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Cloning And Characterization Of A Geranylgeranyl Diphosphate Synthase Gene From *Taxus Chinensis*

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Abstract

Taxol (Paclitaxel) is a diterpene from *Taxus* species and has been used in treatment of various kinds of cancers. Geranylgeranyl diphosphate synthase (GGPPS) catalyzes the formation of geranylgeranyl diphosphate (GGPP, the common precursor for diterpenes and plays a key role in taxol biosynthesis. Here we report a functional GGPPS gene from *Taxus chinensis* (designated *TcGGPPS*). *TcGGPPS* is an intron free gene and has a 1,182-bp open reading frame encoding a polypeptide of 393 amino acid residues with a calculated molecular mass of 42.63 kDa and an isoelectric point of 5.58. The catalytic activity of TcGGPPS for production of GGPP was verified by a color enhancement assay in the *Escherichia coli* cells harboring plasmid pAC-BETA. Multiple sequence alignment indicates that *TcGGPPS* is a little different in sequence from the functional GGPPS genes from other *Taxus* species such as *T. canadensis*, *T. media* and *T. wallichiana*, which are almost identical to each other. Protein structure prediction by using bioinformatics reveals that TcGGPPS consists of 52.2% α-helix, 10.9% extended strand, 8.4% β-turn and 28.5% random coil, and has a three-dimensional structure highly similar to the structurally known *Sinapis alba* GGPPS. In silicon predictions also demonstrate that TcGGPPS has a plastid-targeting peptide at the N-terminus, suggesting it is responsible for the synthesis of GGPP in plastids.

Keywords:Taxus chinensis; Geranylgeranyl Diphosphate Synthase; Gene Cloning; Taxol Biosynthesis

1 INTRODUCTION

Taxol (Paclitaxel, Fig.1) is the most important plant-derived anticancer drug. Since 1992, it has been used to treat breast, ovarian, lung, head and neck, and other metastatic cancers, alone or in combination with other anticancer agents[1].

Taxol was isolated first from *Taxus brevifolia*[2] and then from other *Taxus* species. Phytochemical investigation on *Taxus* plants has led to identification of numerous taxol homologs called taxanes or taxoids[3]. Some taxanes such as baccatin Ⅲ (Fig.1) and 10-deacetyl-baccatin Ⅲ are also pharmaceutically important because they can be used as precursors for semisynthesis of taxol or docetaxel (another taxane drug clinically used for treatment of breast cancer, head and neck cancer, gastric cancer, and non small-cell lung cancer).

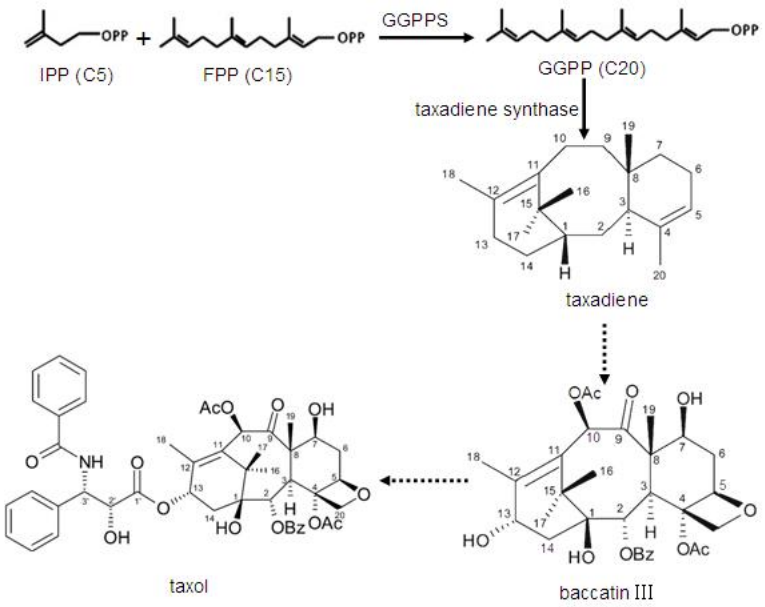

FIG. 1 BRIEF TAXOL BIOSYNTHESIS PATHWAY

IPP: isopentenyl diphosphate; FPP: farnesyl diphosphate; GGPPS geranylgeranyl diphosphate synthase; solid arrow: one-step reaction; dash arrow: multi-step reactions

Since *Taxus* species are slow-growing trees and produce very low amount of taxol, usually ~0.01% of dry weight or lower[3-5], alternative, highly efficient methods to produce taxol have been expected. *Taxus* cell culture has been regarded as the most promising strategy for the sustainable production of taxol and its precursors. However, this method is still empirical and in most cases the yields of useful taxanes in the cell cultures are low and unstable[6-8].

Elucidating the biosynthetic pathway of taxol is very useful for boosting taxane productivity by means of metabolic engineering in the future. In the past two decades, great progress has been made in this respect and some genes involved in the pathway have been cloned[9-11]. Like other diterpenes, all taxanes including taxol are derived from the C20-compound geranylgeranyl diphosphate (GGPP). Usually, GGPPS condenses three molecules of isoprenyl diphosphate (IPP, C5) and one molecule of dimethylallyl diphosphate (DMAPP, the isomer of IPP) in succession to form GGPP[12]. However, *Taxus* GGPPS mainly uses IPP and farnesyl diphosphate (FPP, a C15 compound from two molecules of IPP and one molecule of DMAPP) to produce GGPP[13,14]. In *Taxus*, GGPP can be cyclized to taxa-4(5),11(12)-diene (taxadiene, Fig.1) by taxadiene synthase and taxadiene further undergoes extensive oxidative modification and addition of side chains to form taxol and other taxanes[7,10]. A brief taxol biosynthetic pathway is illustrated in Fig.1. Because GGPP is the precursor of taxanes including taxol, GGPPS plays an important role in taxane biosynthesis. The researches of Hefner et al.[13] and Laskaris et al.[15,16] showed that the yields of taxol and other taxanes in the cells of both *T. canadensis* and *T. baccata* were significantly, positively related to the GGPPS gene expression level and GGPPS activity.

There are more than ten *Taxus* species in the world, but so far genes encoding GGPPS have been isolated only from three species, *T. canadensis*[13], *T. media*[17] and *T. wallichiana*[18]. In this paper, we report the cloning, characterization and functional verification of a GGPPS gene from *T. chinensis*, the main species cultivated for production of taxol and useful taxanes in China.

2 MATERIALS AND METHODS

2.1 Plant material

The young stem sections of *T. chinensis* were used as explants for callus induction on solid 6,7-V medium containing

30 g l^{-1} sucrose, 1 mg l^{-1} 2,4-D, 0.1 mg l^{-1} BA and 8 g l^{-1} agar. The calli were regularly subcultured on the medium every five weeks.

2.2 Extraction of Total RNA and Genomic DNA and cDNA Synthesis

The newly-formed calli two weeks after subculture were used for extraction of total RNA and genomic DNA. Total RNA was extracted using TaKaRa MiniBEST Plant RNA Extraction Kit (Takara, Dalian, China) and genomic DNA was extracted using Plant DNAzol Reagent (Invitrogen, USA) following the manufacturer's instructions. cDNAs were synthesized with 3 μl total RNA using TaKaRa PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (Takara, Dalian, China) according to the user's manual.

2.3 Cloning of T. chinensis GGPPS Gene (TcGGPPS)

All the *Taxus* GGPPS cDNAs deposited in GenBank share 98%-100% identity. Based on the coding sequences of these genes, forward primer TcGGPPS-F (5′-GAAGATCTATGGCTTACACGGCAATGG-3′, the start codon was boxed) and reverse primer TcGGPPS-R (5′-CGAGCTCTCAGTTTTGCCTGAATGC -3′; the stop codon was boxed) were designed to amplify the coding region of *T. chinensis* GGPPS gene by PCR, which was designated *TcGGPPS*. The PCR was conducted in a 20 μl volume containing 2 μ1 10×PCR HIFI buffer, 0.2 μl cDNA, 0.5 μl 10 mM dNTP mix, 0.4 μ1 10 μM TcGGPPS-F, 0.4 μ1 10 μM TcGGPPS-R, 0.2 μl Trans HIFI Taq DNA polymerase (5U μl^{-1}), and 16.3 μl ddH_2O, using the following program: 94 ℃ for 5 min, 30 cycles of amplification (94 ℃ for 30 s, 56 ℃ for 30 s and 72 ℃ for 80 s), and a final extension at 72 ℃ for 7 min. The genomic gene of *TcGGPPS* was amplified in the same way except that 1 μ1 genomic DNA was used as template. The PCR products amplified from cDNA or genomic DNA were separated by electrophoresis, recovered, subcloned into pMD18-T vector and then transformed into *Escherichia coil* strain XL-GOLD competent cells, respectively. Three positive recombinant clones of each transformation were selected for nucleotide sequencing (TsingKe Biological Technology, China).

2.4 Bioinformation Analysis

The ORF of *TcGGPPS* was identified and the amino acid sequence was deduced by ORF Finder on NCBI (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The molecular weight and isoelectric point of the deduced polypeptide of TcGGPPS were calculated using expasy tools (http://expasy.org/tools). Multiple sequence alignment of *Taxus* GGPPS genes and their deduced proteins were done using the DNAMAN program. The presence of signal peptides was predicted by using TargetP 1.1 Server (http://www.cbs.dtu.dk/ services/TargetP-1.1) [19], ChloroP 1.1 Server (http://www.cbs.dtu.dk/services/ChloroP/)[20] and iPSORT software (http://ipsort.hgc.jp/)[21]. Two-dimensional structures were predicted using the SOPMA server (https://npsa-prabi.ibcp.fr/)[22]. Homology modeling of TcGGPPS was performed by Swiss-Model server (http://swissmodel.expasy.org/interactive/)[23,24].

2.5 Functional analysis of TcGGPPS in E. coli

pAC-BETA and pTrcAtIPI plasmids, kindly donated by Dr. Francis X. Cunningham (Department of Cell Biology and Molecular Genetics, University of Maryland, College Park, Baltimore, MD, USA), were used to investigate the biological function of *TcGGPPS*. Plasmid pAC-BETA contains a chloramphenicol resistance gene and four functional genes necessary for β-carotene biosynthesis, *crtE* for GGPPS, *crtB* for phytoene synthase, *crtL* for phytoene desaturase and *crtY* for lycopene cyclase. Plasmid pTrc-AtIPI contains an ampicillin resistance gene and an IPI gene from *Arabidopsis thaliana*.

After the pMD 18-T vector containing *TcGGPPS* was digested with *Bgl* Ⅱ and *Sac* Ⅰ, *TcGGPPS* was recovered and cloned into the plasmid pTrcAtIPI digested by the same enzymes to create the plasmid pTrc-TcGGPPS. The plasmid pTrcAtIPI was also digested by *Pst* Ⅰ to remove AtIPI and then self-ligated by T4 DNA ligase to create the control vector pTrc. The plasmids pTrc-TcGGPPS and pAC-BETA were co-transformed into *E. coli* TOP10F′ competent cells. *E. coli* TOP10F′ cells were also transformed with pTrc-TcGGPPS or with both pTrc and pAC-BETA to be used as controls. Transformants containing both plasmids pTrc-TcGGPPS and pAC-BETA or both plasmids pTrc and pAC-BETA were cultured on solid LB medium containing 100 mg/l ampicillin and 50 mg/l chloramphenicol, and transformants containing pTrc-TcGGPPS were cultured on solid LB medium containing 100 mg/l ampicillin at 37 ℃.

Single colonies of each transformant and the wild type *E. coli* TOP10F′ cells were cultured in 2 ml liquid LB medium with or without antibiotics at 37 ℃ for 1 day. Then, the cultures were streaked on the same plate containing antibiotic-free solid LB medium and incubated at 37 ℃ for 1 day and then at 28 ℃ for 2 days. The color of the transformants on the plate was used as a visible marker to test the function of *TcGGPPS*.

3 RESULTS AND DISCUSSION

3.1 Cloning of T. chinensis GGPPS Gene (TcGGPPS)

PCR was performed with cDNA and genomic DNA as template, respectively. Electrophoresis analysis showed that in the both cases, only a specific band about 1,200 bp was amplified (Fig. 2). The sequencing results indicated that both the PCR products from cDNA and genomic DNA were the same. These data demonstrate that the *T. chinensis* GGPPS gene, *TcGGPPS*, has no introns. Our this founding is consistent with the study of Liao et al.[17] , who cloned a GGPPS gene of *T. media* by genome walking and found that it was intron-free.

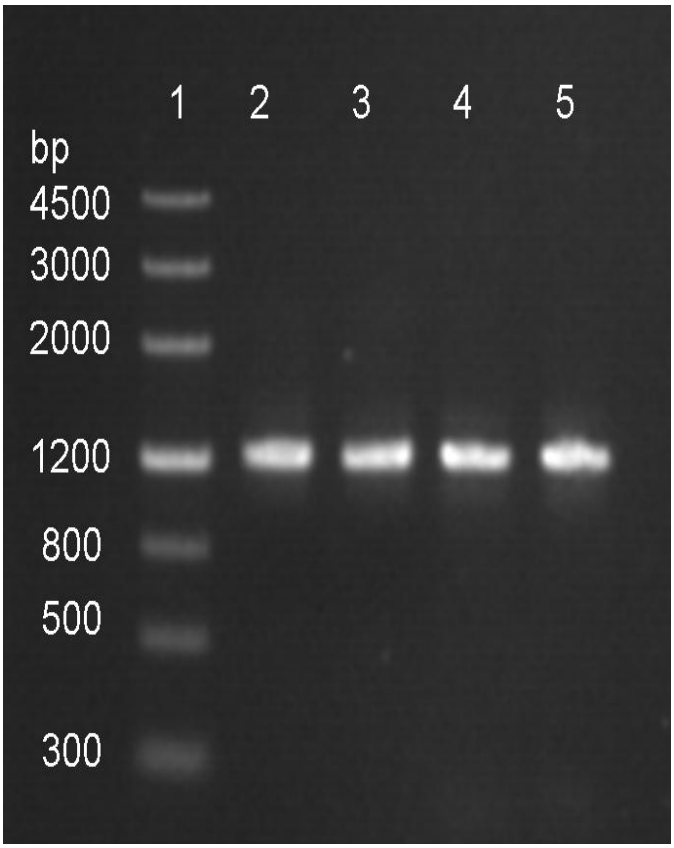

FIG. 2 AMPLIFICATION OF *TcGGPPS* BY PCR

Lane 1: DNA markers. Lanes 2 and 3: PCR product from cDNA. Lanes 4 and 5: PCR product from genomic DNA

3.2 Bioinformatic analysis of TcGGPPS

All the *Taxus* GGPPS genes registered in GenBank have an open reading frame (ORF) of 1,182-bp encoding a polypeptide of 393 amino acids. The ORF analysis showed that *TcGGPPS* had a 1,182-bp ORF, too. The deduced TcGGPPS had a calculated molecular mass of 42.63 kDa and an isoelectric point of 5.58. Multiple sequence alignment analysis indicated that *TcGGPPS* shared very high identity with other *Taxus* GGPPS genes; its nucleic acid sequence and deduced protein sequence had only 11 or 12 bases and 4 amino acid residues that were different from the corresponding sequences of GGPPS genes of *T. canadensis* (GenBank accession no. AF081514), *T. wallichiana* (GenBank accession no. DQ364604) and *T. media* (GenBank accession no. AY453404), respectively (Fig. 3 and 4). The two conserved aspartate-rich motifs identified in GGPPSs and other prenyltransferases, DD(X2–4)D and

FIG. 3 NUCLEOTID SEQUENCE ALIGNMENT OF *TcGGPPS* WITH OTHER TAXUS GGPPS GENES

T. chinensis (KU987429); *T. canadensis* (AF081514); *T. wallichiana* (DQ364604); *T. media* 1 (AY453404); *T. media* 2 (JQ029687).

DDXXD (where ‘‘X’’ is any amino acid) that are crucial in substrate binding and catalysis[25,26], are also present in TcGGPPS. These two motifs are usually called the first aspartate-rich motif (FARM) and the second aspartate-rich motif (SARM), respectively. GGPPSs are categorized into three different types (Ⅰ, Ⅱ and Ⅲ); the FARM of Type Ⅱ GGPPS is DXXXXD, instead of DDXXD in the FARM of the other two types[27]. Fig. 4 shows that all the known *Taxus* GGPPSs have the same FARM (DDLPCMD) and the same SARM (DDILD). Obviously, these *Taxus* GGPPSs belong to Type Ⅱ GGPPS.

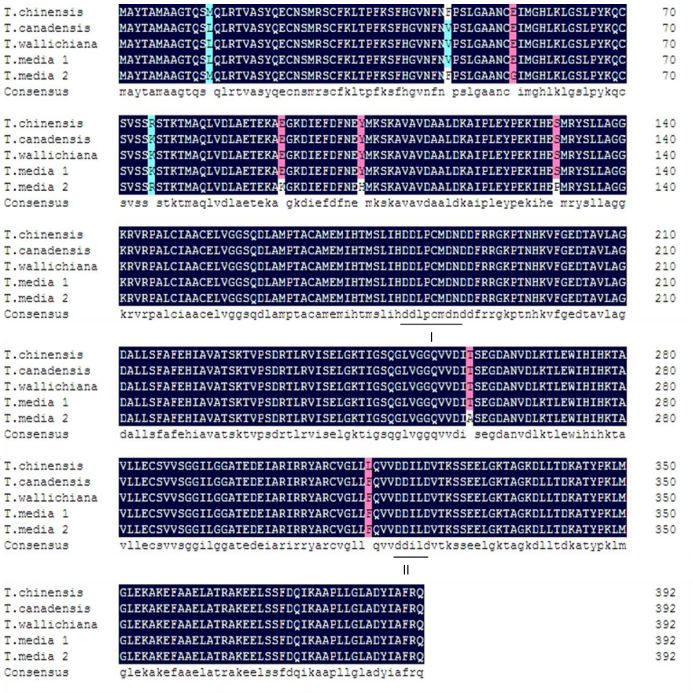

FIG. 4 ALIGNMENT OF THE DEDUCED AMINO ACID SEQUENCES OF TcGGPPS WITH OTHER TAXUS GGPPSs

T. chinensis (AMX21442); *T. canadensis* (AAD16018); *T. wallichiana* (ABC88389); *T. media* 1 (AAS49033); *T. media* 2 (AFD32422). The two conserved aspartate-rich motifs were underlined; I: the FARM (DDXXXXD), and II: the SARM (DDXXD)

Both the TargetP 1.1 server and the iPSORT server are frequently used to predict the subcellular location of eukaryotic proteins. The prediction results from the TargetP 1.1 server showed that TcGGPPS had a chloroplast transit peptide (cTP) and a mitochondrial targeting peptide (mTP), but their prediction reliability classes were low, 0.174 and 0.276, respectively. Signal peptide prediction performed using the iPSORT server conclusively indicated that TcGGPPS had a cTP at the N-terminus, but no mTPs. The ChloroP server is specifically used for prediction of cTPs in protein sequences [20]. When prediction was performed using this server, the results showed that TcGGPPS had a cTP and the prediction reliability score was relatively high (0.541). Based on these predictions, it can be concluded that TcGGPPS has a plastidial signal peptide and is responsible for the synthesis of GGPP in plastids.

Two-dimensional structures prediction performed using the SOPMA server showed that TcGGPPS protein consisted of 52.2% α-helix, 10.9% extended strand, 8.4% β-turn and 28.5% random coil. In general, short-chain prenyltransferases including GGPPS assemble into homodimers with symmetry axes at equivalent positions [28-30]. Laskaris et al. found that *T. baccata* GGPPS existed in homodimers in cells [14]. When homology modeling of TcGGPPS was performed using Swiss-Model server, the results showed that, of the structurally known GGPPs that function as homodimers, *Sinapis alba* (mustard) GGPP synthase was its closest relative (71.92% sequence identity) (Fig. 5), suggesting that these two GGPPS may have a similar catalytic mechanism. The crystal structure of *S. alba* GGPPS was elucidated by multiwavelength anomalous diffraction. The polypeptide of *S. alba* GGPPS is organized in eleven α-helices and contains a large active center pocket that is elongated toward the dimmer interface in order to accommodate the C20 product GGPP, and the substrate binding pockets of the two subunits reach the interface but do not meet there, so that in principle both active centers may work independently [31].

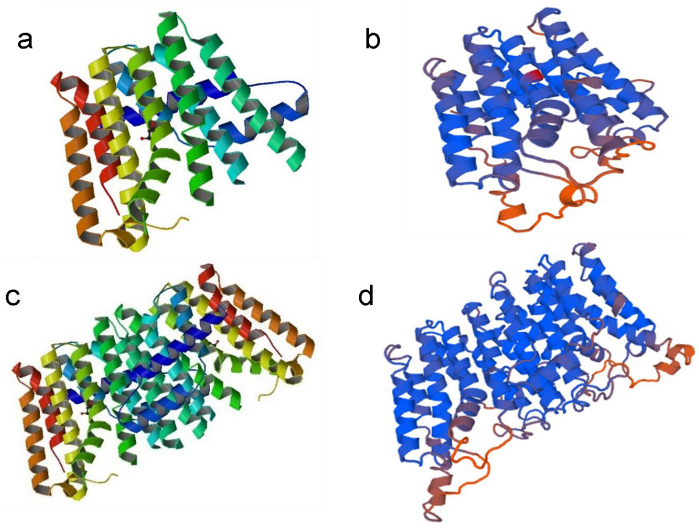

FIG. 5 HOMOLOGY MODELING OF THE THREE-DIMENSIONAL STRUCTURE OF TcGGPPS WITH SINAPIS ALBA GGPPS (PDB CODE 2J1P) AS TEMPLATE

(a) A subunit of *S. alba* GGPPS. (b) A subunit of TcGGPPS. (c) A homodimer of *S. alba* GGPPS. (d) A homodimer of TcGGPPS.

3.3 Biological function analysis of TcGGPPS in E. coli

Hefner et al.[13] and Liao et al.[17] verified the function of *T. canadensis* and *T. media* GGPPS genes through functional complementation of yeast mutants defective for the β-subunit of type Ⅱ geranylgeranyl transferase or GGPP synthase activity, respectively. In this study, we demonstrated that the *E. coli* heterologous system for carotenoid biosynthesis

could be used for identifying *Taxus* GGPPS genes, too.

E. coli has the methylerythritol phosphate (MEP) pathway and can produces IPP and DMAPP, two important building blocks for biosynthesis of isoprenoids including carotenoids, but can not synthesize carotenoids because of lack of other genes necessary for carotenoid biosynthesis. When *E. coli* cells are transformed with plasmid pAC-BETA containing *crtE*, *crtB*, *crtI*, and *crtY* genes, they are able to produce β-carotene and then form yellow colonies[32-34]. We constructed a bacterial expression vector of *TcGGPPS*, pTrc-TcGGPPS, in which *TcGGPPS* was under control of pTrc promoter (a strong bacterial promoter) and used it to transform *E. coli* TOP10F′ competent cells together with pAC-BETA to test the function of *TcGGPPS*. If *TcGGPPS* encodes functional GGPPS, β-carotene synthesis would be enhanced in the cells containing both pAC-BETA and pTrc-TcGGPPS because more GGPP molecules can be produced and these cells should be yellower than those bearing only pAC-BETA or both pAC-BETA and pTrc. As expected, the *E. coli* cells co-transformed with pAC-BETA and pTrc-TcGGPPS were much yellower than the control cells containing both pAC-BETA and pTrc (Fig. 6a, b), demonstrating that *TcGGPPS* expression did accelerate β-carotene production. Like the wild type cells, the cells transformed with pTrc-TcGGPPS formed white colonies due to that they were not able to synthesize carotenoids (Fig. 6c, d).

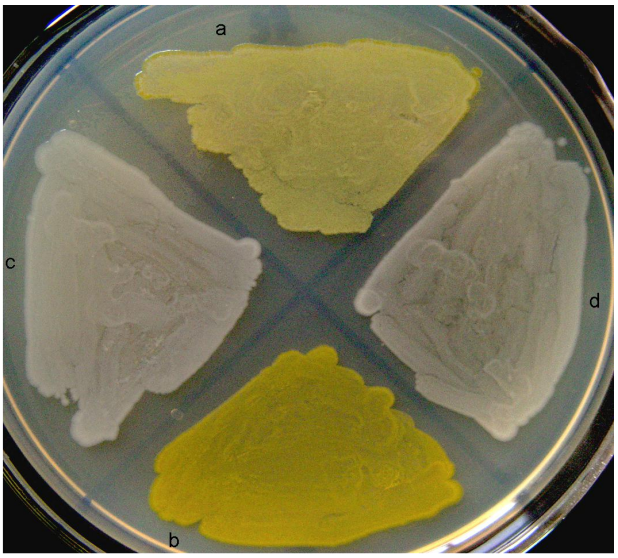

FIG. 6 FUNCTIONAL DEMONSTRATION OF TCGGPPS IN *E.COLI* CELLS

(a) The cells co-transformed with pAC-BETA and pTrc. (b) The cells co-transformed with pAC-BETA and pTrc-TcGGPPS. (c) The wild type cells. (d) The cells transformed with pTrc.

Hefner et al. identified the first functional *Taxus* GGPPS gene (AF081514) by screening cDNA library of *T. canadensis*[13]. Later, Liao et al. cloned a GGPPS gene (AY453404) from *T. media* by genome walking and RACE strategies[17]. These two GGPPS genes have an ORF of 1,182 bp, and their coding sequences are almost the same except that nucleotide C at position 1,041 in *T. canadensis* is replaced by A in *T. media*, but their deduced amino acid sequences were identical (Fig. 3). Lan and Sun amplified a GGPPS cDNA from *T. wallichiana* (DQ364604) by PCR and found it was completely identical to that of *T. canadensis*[18]. Our results show that *TcGGPPS* is a little different in sequence from these *Taxus* GGPPS genes (Fig. 2, 3), but it encodes functional GGPPS. In fact, there is another *T. media* GGPPS gene (JQ029687) in GenBank. It has an ORF of 1,182 bp, too, but its nucleic sequence and deduced protein sequence have 23 nucleotides and 8 amino acid residues different from those of the *T. media* GGPPS gene (AY453404) isolated by Liao et al. [17], respectively (Fig. 2, 3). Though its function verification has not been reported, it should be another *Taxus* GGPPS gene because *T. media* is a hybrid between *T. baccata* and *T. cuspidate*, therefore it should have at least two copies of GGPPS genes. This putative *T. media* GGPPS gene is a little different from *TcGGPPS*, too; there are 19 different nucleotides between their coding regions (Fig. 2) and 6 different amino acid residues between their deduced proteins (Fig. 3).

Since all the known *Taxus* GGPPS genes share very high identity (98%-100%), they should have the same function.

The positive relationship between the expression level of *T. canadensis* GGPPS gene (AF081514) and the taxol content in the suspension cells[13] demonstrates that the known *Taxus* GGPPS genes are involved in the synthesis of taxanes. In this study, we used both the iPSORT server and the TargetP server to predict signal peptides in TcGGPPS and other *Taxus* GGPPSs and found that all of them had a plastid-targeting peptide. These predictions suggest that these *Taxus* GGPPSs catalyze the formation of GGPP in plastids, which is consistent with the results reported by Wildung and Croteau that the cyclization of GGPP to taxadiene (the backbone of taxanes) catalyzed by taxadiene synthase was in plastids[35].

In plants, GGPP synthases function in mitochondria, endoplasmic reticulum (ER) or plastids and play central roles in the synthesis of primary and secondary isoprenoid compounds such as the hormones abscisic acid and gibberellins, chlorophylls, diterpenoids, carotenoids, plastoquinones, ubiquinones, phylloquinones, tocopherols, polyprenols, dolichols and prenylated proteins[36,37]. Usually, higher plants have more than one GGPPS gene[38]. In the case of Arabidopsis, up to ten functional GGPPS genes have been identified, which vary in sizes and sequences; of the ten GGPPSs, one localizes to mitochondria, two to ER and seven to plastids[36]. However, as described above, all the known *Taxus* GGPPS genes seem the same. Whether *Taxus* species have other different GGPPS genes is an interesting issue to be further investigated.

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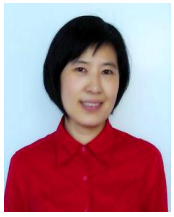

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