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ORIGINAL PAPER

Expression and Localization of Amorpha-4,11-diene Synthase in *Artemisia annua* L.

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Abstract *Artemisia annua* L. is the only commercial source of the widely used antimalarial compound artemisinin. Biosynthesis of artemisinin has been proposed to take place in glandular trichomes. The first committed step in the conversion of farnesyl diphosphate (FDP) to artemisinin is conducted by the amorpha-4,11-diene synthase (ADS). To explore the organ-specific and developmental distributions of ADS, rabbit polyclonal antibodies were raised against recombinant ADS produced in *Escherichia coli* from the corresponding *A. annua* cDNA. Protein gel blot analysis of different *A. annua* organs showed that ADS was most abundant in young leaves and flower buds. Minor amounts of ADS were found in mature leaves. These findings were generally consistent with the analysis of the transcript level of the *ADS* gene. Immunolocalization of ADS showed strong positive staining in apical meristems, young leaves and glandular trichomes. No staining was observed in other cells of the leaf. The whole mount hybridization revealed that ADS was not expressed in all glandular trichomes of mature leaves. Specific staining of ADS could be detected in about 10–40 % of glandular trichomes.

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Introduction

Malaria is a serious endemic disease that threatens more than one third of the global population and kills approximately 1,238,000 people annually (Murray et al. 2012). Artemisinin—an endoperoxidized sesquiterpene originally extracted from the medicinal plant *Artemisia annua* L.—is an effective antimalarial drug, and *A. annua* is still the only commercial source of this drug. However, the low concentrations of artemisinin in *A. annua*, ranging from 0.1 % to 1 % of plant dry weight, makes artemisinin relatively expensive, especially for people in developing countries where most malaria occurs (Wallaart et al. 2000).

Within the plant, the highest concentrations of artemisinin are found in the leaves and inflorescences. The biosynthesis of artemisinin has been proposed to take place in the multicellular glandular secretory trichomes (Duke et al. 1993). Using glanded and glandless biotypes of *A. annua*, Duke et al (1994) showed that all extractable artemisinin is localized in the subcuticular space of trichomes. No artemisinin could be extracted from the glandless biotype, indicating that the sesquiterpenoid is produced and stored in glandular trichomes. The physiological and metabolic specialization of glandular secretory trichomes and their high expression of biosynthetic enzymes have made them valuable targets for molecular biological investigations for natural product biosynthesis. Expressed sequence tag data sets derived from trichome-specific cDNA libraries have been used to identify the enzymes responsible for the synthesis of terpenoids in mint (*Mentha piperita*) (Lange et al. 2000), phenylpropenes in basil

(*Ocimum basilicum*) (Gang et al. 2001), methylketones in tomato (*Solanum lycopersicum*) (Fridman et al. 2005), sesquiterpenes in *A. annua* (Bertea et al. 2005; Teoh et al. 2006; Zhang et al. 2008; Ma et al. 2009), terpenes (Wang et al. 2008) and terpenophenolics (Nagel et al. 2008) in hop (*Humulus lupulus*), and secondary metabolites in pink rockrose (*Cistus creticus*) (Falara et al. 2008).

The first committed step in the conversion of farnesyl diphosphate (FDP) to artemisinin is conducted by amorpha-4,11-diene synthase (ADS) (Mercke et al. 2000). ADS is one of many different terpene cyclases in terpenoid metabolism. The concentrations of artemisinin, artemisinic acid and dihydroartemisinic acid increased by around 100 %, 65 % and 59 %, respectively, in the transgenic plant line overexpressing *ADS* (Ma et al. 2007). In recent years, the expression characteristics and localization of *ADS* have attracted much attention. Using a 2.5-kb-long *ADS* promoter sequence, Kim et al. (2008) showed that *ADSpro*-driven GUS expression was organ-specific, being present mainly in transformed *Arabidopsis* trichomes (Kim et al. 2008). In *A. annua*, under the control of the *ADS* promoter, GUS was expressed specifically in glandular trichomes (Wang et al. 2011). Immunogold labeling (Olsson et al. 2009) and quantitative real time polymerase chain reaction (Olofsson et al. 2011) also showed that *ADS* was most abundant in *A. annua* glandular trichomes, which is in agreement with previous studies on the accumulation of artemisinin in glandular trichomes of this plant (Duke et al. 1994). However, artemisinin was also detected in hairy root cultures of *A. annua*, which has no glandular trichomes (Weathers et al. 1994). In addition, Lommen et al. (2006) showed that, although artemisinin can be detected during the entire life cycle of a leaf, no positive correlation could be expected between the number of glandular trichomes and the quantity of artemisinin. A poor correlation was also found between the glandular trichome index and the artemisinin content of leaves (Liersch et al. 1986).

Therefore, there are still a number of unanswered questions regarding the expression and function of the *ADS* gene and artemisinin biosynthesis. Is *ADS* expressed outside the glandular trichomes? Do all glandular trichomes participate directly in the production of artemisinin? The present paper reports the results of organ-specific expression of ADS in *A. annua* and the expression of *ADS* in trichomes during the life cycle of *A. annua* leaves.

Materials and Methods

Plant Material

A high-artemisinin-yielding strain 001 of *A. annua* (Han et al. 2005) was used in the present work. Cuttings were rooted in a culture chamber using fluorescent lamps (200 μmol photons m^{-2} s^{-1}) 16 h a day at 22°C. After 20 days, the plantlets were transplanted into individual pots (1 L, PVC) filled with growing medium (Voltilon Agriculture Technology, Beijing, China). Pots were kept in an experimental greenhouse at Beijing, WV (39°59′ 23″ N and 116°12′36″ E, 74 m altitude), daily photoperiod was 14 h (08:00–22:00) with controlled temperature (25°C /18°C, day/night) and supplemental light, and a relative humidity of 70 % all day. After 2 months the plants were transferred to controlled chambers at 70 % relative humidity and 22°C with a short-day regime of 8 h light (250 μmol photons m^{-2} s^{-1}) for 8 weeks.

mRNA Isolation and cDNA Preparation for Reverse Transcription-Polymerase Chain Reaction

Total RNA from *A. annua* was isolated using an RNApure Kit (BioTeke, Beijing, China) according to the manufacturer's instructions. The concentration of each RNA sample was measured using a DU 640 UV-VIS-spectrophotometer (Beckman Coulter, Fullerton, CA). The integrity of RNA samples was also assessed by agarose gel electrophoresis. RNA was treated with RNase-free DNase-I (Promega, Madison, WI) for 30 min and purified using an RNeasy MinElute Cleanup kit (Qiagen, Hilden, Germany). Total RNA (1 μg) from each sample was reverse-transcribed for first-strand cDNA synthesis using an M-MLV RT Kit (TianGen, Beijing, China) according to the manufacturer's instructions. Amplification was performed with initial denaturation at 94°C for 5 min, followed by 28 cycles (50 s at 94°C, 50 s at 56°C, and 2 min 20 s or 10 min for the last step at 72°C) using the first-strand cDNA as a template with the primers 5′-GTT TTG AAA ATC ATG TCA CT-3′ and 5′-ACA ACC CAT GAA ACA TAT TC-3′. The cDNA concentrations were then normalized using the conserved actin gene. The primers used for the actin gene amplification were: forward 5′- CAT ACG TCG GAG ACG AGG CA-3′ and backward 5′-ATT GTG GTG CCA CCA CTA AG-3′. The fragment thus obtained from the two primers was 760-bp long.

Generation of Anti-ADS Antibodies

The ORF of the *ADS* cDNA was amplified and cloned into the pET-30b (+) (Novagen, Darmstadt, Germany). The recombinant enzyme contained a hexahistidine tag at the C terminus. After sequencing the ORF on both strands, the recombinant plasmid was introduced into *Escherichia coli* BL21-Rosetta (DE3) (TransGen) and grown at 200 rpm and 37°C in 200 ml Luria-Bertani (LB) medium containing kanamycin (50 μg ml^{-1}) and chloramphenicol (34 μg ml^{-1}). At an absorbance at 600 nm (A_{600}) of 0.6–0.8, 1 mM isopropyl thio-β-D-galactoside (IPTG) was added and the incubation temperature was reduced to 30°C. After

incubation for 6 h, cells were harvested by centrifugation, resuspended in 3 ml 0.1 M potassium phosphate buffer (pH 7.5), and sonicated on ice for 10 min.

The homogenate was centrifuged at 10,000 *g* for 10 min at 4°C. The supernatant was passed through a column of Ni-NTA His-Bind™ Resin (Novagen) containing Ni^{2+} as an affinity ligand. After washing with 0.1 M potassium phosphate buffer (pH 7.5) containing 0.5 M NaCl and 40 mM imidazole, the recombinant ADS was eluted with 0.1 M potassium phosphate buffer (pH 7.5) containing 400 mM imidazole. The efficiency of purification was monitored by SDS-PAGE. Protein concentration was determined by the Bradford method with bovine serum albumin as the standard. Antibodies against the purified protein were raised in New Zealand white rabbits by the Institute of Genetics and Developmental Biology, Chinese Academy of Sciences (Beijing, China).

Protein Isolation from *A. annua* and Protein Gel Blot Analyses

Total proteins of *A. annua* leaves as well as dissected plant organs were isolated by phenol extraction and precipitated with methanol-ammonium acetate. Precipitated proteins were solubilized in an SDS-containing buffer and were then separated by SDS-PAGE (10 % polyacrylamide). After transfer of proteins onto PVDF membranes (PV4HY320F5, GE Osmonics, Minneapolis, MN), immunological detection of ADS was carried out with rabbit polyclonal monospecific antibodies raised against the recombinant protein. The antibodies were used at a dilution of 1:1,000 and visualized by a secondary antibody conjugated with alkaline phosphatase (CB200026, Cali-Bio, Coachella, CA) followed by a staining reaction according to the supplier's instructions. Expression of actin was detected as a loading control.

Whole Mount Immunolocalization

A. annua leaves were analyzed for whole-mount in situ ADS localization, following Sauer et al. (2006) with some modifications. Leaves of *A. annua* (30- to 40-days old) were fixed in 4 % paraformaldehyde in MTSB (50 mM PIPES, 5 mM EGTA, 5 mM $MgSO_4$ (pH 7.0), adjusted with KOH) for 1 h . Samples were washed with MTSB/0.1 % Triton (5×10 min) and with deionized water (3×10 min). Cell walls were digested with 2 % driselase (D9515, Sigma-Aldrich, St. Louis, MO) in MTSB for 30–45 min, and samples were washed with MTSB/0.1 % Triton (5× 10 min). Incubation with 10 % DMSO/3 % NP-40 in MTSB for 1 h followed. After another washing in MTSB/0.1 % Triton (5×10 min), leaves were pre-incubated in 3 % BSA/MTSB (1 h, 37°C) and incubated with the primary antibody in 3 % BSA/MTSB (12 h, 4°C). After extensive washing with MTSB/0.1 % Triton (8×10 min), the leaves were incubated with a secondary antibody conjugated with alkaline phosphatase (Boehringer Mannheim, Mannheim, Germany) in 3 % BSA/MTSB for another 3 h (37°C). Finally, the samples were washed with MTSB/0.1 % Triton (5× 10 min) and deionised water (3×10 min) followed by staining reaction according to the supplier's instructions. Chlorophyll was removed from leaves by immersing in 90 % ethanol. The samples were observed and photographed under a SZX12 anatomy microscope (Olympus, Japan) equipped with a DP70 digital camera system. An ocular grid was used to count the number of glandular trichomes in a 1 mm square under a stereomicroscope at 50 X. Twenty leaflets were examined in this manner for each treatment.

Immunohistochemical Localization of ADS Using Light Microscopy

Tissue was fixed in 4 % formaldehyde, 5 % acetic acid, and 50 % ethanol. Fixed tissue was dehydrated with ethanol, cleared with xylene, and embedded in paraffin (Paraplast Plus; Oxford Labware, St. Louis, MO). Embedded tissue was sliced into 6 μm sections and placed onto slides coated with poly-L-lysine (Sigma). The sections were then dewaxed in xylene and rehydrated by passing through graded alcohols and rinsing in water.

Blocking and antibody reactions were performed in 1 % BSA in phosphate-buffered saline (PBS). The sections were incubated for 5 min with PBS and 0.1 % saponin (Sigma) before incubation with PBS containing 0.1 % saponin, 1 % BSA and 2 % goat serum for 1 h at room temperature. Sections were incubated with rabbit anti-ACBP1 antibody (1:100 [v/v]) at 4°C overnight and with the secondary antibody biotinylated alkaline-phosphatase-conjugated goat anti-rabbit antibodies (1:1,000 [v/v]; BioRad) at room temperature for 2 h. Levamisole (1 mM; Sigma) was included in the alkaline phosphatase reaction to inhibit endogenous phosphatase activity; the reaction was carried out as instructed by the manufacturer (Bio-Rad, Richmond, CA).

Results and Discussion

Organ-Specific Expression of *ADS*

To investigate the organ-specific expression of *ADS* in *A. annua*, total RNA was isolated from root, stem, young leaf, old leaf, flower bud and full blooming flower and reverse transcription polymerase chain reaction (RT-PCR) was performed with *ADS*-specific primers. The results indicated that *ADS* expression was more abundant in flower bud and young leaf followed by mature leaf and blooming flower. A very low level of *ADS* expression was observed in stem whereas no expression were detected in root (Fig. 1). This

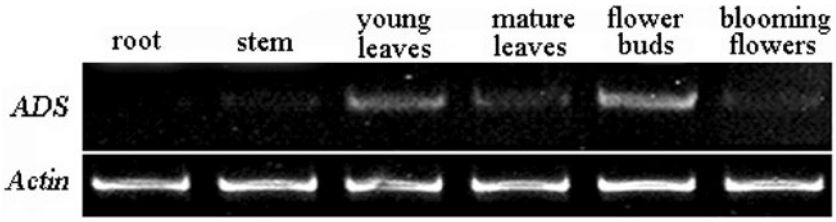

Fig. 1 PCR amplification of various transcripts using cDNA from root , stem, young leaves, mature leaves, flower buds and blooming flowers. *ADS* Amorpha-4,11-diene synthase

ADS expression pattern was further confirmed by protein gel blot with polyclonal antibodies recognizing ADS (Fig. 2).

ADS Expression During Leaf Development

Immunohistological techniques were used to track expression of *ADS* during leaf development. The results indicated that ADS was abundant in apical meristems and young leaves (Fig. 3a). Specific staining of ADS presented in all types of cells, but was stronger in glandular trichomes (Fig. 3b). With development of the leaf, the quantity of ADS decreased and became more specific, mainly in glandular trichomes (Fig. 4a).

Artemisinin was detected in all stages of leaf development, but only very low quantities per leaf were present early after leaf appearance (Ferreira et al. 1995; Lommen et al. 2006). Although the highest artemisinin concentrations were achieved during vegetative stages (Woerdenbag et al. 1994), the total quantity of putative artemisinin precursors (dihydroxyartemisinic acid and other upstream precursors) per leaf increased rapidly 7–14 days after leaf appearance (Lommen et al. 2006). These observations could be well explained by our *ADS* expression results. High level ADS in meristems and young leaves enabled the biosynthesis of artemisinin precursors during the early stage of the leaf life cycle; these compounds might play important role in protecting *A. annua* from biotic or abiotic stresses. It was suggested that dihydroartemisinic acid acts as an antioxidant by quenching singlet oxygen yielding artemisinin as a stable end-product, in which reactive oxygen is stored. High levels of dihydroartemisinic acid will then result in better protection against singlet oxygen. Both night-frost (Wallaart et al. 2000) and water stress (Wallaart et al. 2001), by which relatively high levels of singlet oxygen can be formed in plants, can promote artemisinin biosynthesis in *A. annua*. We also found that salicylic acid induces artemisinin biosynthesis by increasing the conversion of dihydroartemisinic acid into artemisinin caused by the burst of reactive oxygen species (Pu et al. 2009). Because of its toxicity, artemisinin is stored in specialized glandular trichomes in leaves and flowers.

ADS is Expressed Exclusively in Glandular Trichomes in Mature Leaves

With leaf development, ADS signals could be seen only in trichomes (Figs. 4a, 5a), no staining could be observed in other cells of the leaf. Control with pre-immune serum showed no staining (Figs. 4b, 5b). These results indicate that the protective mechanism mentioned above is located exclusively in the trichome of mature leaves, where the biosynthesis of artemisinin precursors and the conversion of these precursors to artemisinin occurs continuously. This is in good agreement with previous studies on the expression of a gene related to artemisinin biosynthesis (Olsson et al. 2009), and the accumulation of artemisinin in the glandular trichomes of the plant (Duke et al. 1994).

Whole mount immunolocalization results further revealed that *ADS* was not expressed in all glandular trichomes in mature leaves. Specific staining of ADS can be detected in about 10–40 % of glandular trichomes (Fig. 5). This might be associated with the physiological and environmental state of the different trichomes or feedback suppression by artemisinin precursors. Liersch et al. (1986) and Lommen et al. (2006) also found that no positive correlation could be expected between the number of glandular trichomes and the quantity of artemisinin. Since the strains of *A. annua* used in these research reports are different, caution should be exercised in directly extrapolating the present results to all *A. annua* cultivars. Nevertheless, these

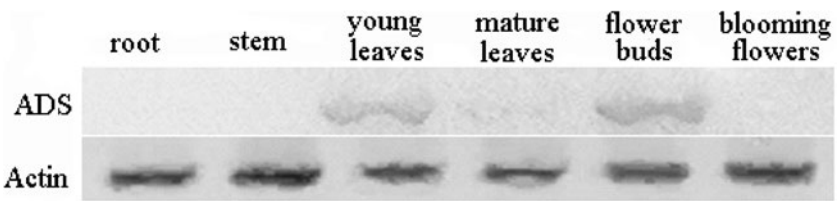

Fig. 2 Protein gel blot analysis of ADS. ADS protein was detected in total protein extracts of tissues. Immunoblotting was performed with a polyclonal serum directed against the peptide sequence of ADS from *Artemisia annua*, followed by an alkaline phosphatase-conjugated second antibody detected by adding nitro blue tetrazolium and bromochloroindolyl phosphate. Actin was used a loading control

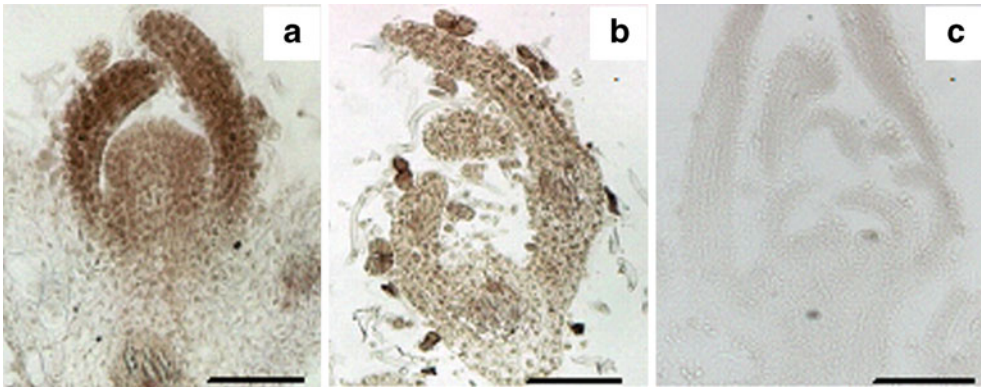

Fig. 3 Distribution of ADS in apical buds of *A. annua*. **a**, **b** Longitudinal section of a young leaf incubated with polyclonal rabbit ADS antibodies and detected using secondary goat anti-rabbit antibodies conjugated with horseradish peroxidase. **c** Longitudinal cut of apical buds incubated with rabbit pre-immune serum. *Bar* 100 μm

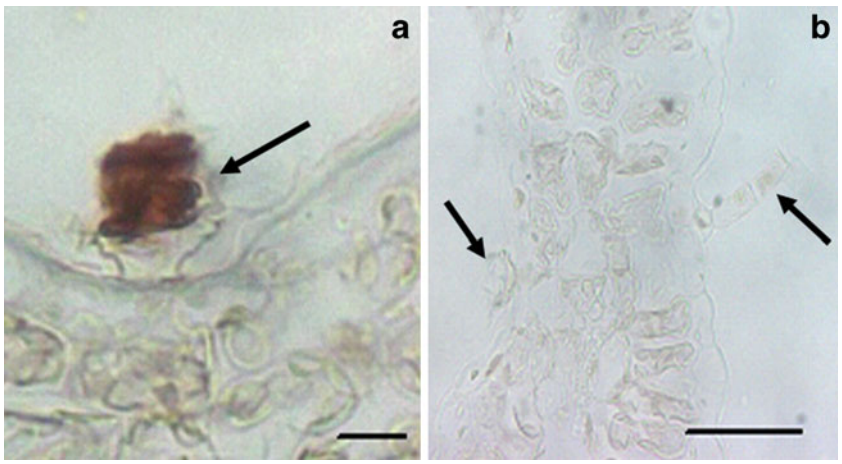

Fig. 4 Immunolabeling of ADS. **a** Longitudinal cut of a young leaf incubated with polyclonal rabbit ADS antibodies and detected using secondary goat anti-rabbit antibodies conjugated with horseradish peroxidase. **b** Longitudinal cut of a young leaf incubated with rabbit pre-immune serum showing unlabeled trichomes. *Arrow* Trichomes. *Bar* 50 μm

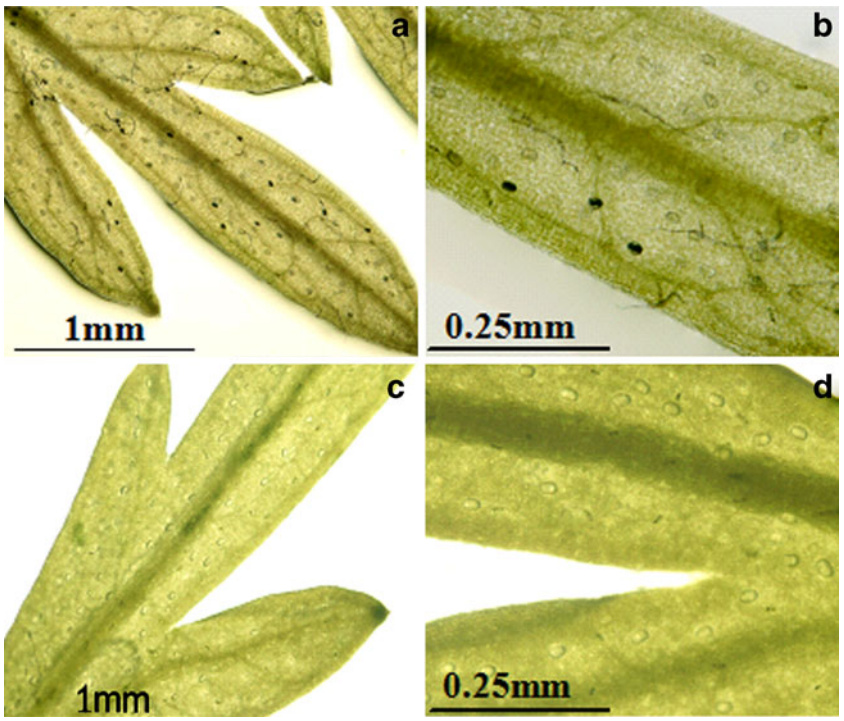

Fig. 5 Whole mount immunolocalization of ADS in *A. annua* leaves. **a**, **b** Young leaf incubated with polyclonal rabbit ADS antibodies and detected by using secondary goat anti-rabbit antibodies conjugated with alkaline phosphatase. **c**, **d** Young leaf incubated with rabbit pre-immune serum showing unlabeled trichomes

results suggest that the capacity for artemisinin biosynthesis differs between glandular trichomes. In addition to increasing the number of trichomes, more effort should be made to activate the biosynthesis of artemisinin in trichomes in order to increase artemisinin content.

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