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Full Length Research Paper

The 1-hydroxy-2-methyl-butenyl 4-diphosphate reductase gene from *Taxus media*: Cloning, characterization and functional identification

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1-Hydroxy-2-methyl-butenyl 4-diphosphate reductase (HDR:EC: 1.17.1.2) is the last and key enzyme involved in the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway, which provides the general precursors for taxol biosynthesis and is the important candidate gene for metabolic engineering of the taxol biosynthetic pathway. The gene encoding HDR from *Taxus media* (designated as *TmHDR*; GenBank accession number: EF541129) was cloned using RACE, characterized at the levels of bioinformatics and tissue expression pattern, and finally functionally identified with the genetic complementation. The full-length cDNA of *TmHDR* was 1987 bp containing an 1425-bp open reading frame (ORF) that encoded a polypeptide of 474 amino acids with a calculated molecular mass of 53.2 kDa and an isoelectric point of 5.65. Comparative and bioinformatic analysis revealed that TmHDR showed extensive homology with HDRs from other plant species. The subcellular prediction showed that TmHDR owned a plastidial transit peptide of 47 amino acids at its N terminus, which directed TmHDR to plastid. The phylogenetic analysis revealed that TmHDR belonged to gymnosperm HDRs. The tissue expression pattern analysis indicated that *TmHDR* expressed in all tested tissues including cortices, stems, roots and leaves but at different levels. The highest expression level of *TmHDR* was found in leaves, followed by roots and cortices; and the expression of *TmHDR* was very low in stems. Finally, *TmHDR* was functionally expressed in lethal *E. coli HDR* mutant and could rescue the mutants. So, the genetic complementation assay demonstrated that *TmHDR* did encode the protein that had the typical activity of HDR proteins. In summary, the present study will be helpful to understand more about the role of HDR involved in the Taxol biosynthesis at the molecular level and provides an important candidate gene for metabolic engineering of the taxol biosynthesis in Taxus species plants.

Key words: 1-hydroxy-2-methyl-butenyl 4-diphosphate reductase (HDR), cloning, genetic complementation, *Taxus media*, tissue expression.

INTRODUCTION

Isoprenoids are the most abundant natural products found in all living organisms (Sacchettini and Poulter, 1997), many of which have pharmaceutical activities. Among them, Taxol should be the most famous one, a member of diterpenes and the most promising anti-tumor agent (Rowinsy et al., 1990). Taxol is the first-line drug widely used in the treatment of a variety of cancers, in-

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cluding carcinomas of the ovary, breast and AIDS-related Kaposi's sarcoma (Alexander, 2001). Taxol is mainly extracted from Taxus plants, but the content of Taxol is extremely low in all Taxus species. The very limited production but the huge commercial demand makes the price of Taxol very high, and many cancer victims cannot afford the drug that is necessary to cure their cancers. It is urgent to find alternative sources of Taxol. Metabolic engineering of Taxol biosynthesis pathway might be a promising way to provide more Taxol, which is based on understanding the Taxol biosynthesis pathway at the level of molecular biology. So, to map the Taxol biosynthetic pathway at the level of molecular biology may lead Taxol production to a bright path.

The diterpenes including Taxol are derived from the five-carbon units, isopentenyl diphosphate (IPP) and its isomer, dimethylallyl diphosphate (DMAPP), both of which are the universal precursors of all isoprenoid compounds (Liao et al., 2006). In plants, IPP and DMAPP are independently synthesized via the cytosolic mevalonate (MVA) pathway and the plastidial methylerythritol phosphate (MEP) pathway (Rohmer, 2003; Kuzuyama and Seto, 2003). The five-carbon precursors including IPP and DMAPP were only provided by the cytosolic MVA pathway according to the classic theory (Zamir et al., 1992); while more and more researches revealed that IPP and DMAPP used for Taxol biosynthesis were mainly provided by the plastidial MEP pathway (Eisenreich et al., 1996). There exists seven enzymatic steps involved in the MEP pathway and the last enzymatic step is catalyzed by 1-hydroxy-2-methyl-butenyl 4-diphosphate reductase (HDR), formerly designated LytB (Altincicek et al., 2002) or IspH protein (Wolff et al., 2003).

HDR directly converts 1-hydroxy-2-methyl-butenyl 4-diphosphate (HMBPP) into a mixture of IPP and DMAPP with the ratio of 5:1 (Altincicek et al., 2002). Only a few full-length cDNAs encoding HDR were cloned from plants including *Adonis aestivalis* (Cunningham et al., 2000), *Arabidopsis thaliana* (Hsieh and Goodman, 2005) and also from medicinal plants such as *Ginkgo biloba* (Kim et al., 2008; Lu et al., 2008), *Camptotheca acuminata* (Wang et al., 2008).Unfortunately, there are no reports on cloning and characterization of the gene encoding HDR from Taxus species. In the present study, we cloned, characterized and functionally expressed the HDR gene from *Taxus media* so that it will facilitate understanding the role of HDR in Taxol biosynthesis and regulating the key enzymatic step at the molecular level.

MATERIALS AND METHODS

Plant materials and RNA isolation

The roots, stems, leaves and cortices were collected from *Taxus media* plant growing in the medicinal plant garden in Southwest University (Chongqing, China). After collection, the plant materials were immediately immersed into liquid nitrogen to store for the future using for total RNAs isolation. Total RNAs from each organ were isolated using RNAplant™, a plant RNA isolation kit (TIAGEN, Beijing, China).

Cloning of the core fragment of TmHDR

Single-strand cDNAs were synthesized from 5 μg of total RNAs with an oligo $(dT)_{17}$ primer and reversely transcribed according to the manufacturer's protocol (PowerScript™, CLONTECH, USA). After RNase H treatment, the single-strand cDNA mixtures were used as templates for PCR amplification of the conserved region of hdr from T. media. Two degenerate primers, FDPHDR (5'-GTTGA(G/A)AAG CA(T/C)AAG(A/C)AGGG-3') and RDPHDR (5'-TCA(G/C)(A/T)(A/G) TCAATCCAGTA(A/T)GA-3'), were designed according to the conserved sequences of HDR genes from other plant and used for the amplification of the core cDNA fragment of *TmHDR* by standard gradient PCR amplification (from 48 to 55 μg) on BioRad My Cycler (USA). The PCR products were purified and sub-cloned into pGEM T-easy vector (Promega, Madison, WI, USA) followed by sequencing.

3`RACE and 5`RACE of TmHDR

The core fragment was subsequently used to design the gene-specific primers for the cloning of cDNA ends of *TmHDR* by RACE. SMART® RACE cDNA Amplification Kit (CLONTECH, USA) was used to clone the 3'-end and 5'-end of *TmHDR* cDNA. The first strand 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) and used as templates for 3'RACE and 5'RACE respectively. The 3'-end of *TmHDR* cDNA was amplified using two 3'-gene-specific primers and the universal primers (UPM and NUP) provided by the kit. For the first PCR amplification of 3'RACE, TMHDR3-1 (5'-CTTATGTTGGTAGTTGGTGG-3') and UPM (5'-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGA GT-3'and 5'-CTAATACGACTCACTATAGGGC-3') were used as the PCR primers, and 3'-RACE-ready cDNA was used as template. For the nested PCR amplification of 3'RACE, TMHDR3-2 (5'-TTCCAGCAATACATCACACC-3') and NUP (5'-AAGCAGTGGTA TCAACGCAGAGT-3') were used as the nested PCR primers, and the products of the first PCR amplification were used as templates.

The 5'-end of *TmHDR* cDNA was also amplified using two 5'-gene-specific primers and the universal primers provided by the kit. For the first PCR amplification of 5'RACE, TMHDR5-1 (5'-TCC TGCAAAAGAGGCGGTAGCAACAG-3') and UPM were used as the first PCR primers, and 5'-RACE-ready cDNA was used as template. For the nested PCR amplification of 5'RACE, TMHDR5-2 (5'-AGGCGGTAGCAACAGTTTCTTCATGAG-3') and NUP were used as the nested PCR primers, and the products of the first PCR amplification were used as templates. For the first and nested PCR amplification of *TmHDR* 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℃ and 3 min at 72℃). The nested 3'RACE and 5'RACE products were purified and sub-cloned into pGEM T-easy vector followed by sequencing.

Amplification of TmHDR full-length cDNA

By assembling the sequences of 3'RACE, 5'RACE and the core fragment on Contig Express (Vector NTI Suite 8.0), the full-length cDNA sequence of *TmHDR* was deduced. According to the deduced *TmHDR* cDNA sequence, two gene-specific primers, FTMHDR (5'-GGAACTGCCATTTTGTTTATTCATC-3') and RTMH

DR (5'-GGTTTATTGATCAATCCTTTATTTGC-3') were designed, synthesized and used to amplified the full-length of *TmHDR* from 5`-RACE-ready cDNA samples through proof-reading PCR. Three independent mono-clones were sequenced to confirm the sequence of *TmHDR*. Finally *TmHDR* was submitted to GenBank to be assigned with an accession number.

Comparative and bioinformatic analysis

Comparative analyses were carried out online at the NCBI websites through BLAST (Altschul et al., 1997). The theoretical molecular mass and isoelectric points were computed at the EXPASY website (http://www.expasy.org). The subcellular location was predicted by TargetP (Emanuelsson et al., 2000). The multiple alignments of TmHDR and HDRs from other plant species were performed with CLUSTAL W (Thompson et al., 1994) using default parameters. A phylogenetic tree was constructed using the neighbor-joining method provided by MEGA3 (Kumar et al. 2004).

Tissue expression pattern analysis

Semi-quantitative one-step RT-PCR was carried out to investigate the expression profile of *TmHDR* in different tissues including roots, stems, leaves and cortices of *T. media*. Aliquots of 0.5 µg total RNA extracted from roots, stems, leaves and cortices of *T. media* were used as templates in one-step RT-PCR reaction with the forward primer FEXTMHDR (5'-GGAACTGCCATTTTGTTTATTCATC-3') and the reverse primer REXTMHDR (5'-GGTTTATTGATCAATC CTTTATTTGC-3') specific to the full-length sequence of *TmHDR* using one-step RNA PCR kit (Takara, Japan). Amplifications were performed under the following conditions: 50°C for 30 min, 94°C for 2 min followed by 25 cycles of amplification (94°C for 30 s, 52°C for 30 s and 72°C for 2 min), then 72°C 10 min. Meanwhile, the RT-PCR reaction for the house-keeping gene (*actin* gene) using specific primers actF (5'-GTGACAATGGAACTGGAATGG-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 in RT-PCR as an internal control. Amplifications were performed under the following conditions: 50°C for 30 min, 94°C for 2 min followed by 25 cycles of amplification (94°C for 30 s, 50°C for 30 s and 72°C for 1 min), then 72°C 10 min. PCR products (15 µL) were separated on 1.0% agarose gels stained with GoldView (SBS company, Beijing, China).

Functional expression of TmHDR in *E. coli* HDR mutant

E. coli hdr mutant strain MG1655*ara*<>*hdr*, which was successfully used to identify the function of Plant HDR gene, was maintained on Luria-Bertani (LB) medium containing 50 µg/mL kanamycin (Kan) and 0.2% (w/v) arabose (Ara) (McAteer et al., 2001). Hsieh and Goodman successfully used the mutant to identify the function of *Arabidopsis* HDR gene (Hsieh and Goodman, 2005). The coding sequence of TmHDR was amplified and the restriction sites including *Bam*H I and *Sac* I were introduced into the ends of the coding sequence of TmHDR; then the coding sequence of TmHDR was introduced into pQE30 through *Bam*H I and *Sac* I. The reconstructed vector was named as pQE-*TmHDR*. The pQE-*TmHDR* plasmid was transformed into the *E. coli hdr* Mutant and selected on LB medium plates containing 50 µg/mL Kan, 50 µg/mL ampicillin (Amp), 0.2% glucose and 0.5 mM IPTG. The presence of the pQE-*TmHDR* plasmid in surviving colonies was verified. As a control, the empty pQE30 vector was transformed into the *E. coli hdr* Mutant and selected on LB medium plates containing 50 µg/mL Kan, 50 µg/mL Amp and 0.5 mM IPTG.

RESULTS

Cloning of the full-length TmHDR cDNA

An approximately 500-bp PCR product was specifically amplified at 58.4°C using two degenerate primers (FDPH DR and RDPHDR), which was sub-cloned and sequenced to generate a 498-bp nucleotide sequence. The BLAST searching results demonstrated that the 498-bp cDNA fragment showed homologous with HDR genes from other plant species, such as *Pinus taeda* (88% identities), *Ginkgo biloba* (88% identities), *Zea mays* (78% identities) and etc. These strongly suggested that the core fragment of *TmHDR* had been obtained, which provided necessary and enough sequence information for isolating the cDNA ends of *TmHDR* by RACE. The 751-bp 3'-end and 848-bp 5'-ends of TmHDR were respectively obtained by 3'RACE and 5'RACE. By assembling the sequences of 3'-end, 5'-end and the core fragment on ContigExpress, the full-length cDNA sequence of *TmHDR* was generated which was 1987 bp. Then, the physical full-length *TmHDR* cDNA was amplified and confirmed by sequencing. *TmHDR* had the 79-bp 5' untranslated region (UTR), the 1425-bp coding sequence and the 483-bp 3' UTR including the poly-A tail (Figure 1). The ORF finding analysis showed that *TmHDR* contained an 1425-bp coding sequence encoding a 474-amino-acid polypeptide with a calculated molecular mass of 53.2 kDa and an isoelectric point of 5.65 that were similar with the reported plant HDRs such as Adonis and rice HDRs (Kim et al., 2008). Then, *TmHDR* was submitted to GenBank and assigned an accession number: EF541129.

Comparative and bioinformatic analysis of TmHDR

The BLAST searching results showed that TmHDR had high similarities with HDRs from other plant species, such as *Oryza sativa* (81% identities), *Stevia rebaudiana* (75% identities) and *Arabidopsis thaliana* (71% identities); and the results indicated that TmHDR belonged to the HDR family. The subcellular prediction analysis by TargetP suggested that TmHDR had a 47-amino-acid transit peptide at its N-terminus that directed TmHDR to plastid. This was consistent with the fact that the MEP pathway was localized in plastids (Lichtenthaler et al., 1997). The multiple alignments results indicated that all HDRs had the four conserved cysteine residues (Figure 2) that might participate in the coordination of the iron-sulfur bridge proposed to be involved in catalysis (Seemann et al., 2002); it is found that all HDRs from plants owned a plastidial transit peptide with no similarity at their N-terminal but the catalytic regions of HDRs had high similarity that followed after the plastidial transit peptides. Based on the multiple alignments, a phylogenetic tree of HDRs was constructed and the tree demonstrated that the plant HDRs might come from the cyanobacterium *Synechocystis* through endosymbiosis (Sprenger et al.,

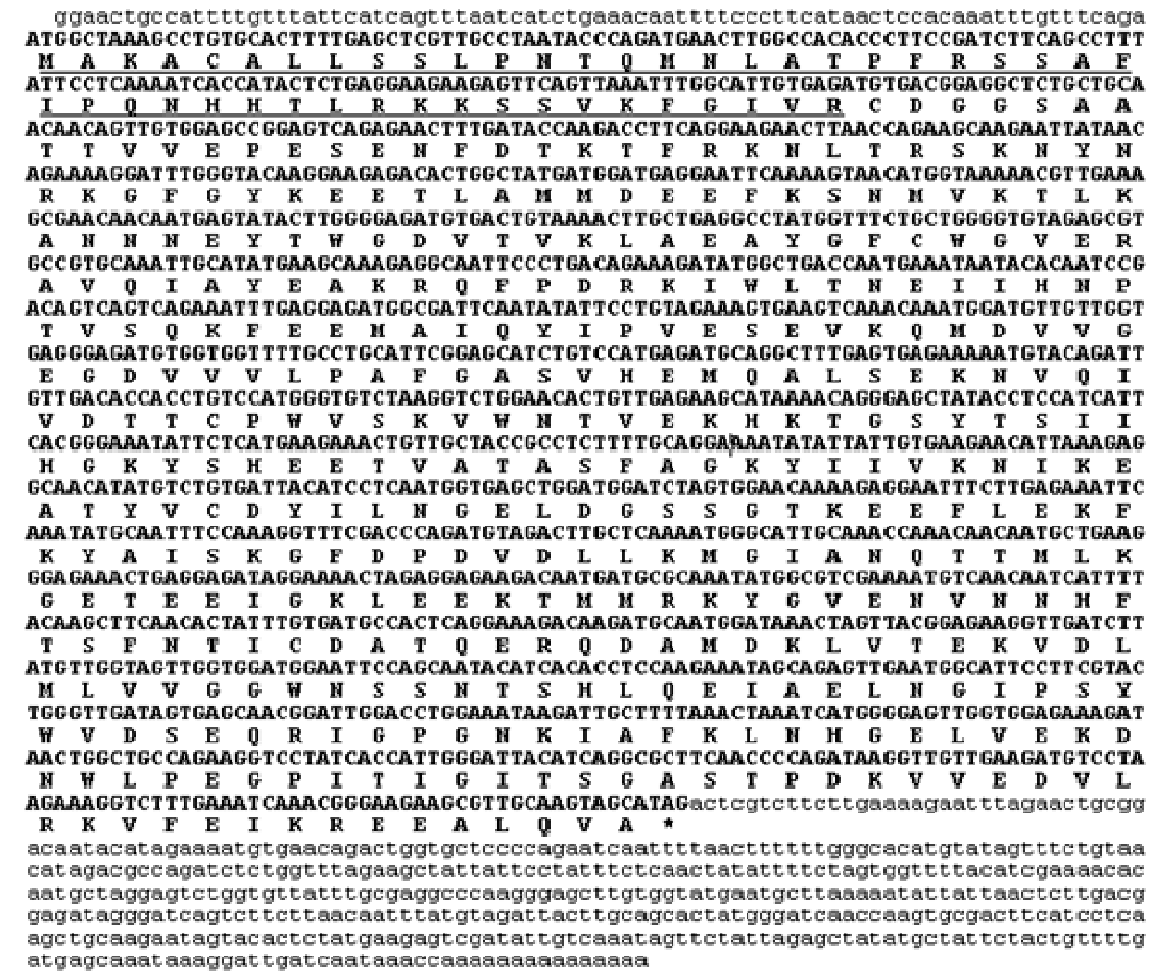

Figure 1. The full-length cDNA sequence and the deduced amino acid sequence of TmHDR. The coding sequence was typed in capital letters; the stop codon was marked with an asterisk; the 5`-and 3`-untranslated regions were shown in normal letters; the transit peptide predicted by TargetP 2.0 was underlined.

1997), like the 1-deoxy-D-xylulose 5-phosphate reductoisomerase gene, which was the secondary gene involved in the MEP pathway (Lange et al., 2000). According to the phylogenetic tree, TmHDR had higher identity with plant HDRs than HDR from *Synechococcus elongates*, the plant HDRs were classified into two clusters. HDRs from angiosperm plants including *Arabidopsis thaliana*, *Lycospersicon esukurentamu*, *Lactuca sativa*, *Stevia rebaudiana* clustered into one group, while HDRs from gymnosperm plants *Ginkgo biloba*, *Pinus densiflora* and *T. media* clustered into another group (Figure 3). The result is consistent with the fact that *T. media* is an ancient plant species which is similar to the *Ginkgo biloba.*

Tissue expression pattern analysis

To investigate the expression profile of *TmHDR* in different tissues including roots, stems, leaves and cortices of *T. media*, total RNAs were respectively isolated from different tissues and subjected to Semi-quantitative one-step RT-PCR using FEXTMHDR and REXTMHDR as primers. The actin gene expression in all the detected tissues was used as internal control with no significant difference. It was found that TmHDR expressed in all the detected tissues including cortices, stems, roots and leaves, suggesting that *TmHDR* was constitutively expressed but at different levels (Figure 4) like that in *Arabidipsis* (Hsieh and Goodman, 2005). The highest expression level of *TmHDR* was found in leaves of *T. media*, the lowest expression level of *TmHDR* was in stems and actually the expression level was extremely low.

TmHDR complements the E. coli hdr mutant

The amino acid sequences of HDR proteins from plant and bacteria are highly conserved. To test whether the TmHDR protein has similar enzymatic activity to its *E. coli*

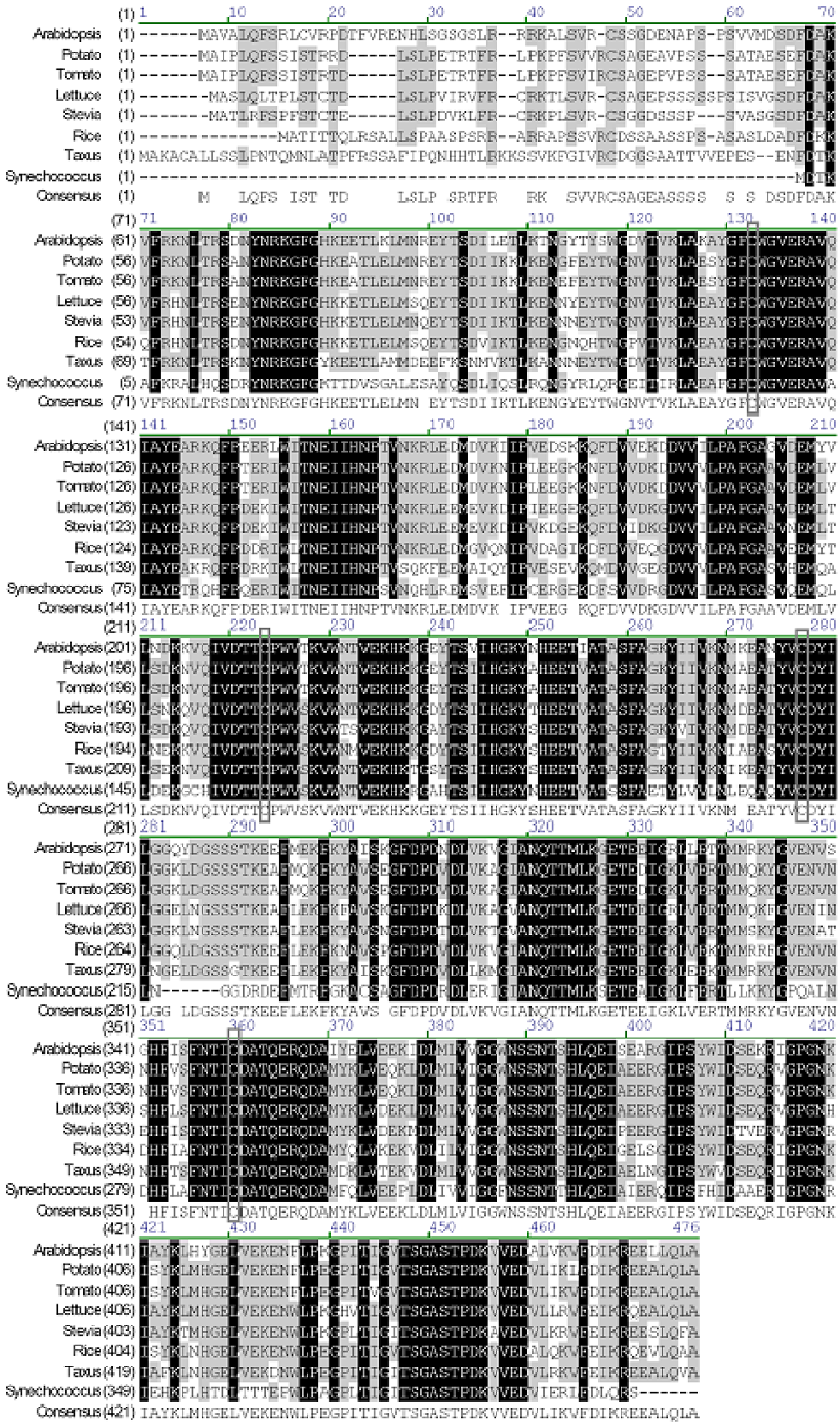

Figure 2. Multiple alignments of amino acid sequences of HDRs. The identical amino acids were showed in white with black background and the conserved amino acids were showed in black with gray background. Numbers indicate the position of amino acid residues.

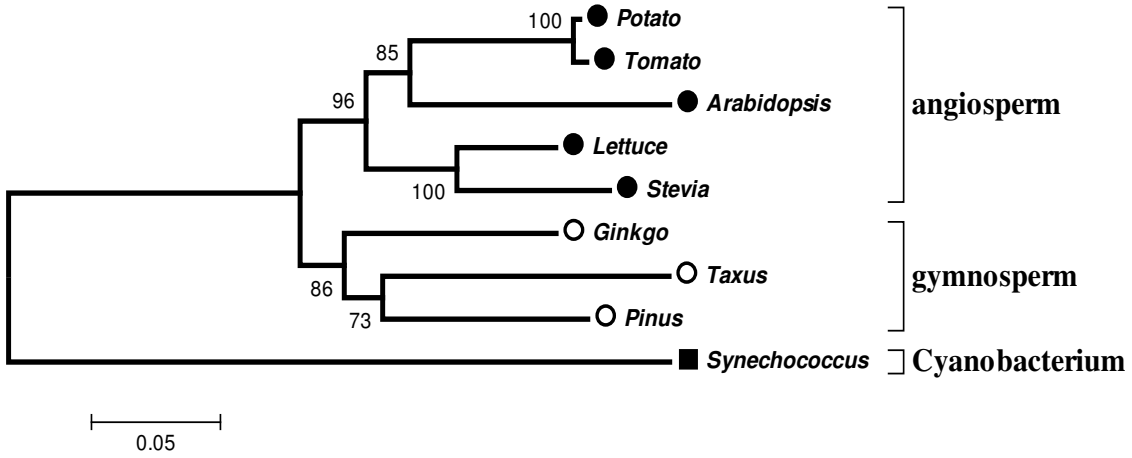

Figure 3. A phylogenetic tree of HDRs from different organisms including plants and bacteria was constructed by the neighbor-joining method on MEGA 4. HDRs from angiosperm plants were marked with●, gymnosperm with O and HDR from bacteria marked with ■.

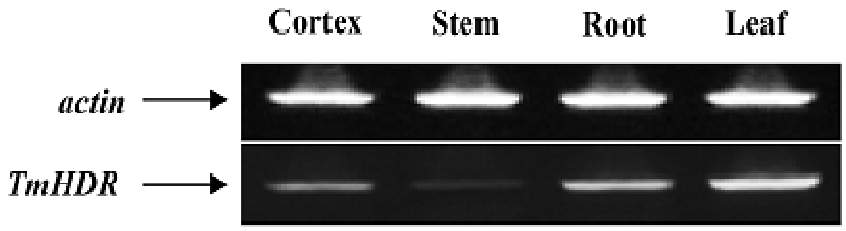

Figure 4. The *hdr* gene expression profile in various tissues of *Taxus x media*. Total RNA samples were isolated from cortices, stems, roots and leaves respectively, and subjected to Semi-quantitative one-step RT-PCR analysis (lower panel). *actin* gene was used as the control to show the normalization of the amount of templates in PCR reactions (upper panel).

counterpart, we performed a complementation assay with an *E. coli hdr* mutant. In *E. coli hdr* mutant strain MG1655 *ara <> hdr,* the endogenous *hdr* gene was replaced by a kanamycin-resistant cassette and a single copy of *hdr* was present on the chromosome under the control of the P_{BAD} promoter (McAteer et al., 2001). Because the *hdr* gene is essential for survival, the *E. coli hdr* mutant cannot grow in the absence of arabinose (Ara) (Figure 5). Growth of the *E. coli* MG1655 *ara <> hdr* mutant on the medium containing glucose (Glc) was restored successfully by transformation of the pQE-*TmHDR* plasmid but not by the empty pQE-30 vector (Figure 5). Therefore, using the complementation strategy it was confirmed that the HDR protein of *T. media* had similar enzymatic activity to its *E. coli* counterpart. The transformants containing the pQE30 empty vector cannot grow on medium containing 0.2% glucose.

DISCUSSION

In the present study, we cloned and characterized a new gene encoding HDR enzyme (TmHDR) involved in the taxol precursor biosynthesis. TmHDR was homologous with a few reported HDRs from other plants, such as ginkgo and pine (Kim et al., 2008). The further bioinformatic analysis indicated that TmHDR had a 47-amino-acid transit peptide that directed it to plastid, that was consistent with the fact that the precursor of taxol was synthesized in plastids (Croteau et al., 2000); the four conserved cysteine residues could be found in TmH DR demonstrated that TmHDR suggested that TmHDR was a functional gene just like its homologies. The phylogenetic analysis revealed that TmHDR belonged to gymnosperm HDR group, which were obtained from cyanobacterium through gene transfer (Lange et al., 2000). The existence of expression of TmHDR in all the detected tissues but at different levels showed that TmHDR was an essential gene that was also highly regulated like the HDR genes from *Arabidopsis* (Hsieh and Goodman, 2005), *Ginkgo* and *Pinus* (Kim et al., 2008). Finally, genetic complementation strategy was applied to identify the function of TmHDR. The HDR gene is an essential gene for survival of *E. coli*. *E. coli hdr*

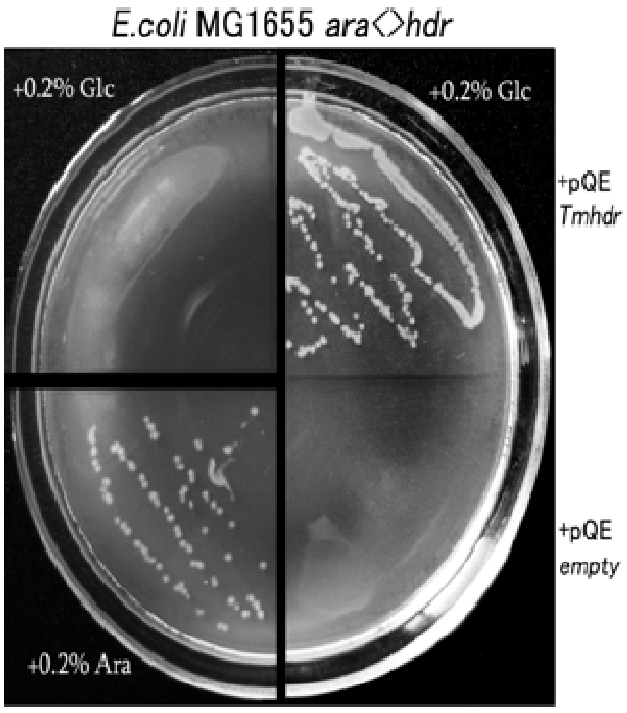

Figure 5. *T.media hdr* complements the *E. coli hdr* mutant. The E. coli *hdr* mutant strain MG1655 *ara<>hdr* was able to grow on Luria-Bertani medium containing 0.2% arabinose (Ara), but not on medium containing 0.2% glucose (Glc) (left). After transformation with the *T. media hdr* cDNA (pQE-*TmHDR*) and, as a control, with the empty vector (pQE) alone, the resulting strains were tested for growth on medium containing 0.2% glucose (right). Expression of *T. media hdr* protein successfully restored the growth of *E. coli hdr* mutant (top right).

mutant strain MG1655 *ara <> hdr* could not grow in the absence of arabinose, which was successfully used to identify the function of plant HDRs (Hsieh and Goodman, 2005). In the present study, TmHDR was introduced into MG1655 *ara<>hdr* and overexpressed, and then the mutant was rescued by TmHDR in media without arabinose. The transformants could grow in media without arabinose. The result of genetic complementation demonstrated that TmHDR did encode the enzyme of HDR. Botella-Pavía et al reported that overexpression of HDR could improve the biosynthesis of taxadiene, which was the first cyclic precursor for taxol (Botella-Pavía et al., 2004); Zhang et al overexpressed the HDR gene in Ginkgo biloba cells and ginkgolides content in transgenic cell lines was 2.5 fold of the untransformed control line (Zhang et al., 2008). It could be concluded that HDR was the rate-limiting enzyme involved in the MEP pathway for diterpenes including taxol, ginkgolides and others. So, cloning, characterization and functional identification of TmHDR can unveil molecular mechanism of the enzymatic step catalyzed by HDR and provide a candidate gene encoding the rate-limiting enzyme of HDR that will facilitate metabolic engineering of taxol.

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