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Molecular cloning and functional identification of a novel borneol dehydrogenase from *Artemisia annua* L.

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ABSTRACT

In order to investigate the physiological functions and biosynthesis regulation of borneol or comphore in *Artermisia annua* L., which is the major source of the anti-malaria drug artemisinin, the full length cDNA of the gene encoding a borneol dehydrogenase (*AaBDH*) was cloned from *A. annua* for the first time by using RACE (rapid amplification of cDNA ends). The completed open read frame of *AaBDH* was 1415 bp and it encoded a 885-amino acid protein with a predicted molecular mass of 31.04 kDa and a pI of 6.16. AaBDH showed 68–70% of amino acid identity to alcohol dehydrogenases from *Solanum lycopersicum*, *Ppulus trichocarpa*, *Morus notabili* and *Ricinus communis*. While it shared 51% and 58% of identity with artemisia alcohol dehydrogenase ADH2 from *A. annua* and borneol dedrogenase LiBDH from *Lavandula x intermedia*, respectively. The recombinant protein was obtained by heterogeneous expression of AaBDH in a strain of *Escherichia coli* BL 21 and purified by affinity chromatography. The function of AaBDH was characterized by using of *in vitro* enzymatic assays, and the results showed that AaBDH had the ability to specifically convert borneol into camphor in the presence of NAD^+ (nicotinamide adenine dinucleotide) or $NADP^+$ (nicotinamide adenine dinucleotide phosphate). The cloning of *AaBDH* laid a significant foundation for further investigation on physiological functions and biosynthesis regulation of plant monoterpenoids.

1. Introduction

Artemisia annua L. (*A. annua*) is a kind of medicinal herb widely cultivated in China for its artemisnin used as anti-malaria drug (Bertea et al., 2005). Beside artemisinin, the major bioactive components in *A. annua* is essential oils (Islamuddin et al., 2014; Rao et al., 2014), which are extensively used in foods (Cavanagh and Wilkinson, 2002), medicines (Zhao et al., 2013), cosmetics (Islamuddin et al., 2014). Though the composition of essential oils in *A. annua* varies in different cultivars, camphor and artemisia ketone are the main components of the essential oil of *A. annua* (Rao et al., 2014; Zhao et al., 2013). Camphor has been found to have many pharmacological functions, including anti-oxidation, radio-sensitizing, and removal of free radicals (Cavanagh and Wilkinson, 2002; Nadeem et al., 2013). However, the exact physiological function of camphor in planta is unclear since it is difficult to produce various ecotypes containing different camphor levels due to the lacking of knowledge on the molecular biosynthetic regulation of camphor (Croteau et al., 2005; Sarker et al., 2012). Based on precursor feeding experiments (Croteau et al., 1978), the biosynthetic pathway for camphor was previously proposed, which suggests that camphor is derived from geranyl diphosphate (GPP), a head-to-tail condensation production of isopentenyl diphosphate (IPP) and dimethylallyl diphosphated (DMAPP) (Croteau and Karp, 1976). The linear GPP is subsequently cyclized and hydrolyzed to form borneol catalyzed by bornel diphosphate synthase and borneol synthase, respectively (Whittington et al., 2002). Camphor is formed directly through the oxidation of borneol under the catalyzation of borneol dehydrogenase (Whittington et al., 2002). Although the biosynthetic pathway for camphor is cleared (Fig. 1), the molecular mechanism and gene regulation involved in the pathway have not be investigated (Croteau et al., 2005). Polichuk et al. reported the cloning of artemisia alcohol dehydrogenase gene *Adh2* that

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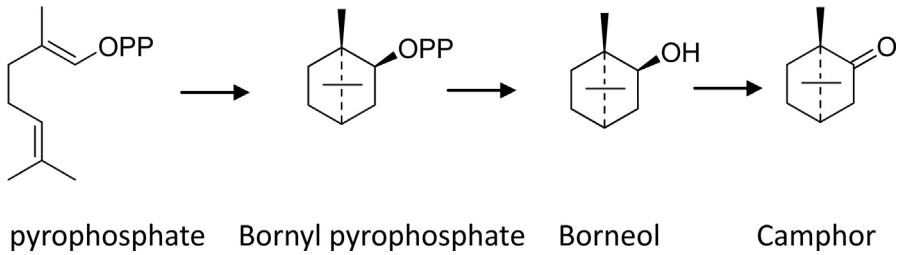

Fig. 1. Proposed camphor biosynthetic pathway.

showed catalytic activity for various monoterpenoid secondary alcohols including carveol and borneol *in vitro* (Polichuk et al., 2010). Recently, a specific borneol dehydrogenase gene *LiBDH* was cloned from the glandular trichome of *Lavandula x intermedia* (Sarker et al., 2012). In order to put more insight on camphor biosynthesis in the *A. annua*, a subtracted differentially expressed library was established by using suppression subtractive hybridization (SSH) between a wild-type and a borneol acuminated mutant of *A. annua*. ESTs were obtained by DNA sequencing of isolated cDNA clones and we have isolated a fragment of *AaBDH* candidate gene from the EST collection. In the present work we described the full-length cDNA cloning and function identification of a cDNA encoding a novel borneol dehydrogenase from *A. annua*.

2. Materials and methods

2.1. Plant material

A. annua plants were grown in greenhouse of Hunan Agricultural University (Changsha, Hunan, China) at 25 °C with 70% relative humidity using fluorescent lamps (with a light intensity of 2800 lx) for 16 h per day.

2.2. Chemicals

Artemisia alcohol and artemisinic alcohol were prepared according to previous method(Bertea et al., 2005; Polichuk et al., 2010). All organic solvents were purchased from Changsha Chemical Reagent Company (Changsha, Hunan, China). All other authentic compounds were from Sigma–Aldrich (Shanghai, China).

2.3. RNA isolation

Total RNA was isolated by using TRIZOL Reagent (Invitrogen) according to the manufacturer's protocol followed by digestion with DNase I at 37 °C for 10 min, and then purified with RNeasy Mini Kit (Qiagen). The quality of total RNA was determined by electrophoresis on 1% agarose gel containing ethidium bromide and the ratio of absorbance at 260/280 nm was measured using UV-spectrometer.

2.4. RACE PCR Cloning

The amplification of the 3′ and the 5′ ends of *AaBDH* was performed using SMART™ RACE cDNA Amplification Kit (Clontech). 5′ RACE PCR reactions were employed with primer UPM (5′-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3″) and gene specific primer BDHSR primer (5′-GGATGTCCGCAATCACGACTT-3′). 3′ RACE PCR reaction was performed by using gene specific primer BDHSF (5′-TTGCCATAATCACCGGCGGAG-3′) and primer UPM (5′-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3′). The applied touchdown PCR program was initially denatured at 94 °C for 30 s, annealing at 72 °C fro 3 min, 5 cycles; denatured at 94 °C for 30 s, annealing at 70 °C for 3 min 5 cycles; 94 °C for 30 s, annealing at 68 °C for 30 s, and extension at 72 °C for 3 min, 22 cycles. The PCR products were sub-cloned into the vector pGEM-T (Promega) and sequenced by using an automatic ABI 377 sequencer (PerkinElmer Applied Biosystems, Forster City, CA, USA) in Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. (Shanghai, China).

2.5. Protein expression and purification

The ORF of *AaBDH* gene was amplified by PCR using forward primer (5′-aaaaagcaggctATGAACGGTGTATATCCCCAC-3′, the lowercase letters represent the adapters) and reverse primer (5′-agaaagctgggtTTATGTTGCATGACTTACACCG-3′), respectively. The amplified PCR product was cloned into the pDONR221 vector (Invitrogen) and transferred to the pDEST17 vector (Invitrogen) by recombination. The resulted plasmid, pDEST17-AaBDH, was used to transform competent *Escherichia coli* BL21 (DE3) strain for recombinant protein expression. Single colony of *E .coli* BL21 (DE3), containing pDEST17-AaBDH expression plasmid, was inoculated in 3 mL of selective Luria-Bertani (LB) medium supplied with 50 mg/L of ampicillin and incubated at 37 °C under vigorous shaking (200 rpm). 500 μL of overnight culture of *E. coli* BL21 (DE3)-pDEST17-AaBDH was transferred to 50 mL of fresh LB medium containing 50 mg/L of ampicillin. The culture was continued to incubated at 37 °C with shaking until the OD600 reached the value of about 0.6. Zero point three micromoles of isopropyl β-D-l-thiogalactopyranoside (IPTG) was added to the culture and it was grown at 28 °C for an additional 8 h. The induced bacteria were harvested by centrifugation at 4000 rpm for 10 min and washed by using binding buffer (300 mM NaCl, 50 mM sodium phosphate buffer, 10 mM imidazole, pH 7.4). The bacteria pellet was suspended in 1 mL binding buffer and the cells were sonicated for 25 times for 10 s at 30 s intervals. The supernatant was collected by centrifugation at 13.500 g for 30 min at 4 °C. AaBDH was further purified by nickel-nitrilotriacetic acid (Ni^{2+}-NTA) agrpse affomotu chromatography. The purified protein was desalted through PD-10 column (GE Healthcare) and stored in 50 mM phosphate buffer (pH 7.5) containing 1 mM dithiothreitol (DTT) and 20% glycerol at −80 °C. The purified protein concentration was determined by the Bradford assay, using a standard curve of bovine serum albumin. The purity of the recombinant enzyme was assessed by standard SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) analysis.

2.6. In vitro enzyme assays

Enzyme assays were performed in 100 μL of 100 mM potassium phosphate buffer, containing 0.5 mM (+)-borneol, 1 mM NAD^+ or $NADP^+$, with addition of 1 μg of enzyme. After the incubation 33 °C for different time, the reaction product was extracted by ethyl acetate and was analyzed by GC (gas-chromatography). Boiled His-tagged protein was used as control. The optimum temperature was determined by performing reactions at 26, 28, 30, 33, 37,