Plant Science 193–194 (2012) 85–95

Contents lists available at SciVerse ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

Biochemical characterization and identification of a cinnamyl alcohol dehydrogenase from *Artemisia annua*☆

Xing Li[a,1], Dongming Ma[a,b,1], Jianlin Chen[a,b,1], Gaobin Pu[c], Yunpeng Ji[a], Caiyan Lei[d], Zhigao Du[b], Benye Liu[b], Hechun Ye[b], Hong Wang[a,*]

[a] *Graduate University of Chinese Academy of Sciences, Beijing 100049, China*
[b] *Key Laboratory of Plant Molecular Physiology, Institute of Botany, The Chinese Academy of Sciences, 100093 Beijing, China*
[c] *Shandong Yingcai University, 250104 Jinan, China*
[d] *College of Plant Protection, Henan Agricultural University, 450002 Zhengzhou, China*

ARTICLE INFO

Article history:
Received 16 February 2012
Received in revised form 2 May 2012
Accepted 21 May 2012
Available online 28 May 2012

Keywords:
Artemisia annua L.
Artemisinic aldehyde
Artemisinin biosynthesis
Cinnamyl alcohol dehydrogenase (CAD)
Coniferaldehyde
Sinapaldehyde

ABSTRACT

It is well known in the literature that cinnamyl alcohol dehydrogenase (CAD) reduces hydroxycinnamyl aldehydes, such as coumaryl, coniferyl, and sinapyl aldehydes, to their corresponding alcohols in the presence of NADPH, and these alcohols act as the precursors of lignin biosynthesis. Here, we report the isolation of a cDNA encoding an $NADP^+$-dependent CAD, designated as *AaCAD*, from the cDNA library using glandular secretory trichomes of *Artemisia annua* as the source of mRNA. A phylogenetic analysis indicated that AaCAD was clustered with AtCAD4 and AtCAD5, which were involved in monolignol biosynthesis from *Arabidopsis*. Semi-quantitative RT-PCR showed that the *AaCAD* transcript was abundant mostly in leaf and root, followed by flower, and lowest in stem. Functional and enzymatic assays showed that the recombinant enzyme was able to reversibly reduce a variety of common CADs substrates, namely geranial, cinnamyl aldehyde, sinapyl aldehyde, coniferyl aldehyde, and a sesquiterpenoid artemisinic aldehyde, to geraniol, cinnamyl alcohol, sinapyl alcohol, coniferyl alcohol, and artemisinic alcohol respectively. Besides, considering that AaCAD was identified from the glandular secretory trichomes of *A. annua*, and that the recombinant enzyme exhibited reductase activity by using artemisinic aldehyde as substrate, some possible role of AaCAD in artemisinin biosynthesis is also discussed.

1. Introduction

The function of cinnamyl alcohol dehydrogenase (CAD, EC 1.1.1.195) in plants has been well characterized. It has been shown to catalyze the conversion of hydroxycinnamyl aldehydes to their corresponding alcohols [1], such as *p*-coumaryl, coniferyl, and sinapyl alcohols. These three alcohols are the precursors of three main lignin units: *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units respectively. The relative proportions of the hydroxycinnamyl alcohols mentioned above are influential factors in the structural and mechanical properties of lignin [2,3]. Lignin is the second major structural component of the plant cell wall in supporting and conducting tissues, and it is used to reinforce mechanical strength and create a chemical and microbial resistant barrier.

Since CAD catalyzes the second reductive step of the lignin committed branch to produce the monomeric precursors [2], its activity is directly involved in lignification, and it has been characterized in many plant species, including alfalfa [3], tobacco [4], loblolly pine [5], Norway spruce [6], *Eucalyptus gunnii* [7], poplar [8], *Eucalyptus globulus* Labill [9], strawberry [10], *Arabidopsis* [11], rice [12], and wheat [13]. The *CAD* genes are considered to be conserved during evolution, they share approximately 80% nucleotide sequence identity with all published angiosperm sequences, and 70% between angiosperms and gymnosperms [10]. However, there are still some differences between angiosperms and gymnosperms, for example, angiosperm CADs usually have high affinity for both coniferyl and sinapyl aldehydes, and have multiple isoforms, whereas gymnosperm CADs are encoded by a single gene and are more specific for the reduction of coniferyl aldehyde than sinapyl aldehyde [13]. Such differences might have given rise to the difference in lignin content and composition between gymnosperms and angiosperms.

Abbreviations: AaCAD, *Artemisia annua* cinnamyl alcohol dehydrogenase; ADS, amorpha-4,11-diene synthase; ALDH1, aldehyde dehydrogenase 1; CAD, cinnamyl alcohol dehydrogenase; CYP71AV1, cytochrome P450 monooxygenase; Dbr2, double bond reductase 2; GC, gas chromatography; GC–MS, gas chromatography–mass spectrometry; HPLC, high performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

☆ The nucleotide sequence of *AaCAD* has been submitted to GenBank under accession number EU417964.

* Corresponding author. Tel.: +86 10 88256585; fax: +86 10 88256080.
E-mail address: hwang@gucas.ac.cn (H. Wang).

[1] These authors contributed equally to this article.

0168-9452/$ – see front matter © 2012 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.plantsci.2012.05.011

Genome-wide analyses of many plants have revealed that *CAD* genes have several isoforms, for example, nine, twelve, and fourteen *CAD* homologues were identified in the genomes of *Arabidopsis* [14], rice [15], and sorghum [16] respectively. However, not all the members (for instance, only *AtCAD5*, *AtCAD4*, and *AtCAD1* in *Arabidopsis*) were responsible for monolignol biosynthesis [14,17,18]. Other isoforms are functionally under-explored. In addition, CAD activity was reported in apparently non-lignified tissues [5].

Artemisia annua L. is a traditional Chinese herb, of which artemisinin is synthesized and sequestered in the glandular secretory trichomes. Artemisinin is the basis for the effective treatment of malaria, and artemisinin-based combination therapies (ACTs) have been recommended by the World Health Organization (WHO) since 2001 to reduce the odds of drug resistance [19]. Low artemisinin content (0.1–0.8% DW) in the leaves and flowers of *A. annua* severely limits its commercialization and has triggered numerous efforts to improve artemisinin production. With the identification of the genes involved in artemisinin biosynthesis, the artemisinin biosynthetic pathway is gradually being understood. The first committed step of artemisinin biosynthesis in *A. annua* is the cyclization of farnesyl diphosphate catalyzed by amorpha-4,11-diene synthase (ADS) to form amorpha-4,11-diene [20,21], which is subsequently transformed into artemisinic alcohol, artemisinic aldehyde, and artemisinic acid by a cytochrome P450 monooxygenase (CYP71AV1) [22,23]. Artemisinic aldehyde is then reduced to dihydroartemisinic aldehyde by Dbr2 (a double bond reductase 2) [24], and oxidized to dihydroartemisinic acid by aldehyde dehydrogenase 1 (ALDH1) [25]. Finally, dihydroartemisinic acid is converted into artemisinin by non-enzymatic reactions. Red1, characterized by Rydén et al., is a broad substrate oxidoreductase with high affinity for dihydroartemisinic aldehyde, and is thought to have a negative effect on artemisinin biosynthesis [26]. However, Olofsson et al. have recently shown that Red1 does not appear to play any role in artemisinin biosynthesis [27].

Here, we describe the cloning and characterization of a cinnamyl alcohol dehydrogenase from *A. annua*. Besides reversibly reducing cinnamyl aldehyde, coniferyl aldehyde, sinapyl aldehyde and geranial to their corresponding alcohols as a typical *bona fide* CAD, the recombinant AaCAD also seems able to utilize artemisinic aldehyde as a substrate to produce artemisinic alcohol *in vitro*. The kinetic characteristics and the reaction aspect of AaCAD, as well as the tissue expression analysis of *AaCAD* are also reported.

2. Materials and methods

2.1. Plant materials

The high artemisinin-yielding strain 001 of *A. annua* from Sichuan Province, China, was used in this study. *A. annua* seedlings were micropropagated as described in Zhang et al. [28]. The rooted plantlets were transferred into pots and grown in a greenhouse with a 16 h/25 °C day and 8 h/20 °C night regimen.

2.2. Chemicals

Coniferaldehyde, coniferalcohol, citral, geraniol, cinnamyl aldehyde, cinnamyl alcohol, and sinapyl aldehyde were purchased from Sigma–Aldrich. All the solvents used for GC and HPLC analysis were from Fisher Scientific, and all the analytical reagents were purchased from Sinopharm Chemical Regent Co., Ltd.

Artemisinic aldehyde, dihydroartemisinic aldehyde, artemisinic alcohol, and dihydroartemisinic alcohol were donated by Dr. Harro J. Bouwmeester (Plant Research International, Wageningen, Netherlands), and the identity and purity were confirmed with GC–MS.

2.3. Isolation of glandular secretory trichomes and cDNA library construction

Leaves from a 4-month-old (preflowering) *A. annua* plant were used for the isolation of glandular trichomes. The trichomes were isolated as described [29]. The collected leaves were chilled in ice-cold water for 10 min, then abraded using a cell disrupter (Bead Beater, Biospec Products, USA). The extraction mixture contained 20–30 g of leaves, 80–100 g of glass beads (0.5 mm diameter), 20–30 g of XAD-4 resin, and 100 ml of isolation buffer [25 mM MOPSO (pH 6.6), 200 mM sorbitol, 10 mM sucrose, 5 mM thiourea, 2 mM dithiothreitol, 5 mM $MgCl_2$, 0.5 mM sodium phosphate, 0.6% (w/v) methylcellulose, and 1% (w/v) polyvinylpyrrolidone (M_r 40,000)]. Total RNA from the glandular secretory trichomes was isolated using the RNeasy Mini Kit (Qiagen, Germany). The cDNA library was constructed with the Creator SMART cDNA Library Construction Kit (Clontech, USA) following the protocols suggested by the manufacturer.

2.4. AaCAD cDNA isolation

Randomly selected clones were sequenced with an ABI 3700 DNA sequencer using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, USA). Sequences were assembled and edited using PHRED/PHRAP [23], with a minimatch score of 35, and all the contigs and singlets were imported into a gap4 database for viewing [22]. The non-redundant unigenes (contigs and singlets) were subjected to similarity searches against the GenBank non-redundant (nr) protein databases using the BLASTX algorithm with default parameters. The EST fragment which contains the consensus cinnamyl alcohol dehydrogenase sequence was identified and subjected to SMART-RACE-PCR (nested PCR) with the first-strand cDNA as the template using a primer binding to the pDNR-LIB vector arm (5′-AAG CAG TGG TAT CAA CGC AGA GT-3′) (forward) and two gene-specific primers (reverse). The gene-specific primers for AaCAD were GSP1 (5′-GAA CTG GCA CCG TGT CAA TAA TG-3′) and GSP2 (5′-CAT CAG CTC CGA GGA CAT CCA GT-3′).

2.5. Heterologous expression and purification of AaCAD

The open reading frame (ORF) of *AaCAD* was amplified with a specific forward and reverse primer, and then fused to the *Bam*HI-*Xho*I sites in the pET-30a (+) vector (Novagen, USA). After sequence confirmation, the pET-*AaCAD* plasmid was transformed into *Escherichia coli* strain BL21 (DE3) competent cells for protein expression. *E. coli* cells containing pET-*AaCAD* were cultured in LB medium supplied with 50 mg/L kanamycin at 37 °C. When the OD_{600} of the cultures reached 0.8, 1 mM IPTG was added and the cultures were incubated for another 4 h at 25 °C. The cells were harvested by centrifugation and resuspended in 10 ml 0.05 M sodium phosphate buffer (pH 7.5) containing 5 mM imidazole, then sonicated on ice for 5 min at 50% pulses using an Ultrasonic Crasher (Ningbo Scientz Biotechnology Co., Ltd., China). The homogenate was centrifuged for 10 min at 10,000 × *g* and 4 °C. The His-tagged protein was isolated through a pre-packed 5 ml Hi-trap Ni column (Pharmacia) following the manufacturer's protocol. The purified recombinant protein was stored at −80 °C until use. The efficiency of purification was monitored by SDS-PAGE. Protein concentration was determined by the Bradford method with bovine serum albumin as the standard.

2.6. Enzyme reaction and product confirmation

To identify the activity of AaCAD on cinnamaldehyde, coniferaldehyde, sinapaldehyde, citral, artemisinic aldehyde, dihydroartemisinic aldehyde, as well as on their corresponding alcohol