Molecular cloning and expression analyses of a new gene encoding 3-hydroxy-3-methylglutaryl-CoA synthase from *Taxus* × *media*

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Abstract

A new full-length cDNA encoding 3-hydroxy-3-methylglutaryl-CoA synthase (designated as *TmHMGS*, GenBank Accession No. AY644708), which catalyses the condensation of acetyl CoA and acetoacetyl CoA to form 3-hydroxy-3-methylglutaryl-CoA as an early step in the taxol biosynthetic pathway, was isolated from young leaves of *Taxus* × *media* by rapid amplification of cDNA ends (RACE) for the first time. The full-length cDNA of *TmHMGS* contained a 1431 bp open reading frame (ORF) encoding a deduced protein of 476 amino acid residues. The deduced protein had an isoelectric point of 5.23 and a calculated molecular mass of about 53 kDa. Amino acid sequence comparison analysis showed that TmHMGS had high similarity with a number of HMGSs ranging from *Schizosaccharomyces pombe* to humans, with much higher identity with other HMGSs from plants than those from yeast and humans. Phylogenic analysis showed that TmHMGS had closest relationship with HMGS from *Pinus sylvestris.* Tissue expression pattern analysis showed that *TmHMGS* expressed in needles and stems at similar level, but no expression could be detected in roots. Expression of *TmHMGS* was all induced by under different elicitors such as silver nitrate, ammonium ceric sulphate and methyl jasmonate, revealed that *TmHMGS* was an elicitor-responsive gene.

Additional key words: elicitor, expression profile, phylogenetic tree analysis, TmHMGS, yew.

Introduction

The isoprenoids, which constitute the most diverse group of natural products, serve as quinones in electron transport chains, components of membranes (sterols), in subcellular targeting and regulation (prenylation of proteins), photosynthetic pigments (carotenoids, side chain of chlorophyll), hormones (gibberellins, brassinosteroids, abscisic acid, cytokinins), plant defense compounds, and attractants for pollinators (monoterpenes, sesquiterpenes, and diterpenes) (Lange *et al*. 2000, Laule *et al*. 2003). In higher plants, there are at least two distinct routes responsible for the biosynthesis of isopentenyl diphosphate (IPP) which is the central five-carbon intermediates of all isoprenoids: the mevalonate (MVA) pathway in cytoplasm and the recently discovered 1-deoxyxylulose 5-phosphate (DXP) pathway in plastid (Bick and Lange 2003). The cytosolic MVA pathway, which involves MVA as a key intermediate, provides the precursor molecules for sterols, ubiquinone, and certain sesquiterpenes, whereas the plastidial MVA-independent pathway with DXP as the first intermediate is involved in the formation of precursors for the biosynthesis of isoprene, monoterpenes, diterpenes, carotenoids, abscisic acid, and the side chains of chlorophylls, tocopherols, and plastoquinone (Bick and Lange 2003, Laule *et al.* 2003).

The 3-hydroxy-3-methylglutaryl-coenzyme A synthase (HMGS, EC 4.1.3.5) catalyses the condensation of acetyl CoA and acetoacetyl CoA to form HMG CoA in MVA pathway. In mammalian tissues, two distinct forms of HMGS, mitochondrial form and cytoplasmic form, encoded by two different genes have been reported (Suwanmanee *et al*. 2002). The mitochondrial enzyme produces HMG-CoA that is converted to ketone bodies.

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Abbreviations: ACS - ammonium ceric sulphate; DXP - 1-deoxyxylulose 5-phosphate; MJ - methyl jasmonate; MVA - mevalonate; ORF - open reading frame; PCR - polymerase chain reaction; pI - isoelectric point; RACE - rapid amplification of cDNA ends; RT-PCR - reverse transcriptase-polymerase chain reaction; SN - silver nitrate; TmHMGS - *T. media* 3-hydroxy-3-methylglutaryl-CoA synthase.
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The HMG-CoA produced by the cytoplasmic enzyme is converted by HMG-CoA reductase (HMGR) to MVA which is subsequently converted to IPP, the universal precursor for the synthesis of isoprenoids including anti-cancer agent taxol from *Taxus* plants.

Taxus × *media* is a very important medical plant, a hybrid presumably arisen from *T. cuspidata* and *T. baccata. T. media* contains comparatively higher content of taxol, a complex diterpenoid with highly effective anticancer activity, in its needles than other *Taxus* species and is one of the major sources currently for commercial production of taxol (Kai *et al*. 2004). Due to the relative scarcity of the tree in the world and the high expense of synthetic processes, the supply of taxol sustained by isolation from the original source is very limited. As a first step in the understanding and the manipulation of *Taxus*, several genes involved in the late steps of taxol biosynthetic pathway from *Taxus* have been isolated (Walker *et al.* 2001, Kai *et al.* 2004). However, little is known about genes encoding the enzymes such HMGS involved in the early steps of taxol biosynthesis in *Taxus* or their regulation mechanism. It is interesting to know about the regulation and expression of the genes involved in taxol biosynthesis.

Until now, there is hardly any information about genes encoding HMGS in *Taxus*. Limited information concerning HMGS genes in other plants is available, whereas much is known about plant HMGR which is encoded by gene families and regulated by light, growth regulators, wounding and treatment with pathogen or elicitors (Alex *et al*. 2000). Recently some HMGS genes have been isolated from some plant species such as *Arabidopsis thaliana* (Montamat *et al*. 1995), *Brassica juncea* (Alex *et al*. 2000), *Pinus sylvestris* (Wegener *et al*. 1997) and *Hevea brasiliensis* (Suwanmanee *et al*. 2002). In this paper, we describe the cloning and characterization of a new HMGS gene from *T. media* (*TmHMGS*), as an initial step to investigate the physiological role in *T. media* in the future. Sequence analysis revealed that TmHMGS belonged to the super family of HMGS. The expression pattern of TmHMGS in various tissues including needles, roots and stems, and phylogenic analysis of TmHMGS with other HMGS were also studied.

Materials and methods

Plants and cell induction treatments: *Taxus* × *media* Rehder plants, provided by Prof. Feng Tan from Southwest Normal University, China, were grown in pots in the greenhouse under 25 °C with 16 h-photoperiod (white fluorescent tubes; irradiance of 350 μmol m^{-2} s^{-1}) and relative air humidity of 50 %. *T. media* cell lines were prepared as described previously (Kai *et al*. 2004a). Four-day-old cells were subjected to various treatments such as 8 μM silver nitrate (*Shanghai Chemical Reagent Co*., Shanghai, China), 80 μM ammonium ceric sulphate (*Shanghai Chemical Reagent Co*.) and 80 μM methyl jasmonate (MJ, *Sigma*, Milwaukee, USA) respectively, using as control cell lines without any treatment. The samples were harvested at 0, 6, 12, 24, 48, 96 and 198 h after treatment and stored at -70 °C for further analyses of *TmHMGS* expression by reverse transcriptase-polymerase chain reaction (RT-PCR). Each treatment was repeated for 3 times.

Isolation of RNA: All tissue materials (1 g) including needles, roots and stems were excised from *T. media* plants, frozen separately in liquid nitrogen, and stored at -70 ºC until use. Due to the high content of phenolic compounds, polysaccharides and the complexity of secondary products in *T. media* tissues, total RNA from *T. media* was extracted using a modification of the cetyltrimethylammonium bromide (CTAB) based RNA isolation procedure (Jaakola *et al.* 2001). Plant materials of 1 g were quickly frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle, transferred to a 50-cm^3 conical tube containing preheated 10 cm^3 extraction buffer contained 2 % (m/v) CTAB, 5 % (m/v) polyvinylpyrrolidone (PVP), 25 mM EDTA, 2.0 M NaCl, 100 mM Tris-HCl (pH 8.0), 0.5 g dm^{-3} spermidine and 5 % (m/v) β-mercaptoethanol, followed by incubation in 65 °C water bath for 15 min with intermittent shaking. After centrifugation (*Eppendorf 5417R*, Hamburg, Germany) at 18 000 *g* for 10 min at 4 °C, the supernatant was removed to new tubes and extracted twice with an equal volume of chloroform, followed by separating the phase at 18 000 *g* at 18 °C for 5 min. A ¼ volume (1.25 cm^3) of 10 M LiCl was added to the supernatant and the mixture was precipitated overnight (16 h) at 4 °C. After centrifugation (18 000 *g*) at 4 °C for 10 min, the RNA pellet was washed twice with 70 % ice-cold ethanol and dissolved in 0.5 cm^3 DEPC-treated water followed by treatment of RNA with RNase-free DNase I (*Promega,* Madison, USA) for removing genomic DNA contamination. The RNA solution was extracted twice with equal volume of chloroform followed by precipitation with two volumes of ice-cold absolute ethanol at -20 °C for 4 h and by centrifugation at 18 000 *g* for 20 min at 4 °C. The RNA pellet was washed twice with ice-cold 70 % ethanol, air dried and suspended in 0.1 cm^3 DEPC-treated water. The quality and concentration of the RNA was checked by agarose gel electrophoresis and spectrophotometer (*WFZUV-2100*, *Unico*™, Shanghai, China) analysis and the RNA samples were stored in -80 °C prior to RACE and RT-PCR analysis.

Cloning of the full-length TmHMGS cDNA by RACE: The 3'-RACE ready and 5'-RACE ready cDNA was synthesized from 5 μg of total RNA extracted from *T. media* leaves using the *SMART*™ RACE cDNA Amplification Kit, according to the manufacturer's instructions (*Clontech*, Palo Alto, USA). A degenerate primer HMGS F2(5'-AGTGA(A/G)AC(T/A)GT

(T/A/G/C)AT(T/A/C)GACAA(G/A)AGCAA-3') was designed and synthesized by *Shanghai Sangon Biotechnological Company* (China) according to the conserved regions of previously reported plant HMGSs (Montamat *et al*. 1995, Wegener *et al*. 1997, Alex *et al*. 2000, Suwanmanee *et al*. 2002). The 3'-RACE was performed in a total volume of 0.05 cm^3 containing 0.002 cm^3 cDNA, 20 μM of HMGS F2 as the forward primer, 20 μM of the Universal Primer A Mix (UPM, Long: 5'-CTAATACGACTCACTATAGGGCAAGCA GTGGTATCAACGCAGAGT; Short: 5'-CTAATACGA CTCACTATAGGGC-3') as the reverse primer, 10 μmol dNTPs, 1 × Ex PCR buffer and 5U Ex *Taq* polymerase (proofreading). PCR was performed using the following protocol: the cDNA was denatured at 94 °C for 3 min followed by 35 cycles of amplification (94 °C for 50 s, 60 °C for 50 s, 72 °C for 150 s) and by 7 min at 72 °C. The 3' PCR product was cloned into pGEM T-easy vector (*Promega*, Madison, WI, USA) followed by sequencing (*Shanghai GeneTech Company*, Shanghai, China). Based on the obtained 3' RACE fragment, a reverse primer HMGS R2 (5'-GCTACATAATCTGCATCTAGAAGAG-3') was designed, synthesized and used in 5' RACE of *TmHMGS* using the similar procedure to the 3' RACE mentioned above. The 5' RACE product was cloned into pGEM T-easy vector (*Promega*) followed by sequencing. By assembling the sequences of the 3' and 5' RACE products, the full-length sequence of *TmHMGS* was deduced, amplified via RT-PCR using a pair of primers F1 (5'-GGCAGAGAGCCATATTTGTTCTCTG-3') and R1 (5'-CTTTTCAAAATGTATATTGATTGATAAG-3'), and sequenced. The PCR amplification and sequencing for the full-length cDNA of *TmHMGS* was repeated three times to avoid PCR and sequencing error. The full-length *TmHMGS* sequence was subsequently analyzed for molecular characterization such as sequence homology and evolution analyses.

Sequence analyses: ORF finder was used to predict coding sequence and BLAST program was used to find similarity of TmHMGS with other HMGSs in the database online (http://www.ncbi.nlm.nih.gov). *Clustal W* and *Clustal X* were used for sequence alignment and phylogenetic analysis (Thompson *et al.* 1994, 1997). Phylogenic tree was constructed by neighbor-joining method and reliability of each node was established by bootstrap methods using MEGA2 software (Kumar *et al*. 2001).

Tissue expression pattern analysis: Semi-quantitative One-step RT-PCR (*Takara Company*, Shiga, Japan) was carried out to investigate the expression of *TmHMGS* in different tissues including needles, roots and stems of *T. media* using the forward primer CF1 (5'- ATGGCGTCCCCTCAAGAAAACG-3') and reverse primer CR1 (5'-CTACAATTCACCATTGCTACTGA-3') using the procedure mentioned before (Kai *et al*. 2004, 2005a). RT-PCR images were captured using a UVP transilluminator with image processed by *Labworks* (*UVP*, Upland, CA, USA).

Results and discussion

Cloning and sequencing of the full-length cDNA of TmHMGS: The full-length cDNA of *TmHMGS* was obtained by using combination of techniques involving 3' RACE, 5' RACE and RT-PCR. Using degenerate primer HMGS F2 designed based on the conserved regions of plant HMGSs and the reverse primer UPM, a 1427 bp PCR product was obtained and sequenced, which had similarity with other plant *HMGSs* (data not shown). The 5' flanking sequence (840 bp) was subsequently obtained by 5' RACE using primer UPM as the forward primer and HMGS R2 as the reverse primer. By aligning and assembling the sequences of 3' RACE and 5' RACE products, the full-length cDNA of *TmHMGS* was obtained, which was 1776 bp long and contained an ORF of 1431 bp, a 5' untranslated region (UTR) of 97 bp and a 3' UTR of 219 bp (Fig. 1). The translation start site (GGA*ATG*G) contained the highly conserved G residues at positions -3 and +4, which was in good agreement with the Kozak ruler (Kozak 1987). The 5' UTR consisted of at least 97 bp with 60 % of A+T content, similar to most leader sequences in plant genes. The G+C content in the coding region was 43 %, which was a little higher than that (33 %) in the non-coding region of the gene. The 3' UTR possessed typical low G+C content (30 %). The deduced protein was 476 amino acids with an isoelectric point (pI) of 5.23 and calculated molecular weight of about 53 kDa, which was very similar to previously reported HMGS (Wegener *et al*. 1997, Suwanmanee *et al*. 2002). The hydrophobicity profile prediction result showed that TmHMGS was a hydrophobic protein (data not shown). The richest amino acid in the deduced TmHMGS was Leu (9 % by frequency), followed by Ala (8 %), Glu (7 %), Gly (7 %) and Lys (6 %). Acidic and basic amino acids constituted 12 and 9 % of the polypeptide, respectively. 32 % of the total amino acids were charged and the percentages of polar and hydrophobic amino acids were 31 and 33 %, respectively.

Sequence analysis of TmHMGS: Sequence comparison by performing Blast Search (http://www.ncbi.nih.gov) showed that TmHMGS had higher homology with other HMGSs such as *Pinus sylvestris* HMGS (PsHMGS) (Wegener *et al*. 1997), *Hevea brasiliensis* HMGS (HbHMGS) (Suwanmanee *et al*. 2002, 2004), *Brassica juncea* HMGS (BjHMGS) (Alex *et al*. 2000) and *Arabidopsis thaliana* HMGS (AtHMGS) (Montamat *et al*. 1995) from plant species, indicating that HMGS belonged to HMGS superfamily. On the protein level, TmHMGS was 85, 75, 74, and 73 % identical to PsHMGS (CAA65250), HbHMGS (AAK73854), BjHMGS

(AAF69804) and AtHMGS (CAA58763) respectively, while TmHMGS was 92, 87, 85, and 85 % similar to PsHMGS, HbHMGS, BjHMGS and AtHMGS respectively (Fig. 2). Interestingly, TmHMGS even

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   1                                                                                                                ggcagag
   8 agccatatttgttctctgtgtttccagcggcaaaaactcggtgcaaggtagaggaactgtaaactatttttgaatttttttcaataggga
  98 atggcgtcccctcaagaaaacgttggtattttggcgatggaggtttactttccgactacttgtgtccagcaggatgccctggaaacattt
     M  A  S  P  Q  E  N  V  G  I  L  A  M  E  V  Y  F  P  T  T  C  V  Q  Q  D  A  L  E  T  F
 188 gatggagtaagtaaaggaaaatatacaattggccttggacaagactgcatgactttctgcacagatttggaagatgtgatttcaatgagc
     D  G  V  S  K  G  K  Y  T  I  G  L  G  Q  D  C  M  T  F  C  T  D  L  E  D  V  I  S  M  S
 278 ttgacagttgtaacgtctcttttggaaaaatatgctattgatccaaaacaaattggccgcttggaagttggtagcgaaactgttattgac
     L  T  V  V  T  S  L  L  E  K  Y  A  I  D  P  K  Q  I  G  R  L  E  V  G  S  E  T  V  I  D
 368 aagagcaagtcaataaagacttggttgatgtgcatttttgagaagtgtggaaatactgaaattgaaggtgtggactcaacaaatgcatgc
     K  S  K  S  I  K  T  W  L  M  C  I  F  E  K  C  G  N  T  E  I  E  G  V  D  S  T  N  A  C
 458 tatggtggaactgcagctctatttaactgtgttaactgggttcaaagtagttcttgggatgggcgatatggtcttgttgttgctacagac
     Y  G  G  T  A  A  L  F  N  C  V  N  W  V  Q  S  S  S  W  D  G  R  Y  G  L  V  V  A  T  D
 548 agcgcagtctatgctgaagggccagctcggcctactgggggagcagctgccattgctatgttgatagggcccaatgctccaatagcattt
     S  A  V  Y  A  E  G  P  A  R  P  T  G  G  A  A  A  I  A  M  L  I  G  P  N  A  P  I  A  F
 638 gagaacagatacaggggaacgcacatggctcacgcatatgacttttataagcccaatcttgctagcgagtacccggttgtagatggaaag
     E  N  R  Y  R  G  T  H  M  A  H  A  Y  D  F  Y  K  P  N  L  A  S  E  Y  P  V  V  D  G  K
 728 ctctcacaaacttgctatctaaaggcactggactcttgctacaaacggttttgtaacaagtttgaaaagggagaaggacatcagttctct
     L  S  Q  T  C  Y  L  K  A  L  D  S  C  Y  K  R  F  C  N  K  F  E  K  G  E  G  H  Q  F  S
 818 cttctagatgcagattatgtagcatttcactctccatacaataagcttgtgcagaagagctttgctcgactattgttcaatgatttttca
     L  L  D  A  D  Y  V  A  F  H  S  P  Y  N  K  L  V  Q  K  S  F  A  R  L  L  F  N  D  F  S
 908 agacatgccagttctgctggaaaggatgcacaagagaagctggaaccctatgctggtttgtctgaagaagagagctatagcagccgtgat
     R  H  A  S  S  A  G  K  D  A  Q  E  K  L  E  P  Y  A  G  L  S  E  E  E  S  Y  S  S  R  D
 998 ctagaaaaggtttctcagcaggctgcgaagccattgtatgatgaaaaagtgcagccatcaactttattgccaaaaaaagaaggcaacatg
     L  E  K  V  S  Q  Q  A  A  K  P  L  Y  D  E  K  V  Q  P  S  T  L  L  P  K  K  E  G  N  M
1088 tatacagcatctctttatgctgcacttgcctcgattatacataacaagtatagcacgctggaaggtcaaagggtgctcatgttctcttat
     Y  T  A  S  L  Y  A  A  L  A  S  I  I  H  N  K  Y  S  T  L  E  G  Q  R  V  L  M  F  S  Y
1178 ggaagtgggcttgcttcgacaatgttctcacttaaaattcgtgaaggtcagcatccttttatcctgtcaaacattgctgaagctatggat
     G  S  G  L  A  S  T  M  F  S  L  K  I  R  E  G  Q  H  P  F  I  L  S  N  I  A  E  A  M  D
1268 ctccaaagcaaacttgaatcccaacatgagttttctcctgaagattttgtggacaacttgaggctgatggagactctatatggagcaaaa
     L  Q  S  K  L  E  S  Q  H  E  F  S  P  E  D  F  V  D  N  L  R  L  M  E  T  L  Y  G  A  K
1358 gacttcgtttcatgtgctcaacataatttgctaaggcctgggacttttatttgactgaagtagattcaatgtaccggcgtttctattcc
     D  F  V  S  C  A  Q  H  N  L  L  R  P  G  T  F  Y  L  T  E  V  D  S  M  Y  R  R  F  Y  S
1448 cagaaattggttagccttgatgataactgtagggagacgaagtttgcaaatggtactatcagtagcaatggtgaattgtagtacatttta
     Q  K  L  V  S  L  D  D  N  C  R  E  T  K  F  A  N  G  T  I  S  S  N  G  E  L  *
1538 cttcgagatggtcagactggtctacatatcatttgtaagaggtcgaatatttgttctaaaatgtaaaatagcatatttttgaggcccccc
1628 ttttatttgttatttatttttatttttatgtgatgtgaaagcattgagaagtgtgaaatgtgcatgagaactgtaccagttaacttggtt
1718 tacttatcaatcaatatacattttgaaaagaaaaaaaaaaaaaaaaaaaaaaaaaaaa
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Fig. 1. The full-length cDNA sequence and deduced amino acid sequence of *Taxus* × *media* 3-hydroxy-3 -methylglutaryl-CoA synthase (TmHMGS). The start codon (ATG) was boxed and the stop codon (TGA) was underlined italically. The conserved motifs were underlined.

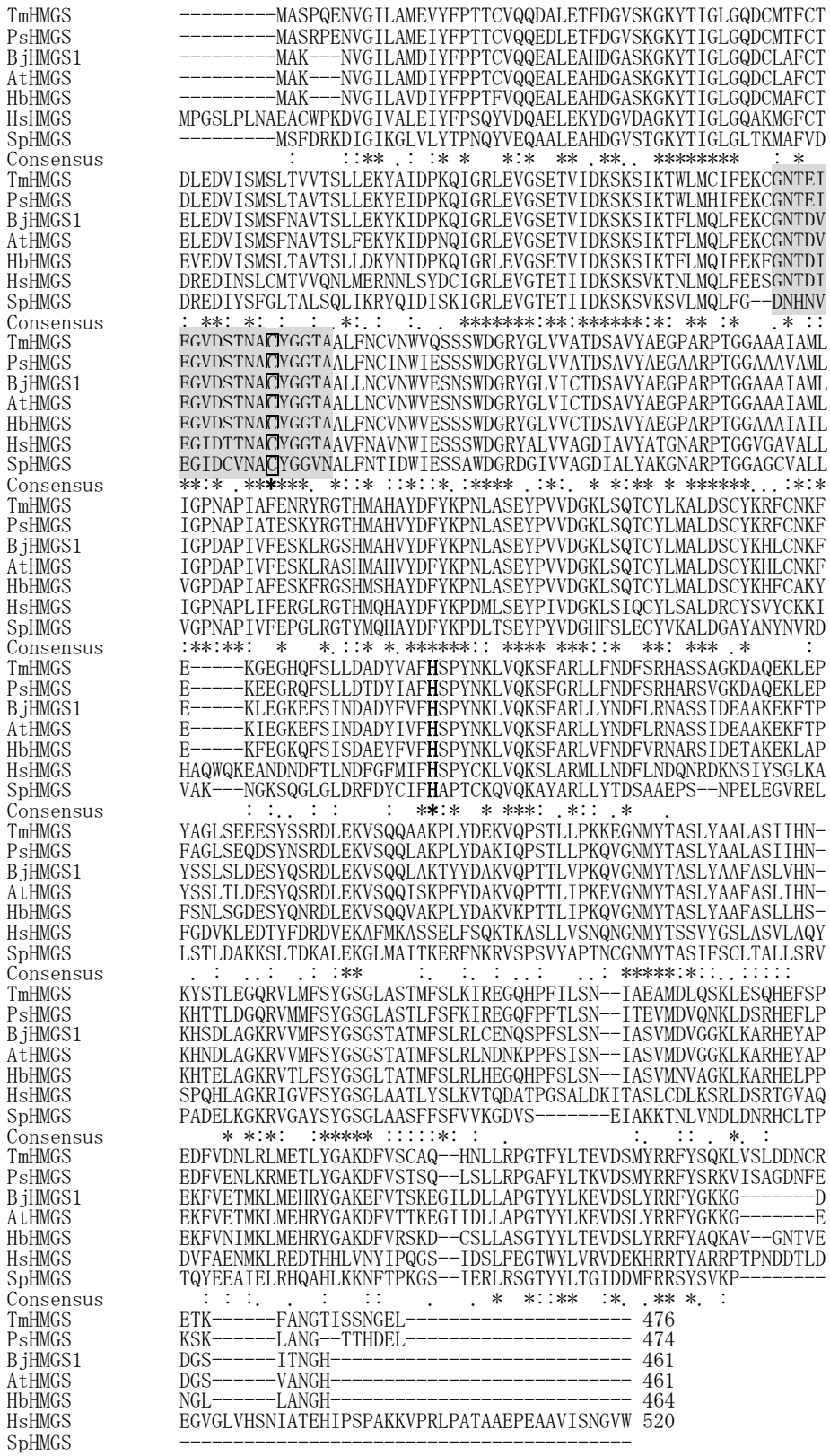

Fig. 2. Multiple alignment of TmHMGS with other HMGSs. The completely identical, conservative and semi-conservative amino acid residues were indicated with "*", ":" and ".", respectively. The conserved motifs were gray-shaded, and conserved cysteine and histidine residues were boxed and bolded, respectively. PsHMGS (*P.sylvestris* HMGS, CAA65250), BjHMGS (*B. juncea* HMGS, AAF69804), HbHMGS (*H. brasiliensis* HMGS, AAK73854), AtHMGS (*A. thaliana* HMGS, CAA58763), HsHMGS (*H. sapiens* cytoplasmic HMGS, CAA47061), SpHMGS (*S. pombe* HMGS, S61875).

showed high similarity to HMGS from vertebrates and fungi, respectively (data not shown).

Multiple sequence alignment of TmHGMS with some homologous HMGSs from plant, yeast and human revealed that comparatively higher conservation exited among various organisms (Fig. 2), suggesting HMGS may play an important role in biological function of providing basal HMG CoA and therefore it was fairly conserved in evolution. The highest conservation with *P. sylvestris* is due to both plants belonging to gymnosperms. TmHGMS also contained the active site peptide for HMGS activity, corresponding to amino acids G^{107}-A^{125}, and showed conservation across species (Fig. 2, marked in gray), which was identical to active region of PsHMGS and showed a 16 out of 21 match with the active region of avian mitochondrial and hamster cytoplasmic HMGS (Wegener *et al*. 1997). Conserved cysteine and histidine residues important for HMGS activity were also identified in TmHGMS (Fig. 2).

The secondary structure of TmHMGS was analyzed by *SOPMA* (Geourjon and Deléage 1995) and the result showed that the putative TmHMGS peptide contained 40 % of alpha helix, 19 % of extended strand, 8 % of beta turn, and 33 % of random coil. The random coil and alpha helix constituted interlaced domination of the main part of the secondary structure.

Molecular evolution analysis: To investigate the evolutionary relationships among TmHMGS and other HMGSs, a phylogenetic tree was constructed based on the deduced amino acid sequences of TmHMGS and other HMGSs (Fig. 3). The result showed that TmHMGS, PsHMGS, *Oryza sativa* HMGS (OsHMGS, No. AAO15287), HbHMGS, BjHMGS and AtHMGS were grouped into a cluster (plants group). HMGSs from human, pig, mouse and rat, *etc.*, formed a cluster (mammalian group), among which mitochondria HMGSs were classified into the one subgroup while cytoplasmic HMGSs formed the other subgroup. *Blattella germanica* HMGS (CAA52032) and *Dendroctonus jeffreyi* HMGS (AAF89580) naturally formed another cluster (insects group). In addition, HMGS from *Phycomyces blakesleeanus* (CAC18553), *Schizosaccharomyces pombe* (S61875) and *Saccharomyces cerevisiae* (NP_013580) were grouped into another cluster (fungi group). The four groups of HMGSs were derived from a common ancestor in the evolution. Our result suggests that HMGSs share a common evolutionary origin based on their similar roles and conserved structural and sequence characteristics such as amino acid homologies and conserved domain motifs. This implies that the conserved motifs may play an important role 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 HMGSs' structures and functions.

Tissue expression pattern analysis: *T. media* is one of the most important sources for commercial production of taxol as its needles contain comparatively higher content of taxol than other *Taxus* species. The aerial parts (needles and stems) of *T. media* plants are usually used as the source for taxol extraction, as taxol content in aerial parts is higher than that in underground parts (roots) (Liao *et al*. 2004). Hence, it is interesting to know if TmHMGS expression is positively correlated with the taxol content in different parts of the *T. media* plant. Using total RNA isolated from different tissues including needles, roots and stems, *TmHMGS* expression in various tissues of *T. media*

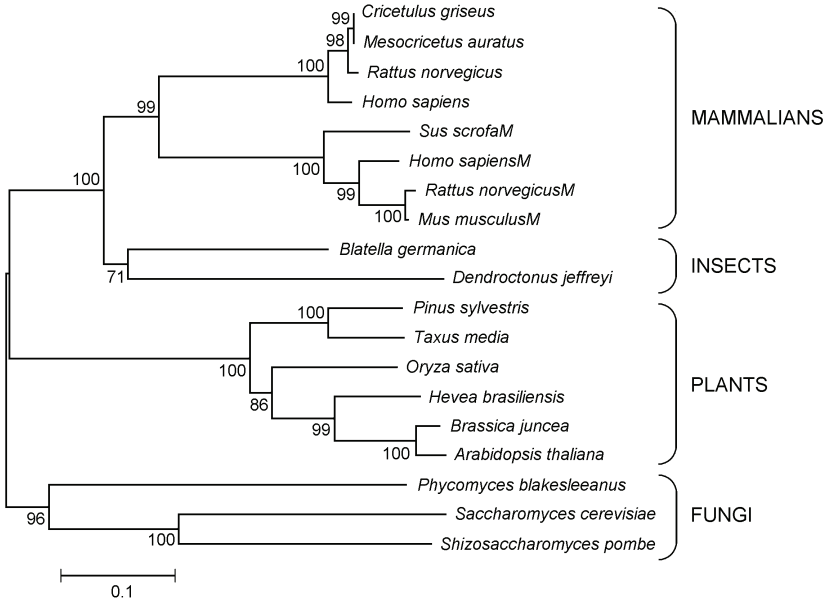

Fig. 3. Phylogenic analysis of TmHMGS and other HMGSs from *B. germanica* (CAA52032), *C. griseus* (A25332), *M. auratus* (AAA37076), *R. norvegicus* (P17425), *S. cerevisiae* (NP_013580), *D. melanogaster* (NP_729119), *K. griseola* (BAB07822), *P. blakesleeanus* (CAC18553), *D. jeffreyi* (AAF89580), *R. norvegicusM* (mitochondrial, P22791), *S. scrofa*M (mitochondrial, O02734), *M. musculus*M (mitochondrial, P54869), *H. sapiens*M (mitochondrial, S71623).

was analyzed by RT-PCR with the primers CF1 and CR1. The result showed that *TmHMGS* expression could be detected in needles and stems at similar level, but no expression could be detected in roots (Fig. 4) similar to *TmTXS* (Kai *et al.* 2005a). Therefore the *TmHMGS* is considered to be a tissue-specific expressing gene, implying that the expression of *Tm-HMGS* correlates with the taxol biosynthesis and reflects the higher taxol contents in the needles and stems than in roots.

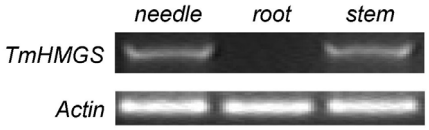

Fig. 4. Expression pattern of *TmHMGS* in different *T. media* tissues. Total RNA (0.5 g dm^{-3}) was isolated from needle, root and stem respectively, and subjected to one-step RT-PCR amplification (*upper panel*). *Actin* gene was used as the control to show the normalization of the templates in PCR reactions (*lower panel*).

TmHMGS expression profiles under elicitor treatments: The use of elicitors, such as fungal elicitors and methyl jasmonate (MJ), for the elicitation of taxol production has been recently documented (Ketchum *et al*. 1996, Robert *et al*. 1997, Yukimune *et al*. 2002). However, 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. In addition, the mechanism of the alternation of gene expression associated with the heavy mental ion such as Ag^{+} and Ce^{4+} which improves the taxol production is unclear. Therefore, it is worthwhile investigating expression profiles of genes involved in taxol biosynthesis induced by various elicitors including MJ, which will be helpful to understand related gene expression regulation and uncover molecular induction mechanism for further improving taxol biosynthesis.

To understand the role of TmHMGS in responses to environmental stresses and plant defense, we treated *T. media* cells with rare earth (Ce^{4+}), heavy mental ion (Ag^{+}) and the plant defense signal molecules (MJ), respectively. For the time-course experiment, four-day-old cells subjected to various kinds of treatments were harvested at 0, 6, 12, 24, 48, 96 and 192 h after elicitor induction followed by extraction of total RNA for further analyses of expression profiles of *TmHMGS* by RT-PCR. RT-PCR analysis revealed that the expression of *TmHMGS* was obviously induced by all the three tested elicitors including SN and ACS and MJ (Fig. 5). Under SN treatment, *TmHMGS* expression was significantly increased 6 h post-treatment and peaked 48 h post-treatment. The expression was then decreased at 96 h of treatment, but still maintained at an increased level at 192 h compared with the control. Under ACS treatment, *TmHMGS* expression was also significantly increased 6 h post-treatment and reached the peak level at 192 h of treatment, which was similar to expression profiling of *T. media* 5-alpha-taxadienol-10-beta-hydroxylase treated by ACS (Kai *et al.* 2005b). It may be explained that Ce^{4+}, an ion with a strong oxidative ability that could induce programmed cell death and result in obvious soluble proteins change (Qiao *et al.* 2003), was toxic to normal cell growth to channel secondary metabolic pathway in *Taxus* including taxol biosynthetic pathway. Consequently, mRNA expression profile of genes encoding related enzymes, involved in taxol biosynthetic pathway, was

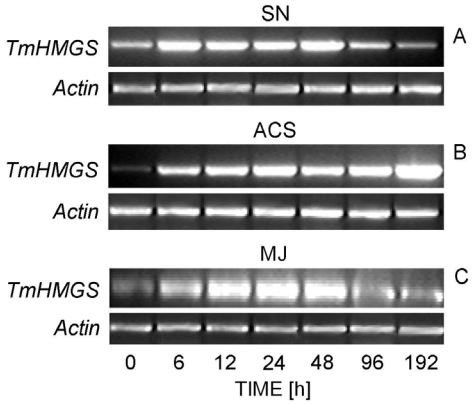

Fig. 5. Expression profile of *TmHMGS* induced by different chemical elicitors in *T. media* cells. Time courses of *TmHMGS* expression profiles under different elicitor treatments in treated cells (all 80 μM) and control. *Actin* gene was used as the control to show the normalization of the amount of templates in PCR reactions (*lower panel*).

influenced by environmental stresses in *Taxus* cells. Under MJ treatment, *TmHMGS* expression was significantly strengthened 6 h post-MJ treatment, reached the peak level at 48 h of treatment and subsequently declined thereafter. This result indicates that *TmHMGS* may play an important role in the processes of plant defense response. Comparing the induction effects by the three elicitors above, we could find that expression profiles of *TmHMGS* under different elicitors were diverse, which might be attributed to their different action mechanisms. For example, 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 (Yuan *et al*. 1998, Wu *et al*. 2001). Our results revealed that *TmHMGS* was elicitor-responsive and could be effectively elicited at least at transcription level, coinciding with their induction effects for improving the taxol production as reported previously (Ketchum *et al*. 1996, Robert *et al*. 1997, Yukimune *et al*. 2002). According to our results (data not shown), almost all the known genes encoding related enzymes involved in taxol biosynthesis were coordinately induced by MJ, providing molecular evidence for improving taxol content by MJ. ORCA-similar transcript factors may exist in *Taxus* cells for central control of several metabolites' biosynthetic genes, as illustrated for terpenoid indole alkaloids (TIA) in *Catharanthus roseus* (Van der Fits and Memelink 2000). The cloning of *TmHMGS*, the MJ-responsive gene, enables us to isolate and analyze its upstream regulation sequence further.

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