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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*

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Camptothecin is an anti-cancer monoterpene indole alkaloid. The gene encoding 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate reductase (designated as *CaHDR*), the last catalytic enzyme of the MEP pathway for terpenoid biosynthesis, was isolated from camptothecin-producing *Camptotheca acuminata*. The full-length cDNA of *CaHDR* was 1686 bp encoding 459 amino acids. Comparison of the cDNA and genomic DNA of *CaHDR* revealed that there was no intron in genomic *CaHDR*. Southern blot analysis indicated that *CaHDR* belonged to a low-copy gene family. RT-PCR analysis revealed that *CaHDR* expressed constitutively in all tested plant organs with the highest expression level in flowers, and the expression of *CaHDR* could be induced by 100 μM methyl-jasmonate (MeJA), but not by 100 mg/L salicylic acid (SA) in the callus of *C. acuminata*. The complementation of *CaHDR* in *Escherichia coli ispH* mutant MG1655 demonstrated its function. [BMB reports 2008; 41(2): 112-118]

INTRODUCTION

Camptothecin (CPT), a plant-derived alkaloid currently in clinical use, was first isolated from *Camptotheca acuminata* (*Nyssaceae*) in 1958 (1) with its structure being elucidated in 1966 (2). Making a cellular target of topoisomerase I (3), the CPT derivatives, irinotecan and topotecan, which are obtained by solvent extraction from bark or seeds of *C. acuminata* or other CPT-producing species, are used throughout the world for the treatment of various cancers (1, 4). Although CPT accumulation occurs in all of the vegetative organs of *C. acuminata*, particularly in young leaves (5, 6), its supply from natural plant materials is unable to meet the demand of clinical use because of its insufficient concentrations. Because of the important role of CPT and its derivatives in clinical use, it is a necessity to develop sustainable and alternative production sources of these compounds (4).

Isoprenoids, besides of their primary roles in membrane structure, redox reactions, light harvesting and photoprotection, and regulation of growth and development, also participate as secondary metabolites in a variety of functions in plants (7). Terpenoid Indole Alkaloids (TIAs) are a group of isoprenoids of important pharmaceutical applications which are found in *Apocynaceae, Loganiaceae, Rubiaceae* and *Nyssaceae* plants (8). Plant isoprenoids biosynthesis occurs by two independent pathways, the mevalonate (MVA) pathway in cytoplasm, and the nonmevalonate (also called methyl-D-erythritol 4-phospate, MEP) pathway in plastids. Although both of the two pathways are operative simultaneously in higher plants, the enzymes of the MVA route are believed to produce precursors for triterpenes, sesquiterpenes and sterols, while enzymes in MEP pathway supply precursors of monoterpenes, some sesquiterpenes, diterpenes and carotenoids (1, 9, 10). Previous study showed that in *C. acuminata*, both MVA and MEP pathway were active, and MVA pathway was responsible for the formation of triterpenes related to ursane- or oleanane-type skeletons (11). In cell cultures of *C. acuminata*, MEP pathway might be inhibited or a possible shift of the isoprenoids from the MEP to the MVA might have occurred (11). Camptothecin is a modified monoterpene indole alkaloid, and is believed to be synthesized in plant via MEP pathway from isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), the universal precursors of all isoprenoids (11, 12). The enzymatic steps and genes of the MEP pathway have been identified in *Escherichia coli* and plants (13). In the initial step of MEP pathway, pyruvate and glyceraldehyde 3-phosphate (G3P) are converted by the enzyme 1-deoxy-D-xylulose-5-phosphate syn-

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thase (*dxs*) to 1-deoxy-D-xylulose-5-phosphate (DXP) (14, 15). Since DXP also serves as a precursor of thiamine and pyridoxal along with MEP, MEP synthesis by DXP reductoisomerase (*dxr* or *ispC*) from DXP is the first committed step of the pathway (13, 16, 17). The following five enzymes including 4-diphosphocytidyl-2-*C*-methyl-D-erythritol synthase (*cms* or *ispD*), 4-diphosphocytidyl-2-*C*-methyl-D-erythritol kinase (*cmk* or *ispE*), 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase (*mcs* or *ispF*), 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate synthase (*hds* or *ispG*) and 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate reductase (*hdr*, also called *lytB* or *ispH*) then complete the synthesis of IPP and DMAPP (Fig. 1). *Hdr* is identified as the last enzyme of the MEP pathway, which converts (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate (HMBPP) into a 5 : 1 mixture of IPP and DMAPP (13, 18). Therefore, the activity of this enzyme could be identified for the branching which is an important difference of the MVA pathway, in which IPP and DMAPP are generated sequentially, the latter arising from the former in a reaction catalyzed by IPP and DMAPP isomerase (*idi*) (13). Previous study showed that *hdr* is essential for *E. coli* survival and related to isoprenoid biosynthesis (19). Cell extract of an *E. coli* strain engineered for hyperexpression of the *lytB* (*ispH*) gene can catalyze the *in vitro* conversion of HMBPP to IPP and DMAPP, the recombinant IspH protein enhanced the intrinsic catalytic activity of *E. coli* wild-type extract up to 4.5 fold and up to 4 fold in *Capsicum annuum* chromoplasts (20). The analysis of double transgenic *Arabidopsis* plants overproducing both the enzyme taxadiene synthase (*txs*) catalyzing the production of the non-native isoprenoid taxadiene from GGPP and either *hdr* or *dxs* showed a twofold effect of *hdr* alone in increasing taxadiene levels (21). A recent study indicates that *hdr* plays a major role in controlling the production of MEP-derived precursors for plastid isoprenoid biosynthesis (21). Considering the importance of *hdr* in TIAs biosynthesis, in the study, we report for the first time the cloning and characterization of the full-length *hdr* gene (*CaHDR*) from *C. acuminata*, which will be helpful in extending our knowledge of the MEP pathway in camptothecin biosynthesis.

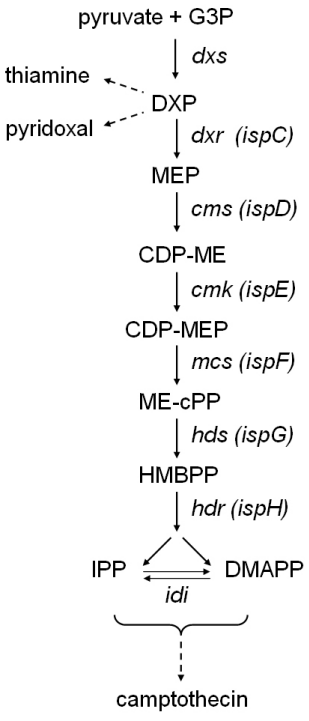

Fig. 1. The MEP pathway in *C. acuminata*.

RESULTS AND DISCUSSION

Cloning and analysis of full-length cDNA of *CaHDR*

The *hdr* genes have been isolated from *E. coli* and some plant species and functional characterized as the last catalytic enzyme of the MEP pathway for isoprenoids biosynthesis (14). However, there is no report of isolation of *hdr* gene in *C. acuminata*. Using total RNA of *C. acuminata* as template, amplification of the core fragment using primers designed from the homologous sequences of plant *hdr* genes resulted in a product of 920 bp in length. Blastn analysis revealed that it had high similarity to plant *hdr* genes, which suggested it might be a conserved fragment of *hdr* gene. Gene specific primers were designed based on this conserved fragment for full-length cDNA isolation.

Amplification of 5'-end and 3'-end of *CaHDR* resulted in 633 bp and 427 bp fragments, respectively. Assembly of 5'-end, conserved fragment and 3'-end cDNA of *CaHDR* using Vector NTI Suit 9.0 resulted in a 1,686 bp full length cDNA sequence including the 5' untranslated region and 3' polyA tail. Analysis of the *CaHDR* coding region using ORF Finder on NCBI indicated a 1,377 bp coding sequence encoding a 459-amino-acid protein with a calculated molecular mass of 51.7 kDa and an pI value of 5.27 (http://cn.expasy.org/tools/protparam.html).

To investigate whether there is any intron in the *CaHDR* gene, genomic sequence of *CaHDR* was amplified and resulted in a fragment of 1.7 kb, which was similar to the size of *CaHDR* cDNA. Different from its counterpart in *Arabidopsis*, whose sequence was interrupted by nine introns (22), sequencing result showed that there was no intron in *CaHDR* (data not shown).

Characterization of *CaHDR*

The protein sequence of CaHDR was submitted to NCBI protein-protein blast (http://www.ncbi.nlm.nih.gov/BLAST) for sequence comparison of CaHDR to other known HDR proteins. The results showed that *CaHDR* had very broad and high local identities and positives to HDRs from species of broad plant classes such as *Adonis palaestina* (AAG21984, Identities 83%, Positives 90%), *Stevia rebaudiana* (ABB88836, Identities 83%, Positives 91%), *Solanum tuberosum* (ABB55395, Identities 83%, Positives 91%), *Picrorhiza kurrooa* (ABM89226, Identities

80%, Positives 91%) and *Ginkgo biloba* (ABC84344, Identities 76%, Positives 87%). Other plant *hdr* gene sequences including *Arabidopsis thaliana* (TC183666), *Vitis vinifera* (TC14300), *Oryza sativa* (TC200700), *Lactuca sativa* (TC12240) and *Lycospersicon esukurentamu* (TC124188) were obtained from TIGR (http://www.tigr.org) and translated into protein sequences for multiple alignments analysis of the homologies of CaHDR to these HDR proteins. The *E. coli lytB* sequence (P22565) was also used for multiple alignments. The results of blast and multiple alignments strongly suggested that CaHDR had high homologies to other HDRs and should be a functional plant HDR protein.

Plant MEP pathway enzymes contain an N-terminal extension that was absent in bacterium and animals (13). All of the MEP genes in *A. thaliana* subjected to the ChloroP algorithm analysis (23) demonstrated that the N-terminal extension was plastid targeting peptide (13). The ChloroP algorithm predicted that all of the plant HDR proteins using in multiple alignment contained a putative chloroplast transit peptide (cTP) of variable length, consistent with their predicted role in plastid isoprenoid biosynthesis. The cleavage site of N-terminal cTP of *CaHDR* was predicted to be located between Arg 33 and Cys 34, comprising of 33 amino acid residues.

Previous study showed that all of the genes in the MEP pathway are highly conserved (24). It was also true for the *hdr* gene, which displays a 30% amino acid identity between *Arabidopsis* and *E. coli* (22). However, the evolution of *hdr* genes had not been elucidated. Although it has been accepted that plant genes in the MEP pathway were acquired during the endosymbiotic event with an ancestral cyanobacteria (15), recent studies demonstrated that both the vertical gene inheritance and lateral gene transfer might play a critical role in the acquisition of MEP pathway genes (24). Thus, a phylogenetic tree was constructed to investigate the evolution position of *CaHDR*. Except for the plant HDRs, in the NCBI database, the search for other HDRs retrieved several deduced amino acid sequences in cyanobateria including *Anabaena variabilis* (YP_323455), *Crocosphaera watsonii WH 8501* (ZP_0051-5051), *Lyngbya sp. PCC 8106* (ZP_01620518), *Nodularia spumigena CCY9414* (ZP_01631570), *Nostoc punctiforme PCC 73102* (ZP_00110421), *Prochlorococcus marinus str. MIT 9313* (NP_895681), *Synechococcus elongatus PCC 6301* (YP_172141), *Synechocystis sp. PCC 6803* (NP_442089) and *Trichodesmium erythraeum IMS101* (YP_723939). Other HDRs from bacteria species such as *Clostridium acetobutylicum ATCC 824* (NP_348471), *Geobacter sp. FRC-32* (ZP_0-1390009), *Pelobacter propionicus DSM 2379* (YP_901027) and *Thermoanaerobacter ethanolicus ATCC 33223* (ZP_00-778736) were also obtained. In the phylogenetic tree, the HDR proteins were divided into two major groups, the plant HDRs and bacteria HDRs (Fig. 2). CaHDR belonged to the plant HDR group, which strongly suggested that CaHDR was a

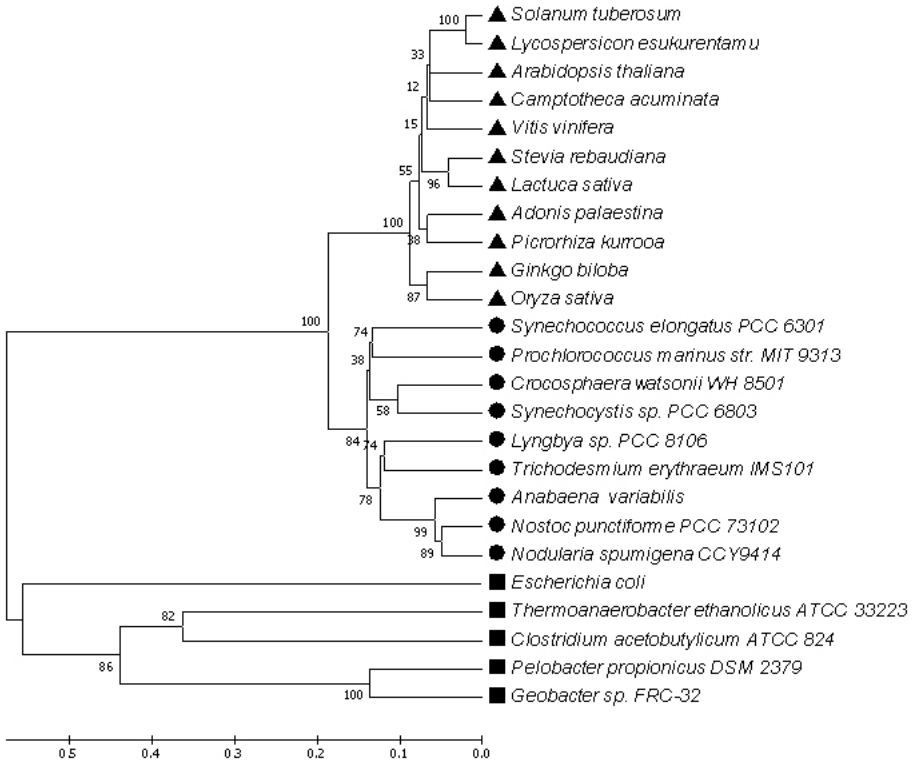

Fig. 2. Phylogenetic tree analysis of HDR proteins. Numbers on branches indicated percentage of support in the bootstrap analysis (1000 replications). Square and dot indicated the bacterial HDRs while dot represented cyanobaterial HDR proteins. Triangle referred to the plant HDRs.

plant HDR protein. According to the phylogenetic analysis, plant HDRs were closer to their cyanobaterial counterpart than other bacterial HDRs in evolution, which supported previous evolutionary studies of plant HDRs (22) and suggested plant *hdr* gene might have evolved from its plastid ancestor.

Southern blot analysis

Among all the MEP pathway enzymes that can be found in the *Arabidopsis* genome, only *dxs* was encoded by three genes (13). Other downstream MEP enzymes including *hdr* gene were present as single copies. In order to investigate the genomic organization of *CaHDR*, genomic DNA was digested with *Bam*HI, *Dra*I and *Hind*III respectively, and subjected to Southern blot analysis. The result showed that only two to three hybridizations signals were present in each lane, indicating that *CaHDR* belongs to a low-copy gene family (Fig. 3A).

Expression of *CaHDR* in different organs

To investigate the expression pattern of *CaHDR* in different organs of *C. acuminata*, total RNA were extracted from roots, stems, leaves, flowers and seeds, and subjected to RT-PCR analysis using 18S rRNA as internal control. The result showed that *CaHDR* expressed, in a constitutive manner, in all tested organs, but at different levels with the highest expression in flowers (Fig. 3B), showing a similar expression pattern to its counterpart in *A. thaliana* (25). However, although HDR protein could be detected in all tissues in *A. thaliana*, it maintained the highest protein level in young leaves (22). The difference between the level of mRNA and protein in different organs was probably because of posttranscriptional regulation, or the expression of *hdr* was developmental dependent. For *C. acuminata*, the highest level of CPT was found in young leaves and declined with tree age and with time during the growing season (1, 26). Thus, further studies are required to investigate the relationship of *CaHDR* expression and CPT biosynthesis.

CaHDR expression under MeJA and SA induction

To examine the expression of *CaHDR* under different elicitor treatments, the 15-day-old *C. acuminata* callus lines were harvested for RNA isolation after treated respectively with 100 μM MeJA or 100 mg/L SA after various duration (0, 6, 12, 24, 48 and 72 h), and used in RT-PCR analysis. MeJA was identified as a signal of altered gene expression in various plant responses to biotic and abiotic stresses as well as of distinct stages of plant development (27, 28). It has been proved that in *Catharanthus roseus*, expression of the first key enzyme *dxs* of the MEP pathway and some other key enzymes for TIAs biosynthesis such as anthranilate synthase (*as*), tryptophan decarboxylase (*tdc*) and strictosidine synthase (*str*) are up-regulated by MeJA (29). In the present study, our result showed that *CaHDR* expression was also up-regulated by 100 μM MeJA and its expression reached the maximum level at 24 h after the treatment (Fig. 3C1). However, though SA is also an important component of signal transduction cascades activating plants' defense response (30), our study showed that the expression of *CaHDR* was not influenced by 100 mg/L SA (Fig.

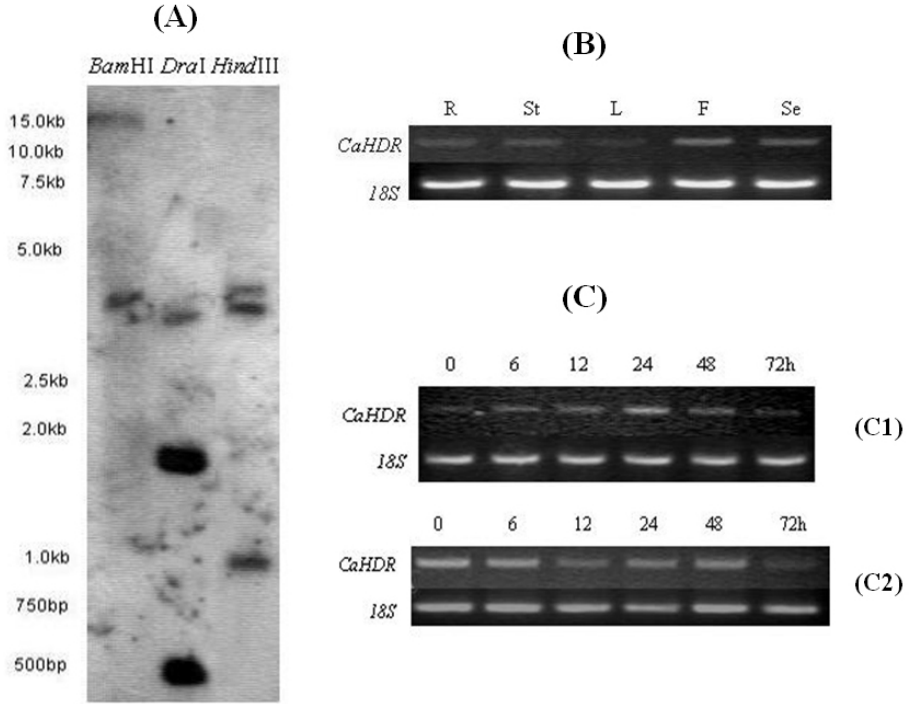

Fig. 3. Expression analysis of *CaHDR*. (A) Southern blot analysis of *CaHDR*. (B) Expression of *CaHDR* in different organs of *C. acuminata*. R, root; St, stem; L, leaf; F, flower and Se, seed. (C) Expression of *CaHDR* under MeJA and SA induction. One set of samples were treated of 100 μM MeJA (C1) and the other set were treated of 100 mg/L SA (C2) under the time gradient from 0 h to 72 h.

3C2). In Fig. 3C1, 5 μL PCR products were used for *CaHDR* expression analysis since less loading amounts made the expression differences more obviously. In Fig. 3C2, 18 μL PCR products were used for analysis. Electrophoresis of equal loading amounts showed that *CaHDR* expression under MeJA and SA treatments at point 0 were similar (data not shown).

Functional complementation of *CaHDR* in *E. coli ispH* mutant

In *E. coli ispH* mutant strain MG1655 *ara*<>*ispH*, the endogenous *ispH* gene was replaced by a kanamycin-resistant cassette and a single copy of *ispH* was present on the chromosome under the control of the P_{BAD} promoter (20). Because the *ispH* gene is essential for survival (11), the *E. coli ispH* mutant cannot grow without arabinose (Fig. 4A and Fig. 4D). To investigate the enzymatic activity of cloned *CaHDR*, a complementation test with its *E. coli* counterpart was performed. The expression plasmid pET32a-*CaHDR* harboring the *CaHDR* coding region was constructed. After transformation of pET32a-*CaHDR*, the *E. coli ispH* mutant strain was restored and grew in Luria-Bertani medium containing glucose, but not with the empty pET32a vector (Fig. 4B, C).

In this paper, the full-length cDNA of *CaHDR* gene was isolated from *C. acuminata* for the first time. For plant, isolation of cDNA coding for enzymes for the biosynthesis of specific secondary metabolite is an important tool to analyze its role in the biosynthesis pathway. Cloning and characterization of the full-length *CaHDR* cDNA from the medicinal *Nyssaceae* plant *C. acuminata* not only constitute an important step of the complete Camptothecin biosynthetic pathway, but also can be used to implement metabolic engineering strategies aimed at the development of efficient and sustainable production sources of biotechnologically interesting secondary metabolites.

MATERIALS AND METHODS

Plant materials

C. acuminata cultured callus lines, initiated from the seeds, were maintained in MS (31) solid medium supplemented with 5.0 mg/L naphthalene-acetic acid, 0.5 mg/L 6-benzyladenine, 0.3 mg/L 2,4-dichlorophenoxy-acetic acid and 30 g/L sucrose at 25°C in dark. Different tissues including roots, stems, leaves, flowers and seeds from *C. acuminata* plant were collected from Fudan University campus (Shanghai, China) for total RNA isolation.

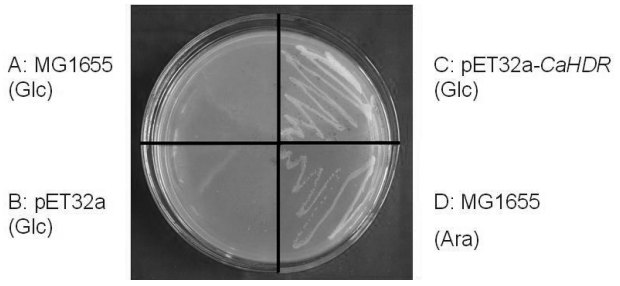

Fig. 4. Complementation of *E. coli ispH* mutant. A, *E. coli ispH* mutant MG1655 on LB media containing glucose (Glc). B, MG1655 with the empty vector pET32a on LB media containing Glc. C, MG1655 with the recombinded plasmid pET32a-*CaHDR* on LB media containing Glc. D, MG1655 on LB media containing arabinose (Ara).

Cloning of *CaHDR* full-length cDNA

Conserved fragment of *CaHDR* was amplified by RT-PCR following the standard RT-PCR program (One Step RNA PCR Kit, TaKaRa). The RT-PCR primers, HDR5-1 (5'-GAGCGTGC[T/A]-GT[T/C]CA[G/A]ATTGC-3') and HDR3-1 (5'-GGGGT[A/T/G]-GA[T/G]GCACC[A/T]GATGT-3'), were designed based on homologous sequences of other *hdr* genes. The amplified PCR product was purified and cloned into pMD18-T vector (TaKaRa) followed by sequencing and was subsequently used for designing gene specific primers (CaH5-1, CaH5-2, CaH3-1 and CaH3-2) for cloning the 5'-end and 3'-end cDNA of *CaHDR* by RACE.

The 5'-end and 3'-end cDNA amplifications were carried out following the manufacturer's protocol (SMART™ RACE cDNA Amplification Kit, User Manual, Clontech, USA). CaH5-1 (5'-CTCCCTTCTTGTGCTTTTCGACA-3'), CaH5-2 (5'-CAACATCGCCTTTATCGACCACATC-3'), CaH3-1 (5'-GTAGA-GAGGACTATGATGCGTAA-3') and CaH3-2 (5'-CCATCTTAC-TGGATTGACAGTGAG-3') were used for the 5'-end primary and nested PCR, 3'-end primary and nested PCR, respectively.

By assembling the 5'-end, conserved-fragment and 3'-end, the full-length cDNA sequence of *CaHDR* was deduced and amplified following the 5'-RACE PCR program. Primers CaHf (5'-CCACGCTCTCCTCGTGCCAACTCAT-3') and CaHr (5'-GC-CTTTCACCATAATTCTTCATTGGC-3') were used for primary PCR. CaHf2 (5'-CAACAGCACCCCTGTGCCGTGAC-3') and CaHr were used for nested PCR.

Genomic DNA of *C. acuminata* was isolated for the cloning of the DNA sequence of *CaHDR*. The full-length DNA sequence was amplified following the same reactions of the full-length cDNA cloning.

Comparative and bioinformatic analyses

The open reading frame and deduced amino acid sequence of *CaHDR* were analyzed online at NCBI website (http://www.ncbi.nlm.nih.gov). Sequences information of other HDRs was obtained by using BLASTP and translating EST sequences from TIGR (http://www.tigr.org). The HDR proteins were aligned by CLUSTALX and a phylogenetic tree of CaHDR and HDRs from other species was constructed by MEGA3 using default parameters. The neighbor-joining method (32) was used to construct the tree. Bootstrap test of the phylogeny was completed by MEGA3 with 1000 replications. Prediction of the signal peptide was carried out using ChloroP (http://www.cbs. dtu.dk /services/ChloroP/).

Southern blot analysis

Genomic DNA of *C. acuminata* was digested with *Bam*HI, *Dra*I

and *Hind*III (30 μg/sample) respectively, electrophoresed on 0.8% agarose gel and transferred to a positively charged Hybond-N^+ nylon membrane (Amersham Pharmacia). The partial sequence product of *CaHDR* generated by PCR using CaHf and CaH5-2 as primers was used as probe in Southern hybridization. Probe labeling (biotin), hybridization and signal detection were performed using *Gene images* random prime labeling module and CDP-Star detection module following the manufacturer's instructions (Amersham Pharmacia). The filter was washed under high stringency condition at 65°C and the hybridized signals were visualized by exposure to Fuji X-ray film at room temperature for 1.5 h.

Expression profile analysis

Total RNA was isolated from 15-day-old *C. acuminata* callus lines and different organs of *C. acuminata*, including roots, stems, leaves, flowers and seeds, to investigate the *CaHDR* expression profile. One set of the callus samples were treated under 100 μM MeJA and the other set under 100 mg/L SA. Callus samples were harvested after 6, 12, 24, 48 and 72 h of treatments respectively using untreated callus as control. Expression profiles of *CaHDR* in different organs and under different treatments were analyzed by RT-PCR using 400 ng RNA as template, CaHf and CaH5-2 as primers. 18S rRNA genes was used as internal control.

Complementation of *E. coli ispH* mutant

The coding region of *CaHDR* was amplified by PCR using primers CaHv5 (5'-GA GGATCCGCGATTTCCCTGCAATTCT-3') and CaHv3 (5'-CCAAGCTTCTATGCCAGTTGTAAGGCTTC-3') with *Bam*HI and *Hind*III digestion site, respectively. rTaq DNA polymerase (TaKaRa) was used in the amplification. The PCR product was cloned into pMD18-T vector (TaKaRa), resulting in vector pMD18-*CaHDR*. Plasmid pMD18-*CaHDR* was confirmed by sequencing, and then double-digested, purified and ligated into the *Bam*HI and *Hind*III-predigested expression vector pET32a (Novagen) to generate the vector pET32a-*CaHDR*, which was subsequently used for functional complementation assay. The *E. coli* ispH mutant MG1655 was maintained on Luria-Bertani medium containing 50 μg/ml kanamycin and 0.2% (w/v) arabinose (19, 25). The pET32a-*CaHDR* was transformed into MG1655 and selected on LB medium containing 50 μg/ml kanamycin, 50 μg/ml carbenicillin, 0.5 mM IPTG and 0.2% (w/v) glucose. The empty vector pET32a was also transformed into MG1655 as a negative control.

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REFERENCES

1. Lorence, A. and Nessler, C.L. (2004) Molecules of interest - Camptothecin, over four decades of surprising findings. *Phytochemistry* **65,** 2735-2749.
2. Wall, M. E., Wani, M. C., Cook, C. E., Palmer, K. H., McPhill, A. T. and Sim, G. A. (1966) Plant antitumour agents.I. The isolation and structure of camptothecin a novel alkoloid leukemia and tumor inhibitor from *Camptotheca acuminata*. *J. Am. Chem. Soc.* **88,** 3888-3890.
3. Hsiang, Y.H., Hertzberg, R., Hecht, S. and Liu, L.F. (1985) Camptothecin induces protein-linked DNA breaks via mammalian DNA topoisomerase I. *J. Biol. Chem.* **260,** 14873-14878.
4. Lorence, A., Medina-Bolivar, F. and Nessler, C.L. (2004) Camptothecin and 10-hydroxycamptothecin from *Camptotheca acuminata* hairy roots. *Plant Cell Rep.* **22,** 437-441.
5. Lopez-Meyer, M., Nessler, C.L. and McKnight, T.D. (1994) Sites of accumulation of the antitumor alkaloid camptothecin in *Camptotheca acuminata*. *Planta Med.* **60,** 558-560.
6. Pasqua, G., Monacelli, B. and Valletta, A. (2004) Cellular localisation of the anti-cancer drug camptothecin in *Camptotheca acuminata* Decne (Nyssaceae). *Eur. J. Histochem.* **48,** 321-327.
7. Gao, S., Lin, J., Liu, X., Deng, Z., Li, Y., Sun, X. and Tang, K. (2006) Molecular cloning, characterization and functional analysis of a 2C-methyl-D-erythritol 2, 4-cyclodiphasphate synthase gene from *Ginkgo biloba*. BMB reports (formerly *J. Biochem. Mol. Biol.*) **39**, 502-510.
8. Memelink, J., Verpoorte, R. and Kijne, J.W. (2001) ORCAnization of jasmonate - responsive gene expression in alkaloid metabolism. *Trends Plant Sci.* **6,** 212-219.
9. Eisenreich, W., Rohdich, F. and Bacher, A. (2001) Deoxyxylulose phosphate pathway to terpenoids. *Trends Plant Sci.* **6,** 78-84.
10. Rohdich, F., Kis, K., Bacher, A. and Eisenreich, W. (2001) The non-mevalonate pathway of isoprenoids: genes, enzymes and intermediates. *Curr. Opin. Chem. Biol.* **5,** 535-540.
11. Pasqua, G., Silvestrini, A., Monacelli, B., Mulinacci, N., Menendez, P. and Botta, B. (2006) Triterpenoids and ellagic acid derivatives from *in vitro* cultures of *Camptotheca acuminata* Decaisne. *Plant Physiol. Biochem.* **44,** 220-225.
12. Cunningham, F.X. and Gantt, E. (1998) Genes and enzymes of carotenoid biosynthesis in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **49,** 557-583.
13. Rodriguez-Concepcion, M. and Boronat, A. (2002) Elucidation of the methylerythritol phosphate pathway for isoprenoid biosynthesis in bacteria and plastids. A metabolic milestone achieved through genomics. *Plant Physiol.* **130,** 1079-1089.
14. Lois, L.M., Campos, N., Rosa Putra, S., Danielsen, K., Rohmer, M. and Boronat, A. (1998) Cloning and characterization of a gene from *Escherichia coli* encoding a transketolase-like enzyme that catalyzes the synthesis of d-1-deoxyxylulose 5-phosphate, a common precursor for isoprenoid, thiamin, and pyridoxol biosynthesis. *Proc. Natl. Acad. Sci. USA* **95,** 2105-2110.
15. Sprenger, G. A., Schorken, U., Wiegert, T., Grolle, S., de Graaf, A. A., Taylor, S. V., Begley, T. P., Bringer-Meyer, S. and Sahm, H. (1997) Identification of a thiamindependent synthase in *Escherichia coli* required for the formation of the 1-deoxy-d-xylulose 5-phosphate precursor to isoprenoids, thiamin, and pyridoxol. *Proc. Natl. Acad. Sci. USA* **94,**

12857-12862.
16. Kuzuyama, T., Takahashi, S., Watanabe, H. and Seto, H. (1998) Direct formation of 2-*C*-methyl-d-erythritol 4-phosphate from 1-deoxy-d-xylulose 5-phosphate by 1-deoxy-d-xylulose 5-phosphate reductoisomerase, a new enzyme in the non-mevalonate pathway to isopentenyl diphosphate. *Tetrahedron Lett.* **39,** 4509-4512.
17. Takahashi, S., Kuzuyama, T., Watanabe, H. and Seto, H. (1998) A 1-deoxy-dxylulose 5-phosphate reductoisomerase catalyzing the formation of 2-*C*-methyl-d-erythritol 4-phosphate in an alternative nonmevalonate pathway for terpenoid biosynthesis. *Proc. Natl. Acad. Sci. USA* **95,** 9879-9884.
18. Rohdich, F., Hecht, S., Gartner, K., Adam, P., Krieger, C., Amslinger, S., Arigoni, D., Bacher, A. and Eisenreich, W. (2002) Studies on the nonmevalonate terpene biosynthetic pathway: Metabolic role of IspH (LytB) protein. *Proc. Natl. Acad. Sci. USA* **99,** 1158-1163.
19. McAteer, S., Coulson, A., McLennan, N. and Masters, M. (2001) The *lytB* gene of *Escherichia coli* is essential and specifies a product needed for isoprenoid biosynthesis. *J. Bacteriol.* **183,** 7403-7407.
20. Adam, P., Hecht, S., Eisenreich, W.G., Kaiser, J., Grawert, T., Arigoni, D., Bacher, A. and Rohdich, F. (2002) Biosynthesis of terpenes: Studies on 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate reductase. *Proc. Natl. Acad. Sci. USA* **99,** 12108-12113.
21. Botella-Pavia, P., Besumbes, O., Phillips, M.A., Carretero-Paulet, L., Boronat, A. and Rodriguez-Concepcion, 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.
22. Guevara-Garcia, A., Roman, C.S., Arroyo, A., Cortes, M.E., de la Luz Gutierrez-Nava, M. and 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.
23. Emanuelsson, O., Nielsen, H., Brunak, S. and von Heijne, G. (2000) Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. *J. Mol. Biol.* **300,** 1005-1016.
24. Lange, B.M., Rujan, T., Martin, W., and Croteau, R. (2000) Isoprenoid biosynthesis: The evolution of two ancient and distinct pathways across genomes. *Proc. Natl. Acad. Sci. USA* **97,** 13172-13177.
25. Hsieh, M.H. and Goodman, H.M. (2005) The *Arabidopsis IspH* homolog is involved in the plastid nonmevalonate pathway of isoprenoid biosynthesis. *Plant Physiol.* **138,** 641-653.
26. Liu, Z.J., Carpenter, S.B., Bourgeois, W.J., Yu, Y., Constantin, R.J., Falcon, M.J. and Adams, J.C. (1998) Variations in the secondary metabolite camptothecin in relation to tissue age and season in *Camptotheca acuminata*. *Tree Physiol.* **18,** 265-270.
27. Creelman, R.A. and Mullet, J.E. (1997) Biosynthesis and action of jasmonates in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **48,** 355-381.
28. Wasternack, C. and Parthier, B. (1997) Jasmonate signalled plant gene expression. *Trends Plant. Sci.* **2,** 302-307.
29. van der Fits, L. and Memelink, J. (2000) ORCA3, a jasmonate-responsive transcriptional regulator of plant primary and secondary metabolism. *Sci.* **289,** 295-297.
30. Durner, J., Shah, J. and Klessig, D.F. (1997) Salicylic acid and disease resistance in plants. *Trends Plant Sci.* **2,** 266-274.
31. Murashige, T. and Skoog, F.A. (1962) Revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant* **15,** 473-479.
32. Saitou, N. and Nei, M. (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4,** 406-425.