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ORIGINAL ARTICLE

Glandular trichome-specific expression of alcohol dehydrogenase 1 (*ADH1*) using a promoter-GUS fusion in *Artemisia annua* L.

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Abstract Artemisinin, isolated from *Artemisia annua* L., is widely known as a functional anti-malaria drug. Due to the low content of artemisinin in *A. annua* plants, great efforts have been made to determine the artemisinin biosynthetic pathway by genetic engineering. *ADH1*, encoding an alcohol dehydrogenase, was cloned from the glandular secretory trichomes (GSTs) in *A. annua*. The gene expression analysis showed that *ADH1* was predominately expressed in buds and young leaves, and the expression of *ADH1* was the highest in the youngest leaves. To further investigate the expression pattern of *ADH1* in *A. annua*, a 1070-bp promoter region of *ADH1* was cloned. We found 14 putative *cis*-elements were presented in the *ADH1* promoter sequence, indicating that *ADH1* is complexly regulated. The *ADH1* promoter sequence was fused to the β-glucuronidase reporter gene (*GUS*) and introduced into *A. annua* plants. GUS signals were only found in the glandular secretory trichomes of young tissues in transgenic *A. annua* plants. Besides, the treatment of *A. annua* seedlings with 100 μM methyl jasmonate (MeJA) and 100 μM abscisic acid (ABA), respectively, increased the *ADH1* transcript levels. The dual luciferase (dual-LUC) assay demonstrated that the reported transcription factors, MYC2 and ERF1, activated the expression of *ADH1* in vivo. Our study shows that *ADH1* gene is exclusively expressed in the glandular secretory trichomes of young tissues of *A. annua*, it implies that the promoter of *ADH1* gene could be used in engineering of *A. annua* for increasing artemisinin content.

Keywords Abscisic acid · Artemisinin · Glandular secretory trichomes · β-Glucuronidase · Methyl jasmonate

Introduction

More than 214 million people are suffering from a threat of malaria all over the world. The case of malaria has led to approximately 4,38,000 deaths in 2015 (WHO 2016). Artemisinin-combination therapies (ACTs), the most effective available antimalarial cure at present, is globally recommended by WHO (Mutabingwa 2005). Artemisinin is an anti-malarial endoperoxide with a peroxide bridge. Unfortunately, the limited artemisinin content in *Artemisia annua* plants (0.01–0.8% DW) results in a worldwide shortage of the antimalarial drug (Abdin et al. 2003). Therefore, it is urgent to adopt new strategies for a large improvement of artemisinin accumulation in *A. annua* to obtain a number of stable high-producing plants. Great efforts have been expended in metabolic engineering to enhance the artemisinin accumulation. Several transcription factors regulating the biosynthetic pathways of artemisinin have been reported. In *A. annua*, WRKY1 is isolated firstly and reported as a positive regulator in artemisinin biosynthesis. Overexpressing *WRKY1* driven by the glandular trichome-specific *CYP71AV1* promoter can lead the artemisinin content to almost 1.9 times higher than that of controls (Han et al. 2014). Previous study revealed that the expressions of *ADS, CYP71AV1*, and *DBR2* were strongly activated in *ORA*-overexpressing transgenic lines, resulting in an increase of artemisinin content by 40–53% compared to control plants (Lu et al. 2013a).

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Numerous efforts have been made to clarify the artemisinin biosynthesis (Fig. 1). The reaction from farnesyl diphosphate (FPP) to amorpha-4,11-diene is catalyzed by amorpha-4,11-diene synthase (ADS), which is considered as the initial step specifically occurred in the glandular secretory trichomes (Banyai et al. 2010; Wang et al. 2011). Next, amorpha-4,11-diene is gradually catalyzed to artemisinic alcohol, and further yield artemisinic aldehyde under the catalysis of both cytochrome P450 monooxygenase (CYP71AV1) and cytochrome P450 reductase (CPR) (Ro et al. 2006; Teoh et al. 2006). Recently, alcohol dehydrogenase 1 (ADH1) has been proved to involve in the formation of artemisinic aldehyde in yeast (Paddon et al. 2013). Subsequently, the artemisinic aldehyde is deoxidized to dihydroartemisinic aldehyde by artemisinic aldehyde Δ11 (13) reductase (DBR2) (Zhang et al. 2008). Then dihydroartemisinic aldehyde is reduced to form dihydroartemisinic acid with the action of aldehyde dehydrogenase 1 (ALDH1) (Teoh et al. 2009). Artemisinin is formed from dihydroartemisinic acid in a photo-oxidative reaction rather than an enzymatic conversion (Sy and Brown 2002; Brown and Sy 2004). Alternatively, the artemisinic aldehyde is catalyzed to artemisinic acid, the precursor of arteannuin B, by the CYP71AV1 and ALDH1 (Ro et al. 2006; Teoh et al. 2009). The conversion of artemisinic acid to arteannuin B is also regarded as a photo-oxidative reaction without any enzyme involved in (Sy and Brown 2002; Brown and Sy 2004).

Previous researches indicate that the glandular secretory trichomes can be considered as storages for artemisinin and infer the precursors of artemisinin biosynthesis are exclusively located in this type of trichomes (Ferreira and Janick 1996; Arsenault et al. 2010; Nguyen et al. 2011). Previous studies proved that ADS, CYP71AV1, DBR2 and ALDH1 are specifically expressed in the glandular secretory trichomes of young tissues (Wang et al. 2011, 2013; Jiang et al. 2014; Liu et al. 2016).

Recently, *ADH1* was isolated from *A. annua* plants. The sequence analysis and in vitro characterization indicate that ADH1 protein, expressed and purified from *Escherichia coli*, encodes alcohol dehydrogenase, suggesting that ADH1 catalyzes artemisinic alcohol to form artemisinic aldehyde in artemisinin biosynthesis (Paddon et al. 2013). Moreover, there was 18% more artemisinic acid than the control when *ADH1* together with *ALDH1, CYP71AV1, CYB5* and *CPR1* were expressed in yeast strain Y1368 (Paddon et al. 2013). However, the localization of ADH1 is not reported in *A. annua*.

An enormous amount of studies to improve the artemisinin content in *A. annua* have been made a great progress. When *FPS* gene was overexpressed in the *A. annua* plants, the transgenic plants exhibited the 2.5-fold artemisinin content compared to that in non-transgenic plants (Banyai et al. 2010). The artemisinin content was improved by 82% in the *ADS* over-expressing lines by GC–MS (Ma et al. 2009). Furthermore, co-overexpressing *ADS, CPR* and *CYP71AV1* genes in *A. annua* led to

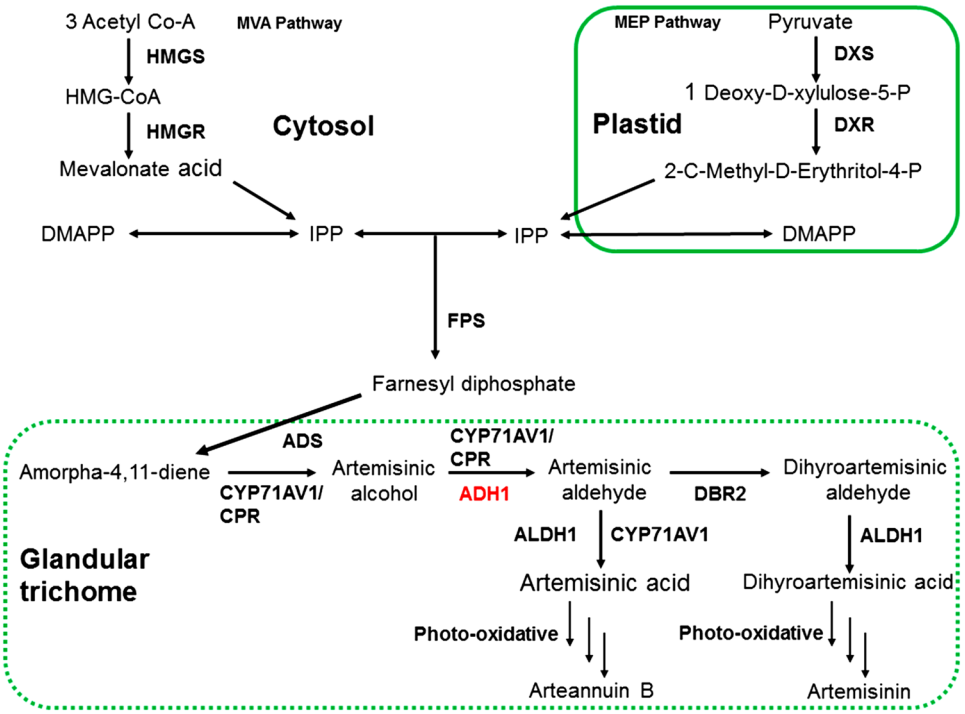

Fig. 1 Artemisinin biosynthetic pathway in *A. annua*. *HMGS* 3-hydroxy-3-methyl-glutaryl coenzyme A synthase, *HMGR* 3-hydroxy-3-methyl-glutaryl coenzyme A reductase, *DXS* 1-deoxy-D-xylulose-5-phosphate synthase, *DXR* 1-deoxy-D-xylulose-5-phosphate reductoisomerase, *FPS* farnesyl diphosphate synthase, *ADS* amorpha-4,11-diene synthase, *CYP71AV1* cytochrome P450 monooxygenase, *CPR* cytochrome P450 reductase, *ADH1* alcohol dehydrogenase 1, *DBR2* artemisinic aldehyde Δ11 (13) reductase, *ALDH1* aldehyde dehydrogenase 1

the greater artemisinin accumulation than that in control plants (Lu et al. 2013b). In another example, *HMGR* and *ADS* genes were co-overexpressed in *A. annua* plants and one of the transgenic lines exhibited more than seven-fold higher artemisinin content than the control lines (Alam and Abdin 2011). Four artemisinin biosynthetic pathway genes (*ADS, CYP71AV1, CPR* and *ALDH1*) were overexpressed in *A. annua* and the transgenic lines showed higher artemisinin content, with 3.4-fold than that detected in wild-type plants (Shi et al. 2017). Therefore, the transgenic approach is vital to improve the production of artemisinin in *A. annua*.

In this study, we analyzed the transcript levels of *ADH1* in different tissues and leaves at leaf development age by quantitative real-time PCR, which indicated that *ADH1* was predominately expressed in buds and young leaves, and the expression of *ADH1* gene was the highest in the youngest leaf and rapidly reduced during leaf development. To further investigate the expression pattern of *ADH1*, the promoter sequence of *ADH1* was cloned. Then the *ADH1* promoter sequence was fused to the *GUS* reporter gene and introduced into *A. annua* plants by *Agrobacterium*-mediated transformation system. GUS signals were only found in the glandular secretory trichomes of young tissues in transgenic *A. annua* plants. Besides, the treatment of seedlings with MeJA and ABA, respectively, increased the *ADH1* transcript levels. Finally, a dual-LUC assay in tobacco leaves indicated that MYC2 and ERF1 activated the expression of *ADH1*, respectively.

Materials and methods

Relative expression analysis by RT-q-PCR

The different organs used in this study [roots, stems, young leaves (the two youngest leaves), old leaves (from the sixth to the ninth leaf), buds and flowers] and the leaves from different nodes were collected from 4-month-old *A. annua* for RNA extraction following the manufacturer's protocol (Tiangen Biotech, Beijing, People's Republic of China). First-strand cDNA was synthesized from the total RNA using the PrimeScript II first Strand cDNA Synthesis Kit (Takara Biotechnology, Dalian, China). We used the β-actin as a standard control (Liu et al. 2015, 2016). Primers involved in this exprriment are listed in Table 1. Amplification was employed using the Roche LightcycleR® 96 (Roche, Mannheim, Germany) with the kit SuperReal PreMix Plus (Tiangen Biotech, Beijing, China) following the manufacturer's instruction as followed: 94 °C for 8 min, followed by 40 cycles of 94 °C (20 s), 55 °C (20 s) and 72 °C (20 s), and a last extension at 72 °C for 8 min. Three technical replications were run for each cDNA sample. The transcription level of *ADH1* was analyzed by RT-q-PCR using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Cloning of the *ADH1* promotor

We extracted genomic DNA from the youngest leaves of *A. annua* plants by the cetyltrimethylammonium bromide method (Fütterer et al. 1995). Based on our genomic

Table 1 Nucleotide sequence of primes used in this study

No	Name	Application	Sequence (5′–3′)
1	ADH1pro-FP1	Cloning	AAAGTATGGAATGTTGGTAAATAGAGGT
2	ADH1pro-RP1	Cloning	CTAACTTCAGATGCTTTTGGCGGAT
3	ADH1pro-HindIII-FP	Recombinant	CCCAAGCTTAAAGTATGGAATGTTGGTAAATA
4	ADH1pro-NcoI-RP	Recombinant	CATGCCATGGTTTAATCAAATCGTTTAGTTAG
5	ADH1-RT-FP	qPCR	AGGAAAAGTGTTTGGAATGACCGAT
6	ADH1-RT-RP	qPCR	AAGTGCGACCGCTAAAGAGTATCAGG
7	ADS-RT-FP	qPCR	AATGGGCAAATGAGGGACAC
8	ADS-RT-RP	qPCR	TTTCAAGGCTCGATGAACTATG
9	CYP-RT-FP	qPCR	CACCCTCCACTACCCTTG
10	CYP-RT-RP	qPCR	GACACATCCTTCTCCCAGC
11	DBR2-RT-FP	qPCR	CTTGGGTTACAAGCTGTGGCTCAAG
12	DBR2-RT-RP	qPCR	ATATAATCAAAACTAGAGGAGTGACC
13	ALDH1-RT-FP	qPCR	GGACTTGCCTCAGGTGTAT
14	ALDH-RT-RP	qPCR	GTGCCTCTAATCCTTGTTC
15	β-actin-F	qPCR	CCAGGCTGTTCAGTCTCTGTAT
16	β-actin-R	qPCR	CGCTCGGTAAGGATCTTCATCA
17	ADH1pro-PstI-Dual -FP	Recombinant	AACTGCAGAAAGTATGGAATGTTGG
18	ADH1pro-BamHI-Dual-RP	Recombinant	CGGGATCCTTTAATCAAATCGT

databases (unpublished), the promoter region of *ADH1* was amplified using high-fidelity PCR (Toyobo, Osaka, Japan) with *ADH1*-specific primers (Table 1). The amplified fragment of *ADH1* promoter was purified and linked with pLB vector (Tiangen Biotech, Beijing, China) to sequence.

Analysis of promoter sequence

The TSSP software (http://linux1.soft-berry.com), the PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html), and the PLACE (http://www.dna.affrc.go.jp/PLACE/) databases were applied for the prediction of transcription start site (TSS) and the *cis*-acting elements of the cloned *ADH1* promoter.

Vector construction

The *ADH1* promoter was amplified with primers containing *Hind*III and *Nco*I restriction sites. The amplified fragment and pCAMBIA1391Z vector (CambiaLabs) carrying the *GUS* were digested with *Hind*III and *Nco*I (Fermentas FastDigest) respectively. The promoter region was inserted into pCAMBIA1391Z vector. Subsequently, the recombinant plasmid was introduced into *Agrobacterium tumefaciens* strain EHA105. The resulting *A. tumefaciens* EHA105 harboring the construct (pCAMBIA1391Z-*proAHD1-GUS*) were prepared for the plant transformation.

Plant materials and transformation of *A. annua*

Seeds called "Huhao 1", originated from Chongqing, were cultivated by our group (Shen et al. 2016). Seeds were immersed in 75% ethanol for 60 s, and then soaked in 25% sodium hypochlorite solution for 8 min, followed by five rinses with sterile distilled water. All the germ-free seeds were plated on MS medium (Murashige and Skoog 1962) [MS powder 4.4 g L^{-1}(Sigma–Aldrich, MO, USA), 30 g L^{-1} sucrose (Sangon Biotech, Shanghai, China), 2.6 g L^{-1} phytagel(Sigma–Aldrich, MO, USA), pH = 5.8] at 25 °C for 3 weeks. 20–25-day-old seedlings were used for transformation. Leaf discs with 0.5 cm diameter were cut and co-cultivated with *A. tumefaciens* strain EHA105 carrying the pCAMBIA1391Z-*pADH1-GU*S on co-cultivation medium in the dark at 28 °C for 72 h. Then the treated explants were transferred to selection medium MS_1 and further to root-inducing medium MS_2 as described previously (Liu et al. 2016). Finally, we got the plantlets and cultivated them for 2–3 months in the greenhouse at room temperature.

GUS assay

The leaf primordia, expanded leaves at different nodes, stems and roots were respectively collected from both transgenic *A. annua* plants and wild-type plants to carry out GUS analysis. GUS staining of these samples was performed according to previous protocol (Jefferson et al. 1987).

Hormonal treatments

MeJA (100 μM) (Sigma–Aldrich) and abscisic acid (100 μM) (Sigma–Aldrich) were prepared for hormone treatments, and 1% DMSO solution was used as a mock

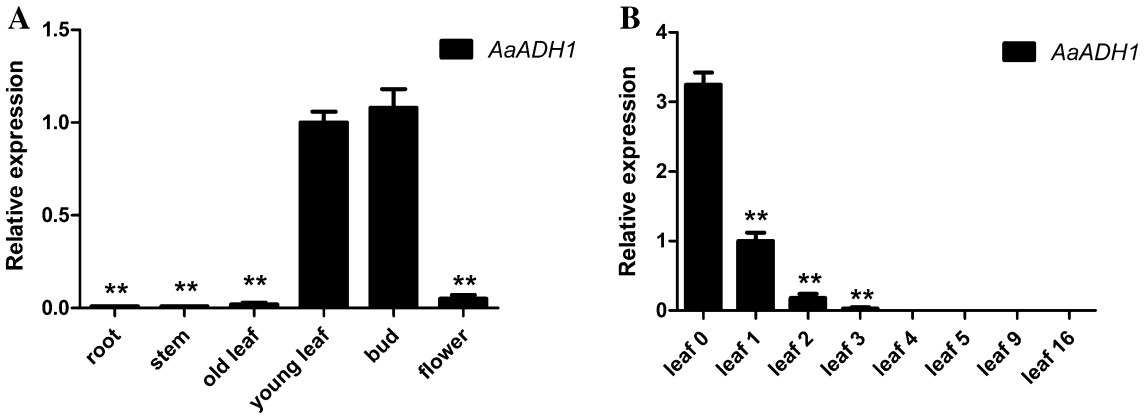

Fig. 2 Expression pattern of *ADH1* in *A. annua* plants: **a** relative expression of *ADH1* in different tissues (root, stem, old leaf, young leaf, flower, and bud). β-actin was used as the control gene. *Error bars* represent ± SD ($n = 3$). Statistical significance was determined by two-tailed Student's *t* test with *P* values; $^{**}P < 0.01$. *Asterisks* above the *bar* represent the significance of the difference between different tissues; **b** relative expression of *ADH1* in expanded leaves at different nodes (leaf 0, leaf 1, leaf 2, leaf 3, leaf 4, leaf 5, leaf 9 and leaf 16). β-actin was used as the control gene. *Error bars* represent ± SD ($n = 3$). Statistical significance was determined by two-tailed Student's *t* test with *P* values; $^{**}P < 0.01$. *Asterisks* above the bar represent the significance of the difference between expanded leaves at different nodes

treatment. The cutting plants were prepared and sprayed with different hormone solutions. After treatment, they were cultivated in the greenhouse with the artificial light at a constant temperature of 25 °C. The youngest leaves (leaves at the first node) of the wild-type *A. annua* plants were respectively collected for RNA extraction at 0, 1, 3, 6, 9, 12 and 24 h after hormonal treatments. Leaves from three independent plants were collected for each determination. In addition, the leaves of transgenic *A. annua* plants were used for GUS staining after the hormonal treatments.

Dual-luciferase activity assay

The promoter fragment of *ADH1* was inserted into pGreenII 0800-LUC vector. The recombinant plasmid was introduced into *A. tumefaciens* strain GV3101 with the plasmid pSoup19 as a reporter construct. For dual-luciferase activity assay, pHB-*ERF1*, pHB-*bZIP1*, pHB-*WRKY1* and pHB-*MYC2* (Ma et al. 2009; Lu et al. 2013c; Zhang et al. 2015; Shen et al. 2016) plasmids were respectively transformed into *A. tumefaciens* strain GV3101 to act as effectors (Luo et al. 2014). The *A. tumefaciens* strains were cultured overnight at 28 °C, collected by centrifugation and further resuspended using MS liquid medium to $OD_{600}=0.6$. Then the mixture were infiltrated into tobacco leaves followed by the *agroinfiltration* procedure (Voinnet et al. 2003). The pHB construct was used as a negative control. The dual-LUC assay was performed using Dual-Luciferase® Reporter Assay System (Promega, Durham, USA). Three biological repeats were valued for each sample.

Statistical analysis

Data are presented as means ± SD unless otherwise stated. To compare group differences, paired or unpaired, two-tailed Student's *t* tests were used. $P<0.05$ was considered significant and $P<0.01$ was considered extremely significant.

Results and discussion

Expression pattern of *ADH1* in *A. annua*

To analyze the expression pattern of *ADH1* in *A. annua*, the different organs were collected for RNA extraction from *A. annua*. The results showed that the *ADH1* expression was the highest in buds and young leaves (Fig. 2a).

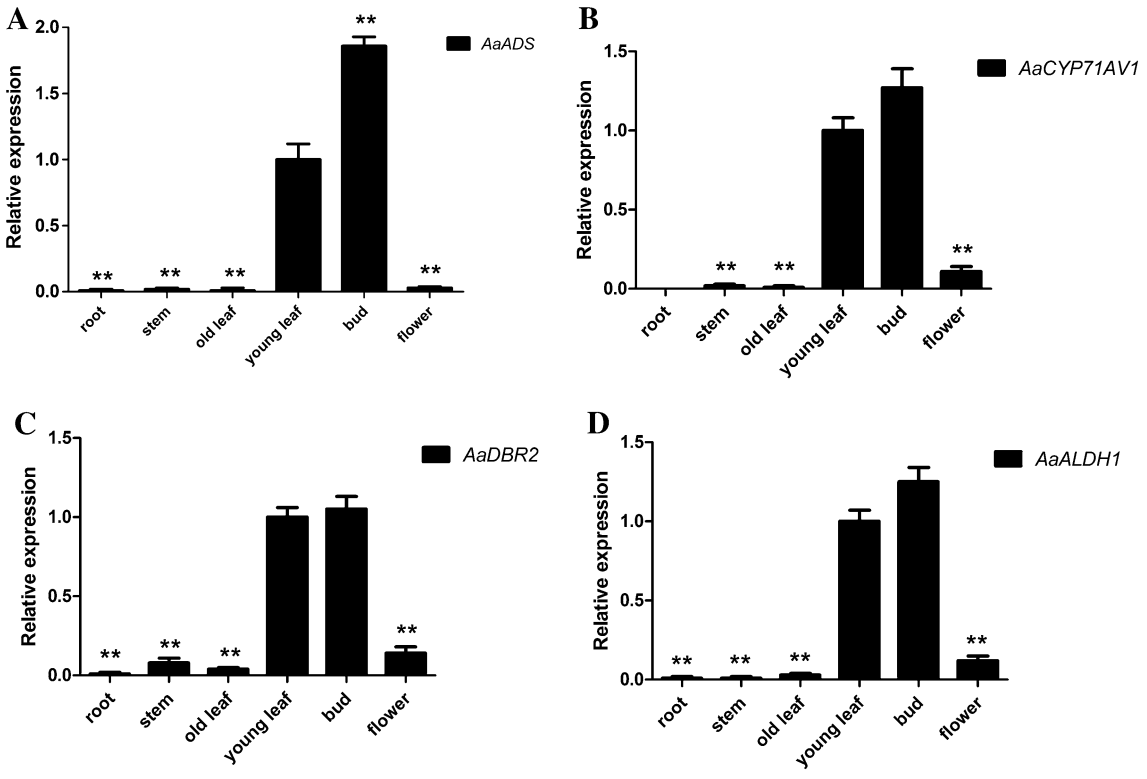

Fig. 3 Expression patterns in different tissues of *A. annua* plants: **a** relative expression of *ADS*; **b** relative expression of *CYP71AV1*; **c** relative expression of *DBR2*; **d** relative expression of *ALDH1*. β-actin was used as the control gene. *Error bars* represent ± SD ($n=3$). Statistical significance was determined by two-tailed Student's *t* test with *P* values; $^{**}P<0.01$. *Asterisks* above the *bar* represent the significance of the difference between different tissues

The *ADH1* expression was poorly detected in roots, old leaves and stems. We analyzed the expression of *ADH1* in leaves at different developmental stages. The transcript level of *ADH1* was the highest in the youngest leaf (leaf 0) and reduced gradually with age of the leaves (Fig. 2b). The *ADH1* expression pattern is similar to that of the artemisinin biosynthetic pathway genes (Figs. 3, 4). Several researches indicate that the artemisinin-specific enzymes (ADS, CYP71AV1, DBR2 and ALDH1) are located to the glandular secretory trichomes, where artemisinin is biosynthesized and stored (Zhang et al. 2008; Wang et al. 2011, 2013; Liu et al. 2016). Our results suggest that ADH1 is also likely to be located to the glandular secretory trichomes.

Cloning and analysis of *ADH1* promoter

To further investigate the expression of *ADH1*, we predicted the *ADH1* promoter sequence from our genomic databases (unpublished) and cloned the 1070-bp *ADH1* promoter.

The TSS of *ADH1* promoter was located 53 bp upstream from the start codon (ATG) at position +1 (labeled +1 in Fig. 5). It was found that a putative TATA-box (TATAA), the well-conserved core promoter sequence, at position from −6 to −10 in the *ADH1* promoter (Fig. 5). A CAAT-box generally accompanied by the consensus sequence (GGCCAATCT) is required for transcription. We found the CAAT-box (CAATT) was located 165–169 bp upstream from the putative TSS of *ADH1* promoter (Fig. 5).

As shown in Table 2, 14 *cis*-acting elements in the *ADH1* promoter were listed.

One G-box (GGTACAC; position −24 to −30) was found in the *ADH1* promoter. The MYC2 transcription factor, one of the bHLH transcription factor family, is capable of binding G-box or G-box-like motifs in the promoter, exhibiting a strong JA-response ability (Shen et al. 2016). There is a strong possibility that the MYC2 binds the G-box of *ADH1* promoter.

Five E-boxes were found at positions −24 to −29 (CAT GTG), −151 to −156 (CATTTG), −237 to −242 (CAT TTG), −370 to −375 (CAACTG), −642 to −647 (CAT ATG), respectively. It was reported earlier that bHLH TFs could induce gene expression through binding to the E-box sequence (5′-CANNTG-3′) (Toledoortiz et al. 2003). Biochemical analysis demonstrates that bHLH TFs of plants

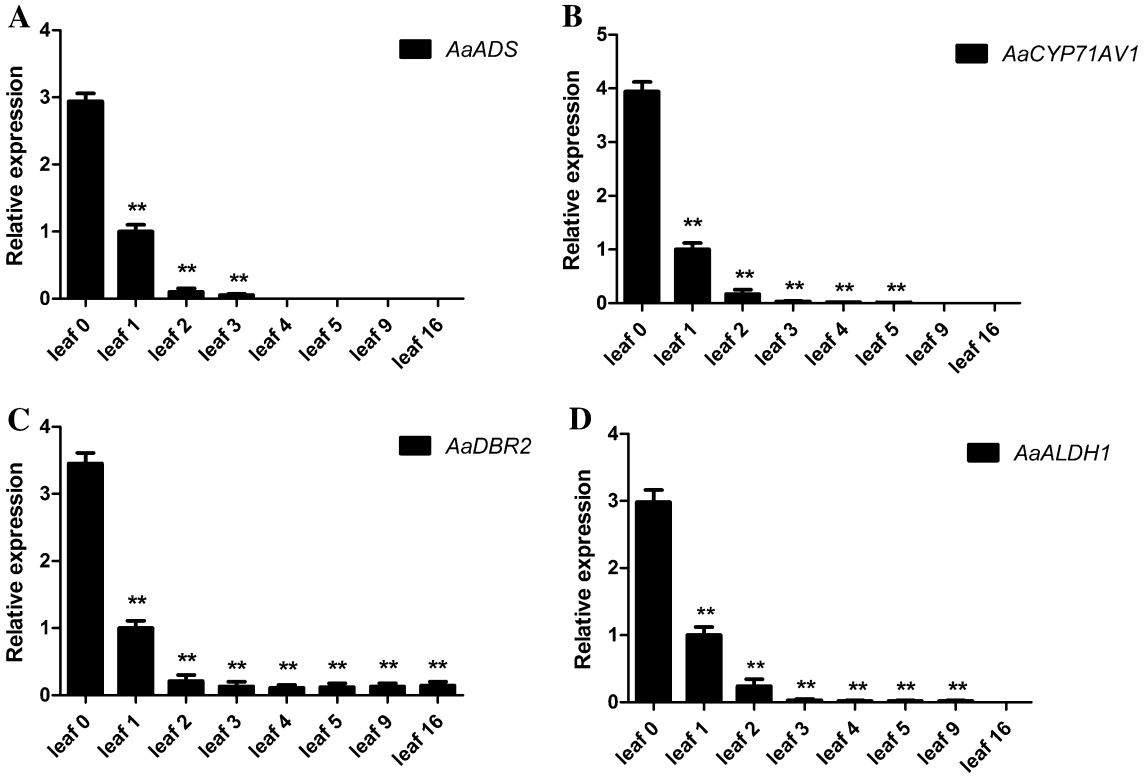

Fig. 4 Expression patterns of leaves at different nodes in *A. annua*: **a** relative expression of *ADS*; **b** relative expression of *CYP71AV1*; **c** relative expression of *DBR2*; **d** relative expression of *ALDH1*. β-actin was used as the control gene. *Error bars* represent ± SD ($n = 3$). Statistical significance was determined by two-tailed Student's *t* test with *P* values; $^{**}P < 0.01$. *Asterisks* above the *bar* represent the significance of the difference between expanded leaves at different nodes

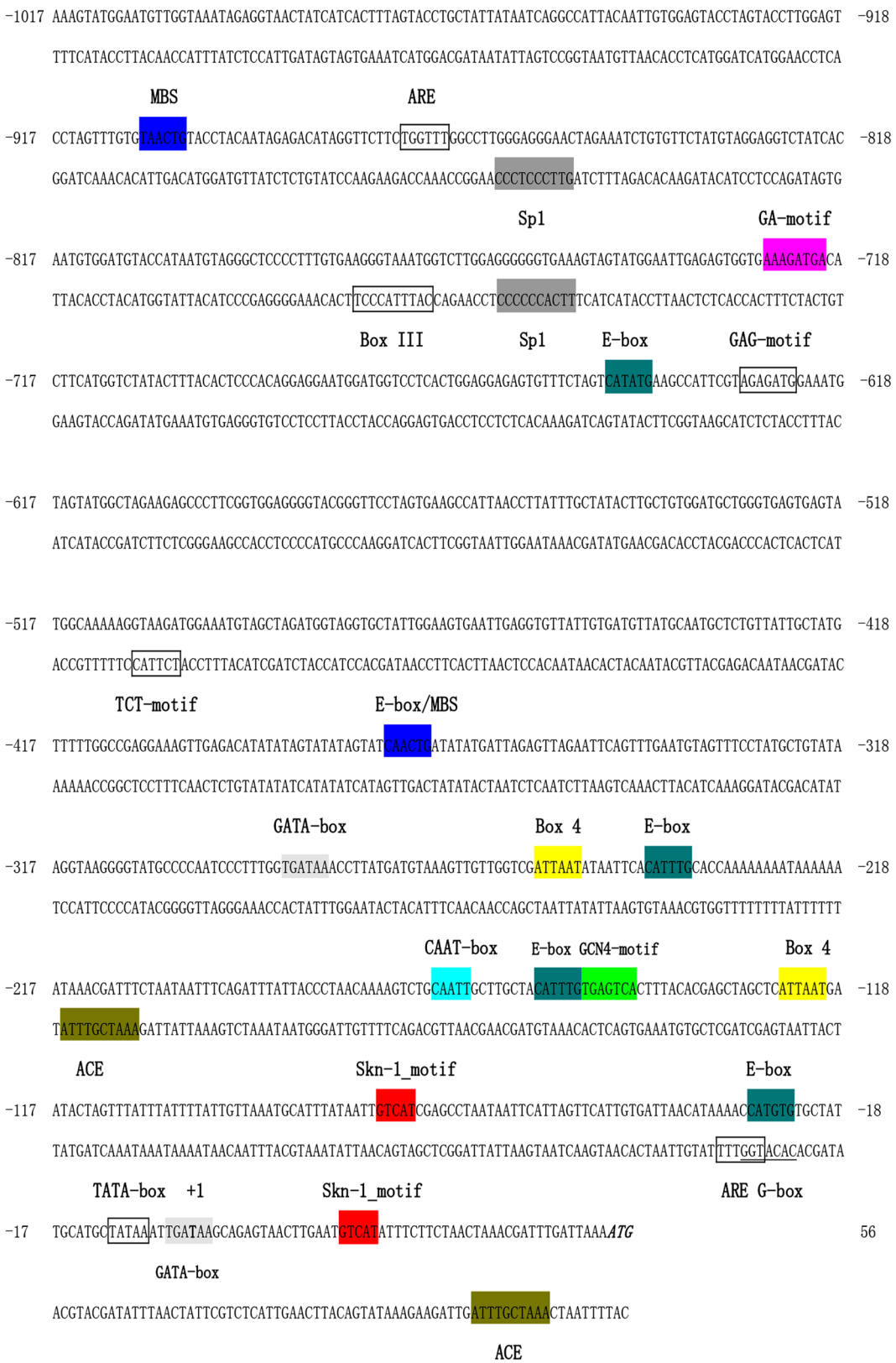

Fig. 5 Nucleotide sequence of *ADH1* promoter with putative *cis*-acting elements. The putative transcript start site is in *bold* and the ATG start codon is in *italic*

make contribution to the JA responsiveness (Miyamoto et al. 2012). Thus, the E-box in the *ADH1* promoter region may act as the JA-responsive *cis*-element. Previous studies also showed that bHLH TFs play positive roles in ABA-mediated response of *Arabidopsis* (Kim and Kim 2006).

Two MBS (MYB binding sites) (CAACTG/TAACTG ; position −370 to −375 and −980 to −985) were predicted in the *ADH1* promoter. Earlier study demonstrated that a MYB-related transcription factor, MYB2, may regulate genes which are induced by water stress in *Arabidopsis* (Urao et al. 1993). It was also reported that *Glabroos1* (*GL1*), another MYB-related gene, is involved in the formation of trichomes of leaves in *Arabidopsis* (Urao et al. 1993). All the above suggests that the *ADH1* may have similar functions in *A. annua*.

In addition, the *ADH1* promoter was predicted to carry other functional *cis*-elements. Two ARE (TGGTTT/TTTGGT) were found at the positions −868 to −863 and −28 to −23, which is the *cis*-element involved in the anaerobic induction. Further analysis of the promoter sequence showed that *ADH1* may relate to the seed development since two Skn-1_motif (GTCAT; position −72 to −76 and 20–24) and one GCN4-motif (TGAGTCA; position −144 to −150), which are essential for endosperm expression, were all detected in the *ADH1* promoter.

A number of light responsive elements are presented in *ADH1* promoter sequence, including two ACE (ATTTGCTAAA; position −216 to −207 and 37–46), two Box 4 (ATTAAT; position −251 to −256 and −120 to −125), two GATA-box (TGATAA; position −283 to −288 and −3 to +3), one TCT-motif (CATTCT; position −502 to −507), one GA-motif (AAAGATGA; position −720 to −727), one GAG-motif (AGAGATG; position −624 to −630), one Box III (TCCCATTTAC; position −770 to −779) and two Sp1 (CCCCCCACTT/CCCTCCCTTG; position −752 to −761 and −852 to −861). This suggests that the *ADH1* may be regulated by light.

Analysis of the activity of *ADH1* promoter

To further analyze the expression pattern of *ADH1* in *A. annua*, the *ADH1* promoter sequence was inserted into the vector pCAMBIA1391Z carrying the *GUS* reporter gene. The recombination plasmid was introduced into *A. annua* plants as described above. The positive transgenic plants were selected by PCR. GUS staining was only observed in the glandular secretory trichomes of leaf primordia (Fig. 6a), young leaves (Fig. 6b–j), and stems of the transgenic *A. annua* plants (Fig. 6m, n). Besides, we did not find any GUS signal in old leaves and roots (Fig. 6k, l, o, p).

Response of the *ADH1* promoter to hormones

It is well established that the expression of *ADS, CYP71AV1, DBR2* and *ALDH1* are induced by MeJA in *A. annua* (Wu et al. 2011; Liu et al. 2016). Besides, the transcript levels of *HMGR, FDS* and *CYP71AV1* were upregulated when *A. annua* plants were exposed to the ABA treatment (Jing et al. 2009).

We treated *A. annua* seedlings with MeJA and ABA separately as described above, and the transcript level of *ADH1* gene was valued using RT-q-PCR. The relative expression levels at different time were normalized to the expression at 0 h. Compared to 0 h, the transcript level of *ADH1* gene reached twofold expression at 9 h after spraying with MeJA (Fig. 7a). The relative expression of *ADH1* gene was induced approximate 2.5-fold of the control at 6 h in the ABA responsiveness experiment (Fig. 7b).

Table 2 Putative *cis*-acting elements involved in the promoter of *ADH1*

Motif	Nucleotide sequence (50–30)	Description
G-box	GGTACAC	*Cis*-acting regulatory element involved in MeJA and light responsiveness
E-box	CATGTG/CATTTG/CAACTG/ CATATG	*Cis*-acting regulatory element involved in MeJA and ABA responsiveness
MBS	CAACTG/TAACTG	MYB binding site involved in drought-inducibility
ARE	TGGTTT/TTTGGT	*Cis*-acting regulatory element essential for the anaerobic induction
Skn-1_motif	GTCAT	*Cis*-acting regulatory element required for endosperm
GCN4-motif	TGAGTCA	*Cis*-acting regulatory element required for endosperm
ACE	ATTTGCTAAA	*Cis*-acting element involved in light responsiveness
Box 4	ATTAAT	Part of a light responsive element
GATA box	TGATAA	Part of a light responsive element
TCT-motif	CATTCT	Part of a light responsive element
GA-motif	AAAGATGA	Part of a light responsive element
GAG-motif	AGAGATG	Part of a light responsive element
Box III	TCCCATTTAC	Part of a light responsive element
Sp1	CCCCCCACTT/CCCTCCCTTG	Part of a light responsive element

In addition, the result showed stronger GUS staining after hormonal treatments compared with the control (Fig. 8a–d). Observation obtained shows that the expression of *ADH1* increased quickly to the peak at a point time and then slowly decreased after hormone treatment. Similarly, it was reported that the expression of *MYC2* was upregulated rapidly and peaked at 6 h after MeJA treatment, and then declined gradually until 48 h (Shen et al. 2016). The transcript level of *MYC2* after ABA treatment increased relatively slowly and declined rapidly (Shen et al. 2016). The *ALDH1* response to MeJA treatment was also quick; it peaked at 3 h and then declined (Liu et al. 2016). The expression pattern of *ADH1* after hormone treatment is similar to that of *MYC2* and *ALDH1*.The results indicate that *ADH1* gene shows hormone–dependent expression, which in agreement with the analysis that the *ADH1* promoter carries a few motifs involved in hormonal induction.

MYC2 and ERF1 activate the transcription of *ADH1* in vivo

To investigate the regulation of *ADH1* transcription, a dual-LUC assay was performed in tobacco leaves. MYC2 activated the expression of *ADH1* promoter by showing a much higher value of relative LUC activity than the control (Fig. 9). ERF1 can also activate the expression of *ADH1* promoter for the value of relative LUC activity reached 2.9 times compared with the control (Fig. 9).Together, these

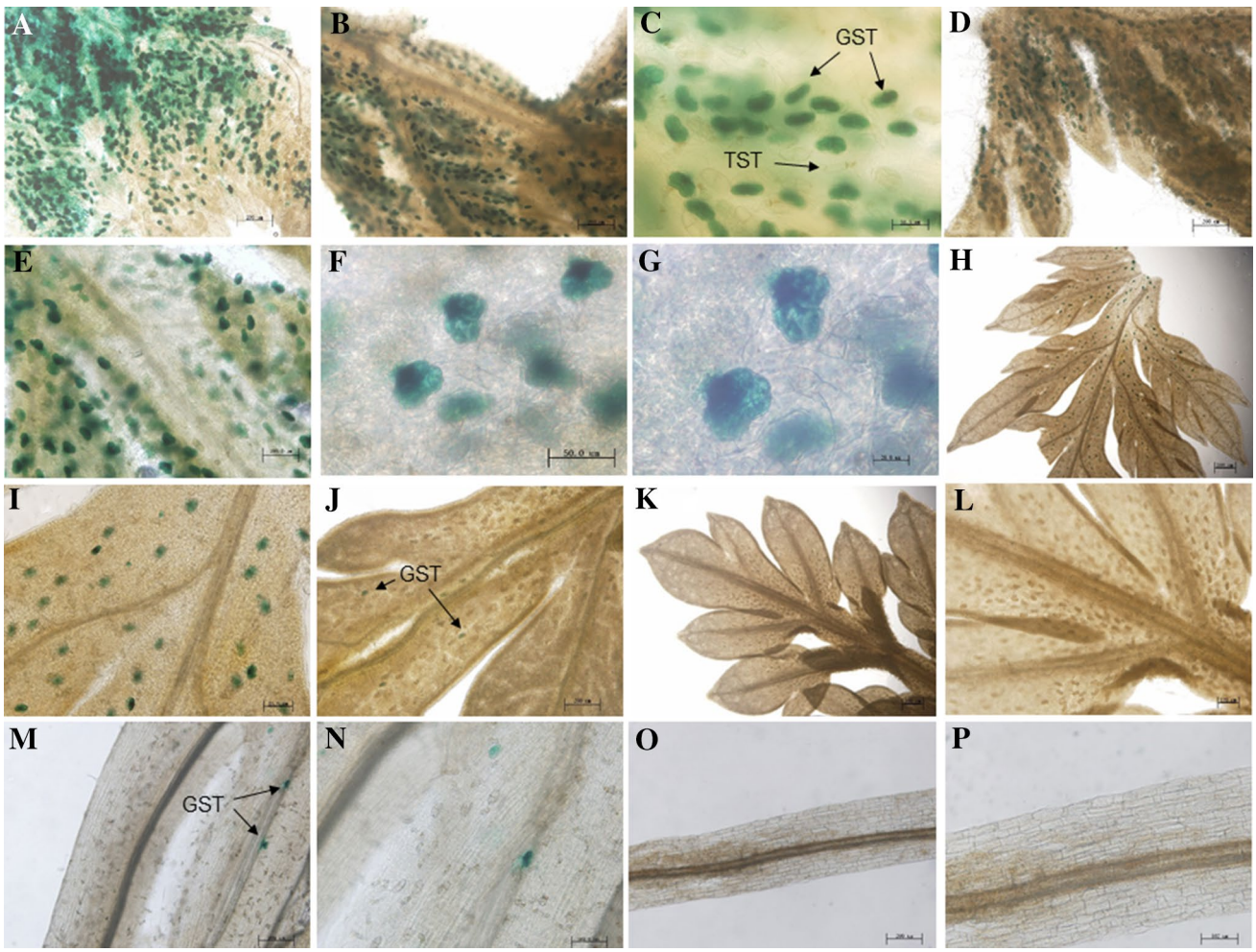

Fig. 6 GUS staining of 1 month-old transgenic *A. annua* plants: **a** leaf primordia of 1 month-old transgenic *A. annua* plant; **b** expanded leaf at node 1 from a 1 month-old transgenic *A. annua* plant; **c** magnification (×4) of expanded leaf at node 1; **d** expanded leaf at node 2 from a 1 month-old transgenic *A. annua* plant; **e** magnification (×2) of expanded leaf at node 2; **f** magnification (×4) of GSTs in expanded leaf at node 2; **g** magnification (×8) of GSTs in expanded leaf at node 2; **h** expanded leaf at node 3 from a 1 month-old transgenic *A. annua* plant; **i** magnification (×2) of expanded leaf at node 3; **j** expanded leaf at node 4 from a 1 month-old transgenic *A. annua* plant; **k** old leaf of 1 month-old transgenic *A. annua* plant; **l** magnification (×2) of old leaf; **m** GSTs in stems; **n** magnification (×2) of GSTs in stems; **o** roots of 1 month-old transgenic *A. annua* plant; **p** magnification (×2) of roots. *GST* glandular secretory trichome and *TST* T-shaped trichome

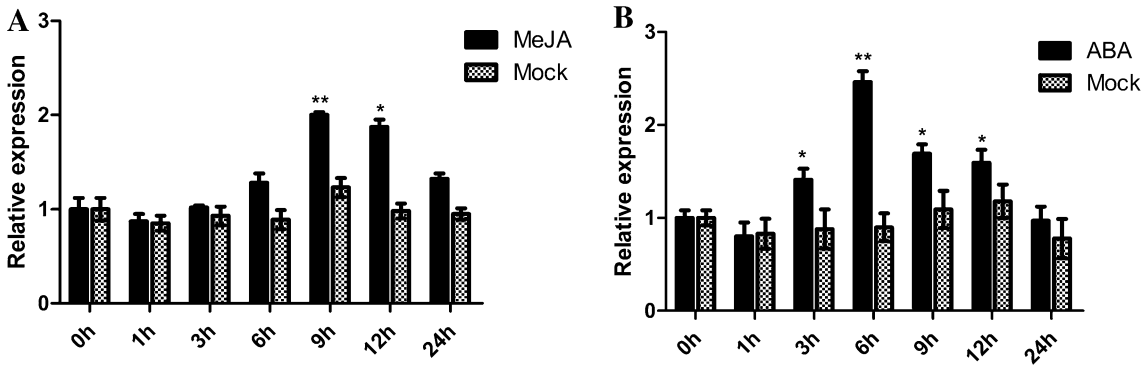

Fig. 7 Expression patterns of *ADH1* in response to hormones: **a** relative expression of *ADH1* in *A. annua* after treatment with MeJA; **b** relative expression of *ADH1* in *A. annua* after treatment with ABA. β-actin was used as the control gene. *Error bars* represent ±SD ($n=3$). Statistical significance was determined by two-tailed Student's *t* test with *P* values; $*P<0.05$; $**P<0.01$. *Asterisks* above the *bar* represent the significance of the difference between hormone-treated and control plants

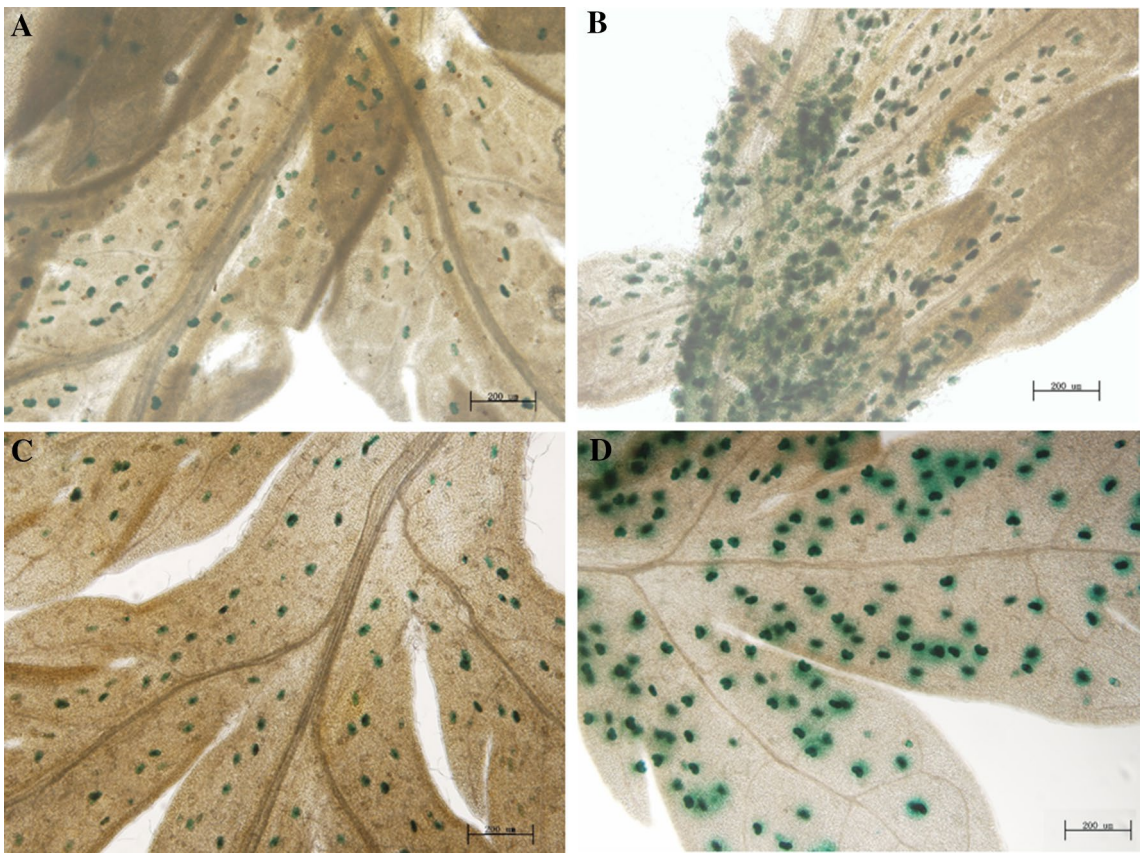

Fig. 8 GUS staining of leaves after the hormonal treatments: **a** leaf of transgenic plant without MeJA treatment (control); **b** leaf of transgenic plant with MeJA treatment; **c** leaf of transgenic plant without ABA treatment (control); **d** leaf of transgenic plant with ABA treatment

results suggest that MYC2 and ERF1 may recognize specific *cis*-regulatory sequences in the *ADH1*promoter, and then activate the transcription of *ADH1*.

Conclusion

Expression pattern of *ADH1* indicates that *ADH1* shows high expression in young leaves as the same as that of other crucial enzyme genes involved in biosynthetic pathways of

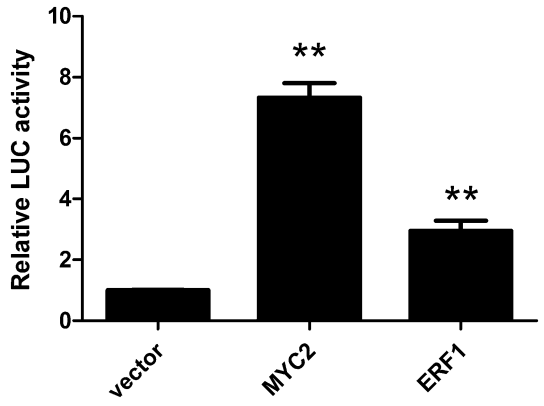

Fig. 9 Transient dual-LUC analysis showing MYC2 and ERF1 activation of *ADH1* in tobacco leaves. The values were obtained by calculating the ratio of LUC activities to REN activities (LUC/REN). *Error bars* represent ±SD ($n=3$). Statistical significance was determined by Student's *t* test; $^{**}P<0.01$

artemisinin. Further analysis of *ADH1* promoter sequence reveals that the expression of *ADH1* may be complexly regulated. The GUS staining analysis shows that the ADH1 is located to the glandular secretory trichomes of young tissues, which are similar to the GUS staining pattern of *ADS* promoter. Not only does the expression of *ADH1* in yeast increase the production of artemisinic acid observably, but also *ADH1* is worth to be considered as an efficient participant in the biosynthesis of artemisinin. Besides, the GST-specific promoter (*proADH1*) may be a good substitute for CaMV 35S promoter to drive the expression of a known gene in the glandular secretory trichomes. Moreover, the expression of *ADH1* can be obviously improved by various hormones, providing an evident for the enhancement of artemisinin content by treating with hormones. We may conclude that overexpression of *ADH1* gene in *A. annua* plants will most likely has a positive effect on artemisinin biosynthesis.

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