

Expression patterns of a cinnamyl alcohol dehydrogenase gene involved in lignin biosynthesis and environmental stress in *Ginkgo biloba*

Hua Cheng · Linling Li · Feng Xu ·
Shuiyuan Cheng · Fuliang Cao · Yan Wang ·
Honghui Yuan · Dezhi Jiang · Conghua Wu

Received: 6 June 2012 / Accepted: 3 October 2012 / Published online: 10 November 2012
© Springer Science+Business Media Dordrecht 2012

Abstract The cinnamyl alcohol dehydrogenase (CAD) is a key enzyme in lignin biosynthesis as it catalyzes the final step in the synthesis of monolignols. A cDNA sequence encoding the CAD gene was isolated from the leaves of *Ginkgo biloba* L., designated as *GbCAD1*. The full-length cDNA of *GbCAD1* was 1,494 bp containing a 1,074 bp open reading frame encoding a polypeptide of 357 amino acids with a calculated molecular mass of 38.7 kDa and an isoelectric point of 5.74. Comparative and bioinformatic analyses revealed that GbCAD1 showed extensive homology with CADs from other gymnosperm species. Southern blot analysis indicated that *GbCAD1* belonged to a multi-gene family. Phylogenetic tree analysis revealed that GbCAD1 shared the same ancestor in evolution with other CADs and had a further relationship with other gymnosperm species. GbCAD1 was an enzyme being pH-dependent and temperature-sensitive, and showing a selected catalyzing. Tissue expression pattern analysis showed that *GbCAD1* was constitutively expressed

in stems and roots, especially in the parts of the pest and disease infection, with the lower expression being found in two- to four-year-old stem. Further analysis showed the change in lignin content had some linear correlation with the expression level of *GbCAD1* mRNA in different tissues. The increased expression of *GbCAD1* was detected when the seedling were treated with exogenous abscisic acid, salicylic acid, ethephon, ultraviolet and wounding. These results indicate that the *GbCAD1* gene may play a role in the resistance mechanism to biotic and abiotic stresses as well as in tissue-specific developmental lignification.

Keywords *Ginkgo biloba* L. · *GbCAD1* · Biotic and abiotic stresses · Lignification

Introduction

Lignins are complex phenolic polymers comprise of guaiacyl (G) units derived from coniferyl alcohol, syringyl (S) units derived from sinapyl alcohol, and *p*-hydroxyphenyl (H) units derived from *p*-coumaryl alcohol. Many enzymes are involved in lignin biosynthesis [1]. Cinnamyl alcohol dehydrogenase (CAD, EC 1.1.1.195), which is a key enzyme in monolignol biosynthesis, catalyses the conversion of the corresponding cinnamyl aldehydes to cinnamyl alcohols; this is the last step in the synthesis of monolignols before their polymerization in cell walls. According to these data, CAD displays distinct features between gymnosperms and angiosperms. Gymnosperm CAD is encoded by a single gene, and is believed to be highly specific for the reduction of coniferyl aldehyde [2], and with lower catalytic activity toward sinapyl aldehyde. On the contrary, angiosperm CAD has multiple isoforms that have significant affinity for both coniferyl and sinapyl aldehydes [3].

Electronic supplementary material The online version of this article (doi:10.1007/s11033-012-2111-0) contains supplementary material, which is available to authorized users.

H. Cheng · L. Li · F. Xu · S. Cheng (✉) · Y. Wang ·
H. Yuan · D. Jiang · C. Wu
Economic Forest Germplasm Improvement and Comprehensive
Utilization of Resources of Hubei Key Laboratories, Hubei
Huanggang 438000, China
e-mail: s_y_cheng@sina.com

H. Cheng · L. Li · S. Cheng · Y. Wang · H. Yuan · D. Jiang ·
C. Wu
College of Chemistry and Life Science, Huanggang Normal
University, Huanggang 438000, China

H. Cheng · S. Cheng · F. Cao
College of Forest Resources and Environment, Nanjing Forestry
University, Nanjing 210037, China

The relationship between CAD and biotic stress has been well-established, the *IbCAD1* gene play a role in the resistance mechanism to biotic and abiotic stresses as well as in tissue-specific developmental lignification [4]. The mRNA level of alfafa *MsCADs* and ryegrass *LpCADs* was induced by wounding treatment, and the addition of fungal elicitor to bean cell cultures led to the rapid accumulation of CAD transcripts [3, 5]. Syringyl lignin was found to be accumulated during the hypersensitive resistance response in wheat [6]. Transgenic plants having reduced levels of lignins are more readily attacked by fungi and bacteria [7]. In addition, CAD genes might also be important for the production of biofuels. Some researchers produced gene mutant poplars with reduced CAD activity and observed higher lignin extractability [8]. All of these indicate that the amount and unit composition of lignin is influenced by gene transcription, developmental and environmental conditions. However, little information is yet available on the role of CAD in relation to abiotic stress.

Plants have to develop a broad range of complex defense systems to struggle against pathogenic infections, because they have being confined to the place where they grow. Recently, *Ginkgo biloba* is one of the most popular functional plants, especially as medicinal plants. Extracts of *G. biloba* leaves contain active compounds such as flavonoids and terpene lactones (ginkgolides and bilobalide) and can therefore be use to increase peripheral and cerebral blood flow [9, 10]. In addition, Ginkgo has strong diseases and pests resistant, grows in widespread area, which has many good characters, such as disease-resistance, drought, low temperature, barren, and nuclear radiation. Flavonoids metabolism and lignans metabolic both belong to phenylalanine metabolism path branch, lignans synthesis might affect the accumulation of *G. biloba* flavonoids. Here we report the cloning and characterization of a *GbCAD1* encoding a putative CADs protein and also discuss its potential function in resistance mechanism to biotic and abiotic stresses. A combination of biochemical and gene expression analysis indicated that *GbCAD1* is the enzyme involved in lignification and defense. Taking into consideration the role of CAD and lignin in imparting disease resistance to the plants, our study was aimed to investigate the possible role of CAD enzyme in imparting disease resistance to the plant, role of lignin deposition in disease resistance against wounding and pathogen infection in Ginkgo and to correlate its transcripts with disease resistance of Ginkgo to pathogen.

Materials and methods

Plant materials and treatments

Fourteen-year old of grafted *G. biloba* seedlings were growing in a greenhouse in Huanggang (E, 111°54′–112°19′,

N, 30°6′–30°39′, Hubei province, central of China) were sampled as plant materials. For gene cloning and tissue expression, diverse tissues including young leaves, mature leaves, ovules, stamens, albumen, gynoecia, stems and roots were collected for DNA and RNA extraction as described by Xu et al. [11]. Tissues were immediately frozen in liquid nitrogen and kept at -80°C prior to total RNA extraction.

Total RNA was isolated from injured stem tissues of 14 years old grafted *G. biloba* seedlings after they were attacked by *Rhizoctonia solani*. For fungal infections blade, the total RNA was isolated from *Ginkgo* leaves cells punctured with an infected agent isolate of *Phyllosticta mortonii* Fairman. One year old cuttings from the same genotypic strain of *G. biloba* were subjected to treatments with UV-B, wounding (WOU), 5-aminolevulinic acid (ALA), abscisic acid (ABA), salicylic acid (SA) and ethephon (ETH). For UV-B treatment, seedlings were exposed to $1,500\ \mu\text{J}/\text{m}^2$ UV-B irradiation in a closed chamber, and the control cuttings were placed in a dark closed chamber. The edges of Ginkgo leaves were cut by about 0.6 cm with scissors for wounding treatment, the intact leaves of Ginkgo was as control. The ALA (100 μM), ABA (10 mM), SA (25 mM), and ETH (20 mM) were dissolved in 0.01 % Tween 20 and sprayed onto young leaves. The control leaves were sprayed with an equivalent volume of 0.01 % (v/v) Tween 20.

Cloning of the full-length cDNA

Given species conservation of the functional domain of the CADs protein and the *Ginkgo* EST database in GenBank, a pair of degenerate primers, CAF and CAR, was designed according to the conserved peptide sequences, ILGLGGVGHM and NDVRYRFVV, to amplify a conservative fragment of the *GbCAD1* gene. The purified PCR product was cloned into the pMD18-T vector (TaKaRa, Dalian China), and the positive clone was confirmed by a BLASTN query of the NCBI Database to verify the homology of this cloned fragment with CAD sequences from other species.

Based on the sequence of cloned *GbCAD1* fragments, the specific primer pairs (CA5R and CA3R) and the nested primer pairs (CA5N and CA3N) were designed to amplify the 5′ and 3′ end of *GbCAD1* using the SMART™ RACE cDNA Amplification Kit (Clontech, USA). The PCR products were purified and cloned into the pMD18-T vector for sequencing. After comparing and aligning the sequence of 5′RACE, 3′RACE, and the internal fragment, the full-length cDNA sequence of *GbCAD1* was obtained.

Southern blot analysis

Genomic DNA (20 $\mu\text{g}/\text{sample}$) was digested overnight at 37°C with *Eco* R I, *Bam*H I, *Hind* III and *Kpn* I. The digested DNA was fractionated by 0.9 % agarose gel

electrophoresis and transferred onto a PVDF membrane (Roche Applied Science, Germany). Specific probes were generated by PCR: *GbCAD1* specific probe was obtained using sense CAT1 and antisense CAT2 primers located respectively at positions 161 and 614 on the *GbCAD1* cDNA. At total of 50 ng purified amplified fragments were used as a template in a total volume of 20 μ l for probe labeling. Probe labeling (DIG), hybridization and signal detection were performed following the manufacturer's instruction for the DIG High Primer DNA Labeling and Detection Starter Kit II (Roche Applied Science, Germany) [12].

Relative quantification by QRT-PCR

The transcription levels of *GbCAD1* were determined in different *G. biloba* tissues, as well as in young seedling leaf samples collected at different time points after stress and hormone treatments. QRT-PCR was carried out using an ABI PRISM 7,500 Sequence Detection System (Applied Biosystems, American) with SYBR Green PCR Master Mix (Applied Biosystems, American) according to the manufacturer's protocol. The *G. biloba* glyceraldehydes-3-phosphate dehydrogenase gene (*GbGAPDH*, L26924) [13], was used as the reference gene as described by Xu [11].

The gene-specific primers (CAQF, CAQR) and reference primers (GAPF, GAPR) for QRT-PCR were listed in Table 1. The QRT-PCR conditions were: 10 min at 95 °C, and 40 cycles (95 °C for 15 s, 60 °C for 1 min). Before performing QRT-PCR, primer efficiency was evaluated using both *GbCAD1* and *GbGAPDH* at 100, 150, 200, 250 and 300 nM combinations. A 120 nM concentration was chosen as most suitable combination for both genes. For each plant sample, aliquots of 150 ng total RNA was analyzed for each gene and the two genes (*GbCAD1* and *GbGAPDH*) were always analyzed simultaneously. Each sample was amplified three times and all reactions were performed on ABI PRISM 7,500 Sequence Detection System. With a housekeeping gene *GbGAPDH*, the relative amount of the *GbCAD1* transcript is presented as $2^{-(\Delta\Delta C_t)}$ according to the C_T method [14]. When comparing the expression of *GbCAD1* in different tissues or treatments, the relative expression of *GbCAD1* was achieved by calibrating its transcription level to that of the reference gene, *GbGAPDH*.

Functional expression of the GbCAD1 in *Escherichia coli*

To clone *GbCAD1* into an expression vector, a pair of primers, CAZF and CAZR, were designed and synthesized to amplify the coding region by RT-PCR with the incorporation of a restriction enzyme site and a protective base

to simplify later vector construction. After confirmation by sequencing, the resulting recombinant plasmid was introduced into BL21 (DE3) by the heat shock method. A single colony of *E. coli* BL21 cells harboring the expression plasmid pET28a-GbCAD1 was inoculated at 37 °C in Luria–Bertani medium containing kanamycin (50 mg L⁻¹) and were grown with shaking (150 rpm) at 37 °C until the optical density (OD 600) reached about 0.6. For induction, Isopropyl β -D-thiogalactoside (IPTG) was added at a final concentration of 1 mM and the cells were further cultured at 30 °C for 2 h. The cells were lysed by sonication for 10 s at 4 °C, and centrifuged at 7,000 g for 15 min. Supernatants and pellets were analyzed by SDS-PAGE and Coomassie Brilliant Blue R250 staining. The recombinant GbCAD1 protein from induced cells was purified using Nickel–CL agarose affinity chromatography (Bangalore Genei) and used for in vitro enzyme assays.

Western blotting was carried out to verify expression of a GbCAD1 protein having a His-tag in the N-terminus. After electrophoresis, the proteins were electro-transferred onto a PVDF membrane and detected with antimouse RGS-His antibody (Santa Cruz, American), and a secondary antibody (goat anti-mouse IgG), conjugated to alkaline phosphatase (AP). Western Blue Stabilized Color Substrate for AP (Promega, USA) was used for the color reaction.

Assay of GbCAD1 activity

CAD1 enzyme activity was measured according to the method of Ma [15] with a little modification. The assay mixture contained about 30 μ g protein in 500 μ l of 0.1 M NaH₂PO₄/Na₂HPO₄ buffer at pH 7.5, 0.2 mM NADPH and 0.02 mM coniferyl aldehyde substrate. Reaction were incubated at different temperature or pH for 15 min and then stopped by adding 50 μ l 25 % trichloroacetic acid (TCA). For each reaction, 10 min of declination at OD₃₄₀ was monitored automatically with 1 min intervals. K_m and V_{max} values were determined by extrapolation from Lineweaver-Burke plots. The effects of pH on enzyme activity were determined after the reaction mixture was incubated in phosphate potassium buffer with the required pH for 15 min. The stability of enzyme was analysed after incubating the reaction mixture at different temperatures.

Lignin content analysis and histo-chemical lignin staining

Lignin content was quantitatively measured by using the Klason method [16]. Briefly, air-dried stem tissues were ground into powder and exhaustively extracted in a Soxhlet apparatus with toluene-ethanol (2:1,v/v), followed by 95 % ethanol and water. Samples were then vacuum dried and 200 mg was hydrolysed in 3 ml of 72 % H₂SO₄ at 25 °C

Table 1 Primers for *GbCAD1*

Primer	Sequence (5'–3')	Description
CAF	TATKGGSTCTTGGAGBTGTDGGTCA	Degenerate primer, forward
CAR	TCGRCCACAAMTCTGTAGCVAACGT	Degenerate primer, reverse
CA5R	CTGGGACAACACCAAGCATAACAAG	Reverse primer for 5' RACE, outer
CA5N	AGCATAACAAGTTTTCCATTCATCT	Reverse primer for 5' RACE, nested
CA3R	GAGCATAGCGGGAAGTTTCATAGGC	Forward primer for 3' RACE, outer
CA3N	AAGGTGTCGTGCATGATTGAGAT	Forward primer for 3' RACE, nested
CAZF	<i>ACAGAGCTCATGGGGAGCGTGGAAATCTGAAAAG</i>	Gene specific primer, forward
CAZR	<i>CAACTCGAGGTTAGTTATGGAGATTGGCAGCGG</i>	Gene specific primer, reverse
CAT1	TCCATAACCATTACACCCTCAGAAAC	Primer for probe forward
CAT2	ATGCTTCATTGGGCTGTAAACTGT	Primer for probe reverse
CAZF	GGGTCATCTATCTCCATACCATTAC	Primer for RQPCR, forward
CAZR	GTGTTTCTGAGGGTGAATGGTATG	Primer for RQPCR, reverse
GAPF	TAGGAATCCCGAGGAAATACC	Primer for RQPCR, forward
GAPR	TTCACGCCAACAACGAACATG	Primer for RQPCR, reverse

Restriction site sequences are indicated in italic letters and underlined

for 3 h with occasional stirring. The hydrolysate was diluted with the addition of 190 ml H₂O and then autoclaved for 1 h. The sample was filtered through a fritted glass crucible, and then washed with hot water. The crucible was dried at 105 °C and weighed. The filtered solution was diluted to 500 ml and A₂₀₅ was determined spectrophotometrically using a 1 cm long cuvette. The Klason lignin was expressed as a percentage of the cell wall residue. The acid-soluble lignin was calculated by following formula: Acid lignin (g L⁻¹) = A₂₀₅/110.

For lignin staining observation, hand-cut sections of different sample were treated by 1 % phloroglucinol ethanol and 35 % HCl, and images were captured by Cannon camera [17].

Bioinformatics analysis and molecular evolution analyses

The obtained sequences were analyzed using bioinformatics tools at websites (<http://www.ncbi.nlm.nih.gov> and <http://www.xpasy.org>). Vector NTI Suite 10 was used for sequence alignment and analysis. Phylogenetic tree analysis of GbCAD1 and known CADs from other plant species retrieved from GenBank were aligned with Mega 4.0 [18]. The phylogenetic tree was constructed by a neighbor-joining (NJ) method and measured by bootstrap analysis with 1,000 replicates. Model of GbCAD1 were created with SWISS-MODEL [19]. The crystal structure of *Arabidopsis* AtCAD5 served as the template for 3D modeling (PDB ID 2CF5) and secondary structure prediction [20]. Structural comparison and analysis were performed with Weblab Viewer Lite 3.5 (Accelrys, San Diego, CA, USA). SPSS 17 was used for statistical analysis and graphing.

Results

Characterization of the *G. biloba* CAD1

A full-length cDNA of the CAD1 gene was obtained from *G. biloba* leaf tissue by degenerate PCR and RACE method. The cDNA sequence was 1494 bp with a poly A tail, and contained a 1,074 bp open reading frame. A 92 bp 5' untranslated region was upstream of the start codon, and the coding region was followed by a 328 bp 3' untranslated region downstream from the stop codon (Fig. 1). The 5' untranslated region conformed to the predicted translation start site for eukaryotic genes (AXXATGG) [21, 22], and two stop codons (TAA, 1,164 and 1,197 bp) found in the 3' UTR proved that the cDNA contained the entire ORF. One potential polyadenylation signal AATAAA was found at 267 bp position downstream from the stop codon. The putative GbCAD1 was 357 amino acid with a theoretical molecular weight of 38.7 kDa and a pI value of 5.74. The subcellular location prediction of GbCAD1 did not find any secretory pathway signal peptide.

Multi-alignment of GbCAD1 with other plant CADs and their homologues showed higher similarities to gymnosperm than other plants (Fig. 2). The amino acid sequence of the GbCAD1 shared 84.9 % identity with that of *Picea abies*, 84.0 % with *Pinus teada*, 83.2 % with *Pinus pinaster*, 82.9 % with *Pinus radiata*, 70.3 % with *Zea mays*, 69.5 % with *Gossypium hirsutum*, *Triticum aestivum* and *Festuca arundinacea*, 69.7 % with *Liriodendron tulipifera*, 69.2 % with *Leucaena leucocephala*, 67.8 % with *Populus nigra*, 67.5 % with *Citrus sinensis*, 67.1 % with *Eucalyptus urophylla*. Homology analysis with CADs from other plants suggested that *GbCAD1* may contain three conserved


```

1  AGCCATTCTC TGTCCTGTCC TGCTTTTTTC CATGGCTGCA ACCAAACGGT CTTAGCAAGG GACATACTCG AGTTTTGAGT TAATAATTCG
   M G S V E S E R R V T G W A A R D S T G H L S P Y H Y T L
91  GAAATGGGGA GCGTGAATC TGAAAGAAGG GTTACAGGAT GGGCAGCAGC TGACTCCACG GGTCACTCAT CTCCATACCA TTACACCCTC
   R N T G P Q D V V L K V I Y C G I C H T D L H Q I H N D M G
181  AGAAACACAG GACCTCAGGA TGTGGTACTC AAGGTCATTT ATTGCGGAAT TTGTCACACT GATTGTCATC AAATTCATAA CGACATGGGA
   M S N Y P M V P G H E V A G V V T E I G E E V K K F K V G G E
271  ATGTCCTAAT ACCCGATGGT CCCCGGGCAT GAAGTGCCGG GAGTCGTAAC TGAAATCGGT GAAGAAGTGA AGAAATTCAA AGTAGGAGAA
   N V G V G C I V G S C R T C Q A C N Q S M E Q Y C S K R I W
361  AATGTGGGTG TGGGTGTGAT TGTGGATCA TGCCGCACCT GCCAGGCCCTG CAACCAAAGC ATGGAACAAT ACTGCAGTAA AAGGATTTGG
   T Y N D V S P D G K P T Q G G F A S T M V V D Q M F V V H I
451  ACTTACAATG ATGTATCTCC TGATGGCAAG CCAACTCAAG GAGGATTTGC AAGCACCATG GTAGTTGATC AGATGTTTGT GGTTCACATT
   P E N L P E L E Q A A P L L C A G G V T V Y S P M K H F G M T E
541  CCTGAGAAC TFCCTCGGA ACAAGCTGCA CCACTTTTAT GTGTGGGTG TACAGTTTAC AGCCCAATG AGAATTTGG AATGACAGAG
   P G K R C G I L G L G G V G H M G V L I A K A F G L H V T V
631  CCTGGCAAAA GATGTGGGAT ATTGGGTCTT GGAGGTGTGG GTCATGGG TGTTTTGATT GCTAAAGCAT TTGGACTTCA TGTAACAGTC
   I S S S D S K K N E A L E V L G A D A Y L V S K D A A Q M E
721  ATAAGTTCAT CAGATAGCAA GAAGAACGAA GCCCTTGAAG TACTCGGTGC AGATGCATAT CTTGTAAGCA AGGATGCCGC ACAGATGGAG
   A A A E S L D Y I L D T I P V V H P L E P Y L S L L K M N G
811  GCAGCAGCAG AGAGCTTGGG CTACATATTG GACACTATTC CGGTTGTGCA TCCTCTTGAG CCATATCTTT CTCTTCTCAA GATGAATGGA
   K L V M L G V V P A P L S F V S P L L I L G R R S I A G S F
901  AAAGTTGTTA TGCTTGGTGT TGTCCCAGCT CCTTTGCTCT TTGTTTACC TCCTCTGATA CTAGGGAGGC GGAGCATAGC GGGAGATTTC
   I G S M E E T Q E T L D F C A E K K V S C M I E I V G L D Y
991  ATAGGCAGCA TGGAGGAAC ACAGGAACT CTGGATTCT GTGCAGAGAA GAAGGTGTCG TGCATGATTG AGATTGTGGG TTTGGATTAT
   V N E A A L K R L E K N D V R Y R F V V D V A A A N L H N
1081  GTGAACGAAG CCTTGAAAAG ACTGGAAAAG AATCAGCTTC GCTACAGATT TGTGGTCCGAC GTCGCCGCTG CCAATCTCCA TAACTAACAA
1171  AATCGCCAC AACAAAGACT CCAACCATGA CTACACTTCT TTTCTAGGTT TTCTTCAAAA TTTTAAGATT AAAAATGTAT TTCCAAATAA
1261  AAAATGTTTG CAATATGTAG AGAAATAGTC TCTGAAATCG ATCACAATGT TTGTGAATGT TCAACATTGA CTACATGTAA TATCAAAGAC
1351  TCCAAAACCT ACCCAACA AAGACTCAA CCATGACTAC ACTTTATCTA GTATTTTGTG ATTATAAGTT TTGCACGATC AAGAAATAAA
1441  TTTGTATGCT TGGTGATGCT AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAA

```

Fig. 1 The partial cDNA sequence and deduced amino acid sequence of *GbCAD1* gene. The nucleotides that encode amino acids of GbCAD1 are given in capital letters. Initiation and termination codon are indicated by underlined. Degenerate primers are indicated with “Square box”

structural domains. The first domain, GHEVAGVV, was a zinc-binding region (zinc coordinating site: His69) that was highly conserved among the analyzed sequences. The second domain, GENVGVGCIV, was also a zinc-binding region and highly conserved. Particularly, the zinc-binding cysteine residues (Cys100, Cys103, Cys106, Cys114) were almost identical across the analyzed species. The third domain, GLGGVG, was a NADPH-binding region that was highly conserved in the analysis.

The three-dimensional (3D) structure of GbCAD1 (Fig. 1S) was examined by homology modeling using SWISS-MODEL (Swiss Institute of Bioinformatics, Lausanne, Switzerland) [19]. The model predicted that GbCAD1 and AtCAD5 may have similar secondary structures, featuring primarily α -helices and β -sheets intermingled with random coils and β -turns. GbCAD1 was predicted to have six α -helices and 12 β -turns.

The amino acid sequences of CADs from various plants were phylogenetically analyzed by the neighbor-joining method [18]. Figure 3 shows the reconstructed tree; the numbers at the nodes show bootstrap support values (1,000 replicates), and the scale line below the tree represents relative evolutionary distance. The CADs of the analyzed plants were divided into three main groups: dicotyledons, monocotyledons, and gymnosperms.

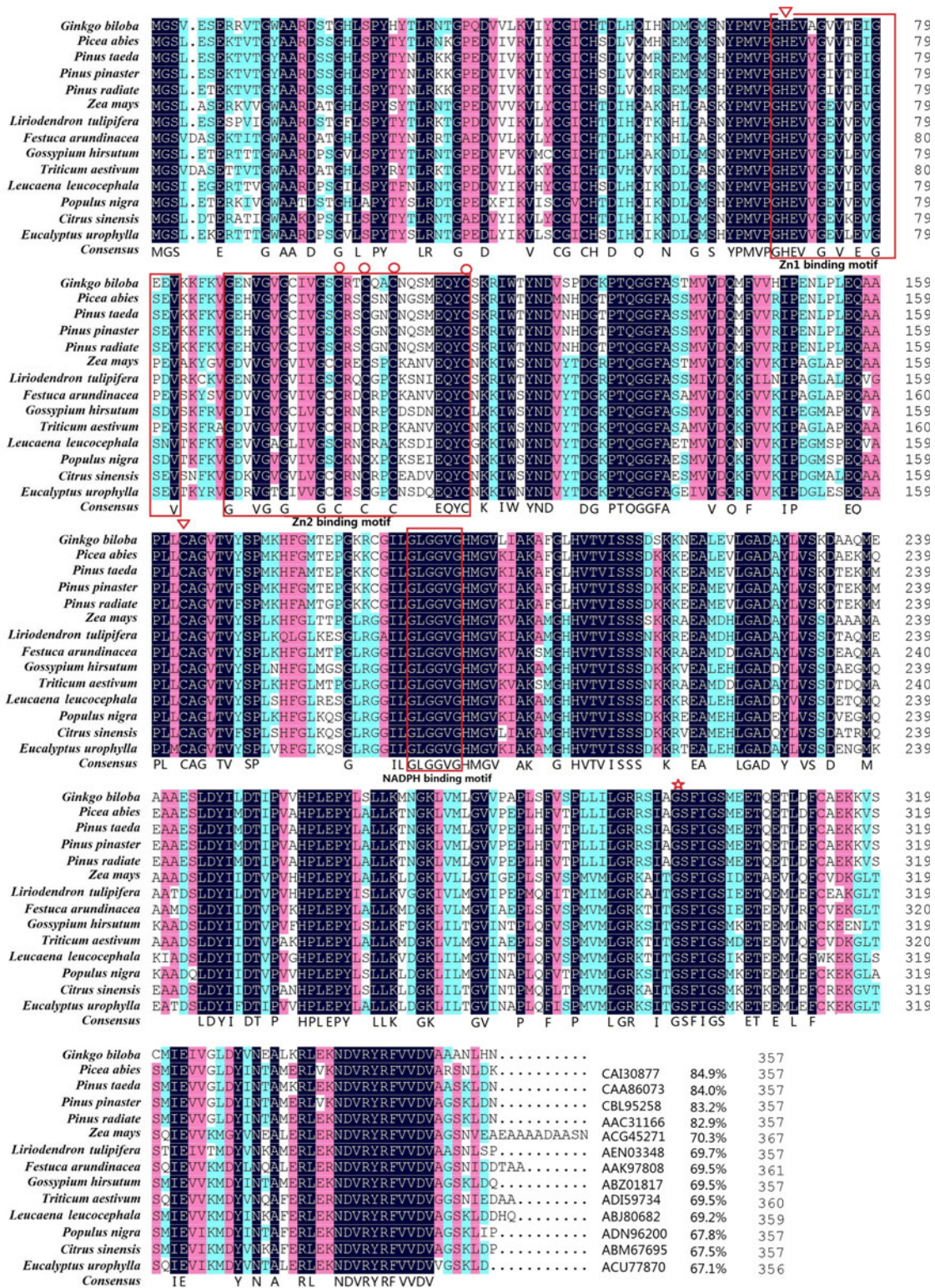
To examine if the *GbCAD1* gene belongs to a multigene family, aliquots of 30 μ g genomic DNA were digested

with *EcoR* I, *Bam* H I, *Kpn* I and *Hind* III, which did not cut within the coding region. These fragments were then hybridized with the coding sequence of GbCAD1 under high stringency conditions. In total, 4–6 specific hybridization bands ranging from 0.5 to 5 kb were recovered in each of the restriction enzyme-digested lanes (Fig. 4), this indicated that *GbCAD1* belonged to a multigene family.

Properties of the recombinant GbCAD1 protein

In order to express *GbCAD1* in *E.coli*, we cloned the coding sequence of *GbCAD1* into pET-28a, an expression vector with the T7 promoter and a His-tag, yielding pET28a-GbCAD1. Upon induction by IPTG, GbCAD1 was expressed as a major soluble protein product. The molecular weight of the expressed recombinant protein was estimated as a 35 kDa band with a His-tag: this size was in agreement with that predicted by bioinformatics methods (33.28 kDa). Western blotting of purified recombinant GbCAD1 protein confirmed its specific immune reactivity to anti-His antibodies (Fig. 5).

GbCAD1 extracted from cell homogenate was purified and cultured in the presence of NADPH or NADH and Coniferyl aldehyde at 25 °C for 1 h. Excessive NADPH was filtered off using microfilters. Absorption spectroscopy revealed that NADPH was bound as a coenzyme to GbCAD1, showing that the enzymatic activity of GbCAD1 is assisted by NADPH instead of NADH.



Because of the potential pH-dependence of plant CAD activities, the enzymatic activity of GbCAD1 was measured (Fig. 6a) at seven points between pH 5.0 and 9.0 (buffered by potassium phosphate) using coniferyl alcohol

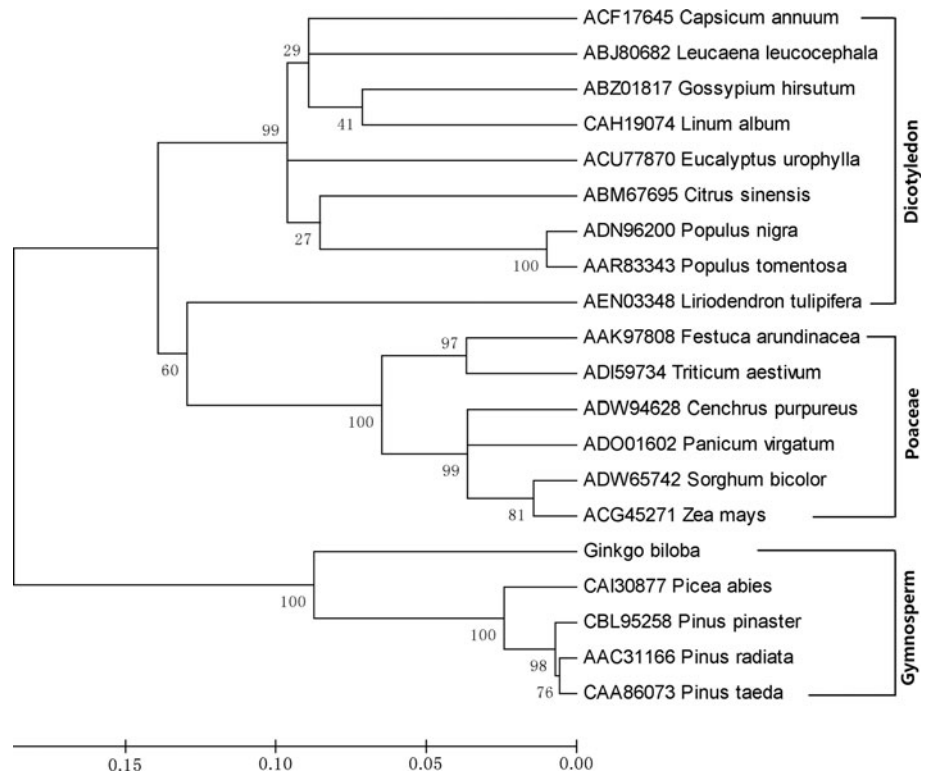
as the substrate and NADPH as the coenzyme. When the pH was raised from 5.0 to 7.0, the enzymatic activity increased substantially and reached a peak at pH 7.0. When the pH was raised from 7.0 to 8.0, the activity decreased.

◀ **Fig. 2** Alignment of amino acid sequences of GbCAD1 and other plant CADs. Conserved important regions identified previously are marked as follows: inverted filled *triangle* denotes catalytic zinc ion coordinating residue, *filled circle* denotes structural Zn ion coordinating residue, *star* denotes key residues for substrate specificity. Locations of the Zn1, Zn2 and NADPH binding domains are shown in *red boxes*. The alignment was performed with the DNAMAN and Vector NTI software programs. The sequence of CADs alignment is homology percentage. *Picea abies* CAI30877, *Pinus taeda* CAA86073, *Pinus pinaster* CBL95258, *Pinus radiata* AAC31166, *Zea mays* ACG45271, *Liriodendron tulipifera* AEN03348, *Festuca arundinacea* AAK97808, *Gossypium hirsutum* ABZ01817, *Triticum aestivum* ADI59734, *Leucaena leucocephala* ABJ80682, *Populus nigra* ADN96200, *Citrus sinensis* ABM67695, *Eucalyptus urophylla* ACU77870. (Color figure online)

At pH 8.0, the enzyme retained approximately 60 % of the peak activity recorded at pH 7.0. When the pH was further raised from 8.0 to 9.0, the enzymatic activity decreased dramatically to 7.6 % of the peak activity. These findings indicated that GbCAD1 was a pH-sensitive enzyme. The optimum environment for this enzyme (pH 7.0) was similar to that in the cytoplasm [15].

GbCAD1 was also sensitive to temperature (Fig. 6b). When the experimental temperature was raised from 5 to 25 °C, the enzymatic activity increased only moderately, with a particularly flat slope between 15 and 25 °C. When the temperature was further raised from 25 to 50 °C, the activity decreased sharply to approach zero. These results indicated 15–25 °C to be the optimum temperature for this enzyme.

Fig. 3 The phylogenetic tree analysis of CADs from several plants. The tree was constructed by the Neighbor-Joining method, MEGA4.1. The *bar* represent evolutionary distance, and the numbers at each node represented the bootstrap value, with 1,000 replicates



The order of substrate, GbCAD1, and NADPH addition also affected the catalytic activity (Fig. 7). When coniferyl aldehyde was premixed with NADPH before the mixture was allowed to interact with GbCAD1, the enzymatic reaction showed an S-shaped profile: a slow reaction from 0–6 min, a fast reaction from 6–8 min, and a subsequent slower reaction after 8 min (Fig. 7a). In comparison, when the substrate was premixed with GbCAD1 before interacting with NADPH, the reaction profile was a typical double-reciprocal curve: an initial fast reaction followed by a decreasing reaction rate (Fig. 7b). Moreover, when GbCAD1 was mixed first with NADPH before interacting with the substrate, the reaction rate was almost constant (zero-order reaction) (Fig. 7c). These results substantially differ from those measured from wheat TaCA1 [15].

GbCAD1 Expression and CAD activity in different tissue

In order to determine if *GbCAD1* gene was related to lignin synthesis or diseases and insect pests defense in *Ginkgo*, the expression pattern of *GbCAD1* was analysed by QRT-PCR. Here we found that *GbCAD1* was expressed in the stems, leaves, roots, sclerotesta, infection tissues and wounding tissues of *Ginkgo*. *GbCAD1* is constitutively expressed, but greatly varies in different tissues (Fig. 8). Of all analyzed tissues, *GbCAD1* was expressed most abundantly in the root, followed by damaged or fungus-infected

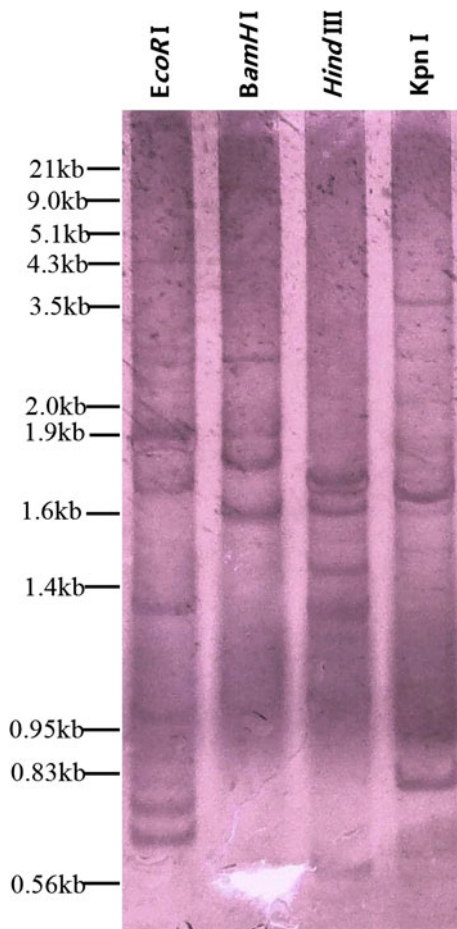


Fig. 4 Southern blot analysis of *GbCAD1* gene. Genomic DNA (25 µg/lane) was digested with *EcoR* I, *BamH* I *Kpn* I and *Hind* III respectively, followed by hybridization with *GbCAD1* coding region sequence as the probe

stem segments, consistent with the patterns of lignin concentration. In actively-growing one-year-old stem tissues, the lignin concentrations were relatively low, and the *GbCAD1* expression levels were high. In contrast to the expression pattern of *GbCCR1* (another lignin synthesis-related gene) [23], *GbCAD1* was weakly expressed in two- to four-year-old stem tissues and essentially non-expressed in leaf veins. Staining experiments revealed abundant lignin in leaf veins and two- to four-year-old stem tissues. *GbCAD1* expression was very low in the epidermis and endosperm, consistent with the presence of lignin in these tissues.

The data obtained are analyzed by linear regression. The results showed that lignin content has a linear correlation with CAD activity (X_1). The correlation coefficient is $R^2 = 0.713$, the correlation curve equation was as follows (Table 2): $Y = (9.632 \pm 0.929) + (15.885 \pm 0.979)X_1$. Similarly, the change in lignin content had some linear correlation with the expression level of *GbCAD1* mRNA (X_2), the correlation coefficient was $R^2 = 0.510$, the

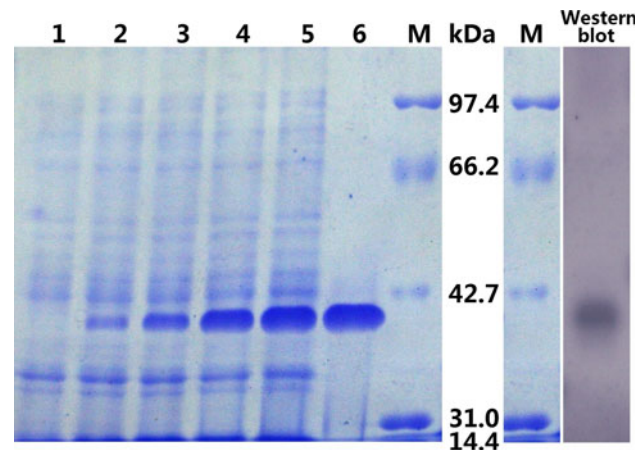


Fig. 5 SDS-PAGE gel and Western blot analysis of *GbCAD1* expressed in *E.coli* BL21 (DE3). After IPTG induction, *E.coli* BL21 cells containing pET28a-CAD1 were grown at 30 °C for 2 h. M, molecular marker; lane 1, protein of total cells without IPTG induction; lane 2, protein of total cells with IPTG induction for 40 min; lane 3, protein of total cells with IPTG induction for 60 min; lane 4, induction for 90 min; lane 5, induction for 120 min; lane 6, purified recombinant *GbCAD1* protein with Nickel-CL agarose affinity chromatography and used for enzyme activity assay; Lane M, protein MW marker; Lane western blot, western blotting of the purified recombinant *GbCAD1* protein with an anti-His-tag primary antibody probe

linear equation (Table 3) was $Y = (11.917 \pm 1.186) + (1.139 \pm 0.108)X_2$. So, the changes of CAD1 expression and the enzyme activity could effect the content of lignin in different tissue of Ginkgo.

UV-B, wounding and hormone regulate the transcription of *GbCAD1*

To examine the effects of hormones on the expression of *GbCAD1*, *gingko* leaves were treated by spraying with ABA, 5-ALA, SA, or ETH. Subsequently, the transcriptional level of this gene was studied by RT-PCR.

ABA spraying induced a sustained elevation of *GbCAD1* transcription, but the response was slower compared with other hormone treatments. The elevation was only moderate 4 h after treatment and became noticeable 8 h after treatment. The transcriptional level reached the peak value (360 % higher than the control level) 12 h after treatment and decreased thereafter. At 96 h after treatment, the transcriptional level remained 170 % higher than the control level (Fig. 9a).

Ginkgo leaves were treated with this concentration of ALA. The *GbCAD1* transcription level (Fig. 9b) increased slightly 2 h after treatment, decreased moderately between 8 and 12 h (reaching 70–80 % of the control level), and increased slowly thereafter. Compared with the hormone treatments, ALA spraying failed to significantly affect *GbCAD1* expression.

Fig. 6 Effects of pH(a) and temperature(b) on the recombinant GbCAD1 activity. Substrates were coniferyl aldehyde and NADPH, and a 200 mM phosphate potassium buffer was used. In **a**, the temperature was 25 °C, and in **b** the pH was 7.0. All reaction mixtures were incubated in water bath for 15 min and then activity was determined. Each value is the mean of three independent samples and vertical bars represent standard errors

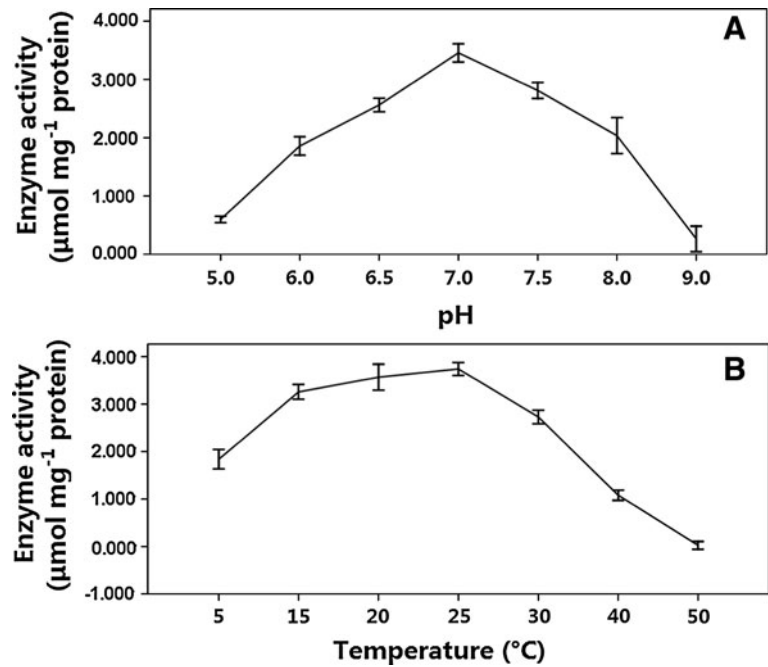


Fig. 7 Analysis of the reaction mechanism catalysed by GbCAD1. Each final reaction mixture contained 100 μM NADPH, 40 mM coniferyl aldehyde, and 1 μg purified recombinant GbCAD1 protein. For each reaction, a two-component mixture was prepared first and a third component was then added. The third (and last) component added was **a** GbCAD1 protein, **b** NADPH, and **c** coniferyl aldehyde. The X axis shows the time lapse after addition of the third component

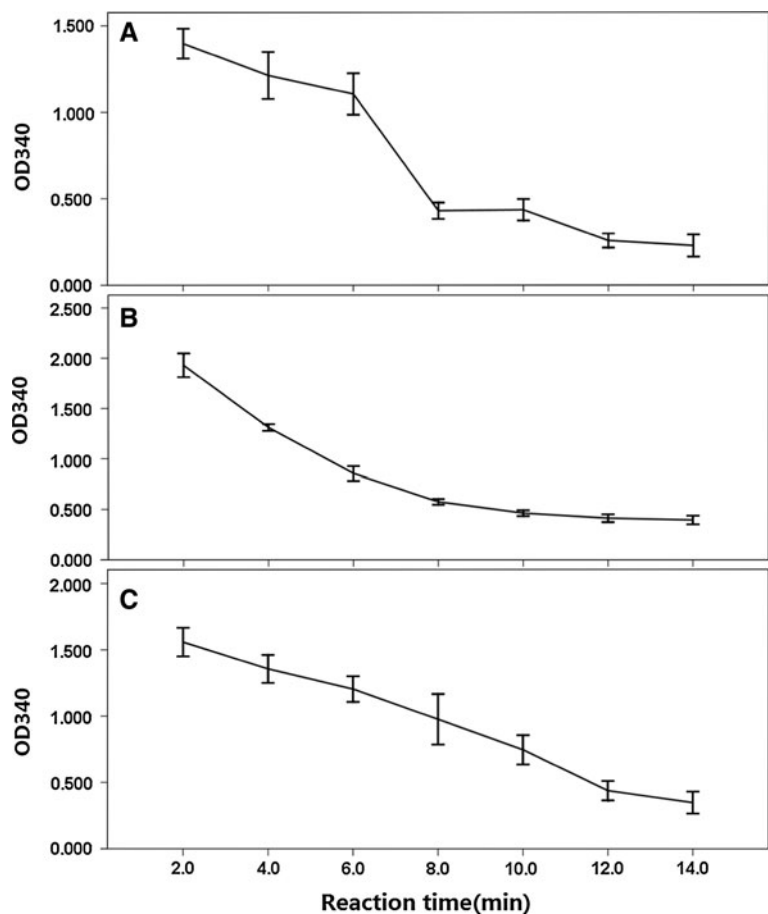
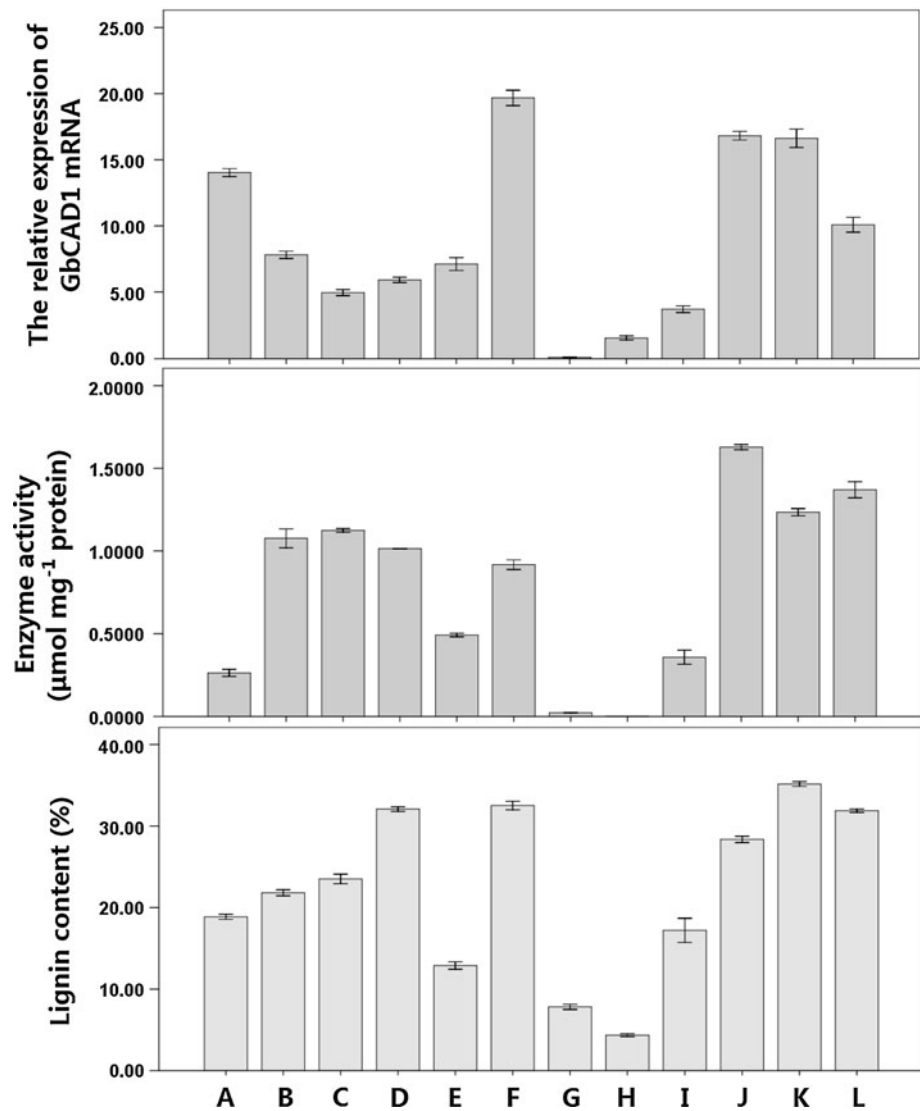
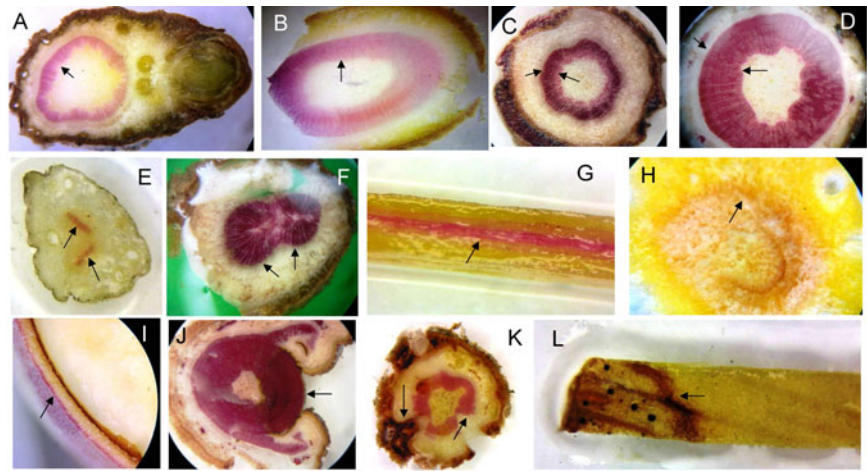


Fig. 8 The expression of *GbCAD1* and enzyme activity in different organs, sections staining with phloroglucinol-HCl. **a** Annual stem; **b** Biennial stem; **c** Triennial stem; **d** Four years stem; **e** Petiole. **f** Root. **g** Leaf veins of the longitudinal cutting. **h** Endosperm. **i** Sclerotesta. **j** Injured stems; **k** Diseases stems; **l** Fungal infections leaves. The arrow indicates histo-chemical lignin staining



Our previous studies have shown that ethephon treatment could effectively increase flavonoid accumulation in *Ginkgo* leaves by elevating the expressions of key genes in

the flavonoid pathway [11, 12, 24–28]. In the present study, *Ginkgo* leaves were treated with the same concentration of ethephon. The *GbCAD1* transcription level (Fig. 9C) was

Table 2 Relationship between lignin content and CAD activity in different tissue of Ginkgo

Model Summary						
Model	Unstandardized Coefficients ^a			Linear regression		
	Regression coefficient	Standard Error	Significance	Multiple correlation coefficient	The coefficient of determination	Adjusted coefficient of determination
Constant	9.632	0.929	0.000	0.844	0.713	0.710
CAD activity	15.885	0.979	0.000			

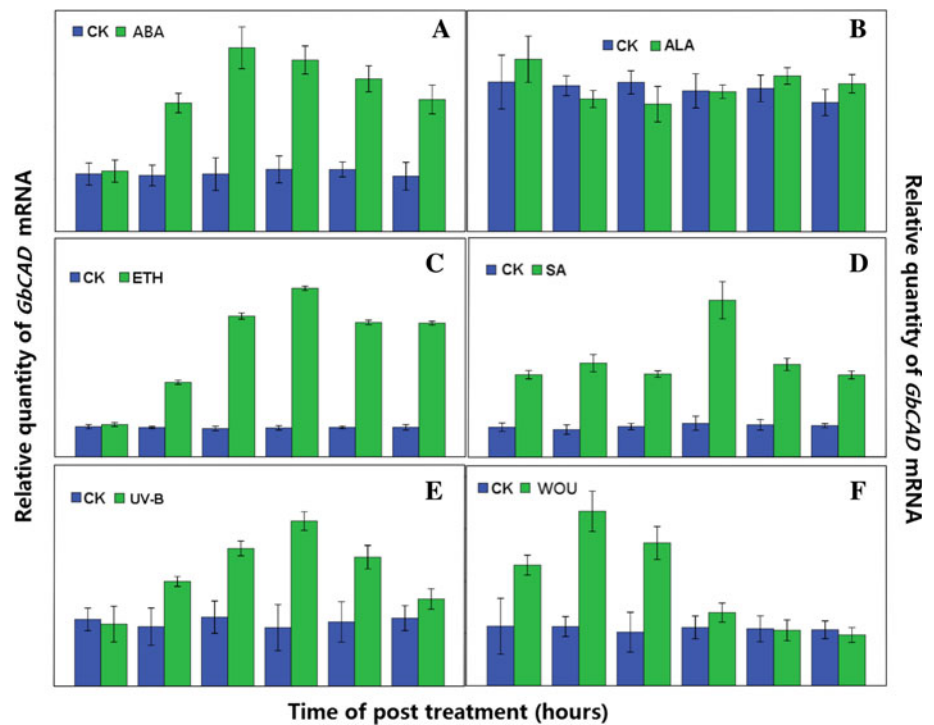
^a Dependent Variable: Lignin content (%)

Table 3 Relationship between *GbCAD1* expression level and lignin content in different tissue of Ginkgo

Model Summary						
Model	Unstandardized Coefficients ^a			Linear regression		
	Regression coefficient	Standard Error	Significance	Multiple correlation coefficient	The coefficient of determination	Adjusted coefficient of determination
Constant	11.917	1.186	0.000	0.714	0.510	0.505
mRNA	1.139	0.108	0.000			

^a Dependent Variable: Lignin content (%)

Fig. 9 Relative quantities of *GbCAD1* mRNA at various time points post-treatment with ABA (a), ALA (b), ETH (c), SA (d), UV-B (e), wounding (f). Each sample was individually assayed in triplicate. Values shown represent the mean reading from three treated plants and the error bars indicated the standard errors of the mean



unchanged 4 h after treatment, started to rapidly increase after 4 h (140 % higher than the control level), and reached a peak value 24 h after treatment (600 % higher than the

control level). Subsequently, it slightly decreased and remained at approximately 420 % higher than the control level.

Following, we treated Ginkgo leaves by spraying with a low concentration of SA. The *GbCAD1* transcription level (Fig. 9d) increased rapidly to 210 % higher than the control level 4 h after treatment and remained stable until 12 h after treatment. It further increased to 500 % higher than the control level 24 h after treatment and subsequently decreased. At 96 h after treatment, the *GbCAD1* transcriptional level was 180 % higher than the control level.

To understand *GbCAD1* expression in Ginkgo leaves under UV-B irradiation, ginkgo seedlings were treated with 260 nm UV-B at 600 $\mu\text{J}/\text{m}^2$ for different periods of time, and the transcriptional levels were analyzed by RT-PCR (Fig. 9E). During the first 24 h of UV-irradiation, *GbCAD1* transcription increased, and then decreased after 24 h. In addition, wound treatment (WOU) also stimulated *GbCAD1* expression (Fig. 9f). The transcription of *GbCAD1* increased rapidly and reached a peak value 8 h after WOU (220 % higher than the control level). Subsequently, it gradually decreased and reached levels similar to the control 48 h after WOU.

Discussion

Cinnamyl alcohol dehydrogenase is a major rate-limiting enzyme in lignin biosynthesis. It converts cinnam aldehydes into cinnamyl alcohol to impart diversity to formation and structure of lignins. More importantly, the CAD gene family is related to stress responses during plant growth and is also closely associated with fruit and seed maturation and aging.

Bioinformatic analysis

Our analyses revealed that *GbCAD1* has high homology to other plant CAD genes, with conserved amino acids throughout the gene sequence. The results suggested that *GbCAD1* is a member of the plant CAD protein family. In the present study, *GbCAD1* was found to be 84 % homologous to the *Pinus taeda* CAD gene, suggesting they have similar CAD functions. All functional CADs have a common structurally conserved Zn-binding domain [Zn1 binding labels, CHE(X)2G(X)5G(X)2V] required for enzymatic catalysis. Additionally, these CADs have another Zn-binding region [GD(X)9,10C(X)2C(X)2C(X)7C] and a glycine-rich NADPH-binding region [GXG(X)2G]. These three domains are hallmarks of the alcohol dehydrogenase superfamily [4]. In *GbCAD1*, the three domains are highly conserved, suggesting that *GbCAD1* belongs to the alcohol dehydrogenase superfamily. Both *SbCAD6* and *OsCAD6* have an evolutionarily conserved SKL sequence at the C-terminus, which may serve as a signal peptide sequence to locate these enzymes in plant cell peroxisomes [29]. Our amino

acid sequence comparison found no homology between the SKL sequence of *GbCAD1* and those of other species, suggesting that *GbCAD1* may not be located in peroxisomes.

Phylogenetic analysis found that the examined CADs formed three clusters: gymnosperms, dicotyledonous, and monocotyledonous. *GbCAD1* was found in the gymnosperms cluster and diverged earlier than CADs from other plants, consistent with the actual evolutionary history of Ginkgo. The CAD genes in higher plants are encoded by a small multigene family. The *Sorghum* genome contains more than 14 CAD-encoding genes [29], and the *G. hirsutum* genome contains more than eight [30]. Similarly, the Ginkgo genome contains multiple homologous CAD genes that belong to a multigene family. Currently, the CAD genes of gymnosperms are believed to exist as single copies. For example, *P. taeda* has only one CAD isozyme, indicating the presence of a single CAD locus [2]. The southern blot results suggested that, the Ginkgo genome may contain multiple CAD genes. These genes may have different functions or may be expressed in different tissues or at different developmental stages. However, the possibility of pseudogenes also exists.

Expression analysis in different tissue

In higher plants, multiple homologous CAD genes may participate in lignin biosynthesis in different tissues or during different developmental stages in one type of tissue. In graminaceous plants, CAD genes are expressed in vascular bundles and are related to the mechanical properties of the stem tissues [31]. *Arabidopsis thaliana* has nine functional CAD genes. Of these, only *AtCAD4* and *AtCAD5* play key roles in lignin biosynthesis. These two genes are expressed in vascular bundles as well as in non-lignified tissues [32, 33]. Tissue-specific expression analysis revealed that *AtCAD4* participated in lignin biosynthesis but was only weakly expressed in stems; In contrast, it was strongly expressed in flowers and roots. The *AtCAD5* gene was expressed in lignified roots and strongly expressed in pathogen-infected tissues [7], which was probably related to pathogen defenses involving lignin [32]. Studies have shown that, in addition to lignin-synthesizing CAD genes, CAD-like genes also participate in plant defense processes [34]. For example, in *Lolium perenne* L., *LpCAD2* is strongly expressed in the stem and is further stimulated by mechanical damage [5]. In Ginkgo, *GbCAD1* is strongly expressed in roots and in stems that are either infected or rapidly growing. Therefore, this gene is involved in both lignin synthesis and pathogen defense. These findings suggest that plant CAD genes may also participate in other unknown functions or have evolved individual functions.

In addition to lignin biosynthesis, stress response, and pathogen defense, plant *CAD* genes are also implicated in vegetable and fruit tissue development and are closely related to tissue ageing. For example, loquats undergo lignification during storage; during this process *CAD* transcripts accumulate, stimulated by ethylene [11]. In the present study, *GbCAD1* transcription started to increase 4 h after ethephon treatment and remained at high levels for a long period, similar to the elevation of *CAD* expression in loquats during maturation. Ethephon treatment during Ginkgo leaf maturation may affect flavonoid concentrations and key enzyme activities in the leaves. Ethephon exerts such functions by releasing ethylene to enhance PAL activity [26, 28, 35]. The biosynthesis of lignin and flavonoids in higher plants originates in the phenylalanine metabolic pathway. Therefore, the induction of *GbCAD1* expression by ethylene may be related to the increase in PAL activity and subsequent product accumulation.

Induced expression analysis

The *CAD* gene participates in lignification defense and is induced by a variety of signals, such as MEJA, mechanical wounding, fungal elicitors, stress conditions, and ethylene [32]. Few studies have investigated the relationship between UV exposure and lignin biosynthesis. However, ultraviolet irradiation has been found to induce the expression of genes (e.g., *PAL*, *CHI*) related to flavonoid metabolism [12, 27]. Bate et al. [36] have shown that PAL inhibition affected lignin biosynthesis. In our study, UV-B treatment induced an initial steady up-regulation of *GbCAD1* transcription followed by a gradual decrease. Based on these observations, the UV-induced *GbCAD1* expression may have resulted from cascade reactions following PAL response to UV irradiation. A previous study reported that the *sweet potato* (*Ipomoea batata*) *IbCAD1* expression was induced by biological and non-biological stresses and responded sensitively to mechanical wounding and chemical signals (SA, JA, ABA) [4]. Another study on the upstream structure of *Picea glauca* *PgCAD* promoter found a MYB transcription factor element (TTAGGTT). This element has been frequently observed in the promoters of genes related to plant stress responses. This finding suggested that *PgCAD* may be responsive to mechanical wounding [37]. Similarly, a MYB-binding cis-element was found in the region upstream of the eucalyptus (*Eucalyptus globulus*) *EgCAD2* promoter. The cis-element has also been frequently observed in genes specifically expressed in the xylem and is inducible by mechanical wounding [38]. In our study, *GbCAD1* expression was initially stimulated by mechanical wounding and subsequently returned to the control level, suggesting that this gene participated in the stress response to mechanical wounding and wound repair. These results may be related to

the upstream regulatory elements or may be attributed to reaction cascades following the mechanical wounding-induced increase in PAL transcripts.

Salicylic acid is a phenolic hormone that regulates the growth of plants and participates in plant defense against pathogens [39]. Moreover, similar to flavonoid and lignin biosynthesis, SA metabolism is also a part of the phenylalanine metabolic pathway [40]. We previously found that SA treatment could significantly increase the expression of Ginkgo flavonoid-related genes [11, 27]. The expression of flavonoid metabolism-related genes is upregulated in plants in response to pathogen invasion [27, 41, 42]. Salicylic acid is biosynthesized via a branch of the phenylalanine metabolic pathway and begins with the transformation of cinnamic acid [40]. Earlier studies have found the SA-response element in the sweet potato *IbCAD1* promoter to be related to SA-induced *CAD1* expression [4]. Similarly, defense-related cis-elements have also been identified in the white spruce *CAD* promoter [37]. Therefore, the SA-induced *GbCAD1* expression observed in the current study may be related to the upstream cis-element or indirectly linked to SA-induced defense responses.

Abscisic acid is a hormone in higher plants that is formed through the degradation of carotenoids. Generally, when plants are exposed to osmotic stresses or other stress conditions, ABA can regulate the expression of relevant genes to increase plant adaptability [43]. Earlier studies have found induced expression of *IbCAD1* in sweet potato after exposure to biological or non-biological stresses. The *IbCAD1* promoter has been confirmed to have response elements to signals such as ABA [4]. The promoter of *P. trichocarpa* *PoptrCAD4* also contains response elements to ABA, mechanical wounding, and light [34]. Studies have suggested that ABA-mediated plant responses to drought stress may be related to the regulation of relevant genes by the MYB transcription factor [44]. Therefore, the ABA-induced *GbCAD1* expression observed in our study may be linked to the upstream MYB response elements. This hypothesis, however, must be verified by future cloning and function analyses of the promoter.

Many studies have confirmed that low-concentration ALA treatments enhanced photosynthesis and, consequently, lignin production [45–47]. It also increases the accumulation of products from the phenylalanine metabolic pathway, such as flavonoids, total phenols, and anthocyanins [48]. Previous studies on lignin synthesis-related genes (*PoptrCAD4* and *PoptrCAD10*) in populus have identified cis-elements in the promoter regions related to MYB, Zn-finger binding regions, and photo- and auxin-response factors [34]. In the present study, the ALA treatment produced no *CAD* induction, suggesting the lack of photo- or auxin-response sequences in the upstream region of *GbCAD1*.

Ethephon accelerates fruit maturation and coloration. It also regulates the expression of flavonoid metabolism-related genes [11, 12, 27, 49] and is related to flavonoid accumulation in Ginkgo leaves [24, 28]. A previous study found that loquat *EjCAD1* expression increased during fruit maturation and ethylene treatment [11]. In the *Brassica chinensis*, *BcCAD1* and *BcCAD2* participated in plant ageing, and were also inducible by ethylene [50]. In the present study, the induction of *GbCAD1* expression by ethylene may be responsible for the ethylene-induced Ginkgo leaf lignification and secondary metabolite accumulation during leaf maturation. Both flavonoids and lignin are metabolized along parallel branches of the phenylalanine metabolic pathway. These parallel branches interact and cooperate to ensure normal plant development. These results show that plant CAD genes respond to various biological and non-biological stresses, and many of them are inducible by mechanical damage and signals for systemic acquired resistance, suggesting their roles in lignin formation and stress response. However, different CAD genes may respond to different stress signals (i.e., stress specificity). A previous study reported that the sixteen CAD genes could be divided into four groups according to their functions [34, 51]. Group 1 participates in lignin biosynthesis and is strongly expressed in the xylem [51, 52]. Group 2 is strongly expressed in the leaves. Group 3 is expressed in the xylem and preferentially expressed in the leaves [53]. Group 4 is related to stress response, mechanical wounding, fungal elicitation, pathogen defense, and ethylene induction [51]. Based on that study, *GbCAD1* is similar to Group 4 with respect to tissue distribution and patterns of induced expression.

In light of these results, we suggest that *GbCAD1* may also be involved in lignifications induced by both abiotic and abiotic stresses and in tissue-specific developmental lignification. Further analysis of the *GbCAD1* gene and its promoter sequence will provide an insight into the signaling pathway involved in the regulation of biotic and abiotic stresses in lignin biosynthesis as well as into the role of CAD protein during cell division.

Acknowledgments This work was supported by Economic Forest Germplasm Improvement and Comprehensive Utilization of Resources of Hubei Key Laboratories (20011BLKF238 and 2011BH0030), the Natural Science Foundation of China (30971974), and University-industry Cooperation Fund of Hubei Educational Office (CXY2009B009).

References

- Rogers LA, Campbell MM (2004) The genetic control of lignin deposition during plant growth and development. *New Phytol* 164(1):17–30
- O'Malley DM, Porter S, Sederoff RR (1992) Purification, characterization, and cloning of cinnamyl alcohol dehydrogenase in loblolly pine (*Pinus taeda* L.). *Plant Physiol* 98(4):1364–1371
- Brill EM, Abrahams S, Hayes CM, Jenkins CLD, Watson JM (1999) Molecular characterisation and expression of a wound-inducible cDNA encoding a novel cinnamyl-alcohol dehydrogenase enzyme in lucerne (*Medicago sativa* L.). *Plant Mol Biol* 41(2):279–291
- Kim YH, Bae JM, Huh GH (2010) Transcriptional regulation of the cinnamyl alcohol dehydrogenase gene from sweetpotato in response to plant developmental stage and environmental stress. *Plant Cell Rep* 29(7):779–791
- Lynch D, Lidgett A, McInnes R, Huxley H, Jones E, Mahoney N, Spangenberg G (2002) Isolation and characterisation of three cinnamyl alcohol dehydrogenase homologue cDNAs from perennial ryegrass (*Lolium perenne* L.). *J Plant Physiol* 159(6):653–660
- Menden B, Kohlhoff M, Moerschbacher BM (2007) Wheat cells accumulate a syringyl-rich lignin during the hypersensitive resistance response. *Phytochemistry* 68(4):513–520
- Tronchet M, Balague C, Kroj T, Jouanin L, Roby D (2010) Cinnamyl alcohol dehydrogenases-C and D, key enzymes in lignin biosynthesis, play an essential role in disease resistance in Arabidopsis. *Mol Plant Pathol* 11(1):83–92
- Marroni F, Pinosio S, Zaina G, Fogolari F, Felice N, Cattonaro F, Morgante M (2011) Nucleotide diversity and linkage disequilibrium in *Populus nigra* cinnamyl alcohol dehydrogenase (*CAD4*) gene. *Tree Genet Genomes* 7(5):1011–1023
- Smith J, Luo Y (2004) Studies on molecular mechanisms of *Ginkgo biloba* extract. *Appl Microbiol Biot* 64(4):465–472
- van Beek TA (2002) Chemical analysis of *Ginkgo biloba* leaves and extracts. *J Chromatogr A* 967(1):21–55
- Shan LL, Li X, Wang P, Cai C, Zhang B, Sun CD, Zhang WS, Xu CJ, Ferguson I, Chen KS (2008) Characterization of cDNAs associated with lignification and their expression profiles in loquat fruit with different lignin accumulation. *Planta* 227(6):1243–1254
- Cheng H, Li L, Cheng S, Cao F, Wang Y, Yuan H (2011) Molecular cloning and function assay of a chalcone isomerase gene (*GbCHI*) from *Ginkgo biloba*. *Plant Cell Rep* 30(1):49–62
- Jansson S, Meyer-Gauen G, Cerff R, Martin W (1994) Nucleotide distribution in gymnosperm nuclear sequences suggests a model for GC-content change in land-plant nuclear genomes. *J Mol Evol* 39(1):34–46
- Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative CT method. *Nat Protoc* 3(6):1101–1108
- Ma QH (2010) Functional analysis of a cinnamyl alcohol dehydrogenase involved in lignin biosynthesis in wheat. *J Exp Bot* 61(10):2735–2744
- Kirk TK, Obst JR (1988) Lignin determination. *Method Enzymol* 161(5):87–101
- Lu X, Liu Y, An J, Hu H, Peng S (2010) Isolation of a cinnamoyl CoA reductase gene involved in formation of stone cells in pear (*Pyrus pyrifolia*). *Acta Physiol Plant* 10(7):1–7
- Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol* 24(8):1596–1599
- Arnold K, Bordoli L, Kopp J, Schwede T (2006) The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. *Bioinformatics* 22(2):195–201
- Youn B, Camacho R, Moinuddin SGA, Lee C, Davin LB, Lewis NG, Kang CH (2006) Crystal structures and catalytic mechanism of the Arabidopsis cinnamyl alcohol dehydrogenases AtCAD5 and AtCAD4. *Org Biomol Chem* 4(9):1687–1697
- Kozak M (1984) Compilation and analysis of sequences upstream from the translational start site in eukaryotic mRNAs. *Nucleic Acids Res* 12(2):857–872

22. Lütcke H, Chow K, Mickel F, Moss K, Kern H, Scheele G (1987) Selection of AUG initiation codons differs in plants and animals. *EMBO J* 6(1):43–49
23. Cheng H, Li L, Cheng S, Cao F, Xu F, Wang Y, Jiang D, Yuan H, Wu C (2012) Characterization of a cinnamoyl-CoA reductase gene in *Ginkgo biloba*: effects on lignification and environmental stresses. *Afr J Biotechnol* 11(26):6780–6794
24. Cheng S, Wang Y, Li J, Fei Y, Zhu G (2004) Study on the relationship between the endogenous hormones and flavonoids in *Ginkgo biloba* leaf. *Scientia Silvae Sinicae* 40(6):45–49 (in Chinese)
25. Cheng S, Wang Y, Liu W, Chen K (2005) Effects of plant growth regulators on phenylalanine ammonia-lyase activities in leaves of *Ginkgo biloba* in vitro. *J Plant Resour Environ* 14(1):20–22 (in Chinese)
26. Wang Y, Cheng S (2002) Studies on the effects of regulating measures on the flavonoids contents in *Ginkgo biloba* leaves. *Hubei Agric Sciences* 25(5):103–105
27. Xu F, Cai R, Cheng S, Du H, Wang Y (2008) Molecular cloning, characterization and expression of phenylalanine ammonia-lyase gene from *Ginkgo biloba*. *Afr J Biotechnol* 7(6):721–729
28. Cheng S, Wang Y, Fei Y, Zhu G (2004) Studies on the effects of different treatments on flavonoids contents in *Ginkgo biloba* leaves and their regulating mechanism. *J Fruit Sci* 21(2):116–119 (in Chinese)
29. Saballos A, Ejeta G, Sanchez E, Kang CH, Vermerris W (2009) A genome-wide analysis of the cinnamyl alcohol dehydrogenase family in sorghum [*Sorghum bicolor* (L.) Moench] identifies *SbCAD2* as the Brown midrib6 gene. *Genetics* 181(2):783–795
30. Fan L, Shi WJ, Hu WR, Hao XY, Wang DM, Yuan H, Yan HY (2009) Molecular and biochemical evidence for phenylpropanoid synthesis and presence of wall-linked phenolics in cotton fibers. *J Integr Plant Biol* 51(7):626–637
31. Li X, Yang Y, Yao J, Chen G, Zhang Q, Wu C (2009) FLEXIBLE CULM 1 encoding a cinnamyl-alcohol dehydrogenase controls culm mechanical strength in rice. *Plant Mol Biol* 69(6):685–697
32. Kim SJ, Kim KW, Cho MH, Franceschi VR, Davin LB, Lewis NG (2007) Expression of cinnamyl alcohol dehydrogenases and their putative homologues during *Arabidopsis thaliana* growth and development: lessons for database annotations. *Phytochemistry* 68(14):1957–1974
33. Sibout R, Eudes A, Pollet B, Goujon T, Mila I, Granier F, Séguin A, Lapierre C, Jouanin L (2003) Expression pattern of two paralogs encoding cinnamyl alcohol dehydrogenases in *Arabidopsis*. Isolation and characterization of the corresponding mutants. *Plant Physiol* 132(2):848–860
34. Barakat A, Bagniewska-Zadworna A, Choi A, Plakatt U, DiLoreto D, Yellanki P, Carlson J (2009) The cinnamyl alcohol dehydrogenase gene family in *Populus*: phylogeny, organization, and expression. *BMC Plant Biol* 9(1):26–41
35. El-Kereamy A, Chervin C, Roustan JP, Cheynier V, Souquet JM, Moutounet M, Raynal J, Ford C, Latché A, Pech JC (2003) Exogenous ethylene stimulates the long-term expression of genes related to anthocyanin biosynthesis in grape berries. *Physiol Plantarum* 119(2):175–182
36. Bate NJ, Orr J, Ni W, Meromi A, Nadler-Hassar T, Doerner PW, Dixon RA, Lamb CJ, Elkind Y (1994) Quantitative relationship between phenylalanine ammonia-lyase levels and phenylpropanoid accumulation in transgenic tobacco identifies a rate-determining step in natural product synthesis. *PNAS USA* 91(16):7608–7612
37. Bedon F, Levasseur C, Grima-Pettenati J, Séguin A, MacKay J (2009) Sequence analysis and functional characterization of the promoter of the *Picea glauca* cinnamyl alcohol dehydrogenase gene in transgenic white spruce plants. *Plant Cell Rep* 28(5):787–800
38. Rahantamalala A, Rech P, Martínez Y, Chaubet-Gigot N, Grima-Pettenati J, Pacquit V (2010) Coordinated transcriptional regulation of two key genes in the lignin branch pathway-CAD and CCR-is mediated through MYB-binding sites. *BMC Plant Biol* 10(1):130
39. Hayat S, Ali B, Ahmad A (2007) Salicylic acid: biosynthesis, metabolism and physiological role in plants. *Salicylic acid: a plant hormone*. Springer. doi:10.1007/1-4020-5184-1
40. Ferrer JL, Austin M, Stewart C Jr, Noel J (2008) Structure and function of enzymes involved in the biosynthesis of phenylpropanoids. *Plant Physiol Biochem* 46(3):356–370
41. Fofana B, McNally DJ, Labbé C, Boulanger R, Benhamou N, Séguin A, Bélanger RR (2002) Milsana-induced resistance in powdery mildew-infected cucumber plants correlates with the induction of chalcone synthase and chalcone isomerase. *Physiol Mol Plant P* 61(2):121–132
42. Meer I, Stuitje A, Mol J, Verma D (1993) Control of plant gene expression. In: Verma D (ed) *Regulation of general phenylpropanoid and flavonoid gene expression*. CABI, Oxford, pp 125–155
43. Skriver K, Mundy J (1990) Gene expression in response to abscisic acid and osmotic stress. *Plant Cell* 2(6):503–512
44. Fujita Y, Fujita M, Shinozaki K, Yamaguchi-Shinozaki K (2011) ABA-mediated transcriptional regulation in response to osmotic stress in plants. *J Plant Res* 124(4):1–17
45. Nishihara E, Takahashi K, Nakata N, Tanaka K, Watanabe K (2001) Effect of 5-aminolevulinic acid (ALA) on photosynthetic rate, hydrogen peroxide content, antioxidant level and active oxygen-scavenging enzymes in spinach (*Spinacia oleracea* L.). *J Jpn Soc Hortic Sci* 70(3):346–352
46. Nishihara E, Kondo K, Parvez MM, Takahashi K, Watanabe K, Tanaka K (2003) Role of 5-aminolevulinic acid (ALA) on active oxygen-scavenging system in NaCl-treated spinach (*Spinacia oleracea*). *J Plant Physiol* 160(9):1085–1091
47. Wang LJ, Jiang WB, Huang BJ (2004) Promotion of 5-aminolevulinic acid on photosynthesis of melon (*Cucumis melo*) seedlings under low light and chilling stress conditions. *Physiol Plantarum* 121(2):258–264
48. Xu F (2008) Cloning and expression of *GbPAL* and *GbANS* genes and effect of ALA on the content of flavonoids in *Ginkgo biloba*. Dissertation, Shandong Agricultural University, Taian
49. Xu F, Li L, Zhang W, Cheng H, Sun N, Cheng S, Wang Y (2012) Isolation, characterization, and function analysis of a flavonol synthase gene from *Ginkgo biloba*. *Mol Biol Rep* 39(3):2285–2296
50. Zhang L, Wang G, Chang J, Liu J, Cai J, Rao X, Zhong J, Xie J, Zhu S (2010) Effects of 1-MCP and ethylene on expression of three CAD genes and lignification in stems of harvested Tsai Tai (*Brassica chinensis*). *Food Chem* 123(1):32–40
51. Barakat A, Bagniewska-Zadworna A, Frost C, Carlson J (2010) Phylogeny and expression profiling of CAD and CAD-like genes in hybrid *Populus* (*P. deltoides* × *P. nigra*): evidence from herbivore damage for sub functionalization and functional divergence. *BMC Plant Biol* 10(1):100–110
52. Li L, Cheng XF, Leshkevich J, Umezawa T, Harding SA, Chiang VL (2001) The last step of syringyl monolignol biosynthesis in angiosperms is regulated by a novel gene encoding sinapyl alcohol dehydrogenase. *Plant Cell* 13(7):1567–1586
53. Goffner D, Van Doorselaere J, Yahiaoui N, Samaj J, Grima-Pettenati J, Boudet AM (1998) A novel aromatic alcohol dehydrogenase in higher plants: molecular cloning and expression. *Plant Mol Biol* 36(5):755–765