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Molecular cloning, expression profiling and functional analysis of a *DXR* gene encoding 1-deoxy-D-xylulose 5-phosphate reductoisomerase from *Camptotheca acuminata*

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Methyl jasmonate;
Rapid amplification of cDNA ends

Summary

As the second enzyme of the non-mevalonate terpenoid pathway for isopentenyl diphosphate biosynthesis, DXP reductoisomerase (DXR, EC: 1.1.1.267) catalyzes a committed step of the MEP pathway for camptothecin (CPT) biosynthesis. In order to understand more about the role of DXR involved in the CPT biosynthesis at the molecular level, the full-length DXR cDNA sequence (designated as *CaDXR*) was isolated and characterized for the first time from a medicinal *Nyssaceae* plant species, *Camptotheca acuminata*. The full-length cDNA of *CaDXR* was 1823 bp containing a 1416 bp open reading frame (ORF) encoding a polypeptide of 472 amino acids. Comparative and bioinformatic analyses revealed that *CaDXR* showed extensive homology with DXRs from other plant species and contained a conserved

Abbreviations: CPT, camptothecin; CTAB, cetyltrimethylammonium bromide; DXP, deoxy-D-xylulose 5-phosphate; DXR, deoxy-D-xylulose 5-phosphate methylerythritol phosphate reductoisomerase; MeJA, methyl jasmonate; MEP, mevalonate-independent plastidial methylerythritol 4-phosphate; ORF, open reading frame; RACE, rapid amplification of cDNA ends

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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. Phylogenetic analysis indicated that CaDXR was more ancient than other plant DXRs. Tissue expression pattern analysis revealed that *CaDXR* expressed strongly in stem, weak in leaf and root. *CaDXR* was found to be an elicitor-responsive gene, which could be induced by exogenous elicitor of methyl jasmonate. The functional color complementation assay indicated that *CaDXR* could accelerate the biosynthesis of carotenoids in the *Escherichia coli* transformant, demonstrating that DXP reductoisomerase plays an influential step in isoprenoid biosynthesis.

Introduction

Camptotheca acuminata of the family *Nyssaceae* is a deciduous and medicinal tree from south China and has been listed as an endangered species in China since 1997 (Li et al., 2002). The main active ingredients of *C. acuminata* extracts are quinoline and indole alkaloids (Wall et al., 1966). The camptothecin (CPT), a terpenoid quinoline alkaloid, and its analogues are the potent topoisomerase I inhibitors, which have been used as anticancer and antiviral indole alkaloids to cure ovarian, lung and colorectal cancers (Li and Adair, 1994; Oberlies and Kroll, 2004). Because of the present and potential clinical uses of CPT and its derivatives, it is a necessity to develop sustainable and alternative production sources of these compounds (Lorence et al., 2004). Based on the reality of limited natural *Camptotheca* plant materials, to map the CPTs biosynthetic pathway at the level of molecular genetics may lead to means to enhance CPTs production (Lorence and Nessler, 2004).

All terpenoids such as CPTs are biosynthesized via recently discovered and mevalonate-independent plastidial methylerythritol 4-phosphate (MEP) pathway (Lichtenthaler et al., 1997a, b; Zeidler et al., 1997; Lichtenthaler, 1999; Rohmer, 1999). The MEP pathway starts with the formation of 1-deoxy-D-xylulose 5-phosphate (DXP) from D-glyceraldehyde 3-phosphate and pyruvate catalyzed by 1-deoxy-D-xylulose 5-phosphate synthase (DXS, EC: 4.1.3.37) (Sprenger et al., 1997; Bouvier et al., 1998; Lange et al., 1998; Lois et al., 1998). Then DXP is converted into MEP and the reaction is catalyzed by a NADPH-dependent reductoisomerase (deoxy-D-xylulose 5-phosphate methylerythritol phosphate reductoisomerase (DXR), EC1.1.1.267) specified by the *DXR* gene. Because DXP is an intermediate not only for isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) biosynthesis, but also for the cofactors thiamine and pyridoxol (Julliard and Douce, 1991; Julliard, 1992; Himmeldirk et al., 1996), the reaction catalyzed by DXR is actually the first committed step of the MEP pathway (Kuzuyama et al., 1998). Moreover, earlier experimental results also showed that DXR participated in the control of isoprenoid accumulation in plants (Veau et al., 2000; Walter et al., 2000; Mahmoud and Croteau, 2001). Earlier investigations showed that overexpression of DXR in transgenic peppermint plants could lead to an increase of the essential oil monoterpenes in the MEP pathway (Mahmoud and Croteau, 2001). DXR generally plays a key regulatory role in the control of terpenoids on the pathway and even in the regulation of CPT biosynthesis just like that in peppermint (Mahmoud and Croteau, 2001). Unfortunately, until now there have been only a few reports on the cloning of genes involved in the MEP pathway from *C. acuminata* (Lu et al., 2005) and no reports on the cloning of *DXR* genes from any *Nyssaceae* species. In the present study, we report, for the first time, the cloning and characterization of the *DXR* gene from the *Nyssaceae* plant *C. acuminata* by rapid amplification of cDNA ends (RACE). The expression profiles of *CaDXR* in various tissues and under the induction by methyl jasmonate (MeJA) elicitor were also investigated, which will facilitate future work to map and regulate this important step involved in CPT biosynthetic pathway at the level of molecular genetics.

Materials and methods

Plant materials and RNA isolation

The cultured callus lines, initiated from young leaves of Chinese happy tree (*C. acuminata* Decne. var. *acuminata*), were maintained on solid Murashige and Skoog (MS) basal medium (Murashige and Skoog, 1962) supplemented with 5.0 mg/L naphthaleneacetic acid (NAA), 0.5 mg/L 6-benzylaminopurine (6-BAP) and 0.3 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) at 25 °C in darkness for 4 weeks and used as the starting material for total RNA isolation. In the experiment of investigating induction by MeJA elicitor, 4-d-old callus

lines were treated by dipping into 100 mM MeJA solution, while the callus lines without treatment were used as control. The callus samples were collected 0, 6, 12, 24, 48 and 72 h after treatment for analysis of *CaDXR* expression profiles by semi-quantitative one-step RT-PCR. Root, stem and leaf tissues of *C. Acuminata*, which were harvested at 2 weeks after the cotyledons emerged, were collected at Fudan University (Shanghai, China) and immediately used for RNA extraction. Total RNA was isolated by cetyltrimethylammonium bromide (CTAB) method and lithium chloride precipitation (Liao et al., 2004).

Cloning of *CaDXR* full-length cDNA by RACE

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, USA). After RNase H treatment, the single-strand cDNA mixtures were used as templates for PCR amplification of the conserved region of DXR from *C. acuminata*. Two degenerate oligonucleotide primers, FDXR (5′-AC(T/C)GG(T/C)TC(A/T/C)AT(A/T)GG(A/G/C)AC(A/T)CAGAC-3′) and RDXR (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 *DXR* genes and used for the amplification of the core cDNA fragment of *CaDXR* by standard gradient PCR amplification (from 52 to 60 °C) on ThermoHybaid PX2 Thermocycler (Hybaid, UK). The core fragment was amplified at the annealing temperature of 55 °C, subcloned into pGEM T-easy vector (Promega, Madison, WI, USA), and transformed into *Escherichia coli* strain DH5α followed by sequencing. The core fragment was subsequently used to design the gene-specific primers for the cloning of the full-length cDNA of *CaDXR* by RACE.

SMART™ RACE cDNA Amplification Kit (Clontech, USA) was used to clone the 3′- and 5′-ends of *CaDXR* cDNA. The first-strand 3′-RACE-ready and 5′-RACE-ready cDNA samples were prepared according to the manufacturer's protocol and used as templates for 3′-RACE and 5′-RACE, respectively.

Two 3′-gene-specific primers were designed for 3′-RACE. For the first cycle of amplification of 3′-end of *CaDXR* cDNA, CaDXR3-1 (5′-GCCAGATATGCGATTGCCGATAC-3′) and UPM (Universal Primer A Mix, 5′-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3′ and 5′-AAGCAGTGGTATCAACGCAGAGT-3′) were used with 3′-RACE-ready cDNA as template. For the nested amplification of 3′-RACE, primers CaDXR3-2 (5′-CAAGGCTCCAGACTGTGTGAAATATCC-3′) and NUP (Nested Universal Primer A, 5′-AAGCAGTGGTATCAACGCAGAGT-3′) were used with the products of the first amplification as templates. The 5′-end of *CaDXR* cDNA was amplified using two 5′-gene-specific primers and the universal primers (UPM and NUP) provided by the kit. For the first amplification of 5′-RACE, CaDXR5-1 (5′-TCCTTCCCTGCTTCTATTGC-3′) and UPM were used with 5′-RACE-ready cDNA as template. For the nested amplification of 5′-RACE, CaDXR5-2 (5′-TGATCTGCAAGTAGAGCCAC-3′) and NUP were used with the products of the first amplification as templates. For the first and nested PCR amplification of *CaDXR* cDNA 3′ and 5′-ends, Advantage™ 2 PCR Kit (Clontech, USA) was used. The first and nested PCRs were carried out under 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, 3 min at 72 °C). The nested 3′-RACE and 5′-RACE products were purified and subcloned into pGEM T-easy vector followed by sequencing. By aligning and assembling the sequences of the core fragment, 3′-RACE and 5′-RACE products on Contig Express (Vector NTI Suite 8.0), the full-length cDNA sequence of *CaDXR* was deduced, and amplified by using 3′-RACE-ready cDNA as the template and a pair of primers, FCaDXR (5′-ACGCGGGATTCAAAGTAGTAAATAG-3′) and UPM. The amplified product was subsequently cloned into pGEM T-easy vector (Promega, Madison, WI, USA), transformed into *E. coli* strain DH5α followed by sequencing. The PCR amplification and sequencing of the full-length cDNA of *CaDXR* was repeated three times to avoid PCR errors. The sequence from this article has been deposited at GenBank under accession number DQ355159.

Comparative and bioinformatic analyses

Comparative and bioinformatic analyses of *CaDXR* were carried out online at the websites (http://www.ncbi.nlm.nih.gov and http://cn.expasy.org). The nucleotide sequence, deduced amino acid sequence and open reading frame (ORF) were analyzed, and the sequence comparison was conducted through database search using BLAST program (NCBI, National Center for Biotechnology Services, http://www.ncbi.nlm.nih.gov). The phylogenetic analysis of CaDXR and DXRs from other plant species was aligned with CLUSTAL W (1.82) using default parameters. A phylogenetic tree was constructed using MEGA version 3.1 (Kumar et al., 2001) from CLUSTAL W alignments. The neighbor-joining method (Saitou and Nei, 1987) was used to construct the tree. The homology-based three-dimensional (3D) structural modeling of CaDXR was accomplished by Swiss-Modeling (Schwede et al., 2003) and WebLab ViewerLite was used for 3D structure displaying (homology-based modeling by Swiss-Model).

Expression profile analysis

Semi-quantitative one-step RT-PCR was carried out to investigate the expression profiles of *CaDXR* in different tissues including root, stem and leaf of *C. acuminata* and under 100 mM MeJA elicitor treatment. Aliquots of 0.5 μg total RNA extracted from each sample were used as templates in one-step RT-PCR reaction with the forward primer fcadxr (5′-CACTGTTCAACTCAGCCACC-3′) and the reverse primer rcadxr (5′-CTCCAGCAATTAAAGTCTCCTT-3′) specific to the coding sequence of *CaDXR* 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, 52 °C for 30 s, 72 °C for 90 s). Meanwhile, the RT-PCR reaction for the house-keeping gene (18S rRNA gene) using specific primers 18SF (5′-GTGACAATGGAACTGGAATGG-3′) and 18SR (5′-AGACGGAGGATAGCGTGAGG-3′) designed according to the conserved regions of plant 18S rRNA genes was also performed to estimate whether equal amounts of RNA among samples were used in RT-PCR as an internal control. PCR products were separated on 1% agarose gels stained with ethidium bromide and intensities of the target bands were measured using Furi FR-200A ultraviolet analyzer (Furi Tech., Shanghai, China).

Functional complementation of CaDXR in *Escherichia coli*

The vectors pTrc-Atipi and pAC-BETA with carotenoid biosynthesizing ability in *E. coli* were kindly provided by Francis X Cunninham Jr. (Department of Cell Biology and Molecular Genetics, University of Maryland, USA). *E. coli* strain DH5α was used to test the activity of CaDXR. The coding region cDNA fragment of *CaDXR* was amplified with two primers, ORFcadxrF (5′-ATGGCACTGAATTTACTTTCAC-3′) and ORFcadxrR (5′-TCAAACCATGGCAGGCTCCAAACTTG-3′), at the annealing temperature of 55 °C, cloned into pGEM T-easy vector (Promega, Madison, WI, USA), and transformed into *E. coli* strain DH5α followed by sequencing. Subsequently, a pair of primers containing *Bgl*II and *Not*I digesting sites were designed and used to amplify the ORF region using the cloned ORF fragment as the template. PCR product was digested with *Bgl*II and *Not*I, and cloned into the *Bgl*II/*Not*I site of pTrc-Atipi vector to generate the expression vector pTrc-CaDXR. As a negative control, the vector pTrc-Atipi was digested with *Pst*I and ligated by T4 DNA ligase. The vector pTrc-CaDXR and pAC-BETA were used to co-transform DH5α. Meanwhile, the vectors pTrc and pAC-BETA were co-transformed and a single vector pAC-BETA was transformed into DH5α as controls. Transformants were selected on solid LB medium containing ampicillin (150 mg/L) and chloramphenicol (50 mg/L) at 37 °C for 2 d.

Results and discussion

Cloning of the full-length cDNA of *CaDXR*

The *DXR* genes have been cloned and characterized from *E. coli* (Takahashi et al., 1998; Kuzuyama et al., 2000; Radykewicz et al., 2000), eubacterium (Jomaa et al., 1999; Grolle et al., 2000) and a few plant species (Lange and Croteau, 1999; Schwender et al., 1999; Veau et al., 2000; Rodriguez-Concepcion et al., 2001; Kim et al., 2006). However, there is no report on the cloning of *DXR* genes from *Nyssaceae* species including *C. acuminata*. Based on the conserved region of plant DXR sequences, two degenerate oligonucleotide primers (FDXR and RDXR) were designed and used for gradient PCR amplification of the core cDNA fragment of *DXR* from *C. acuminata*. An approximately 1000 bp product was amplified and subcloned following by sequencing. The BLAST search revealed this 974 bp

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1       acgcgggattcaaagtagtaaatagtcaggccttacatacattttctgtctgtaaaatc
60      attagagctgctccacggccacatatttttctctctgttccacttgaatttgcctgtcaa
120     agttcatttcaaagagaggtggtggtggtgATGGCACTGAATTTACTTTCACCTACTGAA
1                                   M  A  L  N  L  L  S  P  T  E
180     ATCAAGGCCATTTCGTTCTTGGATTCGTCTAAATCCAACAACAGCTACCTTCTAAAGCTT
11       I  K  A  I  S  F  L  D  S  S  K  S  N  N  S  Y  L  L  K  L
240     CAAGGTGGGATTGCTTTGAAGCGGAAGGATTATGGAGCAACATTTGGGAAGAGAATTCAC
31       Q  G  G  I  A  L  K  R  K  D  Y  G  A  T  F  G  K  R  I  H
300     TGTTCAACTCAGCCACCGCCTCCAGCGTGGCCGGGACGGGCTGTTGCAGAGCCAGGACGT
51       C  S  T  Q  P  P  P  P  A  W  P  G  R  A  V  A  E  P  G  R
360     AAGATTTGGGATGGTCCAAAGCCTATAGCAGTTGTTGGGTCTACCGGTTCCATTGGGACA
71       K  I  W  D  G  P  K  P  I  A  V  V  G  S  T  G  S  I  G  T
420     CAGACTTTGGACATAGTTGCAGAACATCCTGATAAGTTCAGAGTGGTTGCACTGGCAGCG
91       Q  T  L  D  I  V  A  E  H  P  D  K  F  R  V  V  A  L  A  A
480     GGTTCAAATGTGGCTCTACTTGCAGATCAGGTGCGGCAGTTCAAACCTAAACTTGTTGCT
111      G  S  N  V  A  L  L  A  D  Q  V  R  Q  F  K  P  K  L  V  A
540     ATTCGCAATGAATCATTGATCACTGAACTCAAAGCAGCATTGTCAGATTTTGAACCTAAG
131      I  R  N  E  S  L  I  T  E  L  K  A  A  L  S  D  F  E  P  K
600     CCAGAAATAATTTCTGGCGAAGAAGGAATTGCAGAGGTTGCTCGACATCCTGAGGCTGTC
151      P  E  I  I  S  G  E  E  G  I  A  E  V  A  R  H  P  E  A  V
660     AGCGTTATGACAGGCATTGTAGGATGTGCAGGCTTGAAGCCAACTGTTGCAGCAATAGAA
171      S  V  M  T  G  I  V  G  C  A  G  L  K  P  T  V  A  A  I  E
720     GCAGGGAAGGACATAGCACTTGCAAACAAGGAGACTTTAATTGCTGGAGGCCCCTTTGTG
191      A  G  K  D  I  A  L  A  N  K  E  T  L  I  A  G  G  P  F  V
780     CTCCCACTTGCGCACAAACACAAAGTTAAAATTCTACCAGCTGACTCTGAGCATTCTGCT
211      L  P  L  A  H  K  H  K  V  K  I  L  P  A  D  S  E  H  S  A
840     ATATTTCAGTGCATTCAAGGTCTACCAGAAGGTGCTCTGCGGCGTATTATTTTAACAGCT
231      I  F  Q  C  I  Q  G  L  P  E  G  A  L  R  R  I  I  L  T  A
900     TCTGGAGGTGCATTTAGGGATTGGCTAGTTGGGAAATTGAAAGAAGTCAAAGTTTCAGAC
251      S  G  G  A  F  R  D  W  L  V  G  K  L  K  E  V  K  V  S  D
960     GCTTTAAAGCATCCTAACTGGAATATGGGGAAAAAGATCACAGTCGACTCTGCTACCCTG
271      A  L  K  H  P  N  W  N  M  G  K  K  I  T  V  D  S  A  T  L
1020    TTTAATAAGGGTCTTGAGGTGATTGAAGCACACTACCTTTATGGGGCTGACTATGATAAT
291      F  N  K  G  L  E  V  I  E  A  H  Y  L  Y  G  A  D  Y  D  N
1080    ATTGAGATTGTGATTCACCCACAATCAATAGTTCATTCAATGGTGGAGACACAGGATTCC
311      I  E  I  V  I  H  P  Q  S  I  V  H  S  M  V  E  T  Q  D  S
1140    TCGGTTCTAGCTCAACTGGGATGGCCAGATATGCGATTGCCGATACTTTATACAATGTCA
331      S  V  L  A  Q  L  G  W  P  D  M  R  L  P  I  L  Y  T  M  S
1200    TGGCCTGAGAGGGTTCCATGCTCTGAGGTTACATGGCCTCGGCTTGACCTTTGCAAGTCG
351      W  P  E  R  V  P  C  S  E  V  T  W  P  R  L  D  L  C  K  S
1260    AATTCACTAGTGTTCAAGGCTCCAGACTGTGTGAAATATCCTCCCATGGATCTTGCATAT
371      N  S  L  V  F  K  A  P  D  C  V  K  Y  P  P  M  D  L  A  Y
1320    TCTGCAGGTCGAGCAGGTGGTACCATGACTGGAGTCCTCAGCGCTGCTAACGAGAAGGCA
391      S  A  G  R  A  G  G  T  M  T  G  V  L  S  A  A  N  E  K  A
1380    GTTGAGTTATTTATTGATGAAAGAATCTCATATCTGGATATTTTCAAAGTTGTTGAAAAG
411      V  E  L  F  I  D  E  R  I  S  Y  L  D  I  F  K  V  V  E  K
1440    ACATGCGACAAGCACAGAAATGAGCTGGTGCTCCGACCTTCATTGGAAGAGATAATACAT
431      T  C  D  K  H  R  N  E  L  V  L  R  P  S  L  E  E  I  I  H
1500    TATGACCTATGGGCCAGAAAGTATGCTGCATCCCTGGCGCAATCAAGTTTGGAGCCTGCC
451      Y  D  L  W  A  R  K  Y  A  A  S  L  A  Q  S  S  L  E  P  A
1560    ATGGTTTGAgcatgtcaagattggcatatcagtcatataggaaagaatgttttttggtct
471      M  V  *
1620    catttgggaaatttgacttgcatgattgtcttgtgtaaattttgtaattcgtttactctg
1680    ccatagttgattcctcttctctggcgctgaagagaatgaatatcttgaccaggcagttga
1740    gcaaatcagttctagtcatttattttcagtattgtttgtacaaatatctgaaaagaagca
1800    aaagttcagagagagaaaaaaaaa
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Figure 1. The full-length cDNA sequence and the deduced amino acid sequence of *Camptotheca acuminata* 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR). The start codon (ATG) was underlined in bold and the stop codon (TGA) was underlined italically in bold.

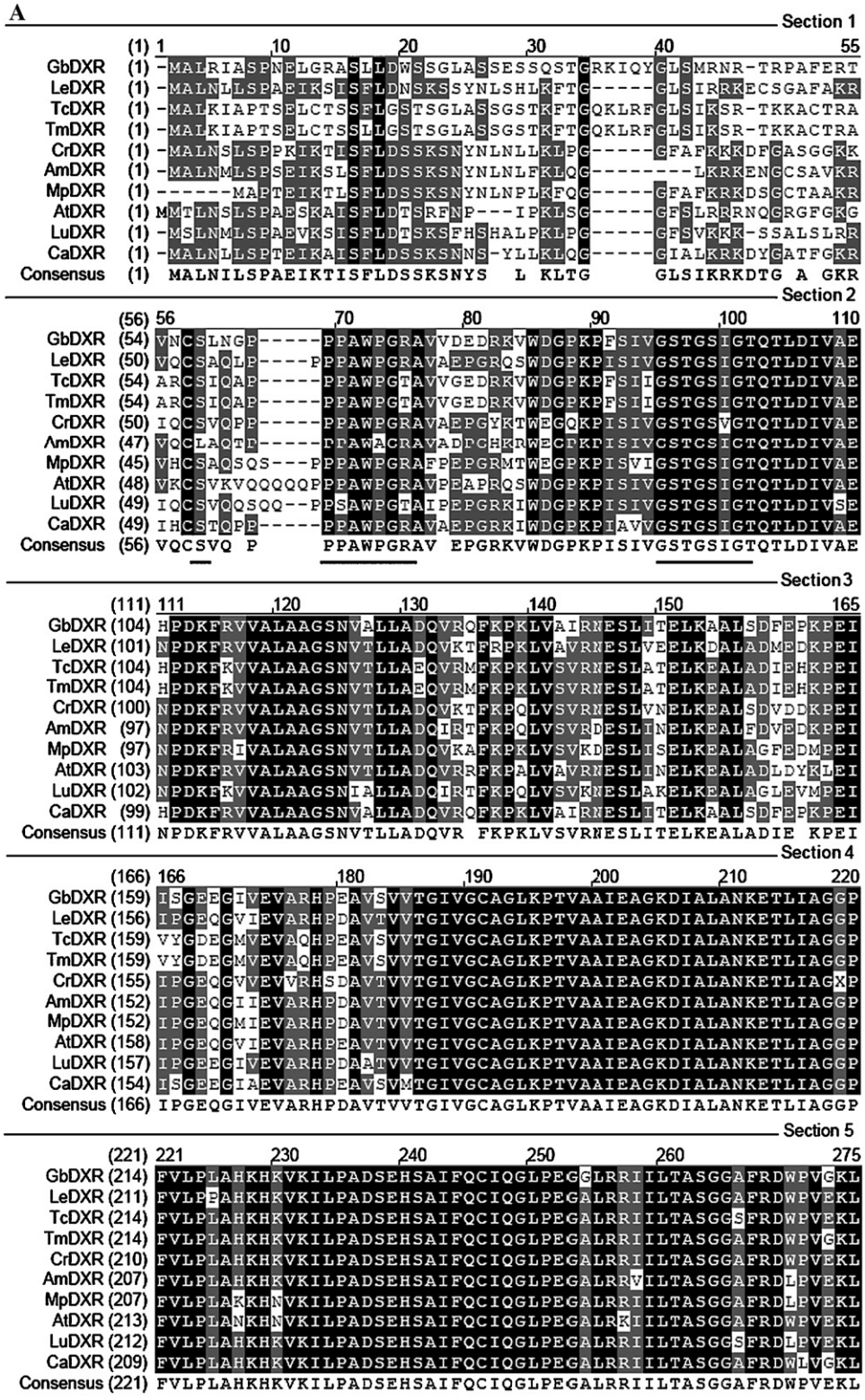

Figure 2. Multi-alignment of amino acid sequences of CaDXR and other plant DXRs. The identical amino acids were showed in white with black background and the conserved amino acids were showed in white with gray background. The highly conserved transit peptide for plastids motif Cys-Ser-(Ala/Met/Val/Thr), an extended Pro-rich motif P(P/Q)PAWPG(R/T) and a conserved NADPH binding motif (GSTGSIGT) were underlined. The aligned DXRs were from *Ginkgo biloba* (GbDXR, GenBank accession no.: AY494186), *Lycopersicon esculentum* (LeDXR, GenBank accession no.: AF331705), *Taxus cuspidata* (TcDXR, GenBank accession no.: AY575140), *Taxus x media* (TmDXR, GenBank accession no.: AY588482), *Catharanthus roseus* (CrDXR, GenBank accession no.: AF250235), *Antirrhinum majus* (AmDXR, GenBank accession no.: AY770406), *Mentha x piperita* (MpDXR, GenBank accession no.: AF116825), *Arabidopsis thaliana* (AtDXR, GenBank accession no.: NM125674), *Linum usitatissimum* (LuDXR, GenBank accession no.: AJ623266) and *Camptotheca acuminata* (CaDXR, GenBank accession no.: DQ355159).

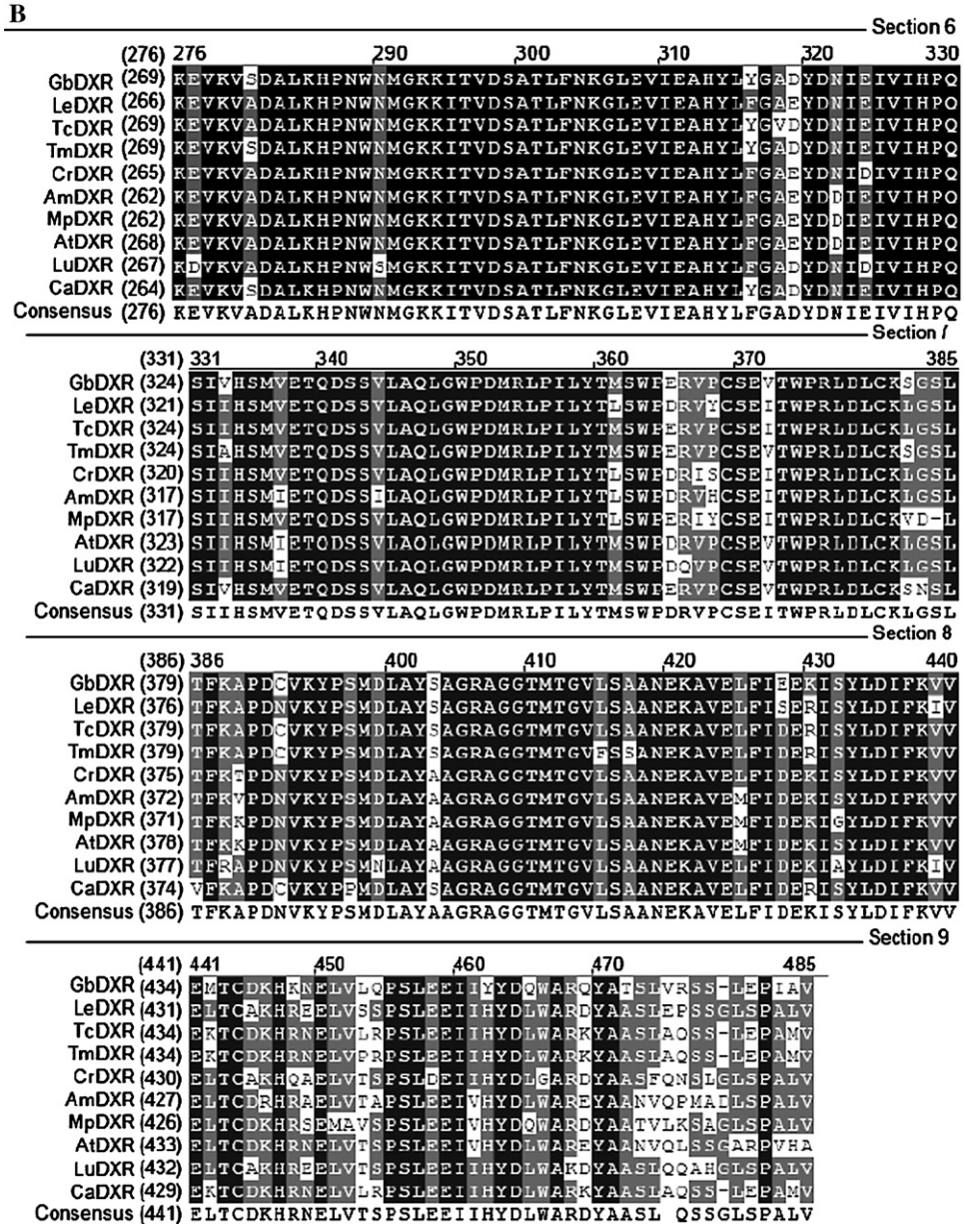

Figure 2. (*Continued*)

cDNA fragment had high homology to *DXR* genes from angiosperm plant species. Thus, this fragment was used to design gene specific primers for both 3′-RACE and 5′-RACE of *CaDXR*.

By 3′-RACE and 5′-RACE, the 570 and 484 bp nested PCR products were, respectively, obtained. Sequencing results revealed the products were the 448 bp 3′-end and 401 bp 5′-end of *CaDXR*, respectively. 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 *CaDXR* was deduced and subsequently confirmed by sequencing, which was 1823 bp. ORF Finding analysis on NCBI showed that the *CaDXR* contained a 1416 bp ORF encoding a peptide of 472 amino acids, with a calculated molecular mass of 51.4 kDa and an isoelectric point of 7.18 (http://cn.expasy.org/tools/protparam.html) (Fig. 1).

Comparative and bioinformatic analyses of CaDXR

The deduced amino acid sequence of *CaDXR* was submitted to NCBI for PSI-BLAST searching (http://www.ncbi.nlm.nih.gov/BLAST/) and the result showed that CaDXR had high homology with DXR sequences from other plant species, with 85%

identity with DXR from *Ginkgo biloba*. CaDXR was also highly similar to DXRs from *Lycopersicon esculentum* (84% identities, 90% positives), *Taxus cuspidata* (82% identities, 88% positives), *Taxus x media* (84% identities, 89% positives), *Catharanthus roseus* (82% identities, 89% positives), *Antirrhinum majus* (81% identities, 90% positives), *Mentha x piperita* (80% identities, 89% positives) and *Arabidopsis thaliana* (81% identities, 89% positives), suggesting that CaDXR belongs to the DXR family.

The amino acid sequences of nine DXRs were aligned according to the algorithm of the CLUSTAL W (Higgins et al., 1994). The result showed that these DXRs had high similarity throughout the entire coding region. Three domains were found in all plant DXR sequences. Domain I was a transit peptide in N-terminal region of CaDXR, which directed the enzyme to plastids where the mevalonate-independent pathway operated in plants and had a conserved Cys-Ser-(Ala/Met/Val/Thr) motif. The second domain contained an extended Pro-rich motif P(P/Q)PAWPG(R/T) at the N terminus of the CaDXR. All plant DXRs cloned so far had a transit peptide for plastids and an extended Pro-rich region at the N terminus of the mature protein, which was not present in prokaryotic DXRs (Carretero-Paulet et al., 2002). The PSI-BLAST also resulted in a highly conserved domain of NADPH binding motif (GSTGSIGT) in CaDXR N-terminal region owned by all plant DXRs (Rane and Calvo, 1997; Lange and Croteau, 1999; Schwender et al., 1999; Kuzuyama et al., 2000). The substrate-binding motifs of nine plant DXRs used in the multiple alignment analysis were picked out and the motif of CaDXR had almost the same amino acid composition with those of other plant DXRs (Fig. 2). These conserved residues may function as important catalytic domains of the enzyme.

CaDXR was the first *DXR* gene cloned form *Nyssaceae* species, therefore it would be interesting to investigate its evolutionary position among the phylogenetic tree of various DXRs. Using MEGA

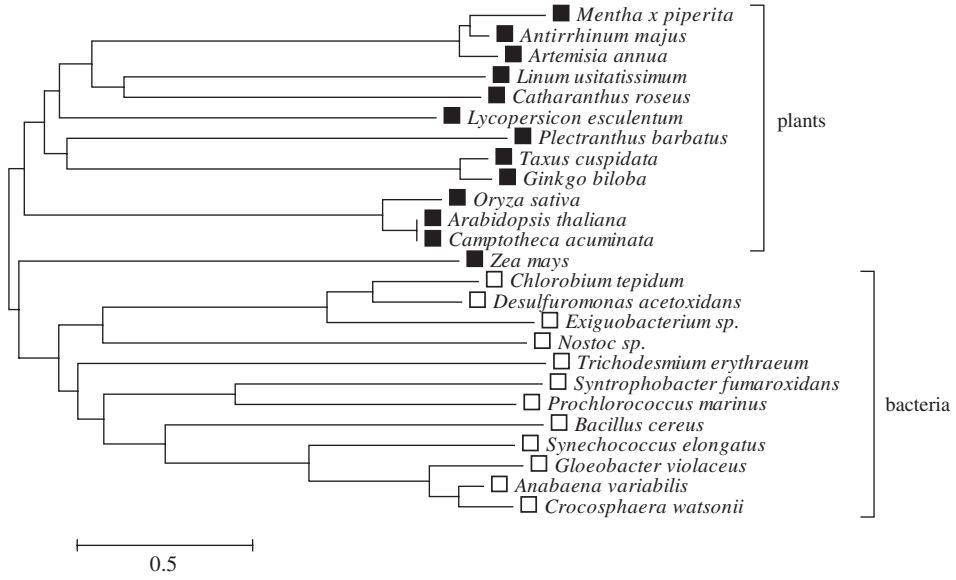

Figure 3. Phylogenetic tree analysis of DXRs from plants and bacteria by MEGA version 3.1 from CLUSTAL W alignments. The neighbor-joining method was used to construct the tree. The DXRs used in phylogenetic tree analysis were from plants including *Ginkgo biloba* (GbDXR, GenBank accession no.: AY494186), *Lycopersicon esculentum* (LeDXR, GenBank accession no.: AF331705), *Taxus cuspidata* (TcDXR, GenBank accession no.: AY575140), *Catharanthus roseus* (CrDXR, GenBank accession no.: AF250235), *Antirrhinum majus* (AmDXR, GenBank accession no.: AY770406), *Mentha x piperita* (MpDXR, GenBank accession no.: AF116825), *Arabidopsis thaliana* (AtDXR, GenBank accession no.: NM125674), *Linum usitatissimum* (LuDXR, GenBank accession no.: AJ623266), *Oryza sativa* (GenBank accession no.: NM183490), *Plectranthus barbatus* (GenBank accession no.: AY515699), *Zea mays* (GenBank accession no.: AJ297566), *Artemisia annua* (GenBank accession no.: AF182287) and *Camptotheca acuminata* (GenBank accession no.: DQ355159), and from bacteria including *Trichodesmium erythraeum* (GenBank accession no.: AABK04000004), *Crocosphaera watsonii* (GenBank accession no.: NZ_AADV02000005), *Nostoc* sp. (GenBank accession no.: BA000019), *Anabaena variabilis* (GenBank accession no.: CP000117), *Synechococcus elongatus* (GenBank accession no.: AJ250721), *Prochlorococcus marinus* (GenBank accession no.: CP000111), *Gloeobacter violaceus* (GenBank accession no.: BA000045), *Desulfuromonas acetoxidans* (GenBank accession no.: NZ_AAEW01000061), *Bacillus cereus* (GenBank accession no.: AE016877), *Syntrophobacter fumaroxidans* (GenBank accession no.: NZ_AAJF01000013), *Chlorobium tepidum* (GenBank accession no.: AE006470) and *Exiguobacterium* sp. (GenBank accession no.: NZ_AADW02000014).

version 3.1 from CLUSTAL W alignments, phylogenetic tree of DXRs was constructed from different organisms including plants and bacteria. The result showed that DXRs were derived from an ancestor gene and evolved into two groups including plant and bacteria DXR groups. According to the phylogenetic tree, CaDXR belonged to plant DXR group, and it was more ancient than other plant DXRs (Fig. 3), indicating that *C. acuminata* was one of the most ancient plant species. All the analysis results strongly suggest that CaDXR is a plant DXR protein involved in the IPP biosynthesis.

Crystal structure of the *E. coli* DXR (Reuter et al., 2002; Yajima et al., 2002; Steinbacher et al., 2003) has been published and the structure of the *Zymomonas mobilis* DXR has also been reported (Ricagno et al., 2004). The homology-based 3D structural modeling of CaDXR was analyzed by Swiss-Modeling on the basis of the *Z. mobilis* DXR crystal structure and displayed by WebLab ViewerLite (Fig. 4). The overall folding of CaDXR, typically built from β-sheets connected by turns and loops, created very tight structural scaffold. CaDXR was found to have many characters commonly possessed by NADPH binding DXR family. Thus, molecular characterization of CaDXR showed that CaDXR was very similar to other DXRs of plant origin, indicating that CaDXR belonged to DXR superfamily. The purification of CaDXR protein from *C. acuminata* and associated analysis will further elucidate the structure and function of CaDXR.

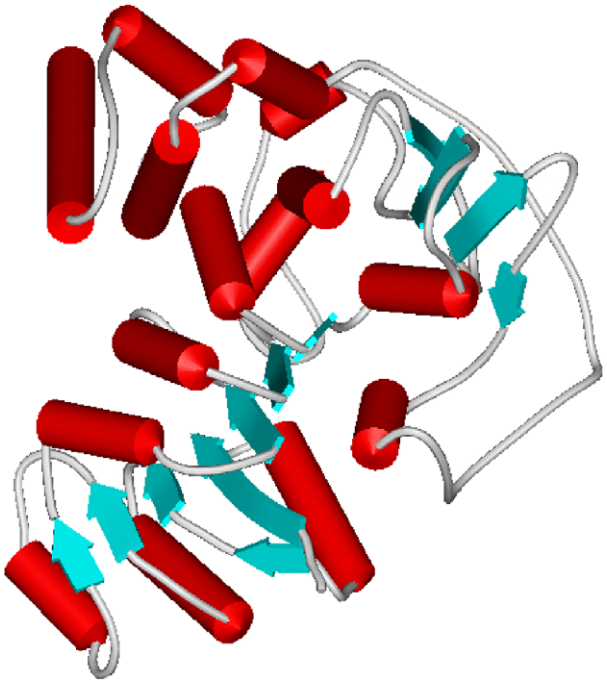

Figure 4. The 3D structure of CaDXR established by homology-based modeling (Ricagno et al., 2004). The α-helix and β-sheet were indicated in red and blue, respectively. Turns and loops were indicated in silver.

Expression profile of *CaDXR* in different tissues of *C. acuminata*

To investigate the expression profile of *CaDXR* in different tissues of *C. acuminata*, total RNA was isolated from root, stem and leaf tissues and subjected to semi-quantitative one-step RT-PCR using primers fcadxr and rcadxr. The result showed *CaDXR* expression could be detected in all tissues, suggesting that *CaDXR* is a constitutively expressed gene but at different expression levels, with the strong expression in stem and weak expression in root and leaf (Fig. 5A), in consistence with the expression pattern of the *DXR* gene from *G. biloba* (Gong et al., 2005).

Expression profile of *CaDXR* under induction of MeJA elicitor

One-step RT-PCR analysis was carried out to monitor the changes of *CaDXR* expression upon MeJA elicitor treatment. The result showed that the expression of *CaDXR* varied along with the treatment time (Fig. 5B). *CaDXR* expression was strongly induced by MeJA, reaching the highest level after 24 h of the treatment, and gradually decreased therefore after, but still maintaining the higher level than the control even after 72 h. Our results provide direct evidence that *CaDXR* is a MeJA elicitor-responsive gene and can be effectively elicited at least at the transcriptional level. Further investigations are required to characterize

Figure 5. Expression profiles of *CaDXR* in different *C.acuminata* tissues and under MeJA induction by semi-quantitative one-step RT-PCR. (A) Expression of *CaDXR* in different tissues including roots, stems and leaves. (B) Expression of *CaDXR* under MeJA (100 mM) induction for different duration. 18S RNA gene was used as the control to show the normalization of the amount of templates in PCR reactions.

the DXR enzyme activity and fully understand its regulatory role in CPT biosynthesis.

Functional complementation of CaDXR in *E. coli* DH5α

Carotenoids are synthesized in plants and cannot be synthesized in *E. coli* because of lacking of carotenogenic genes. However, *E. coli* introduced with foreign carotenogenic gene clusters had the ability to produce carotenoids and accumulated carotenoid pigments (Misawa et al., 1995), which could be as a visible marker for providing an easily screenable phenotype. In our study, the two vectors of pTrc-CaDXR harboring *CaDXR* coding region and pAC-BETA were co-transformed into *E. coli* DH5α to identify if *CaDXR* could enhance carotenoid accumulation. As controls, the vectors pTrc and pAC-BETA were also co-transformed, and the vector pAC-BETA was singly transformed into *E. coli* DH5α. The result showed that *E. coli* DH5α containing pTrc-CaDXR and pAC-BETA accumulated higher saffron yellow carotenoid, while carotenoid in *E. coli* DH5α could not be increased by co-transformation of pAC-BETA and the empty vector pTrc, and *E. coli* DH5α transformed with single vector pAC-BETA could not grow on LB medium containing ampicillin and chloramphenicol (Fig. 6), demonstrating that *CaDXR* encoded a functional protein and played an important role in promoting carotenoid pathway flux.

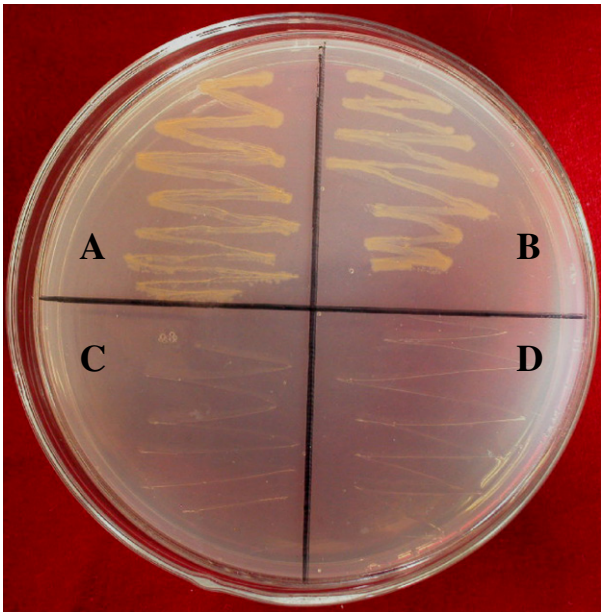

Figure 6. Functional complementation analysis of *CaDXR* in *E. coli*. *E. coli* strain DH5α was transformed with vectors pAC-BETA and pTrc-CaDXR harboring *CaDXR* gene (A), pAC-BETA and an empty vector pTrc (B), pAC-BETA (C) or pTrc-CaDXR (D).

In summary, we successfully isolated and characterized the full-length *CaDXR* cDNA from the deciduous and medicinal *Nyssaceae* plant *C. acuminata*. The cloning and characterization of *CaDXR* will be helpful to understand more about the role of DXR involved in the CPT biosynthesis at the molecular level. Our study indicates that *CaDXR* is responsive to MeJA elicitor. Functional complementation of CaDXR in *E. coli* DH5α demonstrated that *CaDXR* encoded a functional protein and played an important role in promoting carotenoid pathway flux. Plant expression vector containing the *CaDXR* has been constructed and genetic transformation of *C. acuminata* is undergoing in order to test its potential role in improving CPT production by genetic engineering.

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