A New 1-Deoxy-D-xylulose 5-Phosphate Reductoisomerase Gene Encoding the Committed-Step Enzyme in the MEP Pathway from *Rauvolfia verticillata*

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1-Deoxy-D-xylulose 5-phosphate (DXP) reductoisomerase (DXR; EC 1.1.1.267) catalyzes a committed step of the methylerythritol phosphate (MEP) pathway for the biosynthesis of pharmaceutical terpenoid indole alkaloid (TIA) precursors. The full-length cDNA sequence was cloned and characterized from a TIA-producing species, *Rauvolfia verticillata*, using rapid amplification of cDNA ends (RACE) technique. The new cDNA was named as *RvDXR* and submitted to GenBank® to be assigned with an accession number (DQ779286). The full-length cDNA of RvDXR was 1804 bp containing a 1425 bp open reading frame (ORF) encoding a polypeptide of 474 amino acids with a calculated molecular mass of 51.3 kDa and an isoelectric point of 5.88. Comparative and bioinformatic analyses revealed that RvDXR showed extensive homology with DXRs from other plant species and contained a conserved transit peptide for plastids, an extended Pro-rich region and a highly conserved NADPH-binding motif in its *N*-terminal region owned by all plant DXRs. The phylogenetic analysis revealed that DXRs had two groups including a plant and bacterial group; RvDXR belonged to angiosperm DXRs that were obtained from *Synechocystis* through gene transfer according to the phylogenetic analysis. The structural modeling of RvDXR showed that RvDXR had the typical V-shaped structure of DXR proteins. The tissue expression pattern analysis indicated that *RvDXR* expressed in all tissues including roots, stems, leaves, fruits and followers but at different levels. The lowest transcription level was observed in followers and the highest transcription was found in fruits of *R. verticillata*; the transcription level of *RvDXR* was a little higher in roots and stems than in leaves. The cloning and characterization of *RvDXR* will be helpful to understand more about the role of *DXR* involved in *R. verticillata* TIA biosynthesis at the molecular level and provides a candidate gene for metabolic engineering of the TIAs pathway in *R. verticillata*.

Key words: Cloning, Characterization, *DXR* Gene, *Rauvolfia verticillata*

Introduction

Terpenoid indole alkaloids (TIAs) are usually found in the plant families Apocynaceae, Loganiaceae, Rubiaceae and Nyssaceae (Memelink *et al*., 2001). Among them, there are various pharmaceutically important agents such as reserpine and ajmalicine that are widely used for the treatment of hypertension and cardiac disorders because of their antihypertensive and antiarrythmic properties (Li and Ting, 1962). *Rauvolfia verticillata*, a member of the family Apocynaceae, is the main source of reserpine and ajmalicine in China. It is a rare medical shrub that mainly grows in southwest China and produces reserpine and ajmalicine at a very low level. So natural *R. verticillata* can not meet the increasing demands for reserpine and ajmalicine and it is eager for finding an efficient way to provide a source for pharmaceutical TIAs. Therefore, to map TIAs' biosynthetic pathway in *R. verticillata* at the level of molecular genetics is a promising way to increase the pharmaceutical TIAs production.

Biosynthesis of TIAs is initiated by condensation of an indole moiety of tryptamine and a monoterpenoid component of secologanin. Thus, accumulation of TIAs is restricted by the availability of precursors supplied by two convergent indole and monoterpenoid pathways (Hong *et al*., 2003).

Monoterpenoids are derived from 5-carbon isopentenyl diphosphate (IPP) that is synthesized via the plastidial methylerythritol phosphate (MEP) pathway in plants (Memelink *et al.*, 2001). The MEP pathway starts with the formation of 1-deoxy-D-xylulose 5-phosphate (DXP) from D-glyceraldehyde 3-phosphate and pyruvate by the catalytic action of a synthase specified by the DXS gene (Lange *et al.*, 1998; Lois *et al.*, 1998). In the second step, DXP is converted into MEP and the reaction is catalyzed by a NADPH-dependent reductoisomerase (DXR; EC 1.1.1.267) specified by the DXR gene; the reaction catalyzed by DXR is actually the first committed step of the MEP pathway (Kuzuyama *et al.*, 2000). The participation of DXR in the control of terpenoid accumulation in plants is also supported by experimental results (Veau *et al.*, 2000; Mahmoud and Croteau, 2001; Carretero-Paulet *et al.*, 2002). In *Arabidopsis thaliana*, DXR, encoded by a single gene, is a committed enzyme of the MEP pathway proven by the transgenic method and analysis of the metabolites (Carretero-Paulet *et al.*, 2002); in antitumour-TIA-producing *Catharanthus roseus*, the MEP pathway provides isoprene blocks for building the monoterpenoid indole alkaloids and the expression of *DXR* gene isolated from *C. roseus* was up-regulated in parallel with the production of monoterpenoid indole alkaloids (Veau *et al.*, 2000); overexpression of DXR in transgenic peppermint plants has led to an increase of essential oil monoterpenes in peppermint (Mahmoud and Croteau, 2001). All the studies suggest that DXR is an ideal target for metabolic engineering of the biosynthesis of isoprenoids, including TIAs and terpenes. Unfortunately, until now there have been no reports on the cloning of the *DXR* gene from *R. verticillata*. In the present study, we report for the first time the molecular cloning and characterization of the *DXR* gene from *R. verticillata* using the rapid amplification of cDNA ends (RACE) technique, which will enable us to map and regulate an important step involved in the *R. verticillata* TIA biosynthetic pathway at the level of molecular genetics in the future.

Materials and Methods

Plant materials and RNA isolation

Roots, stems, leaves, fruits and followers were collected from *R. verticillata* plants growing in the medicinal plant garden at Southwest University, Chongqing, China. After collection, the materials were immediately immersed into liquid nitrogen to be stored for future total RNA isolation. Total RNAs from leaves and followers were isolated by the TriZOL method provided by Invitrogen (USA) and total RNAs from roots, stems and fruits were isolated by a modified CTAB method and lithium chloride precipitation (Liao *et al.*, 2004).

Cloning of the core fragment of RvDXR

Single-strand cDNAs were synthesized from 5 μg of total RNA with an oligo (dT)17 primer and reversely transcribed according to the manufacturer's protocol (PowerScript, CLONTECH, CA, USA). After RNase H treatment, the single-strand cDNA mixtures were used as templates for PCR amplification of the conserved region of *DXR* from *R. verticillata*. Two degenerate primers, FDPDXR [5′-AC(A/C)GG(T/C)TC(A/T/C)AT-(A/T)GG(A/G/C)AC(A/T)CAGAC-3′] and RDP-DXR [5′-TTCTC(A/G)TT(A/T/G)GC(A/T/G)GC-(A/G)CT(A/TC/G)AG(A/G)ACTCC-3′], were designed according to the conserved sequences of other plant *DXR* genes and used for the amplification of the core cDNA fragment of *RvDXR* by standard gradient PCR amplification (from 52 to 60 °C on a BioRad My Cycler (USA). The PCR products were purified and subcloned into pGEM T-easy vector (Promega, Madison, WI, USA) followed by sequencing.

3′RACE and 5′RACE of RvDXR

The core fragment was subsequently used to design the gene-specific primers for the cloning of cDNA ends of *RvDXR* by RACE. SMART® RACE cDNA Amplification Kit (CLONTECH) was used to clone the 3′-end and 5′-end of *RvDXR* cDNA. The first strand 3′RACE-ready and 5′RACE-ready cDNA samples from *R. verticillata* 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 *RvDXR* cDNA was amplified using a 3′-gene-specific primer and the universal primers provided by the kit. For the PCR amplification of 3′RACE, RVDXR3-1 [5′-CAGATAACATCA-AGTACCCATCC-3′] and Universal Primer A Mix (UPM), [5′-CTAATACGACTCACTATAG-GGCAAGCAGTGGTATCAACGCAGAGT-3′]

and [5′-AAGCAGTGGTATCAACGCAGAGT-3′] were used as the PCR primers, and 3′RACE-ready cDNA was used as template. The 5′-end of *RvDXR* cDNA was amplified using two 5′-gene-specific primers and the universal primers, UPM and NUP, provided by the kit. For the first PCR amplification of 5′RACE, RVDXR5-1 [5′-CATTTCTGACAGCAACTAACTGAGG-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, RVDXR5-2 [5′-CCAGCTGCAAGTGCAACAACTCTAAA-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 *RvDXR* cDNA 3′- and 5′-end, Advantage® 2 PCR Kit (CLONTECH) 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 °C, 30 s at 68 °C and 3 min at 72 °C). The 3′RACE and nested 5′RACE products were purified and subcloned into pGEM T-easy vector followed by sequencing.

Amplification of RvDXR full-length cDNA

By assembling the sequences of 3′RACE, 5′RACE and the core fragment on Contig Express (Vector NTI Suite 6.0), the full-length cDNA sequence of *RvDXR* was deduced. According to the deduced RvDXR cDNA sequence, two gene-specific primers, FRVDXR [5′-ACGCGGGGAATCTCAATTC-3′] and RRVDXR [5′-GATGATCAATATAGATGCTCATA-3′] were designed, synthesized and used to amplify the full length of *RvDXR* from 5′RACE-ready cDNA samples through proof-reading PCR. Three independent mono-clones were sequenced to confirm the sequence of *RvDXR*. Finally RvDXR was submitted to GenBank to be assigned with an accession number.

Comparative and bioinformatic analysis

Comparative and bioinformatic analyses of *RvDXR* were carried out online at the websites http://www.ncbi.nlm.nih.gov and http://www.expasy.org. The nucleotide sequence, deduced amino acid sequence and ORF (open reading frame) encoded by *RvDXR* were analyzed and the sequence comparison was conducted through a database search using the BLAST program (Altschul *et al*., 1997). The multiple alignments of RvDXR and DXRs from other plant species were aligned with CLUSTAL W (Thompson *et al*., 1994) using default parameters. A phylogenetic tree was constructed using MEGA version 3.0 (Kumar *et al*., 2004) from CLUSTAL W alignments. The neighbor-joining method (Saitou and Nei, 1987) was used to construct the tree. The subcellular location was predicted by Predotar (Small *et al*., 2004). The homology-based 3-D structural modeling of RvDXR was accomplished by SWISS-Modeling (Schwede *et al*., 2003). WebLab ViewerLite was used for 3-D structure displaying.

Tissue expression pattern analysis

Semi-quantitative one-step RT-PCR was carried out to investigate the expression profile of RvDXR in different tissues including roots, stems, leaves, fruits and followers of *R. verticillata*. Aliquots of 0.5 μg total RNA extracted from roots, stems, leaves, fruits and followers of *R. verticillata* were used as templates in the one-step RT-PCR reaction with the forward primer fexRvDXR [5′-TTTAGAGTTGTTGCACTTGCAGC-3′] and the reverse primer rexRvDXR [5′-GGATGCTTCAAAGCATCCGCTA-3′] specific to the coding sequence of *RvDXR* using the one-step RNA PCR kit (Takara, Kyoto, 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 50 s, 55 °C for 50 s and 72 °C for 90 s). Meanwhile, the RT-PCR reaction for the house-keeping gene (actin gene) using the 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.

Results and Discussion

Cloning of the full-length RvDXR cDNA

Based on the conserved fragment of plant *DXR* sequences, two degenerate primers (FDPDXR and RDPDXR) were designed and used for gradient PCR amplification of the core cDNA fragment of *DXR* from *R. verticillata*. Following gradient temperature PCR amplification, an approximately 1000 bp product was specifically amplified at

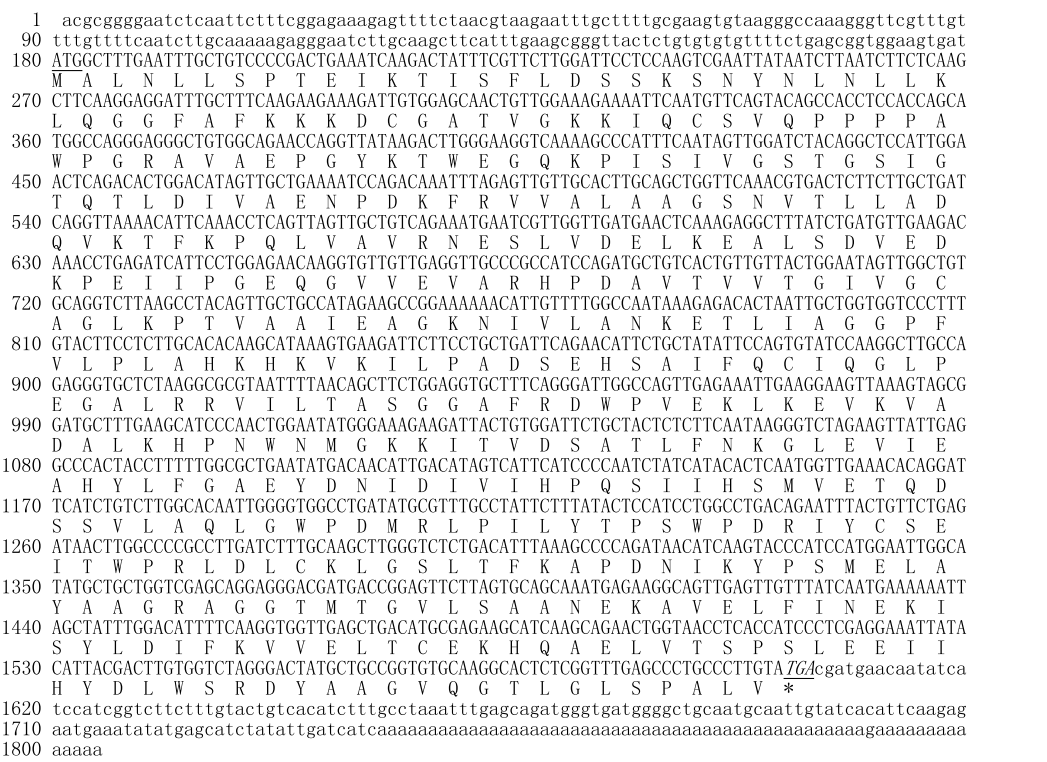

Fig. 1. The full-length cDNA sequence and the deduced amino acid sequence of *RvDXR*. The start codon (ATG) is underlined; the stop codon (TGA) is in italics, underlined and marked with an asterisk; the coding sequence and its deduced amino acid sequence are shown in capital letters.

58.4 °C that was subcloned and sequenced to generated a 974 bp nucleotide sequence. The BLAST search demonstrated that the 974 bp cDNA fragment showed homology with DXR genes from plant species such as *Catharanthus roseus* (Veau *et al*., 2000), *Lycopersicon esculentum* (Rodriguez-Concepcion *et al*., 2001) and *Antirrhinum majus* (Dudarev *et al*., 2005). These strongly suggested that the core fragment of *RvDXR* had been obtained, which provided necessary and enough sequence information for isolating the cDNA ends of *RvDXR* by RACE.

By 3′RACE and nested 5′RACE, the 490 bp 3′-end and 514 bp 5′-end of *RvDXR*, respectively, were obtained. By assembling the sequences of 3′-end, 5′-end and the core fragment on Contig Express (Vector NTI Suite 6.0), the full-length cDNA sequence of *RvDXR* was obtained that was 1804 bp; finally the physical full-length *RvDXR* cDNA was amplified and confirmed by sequencing. The sequencing results showed that *RvDXR* had the 179 bp 5′ untranslated region (UTR), the 1425 bp coding sequence and the 200 bp 3′ UTR including the polyA tail. Then, the full-length *RvDXR* sequence was submitted to GenBank where the accession number DQ779286 was assigned. The ORF finding analysis showed that the *RvDXR* contained a 1425 bp coding sequence encoding a 474-amino-acid polypeptide (Fig. 1) with a calculated molecular mass of 51.3 kDa and an isoelectric point of 5.88 that were similar with the reported plant DXRs such as *Ginkgo biloba* DXR (Gong *et al*., 2005).

Comparative and bioinformatic analysis of RvDXR

The deduced amino acid sequence of *RvDXR* was submitted to NCBI for BLAST searching and the results showed that RvDXR had high similarities with DXRs from other plant species, such as *Catharanthus roseus* (93% identity), *Lycopersicon esculentum* (87% identity), *Antirrhinum majus* (86% identity) and *Picrorhiza kurrooa* (85% iden-

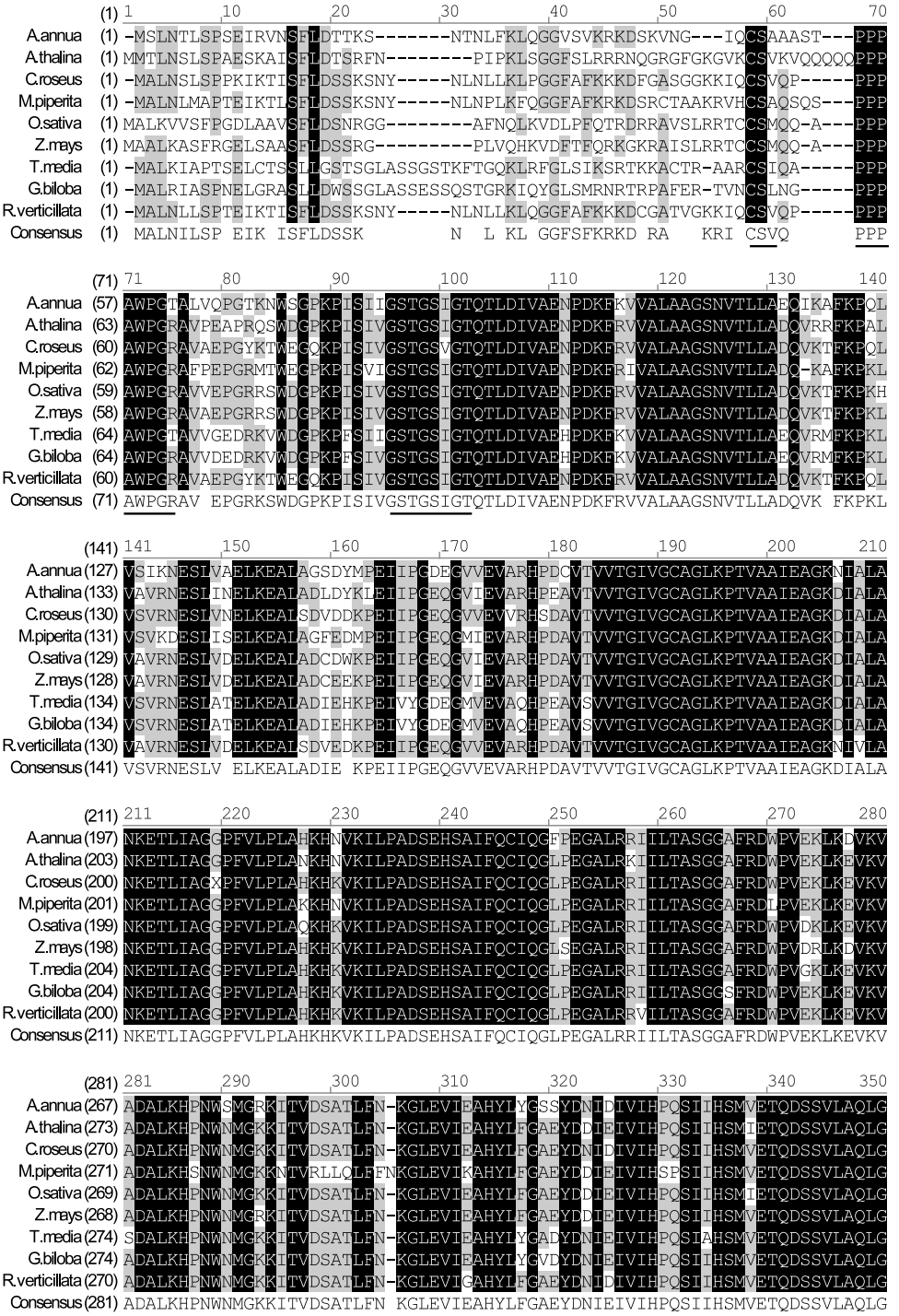

Fig. 2. Multi-alignment of amino acid sequences of *RvDXR* and other plant *DXRs*. The identical amino acids are shown in white with black background and the conserved amino acids are shown in black with gray background. The highly conserved transit peptide for plastids motif Cys-Ser-(Ala/Met/Val), an extended Pro-rich motif PPPAWPG(R/T) and a conserved NADPH-binding motif (GSTGSIGT) are underlined.

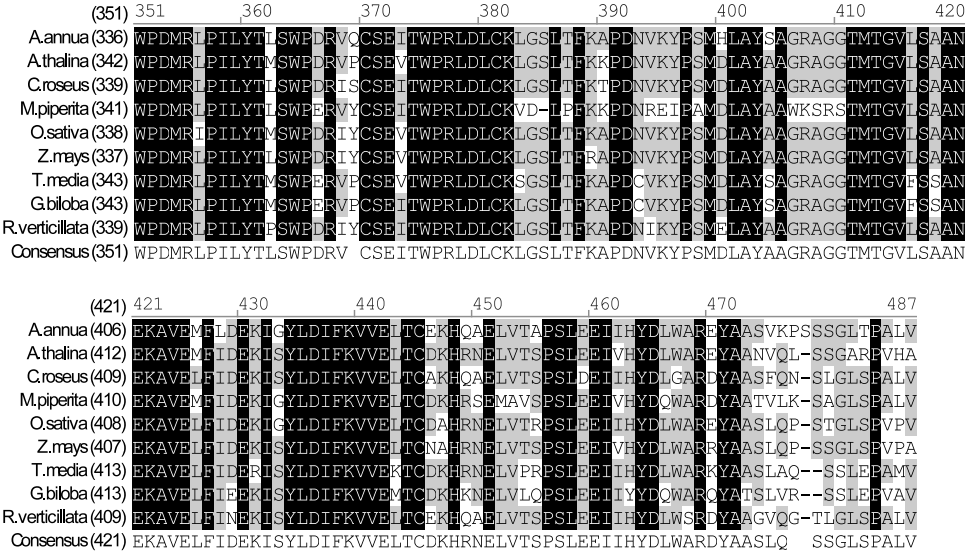

Fig. 2 (continued).

tity). Thus, the BLAST analysis results indicated that RvDXR belonged to the DXR family. The subcellular prediction analysis by Predotar suggested that RvDXR is localized in plastids and this was consistent with the fact that TIAs are synthesized in plastids. Based on the multiple alignments, it was found that all aligned plant DXRs had a plastidial transit peptide at the *N*-terminus, which was not present in prokaryotic DXRs (Carretero-Paulet *et al*., 2002). Furthermore, two conserved domains were found in RvDXR owned by all plant DXRs. A plastidial transit peptide directing the enzyme to plastids where the mevalonate-independent pathway operated in plants was found in the *N*-terminal region of RvDXR and had a conserved Cys-Ser-(Ala/Met/Val) motif and an extended proline-rich motif chatacterized by PPPAWPG(R/T) at the *N*-terminus of the RvDXR; the second highly conserved domain of the NADPH-binding motif (GSTGSIGT) performed the function of binding NADPH in the RvDXR *N*-terminal region owned by all plant DXRs (Lange and Croteau, 1999) (Fig. 2). Using MEGA version 3.0 based on CLUSTAL W alignments, a phylogenetic tree of DXRs was constructed from different organisms including plants and bacteria. The result demonstrated that DXRs were derived from an ancestor gene and evoluted into two groups including plant and bacteria DXR groups. According to the phylogenetic tree, the plant-derived DXRs could be classified into angiosperm and gymnosperm DXR groups (Fig. 3). RvDXR belonged to the angiosperm DXR group, and it beard a closer relationship to DXRs from angiosperm than from gymnosperm. Interestingly, the DXR protein from cyanobacterium *Synechocystis* species was seperated from the bacterial DXRs and gathered within the plant DXRs. This meant that *Synechocystis* DXR had closer relationship with plant DXRs than with bacterial DXRs. This was consistent with the fact that plant nuclear encoded *DXRs* were acquired from *Synechocystis* through gene transfer to the nucleus in the process of the endosymbiotic origin (Lange *et al*., 2000). The homology-based structural modeling of RvDXR was performed by SWISS-Modeling on the basis of the *E. coli* DXR crystal structure (Reuter *et al*., 2002) and displayed by WebLab ViewerLite (Fig. 4). RvDXR displayed a globally V-like shape, which is composed of three domains: an amino-terminal NADPH-binding domain, a carboxy-terminal four-helix bundle domain and a connective domain at the bottom of the V shape. The strictly conserved acidic motifs, $D_{230}X_{231}E_{232}$-motif and $E_{301}X_{302}X_{303}E_{304}$-motif, are clustered at the internal bottom of the V-shaped structure that are involved in the binding of divalent cations mandatory for DXR enzymes (Reuter *et al*., 2002). A conserved NADPH-binding motif (GSTGSIGT) was emerged from the

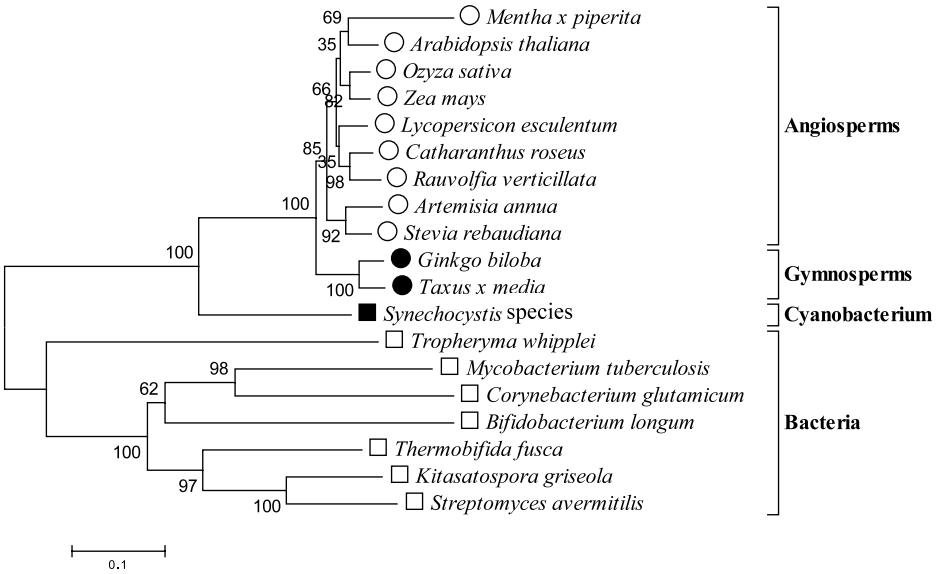

Fig. 3. A phylogenetic tree of DXRs from different organisms including plants and bacteria constructed by the neighbor-joining method on MEGA 3. DXRs from gymnosperms are marked with ●, DXRs from angiosperms are marked with ○ and the others are bacteria-derived DXRs. The numbers on the branches represent bootstrap support for 1000 replicates. The sequences used are listed bellow with their GenBank accession number: *Synechocystis* species, Q55663; *A. thaliana*, CAB43344; *A. annua*, AAD56390; *C. roseus*, AAF65154.1; *L. esculentum*, AAD38941; *O. sativa*, Q22567; *S. rebaudiana*, CAD22156.1; *G. biloba*, AY443101; *Z. mays*, AJ297566; *M.* x *piperita*, AF116825; *T.* x *media*, AY588482; *T. whipplei*, AAO44186.1; *M. tuberculosis*, ZP_00770539.1; *C. glutamicum*, CAF20356.1; *K. griseola*, BAB39759.1; *T. fusca*, AAZ54785.1; *S. avermitilis*, NP_823739.1; *B. longum*, AAN23962.1.

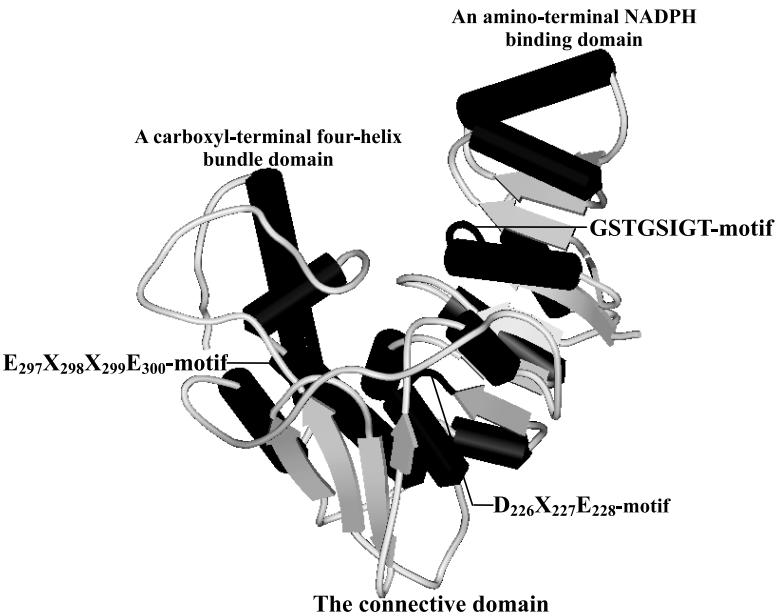

Fig. 4. The 3-D structure of RvDXR established by homology-based modeling. The α-helix, the sheet and the random coil are shown in column-shaped, arrow plate-shaped and rope-shaped structures, respectively. The active motifs, $D_{226}X_{227}E_{228}$ and $E_{297}X_{298}X_{299}E_{300}$, located at the internal bottom of the V-shaped structure are marked with labels; the NADPH-binding site consists of GSTGSIGT and is marked with letters.

convergence of flexible loops in the *N*-terminal region, which was proved to be an important catalytic domain of the RvDXR to the rearrangement and reduction of DXP to yield MEP in a single step (Reuter *et al*., 2002).

Tissue expression pattern analysis

To investigate the expression profile of *RvDXR* in different tissues including roots, stems, leaves, and fruits and followers of *R. verticillata*, total RNA was isolated from different tissues and subjected to semi-quantitative one-step RT-PCR using fexRvDXR and rexRvDXR as primers. The actin gene expression in all the detected tissues was used as internal control that showed no significant difference (Fig. 5). The result showed that *RvDXR* expression could be detected in all tissues including roots, stems, leaves, fruits and followers but at different levels (Fig. 5). This suggested that *RvDXR* was an essential gene for basic physiological and biochemical processes just like the DXR gene in other plants (Liao *et al.*, 2006). The highest expression level of *RvDXR* was found in fruits of *R. verticillata* and the result was consistent with the *DXR* gene expression at the highest level in *Ginkgo biloba* fruits (Gong *et al*., 2005); the lowest expression level of *RvDXR* was in followers and actually the expression level of *RvDXR* in followers was very low. By comparing *RvDXR* expression levels in roots, stems and leaves, it was found that RvDXR expressed at higher level in leaves. The previous studies have also demonstrated that the DXR gene had a lower expression level in leaves of *Arabidopsis* (Carretero-Paulet *et al.*, 2002) and *Ginkgo* (Gong *et al*., 2005). These suggested that the DXR gene was also a highly regulated gene in plants (Dudarev *et al*., 2005).

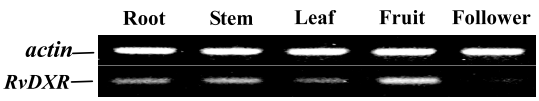

Fig. 5. The *RvDXR* tissue expression profile performed by semi-quantitative one-step RT-PCR. Total RNA samples were isolated from roots, stems, leaves, fruits and followers, respectively, and subjected to semi-quantitative one-step RT-PCR analysis (lower panel). *Actin* gene was used as the control to show the normalization of the amount of templates in PCR reactions (upper panel).

DXR catalyzes the rate-limiting reaction in the MEP pathway and is the ideal target for metabolic engineering of the isoprenoid biosynthetic pathway. There are a few *DXR* genes reported from other plants except *R. verticillata*. So the cloning and characterization of *RvDXR* from *R. verticillata* will facilitate the understanding of the biosynthesis of TIAs including reserpine and ajmalicine and also promote metabolic engineering of the TIA biosynthetic pathway in *R. verticillata*.

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