Journal of Medicinal Plants Research Vol. 4(10), pp. 915-924, 18 May, 2010
Available online at http://www.academicjournals.org/JMPR
DOI: 10.5897/JMPR10.107
ISSN 1996-0875© 2010 Academic Journals

Full Length Research Paper

The HDR gene involved in the TIA pathway from *Rauvolfia verticillata*: Cloning, characterization and functional identification

Jinwu Chen[1], Wanhong Liu[2], Min Chen[1], Guijun Wang[3], Meifang Peng[3], Rong Chen[3], Chunxian Yang[3], Xiaozhong Lan[4], Xingjia Ming[5], Minghsiun Hsieh[6] and Zhihua Liao[3]*

[1]Key Laboratory of Eco-environments in Three Gorges Reservoir Region (Ministry of Education), College of Pharmaceutical Sciences, Southwest University, Chongqing 400715, People's Republic of China.
[2]Department of biology, Chongqing University of Science and Technology, Chongqing 401331, People's Republic of China.
[3]Key Laboratory of Eco-environments in Three Gorges Reservoir Region (Ministry of Education), School of Life Sciences, Southwest University, Chongqing 400715, People's Republic of China.
[4]Agricultural and Animal Husbandry College, Tibet University, Linzhi of Tibet 860000, People's Republic of China.
[5]Chongqing Academy of Chinese Materia Medica, Chongqing 400065, People's Republic of China.
[6]Institute of Plant and Microbial Biology, Academia Sinica, Taipei 11529, Taiwan.

Accepted 2 April, 2010

1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase (HDR, EC 1.17.1.2) catalyzes the last reaction of the methylerythritol phosphate (MEP) pathway. The full-length cDNA sequence of HDR was cloned and characterized from terpenoid-indole-alkaloid-producing *Rauvolfia verticillata.* The new cDNA was named as *RvHDR* and submitted to GenBank® to be assigned with an accession number: EU034699. The full-length cDNA of *RvHDR* was 1679-bp containing a 1389-bp open reading frame (ORF) encoding a polypeptide of 462-amino acids with a calculated molecular mass of 52 kDa and an isoelectric point of 5.26. Comparative and bioinformatic analyses revealed that *RvHDR* had extensive homology with HDRs from other plant species and contained a conserved transit peptide for plastids. The phylogenetic indicated that all HDRs could be divided into three groups and *RvHDR* belonged to plant HDRs family. *RvHDR* was found to be expressed in all tested tissues including roots, stems, leaves, flowers and fruits but at different levels. The highest expression level was found in flowers, and higher expression level in leaves and fruits; the expression level was low in roots and lowest in stems. Expression profiling analyses revealed that *RvHDR* expression was induced by exogenous elicitors including methyl jasmonate, acetyl salicylic acid, abscisic acid and UV, and showed the transcription levels were all up-regulated compared to the control. Finally, *RvHDR* was transformed into the *E. coli* HDR mutant strain MG1655 ara<>HDR, which was able to rescue the lethal phenotype of the *E. coli* HDR mutant. This confirmed that *RvHDR* had the typically function of HDR gene. The cloning, characterization and functional identification of *RvHDR* will be helpful to understand more about the function of HDR at the level of molecular genetics and help to unveil the biosynthetic mechanism of TIAs precursor and provides a candidate gene for metabolic engineering of the TIAs pathway in *R. verticillata.*

Key words: *Rauvolfia verticillata,* HDR gene, cloning, expression profile, functional complementation.

INTRODUCTION

Terpenoid Indole Alkaloids (TIAs), which constitute one of the largest groups of natural products, provide many pharmacologically active compounds. *Rauvolfia alkaloids*, such as ajmalicine and reserpine are therapeutically applied for hypertension and cardiac disorders because of their antihypertensive and antiarrythmic properties (Anitha et al., 2006). *Rauvolfia verticillata* is a rare

*Corresponding author. E-mail: zhliao@swu.edu.cn; Tel: +86-23-68367146. Fax: +86-23-68252365.

medicinal shrub belonging to the family *Apocynaceae*, which is the main source of reserpine and ajmalicine in China (Li et al., 1962). In pharmaceutical industries, reserpine is in great demand. Even though the chemical synthesis of reserpine is possible, it costs more than extracting it from natural resources (Farooqi et al., 2001). So it is eager for finding an efficient way to provide source of pharmaceutical TIAs. Therefore, to map TIAs biosynthetic pathway in *R. verticillata* at the level of molecular genetics is a promising way to increase pharmaceutical TIAs production.

The reserpine of TIAs belongs to isoprenoids is synthesized by condensation of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) units usually synthesized by the methylerythritol phosphate (MEP) pathway in plastids (Ramos-Valdivia et al., 1998).1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase (HDR) simultaneously synthesizes IPP and DMAPP in the last step of the pathway, which is noted a key enzyme in the precursors biosynthesis of isoprenoids. Overexpression studies using sequences from cyanobacteria (*Synechocystis*) and plants (*Adonis aestivalis*) showed that the activity of the HDR enzyme was limiting for isoprenoid biosynthesis in *E. coli* (Cunningham et al., 2000). Studies on overexpression of tomato *HDR* cDNA in *Arabidopsis* plants led to the conclusion that plant HDR protein plays a key role in controlling the biosynthesis of plastid isoprenoids (Botella-Pavia et al., 2004). In *Arabidopsis thaliana*, HDR was able to rescue the lethal phenotype of an *E. coli* HDR mutant, and plants with loss-of-function in the *Arabidopsis HDR* gene are albino (Guevara et al., 2005; Hsieh et al., 2005). All these reports suggested that HDR may be an ideal target enzyme for metabolic engineering of reserpine biosynthesis. Unfortunately, until now there have been no reports on the cloning of the *HDR* gene from *R. verticillata.* In the present study, a new *HDR* gene from *R. verticillata* was cloned, characterized by bioinformatic analysis and the tissue expression profile analysis, and then finally functionally expressed in *Escherichia coli,* which will enable us to map and regulate an important step involved in *R. verticillata* TIAs biosynthetic pathway at the level of molecular genetics in the future.

MATERIALS AND METHODS

Plant materials and treatments

R. verticillata plant was cultured in the plant garden of Southwest University (Chongqing, China). The roots, stems, leaves, fruits and flowers were collected from *R. verticillata i*n September. After collection, the materials were immediately immersed into liquid nitrogen to store for total RNA isolation. Total RNAs were isolated separately using the RNAplant reagent (Tiangen, China) according to the manufacturer's instructions. After isolation, total RNAs was stored in -80°C for future uses. The cell cultures, initiated from young leaves of *R. verticillata*, were maintained on solid MS medium supplemented with 0.5 $mg.L^{-1}$ 6-benzyl aminopuine (6-BA) 5 $mg.L^{-1}$ α-Naphthalene acetic acid (NAA) at 25°C in darkness and subcultured every 4 weeks. In this study for investigating induction by various elicitors, *R. verticillata* cell cultures were respectively dipped into the appropriate treatments such as 100 µM methyl jasmonate (MeJA), 100 $mg.L^{-1}$ acetyl salicylic acid (ASA), 50 µM abscisic acid (ABA) and exposed under UV light, using cell cultures without any treatments as control. Cell cultures were collected after 24 h treatment for analyses of *RvHDR* expression profiles by semi-quantitative one-step RT-PCR.

Cloning of the full-length cDNA of *RvHDR*

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 (PowerScriptTM, 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 *R. verticillata*. Two degenerate primers, dfhdr and drhdr (Table 1), were designed according to the conserved sequences of other plant *HDR* genes and used for the amplification of the core cDNA fragment of *RvHDR* by standard gradient PCR amplification (from 55 – 68°C) on BioRad My Cycler (USA). The core fragment was amplified and subcloned into pGEM T-easy vector (Promega, USA), transformed into *E. coli* strain DH5a followed by sequencing. The core fragment was subsequently used to design the gene-specific primers for the cloning of the full-length cDNA of *RvHDR* by the technology of rapid amplification of cDNA ends (RACE).

SMARTTM RACE cDNA Amplification Kit (Clontech, USA) was used to clone the 3`-end and 5`-end of *RvHDR* cDNA. The first strand 3`-RACE-ready and 5`-RACE-ready cDNA samples from *R. verticillata* were prepared according to the manufacturer's protocol (SMARTTM RACE cDNA Amplification Kit, User Manual, Clontech, USA) and used as templates for 3`-RACE and 5`-RACE, respectively.

Two 3`-gene-specific primers and the universal primers provided by the kit were used to amplify the 3`-end of *RvHDR*. For the first PCR amplification of 3`-RACE of *RvHDR* cDNA, RvHDR3-1 and Universal Primer A Mix (UPM, provided by Clontech) were used as the primers and the 3`-RACE-ready cDNAs were used as templates .Then the first PCR products were used as the templates for the nested amplification of 3`-RACE, RvHDR3-2 and Nested Universal Primer (NUP, provided by Clontech) were used as the second PCR amplification. The 5`-end of *RvHDR* 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, RvHDR5-1 and UPM were used as the first PCR primers, and 5`-RACE-ready cDNAs were used as templates. For the nested PCR amplification of 5`RACE, RvHDR5-2 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 RvHDR cDNA 3' and 5'-ends, 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 (SMARTTM RACE cDNA Amplification Kit, User Manual, Clontech): 25 cycles of amplification (30 sec at 94 °C, 30 s at 68°C, 3 min at 72°C). By 3`-RACE and 5`-RACE, both ends of *RvHDR* were respectively obtained.

By assembling the sequences of 3`RACE, 5`-RACEand the core fragment on Contig Express (Vector NTI Suite 8.0), the full-length cDNA sequence of *RvHDR* was deduced. According to the deduced *RvHDR* cDNA sequence, two gene-specific primers: ffrvhdr and frrvhdr were used to amplified the full-length of *RvHDR* from 5`-RACE-ready cDNA samples through proof-reading PCR. All the PCR amplificons were subcloned into pGEM-T vector and followed by sequencing. Finally *RvHDR* was submitted to GenBank to be assigned with an accession number.

Table 1. The nucleotide sequences of oligonucleotide primers.

Primers	Orientation	Sequence
dfhdr	Sense	5`-GT(C/T)GAGCG(C/T)GC(A/T/C)GT(G/T/C)CAGAT(G/T)GC-3`
drhdr	Antisense	5`-CATC(C/T)TC(A/C)AC(G/A/T)(A/G)CCTT(A/G)TC(A/G/C/T)GG-3`
RvHDR3-1	Sense	5`-GGGTTTCGATCCAGATAATGATC-3`
RvHDR3-2	Sense	5`-GGACGATGATGCGTAAGTATG-3`
RvHDR5-1	Antisense	5`-GCCGCTCTTGAGTTGCATCACAGA-3`
RvHDR5-2	Antisense	5`-GACCTCCAAGGATATAGTCGCAAAC-3`
ffrvhdr	Sense	5`-ACGCGGGGACCTCAAAAAGAAG-3`
frrvhdr	Antisense	5`-ACGAAAAGAGCTATTTATACATCAG-3`
fexRvHDR	Sense	5`-ATGGCTATCTCTCTGCAATTCTCC-3`
rexRvHDR	Antisense	5`-CTATGCCAGTTGCAGGGCTTC-3`
18SF	Sense	5`-ATGATAACT CGACGGATCGC-3`
18SR	Antisense	5`-CTTGGATGTGGTAGCCGTTT-3`
F-cdsRvHDR	Sense	5'-CCGGATCCGCTATCTCTCTGCAATTCTC-3'
R-cdsRvHDR	Antisense	5'-CCAAGCTTCTATGCCAGTTGCAGGGCTT-3'

Comparative and bioinformatic analysis

Comparative and bioinformatic analyses of *RvHDR* were carried out online at the websites (http://www.ncbi.nlm.nih.gov and http://www.expasy.org). The sequence comparison was conducted through database search using BLAST program (Altschul et al., 1997). The subcellular location of *RvHDR* was predicted by TargetP (Emanuelsson et al., 2000). The multiple alignments of *RvHDR* and HDRs from other species were aligned with CLUSTALX (Thompson et al., 1997). A phylogenetic tree was constructed using MEGA 3.0 (Kumar et al., 2004).from CLUSTAL X alignments. The neighbor-joining method (Saitou et al., 1987) was used to construct the phylogenetic tree.

Expression profile analyses of *RvHDR*

Semi-quantitative one-step RT-PCR was carried out to investigate the expression profile of *RvHDR* in different tissues including roots, stems, leaves, flowers and fruits of *R. verticillata* and under different elicitor treatments including 100 µM MeJA, 100 mg.L^{-1} ASA, 50 µM ABA and UV, respectively. Aliquots of 0.4 µg total RNAs extracted from each sample of *R. verticillata* were used as templates in one-step RT-PCR reaction with two primers: fexRvHDR and rexRvHDR specific to the coding sequence of *RvHDR* using one-step RNA PCR kit (TaKaRa, Japan). Amplifications were performed in a volume of 25 µL 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, 58°C for 30 s, 72°C for 2 min). Meanwhile, the RT-PCR reaction for the house-keeping gene (18S rRNA gene) using specific primers 18SF and 18SR designed according to the conserved regions of plant 18S rRNA gene was performed to estimate whether equal control. PCR products (15 µL) were separated on 1.0% agarose gels stained with goldview.

Complementation of the *E. coli* HDR mutant

The *E. coli* HDR mutant strain MG1655 ara<>HDR (HDR, namely IspH) was maintained on Luria-Bertani (LB) medium containing 50 mg.L^{-1} kanamycin (Kan) and 0.2% (w/v) arabinose (Ara) (McAteer et al., 2001), but not able to form colonies on LB medium containing 0.2% (w/v) glucose (Glc) in the absence of Ara. According to the complementation strategy the *E. coli HDR* mutant strain MG1655 ara<>HDR was used to test the biological function of *RvHDR* in this experiment. The coding region of *RvHDR* was amplified by PCR using primers F-cds*RvHDR* and R-cdsRvHDR. Both of the fragment of *RvHDR* and the plasmid pQE30 were digested with BamH I and Hind III for 10 h. Subsequently, the coding region of *RvHDR* was cloned into the expression vector pQE30 to obtain the plasmid pQE30-*RvHDR*. The pQE30-*RvHDR* was transformed into the *E. coli HDR* mutant strain and selected on LB plates containing 50 mg.L^{-1} Kan, 50 mg.L^{-1} ampicillin (Amp), 0.2% Glc, and 0.5 mM IPTG. The presence of the pQE30-*RvHDR* 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 plates containing 50 mg.L^{-1} Kan, 50 mg.L^{-1} Amp, and 0.2% Glc, and 0.5 mM IPTG. The transformants containing the pQE30 empty vector cannot grow on medium containing 0.2% Glc and 0.5 mM IPTG.

RESULTS

Cloning of the full-length cDNA of *RvHDR*

Based on the conserved fragment of other plant HDR sequences, such as *Arabidopsis thaliana, Picrorhiza kurrooa, Vitis vinifera, Hevea brasiliensis* and etc, two degenerate primers (dfhdr and drhdr) were designed and used for gradient PCR-amplification of the core cDNA fragment of *HDR* from *R. verticillata.* Following PCR amplification, an approximately 1000-bp product was obtained at 62.9°C and sequenced. The BLAST search revealed that the 974-bp cDNA core fragment had high homologous with HDR genes from plant species such as *Solanum tuberosum, Nicotiana tabacum, Pinus taeda* and etc. These strongly suggested that the core fragment of *RvHDR* had been obtained. Thus, this fragment was used to design gene specific primers for both 5`-RACE and 3`-RACE. By nested 3`-RACE and 5`-RACE, the 588 bp 3`-end and 929 bp 5`-end of *RvHDR* were respectively

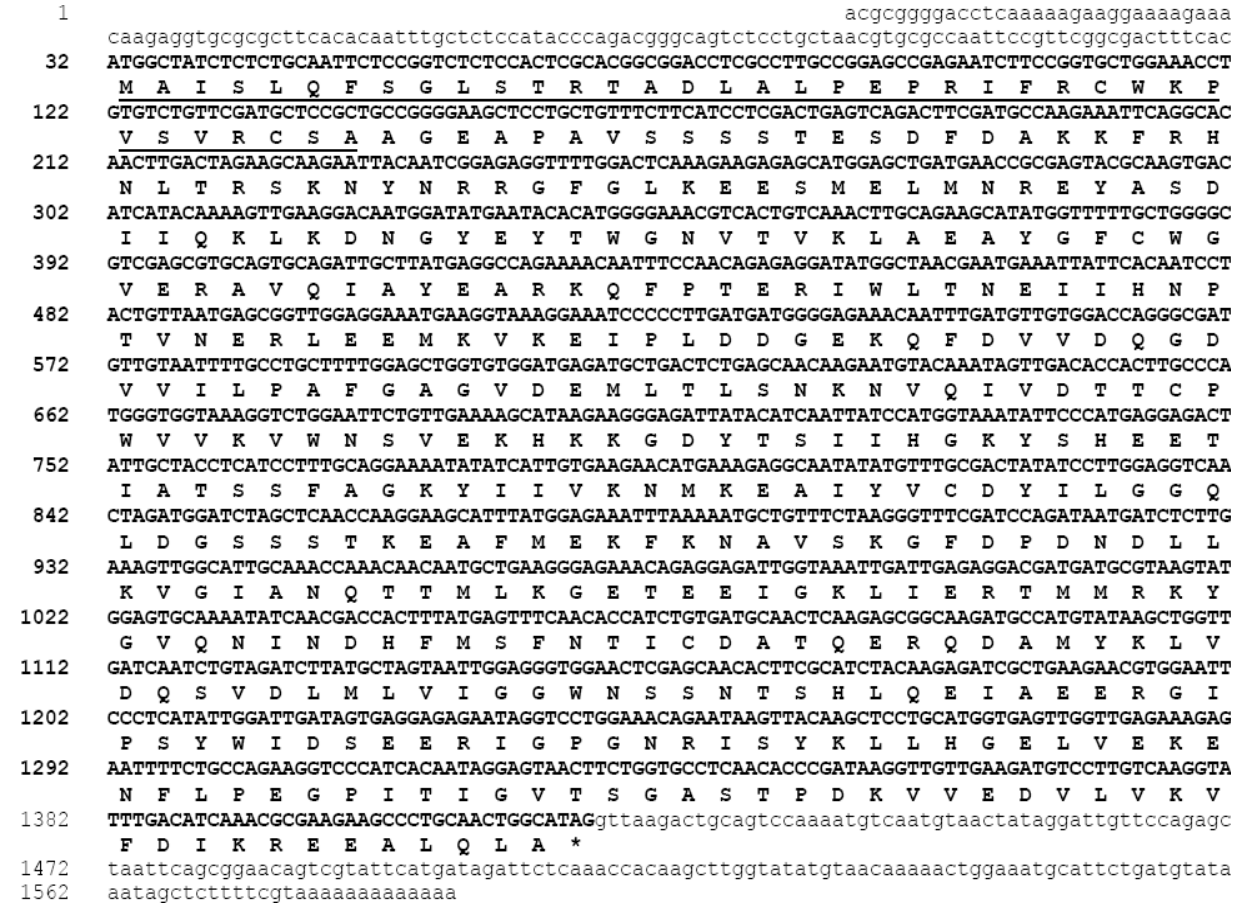


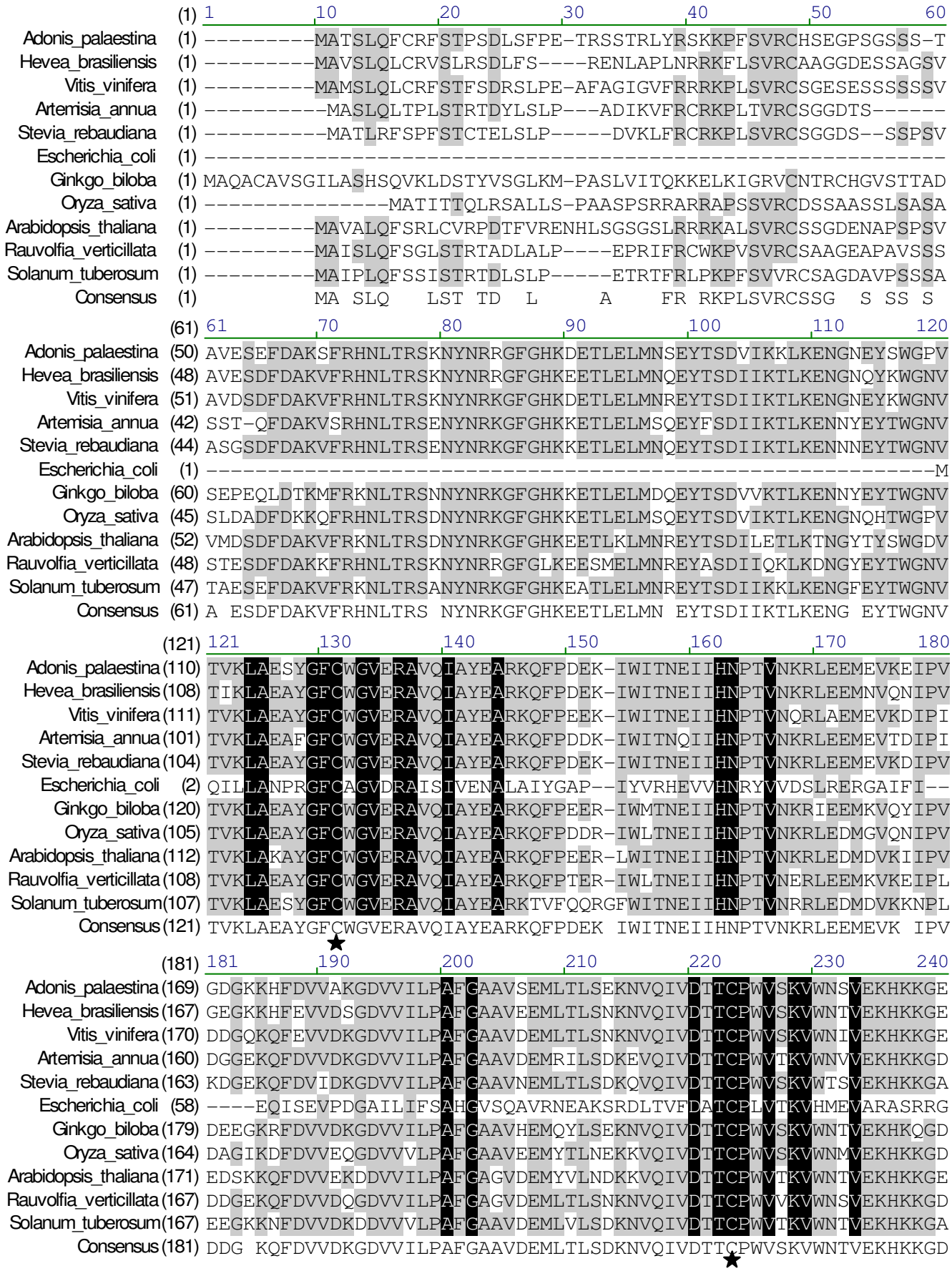
Figure 1. The full-length cDNA sequence and the deduced amino acid sequence of *RvHDR*. The coding sequence and its deduced amino acid sequence were shown in capital letters, and the UTR were shown in small letters. The stop codon (TAG) was marked with an aster, the plastidial transit peptide was underlined.

obtained. By aligning and 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 *RvHDR* with 1679-bp was deduced. Finally the physical full-length *RvHDR* cDNA was amplified and confirmed by sequencing (Figure 1). The sequencing results showed that *RvHDR* had the 121-bp 5` untranslated region (UTR), the1389-bp coding sequence and the 169-bp 3` UTR including the polyA tail. Then, the full-length *RvHDR* sequence was submitted to GenBank and assigned an accession number: EU034699. The ORF Finder program analysis on NCBI showed that the *RvHDR* contained a 1389-bp ORF encoding a protein of 462-amino acid with a calculated molecular mass of 52 kDa and an isoelectric point of 5.26.All dates show that a new full length HDR gene involved in TIAs biosynthesis had been cloned.

Comparative and bioinformatic analysis of *RvHDR*

Sequence BLAST research showed that the deduced amino acids of *RvHDR* from *R. verticillata* had high similarities with *HDRs* from other plant species, such as *Picrorhiza kurrooa* (80% identities), *Vitis vinifera* (81% identities), *Hevea brasiliensis* (79% identities) and *Adonis palaestina* (78% identities). Thus, the BLAST analysis results indicated that *RvHDR* belonged to the HDR family. To analyze the presence of peptide signal, Target P was used to predict the specific plastid targeted.

RvHDR protein was predicted to have plastid localization and had a 37-amino acid sequence with characteristics of plastidial targeting sequences at its N-terminal end. This was consistent with the fact that TIAs was synthesized in plastids (Yamazaki et al., 2003). Based on the multiple alignments, it was found that all aligned plant *HDRs* had a plastidial transit peptide at the N terminus, but the *E. coli* HDR protein lacked the N-terminal extension (Figure 2). This specific N-terminal extension comprised a signal sequence for plastid import, consistent with the subcellular localization of the MEP pathway in plants (Lichtenthaler et al., 1997). Furthermore, four conserved cysteine residues were found in *RvHDR* owned by all plant HDRs that might


(1) 1 10 20 30 40 50 60
Adonis_palaestina (1) ---------MATSLQFCRFSTPSDLSFPE-TRSSTRLYRSKKPFSVRCHSEGPSGSSS-T
Hevea_brasiliensis (1) ---------MAVSLQLCRVSLRSDLFS----RENLAPLNRRKFLSVRCAAGGDESSAGSV
Vitis_vinifera (1) ---------MAMSLQLCRFSTFSDRSLPE-AFAGIGVFRRRKPLSVRCSGESESSSSSSV
Artemisia_annua (1) ----------MASLQLTPLSTRTDYLSLP---ADIKVFRCRKPLTVRCSGGDTS------
Stevia_rebaudiana (1) ----------MATLRFSPFSTCTELSLP-----DVKLFRCRKPLSVRCSGGDS--SSPSV
Escherichia_coli (1) ----------
Ginkgo_biloba (1) MAQACAVSGILASHSQVKLDSTYVSGLKM-PASLVITQKKELKIGRVCNTRCHGVSTTAD
Oryza_sativa (1) ---------------MATITTQLRSALLS-PAASPSRRARRAPSSVRCDSSAASSLSASA
Arabidopsis_thaliana (1) ---------MAVALQFSRLCVRPDTFVRENHLSGSGSLRRRKALSVRCSSGDENAPSPSV
Rauvolfia_verticillata (1) ---------MAISLQFSGLSTRTADLALP----EPRIFRCWKPVSVRCSAAGEAPAVSSS
Solanum_tuberosum (1) ---------MAIPLQFSSISTRTDLSLP-----ETRTFRLPKPFSVVRCSAGDAVPSSSA
Consensus (1) MA SLQ LST TD L A FR RKPLSVRCSSG S SS S
(61) 61 70 80 90 100 110 120
Adonis_palaestina (50) AVESEFDAKSFRHNLTRSKNYNRRGFGHKDETLELMNSEYTSDVIKKLKENGNEYSWGPV
Hevea_brasiliensis (48) AVESDFDAKVFRHNLTRSKNYNRRGFGHKEETLELMNQEYTSDIIKTLKENGNQYKWGNV
Vitis_vinifera (51) AVDSDFDAKVFRHNLTRSKNYNRKGFGHKDETLELMNREYTSDIIKTLKENGNEYKWGNV
Artemisia_annua (42) SST-QFDAKVSRHNLTRSENYNRKGFGHKKETLELMSQEYFSDIIKTLKENNYEYTWGNV
Stevia_rebaudiana (44) ASGSDFDAKVFRHNLTRSENYNRKGFGHKKETLELMNQEYTSDIIKTLKENNNEYTWGNV
Escherichia_coli (1) ----------M
Ginkgo_biloba (60) SEPEQLDTKMFRKNLTRSNNYNRKGFGHKKETLELMDQEYTSDVVKTLKENNYEYTWGNV
Oryza_sativa (45) SLDADFDKKQFRHNLTRSDNYNRKGFGHKKETLELMSQEYTSDVIKTLKENGNQHTWGPV
Arabidopsis_thaliana (52) VMDSDFDAKVFRKNLTRSDNYNRKGFGHKEETLKLMNREYTSDILETLKTNGYTYSWGDV
Rauvolfia_verticillata (48) STESDFDAKKFRHNLTRSKNYNRRGFGLKEESMELMNREYASDIIQKLKDNGYEYTWGNV
Solanum_tuberosum (47) TAESEFDAKVFRKNLTRSANYNRKGFGHKEATLELMNREYTSDIIKKLKENGFEYTWGNV
Consensus (61) A ESDFDAKVFRHNLTRS NYNRKGFGHKEETLELMN EYTSDIIKTLKENG EYTWGNV
(121) 121 130 140 150 160 170 180
Adonis_palaestina (110) TVKLAESYGFCWGVERAVQIAYEARKQFPDEK-IWITNEIIHNPTVNKRLEEMEVKEIPV
Hevea_brasiliensis (108) TIKLAEAYGFCWGVERAVQIAYEARKQFPDEK-IWITNEIIHNPTVNKRLEEMNVQNIPV
Vitis_vinifera (111) TVKLAEAYGFCWGVERAVQIAYEARKQFPEEK-IWITNEIIHNPTVNQRLAEMEVKDIPI
Artemisia_annua (101) TVKLAEAFGFCWGVERAVQIAYEARKQFPDDK-IWITNQIIHNPTVNKRLEEMEVTDIPI
Stevia_rebaudiana (104) TVKLAEAYGFCWGVERAVQIAYEARKQFPDEK-IWITNEIIHNPTVNKRLEEMEVKDIPV
Escherichia_coli (2) QILLANPRGFCAGVDRAISIVENALAIYGAP--IYVRHEVVHNRYVVDSLRERGAIFI--
Ginkgo_biloba (120) TVKLAEAYGFCWGVERAVQIAYEARKQFPEER-IWMTNEIIHNPTVNKRIEEMKVQYIPV
Oryza_sativa (105) TVKLAEAYGFCWGVERAVQIAYEARKQFPDDR-IWLTNEIIHNPTVNKRLEDMGVQNIPV
Arabidopsis_thaliana (112) TVKLAKAYGFCWGVERAVQIAYEARKQFPEER-LWITNEIIHNPTVNKRLEDMDVKIIPV
Rauvolfia_verticillata (108) TVKLAEAYGFCWGVERAVQIAYEARKQFPTER-IWLTNEIIHNPTVNERLEEMKVKEIPL
Solanum_tuberosum (107) TVKLAESYGFCWGVERAVQIAYEARKTVFQQRGFWITNEIIHNPTVNRRLEDMDVKKNPL
Consensus (121) TVKLAEAYGFCWGVERAVQIAYEARKQFPDEK IWITNEIIHNPTVNKRLEEMEVK IPV
(181) 181 190 200 210 220 230 240
Adonis_palaestina (169) GDGKKHFDVVAKGDVVILPAFGAAVSEMLTLSEKNVQIVDTTCPWVSKVWNSVEKHKKGE
Hevea_brasiliensis (167) GEGKKHFEVVDSGDVVILPAFGAAVEEMLTLSNKNVQIVDTTCPWVSKVWNTVEKHKKGE
Vitis_vinifera (170) DDGQKQFEVVDKGDVVILPAFGAAVDEMLTLSNKNVQIVDTTCPWVSKVWNIVEKHKKGE
Artemisia_annua (160) DGGEKQFDVVDKGDVVILPAFGAAVDEMRILSDKEVQIVDTTCPWVTKVWNVVEKHKKGD
Stevia_rebaudiana (163) KDGEKQFDVIDKGDVVILPAFGAAVNEMLTLSDKQVQIVDTTCPWVSKVWTSVEKHKKGA
Escherichia_coli (58) ----EQISEVPDGAILIFSAHGVSQAVRNEAKSRDLTVFDATCPLVTKVHMEVARASRRG
Ginkgo_biloba (179) DEEGKRFDVVDKGDVVILPAFGAAVHEMQYLSEKNVQIVDTTCPWVSKVWNTVEKHKQGD
Oryza_sativa (164) DAGIKDFDVVEQGDVVVLPAFGAAVEEMYTLNEKKVQIVDTTCPWVSKVWNMVEKHKKGD
Arabidopsis_thaliana (171) EDSKKQFDVVEKDDVVILPAFGAGVDEMYVLNDKKVQIVDTTCPWVTKVWNTVEKHKKGE
Rauvolfia_verticillata (167) DDGEKQFDVVDQGDVVILPAFGAGVDEMLTLSNKNVQIVDTTCPWVVKVWNSVEKHKKGD
Solanum_tuberosum (167) EEGKKNFDVVDKDDVVVLPAFGAAVDEMLVLSDKNVQIVDTTCPWVTKVWNTVEKHKKGA
Consensus (181) DDG KQFDVVDKGDVVILPAFGAAVDEMLTLSDKNVQIVDTTCPWVSKVWNTVEKHKKGD

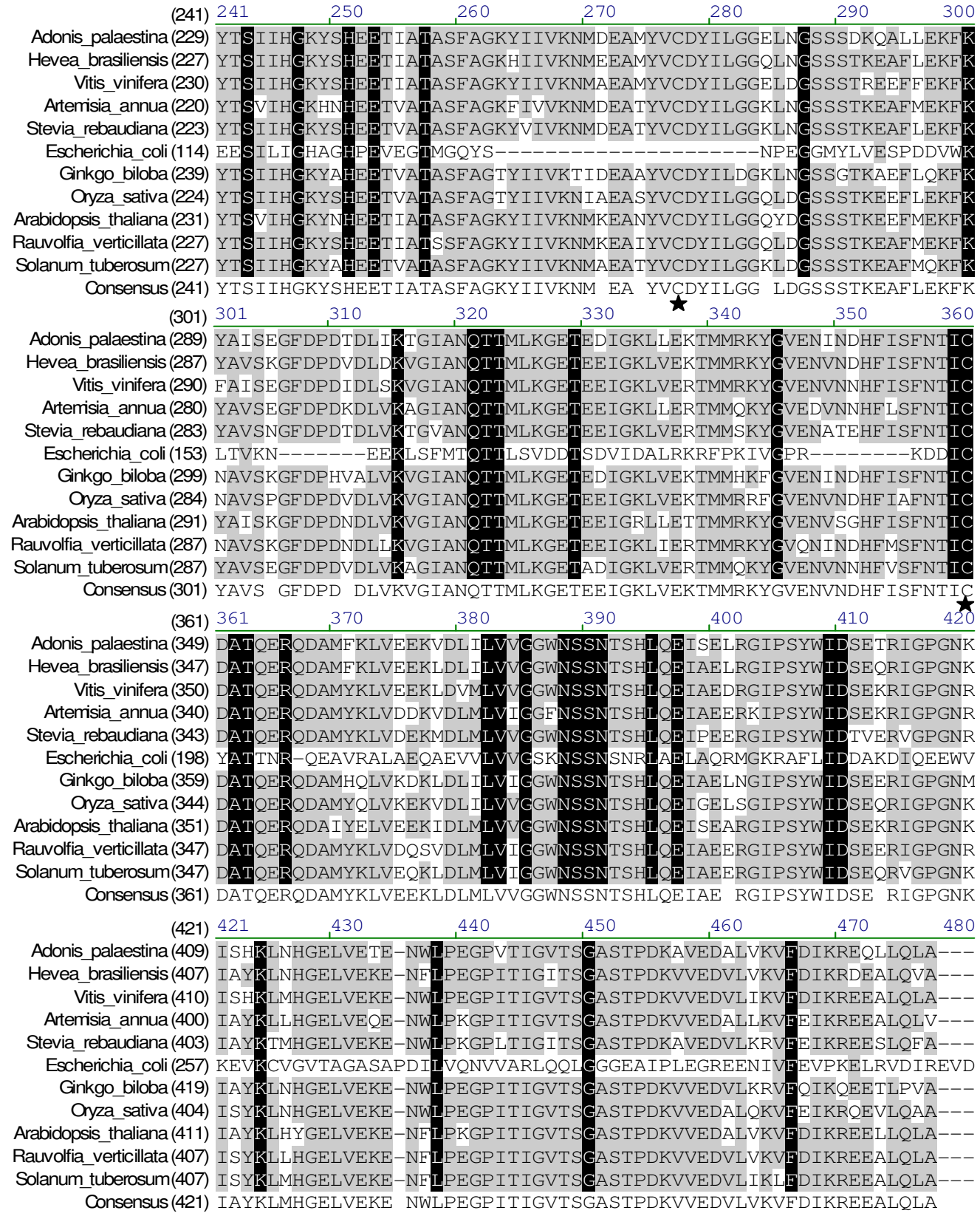


Figure 2. Multi-alignment of amino acid sequences of RvHDR and other HDRs. The identical amino acids were showed in white with black background and the conserved amino acids were showed in black with gray background, other amino acids were showed in black with white background. *Stars* mark the position of conserved cysteine residues.

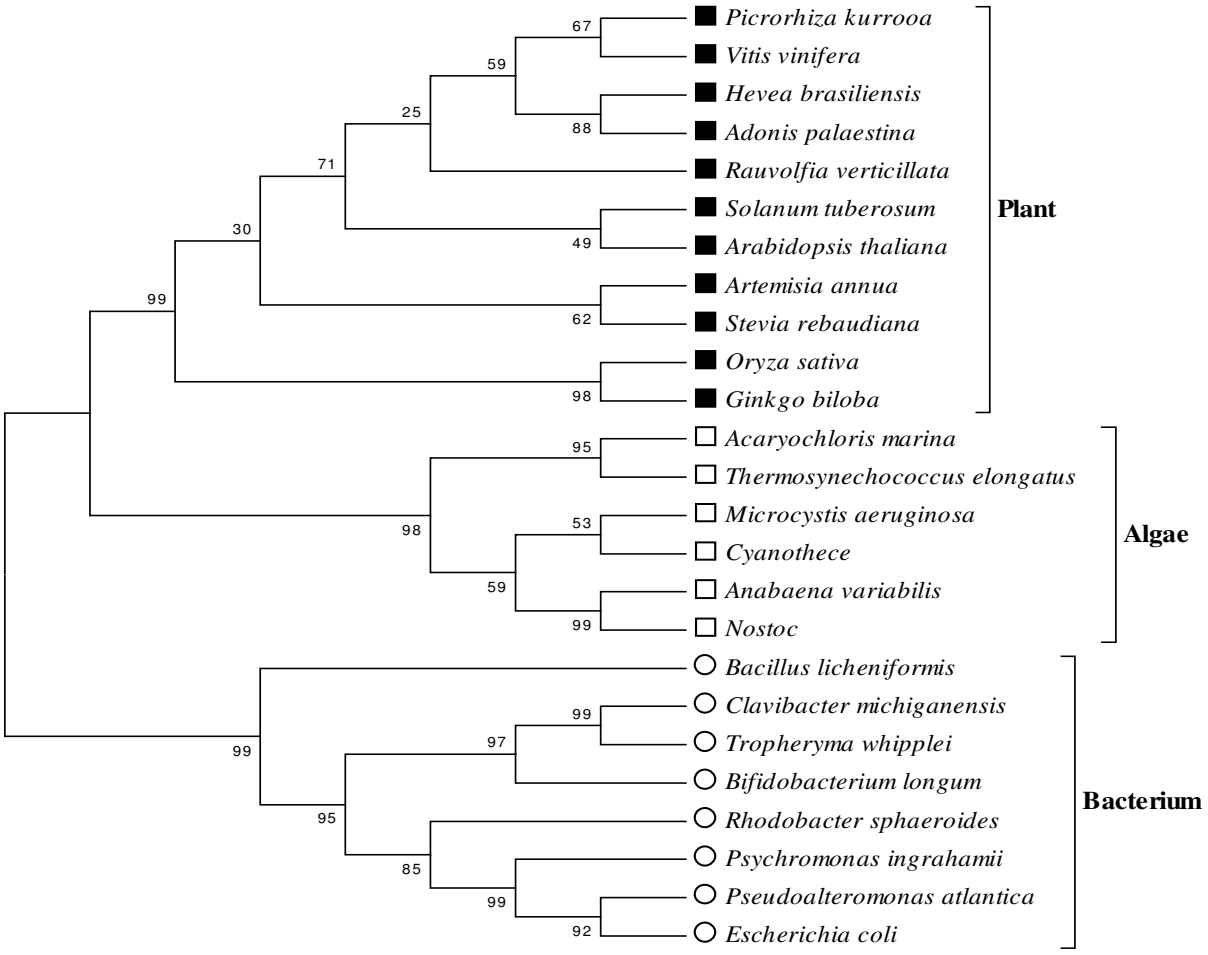


Figure 3. A phylogenetic tree of HDRs from different organisms including plants, algaes and bacteria constructed by the neighbor-joining method on MEGA 3.0. HDRs from plants were marked with ■, HDRs from algaes marked with □, and the others from bacteria marked with○. The numbers on the branches represented bootstrap support for 1000 replicates. The sequences used were listed bellow with Accession number: *Picrorhiza kurrooa*, ABM89226.1; *Vitis vinifera*, CAO47671.1; *Hevea brasiliensis*, BAF98297.1; *Adonis palaestina*, AAG21984.1; *Solanum tuberosum*, ABB55395.1; *Arabidopsis thaliana*, AAN87171.1; *Artemisia annua*, ABY57296.1; *Stevia rebaudiana*, ABB88836.2; *Oryza sativa*, NP_001051167.1; *Ginkgo biloba*, ABC84344.1; *Acaryochloris marina*, YP_001519239.1; *Thermosynechococcus elongatus*, NP_681832.1; *Microcystis aeruginosa*, CAO90213.1; *Cyanothece*, ZP_01731309.1; *Anabaena variabilis*, YP_323455.1; *Nostoc*, NP_485028.1; *Bacillus licheniformis*, YP_079844.1; *Clavibacter michiganensis*, YP_001222973.1; *Tropheryma whipplei*, CAD67323.1; *Bifidobacterium longum*, NP_696525.1; *Rhodobacter sphaeroides*, ABN75415.1; *Psychromonas ingrahamii*, ABM04955.1; *Pseudoalteromonas atlantica*, ABG41681.1; *Escherichia coli*, AAL38655.1.

participate in the coordination of the iron-sulfur bridge proposed to be involved in catalysis (Seemann et al., 2002; Wang et al., 2008). The position of one of these cysteine residues was not conserved in the *E. coli* protein (Figure 2), but it was possible that the cysteine at position 263 in the *E. coli* sequence might participate in the [4Fe-4S] coordination. Using MEGA 3.0 based on CLUSTAL X alignments, a phylogenetic tree of HDRs was constructed from different organisms including plants, algaes and bacteria. The result demonstrated that HDRs were derived from an ancestor gene and evoluted into three groups including plants, algaes and bacteria HDR group. *RvHDR* had higher identity with plant HDRs than bacterium and algae HDRs (Figure 3). All the analysis results strongly suggest that *RvHDR* is a plant HDR protein involved in the mevalonate-independent biosynthesis.

Expression profile analyses

To investigate the expression profile of *RvHDR* in different tissues including roots, stems, leaves, flowers and fruits of *R. verticillata*, total RNAs were isolated from different tissues and subjected to semi-quantitative one-step RT-PCR using fex*RvHDR* and rex*RvHDR* as

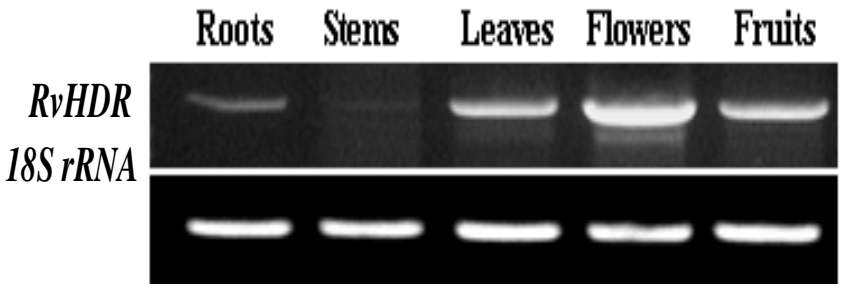


Figure 4. Expression profile of *RvHDR* in different tissues of *R. verticillata*. Total RNA samples were isolated from roots, stems, leaves, flowers and fruits respectively, and subjected to Semi-quantitative one-step RT-PCR analysis (upper panel). *18S rRNA* gene was used as the control to show the normalization of the amount of templates in PCR reactions (lower panel).

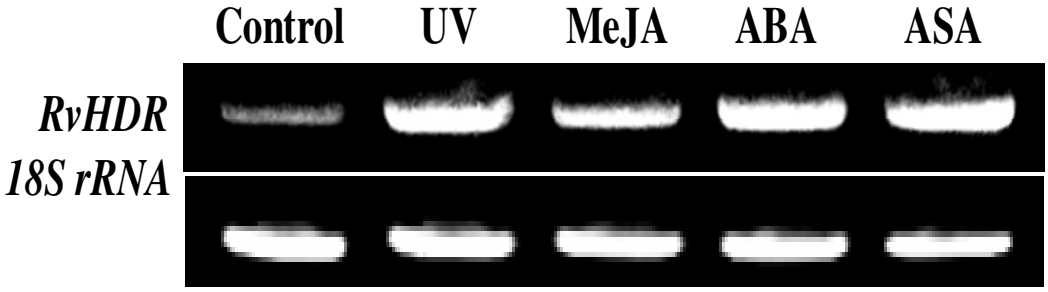


Figure 5. Expression profile of *RvHDR* under induction by elicitors including UV, 100 µM MeJA, 50 µM ABA and 100 mg.L^{-1} ASA. Total RNA samples were isolated from callus treated with UV, MeJA, ABA, ASA and without treatment (as the control), respectively, and analyzed by one-step RT-PCR.

primers. The house-keeping gene (18S rRNA gene) expression in all the detected tissues was used as internal control that showed no significant difference. The result showed *RvHDR* expression could be detected in all tissues, suggesting that *RvHDR* is constitutively expressed but at different levels in different organs. Furthermore, the highest expression level of *RvHDR* was found in flowers of *R. verticillata*, followed by in fruits (Figure 4).

To investigate the induction by various elicitors, one-step RT-PCR analyses were carried out to monitor the changes of *RvHDR* expression levels upon various elicitor treatments including 100 µM MeJA, 100 mg.L^{-1} ASA, 50 µM ABA and UV. The result showed that the expression levels of *RvHDR* were all strongly increased by MeJA, ASA, ABA and UV treatments, among which the highest transcript level of *RvHDR* was found by UV treatment (Figure 5). The result was consistent with the HDR gene expression in Camptotheca acuminata (Wang et al., 2008).and Arabidopsis thaliana (Hsieh et al., 2005) and suggested that *RvHDR* was a highly-regulated gene for basic physiological and biochemical processes in *R. verticillata*.

RvHDR complements the *E. coli* HDR mutant

To test whether the HDR protein of *R. verticillata* has similar enzymatic activity to its *E. coli* counterpart, we used 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 PBAD promoter (McAteer et al., 2001). The *E. coli HDR* mutant cannot form colonies on LB medium in the absence of arabinose (Figure 6), because the HDR gene is essential for survival. When pQE30-*RvHDR* plasmid containing the coding region of *RvHDR* was transformed into the *E. coli HDR* mutant strain MG1655 ara<>HDR, this bacteria can restore successfully on the medium containing 0.2% Glc but cannot by transforming the empty pQE30 vector.

The genetic complementation strategy was applied to identify the function of *RvHDR* in mutant *E-coil* strain MG1655 ara<>HDR. The HDR gene is an essential gene for survival of *E. coil*. In the present study, *RvHDR* was introduced into mutant *E-coil* strain MG1655 ara<>HDR

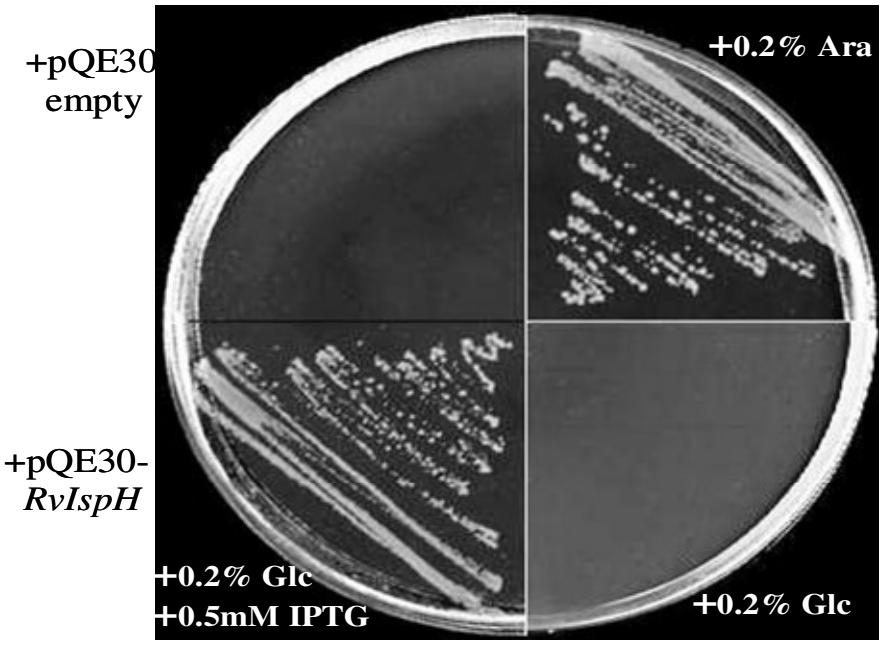


Figure 6. *R. verticillata* HDR complements the *E. coli* HDR mutant. The *E. coli* HDR mutant strain MG1655ara<>*HDR* was able to grow on LB media containing 0.2% Ara, but not on media containing 0.2% Glc (right). After transformation with the *RvHDR* coding region (pQE30-*RvHDR*) and, as a control, with the empty vector (pQE30) alone, the resulting strains were tested for growth on media containing 0.2% Glc and 0.5 mM IPTG (left). Expression of RvHDR protein successfully restored the growth of the *E. coli* HDR mutant (below left).

and over expressed, and then the mutant was rescued by *RvHDR* in media without arabinose. It was confirmed that the HDR protein of *R. verticillata* had similar enzymatic activity to its *E. coli* counterpart.

DISCUSSION

HDR catalyzes the last reaction of the methylerythritol phosphate (MEP) pathway, a branching step that separately produces isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) in a ratio of 5:1 (Altincicek et al., 2002) and is the ideal target for metabolic engineering of the isoprenoid biosynthetic pathway. In our study, we have successfully isolated and characterized the *RvHDR* cDNA from R. verticillata for the first time. The *RvHDR* cDNA contained a 1389-bp ORF coding for protein *RvHDR* with 462-amino acid residues. The further bioinformatics analysis indicated that *RvHDR* contained a 37-amino transit peptide that directed it to plasmid, the result was consistent with the fact that the precursor of TIAs was synthesized in plastids (Yamazaki et al., 2003). The existence of expression of *RvHDR* in all detected tissues but in different levels showed *RvHDR* could be an essential gene that was also highly regulated like the HDR genes from *Arabidopsis* (Hsieh et al., 2005), Ginkgo and Pinus (Kim et al., 2008). In recent years, many studies showed some elicitors were the excellent stimulators of alkaloid biosynthesis exogenously applied to cell suspension cultures, such as MeJA (Sheludo et al.,1998), ASA (Gong et al., 2005).

Our present study indicated that all the elicitors including MeJA, ASA, ABA and UV could up-regulate the expression of *RvHDR*. The results provide direct evidence that *RvHDR* is an elicitor-responsive gene and can be effectively elicited at least at the transcription level. Finally, the RvHDR was been introduced into *E. coil* HDR mutant strain MG1655 ara<>HDR and over expressed, and then the mutant was rescued by *RvHDR* in the media without ara. The result of genetic complementation demonstrated that *RvHDR* gene reported here did encode the active enzyme HDR. Cloning, characterization, and functional identification of *RvHDR* will facilitate the understanding of the biosynthesis of TIAs including reserpine and ajmalicine and also promote metabolic engineering of the TIAs biosynthetic pathway in *R. verticillata.*

ACKNOWLEDGEMENTS

The research is financially supported by the National Science Foundation of China (30771238) and the National High-tech Plan 863 (2010AA100503).

REFERENCES

Altincicek B, Duin EC, Reichenberg A, Hedderich R, Kollas AK, Hintz M, Wagner S, Wiesner J, Beck E, Jomaa H (2002). LytB protein catalyzes the terminal step of the 2-C-methyl-D-erythritol-4-phosphate pathway of isoprenoid biosynthesis. FEBS Lett. 532: 437-440.

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25: 3389-3402.

Anitha S, Ranjitha Kumari BD (2006). Stimulation of reserpine biosynthesis in the callus of Rauvolfia tetraphylla L. by precursor feeding. Afr. J. Biotechnol. 5: 659-661.

Botella-Pavía P, Besumbes Ó, Phillips MA, Carretero-Paulet L, Boronat A, Rodríguez-Concepción M (2004). Regulation of carotenoid biosynthesis in plants: evidence for a key role of hydroxymethylbutenyl diphosphate reductase in controlling the supply of plastidial isoprenoid precursors. Plant J. 40: 188-199.

Cunningham FX, Jr Lafond TP, Gantt E (2000). Evidence of a role for LytB in the nonmevalonate pathway of isoprenoid biosynthesis. J. Bacteriol. 182: 5841-5848.

Emanuelsson O, Nielsen H, Brunak S, Von Heijne G (2000). Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. J. Mol. Biol. 300: 1005-1016.

Farooqi AA, Sreeramu BS (2001). Cultivation of Medicinal and Aromatic Crops. (Pub.) University Press Ltd., India pp. 210-211.

Gong YF, Liao ZH, Guo BH, Sun XF, Tang KX (2005). Molecular cloning and expression profile analysis of Ginkgo biloba DXS gene encoding 1-deoxy-d-xylulose 5-phosphate Synthase, the first committed enzyme of the 2-C-methyl-d-erythritol 4-phosphate pathway. Planta Medica 68: 1-7.

Guevara-Garcia A, San Roman C, Arroyo A, Cortes ME, M de la Luz Gutierrez-Nava, Leon P(2005). Characterization of the Arabidopsis clb6 mutant illustrates the importance of posttranscriptional regulation of the methyl-D-erythritol 4-phosphate pathway. Plant Cell 17: 628-643.

Hsieh MH, Goodman HM (2005). The arabidopsis IspH homolog is involved in the plastid nonmevalonate pathway of isoprenoid biosynthesis. Plant Physiol. 138: 641-653.

Kim SM, Kuzuyama T, Kobayashi A, Sando T, Chang YJ, Kim SU (2008). 1-Hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase (IDS) is encoded by multicopy genes in gymnosperms Ginkgo biloba and Pinus taeda. Planta 227: 1432-2048.

Kumar S, Tamura K, Nei M (2004). MEGA3: Integrated Software for Molecular Evolutionary Genetics Analysis and Sequence Alignment. Brie Bioinform 5: 150-163.

Lichtenthaler HK, Rohmer M, Schwender J (1997). Two independent biochemical pathways for isopentenyl diphosphate and isoprenoid biosynthesis in higher plants. Physiol. Plant. 1016: 43-52.

Li HY, Ting KS (1962). Haemodynamic studies on the total alkaloids of Rauvolfia verticillata. Scientia Sinica 11: 791-804.

McAteer S, Coulson A, McLennan N, Masters M (2001). The lytB gene of Escherichia coli is essential and specifies a product needed for isoprenoid biosynthesis. J. Bacteriol. 183: 7403-7407.

Ramos-Valdivia AC, Van Der Heijden R, Verpoorte R (1998). Isopentenyl diphosphate isomerase and prenyltransferase activities in rubiaceous and apocynaceous cultures. Phytochemistry 48: 961-949.

Saitou N, Nei M (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4: 406-425.

Seemann M, Bui BT, Wolff M, Tritsch D, Campos N, Boronat A, Marquet A, Rohmer M (2002). Isoprenoid biosynthesis through the methylerythritol phosphate pathway: the (E)-4-hydroxy-3-methylbut-2-enyl diphosphate synthase (GcpE) is a [4Fe-4S] protein. Angew. Chem. Int. Ed. Engl. 41: 433-339.

Sheludo YU, Gerasimenko I, Unger M, Kostenyuk I, Stockigt J (1998). Induction of alkaloid diversity in hybrid plant cell lines. Plant Cell Rep. 18: 911-918.

Thompson LD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997). The Clustal X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 24: 4876-4882.

Wang Q, Pi Y, Hou R, Jiang K, Huang Z, Hsieh MS, Sun X, Tang K (2008). Molecular cloning and characterization of 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase (CaHDR) from Camptotheca acuminata and its functional identification in *Escherichia coli*. BMB Rep. 41: 112-118.

Wolff M, Seemann M, Tse Sum Bui B, Frapart Y, Tritsch D, Estrabot AG, Rodriguez-Concepcion M, Boronat A, Marquet A, Rohmer M (2003). Isoprenoid biosynthesis via the methylerythritol phosphate pathway: the (E)-4-hydroxy-3-methylbut-2-enyl diphosphate reductase (LytB/IspH) from Escherichia coli is a [4Fe-4S] protein. FEBS Lett. 541: 115-120.

Yamazaki Y, Urano A, Sudo H, Kitajima M, Takayama H, Yamazaki M, Aimi N, Saito K (2003). Metabolite profiling of alkaloids and strictosidine synthase activity in camptothecin producing plants. Phytochemistry 62: 461-470.