides achieving high levels of phytosterols, this innovative approach also differs from other elicitation procedures in the extraction process. CDs not only act as elicitors but also appear to sequester the plant secondary metabolites that are produced, thus preventing negative feedback loops and allowing successful subcultures (Belchí-Navarro et al., 2012; Sabater-Jara and Pedreño, 2013).

It is well-documented that the combined use of more elicitors enhances secondary metabolite biosynthesis (Zhao et al., 2005). Recent studies have shown that the treatment of plant cell cultures with MeJA and CD can result in the accumulation of secondary metabolites in different plant species (Briceño et al., 2012, Lijavetzky et al., 2008, Sabater-Jara et al., 2010a, 2010b). In fact, Lijavetzky et al. (2008) analyzed the effects of MeJA, CD and a combination of both on *trans*-resveratrol extracellular accumulation and the expression of genes from the stilbene biosynthesis pathway in grapevine cell cultures. The results showed a synergistic effect in *trans*-resveratrol accumulation triggered by the joint action of MJ and CD. Likewise, the expression of these genes was significantly induced but transiently expressed when elicitors were added independently to grapevine cell cultures. Such expression was well-correlated with *trans*-resveratrol production in CD-treated cells but not in MeJA-treated cells. Furthermore, the synergistic interaction of both elicitors on *trans*-resveratrol production seems to be the result of the synergistic effect on the expression of resveratrol biosynthesis-related genes. Similarly, Sabater-Jara (2013) demonstrated that the taxane accumulation in *T. media* cell cultures was significantly increased by the combination of MeJA and CD, and a synergistic effect by the joint action of both elicitors was observed. In addition, the expression of taxol-related genes was determined, observing a strong increase in their expression under elicitation with MeJA and CD. Thus, the synergistic interaction of both elicitors on the production of taxol and related taxanes seems to be the result of a synergistic effect on the expression levels of their biosynthesis-related genes. In the same way, the application of MeJA alone or in combination with CD triggered the accumulation of secondary metabolites in Solanaceae cell cultures, e.g. aromadendrene and solavetivone in *Capsicum annuum* (Sabater-Jara et al., 2010a, 2010b) and taraxasterol in *Solanum lycopersicum* (Briceño et al., 2012). Also, Almagro et al. (2011) demonstrated that the maximum level of ajmalicine produced by *C. roseus*

cells and secreted to the culture media was reached when cells were incubated in the presence of MeJA and CD, the production being around 2.2-fold higher than when cells were treated only with CD, observing again, a synergistic effect by the joint actions of both elicitors.

Rational approaches to the biotechnological production of taxanes

If progress in the biotechnological production of taxanes is to continue, a rational approach to the molecular bioprocesses that take place in the producer cells is essential (Fig. 2). In other words, it is necessary to know how the different empirical factors that increase yields of taxol and related taxanes affect gene expression and metabolic profiles in *Taxus* cell cultures. Such an approach can provide insight into biosynthetic pathways and their regulation.

The taxol biosynthetic pathway consists of 19 metabolic steps (Fig. 3), beginning with the formation of the diterpene, geranylgeranyl diphosphate (GGPP). The enzyme taxadiene synthase (TXS) diverts GGPP from its metabolic pool towards the formation of the first compound bearing the taxane ring, taxadiene. After the action of a variety of hydroxylases and acyl transferases and other enzymes, 10-deacetylbaccatin III (10-DABIII) and baccatin III (BIII) are formed. Both compounds have been used for the semisynthesis of taxol and analogs. A phenylalanine-derived side chain is then attached to BIII, and after two more biosynthetic steps the target molecule is produced (for a detailed description of taxane biosynthesis, see recent reviews: Exposito et al., 2009; Onrubia et al., 2013a). Twelve enzymes involved in this pathway are known, and the genes encoding them have been sequenced and cloned, but 7 steps still remain to be resolved. Omics studies comparing transcriptomic and metabolomic profiles of control and elicited *Taxus* cell cultures may lead to the discovery of new genes encoding other enzymes involved in the metabolic pathway and help to clarify the mechanisms that regulate taxane production in cell cultures.

Transcriptomic profiles for the discovery of new genes involved in taxane metabolism and its regulation

Elicitation of plant cells with jasmonates triggers an extensive reprogramming of metabolism that usually correlates very well with the observed shifts in the accumulation of the metabolites. This has been exemplified repeatedly by genome-wide transcript profiling studies designed to visualize the effect of jasmonates on metabolism-related gene expression (Pauwels et al., 2009). These studies showed that in most cases, (i) the genes involved in a particular biosynthetic pathway are regulated in concert, leading to the coining of the term 'transcriptional regulons', and (ii) that gene induction occurred remarkably fast, usually within 1 to 4 h, indicating that activation of secondary metabolism is one of the prominent targets of jasmonate signaling across the

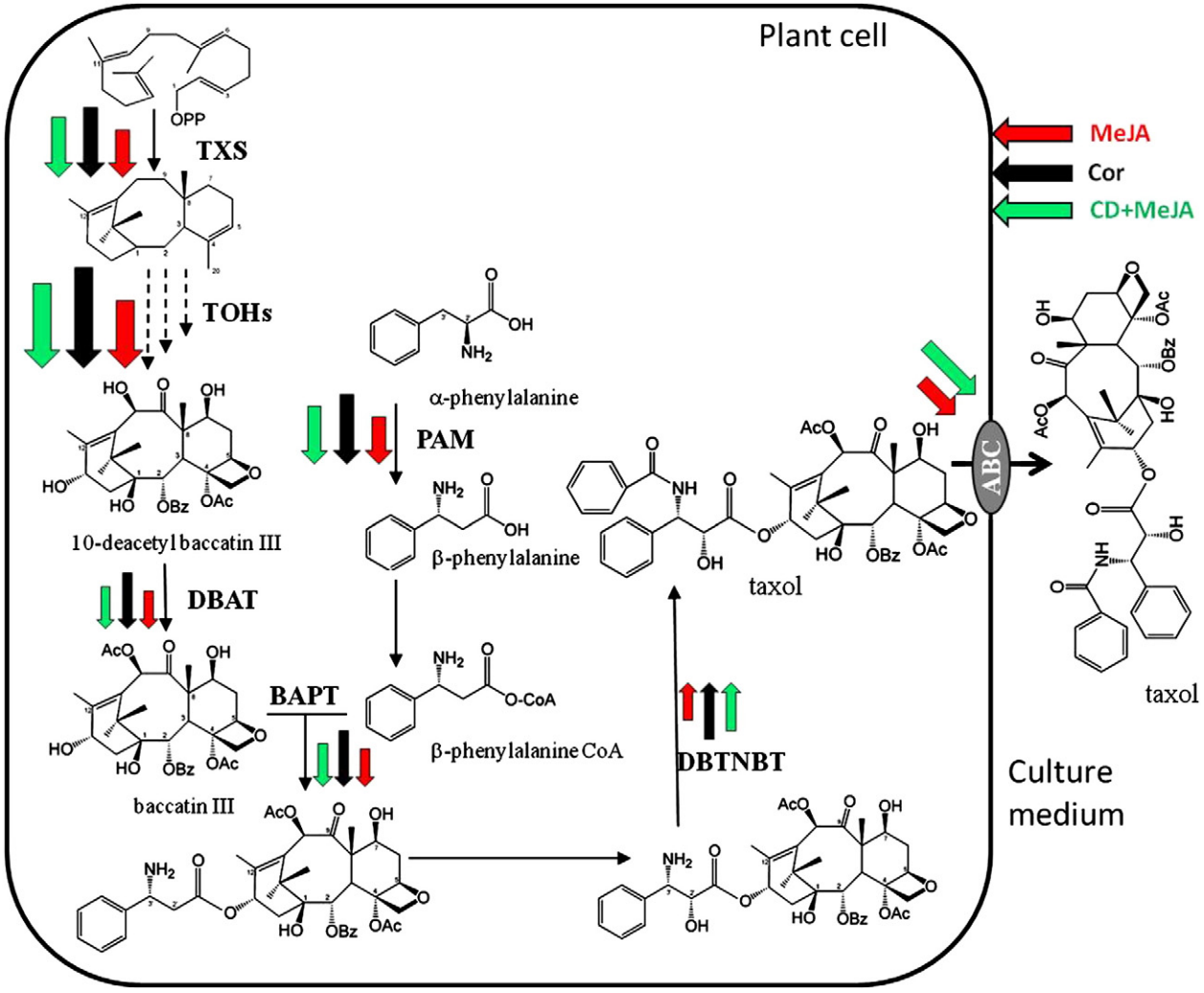

Fig. 3. Gene expression induced by elicitor treatments (MeJA, Cor or CD + MeJA) in *Taxus* sp. cell cultures. Small arrows represent low gene induction. TXS, Taxadiene synthase; TOHs, taxane hydroxylases; PAM, phenylalanine aminomutase; DBTA, 10-deacetylbaccatin III-10-O-acetyltransferase; BAPT, baccatin III-3-amino, 13-phenylpropanoyl-CoA transferase; DBTNBT, Debenzoyl taxol N-benzoyl transferase.

plant kingdom (Pauwels et al., 2009). This conserved feature of plant secondary metabolism allowed gene discovery programs aiming for the identification of the genes encoding the unknown enzymes from a pathway of interest. For instance, a pioneering transcriptome study, consisting of random sequencing of a cDNA library from MeJA elicited *T. cuspidata* cells (Jennewein et al., 2004), (i) revealed all the defined genes of taxol biosynthesis identified at that time — as a matter of fact, most of these had been found by the same authors by screening of the same library, (ii) allowed discovery of 2 novel cytochrome P450 taxoid hydroxylases, and (iii) is expected to contain the candidate genes coding for most of the seven enzymes which remain to be identified (Croteau et al., 2006). The same data set also allowed the identification of enzymes that divert the pathway flux away from taxol production (Hampel et al., 2009).

In jasmonate-mediated induction of secondary metabolism during stress responses, a strong and rapid induction is needed to guarantee that the defense program persists or even intensifies for as long as the plant is under attack. Therefore, plants have evolved a positive feedback system with loops at various control points. One prominent control point that evolved across the plant kingdom is that the transcription factors that drive the expression of the secondary metabolic pathway genes are encoded by jasmonate responsive genes themselves, as well as many other regulatory proteins (De Geyter et al., 2012). As for the enzyme-encoding genes, genes encoding such regulators have become the prime target of transcriptome-based gene discovery programs, in the hope of spotting 'master regulators' capable of activating expression of all the genes encoding the enzymes involved in one particular metabolic pathway. Such programs proved to be fruitful for various metabolic pathways from different plant species and allowed the identification of not only numerous transcription factors that directly regulate expression of metabolic pathway genes (De Geyter et al., 2012) but also E3 ubiquitin ligases that control the activity of rate-limiting pathway enzymes (Pollier et al., 2013) or points of crosstalk between jasmonate and other hormonal pathways (Häkkinen et al., 2007; Lackman et al., 2011).

To date, only two *Taxus* transcription factors have been functionally characterized. Both of them have been 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 gene encoding DBAT and it was shown to be a positive regulator of this gene (Li et al., 2013b). The second, *TcAP2* from *T. cuspidata*, interacted with a jasmonate- and elicitor responsive element (JERE) found in the promoters of the *C. roseus* genes from the terpenoid indole alkaloid pathway (Dai et al., 2009). The role of TcAP2 in the regulation of taxol synthesis has not been investigated yet. Importantly, both factors were found to be jasmonate-responsive, fostering the expectation that the mining of transcriptomes of jasmonate-induced *Taxus* cells or explants with known high taxol levels would indeed be a valuable effort.

Since the pioneering study of Jennewein et al. (2004), several deep sequencing efforts of *Taxus* transcriptomes have been undertaken (Table 1). These cover different *Taxus* species, different types of explants, as well as different types of RNAs, in particular mRNAs (referred to as unigenes in Table 1) and small RNAs such as microRNAs (referred to as small RNAs in Table 1). None of these have led to the discovery of novel enzymes or regulators involved in taxol biosynthesis yet, but undoubtedly will do so in a very near future. Useful information may also be retrieved from genome sequence data but presently only low-coverage draft genome assemblies of *Taxus mairei* and *T. baccata* have been generated (Hao et al., 2011; Nystedt et al., 2013).

Elicitor effects on the expression of genes involved in taxane metabolism

As mentioned above, empirical studies on *Taxus* cell cultures have shown that taxane biosynthesis can be markedly induced by elicitors, but there is scarce information on how the elicitors affect the metabolic pathway (Malik et al., 2011; Onrubia et al., 2013a). To determine whether the presence of elicitors in producing cells increases the expression of genes encoding biosynthetic enzymes or regulatory proteins, and if this in turn is responsible for a higher production, recent transcriptomic studies have analyzed taxane production in different *Taxus* cell systems treated with a variety of elicitors (Onrubia et al., 2010, 2013b; Sabater-Jara, 2013).

In *T. baccata* cell cultures, MeJA (100 μM) increased the production of BIII and taxol, whose levels were 20.5 and 19 times higher, respectively, than in unelicited control cultures. Interestingly, BIII levels were found to be almost double those of taxol (Onrubia, 2012; Onrubia et al., 2010). A qPCR analysis determined the expression level of the *txs* gene as well as some genes encoding different hydroxylases [involved in the hydroxylation of the taxane ring] and three transferases [responsible for forming BIII: 10-deacetylbaccatin III-10β-O-acetyltransferase (DBAT), attaching the lateral chain to BIII: baccatin III 13-O-(3-amino-3-phenylpropanoyl) transferase (BAPT), and the benzoylation of the immediate precursor to taxol: 3′-N-debenzoyl-2′-deoxytaxol-N-benzoyltransferase (DBTNBT)] (Fig. 3).

MeJA clearly increased the expression of the target genes, which peaked between 1 and 2 days after treatment or in some cases after only a few hours. However, while the transcript accumulation for the hydroxylases was considerably higher than the reference value, the increase was far less for the three studied transferases. Under MeJA elicitation, the gene encoding the enzyme phenylalanine amino mutase (PAM) was quite markedly expressed (Fig. 3), as was the gene encoding the first enzyme of the biosynthetic pathway, TXS (Onrubia, 2012).

The particularly high accumulation of BIII may indicate that DBAT is not a limiting enzyme in MeJA-elicited *T. baccata* cell cultures, although its expression was quite low (Fig. 3). The low taxol production in comparison with BIII may be due to the limited expression of the two transferases responsible for connecting the side chain to BIII and/or that are involved in the final step in taxol biosynthesis. For these reasons, it seems that in *T. baccata* cell cultures the more limiting genes are probably those encoding transferases, especially the last two active in the biosynthetic pathway.

As mentioned above, Cor is a powerful new elicitor of taxane production, particularly BIII and taxol, whose levels in *T. media* cell cultures were enhanced 22.2-fold and 9.2-fold, respectively, in comparison with

Table 1
Overview of *Taxus* transcriptome analysis studies.

Species	Explant	Profiling method	Number of transcripts	Reference
T. chinensis	MeJA elicited cells	Illumina deep sequencing	46,581 unigenes	Li et al. (2012a, 2012b)
T. chinensis	MeJA elicited cells	Illumina deep sequencing	1,256,425 sRNAs	Qiu et al. (2009)
T. chinensis	MeJA elicited cells	Random Sanger sequencing of cDNA library	3563 unigenes	Jennewein et al. (2004)
T. cuspidata	MeJA elicited cells	Sanger sequencing of subtractive hybridization library	331 unigenes	Lenka et al. (2012)
T. cuspidata	(Cambial) cells	454 deep sequencing	26,906 unigenes	Lee et al. (2010)
T. cuspidata	Needles	454 deep sequencing	20,557 unigenes	Wu et al. (2011)
T. mairei	Roots, leaves, stems	Illumina deep sequencing	36,493 unigenes	Hao et al. (2011a, 2011b)
T. mairei	Leaves	Illumina deep sequencing	1,190,874 sRNAs	Hao et al. (2012a, 2012b)
T. media	MeJA elicited cells	Illumina deep sequencing	40,348 unigenes	Sun et al. (2013)
T. baccata	MeJA elicited cells	cDNA-AFLP	667 unigenes	Onrubia (2012)

a respective increase of 4.6-fold and 2.5-fold induced by MeJA. Although elicitation with Cor or MeJA resulted in similar transcript levels (Fig. 3), maximum mRNAm occurred 2 days earlier with Cor (Onrubia et al., 2013b). The consequent earlier accessibility of biosynthetic intermediates probably explains the higher accumulation of taxanes in Cor-treated cultures. As in MeJA-treated *T. baccata* cell cultures, which showed a similar transcription profile (Onrubia, 2012), the lower production of taxol compared to BIII in Cor-elicited *T. media* cultures could be due to the low expression of the genes encoding the final transferases, once again suggesting that these genes are ideal targets for metabolic engineering.

In a more recent study, Sabater-Jara (2013) were the first to explore the effect of CD on the expression of genes involved in the biosynthesis of taxol and related taxanes in *T. media* cell cultures, either with or without MeJA. Supplementing the culture medium with MeJA after the addition of CD increased the production of taxol 31-fold and baccatin III 10-fold compared to the control cultures. Moreover, after this dual elicitation, 90% of the taxol had been excreted into the medium, in comparison with 55% in the cultures treated only with MeJA and 25% in the control.

The expression pattern of the target genes after dual elicitation was found to be very similar to that of MeJA-treated cells, despite the greater increase in taxol production (Fig. 3).

Taxol excretion in plant cells depends on a specific mechanism that consumes ATP (Fornale et al., 2002; Naill et al., 2012) involving ABC transporters. Sabater-Jara (2013) showed a remarkable increase in the expression of a gene encoding a putative ABC protein when *Taxus* cells were treated with both CD and MeJA. The *ABC* gene probably encodes an integral and therefore highly stable membrane protein that collaborates in the transfer of CD-taxol inclusion complexes or free taxol from the cell to the culture medium (Fig. 4). This constant release of taxol could promote an active biosynthesis and extracellular accumulation, probably by diminishing the usual feedback inhibition processes and/or toxicity in the cytoplasm when taxol is present (Exposito et al., 2009b), with the ultimate result being the high yields of taxol obtained in this work. The fact that taxol and other taxanes form inclusion complexes with CD, besides decreasing cellular toxicity, could also prevent their possible enzymatic degradation, both intra- and especially extracellular (Fig. 4).

Epigenetic control of taxol production

Plant cell cultures have been used as experimental models to uncover epigenetic mechanisms responsible for modulating gene expression. As well as somaclonal variation and callus capacity to regenerate plants, epigenetic gene regulation is thought to influence plant secondary metabolite production (Law and Suttle, 2005). Understanding the mechanisms underlying epigenetic changes, such as cytosine methylation and core histone multi-acetylation, is therefore a new challenge in biotechnology.

Gradual loss of production of secondary metabolites in plant cell cultures with repeated subcultures is a common obstacle in developing a large-scale production system. Taxane-producing *Taxus* sp. cultures often display significant instability in product accumulation and the lack of response to elicitor treatments is thought to be environmental, genetic, or epigenetic in nature (Kim et al., 2004). A high level of

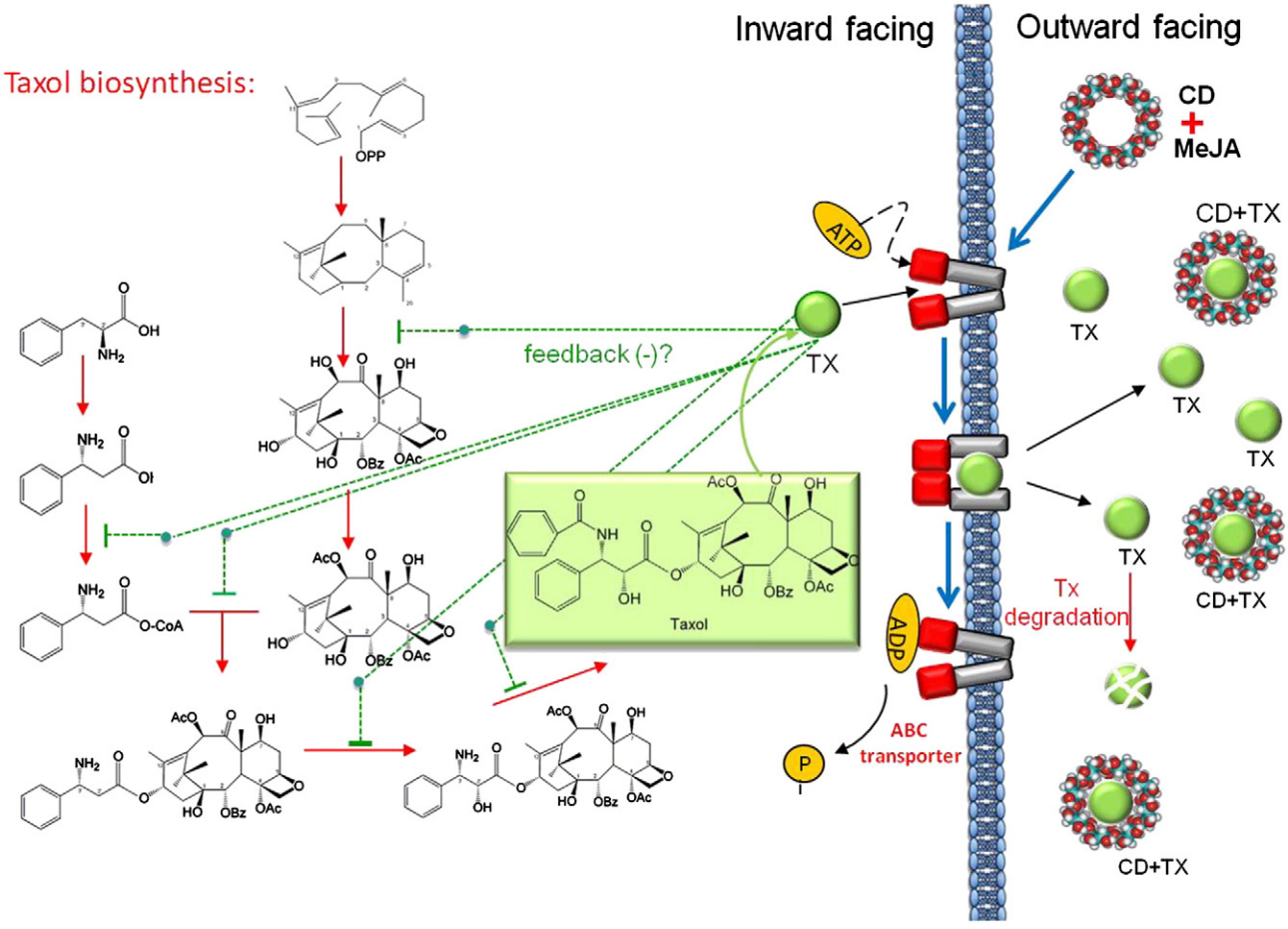

Fig. 4. Suggested mechanism for taxol-release involving an ABC-transporter. CD: cyclodextrin. TX: taxol.

DNA methylation was recently linked to the low production of taxol in 5-year-old *T. media* cell lines (Fu et al., 2012). In confirmation, L.Q. Li et al. (2013) observed that in a repeatedly subcultured *Taxus* cell line a decrease in DNA methylation provoked by treatment with the demethylating agent 5-aza-2′-deoxycytidine coincided with an increase in taxol levels. Similar results have been recently reported in transgenic cell cultures of *Vitis amurensis*, where the treatment of cultures with 5-azacytidine also increased *trans*-resveratrol production (Tyunin et al., 2012). These results are opening the way to a directed manipulation of epigenetic regulators and the application of epigenetic engineering in plant cell platforms for improving secondary metabolite production.

Metabolic engineering for improving taxane production

Although there are only few reports about the genetic transformation of *Taxus* sp., metabolic engineering may be a potent tool for increasing taxane production in *Taxus* cell platforms. The direct approach to metabolic engineering is based on introducing genes into the plant genome that either directly controls a biosynthetic pathway by enhancing a metabolic step or close an unwanted pathway (Fig. 5). Alternatively, in a more efficient holistic approach, the inserted gene encodes a transcription factor or master regulator protein, which enables several biosynthetic pathways to be controlled simultaneously (Capell and Christou, 2004).

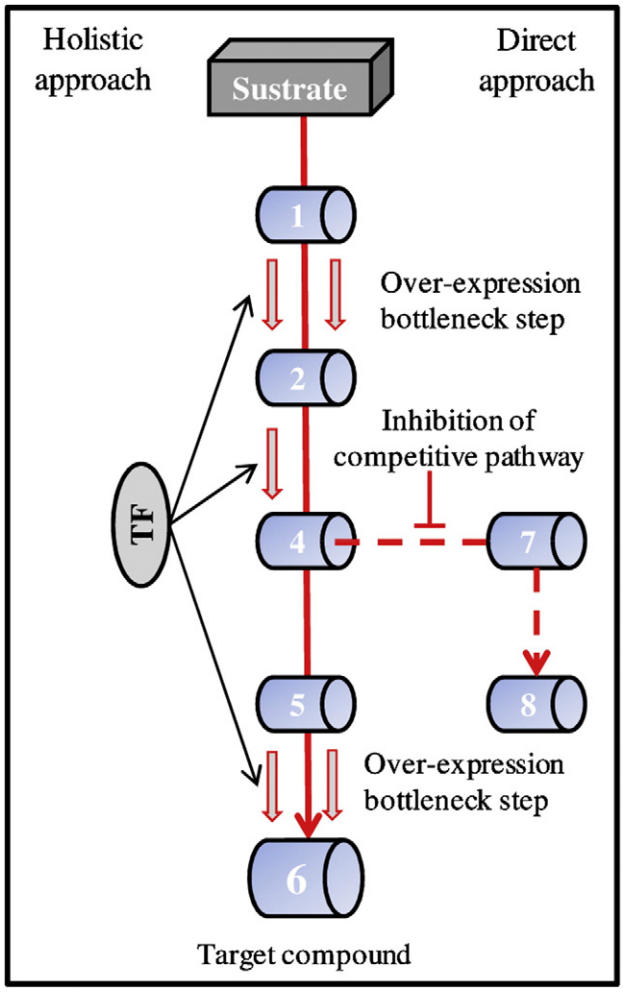

Fig. 5. Summary of a system applying metabolic engineering techniques to improve the biotechnological production of a target compound, taking a direct or holistic approach.

The main problem in metabolically engineering *Taxus* cell cultures to increase taxane production is the difficulty of genetically transforming a gymnosperm plant like *Taxus* and its slow growth capacity. Nevertheless, *T. brevifolia* and *T. baccata* were successfully genetically transformed in 1994, using two wild types of *Agrobacterium tumefaciens* to develop transformed callus cultures (Han et al., 1994), and two years later, Luan et al. (Luan et al., 1996) reported transient expression of the GUS gene in *T. brevifolia* embryos. *Taxus* hairy root cultures were obtained by transformation with *Agrobacterium rhizogenes* for the first time by Furmanowa and Syklowska-Baranek (2000). Although these hairy roots yielded taxanes, the poor growth capacity of this system meant it was unsuitable for industrial scale development. However, feeding experiments in *T.* × *media* hairy root cultures elicited with MeJA showed that the addition of L-phenylalanine and p-amino benzoic acid to the culture medium significantly increased taxane production (Syklowska-Baranek et al., 2009).

Further attempts to obtain *Taxus* hairy root cultures also demonstrated the low growth capacity of transformed organs in hormone-free medium and the necessity of adding plant growth regulators to improve growth (Kim et al., 2009).

The first serious attempt to apply metabolic engineering to the biotechnological production of taxanes using a direct approach was carried out by Ho et al. (2005), who obtained transgenic *T. marei* cell cultures constitutively overexpressing the DBAT gene. Taxane production in those cell lines was found to be dependent on MeJA, and high taxol levels were only achieved in a high-producing line. More recently, *T.* × *media* hairy roots overexpressing the *txs* gene from *T. baccata* have been developed (Expósito et al., 2010). Transformed roots were obtained after the direct inoculation of 3-month-old *Taxus* seedlings with *A. rhizogenes* LBA 9402 and the C58C1 strain of *A. tumefaciens* carrying the pRiA4 and the binary plasmid pCA-TXS-His harboring the *txs* gene. Hairy root lines showed slow growth capacity and turned brown on subculturing, so to overcome this problem, hairy roots were dedifferentiated by hormonal treatment and cell suspensions were obtained (Fig. 6). The results showed that the presence of the *A. rhizogenes* T-DNA in the *Taxus* cell genome increased the capacity of the system to produce taxol, and the overexpression of the *txs* gene in the MeJA-treated cell line also significantly increased TXS activity and taxane production.

The aim of another metabolic strategy was to block branching points in taxol biosynthesis (Li et al., 2011) by the antisense-induced suppression of taxoid 14β-hydroxylase in *T. media* transgenic cell lines. This enzyme catalyzes C-14 oxygenated taxanes, which are not precursors of taxol and are formed by a side-route that may compete with taxol for the same initial precursors.

The metabolic engineering of taxanes has also been approached holistically. It has recently been demonstrated that ozone induces taxane production in *T. chinensis* cell cultures and the response is at least partially dependent on abscisic acid (ABA) signaling (Xu et al., 2011). Taking this into account, *Taxus* transgenic cell lines have been modified to overexpress a 9-*cis*-epoxycarotenoid dioxygenase, an enzyme, responsible for the cleavage of 9-*cis*-epoxycarotenoid, a rate-limiting step in the biosynthesis of ABA. Its overexpression resulted in an increased accumulation of ABA and taxol in the transgenic cell lines (Li et al., 2012a).

The difficulty of genetically transforming *Taxus* sp. has inspired other approaches, such as the production of taxadiene in transformed ginseng roots (Cha et al., 2012) or developing transgenic bacteria that can partially reproduce taxane biosynthesis. The advantages of the latter include a high growth index, and relative ease of transformation and scale-up. Ajikumar et al. (2010) used an engineered *Escherichia coli* strain to overexpress genes encoding initial steps in terpene biosynthesis together with *txs* and taxadiene-5-hydroxylase genes, achieving a production of 1 g L^{-1} of taxadiene and 60 mg L^{-1} of taxadiene-5-ol. These compounds could be used in the future as precursors for the semisynthesis of taxol and its derivatives.

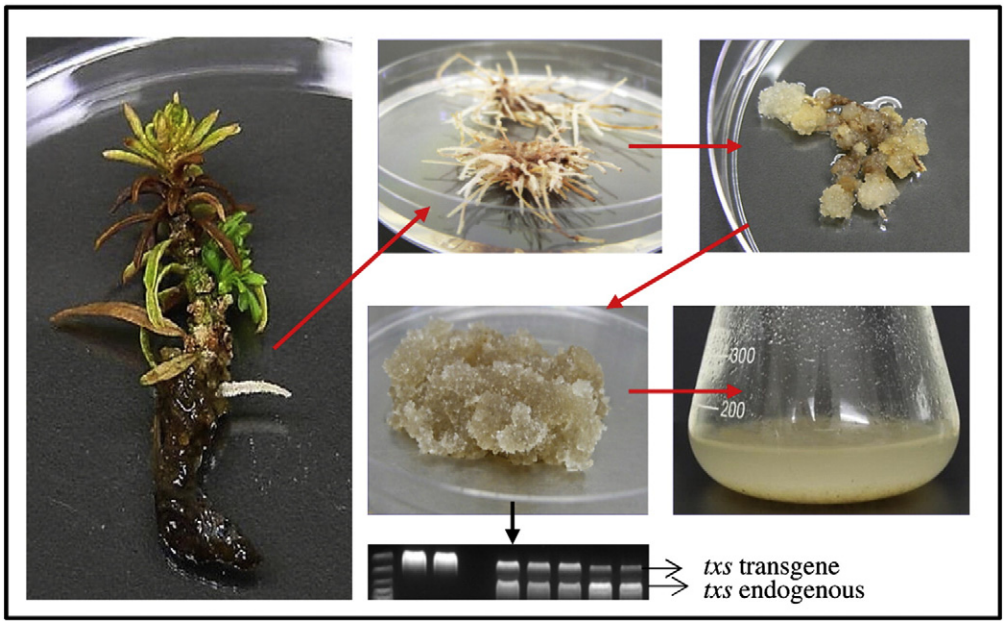

Fig. 6. Induction of hairy roots by *Agrobacterium rhizogenes* carrying the taxadiene synthase gene (*txs*) under the control of the 35-S promoter, their dedifferentiation in calli and the derived cell suspension. Confirmation by PCR of the txs transgene within the *Taxus* cell genome.

Conclusions

The use of elicitors to activate genes involved in taxane metabolic pathways is an effective strategy to increase the biotechnological production of these compounds. Supplementing the medium with elicitors such as MeJA, coronatine and CD (individually or combined) induces an important reprogramming of gene expression in *Taxus* spp. cell cultures, which likely accounts for the observed enhanced production of taxol and related taxanes. Although our knowledge increases, various aspects on the molecular mechanisms behind elicitor action and possible synergy between them remain unknown.

A clear relationship has been observed between the expression profile of the studied genes and the taxane production. From the transcriptome and taxane profiling studies we have shown that taxane biosynthesis in *Taxus* cell cultures is particularly downstream regulated. In a direct metabolic engineering approach, we envisage that the introduction of genes encoding the enzymes involved in the final steps of taxol biosynthesis under the control of a strong promoter is likely to result in high yields of the target taxanes in *Taxus* cultured cells. In a holistic approach, it would also be possible to obtain *Taxus* cell cultures overexpressing genes encoding possible transcription factors or other master regulators of the metabolic pathway, with the ultimate aim of developing new improved biotechnological systems for taxane production. Hence, transcriptome studies carried out so far as well as those planned in the future might find promising application in the identification of such regulatory genes.

Since the transcriptional reprogramming of *Taxus* sp. cells mediated by MeJA can be correlated with significant changes in the metabolome, comparative metabolome studies of control and elicited cells will shed light on the complete taxane profile of the cultures, including all the putative intermediaries involved in the biosynthetic pathway of paclitaxel, as well as other taxanes involved in possible competitive or catabolic branches that may divert the metabolic flux. This information can be used to pinpoint biosynthetic bottlenecks and enable new strategies for metabolic engineering focused on the blocking of competitive pathways. Conversely, combining transcriptomic and metabolomic profiles of elicited cells will give us a better understanding of the connections between primary and secondary metabolism and the role of MeJA. This will potentially lead to the design of synthetic organisms with an optimized capacity for both biomass and taxane production, which is essential for improving the biotechnological production of taxanes.

The formation of CD inclusion complexes with taxol and other taxanes decreases cellular toxicity, but might also prevent the possible intra- and especially extracellular enzymatic degradation to which these compounds may be subjected to. The ability of CD-treated *Taxus* cell cultures to excrete these compounds to the culture medium is crucial for their biotechnological production. The use of high-producing *Taxus* cells that excrete the bioactive compounds into the culture medium allows the establishment of continuous systems in bioreactors without destroying the cell biomass, since the processes required for extracting and purifying of the target compounds will be simpler, more sustainable and economically more viable.

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