# T-shaped trichome-specific expression of monoterpene synthase ADH2 using promoter–$\beta$-GUS fusion in transgenic *Artemisia annua* L.

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## Abstract

Artemisinin, a sesquiterpene lactone isolated from *Artemisia annua* L. (sweet wormwood), is extensively used in the treatment of malaria. In order to better understand the metabolism of terpenes in *A. annua* and the influence of terpene synthases on artemisinin yield, the expression pattern of a monoterpene alcohol dehydrogenase (ADH2) has been studied using transgenic plants expressing promoter–$\beta$-glucuronidase (*GUS*) fusion. ADH2 played a major role in monoterpenoid biosynthesis including carveol, borneol, and artemisia ketone through *in vitro* biochemical analysis. In this study, the *ADH2* promoter was cloned by the genome walking method. A number of putative *cis*-acting elements were predicted in promoter region, suggesting that the ADH2 is driven by a complex regulation mechanism. *ADH2* gene was highly expressed in old leaves, whereas the artemisinin biosynthetic genes were mainly expressed in bud and young leaves. The expression of *ADH2* gene increased quickly during leaf development, revealed by qRT-PCR. GUS expression analysis in different tissues of transgenic *A. annua* demonstrates that ADH2 expression is exclusively located to T-shaped trichome, not glandular secretory trichome.  Volume 00, Number 0, Pages 1–7, 2015

***Keywords:*** *Artemisia annua L., terpenoids, monoterpene synthase, T-shaped trichome, glandular trichome, artemisinin production, promoter activity, cis-acting regulatory element*

## 1. Introduction

Terpenoids constitute the largest class of plant secondary metabolites. Terpenoids play an important role in plant growth and development, such as carotenoids, cytokinins, and abscisic acid. Specialized terpenes are either harmful or beneficial to other organisms [1]. For example, some terpenes have the function of defense against plant pathogens as the phytoalexins [2]. Besides, terpenes are widely used as pharmaceuticals, flavors, and fragrances [3]. In order to understand the biosynthesis of terpenes, a large number of terpene synthases have been cloned and characterized. Terpene synthases can catalyze the formation of just one terpene compound or complex product mixtures [4].

***Abbreviations:*** *ADH2, monoterpene alcohol dehydrogenase; GUS, β-glucuronidase; ADH2 promoter, promoter of the monoterpene alcohol dehydrogenase gene (ADH2).*

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**TABLE 1** *Primers used for PCR application in this study*

| *Primer* | *Purpose* | *Primer sequence (5′–3′)* |
|---|---|---|
| *ADH2*-R1 | Genome walking | TGGCTTTTGTTATTCGGGAGGTT |
| *ADH2*-R2 | Genome walking | CTTGAGCCACTGCACCTGATTCT |
| *ADH2*-RT-F | RT-Q-PCR | AATGCAGGTACTGTCGATGAGCC |
| *ADH2*-RT-R | RT-Q-PCR | GCATGAGATGCAACCCCTCC |
| *ADS*-RT-F | RT-Q-PCR | AATGGGCAAATGAGGGACAC |
| *ADS*-RT-R | RT-Q-PCR | TTTCAAGGCTCGATGAACTATG |
| *CYP71AV1*-RT-F | RT-Q-PCR | CACCCTCCACTACCCTTG |
| *CYP71AV1*-RT-R | RT-Q-PCR | GACACATCCTTCTCCCAGC |
| *DBR2*-RT-F | RT-Q-PCR | CTTGGGTTACAAGCTGTGGCTCAAG |
| *DBR2*-RT-R | RT-Q-PCR | ATATAATCAAAACTAGAGGAGTGACC |
| *β-ACTIN*-F | RT-Q-PCR | CCAGGCTGTTCAGTCTCTGTAT |
| *β-ACTIN*-R | RT-Q-PCR | CGCTCGGTAAGGATCTTCATCA |
| *PROADH2*-1391z-F | Recombinant | CCCAAGCTTATTTTATTTTATATTAAACCTT |
| *PROADH2*-1391z-R | Recombinant | CGGGATCCTATCCTTCAATAAAATTTATA |

Note: *Hin*dIII and *Bam*HI sites are underlined.

Artemisinin is an important sesquiterpene isolated from *Artemisia annua*. Artemisinin-based combination therapies (ACTs) are at present the preferred drug to fight the cerebral and chloroquine-resistant malaria recommended by the World Health Organization [5]. Unfortunately, the supply of artemisinin is greatly limited because of the low content of artemisinin (0.01%–0.8% dry weight) in *A. annua*. Therefore, it is necessary to improve the artemisinin yield. Genetic modification of the artemisinin biosynthetic pathway can increase artemisinin production in transgenic *A. annua*. For example, the artemisinin content was obviously increased by overexpressing *ADS*, *CYP71AV1*, and *CPR* genes [6].

Artemisinin biosynthesis occurs in the glandular trichomes of *A. annua* [7, 8]. Recently, the artemisinin biosynthetic pathway has been clearly studied by several groups. The isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) are provided by the cytosolic mevalonic acid pathway and the plastidial methylerythritol phosphate pathway. Then, IPP and DMAPP are converted to farnesyl diphosphate by farnesyl diphosphate synthase. The first committed step is the conversion of farnesyl diphosphate to amorpha-4, 11-diene by amorpha-4, 11-diene synthase (ADS) in the artemisinin-specific biosynthetic pathway [9]. In the second step, amorpha-4,11-diene is hydroxylated to yield artemisinic alcohol, which is catalyzed by a cytochrome P450-dependent amorpha-4, 11-diene 12-hydroxylase (CYP71AV1) and cytochrome P450 oxidoreductase (CPR) as the native redox partner. CYP71AV1 and CPR can also covert artemisinic alcohol to artemisinic aldehyde and artemisinic acid [10]. A double bond reductase (DBR2) and an aldehyde dehydrogenase (ALDH1) catalyze artemisinin aldehyde to form dihydroartemisinin acid [11, 12]. The conversion of dihydroartemisinic acid to artemisinin is suggested to be a non-enzymatic reaction. These key synthases of artemisinin biosynthetic pathway were localized to glandular secretory trichomes (GSTs) in *A. annua* [10, 13–15].

Some studies on other terpene synthases in *A. annua* have been reported, such as *epi*-cedrol synthase [16], germacrene A synthase [17], monoterpene alcohol dehydrogenase (ADH2) [18], and squalene synthase (SQS) [19]. ADH2, an alcohol dehydrogenase from *Artemisia annua*, was found to show a strong preference for monoterpenoid secondary alcohols including carveol, borneol, and artemisia alcohol, which could participate in the monoterpenoid ketone biosynthesis [18]. And the major constituents of essential oil were oxygenated monoterpenes, artemisia ketone (30.7%), and camphor (15.8%) in *A. annua* [20]. These terpene synthases similarly using FDP as substrate may reduce the yield of artemisinin. The artemisinin production in *A. annua* was effectively increased by downregulating the activity of competitor. The downregulated expression level SQS in transgenic *A. annua* plants led to improved yields of artemisinin by RNAi technique [21]. But the monoterpene synthase linalool synthase expression has little or no effect on artemisinin production because of the expression in T-shaped trichomes (TSTs) [22]. Therefore, the study on the

**TABLE 2** *Putative cis-acting regulatory identified in the ADH2 promoter*

| *cis Element* | *Sequence* | *Number* | *Putative function* |
|---|---|---|---|
| AE-box | AGAAACTT | 1 | Part of a module for light response |
| ARE | TGGTTT | 1 | *cis*-Acting regulatory element essential for the anaerobic induction |
| AT-rich sequence | TAAAATACT | 1 | Element for maximal elicitor-mediated activation (2 copies) |
| AT1-motif | ATTAATTTTACA | 1 | Part of a light responsive module |
| Box 4 | ATTAAT | 2 | Part of a conserved DNA module involved in light responsiveness |
| Box-W1 | TTGACC | 1 | Fungal elicitor responsive element |
| E box | CANNTG | 2 | MYC recognition site conferring abscisic acid, dehydration, cold and freezing induction |
| G-Box | CACGTT | 2 | *cis*-Acting regulatory element involved in light responsiveness |
| MBS | TAACTG | 1 | MYB binding site involved in drought inducibility |
| MRE | AACCTAA | 1 | MYB binding site involved in light responsiveness |
| W box | TTGACC | 1 | WAKY binding site |

activity of terpene enzymes in *A. annua* is very important to increase the artemisinin content.

In this article, the promoter of *ADH2* gene was cloned to better understand the influence of other terpene synthases on artemisinin yield. Here, we study on the expression profile of *ADH2* and the activity of the *ADH2* promoter in different tissues as reported by $\beta$-glucuronidase (GUS) staining.

## 2. Materials and Methods

### 2.1. Plant materials and growth condition

*A. annua* used in this study was obtained from Chongqing, People's Republic of China. Plants were grown in a growth chamber at 24 °C under a 16-H light/8-H dark photoperiod a in the greenhouse at 25 °C [23].

### 2.2. Cloning of the *ADH2* promoter

Genomic DNA was extracted from fresh young leaves of *A. annua* using the cetyltrimethylammonium bromide method. The upstream region of *ADH2* was amplified from genomic DNA using the Genome Walker Kit (Clontech, Mountain View, CA, USA). The *ADH2*-specific primers (*ADH2*-R1 and *ADH2*-R2), Adaptor Prime1 and Adaptor Prime2 were used, following the manufacturer's instructions. PCR was carried out according to the manufacturer's instructions for KOD DNA polymerase (Toyobo, Osaka, Japan). The PCR products were electrophoresed in 1% agarose gel, then purified and cloned into the pJET2.1 vector (Thermo Fisher Scientific, Waltham, MA, USA) and sequenced. All the primers used in the study are listed in Table 1.

### 2.3. Analysis of promoter sequence

A number of putative *cis*-acting regulatory elements were analyzed using PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html) and PLACE (http: //www.dna.affrc.go.jp/PLACE/) databases. The transcription start site (TSS) of the cloned promoter was predicted using the TSSP software (http://linux1.soft-berry.com /berry.phtml).

### 2.4. Expression analysis of *ADH2* by real-time PCR

Real-time quantitative PCR analysis was performed according to the described methods [25]. Different tissues and leaves from different positions were collected for RNA extraction using plant RNA isolation reagent (Tiangen Biotech, Beijing, People's Republic of China) following the manufacturer's instructions. The different tissues (roots, stems, young leaves, old leaves, bud, and flowers) of the plants were collected from 5-month-old *A. annua*. The young leaves contained the two youngest leaves. And the old leaves were collected from the ninth to the sixteenth leaf. The different leaves were collected from the Leaf1, Leaf2, Leaf3, Leaf4, Leaf5, Leaf9, and Leaf16 counted from the apical top of the main stem of 5-month-old *A. annua*. All the tissues and leaves from the three plants were separately pooled for each determination. Amplification was carried out using the SuperReal PreMix Plus (SYBR Green) kit (Tiangen Biotech, Beijing, People's Republic of China) according to the

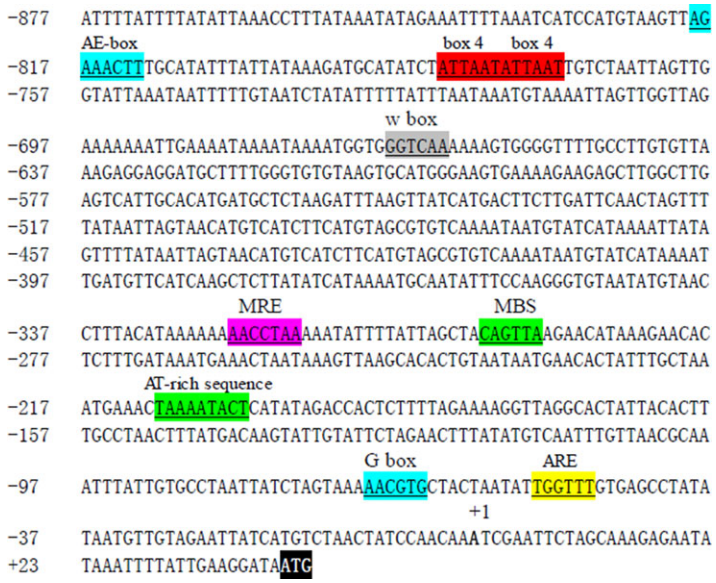


**FIG. 1** *Analysis of cis-acting elements of the ADH2 promoter.*

manufacturer's instructions. The thermal profile for real-time PCR was 95 °C for 10 Min, followed by 40 cycles of 95 °C for 20 Sec, 55 °C for 20 Sec, and 72 °C for 20 Sec, and a final extension at 72 °C for 10 Min. The relative expression levels of genes were normalized to the expression of *A. annua ACTIN*.

Real-time PCR was performed in a Roche lightcycle® 96 (Roche, Mannheim, Germany).

## 2.5. Transformation vector construction

The pCAMBIA1391Z vector (CambiaLabs) carrying the GUS gene was used for the transformation of *A. annua*. The PCR-amplified product was cloned into *Bam*HI and *Hin*dIII sites of pCAMBIA1391Z vector. The construction was introduced into *A. tumefaciens* EHA105.

## 2.6. Plant transformation

The seeds of *A. annua* were surface sterilized in 70% ethanol for 1 Min, then with a 20% sodium hypochlorite solution for 10 Min, and washed three times in sterile distilled water. The seeds were placed on MS solid medium (Sigma–Aldrich, St. Louis, MO, USA) with 2% sucrose and 0.7% agar (pH 5.8) in an environment-controlled growth chamber at 25 ± 2 with 16 H light/8 H dark and light. After 2 weeks, the leaves of seedlings were cut into 0.5-cm-diameter discs used as the explants in *A. tumefaciens*-mediated leaf disk transformation. The leaves and *A. tumefaciens* strain EHA105 were co-cultivated at 25 °C for 3 days. The leaves discs were transferred to selection medium MS1 (MS + 2.5 mg/L 6-benzoyladenine (6-BA) + 0.3 mg/L naphthalene-1-acetic acid (NAA) + 25 mg/L hygromycin + 250 mg/L carbenicillin). After 30 days of the hygromycin selection, some resistant buds were regenerated. Then, the resistant buds were transferred to MS2 (half-strength MS + 250 mg/L carbenicillin). When the roots were formed, the

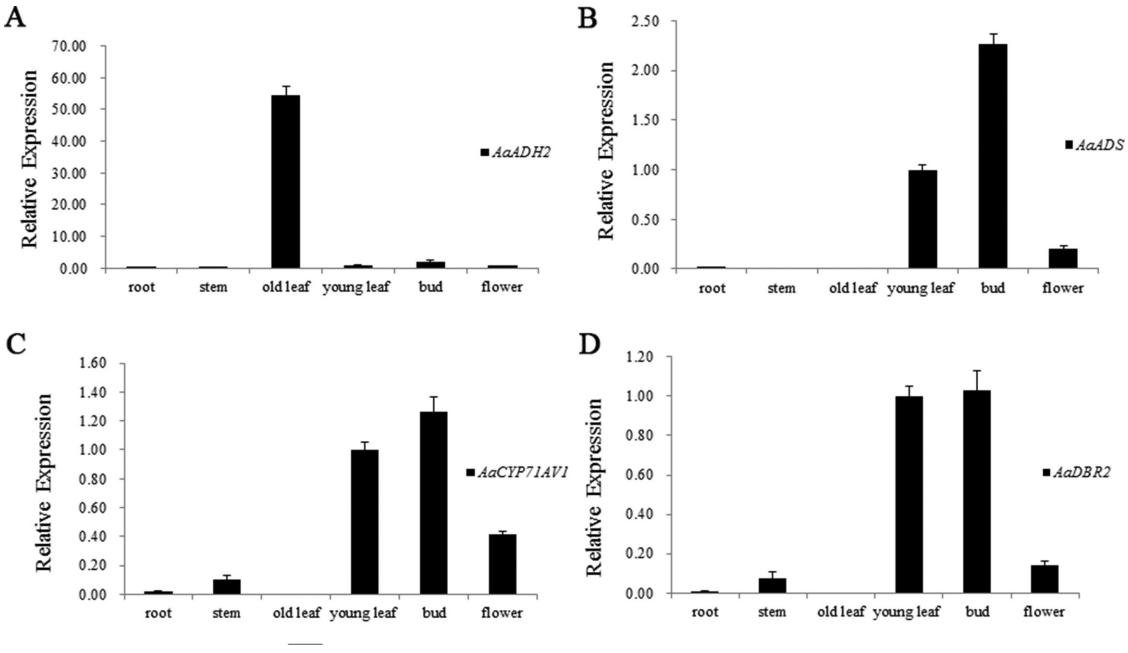


**FIG. 2** *Expression patterns of alcohol dehydrogenase (ADH2) (A), amorpha-4,11- diene synthase (ADS) (B), cytochrome P450-dependent hydroxylase (CYP71AV1) (C), and double bond reductase 2 (DBR2) (D) in different tissues of A. annua.*

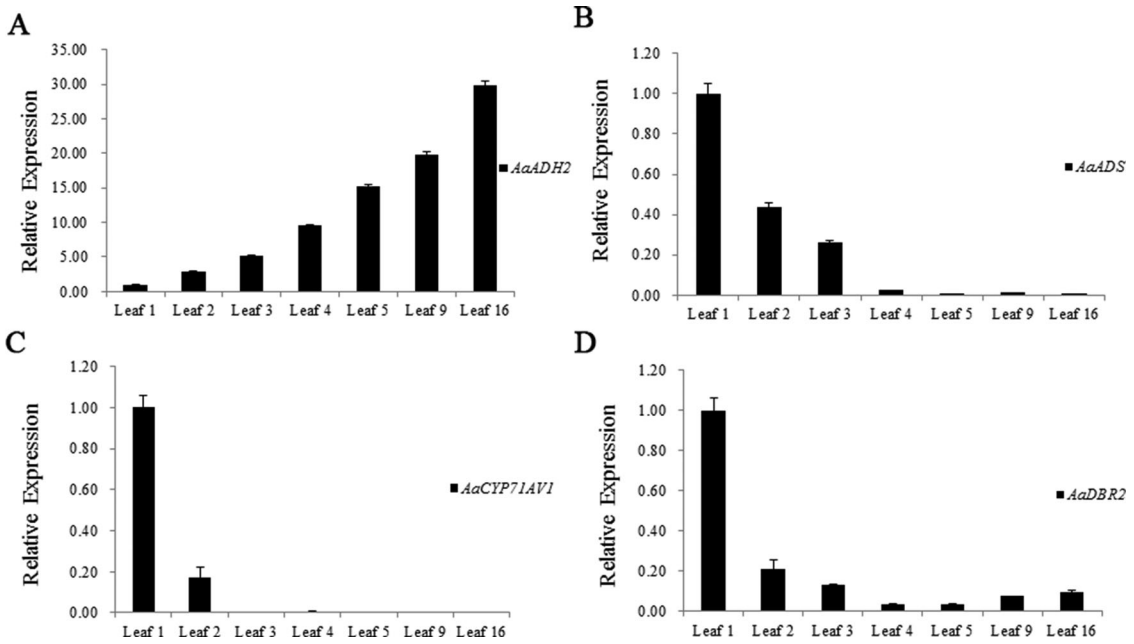


**FIG. 3** *Expression patterns of alcohol dehydrogenase (ADH2) (A), amorpha-4,11- diene synthase (ADS) (B), cytochrome P450-dependent hydroxylase (CYP71AV1) (C), and double bond reductase 2 (DBR2) (D) at different positions of the leaves in A. annua.*

resistant plants were transferred into soil in the greenhouse at 25 °C.

### 2.7. GUS assay

Young leaf, old leaf, stem, and root were sampled from transgenic *A. annua* plants to perform GUS analysis. Histochemical staining for GUS activity was carried out as previously described [24].

## 3. Results and Discussion

### 3.1. Prediction of TSS of the *ADH2* promoter

We cloned a 919 bp DNA fragment upstream of the translation initial codon (ATG) of the *ADH2* gene from the genomic DNA of *A. annua*. A putative TSS of the cloned promoter was predicted 54 bp upstream of the translation initiation codon using the TSSP software. TATA-box was found at positions –29 to –24 (TATATA) in the *ADH2* promoter upstream of the putative TSS.

### 3.2. Prediction of *cis* Elements of Promoter Region of *ADH2*

A number of putative *cis*-acting elements are summarized in Table 2 using PlantCARE and PLACE software (Fig. 1).

The E-box (CANNTG) was found in the studied promoter. Most Basic/helix-loop-helix (bHLH) TFs bind to the E-box sequence, as well as MYB and bZIP TFs. bHLH TFs may be involved in the JA responsiveness of plants [26]. The E-box may be involved in the response of *A. annua* to JA and other hormones.

The WRKY TFs constitute a large family of TFs in plants, binding to W-box elements [27]. Transient expression of the *A. annua* transcription factor AaWRKY in leaves of *A. annua* improved the expression of *ADS*, *HMGR*, *CYP71AV1*, and *DBR2* by binding of *AaWRKY* to W-boxes [28]. In cotton, the GaWRKY1 is regulating the activity of cadinene synthase 1 (CAD1) involved in the biosynthesis of sesquiterpene phytoalexins. The promoter of *CAD1* carries two W-boxes [29]. We found a W-box with sequence TTGACC in the *ADH2* promoter. It seems that the *ADH2* promoter may interact with members of the WRKY transcription factor family to regulate stress reactions and immune response in *A. annua*.

As far as we know, no study on the MYB transcription factors regulating artimisinin metabolism in *A. annua* has been reported. The MYB binding site (MBS) (TAACTG) was found in the *ADH2* promoter, which is involved in drought-inducibility. In *Arabidopsis*, AtMYB1 and AtMYB2 regulate the genes that are responsive to water stress [30]. Two putative MBSs (TAACNG) at positions –697 to –692 and –1,499 to –1,504 were found in the *ADS* promoter of *A. annua* [24].

Moreover, various putative elements involved in light response are abundant in *ADH2* promoter sequence, including the AE-box (AGAAACTT), AT1-motif (ATTAATTTTACA), MRE (AACCTAA), and G boxes (CACGTT). These elements show that this promoter is probably subjected to light regulation. We may conclude that ADH2 is involved in a complex regulation of gene expression.

### 3.3. Expression analysis of *ADH2* in different tissues

To dissect the expression patterns of *ADH2* gene of *A. annua*, the expression of *ADH2* was analyzed by RT-PCR in different tissues. *ADH2* was highly expressed in old leaves, lower in

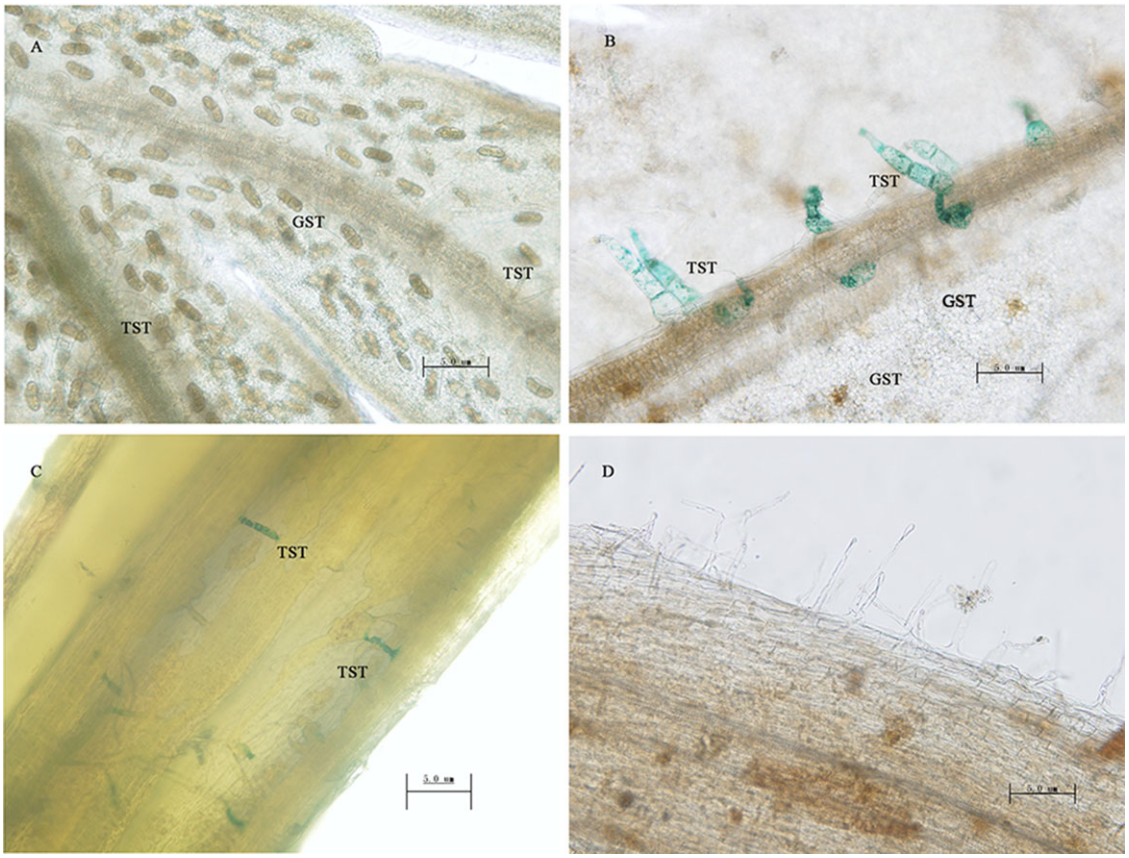


**FIG. 4** *Gus-staining of transgenic A. annua transformed using the pADH2-GUS plasmid. (A) Young leaf, (B) old leaf, (C) stem, and (D) root. GST, glandular secretory trichome; TST, T-shaped trichome.*

young leaves, bud, and flower, and poorly expressed in root and stem (Fig. 2A), which was different than the expression patterns of the key genes in the artemisinin biosynthetic pathway (*ADS*, *CYP71AV1*, and *DBR2*). These key enzyme genes were highly expressed in bud, lower in stem and flower, and poorly expressed in root and old leaves (Figs. 2B–2D).

### 3.4. Expression analysis of *ADH2* at different positions of the leaves

The expression patterns of *ADH2* gene of *A. annua* was investigated at different positions of the leaves by RT-PCR. The key enzyme genes in the artemisinin biosynthesis pathway (*ADS*, *CYP71AV1*, and *DBR2*) were highly expressed in young leaves (Leaf1 and Leaf2). And the expression of these genes was reduced quickly during leaf development (Figs. 3B–3D). However, *ADH2* gene of *A. annua* showed different expression patterns from *ADS*, *CYP71AV1*, and *DBR2*. *ADH2* was highly expressed in old leaves (Leaf9 and Leaf16). The expression of *ADH2* gene increased gradually during leaf development (Fig. 3A).

### 3.5. Activity of the *ADH2* promoter

There are two kinds of trichomes on leaves of *A. annua*, GSTs and non-glandular TSTs. In order to explore the expression pattern of the *ADH2* promoter in *A. annua*, the *ADH2*pro::*GUS* fusion vector was constructed and transformed into *A. annua*. The GUS assay of transgenic *A. annua* showed that GUS staining was only observed in TSTs of old leaves (Fig. 4B) and rarely in TSTs of stem (Fig. 4C) (GSTs not included). No GUS staining was observed in young leaves (Fig. 4A) and roots (Fig. 4D). These results indicate that *ADH2* is almost exclusively expressed in TSTs of old leaves of *A. annua*, which is consistent with the expression patterns of *ADH2* gene in different tissues.

ADH2, as an alcohol dehydrogenase from *A. annua*, could participate in the monoterpenoid ketone (artemisia ketone) biosynthesis in *A. annua*. *ADH2* is almost exclusively expressed in TSTs of *A. annua*. Hence, the artemisia ketone biosynthesis only occurs in the TSTs of *A. annua*, which is quite different with the artemisinin biosynthesis. It seems that ADH2 do not influence the biosynthesis of artemisinin to any great extent because of the artemisinin biosynthesis in the glandular trichomes. Furthermore, the study on the expression patterns of terpene enzymes in *A. annua* and accurately evaluating the potential competitors is very important to increase the artemisinin content. For instance, downregulation of the $\beta$-farnesene synthase (*FP*) gene may have an effect on the yield of artemisinin because of the higher expression level of *FS* in flower buds, which is similar to the key genes in the

artemisinin biosynthesis pathway [31]. We may conclude that downregulation of *ADH2* gene will most likely have little or even no effect on artemisinin production.

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