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Isolation and characterization of a gene encoding cinnamate 4-hydroxylase from *Parthenocissus henryana*

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Abstract Cinnamate 4-hydroxylase (*C4H*, EC 1.14.13.11) plays an important role in the phenylpropanoid pathway, which produces many economically important secondary metabolites. A gene coding for *C4H*, designated as *PhC4H* (GenBank accession no. DQ211885) was isolated from *Parthenocissus henryana*. The full-length *PhC4H* cDNA is 1,747 bp long with a 1,518-bp open reading frame encoding a protein of 505 amino acids, a 40-bp 5′ non-coding region and a 189-bp 3′-untranslated region. Secondary structure of the deduced PhC4H protein consists of 41.78% alpha helix, 15.64% extended strand and 42.57% random coil. The genomic DNA of *PhC4H* is 2,895 bp long and contains two introns; intron I is 205-bp and intron II is 1,172-bp (GenBank accession no. EU440734). DNA gel blot analysis revealed that there might be a single copy of *PhC4H* in *Parthenocissus henryana* genome. By using anchored PCR, a 963-bp promoter sequence was isolated and it contains many responsive elements conserved in the upstream region of *PAL, C4H* and *4CL* including the P-, A-, L- and H-boxes.

Keywords Cinnamate 4-hydroxylase · *Parthenocissus henryana* · Phenylpropanoid pathway · Promoter analysis

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Abbreviations

C4H	Cinnamate 4-hydroxylase
I	Hypoxanthine
PAL	Phenylalanine ammonia-lyase
RACE	Rapid amplification of cDNA ends
RT-PCR	Reverse transcription polymerase chain reaction
4CL	4-Coumarate:coenzyme A ligase

Introduction

Phenylpropanoid pathway produces a wide range of secondary metabolites and many of them have beneficial effects on human health. Resveratrol, one of products in the pathway [1], is the basic skeleton of stilbene, which has many biological properties including anti-cancer, antioxidant and anti-aging [2, 3]. Phytochemical investigations showed that the plants in genus of *Parthenocissus* (Vitaceae family) are rich in stilbene compounds [4, 5]. Though a resveratrol synthase gene has been isolated from *Parthenocissus henryana* [6], little is known on other genes relative to the biosynthesis of stilbene and needed to be further studied.

Cinnamate 4-hydroxylase (*C4H*, EC 1.14.13.11), the second enzyme of the resveratrol biosynthesis pathway, catalyzes the hydroxylation of *trans*-cinnamic acid to *para*-coumaric acid [7]. Besides, *C4H* serves to anchor the enzyme complex, formed by the general phenylpropanoid enzymes (including PAL, C4H and 4CL), to the endoplasmic reticulum membrane through the N-terminal hydrophobic region [8].

The first *C4H* was purified from *Jerusalem artichoke* [9]. And five *C4H* genomic DNA sequences have been isolated from hybrid aspen [10], *Arabidopsis* [11, 12], pea [13] and oilseed rape [14] so far.

To study *C4H* in *Parthenocissus henryana*, we successfully isolated a full-length cDNA coding for *C4H* (*PhC4H*). To further understand *PhC4H* gene structure and regulation in *Parthenocissus henryana*, the genomic DNA sequence and promoter sequence were isolated. DNA gel blot analysis was performed to reveal *PhC4H* copy number in *Parthenocissus henryana* genome.

Materials and methods

RNA and DNA isolation

Total RNA was extracted from young leaves of *Parthenocissus henryana* by Concert Plant RNA Reagent (Invitrogen, USA). Genomic DNA was isolated from young leaves by Plant DNA Extraction Kit (Tiangen, China).

Rapid amplification of 3′ and 5′ cDNA ends of *PhC4H*

Total RNA isolated from *Parthenocissus henryana* was reversely transcribed using RP (5′-GCGGTACCCTTTTTT TTTTTTTTTTTT-3′), and followed by PCR amplification with a pair of degenerate primers dC4HFP (5′-ACTGGCT (G/T/C) CAAGT(A/T/G/C)GG(A/T/C)GA(C/T)G-3′) and dC4HRP (5′-CT(A/G)AA(C/T)T G(C/T)CC(A/T)CC(C/T) TTCTC-3′) based on highly conserved sequences in known *C4H*s from other species. Amplification conditions were performed as follows: 10 min at 94°C, 5 cycles of 30 s at 94°C, 30 s at 42°C and 1 min at 72°C, and followed by 25 cycles of 30 s at 94°C, 30 s at 55°C and 1 min at 72°C and a final extension of 10 min at 72°C. The PCR products were recovered and cloned into pGEM T Easy Vector (Promega, USA) and sequenced.

According to the sequencing result, gene specific primers for 3′ RACE and 5′ RACE were designed. Total RNA was used as templates to generate the first cDNA strand with RP, and then the 3′cDNA end was amplified by PCR with RP and a gene specific primer C4H-3R (5′-GGAGGATCCGTTGT TCGTGAAGC-3′). For 5′ RACE, total RNA was reversely transcribed using a gene specific primer C4H-5R (5′-GTAC TCGAAGCTCTGAGCCAATC-3′), then the 5′ cDNA end was amplified by PCR with 5′ RACE System for Rapid Amplification of cDNA Ends (Gibco-BRL, USA) according to the manual.

Isolation of cDNA and genomic DNA sequences coding for *PhC4H* by PCR method

A pair of gene specific primers C4HFP-C (5′-GCGGATC CGCCATGGATCTCATACTC-3′) and C4HRP-C (5′-GC TCTAGAGGGAAATTCAAGCTTCATTGG C-3′) was designed based on the sequencing results of 3′ and 5′ cDNA ends. PCR was performed under following conditions: 5 min at 94°C, 30 cycles of 30 s at 94°C, 1 min at 55°C and 2 min at 72°C, and a final extension of 10 min at 72°C. Genomic DNA sequence was amplified by replacing cDNA with genomic DNA.

Bioinformatics analysis

Blast was done on NCBI (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). Molecular weight, isoelectric point and structural analysis of the deduced PhC4H protein were predicated on the ExPASy Proteomics Server (http://www.expasy.ch/). The secondary and tertiary structures were predicated by program GOR IV [15] (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_gor4.html) and program CPHmodels [16] (http://www.cbs.dtu.dk/services/CPHmodels/), respectively. The Swiss-Pdb Viewer (v3.7) program is used for the graphical representation of tertiary protein structure.

DNA gel blot analysis

PhC4H gene copy number was analyzed by DNA gel blot using DIG-labeled probe generated by PCR. Thirty microgram genomic DNA extracted from young leaves was digested with *Sph*I, *Xba*I (no cut site within the probe) and *Eco*RI (one recognition site in the probe), respectively. The digested DNA was fractioned on 0.8%-agarose gel electrophoresis and transferred to Hybond-N^+ membrane (Amersham Pharmarcia, USA) by capillary transfer. Hybridization and detection were performed with the DIG system under standard conditions described in the manufacturer's instructions (Roche, Germany).

Isolation of *PhC4H* promoter sequence by anchored PCR

The experiment was carried out according to a recent study [17]. A linear amplification was performed by PCR in four 40-μl reactions consisting of 4 μl 10 × PCR buffer, 0.5 μl dNTPs (10 mM), 2 μl PC4HRP1 (10 mM) (5′-TGCTCTC CGTACACCGTGAACACC-3′), 2 μg genomic DNA, and 2 units of Ex *Taq*-polymerase (TaKaRa, Japan). PCR conditions were as follows: 7 min at 94°C, 25 cycles of 30 s at 94°C, 1 min at 62°C and 2 min at 72°C. The PCR products from four reactions were recovered and dissolved in 16.5 μl sterile water for tailing reaction. The tailing mixture contains 5 μl 5 × tailing buffer, 2.5 μl dCTP (2 mM), and 16.5 μl PCR products. After incubation at 94°C for 5 min, the mixture was hold on ice for 2 min and 1 μl terminal deoxynucleotidyl transferase (TaKaRa, Japan) was added to the mixture. Then it was incubated at

37°C for 15 min and finally at 65°C for 10 min. The poly (dC) tailed DNA was amplified using Abridged Anchor Primer (AAP, 5′-GGCCACGCGTCGACTAGTACGGGII GGGIIGGGIIG-3′) and a gene specific primer PC4HRP2 (5′-CCGGTGAAGATATCGAACACCACG-3′). The PCR products were purified and ligated to pGEM-T Easy Vector (Promega, USA). Transformants were identified by PCR with Abridged Universal Amplification Primer (AUAP, 5′-GGCCACGCGTCGACTAGTAC-3′) and PC4HRP2; the longest fragment was selected for sequencing.

Results

Isolation and analysis of cDNA sequence encoding cinnamate 4-hydroxylase

A cDNA encoding *C4H* was isolated from *Parthenocissus henryana* by RT-PCR and RACE approach. First, a pair of degenerate primers based on other known *C4Hs* was designed and an about 1.2-kb long fragment was amplified from *Parthenocissus henryana*. According to the sequence, primers for 5′ RACE and 3′ RACE were designed. A 574-bp and an 1,115-bp fragment were obtained by 5′ and 3′ RACE, respectively. The 1,115-bp fragment obtained by 3′ RACE contains part of the open reading fragment and 3′-untranslated region, which contains an 18-bp long poly (A) tail and a putative polyadenylation signal (AATAA) positioned at 28 bp upstream of poly (A) tail.

According to the sequencing results of 5′ and 3′ cDNA ends, gene specific primers were synthesized, and a 1,518-bp long coding region was isolated from *Parthenocissus henryana*. Blastn result indicated that the coding region of the cDNA shares highest identity (96%) with a *Vitis* corresponding gene (EMBL accession no. AM468511). Besides, it is identical to many other known *C4Hs* including *C4H* from *Camptotheca acuminata* (AY621152), *Citrus paradisi* (AF378333), and *Gossypium arboreum* (AF286647). The result showed that the gene encodes cinnamate 4-hydroxylase, so it was designated as *PhC4H*.

The *PhC4H* cDNA encodes a 57.9-kDa protein with an isoelectric point of 9.05. The deduced amino acid sequence contains featured motifs found in plant P450s, including a hydrophobic region near the N-terminus, followed by a proline-rich region (PPGPLPVP) [18], a conserved heme-binding motif PFGVGRRSCPG (conserved motif is PFGXGRRXCXG) at C-terminus [19], and an AAIETT sequence (the threonine-containing binding pocket for the oxygen molecule) [20]. Furthermore, it possesses the *C4H* conserved residues N302, I371 and K484 that are essential for substrate recognition and orientation [21].

Secondary and tertiary structure of PhC4H protein

The secondary structure of PhC4H protein (Fig. 1), predicated by GOR IV [15], consists of 41.78% alpha helix, 15.64% extended strand and 42.57% random coil. Alpha helices mainly reside at the N-terminus and the middle region of PhC4H protein. Extended strands mainly distribute at the N-terminus and the C-terminus; while random coils distribute at most region of PhC4H protein.

CPHmodels [16] predicated result showed that PhC4H protein (Fig. 2) is a globular protein. Similar to the results reported by Rupasinghe et al. [22], the tertiary structure of PhC4H protein also contains an alpha-domain and a beta-domain.

Analysis of *PhC4H* DNA sequence

Comparison of *PhC4H* genomic DNA and cDNA sequences revealed that the genomic DNA contains two introns (Fig. 3), which is consistent with the *C4Hs* in other plants. Intron I is 205 bp long, interrupted amino acid 262 and Intron II is 1172 bp long, located between amino acid 307and 308. The known *C4Hs* are different in intron length (Table 1). For example, *AtC4H* has two small introns (Intron I, 85 bp; Intron II, 220 bp) [12], whereas *PsC4H* (*CYP73A9v1*) has two longer introns (Intron I, 162 bp; Intron II, 1726 bp) [13].

The nucleotide sequences of 5′ exon–intron boundaries are AAgt for Intron I and CCgt for Intron II and 3′ sequences are agGA and agGC, which obeys the standard GT-AG rule.

DNA gel blot analysis

Genomic DNA digested with *Sph*I and *Xba*I resulted in a single band when hybridized with the probe (Fig. 4). Genomic DNA digested with *Eco*RI, which has a recognition site within the probe, showed two bands when

Fig. 1 The secondary structure of PhC4H protein. Alpha helix, extended strand and random coil were represented by the *longest, the second longest and the shortest vertical bars* respectively

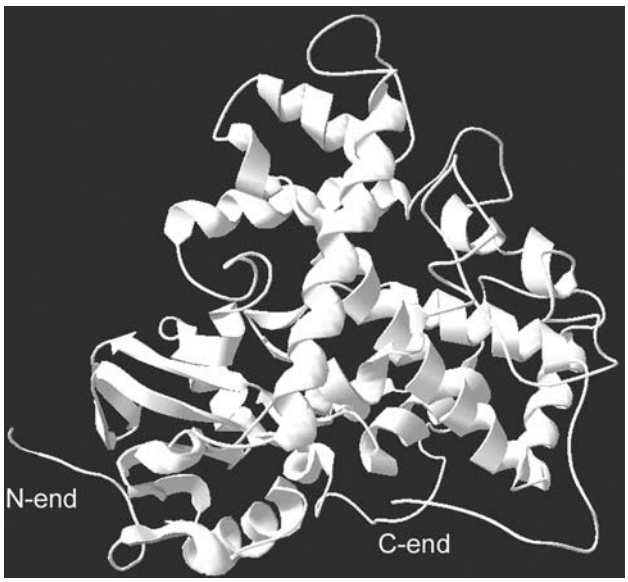

Fig. 2 The tertiary structure of PhC4H protein. The tertiary structure was predicated by program CPHmodels

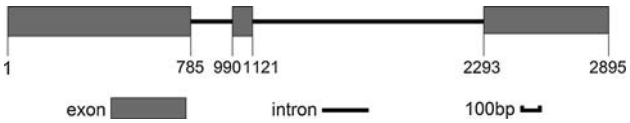

Fig. 3 The structure of *PhC4H* gene

hybridized with the probe. According to the results, we suggested that *PhC4H* is encoded by a single locus in *Parthenocissus henryana* genome.

Analysis of *PhC4H* promoter

PhC4H promoter is 963 bp long. Nucleotide search showed that it contains the TATA box, which is 70 bp upstream from the translation start codon (ATG), as well as the P-, A- and L-boxes conserved in early phenylpropanoid promoters (Table 2) [23]. *PhC4H* promoter also contains two Box IV and two G boxes that are involved in light responsiveness, implicating that the expression of *PhC4H* are regulated by light [24]. In accord with *AtC4H* and *PsC4H*, the P- and L-boxes are overlapped in *PhC4H*

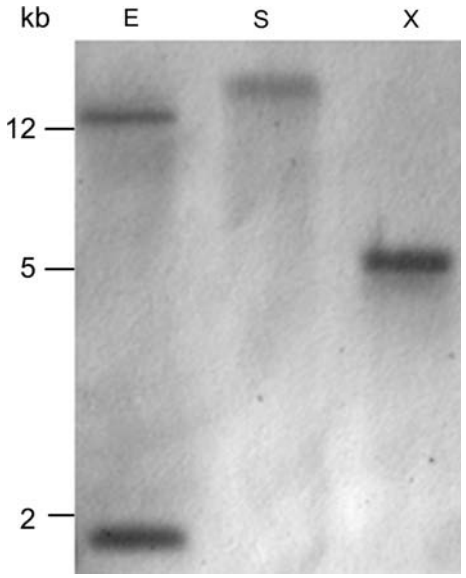

Fig. 4 DNA gel blot analysis of *PhC4H* gene in *Parthenocissus henryana* genome. Genomic DNA was digested with *Eco*RI (E), *Sph*I (S) and *Xba*I (X), fractioned on 0.8%-agarose gel and blotted to Hybond-N^+ membrane, then hybridized with DIG-labeled probe at high stringency

promoter [13]. While *PhC4H* promoter possesses three putative A-boxes, which is absent from *AtC4H* and *PsC4H* promoters. The consensus sequence for A-box is CCGTCC, while all the three A-boxes sequence is CCGTCA, with the sixth position C substituted by A in *PhC4H* promoter.

Discussion

In this work, we have isolated a gene encoding *C4H* from *Parthenocissus henryana.* The blastp result demonstrated that the deduced amino acid sequence of *PhC4H* shares the highest identity with *C4H* from *G. arboreum* (up to 89%). Besides, it contains featured sequences found in plant P450 proteins and conserved residues found in C4H proteins.

Previous studies reported that C4H should be grouped into two classes according to different N-terminus and C-terminus [25]. Sequence alignment revealed that *PhC4H*

Table 1 Introns and genes length of known *C4H* genomic DNA sequences

Accession number	Plant species	Intron I length (bp)	Intron II length (bp)	Gene length (bp)
D82812	*Populus sieboldii* × *P. grandidentata*	686	497	2,700
	Arabidopsis thaliana	85	220	1,823
AF175275	*Pisum sativum*	162	1,726	3,379
DQ485129	*Brassica napus*	71	379	1,968
DQ485131	*Brassica napus*	67	327	1,910
PhC4H	*Parthenocissus henryana*	205	1,172	2,895

Table 2 Putative cis-acting elements on the PhC4H promoter

P box*	A box*	L box*	G box*
CTCTCACCcACtCC (−157)	CCGTCa (−112)	TCTCACCcACt (−158)	CACGTG (−389)
	CCGTCa (−171)		
TTTTCgCCcCCACC (−276)	CCGTCa (−186)	aCCCACCTACt (−257)	CACcTG (−669)

The number in brackets showed the positions relative to translation start codon

*The consensus sequence for P box is $^{c}_{t}T^{tcaa}_{ctcc}C\,^{a}_{c}A\,^{a}_{c}C\,^{cc}_{aa}C$, A box is CCGTCC, L box is $^{t}_{c}C\,^{tc}_{ct}ACC\,^{t}_{a}ACC$ and G box is CACGTG

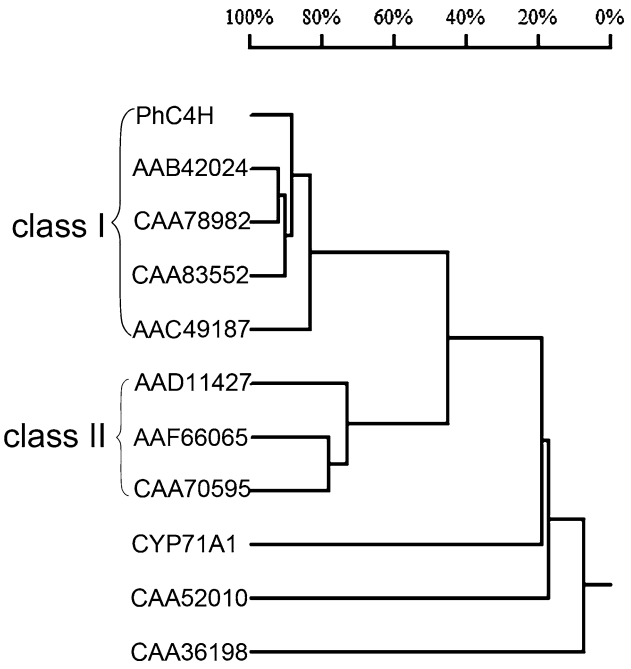

Fig. 5 Homology tree of *PhC4H* and other known *C4Hs*, showing that *PhC4H* belongs to class I. *C4H* from *Zinnia elegans* (AAB42024), *Helianthus tuberosus* (CAA78982), *Catharanthus roseus* (CAA83552), *Pisum sativum* (AAC49187), *Mesembryanthemum crystallinum* (AAD11427), *Citrus sinensis* (AAF66065), *Phaseolus vulgaris* (CAA70595) and P450s from *Persea Americana* (CYP71A1), *Pleuronectes platessa* (CAA52010), *Candida maltose* (CAA36198), respectively

shows higher identity with the C4Hs of class I than with those of class II (Fig. 5), suggesting that *PhC4H* should be grouped into class I.

So far, only five *C4H* DNA sequences were isolated from hybrid aspen [10], *Arabidopsis* [11, 12], pea [13] and oilseed rape [14]. All the DNA sequences contain two introns which located at conserved positions. However, they vary in length because of the differences in intron length.

PhC4H is encoded by a single locus, which is consistent with the *C4H* in *Arabidopsis* [11, 12] and parsley [26], while *C4Hs* are encoded by a small gene family in mung bean [27]. *C4H* plays a key role in both lignin biosynthesis and plant defense in plants which only have one copy of the gene. However, in plants which have two or more copies, each (or some) one plays a specific role in plant physiology and development. For example, three *C4Hs* in *Populus trichocarpa* play different physiological roles, *PtriC4H1* is involved in the biosynthesis of G lignin, and *PtriC4H2* plays a key role in biosynthesis of S lignin, whereas *PtriC4H3* participates in stress responses [28].

The secondary structure of PhC4H protein has lower similarity with those of BnC4H proteins [14]. While the tertiary structure of PhC4H protein is similar to those of BnC4H proteins [14], the AtC4H protein and the CYP84A protein (ferulate-5-hydroxylase, a member of plant P450) [22], which accords with the principle that plant P450 proteins possess conserved tertiary structure [29].

It is reported that the genes of general phenylpropanoid pathway are coordinately regulated [13, 23]. *PhC4H* promoter contains the P-, A-, L-, G- and H-boxes, which were identified in early phenylpropanoid promoters [23], suggesting that the expression of *PhC4H* might be in accord with *PAL* and *4CL* accumulation. In addition, promoter analysis showed that *PhC4H* promoter contains MYB-binding sites and a WRKY-binding site. Up to now, several MYB proteins involved in transcriptional regulation of the genes encoding the general phenylpropanoid enzymes have been identified. Previous research showed that AmMYB305 and AmMYB340 positively regulate the expression of *PAL* [30, 31] and over expression of AmMYB308 and AmMYB330 negatively regulate the expression of *C4H* in snapdragon (*Antirrhinum majus*) [32]; while AtMYB4, the first identified MYB protein functions as a transcriptional repressor, plays a negative role in controlling the expression of *C4H* [33].

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