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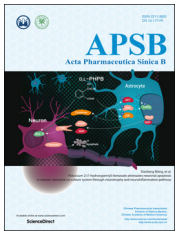

ORIGINAL ARTICLE

Molecular cloning and functional identification of sterol C24-methyltransferase gene from *Tripterygium wilfordii*

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KEY WORDS

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Abstract Sterol C24-methyltransferase (SMT) plays multiple important roles in plant growth and development. SMT1, which belongs to the family of transferases and transforms cycloartenol into 24-methylene cycloartenol, is involved in the biosynthesis of 24-methyl sterols. Here, we report the cloning and characterization of a cDNA encoding a sterol C24-methyltransferase from *Tripterygium wilfordii* (*TwSMT1*). *TwSMT1* (GenBank access number KU885950) is a 1530 bp cDNA with a 1041 bp open reading frame predicted to encode a 346-amino acid, 38.62 kDa protein. The polypeptide encoded by the *SMT1* cDNA was expressed and purified as a recombinant protein from *Escherichia coli* (*E. coli*) and showed SMT activity. The expression of *TwSMT1* was highly up-regulated in *T. wilfordii* cell suspension cultures treated with methyl jasmonate (MeJA). Tissue expression pattern analysis showed higher expression in the phellem layer compared to the other four organs (leaf, stem, xylem and phloem), which is about ten times that of the lowest expression in leaf. The results are meaningful for the study of sterol

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biosynthesis of *T. wilfordii* and will further lay the foundations for the research in regulating both the content of other main compounds and growth and development of *T. wilfordii*.

1. Introduction

Tripterygium wilfordii Hook. F. is a traditional Chinese medicinal plant that has analgesic and anti-microbial properties, and thus it has been widely used to treat inflammatory diseases[1]. Moreover, recent research showed that *T. wilfordii* could treat immune and tumour diseases[2–4].

Isoprenoid compounds are main active ingredients of *T. wilfordii*. Several important enzymes have been cloned and identified for their biosynthetic pathways[5,6]. The isoprenoid compounds in *T. wilfordii* include sterols, chlorophyll, gibberellin, and a variety of terpenes[7]. Among these, sterols are hydrocarbon derivatives that consist of a four-membered cyclopentanoperhydrophenanthrene ring. Plant sterols are essential components of eukaryotic membranes. They help to maintain membrane integrity and permeability[8], participate in mammalian, yeast and plant cell endocytosis and production processes[9], and serve as precursors in the brassinosteroid hormone biosynthesis[10]. In addition, phytosterols can act as signalling molecules in plants , participating in the regulation of various physiological activities, such as photosynthesis, reproduction and immunization[11]. Sterol C24-methyltransferase (SMTs) have been found to play a key role in the synthesis of steroids with its methyltransferase property[12]. The analysis of different amino acid sequences in all the cDNAs suggested that *SMTs* can be separated into two gene families, *SMT1* and *SMT2*[13]. It has been reported that the two compounds play important roles in plant growth and development. The metabolic pathway chart is shown in Fig. 1. It has been indicated that the methylation reactions of cycloartenol and 24-methylene lophenol are catalysed by SMT1 and SMT2, respectively[14]. The two gene families are involved in the biosynthesis of 24-methyl and 24-ethyl sterols, respectively. Thus, cloning of the plant *SMT* genes and characterization of the gene products would provide an alternative approach to addressing some of the important questions regarding *SMTs*, such as the C-24 methylation mechanism and developmental regulation of the enzyme.

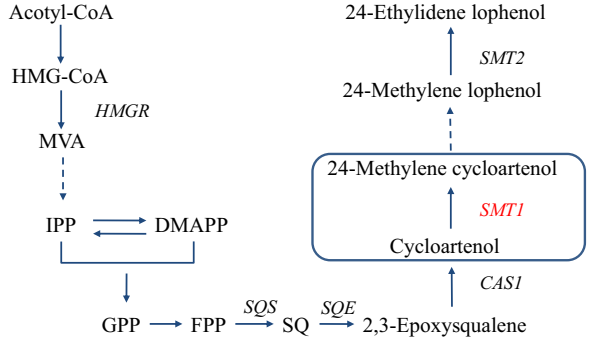

Figure 1 The biosynthetic pathway of phytosterol involving sterol C24-methyltransferase 1 (*SMT1*) gene and sterol C24-methyltransferase 2 (*SMT2*) gene. 3-Hydroxy-3-methylglutary CoA (HMG-CoA); 3-hydroxy-3-methyl glutaryl coenzyme A reductase (*HMGR*); mevalonate pathway (MVA); isopenteny pyrophosphate (IPP); dimethylally pyrophosphate (DMAPP); gerqnyl pyphosphate (GPP); famesyl pyrophosphate (FPP); squalene synthase (*SQS*); squalene (SQ); squalene epoxidase (*SQE*); cycloartenol synthase 1 (*CAS1*).

Molecular cloning of *SMTs* was recently achieved in a number of higher plant species, including *Astragalus bisulcatus*[15], *Arabidopsis thaliana*[16], *Oryza sativa*[17], *Nicotiana tobacum*[18], *Brassica oleracea*[19], and *Camellia sinensis*[20]. Until now, no *SMT* gene from *T. wilfordii* has been cloned. In this paper, we report the isolation and identification of a cDNA encoding SMT1 from *T. wilfordii* for the first time. The polypeptide encoded by the *T. wilfordii* cDNA was expressed in *E. coli* and shown to be an active SMT enzyme. The real-time quantitative PCR analysis of *TwSMT1* expression was found to be promoted upon the methyl jasmonate (MeJA) elicitor treatment.

2. Materials and methods

2.1. Plant materials

Cell suspensions of *T. wilfordii* in the study were cultured in Murashige and Skoog (MS) medium (pH 5.8) containing 2,4-dichlorophenoxyacetic acid (2,4-D, 0.5 mg/L), cytokinin (KT, 0.1 mg/L), indole-3-butytricacid (IBA, 0.5 mg/L), and sucrose (30 g/L), shaking at 120 rpm (Eppendorf, 5810 R, Germany) 25 °C in dark culture and subculture suspension cells (2 g) in the same medium (25 mL) every 20 days. The plants of *T. wilfordii* in the tissue expression analysis were obtained from Fujian province and have grown for seven years.

2.2. Cloning of TwSMT1 from T. wilfordii

Total RNA was extracted from *T. wilfordii* suspension cells stored at –70 °C using the CTAB-LiCl method[21]. The extract was purified using DNase I (Biolabs, Beijing, China) and an RNA cleaning kit (TIANGEN, Beijing, China) to remove contaminating genomic DNAs.

The purified product was reverse transcribed into first-stand 5′-RACE-Ready cDNA and 3′-RACE-Ready cDNA with the SMART RACE cDNA Amplification Kit (Takara Bio Group, Japan). According to mRNA fragments obtained from the transcription data, specific primers (3′-RACE Primer: 5′-TGGATGTAGGATGTGGAATCGGTGGA-3′; 5′-RACE Primer: 5′-TTAGGGCCTCAAGGCATTGTCTGGTC-3′) were designed to amplify 5′ and 3′ cDNA, respectively, followed by ligation into the *pEASY*-T3 vector (TransGen Biotech, Beijing, China) and transfer into *E. coli* Trans5*α* competent cells (TransGen Biotech,

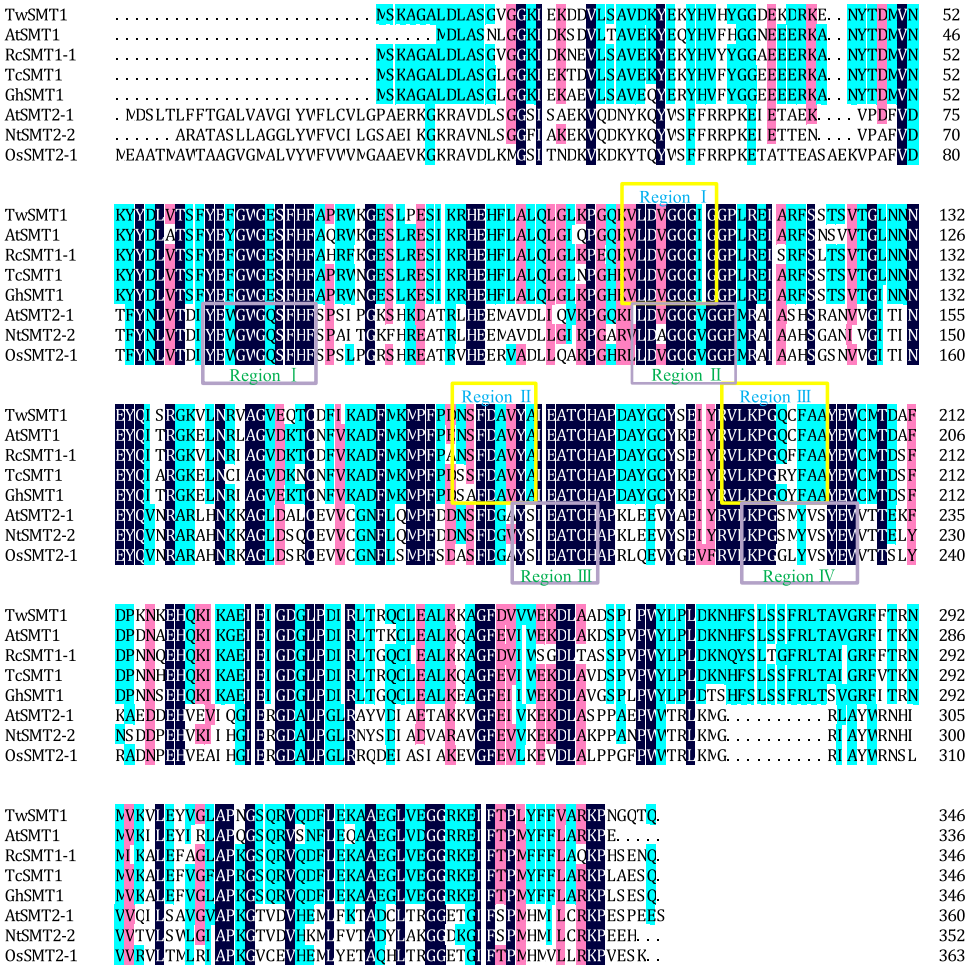

Figure 2 Sequence alignment of the deduced amino acid sequence of TwSMT1 with those of related proteins. The three conserved sterol C24-methyltransferase 1 regions and four conserved sterol C24-methyltransferase 2 regions are boxed and numbered with different colours.

Beijing, China). Transformed cells were plated onto Luria-Bertani (LB) solid medium plates containing ampicillin (Amp) and screened using monoclonal colony PCR. Positive bacterial colonies were selected for sequencing to identify and obtain the *TwSMT1* full-length sequence.

2.3. *Sequence alignments and phylogenetic analyses*

The nucleotide and protein sequences were compared using BLAST at the NCBI (http://www.ncbi.NLM.NIH.gov). The ORF was searched using the ORF Finder (www.ncbi.NLM.NIH.gov/Gorf/Gorf.html). The molecular weight (MW) and theoretical isoelectric point (pI) calculations were performed using the Compute pI/MW tool (http://Web.ExPASy.org/compute_pi/). Multi ple sequence alignments were performed using DNAMAN 8.0, and phylogenetic analysis was carried out using MEGA 7.0 software to build evolutionary trees.

2.4. *Expression of TwSMT1 in E. coli and purification of recombinant protein*

Based on the prokaryotic expression vector pMAL-c2X sequence, the restriction endonuclease sites of *Bam*H I and *Xba* I were selected to design primers from which the stop codon (TAA) has been removed to amplify the *TwSMT1* ORF sequence: *TwSMT1*: 5′-CGGGATCCATGTCGAAGGCTGGGGCGT-3′ (forward) and 5′-GCTCTAGACTGGGTCTGCCCATTAGGCT-3′ (reverse). According to the instructions of Prime STAR GXL DNA Polymerase (Takara Bio Group, Japan), the PCR reaction conditions were set as follows: 98 °C for 3 min; 35 cycles of 98 °C for 10 s, 55 °C for 15 s, and 68 °C for 1 min 20 s; and a final extension at 72 °C for 5 min. After the amplified products were purified, both the vector and the purified products were double-digested with corresponding restriction endonucleases; the enzyme-digested products were purified and ligated with T4 DNA ligase, and then transferred into *E. coli* Trans5*α* competent cells. Transformed cells were cultured in LB solid medium with Amp (100 mg/L) for one night, and then a monoclonal plaque was selected for PCR verification and sequencing to obtain the correct recombinant plaque. The new plasmid extracted from the plaque was named pMAL-c2X-*TwSMT1*.

The recombinant pMAL-c2X-*TwSMT1* was transferred into BL21 (DE3) competent cells along with the same transformation of pMAL-c2X as a control. The detected positive plaques were cultured and induced with 1 mmol/L isopropyl-1-thio-*β*-D-galactopyranoside for protein extraction. The extraction procedure was described in Supplementary information, and the purified extract was used for dodecyl sulfate, sodium salt-polyacrylamide gel electrophoresis (SDS-PAGE) detection.

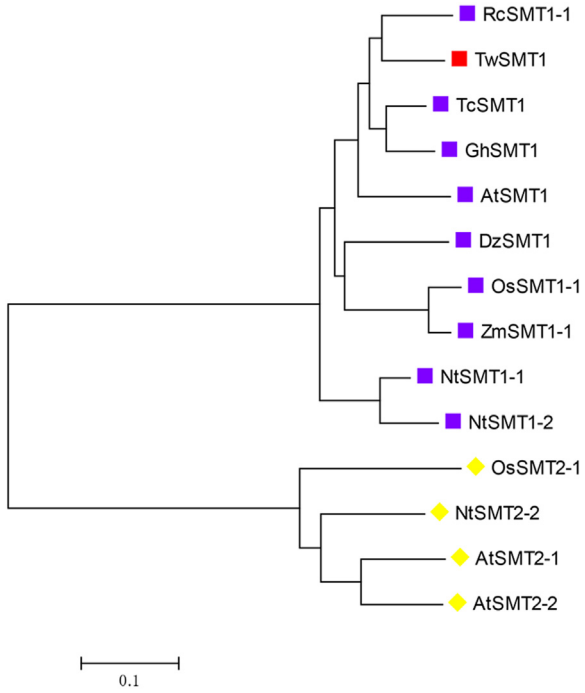

Figure 3 Phylogenetic tree of the amino acid sequences of sterol C24-methyltransferase from different plants constructed by the neighbour-joining method on MEGA 7.0; GenBank accession numbers: *Ricinus communis* (RcSMT1-1 AAB62812.1); *Theobroma cacao* (TcSMT1 XP_007052489.1); *Gossypium hirsutum* (GhSMT1 AAZ83345.1); *Arabidopsis thaliana* (AtSMT1 NP_001078579.1); *Dioscorea zingiberensis* (DzSMT1 CBX33151.1); *Oryza sativa* (OsSMT1-1 AAC34988.1); *Zea mays* (ZmSMT1-1 AAB70886.1); *Nicotiana tabacum* (NtSMT1-1 AAC34951.1); *Nicotiana tabacum* (NtSMT1-2 AAC35787.1); *Oryza sativa* (OsSMT2-1 AAC34989.1); *Nicotiana tabacum* (NtSMT2-2 AAB62807.1); *Arabidopsis thaliana* (AtSMT2-1 CAA61966.1); *Arabidopsis thaliana* (AtSMT2-2 AAB62809.1).

2.5. *Enzyme assays*

To identify *TwSMT1* functions, enzymatic reactions *in vitro* were performed with the purified supplement above using the method reported by Schaeffer et al.[22]. The reaction system contained 0.1 mol/L Tris–HCl (pH 7.5), Tween 80 (0.1%, *w/v*), glycerol (20%, *v/v*)), *β*-mercaptoethanol (1 mmol/L), methyl-3*H*-AdoMet (100 μmol/L), cycloartenol (100 μmol/L) as a substrate, and purified protein (200 μL); the total volume was 500 μL, and the reaction condition was set at 30 °C for 45 min and ethanolic KOH (100 μL of 12%, *w/v*) was used as a quenching agent. The sterol compounds in the mixture solution were extracted three times with 600 μL *n*-hexane, combining the supernatant solution and evaporating the solvent with a pressure blowing concentrator, followed by re-dissolving in *n*-hexane (200 μL) for gas chromatography–mass spectrometer (GC–MS) detection.

The GC–MS detection was performed using a Thermo TRACE 1310/TSQ 8000 gas chromatography (splitless; injector temperature 250 °C) equipped with a DB-5 ms (30 m × 0.25 mm, 0.25 μm) capillary column, and the program condition was set for 1 min at 60 °C, then increased from 60 to 300 °C at a rate of 30 °C/min, and finally held for 15 min at 300 °C; the flow rate was 1 mL/min with He as a carrier gas. The mass spectrometry detection range was from 50 to 500 *m/z*.

2.6. *Expression analysis of TwSMT1 induced by Methyl Jasmonate*

MeJA, as an abiotic elicitor, is widely used in tissue expression analysis for its ability to promote content of secondary metabolites. After the suspension cells of *T. wilfordii* were induced by MeJA (50 μmol/L) at 0, 1, 4, 12, 24, 48, 72, and 120 h, total RNA extracted with the CTAB method subsequently was reversely transcribed to obtain cDNA for qRT-PCR analysis. Primers for the housekeeping gene, *β*-actin, were chosen as described in Tong's paper[23]: *β*-actin F: 5′-AGGAACCACCGATCCAGACA-3′ and *β*-actin R: 5′-GGTGCCCTGAGGTCCTGTT-3′. The specific primers (*TwSMT1* F: 5′-TCTAACCGCTGTTGGACGA-3′ and *TwSMT1* R: 5′-CCCTCAACTAACCCCTCTGC-3′) were designed to amplify the fragment of *TwSMT1*. The reaction solutions were prepared according to the manufacturer's protocol from the KAPA SYBR FAST qPCR Kit, and the amplification conditions were 95 °C for 5 min and 40 cycles of 95 °C for 3 s and 60 °C for 33 s. The experiments were repeated three times for biological and technical replicates, respectively, to ensure the authenticity of the data.

2.7. *Tissue expression pattern analysis of TwSMT1*

Total RNA was extracted from five organs of *T. wilfordii* plants, which were the leaf, stem, phloem, xylem and phellem layer. The purified RNA was reversely transcribed into the First Strand cDNA for relative expression study of *TwSMT1*. The primers for amplifying the housekeeping gene and *TwSMT1*, as well as the reaction condition of RT-PCR were consistent with inducible expression analyses by MeJA, including the operating time.

3. Results

3.1. *Isolation of the cDNA coding for TwSMT1 and sequence analysis*

RT-PCR was performed with total RNA from *T. wilfordii*. *TwSMT1* gene fragments were obtained by 3′ rapid amplification of cDNA ends (3′-RACE-PCR) and 5′-RACE-PCR. The full-length cDNA encoding the SMT1 protein was isolated from *T. wilfordii*. The full-length cDNA of *TwSMT1* was 1530 bp. It had a 1041-bp open reading frame (ORF) encoding a 346-amino-acid polypeptide, with a 177 bp 5′ non-coding-region (NCR) and a 312 bp 3′-NCR including a 19 bp poly (A) tail. The predicted TwSMT1 protein has a calculated molecular mass of 38.99 kDa and a theoretical pI of 6.11 (GenBank accession No. KU885950).

3.2. *Comparison of the deduced amino acid sequence of TwSMT1 with other SMTs*

A Blast search of *TwSMT1* in the NCBI database showed that the deduced amino acid sequence of *TwSMT1* had 75%–86% identity to the SMT1s from *A. thaliana*, *Nicotiana tabacum*, *O sativa*, *Zea mays*, *Ricinus communis*, *Dioscorea zingiberensis*, *Theobroma cacao*, and *Gossypium hirsutum*. Sequence comparison revealed that the deduced amino acid sequence contained three methyltransferase regions identified in diverse sterol C24-methyltransferases[24]. Region I is highly conserved in the protein. Region II contains the invariant central aspartate residue. Region III is located at an interval between the 19-residue C-terminal and region II[25]. The

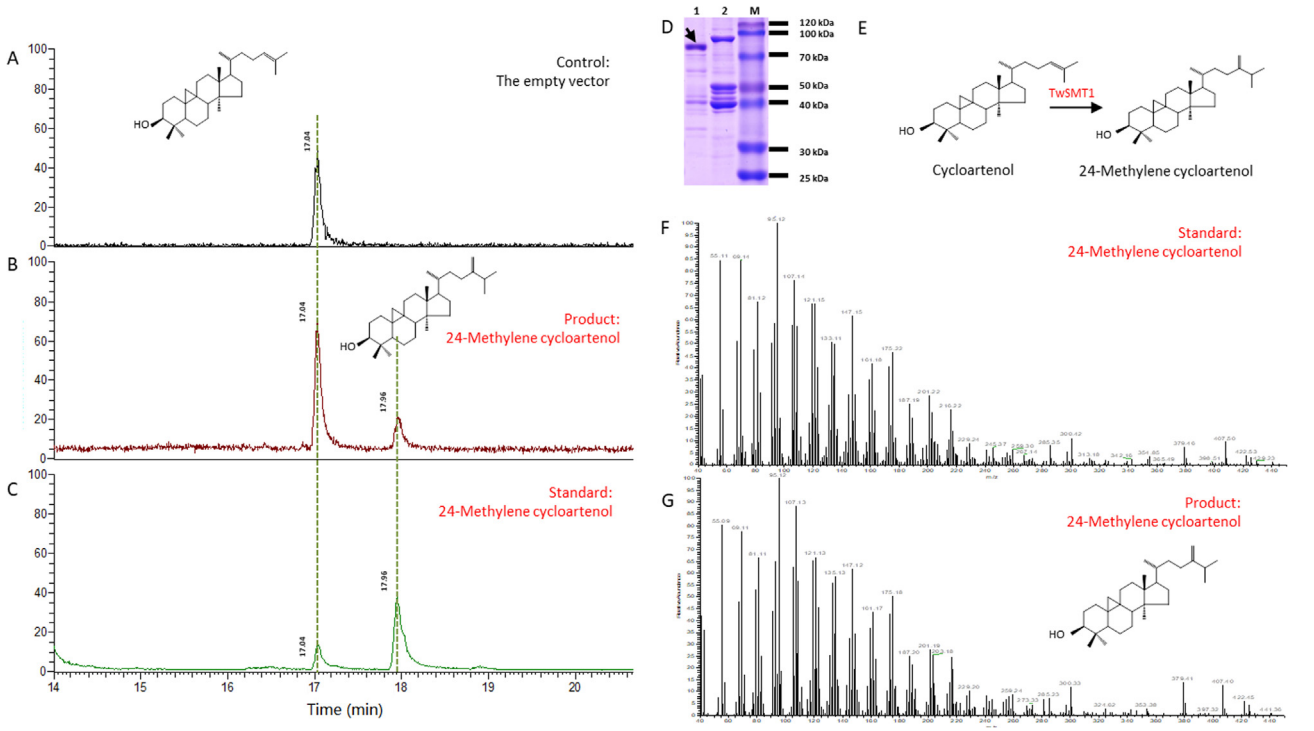

Figure 4 SDS-PAGE analysis and GC–MS detection results of extract from an enzymatic reaction catalysed by purified pMAL-c2X-TwSMT1 protein and pMAL-c2X protein when using cycloartenol as the substrate. (A) The peak of extraction in the empty vector protein reaction system; (B) The peak of extraction in the recombinant pMAL-c2X-TwSMT1 protein reaction mixture; (C) A control using a 24-methylene cycloartenol standard; (D) 1: The recombinant pMAL-c2X-TwSMT1 overexpressed by isopropyl-1-thio-*β*-D-galactopyranoside (IPTG) 2: the empty pMAL-c2X overexpressed by IPTG; (E) The function of *SMT1* from *T. wilfordii*; (F) Mass spectrogram of the 24-methylene cycloartenol standard; (G) Mass spectrogram of the product catalysed by recombinant TwSMT1 protein.

deduced amino acid sequence of *TwSMT1* has 33%–36% identity with the SMT2s from *O. sativa*, *N. tabacum*, and *A. thaliana*. These sequences present highly homologous regions: Region II (IN)LD(A/V)-GCG(V/I)GGP corresponds to the consensus motif described by several authors[26]. Region III IEATCHAP, a second invariant region, is absent in other methyltransferases and is potentially unique for sterol methyltransferases[27]. Those regions marked with boxes suggested that the cDNA of *T. wilfordii* may encode an sterol C24-methyltransferase (Fig. 2).

Comparison of all these amino acid sequences allowed a phylogenetic tree of plant SMTs to be built, which was separated into two main groups (Fig. 3). TwSMT1 clustered with 9 SMT1 sequences and OsSMT2 clustered with 3 SMT2 sequences. Moreover, TwSMT1 and *R. communis* were classified into one cluster. A cluster means that its components had higher homology.

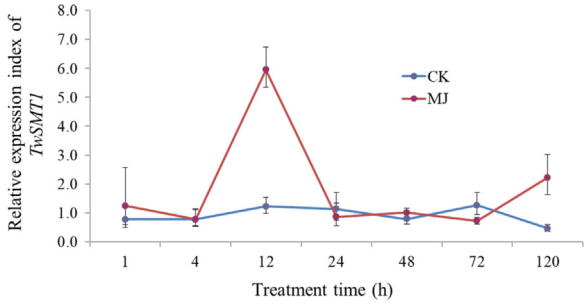

Figure 5 Expression profile of *TwSMT1* when treated with 1 mmol/L methyl jasmonate (MJ) over 120 h. RT-PCR analysis was performed using total RNA isolated from suspension cells of *T. Wilfordii*. CK, the control group; MJ, the MJ-induced group.

3.3. *Functional expression and characterization of TwSMT1*

Fig. 4 shows the results of protein expression and GC–MS detection. SDS-PAGE was used to detect purified proteins extracted from BL21(DE3) strains from which pMAL-c2X or pMAL-c2X-*TwSMT1* was expressed. The electrophoresis results are shown in Fig. 4D. The control vector expressed an MBP-labelled protein with a molecular weight of 42 kDa, whereas owing to the TwSMT1 protein being 39 kDa, the recombinant plasmid expressed the protein at the position of 80 kDa as the sum of the MBP and TwSMT1 proteins. The results suggest that both the *TwSMT1* construct in the pMAL-c2X vector and the empty pMAL-c2X with the MBP label had been successfully expressed in the BL21(DE3) strain, so the extracted protein can be used for further TwSMT1 functional experiments *in vitro*.

The sterol extract from both pMAL-c2X and pMAL-c2X-TwSMT1 in the enzymatic reaction were detected by GC–MS. From the results we can see that, in comparison to a peak of cycloartenol in the pMAL-c2X extract shown in Fig. 4A, a prominent peak whose retention time was 17.96 min was detected in the pMAL-c2X-TwSMT1 protein reaction (Fig. 4B). The experiment was repeated six times, and the same results were

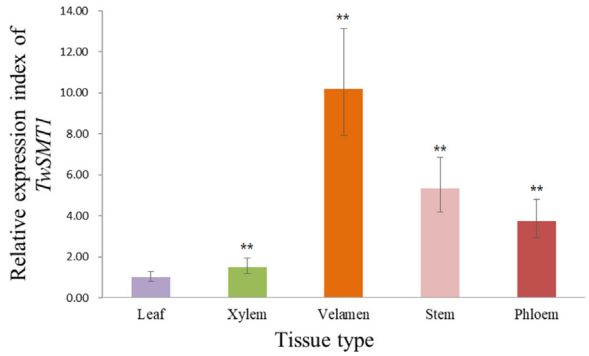

Figure 6 Tissue expression analysis of *TwSMT1* in the leaf, stem, phloem, xylem and phellem layer of *T. Wilfordii* plants. The asterisks mean that the difference is acceptable when the values of other organs take leaf as a standard ($^{**}P < 0.01$).

obtained. Fig. 4C shows the peak of the predicted product, 24-methylene cycloartenol, which is theoretically SMT1's product when cyclaortenol is the substrate, and it shows the same retention time at 17.96 min as the product in Fig. 4C; thus, they were putatively assigned as the same compound. We then compared the mass spectra of the product (Fig. 4G) and the standard (Fig. 4F), and the figures show nearly identical ion peaks, except for a few low intensity peaks. These results demonstrate that TwSMT1 has the function of catalysing the transformation of cycloartenol to 24-methylene cycloartenol, and it is a cycloartenol-C24-methyltransferase.

3.4. *Inducible expression of TwSMT1*

MeJA has the ability to promote the accumulation of secondary metabolites. From Fig. 5, it is clear that the elicitor MeJA works on the expression level of *TwSMT1*. After the suspension cells were elicited by MeJA, the *TwSMT1* transcript levels have an obvious fluctuation especially at 12 h and it is about four times higher than the blank control group. Afterwards, the curve tends to overlap with the control group and stabilize. The methodology can be used to study sterol content accumulation and other sterol genes through transcriptome data mining.

3.5. *Tissue expression pattern of TwSMT1*

Fig. 6 shows the relative expression level of *TwSMT1* in different organs. It shows that phellem layer has the highest expression level, which is about ten times higher than the lowest expression in leaf.

4. Discussion

The enzyme sterol C24-methyltransferase 1 (SMT1) is involved in the biosynthesis of plant sterol, which plays major roles in plant growth and development. In addition, studies have shown that β-sitosterol, a phytosterol belonging to the 24-ethyl sterols, has been isolated from *T. wilfordii*[28], and it has an obvious cholesterol-lowering activity and is widely used in the pharmaceutical industry. In the present study, we have reported the first isolation and characterization of a sterol C24-methyltransferase 1 gene from *T. wilfordii*. The results showed that *TwSMT1* is a 1530-bp cDNA with a 1041-bp ORF predicted to encode a 346-amino acid, 38.62 kDa protein. The results also indicated that the *SMT1* obtained belonged to the family of transferases and catalysed the transformation of cycloartenol to 24-methylene cycloartenol. In order to study the inducible expression of *TwSMT1* from cell suspensions upon MeJA, we analysized the changes of real-time PCR in various stages. The results showed that MeJA caused a significant increase in *TwSMT1* levels in *T. wilfordii* cell suspensions. Tissue expression analysis showed *TwSMT1 h*as a higher expression in velamen compared to other four organs.

Plant sterols play extremely important roles in every stage of plant growth and development. It is important that *SMTs* act on the biosynthesis of plant sterols as many researchers have reported. Researchers often use mutant and enzyme inhibitors to study the functions of plant sterols. Mutants of *SMT1* show abnormal embryoids and cotyledons with different sizes and numbers[29]. This result indicates that plant sterols play a crucial role in the process of embryonic development. Mutants of *SMT1* show cell shrinkage of root epidermis and cortex, stasis phenomenon of the meristem and elongation region cells in the shape of a circle[29], indicating the importance of sterols in normal growth and development of the roots. The *orc* mutation residing in C-24 *SMT1* shows a position disorder of auxin transmission proteins PIN1 and PIN3, indicating that plant sterols could correct the polarity orientation of proteins[30].

Sterols play a vital role in the process of eukaryote growth and development. They are not only structural components, but also have important regulatory functions and are precursors for the synthesis of other compounds. Plant sterols participate in almost all processes of plant growth, from the embryo to post-embryonic development. Therefore, they are indispensable to normal plant growth and development. Studies have also shown that consuming more plant sterols can reduce the absorption of cholesterol, and they may be used as therapeutics in the future.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.apsb.2017.07.001.

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