A MOLECULAR APPROACH TO TAXOL BIOSYNTHESIS

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A mis padres, Raul y Filip.

To all the people that have directed me along the scientific path.

To all the people who wish to follow this route.

The absence of evidence is not evidence of absence.

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Summary

Secondary metabolism in plants produces numerous compounds with wide-ranging activities, including the antineoplastic compound taxol and related taxanes. The biotechnological production of taxol has so far been based on empirical studies. The aim of the present work has been to study how the factors that enhance taxane production affect the metabolic profiles and gene expression in productive cells. As a consequence of this work, new potential candidates have been obtained for unknown taxane biosynthetic genes, some bottle-neck steps of taxane biosynthesis (*in vitro* and *in silico*) have been identified and a master regulator, not only for taxane biosynthesis, but also for other secondary metabolism routes, has been characterized. Coronatine, a powerful and less harmful elicitor than methyl jasmonate, has been successfully assayed and found to increase taxane production. In the different studies of this work, the expression level of genes that participate in taxol biosynthesis has been determined, clarifying their involvement in the production of this anti-cancer agent.

Resumen

El metabolismo secundario de las plantas produce numerosos compuestos con un amplio rango de actividades, entre los que se encuentra el compuesto antineoplásico taxol y los taxanos relacionados, la producción biotecnológica del cual se basa en estudios empíricos. El objetivo de este trabajo ha sido estudiar como los factores que incrementan la producción de taxanos afectan los perfiles metabólicos y la expresión génica en los cultivos. De esta manera se han identificado nuevos genes candidatos que codifican para los genes desconocidos de la biosíntesis, algunos pasos limitantes de ésta y se ha caracterizado un regulador relacionado con el metabolismo secundario. Se ha ensayado la coronatina, un elicitor más eficiente para mejorar la producción de taxanos y menos dañino que el jasmonato de metilo. En los diferentes ensayos de este trabajo han sido determinados los niveles de expresión de genes que participan en la biosíntesis de taxol, ayudando a comprender su papel en la producción de este anticancerígeno.

Index

Abbreviations

AA, amino acid
ABA, Abscisic acid
ACX1, acyl-CoA oxidase 1
ADC, arginine decarboxylase
AFLP-TP, cDNA-AFLP transcript profile
AG, arginase;
AIH, agmatine deiminase
AMP, adenosine monophosphate
AMPs, Antimicrobial peptides
AOC, allene oxidase cyclase
AOS, allene oxidase synthase
BAP, 6-benzylaminopurine
BAPT, baccatin III-3-amino, 13-phenylpropanoyltransferase
BY-2, Bright Yellow-2
CaMV35S, Cauliflower mosaic virus 35S
cDNA-AFLP, cDNA-amplified fragment length polymorphism
Ceph, Cephalomannine
CoA, Coenzyme A
COI1, CORONATINE INSENSTIVE1
Cor, coronatine
CRP, cysteine-rich peptide
CSA, Catalytic Site Atlas
CTRL, control
DABIII, 10-Deacetylbaccatin III
DAT, 10-Deacetyltaxol
DBAT, 10-deacetyl baccatin III-10-*O*-acetyltransferase
DBTNBT, Debenzoyl taxol *N*-benzoyl transferase
DCM, dichloro-methane
DMAPP, Dimethylallyl pyrophosphate
DMSO, dimethylsulfoxide
DW, dry weight
E.C., enzyme class
EST, expressed sequence tag
EtOH, ethanol
FAD, flavin adenine dinucleotide
FDA, U.S. Food and Drug Administration
FMN, flavin mononucleotide
GA_3, gibberellic acid
GCS, Gateway cloning site
GGPP, geranylgeranyl diphosphate
GGPPS, geranylgeranyl diphosphate synthase
GM, Growth medium
GSP, gene specific primer
HPLC, High performance liquid chromatography
HPOT, hydroxyperoxyoctadeca-dienoic acid
HR, hairy root
HR C-, HR negative control
HTH, HOTHEAD protein
IPP, isopentenyl pyrophosphate
JA, jasmonate
JA-Ile, jasmonoyl-isoleucine
JAR1, jasmonate receptor 1
JAZ, jasmonate ZIM domain
JMT, jasmonic acid carboxyl methyltransferase
KAT, L-3-ketoacyl CoA thiolase
LDC, lysine decarboxylase
LOXs, lipoxygenases
LUC, Luciferase
MACiE, Mechanism, Annotation and Classification in Enzymes
MeJ, Methyl jasmonate
MFP, multifunctional protein
MIPS, Munich Information Center for Protein Sequencing
M-MLV RT, Moloney murine leukemia virus reverse transcriptase

MPO, methylputrescine oxidase
mRNA, messenger RNA
MS, Murashige and Skoog
MSA, multiple sequence alignment
MTBE, methyl *tert*-butyl ether
NAA, naphthaleneacetic acid
NCPAH, N-carbamoylputrescine amidohydrolase
NINJA, Novel Interactor of JAZ
ODC, ornithine decarboxylase
OPDA, cis(+)-12-oxophytodienoic acid
OPR, peroxisomal OPDA reductase
ORF, Open reading frame
PAM, Phenylalanine aminomutase
PCR, polymerization chain reaction
PDB, Protein Data Bank
PEG, Polyethylene Glycol
PM, Production medium
PMT, putrescine N-methyltransferase
pRi (Ri plasmid), Root inductor plasmid
PSK-α, phytosulfokine-α
p35S, Cauliflower mosaic virus 35S promoter
QPRT, quinolinate phosphoribosyltransferase
qRT-PCR, quantitative real-time PCR
RACE, Rapid Amplification cDNA Ends
SA, salicylic acid
SCF, Skip/Cullin/F-box
SD, standard deviation
SE, standard error
STR, Strictosidine synthase
tag, temporarily assigned gene
TAT, taxadiene-5α-ol-*O*-acetyl transferase
TBT, taxane-2α-O-benzoyl transferase
TF, transcription factor
TIA, terpenoid indole alkaloids
TXS, Taxadiene synthase
T1βOH (**T1OH**), Taxane 1β-hydroxylase
T2αOH (**T2OH**), Taxane 2α-hydroxylase
T2'αOH (**T2'OH**), Taxane 2'α-hydroxylase from the lateral chain
T5αOH (**T5OH**), Taxane 5α-hydroxylase
T7βOH (**T7OH**), Taxane 7β-hydroxylase
T9αOH (**T9OH**), Taxane 9α-hydroxylase
T10βOH (**T10OH**), Taxane 10β-hydroxylase
T13αOH (**T13OH**), Taxane 13α-hydroxylase
T14βOH (**T14OH**), Taxane-14β-hydroxylase
UV, ultraviolet
VOCs, volatile organic compounds
YEB, Yeast Extract Broth
2,4-D, 2,4-dichlorophenoxyacetic acid

Introduction and Aims

The production of taxol in *in vitro* cultures has become a major challenge for plant physiology due to the great efficiency of this compound in treating various types of tumours, together with its low rate of production in cell cultures, which makes it difficult to meet the growing worldwide demand.

Studies have shown that structural analogues of taxol obtained by semi-synthetic processes from intermediate taxanes in taxol biosynthesis, such as baccatin III, 10-deacetylbaccatin III, cephalomannine, etc., are equally as effective as taxol, or more so, as well as presenting characteristics that facilitate administration and absorption.

Consequently, taxol and its structural analogues have high added value in the chemical-pharmaceutical market and unsurprisingly there is great interest in developing profitable biotechnological processes that can guarantee a high production of these compounds.

Taxol and related taxanes are diterpenoid secondary compounds produced by several species of *Taxus*. Plant secondary metabolism involves a huge variety of biosynthetic processes, each with its own exclusive genetics, which complicates the process of understanding the metabolic steps involved and particularly the controlling molecular mechanisms. Therefore, the biotechnological production of taxol has so far been based on empirical studies, which improve yield by optimizing culture conditions and supplementation with elicitors. However, very little is known about the mechanisms that control taxane biosynthesis and accumulation at the cellular and/or molecular level.

Previous studies carried out by our research group have shown the possibility of obtaining high productivities of taxol and related taxanes in optimized two-phase cell cultures of different *Taxus* species. When an optimized production medium was supplemented with the elicitor methyl jasmonate a far greater taxane yield was obtained than in the corresponding unelicited cell cultures.

Based on these empirical studies, the work of this thesis has been to study how the factors that enhance taxane production affect the metabolic profiles and gene expression in the productive cells.

Due to the lack of extensive genomic data for *Taxus baccata* it is difficult to apply the commonly used microarray-based approach for its transcriptome analysis. However, the cDNA-amplified fragment length polymorphism (AFLP) technology offers a very useful alternative to identifying genes involved in plant secondary metabolism.

Thus, we have applied the cDNA-AFLP technique to study the genetic changes in highly productive cell cultures of *T. baccata* after supplementation with methyl jasmonate, comparing them with the same cultures without elicitation. This technology was used to identify which genes were induced, repressed or without any change in their expression pattern, with the aim of obtaining results that will contribute to the genetic regulation of the taxane biosynthetic pathway.

The genes considered most likely to be involved in specific unresolved steps of taxol biosynthesis were selected and cloned. With the aim of providing data to corroborate whether these genes encode enzymes that are functional proteins in charge of these biosynthetic steps, bioinformatics techniques were applied to narrow down the initial selection of genes, with some being discarded and others chosen as good candidates for further studies.

The use of *in silico* techniques will allow future functional studies to focus on the sequenced and cloned genes most likely to participate in the biosynthesis of taxol and other taxanes of interest.

Another very important aspect of secondary compound biosynthesis, both generally and in the specific case of taxanes, is the production pattern in different parts of the plant during their ontogenetic development, which has been very little studied to date.

Although secondary compounds can be synthesized in the whole plant, they are generally produced in particular organs, tissues or even cells. In many cases, their synthesis does not occur regularly throughout the lifetime of the plant but instead depends on the stage of plant growth. With the intention of gaining more understanding of taxol production at the molecular level, this work has studied the biosynthesis of taxanes and the expression level of the genes involved in this metabolic process in *T. baccata* plantlets during their first year of development. The aim was to assess the relationship between the content of several taxanes (10-deacetylbaccatin III, baccatin III, 10-deacetyltaxol, cephalomannine and taxol) and the expression of the following genes involved in early and late stages of the taxane biosynthetic pathway: *Taxadiene synthase* (*TXS*),

10-deacetylbaccatin III-10β-O-acetyltransferase (*DBAT*), *baccatin III:3-amino,13-phenylpropanoyltransferase* (*BAPT*) and *3'-N-debenzoyl-2'-deoxytaxol-N-benzoyltransferase* (*DBTNBT*).

This study would allow the taxane production in plantlet aerial parts and roots to be correlated with the degree of expression of different genes involved in the taxane biosynthesis, and to identify the existence of any bottlenecks that might limit the formation of these secondary compounds during plant growth.

As mentioned previously, one of the most effective strategies for increasing taxane production in *Taxus* cell cultures is supplementing the culture medium with elicitors, particularly MeJ. Before binding to the receptor and taking effect, MeJ must bind to isoleucine. The jasmonate-promoted chain of signals that lead to secondary metabolism is in fact activated by MeJ-Ile. Coronatine (Cor) is an elicitor that is structurally analogous to MeJ-Ile. In previous studies on different plants and cell cultures, the presence of Cor has enhanced secondary metabolism to a greater degree than MeJ. Thus, in this work taxane production has been studied in Cor-supplemented *T.* x *media* cell cultures. The expression of key genes in taxane biosynthesis was also determined in the first days of elicitation to study their relation with the taxane production pattern.

It was expected that these studies would allow highly productive cell cultures to be established, and that they would provide valuable insight into the genetic mechanisms that control taxane biosynthesis and the role of the different metabolic steps in the production of these valuable compounds.

In light of all the above, the main aims of the work presented in this Thesis were:

1. **To study the transcript profile of elicited cell cultures of *T.baccata* by cDNA-AFLP and shed light on its relationship with taxane production.**
2. **To carry out *in silico* studies on the possible function of t*he protein*s encoded by genes likely to be involved in taxane biosynthesis and previously sequenced and cloned using cDNA-AFLP.**
3. **To study the *relation*ship between TXS, DBAT, BAPT and DBTNBT gene expression and taxane production during the development of *Taxus baccata* plantlets.**

4. **To study the effect of coronatine elicitation on the production of taxol and related taxanes and the expression level of genes that control taxane biosynthesis in *T.* x *media* cell cultures.**

In the first objective of this thesis, the cDNA-AFLP studies of elicited cell cultures of *T. baccata* would provide information not only about the genes known to be involved in taxol biosynthesis, but would also allow the sequencing and cloning of other genes whose expression pattern is altered by the presence of the elicitor. These unknown genes probably encode enzymes involved in taxol biosynthesis as well as transcription factors or master regulators.

The potential of metabolic engineering to improve plant secondary production by modulating individual steps has been demonstrated, albeit with several limitations. In a holistic approach, the ability to switch on an entire metabolic pathway in an engineered cell culture by ectopic expression of a transcription factor or other regulatory peptides opens new possibilities, especially for those pathways not fully elucidated, such as taxane biosynthesis. One of the genes discovered as a consequence of the cDNA-AFLP study was the *Taximin* gene, which encodes a short peptide with high homology with other peptides found in several plant species. We consider that this peptide could act as a master regulator for plant secondary metabolism.

For these reasons, and considering the results obtained during this work, we decided to demonstrate the regulatory role of the *Taximin* gene in the biosynthesis of taxanes as well as other plant secondary compounds. Thus, the final objective of the thesis was:

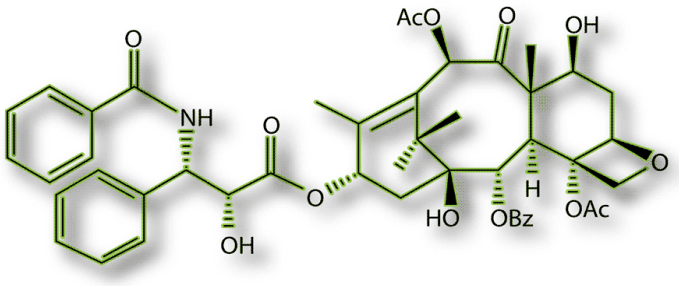
5. **To study the effect of Taximin on the promoter of the genes encoding the TXS and T13OH enzymes, which are involved in taxane biosynthesis, as well as on the promoters of other genes involved in alkaloid biosynthesis in tobacco and *Catharanthus*, using tobacco protoplast cultures. The aim was also to obtain transgenic tobacco material with ectopic expression of the *Taximin* gene in order to verify the effects of this peptide on other secondary metabolic routes.**

We consider that the results derived from this doctoral thesis will be an important contribution to the understanding of the molecular and/or genetic mechanisms that regulate the production of taxol and related

taxanes in *Taxus* cell cultures. These results will also allow the establishment of highly productive cultures that, once optimized, will reveal the molecular mechanisms responsible for this increase in production.

Finally, future studies using metabolic engineering techniques will be able to use these results to establish transgenic cultures capable of over-expressing the key genes in the biosynthesis of taxol and related taxanes or those responsible for bottlenecks in the metabolic pathway.

Considering the above, the perspectives for the establishment of industrial-scale *Taxus* cell cultures able to meet the huge demand for taxol and other taxanes of interest are hopeful.



TAXUS, TAXOL AND TAXANES, A BRIEF INTRODUCTION

1. TAXUS, TAXOL AND TAXANES, A BRIEF INTRODUCTION

1.1. Introduction

Taxus sp. are small- to medium-sized evergreen trees, growing 10-20 m tall, exceptionally up to 28 m. The trees are relatively slow growing, but can be very long-lived, with the maximum recorded trunk diameter of 4 m probably only being reached after more than a thousand years (Lewington and Parker, 1999). Historically, *Taxus* has been used for weapon-making, furniture, gardening and medicine, and is poisonous except for the aril. The species of *Taxus* are distributed in the northern temperate zones of Asia, Asia Minor, India, Europe, North Africa and North America. The genus *Taxus* belongs to the Class Pinopsida, the Order Taxales and the Family Taxaceae. As the species are very similar, they are often easier to separate geographically than morphologically. Typically, eight species are recognized: *T. baccata* (European or English yew), *T. brevifolia* (Pacific or Western yew), *T. canadensis* (Canadian yew), *T. chinensis* (Chinese yew), *T. cuspidata* (Japanese yew), *T. floridana* (Florida yew), *T. globosa* (Mexican yew) and *T. wallichiana* (Himalayan yew). There are also two recognized hybrids: *Taxus* x *media* (= *T. baccata* x *T. cuspidata*) and *Taxus* x *hunnewelliana* (= *T. cuspidata* x *T. canadensis*) (Cope, 1996).

One of the most important compounds synthesized by *Taxus* sp. is taxol (Figure 1.1A), a complex diterpene alkaloid with an intense antitumor activity. In 1992 FDA approved taxol for the treatment of refractory metastatic ovarian cancer and in 1994 for treating metastatic breast cancer (refractory or insensitive to anthracyclines) and subsequently for non-small cell lung cancer and AIDS-related Kaposi's sarcoma (Bristol-Myers Squibb, 2007). Several trials are currently underway for the use of this drug in the treatment of adenocarcinoma of the stomach and upper gastrointestinal tract, urothelial and germ cell cancer, metastatic malignant melanoma or multiple myeloma, among other cancers (Brown, 2003).

In 1979 Schiff and coworkers discovered that the mode of action of taxol is unique, as it exerts a powerful inhibition of cell proliferation and arrests cells in mitosis by stabilization of microtubules. This approach is completely different to that of vinblastine, vincristine or colchicine, which prevent tubuline polymerization. Microtubules are components of the cytoskeleton essential in mitosis and cell division due to their dynamic polymerization. The two subunits required, α-tubulin and β-tubulin, bind

together and allow microtubule polymerization (Jordan and Wilson, 2004). A low concentration of paclitaxel (≤0.05 μM) stabilizes microtubules by binding specifically to the β subunit of the tubulin heterodimers, which induces cell blockage in the mitosis phase and leads to cell death by apoptosis, especially in the proliferating tumour cells. However, a high concentration (≥5 μM) stabilizes microtubules regardless of the cell cycle stage and inhibits cell progression to the S phase, thus inducing cell death by necrosis (Yeung et al., 1999). Due to its characteristic mode of action, taxol has been studied for the treatment of other kinds of illness that require microtubule stabilization and the avoidance of cell proliferation and angiogenesis. Examples include taupathies (affection of tau proteins, as in Parkinson's and Alzehimer's disease linked to chromosome 17) (Zhang et al., 2005), psoriasis (Ehrlich et al., 2004), and multiple sclerosis (Mastronardi et al., 2007). It can also be used in paclitaxel-eluting stents (Stone et al., 2004; device approved by FDA in 2004).

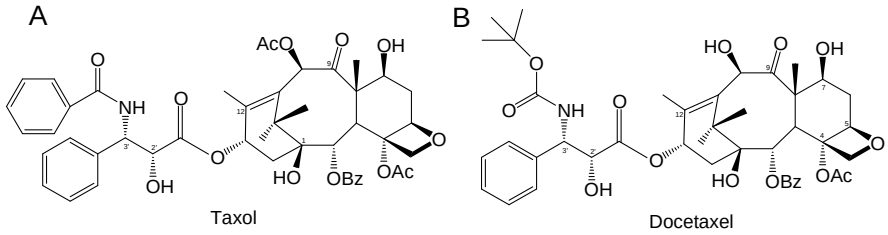


Figure 1.1: Taxol or Paclitaxel (A) and Docetaxel (Taxotere) (B) structures.

An analogous compound named docetaxel (Figure 1.1B) was developed during studies of taxol semi-synthesis from 10-deacetyl baccatin III. Docetaxel, commercialized as Taxotere by Sanofis-Aventis, has the same action mechanism as paclitaxel (Guenard et al., 1993) and is used in the treatment of breast cancer, advanced non-small cell lung cancer, metastatic androgen-independent prostate cancer, advanced gastric/GE junction cancer and locally advanced head and neck cancer (Sanofis-Aventis, Prescribing Information, May 2010).

In 2008, sales of Taxol from Bristol-Myers Squidd and Taxotere from Sanofis-Aventis amounted to more than € 2.3 billion (data corresponding to the 2009 Annual Report from both companies). Due to patent expiration, generic drugs have appeared on the market (paclitaxel from Indena, TEVA genéricos, Actavis, Ferrer, Hospira, Zurich Pharma, etc.), as well as new, more efficient derivatives with fewer secondary effects such as Abraxane (Abraxis Oncology) or Xyotax (Cell Therapeutics). The increase of paclitaxel-derived products reflects the pharmaceutical and economical importance of taxanes as active compounds.

1.2. Obtaining taxol

The richest natural source of taxol is the inner bark of *Taxus brevifolia*, although it represents no more than 0.01% of the dry weight (Cameron and Smith, 2008) and its extraction involves killing the tree. In order to obtain 1Kg of taxol, the inner bark of 3000 yews is necessary (Schippmann, 2001). Taking into account that around 2.5-3 g is required for the treatment of one patient, this translates as the inner bark of 8 adult yews (Bedi et al., 1996). As the natural source of taxol cannot meet the market demand, especially at a competitive price, several methods have been developed for its production. In 1994 the research teams of Holton (1994a y b) and Nicolau (1994) successfully synthesized taxol, but the complexity of the pathway and its low yield make these methods economically unfeasible. Another alternative is semi-synthesis from precursors such as 10-deacetylbaccatin III (Indena S.p.A) or analogue intermediates (Phyton Biotech), but these methods require the extraction of precursors from the natural source. An alternative approach, which does not depend on production-influencing factors such as seasonal variation, climatology or contamination, is cell suspension culture, which is already being used by pharmaceutical suppliers like Phyton Biotech.

1.3. Biotechnological production

The biotechnological production of taxol has been studied since the early 1990s when it was demonstrated that calli and cell suspensions were able to produce the compound and its precursors at least to the same extent as the plant. Highly productive cell cultures can produce up to 0.2 mg of taxol/kg DW, whereas 0.22 mg can be obtained from a kg of dried young shoots, and 0.43 mg of baccatin III can be obtained from 1 kg DW of cell cultures, and only 0.009 mg/kg is produced in the plant organs (Fett-Neto et al., 1992; Wicremesinhe and Arteca, 1993). In order to increase the productivity of taxol and related taxoids in tissue cultures, various strategies have been examined, including optimisation of culture conditions, selection of high-producing cell lines and use of elicitors and precursors.

As secondary metabolite production in plant cell cultures does not usually depend on growth, a two-stage culture system helps to promote growth and production separately (Cusidó et al., 2002). Plant cells are first cultured in a medium optimised for their growth, and then transferred to a

production medium that mainly stimulates the biosynthesis of secondary metabolites. This system has the added advantage of permitting biosynthetic precursors and elicitors to be added when secondary metabolite production is at its highest.

Several elicitors and various strategies have been studied in order to increase taxol production in *Taxus sp.* cell cultures. Some of them include variations in the media, such as different hormone combinations (Cusidó et al., 2002) or the type and combination of carbohydrate sources (Choi et al., 2000; Wang et al., 2001). Others include biosynthetic precursors (Srinivasan et al., 1996; Bentebibel et al., 2005), but there is a whole group of studies using elicitors, either abiotic like lanthanum (Wu et al., 2001) or vannadyl sulfate (Cusidó et al., 1999) or biotic like pathogenic, non-pathogenic and cell extracts from endophytic fungus (Yuan et al., 2002; Lan et al., 2003; Li and Tao 2009), cellulases (Roberts et al., 2003), araquidonic acid (Bonfill et la., 2003), salicylic acid (Wang et al., 2007), ethylene (Mirjalili and Linden, 1996) or even a mix of different kinds of elicitors (Khosroushahi et al., 2006). Other strategies include the use of organic solvents (Wang et al., 2001), low-energy ultrasound (Wu and Ge, 2003), magnetic fields (Shang et al., 2004) or UV-B light (Zu et al., 2011).

Despite these extensive studies, the biotic elicitor methyl jasmonate remains the most effective strategy for increasing taxoid production. Jasmonates are known to play an important role in a signal transduction process that regulates defence genes in plants (Farmer and Ryan, 1990). When exogenously applied, MeJ enhances secondary metabolite production in a variety of plant species (Gundlach et al., 1992; Uppalapati et al., 2005; Rischer et al., 2006). The pioneering study by Yukimune et al. (1996) was the first to use this elicitor with *Taxus baccata*, applying 100 µM MeJ to achieve a 120-130 fold increase in taxoid production, obtaining 48.3 mg/L of taxol and 53.5 mg/L of baccatin III. Bentebibel et al. (2005) achieved the highest productivity rates of taxol to date in immobilized cell suspension cultures of *Taxus baccata*, obtaining values of 2.71 mg L^{-1} day^{-1} in Stirred bioreactors, with an optimized growth medium elicited with 100 µM MeJ and supplemented with the biosynthetic precursors mevalonate (0.38 mM) and N-benzoylglycine (0.2 mM). Ketchum et al. (1999) obtained a 40-fold increase in taxol production in *Taxus canadensis* cell suspension cultures by adding 200 µM MeJ, reporting a production of almost 120 mg/L. Despite these major advances in this field, since 2005 the optimization of media, culture conditions, elicitors and stimulation has not been translated into a real quantitative increase in production (taxane production reviewed by Frense (2007), Malik et al. (2010) and Sabater-Jarra et al. (2010)). At this point, further progress in

taxol production can only be achieved by knowledge of and manipulation of its biosynthesis, which remains only partially understood, while the regulation mechanisms are completely unknown.

1.4. Taxol biosynthesis

Taxol biosynthesis (Figure 1.2) is not completely understood: it has been postulated that 19 enzymes are involved after the formation of geranylgeranyl diphosphate (GGPP) (last reviews by Croteau et al. (2006) and Vongpaseuth and Roberts (2007)). Once GGPP undergoes cyclization into taxadiene, eight oxidations, five acyl/aroyl transferases, one epoxidation, one aminomutase, two CoA esterifications and an N-benzoylation are the steps required for obtaining taxol (all enzymes are listed in Table 1.1).

As a natural diterpenoid, taxol is formed exclusively from GGPP, which is synthesized from three isopenthenyl diphosphate (IPP) molecules and one dimethyl diphsophate (DMAPP) by the enzyme **GGPP synthase** (GGPPS) (Hefner et al., 1998) (Figure 1.3). The origin of IPP and DMAPP is not clear-cut, since both plastidic (Eisenreich et al., 1996) and cytosolic pathways (Lansing et al., 1991; Zamir et al., 1992) exist in plants, and the predominant one can depend on the stage of plant growth. For example, Srinivasan et al. (1996) suggested that cytosolic IPP could play a role in taxol production in the initial growth phase of *Taxus* cells, while Wang et al. (2003), proposed that the translocation of IPP through the plastid membrane only occurs during the late growth phase of the culture. However, research on *T. baccata* cell cultures has shown that taxol biosynthesis is blocked by the addition of fosmidomycin (an inhibitor of the plastidic pathway) and mevinolin (an inhibitor of the cytosolic pathway), indicating that both IPP pathways can be involved (Cusidó et al., 2007).

The first committed step of taxol biosynthesis is the cyclization of GGPP, which leads to the formation of taxa-4(5),11(12)-diene and taxa-4(20),11(12)-diene (Figure 1.4), the first compounds in the taxol biosynthetic pathway bearing the taxoid skeleton (Koepp et al., 1995). This reaction is catalyzed by **taxadiene synthase** (TXS). According to Hezari et al., (1997), who compared the *in vitro* activity of TXS with the production and accumulation of taxol in cell suspension cultures, this step is slow but not rate-limiting, as there is no accumulation of taxadiene in plant tissues.

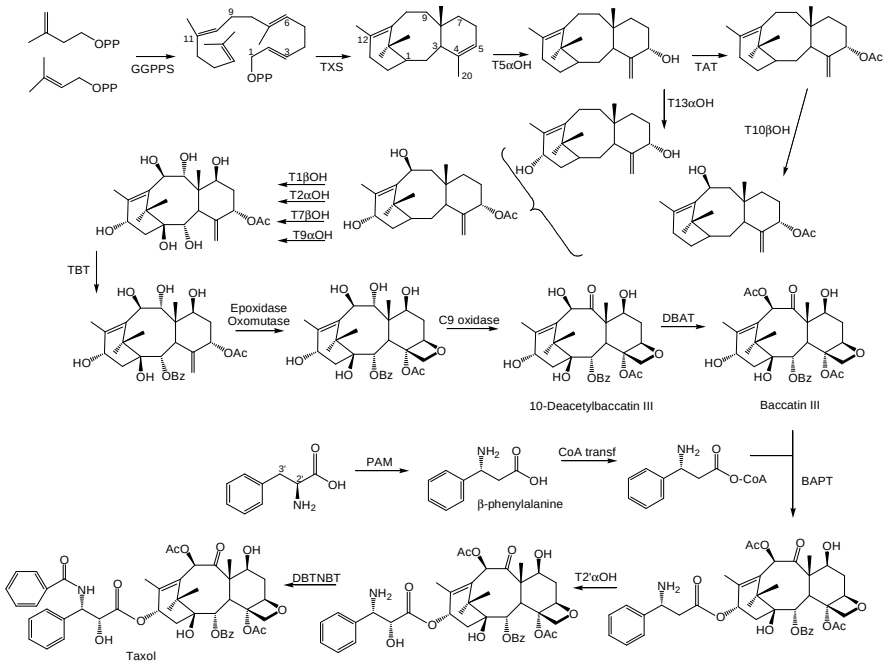


Figura 1.2: A concise depiction of Taxol biosynthesis. GGPPS, Geranylgeranyl diphosphate synthase; TXS, Taxadiene synthase; T5αOH, Taxane 5α-hydroxylase; TAT, taxadiene-5α-ol-*O*-acetyl transferase; T13αOH, Taxane 13α-hydroxylase; T10βOH, Taxane 10β-hydroxylase; T1βOH, Taxane 1β-hydroxylase; T2αOH, Taxane 2α-hydroxylase; T7βOH, Taxane 7β-hydroxylase; T9αOH, Taxane 9α-hydroxylase; TBT, taxane-2α-O-benzoyl transferase; DBAT, 10-deacetyl baccatin III-10-*O*-acetyltransferase; PAM, Phenylalanine aminomutase; CoA transf, CoA transferase; BAPT, baccatin III-3-amino, 13-phenylpropanoyltransferase; T2'αOH, Taxane 2'α-hydroxylase; DBTNBT, Debenzoyl taxol *N*-benzoyl transferase.

Taxadiene is hydroxylated at C5 by **taxadiene-5α-hydroxylase** (T5αOH), giving taxa-4(20),11(12)-dien-5α-ol, which not only undergoes hydroxylation, but also the migration of the double bond from 4(5) to 4(20) (Figure 1.5) (Hefner et al. 1996). This hydroxylase is a cytochrome P450 with an N-terminal membrane (endoplasmic reticulum) insertion sequence, and despite being a slow step, it does not seem rate-limiting either.

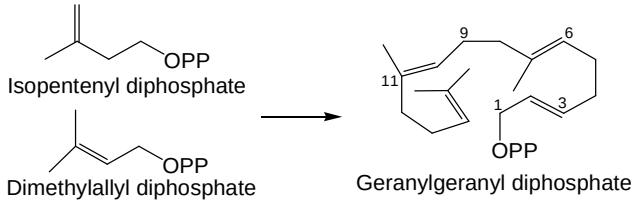
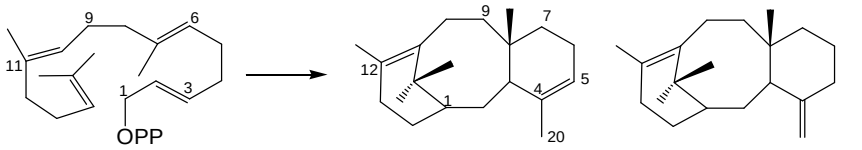


Figure 1.3: GGPP formation from the precursors IPP and DMAPP carried out by GGPP synthase

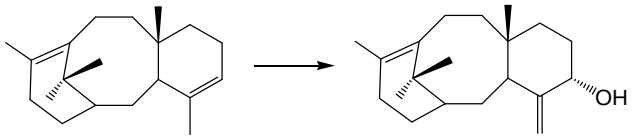
Table 1.1: List of all postulated enzymes involved in taxol biosynthesis, with their abbreviations, GenBank accession number and first published report.

Taxol biosynthesis enzymes	Abbrev.	GenBank acc No	Publication
geranylgeranyl diphosphate synthase	GGPPS	AF081514	Hefner et al., 1998
taxadiene synthase	TXS	AY364469	Wildung et al., 1996
taxadiene-5α-hydroxylase	T5αOH	AY289209	Jennewein et al., 2004
taxadiene-13α-hydroxylase	T13αOH	AY056019	Jennewein et al., 2001
taxadiene-5α-ol-O-acetyl transferase	TAT	AF190130	Walker et al., 2000
taxane-10β-hydroxylase	T10βOH	AF318211	Schoendorf et al., 2001
taxane -14β-hydroxylase	T14βOH	AY188177	Jennewein et al., 2003
taxane-2α-hydroxylase	T2αOH	AY518383	Chau and Croteau, 2004
taxane-7β-hydroxylase	T7βOH	AY307951	Chau et al., 2004
taxan-1β-hydroxylase	T1βOH	----	----
C4β,C20-epoxidase	----	----	----
Oxomutase	----	----	----
taxane-2α-O-benzoyl transferase	TBT	AF297618	Walker and Croteau, 2000a
taxane-10β-O-acetyl transferase or 10-deacetylbaccatin III-10-O-acetyltransferase	DBAT	AF193765	Walker and Croteau, 2000b
taxane-9α- hydroxylase	T9αOH	----	----
phenylalanine aminomutase	PAM	AY582743	Walker et al., 2004
β- phenylalanoyl-CoA ligase	----	----	----
baccatin III: 3-amino, 13-phenylpropanoyltransferase	BAPT	AY082804	Walker et al., 2002a
taxane 2'α-hydroxylase	----	----	----
N-benzoyl transferase	DBTNBT	AF466397	Walker et al., 2002b



Geranylgeranyl diphosphate Taxa-4(5),11(12)-diene Taxa-4(20),11(12)-diene

Figure 1.4: GGPP synthase acts on GGPP and produces the two isomer compounds.



Taxa-4(5),11(12)-diene Taxa-4(20),11(12)-dien-5α-ol

Figure 1.5: C5 hydroxylation from taxadiene. Migration of the double bond from 4(5) to 4(20).

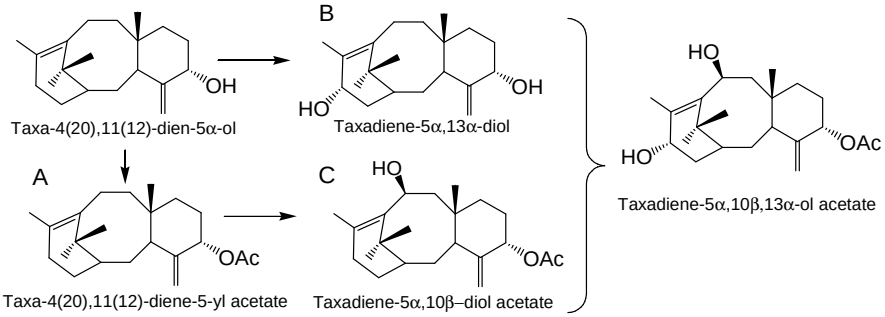


Figure 1.6: First bifurcation in the taxol biosynthetic pathway. Taxadiene-5α-ol can be modified by TAT (A) or T13αOH (B). After acylation, other hydroxylases can act on the substrate, T10βOH (C) and T14βOH.

At this point of the biosynthesis there is a bifurcation and the substrate can be modified by a transferase or a hydroxylase. **Taxadiene-5α-ol-O-acetyl transferase** (TAT), an enzyme without N-terminal organelle targeting information, acylates taxadiene-5α-ol at the C5 position to form taxadiene-5α-yl-acetate (Figure 1.6A) (Walker and Croteau, 2000). On the other hand, **taxadiene-13α-hydroxylase** (T13αOH), a cytochrome P450, hydroxylates taxadiene-5α-ol at C13 (Figure 1.6B) and, less efficiently, taxadiene-5α-yl acetate (Wheeler et al., 2001). Taxadiene-5α-

yl-acetate can easily be hydroxylated by the P450-dependent monooxygenase **taxane 10β-hydroxylase** (T10βOH) at C10 (Figure 1.6C), but due to its less specific nature T10βOH can also modify other taxanes (Schoendorf et al., 2001). The **taxoid 14β-hydroxylase** (T14βOH) is responsible for the formation of taxadiene-5α-acetoxy-10β-14β-diol from taxadiene-5α,10β-diol acetate (Jennewein et al., 2003). T14βOH cannot be involved in the production of taxol, which does not present any hydroxylation at the C14 position, but is responsible for a diversion of the pathway.

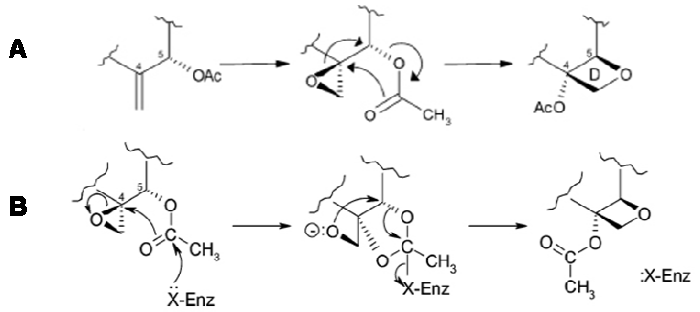


Figure 1.7: The two main theories about oxetane ring formation. Floss and Mocek 1995 (A) and Croteau et al. (2006) (B).

After the formation of taxadiene-5α,10β,13α-triol acetate, the next steps in the taxol biosynthetic pathway include several hydroxylations at the C1, C2, C7 and C9 positions, oxidation of C9 and epoxidation at the C4C5 double bond (Figure 1.7). It is known that the hydroxylations are mediated by Cyt P450 enzymes but not exactly in which order. Taking into account the oxidation frequency of the taxoids found in cell cultures, a probable sequence validated by phylogenetic analyses of previously cloned taxoid P450 oxigenases is: C5, C10, C13, C2, C9, C7 and finally C1 (Vongpaseuth and Roberts 2007). However, rather than intermediates in taxol biosynthesis, some of these taxoids might be commodities of the *in vitro* cultures.

Although various mechanisms have been proposed for the oxetane ring formation (Figure 1.7A and B) (Floss and Mocek, 1995; Croteau et al., 2006), it is currently accepted that the process involves epoxidation of the 4(20) double bond followed by migration of the α-acetoxy group from C5 to C4 together with the expansion of the oxirane to the oxetane group. The enzyme that epoxidates the C4–C20 double bond has not yet been functionally characterized and the oxirane-to-oxetane ring expansion is also an incompletely known step.

After the formation of the hypothetical polyhydroxylated precursor by the activity of the enzyme Taxane 2α-O-benzoyl transferase (TBT) (Walker and Croteau, 2000a), the next compound to be obtained is 10-deacetylbaccatin III (DABIII) (Figure 1.8B). Another identified transacetylation reaction in the taxol biosynthetic pathway involves hydroxylation at the C10 position of the DABIII, which is catalyzed by the enzyme 10-deacetyl-baccatin III-10-O-acetyl transferase (DBAT) (Walker and Croteau, 2000b). The result is the formation of the diterpene intermediate, baccatin III (Figure 1.8C), using DABIII and acetyl CoA as substrates.

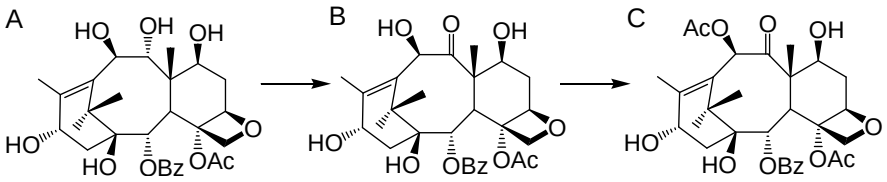


Figure 1.8: From the polyhydroxylated precursor (A) to 10-deacetylbaccatin III (B) and baccatin III (C).

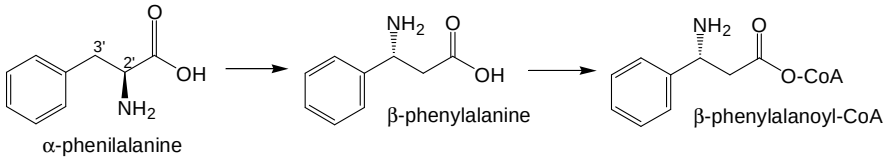


Figure 1.9: Synthesis of β-phenylalanoyl CoA from α-phenyalanine.

An essential step in taxol biosynthesis is the esterification of the C13 hydroxyl group of baccatin III with the β-phenylalanoyl CoA side chain. The side chain is obtained from the amino acid α-phenyalanine by the action of **phenylalanine aminomutase** (PAM) (Floss and Mocek, 1995). An unknown ester CoA ligase probably activates the compound (Figure 1.9) so it can bind to baccatin III. The enzyme that catalyzes the conjugation of the β-phenylalanoyl -CoA side chain to baccatin III is **C-13-phenylpropanoyl-CoA transferase** (BAPT), yielding the compound 3'-*N*-debenzoyl-2'-deoxytaxol (Figure 1.10) (Walker et al., 2002).

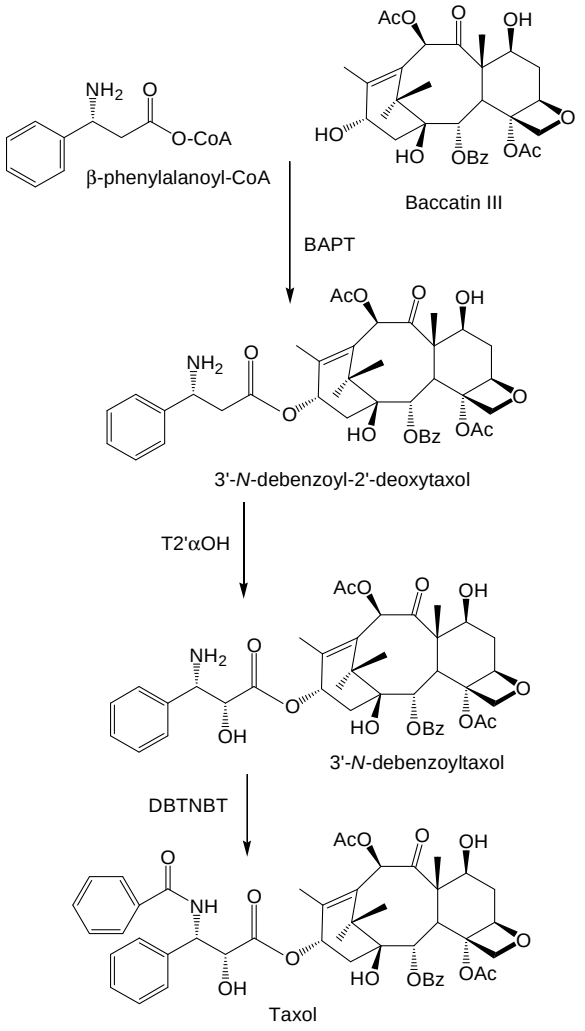


Figure 1.10: End of the pathway, from baccatin III to taxol. Esterification of C13 by BAPT, hydroxylation at C'2 by an unknown hydroxylase and benzoylation at C'3 by DBTNBT.

Although C13-transferase can bind β-phenylalanine and β-phenylisoserine, the unknown **taxane 2'α-hydroxylase** is thought to act at this penultimate point (Figure 1.10). The hydroxylase predicted for the lateral chain should belong to Cyt P450 and share features with other taxane-hydroxylases. According to the studies of Long and Croteau (2005) and Long et al. (2008), it seems that **N-benzoyl transferase** (DBTNBT) (Figure 1.10) is responsible for the last step, as there is no evidence of direct hydroxylation of β-phenylalanine at the 2' position, or in 2'-deoxytaxol, while 3-*N*-debenzoyl-2'-deoxytaxol can be hydroxylated at position 2 of the lateral chain. Moreover, DBTNBT prefers N-debenzoyltaxol over N-debenzoyl-2'-deoxytaxol by a catalytic efficiency factor of two. N-benzoyl transferase is a protein whose deduced amino

acid sequence consists of several signature features of other *Taxus* acyltransferases, such as the absence of N-terminal targeting information (Walker et al., 2002). If other transferases or enzymes modify N-debenzoyl taxol, or even taxol itself, then other compounds can be obtained, such as cephalomannine or 10-deacetyl taxol.

Due to the presence of the N-terminal tag, Wheeler et al. (2001) suggested that significant oxidation steps in taxol biosynthesis are mediated by cytochromes P450 acting in the edoplasmic reticulum. In contrast, all transferases seem to be cytosolic, as they are not tagged. The taxane core is produced in plastids or cytosol, and requires hydroxylations in the endoplasmic reticulum and acylations in the cytosol, which indicates a movement of taxanes inside the cell. However, due to their hydrophobicity, a mechanism for helping or improving the inter-organelle movement should exist. This also indicates that some other modifications of the taxane core might be necessary to improve its solubility.

More than 350 taxanes have been identified (Baloglu and Kingston, 1999) and they differ from taxol on various levels: in the lateral chain, hydroxylations in the core or the side chain and substitutions with acyl or aroyl groups. The most common variations are esterifications with acyl/aroyl at positions that should be modified with other groups, considering that these undesired modifying taxanes reduce the intermediates to taxol. However, these modifications might be necessary for improving the movement of intermediates between organelles (Croteau et al., 2006). These enzymes could be an interesting target for overexpression and knock-out analysis, as they might lead to alternative ends of the pathway or improve the efficiency of the inter-organelle movement.

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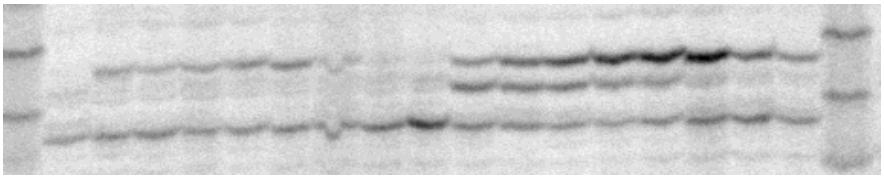


TRANSCRIPTOMIC AND METABOLIC REPROGRAMMING AFTER METHYL JASMONATE TREATMENT OF *TAXUS BACCATA* CELL SUSPENSION CULTURES

2. TRANSCRIPTOMIC AND METABOLIC REPROGRAMMING AFTER METHYL JASMONATE TREATMENT OF *TAXUS BACCATA* CELL SUSPENSION CULTURES

2.1. Abstract

In the last decade considerable effort has been directed to the study of different aspects of secondary metabolism in a wide range of plants, due to the great potential of these metabolites for improving human health. Here we report a comprehensive profiling analysis of *Taxus baccata* (European or English yew), until now the main source of the anticancer drug taxol. Genomewide transcript profiling by cDNA-amplified fragment-length polymorphism combined with metabolic profiling of elicited *Taxus* cell cultures yielded a collection of known and previously undescribed transcript tags. Transcriptome analysis suggested an extensive jasmonate mediated genetic reprogramming of metabolism, which correlated well with the observed shifts in the biosynthesis of the metabolites investigated. This study provides the basis for a better understanding of *Taxus* secondary metabolism and increases the practical potential of metabolic engineering of this important medicinal plant.

2.2. Introduction

Secondary metabolism in plants produces a high number of low molecular weight compounds known as secondary metabolites. Although these molecules are not important for maintaining the basic processes in the plant, they have a very important role in interactions with the environment, defence and reproduction (Oksman-Caldentey and Inzé, 2004; Verpoorte and Memelink, 2002). Generally, these compounds represent less than 1% of the dry weight of each plant, and their quantity depends on the physiological and development state of the plant. Secondary metabolites are characterized by a great variety of complex structures that are usually stored in specific cells or organs, especially in the vacuoles. Although some compounds, such as phenylpropanoids, are found in different taxa, others are distributed in related families, for example, sesquiterpens in Solanaceae, and each plant generally has its own set of secondary

metabolites. Nowadays, around 140,000 different compounds are known (Dixon, 2001).

The complexity and multi-step nature of secondary metabolites biosynthetic pathways make them difficult to characterize. *In vitro* and *in vivo* studies have produced variable results, for instance, unexpectedly high levels of sesquiterpenoid in cotton leaves in response to *Xanthomonas* infection compared to the *in vitro* culture (Pierce et al., 1996). Moreover, some of these pathways have large physically associated enzyme complexes where intermediates are channelled, and a high number of these enzymes can be specific or not, can need activators or cofactors, and some feedback pathways can act as regulators. Also important is the huge number of genes related with biosynthesis, compartmentalization transport or physiological control (Verpoorte and Memelink, 2002). The overexpression of genes involved in secondary metabolism has sometimes had unexpected results, for instance, affecting the production of metabolites other than the target compound. For example, when *Lithospermum* is transformed with *ubiC*, a gene encoding chorismate pyruvate-lyase, the levels of menisdaurin rather than naphtoquinones are increased (Sommer et al., 1999). Taking all these points into account, it can be readily understood why these pathways are so difficult to elucidate, and why the biosynthetic pathway of taxol and its regulation remains virtually unknown despite having been characterized for decades (Dixon, 2001).

Many plants containing high-value compounds are difficult to cultivate while others are becoming endangered because of over-harvesting (Rates, 2001). Furthermore, the chemical synthesis of plant-derived compounds is often not economically feasible, because of their highly complex structures. Thus, the biotechnological production of secondary metabolites in plant cell or organ cultures is an attractive alternative, requiring the selection of high-yielding and stable cultures (Oksman-Caldentey and Inzé, 2004). Cell cultures have been established from many plants and when they do not produce sufficient amounts of the required secondary metabolites, as is often the case, this system allows their production to be enhanced by treatment of the undifferentiated cells with elicitors (Cusidó et al., 1999; Cusidó et al., 2002; Rischer et al., 2006; Häkkinen et al., 2007; Vongpaseuth and Roberts, 2007). Plant cells and organs have been successfully established as factories (Zhang et al., 2004), but the lack of knowledge about biosynthetic pathways and their regulation restricts their use.

Several studies have elucidated considerable information by linking gene expression levels and secondary metabolite production. Goossens et al. (2003) studied the metabolic profile of tobacco BY-2 cells and transcriptomic profile via cDNA-amplified fragment length polymorphism (cDNA-AFLP), showing an extensive jasmonate–mediated genetic reprogramming of metabolism, correlated with alkaloid production. Hirai et al. (2004) integrated the transcriptomic profile of *Arabidopsis* obtained by DNA arrays with metabolic processes from primary and secondary metabolism, showing global and individual regulations under sulphur deficiency conditions. Rischer et al. (2006) combined genome-wide transcript profiling by cDNA-AFLP with metabolic profiling of elicited *Catharanthus roseus* cell cultures, which yielded a collection of known and previously undescribed transcript tags and metabolites associated with terpenoid indole alkaloids. These studies have provided the basis for a better understanding of secondary metabolism and increased the practical potential of metabolic engineering of these important medicinal plants.

As described, several research groups have developed strategies for linking genomic and metabolic data with the aim of increasing our knowledge of genes involved in secondary metabolite pathways. The main problem for these studies is the lack of genomic information, such as the EST data base or cDNA clone collections, which precludes the use of conventional techniques like microarrays. An alternative for studying plant secondary metabolism is cDNA-AFLP (cDNA-amplified fragment length polymorphism) technology, which does not require previous genomic information and provides quantifiable gene expression profiles and allows the identification of new genes (Rischer et al., 2006; Goossens et al., 2003; Vuylsteke et al., 2007).

The cDNA-AFLP transcript profile (AFLP-TP) is a gel-based transcript profiling method that generates quantitative gene expression data for any organism on a genome-wide scale (Vuylsteke et al., 2007). The method has found widespread use as one of the most robust, sensitive and attractive technologies available for gene discovery on the basis of fragment detection. In the cDNA-AFLP technique, after reverse transcription of mRNA into double-stranded cDNA, there is a restriction digestion and a ligation of adaptors. Selective PCR amplification divides the cDNA fragment mixture into small groups, which are subsequently loaded and run on high-resolution gels, and developed using a conventional autoradiography platform. Variations in band intensity in two different conditions should be interpreted as differences in gene expression, and can be semi-quantified and compared. The sequence of

tags of interest can be obtained by purifying bands from cDNA-AFLP gels, before amplifying and sequencing.

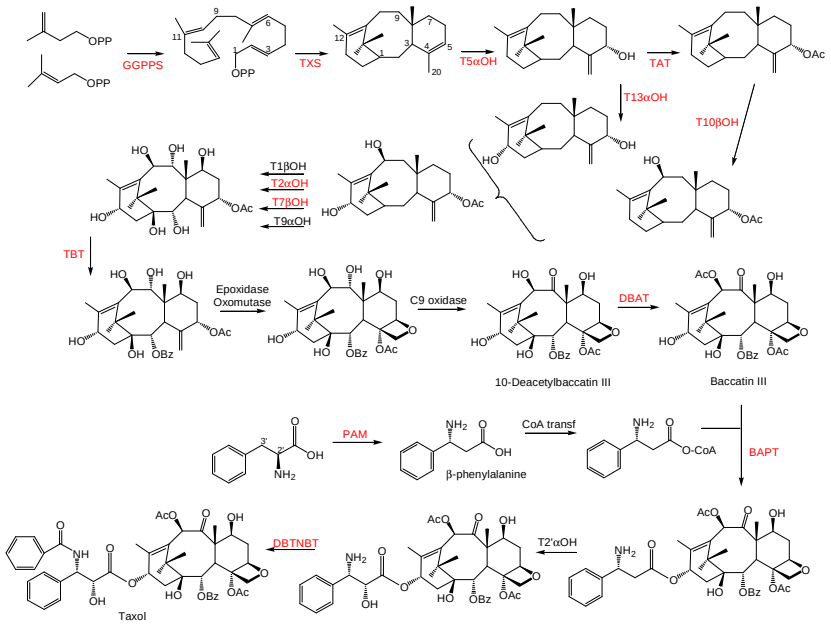


Figure 2.1: A concise depiction of Taxol biosynthesis. GGPPS, Geranylgeranyl diphosphate synthase; TXS, Taxadiene synthase; T5αOH, Taxane 5α-hydroxylase; TAT, taxadiene-5α-ol-*O*-acetyl transferase; T13αOH, Taxane 13α-hydroxylase; T10βOH, Taxane 10β-hydroxylase; T1βOH, Taxane 1β-hydroxylase; T2αOH, Taxane 2α-hydroxylase; T7βOH, Taxane 7β-hydroxylase; T9αOH, Taxane 9α-hydroxylase; TBT, Taxane-2α-*O*-benzoyl transferase; DBAT, 10-deacetyl baccatin III-10-*O*-acetyltransferase; PAM, Phenylalanine aminomutase; CoA transf, CoA transferase; BAPT, baccatin III-3-amino, 13-phenylpropanoyltransferase; T2'OH, Taxane 2'α-hydroxylase; DBTNBT, Debenzoyl taxol *N*-benzoyl transferase. Known genes are in red.

Our group has focused on increasing taxol production in cell suspension cultures of *Taxus* spp. The use of two-media cultures, one for increasing biomass and the other for activating secondary metabolism, together with the addition of elicitors successfully increased taxol levels compared with control cell suspensions (Palazón et al., 2003; Moyano et al., 2004). After this improvement, any further enhancement in production of this anticancer agent would require a greater understanding of taxol biosynthesis. For this reason, we have applied the cDNA-AFLP technique to *Taxus baccata* cell cultures, comparing a highly productive elicited cell line with the same cell line grown in unelicited conditions.

Even though the biotechnological production of taxol is already a reality, and several ways of improving the process have been reported, a greater understanding of the biosynthesis of this anticancer agent and its regulation is required to enhance its production. As indicated in Chapter 1, taxol biosynthesis involves 19 enzymatic steps (Figure 2.1) from the universal diterpenoid geranylgeranyl diphosphate (GGPP) (Croteau et al., 2006): one synthase, eight cytochrome P450 oxygenations, five CoA-dependent acyl/aroyl transfers, an oxidation at C9, an oxetane formation, an aminomutase and two CoA esterifications. Although most of the biosynthetic steps have been described, the possible regulatory and transport systems, cellular compartmentalization or degradation processes remain unclear.

For this reason, our work is focused on an in-depth study of the molecular mechanism that regulates secondary metabolism in targeted *Taxus* cell cultures, the principal objectives being: (1) to determine the taxane production of a selected *T. baccata* cell line when cultured in the optimum medium for production, supplemented with the elicitor MeJ (100 µM); (2) to study the variations in the transcriptomic profile of the selected cell line under MeJ elicitation using cDNA-AFLP; (3) to find the relationship between the metabolic and the transcriptomic profiles in the elicited cell suspensions, always comparing the results with those obtained from the same cell line grown without the addition of the elicitor; and finally, (4) to search for new genes that could be directly or indirectly involved in the metabolism of taxol.

2.3. Material and Methods

2.3.1. Establishment of callus cultures and cell suspension cultures

Callus tissues were obtained from young stem sections of *Taxus baccata* L. plants. After removing the needles, explants were immersed first in 70% ethanol for 1 min, then for 15 min in 0.01% $HgCl_2$ and finally 30 min in sodium hypochlorite (1.5% with a few drops of Tween 20) before being rinsed three times with sterile distilled water. Longitudinally-halved stem sections were placed with the inner, cut surface in contact with the culture medium. The explants were kept on an induction medium, Gamborg's B5 (Gamborg et al., 1968) supplemented with 2x B5 vitamins, 3% sucrose, 4 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 1 mg/L kinetin and 0.5 mg/L

gibberellic acid (GA_3) and solidified with 0.3% Phytagel (Sigma, St Louis, MO, USA). The pH was adjusted to 5.8 prior to autoclaving. After 4–6 weeks, the different callus tissues formed were separated from each explant and cultured on a growth medium, Gamborg's B5 supplemented with 2x B5 vitamins, 0.5% sucrose, 0.5% fructose, 2 mg/L naphthaleneacetic acid (NAA), 0.1 mg/L 6-benzylaminopurine (BAP) and 0.5 mg/L GA_3 and solidified with 0.3% Phytagel. Both explants and calli were maintained at 25℃ in the darkness and subcult ured every 10 days.

Cell suspension cultures were established using stem-derived calli as the inoculum. 1 g of fresh weight of callus pieces was inoculated into 175-mL flasks containing 10 mL liquid growth B5 medium prepared as described above, capped with Magenta B-Cap (Sigma, St Louis, MO, USA) and placed in a rotary shaker (100 rpm) at 25℃ in dark ness.

2.3.2. Increasing taxane production

Two different media were used to improve the productivity of the *T. baccata* cell suspension cultures. Firstly, a growth medium (GM) was used, based on Gamborg's B5 supplemented with 2x B5 vitamins, 0.5% sucrose, 0.5% fructose, 2 mg/L NAA, 0.1 mg/L BAP and 0.5 mg/L GA_3. When a large biomass was achieved, the cells were transferred to a production medium (PM), a basic Gamborg's B5 supplemented with 2x B5 vitamins, 3% sucrose, 0.1mg/L of kinetin, and 2 mg/L picloram. Taxane production was increased by adding 100 μM Methyl jasmonate (MeJ) to this optimum medium for production. MeJ, prepared as described by Yukimune et al. (1996), was added to the production medium prior to inoculation to give the final concentration of 100 μM. For analysis, three flasks were harvested for each treatment at different time points: 0h, 30min, 1h, 2h, 4h, 8h, 16h, 1 day, 2d, 4d, 8d, 12d, 16d, 20d and 24d.

2.3.3. Determination of cell growth and viability

A time course of cell growth was studied in *T. baccata* cell suspension cultures measured as fresh and dry weight. Samples were collected at the different established time points, filtered, weighed and subsequently freeze-dried for determination of dry weight. A small aliquot of each fresh sample was used for the cell viability assay, performed by incubating the cells for 5 min in B5 medium containing 0.01% (P/V) propidium iodide (ICN Biomedicals, Costa Mesa, CA, USA), for the selective labelling of

dead cells, and 0.01% (P/V) fluorescein diacetate (Sigma, St Louis, MO, USA) for the selective labelling of live cells as described by Duncan and Widholm (1990). Fluorescence was observed with a fluorescence microscope (DMRB, Leica Microsystems Inc. Wetzlar, Germany) using specific filters.

2.3.4. Taxane determination

Taxanes were extracted from the culture media as described by Cusidó et al. (1999). 20 mL of media was mixed and vortexed during 2 min with 5 mL of dichloromethane (DCM), followed by 1h sonication at 25ºC. 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 lyophilized material, warmed up during 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 mixed. After adding 4 mL of hexane, the samples were centrifuged at 2500 g during 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 mixed and evaporated together. All samples were resuspended in 500 µL methanol and filtered prior to analysis (0.22 µm PVDF filters, Millipore, Billerica, MA, USA).

Taxane quantification was achieved by high-performance liquid chromatography (HPLC) as described by Richheimer et al. (1992). The chromatographic analyses were performed in an HPLC Agilent 1100 series and the separation was carried out in a SUPELCOSIL LC-F column 25 cm x 4,6mm (Supelco, Bellefonte, USA) using a mobile phase consisting of a mixture of water (A) and acetonitrile (B) with a timed gradient program: Time (min)/%B: 0/25, 38/60, 40/60, 50/25, and 55/25, with a flow rate of 1 mL/min. Criteria for identification included retention time, UV spectra and co-chromatography with standard and peak homogeneity determined by a photodiode array detector when spiked with authentic standard. Quantification was carried out from the calibration curve of each standard: 10-deacetylbaccatin III, baccatin III, cephalomannine, 10-deacetyltaxol and taxol (Chromadex, USA).

2.3.5. cDNA-AFLP Transcript Profiling

cDNA-AFLP-based transcript profiling was performed as described (Vuylsteke et al., 2007). RNA was isolated using the "RNeasy Mini Protocol for isolation of total RNA from Plant cells and tissues and filamentous fungi" (Qiagen, Germany) and cDNA synthesis was obtained after treatment with Superscript® II Reverse Transcriptase (Invitrogen, California, USA) and using a biotin-labelled oligo-dT. The double strand cDNA was recovered with Nucleospin® Extract II (Macherey Nagel, Düren, Germany). After the digestion of each sample with *Bst*YI, the 3'terminal cDNA fragment was recovered by streptavidin-coated Dynabeads. Next, the *Mse*I digestion was performed on the 3' fragment, and adaptors (Bst and Mse) were bound to both extremes. After a first non-selective preamplification with one selective nucleotide (BstC or BstT), messengers were screened with three additional selective nucleotides for the *Bst* / *Mse* templates using radiolabelled primers.

For the gene expression profile study a polyacrylamide gel was analysed. PCR products were separated on a denaturating 5% polyacrylamide gel, after which the gel was autoradiographed by exposure to a standard X-ray film for 2 days and scanned using phosphor imaging technology after 1 day of exposure. Scanned gel images were analysed with AFLP-QUANTARPRO software (Keygene, Wageningen, The Netherlands) in which bands of interest were selected and their size assigned. An AFLP program allowed normalization of data due to PCR or loading differences by the use of a correction factor. The correction factor was calculated based on the sum of the expression levels of all fragments in one lane divided by the highest sum of all lanes within that primer combination. Subsequently, the variance in band intensity was normalized by subtracting the mean of all corrected values of a time course from the corrected expression value, and dividing the result by the standard deviation of all corrected values of the time course. Eventually, for all individual bands, a coefficient of variation (CV) was calculated as the standard deviation of all corrected values of the time course, divided by the average expression over the time course. Gene tags displaying expression values with a CV≥0.5 were considered as differentially expressed. Based on this cut-off value, together with visual inspection of cDNA-AFLP gels, differentially expressed gene tags were selected for further analysis. CLUSTER and TREEVIEW software were used for average linkage hierarchical clustering.

Once the jasmonate-modulated cDNA-AFLP fragments were recognized, the sequences were obtained from re-amplified PCR product, and were

compared with nucleotide and protein sequences in the publicly available databases by BLAST sequence alignments, selecting those with a bit score of over 50 and an e-value lower than 10^{-2}. Finally, tags were classified functionally according to the Functional Catalogue from the Munich Information Centre for Protein Sequencing (MIPS), but due to ambiguity and multifunctionality of some tags, classification was simplified in the following categories: (1) Metabolism and Energy, (2) Cell organisation and defence, (3) Protein synthesis and fate, (4) Cellular transport, transport facilities and transport routes, (5) Transcription, (6) Signal transduction, (7) Transposable elements, (8) Unclassified proteins, and (9) No hits.

2.3.6. Complete sequencing

Cloning of full-length cDNA-sequences was achieved with Rapid Amplification cDNA Ends (RACE) PCR (GibcoBRL-Invitrogen, Carlsbad, USA) following the user guide. An initial amount of 2-3.5 ug of RNA was used and the election primers were AAP (5'-GGCCACGCGTCGACTAGTACGGGIIGGGIIGGIIG-3') and a gene specific primer (GSP1) designed in the known part of our tags. In the following nested-PCR, the two primers were AUAP (5'-GGCCACGCGTCGACTAGTAC-3') and new specific primers designed in the newly sequenced part (GSP2, GSP3 or even GSP4). The chosen polymerase was AmpliTaq polymerase (Applied biosystems, Carlsbad, USA) in a PCR program of 35 cycles of 94℃ 1min, 55℃ 1 min, 72℃ 1min/1Kb to a mplify, followed by 5 min at 72℃.

2.3.7. Quantitative real-time 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, California, USA) and qRT-PCR was performed using 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 2.1) and the amplification efficiency of each primer pair was determined

empirically by 10-fold serial dilutions of cDNA and calculated as described in Qiagen. Only those primer pairs with an efficiency of over 0.8 were used. Expression levels were normalized to the levels of the *18S* from *Taxus baccata*.

Table 2.1: Sequences of the primers used to amplify the genes by quantitative real-time PCR.

Gene		Primer Sequence	Amplicon size	Reference - Acc. Number
18S	Sense	5′-GTGCACAAAATCCCGACTCT-3′	102	Onrubia et al., 2010
	Reverse	5′-GCGATCCGTCGAGTTATCAT-3′		
GGPPS	Sense	5′-CCGGTGTGTGGGGCTTCTGTTT-3′	144	AY566309 (*T.*x *media*)
	Reverse	5′-TTTGCTTTCTCCAGGCCCATCA-3′		
TXS	Sense	5′-TTCGCACGCACGGATACG-3′	115	Onrubia et al., 2010
	Reverse	5′-TTCACCACGCTTCTCAATTCG-3′		
TAT	Sense	5′-TTTCGGTGTTGGGAGATTTGG-3′	88	TB754 (cDNA-AFLP)
	Reverse	5′-AGGTCCACAATGTCTGTATTCG-3′		
T13OH	Sense	5′-GCCCTTAAGCAATTGGAAGT-3′	100	AY866412 (*T.* x *media*)
	Reverse	5′-CAGAGGAATGGCGTTTAGAG-3′		
T2OH	Sense	5′-CGTGCCATTTGGAGGAGGGAGA-3′	122	AY518383 (*T. canadensis*)
	Reverse	5′-CGTGAGGGTCGATTGGCGTGTA-3′		
T7OH	Sense	5′-GGTCCGCCCAAATTGCCAGAA-3′	110	AY307951 (*T. cuspidata*)
	Reverse	5′-CCCTGCAGAGCCCAAAAAACCT-3′		
TBT	Sense	5′-GGCGGACAACGACCTTTC-3′	129	TB766 (cDNA-AFLP)
	Reverse	5′-ACGAGTTACCTGAACAGTTAGAAG-3′		
DBAT	Sense	5′-AGTTGGATTTGGTGATCGAA-3′	92	Onrubia et al., 2011
	Reverse	5′-ATCCATGTTGCACGAGACTT-3′		
PAM	Sense	5′-CCCGGAGGCATGACGTGAAG-3′	99	AY866411 (*T.* x *media*)
	Reverse	5′-CGCCGTCTTCCGCCTTGC-3′		
BAPT	Sense	5′-TAAGCACTCTACAACAACAATGG-3′	111	Onrubia et al., 2010
	Reverse	5′-GCATGAACATTAGTATCTTGATTCC-3′		
DBTNBT	Sense	5′-CGGGGGGTTTGTTGTGGGATTA-3′	104	Onrubia et al., 2011
	Reverse	5′-TTAGCCTCTCCCCTCGCCATCT-3′		

2.3.8. Statistics

Statistical analysis was performed with Statgraphics (Centurion XV) and Excel software. All the data are the average of 3 determinations ± 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.

2.4. Results and Discussion

2.4.1. Cell suspension cultures

Previous studies carried out by our research group on taxane production in *Taxus baccata* cell suspensions have shown that it is possible to increase this production by using a two-phase culture and adding elicitors (Cusidó et al., 2002; Bonfill et al., 2003; Expósito et al., 2010). The cells were cultured for 12 days in a growth medium, then transferred to a production medium for a further 24 days, and growth and viability were analysed. As shown in Figure 2.2A, there was an increase of biomass in unelicited cell cultures, while very little biomass increment was observed when cells were maintained under elicitation. The viability of cultures did not change significantly during the 24 days of the experiment, although it decreased in elicited conditions (Figure 2.2B).

2.4.2. Metabolic analysis, taxane production

The total taxane production (cell associated + extracellular), expressed as mg/L, during 24 days of culture of the studied *T. baccata* cell line, supplemented with MeJ or not (control), is represented in Figure 2.3. The total taxane accumulation in the cell suspensions cultured in the production medium in control conditions was low throughout the experiment, although it began to increase at day 8, decreasing again after day 16. Taxane accumulation increased dramatically when the medium was supplemented with MeJ, the highest level (249 mg/L) being achieved after 24 days of elicitation. At this point, the total taxane contents in the elicited cultures were 10.4 times higher than in the control at the peak of production. The effectiveness of the elicitor MeJ in increasing taxane production in *Taxus* cell cultures has been repeatedly reported (Ketchum et al., 1999; Yukimune et al., 1996; Furmanova et al., 1996; Zhang et al., 2000; Cusidó et al., 2002).

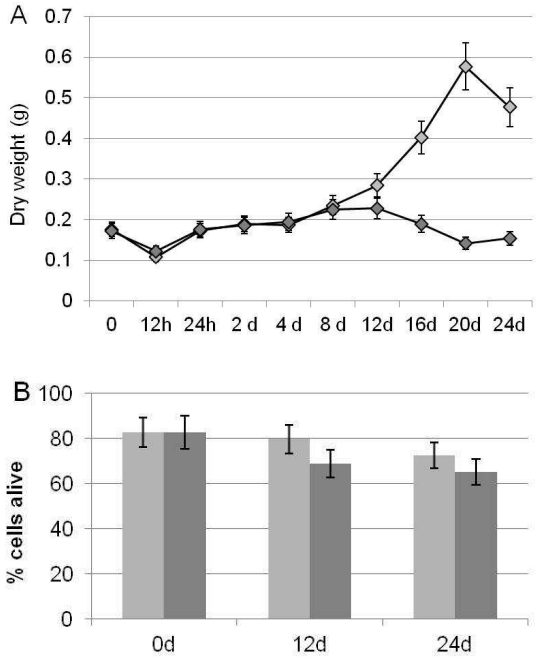


Figure 2.2: Time courses of biomass production expressed as dry weight (A) and viability (B) of the *Taxus baccata* cell line cultured for 24 days in the PM without elicitors (control, light grey) or with the addition of 100 µM MeJ (dark grey). In all cases, the inoculum consisted of 100 g/L of cells. Data represent average values from three separate experiments ± SD.

When the production of individual taxanes in the elicited and control cultures was compared (Figure 2.4), a general increase was observed, but the response to the elicitation was not uniform. The highest accumulation of each taxane was achieved after a minimum of 12 days of culture, the highest levels being most commonly found at day 20. Variable levels of 10-deacetylbaccatin III (DABIII) were found in both elicited and control cell cultures throughout the experiment, the highest being achieved after 12 days. The presence of MeJ in the culture medium did not significantly increase its production. In MeJ-treated cultures, the predominant taxane was baccatin III, whose levels peaked at day 20, decreasing thereafter, while in the control cultures, the highest content was observed at day 16. At its peak in the elicited culture, the content of baccatin III was 20.5 times higher than in the control. It can be inferred from these results that the metabolic step responsible for the formation of baccatin III from its precursor DABIII was not rate-limiting in the elicited *T. ba*ccata cell cultures studied, since baccatin III accumulation was high despite the very low levels of DABIII, indicating that after its formation this precursor is probably immediately transformed into the product baccatin III.

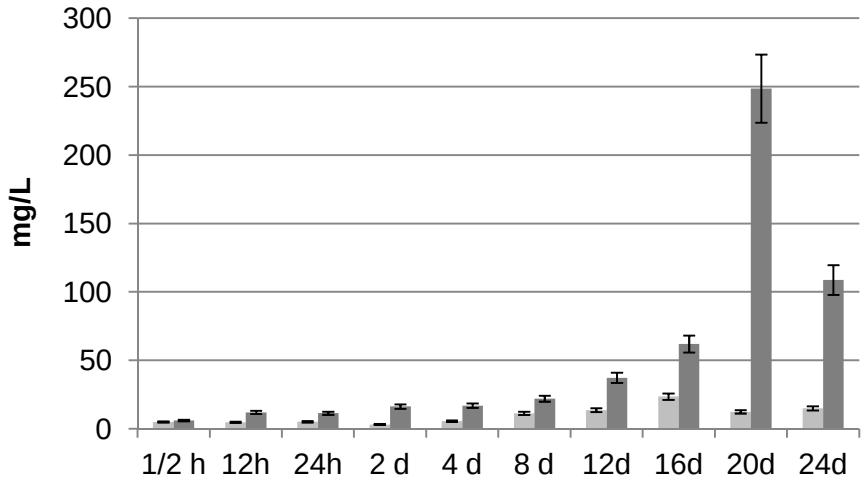


Figure 2.3: Total taxane content (cell associated + extracellular) in *T. baccata* cell cultures growing for 24 days in the production medium with and without the addition of methyl jasmonate (100 µM) to the culture medium. Light grey bars correspond to the control, dark grey bars to MeJ elicited samples. Data are average values from three replicates ± SD.

As observed for baccatin III, the highest accumulation of 10-deacetyltaxol (DAT) was found after 16 days in the control cultures and 20 days in those supplemented with MeJ. However, MeJ exerted a lower effect on the production of this taxane, since DAT levels at their peak were only 2.8 times higher in the elicited culture. It is not clear if DAT is formed as a degradation product of taxol or is involved in a branch or off-pathway competing with the taxol biosynthetic route. In fact, the gene/s involved in the formation of DAT is/are not known.

Taxol accumulation in the *T. baccata* cell cultures increased dramatically after supplementation with MeJ. The highest levels were achieved after 20 days of elicitation, in comparison with 16 days in the control cultures. The addition of MeJ to the culture medium resulted in a very notable increase of this taxane, which at its peak was 19 times higher than in the control. The fact that taxol increased much more than cephalomannine, when both taxanes have a baccatin III moiety, may be due to a lower activation of tigloylation than of benzoylation by the assayed MeJ concentration or the lack of a donor for a side chain, such as tiglic acid. Cephalomannine is very similar to taxol structurally, differing in that it has a tigloyl group at position C-3' of the C-13 side chain instead of a benzoyl group.

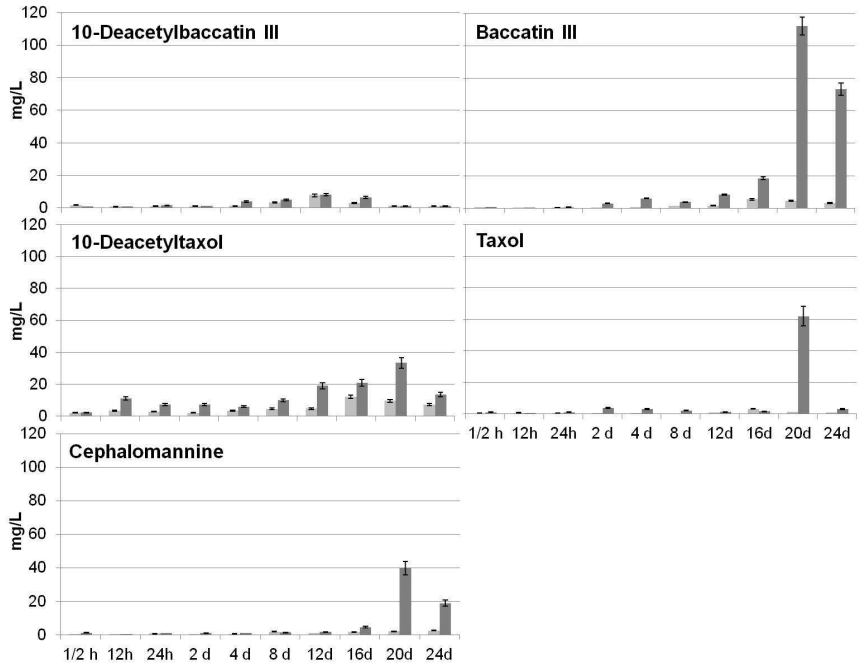


Figure 2.4: 10-deacetylbaccatin III (DABIII), baccatin III, 10-deacetyltaxol (DAT), taxol and cephalomannine (Ceph) content (cell associated + extracellular) in *T. baccata* cell cultures growing for 24 days in the production medium with and without the addition of methyl jasmonate (100 µM) to the culture medium. Light grey bars correspond to control data, dark grey bars to MeJ elicited samples. Data are average values from three replicates ± SD.

In unelicited cultures, only DABIII and DAT were found in concentrations higher than 1 mg/L in the first 8 days of culture. These results agree with those of Nims et al. (2006), who reported that neither baccatin III nor the side-chain taxanes (taxol and cephalomanine) were detected in control cultures over a 7-day time course. However, in the elicited cultures, after 4 days of elicitation, the non-side-chain and side-chain taxanes were found in quantities higher than 1 mg/L. Since the joint amount of taxol and cephalomannine (62 mg/L plus 40 mg/L, respectively) was very similar to that of their precursor, baccatin III (112 mg/L), after 20 days of elicitation, it can be inferred that the metabolic steps leading to these two phenylpropanoyl-side-chain-bearing taxanes from baccatin III were active in this study.

2.4.3. Transcript profile

The cDNA-AFLP technique was applied for genome-wide transcript profiling using the cells cultured for 7 days in the GM, and those maintained for 1h, 2h, 4h, 8h, 16h, 1d, 2d and 4d in the PM, either with or without (control) the addition of the elicitor MeJ. As there is a reprogramming of general metabolism in favour of secondary metabolism when cells are cultured in the PM (Cusidó et al., 2002), the 7d GM sample was considered as the negative control for secondary metabolism gene expression patterns. Using 128 BstY*I* -Mse*I* primer combinations, the quantitative temporal accumulation patterns of 8192 transcript tags were determined and analysed. Tags of interest were those induced or repressed under MeJ elicitation, and therefore differentially expressed in the control and elicited cells. However, as some taxol biosynthetic genes were activated, in both control and elicited conditions and presented differential expression throughout the experiment, their expression pattern was analysed as well. Out of the 858 differentially expressed transcript tags isolated (Figure 2.5), 667 fragments (77.7%) gave good-quality sequences by direct sequencing of the PCR products, indicating that they might represent unique gene tags. Homology searches with the sequences from the unique gene tags revealed that 55.3% (369) were similar to known genes. In Figure 2.6 the selected tags are classified according to the FunCat.

The similarity threshold maintained for BLAST searches was established at a value of $10e^{-2}$; however, due to the small size of some tags, in a few cases a lower *e*-value was accepted when unambiguous matches were observed. 311 (46.3 %) of sequenced tags displayed similarity with genes of known functions and 60 (9.0 %) with genes without allocated functions (Figure 2.6, Unclassified proteins). In contrast, no homology to a known sequence was found for 296 of the sequenced tags (44.7 %, Figure 2.6, No hits). It should be stressed that the percentage of hits using the cDNA-AFLP technique is based upon the amount of information on related organisms available in public databases. Therefore, as available data on gymnosperms is relatively low, high percentages of hits, which have been as much as 75% in previous cDNA-AFPL studies (Rischer et al., 2006; Goossens et al., 2003), cannot be expected. Moreover, with the genome sequencing projects carried out in the last years, there are a lot of tags presenting homology to hypothetical, predicted or unknown proteins, which hinders the possibility of finding functional matches.

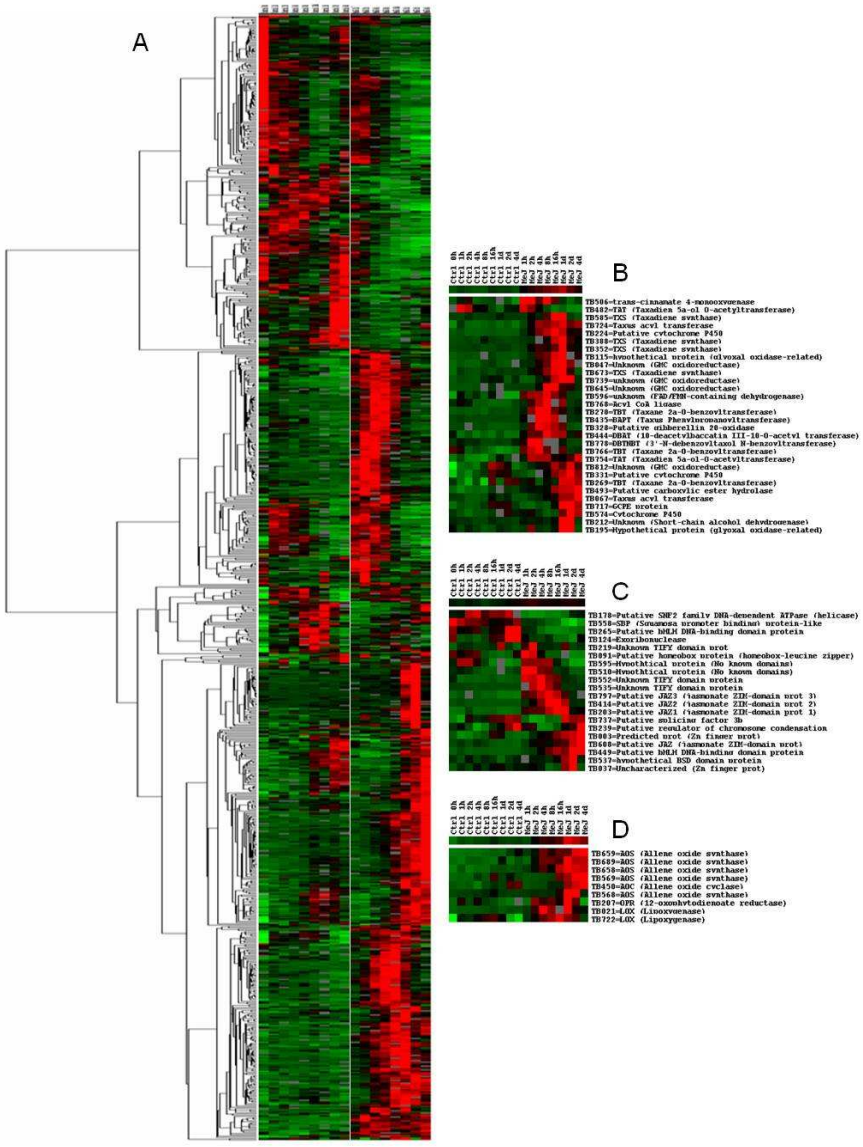
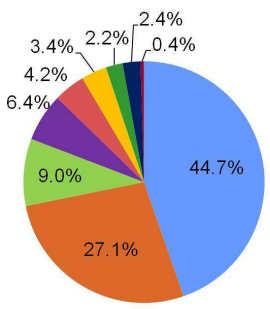


Figure 2.5: Average linkage hierarchical clustering of the transcriptome of selected gene tags from MeJ-elicited *Taxus baccata* cell cultures. (A) Complete transcriptome, (B) tags related to taxol biosynthesis and candidates for unknown steps (Table 1.1 and Table 3.1), (C) tags with homology to transcription factors and (D) tags with homology to putative jasmonate biosynthesis genes. The time points for the cluster analysis are indicated at the top: 12d in GM, and 1h, 2h, 4h, 8h, 16h, 1d, 2d, 4d in PM in control and MeJ-elicited conditions. Red and green boxes reflect transcriptional activation and repression, respectively, relative to the average expression level in control cells. Gray boxes correspond to missing time points. The dendrogram at the left side of the transcriptome indicates the clusters of activated and repressed genes. PM: Production medium.



Function	Nº tags	%
No hits	298	44.7
Metabolism and energy	181	27.1
Unclassified Proteins	60	9.0
Cell organization and defence	43	6.4
Protein synthesis and fate	28	4.2
Cellular transport, transport facilities and transport routes	23	3.4
Signal transduction	15	2.2
Transcription	16	2.4
Transposable elements	3	0.4
Total sequenced tags	**667**	**100**

Figure 2.6: Sequenced tags classified in functional categories according to FunCat classification.

The functional category Metabolism and Energy was the major group, with tags homologous to genes from primary and secondary metabolism, as well as the functional group related with cell organization and defence. In our study we stimulated the cultures with jasmonates, which are involved in plant defence against a wide range of stimuli (Wasternack, 2007). Factors determining taxol production in plant tissue, such as localization and age, and studies involving the treatment of *Taxus* cell suspensions with phytopatogen fungi suggest that taxol is a phytoalexin. Phytoalexins are plant molecules produced to avoid pathogen infections (Mirjalili and Linden, 1996). Elicitors like jasmonates can modify secondary metabolism leading to the production of phytoalexins such as taxol.

There are 14 known genes that encode enzymes involved in the taxol biosynthetic pathway, including those encoding GGPPS, the enzyme responsible for producing the intermediate for taxadiene synthase, and T14OH, an enzyme that controls one of the first steps that picks up intermediates for an alternative pathway. The complete list of genes involved in taxol biosynthesis can be found in Table 1.1 and Figure 2.1. The cDNA-AFLP study yielded tags corresponding to *TXS, TAT, TBT, DBAT, BAPT* and *DBTNBT* genes (42.8 % of the known genes), whereas the remaining 8 genes were not found. Tags corresponding to *T5OH*, *T13OH, T10OH, T7OH* and *PAM* were either too small or too large to be detected, while tags corresponding to *GGPPS* and *T2OH* presented a detectable size (according to *in silico* cDNA-AFLP analysis with known sequences from *Taxus canadensis*, *chinensis*, *cuspidate* and *media*, Table 2.2), but were not determined. The absence of tags can be due to the tag size, an unselected gene expression pattern or the impossibility of sequencing them. If the sequence cannot be cut by Mse*I* and BstY*I*, the

fragment cannot be amplified; if the produced fragments are smaller than 70bp, there is no informative sequence (adaptors constitute more than 40bp), while when the produced fragments are bigger than 700bp, the signal in the gel cannot be distinguished; and if there are problems for amplifying or sequencing, analysis is impossible. Genes without an interesting expression pattern were discarded and those with very low expression could not be observed. The tags homologous to taxol biosynthetic genes had two main expression patterns: the majority were only expressed under MeJ elicitation and few were differentially expressed during culture in the production medium, either in control conditions or under MeJ (Figure 2.5B).

Table 2.2: *In silico* cDNA-AFLP of known taxol biosynthesis genes not found in the experimental cDNA-AFLP analysis. For each gene 2 or more sequences were used. GenBank accession number, species, gene sequence length, 3' terminal part size when cut with BstY*I* and Mse*I* and the size of the final 3' terminal fragment obtained in the cDNA-AFLP are indicated. Canadensis is abbreviated as canaden.

Name	GenBank	Specie	Gene length (bp)	BstY*I* cut (bp)	Mse*I* cut (bp)	3' fragment (bp)
GGPPS	BD241767	canaden.	1889	1182	1773	591
	AY566309	media	3743	3285	3534	249
T5OH	AY289209	cuspidata	1633	1440	1478	38
	AY741375	chinensis	1509	1440	1478	38
T13OH	AY056019	cuspidata	1458	1451	1456	5
	AY959321	chinensis	1853	1847	1852	5
	AY866412	media	1790	1536	1542	6
T10OH	AF318211	cuspidata	1494	1486	-	0
	AY453403	media	1494	1486	-	0
	AF545833	chinensis	1494	1486	-	0
T2OH	AY518383	canaden.	1488	810	943	133
	AY789508	chinensis	1659	810	943	133
T7OH	AY307951	cuspidata	1529	1525	-	0
	AY374652	chinensis	1482	1474	-	0
PAM	AY582743	canaden.	2109	517	1441	924
	AY724735	media	2064	511	1435	924
	AY724735	chinensis	2277	2271	-	0

This study has produced a long list of possible candidate genes for the seven unknown steps of the taxol biosynthetic pathway, which involve hydroxylases, an oxomutase, epoxidase, oxidase and CoA ligase (Croteau et al., 2006). Tags classified in the Metabolism and Energy group, with a clear MeJ induction pattern and homology to genes with functions similar to those expected for the unknown steps of taxol biosynthesis were selected to study their possible involvement in this process. Only some of these gene tags, which are listed in Table 3.1 (Chapter 3), have been fully sequenced. In order to confirm or at least discard some of them, they were subjected to bioinformatics analysis, which are described in Chapter 3.

Additionally, numerous novel genes, either with or without existing homologues, and with or without known functions, were identified as jasmonate-responsive genes. Tags whose expression pattern changed or increased after MeJ elicitation and presented homology to genes encoding transcription factors (TF) or regulatory proteins were studied more deeply (Figure 2.5C). Two main kinds of expression patterns were observed: tags expressed differentially between the MeJ-elicited and unelicited cultures, and tags expressed with a similar expression pattern in both conditions. Two clusters were observed in the former: those repressed or induced after MeJ-treatment. Notable among the putative TF were: putative JAZ proteins or hypothetical proteins with a TIFY domain (TB797, TB414, TB203, TB608, TB219, TB552 and TB535), putative bHLH transcription factor (TB265 and TB449), a hypothetical BSD protein (TB537) and hypothetical proteins without any known domains (TB510 and TB595). Among these gene tags, TB595 was selected due to its annotation, small size and expression pattern. The full sequence of the gene corresponding to this tag was obtained and the protein it encoded was named Taximin. The characterization and functionality of the gene are described in Chapter 5.

Another example of up-regulated genes was provided by a group of genes involved in the biosynthesis of jasmonates (Figure 2.5D). These tags showed a clear induction expression pattern under MeJ treatment, from 1 hour to day 4 of treatment, while no or very low and sporadic expression was observed in the unelicited cultures, supporting the idea that the addition of exogenous jasmonates induces endogenous jasmonate synthesis (Stenzel et al., 2003).

2.4.4. Transcription profiles of genes involved in the taxol biosynthetic pathway

The transcription profiles of the genes already known to be involved in taxol biosynthesis were determined by qRT-PCR in *Taxus baccata* cell suspension cultures with or without MeJ treatment. The pathway has been divided in three parts for its study: a) **early**, b) **intermediate** and c) **late steps**.

The **early steps**, catalyzed by GGPPS, TXS, T5OH, TAT, T10OH and T13OH enzymes, follow the formation of the precursor GGPP and the first branch point after the biosynthesis of taxa-4(20),11(12)-dien-5α-ol, which is the substrate for an acetylation at the C5 position and a subsequent hydroxylation at the C10 position, or for a hydroxylation at 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 (Figure 2.1). ***GGPPS*** gene expression (Figure 2.7) in the control and elicited cultures started at 4h, achieving the highest level in control conditions at day 1, and day 2 in the treated cultures, decreasing thereafter in both cases. The presence of MeJ in the culture medium resulted in an accumulation of *GGPPS* mRNA 5 times higher than the maximum obtained in the control. The ***TXS*** gene expression in the cell cultures maintained in the production medium without elicitors was very low, presenting the highest level at day 4. MeJ treatment caused a rapid and significant increase in *TXS* expression, which peaked 2 days after elicitation. At this point, *TXS* mRNA accumulation was more than 8 times higher than the maximum in the unelicited cultures. These results agree with those obtained by Vongpaseuth and Roberts (2003), who showed that the expression of genes involved in the early steps of taxol biosynthesis (GGPPS and TXS) increased at 6 h after the addition of MeJ to the culture medium, peaking 1–2 days after elicitation. In previous work, we have also shown that the expression of the *TXS* gene is dramatically increased by the presence of MeJ in the culture, and that the highest expression takes place after 1-2 days of elicitation (Expósito et al. 2010; Onrubia et al., 2010).

We did not find any tag homologous to ***T5OH*** or ***T10OH*** in the cDNA-AFLP studies due to the limitations of this technology), but possibly these genes do not significantly change their expression under MeJ elicitation in *Taxus baccata* cell cultures. In fact, Croteau et al. (2006) reported that the *T5OH* gene is not highly induced by MeJ in *T. cuspidata* cells, although the same year Nims et al. (2006) found this gene expressed in *T. cuspidata* cell cultures after 6-12h of elicitation. The induction of ***T13OH***

mRNA started 1 hour after the addition of MeJ to the culture medium and the highest level was achieved 12 h later (1236 times the reference value), clearly decreasing until day 4, when no gene transcription was detected. In contrast, no detectable transcript accumulation was observed in the control cultures until day 4. The expression of the ***TAT*** gene started at 1 h of culture, both in control and elicited conditions. In control cultures the highest transcription levels of the *TAT* gene were achieved after 1 day, decreasing dramatically thereafter. The highest transcript accumulation in the cultures treated with MeJ was obtained at day 2 (82 times the reference value), also decreasing at day 4. These results indicate that MeJ induces a 1.7-fold increase of TAT gene expression compared with unelicited cultures, when comparing peak values.

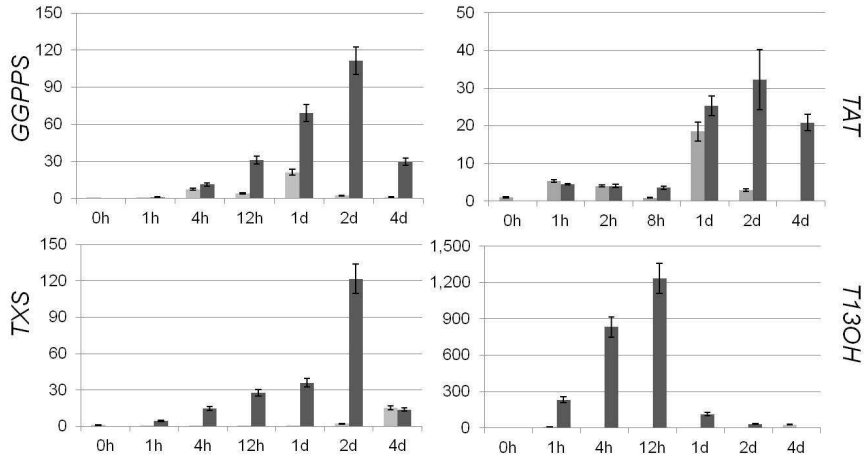


Figure 2.7: Expression levels of the *GGPPS*, *TXS*, *TAT* and *T13OH* genes relative to time zero of the cultures studied. In light grey non-elicited samples, in dark grey MeJ-treated samples.

From these results it can be inferred that the addition of MeJ to the culture medium generally increased the transcription levels of the studied genes to a variable extent. Although the expression of *GGPPS* was notable in MeJ-treated cultures, it cannot be assumed that an ensuing increase of GGPP content was directed to taxane production, since GGPP is the precursor of all diterpenoids found in plants. Several authors (Hezari et al., 1997; Croteau et al., 2006) have shown that TXS is not a rate-limiting enzyme in the taxol biosynthetic pathway, but since its expression was dramatically increased by MeJ, it can be inferred that the metabolic step controlling this enzyme provided enough substrate (taxa-4(5),11(12)-

diene) for a notable increase in taxane production in the elicited cultures studied.

When considering the branch point that exists from the intermediate taxa-4(20),11(12)-dien-5α-ol, and the expression of genes involved in both metabolic pathways, it can be assumed that taxol biosynthesis in the elicited *T. baccata* cultures proceeded mainly through the step catalysed by the enzyme T13OH. Moreover, since the product of TAT did not really increase under elicitation, this branch pathway could be a flux-limiting point for taxane production. Nims et al. (2006) found similar results in elicited *T. cuspidata* cell cultures and suggested a preference for the T13OH-side of the branch pathway. This confirmed metabolic data indicating that the precursor flux leading to taxol is via 5α,13α-diol through T13OH, rather than the 5α-yl acetate derived from the alternative branch controlled by the TAT enzyme (Hefner et al., 1996).

The **intermediate steps** begin with the compounds taxa-4(20),11(12)-dien-5α-13α-diol and taxa-4(20),11(12)-dien-5α-acetoxy-10β-ol to the formation of baccatin III. The middle part of the pathway includes almost all the unknown enzymes (two hydroxylases acting at C1 and C9, a C9 oxidase, a C4β,C20-epoxidase and an oxomutase), the two hydroxylases for C2 and C7 and the transferases TBT and DBAT (Figure 2.1, gene expression in Figure 2.8). The ***T7OH*** gene expression in the control cultures was very low or unnoticeable during the 4 days of study, except at 1h and day 4, when the mRNA accumulation was 19 and 12 times higher, respectively, than the reference value. In contrast, in the elicited cultures the induction of *T7OH* mRNA started 1h after elicitation, achieving the highest levels at 12h, when the mRNA accumulation was 722 times higher than the reference value, and decreasing dramatically thereafter until the end of the study. Comparing peak expression levels, the response of this gene to MeJ was notable, the mRNA accumulation being 37 times higher in the elicited cultures than in the control. In the control cultures, the expression of ***T2OH*** peaked at day 4 (6.4 times the reference value). In the elicited cultures the expression profile of this gene was similar to that observed for *T7OH*, with mRNA levels peaking at 12 h, but differed in that induction decreased dramatically at day 1, before increasing again at day 2 to half the levels observed at 12 h. This gene also responded very actively to elicitation since the maximum mRNA accumulation in the treated cultures was 35 times higher than in the control.

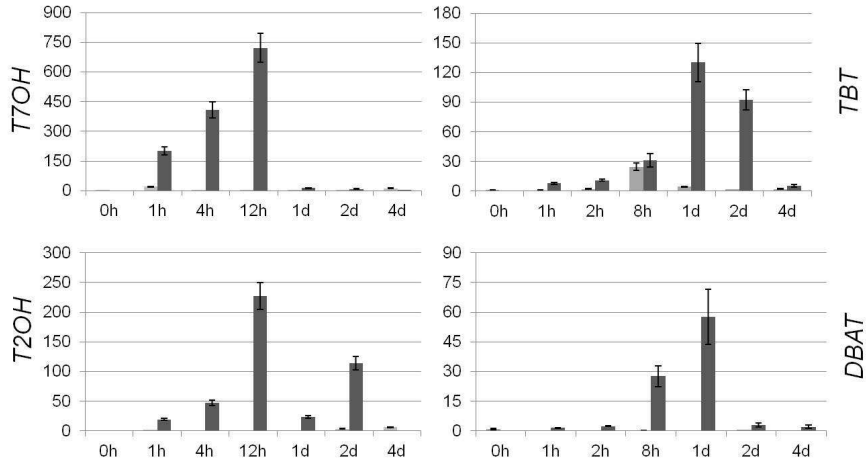


Figure 2.8: Expression levels of the *T7OH, T2OH, TBT and DBAT* genes relative to time zero of the cultures studied. In light grey non-elicited samples, in dark grey MeJ-treated samples.

The expression of the ***TBT*** gene**,** which produces DABIII via an undefined polyhydroxylated intermediate, in unelicited cell suspensions was patent at 8 h of culture, decreasing thereafter until very low levels at the end of the experiment. In the cultures treated with MeJ the accumulation of *TBT* mRNA started at 1 h after elicitation, peaking one day later (130 times the reference value), maintaining high levels for another day and clearly decreasing at day 4. Thus, considering the maximum values in each case, the induction of *TBT* mRNA in the elicited cultures was 5.3 times higher than in the control. Finally, expression of the ***DBAT*** gene was low in unelicited cultures and higher under elicitation, increasing from the first hour and reaching maximum levels at day 1 (58 times the reference value and 100 times the highest expression in control conditions), decreasing thereafter.

The **late steps** are responsible for the phenylpropanol lateral chain formation, its union to baccatin III and the benzoylation of 3'N-debenzoyltaxol to produce the target compound taxol (gene expression represented in Figure 2.9). The highest ***PAM*** gene expression was observed at 12h of MeJ elicitation, when its mRNA accumulation was over 500 times higher than in the control. By day 4 *PAM* transcripts were not present in the elicited cultures. In control conditions *PAM* transcripts were also found but at very low levels. When comparing peak values in elicited and unelicited cultures, it can be observed that the addition of MeJ increased the expression capacity of *PAM* 26-fold. Although the ***BAPT***

gene expression was increased by elicitation, achieving the highest levels at 12h (37 times the reference value), the control cultures also presented high BAPT mRNA accumulation at day 4 (30 times higher than the reference value). At 12 h after elicitation, expression decreased to low levels, increasing thereafter until achieving 13 times the reference value at day 2, before decreasing again at the end of the study. Notably, under elicitation the highest induction of *BAPT* took place three and a half days earlier than in the control cultures.

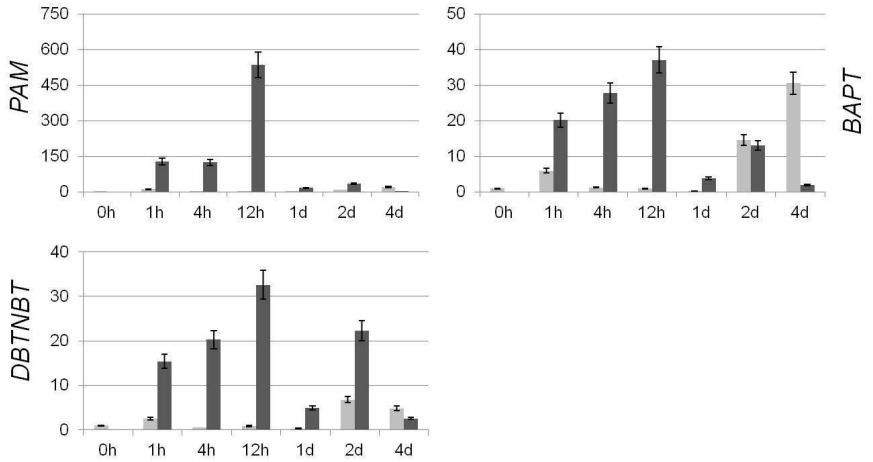


Figure 2.9: Expression levels of the *PAM, BAPT* and *DBTNBT* genes relative to time zero of the cultures studied. In light grey non-elicited samples, in dark grey MeJ-treated samples.

The expression of the last gene implicated in the taxol biosynthesis, ***DBTNBT****,* presented a similar pattern to that of the *BAPT* gene in the elicited cultures, reaching a peak at 12h after elicitation, and another minor peak at day 2. In contrast, the expression of *DBTNBT* in the control cultures was low (6 times the reference value when the transcript accumulation was at its highest). Similar results were obtained by Nims et al. (2006) in elicited *T. cuspidata* cell cultures, although these authors did not find *BAPT* transcripts in control cultures, whereas we observed a significant expression of this gene in unelicited *T. baccata* cell cultures.
The results obtained in this study indicate that the genes *TXS, TAT, TBT, DBAT, BAPT* and *DBTNBT* presented similar expression profiles when analyzed by cDNA-AFLP and qRT-PCR. Moreover, with the exception of *TAT* and *BAPT*, the presence of the elicitor MeJ in the culture medium increased the expression of the genes involved in the taxol biosynthetic pathway, with transcript accumulation peaking in the first hours (12-48 h)

of elicitation. The only genes with a similar expression in both control and elicitation conditions were *TAT a*nd *BAPT*, which suggests they were not responsible for the increase of taxanes observed in the elicited cultures. In contrast, the high expression level observed for the hydroxylase-encoding genes that participate in the conformation of the polyhydroxylated intermediate suggests that these enzymes are not flux-limiting in taxol biosynthesis when cell cultures are elicited with MeJ.

2.4.5. A comparative study of transcriptomic and metabolic profiles

When considering the expression of the studied genes and total taxane production, we can observe that whereas transcript levels peaked soon after elicitation, the highest taxane accumulation took place at day 20. This could be due to slow intermediate biosynthetic steps and the persistence of enzyme activity long after the corresponding mRNAs are not found in the cells.

In this study, the highest levels of DABIII were obtained at day 12 of elicitation, when the baccatin III content had not increased yet. However, while DABIII began to decrease, continuing to do so until the end of the experiment, baccatin III was increasing, reaching a peak at day 20. At this point, baccatin III levels were 14 times higher than the highest content of its immediate precursor. These results suggest that the transformation of DABIII into baccatin III was not a limiting step in the taxol biosynthetic pathway in the *T. baccata* cell line. At the same time, the expression of the *DBAT* gene, which encodes the enzyme catalyzing this metabolic step, was greatly increased by the action of the elicitor MeJ.

In the non-elicited cultures, the transformation of DABIII into baccatin III was also observed, since after reaching a peak at day 12, this first intermediate decreased until the end of the experiment, while baccatin III increased until day 16. However, the low levels of baccatin III accumulated in the control samples could reflect the very low expression level of the *DBAT* gene responsible for the transformation of the precursor.

Since α-phenylalanine is a frequent amino acid in cells, and the expression of the *PAM* gene was very high in the elicited cultures, the formation of the lateral chain is supposed not to be a limiting step in the studied metabolic pathway. Moreover, the earlier increase of *BAPT* under

MeJ treatment could justify a higher and earlier increase of lateral chain-bearing taxanes (taxol, cephalomannine and DAT). The target compound taxol is reached after a specific benzoylation of 3'-*N*-debenzoyltaxol catalyzed by the DBTNBT enzyme. Taxol was found in the elicited cultures at high levels (62 mg/L) 20 days after elicitation, when it was 19 times higher than at day 16 in the control. This could reflect the remarkable increase in *DBTNBT* expression triggered by MeJ-supplementation. The taxane cephalomannine also needs the BAPT enzyme for its formation but not DBTNBT. Since levels of taxol were higher than cephalomannine, it could be inferred that the metabolic steps leading to the latter take place less efficiently than those responsible for taxol formation. Baccatin III and β-phenylalanoylCoA are probably needed for the biosynthesis of DAT, as well as BAPT, which is not a rate-limiting enzyme, as mentioned previously. However, virtually nothing is known about the metabolism of DAT, including whether it is a degradation product of taxol or is formed through a side reaction of the taxol biosynthetic pathway.

Taken as a whole, our results show that MeJ induces a reprogramming of the gene expression in *Taxus* cell cultures, which leads to an enhanced production of taxol and related taxanes. The studied genes involved in the taxane biosynthetic pathway (except *TAT* and *BAPT*) were scarcely expressed under control conditions, which would explain the very low taxane content accumulated in these cultures. In contrast, the elicited cultures showed a notable increase in gene expression, which can generally be correlated with the high taxane production achieved. However, the role of MeJ in the reprogramming of genetic expression is more complex since, as revealed by our cDNA-AFLP study, 103 tags involved in primary metabolism (15.4 % all differentially expressed tags) were also affected by the presence of the elicitor in the cell cultures.

When integrating metabolic and transcriptomic data, it should be taken into account that there was an increase in both biomass and viability in the growth medium, whereas in the production medium there was almost no growth and the viability decreased, especially in elicited cell lines. At the same time, while taxol and baccatin III levels in the growth medium were minimal, they increased in the production medium, especially in elicited lines, with maximum levels obtained in the MeJ cultures. Bearing these observations in mind, all the tags with differential expression in growth and production media can be considered as directly or indirectly related to secondary metabolism. Moreover, as productivity was higher in the elicited lines, all tags with differential expression in control and elicited lines are more likely to be involved in taxane metabolism. As MeJ is an

effective elicitor, those tags induced under MeJ elicitation are expected to play a direct or indirect role in taxane metabolism. The tags with greater expression in the growth medium are probably related with primary metabolism, the cell cycle, its regulation, etc., while tags induced in the production medium are candidates for participation in taxane biosynthesis, the regulation of taxane production, jasmonate signalling pathway, transcription factors related to secondary metabolism, etc.

With this work we have shown that cDNA-AFLP technology is suitable for the analysis of *Taxus baccata* cell cultures. In our comparison of elicited and control conditions, cDNA-AFLP provided a high number of new genes related particularly with secondary metabolism and its regulation, as well as with defence and stress signalling pathways. Although the lack of genomic information in the public database has clearly been a limitation for this research, this technique has proved to be a successful strategy for analysing non-model organisms. The processing of the large quantity of data obtained in this study will probably contribute to a better understanding of the taxol biosynthetic pathway and its regulation. Consequently, once an easy transformation method for *Taxus* cell suspension cultures is established, targeted metabolic engineering of *Taxus* can be attempted to develop superior cell lines for use in bioprocesses to synthesize and supply this anti-cancerigen compound.

2.5. Acknowledgements

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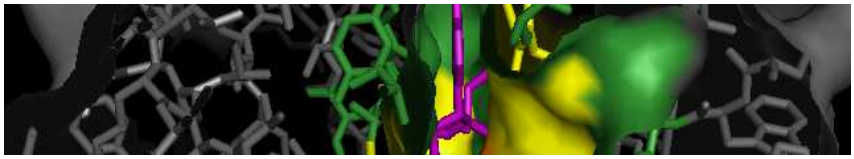


AN *IN SILICO* APPROACH TO ENRICH THE POOL OF CANDIDATE GENES FOR THE UNKNOWN STEPS OF TAXOL BIOSYNTHESIS

3. AN *IN SILICO* APPROACH TO ENRICH THE POOL OF CANDIDATE GENES FOR THE UNKNOWN STEPS OF TAXOL BIOSYNTHESIS

3.1. Abstract

As described in the previous Chapter, with the aim of acquiring a deeper molecular understanding of how the different factors that improve taxane biosynthesis and accumulation affect the metabolic profiles and genetic expression in *Taxus baccata* cell cultures, we applied cDNA-amplified fragment length polymorphism (AFLP) technology to identify genes involved in taxol biosynthesis. Having obtained several candidate genes for the undefined taxol biosynthetic steps, we submitted them to an *in silico* study to highlight those most likely to correspond to these unknown steps. This approach was based on determining only the positions of the candidate enzymes that could be directly involved in the enzymatic action and evaluating the similarity between our candidates and known proteins with the same function that act on the same or similar substrates. Several tools, including protein data bases (MACiE, CSA, PDB, Uniprot), T-Coffee, a multiple sequence alignment software and PyMOL, a graphical resource, were used for the *in silico* approach. Out of 15 candidates for hydroxylases (at C1, C9 on the taxane core and C2 on the lateral chain), the oxidase at the C9 position, the epoxidase and oxomutase of the oxetane ring (D-ring) formation, and also the CoA transferase, we highlighted 3 candidates most likely to be involved in taxol biosynthesis as a hydroxylase, epoxidase and CoA transferase.

3.2. Introduction

The genus *Taxus* has generated considerable interest due to its content of complex diterpene alkaloids, particularly the generic drug paclitaxel or taxol, its trade name registered by Bristol-Myers Squibb. Taxol is an alkaloid with powerful antitumor activity, approved for the treatment of refractory metastatic ovarian cancer, metastatic breast cancer, non-small cell lung cancer and AIDS-related Kaposi's sarcoma (Bristol-Myers Squibb, 2007).

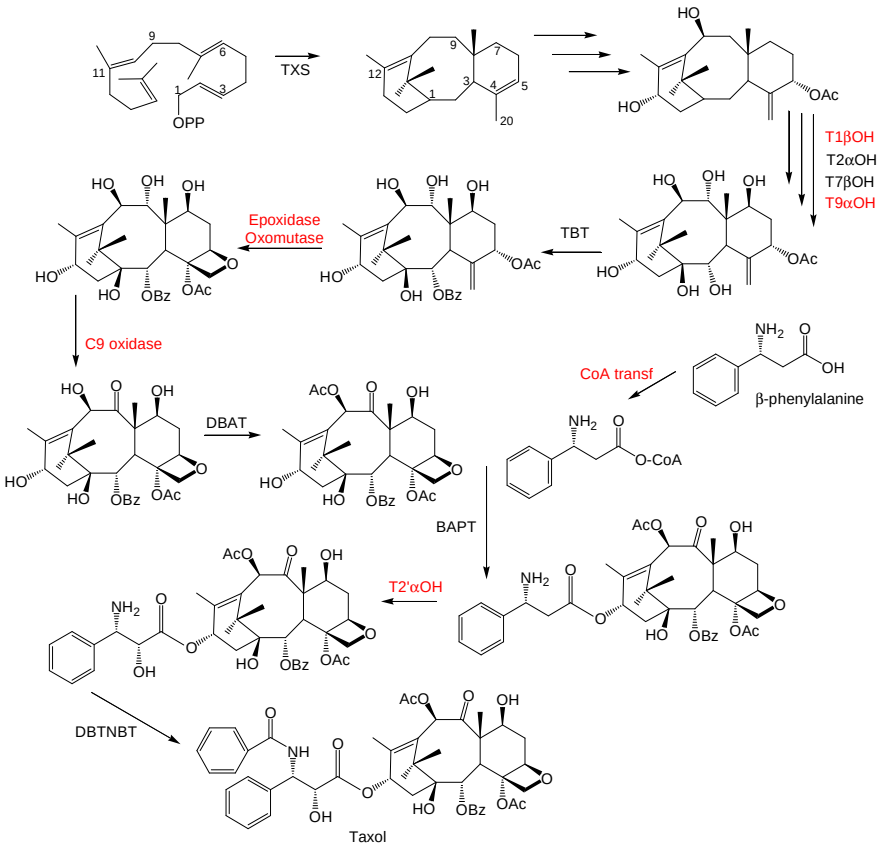


Figure 3.1: Postulated taxol biosynthesis from geranylgeranyl diphosphate. The multiple arrows indicate multiple steps. The unknown genes and corresponding protein are in red. TXS, Taxadiene synthase; T1βOH, Taxane 1β-hydroxylase; T2αOH, Taxane 2α-hydroxylase; T7βOH, Taxane 7β-hydroxylase; T9αOH, Taxane 9α-hydroxylase; TBT, taxane-2α-O-benzoyl transferase; DBAT, 10-deacetylbaccatin III-10-*O*-acetyltransferase; CoA transf., CoA transferase; BAPT, C-13-phenylpropanoyl-CoA transferase; T2'αOH, Taxane 2'α-hydroxylase; DBTNBT, Debenzoyl taxol *N*-benzoyl transferase.

The biosynthesis of paclitaxel has not been completely elucidated (Figure 3.1). As described in Chapter 1, it is believed that 19 enzymes are involved, for which 12 genes and corresponding proteins are known, and 7 steps have been postulated according to the known intermediates and the modified positions in the taxol molecule (last reviews Croteau et al., 2006 and Vongpaseuth and Roberts, 2007). Once GGPP has been cyclized into taxadiene, this intermediate is hydroxylated at C5. In the subsequent pathway to 10-deacetylbaccatin III the order of steps and proteins involved is uncertain, but has been postulated as follows

(Croteau et al., 2006): there is a first bifurcation of the pathway, with C13 hydroxylation on one side and C5 acetylation on the other. After the C5 acetylation there is a C10 hydroxylation, followed by an active taxoid-14β-hydroxylase that yields to an alternative pathway. Once taxane-5,10,13-ol acetate is obtained, it is hydroxylated at C1, C2, C7 and C9, then acetyl/benzoyl groups are added at C2 and C4, an oxetane ring (D-ring) is formed and finally there is a C9 oxidation. An acetyl group is then bound to C10 of 10-deacetyl baccatin III, yielding baccatin III. The remaining step to reach taxol is the addition of the lateral chain. A β-phenylalanoyl-CoA is bound to C13 from the taxane core, hydroxylated at C2 and *N*-benzoylated at C3 (Figure 3.1). The missing genes and proteins from the pathway correspond to hydroxylases at C1 and C9 in the main core and C2 on the lateral chain, a C9 oxidase, the enzymes involved in the formation of ring D, probably an epoxidase and an oxomutase, and the CoA ligase for activation of the lateral chain.

We performed a transcript profiling analysis of *Taxus baccata* (European or English yew) by applying cDNA-amplified fragment-length polymorphism (cDNA-AFLP) combined with the metabolic profiling of elicited *Taxus* cell cultures, which yielded a collection of known and previously undescribed fragments of transcribed genes or temporarily assigned genes (tags) (Chapter 2). Transcriptome analysis suggested an extensive jasmonate-mediated genetic reprogramming of metabolism, which correlated well with the observed shifts in the biosynthesis of taxanes. This study provided the basis for a better understanding of yew secondary metabolism and gave a huge amount of potential tags for those steps of taxol biosynthesis without assigned genes and proteins (Table 3.1).

With the aim of choosing real candidates for specific positions (in Figure 3.1, steps in red) from those obtained in the cDNA-AFLP assay (described in Chapter 2, and summed up in Table 3.1) and discarding others, a common strategy was followed based on carrying out functional alignments. According to Kemena and Notredame (2009), a multiple sequence alignment (MSA) is a way of organizing data where similar sequence features are aligned together, understanding a feature to be any relevant biological information, such as structure or function. The correct alignment should be defined by the chosen features and the way in which these features are described. At the end, in a functionally correct alignment, residues with the same function need to be aligned, even if the origin of their similarity is convergent evolution.

Table 3.1: Complete list of candidates grouped according to their possible function in the taxol biosynthetic pathway (possible function), tag name from the cDNA-AFLP analysis (TAG), best hit for the complete sequenced gene (TAG homology), Swiss-Prot accession number from the best hit (Prot. acc. code) and e-value with that hit.

Possible function	TAG	TAG homology	Prot. Acc. code	e-value
CoA transferase	TB768	Acyl-Coa ligase	B9GQ39	0.0
C1&C9 hydroxylases	TB506	Cinnamate 4-hydroxylase	B2Z6P5	0.0
	TB331	Putative cytochrome P450	D7T4D5	1.0E-118
	TB574	Putative cytochrome P450	D7T4D5	1.0E-118
	TB224	Putative cytochrome P450	D5A7Y6	1.0E-152
C4-C20 epoxidase	TB328	Putative gibberellin 20-oxidase	A9NW35	1.0E-45
C9 oxidase	TB596	Unknown (FAD/FMN-containing dehydrogenase)	B8LR72	8.0E-87
	TB812	Unknown (GMC oxidoreductase)	A9NXU5	1.0E-151
	TB047	Unknown (GMC oxidoreductase)	A9NXU5	1.0E-109
	TB739	Unknown (GMC oxidoreductase)	A9NXU5	1.0E-148
	TB212	Unknown (Short-chain alcohol dehydrogenase)	A9NU86	6.0E-91
	TB645	Unknown (GMC oxidoreductase)	A9NXU5	1.0E-158
Oxomutase	TB493	Putative Carboxylic ester hydrolase	A9SVY3	1.0E-44
	TB195	Hypothetical protein (Glyoxal oxidase-related)	F6GTQ6	1.0E-164
	TB115	Hypothetical protein (Galactose oxidase)	D8RR64	1.0E-163

The candidates were analyzed in groups according to their possible function (first column in Table 3.1). For each function, we first searched for a model protein with a PDB (protein data bank) accession number, since such previously crystallized molecules have available structures (pdb file). Among the several proteins with a PDB entry, we chose those most homologous to our candidates. When necessary, we improved the MSA by adding “intermediate” sequences to the alignment of the candidates and the model protein (with PDB entry). Enzymes of the same functional class, acting on the same or similar substrate, were also added to the alignment. Simultaneously, amino acids related with the active site and involved in binding cofactors, atoms or other molecules obtained from experimental data were mapped in the 3D structure of the model protein. Subsequently, amino acids surrounding the active site were also selected. Finally, all these amino acids were localized in the MSA of the candidates, the model protein and the other sequences. The different sequences were

weighed only according to the similarity with the selected amino acids, allowing us to compare our candidates with enzymes acting on similar substrates, and to highlight the best candidate for each position or rule out those with least potential to be the expected genes. A flow chart of the procedure followed in this study is depicted in Figure 3.2.

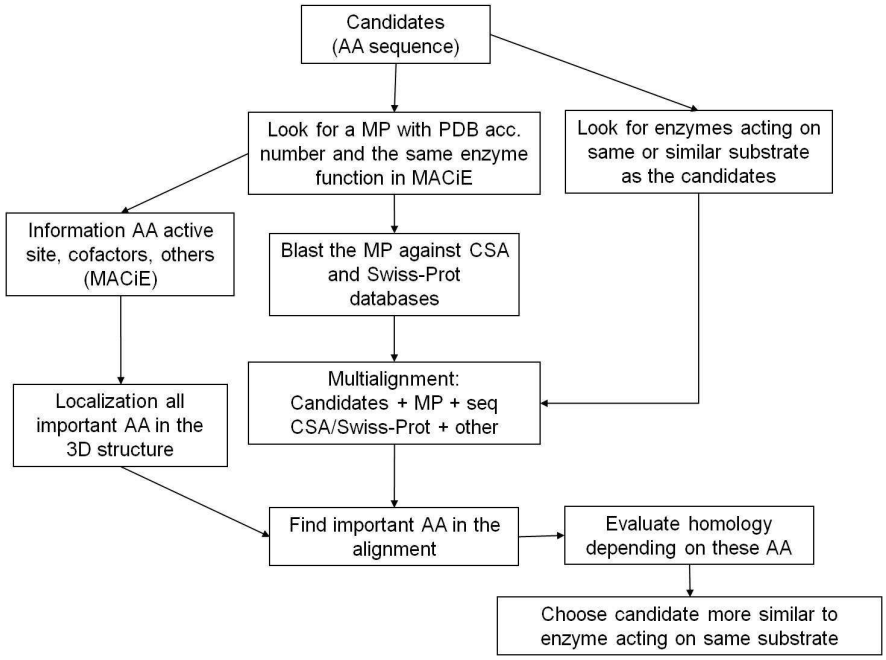


Figure 3.2: A flow chart of the procedure followed in the search for candidate enzymes for the unknown steps of taxol biosynthesis. MP: model protein.

3.3. Material and methods

For this study several candidate genes for several unknown positions were previously selected in the cDNA-AFLP assay described in Chapter 2 (Table 3.1). To determine which candidates should be selected or discarded, our approach was based on picking up the AA most directly related to the enzymatic function and assessing the similarity between the candidates and enzymes with the desired function that act on the same, similar or different substrate at a tridimensional level. The first task was to obtain sequences from public data bases, carry out multiple alignments,

find the AA surrounding the active site in the 3D structure and finally evaluate the similarity between our candidates and known enzymes according to the AA selected as relevant. The relevant AA should include those involved in the enzyme function, such as AA implicated in the active site, AA related to other binding molecules and AA surrounding the active site (flow chart in Figure 3.2).

3.3.1. Databases

The resources used in this approach were the MACiE (Mechanism, Annotation and Classification in Enzymes), CSA (Catalytic Site Atlas), PDB (Protein Data Bank) and Uniprot data bases. In MACiE (http://www.ebi.ac.uk/thornton-srv/databases/MACiE/) proteins are annotated manually, including their structure (pdb file) and experimental data on the amino acids required in the protein function, not only forming the active site, but also involved in the cofactors and other atoms and binding molecules (Holliday et al., 2005 and 2007). CSA (http://www.ebi.ac.uk/thornton-srv/databases/CSA/) contains hand-annotated as well as homologous entries found by PSI-BLAST alignment and a low e-value cut-off of $5x10^{-5}$ (Craig et al., 2004). In the PDB data base (http://www.wwpdb.org/) experimentally-determined structures of proteins, nucleic acids, and complex assemblies can be found in pdb format (Berman et al., 2003). Finally, the Uniprot web page (http://www.uniprot.org/) was also used as it contains a huge data set of entries belonging to the Swiss-Prot and TrEMBL data bases. The former includes hand-annotated proteins, some of them in PDB format, and information related to the catalytic site, while the latter is an automatically annotated and unreviewed protein data base (Apweiler et al., 2004).

For the domain analysis of our candidates we used the Conserved domains database from the NCBI webpage (http://www.ncbi.nlm.nih.gov/-Structure/cdd/cdd.shtml) (Marchler-Bauer et al., 2009 and 2011).

3.3.2. Multiple alignment and alignment manipulation

The multiple sequence alignment (MSA) was carried out with T-Coffee (v7.38) (Notredam et al., 2000), a MSA method that uses global and local pairwise alignment methods. The M-Coffee utility from the T-Coffee package is a meta-method for assembling MSA by combining the output

of several individual aligning methods (ClustalW, T-Coffee, ProbCons, PCMA, Muscle, Dialign-T, MAFFT and POA). T-Coffee compiles libraries previously generated by these aligning programs for producing a final improved MSA (Wallace et al., 2007).

For localizing and mapping in the multiple alignments, and selecting and weighing the already determined important residues for the enzyme function, the “seq_reformat” utilities from the T-Coffee package were used.

3.3.3. Graphics tool

For the 3D study a well-developed molecular graphics tool was used. PyMOL is an open-source molecular modelling package available at http://www.pymol.org/

3.4. Results and discussion

3.4.1. Previously

As described in Material and Methods of the previous Chapter, we grew a cell suspension culture of *Taxus baccata* in two conditions, with and without methyl jasmonate, and we performed a cDNA-AFLP transcript profiling study. From the transcript profile we selected the transcript assigned genes (tags) expressed differentially in the two conditions or along the culture period. From the 858 differentially expressed tags, we were able to sequence 663, of which 309 were homologues to known genes and 60 to genes without allocated functions (392 hits). Considering the functions of the remaining unidentified steps of taxol biosynthesis (hydroxylases, an oxidase, an epoxidase, an oxomutase and a CoA ligase), we selected tags with hits to similar functional proteins for sequencing. Once they were cloned, we determined *in silico* our best candidates for each unknown step.

3.4.2. Bioinformatics study of candidates for hydroxylases

In the cDNA-AFLP analysis 19 tags with homology to cytochrome P450, hydroxylases and monooxygenases were selected. From these, 15 were discarded, due to redundancy, low induction under MeJ elicitation or because it was impossible to completely clone them. Two of the selected tags were TB574 and TB331, which showed homology to each other, cytochrome P450 and taxane hydroxylases, but not enough to be identified as known taxol biosynthetic hydroxylases. The other two selected tags corresponded to TB224 and TB506, with homology to a cytochrome P450 and a Cinnamate 4-hydroxylase (Table 3.1). Thus, TB224, TB331, TB506 and TB574 were the candidates for the unknown taxol biosynthetic hydroxylases, the T1OH and T9OH from the main core and T2OH from the lateral chain.

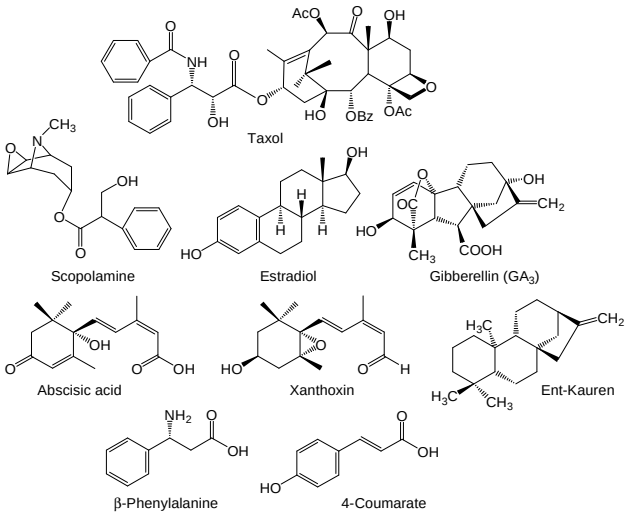


Figure 3.3: Substrates of different enzymes used in the alignments for improving multiple alignments and enzymes acting on the substrates most similar to taxol. Other structures related with this approach are also represented.

Among all the MACiE hydroxylases, the protein with highest homology to the candidates was camphor 5-monooxygenase (1yrc -PDB accession number- and P00183 –Swiss-Prot accession number-). The average homology among the candidates using M-Coffee had a score of 61, which decreased to 49 once the 1yrc was added. When the proteins listed in Table 3.2 were also used to improve the alignment, the score increased to 69. For the unknown hydroxylases two taxol hydroxylases were included

in the alignment: T10OH from *Taxus* x *media* (Q6SPR0) and T13OH from *T. cuspidata* (Q8W4T9). Also, two hydroxylases acting on a similar substrate to taxol, abscisic acid (ABA) (O81077) and gibberellin (GA_3) (Q9C5Y2) (both terpenes), were added (Figure 3.3).

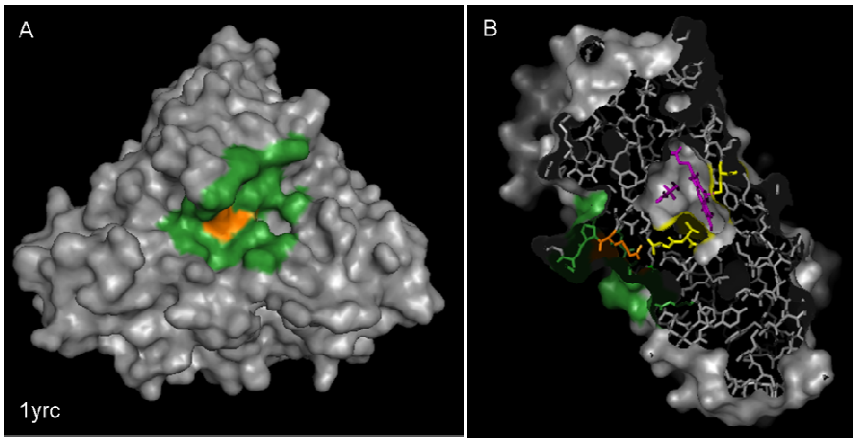


Figure 3.4: PyMOL image of 1yrc with important AA coloured. A) Complete view from one side with smooth surface. B) Section of the protein, with the interior in sticks and smooth surface. Grey: AA discarded for the evaluation; Orange: AA related with the active site; yellow: AA related with active site and/or Cofactor binding; violet: cofactor; green: AA surrounding the active site.

According to the MACiE database, the amino acids involved in the 1yrc function are R186, related with the active site (in orange, Figure 3.4) and D251, T252, C357, L358, and G359, related with the active site and involved in binding cofactors (in yellow, Figure 3.4). Once localized in the 3D structure, these 6 AA (Figure 3.4), Y29, E156, P175, K178, Y179, D182, P187, D188, K392, S393 and Q400, were chosen for their position surrounding the active site (in green, Figure 3.4). Since experimental data about the AA sequences involved in the function of T10OH and T13OH were not available, these could not be used in the next step. When all the important AA were described (Figure 3.4), they were localized in the MSA carried out with M-Coffee, and the position was selected and weighed (Box 3.1A and B).

Table 3.2: Proteins related to the hydroxylase study, indicating the sequence source (MACiE, Swiss-Prot, CSA, TrEMBL), protein name, organism, enzyme class (E.C.) code, protein length and AA with binding function, the file format (3D structure -pdb- or aminoacid sequence -fasta-) and the reason for selection (3D structure, alignment improvement or acting on a similar substrate).

Uniprot access code	Data base	protein name	Organism	E.C. code	AA Seq length	Binding AA	Bind to	pdb	Selection
P00183-1yrc	MACiE	Camphor 5-monooxygenase	*Pseudomonas putida*	1.14.15.1	414	358	iron	pdb	structure
Q9V4T5	Swiss-Prot	Probable cytochrome P450	*Drosophila melanogaster*	1.14.-.-	531	444	iron	fasta	alignment
						307	heme	fasta	
P14762	Swiss-Prot	Cytochrome P450(BM-1)	*Bacillus megaterium*	1.14.14.-	410	356	iron	fasta	alignment
P33006	Swiss-Prot	Cytochrome P450-terp	*Pseudomonas sp.*	1.14.-.-	428	377	iron	fasta	alignment
P46373	Swiss-Prot	Cytochrome P450 FAS1	*Rhodococcus fascians*	1.14.-.-	399	349	iron	fasta	alignment
P48635	Swiss-Prot	Erythromycin B/D C-12 hydroxylase	*Saccharopolyspora erythraea*	1.14.-.-	397	339	iron	fasta	alignment
Q59831	Swiss-Prot	Cytochrome P450 105A3	*Streptomyces carbophilus*	1.14.-.-	410	359	iron	fasta	alignment
Q64410	Swiss-Prot	Steroid 17-alpha-hydroxylase/17,20 lyase	*Cavia porcellus*	1.14.99.9	508	442	iron	fasta	alignment
P15540	Swiss-Prot	Steroid 21-hydroxylase	*Sus scrofa*	1.14.99.10	492	427	iron	fasta	alignment
						341-357	steroid	fasta	
O81077_ABA	Swiss-Prot	Abscisic acid 8'-hydroxylase 2	*Arabidopsis thaliana*	1.14.13.93	482	431	iron	fasta	substrate
Q9C5Y2_GA3	Swiss-Prot	Ent-kaurenoic acid oxidase 2	*Arabidopsis thaliana*	1.14.13.79	489	436	iron	fasta	substrate
Q8W4T9_T13OH	Swiss-Prot	Taxane 13-alpha-hydroxylase	*Taxus cuspidata*	1.14.13.77	485	431	iron	fasta	substrate
Q6SPR0_T10OH	TrEMBL	5-alpha-taxadienol-10-beta-hydroxylase	*Taxus* x *media*	---	497	---	---	fasta	substrate

A

```
P14762                -SYKKDQPY-----ETCLGLTT
P33006                -LPLKQGVHEP-DEDTCLG-TK
P46373                -LDRDAD-------ETCLGRSE
P48635                -FEGDGDIQM----ITCLGF-R
P00183_1yrc           YEPKYDRPD-----DTCLGKSQ
Q59831                -LEQDKQAV-----ETCLGRPN
Q64410                GMERRTNSLSD--DETCVGEGD
P15540                -VHHDQKTWEHWSIETCLGQPK
O81077_ABA            GRD--QRGYNSMPLDTCPGGPK
Q9C5Y2_GA3            GKD--TYGVRAMGIESCPGLPD
Q8W4T9_T13OH_TCUS     GDE--EVGSFSVPLDSCPGKPN
Q6SPR0_T10OH_TMED     GGK--EVGSLSVPLDTCPGDPN
TB224_C1C9            GRSQSADLASHLS-DTCLGIPN
TB331_C1C9            GKE--NKGIWSVPLDTCPGDPM
TB506_C1C9            GLQAQEGDFIPILRETCPGSEA
TB574_C1C9            GQQ--IKAIWSVPLDTCPGEPK
                                    :* *
```

B

Candidate	Compared to	Weight (%)
TB224	Q59831	43.75
TB331	Q6SPR0_T10OH	65.00
	TB574_C1C9	70.00
TB506	P46373	35.71
TB574	Q6SPR0_T10OH	55.00
	O81077_ABA	55.00
	TB331_C1C9	70.00

Box 3.1: After the multiple alignment, the previously determined residues involved in the enzyme function (Figure 3.4) were picked up (Box 3.1A) and the similarities were weighed only according to those residues. Box 3.1B shows the best matches for each candidate. When the best match was another candidate, the second best match is also shown. If there is more than 1 match with the same weight, all of them are summed up in the box.

If the M-Coffee MSA also included T2OH from *T. canadensis* (Q6JD68), T10OH from *T.* x *media* (Q6SPR0), T5OH (Q6WG30), T7OH (Q6JTJ0), T10OH (Q9AXM6), T13OH (Q8W4T9) and T14OH (Q84KI1) from *T. cuspidata*, we observed that the same enzyme in two different species, T10OH from *T.* x *media* and *T. cuspidata*, showed 100% homology (Figure 3.5). When we compared enzymes active at different positions, the similarities ranged from 67% to 40%. The most similar hydroxylases were T13OH and T10OH (independently of species) with 66.67% of similarity, while the most different was T7OH compared to T10OH, T2OH and T14Oh with 40.74% of resemblance. T2OH compared with T14OH also showed this low similarity (Figure 3.5). T10OH and T13OH should be very similar as these two enzymes modify the taxane core in the initial steps, acting on taxadiene-5α-ol and taxadiene-5yl acetate, using the product from the other and finally giving taxadiene-5α,10β-13α-ol acetate (Figure 3.1). T7OH and T2OH act on a polyhydroxylated taxane core and should be very different from T5OH, T10OH, T13OH or T14OH, but not from each other, as Jennewein et al. (2004) found. T13OH was the hydroxylase with the highest similarity with the rest. Bearing in mind that the pathway from taxadiene-5α-ol has not been completely elucidated, this could mean that T13OH uses several taxanes, suggesting that this enzyme is not very selective for its substrate and that it might be able to act on differently modified taxanes.

When a multiple alignment includes a high percentage of similar enzymes, such as when 7 hydroxylases from yew are used, this group of enzymes

has too much weight on the alignment, determining and forcing the alignment itself and modifying the similarity values. The % of homology changed when only hydroxylase pairs were included, the values being about 10% higher: 75% homology for the T10OH-T13OH pair, 68% homology for T2OH-T5OH and 52.63% homology for T2OH-T14OH. Even in the case of T7OH-T14OH they were 20% higher, with 60% homology.

The homology between the known hydroxylases ranged from 67% to 40%, the overall mean of similarity being 51.61%. Based on the evaluated positions (Figure 3.4 and Box 3.1), we were already able to discard the candidates TB506 and TB224 for one of the unknown hydroxylases, as their highest homology was 35.71% to Cytochrome P450 FAS1 (P46373), involved in cytokinin biosynthesis, and 43.75% to Cytochrome P450 105A3 (Q59831), involved in pravastatin biosynthesis. On the other hand, TB574 presented the highest homology to TB331 (70%) followed by T10OH and Abscisic acid 8'-hydroxylase 2 (O81077) (55%). The highest homology shown by TB331 was to TB574 (70%) and T10OH (65%). As TB331 and TB574 showed the most similarity to an enzyme of taxol biosynthesis, with a homology of 40-67%, we can conclude that these are candidates for one of the unknown hydroxylases, but neither T10OH nor T13OH.

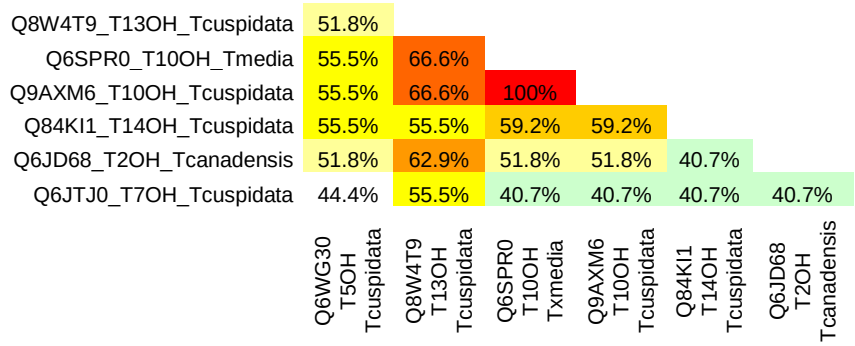


Figure 3.5: Homology between all known hydroxylases of yew involved in taxol biosynthesis. Hydroxylases corresponding to the different positions with their protein code, enzyme name and organism.

When the 7 hydroxylases from taxol biosynthesis were used in the multiple alignment, all together in the same alignment or aligned by pairs, TB331 showed a constant similarity to these hydroxylases, with a homology range of 37.04-62.96% in the first case and 53-65% in the second. This suggests that TB331 could be an unknown hydroxylase in taxol biosynthesis, but not a known one. In the case of TB574, its

homology to the known hydroxylases was 37.04-48.15% in the first case (all hydroxylases aligned together) and 46.67-66.67% in the second case (hydroxylases aligned by pairs). In both kinds of alignments, TB331 was always the first hit, but the second hit was Abscisic acid 8'-hydroxylase (O81077), T10OH or T14OH. With these data, TB331 stands out as a possible candidate, while TB574 is more likely to be related to ABA metabolism.

3.4.3. Bioinformatics study of candidates for C9 oxidase

From the cDNA-AFLP transcript analysis (Chapter 2), 23 tags with homology to oxidoreductases, dehydrogenases and lyases were picked up. Due to redundancy, low induction under MeJ elicitation and impossibility of sequencing, only 6 of those were selected for the determination of the unknown oxidase. The final sequenced tag candidates for the C9 oxidase were TB047, TB212, TB596, TB645, TB739 and TB812 (Table 3.1).

When the candidates were aligned together, there was little agreement between the different M-Coffee methods in the TB212 and TB596 alignment. Moreover, there was a low score for the alignment of TB047, TB645, TB739 and TB812 with the other two (TB212 and TB596), neither of which could be aligned with each other. Consequently, the analysis of the C9 oxidase was done separately: TB212 and TB596 were analysed individually, while TB047, TB645, TB739 and TB812 were analysed in a group.

3.4.3.i. C9 oxidase, TB212

The highest homology to a known protein shown by TB212 was to a short-chain alcohol dehydrogenase (Q9SCU0). Two model proteins were chosen for this analysis: an alcohol dehydrogenase (1mg5-P00334) and a Rhizome secoisolariciresinol dehydrogenase (2bgm-Q94KL8). Both had a short-chain dehydrogenase/reductase (SDR) domain and a high score when aligned with TB212. Although the alignments scored well, other proteins (listed in Table 3.3) were added in order to compare our candidate with enzymes acting on a similar substrate, including dehydrogenases acting on xanthoxin (Q9C826), estradiol (Q5TJF5) or steroid intermediates (P50200) (structures in Figure 3.3).

According to previously obtained experimental data from MACiE and Uniprot, the chosen AA for analysing 1mg5, and consequently our candidate, were S140, due to its substrate-binding function (in orange, Figure 3.6), and D65 and K157 as cofactor-binding (in yellow, Figure 3.6). G15, G130, G133, Y153, and G184 had also been determined experimentally for their relevance in maintaining the function and/or activity of the enzyme (in blue, Figure 3.6). Once all these AA were localized in the 3D structure, the AA surrounding the active site, as well as the cofactor binding surface, were selected: G139, V141, T142, I147, V150, P183, I185 and L190 (in green, Figure 3.6). All these AA were picked up from the multiple alignment and weighed according to their similarity. TB212 showed 78.57% homology to Rhizome secoisolariciresinol dehydrogenase (2bgm) and 73.33 % to a Sex determination protein tasselseed-2 (P50160). This overly high homology to the residues implicated in the function allowed us to discard TB212 as the missing oxidase for C9.

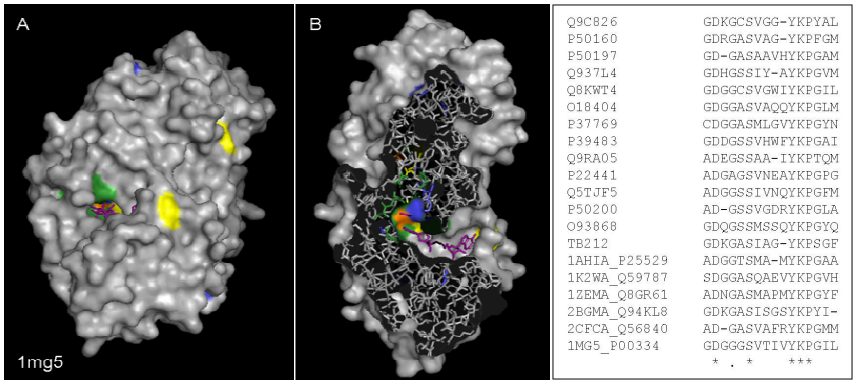


Figure 3.6: PyMOL image of 1mg5 with important AA coloured. A) Complete view from one side with smooth surface. B) Section of the protein, with the interior in sticks. Grey: AA discarded for the evaluation; Orange: AA related with the active site; yellow: AA related with active site and/or Cofactor binding; violet: cofactor; blue: experimentally proven AA important for maintaining the function of 1mg5; green: AA surrounding the active site.

Box 3.2: After the multiple alignment, the previously determined residues involved in the enzyme function (Figure 3.6) were picked up (Box 3.2) and the similarities were weighed only according to these residues

Table 3.3: All proteins used in the TB212 MSA for ruling out or picking up the candidate for this oxidase. Indicated are the sequence source (MACiE, Swiss-Prot, CSA, TrEMBL), protein name, organism, enzyme class (E.C.) code, protein length and AA with binding function, file format (3D structure -pdb- or aminoacid sequence -fasta-) and the reason for being selected (3D structure, alignment improvement or acting on a similar substrate).

Uniprot access code	Data base	Protein name	Organism	E.C. code	AA Seq length	Sites	Bind to	file format	Selection
2BGMA_Q94KL8	CSA / TrEMBL	Rhizome secoisolariciresinol dehydrogenase	*Podophyllum peltatum*	---	278	26-28	NAD	pdb	structure
P00334_1mg5	MACiE	Alcohol dehydrogenase	*Drosophila melanogaster*	1.1.1.1	256	65,157	NAD	pdb	structure
Q9C826	Swiss-Prot	Xanthoxin dehydrogenase	*Arabidopsis thaliana*	1.1.1.288	285	---	NAD	fasta	substrate
P50160	Swiss-Prot	Sex determination protein tasselseed-2	*Zea mays*	---	336	59-83	NAD/NADP	fasta	alignment
P50197	Swiss-Prot	2,5-dichloro-2,5-cyclohexadiene-1,4-diol dehydrogenase	*Pseudomonas paucimobilis*	1.1.1.-	250	---	NAD	fasta	alignment
Q937L4	Swiss-Prot	Cyclopentanol dehydrogenase	*Comamonas testosteroni*	1.1.1.163	250	10-34	NAD	fasta	alignment
Q8KWT4	Swiss-Prot	Bacilysin biosynthesis oxidoreductase bacC	*Bacillus subtilis*	1.-.-.-	253	9-31	NAD/NADP	fasta	alignment
O18404	Swiss-Prot	3-hydroxyacyl-CoA dehydrogenase type-2	*Drosophila melanogaster*	1.1.1.35	255	6-31	NAD	fasta	alignment
P37769	Swiss-Prot	2-deoxy-D-gluconate 3-dehydrogenase	*Escherichia coli*	1.1.1.127	253	14-38	NAD	fasta	alignment
P39483	Swiss-Prot	Glucose 1-dehydrogenase	*Bacillus megaterium*	1.1.1.47	261	11-35	NADP/NAD	fasta	alignment
Q9RA05	Swiss-Prot	(-)-trans-carveol dehydrogenase	*Rhodococcus erythropolis*	1.1.1.n4	277	10-32	NAD/NADP	fasta	alignment
P22441	Swiss-Prot	N-acylmannosamine 1-dehydrogenase	*Flavobacterium sp.*	1.1.1.233	271	20-44	NAD	fasta	alignment
Q5TJF5	Swiss-Prot	Estradiol 17-beta-dehydrogenase 8	*Canis familiaris*	1.1.1.62	259	---	NAD	fasta	substrate
P50200	Swiss-Prot	NADP-dependent 7-alpha-hydroxysteroid dehydrogenase	*Clostridium sordellii*	1.1.1.-	267	10-34	NADP	fasta	substrate
O93868_1h5q	Swiss-Prot/PDB	NADP-dependent mannitol dehydrogenase	*Agaricus bisporus*	1.1.1.138	262	19-44 150-174	NADP	fasta	alignment
1AHIA_P25529	CSA/Swiss-Prot	7-alpha-hydroxysteroid dehydrogenase	*Escherichia coli*	1.1.1.159	255	18-42	NAD	fasta	substrate
1K2WA_Q59787	CSA/Swiss-Prot	Sorbitol dehydrogenase	*Rhodobacter sphaeroides*	1.1.1.14	256	5-34	NAD	fasta	alignment
1ZEMA_Q8GR61	CSA/TrEMBL	Xylitol dehydrogenase	*Gluconobacter oxydans*	1.1.1.9	262		NAD	fasta	alignment
2CFCA_Q56840	CSA/Swiss-Prot	2-(R)-hydroxypropyl-CoM dehydrogenase	*Xanthobacter autotrophicus*	1.1.1.268	250	---	NAD	fasta	alignment

Table 3.4: All enzymes used in the C9 oxidase multiple alignment for ruling out or selecting one of the 4 candidates, TB047, TB645, TB739 and TB812. Indicated are the sequence source (MACiE, Swiss-Prot, CSA, TrEMBL), protein name, organism, enzyme class (E.C.) code, protein length and AA with binding function, file format (3D structure -pdb- or aminoacid sequence -fasta-) and the reason for selection (3D structure, alignment improvement or acting on a similar substrate).

Uniprot access code	Database	Protein name	Organism	E.C. code	AA Seq length	Site	Bind to	file format	Importance
2jbv-Q7X2H8	CSA/TrEMBL	Choline oxidase	*Arthrobacter globiformis*	1.1.3.17	546	44, 71, 232, 465, 500	FAD	pdb	3D structure
1gpe-P81156	CSA/Swiss-Prot	Glucose oxidase	*Penicillium amagasakiense*	1.1.3.4	587	26-55	FAD	pdb	3D structure
1ju2-Q945K2	PDB/TrEMBL	Hydroxynitrile lyase	*Prunus dulcis*	4.1.2.10	563	129, 133, 244, 514	FAD	fasta	alignment
						198	Mannose		
1kdg-Q01738	PDB/Swiss-Prot	Cellobiose dehydrogenase	*Phanerochaete chrysosporium*	1.1.99.18	773	83, 181	Iron	fasta	alignment
P52707	PDB/Swiss-Prot	(R)-mandelonitrile lyase 3	*Prunus serotina*	4.1.2.10	573	55-82	FAD	fasta	alignment
Q9S746	PDB/Swiss-Prot	HOTHEAD	*Arabidopsis thaliana*	---	594	---	FAD	fasta	alignment
P18172	PDB/Swiss-Prot	Glucose dehydrogenase	*Drosophila pseudoobscura*	1.1.99.10	625	66-95	FAD	fasta	alignment
Q8UH55	PDB/Swiss-Prot	Choline dehydrogenase	*Agrobacterium tumefaciens*	1.1.99.1	549	---	FAD	fasta	alignment
Q00593	PDB/Swiss-Prot	Alcohol dehydrogenase	*Pseudomonas oleovorans*	1.1.99.-	558	---	FAD	fasta	alignment
P46371	PDB/Swiss-Prot	Uncharacterized GMC oxidoreductase	*Rhodococcus erythropolis*	1.1.-.-	493	---	FAD	fasta	alignment
Q47944	PDB/Swiss-Prot	L-sorbose 1-dehydrogenase	*Gluconobacter oxydans*	1.1.99.32	531	---	FAD	fasta	alignment
Q92452	PDB/Swiss-Prot	Glucose oxidase	*Talaromyces flavus*	1.1.3.4	605	---	FAD	fasta	alignment
Q9AJD6	PDB/Swiss-Prot	Pyridoxine 4-oxidase	*Microbacterium luteolum*	1.1.3.12	507	---	FAD	fasta	alignment

Table 3.5: Proteins used in the epoxidase analysis. Indicated are the sequence source (MACiE, Swiss-Prot, CSA, TrEMBL), protein name, organism, enzyme class (E.C.) code, protein length and AA with binding function, file format (3D structure -pdb- or aminoacid sequence -fasta-) and the reason for being selected (3D structure, alignment improvement or acting on a similar substrate).

Uniprot access code	Database	Protein name	Organism	E.C. code	AA Seq length	Site	Bind to	file format	Selection
2brt_Q0WWD6	CSA/TrEMBL	Leucoanthocyanidin dioxygenase	*Arabidopsis thaliana*	1.14.11.19	356	---	Iron	pdb	structure
1wa6_Q08506	CSA/Swiss-Prot	1-aminocyclopropane-1-carboxylate oxidase 1	*Petunia hybrida*	1.14.17.4	319	---	Iron, Vit C	pdb	structure
1W9Y_Q08506	CSA/Swiss-Prot	1-aminocyclopropane-1-carboxylate oxidase 1	*Petunia hybrida*	1.14.17.4	319	---	Iron, Vit C	fasta	improve
2BJS_P05326	CSA/Swiss-Prot	Isopenicillin N-synthetase	*Emericella nidulans*	1.21.3.1	331	---	Iron, ascorbate	fasta	improve
P10967	Swiss-Prot	1-aminocyclopropane-1-carboxylate oxidase	*Solanum lycopersicum*	---	363	---	Iron, Vit C	fasta	improve
P51091	Swiss-Prot	Leucoanthocyanidin dioxygenase	*Malus domestica*	1.14.11.19	357	---	Iron, Vit C	fasta	improve
Q9FR99	Swiss-Prot	1-aminocyclopropane-1-carboxylate oxidase	*Musa acuminata*	1.14.17.4	306	---	Iron, Vit C	fasta	improve
Q05964	Swiss-Prot	Naringenin,2-oxoglutarate 3-dioxygenase	*Dianthus caryophyllus*	1.14.11.9	365	---	Iron, Vit C	fasta	improve
P24397	Swiss-Prot	Hyoscyamine 6-dioxygenase	*Hyoscyamus niger*	1.14.11.11	344	---	Iron, Vit C	fasta	improve
O04847	Swiss-Prot	Deacetoxyvindoline 4-hydroxylase	*Catharanthus roseus*	1.14.11.20	401	---	Iron, Vit C	fasta	improve
P27744	Swiss-Prot	Isopenicillin N synthetase	*Nocardia lactamdurans*	1.21.3.1	328	---	Iron, Vit C	fasta	improve
P40902	Swiss-Prot	Sexual differentiation process protein isp7	*S. pombe*	---	397	---	Iron, Vit C	fasta	improve
Q3I411	Swiss-Prot	Gibberellin 3-β-dioxygenase 2-1	*Triticum aestivum*	1.14.11.15	370	---	Iron, ascorbate	fasta	substrate
Q9ZT84	Swiss-Prot	Gibberellin 3-β-dioxygenase	*Arabidopsis thaliana*	1.14.11.15	347	---	Iron, ascorbate	fasta	substrate
Q39110	Swiss-Prot	Gibberellin 20 oxidase 1	*Arabidopsis thaliana*	1.14.11.-	377	---	Iron, ascorbate	fasta	substrate

Table 3.6: Proteins used in the CoA ligase analysis. Indicated are the sequence source (MACiE, Swiss-Prot, CSA, TrEMBL, PDB), protein name, organism, enzyme class (E.C.) code, protein length and AA with binding function, file format (3D structure -pdb- or aminoacid sequence -fasta-) and the reason for selection (3D structure, alignment improvement or acting on a similar substrate).

Uniprot access code	Database	Protein name	Organism	E.C. code	AA Seq length	Site	Bind to	file format	Selection
1v25_Q6L8F0	MACiE	Medium-chain-fatty-acid--CoA ligase	*Thermus thermophilus*	6.2.1.-	541	K439, W444	MG, AMP	pdb	structure
3a9v_Q941M3	TrEMBL/PDB	4-coumarate:CoA ligase	*Populus tomentosa*	---	536	---	AMP	pdb/fasta	structure
1ba3_P08659	Swiss-Prot/PDB	Luciferin 4-monooxygenase	*Photinus pyralis*	1.13.12.7	550	---	AMP	pdb/fasta	alignment
F6H0C0	TrEMBL	Hypothetical protein	*Vitis vinifera*	---	549	---	---	fasta	alignment
B9GQ39	TrEMBL	Acyl CoA ligase	*Populus trichocarpa*	---	554	---	---	fasta	alignment
D7KJ24	TrEMBL	4-coumarate:CoA ligase	*Arabidopsis lyrata*	6.2.1.-	546	---	---	fasta	alignment
B9R8J7	TrEMBL	AMP dependent CoA ligase	*Ricinus communis*	1.13.12.7	549	---	---	fasta	alignment
F6DF01	TrEMBL	O-succinyl benzoate-CoA ligase	*Thermus thermophilus*	6.2.1.26	537	---	---	fasta	alignment

3.4.3.ii. C9 oxidase, TB596

In the cDNA-AFLP assay, the first hit of TB596 was a FAD/FMN-containing dehydrogenase (B8LR72), while Xylitol oxidase (2vfv**-**Q9ZBU1, found in the PDB data base) was the highest scoring PDB sequence aligned to TB596 (with 64). There is no experimental information about 2vfv, not even obtained by similarity to other PDB files. When we tried to improve our alignment by adding more sequences and enzymes acting on substrates similar to taxol, only a few proteins could be incorporated: D-arabinono-1,4-lactone Oxidase (Q7SGY1), L-gulonolactone Oxidase (Q8HXW0) and Xylitol Oxidase (Q9KX73). All these enzymes act on sugar substrates. As it was not possible to find enzymes acting on substrates similar to taxol or to identify the important AA for the function, we could not compare our candidate to known proteins acting on substrates structurally similar to taxol or go any further in its analysis. Due to its good alignment and high score, we considered TB596 to be a sugar oxidoreductase.

3.4.3.iii. C9 oxidase, TB047, TB645, TB739, TB812

The next four candidates, TB047, TB645, TB739 and TB812, exhibited homology to putative glucose-methanol-choline (gmc) oxidoreductases (B9R9I5**,** Q9XI68 and B9RC10) and their high similarity was reflected in an alignment score of 97 and good agreement between all the M-Coffee aligning methods. When blasting TB645 against a PDB data base, a group of gmc oxidoreductases showed the highest similarity: Choline oxidase (2jbv-Q7X2H8), Glucose oxidase (1gpe-P81156), Hydroxynitrile lyase (1ju2-Q945K2) and a Cellobiose dehydrogenase (1kdg-Q01738). Although the alignment was sufficiently good, none of the listed enzymes acted on a substrate similar to taxol, so we looked for more sequences in the Swiss-prot data base, resulting in the chosen proteins listed in Table 3.4. As before, none of the obtained proteins acted on a substrate similar to taxol.

For the model proteins, 2jbv and 1gpe, AA related to the active site are not known from experimental data but had been inferred by PSI-BLAST, being E416, H520 and H563 for 1gpe (Figure 3.7) and H466 and N510 for 2jbv. Once the AA related with the active site were localized in the 3D structure of 1gpe, AA surrounding the active site were also chosen in that model protein. Afterwards, we worked on 2jbv, finding its active site and surrounding AA. With the aim of proving the similarities between 1gpe and

2jbv and using the MSA, we extrapolated all the AA positions relevant for the 2jbv function in the 1gpe model, and only those AA maintained in the surroundings of the active site were chosen as on-going C9 oxidase candidates. Finally, N111, G112, D113, S114, D428, W430, R516, N518, W519, S562, V564 and M565 were also used as AA surrounding the active site (localized in green in Figure 3.7 and selected and weighed in Box 3.3A and B).

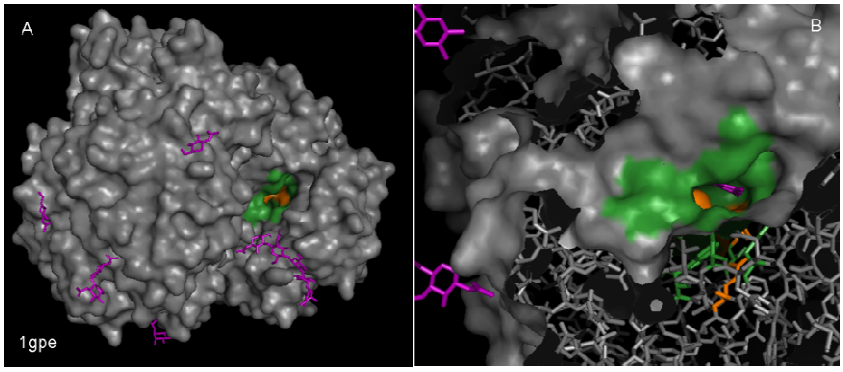


Figure 3.7: PyMOL image of 1gpe with important AA coloured. A) Complete view from one side with smooth surface. B) Section of the protein, with the interior in sticks. Grey: AA discarded for the evaluation; Orange: AA related with the active site; violet: cofactor; green: AA surrounding the active site.

A

```
P52707_MDL              NAGVSVKAYWHSHPQ
Q9S746_HOTHEAD          NAGFTLKVIWHTNPQ
P18172_GDH              NGMMEFAGENHGNTH
Q8UH55_CDH              NGLV-HGEAYHGNLN
Q00593_ADH              NAMV-HCEIYHGNTN
P46371_UncGMCoxred      NGAY-GVGSLHRGPH
Q47944_LSDH             NAEV-NYRSYHSNTN
Q92452_GOX              NGDSEDWRNWHSHVM
Q9AJD6_P4O              HAMGFLGIHHHGPIH
TB645                   NAAFTGKGFFHTNPQ
TB739                   NAGFTFKAHWHTNPQ
TB812                   NGAFTTKAANHTNPQ
TB047                   ----TGKAAFHTNPQ
1GPE                    NGDSEDWRNWHSHVM
1JU2                    NAGVSAKAYWHSHPQ
1KDG                    NGALSVLMSNHGNPQ
2JBV                    NSCIHTNNVYHVNPN
                                    *
```

B

Candidate	Compared to	Weight (%)
TB047	Q9S746_HOTHEAD	63,64
	TB739	72,73
	TB645	81,82
	TB812	
TB645	Q9S746_HOTHEAD	66,67
	TB739	
	TB812	
	TB047	81,82
TB739	TB645	66,67
	TB812	
	TB047	72,73
	Q9S746_HOTHEAD	80
TB812	Q9S746_HOTHEAD	60
	TB645	66,67
	TB739	
	TB047	81,82

Box 3.3: After the multiple alignment, the previously determined residues involved in the enzyme function (Figure 3.7) were picked up (box A) and the similarities weighed only according to those residues (box B).

According to the determined positions (Box 3.3A), our candidates showed a high similarity to each other and to a HOTHEAD (HTH) protein. HTH is related with FAD-containing oxidoreductases implicated in limiting cellular interactions between contacting epidermal cells during floral development (Krolikowski et al., 2003). HTH is a single-domain protein showing sequence similarity to long-chain fatty acid ω-alcohol dehydrogenases and may catalyze the step after cytochrome P450 fatty acid ω-hydroxylases in the ω-oxidation pathway (Kurdyukov et al., 2006). For TB047, TB645 and TB812, the first hit was the other 3 candidates with a high homology of 82.82-66.67%, followed by the HTH protein (63.64, 66.67 and 60%, respectively). For TB739 the first hit was the HTH protein with a homology of 80%, followed by the other candidates. Due to the high homology to the HOTHEAD protein, we could discard these candidates as possible oxidases for C9 of the taxane core.

3.4.4. Oxetane ring formation, Epoxidase and Oxomutase

The oxetane ring (ring D) formation has not been defined but two different mechanisms have been proposed (Figure 3.8A and B) (Floss and Mocek, 1995 and Croteau et al., 2006). It is currently accepted that the process involves epoxidation of the 4(20) double bond followed by migration of the α-acetoxy group from the C5 to the C4 position together with the expansion of the oxirane to the oxetane group. The epoxidase acting between C4–C20 and the possible oxomutase involved in the oxetane ring formation are other unknown enzymes.

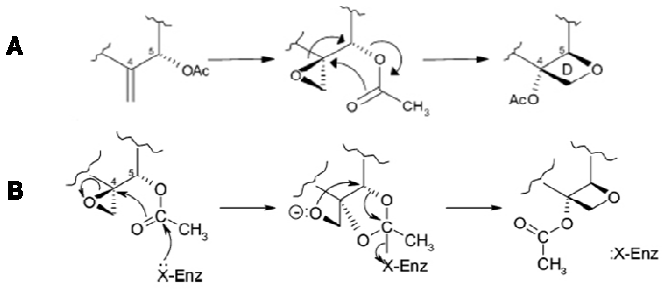
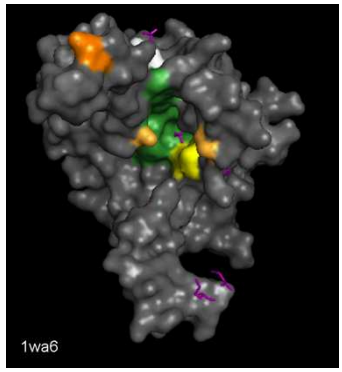


Figure 3.8: The main theories of oxetane ring formation. A) Floss and Mocek, 1995. B) Croteau et al., 2006.

3.4.4.i. Epoxidase

Only 1 tag from the cDNA-AFLP assay was sequenced as a possible epoxidase, TB328, whose best hit was a putative gibberellin 20-oxidase (A9NW35). The chosen model protein for the 3D structure analysis was a Leucoanthocyanidin dioxygenase (2brt-Q0WWD6) found in the PDB data base. Also a 1-aminocyclopropane-1-carboxylate oxidase 1 (1wa6-Q08506) was used as a support for the 3D structure analysis. Several other proteins listed in Table 3.5 were used to improve the alignment, including oxidases from gibberellin biosynthesis, such as the Gibberelline 3-dioxygenase, due to their capacity to epoxidate (Kwak et al., 1988).




```
2BJS                eyviypfhdtlqlnhrrspf
P27744|IPNS_NOCLA   nysiypfhdtlqlnhrrspf
P40902|ISP7_SCHPO   rvrlypvhdtmqinhrrtpf
Q3I411_GA3bdio      c-hnyplhdtvqvnhrrsgf
Q9ZT84_G3OX2        s-qnyplhdtlqvnhrrsal
1W9Y                -qksyplhdilqvnhrrsaf
1wa6_Q08506         -qksyplhdilqvnhrrsaf
Q9FR99|ACCO_MUSAC   -qksyplhdilqvnhrrsaf
O04847|DV4H_CATRO   nllhypthdtlqvnhrrsaa
P10967|ACCH3_SOLLC  kksnypthdtlqvnhrrstf
P24397|HY6H_HYONI   kllnypshdtlqvnhrrsal
Q05964|FL3H_DIACA   glvnyplhdtlqvnhqrsaf
Q39110|GAOX1_ARATH  vgrnypthdtlqvnhrrsaf
2brt_Q0WWD6         inknypvhdtihvhhrrsaf
P51091|LDOX_MALDO   inknypvhdtihvhhrrsaf
TB328               --hnyplhdtlnvnhrrsps
                       ** ** ::::*:*:
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Figure 3.9: Complete view from one side with smooth surface with PyMOL of 1wa6 with important AA coloured. Grey: AA discarded for the evaluation; Orange: AA related with the active site in 1wa6; light orange: AA related to the active site in 2brt; white: AA related to the active site in Q3I411; yellow: AA related with the active site and or cofactor binding; violet: cofactor; green: AA surrounding the active site.

Box 3.4: After the multiple alignment, the previously determined residues involved in the enzyme function (Figure 3.9) were picked up.

There are several residues known to be involved in the 1wa6 enzyme function. According to CSA, Q78 is related with the active site (bright orange in Figure 3.9) and according to Uniprot, H177, D179, and H234 bind cofactors (yellow in Figure 3.9). Information related to the active site of 2brt was also used for the analysis. According to CSA, I122, K213 and V229 are related with the activity, the latter two corresponding to K158 and L174 from 1wa6 when aligned with 2brt (light orange in Figure 3.9). Information related to the Gibberellin 3β-dioxygenase (Q3I411) from Uniprot was also used. The AA involved in the enzyme function

corresponded to H177, R179, H234 and R244 from 1wa6 when aligned with Q3I411. The first three had already been described as AA related to 1wa6 activity, a coincidence that confirmed the relevance of these positions. Only R244 was new, and we also included it in the analysis (illustrated in white in Figure 3.9). Once all AA related to the active site and cofactor binding were mapped, surrounding AA were selected. We checked all AA surrounding the active site from 2brt and 1wa6. Only those related with the active site in both proteins were finally chosen, corresponding in 1wa6 to S160, Y162, P163, I184, L186, Q198, V214, N216, R235, S246, A248 and F250 (in green in Figure 3.9).

According to the determined positions (Box 3.4), the highest homology displayed by our candidate TB328 was to two gibberellin 3-oxidases (Q3I411 and Q9ZT84), with 77.78% similarity. The homology between the three gibberellin oxidases were above and below this value, being 78.95% between Q9ZT84 and Q39110, and 73.68% between both of them and Q3I411. That could mean that TB328 was a gibberellin oxidase, but bearing in mind that Q9ZT84 showed more resemblance to P24397, a Hyoscyamine 6-dioxygenase (whose final product is scopolamine, represented in Figure 3.3), rather than a gibberellin oxidase, TB328 might not be related to gibberellin oxidation. Moreover, if we consider that gibberellin 3-oxidase can epoxidate GA_5 between C2 and C3 (Kwak et al., 1988), TB328 could act as an epoxidase of a substrate similar to gibberellins, such as the taxane core (Figure 3.3).

3.4.4.ii. Oxomutase

In the oxetane ring formation of taxol, both the acyl group and an oxygen have to be transferred to another position. There are only two known classified acyl mutases, lysolecithin acylmutase (E.C.=5.4.1.1) and precorrin-8X methylmutase (E.C.=5.4.1.2), and they correspond to the isomerase group (E.C.=5). However, according to Croteau et al. (2006), the unknown oxomutase involved in taxol biosynthesis should correspond to a hydrolase, protease, esterase, or even a new type of enzyme. Three tags from our cDNA-AFLP analysis were sequenced and used as possible oxomutases: TB493, TB115 and TB195. Once sequenced, their best hits were a Putative carboxylic ester hydrolase and two hypothetical proteins, the first characterized protein hits being Galactose oxidase and Glyoxal oxidase. The alignment of TB115 and TB195 had a score of 98, which decreased to 74 when TB493 was added, while TB493 was aligned with a score of 35. Consequently, these candidates were analysed separately.

Oxomutase, TB115 and TB195

Due to the low score with precorrin-8X methylmutase (33) and the lack of other mutases, the candidates were blasted against a PDB data base, giving a Galactose oxidase (2eic-Q01745) as the best hit. When we looked for more homologue proteins in Uniprot only Galactose oxidases were found. We could not enrich our multiple alignment with proteins with other functions. We searched for conserved domains from both candidates in the Conserved Domains Database from NCBI, and as they showed a high similarity (e-value lower than e^{-50}) to glyoxal oxidase N-terminal domain, their function as oxomutases was discarded.

Oxomutase, TB493

With the low score between TB493 and precorrin-8X methylmutase (28) and the impossibility of finding any match on PDB databases for this candidate, we searched for more matches in Uniprot. Four unique matches were found, a α-fucosidase 3-precursor (Q9FXE5), an Anter-specific Proline-rich protein (P40602), an Esterase precursor (Q7Y1X1) and a Thermolabile hemolysin precursor (Q99289). A Blast search of these homologues against a PDB data base gave no matches and the same kind of enzymes appeared in Uniprot. Although these four proteins contain esterases SGNH and/or the lipase GDSL domain, when the domains from TB493 were analyzed, they presented high similarity to the SGNH lipase domain (e-value lower than e^{-50}), which is characteristic of the SGNH-family of hydrolases, a diverse family of lipases and esterases. If the unknown oxomutase corresponds to one of the enzyme types defined by Croteau et al. (2006), our results did not allow us to choose or discard TB493 as a possible oxomutase.

3.4.5. Bioinformatics study of candidates for Aroyl CoA transferase

The unknown aroyl CoA ligase related to taxol biosynthesis is the enzyme that activates the lateral chain bound to the taxane core, the reaction substrate being a β-phenylalanine. Only 1 tag from our cDNA-AFLP analysis, TB768, could fit as a CoA ligase. Its first hits were an Acyl CoA ligase (B9GQ39), a 4-Coumarate CoA ligase (D7KJ24), a Hypothetical protein (F6H0C0) and an AMP-dependent CoA ligase (B9R8J7), all of

them with the same e-value (0.0). Out of these CoA ligases, only the enzyme class for 4-coumarate CoA ligase was known (E.C.=6.2.1). In MACiE only one protein with this enzyme class was found, a long-chain fatty acid CoA ligase (1v25 - Q6L8F0). After looking for 1v25 matches in the PDB data base, two more enzymes with PDB files were added to our list, 4-coumarate-CoA ligase (3a9v-Q941M3) and Luciferin 4-monooxigeanse (1ba3-P08659). The complete list of enzymes used is given in Table 3.6.

Experimental data related to the catalytic AA of 1v25 was found in MACiE, K439 and W444 being highlighted, while G302, S303, G323, T327, D418, K435, and K439 were also found to be involved in the enzyme function according to Uniprot. Once the functional AA were localized, the surrounding AA were also localized in the 3D structure: N103, A182, Y183, T184, T185, G186, T187, H230, V231, W234, V300, G301, A304, Q322, Y324, G325, L326, E328, P331, A355, I430, R433, G442, Q474, E475 (Figure 3.10). Using the MSA it was possible to infer the position of all these AA in the 3a9v structure, and once mapped (through PyMOL) we found that the positions were maintained in the same region. The AA described by MACiE and Uniprot were in contact with the AA surrounding the active site.

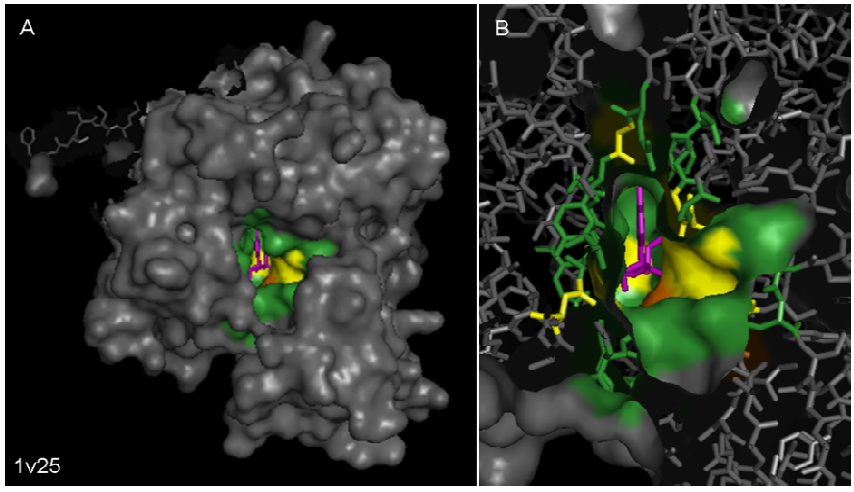

Figure 3.10: PyMOL image of 1v25 with important AA coloured. A) Complete view from one side with smooth surface. B) Section of the protein, with the interior in sticks. Grey: AA discarded for the evaluation; orange: AA related with the active site; yellow: AA related with active site and or Cofactor binding; violet: cofactor; green: AA surrounding the active site.

```
1v25            NAYTTGTHVWVGGSAQGYGLTEPADIRKKGWQE
F6DF01          NAYTTGTHVWVGGSAQGYGLTEPADIRKKGWQE
D7KJ24          NLYSSGTHILCGGAPQGYGLTEGGDVRKKGQGQ
B9R8J7          NLYSSGTHILSGGAPQGYGLTEGGDVRKKGQGQ
F6H0C0          NLYSSGTHILSGGAPQGYGLTEGGDIRKKGQGQ
B9GQ39          NLYSSGTHILSGGAPQGYGLTEAGDVRKKGQGQ
1ba3_P08659     NIMSSGSHGMSGGAPQGYGLTESGDIRKKGQGE
3a9v_Q941M3     NPYSSGTHILSGGAPQGYGMTEPGDIRKKGQGE
TB768           NLYSSGTHILCGGAPQGYGLTEAGDVRKKGQGQ
                *  ::*:*   **:.****:** .*:****  :
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Box 3.5: After the multiple alignment, the previously determined residues involved in the enzyme function (Figure 3.10) were picked up and weighed.

When the positions were extracted from the MSA (Box 3.5) and weighed, we found that TB768 presented 96.97% similarity to B9GQ39 and D7KJ24, an acyl CoA ligase and 4-coumarate CoA ligase, respectively. These two proteins also showed 96.97% similarity with each other, but when we compared two 4-coumarate CoA ligases from different species (D7KJ24 and B941M3 from *Arabidopsis* and *Populus*) their resemblance was only 81.82%. These data indicate that the two different enzymes showed more similarity (96.97%) between them than the same enzyme from two different species (81.82%). Another limitation of this study was the lack of any ligase related to phenylalanine, but luckily 4-coumarate is structurally similar to β-phenylalanine (Figure 3.3). Despite the high homology of our candidate to an acyl CoA ligase or a 4-coumarate CoA ligase and the similarity between the 4-coumarate and β-phenylalanine, our data did not allow us to choose or rule out TB768 as the candidate for the aroyl CoA ligase.

3.4.6. About our approach: advantages and limitations

At the start of these studies on undefined aspects of taxol biosynthesis, we were aware of various limitations. Firstly, our data was based on the limited results of our previous cDNA-AFLP assay (Chapter 2), as well as the incomplete information available in public data bases. Then there were the limitations of the *in silico* approach itself.

Besides the incomplete elucidation of the taxol biosynthetic pathway, another major handicap is not knowing which genes can be expressed when the pathway is activated. As reported by Baloglu and Kingston

(1999), there are more than 350 known taxanes, meaning that there are probably many more genes related to taxane biosynthesis than those involved directly in taxol biosynthesis. In other words, the candidates picked up by the *in silico* approach to the unknown steps could be related to modifications other than those found in taxol. For example, while TB331 could be the hydroxylase for C1 and C9 from the main taxane core, it might also be another taxane hydroxylase like T14OH, which kidnaps substrates to taxanes other than taxol.

Although it did not limit our approach, the quantity and quality of the data set used affected the results. If the complete genome had been available, the pool of candidates for each step would have been bigger and we would probably have been able to find a small quantity of candidates corresponding to each unknown position. But as data came from a transcript profile, only differentially expressed genes were available, and it was necessary for the tags to be of sufficient size for sequencing and to present similarities to known genes. When no conserved domains or high similarity to known genes were observed, the tags did not enrich our initial pool, and probably some real candidates were missed.

The biggest limitation, when *in silico* analyses are carried out, is the quantity and quality of information in public databases. Studies would more reliable if information came principally from experimental rather than automatically annotated data. Moreover, the use of hypothetical proteins and proteins with unknown or uncharacterized function does not help. The size of the MACiE data base limits the number of enzymes with concrete functions (or model proteins) available for studies. Another problem is the lack of information about the AA involved in the enzyme function.

Once the most appropriate PDB file has been selected as a model protein, the active site and surrounding AA in the 3D view are localized. Afterwards, these AA are localized in the MSA and picked up for evaluation. If the alignment is not correct, then all that work is invalid. The strong point of the M-Coffee utility is the use of 8 aligning programs that give information about the agreement between them. If there is a general agreement, then the alignment is reliable, providing a solid base for the following work. The regions with low agreement between the different aligning M-Coffee programs cannot be studied with reliable results.

The advantage of the *in silico* approach is being able to focus on a minimum number of candidates while maintaining a high probability of finding the candidate for the unknown steps. In short, with this approach we hoped to maximise the efficiency of our subsequent experimental

work, which would consequently be less time-consuming and require fewer resources.

Summing up, out of our 15 gene candidates for enzymes involved in 5 unknown steps of the taxol biosynthetic pathway, 4 of them were selected and 11 were completely discarded after the multiple alignment studies. Considering the unknown enzymes (hydroxylases, C9 oxidase, epoxidase, oxomutase and CoA ligase), we were able to confirm that the TB331 protein is a strong candidate for a hydroxylase involved in taxane biosynthesis and the TB328 protein is a candidate for the epoxidase related to the oxetane ring formation. Moreover, the TB768 protein is a contender for the CoA transferase related to the lateral chain activation, while TB496 cannot be discarded as the unknown oxomutase.

However, further functional studies using a suitable cell system are needed in order to confirm the functionality of the selected enzymes and their involvement in taxol biosynthesis.

3.5. Acknowledgements

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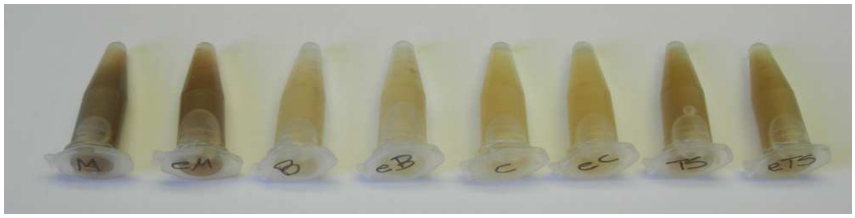

CORONATINE, AN IMPROVEMENT IN TAXANE PRODUCTION IN *TAXUS* X *MEDIA* CELL SUSPENSION CULTURES

4. CORONATINE, AN IMPROVEMENT IN TAXANE PRODUCTION IN *TAXUS* X *MEDIA* CELL SUSPENSION CULTURES SUSPENSION

4.1. Abstract

Coronatine is a pathogenic toxin produced by *Pseudomoneas syringae* that has received much attention in recent years as a plant growth regulator. It has been shown that this compound is a natural structural analogue of jasmonates conjugated to isoleucine, the intracellular switch of the jasmonate pathway. In order to gain more insight into the action mechanism of elicitors in the biotechnological production of taxanes at the cellular-molecular level and the control they exert on the taxane biosynthetic pathway, we have studied the effect of adding coronatine (Cor) and methyl jasmonate (MeJ) to a *Taxus* x *media* cell line carrying the taxadiene synthase gene under the control of the CaMV35S promoter (Expósito et al., 2010). For this study, a two-stage cell culture was established, in which cells were first cultured for 12 days in an optimum medium for growth, and then the biomass was transferred to the optimum medium for secondary metabolite production. The two elicitors assayed were added to the medium at the beginning of the second stage. Taxane production of the cell suspension was significantly increased by the elicitors, since in control conditions the highest level was 8.14 mg/L, whereas the highest taxane production achieved by the cultures treated with MeJ and Cor was 21.48 mg/L (day 12) and 77.46 mg/L (day 16), respectively. In addition, the expression of *TXS*, *T13OH, T2OH, T7OH, DBAT*, *PAM, BAPT* and *DBTNBT* genes was determined, being observed that under elicitation these genes increased their expression level between 8- to more than 100-fold.

4.2. Introduction

The demand for paclitaxel, a powerful antitumor agent produced by *Taxus* sp. used in the treatment of several cancers, is growing, although its production remains low. Obtaining paclitaxel from its natural source or by complete synthetic synthesis is not economically feasible, although it is currently being produced by semi-synthesis or in cell culture systems.

Several groups have reported high production levels of taxanes in cell suspension cultures treated with different elicitors, methyl jasmonate being the most successful (Yukimune et al., 1996; Ketchum et al, 1999; Cusidó et al., 1999 and 2002; Vongpaseuth and Roberts, 2007; Expósito et al., 2010), but major advances will only arrive with the understanding and regulation of taxol biosynthesis. Although taxol biosynthesis (Figure 4.1) is not completely known, Croteau et al. (2006) have postulated that there are 19 enzymatic steps involved from the universal diterpenoid geranylgeranyl diphosphate (GGPP). After GGPP cyclization, 8 hydroxylations, 5 acyl/aroyl transformations, one epoxidation, one mutation and 2 CoA esterifications, taxol is reached.

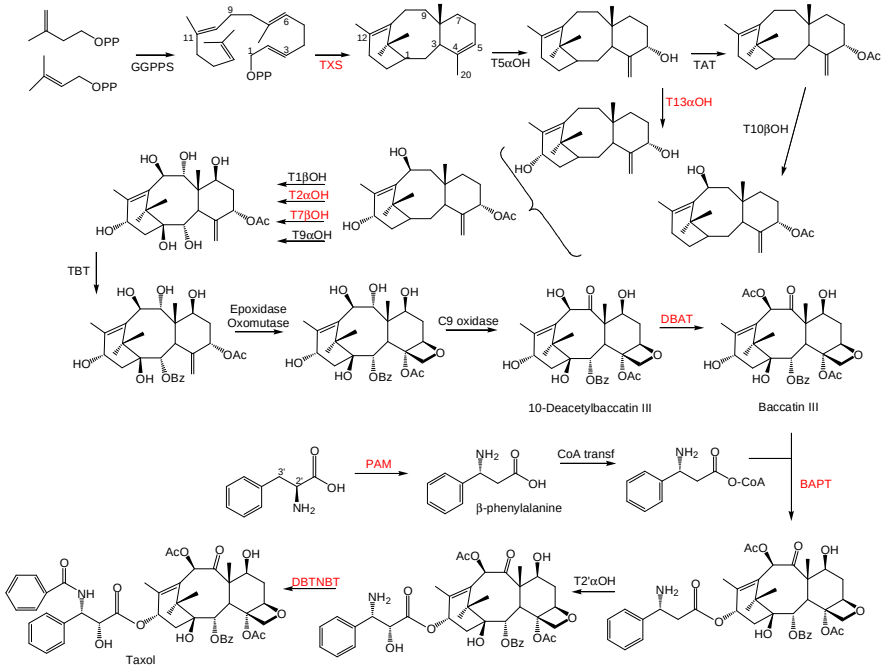

Figure 4.1: A concise depiction of the Taxol biosynthesis. The genes whose expression level has been determined in this study are in red. GGPPS, Geranylgeranyl diphosphate synthase; TXS, Taxadiene synthase; T5αOH, Taxane 5α-hydroxylase; TAT, taxadiene-5α-ol-*O*-acetyl transferase; T13αOH, Taxane 13α-hydroxylase; T10βOH, Taxane 10β-hydroxylase; T1βOH, Taxane 1β-hydroxylase; T2αOH, Taxane 2α-hydroxylase; T7βOH, Taxane 7β-hydroxylase; T9αOH, Taxane 9α-hydroxylase; TBT, Taxane-2α-*O*-benzoyl transferase; DBAT, 10-deacetyl baccatin III-10-*O*-acetyltransferase; PAM, Phenylalanine aminomutase; CoA transf, CoA transferase; BAPT, baccatin III-3-amino, 13-phenylpropanoyltransferase; T2'αOH, Taxane 2'α-hydroxylase; DBTNBT, Debenzoyl taxol *N*-benzoyl transferase.

Over the last two decades jasmonates have attracted considerable interest due to their role in plant growth and response to biotic or abiotic stress and defence activation. Jasmonates are critically involved in decreasing primary metabolism, arresting the cell cycle and growth, and directing resources for defence mechanisms (Herms and Mattson, 1992). Jasmonates (JA), including methyl jasmonate, jasmonic acid, and their derivatives and precursors, are oxylipins, a group of compounds derived from α-linolenic acid. Vick and Zimmermann (1984) elucidated the jasmonate biosynthesis but its mechanism of action is still not fully understood. In 1962 methyl jasmonate (MeJ) was isolated and determined for the first time from *Jasminum grandiflorum* (Demole et al. 1962), but it was not related with defence and secondary metabolism until 1992. Gundlach et al. (1992) studied the effect of a fungal elicitor in cell suspension cultures of several angiosperm species. The resulting increase in endogenous MeJ levels and an exogenous application of MeJ successfully enhanced the transcription of genes encoding enzymes related with secondary metabolism, increasing their activity and consequently the biosynthesis of secondary metabolites. The studies also revealed how MeJ acts as a signal molecule between external stimuli and secondary metabolism.

There are three main parts in the jasmonate biosynthetic pathway: α-linolenic acid is released and modified to oxylipin intermediates, which are eventually converted to jasmonates (Figure 4.2). α-linolenic acid is released from chloroplast membranes by lipid-hydrolysing enzymes, and lipoxygenases (LOXs) form hydroperoxides, (13S)-hydroxyperoxyoctadecadienoic acid (13-HPOT) being the intermediate for jasmonate biosynthesis. 13-allene oxidase synthase (13-AOS) carries out the conversion to the unstable allene oxide, metabolized by allene oxidase cyclase (AOC) to establish cis(+)-12-oxophytodienoic acid (OPDA), which bears the cyclopentenone ring characteristic of jasmonates. The subsequent step, the reduction of the cyclopentenone ring, is catalysed by a peroxisomal OPDA reductase (OPR), which needs to be transported from the chloroplasts to the peroxisomes. Esterification with CoA occurs prior to β-oxidation, as the 3 β-oxidation steps only occur in OPDA-CoA. The esterification step is unknown, although a 4-coumarate CoA ligase-like is supposed to activate OPDA in the peroxisome (Schneider et al., 2005). Jasmonic acid is obtained after β-oxidation, which is carried out by acyl-CoA oxidase (ACX1), a multifunctional protein (MFP) and L-3-ketoacyl CoA thiolase (KAT), (Vick and Zimmermann, 1984; reviewed in Wasternack, 2007). Multiple derivatives can be obtained from jasmonic acid by other enzymes, for

example, MeJ, produced by jasmonic acid carboxyl methyltransferase (JMT) (Seo et al., 2001) (Figure 4.3).

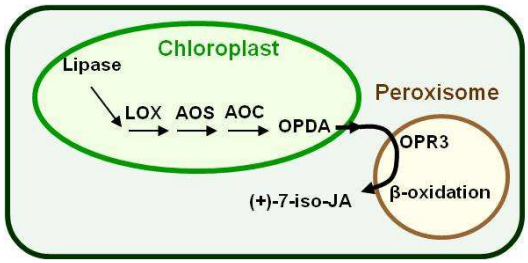

Figure 4.2: Localization and enzymes implicated in jasmonate biosynthesis. LOX, lipoxygenases; AOS, allene oxidase synthase; AOC, allene oxidase cyclase; OPDA, cis(+)-12-oxophytodienoic acid; OPR3, peroxisomal OPDA reductase.

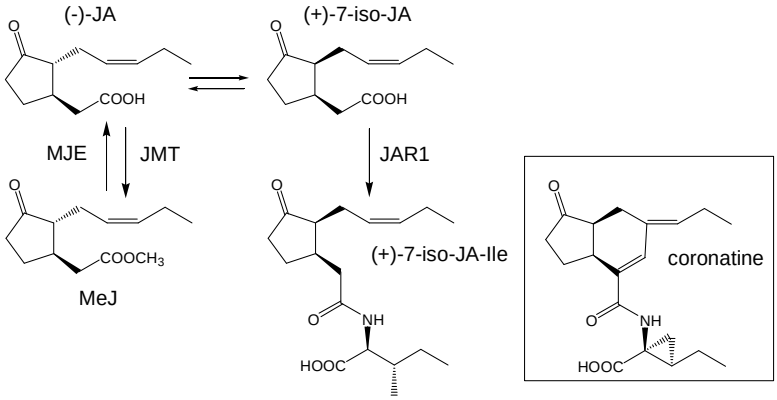

Figure 4.3: Main structures of active molecules in jasmonate signalling. (+)-7-iso-JA, (-)-JA, MeJ or (+)-7-iso-JA-Ile and also the analogue phytotoxin coronatine (Cor). The known implicated enzymes correspond to: MJE, methyl jasmonate esterase; JMT, jasmonic acid carboxyl methyltransferase; JAR1, jasmonic acid-amino synthase.

The expression of early jasmonate-responsive genes is controlled by several transcription factors, including MYC2, MYC3 and MYC4 (Lorenzo et al., 2004; Fernández-Calvo et al., 2011), and under non-induced conditions these transcription factors are repressed by JAZ family proteins (Chini et al., 2007), NINJA and TOPLESS (Pauwels et al., 2010). An E3 ubiquitin ligase is related to the release of transcription factors by ubiquitinating JAZ proteins at the appropriate signal. In this SCF (Skip/Cullin/F-box) E3 ubiquitin ligase, the F-box component is COI1 (SCF^{COI1}) (Xu et al., 2002), which recognizes and recruits JAZ. Under stress conditions, JAR1 (Staswick and Tiryaki, 2004) conjugates jasmonic

acid to isoleucine (JA-Ile). The subsequent increase in Ja-Ile levels promotes the formation of JAZ-COI1 complexes, involving the recruitment of JAZ by SCF^{COI1} and its posterior ubiquitination and degradation via the 26S proteasome. The transcription of early jasmonate-responsive genes can be activated due to the release of MYC2 (reviewed in Fonseca et al., 2009 and Browse, 2009) (Figure 4.4).

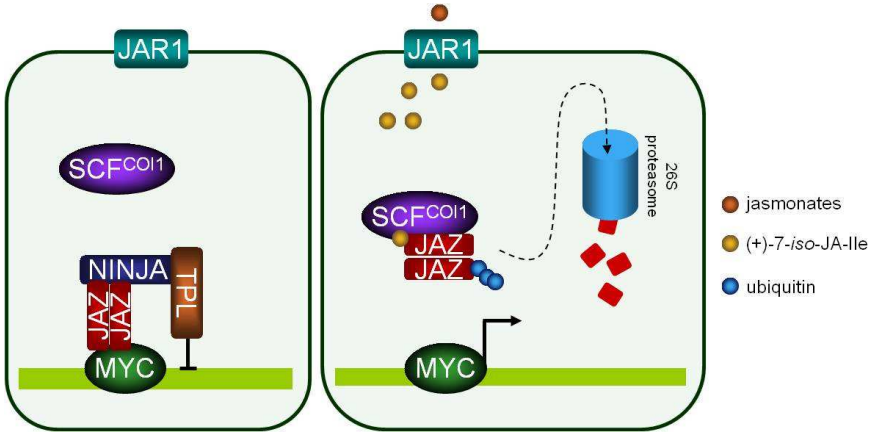

Figure 4.4: Jasmonate signalling pathway. In basal conditions JAZ proteins maintain inactive MYC transcription factors, while under stress JAR1 conjugate JAs (orange circle) to isoleucine to obtain JA-Ile (yellow circle). The presence of JA-Ile favours the formation of SCF^{COI1}-JAZ complexes, ubiquitinating JAZ to be degraded by the proteasome. The released MYC can activate transcription of early JA-responsive genes.

This complex signalling pathway is highly regulated. Jasmonates can be inactivated by hydroxylation and subsequent sulfonation and glycosylation (review Fonseca et al., 2009). Some of the early jasmonate-responsive genes controlled by MYC2 are JAZ proteins, repressors of this transcription factor, but MYC2 also controls genes encoding itself and genes related with JA-Ile synthesis. In this way, SCF^{COI1}-dependent removal of pre-existing JAZ proteins can provide a rapid activation of defences and other jasmonate-dependent processes, whereas rapid synthesis of new JAZ proteins can locally dampen jasmonate signals, ensuring attenuation of the jasmonate response. The synthesis of MYC2 and expression of JA-Ile-related genes can help activation on a systemic level (Thines et al., 2007; reviewed in Browse, 2009).

JA-Ile has a natural analogue, coronatine, which is a phytotoxin produced by plant pathogenic strains of *Pseudomonas syringae* (Bender et al., 1999) (Figure 4.3). Several lines of evidence indicate that Cor exerts its

virulence effects by activating the host's jasmonate signalling pathway (Feys et al., 1994; Zhao et al., 2003) and plants insensitive to Cor, like Arabidopsis *coi1* mutant, exhibit resistance to Cor-producing strains like *P. syringae* (Zhao et al., 2003).

Biological assays, and ultrastructural and gene expression studies have described Cor effects. It induces JA biosynthesis, affects signalling 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 and accumulation (anthocyanins, alkaloids, flavonoids, phytoalexins), ethylene emission, accumulation of proteinase inhibitors and apoptotic cell death (Farmer and Ryan, 1990; Tamogami and Kodama, 2000; Yao et al., 2002; summed up in Uppalapati et al., 2005).

Ethylene and JA positively regulate susceptible interactions between tomato/*Arabidopsis* and *P. syringae* (Kunkel and Brooks, 2002). Cor could act as a suppressor of defence responses, possibly by suppressing salicylic acid (SA)-dependent defences in *Arabidopsis* and tomato (Kloek et al., 2001; Zhao et al., 2003). However, exogenously applied Cor does not suppress SA-mediated defences, while inducing genes involved in JA biosynthesis and responsiveness (Uppalapati et al., 2005). As mutual antagonism between JA- and SA-mediated defence pathways is well documented (Kunkel and Brooks, 2002), Cor may stimulate the JA pathway at the expense of SA-dependent defence responses (Uppalapati et al., 2005).

The aim of this work is to describe the effect of Cor, an analogue of an active jasmonate derivative, and prove its efficiency in increasing taxol production. A selected *Taxus* x *media* cell line was elicited with 100 µM MeJ, or 1 µM Cor at the beginning of the second culture stage. The effect of MeJ and Cor on genes related with taxol biosynthesis and the differing taxane production levels have allowed us to further our understanding of the molecular changes that take place in producer cells induced by elicitors.

4.3. Material and Methods

4.3.1. Plant material

A transgenic *Taxus* x *media* cell line carrying the gene encoding taxadiene synthase (TXS) of *T. baccata* under the control of p35S was obtained as described by Expósito et al. (2010) and routinely subcultured every 2 weeks for more than 3 years. In this experiment, cells were grown in Gamborg's B5 medium (Gamborg et al., 1968) supplemented with 2x B5 vitamins, 0.5% sucrose, 0.5% fructose, 2 mg/L picloram, 0.1 mg/L kinetin and 0.5 mg/L gibberellic acid (GA_3) (B5 growth medium). The pH was adjusted to 5.8 prior to autoclaving. After 14 days of cultivating 4 g of cells in 20 mL of growth media in a 175-mL flask capped with Magenta B-Cap (Sigma St Louis, MO, USA), cells were transferred to the production media in the same proportion and conditions. The production media was a basic Gamborg's B5 medium supplemented with 2x B5 vitamins, 3% sucrose, 2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 0.1 mg/L of benzyladenine (BAP) and 0.5 mg/L GA_3. Taxane production was increased by adding elicitors to this production media.

Previous studies on *Taxus* spp. have shown that the best elicitor is MeJ at a concentration of 100 µM (Yukimune et al., 1996; Cusidó et al., 2002) but recent studies on dicotyledonous plants have also demonstrated the suitability of Cor as an elicitor (Uppalapati et al., 2005; Tamogami and Kodama, 2000). Due to the lack of previous studies on *Taxus* spp. with Cor, a variety of concentrations were initially checked (0.05 µM, 0.1 µM, 0.5 µM and 1 µM), but after observing that cell viability was not severely affected, 1 µM was finally chosen as a suitable concentration for the experiment. All compounds were filter-sterilized (0.22 µm sterile PES filters, Millipore, Billerica, MA, USA) and added to the production medium prior to inoculation to give the final concentrations considered. For analysis, three flasks were harvested for each treatment at different time points: 0h, 30min, 1h, 2h, 4h, 8h, 12h, 1d, 2d, 4d, 8d, 12d, 16d, 20d and 24d. Cell growth and viability were determined as previously described in Chapter 2.

4.3.2. Taxane determination

Taxanes were extracted from the culture media and quantified as described in Material and methods from Chapter 2.

4.3.3. Quantitative real-time 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, California, USA) and qRT-PCR was performed using 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 normalized to the levels of the *18S* from *Taxus baccata*.

Table 4.1: Sequences of the primers used to amplify the genes by quantitative real-time PCR.

Gene		Primer Sequence	Amplicon size	Reference - Acc. Number
18S	Sense	5′-GTGCACAAAATCCCGACTCT-3′	102	Onrubia et al., 2010
	Reverse	5′-GCGATCCGTCGAGTTATCAT-3′		
TXS	Sense	5′-TTCGCACGCACGGATACG-3′	115	Onrubia et al., 2010
	Reverse	5′-TTCACCACGCTTCTCAATTCG-3′		
T13OH	Sense	5′-GCCCTTAAGCAATTGGAAGT-3′	100	AY866412 (*T.* x *media*)
	Reverse	5′-CAGAGGAATGGCGTTTAGAG-3′		
T2OH	Sense	5′-CGTGCCATTTGGAGGAGGGAGA-3′	122	AY518383 (*T. canadensis*)
	Reverse	5′-CGTGAGGGTCGATTGGCGTGTA-3′		
T7OH	Sense	5′-GGTCCGCCCAAATTGCCAGAA-3′	110	AY307951 (*T. cuspidata*)
	Reverse	5′-CCCTGCAGAGCCCAAAAAACCT-3′		
DBAT	Sense	5′-AGTTGGATTTGGTGATCGAA-3′	92	Onrubia et al., 2011
	Reverse	5′-ATCCATGTTGCACGAGACTT-3′		
PAM	Sense	5′-CCCGGAGGCATGACGTGAAG-3′	99	AY866411 (*T.* x *media*)
	Reverse	5′-CGCCGTCTTCCGCCTTGC-3′		
BAPT	Sense	5′-TAAGCACTCTACAACAACAATGG-3′	111	Onrubia et al., 2010
	Reverse	5′-GCATGAACATTAGTATCTTGATTCC-3′		
DBTNBT	Sense	5′-CGGGGGGTTTGTTGTGGGATTA-3′	104	Onrubia et al., 2011
	Reverse	5′-TTAGCCTCTCCCCTCGCCATCT-3′		

4.3.4. Statistics

Statistical analysis was performed with Statgraphics (Centurion XV) and Excel software. All the data are the average of 3 determinations ± 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

4.4. Results and discussion

As previously observed (Ketchum et al, 1999; Bonfill et al., 2006), cell lines with a low taxane production capacity are more capable of responding to elicitation, resulting in a greater improvement in taxane production. As described by Expósito et al. (2010), the TXS cell line is a low taxane production culture in a non-elicited growth media. However, this cell line is able to increase taxane production after elicitation with MeJ in a production media environment. These characteristics would help to analyse the effect of Cor, an analogue of Ja-Ile, an active form of jasmonate, when applied to this cell suspension line.

Two-stage suspension cultures of the studied cell line were established as previously described by Cusidó et al. (2002). The first stage, using an optimum cell growth medium, lasted until day 14, when the cells entered their stationary growth phase. In the second stage, a medium for optimum production was used, with or without the addition of the elicitors, 100 µM MeJ or 1 µM Cor.

4.4.1. Preliminary studies

A preliminary assay was carried out to find the highest non-toxic Cor concentration for the TXS cell line. 4 g of cells were incubated in 20 mL production media in a 100 mL-shake flask in darkness. We had previously observed that 0.5 µM Cor was toxic for other species like *Ruscus aculeatus* (unpublished results), so we now tried different concentrations: 0 µM (control), 0.05 µM, 0.1 µM, 0.5 µM and 1 µM. Samples were taken for viability analysis on the first day after treatment and then every 4 days until 28 days. The data obtained revealed no statistical differences between the control conditions and the three concentrations. Viability, counted as live cells over total cells, remained over 60 % until the last day (data not shown). Finally, since these preliminary tests resulted in an unexpectedly high viability**,** the Cor concentration chosen for the cell suspension assay was 1 µM.

4.4.2. Characterization, growth and cell viability of suspension cell cultures

The TXS cell line was grown in the optimum medium for biomass formation until day 14. Then cells were transferred to the production media, and immediately elicited with 100 µM MeJ or 1 µM Cor. Samples were taken during this second stage of culture. Despite being in production media, a good growth was observed in the three conditions (Figure 4.5).

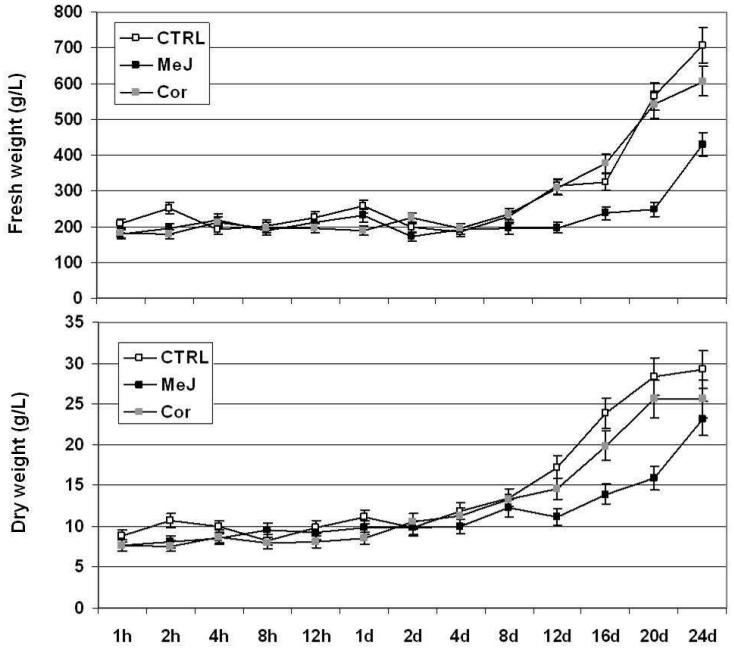

Figure 4.5: Time courses of biomass production (expressed as fresh weight or dry weight) of the transformed TXS cell line cultured for 24 days in the production medium without elicitors (CTRL, control) or with the addition of 100 µM methyl jasmonate (MeJ) or 1 µM coronatine (Cor). In all cases, the inoculum consisted of 200 g L^{-1} of cells. Data represent average values from three separate experiments ± SD.

The capacity for biomass formation of Cor-elicited and unelicited cells was similar except at the end of the experiment when the growth of the former slowed down while the latter continued to grow actively. The growth observed in MeJ-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 MeJ 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 (Figure 4.5).

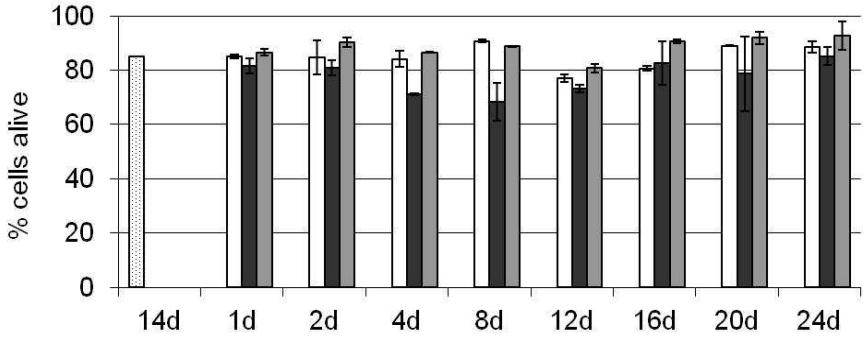

Figure 4.6: Viability of the TXS cell line growing for 24 days in the PM without the addition of the elicitors (CTRL), or with 100 µM methyl jasmonate (MeJ) and 1 µM coronatine (Cor). Bars represented in dots, GM; white, CTRL; black, MeJ; grey, Cor. Data represent average values from three separate experiments ± SD

Viability (Figure 4.6), 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, and MeJ- and coronatine- treated cultures, respectively. These results agreed with those described by Expósito et al. (2010) for experiments with control and MeJ-treated cultures.

The fact that the fresh and dry weight results followed the same pattern suggests that the lower fresh weight of cell cultures treated with MeJ was not due to osmotic changes but to a decrease of living cells, as shown by the viability study.

4.4.3. Taxane production

Total taxane production increased significantly when cells were transferred to the production medium from the growth medium. The highest taxane levels in the control cultures were observed in the second part of this second culture stage (8.8 mg/L at day 24), although an

increase was also observed after 4 and 8 days (8.2 mg/L at day 4) (Figure 4.7). At the end of the culture, the production obtained was almost 6 times higher than at the beginning. Under MeJ elicitation, a significant increase in total taxane production was observed at day 4, the highest being achieved at day 12 (24.7 mg/L). At its peak, the total taxane production of MeJ-treated cultures was 2.8-fold higher than the maximum in the control cultures. The increase of taxane production after MeJ elicitation is well documented (Yukimune et al., 1996; Ketchum et al., 1999; Cusidó et al., 2002; Bentebibel et al., 2005). However, if the taxane production achieved by the control and MeJ-elicited cultures in this study is compared with the results obtained by Expósito et al (2010), working with the same cell line, it can be observed that although productivity under MeJ is similar, the unelicited cultures have clearly increased their capacity to accumulate taxanes. It should be stressed, as reported by Ketchum et al. (1999) and Kim et al. (2006), that *Taxus* cell cultures show great variability in production, which in some cases can fall sharply, only to increase again after various subcultures. Sometimes productivity can decrease irreversibly due to epigenetic instability (Hirasuna et al., 1996). Such periodic variation is not always a completely random process since it can reflect underlying intracellular dynamics. Fluctuations in productive capacity can also be due to a cell line being highly sensitive to culture conditions. Hirasuna et al. (1996) offer a clear example of this variability in *Taxus*, since they obtained several *T. baccata* cell lines whose production of taxol ranged between 0.1 mg/L and 13 mg/L, after 27 days of culture.

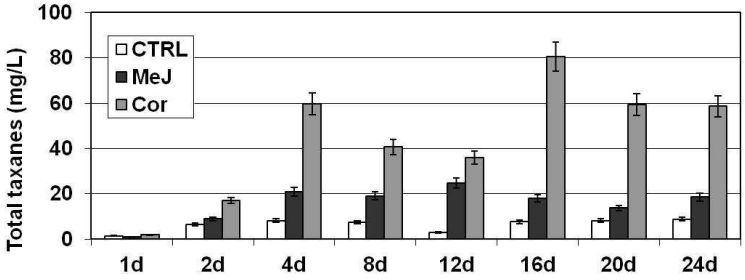

Figure 4.7: Total taxane content (cell associated + extracellular) in the TXS cell line growing for 24 days in the production medium without (CTRL) and with the addition of 100 µM methyl jasmonate (MeJ) or 1 µM coronatine (Cor). Data are average values from three replicates ± SD.

Under Cor elicitation, production peaked at 80.6 mg/L at day 16, which represents a 40.1-fold increase compared to the first day of culture (Figure 4.7). At its peak, total taxane production in Cor-treated cultures was 9.2- and 3.3- fold higher than in the control and MeJ-treated cell

cultures, respectively. The productivity reached by the Cor-treated cultures corresponded to 5 mg L^{-1} day^{-1}.

The results shown in Figure 4.8 suggest that the cell line, growing in either an elicited or unelicited production medium, has a high capacity for taxane excretion from the producer cells to the medium. Excretion, calculated as the total taxane content in the media over the total taxane content per day, was extremely high: in the control conditions, over 60% of taxanes were found in the media, remaining over 91% until day 16. Under MeJ and Cor treatment, over 80 % of total taxanes were excreted, with maximum values reaching over 99%. It is known that cell capacity for taxane excretion fluctuates during the culture period and depends largely on the cell line and species (Seki et al., 1997, Navia-Osorio et al., 2002). The release of accumulated taxanes from cells to the medium is important, since toxic effects on the cells are avoided and biosynthesis is enhanced (Seki et al., 1997). Additionally, the excretion of compounds of interest to the medium is essential in a continuous system for large-scale industrial production.

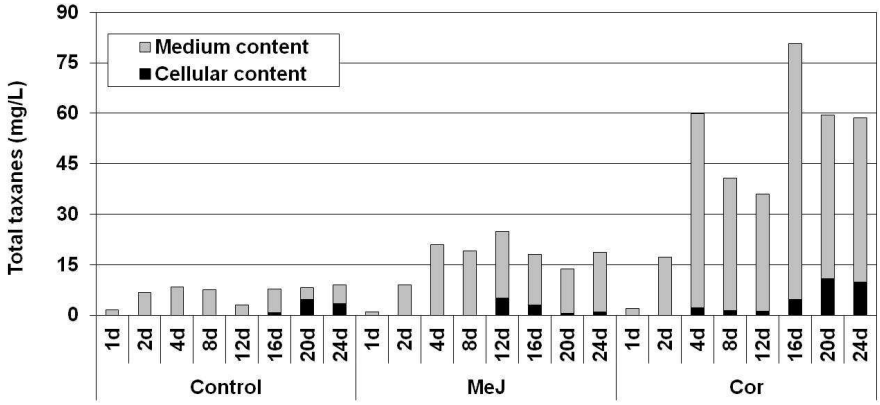

Figure 4.8: Capacity of taxane excretion from the producer cells to the medium in the TXS cell line, cultured for 24 days in control conditions and with the addition of 100 µM MeJ or 1 µM Cor. Medium content: Taxanes found in the culture medium; Cellular content: Cell-associated total taxanes.

The contents of 10-deacetyl baccatin III (DABIII), 10-deacetyltaxol (DAT), baccatin III, cephalomannine and taxol in the studied cell cultures were also determined individually (Figure 4.9). As shown in Figures 4.9A and B, the major taxane in the control and MeJ-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 MeJ-elicited cell cultures, respectively. This result was expected, since during the first days of culture, when the level of total taxanes is at its lowest, taxanes without a lateral chain are the most abundant, 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 *Taxus cuspidata* cell culture, both unelicited and elicited with 100 µM MeJ. In contrast, in Cor-treated cultures, DABIII was found at a very low level: 9 mg/L at day 16, which represented on average 7% of the total taxanes considered. This taxane is usually found in low proportions in highly productive *Taxus* cell cultures (Nims et al., 2006).

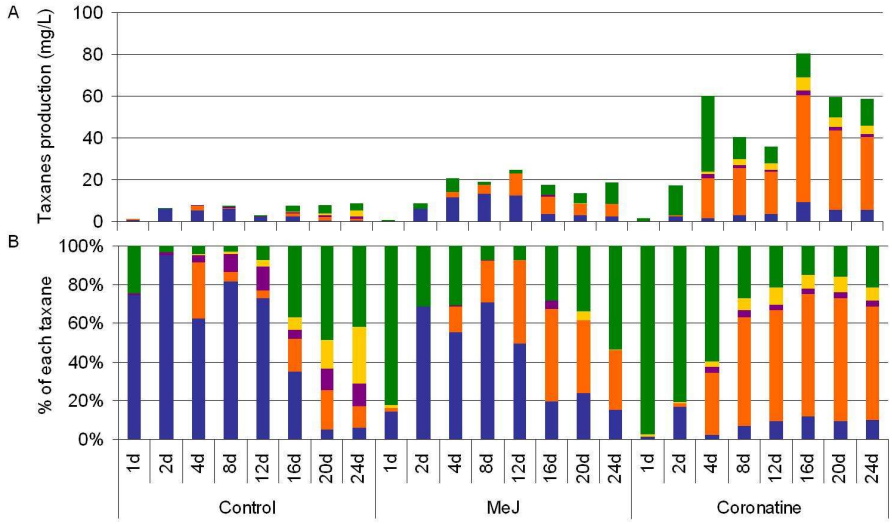

Figure 4.9: Total taxane content in the TXS cell line when maintained for 24 days in the optimum medium for production with and without the presence of elicitors. A, Taxane contents expressed as mg/L. B, Proportion of the taxanes determined, expressed as a percentage of the total taxane content. MeJ: 100 µM methyl jasmonate; Coronatine: 1 µM coronatine. Blue, DABIII; orange, Baccatin III; Violet, DAT; yellow, Cephalomannine; green, Taxol.

Baccatin III was the main taxane in the elicited cultures, especially when the total production was at its highest. Cell cultures treated with MeJ achieved a baccatin III production of 10.7 mg/L at day 12 (Figure 4.9A), 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 (Figure 4.9A), which represented more than 63% of the

total taxanes produced (Figure 4.9B). In control cultures, baccatin III was found in low or very low amounts throughout the experiment.

Taxol in the unelicited cultures increased with time, peaking at day 20, when production was almost 4 mg/L (Figure 4.9A). The highest taxol contents in the MeJ-treated cultures, almost 10 mg/L, were found at day 24. Taxol 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 taxol content reached 36 mg/L, and the second was at the end, when almost 13 mg/L taxol was obtained (Figure 4.9A). The main taxane determined (Figure 4.9B) was taxol, which was succeeded by baccatin III after 10 days of elicitation, as mentioned previously.

The content of DAT was not detectable or very low, both in the control and elicited cell cultures. Since no information about the biosynthesis of DAT is available, it is difficult to interpret this result. Cephalomannine was found in significant levels at day 24 and 8 under control and Cor conditions, corresponding to 2.6 mg/L and 6 mg/L, respectively. It is known that cephalomannine differs from taxol in having 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 this precursor in Cor-treated cultures was taxol, a small amount of the very high baccatin III content 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 still not understood.

When comparing the maximum levels of taxane production in the transformed cell line cultured in the three conditions, it can be seen that the content of baccatin III in the Cor-treated cultures was 21.6- and 4.8-fold higher than in the control and MeJ-treated cultures, respectively, whereas MeJ-elicitation increased baccatin III production 4.5-fold compared to the control. Taxol production under Cor treatment was 3.6- and 9.0-fold greater than in MeJ-treated and control cultures, respectively.

It is notable that the highest taxol level under Cor elicitation was obtained after only 4 days, with a productivity rate of 9 mg L^{-1} day^{-1}, whereas in MeJ-treated cultures the highest was achieved at the end of the experiment, 24 days after elicitation (0.4 mg L^{-1} day^{-1}). The time factor is particularly important when scaling up the culture for industrial production. Moreover, to our knowledge, this is the highest taxol productivity achieved by an academic laboratory.

Also worth emphasising is the high baccatin III level accumulated in the Cor-treated cultures (52 mg/L) at day 16, when the productivity rate reached 3.3 mg L^{-1} day^{-1}. This result is very important since new taxol-related compounds with improved efficacy and less toxicity are currently being sought, and most of them are obtained semi-synthetically from the natural precursor baccatin III (Expósito et al., 2009).

The improvement of taxol and baccatin III production by supplementing different *Taxus* cell cultures with MeJ has been repeatedly shown (Yukimune et al., 1996; Ketchum et al., 1999; Cusidó et al., 2002; Bentebibel et al., 2005). However, to our knowledge, this is the first time that a noticeable increase in taxol and baccatin III production has been reported after elicitation with coronatine.

4.4.4. Gene expression profile

With the aim of understanding the action mechanism of elicitors in taxane biosynthesis and the relationship between gene expression and the pattern of taxane production, changes in the transcript profiling of genes involved in the taxol biosynthesis were determined by qRT-PCR. The studied genes were: *TXS* (taxadiene synthase), *T13αOH* (taxadiene 13α-hydroxylase), both involved in early steps; *T7βOH* (taxane 7β-hydroxylase), *T2αOH* (taxane 2α-hydroxylase) and *DBAT* (10-deacetylbaccatin III-10β-*O*-acetyltransferase), which control intermediate steps, and *PAM* (phenylalanine aminomutase), *BAPT* (baccatin III-3-amino-13-phenylpropanoyltransferase) and *DBTNBT* (3'*N*-benzoyl transferase), which are involved in the last steps. TXS controls the first committed step of the taxol biosynthetic pathway (Hezari et al., 1995) and is highly regulated in cell suspension cultures (Onrubia et al., 2010). T13αOH hydroxylates taxa-4(20),11(12)-dien-5α-ol to taxa-4(20),11(12)-dien-5α-13α-diol (Jennewein et al., 2001) and is expressed most during the first hours after MeJ treatment (Nims et al., 2006; Chapter 2). In the intermediate steps of taxol biosynthesis, the enzymes T2αOH and T7βOH catalyse the hydroxylation of the 2C and 7C positions, respectively, of the taxane skeleton (Chau et al., 2004a and b). DBAT is the enzyme responsible for the conversion of 10-deacetylbaccatin III to baccatin III (Walker et al., 2000) and the codifying gene is activated under MeJ elicitation during the first 24h (Nims et al., 2006). In the final steps of the metabolic pathway, BAPT is responsible for binding the lateral chain to baccatin III, leading to taxanes with β-phenylalanine as a lateral chain

(Walker et al., 2002), and its gene expression is activated under MeJ treatment (Nims et al., 2006; Onrubia et al., 2010). PAM is necessary for the formation of the phenylisoserine lateral chain since it is responsible for the conversion of the amino acid α-phenylalanine to β-phenylalanine (Walker et al., 2004), being induce its transcription under MeJ elicitation for the first 18h (Nims et al., 2006). Finally, DBTNBT is involved in the last metabolic step leading to taxol (Long et al., 2008) (Figure 4.1). In order to standardize the results, we took as the reference value (gene expression = 1) the accumulation level of each gene mRNA at day 14 of culture in the growth medium (Figure 4.10).

In this study, the expression of the considered genes was determined from 1h to 4 days after elicitation. Subsequent transcript accumulation was not quantified because in previous studies we had observed that the highest expression was found in this period, decreasing thereafter (Expósito et al. 2010; Onrubia et al. 2010). Similar results have been achieved by other research groups (Nims et al. 2006; Hu et al., 2006).

The expression of the first gene involved in the taxol biosynthetic pathway, *TXS*, was greatly enhanced in the cell line by the presence of elicitors in the culture medium. The accumulation of *TXS* transcripts under MeJ-treatment started 12 h after elicitation, reaching a maximum at days 1 to 4. At their peak (day 4), *TXS* mRNA levels were 5.2 times higher in the MeJ-treated cultures than in the control. Under Cor, *TXS* mRNA levels clearly increased after 12 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. Although values were similar under both elicitors (140 and 130 times higher than the reference value) ($p<0.05$), it is notable that *TXS* mRNA levels peaked 3 days earlier in Cor-treated cultures.

Expression of the *T13OH* gene also increased after elicitation. As shown in Figure 4.10, transcript accumulation of this gene was significantly higher in Cor-treated cultures than in those supplemented with MeJ. At the same time, both cultures presented significantly higher *T13OH* mRNA levels than unelicited cultures. Expression under MeJ began 12 h after elicitation, peaking at day 4, while under Cor it increased from 12 h to day 2, decreasing thereafter. At their maximum, *T13OH* mRNA levels were 55.4-times and 82.8-times higher in the MeJ- and Cor-treated cells, respectively, than the reference value. Levels under Cor were 4-fold higher than under MeJ.

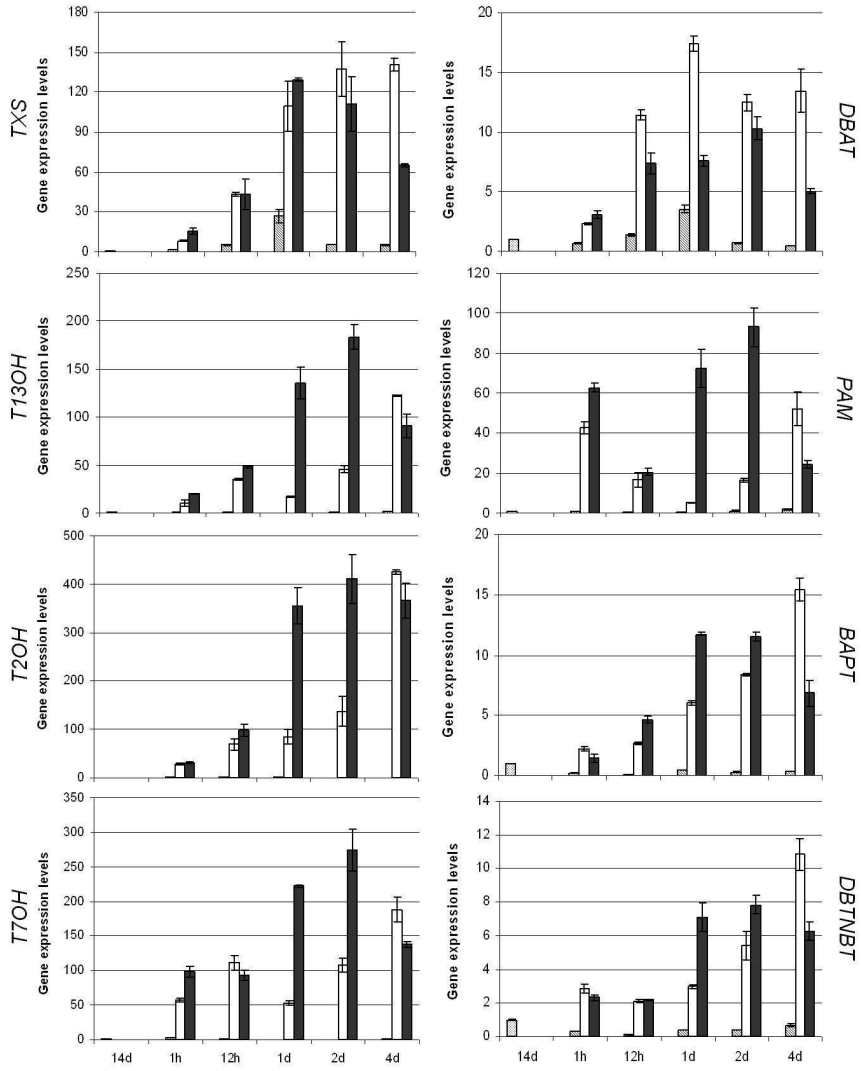

Figure 4.10: Gene expression in the TXS cell line maintained in the production media (PM) for 24 days with the three different treatments: Control, 100 µM MeJ and 1 µM Cor. Y-axis: Gene expression level compared to 14d in growth medium; X-axis, time of culture, 14d in growth medium (GM), posterior time points in PM with or without treatment. Dot boxes: GM; striped boxes: PM control; white boxes: PM with MeJ; black boxes: PM with coronatine. *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.

It is known that there is a branch point after the biosynthesis of taxa-4(20),11(12)-dien-5α-ol, which is the substrate either for an acetylation at C5 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. 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, the high levels of the *T13OH* gene observed in our TXS cell line could indicate that taxol biosynthesis, after elicitation with MeJ and to a higher extent with Cor, proceeds mainly through the step catalysed by the T13αOH enzyme. Nims et al. (2006) suggested a preference for the T13αOH-side of the branch pathway in elicited *T. cuspidata* cell cultures, confirming metabolic data (Ketchum et al., 2003) that show precursor flux leading to taxol is through the 5α,13α-diol via T13α-OH, rather than the 5α-yl acetate derived from the alternative branch controlled by the TAT enzyme.

The two genes involved in the intermediate steps of taxol biosynthesis encoding hydroxylases *T2αOH* and *T7βOH* showed very similar expression patterns, comparable with that of the aforementioned *T13αOH*. The hydroxylases codified by these three genes are Cytochrome P450-dependent enzymes with similar mechanisms of action. This probably explains why the *T. media* cells in this study showed a similar response to elicitation, that is, the highest transcript accumulation in the Cor-treated cultures was observed at day 2 and in MeJ-treated cultures at day 4.

At its highest, the expression level of the *T2αOH* gene in the cell cultures treated with MeJ or Cor was 425-times and 411-times higher, respectively, than the reference value, and 360 and 350 times higher, respectively, than the peak expression in unelicited cultures. When considering the effect of elicitation on the *T7βOH* gene expression, it can be observed that the maximum transcript accumulation was 188-times and 274-times higher in the cultures treated with MeJ and Cor, respectively, than the reference value, and 85- and 123-times higher, respectively, than in the control.

The expression level of the *DBAT* gene, responsible for the transformation of DABIII into baccatin III, in the unelicited cultures peaked after 1 day of culture, being 3.6 times higher than the reference value. Elicitation with MeJ resulted in a higher induction of the gene than with Cor, respective values being 17.4 and 10.3 times higher than the reference value. Levels of *DBAT* mRNA in MeJ-treated cell cultures were high at 12 h, increasing until day 1 and decreasing to values similar to those observed at 12 h until

the end of the study. Treatment with Cor also caused an increase in *DBAT* mRNA levels 12 h after elicitation, although in this case the highest value was observed at day 2, decreasing thereafter. The highest values corresponding to MeJ- and Cor- supplemented cultures were 5 and 3 times higher, respectively, than in the control.

PAM gene expression in the control cells increased from the beginning of the culture until day 4, whereas in the elicited cultures two peaks of expression were observed. The first occurred only 1 h after elicitation, with *PAM* transcript levels in the MeJ- and Cor-treated cultures being 43 and 63 times higher, respectively, than the reference value. The second peak, which was the maximum *PAM* mRNA level achieved, was observed at day 4 under MeJ and day 2 under Cor, 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 MeJ for the induction of the *PAM* gene.

The last two genes we studied encode two transferases: the *BAPT* gene and *DBTNBT* gene. Control cultures grown in the production medium presented only a slight increase in the expression of these two genes in the 4 days considered. When comparing elicitor treatments, the maximum expression level of both genes in the MeJ-elicited cultures was 1.3 times higher than the maximum observed in the Cor-supplemented cultures. However, the two genes were activated earlier under Cor, as shown in Figure 4.10. In MeJ-elicited cultures, the highest transcript levels of both genes were observed at day 4, while in the Cor-treated cells the highest levels were obtained at day 1 and maintained until day 4 after the elicitation.

When considering taxane production in relation with the expression of genes involved in their biosynthesis, it is striking that the highest expression was observed during the first 2-4 days after elicitation whereas the highest taxane production was achieved much later. Nims et al. (2006) reported that a few days after their expression, the transcripts of several genes were not found in the cells, indicating that enzyme activity may persist long after their cognate mRNAs are absent from the cells.

The expression of the *TXS* gene in cell cultures supplemented with MeJ or Cor was much higher than in control conditions. Previous studies have shown that although *TXS* controls the first committed step in the taxol biosynthetic pathway, it is not a limiting enzyme, since taxadiene is not a limiting substrate in taxol 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 polyhydroxylated intermediate, which is the substrate for the formation of DABIII and baccatin III. In our study, these three genes were the most receptive to induction by the assayed elicitors. Maximum transcription of *T13αOH*, *T2αOH* and *T7βOH* under Cor was 183-, 411- and 274-fold higher than the reference value and 122-, 425- and 188-fold higher under MeJ. It is also worth noting that the highest transcript levels occurred two days earlier under Cor than MeJ. This suggests that the substrates for the formation of the following taxanes were available earlier for the downstream metabolic steps in the cell cultures supplemented with Cor than in those treated with MeJ, consequently leading to the higher and earlier taxane accumulation observed in the Cor-elicited cells.

As mentioned before, the *DBAT* gene, responsible for the transformation of DABIII into baccatin III, was more effectively induced by MeJ than Cor, although in both cases the induction was much lower than for the aforementioned hydroxylases, only 17.4 and 10.3, respectively. However, the content of baccatin III in the Cor-treated cultures was much higher than in those elicited with MeJ. These results suggest that the *DBAT* transcript accumulation was enough for a high baccatin III production, and that the DBAT enzyme probably does not control a flux-limiting step. Consequently, the formation of the product baccatin III may depend more on the amount of precursors than on the quantity of the enzyme formed.

The next three genes studied are only involved in the biosynthesis of taxanes with a phenylisoserine lateral chain attached to the C13 of baccatin III. *PAM* was induced mainly 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 taxol in the Cor-treated cultures. The last two genes controlling taxol formation, *BAPT* and *DBTNBT*, were not induced to a great extent by neither MeJ nor Cor, the highest *BAPT* and *DBTNBT* transcript accumulation in MeJ-elicited cultures being only 15.4 and 10.8 times higher, respectively, than the reference value, and 11.8 and 7.8 times higher in the Cor-treated cells. This could explain the limited accumulation of taxol in the elicited cultures.

It is worth noting that in the Cor-treated cultures, when total taxane production was at its peak (days 16, 20 and 24 after elicitation), the average of 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 taxol or lateral chain-bearing taxanes, which is probably due to the low induction of the *BAPT* and *DBTNBT* genes by Cor. Conversely, the average baccatin III accumulation in MeJ-elicited cells was lower than the average accumulation of lateral chain-bearing taxanes. In this case, since the amount of the precursor baccatin III was low, and the expression of the *BAPT* and *DBTNBT* genes 4 days after elicitation was higher under MeJ than Cor, a sufficient amount of the corresponding enzymes had consequently been formed. It is also notable that when considering taxol contents, although the total taxane production was higher in the Cor-treated cells, the proportion of taxol among the total taxanes (Figure 4.9B) was higher in MeJ-treated cultures. This could reflect the aforementioned enhanced *BAPT* and *DBTNBT* expression levels in the MeJ cultures, particularly during the second part (days 16, 20 and 24).

Taking the results as a whole, it can be inferred that all the studied genes were induced to a lower or higher extent by the presence of the elicitors MeJ (100 µM) and Cor (1 µM). In general, elicitors notably increased the expression of the genes involved in the formation of both parts of the taxol molecule, the polyhydroxylated precursor of taxol and the phenylalanoil CoA chain, but their effect was more limited in the last steps of the taxol biosynthetic pathway (Figure 4.1). It is not clear if the genes were more actively induced by Cor, but their highest expression levels were observed earlier than under MeJ elicitation, except in the case of the *DBAT* gene. This earlier induction could be partly responsible for the higher total taxane production achieved in cultures treated by Cor rather than MeJ (3.3 times higher). It also suggests that the Cor action mechanism differs from that of MeJ, even though Cor structurally resembles the active jasmonate JA-Ile.

Studies carried out by Weiler et al. (1994) report that 1 µM Cor conditioned more accumulation of indole alkaloids by *Rawvolfia serpentina* cells than 100 µM MeJ. 100 nM Cor also elicited a maximum accumulation of isoflavones in cell cultures of *Glycine max*. 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- to 100-fold lower than for MeJ in *Eschscholtzia californica* cell cultures, species that have shown a several-hundred-fold rise in JA-levels, the most dramatic response to elicitation reported so far.

At a production and growth level, Uppalapati et al. (2005) demonstrated that a 10,000-fold higher concentration of MeJ was necessary to have the

same effect as Cor on anthocyanin content in tomato. Moreover, treatment with 2 to 2000 nmol of Cor had the same effect on root growth and anthocyanin accumulation (Uppalapati et al. 2005), demonstrating that a saturation occurs from 2 nmol Cor onwards in the indicated system..

The formation of the complex COI1–JAZ1 in *Arabidopsis* and tomato is stimulated by JA–Ile but not by jasmonic acid, methyl-JA (MeJ) or the JA precursor 12-oxo-phytodienoic acid (OPDA) (Thines et al., 2007), meaning that JA-Ile, or an analogue like Cor, could be more effective at switching on the JA pathway than another molecule that requires processing. For example, MeJ needs to be demethylated and conjugated to an aminoacid to become an active jasmonate. As Cor is a stable compound, in contrast with JA-Ile, it is able to keep active the SCF^{COI1}, which controls genome expression and promotes JA responses (Feng et al., 2003), allowing longer transcription processes.

Although it has been shown that JA-Ile and Cor have the COI1 protein as a receptor (Yan et al., 2009), the differing response we have obtained in our *T.* x *media* cell cultures elicited with Cor or MeJ could be explained by the existence of different metabolic steps downstream of the corresponding signalling pathway. Uppalapati et al. (2005) found that more genes in tomato leaf tissue responded to MeJ than to Cor treatment: out of 448 non-redundant genes differentially expressed after elicitation compared with an unelicited control, 42.8% were induced only by MeJ, 28.6% only by Cor and 28.6% by both treatments. However, since studies on Cor structure, mode of action and effects on secondary metabolism are still at an early stage, it is not possible to know which steps are controlled by this elicitor. Further research on the action mechanism of both elicitors, MeJ and Cor, is necessary in order to gain more insight into the activation or repression of other genes directly involved in taxol biosynthesis as well as genes encoding transcription factors, or other regulatory proteins.

4.5. Acknowledgements

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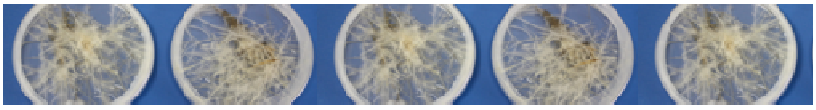

TAXIMIN: A NOVEL SMALL PEPTIDE FOUND IN *TAXUS BACCATA* RELATED WITH SECONDARY METABOLISM

5. TAXIMIN: A NOVEL SMALL PEPTIDE FOUND IN *TAXUS BACCATA* RELATED WITH SECONDARY METABOLISM

5.1. Abstract

Phytopharmaceuticals are compounds obtained from plant secondary metabolism that can be used as pharmaceuticals. One example is the powerful anticancer agent paclitaxel, a diterpene produced in species of yew (*Taxus* spp.). It has been shown in yew plant cell cultures that the vast majority of the known paclitaxel biosynthetic genes are sensitive to methyl jasmonate (MeJ), which allows production of this valuable metabolite to be increased. An unexplored alternative approach is to boost the biotechnological production of diterpenes by overexpressing genes that activate several biosynthetic steps. In previous studies we carried out a cDNA-AFLP analysis to compare the transcriptomic profile of high and low taxane-producing cell cultures, and obtained the sequence of previously undescribed genes related with high production. One of these new genes encoded the protein Taximin, which presented homology to known proteins without known domains. In the present study Taximin was functionally analysed by a BY-2 protoplast transient expression assay, and alkaloid production in Taximin-transformed tobacco hairy roots was studied. The results show that the overexpression of *Taximin* in the presence of MeJ clearly had a positive effect on secondary metabolism gene expression and alkaloid production.

5.2. Introduction

Plant secondary metabolism produces around 100,000 low weight molecules known as secondary metabolites (Dixon, 2001). Although these molecules are not important for maintaining the basic processes in the plant, they play a crucial role in plant interactions with the environment, defence and reproduction (Oksman-Caldentey and Inzé, 2004; Verpoorte and Memelink, 2002). Generally, these compounds represent less than 1% of the dry weight of each plant, and their quantity depends on the plant's physiological and developmental state. Secondary metabolites are characterized by a huge variety of complex structures that are usually

stored in specific cells or organelles, especially in the vacuoles. New medicinal uses for secondary metabolites are being found everyday, and many of them are already important pharmaceuticals, including paclitaxel (Taxol), an anticancer agent produced by *Taxus* spp.

Taxol is a complex diterpene with several rings and functional groups, and a modified lateral chain. Croteau et al. (2006) have postulated that its biosynthetic pathway is composed of 19 steps (Figure 5.1 and Chapter 1), from which 12 genes have been sequenced and functionally expressed, confirming their involvement in different modifications of the taxane core. Due to the diterpenic nature of taxol, the IPP and DMAPP precursors as well as the GGPP are formed in the plasts. At the same time, some of the sequenced genes have terminal tags that drive the corresponding protein to a determined organelle. Consequently, each kind of chemical modification (hydroxylation, acylation, etc.) occurs in different organelles, involving the movement of taxane precursors across the cell. The lack of knowledge about the relevant enzymes and their order of action, as well as about the genes that control the side-routes, flux-limiting steps, regulation mechanisms and genes involved in modifications that facilitate movement in the cell, highlights the need to find a general mechanism to easily activate taxol production.

Studies have shown that taxol production in *Taxus* sp. cell suspension cultures can be increased by elicitation, MeJ being the most effective elicitor known to date (Yukimune et al., 1996; Cusidó et al., 1999 and 2002; Expósito et al., 2010). Studies have also demonstrated the effect of MeJ on the expression of taxol biosynthetic pathway genes, including those that encode the first specific enzyme taxadiene synthase (TXS), several known hydroxylases (T13OH, T7OH or T2OH), and almost all the transferases involved in the pathway, including TBT, DBAT, PAM, BAPT, and DBTNBT (Nims et al., 2006; Expósito et al., 2010; Onrubia et al., 2010; Chapter 2 and 4).

Another mechanism for enhancing secondary metabolism production is the overexpression of genes encoding transcription factors that activate several genes of a biosynthetic pathway. Van der Fits and Memelink (2000) proved that ORCA3, an AP2 domain transcription factor from *Catharanthus roseus*, increases the expression of several genes involved in vinblastine biosynthesis (*TDC*, *CPR*, *STR*, *SGD* and *D4H*) and some primary metabolism genes related with precursors for this pathway (*AS* and *DXS*). Deluc et al. (2008) demonstrated that VvMYB5b, a MYB transcription factor from *Vitis vinifera*, can activate the promoters of several flavonoid biosynthetic genes (*VvCHI*, *VvF3'5'H*, *VvANS*) and

some prostaglandine genes (*VvLAR1*, *VvANR*) in *Vitis* and tobacco. Since current knowledge about the *Taxus* genome is limited, only one transcription factor from *Taxus cuspidata* has been described: TcAP2 with an AP2/EREBP (ethylene responsive element binding protein) domain (Dai et al., 2009), which increases its expression when *Taxus* seedlings are treated with NaCl, cold or MeJ with salicylic acid. No further work has been done linking this gene with secondary metabolism.

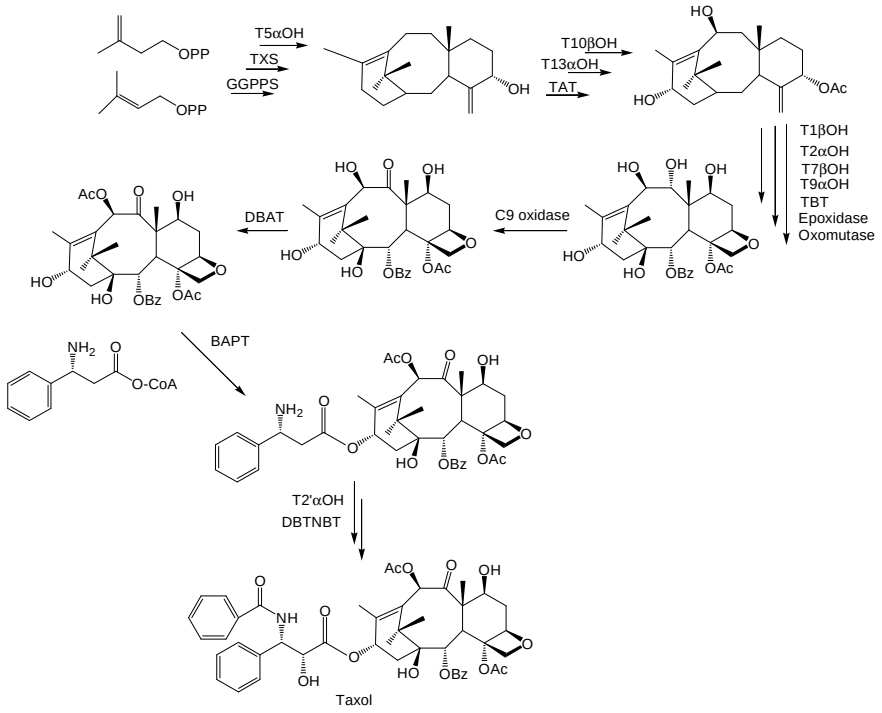

Figure 5.1: Brief depiction of the taxol biosynthetic pathway from IPP and DMAPP, including the first hydroxylation of the taxane core, the polyhydroxylated intermediate, the first commercially available intermediates 10-deacetylbaccatin III and baccatin III, the combination with the lateral chain β-phenylalanoyl CoA, which requires two more enzymes, a mutase (PAM) and a CoA transferase, and finally taxol. GGPPS, Geranylgeranyl diphosphate synthase; TXS, Taxadiene synthase; T5αOH, Taxane 5α-hydroxylase; TAT, taxadiene-5α-ol-*O*-acetyl transferase; T13αOH, Taxane 13α-hydroxylase; T10βOH, Taxane 10β-hydroxylase; T1βOH, Taxane 1β-hydroxylase; T2αOH, Taxane 2α-hydroxylase; T7βOH, Taxane 7β-hydroxylase; T9αOH, Taxane 9α-hydroxylase; DBAT, 10-deacetyl baccatin III-10-*O*-acetyltransferase; PAM, Phenylalanine aminomutase; CoA transf, CoA transferase; BAPT, baccatin III-3-amino, 13-phenylpropanoyltransferase; T2'αOH, Taxane 2'α-hydroxylase; DBTNBT, Debenzoyl taxol *N*-benzoyl transferase.

Although plant hormones mediate cell-to-cell interaction in higher plants, recent findings indicate that many secretory and non-secretory peptides are also involved in various aspects of plant growth regulation, including callus growth, meristem organization, root growth, leaf-shape regulation, nodule development, organ abscission, self-incompatibility and defence responses (Matsubayashi and Sakagami 2006; Butenko et al., 2009; and Marshall et al., 2011). One of the most studied signal peptides is systemin, which is synthesized as pro-systemin and shortly after a herbivore or pathogen attack is processed (Rocha-Granados et al., 2005). The attack initiates a defence signal cascade that activates jasmonate (JA) biosynthesis and subsequently the synthesis and release of direct (proteinase inhibitor proteins) and indirect defence substances (volatile organic compounds (VOCs)) (Sun et al., 2011; Corrado et al., 2007; Degenhardt et al., 2010). Systemin can activate the synthesis of VOCs, which are mainly terpenoids, demonstrating a link between this plant peptide and secondary metabolism. Phytosulfokine (PSK-α), another 5-aminoacid peptide, was tested as an elicitor in *Atropa belladona* hairy roots (Sasaki et al., 2001), resulting only in increased growth, and *Taxus* cell suspension cultures (Kim et al., 2006), in which it considerably enhanced taxol production but only when added simultaneously with MeJ (Kim et al., 2006).

In the cDNA-AFLP study of *Taxus baccata* cell suspension cultures, we studied genes differentially expressed under MeJ elicitation (Chapter 2). Several genes homologous to those encoding transcription factors and some completely unknown genes were obtained. The gene encoding Taximin, a small protein of 73 aminoacids, was isolated, sequenced and cloned from our cDNA-AFLP library. Taximin presents homology to predicted proteins from several organisms (*Vitis vinifera*, Arabidopsis, *Poplus trichocarpa*, *Ricinus communis* or *Glycine max*), none of them with known conserved domains or previously studied.

The differential expression pattern of *Taximin* in MeJ-elicited and unelicited cultures and the different taxane productivities led us to expect a link between *Taximin* and taxane production. In order to lay bare the relationship between *Taximin* and secondary metabolism, several assays were carried out. Due to the difficulty of obtaining transgenic *Taxus* material, tobacco was chosen as a model plant. A protoplast transient expression assay (De Sutter et al., 2005) was performed to test the effect of *Taximin* on the activity of several promoters of genes controlling taxol biosynthesis (promoters of taxadiene synthase and taxadiene-13α-hydroxylase genes), the pyridine alkaloid biosynthesis in *Nicotiana* spp. (promoters of putrescine *N*-methyltransferase and quinolinate

phosphoribosyltransferase genes) and the terpenoid indole alkaloids formation in *Catharanthus roseus* (promoter of the strictosidine synthase gene). In addition, Taximin expression and alkaloid production was screened in different hairy root lines from tobacco carrying the construction p35S::*Taximin*. We observed that *Taximin* can activate several secondary metabolism genes from yew, tobacco and *Catharanthus roseus*, and trigger an increase in alkaloid production in tobacco hairy roots.

5.3. Material and methods

5.3.1. Plant material

BY-2 tobacco (*Nicotiana tabacum* L.) cell suspensions were grown in BY-2 medium (Nagata et al., 1992) in the dark at 25ºC on an orbital shaker (150 rpm). A 5-ml aliquot of saturated culture was used weekly for incubation in fresh medium (100 ml in a 500-ml flask). The BY-2 medium was Murashige and Skoog (MS) (Murashige and Skoog, 1962) basal salts with minimal organics with 30 g/L sucrose, 0.2 g/L KH_2PO_4, pH 5.7, supplemented with 500 µg/L α-naphtaleneacetic acid and 50 µg/L kinetin at 25ºC on an orbital shaker (150 rpm).

Transgenic hairy roots were obtained from *Nicotiana tabacum* cv Xanthi plantlets, maintained *in vitro* on MS medium, supplemented with 30 g/L sucrose and pH 5.7 with a photoperiod of 16 h light and 8 h darkness at 25ºC and subcultured monthly.

5.3.2. Recombinant DNA constructs

Recombinant DNA constructs were obtained by the Gateway system (Invitrogen, Carlsbad, CA, USA). The Taximin-expression vector $p2B_1TaximinB_27$ was made by PCR amplification of the complete sequence tag TB595 (Chapter 2), using specific primers extended with the *att*B1 and *att*B2 recombination sites, transferred to pDONR221 by BP clonase and to p2GW7 or pK7WG2D by LR clonase. Once the expression constructs, P2GW7 and pK7WG2D, were obtained, they were introduced in *E.coli* and *Agrobacterium rhizogenes* LBA9402, respectively. Reporter vectors were generated with the promoters of Taxadiene synthase (*TXS*),

taxadiene-13α-hydroxylase (*T13OH*), putrescine *N*-methyltransferase (*PMT*), quinolinate phosphoribosyltransferase (*QPRT*) and Strictosidine synthase (*STR*). *TXS* and *T13OH* promoters (EMBL accession number EF153471, EF153469) were provided by GenScript (Piscataway, NJ, USA) in the pDONR221 vector and afterwards transferred to the destination vector pGWL7 (pm43GWfL7) by LR clonase, having obtained the expression vectors $pB_4PromoterB_3fL7$. The expression vectors with *PMT*, *QPRT* and *STR* promoters were generated previously (De Boer et al., 2011). Before being used in the experiments, the constructs were validated by amplification and sequencing using the specific universal primers M13 sense and reverse from the Gateway system (as described in the manufacturer's manual). All vectors used during the protoplast transient expression assays and in the hairy roots procedure are described in the table below (Table 5.1).

All plasmid DNAs used in the protoplast transient expression assay were prepared using the Plasmid Maxi Kit (Qiagen, Hilden, Germany) and DNA quantifications were performed with a ND-1000 Spectrophotometer (NanoDrop Technologies, Rockland, DE, USA).

Table 5.1: Vectors used during the protoplast transient expression assay. pro, promoter; ter, terminator; 35S, CaMV 35S; GCS, Gateway cloning site; rLUC, *Renilla* Luciferase ORF; fLUC, firefly Luciferase ORF; *TXS*, *Taxadiene synthase*; *T13OH*, *Taxadiene-13α-hydroxylase*; *PMT*, *Putrescine N-methyltransferase*; *QPRT*, *Quinolinate phosphoribosyltransferase*; *STR*, *Strictosidine synthase*.

Vector	Type		Composition	Reference
p2GW7	effector	destination	35Spro::GCS::35Ster	Karimi et al. (2002)
$p2B_1TaximinB_27$	effector	expression	35Spro::*Taximin*::35Ster	This work
$p2B_1GUSB_27$	Internal control		35Spro::*GUS*::35Ster	De Sutter et al. (2005)
$p2B_1rLB_27$	Internal standard		35Spro::*rLUC*::35Ster	De Sutter et al. (2005)
pm43GWfL7	reporter	destination	GCS::*fLUC*::35Ster	De Sutter et al. (2005)
pB_4TXSB_3fL7	reporter	expression	*TXS*pro::*fLUC*::35Ster	This work
$pB_4T13OHB_3fL7$	reporter	expression	*T13OH*pro::*fLUC*::35Ster	This work
pB_4PMTB_3fL7	reporter	expression	*PMT*pro::*fLUC*::35Ster	De Boer et al., 2011
pB_4QPRTB_3fL7	reporter	expression	*QPRT*pro::*fLUC*::35Ster	De Boer et al., 2011
pB_4STRB_3fL7	reporter	expression	*STR*pro::*fLUC*::35Ster	GenBank: Y10182
pK7WG2D	effector	destination	35Spro::GCS::35Ster	Karimi et al. (2002)
$pK7B_1TaximinB_22D$	effector	expression	35Spro::*Taximin*::35Ster	This work

5.3.3. Protoplast preparation

BY-2 protoplasts were prepared as described by De Sutter et al. (2005) from 100 ml 3-day-old BY-2 cell suspension cultures. Cells were poured into 2 x 50-ml tubes (Greiner Bio-One, Wemmel, Belgium) and stored on ice until they had settled down. The supernatant was removed and the soft cell pellet was mixed with a total of 100 ml cell wall lysis enzyme solution [1% cellulase Y-C, 0.1% pectolyase (Kyowa Chemical Products, Osaka, Japan), 0.4 M mannitol, 5 mM MES, pH 5.7, filter sterilized]. The mixture was incubated in a large Petri dish (145/20 mm, Greiner Bio-One) at 30ºC in the dark on an orbital shaker (Bellco Glass, Vineland, NJ, USA) at 60 rpm, and every 30 min a small fraction was observed under the microscope. Protoplasts were then transferred to 2 x 50-ml tubes and centrifuged at low speed (145 g; Eppendorf 5810R) for 4 min. After removal of the supernatant, the protoplasts were washed three times in 20 ml wash buffer (0.4 M mannitol, 2.5 mM $CaCl_2$, 1 mM MES, pH 5.7) via consecutive resuspension and centrifugation. Finally, the protoplasts were resuspended in MaMg solution (0.4 M mannitol, 15 mM $MgCl_2$, 5 mM MES, pH 5.7) with a variable volume of 12-20 mL to ensure a concentration of around $5x10^4$ to $5x10^3$ protoplasts/mL.

5.3.4. Automated protoplast transfection and lysis

All liquid handling of samples was performed on a Genesis Workstation 200 (Tecan, Mannedorf, Switzerland) robotic platform as previously described by De Sutter et al. (2005). Transfection pipetting steps were carried out with wide-bore liquid-sensing tips (WB-LS, 200 ll, Pure BioRobotix Tips, Genomics; Molecular BioProducts, San Diego, CA, USA); normal fine tips were used in other pipetting operations, either with or without liquid-sensing capability [FT-LS(1000) or FT(200); BIOplastics, Landgraaf, The Netherlands]. The procedure was performed in 48-well plates (flat bottom; Falcon, Becton Dickinson, Franklin Lakes, NY, USA) in which 6 different transfections with 8 replicates were performed. Solutions were dispensed successively as follows: 200 ng plasmid mix, 100 µL protoplast suspension, 120 µL PEG/$Ca2^+$ solution (40% Polyethylene glycol, 0.4 M mannitol, 0.1 M $Ca(NO_3)_2$, pH 6.0, filter sterilized) and 800 µL W5 dilution buffer (0.4 M mannitol, 154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 5 mM glucosa, 1.5 mM MES, pH 5.8, filter sterilized). After removal of the supernatant, the protoplasts were resuspended in 800 µL BY-2 medium with or without 100µM MeJ, and then incubated for 24 h at

23ºC with gentle shaking. The next day, the incubation buffer was removed and protoplasts were lysed in cell culture lysis reagent (CCLR) buffer (Promega) on the robot platform by mixing at high speed with aspiration/dispensing cycles. Finally, the lysate was transferred to 96-well Maxisorp reading plates (Nunc A/S, Roskilde, Denmark).

5.3.5. Dual luciferase assay

For each sample, firefly luciferase activity values were normalized with the luciferase activity derived from an internal standard plasmid encoding the *Renilla* luciferase gene under control of the 35S CaMV promoter. Both luciferase activities were measured subsequently on a LUMIstar Galaxy luminometer with double injectors (BMG Labtechnologies, Offenburg, Germany), using reagents from the Dual-Luciferase Reporter 1000 Assay System, as described in the provider's manual (Promega, Madison, WI, USA).

5.3.6. Establishment and culturing of hairy root lines

Leaves of *Nicotiana tabacum* cv. Xanthi plantlets grown as described previously (Plant Material section) were used for the establishment of hairy roots, after their infection with *Agrobacterium rhizogenes* LBA9402 carrying the pK7WG2D expression vector with *Taximin* or *GUS* under the control of the CaMV 35S promoter, and the *nptII* gene as a marker. The *Agrobacterium* was cultured on solid YEB medium supplemented with 100 mg/L spectinomycin, 300 mg/L streptomycin and 100 mg/L rifampicin in the dark at 28ºC during 48 h. Leaves were infected by wounding the mid-ribs with a sterile needle inoculated with the desired *Agrobacterium* strain. Hairy roots appeared 2-4 weeks after the infection and they were excised and cultured individually on MS medium supplemented with 30 g/L sucrose and 500 mg/L cefotaxime to eliminate the bacteria and 100 mg/L kanamycin for hairy root selection. For better growth, 1 ± 0.5 g of the root lines were grown in half-strength Gamborg's B5 salts and full strength Gamborg's vitamins (Gamborg et al. 1968) (B5/2) solid medium. Hairy root lines were kept in the dark at 25 °C and routinely subcultured every 3-4 weeks.

5.3.7. Elicitor treatment

2.5 ± 0.5 g of *N. tabacum* hairy roots p35S::Taximin and of the control were inoculated in plates with B5/2 solid culture medium and supplemented at the beginning of the culture with 100 µM methyl jasmonate. The cultures were kept in the dark at 25°C. Samples were taken after 8 days.

5.3.8. Polymerase chain reaction (PCR) analysis

The transformed nature of hairy root lines was checked by PCR. Genomic DNA was isolated from hairy root samples according to Dellaporta et al. (1983). PCR analysis was performed using the preformulated, predispensed single dose reaction 'Illustra PuReTaq Ready-to-go PCR beads' (GE Healthcare, Buckinghamshire, UK) with 500 ng DNA. Gene-specific primers were designed with Primer3 software version 0.4.0 (Table 5.2). PCR amplification of the *virD* gene was as follows: 5 min at 95ºC for denaturation, 35 cycles of amplification (45 sec at 95ºC, 45 sec at 58ºC, 45 sec at 72ºC) and finally 5 min at 72ºC. For the detection of the *rolC* gene, the template was denatured at 95ºC for 5 min followed by 35 cycles of amplification (20 sec at 95ºC, 40 sec at 60ºC, 1min and 30 sec at 72ºC) and 10 min at 72ºC. The *Taximin* gene was amplified with 95ºC for 5 min followed by 30 cycles of amplification (30 sec at 95ºC, 30 sec at 62ºC, 30 sec at 72ºC) and 5 min at 72ºC.

Table 5.2: Sequences of the primers used to amplify the genes by PCR.

Gene		Primer Sequence	Amplicon size	Reference
virD	Sense	5'-ATGTCGCAAGGCAGTAAGCCCA-3'	438	Hirayama et al., 1988
	Reverse	5'-GGAGTCTTTCAGCATGGAGCAA-3'		
rolC	Sense	5'-CTGTACCTCTACGTCGACT-3'	1136	Slightom et al., 1986
	Reverse	5'-TCAGTCGAGTGGGCTCCTTG-3'		
Taximin	Sense	5'-TGAGCTGCATATGTCCGTGTTGTA-3'	88	TB595 (cDNA-AFLP)
	Reverse	5'-TCGCATGATTTTGAGAGGAAGCTT-3'		

5.3.9. Alkaloid extraction and analysis

Nicotine alkaloids were extracted using a modified version of the protocol of Sheng et al. (2005). 100 ± 20 mg powdered lyophilized roots were resuspended in 200 µL 6% NH_3-H_2O and 4 mL methanol:dichloromethane in 1:3 (v:v), kept in an ultrasound bath for 15 min and filtered. After evaporation, the supernatant was washed 3 times with methyl *tert*-butyl ether:dichlormethane (MTBE:DCM) in 1:0.3 (v:v), evaporated again and kept at -20ºC. Finally, just before its analysis, the extract was resuspended in 400 µL MTBE:DCM 1:0.3 (v:v). The samples were determined using a Flame Ionization Detector (FID) Gas Chromatography system (Hewlett-Packard HP5890 Series II Plus). The analyses were performed on an RTX-35 amine column (30 m, 0.25 mm i.d. x 0.5 µm d.f.) using split mode, with 1mL/min of Helium flux and a programmed oven temperature from 60ºC to 240ºC, increasing by 10ºC/min. Injector and detector temperatures were kept at 270ºC. Aliquots of 1 µL were injected by a split-splitless 6890 AutoSampler (Agilent Technologies). The nicotine alkaloids were identified by comparing retention times with the standards: Nicotine, Nornicotine (LGC Promochem, Barcelona, Spain), Anabasine (Chromadex, Irvine, CA, USA) and Anatabine (TransMIT, Marburg, Germany).

5.3.10. Quantitative real-time PCR

RNA was isolated using the "REAL ARNzol SPIN KIT" (REAL, Valencia, Spain), cDNA was prepared from 1 µg of RNA with SuperscriptII reverse transcriptase (Invitrogen, California, USA) and qRT-PCR was performed using 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 5.3) 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 normalized to the levels of the *β-ATPase* from *Nicotiana tabacum*.

Table 5.3: Sequences of the primers used to amplify the genes by quantitative real-time PCR.

Gene		Primer Sequence	Reference
β-ATPase	Sense	5'-CCATCAACACCACCGAAGTCC-3'	Häkkinen et al., 2007
	Reverse	5'-GATGACCTGGCACACCTTCC-3'	
PMT	Sense	5'-TGGATGGAGCAATTCAACA-3'	Häkkinen et al., 2007
	Reverse	5'-AACCAATTCCTCCGCCGATG-3'	
QPRT	Sense	5'-ATACGGAGGGCTTCAGGAAATG-3'	Häkkinen et al., 2007
	Reverse	5'-GTCAAGTGCTTTCACGGAATGC-3'	
Taximin	Sense	5'-TGAGCTGCATATGTCCGTGTTGTA-3'	TB595 (cDNA-AFLP)
	Reverse	5'-TCGCATGATTTTGAGAGGAAGCTT-3'	

5.3.11. Statistics

The statistical analysis was performed with Statgraphics (Centurion XV) and Excel software. The average of 3 to 6 samples ± SD was used for all data. 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.

5.4. Results and discussion

5.4.1. Molecular cloning and sequence analysis

TB595, referred to as Taximin, was selected from all the differentially expressed tags obtained by cDNA-AFLP because of its expression pattern. In *Taxus baccata* cell suspension cultures it was induced for a longer period under MeJ than in non-elicited conditions (Figure 5.2). The complete sequence (Box 5.1) was achieved by RACE PCR (Chapter 2) and the corresponding translated protein was deduced by GeneTool Lite software (Box 5.1). The interest of the *Taximin* gene lay in the small size of its transcript (222 nucleotides), the translated peptide, 73 amino acids, and a lack of similarity to proteins with known domains.

According to the parameters analyzed by Signal peptide v4.0 (Petersen et al., 2011), *Taximin* is likely to have a signal peptide that comprises the first 27 AA, starting at the mature protein in V28 (Figure 5.3). The *C-score*,

which indicates probable AA involved in the cleavage site, is over the cut-off for A24, A27 and V28. At the same time, the *S-score*, with values over the cut-off for signal peptide AA, is high until C40, and the *Y-score* is high for the positions with a higher *C-score*. Moreover, the *Y-max*, which is a better parameter than the *C-score* for predicting the unique cleavage site, as it combines the *C* and *S-scores*, pointed to position 28 as the cleavage point. Finally, the *S* and *D-mean* parameters are over the cut-off for positions between the N-terminal and the end of the signal peptide (A27), supporting the idea that the signal peptide goes from the N-terminal to A27, V28 being the first AA from the mature peptide. The destination of that protein is not clear, as the plasma membrane (PSORT -Nakai and Kanehisa, 1991-) and vacuole (WoLFPSORT -Horton et al., 2006-) were predicted as the most probable subcellular destinations, but chloroplast and endoplasmic reticulum were also picked up by WoLFPSORT.

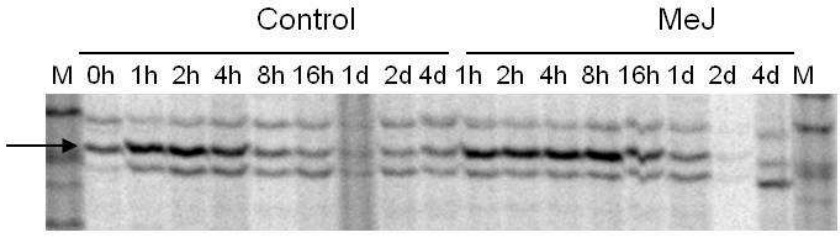

Figure 5.2: cDNA-AFLP acrylamide gel image. *Taximin* is signalled with an arrow. First and last lanes correspond to Marker (M).

```
atgggggagtgcagaccattgggatttcttttgggcctgccctttgcat
tgttatctctcgtttttgctgctgtgggtgcagtcgtctggatcttagg
gacattgttgagctgcatatgtccgtgttgtatatgcttttctggcctg
gcaaacatggcggtgggcctcatgaagcttcctctcaaaatcatgcgat
ggtccattcatcaaataccctgctga

MGECRPLGFLLGLPFALLSLVFAAVGAVVWILGTLLSCICPCCICFSGL
ANMAVGLMKLPLKIMRWSIHQIPC*
```

Box 5.1: Nucleotide and deduced protein sequence of Taximin. In bold the 7 cysteines from the pro-peptide.

Taking into account the presence of a signal peptide, the mature Taximin has 46 AA, of which 6 correspond to cysteines and 24 are hydrophobic AA. The small size and lack of homology to known domains suggest that Taximin is a pathogenesis-related peptide, and although highly

hydrophobic, it is probably a cysteine-rich peptide (CRP) as it has less than 160 AA, a signal peptide in the N-terminal and contains between 4 and 16 cysteines in the C-terminal (Marshall et al., 2011).

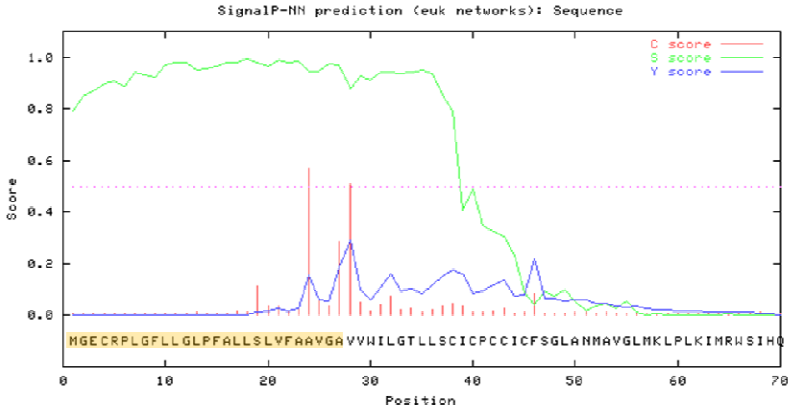

Figure 5.3: Figure obtained by Signal peptide 4.0, showing the different parameters that evaluate the presence of signal peptides in peptide sequences. The predicted signal peptide is highlighted in yellow. The aminoacids from Taximin are represented in 1 letter code.

It has previously been described that CRPs are produced as a front-line defence in peripheral cell layers of nutrient-rich structures such as flowers or seeds and are induced by various defence or stress-signalling pathways, particularly the jasmonate/ethylene pathway (Silverstein et al., 2007). As well as an active microbicidal defence function, reproductive regulatory roles have been observed, as in the case of the stigma-specific STIG1 (Goldman et al., 1994) or defensin-like S-locus cysteine-rich (SCR) (Schopfer et al., 1999; Takayama et al., 2000) proteins and other functions related to plant growth regulation and development, such as the rapid alkanization factor (RALF) proteins (Pearce et al., 2001), lipid transfer protein (LTP)-like xylogens (Motose et al., 2004) or the gibberellin-stimulated GASA/GAST family of proteins (Aubert et al., 1998). According to Silverstein et al. (2007), although the number and diversity of characterized plant CRPs is large, it is thought that many more members of this family remain to be discovered. Thus, as Taximin is a small peptide, possessing a signal peptide and 6 cysteines, and is transcribed for a longer period under MeJ elicitation, it could be included in a new family of proteins. The results obtained in the assays planned in this work should clarify the functionality of this peptide and its involvement in the

general secondary metabolism and, concretely, in the taxol biosynthetic pathway.

5.4.2. Promoter analysis

In order to identify the potential of Taximin to regulate the taxol biosynthetic pathway, we studied its effect on the promoter activity and consequently on the transcription level of genes highly inducible by MeJ. The taxol biosynthetic genes selected were those encoding taxadiene synthase (*TXS*) and C13 hydroxylase (*T13OH*). Previous work on *Taxus* cell suspension cultures has shown how MeJ elicitation increases taxane production (Yukimune et al., 1996; Cusidó et al., 1999 and 2002; Expósito et al., 2010, Chapters 2 and 4). Also, when the expression of taxol biosynthetic genes was analyzed, a high expression of *TXS* and *T13OH* was observed under MeJ elicitation (Nims et al., 2006; Onrubia et al., 2010; Chapters 2 and 4), suggesting a relationship between the activation of gene transcription by MeJ and a clear increase in taxane production. When *Taxus baccata* cell suspension cultures were analyzed, the maximum expression of the *TXS* and *T13OH* genes took place 2 days and 12 h after MeJ elicitation, respectively (Onrubia et al., 2010; Chapters 2 and 4).

To test Taximin as a general regulator of secondary metabolism, we also used promoters of genes encoding enzymes involved in the biosynthesis of other secondary compounds characteristic of other plants. The putrescine *N*-methyltransferase (PMT) protein catalyses the first committed step in nicotine biosynthesis. In BY-2 cells, *PMT* transcription is induced early, within 1–2 h of jasmonate elicitation (Goossens et al., 2003), and is an excellent reporter for nicotine biosynthesis (De Sutter et al., 2005). Anatabine has been found to be a major constituent in MeJ-elicited BY-2 cell suspension cultures, (Goossens et al., 2003). The first specific enzyme related with anatabine is quinolinate phosphoribosyltransferase (QPRT), whose corresponding gene increased its expression at 2 h to 1 day after the addition of MeJ to the BY-2 cell cultures (Häkkinen et al., 2007). The biosynthetic pathway of terpenoid indole alkaloids (TIA), secondary metabolites from *Catharanthus roseus,* is well known. Strictosidine synthase (STR) is the enzyme coupling the iridoid glycoside secologanin and tryptamine to form strictosidine, the central intermediate in the biosynthesis of TIA (El-Sayed and Verpoort, 2007). In *Catharanthus roseus* cell suspension cultures, *STR* gene

expression is inducible by MeJ, increasing at 2 h after the treatment and reaching a peak at 4-8 h (Menke et al., 1999).

With the aim of studying the activity of the peptide Taximin in the regulation of the secondary metabolism in the aforementioned pathways, a protoplast transient expression assay with Bright-Yellow 2 (BY-2) tobacco cells was performed. *Taximin* ORF was cloned in a p2GW7 to test the effect of the cognate protein production on *TXS, T13OH, PMT, QPRT* and *STR* promoters, which control the expression of the firefly *luciferase* gene reporter, when co-transfected in BY-2 protoplasts. The screening consisted of 8 replicates for each combination of one of the five promoters and *Taximin*. These assays were performed with an internal standard and a GUS control in multiple 48-well plates (as indicated in Table 5.4). Plasmid DNA mixes, PEG transfection, cell washes, and incubation with 100 µM MeJ or DMSO were completed on day 1; cell lyses and luciferase measurements were performed on day 2. Reporter activity is presented as normalized LUC activity between firefly luciferase and *Renilla* luciferase. The experiments are summarized in Table 5.4.

Table 5.4: All programmed assays with the negative control GUS or Taximin and the reporter genes with the 5 promoters considered. Boxes in grey: negative control; boxes with vertical stripes: mock condition. DMSO: dimethylsulfoxide, MeJ: 100 µM methyl jasmonate, fLUC: firefly luciferase, pro: promoter, *TXS*: *Taxadiene synthase*, *T13OH*: *taxane-13α-hydroxylase*, *PMT*: *putrescine N-methyltransferase*, *QPRT*: *Quinolinic acid phosphoribosyltransferase*, *STR*: *strictosidine synthase*.

Constructions	Reporter genes	Internal standard	DMSO	MeJ
35Spro::*TB595*::35Ster	*TXS*pro::*fLUC*::35Ster	35Spro::*rLUC*::35Ster	|||	
35Spro::*GUS*::35Ster			|||	
35Spro::*TB595*::35Ster	*T13OH*pro::*fLUC*::35Ster	35Spro::*rLUC*::35Ster	|||	
35Spro::*GUS*::35Ster			|||	
35Spro::*TB595*::35Ster	*PMT*pro::*fLUC*::35Ster	35Spro::*rLUC*::35Ster	|||	
35Spro::*GUS*::35Ster			|||	
35Spro::*TB595*::35Ster	*QPRT*pro::*fLUC*::35Ster	35Spro::*rLUC*::35Ster	|||	
35Spro::*GUS*::35Ster			|||	
35Spro::*TB595*::35Ster	*STR*pro::*fLUC*::35Ster	35Spro::*rLUC*::35Ster	|||	
35Spro::*GUS*::35Ster			|||	

When MeJ-elicited and mock cultures were compared (Figure 5.4), it could be observed how the normalized LUC activity of the negative controls (grey boxes in Table 5.4) differed according to the promoters analyzed. When *GUS* was co-transfected with *TXS*, *PMT*, and *STR* constructions and elicited, LUC activity presented a slight increase of 1.7, 2.0, and 2.0-fold respectively, while with *QPRT* the increase was 13.3-fold. In contrast, LUC activity was reduced 0.7-fold in the *T13OH-GUS* co-transfection and several independent assays confirmed the reduction of LUC activity in that combination.

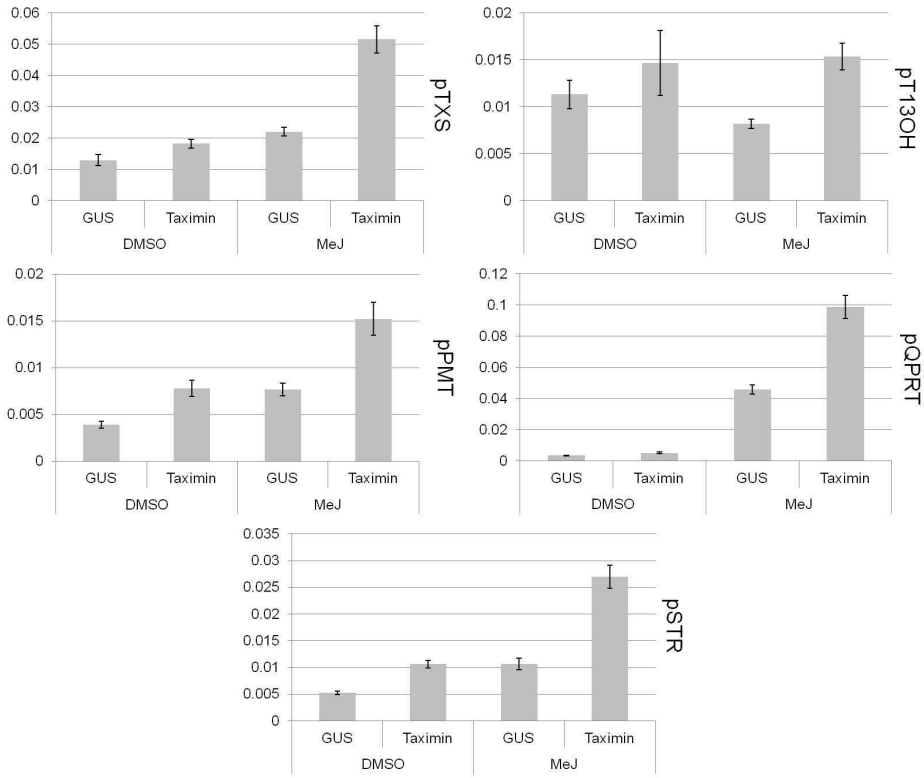

Figure 5.4: Protoplast transient expression assay in BY-2 tobacco protoplast comparing the negative control, *GUS,* and *Taximin* with DMSO (negative condition) or MeJ. Y-axis: Normalized LUC activity. DMSO: dimethylsulfoxide, LUC: luciferase, p: promoter, *TXS*: *taxadiene synthase*, *T13OH*: *taxane-13α-hydroxylase*, *PMT*: *putrescine N-methyltransferase*, *QPRT*: *Quinolinic acid phosphoribosyltransferase*, *STR*: *strictosidine synthase*. Values are mean of ± SE of 8 samples.

For each tested promoter the normalized LUC activity increased when the *Taximin* gene was used instead of *GUS* and there was no elicitation (Table 5.4, boxes with vertical stripes). The increase ranged from 1.4, 1.5,

2.0 and 2.0-fold for *TXS, QPRT, PMT* and *STR* gene promoters, respectively (Figure 5.4). In the co-transfection of *Taximin* and the promoters (white boxes in Table 5.4), there was an increment of normalized LUC activity when MeJ was applied instead of DMSO, ranging from 1.9, 2.5 and 2.8 to 19.3-fold when using *PMT, STR, TXS* and *QPRT* promoters, respectively.

When comparing the normalised LUC activity for each promoter under MeJ (Table 5.4, boxes without stripes), *Taximin* doubled the signal compared to *GUS*, with the increase ranging from 2, 2.2, 2.3 to 2.5-fold in the *PMT, QPRT, TXS and STR* gene promoters, respectively. The increase of the normalised LUC activity was notable when the samples corresponding to each promoter in GUS-mock conditions were compared with those carrying the *Taximin* gene elicited with MeJ. The LUC activity increased 3.9, 4.0, 5.1 and 28.7-fold for the *PMT, TXS, STR* and *QPRT* gene promoters, respectively.

Transfections with *T13OH*pro::*fLUC* generally gave different results from those obtained with the other 4 promoters studied. In the co-transfection of the promoter with *GUS*, *T13OH* promoter did not increase transcription under MeJ compared to mock conditions. Neither was there an increment of normalized LUC activity when *Taximin* was co-transfected with *T13OH* promoter.

From these results we could infer that the presence of the peptide Taximin increased the activity of the *TXS, PMT, QPRT* and *STR* gene promoters, although the greatest enhancement of promoter activation was observed in *Taximin* transfected cells elicited with MeJ, highlighting the synergism between the presence of Taximin and MeJ elicitation.

5.4.3. Transgenic characterization, growth of tobacco hairy roots and elicitor treatment

Several studies have shown that *Nicotiana* spp. is an appropriate model plant for studying the functionality of foreign genes, either by their overexpression under the control of the 35S promoter or their down-regulation by RNA interference (Palazón et al., 1998; Shoji et al., 2008; Lackman et al., 2011). In the present study, *Nicotiana tabacum* leaf disks were infected with *A. rhizogenes* LBA9402 carrying the plasmid pK7WG2D with the *Taximin* gene (HR) or with *GUS* (HR C-) under the control of the CaMV 35S promoter. Hairy roots appeared after 2-4 weeks,

were excised 3-4 cm from the apex and cultured individually on MS medium supplemented with 30 g/L sucrose and 500 mg/L cefotaxime (to eliminate the bacteria), as well as 100 mg/L kanamycin in the case of hairy roots obtained after infection with transgenic bacteria. Polymerase chain reaction with primers for the *rolC* gene confirmed the integration of the T-DNA fragment of the Ri plasmid and the presence of the *Taximin* gene confirmed the integration of the T-DNA fragment of the pK7WG2D vector (Figure 5.5). Three control (C-1, C-2 and C-11) and seven transgenic (L1, L6, L7, L9, L10, L11 and L12) root lines were selected.

After several subcultures hairy root lines were grown on B5/2 solid medium and presented characteristic traits of high branching and plagiotropic growth (David et al., 1984). However, in the case of root lines carrying the *Taximin* gene, visualization of morphological traits such as length of secondary roots or branching showed a slow growth rate when compared with control lines (Figure 5.6).

Since the results of the protoplast transient expression assay showed that the studied secondary metabolite biosynthetic gene promoters were most active when the *Taximin* gene-carrying cells were elicited with MeJ, the root lines were also elicited with the same concentration of this elicitor (100 µM). For the elicitation assay, 2.5 ± 0.5 g of *N. tabacum* hairy roots carrying the *Taximin* gene and the control roots were inoculated into plates with B5/2 solid culture medium and supplemented with 100 µM MeJ or ethanol, respectively, at the beginning of the culture. At day 8 the root lines grown under MeJ reduced their growth and root apexes started to be necrotic (Figure 5.7A).

The most notable differences in biomass production observed in the hairy root assay were related with the presence of MeJ in the medium. Not only were morphological traits affected, but the fresh and dry weights also decreased significantly. After 8 days on B5/2 solid culture medium, non-elicited hairy roots increased their weight by around 116±19%, while when hairy roots were supplemented with MeJ the fresh weight was reduced by 70±8% compared to mock-grown hairy roots. When dry weight was measured after 8 days of culture (Figure 5.7B), the MeJ-grown hairy roots had decreased their weight by 75±3% compared to the weight in the mock medium, except for lines HR 7, 9 and 11, which decreased by 56±4%.

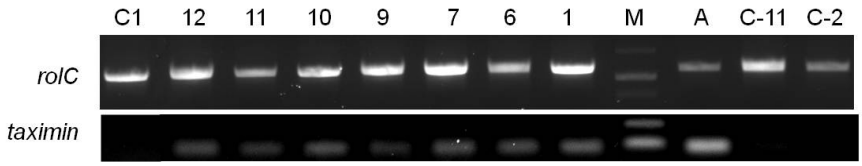

Figure 5.5: Molecular analysis of obtained hairy roots. PCR for analysing the presence of the *rolC* and *Taximin* genes in transgenic hairy root lines. 1, 6, 7, 9, 10, 11 and 12 correspond to hairy roots carrying 35Spro::*Taximin*; M, marker; A, *A. rhizogenes* LBA9402; C-1, C-2 and C-11, negative control lines.

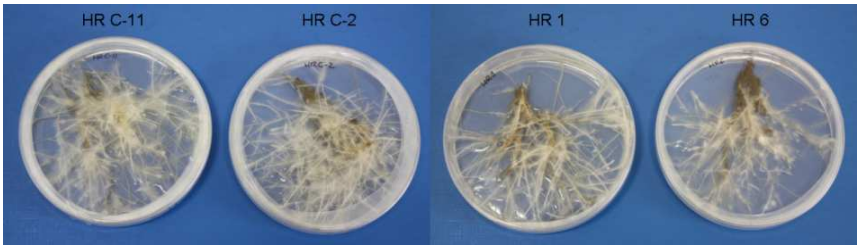

Figure 5.6: 20 day-old hairy roots carrying the 35Spro::*Taximin* construction (HR 1 and 6) and control (HR C-2 and 11) lines grown on solid B5/2 medium.

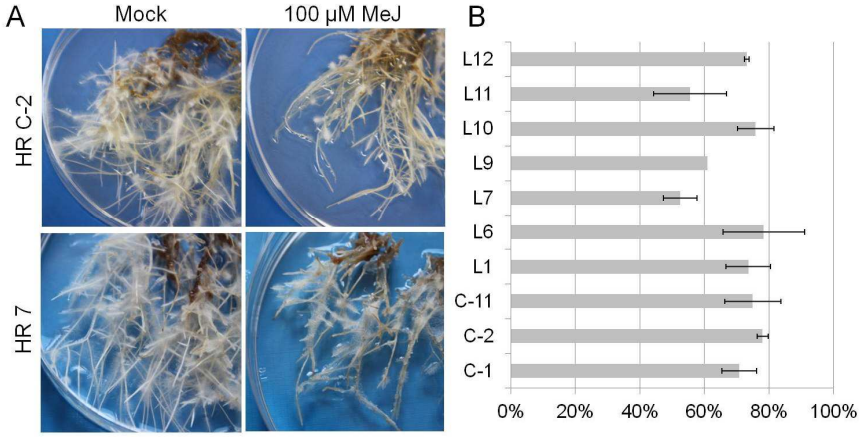

Figure 5.7: Differences in growth of hairy roots cultured on B5/2 supplemented with 100 µM MeJ or ethanol. A) Morphological traits from HR 7 (hairy root line 7 obtained by infection with *A. rhizogenes* LBA9402 with pK7WG2D-*Taximin*) and HR C-2 (control line 2 obtained by infection with *A. rhizogenes* LBA9402). B) % decrease of dry weight when comparing hairy roots grown in mock and MeJ medium. Values are mean ±SD of 3 to 6 samples.

Comparing control (HR C-) and *Taximin*-expressing lines (HR), there were no significant differences in the increase or reduction of fresh and dry weight, except, as indicated previously, in the lines HR 7, 9, 11, whose growth was clearly lower. The reduction of growth caused by the addition of MeJ to the medium is a detrimental effect of the elicitation on biomass production observed in other hairy root systems. In previous studies we found that *Panax ginseng* hairy roots reduced their growth more than 4-fold when elicited with the same concentration of MeJ (Palazón et al., 2003), although the ginsenoside production was enhanced. *Taxus media* hairy roots also significantly reduced their growth when treated with MeJ, while the taxol production increased dramatically (Syklowska-Baranek et al., 2009).

5.4.4. Alkaloid production in hairy roots from tobacco

Hairy roots growing on B5/2 solid culture medium supplemented with 100 µM MeJ or ethanol (control conditions) were harvested after 8 days, and immediately frozen and lyophilized until the alkaloid extraction. Nicotine and nornicotine are the main alkaloids in tobacco plants, but anabasine and anatabine were also determined (Figure 5.8). Although maximum alkaloid accumulation in transformed hairy roots is usually observed after 28 days of cultivation (Jouhikainen et al., 1999), day 8 was chosen for sample collection and alkaloid profiling due to the reduced growth under MeJ conditions.

As reported previously (Häkkinen et al., 2005; Palazón et al., 1998), nicotine is the main alkaloid found in tobacco hairy roots. In the present study, nicotine levels in all root lines were much higher than any other studied alkaloid, followed by anatabine or anabasine, depending on the line (Figures 5.9A and B), whereas nornicotine was only detected in traces. Anabasine was also found only in minor amounts, most probably due to its rapid metabolization to anatalline (Goossens et al., 2003). As observed in previous studies (Palazon et al., 1998; Häkkinen et al., 2005), production varied greatly between the different hairy root lines since each one responds to a different transformation event. The addition of the elicitor MeJ to the culture medium not only affected hairy root growth, as described previously, but also generally caused a significant increase in the production of the alkaloids nicotine, anabasine and anatabine.

Comparing the transgenic lines that constitutively expressed the gene encoding the peptide Taximin with the control lines, we found important

differences in alkaloid production. As shown in Figure 5.9A, the nicotine level clearly reflect the total alkaloid contents found in each root line, since this alkaloid was produced in quantities 30-50 times higher than the other alkaloids studied. The differences in total alkaloid production among the control root lines and those overexpressing the *Taximin* gene were not statistically significant and followed the usual production variability found in different hairy root lines established from the same plant. However, the addition of MeJ to the culture medium conditioned a high increase of total alkaloid production, and this effect was especially remarkable in the root lines carrying the gene encoding the peptide Taximin.

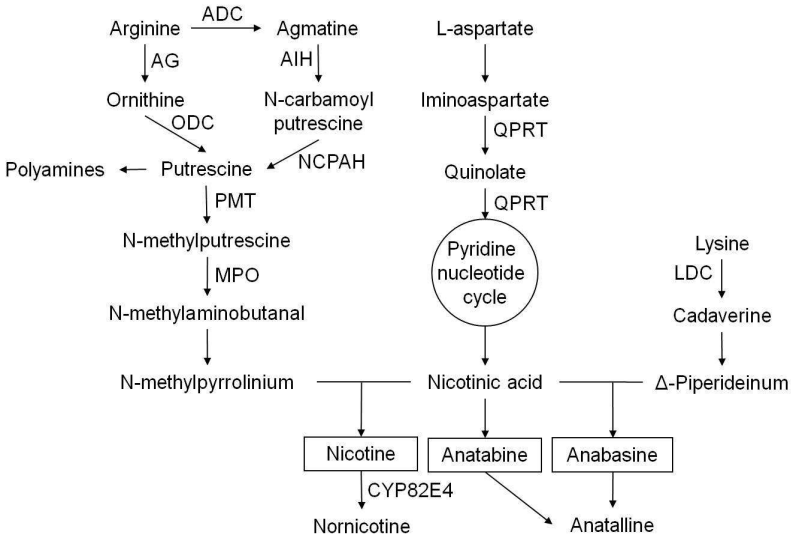

Figure 5.8: Pyridine alkaloid biosynthesis with known enzymes and intermediates in *Nicotiana tabacum.* The four analyzed alkaloids are inside boxes. ADC, arginine decarboxylase; AIH, agmatine deiminase; NCPAH, *N*-carbamoylputrescine amidohydrolase, AG, arginase; ODC, ornithine decarboxylase; PMT, putrescine *N*-methyltransferase; MPO, methylputrescine oxidase; QPRT, quinolinate phosphoribosyltransferase; LDC, lysine decarboxylase; CYP82E4, nicotine *N*-demethylase.

The nicotine levels found in the elicited transgenic Taximin root lines ranged from 4.5 mg/g DW (root line HR 7) to 9.7 mg/g DW (root line HR 9), the overall average production being 6.3 mg/g. The nicotine production in the elicited control root lines ranged between 2.7 and 4 mg/g DW, with an average of 3.5 mg/g DW. The nicotine levels in the elicited root lines HR 1, HR 6 and HR 11 was notable, being more than 4 times higher than in the same root line grown without elicitation. Root lines HR 9 and HR 10 increased their nicotine contents 3.5-fold when treated with the elicitor. The improvement in nicotine production caused by MeJ in root lines HR 7

and HR 12 was lower (2.5-fold) when compared with the same root lines grown in control conditions. Taking these results as a whole, we can observe that the improvement of nicotine production in response to elicitation was significantly higher in the *Taximin* root lines, in which the content of this alkaloid increased, on average, 3.6-fold after elicitation, compared to only 2.1-fold in the control. These results clearly showed that the response to MeJ elicitation was much higher in the root lines transgenic for the *Taximin* gene than in the control roots.

The other alkaloids studied (anabasine and anatabine) were only found at very low levels in the hairy root lines: no more than 0.15 mg/g DW in the case of anabasine, and 0.5 mg/g DW in the case of anatabine. As previously mentioned, nicotine is the main alkaloid in tobacco transformed roots, and the others generally accumulate to a very low extent. As with nicotine, the addition of MeJ to the culture medium caused a significant increase in anabasine and anatabine production in all the root lines (both control and those producing Taximin). However, also as with nicotine, their elicitation-enhanced levels were greater in the root lines transgenic for *Taximin* than in the controls. Whereas the average increase of anabasine and anatabine in the elicited control root lines was 2- and 1.7-fold higher than in non-elicited control roots, in the MeJ-treated *Taximin* root lines it was 3- and 5.5-fold higher, respectively.

As described previously, *Taximin* is a gene found in *T.baccata* cell cultures after elicitation with MeJ, whose presence was determined after the cDNA-AFLP studies. Although this gene has not been reported in any other plant cell cultures, the presence and activity of other proteins that act as master regulators in secondary metabolite production have been studied. Van der Fits et al. (2001) described the presence of *Orca 3* in *Catharanthus roseus* (L.) G. Don cell cultures, a gene encoding an AP2/ERF-domain transcription factor that acts as a master regulator of primary and secondary metabolism. More recently, Perrin et al., (2008) described the nuclear master regulator LaeA in *Aspergillus nidulans* cells that control 20% to 40% of major classes of secondary metabolite biosynthetic genes such as *nonribosomal peptide synthetases* (*NRPSs*), *polyketide synthases*, and *P450 monooxygenases*.

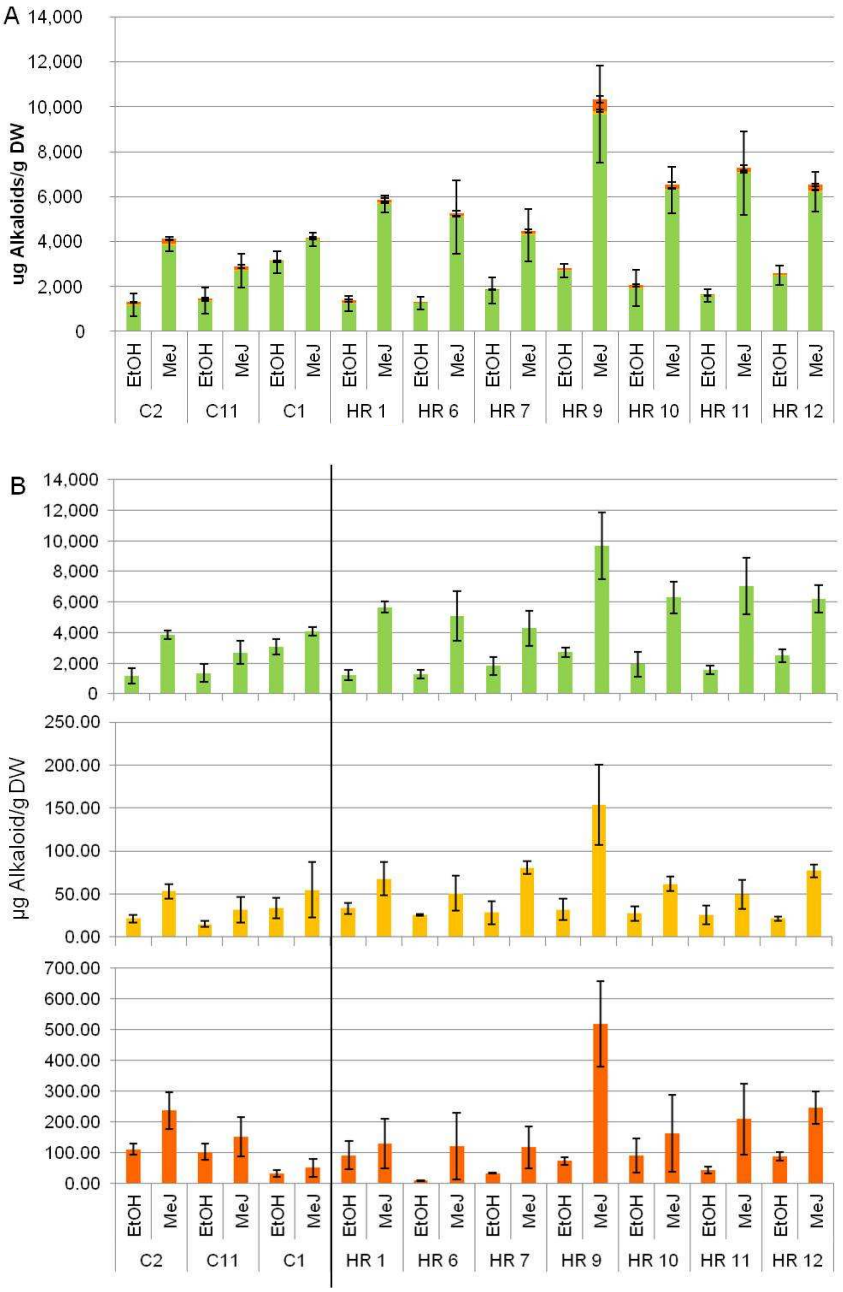

Figure 5.9: Alkaloid accumulation in control (HR C-) and transgenic lines (HR). A) The three main alkaloids represented together, B) each alkaloid represented separately. Alkaloids correspond to: green, Nicotine; yellow, Anabasine; orange, Anatabine. Values are mean ±SD from 3 to 6 independent samples.

When considering the results of the BY-2 tobacco (*Nicotiana tabacum* L.) protoplast transient expression carried out in this study and the alkaloid production in the tobacco hairy root lines overexpressing the *Taximin* gene, we could conclude that Taximin controls the expression of genes involved in secondary metabolism and consequently secondary compound production, and that the activation of this gene requires the presence of the elicitor MeJ. However, the results obtained so far do not permit us to detail the relationship between the presence of the Taximin peptide and the MeJ signalling pathway. Further analyses are needed in order to determine at what genetic level Taximin acts and the coordination of the secondary pathways in response to the presence of MeJ and Taximin.

5.4.5. Gene expression in tobacco hairy roots

With the aim of exploring the relationship between Taximin and secondary metabolism, the expression of genes closely related to nicotine biosynthesis was analyzed. The transcript profiling of *PMT* and *QPRT*, the first specific genes related to the pyrrolinium and pyridine part of tobacco alkaloid biosynthesis, was determined by qRT-PCR. We had previously observed a high activation of their promoters when *Taximin* was also expressed and cultures were supplemented with MeJ (Figure 5.4). *Taximin* expression was also studied in order to confirm the constitutive expression of the transgene in all HR lines.

When comparing the transcript accumulation of the genes related with tobacco alkaloid biosynthesis, *PMT* and *QPRT* (Figure 5.10), we could observe a basal expression in unelicited conditions and a clear induction related to MeJ elicitation in all lines, including control and transgenic ($P<0.05$). When MeJ was present in the media, *PMT* transcript accumulation increased 4.6 to 11.7-fold (for HR 10 and C-2) compared to the hairy roots grown in mock conditions, while the increase in *QPRT* transcript accumulation was lower, ranging from 2.8 to 5.7-fold (for HR 10 and C-11). No differences in *PMT* and *QPRT* transcript levels were observed between control and transgenic lines. The maximum increase in *PMT* transcripts in basal conditions was observed in line HR 10, where the gene was 2.5 or 3 times more expressed than in C-2 and C-11, while under MeJ treatment the maximum increase was in HR 7, being 1.4 and 1.8 times higher than in C-2 and C-11. Maximum increments in *QPRT* transcript levels were lower, being 1.9 and 1.8-fold higher in HR 10 than

C-2 and C-11, respectively, in mock cultures, and,1.3 and 1.1-fold higher in HR 1 and HR 7 than C-2 and C-11, respectively, in MeJ cultures.

The lines with the construction 35Spro::*Taximin* (HR 1, 6, 7, 9, 10, 11 and 12) showed variable expression of *Taximin*, which was not expressed in the control lines (Figure 5.10). HR 11 presented the highest *Taximin* transcript levels, followed by HR 1, while the lowest were found in HR 6, 7, 9, 10 and 12. As expected, due to having a constitutive promoter, the expression of *Taximin* was not significantly increased by MeJ compared with unelicited cultures.

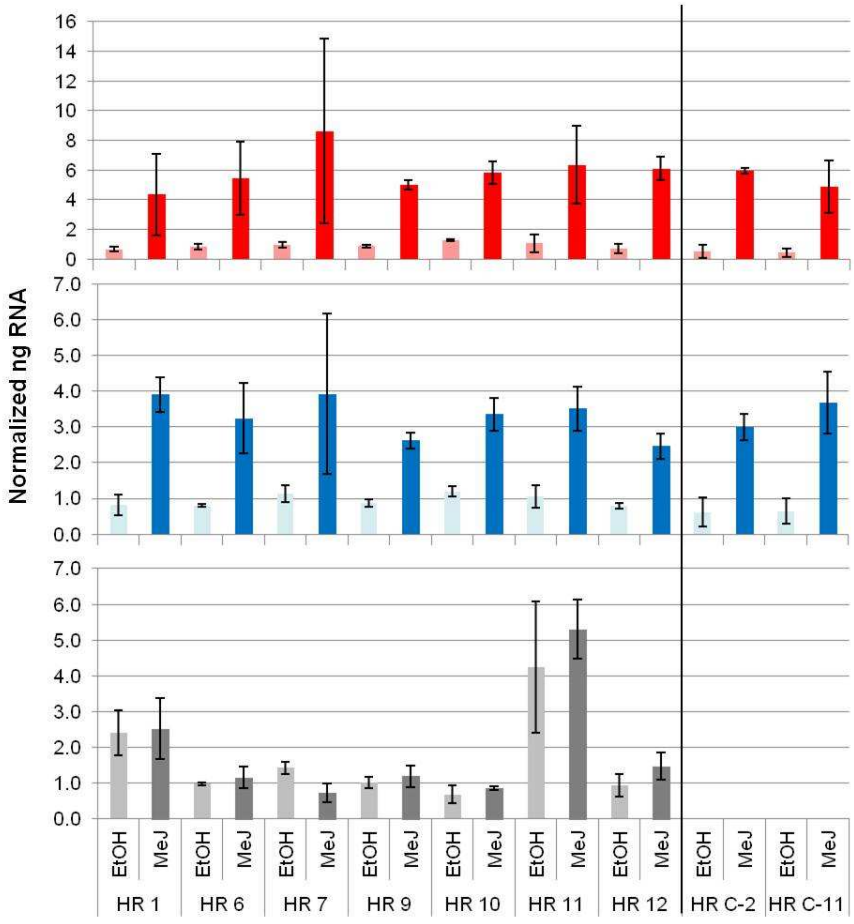

Figure 5.10: *PMT*, *QPRT* and *Taximin* gene expression in control (HR C-) and transgenic lines (HR). In light colour, gene expression in untreated hairy roots; in bright colour gene expression in MeJ treated cultures. Red: PMT (putrescine N-methyltransferase); blue: QPRT (quinolinate phosphoribosyltransferase); Grey: Taximin. Values are mean ±SD of 3 independent samples.

Quantitative real-time PCR analysis did not confirm that higher alkaloid levels correlate with higher *PMT* and *QPRT* expression levels. For example, transgenic root line HR 7, which had one of the highest levels of *PMT* expression, was among the least productive transgenic root lines (Figures 5.10 and 5.9), while HR 9, the most productive root line, accumulated nearly 10 mg/g DW of nicotine and presented one of the lowest levels of gene expression (Figure 5.10).

According to the obtained results, the presence of *Taximin* clearly affected the promoter activity of the studied genes involved in secondary metabolism (*TXS*, *PMT*, *QPRT*, *STR*) during the first 24h after MeJ treatment (Figure 5.4). At the same time, the presence of the gene encoding this peptide in the hairy root lines also affected the alkaloid contents under MeJ culture conditions. However, as expected from the promoter analysis carried out before, the *PMT* and *QPRT* gene expression was not differentially activated. It is possible that the gene expression varied most in the first hours after elicitation, and the increase in alkaloid content of the *Taximin*-overexpressing root lines followed an early gene expression. In fact, if Taximin is a new Cys-rich peptide (CRPs, antimicrobial peptide), its purpose should be neutralizing microbial infection, and as most AMPs are mobilized shortly after pathogen attack (Broekaert et al., 1997; Boman, 2003), this would explain its effect during the first 24 h after infection. Similarly to other AMPs, the expression of this gene probably diminishes in the following hours, as has been observed in genes encoding thionins, whose expression in light conditions dropped to minimal levels 96 h after stress (Kitanga et al., 2006). Moreover, as with thionins and defensins, *Taximin* expression is activated by jasmonates and it probably accumulates in vacuolar membranes or the plasmalemma (Broekaert et al., 1997). All these data clearly suggest that Taximin is a novel Cys-rich peptide involved in plant defence systems.

5.5. Acknowledgements

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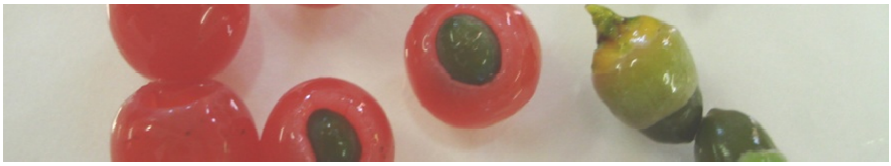

THE RELATIONSHIP BETWEEN *TXS*, *DBAT*, *BAPT* AND *DBTNBT* GENE EXPRESSION AND TAXANE PRODUCTION DURING THE DEVELOPMENT OF *TAXUS BACCATA* PLANTLETS

Onrubia, M., Moyano, E., Bonfill, M., Palazón, J., Goossens, A., Cusidó, R.M. (2011) The relationship between *TXS*, *DBAT*, *BAPT* and *DBTNBT* gene expression and taxane production during the development of *Taxus baccata* plantlets. Plant Science 181:282–287.

CONCLUSIONS

7. CONCLUSIONS

Taking an empirical approach, we developed a two-phase system optimized for taxol production. *Taxus baccata* cells grew actively during the first phase of the culture in the growth medium (B5 + 2 mg/L NAA + 0.1 mg/L BAP + 0.5 mg/L GA_3), but slowed down when transferred to the production medium (B5 + 2 mg/L Picloram + 0.1 mg/L kinetin). This lower growth was not a consequence of cell lysis, since the viability of cultured cells was higher than 70% throughout the experiment. Conversely, the taxane production was much higher during the second stage of culture. The addition of MeJ to the production medium, although detrimental for growth, triggered a significant increase in taxane production.

In a rational approach and with the help of "omics" tools, we have demonstrated the effectiveness of the cDNA-AFLP technology for analysing the *Taxus baccata* transcriptome. More than 650 gene tags, presenting a different expression pattern in unelicited and elicited (MeJ treated) culture conditions, were visualized and sequenced. Within the Metabolism and Energy functional category, gene tags corresponding to 42.8% of the known genes involved in taxol biosynthesis were found. This study also produced a long list of possible candidates for the 7 supposed functions without known genes related with the taxol biosynthetic pathway, as well as genes related with secondary metabolism and its regulation, which have been the subject of different chapters of this research work.

Our *in silico* studies showed that among the 15 gene tags that potentially correspond to genes encoding enzymes involved in the 5 unknown steps of the taxol biosynthetic pathway, there is a strong possibility that the TB331 protein is a hydroxylase involved in taxane biosynthesis and the TB328 protein is a candidate for the epoxidase responsible for the oxetane ring formation. In addition, the TB768 protein is a candidate for the CoA transferase involved in activating the lateral chain, while TB496 cannot be ruled out as the unknown oxomutase. Functional studies, using a suitable cell system, would confirm the functionality of these *in silico* selected candidates, and their involvement in taxol biosynthesis

Taximin is a new Cys-rich peptide activated by jasmonates, which was obtained after the cDNA-AFLP studies. Transient expression in transfected tobacco protoplasts showed that this peptide significantly increased the activity of the promoters of the following genes: *TXS (*involved in taxol biosynthesis*), PMT* and *QPRT* (both involved in tobacco

alkaloid biosynthesis) and *STR* (active in terpenoid indole alkaloids production). This indicated the capacity of Taximin to regulate not only taxol biosynthesis, but also other secondary metabolism pathways. Tobacco hairy roots over-expressing Taximin showed an enhanced nicotine production (3.6-fold) after MeJ elicitation, proving much more sensitive to elicitation than the control root lines in which the corresponding increase was only 2.1-fold.

In *Taxus* x *media* cell cultures carrying the *TXS* gene of *T. baccata* under the control of the 35SCaMV promoter, the total taxane production was 4 times higher under treatment with 1 µM Coronatine than with 100 µM MeJ, and almost 8 times higher than in the unelicited cell cultures, demonstrating the effectiveness of this new elicitor for improving taxane production. Although cultures grown with MeJ and Cor both showed a higher gene expression compared to the control, taxol biosynthetic genes were expressed earlier under Cor elicitation. This probably led to an earlier accumulation of intermediates and consequently a higher taxane production.

When comparing transcriptomic and metabolomic profiles in control and elicited conditions, we can conclude that the *TXS gene* and those encoding the different P450-dependent hydroxylases are highly induced under elicitation, not limiting taxane biosynthesis. The same behaviour was observed for the *PAM* gene, which converts α-phenylalanine into β-phenylalanine in the taxol lateral chain formation. Conversely, the genes encoding transferases *TBT*, *DBAT*, *BAPT*, and *DBTNBT*, responsible for DABIII formation, baccatin III formation, the union of the lateral chain to baccatin III and taxol formation, respectively, although also induced, generally showed a lower response to the elicitors. Consequently, these genes are probably involved in the more limiting steps of taxol formation.

In an organized biological system, *T. baccata* plantlets growing *in vitro* showed lower *TXS, DBAT, BAPT* and *DBTNBT* gene expression in roots than in the aerial part throughout the first year of development. This was reflected in the lower taxane levels found in the roots, where only the early pathway gene TXS was induced during the 1-year growth period. In the aerial part of *T. baccata* plantlets, 10-deacetylbaccatin III was poorly converted into baccatin III, which might be due to the low expression level of the *DBAT* (10-deacetylbaccatin III-10β-O-acetyltransferase) gene responsible for this conversion. From these results, we can infer that in *T. baccata* plantlets, the *DBAT* gene controls a rate-limiting step in the taxane biosynthetic pathway during the first year of development.

Although further studies about the activity of the enzymes involved in the studied metabolic steps are needed, the results obtained in this rational approach to the biotechnological production of taxol, both in *Taxus* cell cultures and *in vitro* plantlets, suggest that the major bottlenecks are found mainly in the last steps of the taxol biosynthetic pathway. The genes involved in the first and intermediate steps of the pathway are clearly activated by elicitors or are naturally active, whereas the last steps of the taxol biosynthetic pathway are poorly activated. It could thus be inferred that these late steps are probably responsible for the limited taxol production in the biological systems tested. Consequently, the genes involved at the end of the pathway could constitute potential targets of choice for metabolic engineering in the search for new highly productive *Taxus* cell lines and transgenic plants.

PUBLISHED PAPERS

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