Phytochemistry 71 (2010) 1264–1269

Contents lists available at ScienceDirect

Phytochemistry

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

A glandular trichome-specific monoterpene alcohol dehydrogenase from *Artemisia annua*

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ARTICLE INFO

Article history:
Received 2 January 2010
Received in revised form 30 March 2010
Available online 4 June 2010

Keywords:
Artemisia annua
Asteraceae
Monoterpene
Trichome
Dehydrogenase

ABSTRACT

The major components of the isoprenoid-rich essential oil of *Artemisia annua* L. accumulate in the subcuticular sac of glandular secretory trichomes. As part of an effort to understand isoprenoid biosynthesis in *A. annua*, an expressed sequence tag (EST) collection was investigated for evidence of genes encoding trichome-specific enzymes. This analysis established that a gene denoted *Adh2*, encodes an alcohol dehydrogenase and shows a high expression level in glandular trichomes relative to other tissues. The gene product, ADH2, has up to 61% amino acid identity to members of the short chain alcohol dehydrogenase/reductase (SDR) superfamily, including *Forsythia* × *intermedia* secoisolariciresinol dehydrogenase (49.8% identity). Through *in vitro* biochemical analysis, ADH2 was found to show a strong preference for monoterpenoid secondary alcohols including carveol, borneol and artemisia alcohol. These results indicate a role for ADH2 in monoterpenoid ketone biosynthesis in *A. annua* glandular trichomes.

1. Introduction

Artemisia annua L. is an aromatic and medicinal plant that belongs to the Asteraceae family (Bertea et al., 2005). The major components of *A. annua* essential oil are mono- and sesquiterpenes (Ma et al., 2007), and they are thought to be biosynthesized within glandular trichomes (Duke and Paul, 1993; Olsson et al., 2009; Tellez et al., 1999). The sesquiterpenes in *A. annua*, in particular, the anti-malarial compound artemisinin and related compounds, have been studied extensively (Bertea et al., 2005; Covello et al., 2007; Ro et al., 2006; Teoh et al., 2006; Zhang et al., 2008). The proportion of the major essential oil components varies widely in different lines (or ecotypes) of *A. annua*. Camphor and germacrene D were determined to be the main components of the essential oil of *A. annua* in a Vietnamese biotype, while artemisia ketone, was the major constituent of the oil from a Chinese line (Woerdenbag et al., 1994). Artemisia ketone, is an irregular monoterpene that is apparently formed via artemisia alcohol (Fig. 1) in an unusual head-to-head condensation of IPP and DMAPP. Although the biosynthetic pathway for artemisia ketone was proposed almost four decades ago by Epstein and Poulter (1973), the genes for the enzymes involved in the pathway have never been isolated and characterized. In an effort to understand isoprenoid biosynthesis in the glandular trichomes of *A. annua*, an existing EST collection (Covello et al., 2007; Teoh et al., 2006) was investigated. The collection was developed from two related tissue sources – glandular secretory trichomes isolated from flower buds, and intact flower buds. Two unsubtracted cDNA libraries were prepared from these tissues and a "trichome-minus-flower bud" cDNA library was also prepared. ESTs were obtained from Sanger type DNA sequencing of randomly isolated cDNA clones (Covello et al., 2007; Teoh et al., 2006). This EST collection has proven to be an important resource in identifying genes encoding enzymes involved in trichome-dependent biosynthesis of natural products in *A. annua* (Covello et al., 2007; Covello, 2008; Teoh et al., 2009; Zhang et al., 2008). Indeed some of the largest contigs in the trichome-derived EST collection, i.e., the ones representing high expression, correspond to genes involved in isoprenoid biosynthesis (see Table 1). As part of an ongoing EST-based study of trichome-expressed genes in *A. annua*, we have investigated and report here on a cDNA encoding a monoterpene alcohol dehydrogenase which appears to be involved in the biosynthesis of monoterpenoid ketones.

Abbreviations: AAFB, *A. annua* flower bud cDNA library; AAGST, *A. annua* glandular trichome cDNA library; ADH2, *A. annua* alcohol dehydrogenase 2; DMAPP, dimethylallyl diphosphate; EST, expressed sequence tag; GSTSUB, *A. annua* glandular-trichome-minus-flower-bud cDNA library; IPP, isopentenyl diphosphate; MDR, medium chain dehydrogenase/reductase; SDR, short chain alcohol dehydrogenase/reductase.

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2. Results

2.1. Isolation of a cDNA encoding A. annua alcohol dehydrogenase 2

The *A. annua* EST collection originally described by Teoh et al. (2006) was recently re-analyzed, during which EST from three libraries were clustered together (see Table S1). The analysis quali-

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doi:10.1016/j.phytochem.2010.04.026

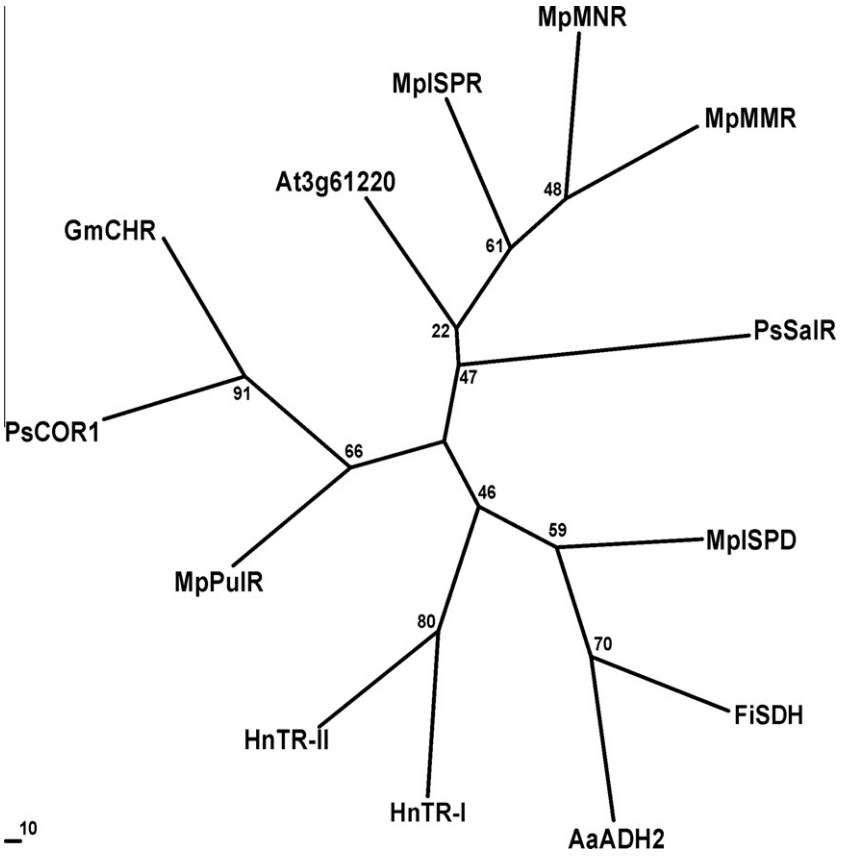

Fig. 1. Substrate specificity of ADH2.

Table 1
Kinetic parameters for ADH2.

Substrate	km (μM)	V_{max} (pkat/μg)	V_{max}/km (pkat/μg/μM)
(−)-Artemisia alcohol (**1b**)	86 ± 10[a]	2.39 ± 0.02[a]	0.03
(−)-*cis*-Carveol (**3a**)	27 ± 7[a]	9.4 ± 0.6[a]	0.34

[a] Values represent mean ± SE (n = 3) of replicate measurements of a single enzyme preparation.

fied 1625, 4085 and 3612 ESTs from the AAFB, AAGST and GSTSUB libraries, respectively, of which 894, 2508 and 2958 fell into contigs. The *A. annua* ESTs were submitted to Genbank as Accession Numbers GW328054–GW337375.

As part of the EST analysis, a putative alcohol dehydrogenase was found to be very highly represented in trichome-derived ESTs as a contig called CL1Contig2. The corresponding gene, designated *Adh2*, was associated with 12.4%, 1.9% and 0.12% of ESTs in the "trichome-minus-flower-bud" (GSTSUB), glandular trichome (AAGST) and flower bud (AAFB) collections. A full-length *Adh2* cDNA was isolated from the *A. annua* and the nucleotide sequence was submitted to GenBank as ID: GU253890. The *Adh2* gene has an open reading frame encoding a polypeptide of 265 amino acids (Fig. S1) with a molecular mass of 28,127. The predicted subcellular localization of Adh2 was investigated by amino acid sequence analysis using IPSORT (Bannai et al., 2002), PREDOTAR (Small et al., 2004) and TARGETP (Emanuelsson et al., 2007). IPSORT predicted a mitochondrial location, PREDOTAR a possible mitochondrial location and TARGETP did not predict a transit peptide.

Based on sequence similarities, ADH2 is a member of the short chain alcohol dehydrogenase/reductase superfamily (SDR) (Krozowski, 1994). A BLASTP search showed that ADH2 was most closely related to a hypothetical protein from *Vitis vinifera* (61% amino acid sequence identity to Genbank XP_002272206). ADH2 also shows amino acid sequence similarity to *Forsythia x intermedia* secoisolariciresinol dehydrogenase (FiSDH; Genbank AAK38665; 49.8% amino acid identity) (Xia et al., 2001), 3-β-hydroxysteroid dehydrogenase from *Digitalis lanata* (Genbank Q93Y47; 43.8% amino acid identity) (Finsterbusch et al., 1999), short chain alcohol dehydrogenase from *Pisum sativum* (Genbank AF097651, 39.6% amino acid identity) and (−)-isopiperitenol/(−)-carveol dehydrogenase (ISPD) from *Mentha x piperita* (Genbank AY641428; 37.5% amino acid identity) (Ringer et al., 2005). A phylogenetic tree was constructed to examine how ADH2 relates to other plant oxidoreductases (Ziegler et al., 2006) (Fig. 2). ADH2 lies within a branch that includes secoisolariciresinol dehydrogenase (FiSDH) from *Forsythia x intermedia* and (−)-isopiperitenol/(−)-carveol dehydrogenase (ISPD) from *Mentha x piperita*.. The sequence motif common to the active site of SDR's, $Y^{161}XXSK^{165}$ (ADH2 numbering) was found in ADH2. In common with other SDRs, ADH2 also has a conserved domain, $G^{19}GARGIG^{25}$, which is known to participate in the binding of the dinucleotide cofactor. An aspartate at position 43 is indicative of a preference for NAD over NADP (Ringer et al., 2005).

2.2. Functional analysis of the recombinant ADH2

ISPD participates in the glandular trichome-dependent biosynthesis of monoterpenoid ketones in *M. piperita* (Ringer et al., 2005). The sequence similarity between ISPD and ADH2 led us to investigate ADH2 as a monoterpene alcohol dehydrogenase. Previous chemical analyses of *A. annua* essential oils suggest compounds such as camphor, carvone and artemisia ketone as possible prod-