INCREASED TAXOL PRODUCTION IN *TAXUS X MEDIA* THROUGH METABOLIC ENGINEERING APPROACHES

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LIST OF ABBREVIATIONS

2,4-D	2,4-dichlorophenoxyacetate
10-DAB III	10-deacetylbaccatin III
Å	angstrom
AFLP	amplified fragment length polymorphism
ATA	aurintricarboxylic acid
BAP	benzylaminopurine
BAPT	baccatin III:3-amino-3-phenylpropanoyltransferase
bp	base pair
BLAST	basic local alignment search tool
CAMBIA	Center for the Application of Molecular Biology to International Agriculture, Australia
cDNA	complementary DNA
CoA	coenzyme A
CTAB	hexadecyltrimethylammonium bromide
DBAT	10-deacetylbaccatin III 10-*O*-acetyltransferase
DBTNBT	3'-N-debenzoyl-2'-deoxytaxol N-benzoyltransferase
DD	differential display
DEGeF	differentially expressed gene fragment
DEPC	diethyl pyrocarbonate
ds	double-stranded

DTT	dithiothreitol
E-value	expect value
EDTA	ethylenediaminetetraacetic acid
EDW	extracted dry weight
EST	expressed sequence tag
FDA	U.S. Food and Drug Administration
FPP	farnesyl diphosphate
GFP	green fluorescent protein
GGPP	geranylgeranyl diphosphate
h	hour
h3p	*Arabidopsis* histone-3 gene promoter
HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
HPLC	high performance liquid chromatography
IPP	isopentenyl diphosphate
kb	kilobases
kD	kilodalton
kg	kilogram
LB	Luria-Bertani medium
m5p	*Arabidopsis* meri-5 gene promoter
MeJA	methyl jasmonate
min	minute
MMLV	moloney murine leukemia virus
mRNA	messenger RNA

ms	millisecond
MS	Murashige and Skoog
MW	molecular weight
NCBI	The National Center for Biotechnology Information (U.S.)
NCI	National Cancer Institute (U.S.)
ng	nanogram
nm	nanometer
NMR	nuclear magnetic resonance
NAA	naphthaleneacetic acid
OD	optical density
ORF	open reading frame
p.	page
PCR	polymerase chain reaction
PVP	polyvinylpyrrolidone
RDA	representational difference analysis
RSD	relative standard deviation
RT-PCR	reverse transcriptase-polymerase chain reaction
SA	salicylic acid
SAGE	serial analysis of gene expression
SDS	sodium dodecyl sulfate
SPE	solid phase extraction
SSC	standard saline citrate
SSH	suppression subtraction hybridization

T-DNA	transfer DNA (from *Agrobacterium*)
TAT	taxadien-5α-ol *O*-acetyltransferase
TBT	taxane 2α-*O*-benzoyltransferase
TE	Tris-EDTA
Ti	tumor-inducing
Tris	tris(hydroxymethyl)aminomethane
TS	taxadiene synthase
ts	taxadiene synthase encoding gene
UV	ultraviolet

CHAPTER I

GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 WHAT IS TAXOL?

Taxol is one of the best medicines developed from plant source (Rowinsky et al., 1992; Huizing et al., 1995). Its first clinical use against ovarian cancer was approved by the U.S. Food and Drug Administration (FDA) in 1992. Because of its effective anti-cancer function, the FDA has extended its clinical use to breast cancer, non-small-cell lung cancer and AIDS-related Kaposi's sarcoma. Current clinical trials also show its effectiveness in the treatment of a series of other cancers, such as colon, melanoma, bladder, cervix, pancreatic and prostate cancers.

1.1.1 First discovery of Taxol

In 1958, the U.S. National Cancer Institute (NCI) launched an extensive program to screen 35,000 plant species around the world for compounds with anti-cancer activity. In 1963, as part of this program, the U.S. Forest Service collected the bark of Pacific yew (*Taxus brevifolia* Nutt., a slow-growing understory tree) and sent it to the NCI for analysis. The crude extract from the yew bark was found to be cytotoxic. In 1971, Drs. Monroe Wall and M. C. Wani, then at the Research Triangle Institute of North Carolina, identified Taxol as the active ingredient (Wani et al., 1971). Its structure was elucidated by chemical, spectroscopic and X-ray crystallographic techniques.

1.1.2 Unique antitumor mechanism of Taxol

Taxol treatment arrests cell division. Microtubules, which are formed by polymerization of tubulin monomers, play an important role on cell division. Most of the previously known anticancer agents, such as vincristine, vinblastine, colchicine and podophyllotoxin, act as cell mitosis poisons by inhibiting assembly of microtubules. In

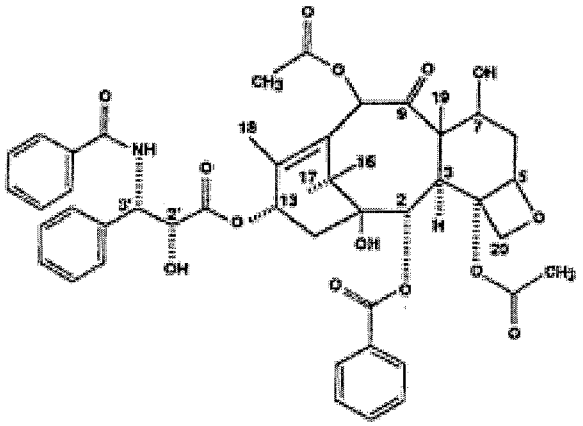
contrast, Taxol inhibits mitosis by promoting assembly and preventing disassembly of microtubules. This unique mode of action was first reported by Dr. Susan Horwitz at Albert Einstein College of Medicine in 1979 (Schiff et al., 1979).

1.1.3 Chemical structure of Taxol

The mode of action of Taxol stems from its unusual chemical structure. Taxol is composed of two principal parts: the diterpenoid core and the N-benzoyl-β-phenylisoserine side chain (Figure 1, next page). In the diterpenoid core, the oxetane ring at C-4-C-5 and the benzoyloxy group at C-2 are essential for its cancer-fighting activity. For the side chain, the 3'-phenyl group and N-acyl group are also indispensable for its unique function. Taxol belongs to a large family of taxoid molecules, which has over 350 members (Baloglu and Kingston, 1999). Some molecules in this family, such as Taxotere (generic name: docetaxel, Figure 1), also demonstrate anti-tumor activity.

1.2 SUPPLY PROBLEM OF TAXOL

The supply of Taxol has been problematic since its discovery. Its content is very low in yew bark, generally varying from 0.001% to 0.01% of the dry bark weight. Statistically, to obtain 1 kg of Taxol, 13,500 kg of *Taxus brevifolia* bark is needed. Moreover, yew bark is not a renewable source; after removing the bark the tree dies. To make this situation worse, the distribution of Pacific yew is very limited (Figure 2, p.5). In North America, this species occurs along the northwestern Pacific Coast, from southeastern Alaska to central California through British Columbia. In the Rocky Mountain region, it spreads from southeastern British Columbia into eastern Washington and Oregon through northwestern Montana and northern Idaho. Because of its scarcity,



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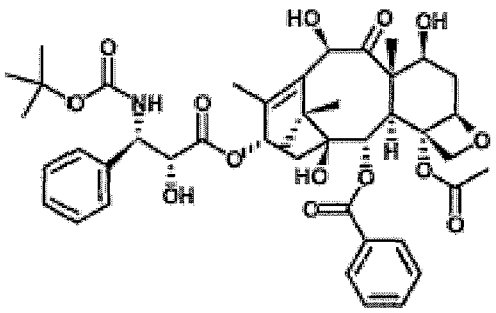

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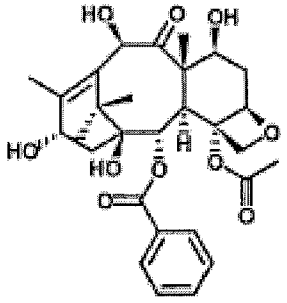

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Figure 1. Chemical structures of Taxol (A), Taxotere (B) and 10-DAB III (C).

Figure 2. Distribution of Pacific yew in North America.

the early-stage trial of Taxol had once been suspended in 1971 until the discovery of its unique mode of action.

Since the discovery of this precious drug, large-scale harvesting of yew bark for Taxol isolation threatened the existence of the Pacific yew. In order to protect this species, some environmental group lobbied the U.S. Congress to ban the use of Taxol despite its strong anti-cancer functionality. Although its use is not banned, the environmental impact caused by this supply crisis has been widely recognized and discussed throughout the world.

Short supply means high prices. A standard course of Taxol treatment ranges from $6,000 to $10,000 per patient. Many patients are unable to afford this treatment. In order to increase the affordability of this precious drug and to alleviate the environmental impact, the Taxol supply problem has to be solved by alternative means.

1.3 How to Solve the Taxol Supply Problem?

Several alternative approaches to Taxol production have been conceived and studied extensively. These approaches include: 1) genotype selection and cultivation; 2) total synthesis; 3) semi-synthesis from relatively abundant intermediates of the Taxol biosynthetic pathway; 4) identification of other more abundant taxoid family molecules with equivalent or better function; 5) cell culture and large-scale bioreactor production; and 6) metabolic engineering.

The total synthesis of Taxol was first achieved by two independent groups simultaneously, the Holton group (Holton et al., 1994a; Holton et al., 1994b) and the Nicolaou group (Nicolaou et al., 1994). Currently many other groups around the world are continuing to work in this area, with the purpose of not only developing shorter and

newer Taxol synthetic routes but also creating novel taxoid molecules with improved biological activity. However, to date none of these total synthetic routes are commercially viable (Borman, 1994).

Although the content of Taxol in bark is low, some precursors of Taxol biosynthesis, such as 10-deacetyl baccatin III (10-DAB III, see Figure 1, p.4), are relatively abundant in more renewable parts of the tree, such as needles. The approach of semi-synthesis utilizes these precursors to synthesize Taxol in order to increase its supply. Semi-synthesis of Taxol from 10-DAB III involves the attachment of the N-benzoyl-3-phenylisoserine side chain to the C-13 hydroxyl group of 10-DAB III and the acetylation of the 10β-hydroxyl position (Holton et al., 1995). Since the semisynthetic form of Taxol received marketing clearance from FDA in 1995, a high percentage of Taxol in the market is from semi-synthesis. However, this approach still depends on collected yew tree parts for precursor extraction. Moreover, the concentration of these precursors can vary dramatically due to environmental factors (Wheeler et al., 1992). Therefore, semi-synthesis alone cannot meet the increasing demand for Taxol.

The development of Taxotere (*N*-debenzoyl-*N-tert*-butoxycarbonyl-10-deacetyl Taxol, see Figure 1, p.4) is a result of the fourth approach. Taxotere is a Taxol analogue with improved solubility and increased potency. Structurally, Taxotere is very similar to Taxol, except at the C-10 position of the baccatin ring and the C-3' position of the side chain. Besides structural similarity, Taxotere and Taxol have the same mode of action against cancer cells. Taxotere is also obtained by semi-synthesis from 10-DAB III. Consequently, Taxotere faces the same supply limitation as Taxol. Although some other

taxoids have also shown similar activity to Taxol (Altstadt et al., 2001; Plummer et al., 2002), they are not currently available for clinical use due to low abundance.

Cell culture of *Taxus spp.* holds promise for future production of Taxol (Gibson et al., 1995; Zhong, 1995; Jaziri et al., 1996; Zhong, 2002). This approach has advantages over other above-mentioned alternative technologies. As a sustainable way of Taxol production, cell culture does not depend on collection and extraction of tree parts. In addition, a large-scale cell culture bioreactor can realize continuous production of Taxol. Since the first report of Taxol production by *Taxus* cell culture in 1989 (Christen et al., 1989), intensive research efforts have been focused on increasing Taxol production by this approach. A number of strategies have been found to be effective to improve Taxol or other taxoids yield in *Taxus spp.* cell culture. These strategies include: 1) modification of culture conditions such as medium composition, pH, temperature and light (Zhong, 1995; Jaziri et al., 1996); 2) elicitation (Yukimune et al., 1996); 3) feeding of precursors (Fett-Neto et al., 1994) or sugars (Choi et al., 2000; Wang et al., 2001); 4) adjustment of osmotic pressure (Kim et al., 2001); 5) control of gas composition (Mirjalili and Linden, 1995); and 6) bioengineering strategies such as semi-continuous culture (Phisalaphong and Linden, 1999; Choi et al., 2001), two-stage (Cusido et al., 2002) and two-phase (Wu et al., 2000) cultivation. Synergistic effects resulting from simultaneous application of multiple strategies further improve production of Taxol. Currently pilot-scale culture of yew cells is being performed in academia and industry throughout the world. However, despite of all these efforts, production of Taxol by cell culture is still not commercially viable at the present. In order for cell cultures to be cost-effective, further improvement

of productivity is essential, which will be dependent on other biotechnological methodologies such as metabolic engineering.

Metabolic engineering is the key for solving the Taxol supply problem (Verpoorte et al., 2000; Verpoorte and Memelink, 2002). As an integrating methodology for low cost production of industrial-use chemicals, metabolic engineering has been proved to be successful (Chartrain et al., 2000). By genetically manipulating certain relevant metabolic pathways, production of target compounds can be increased significantly. Commonly employed metabolic engineering approaches include: 1) overcoming rate-limiting steps; 2) overexpressing relevant regulatory genes; 3) reducing catabolism and 4) avoiding flux of precursor(s) or intermediate(s) into competitive metabolic pathways.

Taxol metabolic engineering relies on full understanding of the Taxol biosynthesis route in yew cells. Some of the enzymes, and their encoding genes, in this pathway have been isolated and characterized. With discovery of more and more enzymes and their genes, there will be a better understanding of the entire biosynthetic pathway and its regulation. Based on such information, a metabolic engineering approach could be employed to increase the production of this valuable pharmaceutical.

An important preliminary step for nearly all Taxol production alternatives is screening of different *Taxus* species and cultivars for high-yielding plants to provide good starting materials not only for direct Taxol and other taxoids extraction, but also for biotechnological approaches such as cell culture and metabolic engineering.

1.4 SCREENING OF HIGH-TAXOID-YIELDING *TAXUS* SPECIES AND CULTIVARS

1.4.1 Screening methodology: separation and quantitation of taxoids

High performance liquid chromatography (HPLC) is by far the most commonly used method for separation and quantitation of taxoids (Theodoridis and Verpoorte, 1996). Crude extracts from yew materials are often pre-purified prior to HPLC analysis to remove some interfering molecules, by which separation of taxoids is improved and column life is also extended. One of the most efficient and widely used methods for pre-purification is solid phase extraction (SPE) (Mattina and MacEachern, 1994). Almost all previous HPLC separation and quantitation of taxoids were conducted in reverse phase mode, with the utilization of C_8, C_{18}, phenyl or some proprietary Taxol specialty matrix such as Curosil^{TM} columns. Most mobile phases contain methanol, acetonitrile, water or aqueous buffer. The detection of taxoids is usually achieved by UV analysis, with the wavelength set at 227 or 228 nm. The identity of separated taxoid molecules is determined by elution time, spiking, mass spectrometry (MS) and nuclear magnetic resonance (NMR). Although HPLC is the most widely used separation method, other methods, such as enzyme linked immunoassay (ELISA) (Jaziri et al., 1991), thin layer chromatography (TLC) (Stasko et al., 1989) and micellar electrokinetic chromatography (MEKC) (Chan et al., 1994) have also been used.

1.4.2 Screened *Taxus* species and cultivars

Besides *Taxus brevifolia* Nutt., taxoids have been found in nearly all *Taxus* species (Vidensek et al., 1990; Zamir et al., 1992; Fang et al., 1993; Fuji et al., 1993;

Elsohly et al., 1995; Kitigawa et al., 1995; Zhang et al., 1995; van Rozendaal et al., 2000; Parc et al., 2002). These species include *T. baccata* L., *T. cuspidata* Siebold & Zucc., *T. Canadensis* Marshall, *T. chinensis* Rehder, *T. globosa* Schltdl., *T. x media* Rehder, *T. wallichiana* Zucc., *T. yunnanensis* C. Y. Cheng, W. C. Cheng & L. K. Fu, *T. sumatrana* (miq.) de Haub., *T. floridana* Champ., *T. celebica* (Warb.) Li and *T. x hunnewelliana* Rehder. Taxol and other taxoids were not only found in yew bark, but also in other renewable parts of these plants, like needles, twigs and roots. Taxol concentration in these renewable parts sometimes is even higher than that in the original source, bark of the Pacific yew. Species in other genera of the Taxaceae family, such as *Amentotaxus formosana* Li, *Cephalotaxus fortunei* Hook, *Cephalotaxus harringtonia* (Forbes) K. Koch, *Pseudotaxus chienii* (Cheng) Cheng, *Torreya grandis* Fort. and *Torreya californica* Torr., have also been screened. However, taxoids have not been detected in these species.

1.4.3 Variation of Taxol content in screened species and cultivars

Taxoids are secondary metabolites and play an indirect role on *primary* growth and development of yew plants. Therefore, there is large variation in content among different species and populations, within individual trees, and in different tissues during different seasons. Some of this variation is caused by defense responses against external invasion, while the rest is considered to be an intrinsic trait. In order to get a reliable results on evaluation of taxoids content among different *Taxus* species or cultivars, influences of external conditions, such as sampling season, sample treatment, etc., should be minimized. Yew plants with intrinsically high Taxol content should be a good starting point for biotechnological approaches to increase Taxol production.

1.5 BIOSYNTHESIS OF TAXOL

Taxol is synthesized in the plastid of differentiated *Taxus* stem cells *in vivo*. Its biosynthesis also occurs in de-differentiated cells cultured *in vitro*. The biosynthetic pathway of Taxol involves approximately 20 enzymatic reactions. These reactions can be divided into 2 phases: the biosynthesis of the Taxol diterpene core and the later attachment of the side chain (Figure 3, next page). Some steps in this pathway are still not fully understood to date (Walker and Croteau, 2001).

Little is known regarding the regulation of Taxol production at the physiological, biochemical and gene expression levels, with the exception that certain elicitors, such as methyl jasmonate and salicylic acid, can increase Taxol production in *Taxus* suspension culture cells (Yukimune et al., 1996).

It should also be noted that the production of Taxol is a dynamic process, involving not only biosynthesis but also degradation. Although more and more details of the Taxol biosynthesis pathway have been established, there is virtually no information about its degradation.

1.5.1 Biosynthesis of the diterpene core

The biosynthesis of the Taxol diterpene core starts with geranylgeranyl diphosphate (GGPP) and finishes with baccatin III. Currently, 8 enzymes in this process, including GGPP synthase, have been defined and characterized.

1.5.1.1 Biosynthesis of GGPP by GGPP synthase

The diterpene core of Taxol is derived from GGPP, a precursor for the biosynthesis of terpenoid compounds. GGPP synthase, a prenyltransferase, catalyzes the

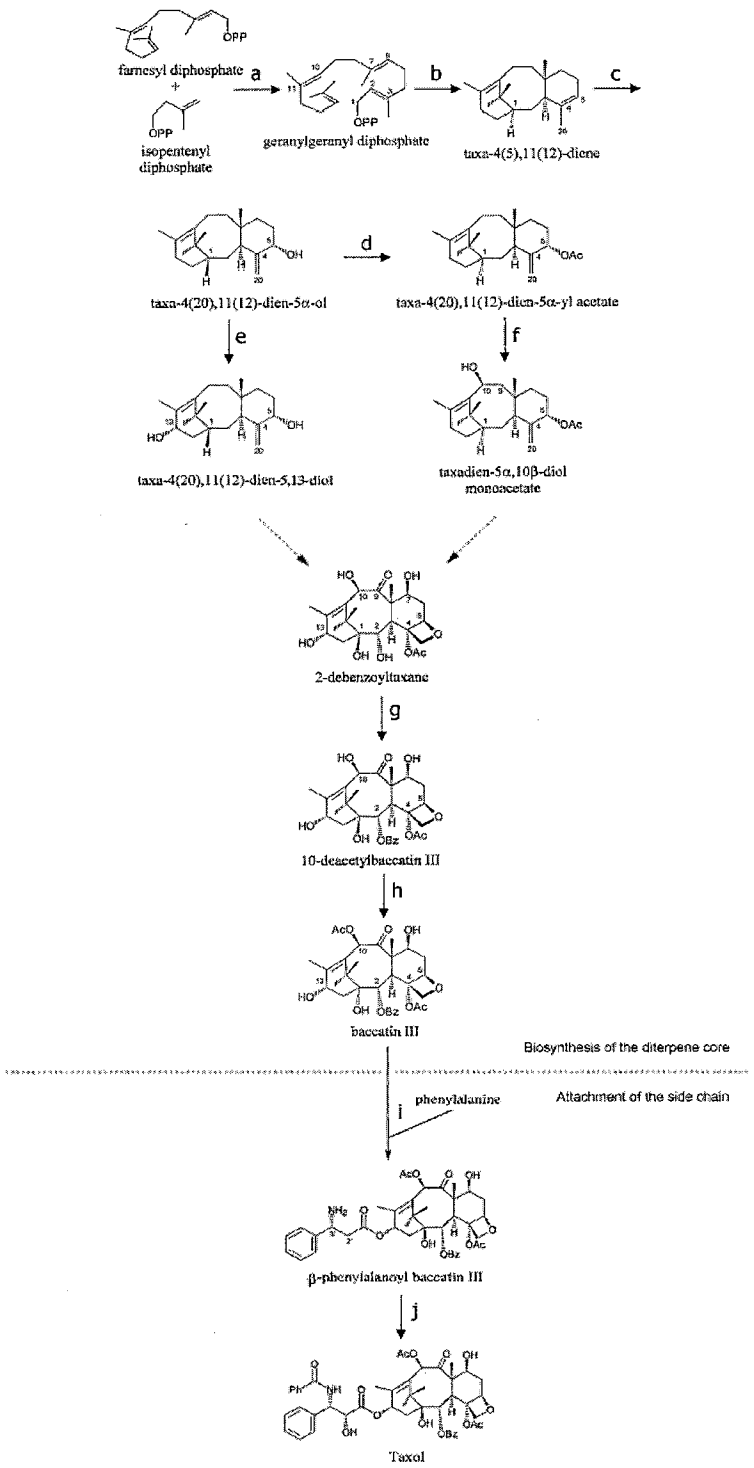

Figure 3. Outline of the Taxol biosynthesis pathway known to date. Some steps in this pathway are still unknown, which are indicated by the broken arrows. The enzymes involved in this pathway are: a. GGPP synthase; b. taxadiene synthase; c. taxadiene 5α-hydroxylase; d. taxadien-5α-ol *O*-acetyltransferase (TAT); e. taxane 13α-hydroxylase; f. taxane 10β-hydroxylase; g. taxane 2α-*O*-benzoyltransferase (TBT); h. 10-deacetylbaccatin III 10-*O*-acetyltransferase (DBAT); i. baccatin III:3-amino-3-phenylpropanoyltransferase (BAPT); j. 3'-N-debenzoyl-2'-deoxytaxol N-benzoyltransferase (DBTNBT).

formation of GGPP from farnesyl diphosphate (FPP) and isopentenyl diphosphate (IPP). The GGPP synthase cDNA, which has an open reading frame (ORF) of 1179 nucleotides, has been isolated (Hefner et al., 1998). The deduced enzyme has 393 amino acid residues, with a molecular weight (MW) of 42.6 kD. This enzyme contains an N-terminal transit peptide, which leads the gene translation product into plastid and is removed afterwards to form the mature form of the enzyme. This protein shows a significant identity (62-75%) to other GGPP synthases of plant origin.

1.5.1.2 Biosynthesis of taxa-4(5), 11(12)-diene by taxadiene synthase

This is the first Taxol-biosynthesis-committed step, however this reaction is very slow. The taxa-4(5),11(12)-diene is made through the cyclization of GGPP by taxadiene synthase (TS) (Koepp et al., 1995). The full-length plastid-transit-peptide-containing preprotein has 862 residues, with a MW of 98.3 kD. The mature enzyme, which is formed by cleaving the transit peptide, is monomeric with a MW of 79 kD. It also contains the typical terpenoid synthase DDXXD motif for the binding of divalent metal ion-substrate complex. This protein shows significant homology to other plant terpenoid synthases, among which it shares closest identity (46%) and similarity (67%) to abietadiene synthase. Its cDNA, cloned from *T. brevifolia* and *T. baccata*, specifies an ORF of 2586 nucleotides (Wildung and Croteau, 1996; Liu, 1997). Heterologous overexpression of this enzyme in *E. coli* resulted in functional products. Since this step is the first committed step but a slow reaction, it is suggested to be one of the rate-limiting key steps in the Taxol biosynthesis pathway (Croteau et al., 1995a; Croteau et al., 1995b).

1.5.1.3 First hydroxylation of the taxadiene core by taxadiene 5α-hydroxylase

The conversion of taxa-4(5),11(12)-diene to taxa-4(20),11(12)-dien-5α-ol is the second step committed to Taxol biosynthesis. The taxadiene 5α-hydroxylase for this conversion is a cytochrome P450 monooxygenase. Although this enzyme has been purified and characterized (Hefner et al., 1996), its encoding cDNA has not been cloned to date. Oxygenation reactions occur in six positions of the Taxol molecule; this is the first oxygenation step.

1.5.1.4 Acetylation and hydroxylation of taxa-4(20),11(12)-dien-5α-ol

The acetylation of taxa-4(20),11(12)-dien-5α-ol is believed to be the next step along the Taxol biosynthesis pathway. The enzyme responsible for this reaction, taxadien-5α-ol *O*-acetyltransferase (TAT), has been purified and characterized (Walker et al., 1999). Based on the internal partial amino acid sequence of this protein, its encoding cDNA has been isolated from a cDNA library constructed from poly(A)$^+$ RNA isolated from methyl jasmonate-induced yew cells (Walker et al., 2000). The encoding cDNA has an ORF of 1317 nucleotides, corresponding to a deduced amino acid sequence of 439 residues (MW: 49 kD), which is consistent with the size of the native enzyme. There is no N-terminal organellar targeting signal sequence found in this enzyme. It shares a significant similarity with other acetyl transferases of plant origin.

In 2001, a cytochrome P450-dependent monooxygenase was found to be able to catalyze the hydroxylation of taxa-4(20),11(12)-dien-5α-ol at the C-13 position (Jennewein et al., 2001). This enzyme, i.e., taxane 13α-hydroxylase, was actually one of several expression products from a set of full-length cytochrome P450 cDNA clones, which were obtained by a PCR-based differential display-cloning approach from methyl

jasmonate-induced *Taxus* culture cells. This enzyme has 485 amino acid residues and shares similarity with other cytochrome P450 oxygenases. The finding of taxane 13α-hydroxylase may indicate the existence of more than one biosynthetic route toward Taxol.

1.5.1.5 Hydroxylation of taxadien-5α-yl acetate

Functional assays of the aforementioned expression products of the full-length cytochrome P450 cDNA clone set, which allowed the identification of taxane 13α-hydroxylase, also led to the discovery of taxane 10β-hydroxylase (Schoendorf et al., 2001). This enzyme catalyzes the conversion of taxadien-5α-yl acetate to taxadien-5α, 10β-diol monoacetate. The ORF of its cDNA clone has 1494 nucleotides, which encodes 498 amino acid residues of this protein (MW: 56.7 kD). It shares 63% sequence identity with taxane 13α-hydroxylase and bears all the structural motifs found in other cytochrome P450 monooxygenases.

1.5.1.6 Benzoylation of 2-debenzoyltaxane

The reaction steps of Taxol biosynthesis pathway from taxadien-5α, 10β-diol monoacetate to 2-debenzoyltaxane are still unknown. The benzoylation of 2-debenzoyltaxane to 10-DAB III by taxane 2α-*O*-benzoyltransferase (TBT) is the second last step before the completion of the diterpene core. The TBT-encoding cDNA clone has been isolated by a homology-based PCR cloning strategy (Walker and Croteau, 2000b). This full-length cDNA clone has an ORF of 1320 nucleotides, which correspond to 440 residues of the protein (MW: 50 kD). The functionality of the *in vitro* expressed protein

has been confirmed. This enzyme bears the highly conserved HXXXDG motif found in other transacylases.

1.5.1.7 Completion of the diterpene core

The acetylation of 10-DAB III to baccatin III by 10-deacetylbaccatin III 10-*O*-acetyltransferase (DBAT) completes the Taxol diterpene core biosynthesis. The cDNA clone encoding this enzyme has been isolated by homology-based PCR cloning and library screening (Walker and Croteau, 2000a). This cDNA clone has an ORF of 1320 nucleotides, corresponding to the 440 amino acid residues of the deduced enzyme (MW: 49 kD). This size is consistent with that of the functional native enzyme. Compared to taxadiene-5α-ol-*O*-acetyltransferase and other acyl transferases of plant origin, the amino acid sequence of this enzyme shares a significant similarity.

1.5.2 Attachment of the side chain

The 13-*O*-(N-benzoyl-3-phenylisoserinoyl) side chain of Taxol is essential for its tubulin binding function. The attachment of this side chain takes fewer steps than the synthesis of the diterpene tricyclic core. It includes 1) transfer of the aminophenylpropanoyl group to C-13 of baccatin III, 2) benzamidation and 3) C-2' hydroxylation of the side chain. The enzymes catalyzing the first two steps, along with their cDNA clones, have been isolated and characterized. However, little is known about the third step to date.

1.5.2.1 Formation of β-phenylalanoyl baccatin III

As the first step of Taxol side chain attachment, baccatin III:3-amino-3-phenylpropanoyltransferase (BAPT) catalyzes the acylation of baccatin III by using β-

phenylalanoyl CoA as the acyl source. The cDNA encoding this enzyme has been isolated, and the catalytic function of its *in vitro* expressed product has been confirmed (Walker et al., 2002). This cDNA clone has an ORF of 1335 nucleotides, which encode 445 amino acids of the protein (MW: 50.5 kD). At amino acid level, this enzyme shares a similarity of 71-74% with the other four acyl/aroyltransferases in the Taxol biosynthesis pathway. Surprisingly, this enzyme contains a $G_{163}XXXDA_{168}$ instead of the typical HXXXDG acyltransferase motif.

1.5.2.2 Benzamidation of the side chain

The benzamidation of β-phenylalanoyl baccatin III to 2'-deoxytaxol is catalyzed by 3'-N-debenzoyl-2'-deoxytaxol N-benzoyltransferase (DBTNBT). The DBTNBT-encoding cDNA was isolated recently (Walker et al., 2002). It specifies an ORF of 1,323 nucleotides, encoding the 441 amino acid residues of the enzyme (MW: 49 kD). At the amino acid level, this protein shows common characteristics shared by other Taxol biosynthetic acyltransferases, like sequence similarity, the conserved HXXXDG motif, lack of an N-terminal targeting signal and a molecular size around 50 kD.

1.6 GENE EXPRESSION PROFILING OF *TAXUS* CELLS

1.6.1 What is gene expression profiling?

Life is a dynamic process. The pattern of gene expression in any organism is constantly changing due to alterations of growth and developmental phase, or response to internal or external environmental stimuli. Gene expression profiling, or transcript profiling, is used to determine the expression level of genes. Usually it involves a series

of techniques to detect alterations of gene expression pattern by comparing two samples, generally a control and a treatment. Detection of these alterations leads to deeper and better understanding of life processes. In this post-genomics era, expression profiling also becomes a powerful tool to determine function of sequenced genes, or to identify novel genes (Donson et al., 2002).

Gene expression profiling is not a brand new term specially coined in this functional genomics era. Traditional methods, such as northern blot, S1 nuclease analysis and *in situ* hybridization, are all earlier techniques of gene expression profiling. However, these techniques are best for in-depth parallel analysis of individual genes. Since the completion of whole-genome-sequencing projects, it becomes increasingly necessary to analyze expression profiles of a large number of genes simultaneously. As a result, various approaches toward high-throughput transcript profiling have been designed and carried out by both industry and academia. Most of these approaches can be categorized into three groups: 1) nucleotide sequencing; 2) fragment sizing or 3) hybridization based.

EST (expressed sequence tag) sequencing and SAGE (serial analysis of gene expression) are the two main nucleotide sequencing-based gene expression profiling methods. Discovery of new genes can be achieved by EST sequencing; meanwhile, expression level of target gene(s) can also be assessed by this approach, since the frequency of occurrence of their ESTs in a cDNA library is a good indicator of their expression levels (Mekhedov et al., 2000; White et al., 2000). However, if a statistically significant in-depth analysis is to be achieved by this method, it can be very expensive (Audic and Claverie, 1997; Ohlrogge and Benning, 2000). Some other methodologies, like SSH (suppression subtraction hybridization) and RDA (representational difference

analysis), can help reduce the amount of sequencing involved (Sargent, 1987; Hubank and Schatz, 1994). SAGE, based on concatenation and quantitation of transcript-specific short sequence tags (10-14bp), is another broadly applicable yet more cost-efficient technique for quantitative cataloging and comparison of expressed genes in different tissues at different states (Velculescu et al., 1995). One disadvantage of this technique, however, is the redundancy of matches in database since the sequence tag is short.

Oligonucleotide and cDNA microarray are the currently most widely used high-throughput hybridization-based transcript profiling technologies (Schena et al., 1995; Lockhart and Winzeler, 2000). Different from traditional hybridization based methods, such as Northern blot, a microarray has the capability of analyzing large numbers of genes at one time, and thereby offers a more holistic approach for expression profiling (Duggan et al., 1999; Lipshutz et al., 1999). Oligonucleotide microarray requires a comprehensive reference sequence database and accurate gene annotation. However, it can avoid cross-hybridization by utilizing proper oligonucleotide design (Lockhart et al., 1996). The cDNA microarray requires extensive clone and PCR fragment curation and has potential cross-hybridization problem. However, it does not require existing gene sequence information. In addition, the cDNA microarray is ratio-based, i.e., both treatment and control samples are hybridized to microarrayed cDNA clones at the same time, which is an obvious advantage over radioactive nylon or nitrocellulose-based arrays (Desprez et al., 1998). Since both microarray approaches require special equipment and software for microarray fabrication, image scanning and analysis, the cost of microarrays is higher than other expression profiling methodologies.

The fragment sizing-based methods, including DD (differential display) and cDNA-AFLP (amplified fragment length polymorphism), have the advantages of being easy to set up and more cost-effective. DD was first introduced by Liang and Pardee (1992). This approach is based on random amplification of cDNA fragments, side-by-side comparison of polyacrylamide gel electrophoresis pattern and isolation of differential bands. Since this approach is much more economical than most other approaches, it has been used extensively in transcript profiling analyses. However, this technique has the inherent disadvantages of the appearance of a high-percentage of false positives (Sun et al., 1994; Sompayrac et al., 1995), low sensitivity (Bertioli et al., 1995) and poor reproducibility (Haag and Raman, 1994; Zhang et al., 1998). Introduction of cDNA-AFLP (Bachem et al., 1996) solves these problems through combining restriction digestion, ligation of adaptors to restriction fragments and selective cDNA fragment amplification (Figure 4, next page). Because of its high reliability, good reproducibility and high sensitivity, this technique is an ideal approach for systemic transcriptome analysis. In this research, the cDNA-AFLP technique was chosen for the expression profiling of elicited yew suspension cell.

1.6.2 Gene expression profiling of *Taxus* cells

Plant secondary metabolism is a complicated network, involving a large number of intertwined processes. Therefore, it is very difficult to analyze a certain secondary metabolite pathway without interference from other metabolic pathways. Furthermore, it is actually impossible to clearly distinguish primary metabolism from secondary metabolism. Therefore, it is very challenging to apply gene expression profiling to plant secondary metabolism. As a result, there are few reports on the application of gene

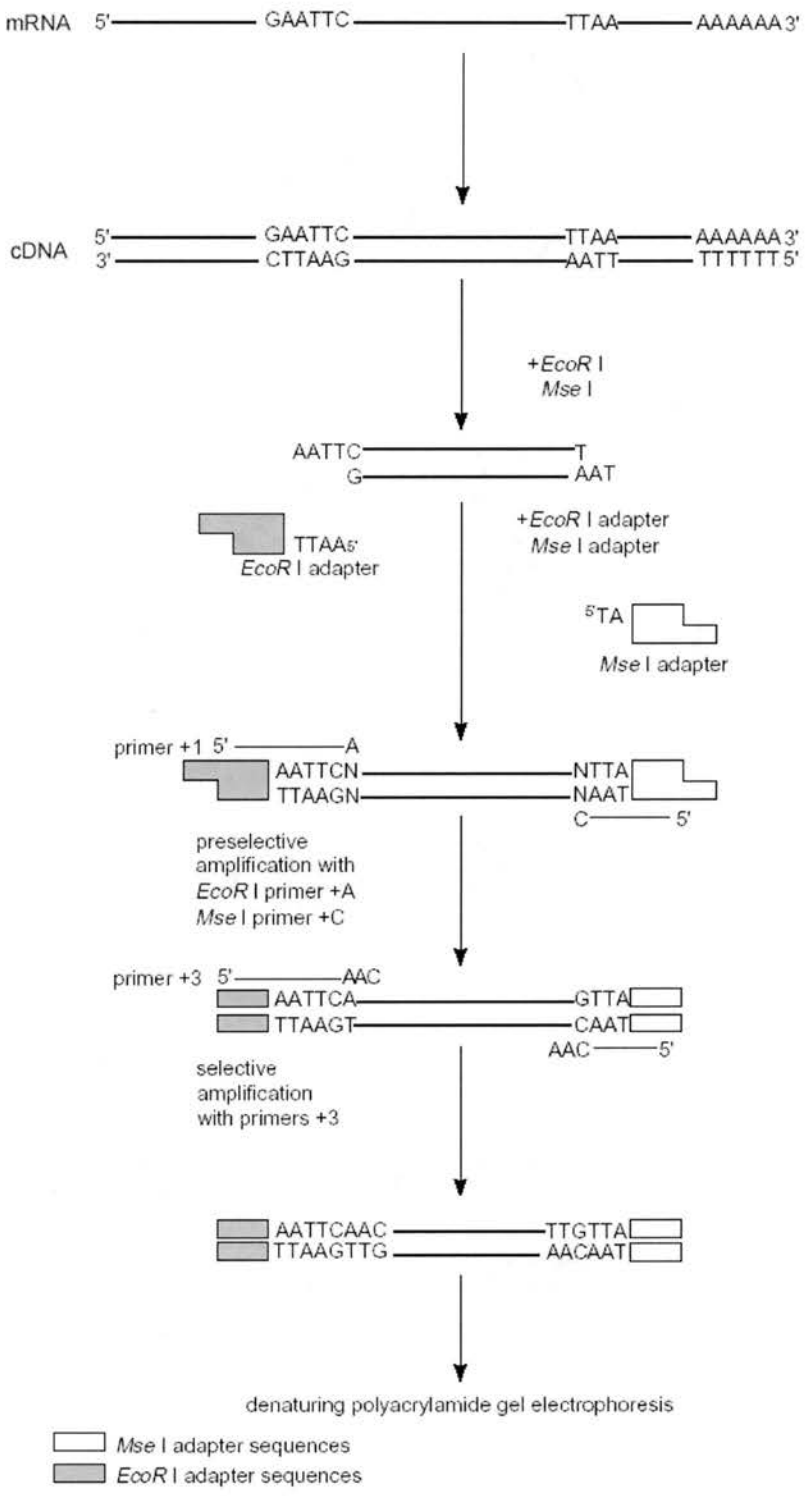

Figure 4. A simplified representation of the cDNA-AFLP process. (modified from Invitrogen AFLP® Analysis System I instruction manual)

expression profiling on plant secondary metabolism to date. However, it worth trying to apply this powerful technology on certain plant secondary metabolites of high industrial value, such as Taxol, which will undoubtedly shed more light on their metabolic network at the molecular level.

It is well known that certain elicitors, such as methyl jasmonate (MeJA) and salicylic acid (SA), can increase Taxol production in yew suspension culture cells (Yukimune et al., 1996). This increased Taxol production is presumably due to increased activity of at least some biosynthetic enzymes or some regulatory elements of the Taxol biosynthetic pathway. Therefore, Taxol biosynthesis genes or related regulatory genes might be found among elicitation-triggered differentially expressed genes.

Methyl jasmonate and salicylic acid are not only Taxol production elicitors, they are actually universal plant endogenous messenger molecules, playing key roles in a wide range of physiological events such as flowering, senescence and defense responses. Expression of a large number of genes is regulated by MeJA and SA through a series of intercellular and intracellular signaling pathways. Production of many classes of plant secondary metabolites has been reported to be induced or increased by these two signaling molecules.

There are few reports on the application of gene expression profiling to *Taxus* cells to date. Schoendorf et al. (2001) employed a DD-RT-PCR based approach to identify cytochrome P450 oxygenases in the Taxol biosynthesis pathway using cytochrome P450-specific forward primers instead of random primers. Hu et al. (2002) reported the application of differential display in searching for unknown genes in the Taxol biosynthetic pathway. In the latter report, 8 cDNA clones were found to be up-

regulated by MeJA, among these was a cDNA clone (named TS-4) believed to encode an acyltransferase specific to Taxol biosynthesis. The coding sequence of this cDNA clone shared significant homology to known acyltransferase and contained the conserved acyltransferase HXXXDG motif. Although the catalytic function of the corresponding enzyme still needs to be further confirmed by biochemical experiments, the gene expression profiling approach has proved to be an effective way to explore the Taxol biosynthetic pathway.

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CHAPTER II

VARIATION OF TAXOID CONTENT IN NEEDLES OF *TAXUS X MEDIA* CULTIVARS WITH DIFFERENT GROWTH CHARACTERISTICS

2.1 INTRODUCTION

The efficacy of Taxol as a potent drug against multiple cancers has been well proven over the years. However, the demand for it has far exceeded its supply for a long time. Solving the Taxol supply problem is a huge challenge for both industry and academic researchers worldwide. Since the content of Taxol in the original source, i.e., the bark of Pacific yew, is only 0.01%, harvesting of high-Taxol-yielding species or cultivars for Taxol extraction seems to be the quickest way to solve this problem. Ideally, higher concentration of Taxol can be found in more renewable parts of the tree, such as needles or twigs, other than the irreplaceable bark. In addition to direct Taxol extraction, high-Taxol-yielding plants are also good starting materials for biotechnological approaches, such as cell culture and metabolic engineering. Because of these reasons, a large amount of effort has been focused on screening different yew species and cultivars, which is reviewed in Chapter I.

In addition to variable taxoid content, different *Taxus* species and cultivars have different growth characteristics, like growth rate and growing habit. Fast-growing yew trees accumulate larger amount of biomass in their renewable parts (like needles) in a given period of time than those of slow growth rate. Since it was reported that needles contain taxoid with an amount comparable to that in bark (Witherup et al., 1990), it is desirable to identify cultivars that can combine both high taxoid content in their needles and fast biomass accumulation.

Every year large numbers of cultivated yew plants are clipped in nurseries around the world. Mechanical collection of high-Taxol-content clippings from these nurseries for Taxol extraction would certainly lower the production cost of this costly medication.

Different growing habits of yew species and cultivars, like upright or spreading, would play an important role in mechanical collection of these clippings. Upright-growing shoots would make mechanical collection of these renewable parts easier.

In addition to the advantage of being easy to collect by mechanical means, upright-growing yew species and cultivars can also be grown at higher density than those of spreading growth. Consequently, more materials could be collected per unit area for Taxol extraction purpose.

2.2 OBJECTIVE

Although Taxol content variation among different yew samples is partially due to environmental factors, part of this variation is genetic. To quantify genetic variation, needle samples were collected from 17 widely cultivated *Taxus x media* cultivars with different growth characteristics. The negative influences of environmental factors on taxoid content were minimized during the sample collection process. In addition to Taxol, three other important taxoids (10-DAB III, baccatin III and cephalomannine) were also analyzed, all of which can be used as substrates for semi-synthesis of Taxol.

The objective of this research was to identify those *Taxus x media* cultivars combining fast growth rate, upright growing habit and high taxoids content in their needles. These cultivars make good candidates for direct taxoids extraction from clippings; meanwhile, they served as good starting materials for the rest of my dissertation research: genetic transformation and expression profiling.

2.3 MATERIALS AND METHODS

2.3.1 Taxoid standards

The Taxol standard (>95% purity) was provided by the National Cancer Institute (NCI), Bethesda, MD, USA. Standards for 10-DAB III, baccatin III and cephalomannine were donated by Dr. David G. I. Kingston of the Chemistry Department, Virginia Polytechnic Institute and State University, USA.

2.3.2 Plant materials

Twigs from 17 *Taxus x media* cultivars (see Table II, p.44) were collected from the Secrest Arboretum, Ohio Agricultural Research and Development Center, The Ohio State University. Arboretum accession numbers are available upon request. Immediately after twig collection in February 2001, fresh needles were removed from the branches for taxoids content analysis by HPLC.

2.3.3 Taxoids extraction

Ten grams of fresh needles were first chopped with a scalpel. The chopped needles were then mixed with 1.5 g glass beads (425-600 microns, from Sigma) and ground in a mortar. The ground tissues were soaked in 45 ml of hexane with occasional shaking to remove nonpolar components like wax. After 18 h, the hexane solution was carefully discarded and the remaining hexane in the extracted plant material was allowed to evaporate overnight at room temperature. Then 100 ml of methanol was added to extract taxoids by constant stirring at room temperature in the dark for 18 h. The resulting methanol extraction solution was then filtered through a glass filter and evaporated to

dryness. The needle tissue residue was dried at 45°C until constant weight to determine the extracted dry weight (EDW). The methanol extract residue was re-dissolved in 15 ml methanol and subjected to pre-HPLC treatment using a solid phase extraction (SPE) cartridge to remove part of the interfering non-taxoid compounds.

2.3.4 SPE purification

An aliquot of 1.5 ml of the methanol extract from the above step was brought to 5 ml with H_2O and subject to SPE. The Supelco LC-18 SPE cartridge (Supelco, Bellefonte, PA, USA) was first conditioned with 2 ml of methanol followed by 2 ml of H_2O. After loading 5 ml of the diluted methanol extract, the column was washed with 2 ml of H_2O twice, followed by 2 ml of 20% methanol (in H_2O, v/v). The taxoids were then eluted with 2 ml of methanol from the column and the eluate was dried in a speed vac. The dried residue was re-dissolved in 200 µl acetonitrile and 10 µl of this sample was applied to the HPLC column.

2.3.5 HPLC

The taxoid HPLC analyses were performed on a Novapak Phenyl column (4µm, 60 Å, 150 x 3.9 mm) from Waters Corporation (Milford, MA, USA). The mobile phase was composed of two solvents: A) 0.05 M ammonium acetate-acetonitrile (7:3, v/v) and B) 0.05 M ammonium acetate-acetonitrile (1:9, v/v). For quantitation of 10-DAB III, baccatin III and cephalomannine, the gradient elution program of Theodoridis et al. (1998) was followed. For Taxol analysis, a modified program (Table I, next page) was used for improved separation. The flow rate of the mobile phase was 0.8 ml/min. The injection volume was 10 µl. All peaks were detected at 227 nm. The retention times for

10-DAB III, baccatin III, cephalomannine and Taxol were 3.34, 7.15, 22.60 and 29.12min, respectively. Every sample was injected 3 times and the relative standard deviation (RSD) for all samples was 1-2%. The identities of taxoid peaks in the chromatogram were further confirmed by spiking using taxoid standards. Linearity of the detector response was established for all 4 standards ranging from 0.5 to 250 μg/ml. For HPLC analysis, all solvents and samples were filtered through 0.45 μm filters.

Table I. HPLC gradient elution program for Taxol quantitation

Time (min)	A[1](%)	B[2](%)
0	95	5
1	95	5
36	82.8	17.2
38	100	0

1: 0.05 M ammonium acetate-acetonitrile (7:3, v/v)
2: 0.05 M ammonium acetate-acetonitrile (1:9, v/v)

2.4 RESULTS

For the screening of cultivars with different growth characteristics for their taxoid content, needle samples from 17 different cultivars of *Taxus x media* were collected from the Secrest Arboretum at The Ohio State University, in which many *Taxus* species and cultivars are collected and planted for research purposes. All needle samples were mixed from multiple trees (3-5 trees) of each cultivar. The classification of these 17 cultivars is

based on the comparison of their growth rate and growing habit. Since our samples were collected from trees growing in similar environment, the influence of environmental factors on taxoid production was minimized.

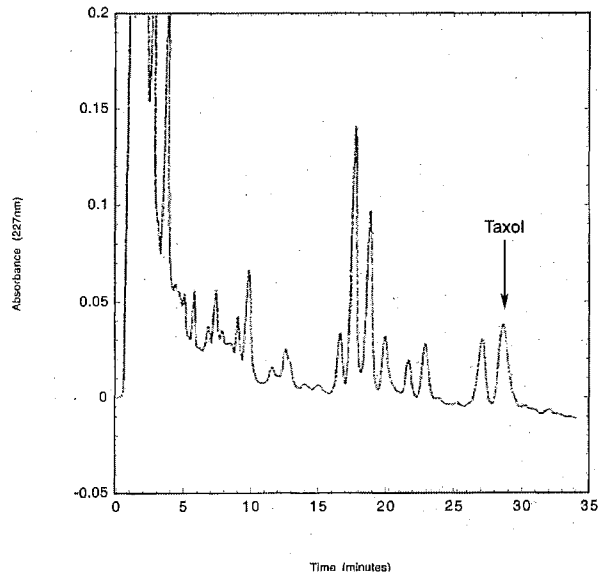
A well-developed taxoid HPLC quantitation system (Theodoridis et al., 1998) was employed to analyze taxoid content of these samples. By removing some of the interfering compounds, the SPE pre-purification step facilitated HPLC separation and quantitation of taxoids. Figure 5 (next page) shows an example of the HPLC separation of Taxol (the separation of the other three taxoids are not shown here).

2.4.1 Variation of Taxol content

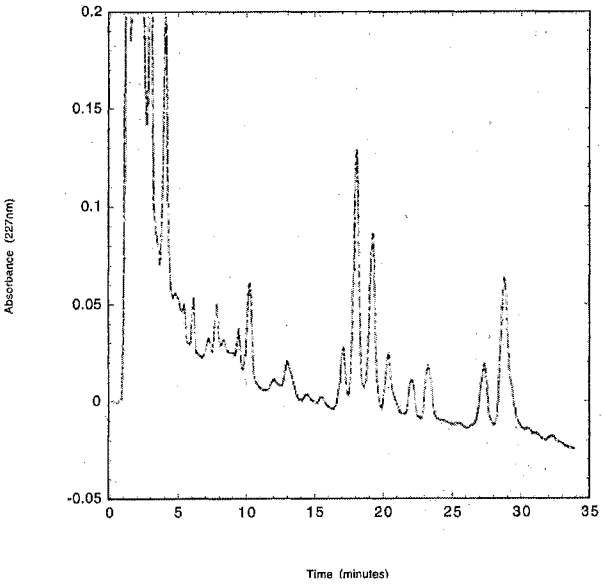
Large variation was found in the content of 10-DAB III, baccatin III, cephalomannine and Taxol among the 17 *Taxus x media* cultivars studied (Table II, p.44 and Figure 6, p.45). For Taxol, the content ranged from 108 μg/g EDW ('Natorp', Group 4) to 378 μg/g ('Coleana', Group 1). The top three cultivars with the highest Taxol content were all found in Group 1, the fast/upright growth group. Following 'Coleana', another cultivar in Group 1, 'Hicksii', contained the second highest Taxol content (322 μg/g), while 'Stovekenii' had the third highest Taxol content (309 μg/g).

2.4.2 Content variation of 10-DAB III, baccatin III and cephalomannine

As the main precursor for Taxol semi-synthesis, 10-DAB III is also of interest in this analysis. The range of its content was from 203 μg/g ('Hillii' in Group 2 and 'Hicksii' in Group 1) to 543 μg/g ('Viridis', Group 2). The three cultivars with the highest yield of 10-DAB III were all found in upright-growing groups. 'Stovekenii' had the second highest 10-DAB III content (482 μg/g) and 'Coleana' the third (463 μg/g).



A



B

Figure 5. Separation and quantitation of Taxol in *Taxus x media* needle extracts by HPLC. Shown here were the HPLC chromatograms for the 'Andorea' needle extract without spiking (A), and with spiking of Taxol standard (B) in order to confirm the identity of the Taxol peak (indicated by arrow).

Table II. Taxoid content in needles of 17 *Taxus x media* cultivars in four different growth groups

Growth Group No. and Characteristics	Cultivar Name	Taxane Content (μg/g)			
		10-DAB III	Baccatin III	Cephalo-mannine	Taxol
1 Fast/Upright	Andorea	**291**	**146**	**125**	**261**
	Coleana	**463**	**360**	**390**	**378**
	Hicksii	**203**	**88**	**346**	**322**
	Stovekenii	**482**	**293**	**60**	**309**
	Wellesleyana	**369**	**72**	**421**	**152**
2 Slow/Upright	Grandfolia	**291**	**55**	**106**	**163**
	Green Candle	**270**	**248**	**179**	**197**
	Hillii	**203**	**135**	**194**	**234**
	Viridis	**543**	**139**	**169**	**189**
3 Fast/ Spreading	Fairview	**275**	**102**	**181**	**143**
	Roseco	**238**	**122**	**N/D***	**208**
	Runyan	**460**	**51**	**276**	**240**
	Sebian	**422**	**98**	**226**	**148**
4 Slow/ Spreading	Everlow	**230**	**79**	**188**	**123**
	Kobel	**330**	**134**	**190**	**191**
	Natorp	**378**	**269**	**170**	**108**
	Wardii	**285**	**49**	**75**	**235**

* Not Detected

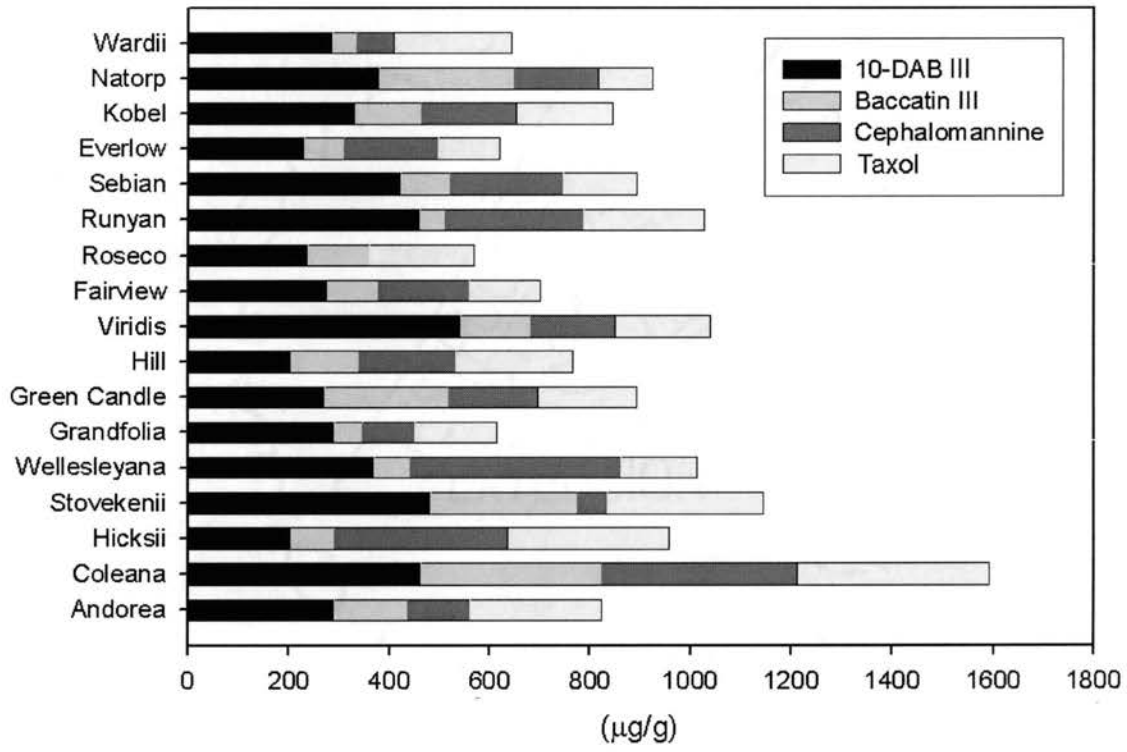


Figure 6. Content of 10-DAB III, baccatin III, cephalomannine and Taxol in *Taxus x media* cultivars with different growth characteristics.

Compared to Taxol and 10-DAB III, the variation of baccatin III and cephalomannine content was larger. The content range for baccatin III was from 49 μg/g ('Wardii', Group 4) to 360 μg/g ('Coleana'). For cephalomannine, content ranges from 0 (not detected in 'Roseco' of Group 3) to 390 μg/g ('Coleana').

2.4.3 Average taxoid content of the four growth groups

On average, Group 1 cultivars were found to contain the highest content of all four taxoids assayed in this report (Figure 7, next page). Since the cultivars in this group offer advantages over those in other groups, i.e., faster biomass accumulation and applicability of mechanical clippings collection, several cultivars in this group make good candidates for taxoid extraction. 'Coleana' contained the highest content of Taxol, baccatin III and cephalomannine, as well as the third highest content of 10-DAB III. 'Stovekenii' contained the third highest content of Taxol and the second highest content of 10-DAB III. The second highest Taxol content in 'Hicksii', along with its wide availability, makes this cultivar another attractive target.

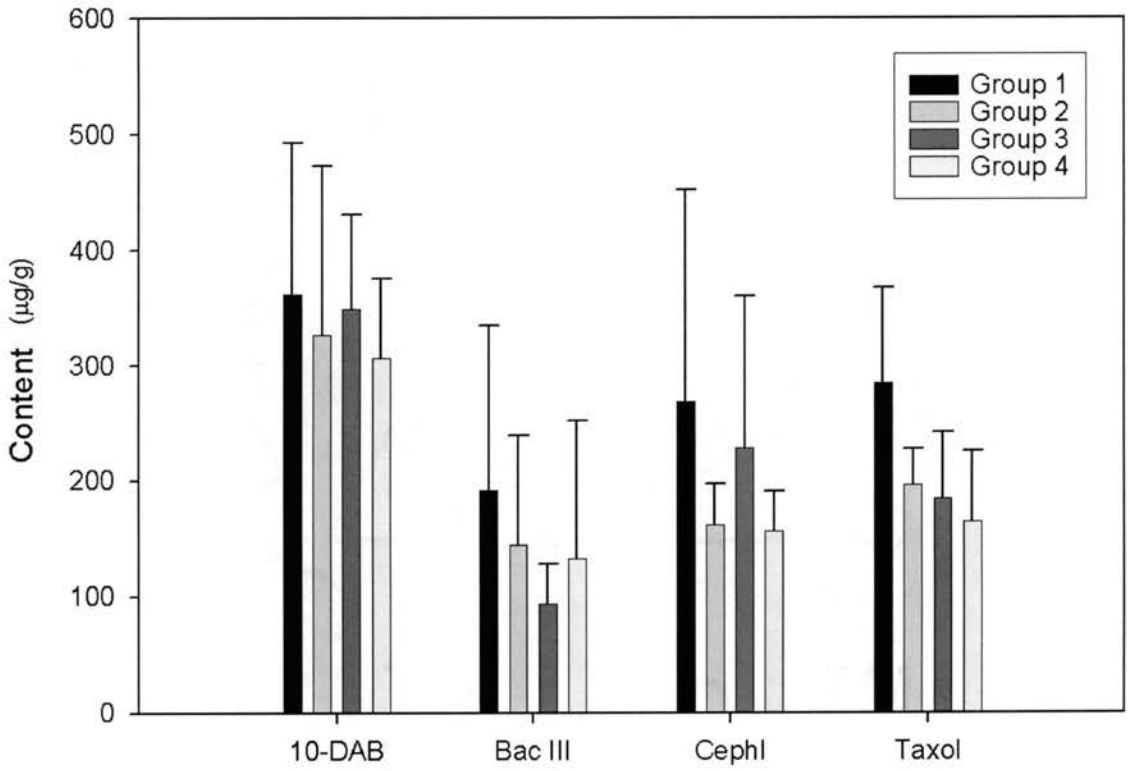


Figure 7. Average taxoids content in the four growth groups. Group 1: fast/upright; Group 2: slow/upright; Group 3: fast/spreading; Group 4: slow/spreading.

2.5 DISCUSSION

In this report, *fresh* needles were utilized as starting materials for taxoids extraction instead of the commonly used *dried* needles. The taxoid content data here were expressed on the basis of extracted dry weight (EDW). This EDW-based taxoid content expression was first used by Fett-Neto and DiCosmo (1992). Since degradation of Taxol during the needles drying process was indicated in their report, the EDW-based taxoid content offers more accuracy than the commonly used dry-weight-based content. The Taxol content obtained in this report is also comparable to the data in their report. The EDW-based content of Taxol in fresh needles of most cultivars in this analysis was higher than that in its original source, i.e. the bark of *T. brevifolia* (100 μg/g) on the dry weight basis. 'Hicksii' was found in this report to contain a high amount of Taxol, which is in agreement with other authors who based their results on the dry weight basis (Hansen et al., 1999; van Rozendaal et al., 2000).

Cultivars of *Taxus x media* are very common garden and yard plants. Every year large amount of clippings is pruned from these plants in nurseries. Since needles of some cultivars were found by this study to contain Taxol at a concentration even higher than its original source (the bark of Pacific yew), these clippings could be utilized to extract Taxol and 10-DAB III to treat cancer patients. By estimation, Taxol extracted from 'Hicksii' clippings alone could treat 3,000 to 4,000 ovarian cancer patients annually (Hansen et al., 1999). The high-Taxol-yielding cultivars identified here could be widely planted for gardening and taxoid extraction simultaneously.

2.6 CONCLUSION

Taxus x media cultivars were identified with regard to Taxol content, growth rate, and growing habit. The three cultivars with highest Taxol content ('Coleana', 378 μg/g; 'Hicksii', 322 μg/g; and 'Stovekenii', 309 μg/g) were also fast growing with an upright form. These traits make them ideal candidates for Taxol extraction through clipping collection and for genetic manipulation through metabolic engineering. Because of its second highest Taxol content and *wide availability*, 'Hicksii" was used as starting material in the following studies of genetic transformation and expression profiling.

2.7 REFERENCES

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CHAPTER III

INCREASED TAXOL PRODUCTION BY OVEREXPRESSING TAXADIENE SYNTHASE

3.1 INTRODUCTION

Although screening for high-Taxol-yielding *Taxus* species and cultivars can help to alleviate the Taxol supply problem, it can not solve the problem completely because Taxol productivity is inherently low even in the species or cultivars of *relatively* high Taxol content. Plant biotechnology is believed to hold promise for ultimately solving this problem. As reviewed in the first chapter, metabolic engineering will be the main direction for future Taxol biotechnology.

By definition, metabolic engineering is "the directed improvement of product formation or cellular properties through the modification of specific biochemical reactions or introduction of new ones with the use of recombinant DNA technology" (Stephanopoulos, 1999). Production of many phytochemicals, which can be used as valuable pharmaceuticals, fine chemicals, dyes and spices, nutraceuticals, pesticides, etc., can be increased by metabolic engineering (Verpoorte et al., 1999; Chartrain et al., 2000). The application of plant metabolic engineering to improve production of these valuable phytochemicals has already been proved by previous research efforts, such as increased accumulation of essential oil in Peppermint (*Mentha x piperita* L.) (Mahmoud and Croteau, 2001), accumulation of epoxy fatty acids in transgenic tobacco callus or somatic soybean embryos transformed by a cytochrome P450 enzyme encoding gene from *Euphorbia lagascae* (Cahoon et al., 2002) and significant increase of flavor volatiles in transgenic tomato leaves by expression of a yeast desaturase gene (Wang et al., 2001).

There are several possible approaches to increase Taxol production by metabolic engineering. First, this could be achieved by overexpressing genes controlling rate-limiting steps. Overexpression of these genes could lead to an elevated level of these

reactions and thus increased Taxol productivity of the whole pathway. Second, by suppressing expression of genes in other competitive pathways, more precursors and energy could be supplied for the biosynthesis of Taxol. Third, novel or better taxoid medicinal compounds might be produced in increased amounts by modifying the existing biosynthesis pathway. With the availability of more and more details about the Taxol biosynthesis pathway, more approaches will be devised.

Successful application of metabolic engineering to improve Taxol production relies on a better understanding of the Taxol biosynthesis pathway and its relationship with other relevant metabolic routes. As reviewed in the first chapter, not all the steps of this pathway are known to date. However, about ten enzymes and their encoding genes involved in this pathway have been isolated and characterized. Based on the available information, possible rate-limiting steps can be targeted by metabolic engineering approaches.

Taxadiene synthase is the first committed enzyme in the Taxol biosynthesis pathway. The reaction that it catalyzes is regarded as one of the rate-limiting steps of the pathway. This enzyme was first purified and characterized from Pacific yew (*Taxus brevifolia*) by Hezari et al. (1995). The full-length taxadiene synthase preprotein contains 862 amino acid residues, with a long plastidial transit signal peptide, which is removed later to form the mature enzyme. Bearing the typical terpenoid synthase DDXXD motif, taxadiene synthase shares significant homology to other plant terpenoid synthases. Its full-length cDNA, with an ORF of 2586 nucleotides, was obtained from *T. brevifolia* by Wildung and Croteau (1996). In 1997, the same gene was isolated by Liu from European yew (*T. baccata*). Sequence comparison of this gene between these 2 *Taxus* species

showed identities of 98% in DNA sequences and 97% in protein amino acid sequences (Liu, 1997). Therefore, this enzyme is an ideal target to increase Taxol production by metabolic engineering through genetic transformation.

3.2 HYPOTHESIS

The conversion of GGPP to taxa-4(5),11(12)-diene by taxadiene synthase is the first committed yet rate-limiting step of the Taxol biosynthesis pathway. It is hypothesized that the low expression level of this enzyme is one factor underlying the slow reaction rate of this step. If this enzyme is overexpressed, the bottleneck set by this step might be overcome. Consequently, the production of Taxol would be increased.

3.3 OBJECTIVE

The objective of this research was to increase Taxol production by overexpressing taxadiene synthase. In order to do so, two high-efficiency tissue-specific promoters from *Arabidopsis* (meri-5 and histone-3 promoter) were used to direct high-level expression of the taxadiene synthase gene (*ts*) in continuously dividing cells or tissues, such as the exponential growth phase of yew suspension culture cells. In *Arabidopsis*, the meri-5 gene promoter (*m5p* hereafter) directs gene expression mostly in the meristematic dome, while the histone-3 gene promoter (*h3p* hereafter) drives gene expression in the peripheral zone of meristem in transgenic plants (Medford et al., 1991).

3.4 MATERIALS AND METHODS

3.4.1 Vector construction

The vector pCAMBIA1302 was obtained from CAMBIA (Center for the Application of Molecular Biology to International Agriculture, Australia). As illustrated in Figure 8 (next page), the integration of the *ts* gene as well as the *h3p* or *m5p* promoter into this vector was divided into 2 steps: 1) ligation of both promoters into pCAMBIA1302 to create the intermediate vectors pCM1302 (for the *m5p* promoter) and pCH1302 (for the *h3p* promoter); 2) integration of the *ts* gene into the two intermediate vectors to make pM5TS1302 (containing *m5p*) and pH3TS1302 (containing *h3p*). Both constructs contain the hygromycin B resistance gene as a selectable marker and the modified GFP (green fluorescent protein) gene (*mgfp5*) as an expression marker.

3.4.1.1 Ligation of m5p and h3p into vector pCAMBIA1302

The pCAMBIA1302 vector was first digested by HindIII and XbaI. The digestion product was then purified using the Promega Wizard gel purification kit.

The two promoters, *m5p* and *h3p*, were amplified by PCR from pMON11004 and pMON672 (Medford et al., 1991), respectively. Meanwhile, two corresponding restriction sites, i.e. HindIII and XbaI, were introduced into both amplified promoters for later integration. The primers for *m5p* amplification were: forward primer 5'-GGC CAA GCT TCC TCT GGT TTC TTC T-3' (underlined nucleotides show HindIII site) and reverse primer 5'-GGC CTC TAG ATG TGT GTG TGC GTA-3' (underlined nucleotides show XbaI site). The PCR was performed by *Pfu* DNA polymerase on a

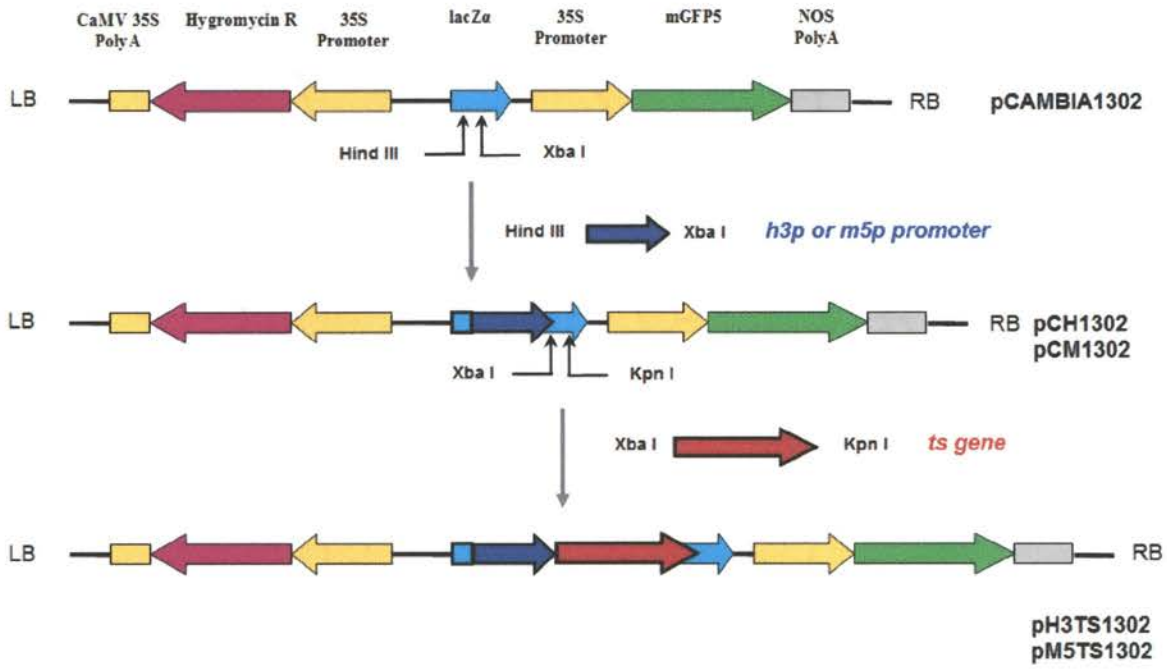


Figure 8. Illustration of the construction of the transformation vectors pH3TS1302 and pM5TS1302. The *h3p* and *m5p* promoters were first integrated into pCAMBIA1302 to construct pCH1302 and pCM1302; then the *ts* gene was inserted downstream of both promoters to form pH3TS1302 and pM5TS1302. In this process PCR was employed to create designated restriction sites in the 2 promoters and the *ts* gene. Only the T-DNA part of pCAMBIA1302 is shown here.

PTC-100® Thermal Cycler (MJ Research, Inc., Waltham, Massachusetts) with the following program: initial denaturation step at 95°C for 3 min followed with 5 cycles of 1 min at 95°C, 30 seconds at 31°C and 2 min at 72°C; then 30 cycles of 1 min at 95°C, 30 seconds at 69°C and 2 min at 72°C; and a final extension for 5 min at 72°C. The primers for *h3p* amplification were: forward 5'-GGC CAA GCT TTG CGG AAT CTG ATG-3' (underlined nucleotides show HindIII site) and reverse 5'-GGC CTC TAG AAA ATG TTA TTA AG-3' (underlined nucleotides show XbaI site). The PCR program for *h3p* amplification is: initial denaturation step at 95°C for 3 min followed with 5 cycles of 1 min at 95°C, 30 seconds at 31°C and 2 min at 72°C; then 30 cycles of 1 min at 95°C, 30 seconds at 57°C and 2 min at 72°C; and a final extension for 5 min at 72°C. After PCR, the reactions were purified utilizing the Promega Wizard PCR purification kit. Then the purified products were digested by HindIII and XbaI and the digestion products were purified from low-melting-point agarose gel utilizing the Promega Wizard gel purification kit.

The PCR-amplified and digested promoters of *m5p* and *h3p* were ligated into the digested pCAMBIA1302 using a Roche ligation kit. All manufacturer instructions were followed throughout the ligation process. The molar ratio of vector to promoter was 1:3.

Upon completion of the ligation, the reaction products were transformed into competent *E. coli* cells by following instructions on the Roche ligation kit manual. Transformed *E. coli* cells were grown on LB plates with kanamycin 50 mg/l overnight at 37°C. The identity of transformants was established by both restriction digestion and PCR amplification. For the restriction digestion confirmation, plasmids were isolated from single colonies utilizing the alkaline method and then digested by HindIII and XbaI.

For the PCR confirmation, the primers used for the amplification of the two promoters above were employed here for amplification of both promoters from the isolated plasmids.

3.4.1.2 Ligation of the ts gene into pCM1302 and pCH1302

The *ts* gene was first amplified from pBlueScript-*ts* plasmid, which was constructed previously. Two restriction sites, XbaI and KpnI, were integrated during the PCR process. The forward primer for the PCR was 5'-GGC CTC TAG AAT GGC TCA GCT C-3' (underlined nucleotides show XbaI site); and the reverse primer was 5'-GGC CGG TAC CTC ATA CTT GAA TTG-3' (underlined nucleotides show KpnI site). The PCR was performed by *Pfu* DNA polymerase on a PTC-100® Thermal Cycler (MJ Research, Inc., Waltham, Massachusetts) with the following program: initial denaturation step at 95°C for 3 min followed with 5 cycles of 1 min at 95°C, 1 min at 47°C and 6 min at 72°C; then 30 cycles of 1 min at 95°C, 1 min at 65°C and 6 min at 72°C; and a final extension for 5 min at 72°C. Next the PCR reactions were purified using the Promega Wizard PCR purification kit. The purified products were then digested by XbaI and KpnI. The XbaI-KpnI-digested full-length *ts* gene was purified using the Promega Wizard gel purification kit from low-melting-point agarose gel.

The two intermediate vectors, pCM1302 and pCH1302, were also digested with XbaI and KpnI. The digestion products were also purified from low-melting-point agarose.

The XbaI-KpnI-digested full-length *ts* gene, along with the purified digested pCM1302 and pCH1302 vectors, were then subject to ligation to construct pM5TS1302 and pH3TS1302 using the Roche ligation kit. All manufacturer instructions were

followed throughout the whole ligation process. The molar ratio of vector to insert was 1:3.

Upon completion of the ligation, the reaction products were transformed into competent *E. coli* cells by following instructions on the Roche ligation kit manual. Transformed *E. coli* cells were grown on LB plates with kanamycin 50 mg/l overnight at 37°C. The identity of transformants was established by both restriction digestion and PCR amplification. For the digestion confirmation, plasmids were isolated from single colonies using the alkaline method and then digested by XbaI and KpnI. For PCR confirmation, primers utilized for the amplification of the *ts* gene above were employed.

3.4.2 Introduction of constructed vectors into *Agrobacterium tumefaciens* by electroporation

Two strains of *A. tumefaciens*, Bo542 and LBA4404, were chosen as vector construct recipients. LBA4404 is a disarmed strain, while Bo542 still contains tumor producing genes in its Ti plasmid. Electroporation was employed to introduce the constructed vectors into competent cells of both strains.

3.4.2.1 Preparation of electroporation-competent Agrobacterium tumefaciens cells

The two strains of *A. tumefaciens* were first grown in 10 ml YM broth (0.04% yeast extract, 1% mannitol, 0.01% NaCl, 0.02% $MgSO_4 \cdot 7H_2O$, 0.05% $K_2PO_4 \cdot 3H_2O$, pH 7.0) with kanamycin 50 mg/l overnight at 29°C, until OD_{600} reached 0.5-0.7. Then the culture was chilled on ice for 15 min and subsequently centrifuged at 5,000 g for 15 min at 4°C. The pellet was washed in 7 ml 1mM HEPES (pH 7.0) twice. Finally, it was resuspended in 200 μl sterile HEPES containing 10% glycerol at a final concentration of

10^{11} bacteria/ml. These electroporation-competent cells were kept frozen at -80°C until use.

3.4.2.2 Electroporation

First the competent cells prepared above were thawed on ice. Then 1 μl construct plasmid (1-2 ng dissolved in sterile H_2O) was added to 45 μl thawed competent cells and the mixture was incubated on ice for 2 min. Next the mixture was transferred into a prechilled electroporation cuvette (0.2 cm gap), which was then put into the Gene Pulser (Bio-Rad). An electric pulse of 5 ms at field strength of 3 kV/cm was applied. Immediately after the electroporation, the cuvette was removed from the Gene Pulser and 1 ml of prechilled YM broth was added. The mixture was then transferred into a sterile centrifuge tube and incubated for 2 h at 28°C with gentle shaking. Finally the culture was spreaded onto YM broth plate with kanamycin 50 mg/l and incubated at 29°C for 2-3 days. Then colonies grown on the plate were checked for existence of the constructed vectors by restriction digestion and PCR amplification.

3.4.3 *In vitro* transformation of tobacco leaf disk

3.4.3.1 Leaf disk transformation

Preparation of *Agrobacterium tumefaciens* strains: Four to five colonies were first picked from the plate and resuspended in 25 ml YM broth contained in a 250 ml flask. The culture was incubated at 26-28°C overnight and the cell density was determined the next day using 1 $OD_{620} = 5 \times 10^8$ cells. The cells were then collected by centrifugation at 5,500 g for 10 min. The pellets were resuspended in MS medium at a concentration of 10^{10} cells/ml for leaf disk transformation.

Co-culture of leaf disks with *Agrobacterium tumefaciens* cells: Tobacco leaf disks were obtained from sterile plants by using paper punch. These leaf disks were placed in a sterile Petri dish. The *A. tumefaciens* strain resuspension inoculum was then added to the Petri dish to cover all the explants for 10 min. Next the leaf disks were sandwiched between 2 pieces of sterile, dry filter paper to remove excess *A. tumefaciens* cells. These explants were co-cultured with *Agrobacterium* cells for 2-3 days on co-culture medium (consisted of MS basal salts, Gamborg's B5 vitamins, 30 g/l sucrose, 0.1 mg/l NAA, 1.0 mg/l BAP) at room temperature.

Transgenic plant regeneration or tumorous callus development: After co-culture, the leaf disks were transferred to shoot induction medium (consisted of MS basal salts, Gamborg's B5 vitamins, 30 g/l sucrose, 0.1 mg/l NAA, 1.0 mg/l BAP, 50 mg/l hygromycin B, and 200 mg/l Timentin). The culture was placed at room temperature with a 16 h light/8 h dark cycle. For root regeneration, the regenerated shoots were cut from the leaf disks and placed onto root induction medium (consisted of MS basal salts, Gamborg's B5 vitamins, 30 g/l sucrose, 50 mg/l hygromycin B and 200 mg/l Timentin).

3.4.3.2 Isolation of genomic DNA from transgenic tobacco tissues

Genomic DNA was isolated from transgenic tobacco leaves and tumorous calli by a modification of the method of Murray and Thompson (1980). Fresh tissue (0.5-1.5 g) was ground to a fine powder in liquid nitrogen using a chilled pestle and mortar. The powder was then transferred, while still frozen, to a 30 ml centrifuge tube and 10 ml pre-warmed (65°C) 1X CTAB extraction buffer (50 mM Tris-HCl, pH 8.0, 0.7 M NaCl, 10 mM EDTA, pH 8.0, 3% CTAB, 20 mM 2-mercaptoethanol) was added. The mixture was swirled gently several times and then incubated at 65°C for 1 h with occasional swirling.

It was then cooled to room temperature and an equal volume of chloroform/isoamyl alcohol (24:1, v/v) was added. The tube was inverted repeatedly but gently to form an emulsion. Next it was centrifuged at 8,000 g for 10 min at room temperature to separate the phases. After the upper phase was transferred to a clean tube, an equal volume of 4 M NaCl was added to help remove polysaccharides from genomic DNA. After the addition of 4 M NaCl, 0.7 volume of isopropanol was added and mixed. The mixture was put into a -20°C freezer for 30 min to allow the DNA to precipitate. Then the DNA was pelleted by centrifugation at 12,000 g for 20 min. The DNA pellet was washed with 70% ethanol once, dried and resuspended in TE buffer. In order to remove RNA contamination, RNase A was added to a final concentration of 10μg/ml.

3.4.3.3 Isolation of total RNA from transgenic tobacco tissues

Fresh transgenic tobacco leaves and tumorous calli (1-2 g) were ground in liquid nitrogen to a fine powder by using a mortar and pestle. Immediately after grinding, the frozen powder was transferred to a centrifuge tube containing the extraction buffer (1X TE (pH 8.0), 1 mM ATA (aurintricarboxylic acid), 4% PAS (p-aminosalicylate), 1% TPN (tri-isopropylnaphthalene), 2% β-mercaptoethanol). After mixing thoroughly, phenol: chloroform: isoamyl alcohol (25:24:1, v/v/v) was added to the mixture. The tube was inverted repeatedly to form an emulsion. Then the mixture was centrifuged at 10,000 g for 15 min. The upper phase was collected into another clean tube and fresh ATA was added to a final concentration of 1 mM. Then RNA was precipitated overnight by adding 12 M LiCl to a final concentration of 2 M and thorough mixing. The next day RNA was pelleted by centrifugation at 10,000 g for 20 min at 4°C. The RNA pellet was dissolved in 200 μl 0.1 mM ATA. After the RNA pellet was completely dissolved, the RNA was

precipitated again by adding 100 μl 7.5 M NH_4OAc and 600 μl ice-cold ethanol. After incubating at -70°C for at least 30 min, the RNA pellet was collected at 12,000 g for 30 min at 4°C. Finally the RNA was dissolved in DEPC-dH_2O containing 50 μM ATA.

3.4.3.4 PCR confirmation of tobacco transformation

For PCR confirmation, two *ts*-specific primers (forward primer: 5'-GGC CTC TAG AAT GGC TCA GCT C-3'; reverse primer: 5'-GCA GAA ACA TCT GTA AGC CTG G-3') were used to amplify an 800 bp *ts* fragment from the isolated tobacco genomic DNA. Additionally, two *mgfp5*-specific primers (forward primer: 5'-AAC TAC CTG TTC CAT GGC CA-3'; reverse primer: 5'-ATG TGT AAT CCC AGC AGC TGT-3') were also employed to amplify a 500 bp target fragment, in order to further confirm the existence of the modified GFP gene in the transgenic tissue.

3.4.3.5 Southern blot confirmation of tobacco transformation

The isolated tobacco genomic DNA was first digested by EcoRI and XbaI overnight at 37°C. The next day the digested DNA was loaded and separated in a 0.8% agarose gel. After separation, the gel was stained in 1μg/ml ethidium bromide solution. The DNA bands were visualized and photographed along with a ruler as a size reference under UV light. Next, the gel was incubated in 500 ml of 0.25 M HCl for 15-30 min until the blue dye turned yellow and washed twice with deionized water. The gel was then incubated in 0.4 M NaOH for 15-30 min. A downward capillary blot procedure developed by Chomczynski (1992) was followed for maximal transfer of DNA from the gel to a positively charged nylon membrane. After transfer, the gel was restained in ethidium bromide to check the transfer efficiency. The nylon membrane was subjected to

prehybridization directly. The prehybridization was carried out in a plastic heat-sealable bag on a shaking incubator at 65°C for 4-5 h. For hybridization, an 800 bp *ts* gene fragment was labeled with ^{32}P by random labeling using the Roche High Primer DNA labeling kit. The hybridization was performed at 65°C overnight in a shaker. The next day the membrane was first washed at room temperature in 2X SSC/0.1% SDS twice with 5 min each. Next, the membrane was washed at room temperature in 0.2X SSC/0.1% SDS twice, also 5 min each time. Further washing was done in 0.1X SSC/0.1% SDS at 65°C. Finally the membrane was wrapped in a Saran film and exposed to X-ray film at -80°C.

3.4.3.6 Northern blot confirmation of tobacco transformation

The isolated total RNA was first separated in 1% formaldehyde gel. The RNA bands were then visualized and photographed under UV light. To remove formaldehyde, the gel was soaked in DEPC-dH_2O for 30 min with several changes of water. Next the gel was subject to hydrolysis by soaking in 0.05 M NaOH/1.5 M NaCl for 30 min. The gel was then rinsed with 20X SSC and subject to downward capillary blot procedure as described for the Southern blot. After the transfer, RNA was UV-crosslinked to the nylon membrane using the Stratalinker® UV Crosslinker (Stratagene Corp., La Jolla, California). The prehybridization and hybridization were carried out at 42°C. The hybridization probe, as well as the later washing steps, was the same as that used for the Southern blot.

3.4.4 *In vitro* transformation of yew suspension cells

3.4.4.1 Establishment of yew suspension culture

Young stems of *Taxus x media* cv. 'Hicksii' were subjected to callus induction. Young stems cut from yew plants were first cleaned with soapy water for 15 min. Then

they were soaked in 75% ethanol for 1 min and subsequently 30% commercial bleach for 10 min. After sterilization, these young stems were cut into pieces of 1-2 cm in length and put onto callus induction medium (Gamborg's B5 basic medium supplemented with 2,4-D 2.0 mg/l and BAP 0.1 mg/l). Callus was induced in 2 weeks. These calli were then used to initiate suspension cell cultures by crushing them into fine particles, passing the particles through a CELLECTOR 85 ml tissue sieve (EC582, E-C Apparatus Corp., St. Petersburg, Florida) and culturing the cells in 50 ml liquid medium with the same components as the callus-induction medium.

3.4.4.2 Transformation of yew suspension cells

Before transformation, the medium for the suspension culture was changed to co-inoculation medium (Gamborg's B5 basic medium supplemented with vitamin C 50 mg/l, BAP 0.1 mg/l, 2,4-D 2.0 mg/l, glucose 10 g/l and acetosyringone 100 μM). Meanwhile, the transformed *A. tumefaciens* strains were grown in 1.5 ml LB liquid medium with kanamycin 50 mg/l for 2 days at 28°C. Then the *A. tumefaciens* cells were collected at 5,000 g and the pellet was washed 3 times with the co-inoculation solution. After washing, the cells were resuspended in 1 ml co-inoculation medium and the resuspended cells were added into the yew suspension cell cultures. After 2 days of co-inoculation, the culture cells were washed twice with selection medium (Gamborg's B5 basic medium supplemented with vitamin C 50 mg/l, BAP 0.1 mg/l, 2,4-D 2.0 mg/l and cefotaxime 250 mg/l) and then resuspended in 50 ml selection medium. The expression of GFP protein in the yew suspension cells was detected by microscopy 2 days later.

3.4.4.3 Visualization of GFP expression

A Nikon E600 epifluorescence microscope with a blue excitation filter (420–490 nm) was utilized to visualize GFP expression in the transformed yew suspension culture cells. The photos were captured by Optronics Magnafire camera (Intelligent Imaging Innovations, Inc., Denver, CO).

3.4.5 *In planta* transformation of *Taxus x media* cv. 'Hicksii'

3.4.5.1 Source of yew plants

The plants of *T. x media* cv. 'Hicksii' were obtained from a local nursery. The yew plants were grown in green house conditions.

3.4.5.2 Inoculation of yew plants with Agrobacterium tumefaciens strains

The transformation vector-harboring strains of *A. tumefaciens*, Bo542 and LBA4404, were first streaked to fresh YM medium with kanamycin 50 mg/l and grown for 2-3 days. The fully grown *Agrobacterium* cells were picked by scalpel blades from the plates and then applied to yew plants by cutting wounds in branches and inoculating the *Agrobacterium* cells into the wounds.

3.4.5.3 Determination of Taxol content in Agrobacterium-induced gall tissue

After galls were induced from the *A. tumefaciens*-inoculated wounds, they were cut off from the host plants and dried at 45°C until a constant weight was reached. Next the dried gall tissues were ground into fine powders by using a pestle and mortar. The subsequent extraction, SPE purification and HPLC analyses followed protocols described in the "materials and methods" part of Chapter 2.

3.5 RESULTS

3.5.1 Vector construction

The vector construction process, for the integration of high-efficiency promoter (*h3p* and *m5p*) and *ts* gene into the binary vector pCAMBIA1302, involved two phases: first incorporation of the promoter and later integration of the *ts* gene. Since the incompatibility of restriction sites among the original vector, the promoters and the *ts* gene, PCR was employed to introduce designated restriction sites into the *ts* gene and the two promoters, *h3p* and *m5p*. For the two promoters, a HindIII restriction site was introduced into the 5' end while a XbaI site was integrated into the 3' end. For the *ts* gene, XbaI and KpnI restriction sites were incorporated into the 5' and 3' ends, respectively.

The identity of the intermediate vector constructs (pCM1302 and pCH1302) and the final vector constructs (pM5TS1302 and pH3TS1302), was checked by restriction digestion (Figures 9 and 10, next page) and the PCR amplification (data not shown here). The appearance of the expected-sized bands in both analyses confirmed the integration of both the promoters and the *ts* gene.

3.5.2 *In vitro* transformation of tobacco leaf disk

3.5.2.1 Regeneration of transgenic plants and formation of tumorous callus

After 2-day co-culture of tobacco leaf disks with LBA4404 and Bo542 harboring the designated vector constructs, the leaf disks were put onto shoot induction medium with 50 mg/l hygromycin B as selection agent. In 3 weeks, shoots were formed on LBA4404-infected leaf disks, while tumorous calli were developed from those infected

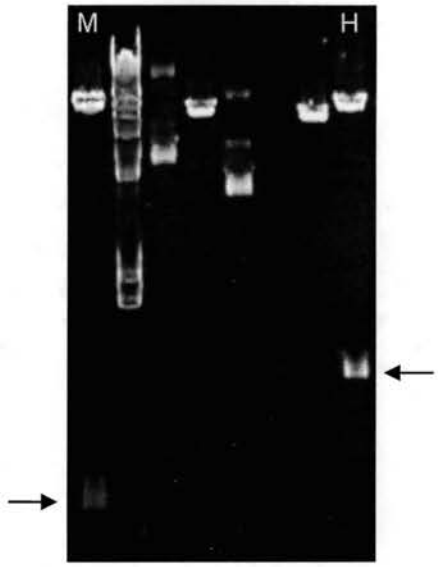


Figure 9. Restriction digestion confirmation of the identities of pCM1302 (M) and pCH1302 (H). Arrows indicate the digested *h3p* (1.4 kb) and *m5p* (730 bp) fragments. The larger band at the upper part was the original vector. The 6 lanes in the middle were molecular marker and negatives.

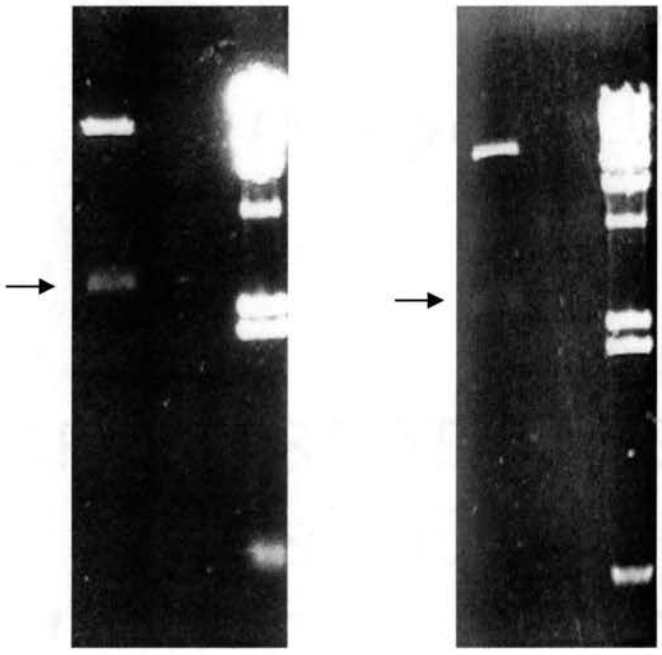


Figure 10. Confirmation of the identities of pH3TS1302 (A) and pM5TS1302 (B) by restriction digestion. Arrows indicate the digested *ts* gene fragment (2.8 kb). The larger band is the half-finished vector (pCH1302 and pCM1302) part.

by Bo542. This difference is due to the fact that LBA4404 is a disarmed strain, while Bo542 is not. The negative control leaf disks, which were inoculated with blank culture medium, gradually died on the selection medium (Figure 11, next page).

For LBA4404-mediated transformation, the young shoots formed on the edges of leaf disks were transferred to root induction medium, which also contained 50 mg/l hygromycin B. Roots developed in this medium in about one week. After the formation of a well-developed root system, the regenerated plantlets were transferred into soil (Figure 12, p.71).

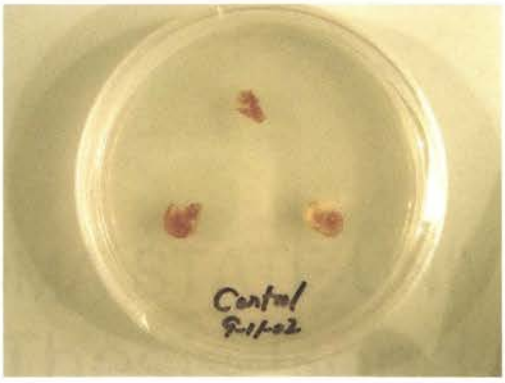
For Bo542-mediated transformation, no shoots developed from the induced tumorous callus. These tumorous calli were subcultured onto new medium every 10 days. Since no regenerated transgenic plants were available for the Bo542-mediated transformation, later analyses were performed on these tumorous calli.

3.5.2.2 Confirmation of transformation

As a preliminary confirmation of the integration of the *ts* gene in the transformed tobacco tissue, PCR was performed by using *ts*-specific primers. The appearance of the expected-sized band (800 bp) proved the existence of the *ts* gene in the transgenic tobacco genome. In addition, the expected 500 bp band in the *mgfp5*-specific PCR amplification further proved the integration of the T-DNA region of the vector constructs in the tobacco genome. Southern and Northern blot were then carried out to further confirm the integration and transcription of the *ts* gene in the tobacco cells. Figure 13 (p.72) shows an example of these analyses on the pM5TS1302-harboring Bo542 induced tumorous calli.

A

B



C

Figure 11. Transformation of tobacco leaf disk. (A) Shoots developed on leaf disks infected by LBA4404; (B) Tumorous calli formed on leaf disks inoculated with Bo542; (C) Leaf disks inoculated with blank culture medium (negative control) gradually died on selection medium.

A

B

Figure 12. Transgenic tobacco plants on root-induction medium (A) and transferred into soil (B).

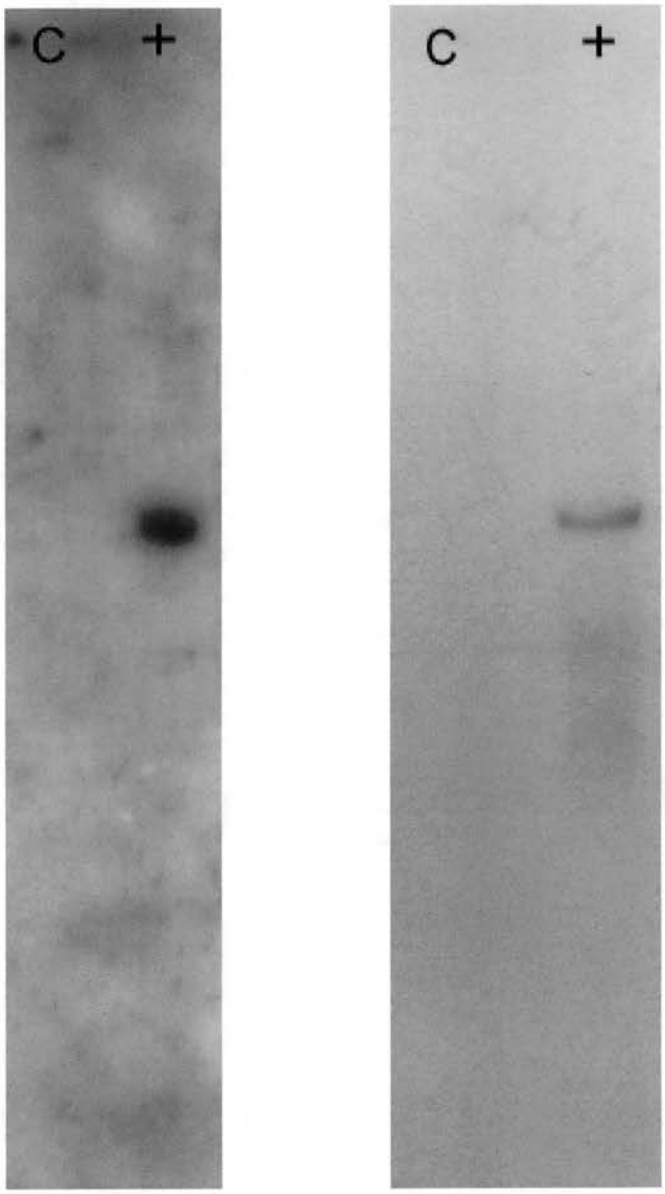


Figure 13. Autoradiographs from the Southern (A) and Northern (B) blot analyses of the tobacco leaf disk transformation. The result was obtained from the pM5TS1302-harboring Bo542 mediated transformation (The lane marked with "C" was negative control).

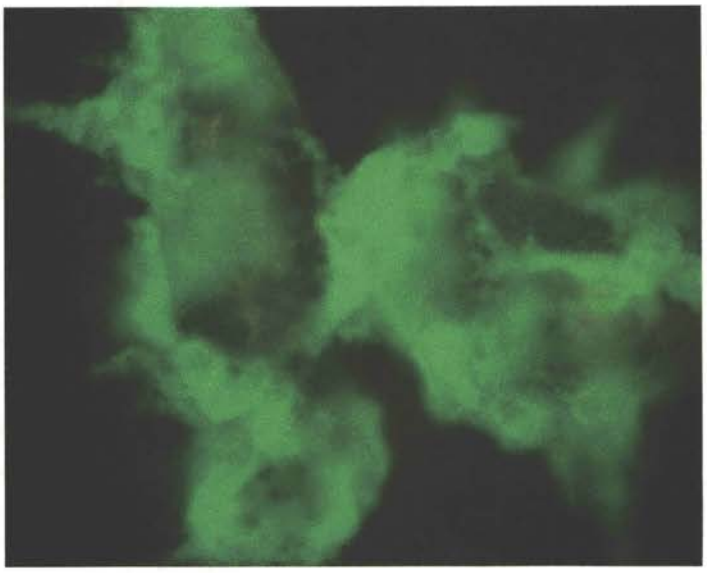
3.5.3 *In vitro* transformation of yew suspension cells

The transformation of tobacco leaf disks by the designated *ts*-containing vector constructs as described above indicates that this *A. tumefaciens*-mediated transformation system worked as anticipated. Since there is no Taxol biosynthesis pathway in tobacco, the next logic step is to transfer the high-efficiency-promoter-driven *ts* gene back into *Taxus* cells. Because of its high potential for industrial bioreactor Taxol production, suspension cell culture established from *T. x media* cv. 'Hicksii' was employed here as the target for *in vitro A. tumefaciens*-mediated transformation.

Both vector constructs, pH3TS1302 and pM5TS1302, contain *mgfp5* gene (a modified form of GFP) as an expression marker. Its expression was detected in yew suspension cells two days after the *Agrobacterium* cell inoculation. The existence of the GFP-specific green fluorescence in suspension cells demonstrated the integration and expression of the T-DNA region gene in both vector constructs (Figure 14, next page).

3.5.4 *In planta* transformation of *Taxus x media* cv. 'Hicksii'

Under natural conditions, when a host plant is attacked by *A. tumefaciens*, gall develops. Here the efforts had been focused on inducing gall formation by inoculating yew plants with *A. tumefaciens* strains harboring pH3TS1302 and pM5TS1302, with the aim of improving Taxol production in the transformed tissues. After inoculation, galls gradually formed at some of the Bo542-infected wounding sites (Figure 15, p.75). The first formation of gall tissues was observed two months after the inoculation. More galls were formed over the time. Generally, the growth rate of these galls was relatively slow.



A

B

Figure 14. Visualization of GFP expression in yew suspension cells transformed by pH3TS1302 (A). Those transformed by pM5TS1302 displayed the same GFP expression pattern (not shown here). Picture B was yew suspension cells transformed by blank culture medium as a negative control.

Figure 15. *In planta* transformation of *Taxus x media* cv. 'Hicksii'. After inoculating the plants with *Agrobacterium tumefaciens* strains carrying pH3TS1302 and pM5TS1302, galls gradually formed at the Bo542-infected wounding sites. Significant improvement of Taxol production was detected in these gall tissues by later HPLC quantitation analysis.

HPLC quantitation were performed on these gall tissues to determine their Taxol content. As shown in Table III, Taxol production was remarkably increased in these tissues. This improvement is a good indication of increased activity of the Taxol biosynthesis pathway. This, in turn, suggests the feasibility of increasing Taxol production by overexpressing taxadiene synthase.

Table III. Taxol content in the gall tissues induced on *Taxus x media* cv. 'Hicksii' by *in planta Agrobacterium tumefaciens*-mediated transformation with *ts* gene

Gall Sample	Gall Source	Taxol Content (μg/g gall dried weight)
1	Bo542/pH3TS1302	**16664**
2	Negative control for Bo542/pH3TS1302[a]	**n/d[b]**
3	Bo542/pM5TS1302	**10233**
4	Negative control for Bo542/pM5TS1302[a]	**805**

[a] Uninoculated *Taxus x media* cv. 'Hicksii' stem of the same age as those used for *in planta* transformation

[b] Not detected

3.6 DISCUSSION

It is quite a challenge to apply metabolic engineering to improve Taxol production at the present, since detailed information on the entire Taxol biosynthetic pathway is still not available. By estimation, there are around 20 steps in this pathway (Walker and Croteau, 2001). As reviewed in the first chapter, 10 steps have been characterized to date. Therefore, about one half of the steps along the Taxol biosynthetic pathway are still

unknown. Under this limited understanding of the pathway, this research has been focused on taxadiene synthase, the enzyme catalyzing the first committed reaction of Taxol biosynthesis *in vivo*, which is a possible key rate-limiting step in the pathway. High-efficiency promoters were designed to drive the overexpression of its encoding gene; when the completed gene constructs were used to transform the yew plant *in planta*, the production of Taxol was shown to be greatly increased. This fact clearly proves that the conversion of GGPP to taxa-4(5),11(12)-diene is a bottleneck step in the Taxol biosynthesis pathway.

This exciting result suggests significant potential to improve Taxol production by metabolic engineering . Although the growth rate of the *Agrobacterium tumefaciens*-induced gall was very slow in this research and thus is hardly a practical way to supply Taxol, the approach of overexpressing a rate-limiting enzyme to increase Taxol production has been proved successful. The production of Taxol in transformed suspension cell cultures was not quantitated, because the cultures did not survive long enough after the *A. tumefaciens*-mediated transformation.

This study demonstrated that the taxadiene synthase-catalyzed first committed step of Taxol biosynthesis is a rate-limiting step, which seemingly disagrees with some previous research. Hezari et al. (1997) reported that the appearance of taxadiene synthase activity preceded Taxol accumulation by 3 days, and persisted into the stationary phase (see Figure 16, p.79). The authors concluded that rate-limiting steps lay farther down the pathway than the cyclization step in their *Taxus canadensis* suspension culture system. This inconsistency may be due to two factors. First, it may result from differences between the *A. tumefaciens*-induced gall used in this research and the *T. canadensis*

suspension culture system in the previous report. Second, it is possibly due to species differences between *T. x media* cv. 'Hicksii' used in this study and *T. canadensis* in the previous report. In addition, in Figure 16, although taxadiene synthase activity preceded Taxol accumulation by 3 days, their overall dynamics had certain degree of similarity, which indicated a possible correlation between them. The time gap between taxadiene synthase activity and Taxol accumulation may indicate the possible existence of more than one rate-limiting step in this pathway. Overcoming the first rate-limiting step would speed up the whole process to some degree depending on the limits placed by other rate-limiting steps. With the discovery of more rate-limiting steps in the Taxol biosynthesis pathway, further improvement of Taxol production is possible by metabolic engineering approaches.

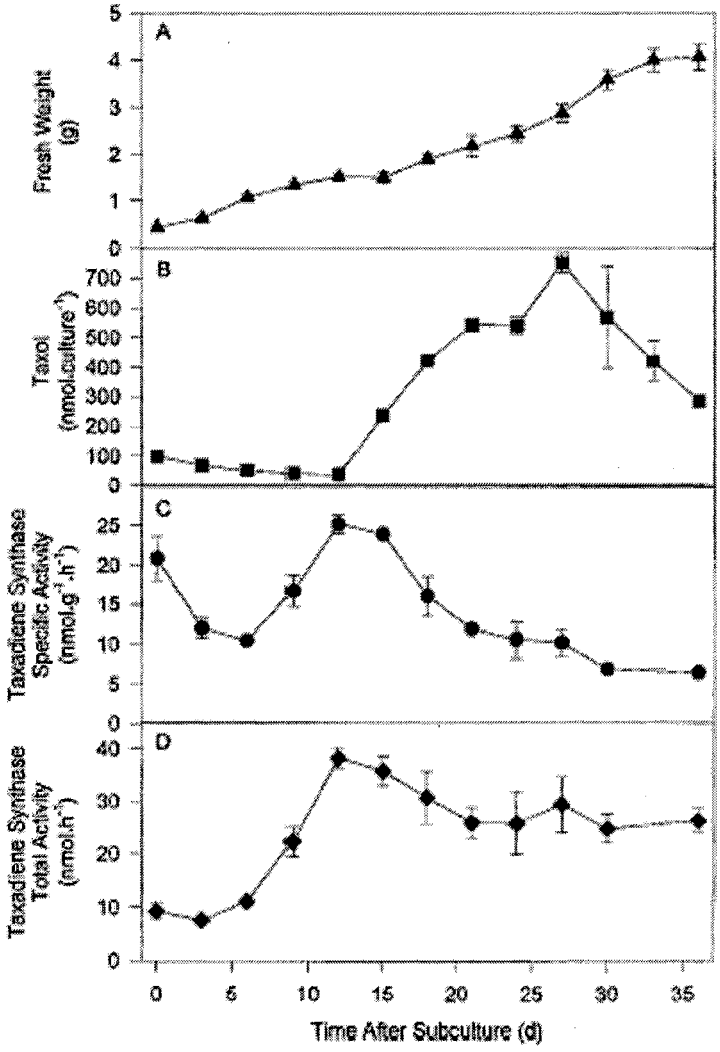


Figure 16. Time courses of Taxol content and taxadiene synthase activity, as well as fresh weight of *Taxus canadensis* suspension cells. Reprinted from Archives of Biochemistry and Biophysics, Vol 337, Hezari et al., "Taxol Production and Taxadiene Synthase Activity in *Taxus canadensis* Cell Suspension Cultures", pp 185-190, Copyright 1997, with permission from Elsevier.

3.7 REFERENCE

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CHAPTER IV

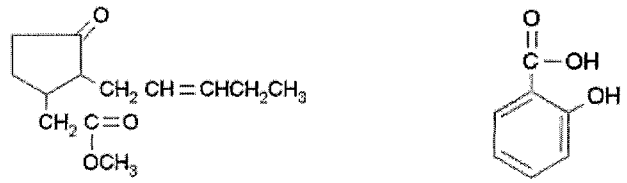
GENE EXPRESSION PROFILING OF YEW SUSPENSION CELLS IN RESPONSE TO ELICITATION BY METHYL JASMONATE AND SALICYLIC ACID

4.1 INTRODUCTION

The biosynthesis of Taxol is a sophisticated process, involving the synthesis of the diterpene core and the later attachment of the side chain. By present estimation, this whole process includes about 20 steps, half of which have been characterized to date (Jennewein et al., 2001; Walker and Croteau, 2001; Walker et al., 2002a; Walker et al., 2002b). Taxadiene synthase catalyzes the first committed step of Taxol biosynthesis and is supposed to be one of the rate-limiting steps in this pathway (Koepp et al., 1995; Wildung and Croteau, 1996). In chapter III, overexpression of this enzyme led to a significant increase of Taxol production. To further increase Taxol production by metabolic engineering, presently unknown enzymes and their encoding genes, along with the entire Taxol production regulatory network, must be illuminated. Reverse genetics and homology-based PCR cloning are currently the two main approaches for the cloning of genes in Taxol biosynthesis pathway, and account for the discovery of most genes to date. However, both methods have their limitations. For reverse genetics, a sufficient amount of purified protein, which is not always readily available, is needed for peptide sequencing before designing degenerate primers for gene cloning. The homology-based PCR cloning is designed to identify genes sharing some common characteristics; completely new genes are missed by this method.

As universal plant endogenous messenger molecules, methyl jasmonate (MeJA) and salicylic acid (SA) (Figure 17, next page) play key roles in a wide range of physiological events such as flowering, senescence and defense responses (Delaney et al., 1994; Reinbothe et al., 1994). It is also well known that the elicitation of yew suspension culture by MeJA and SA leads to increased production of Taxol (Yukimune et al., 1996).

In this elicitation process, expression of a large number of genes is regulated by MeJA and SA through a series of intercellular and intracellular signaling pathways, among which are those genes related to Taxol production.



A B

Figure 17. Molecular structures of methyl jasmonate (A) and salicylic acid (B).

Gene expression profiling, or transcript profiling, is a powerful approach for identifying novel genes by analyzing alterations of gene expression patterns (Donson et al., 2002). For yew cell suspension cultures, genes showing differential expression pattern can be identified by comparing gene expression profiles between elicited and non-elicited yew cells. The identification of presently unknown Taxol-production-related genes should be possible by screening these differentially expressed genes.

The cDNA-AFLP expression profiling is the method of choice here for the isolation of differentially expressed genes because of its high reliability, good reproducibility and high sensitivity. The effectiveness of cDNA-AFLP to identify differentially expressed genes has been fully proven by previous reports (Bachem et al., 1996; Qin et al., 2000; van der Biezen et al., 2000; Bachem et al., 2001; Campalans et al., 2001; Ditt et al., 2001; Milioni et al., 2001; Noel et al., 2001; Petters et al., 2002; Simoes-Araujo et al., 2002; Dubos and Plomion, 2003).

4.2 HYPOTHESIS

Since the elicitation of *Taxus* suspension cell cultures by MeJA and SA increases Taxol production, it is logical to assume that the expression of genes related to Taxol production, as well as other genes, is regulated by this elicitation. By comparing elicited and non-elicited yew cells, differentially expressed genes can be identified through gene expression profiling techniques (such as cDNA-AFLP). Those currently unknown Taxol-production-related genes should be among these differentially expressed genes.

4.3 OBJECTIVE

Based on the above hypothesis, the purpose of this research was to isolate differentially expressed elicitation-related genes by cDNA-AFLP, among which currently unknown Taxol-production-related genes may be discovered.

4.4 MATERIALS AND METHODS

4.4.1 Elicitation of yew suspension cells

Taxus suspension cell cultures were established as described in Chapter III. For elicitation, MeJA and SA were added into the suspension medium to a final concentration of 100 μM and 0.1 mg/l, respectively. After 48 h of elicitation, the suspension cells were collected by centrifugation at 3,000 g and stored at -80°C for later RNA isolation. In addition to the suspension cells, callus induced from young stems of *Taxus x media* cv. 'Hicksii', which were used to initiate the suspension cultures, was subjected to the same elicitation treatment for later cDNA-AFLP analysis.

4.4.2 Isolation of total RNA

Total RNA was isolated from the elicited and non-elicited (control) cells by first grinding 1 g of the collected cells in liquid nitrogen to a fine powder using a pestle and mortar. After grinding, the frozen powder was immediately mixed with 10 ml extraction buffer (containing 2% CTAB, 2% PVP (K30), 100 mM Tris-HCl (pH 8.0), 25 mM EDTA, 2.0 M NaCl, 0.5 g/l spermidine, 2% 2-mercaptoethanol) and the mixture was incubated at 65°C for 15 min. This mixture was extracted twice with an equal volume of chloroform:isoamyl alcohol (24:1, v/v), with the phases separated at 10,000 g for 10 min at room temperature. After adding 1/4 volume 10 M LiCl to the supernatant and gentle mixing, total RNA was precipitated overnight at 4°C. The next day the RNA was pelleted by centrifugation at 12,000 g for 20 min at 4°C. The RNA pellet was then dissolved in 500 μl SSTE (0.6 M NaCl, 0.2% SDS, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA) and extracted once more with an equal volume of chloroform:isoamyl alcohol (24:1, v/v). Two volumes of ethanol were added to the aqueous phase to precipitate RNA at -80°C for at least 30 min. Finally the RNA pellet was spun down, dried and resuspended in 50 μl DEPC-H_2O.

4.4.3 Synthesis of cDNA

4.4.3.1 Purification of mRNA from total RNA

The PolyATtract® mRNA Isolation System III from Promega was used to purify mRNA from total RNA. The procedure was carried out according to the product manual. The isolation of mRNA from total RNA was achieved by first hybridizing the 3' poly(A)$^+$ region of mRNA with biotinylated oligo(dT) primer, and subsequently binding the

formed hybrid to streptavidin-coupled paramagnetic particles, which were then captured by a magnetic stand. The mRNA was obtained by washing from the binding complex at high stringency.

4.4.3.2 Synthesis of the first-strand cDNA

The first-strand cDNA synthesis reaction mix included the following reagents:

10 µl	polyA$^+$ RNA (from 4.4.3.2 above)
3 µl	Oligo(dT)$_{12-18}$ Primer (Invitrogen)
5 µl	10X cDNA-1 buffer (500 mM Tris-HCl, pH 8.3, 800 mM KCl, 100 mM $MgCl_2$, 40 mM DTT)
2 µl	dNTPs (25 mM each, Invitrogen)
0.6 µl	MMLV Reverse Transcriptase (GenHunter)
29.4 µl	RNase-free H_2O
---	---
50 µl	Total reaction mixture volume

Reaction condition: incubation at 42°C for 2 h.

4.4.3.3 Synthesis of the second-strand cDNA

For the second-strand cDNA synthesis, the following reagents were added to the first-strand reaction mix:

15 µl	10X cDNA-2 buffer (350 mM Tris-HCl, pH 7.4, 40 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 30 mM DTT)
3.5 µl	DNA Polymerase I (Promega)

1.5 µl	RNase H (Invitrogen)
1 µl	dNTPs (25 mM each, Invitrogen)
79 µl	H_2O
150 µl	Total reaction mixture volume

Reaction condition: incubation at 16°C for 2 h.

The reaction was extracted by chloroform:isoamyl alcohol (24:1, v/v), followed by precipitation of the ds cDNA by ethanol. After washing, the cDNA pellet was dissolved in 40 µl H_2O.

4.4.4 cDNA-AFLP

The cDNA-AFLP (for illustration see figure 4, p.22) was implemented using the Invitrogen AFLP® Analysis System I kit according to the procedure given in the product manual. The cDNA was first digested by EcoRI and MseI. Then the adapters were ligated to the digested cDNA fragments. Next, preamplification was performed, followed by selective amplification. For selective AFLP amplification, the EcoRI primers were labeled with [γ-^{33}P]ATP. All the 64 primer combinations in this kit were utilized to ensure comprehensive coverage. The selective AFLP amplification products were separated on 6% polyacrylamide gel. The gel was dried and then exposed to X-ray film at -70°C.

4.4.5 Re-amplification and cloning of differentially expressed gene fragments

Bands showing differential expression patterns were identified and marked by comparing bands side by side on the developed films. By aligning the film and the gel,

hydrated by soaking in 100 µl water at 95°C for 15 min. Then, after brief centrifugation, the supernatants were amplified with the same primers and under the same conditions as for the earlier cDNA-AFLP selective amplification. The re-amplified Differentially Expressed Gene Fragment (DEGeF) bands were then ligated into the pCR® 2.1 vector by using the TOPO TA Cloning® Kit from Invitrogen.

4.4.6 DNA sequencing and BLAST search

The cloned DEGeFs were sequenced on a Perkin-Elmer Applied Biosystems ABI 373A automated DNA sequencer using the Big Dye Terminator technology. Each fragment sequence was then compared against the NCBI protein databases by using the BLASTX program. For fragment sequences with no significant homology found by BLASTX, BLASTN was employed to compare against the NCBI nucleotide databases. The criteria for a hit with *significant* similarity was the expect value (E-value) being less than 0.0001, and for these analyses only the most significant hits were examined.

4.4.7 Dot blot confirmation

The differential expression patterns of the DEGeFs found by cDNA-AFLP were confirmed by dot blot. The re-amplified DNA fragments showing differential patterns were first purified and then applied to positively charged nylon membrane. The cDNA probes for the hybridization were prepared from the total RNA isolated from the elicited and non-elicited cells, with the SMART™ PCR cDNA Synthesis Kit (Clontech, Palo Alto, California).

4.5 RESULTS

4.5.1 Isolation of differentially expressed genes during MeJA and SA elicitation

A total of 157 MeJA and SA-elicitation-related DEGeFs were isolated using the cDNA-AFLP approach. Only bands showing the most distinctive differential expression pattern were isolated and re-amplified for sequencing (Figure 18, next page). These DEGeFs were categorized into 4 groups: 1) MeJA-elicitation-specific: genes represented in this group are up-regulated by MeJA (but not SA) elicitation; 2) SA-elicitation-specific: those up-regulated by SA (but not MeJA) elicitation; 3) MeJA&SA-elicitation-specific: those up-regulated by both MeJA and SA elicitation; 4) Control-specific: genes represented by this group are down-regulated by MeJA and SA elicitation. The distribution of these 157 DEGeFs are shown in Table IV.

Table IV. The distribution of differentially expressed gene fragments (DEGeFs) in *Taxus* suspension cell cultures and callus during MeJA and SA elicitation

	Control	MeJA	SA	MeJA&SA	Total
Suspension Cell	**16**	**20**	**10**	**19**	**65**
Callus	**29**	**10**	**37**	**16**	**92**

4.5.2 Expression pattern confirmation of the isolated DEGeFs

Dot blots, which are virtual reverse Northerns, were carried out to confirm the expression pattern of all the re-amplified DEGeFs. Compared to other methods, the dot

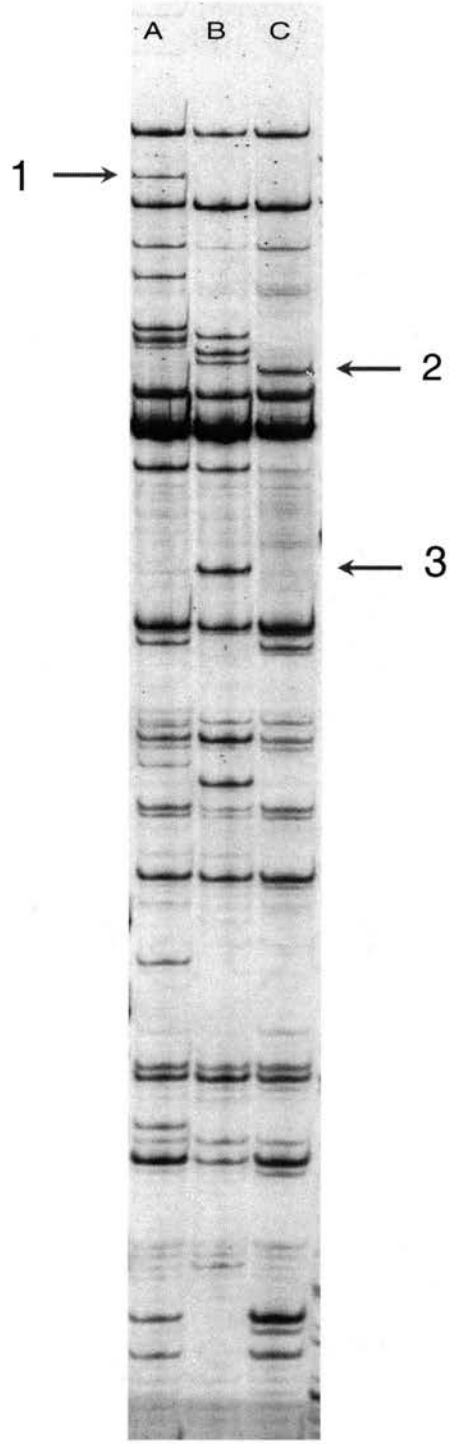


Figure 18. The cDNA-AFLP polyacrylamide gel autoradiography pattern. The 3 lanes represent negative control (A), MeJA (B) and SA (C) elicitation. Only bands showing the most distinctive differential expression pattern were identified and re-amplified, such as the 3 bands shown here (1: control-specific, 2: SA-specific and 3: MeJA-specific).

blot method has the advantage of confirming multiple samples at one time from a small amount of total RNA (as low as 2 μg). Figure 19 (next page) shows an example of this confirmation for DEGeFs isolated from suspension cells elicited by MeJA.

4.5.3 Sequence analysis and BLAST search

Of the 157 DEGeFs isolated 105 were re-amplified, of which 70 were sequenced. The size of these sequenced DEGeFs was from 120 bp to 500 bp, with most of them in the 200-400 bp range. Among the 70 sequenced DEGeFs, 15 were found by BLASTX to encode proteins that showed significant similarity to previously published sequences in the NCBI peptide sequence databases. For the rest, BLASTN searches showed that 2 had similarity with entries in the EST databases. Table V (p.94) is a full list showing the homologies of the DEGeF sequences to those in the protein and nucleotide databases. Most of the genes in the significant hits list were related to pathogenesis or other stresses such as heat shock. This is consistent with the roles MeJA and SA play in plant cells. However, no matches were directly related to the Taxol biosynthesis pathway based on these BLAST searches.

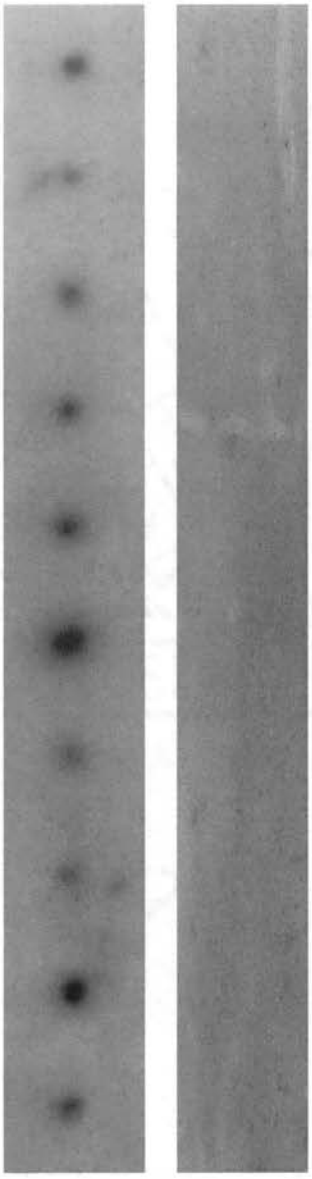


Figure 19. Dot blot confirmation of the expression pattern of the differentially expressed gene fragments (DEGeFs) identified by cDNA-AFLP. Data shown here were the confirmation of the 10 DEGeFs up-regulated by SA in suspension culture (Panel A: the re-amplified and purified DEGeFs hybridized with SMART^{TM} cDNA synthesized from total RNA of SA-elicited suspension cells; Panel B: DEGeFs hybridized with SMART^{TM} cDNA synthesized from total RNA of negative control suspension cells).

Table V. Sequenced differentially expressed gene fragments (DEGeFs) showing homologies to entries in the NCBI protein and nucleotide databases

DEGeF	Group	Length (bp)	Homology	E-Value
C4	Control	340	Putative *Arabidopsis* protein (gi|15229628)	9e-13
C9	Control	264	Translation initiation inhibitor (gi|21232668)	5.4e-24
C15	Control	332	*Pinus taeda* cDNA clone (gi|12125849)	1.2e-8
C46	SA	132	*Pinus taeda* cDNA clone (gi|12482945)	2.7e-4
C59	SA	280	Putative *Arabidopsis* polyprotein (gi|13699782)	3.7e-6
C63	SA	262	Pectate lyase (gi|20149058)	5e-44
C71	SA	284	14-3-3 protein (gi|8515888)	2.8e-30
C72	SA	329	Auxin down-regulated (ADR) 11-2 protein (gi|485514)	4.7e-13
C87/C88	MeJA&SA	471	Unknown *Arabidopsis* protein (gi|15225276)	2.3e-46
S6	Control	218	Putative *Arabidopsis* protein kinase (gi|15222512)	3.5e-27
S8	Control	496	Putative intracellular pathogenesis-related protein (gi|6466176)	1.2e-27
S9	Control	287	Putative *Arabidopsis* heat shock protein (gi|15228059)	2.3e-21
S32	MeJA	384	Cation efflux system transmembrane protein (gi|17549149)	2.5e-45
S34	MeJA	342	Unknown *Arabidopsis* protein (gi|21593282)	1.6e-6
S45/S46	MeJA&SA	264	Translation initiation inhibitor (gi|21232668)	5.4e-24

4.6 DISCUSSION

As important plant signaling molecules, methyl jasmonate and salicylic acid induce a series of responses in *Taxus* cells *in vitro*. In an attempt to characterize these responses, a recently developed transcript profiling approach, cDNA-AFLP, was employed to identify genes whose expression pattern was altered by MeJA and SA. Compared to other approaches such as the widely applied differential display, this method has the advantages of high reliability, high sensitivity, and good reproducibility, which were validated by this research. Replicated reactions on the same sample generated highly reproducible gene expression patterns.

Since the MeJA and SA elicitation increases Taxol production (Yukimune et al., 1996), genes related to Taxol production are assumed to be included in the MeJA and SA signaling network. However, none of the currently known Taxol biosynthetic genes were found in the 70 sequenced differentially expressed gene fragments (DEGeFs) in this study. There are several reasons for this. First, this is possibly due to the limited number of entries in the current DNA and protein sequence databases. By estimation, only about half of the Taxol biosynthesis pathway genes and proteins sequence information are deposited in the databases. Furthermore, there are no genes or proteins currently known to be directly related to Taxol *degradation* and production *regulation* in GenBank. Therefore, these DEGeFs may represent completely new genes related to the biosynthesis, degradation or production regulation of Taxol. Second, the distribution of the two restriction enzyme cutting sites employed in this study, EcoRI and MseI, is not uniform among different genes. Some genes may not have both restriction sites in their encoding sequence, which can be exemplified by several genes in the Taxol biosynthesis

pathway, like those encoding taxadiene synthase (Genbank Accession No. U48796), DBNT (AF193765), BAPT (AY082804) and DBTNBT (AF466397). These genes are not amplified by the EcoRI-MseI cDNA-AFLP process. Lastly, in order to have complete coverage of differentially expressed gene upon MeJA and SA elicitation, all the possible 64 primer combinations were utilized. However, due to the overwhelming number of differential bands (including both strong and weak bands showing differential signal intensities) on the cDNA-AFLP polyacrylamide gel, only bands showing the most distinctive differential expression pattern were re-amplified and sequenced. Most of these DEGeFs are anticipated to represent freshly activated genes with no or minimum expression prior to elicitation. Since Taxol production related genes are assumed to be expressed at low level prior to elicitation, these genes may have been missed.

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