ISOLATION OF A GENE CODING FOR TAXADIENE SYNTHASE IN TAXOL PRODUCTION

By

BAOXIAN LIU

Bachelor of Science

Beijing Agricultural University

Beijing, P. R. China

1992

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
July, 1997

ISOLATION OF A GENE CODING FOR TAXADIENE SYNTHASE IN TAXOL PRODUCTION

Thesis Approved:

Thesis Adviser

Dean of the Graduate College

ACKNOWLEDGEMENTS

Completion of this work was made possible by the joint efforts of several people to whom I am deeply indebted. First, I wish to thank my thesis adviser, Dr. Yinghua Huang, for his guidance and encouragement offered generously throughout this study. I would also like to extend my sincere appreciation to Dr. Chuck Tauer for his support and valuable suggestions. Many thanks are due to my committee member, Dr. Andrew J. Mort for his time and support.

I sincerely appreciate the Department of Forestry, OSU, for its financial support throughout my graduate study, and also for the Afanasiev Distinguished Graduate Fellowship.

I appreciate the encouragement and support from the many of my friends at OSU. Being side by side with them simply alleviated some of my home sickness and together we benefited from our endeavor in the totally new environment. I am satisfied I can fulfill my M. Sc. degree in this time frame.

As their only daughter, I wish to dedicate this work to my parents, whose value instilled me. I will be always thankful to their love and encouragement.

TABLE OF CONTENTS

Part | Page

I. INTRODUCTION 1

1.1 Goals and Objectives of the Study 1
1.2 Significance of the Study 3
1.3 Literature Review 5
1.3.1 Taxol and Related Taxanes 5
1.3.2 Taxa-4(5), 11(12)-diene Synthase 15
1.3.3 *Taxus baccata* 17
1.3.4 Transformation Using *Agrobacterium* System 18

II. MATERIALS AND METHODS 22

2.1 Isolation of the Taxadiene Synthase Gene from a cDNA Library of *Taxus baccata* 22
2.1.1 Plant Material 22
2.1.2 Probe Generation 22
2.1.3 Screening of the cDNA Library 30
2.1.4 *In vivo* Excision 34
2.1.5 Sequencing of the cDNA Clones 35
2.1.6 Completion of Cloning the Full-length Gene 36
2.1.7 Northern Blot Analysis 37
2.2 *Agrobacterium*-mediated Transformation of *Taxus* 40
2.2.1 Materials 40
2.2.2 Stem Cuttings and Inoculation 41
2.2.3 Seedlings and Inoculation 42

III. RESULTS AND DISCUSSION 44

3.1 The Taxadiene Synthase Gene Cloned from a cDNA Library of *Taxus baccata* 44
3.1.1 Isolation of the Taxadiene Synthase cDNA Clone 44
3.1.2 Nucleotide Sequence of the Taxadiene Synthase cDNA from *Taxus baccata* 47
3.1.3 Sequence Comparison with Other Terpenoid Cyclases 58
3.1.4 RNA Blot Analysis 59
3.2 Transformation of *Taxus* 62

Part Page

IV. CONCLUSION 68

REFERENCES 70

LIST OF TABLES

Table | Page

I-1. Chronology of taxol development 6

I-2. Advantages of using Ri over Ti plasmids for transformation 20

II-1. Characteristics of the *Agrobacterium rhizogenes* strains used for *Taxus* species susceptibility test 40

III-1. Root induction in stem cuttings of *Taxus x media* Runyan eight weeks after infection with *Agrobacterium rhizogenes* 66

III-2. Root induction in seedlings of *Taxus baccata* four weeks after infection with *Agrobacterium rhizogenes* TR105 66

LIST OF FIGURES

Figure | Page

I-1. Structure of taxanes: (A) taxol and (B) taxotere 8

I-2. Structure of 10-deacetylbaccatin III (10-DAB) 11

I-3. Semisynthesis of taxotere 11

I-4. Biosynthetic pathway of taxol and related taxanes 14

I-5. Biosynthetic pathway of 10-deacetylbaccatin III (10-DAB) 18

III-1. Nucleotide and predicted amino acid sequences of *Taxus baccata* taxadiene synthase cDNA 49

III-2. Comparison of the DNA sequences of taxadiene synthase of *Taxus baccata* and *T. brevifolia* 52

III-3. Comparison of the deduced amino acid sequences of taxadiene synthase of *Taxus baccata* and *T. brevifolia* 56

III-4. Expression pattern of taxadiene synthase at the transcriptional level 61

III-5. Roots induced at the wound site of an *in vitro* cultured stem cutting of *Taxus x media* Runyan two months after infection with *Agrobacterium rhizogenes* 65

III-6. Roots induced at the wound site of an *in vitro* cultured seedling of *Taxus baccata* four weeks after infection with *Agrobacterium rhizogenes TR105* 67

PART I

INTRODUCTION

1.1 Goals and Objectives of the Study

Taxol and other taxanes are antimicrotubule antitumor agents that are effective on many advanced and early stages of cancers, but the supply of taxol is limited since it is produced mainly by the bark of *Taxus brevifolia* Nutt. (Pacific yew). Alternative approaches for taxol production include: plant cell culture, yew cultivation, total synthesis, semisynthesis of the taxol analog taxotere, biosynthesis, and genetic engineering (Suffness, 1995). Among these alternatives, semisynthesis is the most promising approach. Semisynthesis depends on the precursor 10-deacetylbaccatin III (10-DAB) produced by *T. baccata* L. (European yew). Defining the taxane biosynthetic pathway and manipulating the rate-limiting enzymatic steps by genetic engineering will greatly assist in efforts to develop and improve the biological routes for taxane production.

The hypothesis for this research is that taxane production in yew cells is under genetic control, and the expression of taxol and related taxanes is due to the activities of some specific genes involved in the biosynthetic pathway. Production of these antitumor

compounds can be improved through over-expression of these genes and optimization of other regulatory factors affecting the biosynthetic pathway.

The long term goal of our research is to develop a bioengineering system for large-scale production of taxol and related taxanes *in vitro* at a reasonable cost. The success is dependent on exploring molecular mechanisms underlying biosynthesis of taxol and related taxanes in yew cells, and on developing an efficient transformation system for *Taxus*.

Taxadiene synthase is considered to be an enzyme that catalyzes the committed step in the biosynthetic pathway(Koepp et al., 1995). This enzyme has been isolated (Hezari et al., 1995) and its role in taxol biosynthesis was reported (Lin et al., 1996). To achieve our long term goal, the specific objectives of this research were to identify, clone, and characterize the gene coding for taxadiene synthase from *T. baccata* which has high concentration of 10-DAB. To develop an efficient genetic engineering system for yew trees focused on testing the virulence of *Agrobacterium rhizogenes* on various yew explants.

OKLAHOMA STATE UNIVERS

1.2 Significance of the Study

Taxanes are such effective anti-cancer drugs that they are being used in more than 50 countries. However, the supply of the drugs limits their extensive use. The original source of supply, isolation of taxol from bark of *Taxus brevifolia* is being replaced by the semisynthesis of its analog taxotere using 10-DAB from *T. baccata*.

The objective of this study is to improve the amount of 10-DAB produced per gram in *T. baccata* tissue. After cloning the gene coding for taxadiene synthase, this gene could be ligated to promoter sequences and transferred back into yew by *Agrobacterium rhizogenes*, leading to an increase in copy number of the gene, and enhancement in the production of the enzyme. Since taxadiene synthase catalyzes the rate-limiting step in the taxane biosynthetic pathway, the concentration of 10-DAB should be increased by overexpression of this enzyme. More precursors will improve the yield of the taxol analog using semisynthesis. Thus, more taxol analog can be produced to meet demand, the drug will be cheaper, and more people may be cured of cancer in the future.

The major significance of the present research, however, is more fundamental in nature. This research will expand the knowledge about the biosynthetic pathway of taxol and related taxanes. The taxadiene synthase gene can be functionally expressed in microorganisms. The availability of the gene and overexpressed taxadiene synthase will permit details of the action mechanism of taxol and the relationship of its structure and function to be deduced at a level previously impossible.

The knowledge of the terpenoid cyclase family can also be extended. Taxadiene synthase belongs to this family, having homology at amino acid sequence level. Comparison of the sequence and mechanism of the cyclases should provide evidence concerning their origin

OKLAHOMA STATE UNIVERSITY

and evolutionary development that has resulted in a large number of plant products in this family.

The understanding of conifers at the molecular level is very limited. The homology of taxadiene synthase in both *T. baccata* and *T. brevifolia* can be revealed by comparing the DNA and amino acid sequences of the enzyme from these two species.

Since genetic transformation in *Taxus* species is still in its infancy, this research will augment knowledge of the host range of *Agrobacterium* for *Taxus* species. Knowledge of the interaction between *Agrobacterium* and *Taxus* will provide the basic information needed to genetically engineer *Taxus* genomes with desirable traits using this *Agrobacterium rhizogenes*-mediated gene transformation system.

1.3 Literature Review

1.3.1 Taxol and Related Taxanes

The taxane class of antimicrotubule anticancer drugs is probably the most important member of the chemotherapeutic armamentarium against cancer developed in the past several years. After only a brief period, the taxanes have not only shown a unique effectiveness to assuage the symptoms of many types of advanced cancers such as carcinoma of the ovary, lung, head and neck, bladder, and esophagus, they have also demonstrated effectiveness in initial therapy fighting earlier stages of cancer (Rowinsky et al., 1992).

History and Chemical Structure

The prototypic taxane, taxol, was the first agent in the taxane class used in clinical trials. The bioassay-directed analysis of *T. brevifolia* and other *Taxus* species resulted in the isolation of taxol (Cancer Chemotherapy National Service Center, 1961). The structure of taxol was determined in 1971 (Wani et al., 1971). In 1979, it was found that taxol killed cancer cells in a unique way, by binding to tubulin (Schiff et al., 1979). Taxol's novel structure and unique mechanism of action made it a high priority for clinical trials at the National Cancer Institute (NCI). The progress of the early clinical trials was extremely slow because only 0.5 kilogram of taxol was produced over two years. Although trials were started in 1983, it took 6 years to demonstrate clinical activity in solid tumors. The first success was with refractory ovarian cancer (McGuire et al., 1989). The NCI contracted with Bristol-Myers Squibb (BMS) to further develop the drug in 1991. BMS then established cooperative agreements with multiple government agencies to continue drug development. In 1992 the Food and Drug

Administration (FDA) approved taxol for marketing. The initial shortage of taxol for general clinical use had been alleviated by this time. The major events of taxol development are listed in Table I-1, and details of these studies can be found in some review papers (Suffness, 1993; Wall and Vani, 1995).

Table I-1. Chronology of taxol development.

Major events	Time
Isolation and structure/P388 and L1210 activity	1971
Activity in a panel of tumor systems	1975-1976
Preclinical development	1977
Mechanism of action	1979
Animal toxicology	1982
Phase I clinical trials	1983-1984
Phase II clinical trials	1985-1986
Synthesis of taxol side chain	1986
Semisynthesis of taxol	1988
Activity in refractory ovarian cancer	1989
Improved syntheses of taxol side chain	1990-1993
Bristol-Myers Squibb receives CRADA* from NCI	January 1991
NDA* filed with FDA	December 1992
Total synthesis of taxol/Holton-Nicolaou	1994
Biosynthetic pathway: taxadiene synthase	1995

* CRADA: Cooperative Research and Development Agreement

NDA: New Drug Application

There are two major taxanes, taxol (paclitaxel, BMS) and taxotere (docetaxel, Rhone-Poulenc Rhorer). Taxol is a diterpenoid (diterpene ester) of molecular weight 853 ($C_{47}H_{51}NO_{14}$) (Figure I-1). Taxotere, an analog of taxol, is derived semisynthetically from 10-deacetylbaccatin III (10-DAB) and is slightly more water soluble than taxol (Bissery, 1995). Both taxol and taxotere consist of a complex taxane ring (geranylgeranyl pyrophosphate-originated) which is linked to an ester (phenylalanine-originated) at the C-13 position (Figure I-1). The moieties at the C-2' and C-3' position on the C-13 side chain are essential for their antimicrotuble activity. The structure of taxotere is different from the structure of taxol in that a *tert*-butoxycarbonyl group instead of a benzoyl group is found on the nitrogen atom at C-3' of the C-13 side chain and a hydroxyl group instead of an acetate at the C-10 position of the taxane B-ring.

Mechanism of Action

Most natural antimitotic agents, such as colchicine, podophyllotoxin, vincristine and vinblastine, bind to soluble tubulin and freeze the polymerization of tubulin to inhibit the formation of microtubules (Horwitz et al., 1981; Horwitz, 1992). Due to their unique structures, taxanes have a mechanism of action different from the above agents. Taxanes are mitotic inhibitors. They bind to the N-terminal 31 amino acids of the ß-tubulin subunit of tubulin polymers (Rao et al., 1994), and catalyze the formation of discrete bundles to stabilize the microtubules from depolymerization to tubulin (Schiff et al., 1979; Schiff and Horwitz, 1981). Therefore, taxanes disrupt the equilibrium of microtubules and tubulin dimers toward polymerization. These effects occur even in the absence of GTP- and microtubule-associated proteins, which are usually essential for this function.

Figure I-1. Structure of taxanes: (A) taxol and (B) taxotere.

The taxanes prevent cell proliferation via formation of a sustained mitotic block at the metaphase/anaphase boundary, and via inducing an incomplete metaphase plate of chromosomes and an aberrant organization of spindle microtubules (Jordon et al., 1996). Taxol acts to inhibit inherent microtubule processes, which involve the balance between tubular dimers and microtuble polymers. The stabilization of microtuble dynamics causes abnormal mitotic spindles and mitotic block. After taxane treatment, tumor cells have been documented to show morphologic features and DNA fragmentation patterns characteristic of programmed cell death or apoptosis (Jordon et al., 1996). These apoptotic effects are due to the phosphorylation of *bcl-2*, an antiapoptotic protein, which interrupts the equilibrium between the dimerization of *bcl* and *bax* proteins (Haldar et al., 1996). Both taxol and taxotere have also been shown to enhance the cytotoxic effects of ionizing radiation in vitro at clinically attainable concentrations, which may result from the inhibition of cell-cycle procession in the G2 and M phases, the most radiosensitive phases of the cell cycle (Hajek et al., 1996).

Alternative Sources for Taxane Production

Taxanes have become more and more important for people today. Taxol is approved for use in the therapy of both breast and ovarian cancer. Taxol is currently in use in more than 50 countries worldwide. Therefore, the demand for taxanes is increasing year by year, but the original supply of taxol has limited its extensive use (Cragg and Snader, 1991) since its natural source is the bark of *T. brevifolia*. *T. brevifolia* is a small slow-growing evergreen, and harvesting bark tissue from the tree causes the yew to die. The drug is too expensive for people to afford because the yield is very low—about 100 mg taxol per kg of dried bark (Huang et al., 1986). Therefore, alternative techniques are needed to ensure sufficient drug supply and reasonable cost when the drug is approved for clinical use. Although taxol

production from bark is an immediate source, yew bark is a finite resource. The long-term, commercially viable sources of taxanes might include precursor isolation and semisynthesis, yew cultivation for biomass production, plant cell culture, and biosynthesis and genetic engineering (Suffness, 1995). Interestingly, a fungus, *Taxomyces andreanae* that was isolated from the inner bark of *Taxus* can produced taxol (Stierle et al., 1993; Strobel et al., 1993), but the significance of this fungus is not elucidated yet.

Semisynthesis

Although the supply of most drugs rely on total synthesis and taxol has also been prepared by this approach (Nicolaou et al., 1994; Holton et al., 1994a; 1994b), total chemical synthesis of taxanes, at present, is not commercially viable since the yield is too low (Borman, 1994). Semisynthesis of taxol and the analog taxotere from more abundant taxane diterpenoids (taxoids), for example via the conversion of cephalomannine (Kingston, 1995) or by C-13 side-chain attachment to 10-DAB (Figure I-2, Figure I-3) (Guénard et al., 1993; Kingston et al., 1993; Nicolaou et al., 1994), has provided an immediate alternative source of the drug.

The original source of taxol, *T. brevifolia,* does not appear to be a long term source, and indeed is already being replaced by semisynthesis of taxotere using 10-DAB which is produced from leaves of *T. baccata* and its close ally *T. wallichiama* Zucc. (considered as a sub-species of *T. baccata*) (Suffness, 1995). Comparison of the characteristics of taxol with its analog taxotere shows that taxotere induces tubulin polymerization at a 2.1-fold lower critical tubulin concentration, and its affinity for the N-terminal 31 amino acids site of the ß-tubulin subunit is 1.9-fold higher than taxol's affinity (Diaz and Andreu, 1993). Taxotere also has a greater initial slope of the assembly reaction and a greater amount of polymer is formed. In addition, the ability of inducing cytotoxicity *in vitro* and in tumor xenografts is greater for

taxotere (Ringel and Horwitz, 1991). These differences do not imply that taxotere has a greater therapeutic index because greater potency may also portend greater toxicity.

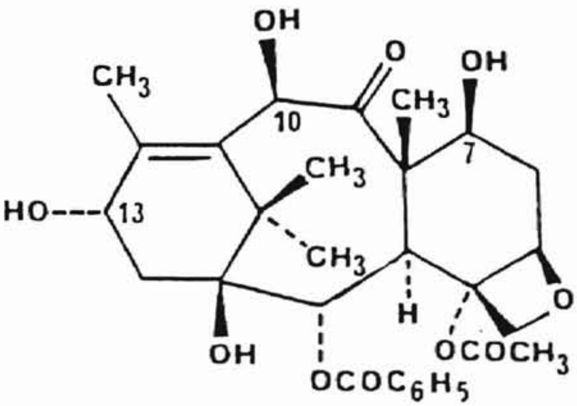


Figure I-2. Structure of 10-deacetylbaccatin III (10-DAB).

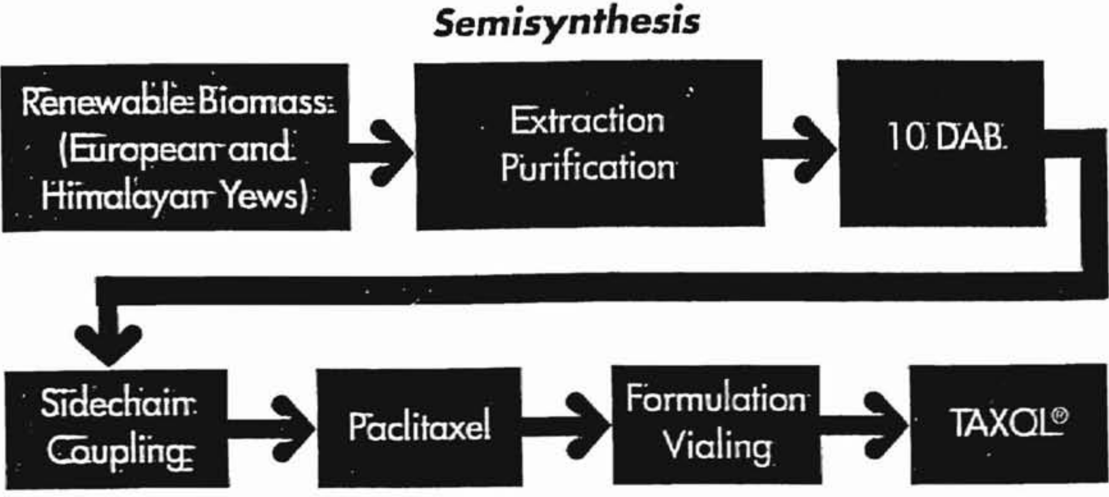


Figure I-3. Semisynthesis of taxotere (Adapted from DeFuria, 1996).

Although production of taxol has the distinct advantage in that it is the final marketed compound, there are three notable disadvantages: purity, flexibility, and cost. Minor compounds are co-eluted with taxol, and they are variable with climatic changes, location of plantations, time of harvest, etc. This would not be a problem if the end product of cultivation was 10-DAB and the last several steps in taxol production were synthetic steps with more defined and reproducible chemical byproducts. Improvement of the biomass of 10-DAB is a more flexible approach since 10-DAB is a key intermediate for a broad diversity of compounds with alteration in the C-13 side chain, including taxotere. The yields for 10-DAB from *T. baccata* are 200 to 1,000 milligrams per kg of fresh leaves while the yields for taxol from *T. brevifolia* bark are 50 to 200 milligram per kg of dried bark, thus the production of 10-DAB costs much less than the production of taxol (Suffness, 1995). How long taxol will remain on the market before it is replaced by newer analogues is a critical issue.

Biological Methods

The semisynthesis approach must rely on biological methods for the production of suitable advanced precursors, thus requiring the sustained, large-scale growth of *Taxus* species or, ultimately, the generation of taxol and its progenitors in cell culture.

Yew cultivation includes strain selection and strain improvement. The most obvious object of yew cultivation is to determine the best yew species or genotypes for yield of taxanes: taxol and/or 10-DAB. Rapid growth, resistance to diseases, and suppression of other metabolites are also parameters of selection. Moreover, it is important to examine individual plants of the highest yielding populations, and to clone the best of these using tissue culture methodology as starting stock for plantations.

Plant cell culture has many advantages over other alternatives for taxol production. It is a more environmentally favorable process, generates product faster than cultivation, it is a controlled, reliable source of high-quality material, and production levels can be matched to commercial demand. The cell culture conditions are more easily controlled to assure the fermentation of the product of choice and its high quality. Downstream processing for extraction and purification of the wanted product often can be simplified. Cell culture provides the opportunity for additional novel metabolites to be identified that may have a broader spectrum of activity or an increased potency. There is no doubt that taxanes can be produced by cell culture, the only question is whether the cost will be competitive with current method of semisynthesis.

Biosynthetic Pathway

Understanding biosynthesis of taxanes will greatly assist efforts to develop and improve biological routes for taxane production. Such understanding includes many possibilities, such as 1) enhancement of production via feeding of precursors presently shown in limiting quantities; 2) enhancement of yield through manipulation of the genes coding for the enzymes involved in biosynthesis, through activation of gene transcription, increase in copy number of genes, introduction of promoter sequences to enhance expression, or transfer of the entire biosynthetic pathway to a fast growing bacterium such as *E. coli*; 3) increased yield in plant cell culture, and 4) the use of mutational techniques to modify key genes and enhance the efficiency of their derived enzymes. The results most needed for increasing taxane production are to determine the rate limiting steps in the biosynthetic pathway and to apply genetic technologies to increase these rates (Suffness, 1995).

The biosynthetic pathway of taxol is not yet well defined. Based on structures of previously isolated taxanes, the biosynthesis of the side chain and nucleus of taxol are separate. The study of enzymatic formation of taxanes can be divided into three parts: the formation of the taxane ring system and substitutions, the synthesis of the sidechain, and the attachment of the sidechain to the diterpene moiety (Figure I-4) (Heinstein and Chang, 1994).

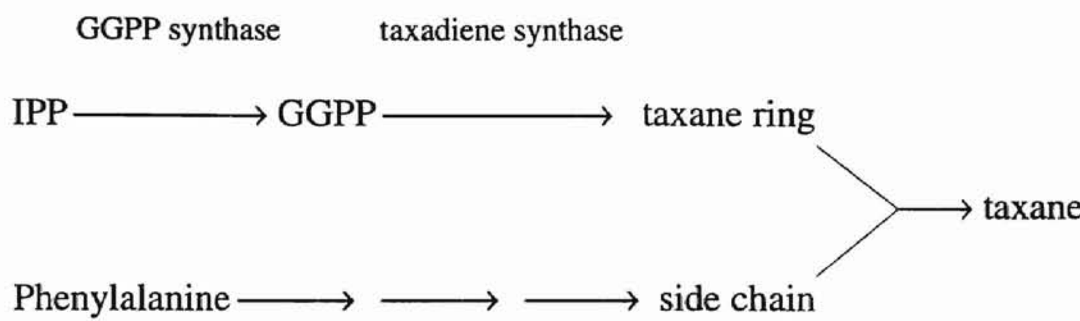


Figure I-4. Biosynthetic pathway of taxol and related taxanes.

The entire pathway for the side chain has been elucidated (Fleming et al., 1993; Lansing et al., 1991).

The obvious precursor of the taxane ring is geranylgeranyl pyrophosphate (GGPP), the ubiquitous C-20 isoprenoid intermediate (Mutton, 1962). GGPP is synthesized through a mevalonate pathway: GGPP synthase, a member of the prenyltransferase family, catalyzes the successive 1'-4 condensation of three molecules of isoprentenyl pyrophosphate (IPP) with homoallylic demethylallyl pyrophosphate (DMAPP) to generate GGPP (Dogbo and Camara, 1987). First GGPP synthase catalyzes the combination of two molecules: IPP and DMAPP, a multi-step chemical process, providing the simplest compound of terpene—the monoterpene geranylgeranyl pyrophosphate (GPP, C10). Then, it catalyzes the combination of GPP and IPP

providing the sesquiterpene farnesyl pyrophosphate (FPP, C15). Finally, the combination of FPP and IPP produces GGPP. Since mevalonate is a precursor for an extremely wide set of terpenoids (Mutton, 1962; Coates, 1976; Bartley et al., 1994; Sandonann, 1994), sterols (Goodwin, 1971; Kuntz et al., 1992), and prenylated compounds (Rilling et al., 1990), and is readily produced, the availability of GGPP is unlikely to be a rate limiting step.

The initial folding and cyclization, which gives rise to the specific diterpenoid nucleus, have been demonstrated to often be a slow step along the pathway. The cyclization of GGPP to the taxane skeleton which is catalyzed by a diterpene olefin cyclase, taxa-4(5), 11(12)-diene synthase (Hezari et al., 1995; Gueritte-Voegelein et al.; 1987; Lewis and Croteau, 1992), is considered to be a rate limiting step (Koepp et al., 1995), thus if the taxadiene synthase gene could be characterized, and cloned, it could be manipulated to improve the efficiency of the whole pathway.

1.3.2 Taxa-4(5), 11(12)-diene Synthase

Taxadiene Synthase

The mechanism of cyclization of GGPP to taxa-4(5), 11(12)-diene has been elucidated (Lin et al., 1996). C-C bond formation between C-1 and C-14 of the substrate is produced by ionization of the GGPP ester and closure of the A ring via *re*-face attack at C-10 occurs. Then 1S-verticillene is afforded through deprotonation of the resulting 11α-verticillyl cation by removal of the 11α-hydrogen. This bound intermediate is rapidly reprotonated at C-7 through the same enzyme base responsible for the earlier deprotonation step. The reprotonation then initiates transannular cyclization to yield the taxenyl cation, which produces the endocyclic double bond of the taxadiene product upon deprotonation at C-5.

Taxadiene synthase (cyclase) which catalyzes the transformation of GGPP to taxa-4(5), 11(12)-diene has been partially purified from bark of *Taxus brevifolia* and characterized (Hezari et al., 1995). The enzyme is a monomer of 79 kDa. In general characteristics including operational solubility, requirement for Mg^{2+}, and Km for GGPP, taxadiene synthase is similar to several other diterpenoid cyclases from various higher plants, such as *cis*-abienol synthase of *Nicotiana tabacum* (tobacco) (Guo et al., 1994), kaurene synthase A and B in biosynthesis of gibberellin (Frost and West, 1977; Duncan and West, 1981), casbene synthase of phytoalexin formation in *Ricinus communis* (castor bean) (Dueber et al., 1978), and abietadiene synthases of resin acid production in conifers (LaFever et al., 1994). Among them, taxadiene synthase is most like casbene synthase in enzyme and cyclization reaction (Hezari et al., 1995). However, these two diterpenoid cyclases are distinguished in optimum pH and response to inhibitors, which implies a difference in active site arrangement. A notable distinction of the taxadiene synthase is the low tissue levels of activity, and the apparent lack of response to stem injury or elicitor challenge.

Recently, the cDNA sequence of taxadiene synthase has been reported for *Taxus brevifolia* (Wildung and Croteau, 1996). The open reading frame of the sequence contains 2586 nucleotides. The deduced polypeptide has 862 amino acid residues, and the molecular weight is 98303. This polypeptide contains a long presumptive plastidial transit peptide which is about 137 residues, and the mature protein is approximately 725 amino acids of 82.5 kDa. The cDNA sequence has a high similarity with other plant terpenoid cyclases.

Terpenoid Cyclases

Taxadiene synthase belongs to the terpenoid cyclase family. This class of cyclases have a common ancestry. Most of the cyclases have the DDXXD motif(s) in their amino acid

sequences which is thought to be a substrate binding site (Chappell, 1995; McGarvey and Croteau, 1995). Many of the enzymes and DNA clones have been isolated, such as abietadiene cyclase from *Abies grandis* (Dougl. C x D. Don) Lindl. (grand fir) (Vogel et al., 1996), *ent*-kaurene synthase A from *Arabidopsis thaliana* (Sun and Kamiya, 1994) and *Zea mays* (maize) (Bensen et al., 1995), kaurene synthase B from *Cucurbita maxima* (pumpkin) (Yamaguchi et al., 1996), (–)-limonene synthase from *Mentha specata* (spearmint) (Colby et al, 1993), 5-*epi*-aristolochene synthase from *Nicotiana tabacum* (Facchini and Chappell, 1992), and casbene synthase from *Ricinus communis* (Mau and West, 1994). Among them, the taxadiene cyclase most closely resembles abietadiene synthase from *Abies grandis* (Vogel et al., 1996).

1.3.3 *Taxus baccata*

Taxol and related taxanes are produced not only by *Taxus brevifolia*, but also by other *Taxus* species (Vidensek et al., 1990; Witherup et al., 1990; Fett-Neto and DiCosmo, 1992). In some species, the yield of taxol is higher than in *T. brevifolia* (Choi et al., 1995).

T. baccata is a yew species which has been cultivated as ornamental trees for a long time, and the knowledge about this species is greater than that about *T. brevifolia*. Moreover, the supply of 10-DAB in semisynthesis of taxotere is dependent on *T. baccata*. It will greatly assist the production of 10-DAB if the biosynthetic pathway in this species is elucidated. Taxadiene synthase catalyzes the committed step in the pathway (Figure I-5). Thus if this enzyme can be cloned from *T. baccata*, it could be manipulated to result in enhancement of the entire pathway. Although the gene coding for taxadiene synthase was cloned from *T. brevifolia* (Wildung and Croteau, 1996), the degree of homology in taxane biosynthesis among *Taxus* species is unknown because little is known about these conifers at the molecular level.

1.3.4 Transformation Using *Agrobacterium* System

Genetic engineering, with abundant knowledge about the genes involved in the rate-limiting steps in the taxol biosynthetic pathway, promises to augment the *in vitro* production system of taxol and related taxoids by direct manipulation of the plant genome (Suffness, 1995). For this approach to be successful, a stable genetic transformation system has to be developed for *Taxus* species.

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

→ → → 10-deacetylbaccatin III

Figure I-5. Biosynthetic pathway of 10-deacetylbaccatin III (10-DAB).

Agrobacterium is a pathogenic gram-negative soil bacterium which has been studied widely due to its natural ability of transferring part of its genetic information into plant genomes (for review see: Hooyklaas and Beijersbergen, 1994; Zupan and Zambryski, 1995). The Ti (tumor-inducing) plasmid of *Agrobacterium tumefaciens* and the Ri (root-inducing) plasmid of *A. rhizogenes*, which are 200 to 500 kb circular extrachromosomal plasmids, are the agents involved in transformation. The chemotactic compounds produced by wounded plant cells attract *Agrobacterium* to plants. The net result of this attraction is the movement of a segment of the plasmids called the transfer DNA (T-DNA), which contains genes that are responsible for overproduction of plant hormones, into the infected cell. The T-DNA intergrates into the plant chromosome, causing crown gall disease, induced by *A. tumefaciens*, or hairy root disease, induced by *A. rhizogenes*. Exploitation of the natural gene transfer system of *Agrobacterium* is the most widely used strategy for plant transformation, an additional tool for plant breeding.

Agrobacterium rhizogenes

Agrobacterium rhizogenes is able to cause hairy root induction in a large number of plants (Tepfer 1989). Insertion of pRi T-DNA into the plant genome and its subsequent expression results in this hairy root formation (Chilton et al., 1982). Use of the Ri plasmid has a number of advantages over the Ti plasmid for secondary metabolite production, including possible straightforward regeneration, genetic uniformity because of single cell self-cloning (Table I-2).

Root cultures show several advantages over crown gall tumor growth, such as a natural defense against infection and abundant biomass to begin earlier secondary metabolite production, since hairy roots grow fast. Hairy roots are a proprietary phytoproduction system

for secondary metabolites. Using hairy roots to improve plant secondary metabolites is increasing, and the technology was successful in ginseng root culture to improve the production of ginsenoside (Inomata et al., 1993). Since Taxol is a secondary product, and roots contain the secondary highest concentration of taxol after bark (Vidensek, 1990), roots should be a suitable yew tissue to produce taxol and related taxanes.

Table I-2. Advantages of using the Ri over Ti plasmids for transformation.

	Ri	Ti
Differentiation	organ, root	unorganized cells
Originated from	single cells	multiple cells
	self-cloning	callus formation
	genetically uniform	somaclonal variation
Morphology	abundant roots	crown galls
	root system	tumor growth
Regeneration	possible, straightforward	difficult
Industrial use	production of secondary metabolites from mature organ	extremely limited potential
Agricultural importance	1) plant establishment	none reported
	2) water uptake	
	3) adaptable to arid environment	

Most *Agrobacterium rhizogenes* strains induce hairy roots on a wide range of dicotyledonous plant species, including at least 193 plant species belonging to 83 families (De Cleene and De Ley, 1981). Although woody species can be transformed using the genes harbored by the Ri plasmid and induced adventitious rooting was reported in their explants, the

host range of *A. rhizogenes* is narrower. Successful *Agrobacterium*-mediated transformation of commercially and ecologically important forest tree species has been reported, and some transformations were with *A. rhizogenes* (Ellis et al., 1989; Huang et al., 1991; McAfee et al., 1993; Magnussen et al., 1994).

Mature tissue (stems) of *Taxus brevifolia* and *T. baccata* has been transformed by *Agrobacterium tumefaciens* and taxol production was reported in the transformed tissue (Han et al., 1994). However, the author could find no report of the infection of *Taxus* with *A. rhizogenes*.

PART II

MATERIALS AND METHODS

2.1 Isolation of the Taxadiene Synthase Gene from a cDNA Library of *Taxus baccata*

2.1.1 Plant Material

A yew cDNA library served as the gene pool for isolation of a cDNA coding for taxadiene synthase. The cDNA library was constructed by Dr. Yinghua Huang using mRNA isolated from actively growing mixed tissues (barks, needles, and shoot tips) of *Taxus baccata* collected from the OSU campus.

2.1.2 Probe Generation

Before screening the cDNA library to identify the taxadiene synthase gene, the probe DNA used for screening was synthesized via reverse transcription-PCR (RT-PCR).

A. Isolation of total RNA

Total RNA was isolated from the mixed tissues (barks, needles, and shoot tips) of *T. baccata*. This total RNA served as template for first strand cDNA synthesis in the next step. To avoid or eliminate any RNase contamination, all solutions and plasticware were treated with 0.1% (v/v) DEPC-treated water overnight and autoclaved for at least 30 minutes. The glassware was also soaked in DEPC water and baked at 200°C for 8 hours. Disposable gloves were worn and frequently changed during handling RNA to minimize RNA degradation by RNases.

RNA isolation protocol:

1. 4 g tissue (barks, needles, shoot tips) of *T. baccata* were ground in liquid nitrogen with a mortar and pestle, and placed in a 50 ml plastic centrifuge tube. The prewarmed extraction buffer (65°C) was added to the ground tissue, and mixed completely by inverting the tube.
2. An equal volume of chloroform:isoamyl alcohol (CI, 24:1) was added to the homogenate, mixed well, and centrifuged at 3,000 rpm at room temperature for 10 minutes to remove insoluble materials. The supernatant was collected to a new tube, and this step was repeated once.
3. 1/4 volume of 10 M LiCl was added to the resulting supernatant and mixed. The RNA was precipitated overnight at 4°C and collected by centrifugation at 10,000 rpm for 20 minutes at room temperature.
4. The RNA pellet was dissolved into 500 μL of SSTE buffer. An equal volume of CI was added and centrifuged as in step 3. The supernatant was transferred to an 1.5 ml microcentrifuge tube. Two volumes of ethanol were added to the supernatant. The RNA was precipitated at -70°C for at least 30 minutes or 2 hours at -20°C.

5. The precipitated RNA was harvested by centrifugation at 10,000 rpm for 20 minutes at room temperature. The supernatant was discarded. The pellet was washed with 70% ethanol, then air dried. The RNA was resuspend in DEPC-treated water and stored at -70°C.

Extraction buffer:

2% CTAB (hexadecyltrimethylammonium bromide)

2% PVP (polyvinylpyrrolidinone K30)

100 mM Tris-HCl, pH 8.0

25 mM EDTA, pH 8.0

2.0 M NaCl

0.5 g/l spermidine

2% β-mercaptoethanol (add just before use)

SSTE:

1.0 M NaCl

0.5% SDS

10 mM Tris-HCl, pH 8.0

1 mM EDTA, pH 8.0

B. Synthesis of first strand cDNA

The first strand cDNA was reversal transcribed using 1 μg total RNA as template following the manufacturer's instructions (Gibco BRL, SuperScript Preamplification system kit). This synthesized cDNA was the template for PCR amplification in the next step.

cDNA synthesis protocol:

1. 1 μg of total RNA in 13 μl of DEPC-treated water was added to an autoclaved 0.5-ml microcentrifuge tube, then 1 μl of random hexamer solution was added to the tube and mixed gently. The mixture was heated at 70°C for 10 minutes, then incubated on ice for 1 minute.
2. The following compounds were added to the mixture:

10x synthesis buffer	2 μl
10 mM dNTP mixture	1 μl
0.1 M DTT	2 μl
SuperScript II Reverse Transcriptase (200 U/μl)	1 μl.

Total volume of the reaction was 20 μl.

3. The reaction was incubated at room temperature for 10 minutes. Then the tube was transferred to a 42°C heat block and incubated for 50 minutes. The reaction was terminated by incubating the tube at 70°C for 15 minutes.
4. 1 μl of RNase H was added to the tube and incubated at 37°C for 20 minutes to degrade the RNA. The first strand cDNA was used for the subsequent experiments.

C. PCR Amplification

PCR was performed to generate a specific fragment for library screening using the cDNA as template. Based on the published cDNA sequence of taxadiene synthase in *T. brevifolia* and available sequences for terpenoid cyclases from other higher plants, four specific primers and three consensus degenerate primers were designed from the homologous regions. The sequences of the designed primers were:

primer CYC31: MRCATCRWWWRTRTCATC Tm = 44°C

primer CYC51: GGYRARTTCAARGARWSYCT Tm = 50°C

primer CYC52: TWYGAAGCTTCMYWTST Tm = 42°C

primer PY31: GTCAGCCATATCATCAA Tm = 48°C

primer PY51: TGATTCATATGACGACA Tm = 46°C

primer PY52: GAATTGGCAAAATTGGACTTCAAT Tm = 57°C

primer PY53: ATGGTGGAAGGAATCCCG Tm = 59°C

CYC31, CYC51, and CYC52 were degenerate primers, the others specific primers. Among them, the primers were paired as CYC31 (reverse) and CYC51 (forward), CYC31 (reverse) and CYC52 (forward), PY31 (reverse) and PY51 (forward), PY31 (reverse) and PY52 (forward), and PY31 (reverse) and PY53 (forward).

PCR amplification protocol:

1. The following compounds were added to a microcentrifuge tube:

first strand reaction	5 μl
10x PCR buffer	10 μl
25 mM Mg^{2+}	10 μl
10 mM dNTP	10 μl
dH_2O	70 μl
reverse primer	1 μl (100 ng)
forward primer	1 μl (100 ng)
Taq DNA polymerase (5 U/μl)	1 μl

The mixture was layered with ~50 μl of mineral oil.

2. The reaction was heated at 94°C for 4 minutes to denature the RNA/cDNA hybrid.

3. 40 cycles of denaturation, annealing, and extension were performed to amplify the target fragment using the following program:

denaturation	94°C	2 minutes
annealing	*	2 minutes
extension	72°C	2 minutes

*: The annealing temperature was dependent on the Tm value of specific primers.

4. Following amplification, 20 μl of the samples were analyzed through agarose gel electrophoresis.

D. Cloning of the PCR product into pCR®2.1 vector

After PCR, the DNA fragment amplified by primers PY31 and PY53 was cloned into pCR®2.1 vector (Invitrogen, Original TA cloning kit) and transformed into *E. coli*. Taq polymerase has a nontemplate-dependent activity which adds a single deoxyadenosine (A) to the 3' ends of PCR products. The linearized pCR®2.1 vector has single 3' deoxythymidine (T) residues. This allows PCR inserts to ligate efficiently with the vector.

E. Isolation of the plasmid containing insert.

Plasmid DNA was isolated from *E. coli* using the boiling mini-prep method (Sambrook et al., 1989). The DNA was then digested by EcoR I, and electrophoresed on an agarose gel to determine if the plasmid contained insert.

F. Sequencing of the cDNA fragment

The insert DNA was sequenced (Applied Biosystems machine, Department of Biochemistry Core Facility) using the automated sequencing fluorescent method. This method automates the gel electrophoresis step, detection of DNA band pattern, and analysis of bands. Currently, all commercially available automated sequencers are enzymatic sequencing reactions. Fluorescent label can be incorporated into the sequencing products either through the primer or the ddNTPs. Because the primers or ddNTPs fluoresce at different wavelengths, all four reaction products can be separated in a single lane.

Before automated fluorescent sequencing, the plasmid was purified using Wizard™ *Plus* Minipreps System (Promega). Purified plasmid DNA must be within the proper concentration range for successful automated cycle sequencing (ideally 0.2 μg/μl, not less than 0.1 μg/μl).

G. Purification of the insert

The plasmid DNA containing the suitable insert was digested by EcoR I. Then the reaction was electrophoresed on an agarose gel. The DNA fragment band was excised from ethidium bromide stained agarose gel, and purified using GLASSMILK (Geneclean).

H. Labeling of the insert

To screen the cDNA library, 400 ng of purified DNA probe was labeled using the random primer DNA labeling protocol (Feinberg and Vogelstein, 1983). One hundred nanogram DNA was labeled in one reaction.

Random primer labeling protocol:

1. 100 ng of DNA in a total volume of 7.1 μl sterile water was denatured at 95°C for 5 minutes, then quenched on ice immediately.

2. The following components were added to the DNA to a final volume of 25 μl:

10 mg/ml BSA	1.0 μl
RPL buffer	11.4 μl
50 μCi α-32P dCTP (3,000 Ci/mM)	5.0 μl
Klenow fragment (2.5 u)	0.5 μl

3. The reaction was performed at 37°C for at least 3 hours, and was stopped by adding 2 μl of 0.2 M EDTA (pH 8.0).

4. The incorporated and unincorporated nucleotides were separated by spin-column procedure:

   a. Preparation of Sephadex column: The bottom of a 1-ml disposable syringe was plugged with sterile glass wool. Sephadex G-50 (equilibrated in TE buffer, pH 8.0) was loaded in the syringe, and the syringe was centrifuged at 1,500 rpm for 4 minutes in an IEC clinical centrifuge. Sephadex was loaded again until the packed column volume was 0.9 ml. Then 0.1 ml of STE buffer was added in the syringe to wash the column by centrifuging at 1,500 rpm for 4 minutes.

   b. The DNA labeling reaction was loaded into the column in a total volume of 0.1 ml (STE was added to make up the volume). The column was inserted into a microcentrifuge tube and recentrifuged at the same speed and for the same time. The effluent from the syringe in the tube was the labeled DNA.

5. 1 μl of labeled DNA was mixed with 3 ml of cocktail, and the sample was counted in a scintillation counter. For screening, the specific activity of the probe should be greater than 10^8 CPM/μg.

2.1.3 Screening of the cDNA Library

To obtain the cDNA clones coding for taxadiene synthase, the cDNA library was screened using α-^{32}P labeled DNA probe followed the manufacturer's instructions (Stratagene, ZAP-cDNA synthesis kit).

A. First screening

<u>First screening protocol</u>:

1. Before screening, the cDNA library was titered to determine the concentration.
2. Preparation of bacterium cells: A single colony of XL1-Blue MRE' bacterium was picked and grown in 50 ml of LB medium containing 0.2% maltose and 10 mM $MgSO_4$ at 30 °C overnight. The cells were collected by centrifugation, and then resuspended in 10 mM $MgSO_4$ to an OD_{600} of 0.5.
3. Plating of the library: 600 μl of XL1-Blue MRF' cells was added to each of 20 tubes containing about 50,000 phages/tube, and the tubes were co-incubated at 37°C for 15 minutes. Seven to eight ml of NZY top agar (48°C preheated) was then added to each tube, and plated immediately onto NZY agar plates (150 mm). To screen 10^6 phages, 20 NZY plates were used in total.

4. The plates were set at room temperature for 10 minutes, and incubated at 37°C for ~8 hours for phage growth. Then the plates were chilled for 2 hours at 4°C to prevent the NZY top agar from sticking to the Nylon membrane.
5. The plaques were transferred onto Nylon membranes (two set of membranes for each plate). The blotting transfer was 2 minutes for the first membranes, and 4 minutes for the second membranes. Waterproof ink in a syringe needle was used to puncture the membranes through the agar for orientation. After lifting, the plates were stored at 4°C for picking up the positive plaques after screening.
6. The membranes were first denatured for 5 minutes by submerging the membranes in denaturation solution, then neutralized for 7 minutes in neutralization solution, finally rinsed for 2 minutes in 2x SSC buffer solution. The treated membranes were bolt-dried briefly on an absorbance paper. The DNA was crosslinked to the membranes using the autocrosslink setting on the Stratalinker® UV crosslinker (120,000 μJ of UV energy).
7. Prehybridization: The prehybridization solution without salmon sperm DNA was preheated to ~50°C. The salmon sperm DNA was preboiled for 5 minutes and then added to the warm prehybridization solution. The membranes were wet in 2x SSC for 2 minutes, then were prehybridized in prehybridization solution at 42°C for at least 2 hours. Four ml of prehybridization solution was needed for each membrane.
8. Hybridization: 400 ng of α-^{32}P dCTP labeled double-stranded DNA probe prepared above was boiled for 5 minutes and then added to the prehybridization tubes. The concentration of counts of probe were ~1x 10^7 counts/membrane. The membranes were hybridized at 42°C (low stringency) overnight.

9. Washes: After hybridization, the membranes were washed at 52°C in 500 ml of washing solution for 30 minutes 2~3 times to remove the unhybridized radioactive dNTP and DNA. When the count on the membranes reached 200~300 CPM, the membranes were rinsed in 2x SSC and dried briefly on an absorbance paper.

10. Exposure to film: The membranes were placed between two sheets of plastic wrap in a cassette with intensifying screens, and exposed to X-ray films for 2 days at -80°C. Then the films were developed.

Denaturation solution:

1.5 M NaCl

0.5 M NaOH

Neutralization solution:

1.5 M NaCl

0.5 M Tris-HCl, pH 8.0

Prehybridization and hybridization solution (low stringency):

5x SSPE buffer

0.1% (w/v) sodium dodecyl sulfate (SDS)

5x Denhardt's reagent

200 μg/ml of denatured, sonicated salmon sperm DNA

Washing solution:

5x SSPE

0.1% SDS

B. Secondary screening

Fifty four putative plaques were obtained after first screening. Since some of them may not contain the cDNA coding for taxadiene synthase, one additional cycle of hybridization at high stringency was carried out to verify these putative plaques.

Secondary screening protocol:

1. To orient the membranes, the films were lined up and marked where the needle poked through. The strongest putative clones were determined. These putative clones were picked with a glass Pasteur pipette, and transferred into 1 ml of SM buffer. The clones were shaken at room temperature for 4~6 hours to release the phages. After adding 20 μl of chloroform, the clones were stored at 4°C.
2. After dilution and titering, the clones were plated with host cells XL1-Blue MRF' on small NZY agar plates (100 mm) so that each plate had ~500 plaques. The plates were incubated at 37°C for 6~8 hours.
3. Two sets of nylon membrane lifts were made from prechilled plates as before.
4. Prehybridization and hybridization were performed at 62°C for high stringency. The membranes were washed at 65°C three times with 2x SSC/0.1% SDS, 1x SSC/0.1% SDS, and 0.1x SSC/0.1% SDS solutions, respectively. The membranes were then exposed to X-ray films as before.

Prehybridization and hybridization buffer (high stringency):

3x SSC

0.5% SDS

2x Denhardt's reagent

50 μg/ml salmon sperm DNA

10 μg/ml polyuridylic acid (optional)

2.1.4 *In vivo* Excision

Since pBluescript phagemids which contained cloned inserts were packaged as filamentous phage particles, they were *in vivo* excised and recircularized using *E. coli* XL1-Blue MRF' and ExAssist helper phage. Then the phagemids were plated on LB-ampicillin plates using *E.coli* SOLR cells. The colonies arising on the plates would consist of the pBluescript double-stranded phagemids having the cloned DNA insert. Helper phages would not grow since they could not replicate in the SOLR strain.

<u>*In vivo* excision protocol</u>:

1. Thirty-eight positive clones were picked from secondary screening, and transferred into 500 μl of SM buffer and 20 μl of chloroform. The phage particles were released by shaking the tubes at room temperature as before.
2. XL1-Blue MRF' and SOLR cell cultures were grown overnight at 30°C in 50 ml of LB broth.
3. The overnight culture of XL1-Blue MRF' cells was centrifuged at 1,500x *g* for 10 minutes, the cells were then resuspended in 10 mM $MgSO_4$ at an OD_{600} of 1.0 for single-clone excision. The SOLR cells continued growth to an $OD_{600} \geq 1.0$.
4. The following components were combined into a 2.0-ml microcentrifuge tube:

    100 μl of XL1-Blue MRF' cells ($OD_{600} = 1.0$)

    125 μl of phage stock (containing > 10^5 phage particles)

    0.5 μl of the ExAssist helper phage (> 5x 10^5 pfu/μl)

3. Seven phagemids containing large inserts were purified using the Wizard™ *Plus* Minipreps System (Promega).
4. The purified cDNA clones were sequenced using an automated fluorescent sequencing method (Biochemistry Department Core Facility).
5. The obtained cDNA sequences were compared with known taxadiene synthase gene sequences from *T. brevifolia* and cyclase genes from other higher plants.

2.1.6 Completion of Cloning the Full-length Gene

The largest cDNA clone 12b-1a was 2031 bp in length. Since all the cDNA clones obtained above did not contain the 5' open reading frame of the taxadiene synthase gene, the remaining region was cloned via RT-PCR.

Full-length gene cloning protocol:

1. The first strand cDNA was synthesized using 1 μg total RNA as template following the manufacturer's instructions (Gibco BRL, SuperScript Preamplification system kit).
2. Amplification of the target cDNA: Based on the sequences of the obtained cDNA clones and the reported sequence of the taxadiene synthase gene from *T. brevifolia*, three specific primers were synthesized:

   primer 3021: GCCTCCACTGCCTGTCTTACTGATG   Tm = 70°C

   primer 3022: CCAGGCTTACAGATGTTTCTGC   Tm = 66°C

   primer 3079: CCTGCCTCTCTGGAGAAATG   Tm = 60°C

   They were paired as primer 3021 (reverse) & 3079 (forward), and primer 3022 (reverse) & 3079 (forward). PCR was performed with these two pairs of primers

using first strand cDNA as template. Forty repeated cycles of denaturation, annealing, and extension were performed. The temperatures for each step were:

denaturation	94°C	2 minutes
annealing	55°C	2 minutes
extension	72°C	2 minutes

3. After analysis of the PCR products by agarose gel electrophoeresis, the amplified DNA fragment was cloned into pCR®2.1 vector (Invitrogen, Original TA cloning kit).
4. The plasmid DNA containing PCR product was isolated using the boiling miniprep method (Sambrook et al., 1989), purified with Wizard™ *Plus* Minipreps System (Promega), and sequenced by the automated fluorescent sequencing technique (Department of Biochemistry Core Facility).
5. The full-length gene coding for taxadiene synthase was obtained by combining the sequence of the cDNA clone with the sequence of the RT-PCR product.

2.1.7 Northern Blot Analysis

A. Isolation of total RNA

For Northern blot, total RNA was isolated from different tissues: bark, needle, root, and mixed tissue (bark, needles, and shoot tips) from *Taxus x media* Runyan growing in the green house, and the mixed tissue (barks, needles and shoot tips) from *T. baccata*. The protocol for RNA isolation was the same as before (p 23).

B. RNA formaldehyde gel electrophoresis

One hundred micrograms of total RNA was electrophoretically size-separated through a 1% agarose gel containing formaldehyde, after denaturation with formaldehyde/ formamide (Sambrook et al., 1989). The gel was run at 80 v for about 3 hours.

C. RNA blot

RNA was blotted on a Hybond N^+ membrane (Amersham) using the protocol recommended by Amersham.

Northern blotting protocol:

1. A sterile glass dish was filled with 20x SSC as blotting buffer. A platform was covered with a wick made from two sheets of Whatman 3MM filter paper, saturated with blotting buffer.
2. The unused areas of the gel were trimmed away, and the gel was placed on the wick without any air bubbles beneath it.
3. A sheet of Hybond-N^+ membrane of the exact size of the gel was placed on top of the gel. Any bubbles trapped between the gel and the membrane were squeezed out using a glass rod.
4. A double layer of 3MM paper was cut to size, wetted with blotting buffer, and placed on top of the membrane.
5. A stack of absorbent paper towels was placed on top of the 3MM paper. A glass plate was placed on top of the paper towels, and a 500 g weight was put on top.
6. The transfer of RNA proceeded for at least 12 hours. After blotting, the apparatus was carefully dismantled. The membrane was removed and marked.

7. The membrane was washed briefly in 2x SSC to remove any adhering agarose, then was soaked in 50 mM NaOH for 10 minutes to fix the RNA. The membrane was briefly rinsed in 5x SSC and air dried.

D. Preparation of DNA probe

The probe for Northern hybridization was the cDNA clone 12b-1a obtained from library screening. This probe contains the open reading frame for taxadiene synthase. The insert of 12b-1a was cut by EcoR I and Xho I, recovered from an agarose gel, then labeled with ^{32}P by the random primer DNA labeling method (Feinberg and Vogelstein, 1983) as mentioned above.

E. Northern hybridization

Northern hybridization was performed based on the protocol described by Church and Gilbert (1984). The blotted membrane was first hybridized with ^{32}P labeled probe at 60°C for about 24 hours for high stringency, and finally washed with 0.1 x SSC containing 0.1% SDS at 60°C for 5 minutes.

Hybridization solution:

0.5 M $NaHPO_4$, pH 7.2

1% BSA

7% SDS

10 μg/ml salmon sperm DNA

2.2 *Agrobacterium*-mediated Transformation of *Taxus*

To test the transformation efficiency, several *A. rhizogenes* strains were used to inoculate *Taxus* species.

2.2.1 Materials

Plant material: Actively growing stem cuttings of *Taxus x media* Runyan were obtained from the green house. Seeds of *Taxus baccata* were collected from plants growing on campus in 1994. Before use, the seeds were stratified at 4°C for one year to stimulate germination.

Agrobacterium strains: For the susceptibility tests, four *A. rhizogenes* strains: 9405, E15, S6, and TR105 were used on stem cuttings of Runyan yew and one strain TR105 was used on seedlings of *T. baccata* (see Table II-1). Before inoculation of explants, *Agrobacteria* were grown on AB liquid or solid medium (Chilton et al., 1974) at 28°C for 48 hours (Petit et al., 1983).

Table II-1. Characteristics of the *Agrobacterium rhizogenes* strains used for *Taxus* species susceptibility test.

Strain	Plasmid	Characteristics
9405	binary	GUS, Kan
E15	binary	*rolA, rolB; rolC*
S6	binary	*Aux1, Aux2*
TR105	wild type	wild type Ri

2.2.2 Stem Cuttings and Inoculation

Plant explants and inoculation: Stems of Runyan yew were cut into about 3 cm in length, sterilized, and precultured on solid DCR medium (Gupta and Durtan, 1985). After 2-3 weeks, fresh cuts were made at the stem base, which was then inoculated with a fresh culture of *A. rhizogenes*. The inoculated cuttings were cultivated on DCR medium about 2 months for hairy root development.

A. Preparation of plant stem cuttings

1. The leaves and buds were removed from the stems of Runyan yew. Then the stems were cut into about 3 cm in length.
2. The stem cuttings were rinsed with running water for at least 30 minutes, washed with detergent, and rinsed with running water again for 30 minutes or longer.
3. The stem cuttings were soaked in a fungicide solution containing Benomyl and Captan (6.25-50 mg/l) for 6 hours with shaking to kill the fungi on the surface of cuttings, then rinsed with sterile water three times for 1 minute, 5 minutes, and 1 minutes, respectively.
4. The cuttings were washed with acid-ethanol (70% ethanol with a few drops of 2.5 N HCl) for 1 minute, and rinsed with sterile water three times.
5. The cuttings were washed again with 30% chlorox with a few drops of Twean 20 for 15 minute, and rinsed with sterile water four times.
6. The sterilized stem cuttings were placed on solid DCR medium with indole-3-butyric acid (IBA) and naphthalene acetic acid (NAA), and cultured for 2 weeks.

B. Inoculation

The bottoms (about 0.5 cm in length) of the precultured stem cuttings were removed to make new wounds. The wound sites were infected by freshly prepared and actively growing solid or suspension cultures of *Agrobacteria* (Table II-1). The inoculated stem cuttings were then co-cultured with the *Agrobacteria* on DCR solid medium without hormones for about 2 months waiting for hairy root development. Unwounded and wounded but uninoculated cuttings were used as controls. The cuttings were transferred to new medium every two weeks.

2.2.3 Seedlings and Inoculation

Seeds of *Taxus baccata*, collected from campus and stratified at 4°C for one year, were aseptically germinated and grown *in vitro* (Flores and Sgrignoli, 1991). Small linear wounds were made on the seedling hypocotyls, and the wound sites were aseptically inoculated with *A. rhizogenes* TR105 (Huang et al., 1991; Huang and Tauer, 1993).

A. Preparation of seeds

1. Seedcoats were removed from the seeds of *Taxus baccata*. Then the seeds were soaked in 0.5% Mercuric chloride for 10 minutes with agitation, and rinsed with sterile water three times (1-5-1 minutes).
2. The seeds were further sterilized by treating with 15% Chlorox (commercial bleach) for 10 minutes with agitation, then rinsed with sterile water three times (1-5-1 minutes).
3. The sterilized seeds were placed on water-agar medium for germination. The germinated seedlings were then transferred to DCR medium for further growth.

B. Inoculation and incubation

2 weeks old seedlings were used for the in vitro inoculation experiment. Small vertical liner wounds were made on the hypocotyl (one or two wounds per seedling) with a sterile scalpel. The wounding sites were inoculated aseptically with *Agrobacterium* strain TR105 culture grown on solid AB medium. The infected seedlings were co-cultured on solid DCR medium for one month to develop the hairy roots. Unwounded and wounded but uninoculated seedlings were used as controls.

PART III

RESULTS AND DISCUSSION

3.1 The Taxadiene Synthase Gene Cloned from a cDNA Library of *Taxus baccata*

3.1.1 Isolation of the Taxadiene Synthase cDNA Clone

Previously, the taxadiene synthase protein was purified from bark of *Taxus brevifolia* (Hezari et al, 1995). Taxadiene synthase is known to be a key enzyme in the taxanes pathway in this tissue (Koepp et al., 1996). A full-length cDNA clone coding for this protein was also isolated from a cDNA library of this species, and expressed in *E. coli* as a fusion protein (Wildung and Croteau, 1996). The extract of the recombinant *E. coli* demonstrated cyclase activity in an enzyme assay using standard protocols with [1-^{3}H]GGPP as substrate (Hezari et al., 1995). Taxa-4(5), 11(12)-diene was detected by combined capillary gas chromatography-mass spectrometry. This confirmed that the isolated cDNA from *Taxus brevifolia* encodes taxadiene synthase (Wildung and Croteau, 1996).

From the conserved cDNA sequences of taxadiene synthase from *T. brevifolia,* four specific oligonucleotides (primer PY31: GTCAGCCATATCATCAA, primer PY51: TGATTCATATGACGACA, primer PY52: GAATTGGCAAAATTGGACTTCAAT, primer PY53: ATGGTGGAAGGAATCCCG) were synthesized for use as polymerase chain reaction (PCR) primers. By comparison of the deduced amino acid sequences between the taxadiene synthase from *T. brevifolia* and six other higher plant terpenoid cyclases, including monoterpenoid cyclase—(–)-limonene synthase from *Mentha spicata* (Colby et al., 1993), sesquiterpenoid cyclase—*epi*-aristolochene synthase from *Nicotiana tabacum* (Facchini and Chappell, 1992; Back and Chappell, 1995), three diterpenoid cyclases—casbene synthase from *Ricinus communis* (Mau and West, 1994), kaurene synthase A from *Arabidopsis thaliana* (Sun and Kamiya, 1994) and *Zea mays* (Bensen et al., 1995), and (–)-abietadiene synthase from *Abies grandis* (Vogel et al., 1996), three degenerate primers (primer CYC31: MRCATCRWWWRTRTCATC, primer CYC51: GGYRARTTCAARGARWSYCT, primer CYC52: TWYGAAGCTTCMYWTST) were also synthesized for PCR cloning. Reverse transcription PCR (RT-PCR) was performed using these synthetic oligonucleotides as primers to amplify cDNA fragments from total RNA isolated from a tissue mixture (barks, needles, and shoot tips) of *Taxus baccata.* One cDNA fragment was obtained from each of the following combination of primers; PY52 (forward) and PY31 (reverse), and primers PY53 (forward) and PY31 (reverse). The amplified DNA fragment resulting from primer set PY53 with PY31 was sequenced, 160 bp in length. Primer PY53 sequence represents a strong homology region among (–)-limonene synthase (Colby et al., 1993), *epi*-aristolochene synthase (Facchini and Chappell, 1992; Back and Chappell, 1995), and casbene synthase (Mau and West, 1994). Wildung and Croteau (1996) also used this region for identifying the taxadiene synthase cDNA clone from *T. brevifolia.* Primer PY31 represents a DDXXD motif in the taxadiene synthase

from *T. brevifolia*. The 160-bp fragment was cloned and sequenced and showed identical sequence to the cDNA clone of taxadiene synthase from *T. brevifolia* (Wildung and Croteau, 1996). This result demonstrated that the PCR-amplified fragment is a fraction of the taxadiene synthase cDNA in *T. baccata*. Thus the second fragment porduced by primers PY31 and PY52 was not cloned and sequenced.

To obtain a full-length cDNA, a cDNA library was constructed from poly(A)$^+$ RNA of a *Taxus baccata* tissue mixture (barks, needles, and shoot tips) using the λZAP II vector. After amplification of the library, 1x 10^6 clones were screened at low stringency using the ^{32}P-labeled 160-bp PCR product, yielding 54 positive clones. All of these clones were verified through one additional round of screening in high stringency. Thirty-eight positive plaques were identified after secondary screening and *in vivo* excised. The inserts sizes were determined by gel electrophoresis. The seven clones containing the largest inserts were partially sequenced. Sequence results demonstrated that all clones characterized the same gene and were similar to the sequence of taxadiene synthase from *T. brevifolia* (Wildung and Croteau, 1996). Of these clones, cDNA clone 12b-1a contained the largest insert, 2031 bp in length, but as did all other clones, it lacked 5'-region of the gene. Comparison of the size of the cDNA with that of the cDNA clone coding for taxadiene synthase from *T. brevifolia* (2700 bp in length) supported this conclusion. These results implied that the cDNA library does not contains a full length cDNA, probably due to secondary structure of the mRNA of the taxadiene synthase gene which was used to make the cDNA library.

To clone the 5'-region of 12b-1a, RT-PCR was performed. Based on the sequences of obtained clones and the sequence of the taxadiene synthase gene from *T. brevifolia* (Wildung and Croteau, 1996), 3 regions were selected to design the PCR primers (p36). The primer 3079 was paired with 3021, and 3022, respectively. Only the primer set 3079 (forward) with

3022 (reverse) yielded a specific DNA fragment (841 bp), based on product analysis via gel electrophoresis after PCR amplification. The 841-bp fragment was cloned and sequenced, the results indicated that this fragment contains an extra 654 bp in the 5'-region, and an overlapping 187 bp with the insert of 12b-1a. The overlapping sequences of the clones were identical. After combining the above two fragments, a 2685-bp full-length sequence of the taxadiene synthase cDNA from *T. baccata* was obtained.

3.1.2 Nucleotide Sequence of the Taxadiene Synthase cDNA from *Taxus baccata*

The nucleotide and deduced amino acid sequences of the taxadiene synthase cDNA from *Taxus baccata* were characterized (Figure III-1). This taxadiene synthase cDNA contains 2685 nucleotides (nt). The PCR amplified fragment used for the library screening represents an internal segment of the cDNA since they have an identical sequence (nucleotides 1709-1868). The complete open reading frame (ORF) of the cDNA is 2586 nt in length, and contains the start codon ATG (nucleotide 18), and the stop codon TGA at position 2604. There also is an AATAAA sequence, which is a putative polyadenylation signal, at 27-32 nt downstream from the stop codon. The ORF consists of 862 amino acids, including an approximately 137 amino acid putative plastidial transit peptide. According to Wildung and Croteau (1996), the reasons this sequence might contain a plastid transit peptide are: 1) the size of the native (mature) enzyme (~79kDa), estimated by gel permeation chromatography and SDS-polyacrylamide gel electrophoresis (Hezari et al., 1995), is smaller than that of the deduced amino acid sequence, 2) the deduced taxadiene protein implies the common characteristic features of the amino-terminal targeting transit peptide sequence in their amino acid content, structure, and their cleavage site (Keegstra et al., 1989; von Heijne et al., 1989),

and 3) plastids are always the place where diterpene biosynthesis occurs (West et al., 1979; Kleinig, 1989). The position where the transit peptide and the mature protein connect, and thus, the precise lengths of both parts are not clear.

Sequences comparison of taxadiene synthase from *Taxus baccata* with that from *T. brevifolia* indicated a significant degree of identity at both the DNA level and the amino acid level. There were only 48 nucleotide and 19 amino acid differences between the two species. The DNA sequence identity was 98% (Figure III-2) and the amino acid sequence identity was 97% (Figure III-3). The similarity between the amino acid sequences was 98%. These characteristics confirm that the cDNA isolated from *T. baccata* is the gene that encodes taxadiene synthase.

Figure III-1. Nucleotide and predicted amino acid sequences of *Taxus baccaca* taxadiene synthase cDNA. The start and stop codons, and the polyadenylation signal are underlined, the location for primer synthesis are double underlined, and the DDMAD and DSYDD motifs are in boldface.

```
        10         20         30         40         50         60
CCTGCCTCTC TGGAGAAATG GCTCAGCTCT CATTTAATGC AGCGCTGAAG ATGAATGCAT
                   M   A  Q  L   S  F  N  A   A  L  K   M  N  A>

        70         80         90        100        110        120
TAGGGAACAA GGCAATCCAC GATCCAACGA ATTGCAGAGC CAAATCTGAG GGCCAAATGA
L  G  N  K   A  I  H   D  P  T   N  C  R  A   K  S  E   G  Q  M>

       130        140        150        160        170        180
TGTGGGTTTG CTCCAAATCA GGGCGAACCA GAGTAAAAAT GTCGAGAGGA AGTGGTGGTC
M  W  V  C   S  K  S   G  R  T   R  V  K  M   S  R  G   S  G  G>

       190        200        210        220        230        240
CTGGTCCTGT CGTAATGATG AGCAGTAGCA CTGGCACTAG CAAGGTGGTT TCCGAGACTT
P  G  P  V   V  M  M   S  S  S   T  G  T  S   K  V  V   S  E  T>

       250        260        270        280        290        300
CCAGTACCAT TGTGGATGAT ATCCCTCGAC TCTCCGCCAA TTATCATGGC GATCTGTGGC
S  S  T  I   V  D  D   I  P  R   L  S  A  N   Y  H  G   D  L  W>

       310        320        330        340        350        360
ACCACAATGT TATACAAACT CTGGAGACAC CATTTCGTGA GAGTTCTACT TTCCAAGAAC
H  H  N  V   I  Q  T   L  E  T   P  F  R  E   S  S  T   F  Q  E>

       370        380        390        400        410        420
GGGCAGACGA GCTGGTTGTG AAAATTAAAG ATATGTTCAA TGCGCTCGGA GACGGAGATA
R  A  D  E   L  V  V   K  I  K   D  M  F  N   A  L  G   D  G  D>

       430        440        450        460        470        480
TCAGTCCGTC TGCATACGAC ACTGCGTGGG TGGCGAGGTG GCGGACCGTT TCCTCTGATG
I  S  P  S   A  Y  D   T  A  W   V  A  R  W   R  T  V   S  S  D>

       490        500        510        520        530        540
GATCTGAGAA GCCACGGTTT CCTCAGGCCC TCAACTGGGT TTTAAACAAC CAGCTCCAAG
G  S  E  K   P  R  F   P  Q  A   L  N  W  V   L  N  N   Q  L  Q>

       550        560        570        580        590        600
ATGGATCATG GGGTATCGAA TCGCACTTTA GTTTATGCGA TCGATTGCTT AACACGGTCA
D  G  S  W   G  I  E   S  H  F   S  L  C  D   R  L  L   N  T  V>

       610        620        630        640        650        660
ATTCTGTTAT CGCCCTCTCG GTTTGGAAAA CAGGGCACAG CCAAGTAGAA CAAGGTACTG
N  S  V  I   A  L  S   V  W  K   T  G  H  S   Q  V  E   Q  G  T>

       670        680        690        700        710        720
AGTTTATTGC AGAGAATCTA AGATTACTCA ATGAGGAAGA TGAGTTGTCC CCGGATTTCG
E  F  I  A   E  N  L   R  L  L   N  E  E  D   E  L  S   P  D  F>

       730        740        750        760        770        780
AAATAATCTT TCCTGCTCTG CTGCAAAAGG CAAAAGCGTT GGGGATCAAT CTTCCTTACG
E  I  I  F   P  A  L   L  Q  K   A  K  A  L   G  I  N   L  P  Y>
```

```
       790        800        810        820        830        840
ATCTTCCATT TATCAAATCT TTGTCGACAA CACGGGAAGC CAGGCTTACA GATGTTTCTG
D  L  P  F   I  K  S   L  S  T   T  R  E  A   R  L  T   D  V  S>

       850        860        870        880        890        900
CGGCAGCAGA CAATATTCCA GCCAACATGT TGAATGCGTT GGAGGGTCTC GAGGAAGTTA
A  A  A  D   N  I  P   A  N  M   L  N  A  L   E  G  L   E  E  V>

       910        920        930        940        950        960
TTGACTGGAA CAAGATTATG AGGTTTCAAA GTAAAGATGG ATCTTTCCTG AGCTCCCCTG
I  D  W  N   K  I  M   R  F  Q   S  K  D  G   S  F  L   S  S  P>

       970        980        990       1000       1010       1020
CCTCCACTGC CTGTGTACTG ATGAATACAG GGGAGGAAAA ATGTTTCACT TTTCTCAACA
A  S  T  A   C  V  L   M  N  T   G  E  E  K   C  F  T   F  L  N>

      1030       1040       1050       1060       1070       1080
ACCTGCTCGA CAAATTCGGC GGCTGCGTGC CCTGTATGTA TTCCATCGAT CTGCTGGAAC
N  L  L  D   K  F  G   G  C  V   P  C  M  Y   S  I  D   L  L  E>

      1090       1100       1110       1120       1130       1140
GCCTTTCGCT GGTTGATAAC ATTGAGCATC TCGGAATCGG TCGCCATTTC AAACAAGAAA
R  L  S  L   V  D  N   I  E  H   L  G  I  G   R  H  F   K  Q  E>

      1150       1160       1170       1180       1190       1200
TCAAAGTAGC TCTTGATTAT GTCTACAGAC ATTGGAGTGA AAGGGGCATC GGTTGGGGCA
I  K  V  A   L  D  Y   V  Y  R   H  W  S  E   R  G  I   G  W  G>

      1210       1220       1230       1240       1250       1260
GAGACAGCCT CGTTCCAGAT CTCAACACAA CAGCCCTCGG CCTGCGAACT CTTCGCACGC
R  D  S  L   V  P  D   L  N  T   T  A  L  G   L  R  T   L  R  T>

      1270       1280       1290       1300       1310       1320
ACGGATACGA TGTTTCTTCA GATGTTTTGA ATAATTTCAA AGATGAAAAC GGGCGGTTCT
H  G  Y  D   V  S  S   D  V  L   N  N  F  K   D  E  N   G  R  F>

      1330       1340       1350       1360       1370       1380
TCTCCTCTGC GGGCCAAACC CATGTCGAAT TGAGAAGCGT GGTGAATCTT TTCAGAGCTT
F  S  S  A   G  Q  T   H  V  E   L  R  S  V   V  N  L   F  R  A>

      1390       1400       1410       1420       1430       1440
CCGACCTTGC ATTTCCTGAC GAAGGAGCTA TGGACGATGC TAGAAAATTT GCAGAACCAT
S  D  L  A   F  P  D   E  G  A   M  D  D  A   R  K  F   A  E  P>

      1450       1460       1470       1480       1490       1500
ATCTTAGAGA CGCACTTGCA ACAAAAATCT CAACCAATAC AAAACTATTC AAAGAGATTG
Y  L  R  D   A  L  A   T  K  I   S  T  N  T   K  L  F   K  E  I>

      1510       1520       1530       1540       1550       1560
AGTACGTGGT GGAGTACCCT TGGCACATGA GTATCCCACG CCTAGAAGCC AGAAGTTATA
E  Y  V  V   E  Y  P   W  H  M   S  I  P  R   L  E  A   R  S  Y>

      1570       1580       1590       1600       1610       1620
TTGATTCGTA TGACGACGAT TATGTATGGC AGAGGAAGAC TCTATACAGA ATGCCATCTT
I  D  S  Y   D  D  D   Y  V  W   Q  R  K  T   L  Y  R   M  P  S>

      1630       1640       1650       1660       1670       1680
TGAGTAATTC AAAATGTTTA GAATTGGCAA AATTGGACTT CAATATCGTA CAATCTTTGC
L  S  N  S   K  C  L   E  L  A   K  L  D  F   N  I  V   Q  S  L>

      1690       1700       1710       1720       1730       1740
ATCAAGAGGA GTTGAAGCTT CTAACAAGAT GGTGGAAGGA ATCCGGCATG GCAGATATAA
H  Q  E  E   L  K  L   L  T  R   W  W  K  E   S  G  M   A  D  I>
```

```
      1750       1760       1770       1780       1790       1800
ATTTCACTCG ACACCGAGTG GCGGAGGTTT ATTTTTCATC AGCTACATTT GAACCTGAAT
N  F  T  R   H  R  V   A  E  V   Y  F  S  S   A  T  F   E  P  E>

      1810       1820       1830       1840       1850       1860
ATTCTGCCAC TAGAATTGCC TTCACAAAAA TTGGTTGTTT ACAAGTCCTT TTTGATGATA
Y  S  A  T   R  I  A   F  T  K   I  G  C  L   Q  V  L   F  D  D>

      1870       1880       1890       1900       1910       1920
TGGCTGACAT CTTTGCAACA CTAGATGAAT TGAAAAGTTT CACTGAGGGA GTAAAGAGAT
M  A  D  I   F  A  T   L  D  E   L  K  S  F   T  E  G   V  K  R>

      1930       1940       1950       1960       1970       1980
GGGATACATC TTTGCTACAT GAGATTCCAG AGTGTATGCA AACTTGCTTT AAAGTTTGGT
W  D  T  S   L  L  H   E  I  P   E  C  M  Q   T  C  F   K  V  W>

      1990       2000       2010       2020       2030       2040
TCAAATTAAT GGAAGAAGTA AATAATGATG TGGTTAAGGT ACAAGGACGT GACATGCTCG
F  K  L  M   E  E  V   N  N  D   V  V  K  V   Q  G  R   D  M  L>

      2050       2060       2070       2080       2090       2100
CTCACATAAG AAAACCTTGG GAGTTGTACT TCAATTGTTA TGTACAAGAA AGGGAGTGGC
A  H  I  R   K  P  W   E  L  Y   F  N  C  Y   V  Q  E   R  E  W>

      2110       2120       2130       2140       2150       2160
TTGAAGCTGG GTATATACCA ACTTTTGAAG AGTACTTAAA GACTTATGCT ATATCAGTAG
L  E  A  G   Y  I  P   T  F  E   E  Y  L  K   T  Y  A   I  S  V>

      2170       2180       2190       2200       2210       2220
GCCTTGGACC ATGTACCCTA CAACCAATAC TACTAATGGG TGAGCTTGTG AAAGATGATG
G  L  G  P   C  T  L   Q  P  I   L  L  M  G   E  L  V   K  D  D>

      2230       2240       2250       2260       2270       2280
TTGTTGAGAA AGTGCACTAT CCCTCAAATA TGTTTGAGCT TGTATCCTTG AGCTGGCGAC
V  V  E  K   V  H  Y   P  S  N   M  F  E  L   V  S  L   S  W  R>

      2290       2300       2310       2320       2330       2340
TAACAAACGA CACCAAAACA TATCAGGCTG AAAAGGCTCG AGGACAACAA GCCTCAGGCA
L  T  N  D   T  K  T   Y  Q  A   E  K  A  R   G  Q  Q   A  S  G>

      2350       2360       2370       2380       2390       2400
TAGCATGCTA TATGAAGGAT AATCCAGGAG CAACTGAGGA AGATGCCATC AAGCACATAT
I  A  C  Y   M  K  D   N  P  G   A  T  E  E   D  A  I   K  H  I>

      2410       2420       2430       2440       2450       2460
GTCGTGTTGT TGACCGGGCC TTGAAAGAAG CAAGCTTTGA ATATTTCAAA CCATCCAATG
C  R  V  V   D  R  A   L  K  E   A  S  F  E   Y  F  K   P  S  N>

      2470       2480       2490       2500       2510       2520
ATATCCCAAT GGGTTGCAAG TCCTTTATTT TTAACCTTAG ATTGTGTGTC CAAATCTTTT
D  I  P  M   G  C  K   S  F  I   F  N  L  R   L  C  V   Q  I  F>

      2530       2540       2550       2560       2570       2580
ACAAGTTTAT AGATGGGTAC GGAATTGCCA ATGAGGAGAT TAAGGATTAT ATAAGAAAAG
Y  K  F  I   D  G  Y   G  I  A   N  E  E  I   K  D  Y   I  R  K>

      2590       2600       2610       2620       2630       2640
TTTATATTGA TCCAATTCAA GTATGATATA TCATGTAAAA CCTTTTTTTC ATAATAAATT
V  Y  I  D   P  I  Q   V>

      2650       2660       2670       2680
GACTTATTAT TGTATTGGCA AAAAAAAAAA AAAAAAAAAA AAAAA
```

Figure III-2. Comparison of the DNA sequences of taxadiene synthase of *Taxus baccata* and *T. brevifolia.*

```
  1 CCTGCCTCTCTGGAGAAATGGCTCAGCTCTCATTTAATGCAGCGCTGAAGATGAATGCAT 60
    ||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||
  5 CCTGCCTCTCTGGAGAAATGGCTCAGCTCTCATTTAATGCAGCGCTGAAGATGAACGCAT 64

 61 TAGGGAACAAGGCAATCCACGATCCAACGAATTGCAGAGCCAAATCTGAGGGCCAAATGA 120
    | |||||||||||||||||||||||||||||||||||||||||||||||| |||||||||
 65 TGGGGAACAAGGCAATCCACGATCCAACGAATTGCAGAGCCAAATCTGAGCGCCAAATGA 124

121 TGTGGGTTTGCTCCAAATCAGGGCGAACCAGAGTAAAAATGTCGAGAGGAAGTGGTGGTC 180
    ||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
125 TGTGGGTTTGCTCCAGATCAGGGCGAACCAGAGTAAAAATGTCGAGAGGAAGTGGTGGTC 184

181 CTGGTCCTGTCGTAATGATGAGCAGTAGCACTGGCACTAGCAAGGTGGTTTCCGAGACTT 240
    ||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
185 CTGGTCCTGTCGTAATGATGAGCAGCAGCACTGGCACTAGCAAGGTGGTTTCCGAGACTT 244

241 CCAGTACCATTGTGGATGATATCCCTCGACTCTCCGCCAATTATCATGGCGATCTGTGGC 300
    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
245 CCAGTACCATTGTGGATGATATCCCTCGACTCTCCGCCAATTATCATGGCGATCTGTGGC 304

301 ACCACAATGTTATACAAACTCTGGAGACACCATTTCGTGAGAGTTCTACTTTCCAAGAAC 360
    ||||||||||||||||||||||||||||||| ||||||||||||||||||| ||||||||
305 ACCACAATGTTATACAAACTCTGGAGACACCGTTTCGTGAGAGTTCTACTTACCAAGAAC 364

361 GGGCAGACGAGCTGGTTGTGAAAATTAAAGATATGTTCAATGCGCTCGGAGACGGAGATA 420
    ||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
365 GGGCAGATGAGCTGGTTGTGAAAATTAAAGATATGTTCAATGCGCTCGGAGACGGAGATA 424

421 TCAGTCCGTCTGCATACGACACTGCGTGGGTGGCGAGGTGGCGGACCGTTTCCTCTGATG 480
    ||||||||||||||||||||||||||||||||||||||  |  |||| ||||||||||||
425 TCAGTCCGTCTGCATACGACACTGCGTGGGTGGCGAGGCTGGCGACCATTTCCTCTGATG 484

481 GATCTGAGAAGCCACGGTTTCCTCAGGCCCTCAACTGGGTTTTAAACAACCAGCTCCAAG 540
    ||||||||||||||||||||||||||||||||||||||||||| |||||||||||||| |
485 GATCTGAGAAGCCACGGTTTCCTCAGGCCCTCAACTGGGTTTTCAACAACCAGCTCCAGG 544

541 ATGGATCATGGGGTATCGAATCGCACTTTAGTTTATGCGATCGATTGCTTAACACGGTCA 600
    ||||||| ||||||||||||||||||||||||||||||||||||||||||||||||  ||
545 ATGGATCGTGGGGTATCGAATCGCACTTTAGTTTATGCGATCGATTGCTTAACACGACCA 604

601 ATTCTGTTATCGCCCTCTCGGTTTGGAAAACAGGGCACAGCCAAGTAGAACAAGGTACTG 660
    ||||||||||||||||||||||||||||||||||||||||||||||| |||||||| |||
605 ATTCTGTTATCGCCCTCTCGGTTTGGAAAACAGGGCACAGCCAAGTACAACAAGGTGCTG 664
```

```
 661 AGTTTATTGCAGAGAATCTAAGATTACTCAATGAGGAAGATGAGTTGTCCCCGGATTTCG 720
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||| 
 665 AGTTTATTGCAGAGAATCTAAGATTACTCAATGAGGAAGATGAGTTGTCCCCGGATTTCC 724

 721 AAATAATCTTTCCTGCTCTGCTGCAAAAGGCAAAAGCGTTGGGGATCAATCTTCCTTACG 780
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
 725 AAATAATCTTTCCTGCTCTGCTGCAAAAGGCAAAAGCGTTGGGGATCAATCTTCCTTACG 784

 781 ATCTTCCATTTATCAAATCTTTGTCGACAACACGGGAAGCCAGGCTTACAGATGTTTCTG 840
     |||||||||||||||||| |||||||||||||||||||||||||||||||||||||||||
 785 ATCTTCCATTTATCAAATATTTGTCGACAACACGGGAAGCCAGGCTTACAGATGTTTCTG 844

 841 CGGCAGCAGACAATATTCCAGCCAACATGTTGAATGCGTTGGAGGGTCTCGAGGAAGTTA 900
     ||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||
 845 CGGCAGCAGACAATATTCCAGCCAACATGTTGAATGCGTTGGAAGGTCTCGAGGAAGTTA 904

 901 TTGACTGGAACAAGATTATGAGGTTTCAAAGTAAAGATGGATCTTTCCTGAGCTCCCCTG 960
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
 905 TTGACTGGAACAAGATTATGAGGTTTCAAAGTAAAGATGGATCTTTCCTGAGCTCCCCTG 964

 961 CCTCCACTGCCTGTGTACTGATGAATACAGGGGAGGAAAAATGTTTCACTTTTCTCAACA 1020
     |||||||||||||||||||||||||||||||||| |||||||||||||||||||||||||
 965 CCTCCACTGCCTGTGTACTGATGAATACAGGGGACGAAAAATGTTTCACTTTTCTCAACA 1024

1021 ACCTGCTCGACAAATTCGGCGGCTGCGTGCCCTGTATGTATTCCATCGATCTGCTGGAAC 1080
     | ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1025 ATCTGCTCGACAAATTCGGCGGCTGCGTGCCCTGTATGTATTCCATCGATCTGCTGGAAC 1084

1081 GCCTTTCGCTGGTTGATAACATTGAGCATCTCGGAATCGGTCGCCATTTCAAACAAGAAA 1140
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1085 GCCTTTCGCTGGTTGATAACATTGAGCATCTCGGAATCGGTCGCCATTTCAAACAAGAAA 1144

1141 TCAAAGTAGCTCTTGATTATGTCTACAGACATTGGAGTGAAAGGGGCATCGGTTGGGGCA 1200
     |||||| |||||||||||||||||||||||||||||||||||||||||||||||||||||
1145 TCAAAGGAGCTCTTGATTATGTCTACAGACATTGGAGTGAAAGGGGCATCGGTTGGGGCA 1204

1201 GAGACAGCCTCGTTCCAGATCTCAACACAACAGCCCTCGGCCTGCGAACTCTTCGCACGC 1260
     |||||||||| ||||||||||||||||| |||||||||||||||||||||||||||| ||
1205 GAGACAGCCTTGTTCCAGATCTCAACACCACAGCCCTCGGCCTGCGAACTCTTCGCATGC 1264

1261 ACGGATACGATGTTTCTTCAGATGTTTTGAATAATTTCAAAGATGAAAACGGGCGGTTCT 1320
     |||||||| ||||||||||||| |||||||||||||||||||||||||||||||||||||
1265 ACGGATACAATGTTTCTTCAGACGTTTTGAATAATTTCAAAGATGAAAACGGGCGGTTCT 1324

1321 TCTCCTCTGCGGGCCAAACCCATGTCGAATTGAGAAGCGTGGTGAATCTTTTCAGAGCTT 1380
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1325 TCTCCTCTGCGGGCCAAACCCATGTCGAATTGAGAAGCGTGGTGAATCTTTTCAGAGCTT 1384

1381 CCGACCTTGCATTTCCTGACGAAGGAGCTATGGACGATGCTAGAAAATTTGCAGAACCAT 1440
     ||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
1385 CCGACCTTGCATTTCCTGACGAAAGAGCTATGGACGATGCTAGAAAATTTGCAGAACCAT 1444

1441 ATCTTAGAGACGCACTTGCAACAAAAATCTCAACCAATACAAAACTATTCAAAGAGATTG 1500
     |||||||||| ||||||||||| |||||||||||||||||||||||||||||||||||||
1445 ATCTTAGAGAGGCACTTGCAACGAAAATCTCAACCAATACAAAACTATTCAAAGAGATTG 1504
```

```
1501 AGTACGTGGTGGAGTACCCTTGGCACATGAGTATCCCACGCCTAGAAGCCAGAAGTTATA 1560
     ||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||
1505 AGTACGTGGTGGAGTACCCTTGGCACATGAGTATCCCACGCTTAGAAGCCAGAAGTTATA 1564

1561 TTGATTCGTATGACGACGATTATGTATGGCAGAGGAAGACTCTATACAGAATGCCATCTT 1620
     ||||||| ||||||||| |||||||||||||||||||||||||||| |||||||||||||
1565 TTGATTCATATGACGACAATTATGTATGGCAGAGGAAGACTCTATATAGAATGCCATCTT 1624

1621 TGAGTAATTCAAAATGTTTAGAATTGGCAAAATTGGACTTCAATATCGTACAATCTTTGC 1680
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1625 TGAGTAATTCAAAATGTTTAGAATTGGCAAAATTGGACTTCAATATCGTACAATCTTTGC 1684

1681 ATCAAGAGGAGTTGAAGCTTCTAACAAGATGGTGGAAGGAATCCGGCATGGCAGATATAA 1740
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1685 ATCAAGAGGAGTTGAAGCTTCTAACAAGATGGTGGAAGGAATCCGGCATGGCAGATATAA 1744

1741 ATTTCACTCGACACCGAGTGGCGGAGGTTTATTTTTCATCAGCTACATTTGAACCTGAAT 1800
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||
1745 ATTTCACTCGACACCGAGTGGCGGAGGTTTATTTTTCATCAGCTACATTTGAACCCGAAT 1804

1801 ATTCTGCCACTAGAATTGCCTTCACAAAAATTGGTTGTTTACAAGTCCTTTTTGATGATA 1860
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1805 ATTCTGCCACTAGAATTGCCTTCACAAAAATTGGTTGTTTACAAGTCCTTTTTGATGATA 1864

1861 TGGCTGACATCTTTGCAACACTAGATGAATTGAAAAGTTTCACTGAGGGAGTAAAGAGAT 1920
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1865 TGGCTGACATCTTTGCAACACTAGATGAATTGAAAAGTTTCACTGAGGGAGTAAAGAGAT 1924

1921 GGGATACATCTTTGCTACATGAGATTCCAGAGTGTATGCAAACTTGCTTTAAAGTTTGGT 1980
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1925 GGGATACATCTTTGCTACATGAGATTCCAGAGTGTATGCAAACTTGCTTTAAAGTTTGGT 1984

1981 TCAAATTAATGGAAGAAGTAAATAATGATGTGGTTAAGGTACAAGGACGTGACATGCTCG 2040
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1985 TCAAATTAATGGAAGAAGTAAATAATGATGTGGTTAAGGTACAAGGACGTGACATGCTCG 2044

2041 CTCACATAAGAAAACCTTGGGAGTTGTACTTCAATTGTTATGTACAAGAAAGGGAGTGGC 2100
     |||||||||||||||| |||||||||||||||||||||||||||||||||||||||||||
2045 CTCACATAAGAAAACCCTGGGAGTTGTACTTCAATTGTTATGTACAAGAAAGGGAGTGGC 2104

2101 TTGAAGCTGGGTATATACCAACTTTTGAAGAGTACTTAAAGACTTATGCTATATCAGTAG 2160
     ||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
2105 TTGAAGCCGGGTATATACCAACTTTTGAAGAGTACTTAAAGACTTATGCTATATCAGTAG 2164

2161 GCCTTGGACCATGTACCCTACAACCAATACTACTAATGGGTGAGCTTGTGAAAGATGATG 2220
     |||||||||| |||||||||||||||||||||||||||||||||||||||||||||||||
2165 GCCTTGGACCGTGTACCCTACAACCAATACTACTAATGGGTGAGCTTGTGAAAGATGATG 2224

2221 TTGTTGAGAAAGTGCACTATCCCTCAAATATGTTTGAGCTTGTATCCTTGAGCTGGCGAC 2280
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
2225 TTGTTGAGAAAGTGCACTATCCCTCAAATATGTTTGAGCTTGTATCCTTGAGCTGGCGAC 2284

2281 TAACAAACGACACCAAAACATATCAGGCTGAAAAGGCTCGAGGACAACAAGCCTCAGGCA 2340
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
2285 TAACAAACGACACCAAAACATATCAGGCTGAAAAGGCTCGAGGACAACAAGCCTCAGGCA 2344
```

```
2341 TAGCATGCTATATGAAGGATAATCCAGGAGCAACTGAGGAAGATGCCATCAAGCACATAT 2400
     ||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||
2345 TAGCATGCTATATGAAGGATAATCCAGGAGCAACTGAGGAAGATGCCATTAAGCACATAT 2404

2401 GTCGTGTTGTTGACCGGGCCTTGAAAGAAGCAAGCTTTGAATATTTCAAACCATCCAATG 2460
     ||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
2405 GTCGTGTTGTTGATCGGGCCTTGAAAGAAGCAAGCTTTGAATATTTCAAACCATCCAATG 2464

2461 ATATCCCAATGGGTTGCAAGTCCTTTATTTTTAACCTTAGATTGTGTGTCCAAATCTTTT 2520
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
2465 ATATCCCAATGGGTTGCAAGTCCTTTATTTTTAACCTTAGATTGTGTGTCCAAATCTTTT 2524

2521 ACAAGTTTATAGATGGGTACGGAATTGCCAATGAGGAGATTAAGGATTATATAAGAAAAG 2580
     ||||||||||||||||||||||||| |||||||||||||||||||| |||||||||||||
2525 ACAAGTTTATAGATGGGTACGGAATCGCCAATGAGGAGATTAAGGACTATATAAGAAAAG 2584

2581 TTTATATTGATCCAATTCAAGTATGATATATCATGTAAAACCTTTTTTTCATAATAAATT 2640
     |||||||||||||||||||||||||||||||||||||||||| |||||||| ||||||||
2585 TTTATATTGATCCAATTCAAGTATGATATATCATGTAAAACCTCTTTTTCATGATAAATT 2644

2641 GACTTATTATTGTATTGGCAAAAAAAAAAAAAAAAAAAAAAAAAA 2685
     |||||||||||||||||||||||||||||||||||||||||||||
2645 GACTTATTATTGTATTGGCAAAAAAAAAAAAAAAAAAAAAAAAAA 2689
```

Figure III-3. Comparison of the deduced amino acid sequences of taxadiene synthase of *Taxus baccata* and *T. brevifolia*.

```
  1 MAQLSFNAALKMNALGNKAIHDPTNCRAKSEGQMMWVCSKSGRTRVKMSR  50
    ||||||||||||||||||||||||||||||| ||||||| ||||||||||
  1 MAQLSFNAALKMNALGNKAIHDPTNCRAKSERQMMWVCSRSGRTRVKMSR  50

 51 GSGGPGPVVMMSSSTGTSKVVSETSSTIVDDIPRLSANYHGDLWHHNVIQ 100
    ||||||||||||||||||||||||||||||||||||||||||||||||||
 51 GSGGPGPVVMMSSSTGTSKVVSETSSTIVDDIPRLSANYHGDLWHHNVIQ 100

101 TLETPFRESSTFQERADELVVKIKDMFNALGDGDISPSAYDTAWVARWRT 150
    ||||||||||| |||||||||||||||||||||||||||||||||||  |
101 TLETPFRESSTYQERADELVVKIKDMFNALGDGDISPSAYDTAWVARLAT 150

151 VSSDGSEKPRFPQALNWVLNNQLQDGSWGIESHFSLCDRLLNTVNSVIAL 200
     ||||||||||||||||| |||||||||||||||||||||||| ||||||
151 ISSDGSEKPRFPQALNWVFNNQLQDGSWGIESHFSLCDRLLNTTNSVIAL 200

201 SVWKTGHSQVEQGTEFIAENLRLLNEEDELSPDFEIIFPALLQKAKALGI 250
    |||||||||| || |||||||||||||||||||| |||||||||||||||
201 SVWKTGHSQVQQGAEFIAENLRLLNEEDELSPDFQIIFPALLQKAKALGI 250

251 NLPYDLPFIKSLSTTREARLTDVSAAADNIPANMLNALEGLEEVIDWNKI 300
    |||||||||| |||||||||||||||||||||||||||||||||||||||
251 NLPYDLPFIKYLSTTREARLTDVSAAADNIPANMLNALEGLEEVIDWNKI 300

301 MRFQSKDGSFLSSPASTACVLMNTGEEKCFTFLNNLLDKFGGCVPCMYSI 350
    ||||||||||||||||||||||||| ||||||||||||||||||||||||
301 MRFQSKDGSFLSSPASTACVLMNTGDEKCFTFLNNLLDKFGGCVPCMYSI 350

351 DLLERLSLVDNIEHLGIGRHFKQEIKVALDYVYRHWSERGIGWGRDSLVP 400
    |||||||||||||||||||||||||| |||||||||||||||||||||||
351 DLLERLSLVDNIEHLGIGRHFKQEIKGALDYVYRHWSERGIGWGRDSLVP 400

401 DLNTTALGLRTLRTHGYDVSSDVLNNFKDENGRFFSSAGQTHVELRSVVN 450
    ||||||||||||| ||| ||||||||||||||||||||||||||||||||
401 DLNTTALGLRTLRMHGYNVSSDVLNNFKDENGRFFSSAGQTHVELRSVVN 450

451 LFRASDLAFPDEGAMDDARKFAEPYLRDALATKISTNTKLFKEIEYVVEY 500
    |||||||||||| |||||||||||||| ||||||||||||||||||||||
451 LFRASDLAFPDERAMDDARKFAEPYLREALATKISTNTKLFKEIEYVVEY 500

501 PWHMSIPRLEARSYIDSYDDDYVWQRKTLYRMPSLSNSKCLELAKLDFNI 550
    ||||||||||||||||||||| ||||||||||||||||||||||||||||
501 PWHMSIPRLEARSYIDSYDDNYVWQRKTLYRMPSLSNSKCLELAKLDFNI 550
```

```
551 VQSLHQEELKLLTRWWKESGMADINFTRHRVAEVYFSSATFEPEYSATRI 600
    ||||||||||||||||||||||||||||||||||||||||||||||||||
551 VQSLHQEELKLLTRWWKESGMADINFTRHRVAEVYFSSATFEPEYSATRI 600

601 AFTKIGCLQVLFDDMADIFATLDELKSFTEGVKRWDTSLLHEIPECMQTC 650
    ||||||||||||||||||||||||||||||||||||||||||||||||||
601 AFTKIGCLQVLFDDMADIFATLDELKSFTEGVKRWDTSLLHEIPECMQTC 650

651 FKVWFKLMEEVNNDVVKVQGRDMLAHIRKPWELYFNCYVQEREWLEAGYI 700
    ||||||||||||||||||||||||||||||||||||||||||||||||||
651 FKVWFKLMEEVNNDVVKVQGRDMLAHIRKPWELYFNCYVQEREWLEAGYI 700

701 PTFEEYLKTYAISVGLGPCTLQPILLMGELVKDDVVEKVHYPSNMFELVS 750
    ||||||||||||||||||||||||||||||||||||||||||||||||||
701 PTFEEYLKTYAISVGLGPCTLQPILLMGELVKDDVVEKVHYPSNMFELVS 750

751 LSWRLTNDTKTYQAEKARGQQASGIACYMKDNPGATEEDAIKHICRVVDR 800
    ||||||||||||||||||||||||||||||||||||||||||||||||||
751 LSWRLTNDTKTYQAEKARGQQASGIACYMKDNPGATEEDAIKHICRVVDR 800

801 ALKEASFEYFKPSNDIPMGCKSFIFNLRLCVQIFYKFIDGYGIANEEIKD 850
    ||||||||||||||||||||||||||||||||||||||||||||||||||
801 ALKEASFEYFKPSNDIPMGCKSFIFNLRLCVQIFYKFIDGYGIANEEIKD 850

851 YIRKVYIDPIQV 862
    ||||||||||||
851 YIRKVYIDPIQV 862
```

3.1.3 Sequence Comparison with Other Terpenoid Cyclases

The deduced amino acid sequence of the taxadiene synthase cDNA from *Taxus baccata* was compared with those of other terpenoid cyclases. In addition to its notable homology with the taxadiene synthase from *T. brevifolia*, it shows considerable similarity to other terpenoid cyclases such as abietadiene cyclase from *Abies grandis* (57% identity, 71% similarity, Vogel et al., 1996), *ent*-kaurene synthase A from *Arabidopsis. thaliana* (47% identity, 67% similarity, Sun and Kamiya, 1994) and *Zea mays* (47% identity, 69% similarity, Bensen et al., 1995), kaurene synthase B from *Cucurbita maxima* (37% identity, 61% similarity, Yamaguchi et al., 1996), (–)-limonene synthase from *Mentha spicata* (26% identity, 52% similarity, Colby et al, 1993), and 5-*epi*-aristolochene synthase from *Nicotiana tabacum* (26% identity and 49% similarity, Facchini and Chappell, 1992). Sequence alignments of these terpenoid cyclases revealed that taxadiene synthase from *Taxus baccata* contains the DDXXD motif (amino acid 613-617, Figure III-1), which is conserved in most prenyltransferases and terpenoid cyclases, and a related DXXDD motif (amino acid 516-520, Figure III-1). These two conserved motifs are also present in the amino acid sequence of taxadiene synthase from *T. brevifolia*. The aspartate-rich DDXXD motif is suggested to function as a divalent metal ion-diphosphate complex binding site in the terpenoid cyclases (Chappell, 1995; McGarvey and Croteau, 1995). Comparison with other terpene cyclases from various plant families indicate approximately homogeneous levels of identity (30-40%) and similarity (50-60%). These results imply that the terpenoid cyclases are evolutionarily originated from the same pedigree (Colby et al., 1993; Mau and West, 1994; Back and Chappell, 1995; McGarvey and Croteau, 1995; Chappell, 1995).

In the GeneBank databases, taxadiene synthase from yew (*Taxus baccata* and *T. brevifolia*) revealed the highest homology with the abietadiene cyclase from a conifer, *Abies grandis* (Vogel et al., 1996). Many other terpenoid cyclases are in this database. Other than taxadiene synthase, however, abietadiene cyclase is the only available terpenoid cyclase sequence from a gymnosperm. These two diterpenoid cyclases from conifers have several regions showing significant sequence homology.

Some other plant terpenoid cyclases have the identical reaction mechanisms to taxadiene synthase in the cyclization of the particular GPP (C_{10}), FPP (C_{15}), and GGPP (C_{20}) substrates (Lin et al., 1996). This kind of enzymes include casbene synthase from *Ricinus communis* (Mau and West, 1994), (–)-limonene synthase from *Mentha spicata* (Colby et al, 1993) and 5-*epi*-aristolochene synthase from *Nicotiana tabacum* (Facchini and Chappell, 1992). Comparison of the primary structure of taxadiene synthase from yew with these of plant terpenoid cyclases shows that it is less similar to these enzymes that than it is to abietadiene cyclase.

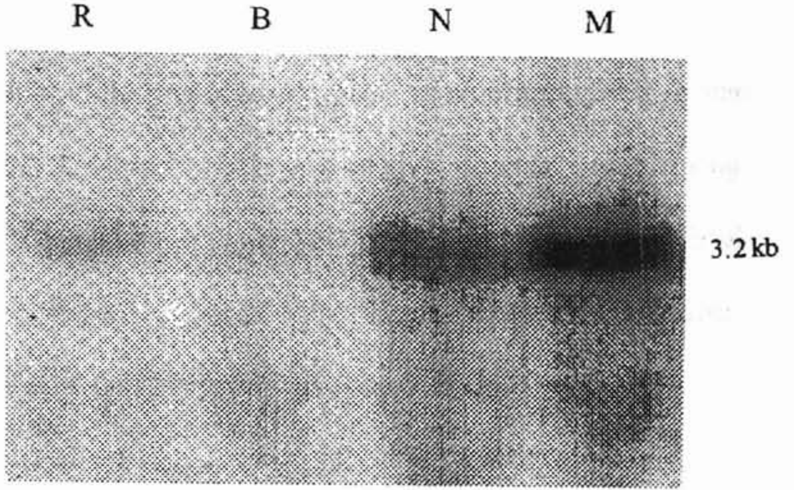
3.1.4 RNA Blot Analysis

RNA blot analysis was carried out using the isolated taxadiene synthase cDNA 12b-1a as a probe under high stringency conditions. The distribution of the taxadiene synthase transcript was investigated using total RNAs isolated from different tissues of Runyan yew and mixed tissue from *T. baccata*. The taxadiene synthase transcript was detected as a single band of about 3260 nt in all of the tested tissues of Runyan yew (Figure III-4) which suggested that the structural gene for the taxadiene synthase from *Taxus baccata* has been successfully cloned. Our cDNA clone, which is 2685 nt in length, is probably still lacking part of the 5'

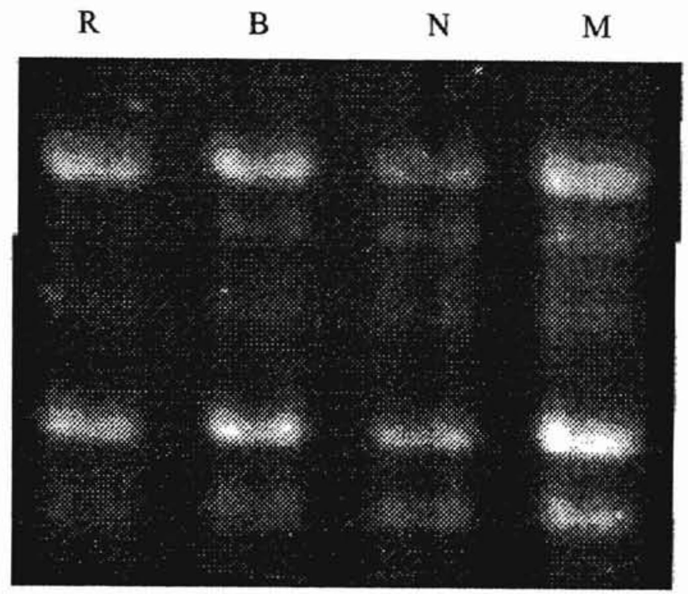
untranslated region, although it contains a complete ORF. *T. baccata* did not show any signal which need repeat experiment.

In Runyan yew, the transcript was abundant in mixed tissues (lane M) and needles (lane N). Only a small amount of the transcript was detected in roots (lane R) and bark (lane B). This result supported the hypothesis that the synthesis of taxa-4(5), 11(12)-diene occurs in plastids because green tissues (needles and mixture) contain more plastids than non-green tissues (roots and bark). The synthesis of taxadiene in plastids is further supported by the fact that the synthesis of the taxadiene precursor GGPP, which was catalyzed by GGPP synthase, occurs in plastids (Kuntz et al., 1992; Scolnik and Bartley, 1994). Further examination of the localization of taxadiene synthase may be carried out by immunocytochemical analysis with antibodies.

According to Vidensek (1990), bark contains the highest concentration of taxol, and roots contain the second highest concentration. Taxol levels in needles are variable because of environmental effects such as climate, the place for planting (Choi et al., 1995). This may be explained by the fact that the synthetic site and storage site of taxol and related taxanes are different, or at least the synthetic sites of taxol and taxadiene are different. Further studies are needed to determine whether the synthesis and storage of taxol are at different sites.



A. Northern hybridization result.



B. RNA pattern for Northern hybridization

Figure III-4. Expression pattern of taxadiene synthase at the transcriptional level.

R, roots; B, barks; N, needles; M, mixture (shoot tips, barks, and needles) of *Taxus x media* Runyan.

3.2 Transformation of *Taxus*

All four *Agrobacterium* strains were capable of inducing rooting in stem cuttings of Runyan yew (Figure III-5). However, efficiency of root induction varied among the different bacterial strains tested (Table III-1). Strain 9405 demonstrated the highest level of virulence based on the number of cuttings forming roots. Strain TR105 was most virulent based on counting the maximum number of roots on one cutting.

After two weeks of preculture, most stem cuttings showed white callus proliferation around the wound sites. Adventitious roots were produced within 8-10 weeks after inoculation. Controls also grew roots, but with less frequency. Roots originated at the bottom of the cuttings where the wounds were made, and proceeded from swelling at the wound site. Comparing the controls with cuttings from each of the four *Agrobacterium* strains, the pattern of root initiation in the cuttings was roughly the same. They all had a natural root phenotype.

Agrobacterium strain TR105 was chosen to inoculate seedlings of *Taxus baccata*. Seedlings were susceptible to infection by this bacterial strain (Table III-2). Adventitious roots formed in 86% of the seedlings within four weeks after inoculation. Roots were initiated at the surface or near the surface of the basal cut with visible callus proliferation (Figure III-6). Seedlings had a hairy root phenotype and roots were formed within callus at the wound sites. All the seedlings with one wound grew roots. Six out of eight plants with two cuts (one was upper, another was lower on the hypocotyl) developed roots at both wound sites, while the remaining two plants developed roots at only one wound site, only callus grew at the other cut. The transformation efficiency appears to be similar at both wound sites.

The generated roots of the seedlings had a hairy root phenotype typical of herbaceous plants, but the developed roots of stem cuttings had a normal root phenotype. Magnussen et al.

(1994) recognized both normal and hairy roots in transformed pines: normal roots were produced on 5-10% of *A. rhizogenes* (R1600) treated seedlings of *Picea abies*, *Pinus sylvestris* and *P. contorta*. Diner and Karnosky (1987) have reported hairy root induction and development on *Lairx decidua*. Based on the current available information, the hairy root phenotype in conifers is not yet clear. In other conifers, transgenic roots (Huang et al., 1991; McAfee et al., 1993; Mihaljevie et al., 1996) or transgenic shoots (Shin et al., 1994) showed normal phenotypes. According to Huang et al. (1991), although the genetic basis of why the pRi plasmid functions to yield the normal phenotype in transformed conifer tissues is not yet certain, there is no doubt it depends on a number of variables including the endogenous hormones levels in the host conifers and on *Agrobacteria* genes.

It was reported that root induction on *in vitro* adventitious *Pinus monticola* Dougl. shoots and seedling shoots of *P. banksiana* and *Larix laricina* was improved by the prolonged presence of *Agrobacteria*, compared with the control (McAfee et al., 1993). This experiment also showed that extended co-cultivation with bacteria enhanced root induction, and the root growth pattern in the stem cuttings of Runyan yew did not vary. Roots were always produced at the wound site.

The efficiency of *Agrobacterium* infection can be affected greatly by several factors, such as genotype, age of plant, season of the year, and nutrition, factors which can change the physiological state of the host (Tepfer, 1989). In this experiment, it was observed that seedlings were more susceptible to *Agrobacterium* transformation than stem cuttings, and mature stem cuttings (1-2 years old sprouts) were more sensitive than young cuttings. However, younger seedlings were more susceptable than older seedlings (> 2-3 months), similar to reports for most herbaceous plants (Tepfer, 1989). Han et al. (1994) also used mature *Taxus* stems for *Agrobacterium tumefaciens* transformation. Using mature tissues has

an advantage over juvenile tissues in that mature tissues would provide the ability to incorporate proven and/or selected genotypes in a genetic improvement program. For example, a yew tree which has a high level taxol or 10-DAB production can be selected via a progeny test, then the selected genotype can be genetically transformed for further improvement (Han et al., 1994). It was also observed in this study that transformation efficiency was higher in the spring (March to May) than in other seasons, probably because the period of high cambial activity during the spring vegetative growth yields a greater proportion of the cells competent for transformation and suitable for hairy root formation (Sangwan et al., 1992). Rapid plant cell division at the wound site is an essential element for effective infection (Guivarch et al., 1993). It was also observed that bacterial and fungal contamination were less in the spring.

Figure III-5. Roots induced at the wound site of an *in vitro* cultured stem cutting of *Taxus x media* Runyan two months after infection with *Agrobacterium rhizogenes*.

Table III-1 Root induction in stem cuttings of *Taxus x media* Runyan eight weeks after infection with *Agrobacterium rhizogenes*.

Bacterial strain	Total number of inoculated cuttings	Cuttings forming roots	Maximum roots on one cuttings
Control	40	2	2
9405	20	8	5
E15	19	2	2
S6	19	6	4
TR105	20	3	6

Table III-2. Root induction in seedlings of *Taxus baccata* four weeks after infection with *Agrobacterium rhizogenes* TR105.

	One wound site per seedling		Two wound sites per seedling	
Bacterial strain	Number of inoculated seedlings	Seedlings forming roots	Number of inoculated seedlings	Seedlings forming roots
TR105	6	6	8	6
Control	5	0	5	0

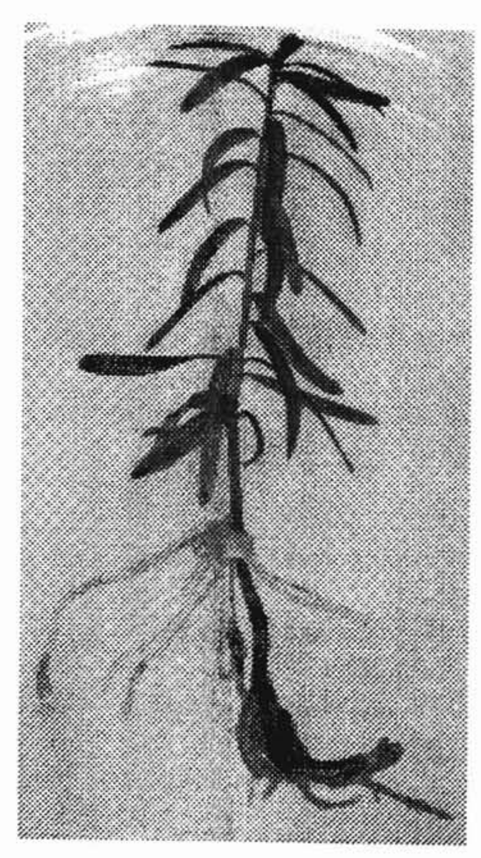


Figure III-6. Roots induced at the wound site of an *in vitro* cultured seedling of *Taxus baccata* four weeks after infection with *Agrobacterium rhizogenes* TR105.

PART IV

CONCLUSION

A taxadiene synthase cDNA was isolated from *Taxus baccata*. The nucleotide sequence and deduced amino acid sequence have a significant homology with that of the enzyme from *T. brevifolia*.

A. rhizogenes-mediated transformation of *Taxus* species has not been described before. This experiment showed that *A. rhizogenes* has the ability to transform *Taxus* spp. The transformation was phenotypically evidenced by root production at the inoculated sites. Confirmation of the transformation at the molecular level and an assay of the taxol production level are the next step.

The root of *Taxus* is the tissue with the second highest concentration of taxol after bark (Vidensek, 1990); thus, culture of roots induced by *Agrobacterium* transformation offers a promising alternative approach for taxol production (Edgington, 1991). Since taxadiene synthase catalyzes the committed cyclization step in the taxane biosynthetic pathway (Koepp et al., 1995; Hezari et al., 1995), the cDNA taxadiene synthase clone will be fused with a strong promoter, and then reintroduced into yew tissues by *Agrobacterium rhizogenes*. With an increase in the copy number of this enzyme, and enhancement of its expression, the yield of

taxa-4(5), 11(12)-diene can be improved, thus the amount of 10-DAB can also be improved, which could provide more biomass for the semisynthesis of taxotere.

REFERENCES

Back K, Chappell J (1995) Cloning and bacterial expression of a sesquiterpene cyclase from *Hyoscyamus muticus* and its molecular comparison to related terpene cyclases. *J Biol Chem* 270: 7375-7381

Bartley GE, Scolnik PA, Gioliano G (1994) Molecular biology of carotenoid biosynthesis in plants. *Annu Rev Plant Physiol Plant Mol Biol* 45: 287-301

Bensen RJ, Johal GS, Crane VC, Tossberg JT, Schnable PS, Meeley RB, Briggs SP (1995) Cloning and characterization of the maize *An1* gene. *Plant Cell* 7: 75-84

Bissery M-C, Nohynek G, Sanderlink G-J, Lavelle F (1995) Docetaxel (Taxotere®): a review of preclinical and clinical experience. Part I: Preclinical experience. *Anti-Cancer Drugs* 6: 339-355

Borman S (1994) Taxol synthesis of anticancer agent, taxol achieved by two different routes. *Chem Eng News* 72: 32-34

Cancer Chemotherapy National Service Center (1961) Protocols for screening chemical agents and natural products against animal tumors and other biological systems. *Cancer Chem Rep* 25: 1-66

Chappell J (1995) The biochemistry and molecular biology of isoprenoid metabolism. *Plant Physiol* 107: 1-6

Chilton MD, Farrand SK, Bendich AJ, Gordon MP, Nester EW (1974) *Agrobacterium tumefaciens* DNA and PS8 bacteriophage DNA not detected in crown gall tumors. *Proc Nat Acad Sci USA* 71: 3672-3676

Chilton MD, Tepfer DA, Petit A, David C, Casse-Delbart F, Tempe J (1982) Agrobacterium rhizogenes inserts T-DNA into the genomes of host plant root cells. *Nature* 295: 432-434

Choi M, Kwak S, Liu J, Park Y, Lee M, An N (1995) Taxol and related compounds in Korean native yews (*Taxus cuspidata*). *Planta Med* 61: 264-266

Church GM, Gilbert W (1984) Genomic sequencing. *Proc Natl Acad Sci USA* 81: 1991-1995

Coates RM (1976) Bioigenetic-type rearrangements of terpenes. *Prog Chem Org Nat Prod* 33: 73-230

Colby SM, Alonso WR, Katahira EV, McGarvey DJ, Croteau R (1993) 4S-Limonene synthase from the oil glands of spearmint (*Mentha spicata*): cDNA isolation, characterization, and bacterial expression of the catalytically active monoterpene cyclase. *J Biol Chem* 268: 23016-23024

Cragg GM, Snader KM (1991) Taxol: the supply issue. *Cancer cells*. 3: 233-235

De Cleene M, De Ley J (1981) The host range of infectious hairy root. *Bot Rev* 47: 147-194

DeFuria MD (1996) Plant cell culture technology. *In* RWF Hardy, JB Segelken, eds, Agricultural Biotechnology: Novel Products and New Partnerships, NABC Reports 8. National Agricultural Biotechnology Council, Ithaca, NY, pp77-85

Diaz JF, Andreu JM (1993) Assembly of purified GDP-tubulin into microtubules induced by taxol and taxotere: reversiblity, ligand stoichiometry and competition. *Biochem* 32: 2747-2755

Diner AM, Karnosky DF (1987) Tissue culture application to forest pathology and pest control. *In* JM Bonga, DJ Durzan, eds, Cell and Tissue Culture in Forestry, Vol 2. Nijhoff Publishers, Dordrecht, pp351-373

Dogbo O, Camara B (1987) Purification of isopentenyl pyrophosphate isomerase and geranylgeranyl pyrophosphate synthase from *Capsicum* chromoplasts by affinity chromatography. *Biochim Biophys Acta* 920: 140-148

Dueber MT, Adolf W, West CA (1978) Biosynthesis of the diterpene phytoalexin casbene. *Phant Physiol* 62: 598-603

Duncan JD, West CA (1981) Properties of kaurene synthetase from March macrocarpus endosperm: Evidence for the participation of separate but interacting enzymes. *Plant Physiol* 68: 1128-1134

Edgington SM (1991) Taxol: out of the woods. *Bio/Technology* 9: 933-936

Ellis D, McCabe D, Russell D, McCown B, Martinell B (1989) A transient assay for heterologous promoter activity in *Picea glauca*. *In* MR Ahuja, ed, Woody Plant Biotechnology. Plenum Press, New York, pp269-281

Facchini PJ, Chappell J (1992) Gene family for an elicitor-induced sesquiterpene cyclase in tobacco. *Proc Natl Acad Sci USA* 89: 11088-11092

Feinberg AP, Vogelstein B (1983) A technique for radiolabeling DNA restriction endonulease fragments to high specific activity. *Anal Biochem* 132: 6-13

Fett-Neto AG, DiCosmo F (1992) Distribution and amounts of taxol in different shoot parts of *Taxus cuspidata*. *Planta Med* 58: 464-466

Fleming PE, Mocek U, Floss HG (1993) Biosynthesis of taxoids: mode of formation of the taxol side chain. *J Am Chem Soc* 115: 805-807

Flores HF, Sgrignoli PJ (1991) In vitro culture and precious germination of *Taxus* embryos. *In Vitro Cell Develop Biol* 27P: 139-142

Frost RG, West CA (1977) Properties of kaurene synthetase from *March macrocarpus*. *Plant Physiol* 59: 22-29

Goodwin TW (1971) Biosynthesis of carotenoids and plant triterpenes. *Biochem J* 123: 293-329

Guénard D, Guéritte-Voegelein F, Potier P (1993) Taxol and taxotere: discovery, chemistry, and structure-activity relationships. *Acc Chem Res* 26: 160-167

Gueritte-Voegelein F, Guenard D, Potier P (1987) Taxol and derivatives: a biogenetic hypothesis. *J Nat Prod* 50: 9-18

Guivarch A, Caissard J-C, Brown S, Marie D, Dewitte W, Van Onckelen H, Chriguw D (1993) Localization of target cells and improvement of *Agrobacterium*-mediated transformation efficiency by direct acetosyringone pretreatment of carrot root discs. *Protoplasma* 174: 10-18

Guo Z, Severson RF, Wagner GF (1994) Biosynthesis of the diterpene *cis*-abienol in cell-free extracts of tobacco trichomes. *Arch Biochem Biophys* 308: 103-108

Gupta P, Durzan D (1985) Shoot multiplication from mature trees of Douglas fir (*Pseudotsuga menziesii*) and Sugar pine (*Pinus lambertiana*). *Plant Cell Rep* 2: 177-179

Hajek R, Vorlicek J, Slavik M (1996) Paclitaxel (Taxol®): A review of its antitumor-activity in clinical studies—minireview. *Neoplasma* 43: 141-154

Haldar S, Chintapalli J, Croce CM (1996) Taxol induces *bcl*-2 phosphorylation and death of prostate cancer cells. *Cancer Res* 56: 1253-1255

Han K-H, Fleming P, Walker K, Loper M, Chilton WS, Mocek U, Gordon MP, Floss HG (1994) Genetic transformation of mature *Taxus*: an approach to genetically control the in vitro production of the anticancer drug, taxol. *Plant Sci* 95: 187-196

Heinstein PF, Chang CJ (1994) Taxol. *Annu Rev Plant Physiol Plant Mol Biol* 45: 663-674

Hezari M, Lewis NG, Croteau R (1995) Purification and characterization of taxa-4(5), 11(12)-diene synthase from Pacific yew (*Taxus brevifolia*) that catalyzes the first committed step of taxol biosynthesis. *Arch Biochem biophys* 322: 437-444

Holton RA, Somoza C, Kim H-B, Liang F, Beidiger RJ, Boatman PD, Nadizadeh M, Suzuki Y, Tao C, Vu P, Tang S, Zhang P, Murthi KK, Gentile LN, Liu JH (1994a) First total synthesis of taxol: 1. Functionalization of the B ring. *J Am Chem Soc* 116: 1597-1598

Holton RA, Somoza C, Kim H-B, Liang F, Beidiger RJ, Boatman PD, Nadizadeh M, Suzuki Y, Tao C, Vu P, Tang S, Zhang P, Murthi KK, Gentile LN, Liu JH (1994b) First total synthesis of taxol: 2. Completion of the C and D rings. *J Am Chem Soc* 116: 1599-1600

Hooykaas PJJ, Beijersbergen AGM (1994) The virulence system of *Agrobacterium tumerfaciens*. *Annu Rev Phytopathol* 32:157-179

Horwitz SB, Parness J, Schiff PB, Manfredi JJ (1981) Taxol: a new probe for studying the structure and function of microtubules. *Cold Spring Harbor Symp Quant Biol* 46: 219-226

Horwitz SB (1992) Mechanism of action of taxol. *Trends Pharmacol Sci* 13: 134-13

Huang CHO, Kingston DGI., Magri NF, Samaranayake G, Boettner FE (1986) New taxanes from *Taxus brevifolia*. *J Nat Prod* 49: 665-669

Huang Y, Diner AM, Karnosky DF (1991) *Agrobacterium rhizogenes*-mediated genetic transformation and regeneration of a conifer: *Larix decidua*. *In vitro Cell Devel Biol.* 27: 201-207

Huang Y, Tauer CG (1993) Another tool for gene transfer in pine species: *Agrobacterium rhizogenes*. *In Vitro Cell Develop Biol* 29: 65A

Inomata S, Yokoyams M, Gozu Y, Shimizu T, Yanagi M (1993) Growth pattern and ginsenoside production of *Agrobacterium*-transformed *Panax ginseng* roots. *Plant Cell Rep* 12: 681-686

Jordon MA, Wendell K, Gardiner S, Derry WB, Copp H, Wilson L (1996) Mitotic block induced in HeLa cells by low concentrations of paclitaxel (Taxol) results in abnormal mitotic exit and apoptotic cell death. *Cancer Res* 56: 816-825

Keegstra L, Olsen LJ, Theg SM (1989) Chloroplastic precursors and their transport across the envelope membranes. *Ann Rev Plant Physiol Plant Mol Biol* 40: 471-501

Kingston DGI, Molinero AA, Rimoldi JM (1993) The taxane diterpenoids. *Prog Chem Org Nat Prods* 61: 1-206

Kingston DGI. (1995) Resent advances in the chemistry and structure-activity relationships of Paclitacel. *In* GI Georg, TT Chen, I Ojima, DM Vyas, eds, Taxane Anticancer Agents: Basic Science and Current Status. American Chemical Society, Washington, D.C., pp203-217

Kleinig H (1989) The role of plastids in isoprenoid biosynthesis. *Ann Rev Plant Physiol Plant Mol Biol* 40: 39-59

Koepp AE, Jezari M, Zajicek J, Vogel BS, LaFever RE, Lewis NG, Rodney C (1995) Cyclization of geranylgeranyl diphosphate to taxa-4(5), 11(12)-diene is the committed step of taxol biosynthesis in Pacific yew. *J Biol Chem* 270: 8686-8690

Kuntz M, Romer S, Hugueney P, Weil J H, Camara B (1992) Identification of a cDNA for the plastid-located geranylgeranyl pyrophosphate synthase from *Capsicum annuum*: correlative increase in enzyme activity and transcript level during fruit ripening. *Plant J* 2: 25-34

LaFever RE, Stofer Vogel B, Croteau R (1994) Diterpenoid resin acid biosynthesis in conifers: Enzymatic cyclization of geranylgeranyl pyrophosphate to abietadiene, the precursor of abietic acid. *Arch Biochem Biophys* 313: 139-149

Lansing A, Haaertel M, Gordon M, Floss HG (1991) Biosynthetic studies on taxol. *Planta Med*, Suppl 2. 57: A83

Lewis N, Croteau R (1992) Taxol biosynthesis. *In* Second National Cancer Institute Workshop on Taxol and *Taxus*. Alexandria, VA, pp23-24

Lin X, Hezari M, Koepp AE, Floss JG, Croteau R (1996) Mechanism of taxadiene synthase, a diterpene cyclase that catalyzes the first step of taxol biosynthesis in Pacific yew. *Biochem* 35: 2968-2977

Magnussen D, Clapham D, Gronroos R, von Amold S (1994) Induction of hairy and normal roots on *Picea abies*, *Pinus sylvestris* and *Pinus contorta* by *Agrobacterium rhizogenes*. *Scand J For Res* 9: 46-51

Mau CJD, West CA (1994) Cloning of casbene synthase cDNA: Evidence for conserved structural features among terpenoid cyclases in plants. *Proc Natl Acad Sci USA* 91: 8497-8501

McAfee BJ, White EE, Pelcher LE, Lapp MS (1993) Root induction in pine (*Pinus*) and larch (*Larix*) spp. using *Agrobacterium rhizogenes*. *Plant Cell Tissue Organ Cult* 34: 53-62

McGarvey DJ, Croteau R (1995) Terpenoid metabolism. *Plant Cell* 7: 1015-1026

McGuire WP, Rowinsky EK, Rosenheim N.B, Grumbine FC, Etinger DS, Armstrong DK, Donehower RC (1989) Taxol: a unique antineoplastic agent with significant activity in advanced ovarian epithelial neoplasms. *Ann Intern Med* 111: 273-279

Mihaljevic S, Stipkovic S, Jelaska S (1996) Increase of root induction in *Pinus nigra* explants using agrobacteria. *Plant Cell Rep* 15: 610-614

Mutton DB (1962) Wood Resins. *In* WE Hillis, ed, Wood Extractives and Their Significance to the Pulp and Paper Industries. Academic Press, New York, pp331-363

Nicolaou KC, Yang Z, Liu JJ, Ueno H, Nantermet PG, Guy RK, Claiborne CF, Renaud J, Couladouros EA, Paulvannan K (1994) Total synthesis of taxol. *Nature* 367: 630-634

Petit A, David C, Dahl GA, Ellis JG, Guyon P, Casse-Delbart F, Tempe J (1983) Further extension of the opine concept: Plasmids in *Agrobacterium rhizogenes* cooperate for opine degradation. *Mol Gen Genet* 190:204-214

Rao S, Krauss N E, Heerding J M, Swindell CS, Ringel I, Orr GA, Horwitz SB (1994) 3'-(p-Azidobenzamido)taxol photolabels the N-terminal 31 amino acids of β-tubulin. *J Biol Chem* 269: 3132-3134

Rilling HC, Breunger E, Epstein WW, Crain P (1990) Prenylated proteins: the structure of the isoprenoid group. *Science* 247: 318-320

Ringel I, Horwitz SB (1991) Studies with RP56976 (Taxotere): a semisynthetic analogue of taxol. *J Natl Can Inst* 83: 288-291

Rowinsky EK, Onetto N, Canetta RM, Arbuck SG (1992) Taxol: the prototypic taxane, an important new class of antitumor agents. *Sem Oncol* 19: 646-662

Sambrook J, Fritsch, EF, Maniatis T (1989) In Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sandonann G (1994) Carotenoid biosynthesis in microorganisms and plants. *Euro J Biochem* 223: 7-24

Sangwan RS, Bourgeois Y, Brown S, Vasseur G, Sangwan-Norreel BS (1992) Characterization of competent cells and early events of *Agrobacterium*-mediated genetic transformation in *Arabidopsis thaliana*. *Planta* 188: 439-456

Schiff PB, Fant J, Horwitz SB (1979) Promotion of microtubule assembly in vitro by taxol. *Nature* 22: 665-667

Schiff PB, Horwitz SB (1981) Taxol assembles tubulin in the absence of exogenous guanosine 5' triphosphate or microtubule-associated proteins. *Biochem* 20: 3242-3252

Scolnik PA, Bartley GE (1994) Nucleotide sequence of an *Arabidopsis* cDNA for geranylgeranyl pyrophosphate synthase. *Plant Physiol* 104: 1469-1470

Shin D-J, Podila GK, Huang Y, Karnosky DF (1994) Transgenic larch expressing genes for herbicide and insect resistance. *Can J For Res* 24: 2059-2067

Stierle A, Strobel G, Stierle D (1993) Taxol and taxane production by *Taxomyces andreanae*, an endophytic fungus of Pacific yew. *Science* 260: 214-216

Strobel G, Stierle A, Stierle D, Hess WM (1993) *Taxomyces andreanae*, a proposed new taxon for a bulbilliferous hyphomycete associated with Pacific yew (*Taxus brevifolia*). *Mycotaxon* 47: 71-80

Suffness M (1993) Taxol: from discovery to therapeutic use. *In* JA Bristol, ed, Ann Repts Med Chem. Academic Press, San Diego, CA, pp305-314

Suffness M (1995) Overview of paclitaxel research, progress on many fronts. *In* GI Georg, TT Chen, I Ojima, DM Vyas, eds, Taxane Anticancer Agents: Basic Science and Current Status. American Chemical Society, Washington, DC, pp1-17

Sun T-P, Kamiya Y (1994) The *Arabidopsis GA1* locus encodes the cyclase *ent*-kaurene synthetase A of gibberellin biosynthesis. *Plant Cell* 6: 1509-1518

Tepfer D (1989) Plant-microbe interactions. Kosuge T, Nester EW, eds. McGraw Hill, New York, pp294-342

Vidensek N, Lim P, Campbell A, Carlson C (1990) Taxol content in bark, wood, root, leaf, twig, and seedling from several *Taxus* species. *J Nat Prod* 53: 1609-1610

Vogel BS, Wildung MR, Vogel G, Croteau R (1996) Abietadiene synthase form Grand fir (*Abies grandis*): cDNA isolation, characterization, and bacterial expression of a bifunctional diterpene cyclase involved in resin acid biosynthesis. *J Biol Chem* 271: 23262-23268

von Heijine G, Steppuhn J, Herrmann RG (1989) Domain structure of mitochondrial and chloroplast targeting peptides. *Eur J Biochem* 180: 535-545

Wall ME, Wani MC (1995) Paclitaxel: form discovery to clinic. *In* GI Georg, TT Chen, I Ojima, DM Vyas, eds, Taxane Anticancer Agents: Basic Science and Current Status. American Chemical Society, Washington, DC, pp18-30

Wani MC, Taylor HL, Wall ME, Coggon P, McPhail AT (1971) Plant antitumor agent, VI. The isolation and structure of taxol, a novel antileukemic and antitumor agent from *Taxus brevifolia*. *J Am Chem Soc* 93: 2325-2326

West CA, Dudley MW, Dueber MT (1979) Regulation of terpenoid biosynthesis in higher plants. *Rec Adv Phytochem* 13: 163-198

Wildung MR, Croteau R (1996) A cDNA clone for taxadiene synthase, the diterpene cyclase that catalyzes the committed step of taxol biosynthesis. *J Biol Chem* 271: 9201-9204

Witherup KM, Look SA, Stasko MW, Ghiorzi TJ, Muschik GM, Cragg GM (1990) *Taxus* spp. needles contain amounts of taxol comparable to the bark of *Taxus brevifolia*: analysis and isolation. *J Nat Prod* 53: 1249-1255

Yamaguchi S, Saito T, Abe H, Yamane H, Murofushi N, Kamiya Y (1996) Molecular cloning and characterization of a cDNA encoding the gibberellin biosynthetic enzyme *ent*-kaurene synthase B from pumpkin (*Cucurbita maxima* L.) *Plant J* 10: 203-213

Zupan JR, Zambryski P (1995) Transfer of T-DNA from *Agrobacterium* to the plant cell. *Plant Physiol* 107:1041-1047

VITA

Baoxian Liu

Candidate for the Degree of

Master of Science

Thesis: ISOLATION OF A GENE CODING FOR TAXADIENE SYNTHASE IN TAXOL PRODUCTION

Major Field: Forest Resources

Biographical:

Personal Data: Born in Beijing, P. R. China.

Education: Graduated from Tiedao High School, Beijing, P. R. China in July 1988; received Bachelor of Science degree in Biochemistry and Animal Physiology from Beijing Agricultural University, Beijing, P. R. China in July 1992. Completed the requirements for the Master of Science degree with a major in Forest Resources at Oklahoma State University in July 1997.

Experience: Employed by Institute of Botany, Chinese Academy of Science as a research assistant, 1992 to 1995; employed as a graduate research assistant, Oklahoma State University, Department of Forestry, 1995 to present.