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# Characterization and Expression Profile Analysis of a New cDNA Encoding Taxadiene Synthase from *Taxus media*

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**A full-length cDNA encoding taxadiene synthase (designated as TmTXS), which catalyzes the first committed step in the Taxol biosynthetic pathway, was isolated from young leaves of *Taxus media* by rapid amplification of cDNA ends (RACE). The full-length cDNA of TmTXS had a 2586 bp open reading frame (ORF) encoding a protein of 862 amino acid residues. The deduced protein had isoelectric point (pI) of 5.32 and a calculated molecular weight of about 98 kDa, similar to previously cloned diterpene cyclases from other *Taxus* species such as *T. brevifolia* and *T. chinenisis*. Sequence comparison analysis showed that TmTXS had high similarity with other members of terpene synthase family of plant origin. Tissue expression pattern analysis revealed that TmTXS expressed strongly in leaves, weak in stems and no expression could be detected in fruits. This is the first report on the mRNA expression profile of genes encoding key enzymes involved in Taxol biosynthetic pathway in different tissues of *Taxus* plants. Phylogenetic tree analysis showed that TmTXS had closest relationship with taxadiene synthase from *T. baccata* followed by those from *T. chinenisis* and *T. brevifolia*. Expression profiles revealed by RT-PCR under different chemical elicitor treatments such as methyl jasmonate (MJ), silver nitrate (SN) and ammonium ceric sulphate (ACS) were also compared for the first time, and the results revealed that expression of TmTXS was all induced by the tested three treatments and the induction effect by MJ was the strongest, implying that TmTXS was high elicitor responsive.**

**Keywords:** Elicitors, Induction expression, RACE, RT-PCR, Taxadiene synthase, *Taxus media*

## Introduction

Taxol (paclitaxel), a kind of complex diterpenoid from the bark of yew, is a potent antimitotic agent with excellent activity against breast and ovarian cancers (Dieras, 1998), as well as AIDS-related Kaposi's sarcoma (Sgadari *et al*., 2000). It binds tubulin heterodimers (Rowinsky *et al*., 1993), promotes and stabilizes, microtubule assembly, disrupts the over-rapid division of cancer cell. The unique mode of action by this drug ultimately lead to the development of Taxol as one of the most effective anticancer agents approved by FDA (the Food and Drug Administration) in current use (Suffness, 1994). The limited supply of this drug from the bark of natural yew prompted intensive effort to develop alternate means of production, including isolation from plantation-grown Taxus species, biosynthesis in tissue culture systems, semisynthesis of Taxol from 10-deacetyl baccatin III that is more readily available in the needle of yew, and total synthesis of Taxol (Hefner *et al.,* 1998) to meet the needs of over 250 kg Taxol/year to treat multiple types of cancers (Kwon *et al*., 1998). It is clear that in the foreseeable future the supply of Taxol and its synthetically useful progenitors must depend on biological methods of production. Several genes coding enzyme responsible

**Abbreviations:** ACS = ammonium ceric sulphate, MJ = methyl jasmonate, ORF = open reading frame, RACE = rapid amplification of cDNA ends, SN = silver nitrate, *T. mediar* = *Taxus media*, TmTXS = *Taxus media* taxadiene synthase, PCR = Polymerase chain reaction, pI = isoelectric point, RT-PCR= reverse transcriptase-polymerase chain reaction

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for the biosynthesis of Taxol in Taxus have been cloned as the result of a movement towards detailed understanding of the pathway for Taxol biosynthesis (Wildung *et al.,* 1996; Walker *et al.,* 2001). It has been reported that the first committed step of Taxol biosynthesis is the initial cyclization of geranylgeranyl diphosphate (GGDP, the universal precursor of diterpenoids) to taxa-4(5)-11(12)-diene to establish the taxane core structure (Hezari *et al.,* 1995; Wildung *et al.,* 1996). The biosynthetic route to Taxol, therefore, can be divided into three steps (Walker *et al.,* 2001): the synthesis of GGDP, the cyclization of GGDP to taxa-4(5), 11(12)-diene (the unique taxane skeleton), and the hydroxylation and acylation of taxane skeleton to produce Taxol.

Being a key enzyme in Taxol biosynthetic pathway, taxadiene synthase has recently been cloned from *T. brevifolia* and *T. chinensis* and functionally expressed in *Escherichia coli* (Wildung *et al.,* 1996; Wang *et al.,* 2002). However, until now there is no report on the cloning of taxadiene synthase gene (*TXS*) from *T. media* (*TmTXS*), which is a *Taxus* species with its needles containing higher content of Taxol and is one of the major sources currently for commercial production of Taxol. To our knowledge, being a promising method, *in vitro* cell culture of *Taxus* to improve Taxol production by various kinds of treatments such as MJ elicitor has been most extensively studied and well documented recently (Laskaris *et al.,* 1999; Wu *et al.,* 2001), but few report on mRNA expression profile of genes encoding key enzymes involved in Taxol biosynthetic pathway either in different tissues of *Taxus* plants or under various kinds of elicitor treatments in *Taxus* cells.

In this work, we report the cloning and characterization of taxadiene synthase gene from *T. media*. *TmTXS* gene expression profiles induced by different elicitors such as methyl jasmonate, silver nitrate and ammonium ceric sulphate treatment were compared firstly. The phylogenetic analysis of *TXS* gene family in different *Taxus* species and the expression pattern of TmTXS in various tissues including leaves, fruits and stems of *T. media* plant were also studied for the first time.

## Materials and Methods

**Plant materials and elicitor treatments** *T. media* Rehder plants, provided by Professor Feng Tan at Southwest Normal University in China, were grown in pots in the greenhouse under 25°C with a photoperiod of 16 h light and 8 h dark. All tissue materials (1 g) including leaves, fruits and stems were excised from *T. media* plants and immersed separately in liquid nitrogen for RNA extraction.

*T. media* cell line, initiated from zygotic embryo, maintained in 1/2 modified $B_5$ medium supplemented with 25 g/L sucrose, 2 mg/L a-naphthalene acetic acid (NAA), 0.15 mg/L 6-BA, and 1 g/L enzymatic hydrolyzed casein and subcultured every 4 weeks. The cells were grown on the rotary shaker (120 rpm) at 25°C in the dark. Four-day-old cells were subjected to various kinds of treatments such as 8 μmol/l SN (Shanghai chemical reagent Co. LTD, China), 80 μmol/l ACS (Shanghai Chemical Reagent Co. LTD, China) and 80 μmol/l MJ (Sigma Chemical Co., St. Louis, USA) respectively (Kai *et al*., 2005a), using cell lines without any treatment as control at the same time. The samples were then harvested at different time points (0, 6, 12, 24, 48, 96, 192 h) after treatments and stored at −70°C respectively for further analyses of expression profile of TmTXS by RT-PCR. For the time-course experiment, three independent samples for each time point were assayed in triplicate. All the data were presented as means ± standard deviation (SD).

**Molecular cloning of *TmTXS*** The first strand cDNA was synthesized from 5 μg of total RNA according to the protocol of the 3' RACE System for Rapid Amplification of cDNA Ends (GIBCO BRL, Gaithersburg, USA) using the adapter primer (AP, 5'-GGCC ACGCGTCGACTAGTAC$(T)_{16}$-3') provided within the kit. For the amplification of 3' end of *TmTXS*, a specific primer F2 (5'-CGCTG AAGATGAACGCATTGG-3') was designed and synthesized according to the conserved region of known *TXS* sequences from other *Taxus* species such as *T. brevifolia* and *T. chinensis*. The 3' RACE was performed using primer F2 as the forward primer and the Abridged Universal Amplification Primer (AUAP, 5'-GGCCAC GCGTCGACTAGTAC-3') as the reverse primer in a total volume of 50 μl containing 2 μl cDNA, 10 pmol of F2, 10 pmol of AUAP, 10 μmol dNTPs, 1 × Ex PCR buffer and 5U Ex *Taq* polymerase. PCR was performed using the following protocol: the template was denatured at 94°C for 3 min followed by 35 cycles of amplification (94°C for 50 sec, 58°C for 50 sec, 72°C for 180 sec) and by 10 min at 72°C. The amplified product was purified and subcloned into pGEM-T vector (Promega, USA), and transformed into *E. coli* DH5α. Based on the color reaction using Xgal-IPTG System and PCR identification, the positive clones were picked out and sequenced by ABI 377 Sequencer (Perkin-Elmer, Norwalk, USA).

The 5' end of the cDNA sequence was obtained according to the protocol of the 5' RACE System for Rapid Amplification of cDNA Ends (GIBCO BRL, USA). Complementary reverse gene specific primer R2 (5'-CATCCTGGAGCTGGTTGTTGA-3') was designed and synthesized based on the 3' RACE product. Total RNA was reversely transcribed followed by tailing cDNA with oligo (dC). 5' RACE-PCR was performed using R2 as the reverse primer and Abridged Anchor Primer (AAP, 5'-GGCCACGCGTCGACTAGTA CGGGIIGGGIIGGGIIG-3') as the forward primer. PCR was carried out by denaturing cDNA at 94°C for 2 min followed by 35 cycles of amplification (94°C for 50 sec, 56°C for 50 sec and 72°C for 2 min) and by 7 min at 72°C The PCR product was purified and cloned into pGEM-T vector followed by sequencing.

By aligning and assembling the sequences of the 3' RACE and 5' RACE products, the full-length cDNA sequence of TmTXS was obtained which was subsequently amplified via PCR using a pair of primers F1 (5'-TTTAAAGTGGGTATTCAACGC-3') and AUAP, and sequenced. The PCR amplification and sequencing for the full-length cDNA of TaTXS was repeated three times. The full-length sequence of TmTXS was subsequently analyzed for molecular characterization such as sequence homology, the presentation of conserved motifs, and secondary and three-dimensional structures etc.

**Computer analysis** DNA sequences and associated molecular information were analyzed using programs from PSI-Blast, SOPMA, SWISS-Model and CLUSTAL W1.82. The phylogenetic tree of

TmTXS and TXS from other *Taxus* species retrieved from GenBank was constructed by the neighbor-joining method (Thompson *et al.,* 1994).

**RNA isolation and RT-PCR analysis** Total RNA was extracted by the cetyltrimethylammonium bromide (CTAB) based RNA isolation method (Jaakola *et al.,* 2001; Kai *et al*., 2005b), because of the high concentration of phenolic components, polysaccharides and the complexity of secondary products in *T. media* tissues. The quality and concentration of the extracted RNA were checked by agarose gel electrophoresis and by spectrophotometer (DU-640, Beckman, USA) analysis and the RNA samples were stored at −70°C prior to RT-PCR analysis.

Tissue expression pattern analysis by semi-quantitative One-step RT-PCR was carried out to investigate the expression of *TmTXS* in different tissues including leaves, fruits and stems of *T. media*. Expression profiles of *TmTXS* under different kinds of elicitors including MJ, SN and ACS were also investigated. Aliquots of total RNA (0.5 μg) extracted from leaves, fruits, stems and cells of *T. media* were used as templates in one-step RT-PCR reaction with the forward primer KF1 (5'-ATGGCTCAGCTCTCATTTAATG-3') and reverse primer KR1 (5'-TCATACTTGAATTGGATCAATA-3') specific to coding sequence of *TmTXS* using one-step RNA PCR kit (Takara, Japan) respectively. Meanwhile, the RT-PCR reaction for the house-keeping gene (actin gene, which is highly conserved in plants) using specific primers actF (5'-GTGACAATGGAACTGGA ATGG-3') and actR (5'-AGACGGAGGATAGCGTGAGG-3') designed according to the conserved regions of plant actin genes was performed to estimate if equal amounts of RNA among samples were used as an internal control in RT-PCR (Kai *et al.*, 2004). Amplifications were performed under the following condition: 50°C for 30 min, 94°C for 2 min followed by 25 cycles of amplification (94°C for 30 sec, 60°C for 30 sec and 72°C for 3 min). The amplified products were separated on 1% agarose gel and analyzed with Gene analysis software package (Gene company, USA).

## Results

**Molecular cloning of the full-length cDNA of *TmTXS*** A single fragment of about 2.6 kb was obtained by 3' RACE-PCR using cDNA reverse transcribed from leaves RNA. A 3' untranslated region (UTR) of 53 bp was found downstream from the stop codon containing a typical poly (A) adenylation site (AATAAA). Complementary reverse specific primer R2 was designed based on the sequence of 3' RACE product and used in the 5' RACE. A single and specific fragment of about 650 bp was amplified in which a 5' UTR of 112 bp was found upstream of the first start codon. Based on the sequences of the 3' and 5' RACE products, the full-length cDNA of *TmTXS* was deduced, amplified using a pair of primers F1 and AUAP and confirmed by sequencing. The cloned full-length cDNA of taxadiene synthase (*TmTXS,* Genbank Accession No. AY461450) from *T. media* was 2771 bp, whose size was similar to those of other *Taxus* species. The cDNA contained a 2586 bp ORF encoding a protein of 862 amino acids with an isoelectric point of 5.32 and calculated molecular weight of about 98 kDa (Fig. 1). The richest amino acid in deduced TmTXS was Leu (10% by frequency), followed by Ser (8%), Glu (7%), Ala (7%) and Val (7%). Acidic and basic acids constituted 14% and 10% of the polypeptide respectively. A 32% of the total amino acids were charged and the percentages of polar and hydrophobic amino acids were 26% and 35% respectively.

**Sequence analysis of TmTXS** Sequence comparison (http://www.ncbi.nih.gov) revealed that TmTXS had high similarity with many other terpene synthase from higher plants at the amino acid level, indicating that TmTXS belonged to terpene synthase superfamily. TmTXS showed identity of 47, 45, 45 and 45% to E-alpha-bisabolene synthase and abietadiene cyclase from *Abies grandis*, levopimaradiene synthase from *Ginkgo biloba* and (-)-alpha-pinene synthase from *Pinus taeda* respectively. However, TmTXS had much higher identity to TXS proteins from other *Taxus* species with the identity of 98, 96 and 96% to TXS proteins from *T. baccata* (AAR02861), *T. chinensis* (AAG02257) and *T. brevifolia* (AAC49431, AAK83566) respectively (Fig. 2), indicating protein's key structure and functional sites of this type of cyclase was strongly conserved. NCBI conserved domain search results suggested that TmTXS belonged to terpene synthase superfamily (split into six subgroups on the basis of phylogeny called tpsa-tpsf) and also contained the specific conservative DXXDD motif.

A search of the aspartate-rich consensus sequence motifs (XDDXXD) revealed that there were also two motifs (namely DDMAD and DSYDD motifs) in TmTXS, which was in good agreement with those in other TXS proteins previously reported (Wildung *et al.,* 1996). The specific conservative DXXDD motifs were found in most terpene cyclases and involved in substrate binding, which may play an important role in biological function of terpenoid cyclases and therefore it was fairly conserved in evolution, as is a related DXXDD motif (Fig. 2).

The secondary structure of TmTXS was analyzed by SOPMA (Geourjon *et al.,* 1995) and the result showed that the putative TmTXS peptide contained 56% of alpha helices, 10% of extended strands, 7% of beta turns and 27% of random coils (Fig. 3a). The random coil and alpha helix constituted interlaced domination of the main part of the secondary structure, while alpha helix was the basic element of both N and C terminal parts.

Swiss-Model structure prediction (Guex *et al.,* 1997) for the three-dimensional structure revealed that TmTXS had similar modeling to terpene synthases from plant origin on the structures and functions, providing evidence for an evolutionary relationship among the terpene synthases (Fig. 3b). Through comparison of structures of TmTXS and other plant terpene synthases, TmTXS was found to have many characters commonly possessed by terpene synthase superfamily. This implies that the conserved motifs may play an important role

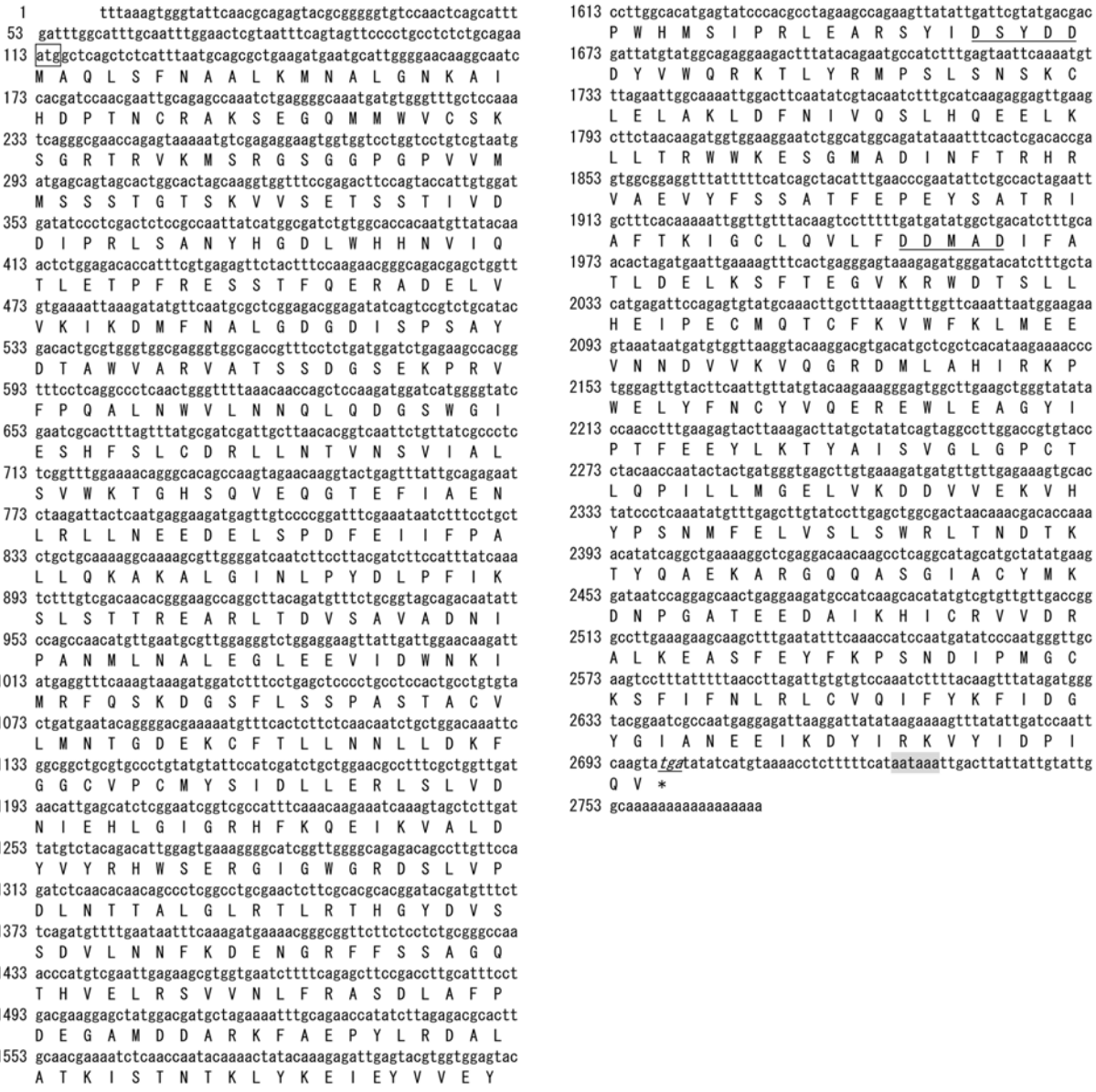

**Fig. 1.** The full-length cDNA sequence and deduced amino acid sequence of *T. media* taxadiene synthase (TmTXS). The start codon (ATG) was boxed and the stop codon (TGA) was underlined italically. The signal for poly(A) tail-addition (AATAAA) was grey-shaded and the conserved motifs were underlined.

in the biological functions and thus are preserved in evolution, while some variations on un-conserved domain can form the molecular foundation for the diversity of the structures and functions. The X-ray crystal structure analysis will further help to elucidate the detailed structure of TmTXS in the future.

**Tissue expression pattern analysis** To investigate *TmTXS* expression pattern in various tissues of *T. media,* total RNA was isolated from different tissues including leaves, fruits and stems, and subjected to one-step RT-PCR analysis using the primers KF1 and KR1. The result showed *TmTXS* expression could be detected in leaves with highest signal and in stems with weaker signal, but no signal was detected in fruits (Fig. 4). Therefore, the *TmTXS* was considered to be a non-constitutively expressing gene.

**Molecular evolution analysis** A phylogenetic tree was constructed based on the deduced amino acid sequences of TmTXS and other taxadiene synthases, in order to investigate the evolutionary relationships among different taxadiene synthases. The result showed that TmTXS and taxadiene synthase from *T. baccata* (AAR02861) formed a cluster with the shortest distance and two taxadiene synthases from *T. brevifolia* (AAC49310 and AAK83566) naturally formed a close cluster and then joined taxadiene synthase from *T.*

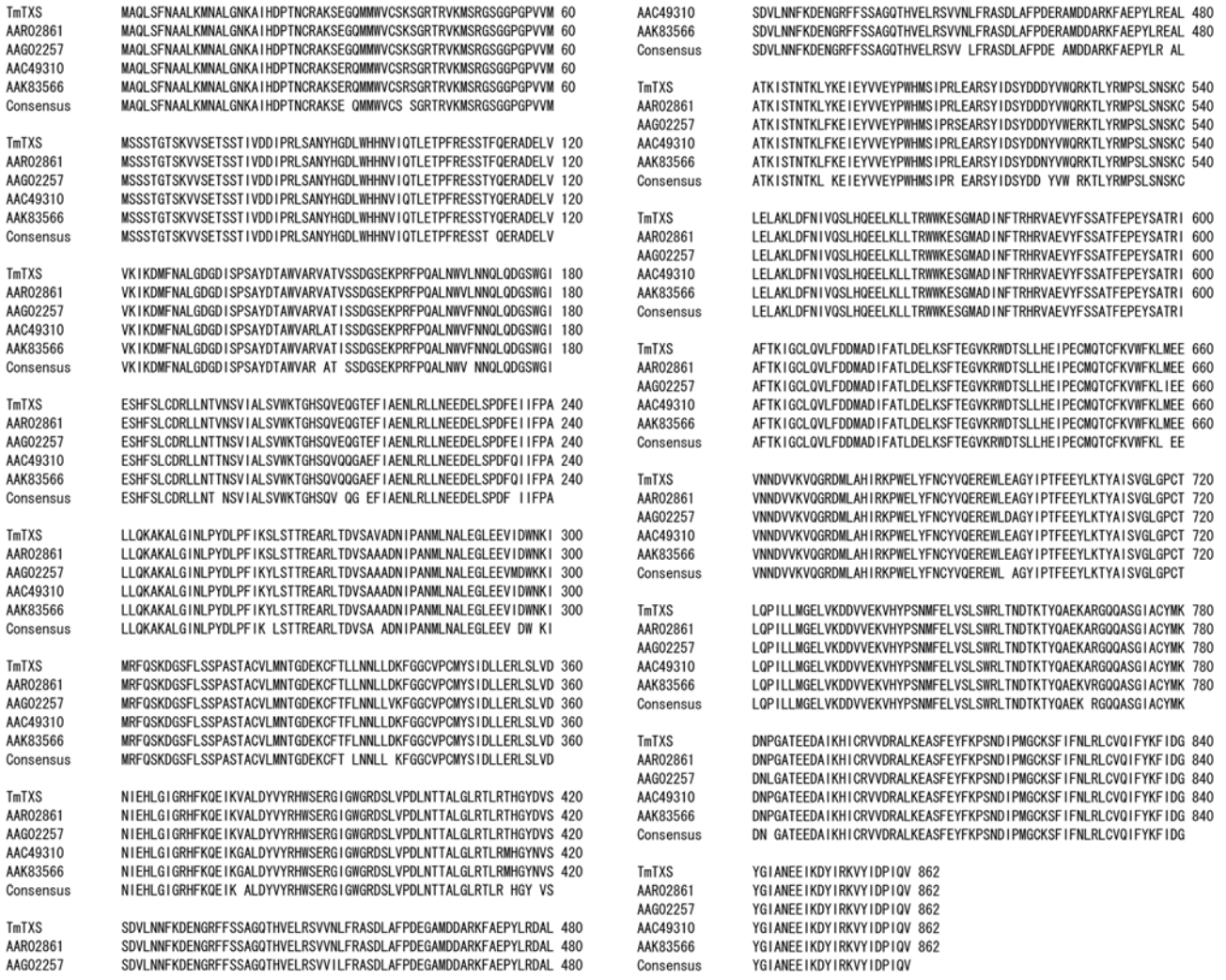


**Fig. 2.** Multiple alignment of TmTXS with other taxadiene synthases. The completely identical amino acids were indicated with single capital letter. AAR02861, *T. baccata* taxadiene synthase; AAC49310 and AAK83566, *T. brevifolia* taxadiene synthases; AAG02257, *T. chinensis* taxadiene synthase.

*chinensis* (AAG02257) to form a higher group (Fig. 5). The three groups of taxadiene synthases were derived from a common ancestor in evolution, suggesting that taxadiene synthases share a common evolutionary origin based on their similar roles and conserved structural and sequence characteristics such as amino acid homologies, conserved domain motifs and signal cleavage sites.

**Induction expression profile under different elicitors** RT-PCR was performed to investigate the expression profile of *TmTXS* under various kinds of elicitors including MJ (80 μmol/l), SN (8 μmol/l) and ACS (80 μmol/l) treatments. The results revealed that all the given elicitors could effectively induce *TmTXS* mRNA expression, and the accumulation of *TmTXS* mRNA uniformly reached the peaks at 24 h under all the treatments (Fig. 6). Significant differences ($p < 0.05$) of *TmTXS* mRNA expression were detected between the treated cells and the control.

In the MJ-induction experiment, *TmTXS* mRNA expression was clearly induced within 6 h after the start of MJ treatment with a rapid increase up to 24 h, and reached the peak at 24 h and then decreased. At 192 h the expression of *TmTXS* was very weak. The variation tendency of *TmTXS* mRNA expression induced by SN and ACS were very similar to those by MJ. Comparing the induction effects by these three elicitors above, we could find that the MJ-induced strength of the *TmTXS* mRNA was the highest among all the given elicitors, implying that *TmTXS* was probably elicitor responsive and especially MJ-responsive gene. Our results revealed that MJ was one of most effective elicitors for improving *TmTXS* expression at least at the transcriptional level, which was in good agreement with its great induction effect for improving the *Taxol* production as reported previously (Ketchum *et al.,* 1996; Robert *et al.,* 1997; Yukimune *et al.,* 2002). The induction effects of the two metal ions were weaker compared to that of MJ, which may be associated with their comparatively higher

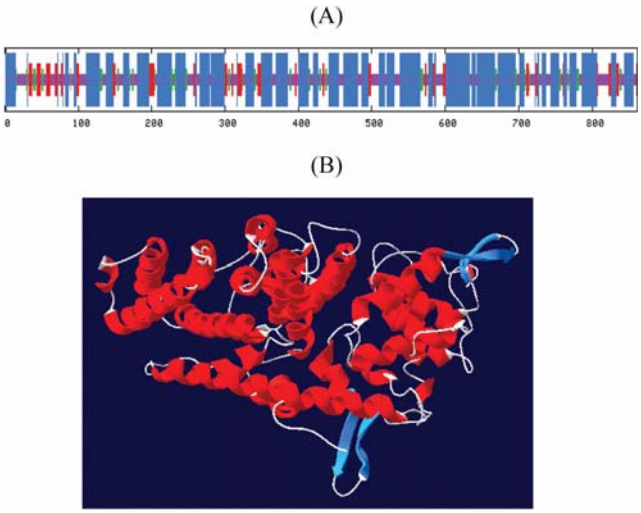


**Fig. 3.** Predicted secondary and three-dimensional structures of TmTXS. (a) The secondary structure of TmTXS. The helix, sheet, turn and coil were indicated respectively with blue, red, green and yellow vertical lines. (b) The three-dimensional structure of TmTXS. α-helix and â-sheet were indicated as red ribbon and blue patch respectively. The turn and loop were indicated by lines in white.

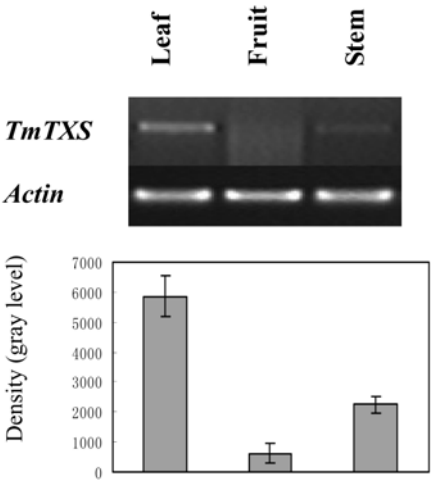


**Fig. 4.** Expression pattern of *TmTXS* in different *T. media* tissues. Total RNA (0.5 μg/sample) was isolated from the leaf, fruit and stem respectively, and subjected to one-step RT-PCR amplification (upper panel). The a*ctin* gene was used as the control to show the normalization of the amount of templates used in PCR reactions (lower panel). Data represented the means of three replicates ± SD.

treatment concentrations and action mechanisms (Wu *et al.,* 2001).

## Discussion

Plants develop different complex and effective self-protection mechanisms to adapt to environmental stresses including

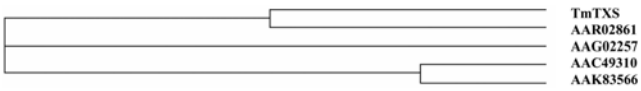


**Fig. 5.** Phylogenetic tree analysis of TmTXS and other taxadiene synthases. AAR02861, *T. baccata* taxadiene synthase; AAC49310 and AAK83566, *T. brevifolia* taxadiene synthases; AAG02257, *T. chinensis* taxadiene synthase.

abiotic and biotic factors. Taxol is a kind of phytoalexin produced by *Taxus* cells as part of the plants defense responses to pathogenic attack, so the production of Taxol may be directly associated with outer stimulus. Nowadays *Taxus* cell culture is an alternative source of paclitaxel and related taxane production, and the use of elicitors, such as fungal elicitors and MJ, has been recently one of the most effective strategies for the elicitation of taxol production which is well documented (Ketchum *et al.,* 1996; Robert *et al.,* 1997; Yukimune *et al.,* 2002), while there are a few studies reporting on the effects of heavy metals such as $La^{3+}$ and $Ce^{4+}$ (Yuan *et al.,* 1998; Wu *et al.,* 2001). However, very little is known about mRNA expression profile of genes encoding related enzymes involved in Taxol biosynthetic pathway either in different tissues of *Taxus* plants or under various kinds of elicitors treatments in *Taxus* cells, which is very important for the understanding of the expression and regulation of those genes.

In the present study, we successfully isolated the *TXS* gene from *T. media* whose deduced amino acid sequence showed high similarities to those genes from terpene synthase superfamily. The purification of TmTXS protein from *T. media* and associated analysis will further elucidate the structure and function of TmTXS. Furthermore, the transcript levels of the *TmTXS* under the three kinds of common elicitors including MJ, SN and ACS were also measured and compared in suspension cultures of *T. media*, and our results revealed that all the tested treatments could effectively induce *TmTXS* mRNA expression at least at the transcriptional level. Among the given treatments, MJ was found to be significantly the most effective in activating gene transcription in cells, compared to SN and ACS. The elicitation effect of SN and ACS may be involved with an increase in the membrane permeability due to interactions of the cells with metal ions (Wu *et al.,* 2001). As taxadiene synthase, dependent on divalent positive metal ions especially $Mg^{2+}$, catalyzes the first committed step of taxol biosynthesis (Wildung *et al.*, 1996), its enzymatic activity directly influence the Taxol production. Some metal ions such as $Ce^{2+}$ with low concentration have been reported to effectively improve Taxol production through affecting some key enzymes' activities (Yuan *et al.,* 1998). Sometimes, the induction effects were influenced by several factors such as the type of elicitors, the treatment concentration and period, the growth state of cells and the composition of culture medium *etc*. We ever investigated the effects on the mRNA expression of different elicitors such as MJ, arachidonic acid (AA) and salicylic acid (SA) with different concentrations,

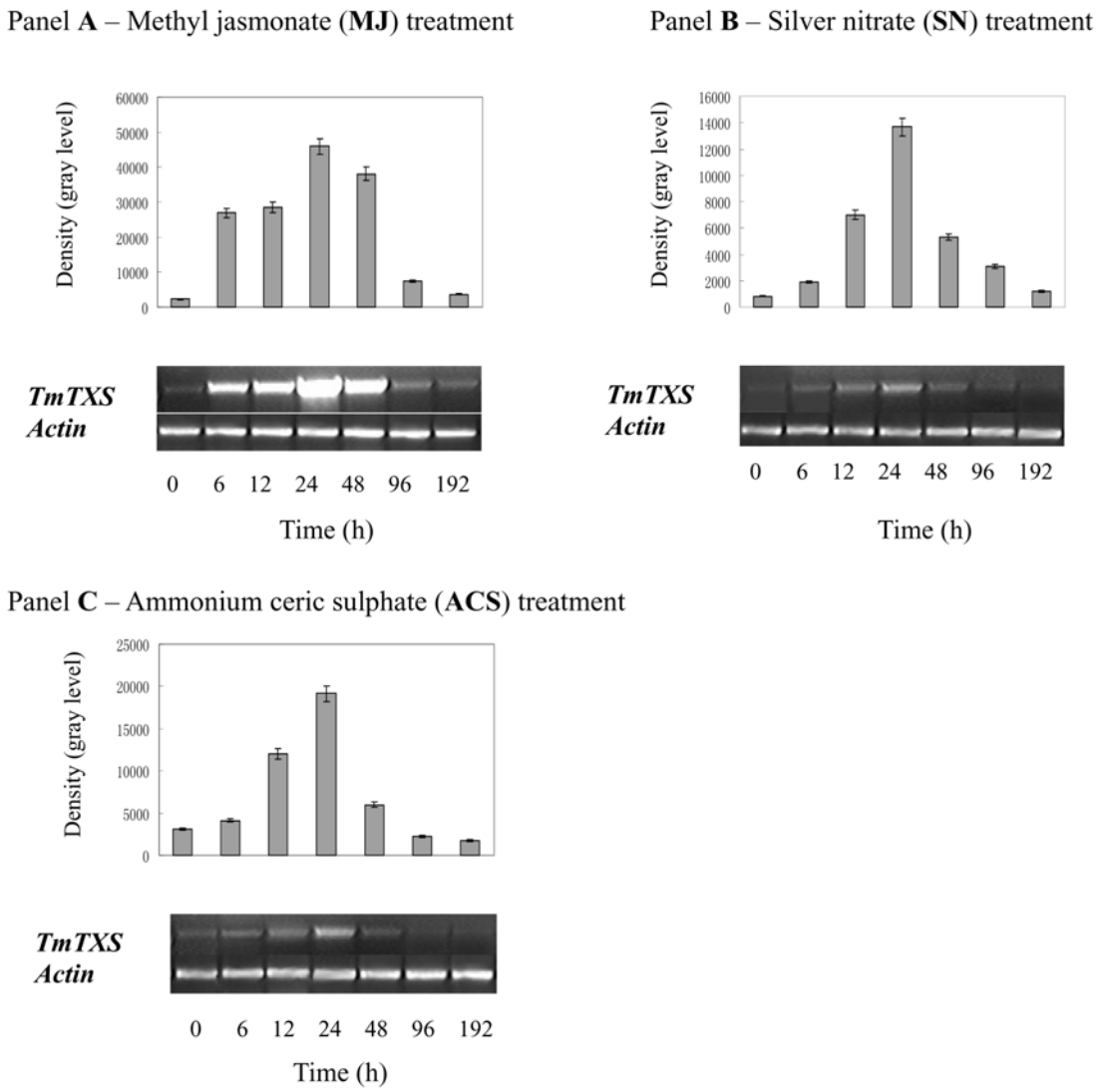


**Fig. 6.** Time courses of *TmTXS* expression profiles under different treatments in treated cells and the control. Total RNA (0.5 μg/sample) was isolated from cells after various treatments respectively, and subjected to one-step RT-PCR amplification (upper panel). The a*ctin* gene was used as the control to show the normalization of the amount of templates used in PCR reactions (lower panel). Data represented the means of three replicates ± SD.

and the results indicated that too-high concentration of elicitors inhibited the transcription while too-low concentration could not induce the transcription effectively (data not shown).

Our current study revealed that the MJ-induced strength of the *TmTXS* mRNA was the highest among all the three kinds of elicitors, indicating that MJ was a more effective elicitor than heavy metal ions, implying that *TmTXS* was MJ-responsive. MJ is a kind of plant stress hormones that act as global regulators of defense response. The mechanisms whereby jasmonate signaling triggers gene expression are just starting to be elucidated (Van der Fits *et al.,* 2001). Recently ORCA3 in *Catharanthus roseus*, as a master regulator of metabolism, was overexpressed with greatly increased TIA production (Van der Fits *et al.,* 2000), implying that transcription factor can act as another promising tool for metabolic engineering in plants in the future. The cloning of *TXS* gene will enable us to isolate its promoter. Once special element involved in jasmonate- and elicitor-responsive gene expression was identified, we could use yeast one-hybrid screening system with this element as a bait to clone the transcription factor, which is important and interesting for *Taxus* metabolic engineering in the future.

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