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

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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 tags (EST) collection was investigated for evidence of genes encoding trichome-specific enzymes. This analysis revealed a gene denoted *Adh2,* that encodes an alcohol dehydrogenase and shows a high expression level in glandular trichomes relative to other tissues. The gene product, ADH2, shows up to 61% amino acid identity to members of the short chain alcohol dehydrogenase/reductase (SDR) superfamily, including *Forsythia x 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.

Keywords: *Artemisia annua*; monoterpene; trichome; dehydrogenase

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.

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 et al., 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 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 (Epstein et al., 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.

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 qualified 1625, 4085 and 3612 ESTs from the AAFB, AAGST and GSTSUB librairies, 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 CL8Contig1. 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. 1). 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 products of an alcohol dehydrogenase (Bhakuni et al., 2001, Ma et al., 2007, Tellez et al., 1999). The corresponding alcohols and other substrates were tested with 6XHis tagged full length recombinant ADH2 purified from *E. coli* utilizing NAD as the cofactor (Fig. S2). All enzyme reactions were extracted with ethyl acetate and product formation was evaluated by GC/MS using octadecane as an internal standard. ADH2 showed dehydrogenase activity with monoterpene alcohol substrates including (-)-artemisia alcohol, (+)-borneol, (-)-borneol, (-)-*cis*-carveol, (-)-*trans*-carveol, and (-)-*trans*-pinocarveol (Figs. 2 and S3). On the other hand, no activity was found with other monoterpenes including citronellol, lavandulol, myrtenol, and (*S*)-(-)-perillyl alcohol, nor with sesquiterpenes including artemisinic alcohol, farnesol, khusinol, and santalol, nor with other alcohols including secoisolariciresinol, larixol, phytol, retinol, benzyl alcohol, cinnamyl alcohol, 2-cyclohexen-1-ol, and 3-methyl-2-buten-1-ol (data not shown). Thus, ADH2 appears to be a monoterpene alcohol dehydrogenase with highest activity on (-)-*cis*-carveol and (-)-artemisia alcohol and to a lesser extent on (+)-borneol, (-)-borneol, (-)-*trans*-carveol, and (-)-*trans*-pinocarveol.

When assayed with (-)-artemisia alcohol and NAD, recombinant ADH2 showed a pH optimum of 10.0 (Fig. S4), which is similar to the pH reported for ISPD and within the range expected for short chain alcohol dehydrogenases (Ringer et al., 2005). Using 1 mM NADP as the cofactor, oxidation of artemisia alcohol to artemisia ketone by ADH2 was not detected (data not shown). The kinetic parameters of the pure recombinant (His-tagged) ADH2 with artemisia alcohol and (-)-*cis*-carveol were measured using 1 mM NAD as the cofactor (Fig. S5). The kinetic parameters for ADH2 are shown in Table 1.

2.3. Tissue specific expression analysis of ADH2 in A. annua

The glandular trichomes of *A. annua* and *M. piperita* accumulate a variety of terpenoid compounds, particularly monoterpenes. In the case of *M. piperita*, ISPD was shown to be localized to the secretory cells of the glandular trichomes. The *in vitro* activity of the enzyme and its localization coincide with its proposed function in the biosynthesis of monoterpenes found in the essential oil of *M. piperita*. ADH2 was shown to participate in the oxidation of monoterpenes, so it was hypothesized that it should also be found in a similar location, the glandular trichomes of *A. annua*. To evaluate this possibility, the gene expression pattern of ADH2 was determined using quantitative RT-PCR. As expected from EST data, *Adh2* was highly expressed in the glandular trichomes of *A. annua* with lower expression in the flower buds and leaves, and no significant expression was detected in the roots (Fig. 3). The expression pattern matches the presence of ESTs corresponding to ADH2 from flower bud (AAFB), glandular trichome (AAGST), and "trichome-minus-bud" (GSTSUB) libraries (see 2.1). This expression pattern strongly

suggests a role in terpenoid biosynthesis for ADH2 and has been seen for other enzymes proposed to have functions in trichome-specific terpenoid biosynthesis in *A. annua* (Teoh et al., 2009, Zhang et al., 2008).

3. Discussion

Isolated gland secretory trichomes are a valuable resource for studying isoprenoid metabolism in *A. annua*. The generation of an EST collection from trichomes has facilitated the cloning of several key genes involved in artemisinin (sesquiterpenoid) biosynthesis in *Artemisia annua* (Teoh et al., 2006, Teoh et al., 2009, Zhang et al., 2008). In this paper, we report the use of the EST collection in the cloning and characterization of an alcohol dehydrogenase, ADH2. Based on the carbonyl compounds associated with trichomes, the properties of ADH2 as measured *in vitro*, and its highly preferential expression in glandular trichomes, it is possible to deduce the *in vivo* function of ADH2. Since most terpenoid alcohols are produced either directly from terpene synthases, or by hydroxylation of terpenes, ADH2 is not likely to be involved in the production of alcohols, but rather, carbonyl compounds. In terms of aldehydes and ketones, the most prominent compounds in *A. annua* essential oil include the monoterpenoid ketones artemisia ketone, camphor, pinocarvone (Tellez et al., 1999) in amounts that vary with chemotype. On the other hand, carvone is reported to be present, but typically in very low amounts. Given this, and the substrate specificity of ADH2 (Table 1 and 2), it would appear that ADH2 plays an important role in the production of artemisia ketone (from (-)-artemisia alcohol), (1*S*)-(-)-camphor (from (1*S*)-(-)-borneol) and pinocarvone (from trans-pinocarveol), and to a much lesser extent carvone (from carveol) in the glandular

trichomes of *A. annua*. Therefore, ADH2 can be considered a NAD-dependant monoterpenoid alcohol dehydrogenase (of the SDR superfamily) with a strong preference for secondary alcohols. The substrates for ADH2 are likely to be produced in *A. annua* from prenyl diphosphates by a putative artemisia alcohol synthase (Epstein et al., 1973, Umlauf et al., 2004), bornyl diphosphate synthase (Croteau et al., 1977, Whittington et al., 2002) and a hydrolase (to give borneol)(Croteau et al., 1979), and pinene synthase and pinene hydroxylase (to give pinocarveol)(Karp et al., 1992).

The phylogenetic analysis of Adh2-related enzymes (Fig. 1) gives some insight into their evolution. Clearly Adh2 is related to other dehydrogenases and reductases which act on monoterpenoids in a variety of plant families. As well, Adh2 shows close relationship to alcohol dehydrogenases whose substrate specificity extends to lignans and alkaloids. The observed pattern is consistent with a relatively early divergence from dehydrogenases/reductase of primary metabolism and recruitment in a variety of secondary metabolic pathways in different lineages.

ADH2 shares a number of features with *M. piperita* ISPD. ADH2 and ISPD are among the very few known dehydrogenases directly involved in monoterpenoid ketone biosynthesis. In terms of substrate specificity, both enzymes oxidize carveol isomers, although ADH2 shows a preference for (-)-*cis*-carveol and ISPD exclusively acts on the *trans* isomer. ISPD is reported to be localized to the mitochondrion (Turner et al., 2004). The sequence analysis for Adh2 is not definitive about its subcellular location and it

would help to test for a transit peptide by the appropriate fusion protein expression experiments.

A borneol dehydrogenase has been partially purified from tansy (*Tanacetum vulgare*) and shown to be distinct from a sabinol dehydrogenase involved in thujone biosynthesis (Dehal et al., 1987). Given the close taxonomic relationship between *T. vulgare* and *A. annua* (same tribe, Anthemideae, within the Asteraceae), it is tempting to speculate that ADH2 is homologous to the *T. vulgare* borneol dehydrogenase. However, unlike ADH2, the borneol dehydrogenase from tansy is reported to be functional in the presence of NADP. Moreover, the reported M_r (~70,000) of the enzyme from tansy doesn't match the predicted M_r for the possible monomeric, dimeric or tetrameric forms of ADH2. Thus, it would appear that a dehydrogenase distinct from ADH2 is responsible for camphor biosynthesis in tansy. It follows that there may also be a separate enzyme in *Artemisia* spp. that plays a major role in camphor biosynthesis.

Comparison of monoterpene metabolism in *A. annua* with other species raises some interesting questions about monoterpenoid dehydrogenases. Within the *Artemisia* genus, *A. herba-alba* is unusual in containing (+)-enantiomer of artemisia alcohol (Segal et al., 1980). Furthermore, the species contains artemisia ketone. Given the stereoselectivity of ADH2 for (-)-artemisia alcohol, it would seem that artemisia ketone is formed from an enzyme with substrate specificity distinct from ADH2 in *A. herba-alba*. ADH2 also shows similarity to carveol dehydrogenase, which produces (+)-carvone in caraway (*Carum carvi* L., Apiaceae)(Bouwmeester et al., 1998). The enzymes share similar pH

optima, a requirement for NAD, and a preference for (-)-*cis*-carveol over (-)-*trans*-carveol. The molecular cloning of additional monoterpene alcohol dehydrogenases should shed additional light on the evolution of these enzymes in plants.

In terms of cellular localization within glandular trichomes, the expression of genes for artemisinin biosynthesis was recently investigated at the cellular level (Olsson et al., 2009). Such genes were found to be expressed specifically in the apical cells of glandular trichomes. It would, of course, be interesting to investigate cell-specific expression of monoterpene-related genes, including ADH2.

In conclusion, the molecular cloning of ADH2 from *A. annua* provides further insight into the nature and evolution of the oxidoreductases involved in monoterpenoid biosynthesis. This information may be important in the use genetic manipulation for the suppression or engineering of monoterpene biosynthesis in various organisms.

4. Experimental

4.1. Chemicals

Artemisinic alcohol was prepared as described previously (Chang et al., 2000, Teoh et al., 2006). (-)-Carveol (mixture of *cis*- and *trans*-isomers) was obtained from Sigma Chemical (St. Louis) and the *cis* and *trans* isomers were separated by HPLC using a Gemini (5 micron) 25cm X 10 mm C18 column (Phenomenex) with a gradient of acetonitrile in water from 10% to 100% acetonitrile in 90 mL. Separate fractions containing one of the 2 isomers were isolated and *cis*-(-)-carveol and *trans*-(-)-carveol

were determined to be >99% pure by GC/MS. Artemisia alcohol was prepared by the reduction of artemisia ketone (Sigma). Artemisia ketone (50 mg), dissolved in 1 mL of methanol and 100 mg of sodium borohydride was added slowly at room temperature with stirring and the reaction was allowed to stir for an additional 15 minutes. Two mL of water was added to the reaction and the artemisia alcohol mixture was extracted with 2 X 5 mL dichloromethane and the solvent was then evaporated carefully under a nitrogen flow. The (+) and (-) isomers of artemisia alcohol were separated on an R,R-Whelk-01 25cm X 4.6 mm chiral HPLC column (Regis Technologies) using an isocratic mixture of 1% isopropyl alcohol in hexane. The 2 separated isomers were collected separately and were found to be >98% pure by GC/MS. The first eluting peak from the chiral HPLC column was determined to be the (-)-artemisia alcohol isomer and the second peak was (+)-artemisia alcohol using a Perkin Elmer 341 polarimeter (data not shown). Santalol, myrtenol, thujyl alcohol and citronellol were obtained from the Plant Biotechnology Institute chemical stocks. All other chemicals were from Sigma-Aldrich (Oakville, ON, Canada). Unless otherwise specified, all commercial enzymes were obtained from New England Biolabs (Cambridge, MA, USA).

4.2. Plant materials

A. annua seeds were obtained from Elixir Farm Botanicals (Brixey, MO, USA). Seeds were germinated and grown in soil in a controlled chamber with 16 h, 24 ºC light (150 μmol m^{-2} s^{-1}) / 8 h, 21 ºC dark cycle. Plants that reached the height of approximately 1.2 m (about 3 months) were transferred to a flowering chamber with 12 h light / 12 h dark. Flower buds that developed after 19-21 days in the flowering chamber were collected for trichome isolation(Teoh et al., 2006, Zhang et al., 2008). Roots, leaves, flower buds and

isolated trichomes from the same plant were harvested and frozen in – 80 ºC for later RNA isolations for qPCR analysis.

4.3 EST analysis

Initial EST analysis was performed as described in Teoh et al. (2006) using PHRED, LUCY, STACKPACK and BLAST. The *A. annua* EST collection was recently re-analyzed using PHRED to read DNA sequencer trace data, call bases and assign quality values (Ewing et al., 1998), LUCY for trimming low quality sequence (Chou et al., 2001), CROSSMATCH to identify and mask contaminating cloning, vector and bacterial sequences (http://www.phrap.org), VMATCH (http://www.vmatch.de) to identify repetitive elements by comparison of the sequences to TIGR plant repeat databases (http://www.tigr.org/tdb/e2k1/plant.repeats/), and TIGR Gene Indices sequence cleaning protocols (Quackenbush et al., 2001) implemented in the SeqClean tool (http://www.tigr.org/tdb/tgi/software) to identify and trim poly(A/T) tails. After trimming the low quality, vector and other contaminants, ESTs of at least 100 bp were clustered using TGICL software (Pertea et al., 2003). This clustering was performed by a modified version of NCBI's MEGABLAST, and the resulting clusters are subsequently assembled using CAP3 assembly program. For each contig and singleton a BLASTX search was performed against a filtered version of the UniProt plant database that excludes sequences with poor annotation, e.g. "unknown protein".

4.4. Isolation of full-length ADH2 cDNA and plasmid construction.

One of the largest contigs in the EST collection, CL8Contig1, showed sequence similarity to alcohol dehydrogenases. The corresponding *A. annua* gene was named *Adh2*. The complete open reading frame of *Adh2* was amplified by RT-PCR from the *Artemisia*

annua glandular trichome cDNA using the gene-specific primers 5'-CATATGGCTTCTTTAACTCCAAAAGC-3' and 5'-GGATCCTTACGGTTTCCATGAAAATAAACC-3' and Taq DNA polymerase (Invitrogen). The resulting PCR product was cloned into PCR 2.1-TOPO (Invitrogen) and sequenced. The DNA insert was transferred into the pET15b vector at the *NdeI* and *BamHI* sites to generate a bacterial expression plasmid pDP001. The *Adh2* ORF was arranged in-frame with the 6XHIS tag region corresponding to the N-terminus of the expression product.

4.5. Analysis of ADH2 expression in the different tissues of A. annua.

Total RNA was isolated from roots, leaves, flower buds, and trichomes using Trizol reagent (Invitrogen). First strand cDNA synthesis was performed using Superscript II reverse transcriptase (Invitrogen) utilizing 2 µg of total RNA as the template. The oligonucleotides 5'- AATGCAGGTACTGTCGATGAGCC -3'and 5'- GCA TGAGATGCAACCCCTCC-3' were used to amplify a 200-bp fragment of the ADH2 transcript. The oligonucleotides 5'- CAGCACCAATGGTGATGACC TG -3' and 5'- CAGCAGAGCGGGAAATTGTG-3' were used to amplify a 150-bp fragment of *A. annua actin1* cDNA. Quantitative RT-PCR was performed using an Applied Biosystems step one real-time PCR system with a Power SYBR^{R} Green PCR Master Mix (Applied Biosystems, Forster city, USA). The thermal cycling conditions were as follows: 50 ºC for 2 min, 95 ºC for 4 min, followed by 40 cycles of 95 ºC for 30 s, 60 ºC for 1 min, and 72 ºC for 30 s.

4.6. Preparation of recombinant ADH2 protein

The plasmid pDP001 was transformed into competent BL21 (DE3) pLysS *E. coli* cells (Novagen). Fifty mL liquid LB medium containing 100 mg/l ampicillin was inoculated with 0.5 mL of an overnight culture and grown at 37 ºC for about 3 h (to OD_{600} of 0.5-0.7). The temperature was changed to 30 ºC and expression of ADH2 was induced with 1 mM IPTG. Cells were allowed to grow for 12 h and harvested by centrifugation at 10000 g for 10 minutes at 4 ºC. The cell pellets were resuspended in 3 mL of lysis buffer (20 mM sodium phosphate, 500 mM NaCl, 50 mM imidazole, 3 mM DTT, 10% glycerol, pH 7.4) and lysed with a French Press. The resulting suspension was centrifuged at 10,000 g at 4 ºC for 15 minutes and the supernatant was loaded onto a HIS-trap FF column (Amersham Bioscience, NJ) equilibrated with binding buffer (20 mM sodium phosphate, 500 mM NaCl, 50 mM imidazole, pH 7.4). The column was washed with 5 column volumes of binding buffer at the rate of 1 mL/min and the recombinant ADH2 was eluted with elution buffer (20 mM sodium phosphate, 500 mM NaCl, 400 mM imidazole, pH 7.4) at the rate of 0.1 mL/min. Fractions were collected at 10 min intervals. The purity of the recombinant ADH2 from each fraction was estimated by SDS-PAGE gel stained with Rapid Stain (Biosciences, St. Louis, MO) (Fig. S2). Fractions 3-7 were pooled and loaded onto a PD-10 desalting column (GE Healthcare, USA) following manufacturer's instructions and the recombinant ADH2 was eluted using 100 mM CHES buffer (pH 9.5) with 10% glycerol. The eluted ADH2 protein was concentrated in centrifugal filter devices (Amicon Ultra-15, Millipore, MA) and the purified protein concentration was determined by Bradford assay (Bio-Rad).

4.7. In vitro enzyme assay

Unless otherwise stated, ADH2 enzyme assays included 100 mM CHES buffer (pH 9.5), 0.5 mM substrate, 1 mM NAD, 5 μg of octadecane as internal standard, and 3 μg of

recombinant ADH2 in a total volume of 300 μL. Negative controls were carried out in the absence of NAD. Reactions were allowed to proceed for 30 minutes at 30 ºC with shaking (600 rpm), and immediately stopped by the extraction with 150 μL of ethyl acetate prior to GC-MS analysis.

4.8. Characterization of the purified recombinant ADH2

The linear range of the ADH2 assay with respect to time was tested by the reactions in 100 μL assay containing 100 mM CHES buffer (pH 9.5), 0.5 mM artemisia alcohol, 1 mM NAD, 5 μg of octadecane as internal standard, and 2 μg of purified recombinant ADH2. The pH optimum of the purified ADH2 was determined to be 10.0 based on a series of 10 min assays containing 0.5 mM artemisia alcohol, 2 μg of purified recombinant ADH2, 5 μg of octadecane as the internal standard, and 100 mM buffer (BICINE, CHES, or CAPS) with the pH range from 8.0-11 in 0.5 intervals (Fig. S4). Substrate specificity was determined in 10 min reactions with 0.5 mM of the test substrates, 1 mM NAD, and 2 μg of purified recombinant ADH2 in 100 mM CHES buffer (pH 9.5). Kinetic parameters were determined by varying the concentrations of substrates (3.9-500 μM) in an assay using 1.0 μg ADH2, 1 mM NAD, and an incubation time of 10 min in 100 mM CHES buffer (pH 9.5). Octadecane was used as an internal standard to quantify the products from the reactions using response factors determined by using standards for each of the enzyme products. Kinetic constants were determined by fitting the data to the Michaelis-Menten equation using non-linear regression and GraphPad software (GraphPad Software Inc., San Diego, CA) and the results presented represent the means of three independent experiments

4.9. GC-MS analysis

The ethyl acetate extracts from ADH2 reactions were directly analyzed by GC-MS using an Agilent 6890N GC connected to an Agilent 5973N mass selective detector equipped with a DB-5 column (30m x 0.25mm i.d., J&W Scientific) split 25:1 using an initial temperature of 70 ºC except for extracts containing artemisia alcohol, (-)-carveol, benzyl alcohol, 3-methyl-2-buten-1-ol, 2-cyclohexen-1-ol, and 1-octen-3-ol, for which 40 ºC was used, and farnesol, artemisinic alcohol, and phytol, for which 125 ºC was used, for 1 min. This was followed by an increase to 240°C at 5ºC/min. The samples were analyzed in electron impact mode under standard conditions (70eV).

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Table 1. Kinetic parameters for ADH2.

Substrate	Km (μM)	Vmax (pkat/μg)	Vmax /Km (pkat/μg/μM)
(-)-artemisia alcohol	86 ± 10[a]	2.39 ± 0.02[a]	0.03
(-)-*cis*-carveol	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.

Figure legends

Fig. 1. Phylogenetic tree of selected oxidoreductase enzymes involved in plant secondary metabolism. The tree was constructed using ClustalW and Phylip available at the Mobyle Web Portal and visualized with TreeView. One hundred bootstrap iterations were performed. The sequences and associated Genbank accession numbers are AaADH2, *A. annua* alcohol dehydrogenase 2 (this work); At3g61220 *Arabidopsis thaliana* alcohol dehydrogenase (some (-)-menthone:(+)-neomenthol reductase activity), NM115986; PsSalR, *Papaver somniferum* salutaridine reductase, DQ31621; MpISPR, *M. piperita* (-)-isopiperitenone reductase, AY300162; MpMNR, *M. piperita* (-)-menthone/(+)-neomenthol reductase, AAQ55959; MpMMR, *M. piperita* (-)-menthone:(-)-menthol reductase, AAQ55960; MpPulR mt, *M. piperita* (+)-pulegone reductase, AY300163; MpISPD, *M. piperita* (-)-isopiperitenol dehydrogenase, AY641428; HnTR-I, *Hyoscyamus niger* tropinone reductase I, D88156; HnTR-II, *Hyoscyamus niger* tropinone reductase II, L20485; FiSDH, *Forsythia x intermedia* secoisolariciresinol dehydrogenase, AAK38665; PsCOR1, *Papaver somniferum* codeinone reductase 1, AF108432; GmCHR, *Glycine max* chalcone reductase, X55730.

Fig. 2. Substrate specificity of ADH2.

Fig. 3. *Adh2* expression in different tissues measured by quantitative RT-PCR is indicated as the means and standard deviations of two independent experiments performed in four technical repeats.

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