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A cDNA Clone for Taxadiene Synthase, the Diterpene Cyclase That Catalyzes the Committed Step of Taxol Biosynthesis*

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The committed step of taxol (paclitaxel) biosynthesis is catalyzed by taxa-4(5),11(12)-diene synthase, a diterpene cyclase responsible for transforming the ubiquitous isoprenoid intermediate geranylgeranyl diphosphate to the parent olefin with a taxane skeleton. To obtain the corresponding cDNA clone, a set of degenerate primers was constructed based on consensus sequences of related monoterpene, sesquiterpene, and diterpene cyclases. Two of these primers amplified a 83-base pair fragment that was cyclase-like in sequence and that was employed as a hybridization probe to screen a cDNA library constructed from poly(A)$^+$ RNA extracted from Pacific yew (*Taxus brevifolia*) stems. Twelve independent clones with insert size in excess of 2 kilobase pairs were isolated and partially sequenced. One of these cDNA isolates was functionally expressed in *Escherichia coli*, yielding a protein that was catalytically active in converting geranylgeranyl diphosphate to a diterpene olefin that was confirmed to be taxa-4(5),11(12)-diene by combined capillary gas chromatography-mass spectrometry. The sequence specifies an open reading frame of 2586 nucleotides, and the complete deduced polypeptide, including a long presumptive plastidial targeting peptide, contains 862 amino acid residues and has a molecular weight of 98,303, compared with about 79,000 previously determined for the mature native enzyme. Sequence comparisons with monoterpene, sesquiterpene, and diterpene cyclases of plant origin indicate a significant degree of similarity between these enzymes; the taxadiene synthase most closely resembles (46% identity, 67% similarity) abietadiene synthase, a diterpene cyclase from grand fir.

The highly functionalized diterpenoid taxol (paclitaxel)[1] (1) is well established as a potent chemotherapeutic agent (2, 3). The limited supply of this drug from the original source, the bark of the Pacific yew (*Taxus brevifolia* Nutt.; Taxaceae), prompted intensive efforts to develop alternate means of production, including isolation from the foliage and other renewable tissues of plantation-grown *Taxus* species, biosynthesis in tissue culture systems, and semisynthesis of taxol and its analogs from advanced taxane diterpenoid (taxoid) metabolites that are more readily available (4). Total synthesis of taxol, at present, is not commercially viable (5), and it is clear that in the foreseeable future the supply of taxol and its synthetically useful progenitors must rely on biological methods of production, either in *Taxus* plants or in cell cultures derived therefrom (6). The development of improved biological processes should be based upon a detailed understanding of the pathway for taxol biosynthesis, the enzymes catalyzing the reaction sequence, especially the slow steps, and the genes encoding these proteins.

The biosynthesis of taxol involves the initial cyclization of geranylgeranyl diphosphate, the universal precursor of diterpenoids (7), to taxa-4(5),11(12)-diene (8) followed by extensive oxidative modification of this olefin (8, 9) and elaboration of the side chains (Scheme I) (10). The mechanism of the cyclization, which delineates the taxane skeleton, has been examined (11), and the responsible enzyme, taxadiene synthase, has been isolated from *T. brevifolia* stem tissue, partially purified, and characterized (12). This committed step of the pathway is slow and perhaps rate-limiting in the extended biosynthetic sequence leading to taxol and related taxoids (8, 12). For this reason, the cyclization reaction has clear implications for improving taxol yield, and the taxadiene synthase is an obvious target for gene isolation. Although taxadiene synthase resembles other plant terpenoid cyclases in general properties (12), the enzyme has proved extremely difficult to purify in sufficient amounts for antibody preparation or microsequencing, thwarting this approach toward cDNA cloning. In this communication, we describe a homology-based PCR[2] cloning strategy that was successfully employed to isolate a cDNA encoding taxadiene synthase.

MATERIALS AND METHODS

Plants, Substrates, and Standards—Four-year-old *T. brevifolia* saplings in active growth were obtained from the Weyerhaeuser Research Center, Centralia, WA, and maintained in a greenhouse. [1-^{3}H]Geranylgeranyl diphosphate (120 Ci/mol) was prepared as described previously (13), and authentic (±)-taxa-4(5),11(12)-diene, prepared by total synthesis (14), was a gift from Robert M. Williams, Colorado State University, Fort Collins, CO.

Library Construction—Total RNA was extracted from *T. brevifolia* stem using the procedures of Lewinsohn and associates (15) developed for woody gymnosperm tissue. Poly(A)$^+$ RNA was purified by chromatography on oligo(dT)-cellulose (Pharmacia Biotech Inc.), and 5 μg of the resulting mRNA was utilized to construct a λZAP II cDNA library according to the manufacturer's instructions (Stratagene).

PCR-based Probe Generation and Library Screening—Comparison of six available sequences for monoterpene, sesquiterpene, and diterpene cyclases from higher plants (16–21) allowed definition of 11 homologous regions for which consensus degenerate primers were synthesized. All 20 primers (the most carboxyl-terminal primer, the most amino-termi-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) U48796.

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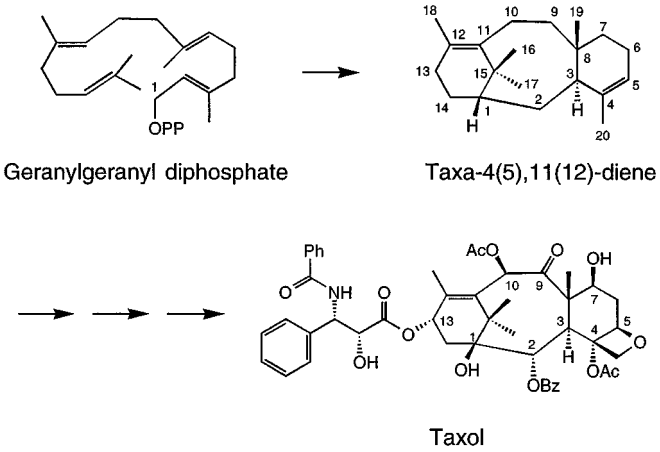
[1] Paclitaxel is the generic name for taxol, which is now a registered trademark of Bristol-Myers Squibb. Because of the far greater familiarity with the word taxol, we use it in this paper in lieu of paclitaxel.

[2] The abbreviations used are: PCR, polymerase chain reaction; GC, gas chromatography; MS, mass spectrum/spectrometry; bp, base pair(s); kb, kilobase pairs.



SCHEME I

nal primer, and nine internal primers in both directions) were deployed in all possible combinations with a broad range of amplification conditions using CsCl-purified *T. brevifolia* stem library phage DNA as template (22, 23). Analysis of PCR products by gel electrophoresis (23) indicated that only the combination of primers CC7.2F and CC3R (see Fig. 2) generated a specific fragment (~80 bp). This DNA fragment was cloned into pT7Blue (Novagen) and sequenced (DyeDeoxy Terminator Cycle Sequencing, Applied Biosystems) and shown to be 83 bp in length. PCR was used to prepare approximately 1 μg of this material for random hexamer labeling with [α-^{32}P]dATP (24) and for use as a hybridization probe to screen filter lifts of 3×10^5 plaques grown in *Escherichia coli* LE392 using standard protocols (25).

Of the plaques affording positive signals (102 total), 50 were purified through two additional cycles of hydridization. Thirty-eight pure clones were *in vivo* excised as Bluescript phagemids, insert size was determined by PCR using T3 and T7 promoter primers, and the 12 largest clones (insert, >2 kb) were partially sequenced.

cDNA Expression in E. coli—All of the partially sequenced, full-length inserts were either out of frame or bore premature stop sites immediately upstream of the presumptive methionine start codon; the latter complication likely resulted from hairpin primed second strand cDNA synthesis (26). The 2.7-kb insert from pTb42 was cloned into frame by PCR using the thermostable, high fidelity, blunting polymerase *Pfu1* (Stratagene) and primers FRM42 (downstream of false stop codons) and T7 promoter primer. The resulting blunt fragment was ligated into *Eco*RV-digested pBluescript SK(−), yielding pTb42.1, and transformed into *E. coli* XL1-Blue.

To evaluate functional expression of terpene cyclase activity, *E. coli* XL1-Blue harboring pTb42.1 was grown (to A_{600} = 0.4) in 5 ml of LB medium supplemented with 100 μg of ampicillin/ml and 12.5 μg of tetracycline/ml before induction with 200 μM isopropyl-1-thio-β-D-galactopyranoside and subsequent growth for 4 h at 25 °C. Bacteria were harvested by centrifugation ($1800 \times g$, 10 min), resuspended in taxadiene synthase assay buffer (12), and disrupted by brief sonication at 0–4 °C, and the resulting suspension was centrifuged ($18{,}000 \times g$, 10 min) to pellet debris. The supernatant was assayed for taxadiene synthase activity by an established protocol (12), in the presence of 15 μM [1-^{3}H]geranylgeranyl diphosphate and 1 mM $MgCl_2$, with incubation at 31 °C for 4 h. The reaction products were extracted with pentane and the extract purified by column chromatography on silica gel as described previously (12) to afford the olefin fraction, an aliquot of which was counted by liquid scintillation spectrometry to determine ^{3}H incorporation. Control experiments with transformed *E. coli* bearing the plasmid with an out of frame insert were also carried out.

The identity of the olefin product of the recombinant enzyme was verified by capillary radio-GC (27) as well as capillary GC-MS using methods described previously (8, 11) and authentic taxa-4(5),11(12)-diene (14). For GC-MS analysis (Hewlett-Packard 6890 GC-MSD), selected diagnostic ions were monitored: *m/z* 272 [P^+]; 257 [P^+-15(CH_3)]; 229 [P^+-43(C_3H_7)]; 121, 122, 123 (C-ring fragment cluster); and 107 (*m/z* 122 base peak − 15(CH_3)). The origin of the highly characteristic C-ring double cleavage fragment ion (base peak, *m/z* 122(C_9H_{14})) has been described (8, 11).

RESULTS AND DISCUSSION

cDNA Isolation and Characterization—In general characteristics (molecular weight, divalent metal ion requirement, kinetic constants, etc.), taxadiene synthase resembles other terpenoid cyclases from higher plants; however, the low tissue titers of the enzyme and its instability under a broad range of fractionating conditions impeded purification of the protein to homogeneity (12). A 10-μg sample of the electrophoretically purified cyclase, prepared by standard analytical procedures (28, 29), failed to provide an amino-terminal sequence via Edman degradation. Repeated attempts at trypsinization and CNBr cleavage of comparable protein samples also failed to provide sequenceable peptides, in large part because of very low recoveries.

As an alternate approach to cDNA library screening using protein-based oligonucleotide probes, a PCR-based strategy was developed that was founded on a set of degenerate primers for PCR amplification designed to recognize highly conserved regions of six higher plant terpene cyclases whose nucleotide sequences are known. Three of these cyclases, (−)-limonene synthase (a monoterpene cyclase from spearmint) (17), *epi*-aristolochene synthase (a sesquiterpene cyclase from tobacco) (16, 19), and casbene synthase (a diterpene cyclase from castor bean) (18), exploit reaction mechanisms similar to taxadiene synthase in the cyclization of the respective geranyl (C_{10}), farnesyl (C_{15}), and geranylgeranyl (C_{20}) diphosphate substrates (11). The remaining two diterpene cyclases, kaurene synthase A from *Arabidopsis thaliana* (20) and maize (21) and (−)-abietadiene synthase from grand fir (*Abies grandis*),[3] exploit a quite different mechanism that involves protonation of the terminal double bond of geranylgeranyl diphosphate to initiate cyclization to the intermediate copalyl diphosphate followed, in the case of abietadiene synthase, by the more typical ionization of the diphosphate ester function to initiate a second cyclization sequence to the product olefin (13). The latter represents the only gymnosperm terpene cyclase sequence presently available.[3]

Comparison of deduced amino acid sequences between all of the cyclases targeted 11 regions for PCR primer construction. Testing of all 20 primers in all combinations under a broad range of amplification conditions, followed by product analysis by gel electrophoresis, revealed that only one combination of primers (CC7.2 (forward) with CC3 (reverse), see Fig. 2 for locations) yielded a specific DNA fragment (83 bp) using *T. brevifolia* library phage as template. Primer CC3 delineates a region of strong homology between (−)-limonene synthase (17), *epi*-aristolochene synthase (16), and casbene synthase (18), whereas primer CC7.2 was selected based on sequence comparison of the angiosperm diterpene cyclases (18, 20, 21) to the recently acquired cDNA clone encoding a gymnosperm diterpene cyclase, (−)-abietadiene synthase, from grand fir.[3]

The 83-bp fragment was cloned and sequenced and thus demonstrated to be cyclase-like. This PCR product was ^{32}P-labeled for use as a hybridization probe and employed in high stringency screening of 3×10^5 plaques, which yielded 102 positive signals. Fifty of these clones were purified through two additional rounds of screening, *in vivo* excised, and the inserts sized. The 12 clones bearing the largest inserts (>2.0 kb) were partially sequenced, indicating that they were all representations of the same gene. Four of these inserts appeared to be full-length.

cDNA Expression in E. coli—All four of the full-length clones that were purified were out of frame or had stop sites immediately upstream of the starting methionine codon resulting from hairpin-primed second strand cDNA synthesis. The insert from pTb42 was cloned into frame by PCR methods, and the blunt

[3] B. Stofer Vogel, M. Wildung, G. Vogel, and R. Croteau, manuscript in preparation.

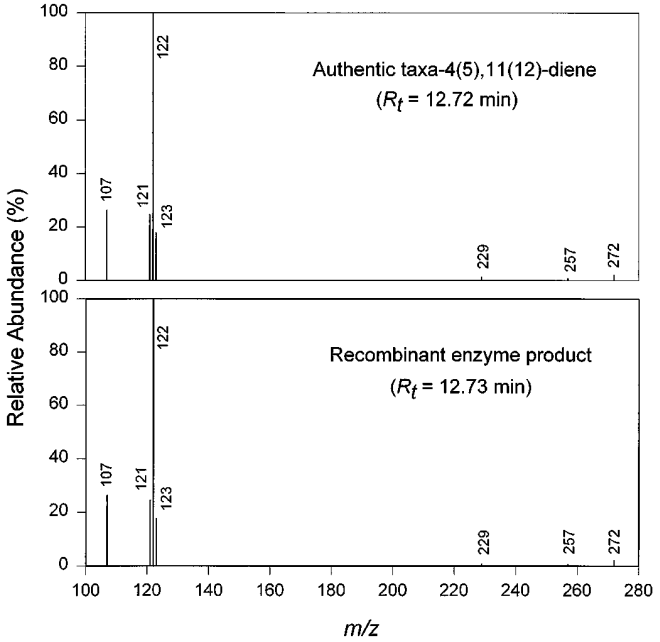


FIG. 1. **GC-MS analysis of the product of the recombinant protein.** Selected ion mass fragmentation patterns are illustrated for authentic (±)-taxa-4(5),11(12)-diene (R_t = 12.72 min) and for the diterpene (R_t = 12.73 min) obtained from geranylgeranyl diphosphate when incubated with a cell-free extract of *E. coli* harboring pTb42.1. The origin of the selected diagnostic ions, which account for most of the full spectrum abundance, is described under "Materials and Methods" and elsewhere (8, 11).

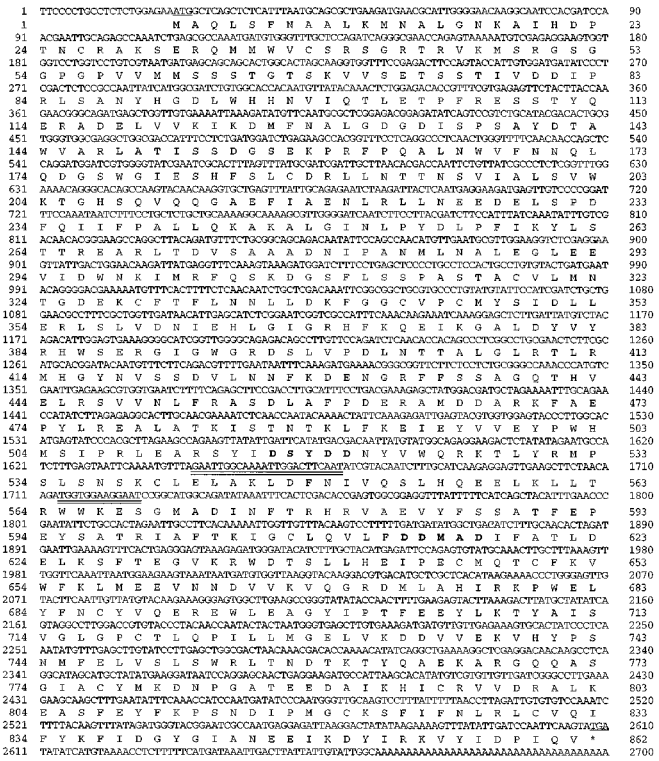


FIG. 2. **Nucleotide and predicted amino acid sequence of Pacific yew taxadiene synthase clone pTb42.1.** The start and stop codons are *underlined*, the locations of regions employed for primer synthesis are *double underlined*, and the DDMAD and DSYDD motifs are in *boldface*.

fragment was ligated into the *Eco*RV site of pBluescript SK(−), yielding pTb42.1, and transformed into *E. coli* XL1-Blue.

Transformed *E. coli* were grown in LB medium supplemented with antibiotics and induced with isopropyl-1-thio-β-D-galactopyranoside. The cells were harvested and homogenized, and the extracts were assayed for taxadiene synthase activity using standard protocols with [1-^{3}H]geranylgeranyl diphosphate as substrate (12). The olefin fraction isolated from the reaction mixture contained a radioactive product (~1 nmol) that was coincident on capillary radio-GC with authentic taxa-4(5),11(12)-diene (R_t = 19.40 ± 0.13 min). The identification of this diterpene olefin was confirmed by capillary GC-MS analysis, which showed the diterpene olefin product to possess the identical retention time (12.72 ± 0.01 min) and selected ion mass spectrum (Fig. 1) as an authentic sample of (±)-taxa-4(5),11(12)-diene (14). Since identically prepared extracts of control cultures of *E. coli*, transformed with pBluescript bearing an out-of-frame insert, were incapable of transforming geranylgeranyl diphosphate to detectable levels of diterpene olefin, these results confirm that clone pTb42.1 encodes the taxadiene synthase from Pacific yew.

Sequence Analysis—Both strands of the inserts from pTb42 and pTb42.1 were sequenced; no mistakes were incorporated by *Pfu* polymerase. The pTb42.1 taxadiene synthase cDNA is 2700 nucleotides in length and contains a complete open reading frame of 2586 nucleotides (Fig. 2). The deduced amino acid sequence indicates the presence of a putative plastidial transit peptide of approximately 137 amino acids and a mature protein of about 725 residues (~82.5 kDa), based on the size of the native (mature) enzyme (~79 kDa) estimated by gel permeation chromatography and SDS-polyacrylamide gel electrophoresis (12), the characteristic amino acid content and structural features of such amino-terminal targeting sequences and their cleavage sites (30, 31), and the fact that diterpene biosynthesis is localized exclusively within plastids (32, 33). The transit peptide/mature protein junction and, thus, the exact lengths of both moieties are unknown, because the amino terminus of the mature protein is apparently blocked and has not yet been identified.

Pairwise sequence comparison (34, 35) with other terpene cyclases from higher plants revealed a significant degree of sequence similarity at the amino acid level. The taxadiene synthase from yew showed 32% identity and 55% similarity to (−)-limonene synthase from spearmint (17), 30% identity and 54% similarity to *epi*-aristolochene synthase from tobacco (16), 31% identity and 56% similarity to casbene synthase from castor bean (18), 33% identity and 56% similarity to kaurene synthase A from *A. thaliana* and maize (20, 21), and 45% identity and 67% similarity to (−)-abietadiene synthase from grand fir.[3] Pairwise comparison of other members within this group show roughly comparable levels of identity (30–40%) and similarity (50–60%). These terpenoid synthases represent a broad range of cyclase types from diverse plant families, supporting the suggestion of a common ancestry for this class of enzymes (17–19, 36, 37).

The sequence of taxadiene synthase does not closely resemble (identity, ~20%; similarity, ~40%) those of any of the microbial sesquiterpene cyclases that have been determined recently (38–40) nor does the taxadiene synthase sequence resemble any of the published sequences for prenyltransferases (41–43), a group of enzymes that, like the terpenoid cyclases, employ allylic diphosphate substrates and exploit similar electrophilic reaction mechanisms (44). The aspartate-rich (I,L,V)*XDDXX*(*XX*)D motif(s) found in most prenyltransferases and terpenoid cyclases (16–19, 38–43, 45) and thought to play a role in substrate binding (41, 45–48) is also present in taxadiene synthase, as is a related D*XX*DD motif (Fig. 2). Histidine and cysteine residues have been implicated at the active sites of several terpenoid cyclases of plant origin (49, 50). A search of the aligned sequences revealed that three histidines (at posi-

tions 370, 415, and 793) and three cysteines (at positions 329, 650, and 777) of taxadiene synthase are conserved among the plant terpenoid cyclase genes; however, the significance of this observation is presently uncertain. The taxadiene synthase from yew most closely resembles the abietadiene synthase from grand fir rather than the casbene synthase from castor bean (18), which catalyzes a similar type of cyclization reaction (11) but is phylogenetically quite distant. The abietadiene synthase from grand fir is the only other terpenoid cyclase sequence from a gymnosperm now available,[3] and these two diterpene cyclases from the coniferales share several regions of significant sequence homology, one of which was fortuitously chosen for primer construction and proved to be instrumental in the acquisition of a PCR-derived probe that led to the cloning of taxadiene synthase.

Agrobacterium tumefaciens-mediated transformation of *Taxus* species has been described and the resulting callus cultures shown to produce taxol (51). Given the slowness of the committed cyclization step of the target pathway (8, 12), it seems likely that incorporation of the taxadiene synthase gene under the influence of a strong constitutive promoter would increase the yield of taxol and related taxoids produced by such transformed cells.

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