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Full Length Research Paper

A new 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase gene from *Taxus media*: Cloning, characterization and functional complementation

Wanhong Liu[1,2], Min Chen[3], Chunxian Yang[1], Yijiang Yang[1], Xiaozhong Lan[4] and Zhihua Liao[1]*

[1]Laboratory of Natural Products and Metabolic Engineering, Key Laboratory of Eco-environments in Three Gorges Reservoir Region (Ministry of Education), School of Life Sciences, Southwest University, Chongqing 400715, People's Republic of China.
[2]Department of Biology, Chongqing University of Science and Technology, Chongqing 401331, People's Republic of China.
[3]College of Pharmaceutical Science, Southwest University, Chongqing 400715, People's Republic of China.
[4]Agricultural and Animal Husbandry College, Tibet University, Linzhi of Tibet 860000, People's Republic of China.

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2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (MECPS, EC: 4.6.1.12) is the fifth enzyme of the methylerythritol phosphate (MEP) pathway of Taxol biosynthesis. The full-length cDNA sequence (designated as TmMECPS) was cloned and characterized from *Taxus media*. The full-length cDNA of *TmMECPS* was 899 bp containing a 723 bp open reading frame (ORF) encoding a polypeptide of 241 amino acids with a calculated molecular mass of 26.1 kDa and an isoelectric point of 8.22. Comparative analysis indicated that *TmMECPS* was similar with other plant MECPSs with the conserved Zn^{2+} ligands and CDP-binding residues, the subcellular prediction showed that *TmMECPS* owned a 60 amimo-acid plastidial transit peptide at it N-terminus. In the phylogenetic analysis, plant MECPSs were divided into 2 groups including angiosperm MECPSs and gymnosperm MECPSs. Tissue expression pattern analysis indicated that *TmMECPS* expressed in all tested tissues including tender barks, leaves, roots and stems but at different levels, *TmMECPS* had higher expression levels in tender barks and leaves than that in roots and stems. The genetic complementation assay demonstrated that *TmMECPS* did encode the enzyme that had the MECPS activity like Arabidopsis MECPS. The cloning and characterization of TmMECP*S* will be helpful to understand more about the role of MECPS involved in the taxol precursor biosynthesis at the molecular level.

Key words: MECPS gene, cloning, color complementation assay, *Taxus media*, expression.

INTRODUCTION

The diterpenoid taxol (generic name *Paclitaxel*) from the yew (Taxus) species has been used effectively in the treatment of ovarian, breast and lung cancers, as well as of Kaposi's sarcoma (Alexander, 2001). This drug, with its unique functioning mechanism, has been approved by FDA as one of the most effective anticancer agents (Suffness, 1994). Due to the scarcity of Taxus trees and its low content, the amount of taxol that can be isolated from the original plant remains very limited, which makes it necessary to find others ways to increase its production. The total synthesis strategy, once conceived to settle the problem, has failed because of its innate weakness such as low efficiency, unaffordable expenditure and insufficient yielding volume. It is clear that, in the foreseeable future, the supply of taxol must continue to rely on biological methods (Hefner et al., 1998). Renovation of the biosynthetic methods in intact yew or derived cell cultures should be based upon a full understanding of the

*Corresponding author. E-mail: zhliao@swu.edu.cn. or zhihualiao@163.com. Tel.: 86-23-68367146. Fax: 86-23-68252365.

taxol formation pathway, such as the enzymes that catalyze this extended sequence of reactions and their function mechanisms and the structural genes encoding these enzymes (Jennewein et al., 2004).

Taxol, as a highly functional diterpene, is confirmed to be mainly synthesized via the newly discovered plastidial MEP pathway (Palazón et al., 2003). The protein 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (MECPS EC:4.6.1.12) is the fifth enzyme in the MEP pathway that transforms 2-phospho-4-(cytidine5'-diphospho)-2-C-methyl-D-erythritol into 2-C-methyl-D-erythritol 2,4-cyclodiphosphate. In this study, we report the molecular cloning and characterization of the MECPS gene from gymnosperm species *Taxus media* and its functional identification in *Escherichia coil*, which will help us to map and regulate an enzymatic step involved in the taxol biosynthetic pathway at the molecular level in the future.

MATERIALS AND METHODS

Materials

The plant materials of *T. media* were collected from the medical plant garden in Southwest University (Chongqing, China). RNA isolation reagent and reverse transcriptase were purchased from Tiangen (Beijing, China) and TaKaRa (Dalian, China) respectively. RACE Kit was purchased from Clontech (CA, USA). The pGEM® T-easy vector was purchased from Promega (WI, USA). The Taq polymerase was purchased from TaKaRa (Dalian, China). Other chemicals were purchased from Sigma (USA). Primers used in the present study were synthesized by invitorgen (Shanghai, China).

Isolation of the total RNA

All testing tissues including leaves, tender barks, stems, roots were excised from *T. media* plants and immersed separately in liquid nitrogen for RNA extraction. Total RNAs were isolated from the plant materials using RNAPlant isolation system (Tiangen, China). After isolation, total RNAs were stored in -70℃ for future using.

Isolation of a core fragment of TmMECPS

Single-stranded cDNAs were synthesized from 5 µg of total RNAs with an oligo(dT)17 primer that were reversely transcribed according to the manufacturer's protocol (PowerScript™, Clontech, USA). After RNaseH treatment, the single-stranded cDNA mixture was used as templates for polymerase chain reaction (PCR) amplification of the conserved region of MECPS from *T. media*. 2 degenerate oligonucleotide primers (dfmecps: 5'-GAGGATGCGA (G/A/C)GCTCACTCTG-3' and drmecps: 5'-C(G/C)CCAAGACTGTC (A/G)ACCTTCTC-3') were used for standard gradient PCR amplification (from 55 to 65℃). The gradient PCR was carried out by denaturing the cDNA at 94℃ for 3 min followed by 30 cycles of amplification (94℃ for 45 s, 55 - 65℃ for 45 s and 72℃ for 1 min) and by extension at 72℃ for 6 min. The core fragment of TmMECPS was amplified and subcloned into pGEM® T-easy vector followed by sequencing, which was confirmed to be similar to other plant MECPS genes by blast-n search. The core fragment was subsequently used to design the gene-specific primers for the cloning of full-length cDNA of TmMECPS by the technology of rapid amplification of cDNA ends (RACE).

Cloning of the full-length cDNA of TmMECPS

SMART™ RACE cDNA amplification kit (Clontech, USA) was used to isolate TmMECPS cDNA 3'-end and 5'-end. Firstly, the first-Stranded 3'-RACE-ready and 5'-RACE-ready cDNA samples from *T. media* were prepared according to the manufacturer's protocol (SMART™ RACE cDNA Amplification Kit, User Manual, Clontech). The 3'-RACE-ready cDNA and 5'-RACE-ready cDNA were used as templates for 3'-RACE and 5'-RACE respectively. TmMECPS cDNA's 3'-end was amplified using 3'-gene-specific primers and the universal primers provided by the kit. For the first PCR amplification of 3'-RACE, TmMECPS3-1 (5'-AGGAAACGATTCGAAGCAATTTA-3') and UPM (Universal Primer Mix, provided by Clontech) were used as the first PCR primers (3'-RACE), and 3'-RACE-ready cDNAs were used as templates. For the nested PCR amplification of 3'-RACE, TmMECPS3-2 (5'-GACTCATGAGAAGGTTGACA-3') and NUP (Nested universal primer, provided by Clontech) were used as the nested PCR primers (3'-RACE), and the products of the first PCR amplification were used as templates. TmMECPS cDNA 5'-end was amplified using 5'-gene-specific primers and the universal primers provided by the kit. For the first PCR amplification of 5'-RACE, TmMECPS5-1 (5'-GCTTCGAATCGTTTCCTTATGA GGG-3') and UPM were used as the first PCR primers (5'-RACE) and 5'-RACE-ready cDNAs were used as templates. For the nested PCR amplification of 5'-RACE, TmMECPS5-2 (5'-CTTACCGCCT CTGTCAAGAAGAC-3') and NUP were used as the nested PCR primers (5'-RACE) and the products of the first PCR amplification were used as templates. For the first and nested PCR amplification of TmMECPS cDNA 3' and 5'-end, Advantage™ 2 PCR Kit (Clontech, USA) was used. The first and nested PCR procedures were carried out at the same conditions described in the protocol (SMART™ RACE cDNA amplification kit, user manual, Clontech), 25 cycles (30 s at 94℃, 30 s at 68℃, 3 min at 72℃). By 3'-RACE and 5'-RACE, both ends of TmMECPS were respectively obtained. The products were subcloned into pGEM® T-easy vector followed by sequencing. By assembling the sequences of 3'-RACE, 5'-RACE and the core fragment on ContigExpress (Vector NTI Suite 6.0), the full-length cDNA sequence of TmMECPS was obtained. The open reading frame (ORF) of *TmMECPS* was predicted by ORF finder on NCBI (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The full-length cDNA of TmMECPS was isolated by PCR amplification with a pair of primers: ftmMECPS (5'- ATTTTTCAACTTTCCCTAAATATTC-3') and rtmMECPS (5'- AGATGGAAATTTCACTGTAATGTG-3'). The PCR procedure was conducted under the following conditions: 3 min at 94℃, 29 cycles (50 s at 94℃, 50 s at 53℃, 2min at 72℃) and 10min at 72℃. The amplified PCR product was purified and cloned into pGEM® T-easy vector and then sequenced.

Bioinformatic analysis

The subcellular location was predicted by TargetP (Emanuelsson et al., 2000). CLUXTALX was used for multiple alignment analysis of the full-length MECPS amino acid sequences (Thompson *et al*., 1997). The sequence's homology-based structural modeling was performed by Swiss-model (Arnold et al., 2006) and WebLab ViewerLite was used for 3-D structure displaying. The phylogenetic tree of MECPS proteins was constructed using MEGA version 3.0 (Kumar et al., 2001) by the neighbor-joining method (Saitou and Nei, 1987).

The tissue expression pattern of TmMECPS

The tissue expression pattern of TmMECPS was investigated with semi-quantitative RT-PCR. Aliquots of 0.5 µg total RNA extracted from each sample were used as templates in the one-step RT-PCR reaction with the forward primer TEF1 (5`-ATGTCTGCTGGAGCATTTTCTGC-3`) and the reverse primer

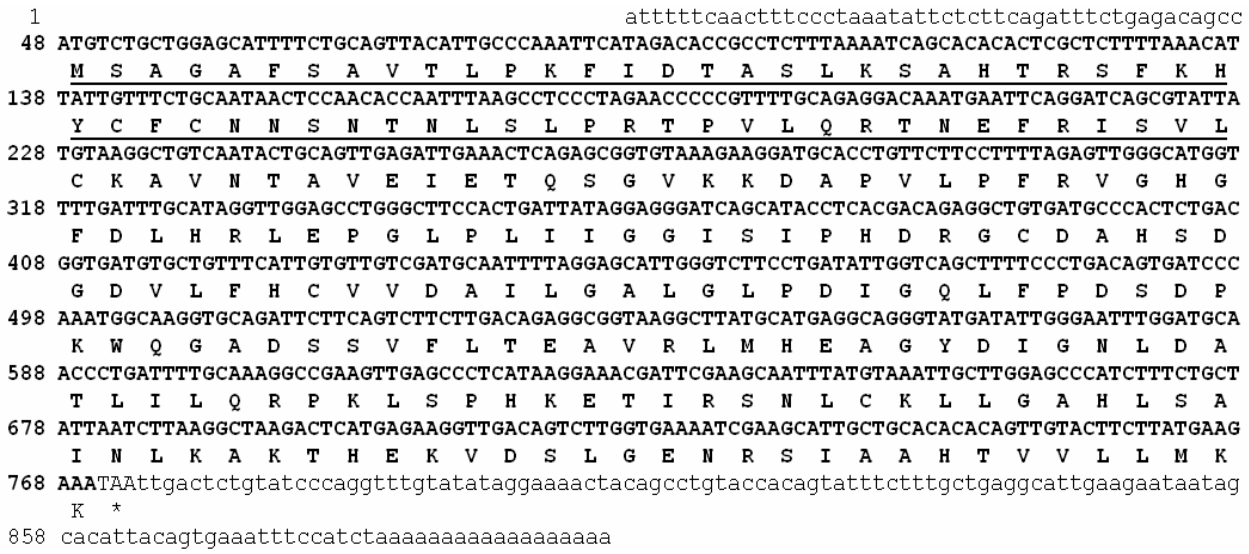

Figure 1. The full-length cDNA sequence and the deduced amino acid sequence of TmMECPS. The coding sequence was typed in capital letters, the stop codon (TAA) was marked with an aster; the 5`- and 3`-untranslated regions were shown in normal letters; the plastidial transit peptide predicted by TargetP 2.0 was underlined.

TER1 (5`- TTATTTCTTCATAAGAAGTACAAC -3`) specific to the coding sequence of TmMECPS using one-step RT-PCR kit (TaKaRa, Japan). Amplifications were performed in a volume of 25 µl under the following conditions: 50℃ for 30 min, 94℃ for 2 min followed by 25 cycles of amplification (94℃ for 50 s, 55℃ for 45 s, 72℃ for 1 min). Meanwhile, the RT-PCR reaction for the house-keeping gene (18S rRNA gene), using specific primers 18sf (5`-GTGACAATGGAACTGGAATGG-3`) and 18sr (5`-AGACGGAGGAT AGCGTGAGG-3`), was used to estimate whether the equal amounts of total RNA among samples were used in RT-PCR reaction.

Color complementation assay of TmMECPS

The color complementation assay was successfully used to identify the function of MECPS by using 2 plasmids including pAC-BETA and pTrcAtipi (Gao et al., 2006). The β-carotene biosynthetic pAC-BETA (Cunningham et al., 1996) was introduced into *E. coli* XL1–Blue to reconstruct the biosynthetic pathway of β-carotene. The transformants harboring pAC-BETA were screened out with chloromycetin (50 µg/ml). pTrcAtipi harboring the Arabidopsis IDI gene (Cunningham et al., 2000) was digested with Pst I to get rid of Arabidopsis IDI gene and then recycled with T4 ligase; the modified plasmid was designated as pTrc that had the resistance of ampicillin (150 µg/ml). The coding region of TmMECPS was amplified with a pair of primers, F-funMECPS (5`-CCAGATCT ATGTCTGCTGTAGCA-3`) and R-funMECPS (5`-CCGCGGCCGC TTATTTCTTCATAAG-3`) and then introduced into pTrc through "*Bgl*" and "*Not"* restriction enzymes to substitute Arabidopsis IDI gene, namely pTrcTmMECPS. Meanwhile, Arabidopsis MECPS gene (AtMECPS) (Hsieh and Goodman, 2006) was used as positive control in the color complementation assay. The coding region of AtMECPS was amplified by PCR using primer F-AtMECPS (5`-CCAGATCTATGGCTACTTCTTCTACTC-3`) and R-AtMECPS (5`-CCGCGGCCGCCTATTTCTTCATGAGGA-3`). AtMECPS was also introduced into pTrc through "*Bgl"* and "*Not"* restriction enzymes to obtain the recombinant pTrcAtMECPS (positive control). pTrc, pTrcTmMECPS and pTrcAtMECPS were respectively introduced into *E. coli* XL1-Blue harboring pAC-BETA and the transformants were selected by ampicillin (150 µg/ml) and chloromycetin (50 µg/ml); pTrcTmMECPS was introduced into XL1-Blue and the transformants were selected by chloromycetin (50 µg/ml). Finally, the 6 types of XL1-Blue were streaked into the LB medium with chloromycetin (50 µg/ml), ampicillin (150 µg/ml) and IPTG (100 µM) to observe the growth and β-carotene production of the bacteria after 48 h at 28℃.

RESULTS

Molecular Cloning of the Full-Length cDNA of *TmMECPS*

A 339 bp core fragment of TmMECPS was amplified by using *T. media* cDNA as template with 2 degenerate primers, dfmecps and drmecps, at 55 - 65℃ by standard gradient PCR. Confirmed by sequencing, the 339 bp fragment showed similarity to other plant MECPS genes through blast-n search. Based on the 339 bp core sequence, the 544 bp 5'-end and 222 bp 3'-end cDNA fragments were amplified by RACE respectively. Then the deduced full-length TmMECPS cDNA was obtained and subsequently confirmed by sequencing. The full-length cDNA of TmMECPS (GenBank accession No. EF534009) was 899 bp with 5', 3' untranslated regions and a polyA tail, which contained a 723 bp open reading frame encoding 241-amino-acid protein (Figure 1). The predicted TmMECPS protein had a calculated molecular mass of 26.1 kDa and a theoretical pI of 8.22 that were similar with the reported Ginkgo MECPS (Gao et al., 2006).

Bioinformatic analysis of TmMECPS

Sequence comparison revealed that TmMECPS was

similar with MECPSs from other plant species at the amino acid level, such as *Ginkgo biloba* MECPS (75% identity, 84% positive), *Hevea brasiliensis* MECPS (73% identity, 80% positive) and *Arabidopsis thaliana* MECPS (84% identity, 90% positive). According to the TargetP analysis, the TmMECPS protein had a 60 amino-acid transit peptide in the N-terminal region (Figure 1).

The multiple alignment results indicated that the plastidial transit peptides were similar in length but were highly varied at the level of amino acid sequence, while the following catalytic regions were conserved (Figure 2). The search of the two CDP-binding subunits (Asp140Gly142 and Leu184Leu189Ser190Lys216Thr217 Glu219) was conserved amino acid in most others MECPS. The active site residues of EcMECPS (Steinbacher et al., 2002), such as Asp8, His10, and His42 (Zn^{2+} ligands), and Asp56Gly58 (one of CDP-binding subunit) were conserved in TmMECPS.

The phylogenetic result demonstrated that plant MECPSs were divided into 2 groups including angiosperm group and gymnosperm group. TmMECPS belonged to gymnosperm groups (Figure 3).

The homology-based 3-D structural modeling of TmMECPS was given by Swiss-Modeling on the base of the *E. coil* MECPS crystal structure (Steinbacher et al., 2002). The results showed that the overall structure of TmMECPS contained 7 β-sheets and 3 α-helices and had high similarity with the known EcMECPS structures, except for one extra β-sheet and a very small α-helix (Figure 4). Additionally, the active sites including CDP-binding sites (Asp140Gly142 and Leu184Leu189Ser190 Lys216Thr217Glu219) and Zn^{2+} ligand (Asp92-His94-His 126) could be found in the modeled TmMECPS, whose spatial position were similar with those in EcMECPS.

TmMECPS expression pattern in different tissues

Semi-quantitative RT-PCR analysis was employed to reveal the expression profile of TmMECPS in different tissues including tender barks, leaves, roots and stems. The results showed that TmMECPS expressed in all the 4 types of tissues but at different levels, and the constitutive 18S rRNA gene had the almost same expression level in all the tissues. The lowest-level expression of TmMECPS was found in stems and the roots had the middle-level expression of TmMECP, the highest-level expression of TmMECPS was found in leaves and tender barks (Figure 5). Similarly, GbMECPS had higher expression level in leaves (Gao et al., 2006).

Functional identification of TmMECPS

Normal *E. coli* cell can produce the essential precursors IPP and DMAPP for carotenoids, but can not produce β-carotene itself because of the absence of carotenoid pathway (Rohmer et al., 1993). The plasmid (pAC-BETA) could reconstruct β-carotene pathway in *E. coli*, which retained functional genes for geranylgeranyl pyrophosphate synthase (crtE), phytoene synthase (crtB), phytoene desaturase (crtL), lycopene cyclase (ctrY) and a chloramphenicol resistance gene (Cunningham et al., 1994). However, engineered E. coli harboring pAc-BETA could only produce β-carotene in very low amount, this was due to the existence of some committed steps involved in the MEP biosynthetic pathway (Cunningham and Gantt, 2000). pAC-BETA used pAC-BETA successfully to identify the function of GbMECPS. In the present research, the system was modified based on Gao's work to ascertain the catalytic function of the TmMECPS. In the selected medium, *E. coli* harboring both of the plasmids, pAC-BETA and pTrc or pTrcTmMECPS or pTrcAtMECPS could grow well, while *E. coli* without exogenous plasmid or harboring either of the plasmids could not grow. In the selected medium, the bacterial clones of *E. coli* harboring pAC-BETA and pTrc were the raw color, while *E. coli* harboring pAC-BETA and pTrcTmMECPS or pTrcAtMECPS were orange that was given by β-carotene (Figure 6).

DISCUSSION

The MECPS genes had been cloned and characterized from several species such as bacterium (Steinbacher et al., 2002), angiosperm (Veau et al., 2000) and gymnosperm (Gao et al., 2006), However there no reports on the cloning of MECPS gene from *T. media* which have a important commerce value. In this article we report for the first time the cloning and characterization the gene encoding the MECPS from *T. media* (TmMECPS) using the RACE technique. The deduced amino acid sequence of TmMECPS showed significant similarity to MECPS from other species. TargetP analysis showed that the TmMECPS localization to the plastid. This was consistent with the subcellular localization of the MEP pathway for IPP biosynthesis in plants (Bick and Lange, 2003) and taxol synthesis in plastid (Eisenreich et al., 1996). The phylogenetic result was consistent with the previous research that the other MEP pathway genes such as TcDXR (Zheng et al., 2004) and TmGGPPS were separated from angiosperm group (Liao *et al*., 2005). Homology-based 3-D structural modeling of TmMECPS showed that TmMECPS had the typical spatial structure of MECPSs. Structure analysis and molecular modeling showed that TmMECPS strongly resembled the EcMECPS suggested that they had potential catalytic similarities. MECPS is an important potential target for monoterpenoid indole alkalodis (MIAs) biosynthesis in *Catharanthus roseus* (Veau et al., 2000). Overexpression of MECPS may induce to an increase of the metabolic flux in terpenoid biosynthesis. Color complementation assay showed TmMECPS can accelerate the accumulation of β-carotene, this phenomenon was better than that in *G. biloba* without the positive control (Gao et al., 2006). The result demonstrated that TmMECPS had the

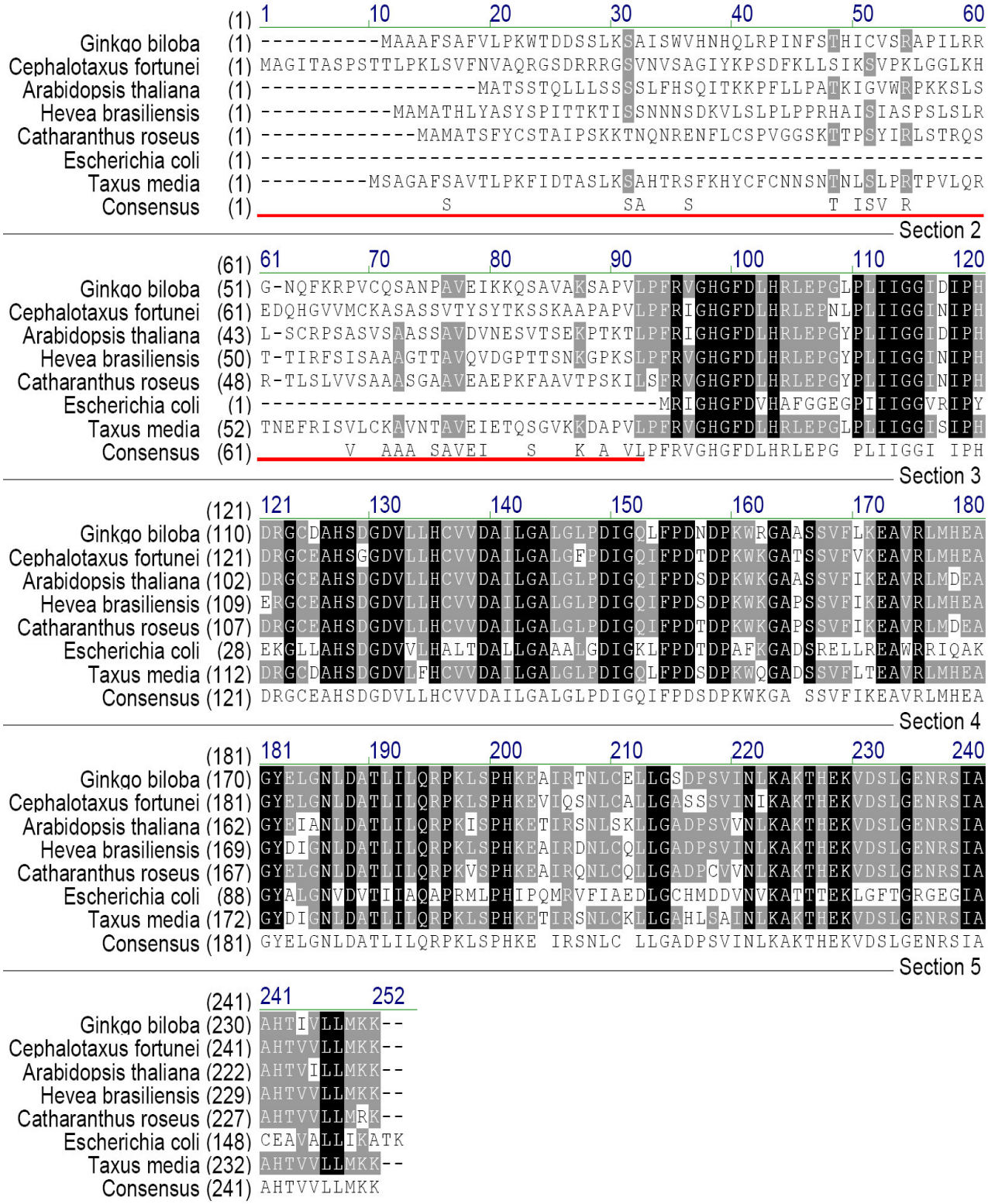

Figure 2. Alignment of the deduced amino acid sequences of MECPSs. The identical amino acids were showed in white with black background, and the conserved amino acids in white with gray background. the plastidial transit peptide predicted by TargetP 2.0 was underlined The aligned MECPSs were from *Ginkgo biloba*, AAR95701.1; *Cephalotaxus fortunei*, ABD73009.1; *Arabidopsis thaliana*, AAM62786.1; *Hevea brasiliensis*, AAS94122.1; *Catharanthus rouseus*, AAF65155.1; *Escherichia coli*, YP_670618.1.

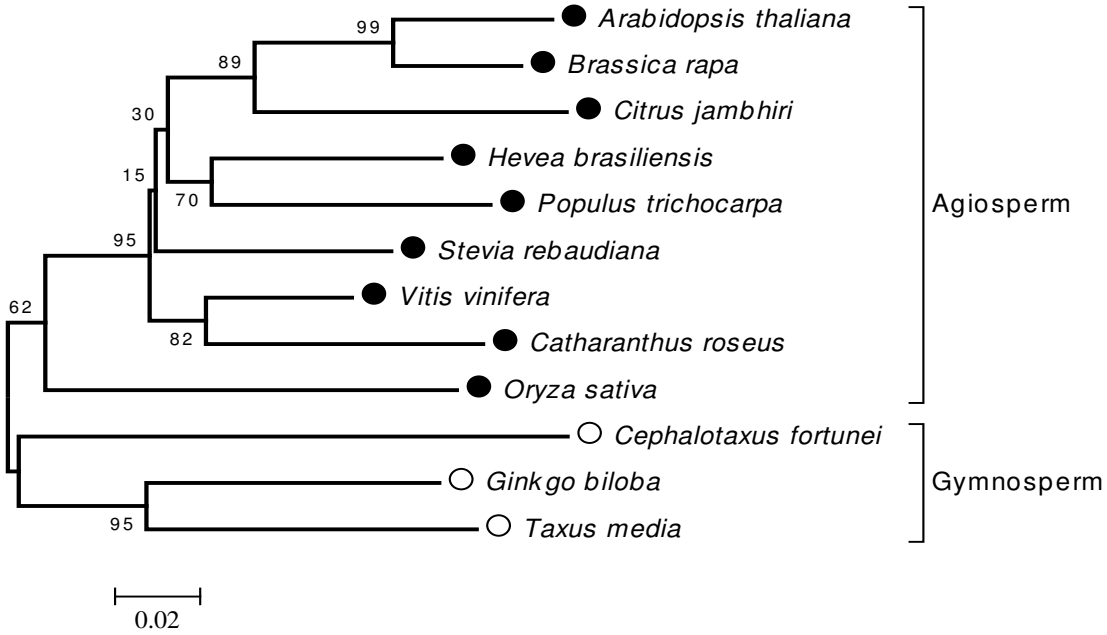

Figure 3. A phylogenetic tree of MECPSs from different plants was constructed by the neighbor-joining method on MEGA 3. MECPSs from gymnosperms were marked with○, MECPSs from angiosperms marked with ●". The numbers on the branches represented bootsrap support for 1000 replicates. The sequences used were listed bellow with DateBase Accession number. *Arabidopsis thaliana*, AAM62786.1; *Brassica rapa*, AB300309.1; *Citrus jamhhiri*, AB266583.1; *Hevea brasiliensis*, AAS94122.1; *Populus trichocarpa*, EF145458.1; *Stevia rebaudiana*, ABG23395.1; *Vitis vinifera*, CAO65908.1; *Catharanthus rouseus*, AAF65155.1; *Oryza sativa*, BAD29384.1; *Cephalotaxus fortunei*, ABD73009.1; *Ginkgo biloba*, AAR95701.1.

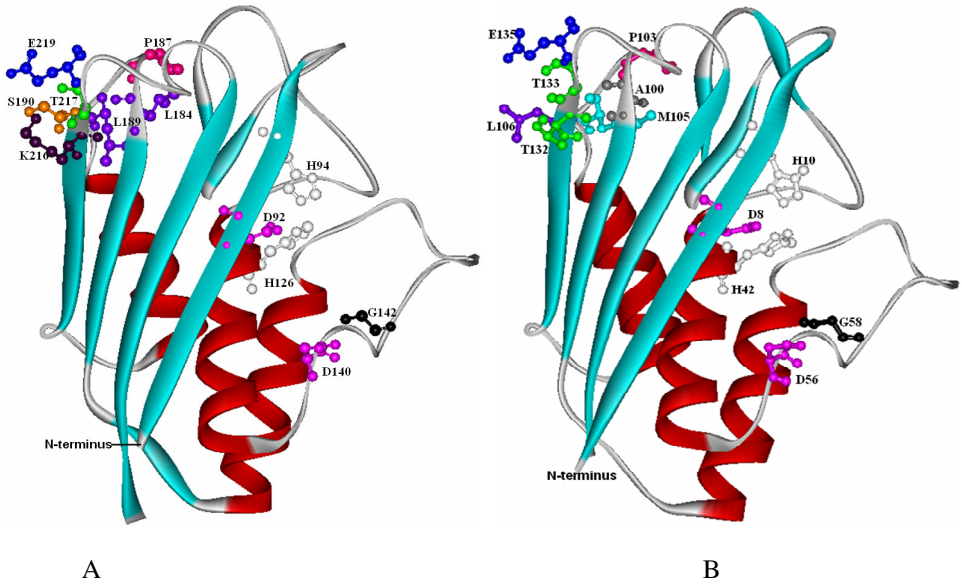

Figure 4. The three-dimensional structure of *TmMECPS* (A) and *EcMECPS* (B) monomer established by homology-based modeling. The α-helix, β-pleated sheet and random coil are indicated by the helix, sheet and rope-shape, respectively. The sites of metal ion co-coordinated, Asp92-His94-His126 and ADP binding site Asp140-Gly142-Leu184-Leu189-Ser190-Lys216-Thr217-Glu219, were indicated by balls.

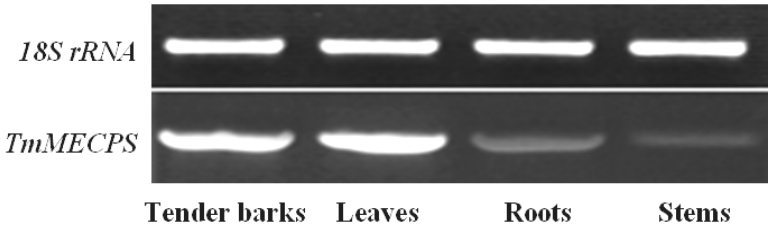

Figure 5. *TmMECPS* expression profile in various tissues.

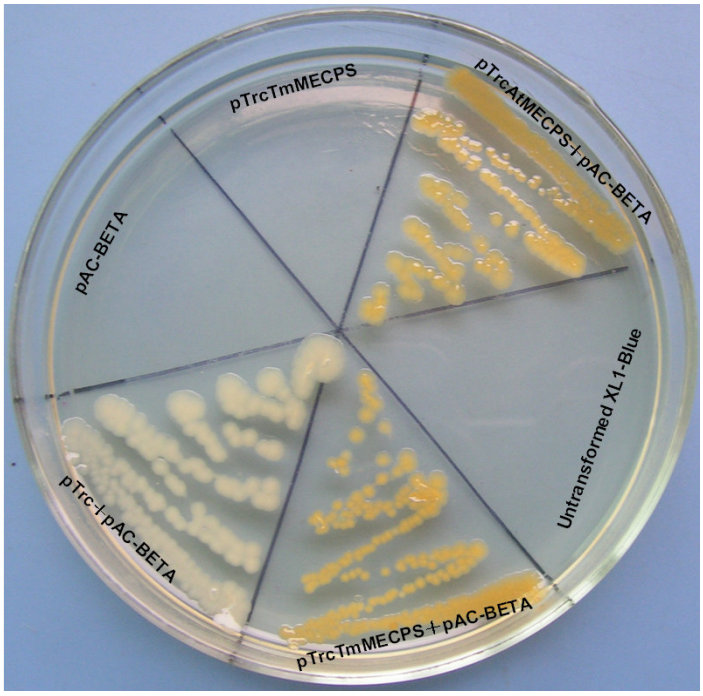

Figure 6. Functional complementation of TmMECPS activity, using *E. coil* strain XL1-Blue.

same catalytic activity as MECPS from Arabidopsis plants.

ACKNOWLEDGEMENT

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