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Molecular cloning, characterization, and promoter analysis of the isochorismate synthase (*AaICS1*) gene from *Artemisia annua*[*]

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Abstract: Isochorismate synthase (ICS) is a crucial enzyme in the salicylic acid (SA) synthesis pathway. The full-length complementary DNA (cDNA) sequence of the *ICS* gene was isolated from *Artemisia annua* L. The gene, named *AaICS1*, contained a 1710-bp open reading frame, which encoded a protein with 570 amino acids. Bioinformatics and comparative study revealed that the polypeptide protein of *AaICS1* had high homology with *ICS*s from other plant species. Southern blot analysis suggested that *AaICS1* might be a single-copy gene. Analysis of the 1470-bp promoter of *AaICS1* identified distinct *cis*-acting regulatory elements, including TC-rich repeats, MYB binding site (MBS), and TCA-elements. An analysis of *AaICS1* transcript levels in multifarious tissues of *A. annua* using quantitative real-time polymerase chain reaction (qRT-PCR) showed that old leaves had the highest transcription levels. *AaICS1* was up-regulated under wounding, drought, salinity, and SA treatments. This was corroborated by the presence of the predicted *cis*-acting elements in the promoter region of *AaICS1*. Overexpressing transgenic plants and RNA interference transgenic lines of *AaICS1* were generated and their expression was compared. High-performance liquid chromatography (HPLC) results from leaf tissue of transgenic *A. annua* showed an increase in artemisinin content in the overexpressing plants. These results confirm that *AaICS1* is involved in the isochorismate pathway.

Key words: Salicylic acid; *Artemisia annua* L.; Quantitative real-time polymerase chain reaction (qRT-PCR); Isochorismate synthase

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1 Introduction

The annual plant *Artemisia annua* L., belonging to the Asteraceae family, has attracted attention as the source of artemisinin, which is used as an alternative to quinoline drugs to treat malaria (Yin *et al*., 2012). At present, *A. annua* is the sole commercial source of artemisinin, but the content of artemisinin in its flowers and leaves is just 0.05%–1.00% of the dry weight depending on the species (Duke *et al*., 1994; Delabays *et al*., 2001; Abdin *et al*., 2003; Shen *et al*., 2012; Patra and Srivastava, 2014). Because of its low concentration, numerous efforts have been made to improve artemisinin production to facilitate the commercialization of *A. annua*.

Salicylic acid (SA) is a type of phenolic phytohormone, which has been shown to be a significant player in plant growth and development. Compared with controls, 1.0 mmol/L SA could increase the content

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of artemisinic acid, artemisinin, and dihydroartemisinic acid (Pu *et al*., 2009; Aftab *et al*., 2010; Guo *et al*., 2010). In addition, the artemisinin content of cell suspensions of *A. annua* treated with 20–50 mg/L (140–360 mmol/L) SA increased significantly, and the highest content was nearly 6-fold higher than that of controls (Baldi and Dixit, 2008). Genes in the SA biosynthetic pathway are considered to be involved in SA production, plant development, and secondary metabolism (Loake and Grant, 2007).

In the biosynthesis of SA, isochorismate synthase (ICS; EC 5.4.4.2) catalyzes the conversion of isochorismate to yield SA (van Tegelen *et al*., 1999). There are two very similar *ICS* genes in *Arabidopsis thaliana*, *AtICS1* (AT1G74710) and *AtICS2* (AT1G 18870) (Haas *et al*., 2004). These genes are differently regulated, and only *AtICS1* is induced by SA-mediated defense (Wildermuth *et al*., 2001; Garcion *et al*., 2008). From studies of the *ics1* mutant, it is clear that the absence of *ICS1* would lead to a low accumulation of SA (Wildermuth *et al*., 2001).

The effect of *AaICS1* on the growth process is not fully understood. Therefore, it is important to determine how *AaICS1* influences the growth of *A. annua* in terms of plant biochemistry and physiology. With the further aim to improve SA content and overcome the low yield of artemisinin, it is crucial to determine how *AaICS1* affects the secondary metabolic products by way of prospective metabolic engineering in *A. annua*.

In this study, we isolated the promoter and full-length complementary DNA (cDNA) of *AaICS1*. The transcription profiles of *AaICS1* under ambient stresses including wounding, drought, salinity, and SA treatments were analyzed. *AaICS1* overexpression and RNA interference transgenic plants were also studied.

2 Materials and methods

2.1 Plant materials and growth condition

Seeds of *A. annua* were provided by the School of Life Sciences, Southwest University, Chongqing, China. The parent sweet *A. annua* was used to provide cuttings for asexual reproduction. The branches were allowed to grow at (25±2) °C at 70%–80% relative humidity and with a 16-h photoperiod in plastic potted trays in the greenhouse. The cuttings were irrigated with distilled water.

Total RNA was isolated from leaves, flowers, stems, and buds of *A. annua* using plant RNA isolation reagent (TaKaRa, Japan) in accordance with the manufacturer's instructions. A NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, MA, USA) was used to measure and examine the quality and concentration of RNA samples. Total RNA was used for reverse transcription to obtain cDNA.

2.2 Generation of the full-length cDNA of *AaICS1*

Total RNA extracted from the leaves was reverse-transcribed to cDNA. The reverse transcription was performed in accordance with the following conditions: 20 min at 37 °C and 5 s at 85 °C. The cDNA was then stored as the polymerase chain reaction (PCR) template for the full length of *AaICS1* amplification.

Based on the data of transcriptome sequencing of *A. annua* in our lab and the nucleotide sequences of *A. thaliana* published by the National Center for Biotechnology Information (NCBI), two primers were designed (*AaICS1*-R and *AaICS1*-F; Table 1). *AaICS1* cDNA sequence was isolated under the following conditions: initial denaturation of template DNA for 5 min at 94 °C, followed by 35 cycles of amplification (94 °C for 30 s, 55 °C for 40 s, and 68 °C for 2 min) and 68 °C for 10 min. KOD-Plus-neo (TOYOBO, Japan) enzyme was used in the PCR. The final products were purified and ligated into pJET1.2 vectors (Thermo Scientific-Fermentas, USA) and sequenced. The primers involved in the study are listed in Table 1.

2.3 Isolation of the promoter of *AaICS1*

Two primers (*AaICS1*-promoter-R and *AaICS1*-promoter-F; Table 1) were designed according to the genome sequencing data of *A. annua*, to obtain the promoter sequences of *AaICS1*. Genomic DNA was extracted from the leaves. PCR was performed for 5 min at 94 °C, followed by 35 cycles of amplification (94 °C for 30 s, 55 °C for 40 s, and 68 °C for 2 min 30 s) and 68 °C for 10 min. The PCR amplification was implemented using a KOD-Plus PCR kit (TOYOBO, Japan). Then the purified PCR products were ligated into a pJET1.2 vector and sequenced. Table 1 lists all the primers involved in this study.

Table 1 Primers used for PCR amplification in this study

Primer	Purpose	Primer sequence (5'→3')
AaICS1-F	Clone	TCAAGCCTACTCACATGGGACAT
AaICS1-R	Clone	CGTAAAATCTAAGGCTATCTACCATAAATC
AaICS1-promoter-F	Clone	AATGATTTGGATGGAAAGGAGAC
AaICS1-promoter-R	Clone	AATGGATTTTGGTTGTGGCTG
AaICS1-Rt-F	qRT-PCR	AGAACTCCTGTTGGGATGACCGAAA
AaICS1-Rt-R	qRT-PCR	TGTTGATTCGTGCTTGTCGTCTTCC
β-Actin-F	qRT-PCR	CCAGGCTGTTCAGTCTCTGTAT
β-Actin-R	qRT-PCR	CGCTCGGTAAGGATCTTCATCA
AaICS1-southern-F	Southern	TCCAATAGAAGAGGAAGTAGAA
AaICS1-southern-R	Southern	TTGATAGAACGCCAATCATGAAGT
AaICS1-pET-F	Recombinant	CGCGAGCTCATGGCAGCCACAACCAAAATCCA
AaICS1-pET-R	Recombinant	CCGCTCGAGCTTTTCGGACAACAGTGCCTTCTT

*Sac*I and *Xho*I sites are underlined

2.4 Bioinformatics and comparative genome analysis

Multiple sequence alignments were determined using the software Vector NTI Advance® 11.5. Deduced amino acids and nucleotide sequences were predicted using the NCBI website. Phylogenetic analysis of *AaICS1* and its homologues was carried out with MEGA 5.2 software. The *cis*-acting elements were forecasted after submitting *AaICS1* 5' sequence data to PLANTPAN, PLACE, and PlantCare databases.

2.5 Analysis of *AaICS1* expression by real-time PCR

Two-month-old *A. annua* cuttings were used for stress treatments. The leaves were punched using sterile blunt-nosed forceps to make wounds. Drought stress was induced by immersing the roots in 15% (0.15 g/ml) polyethylene glycol 6000 (PEG6000) during the experiment, with double distilled water added to the controls. For salt treatment, the cuttings were irrigated with 100, 200, or 300 mmol/L NaCl solutions while the control treatment was served with double distilled water. For SA treatment, the leaves of the cuttings were sprayed with either 0.1 or 1.0 mmol/L SA. The control treatment was served with double distilled water. Leaves were collected at different time during all stress treatments, then immediately frozen in liquid nitrogen and stored at −80 °C.

Quantitative PCR was designed using a Lightcycler® 96 PCR system (Roche, Germany) according to the manufacturer's protocol (Zhang *et al*., 2013). All samples were used to obtain the first-strand cDNA. Quantitative real-time PCR (qRT-PCR) was carried out with a Super Real PreMix Plus (SYBR Green) kit (Tiangen Biotech, Beijing, China). Every sample was denatured for 10 min at 95 °C, followed by 40 cycles of PCR amplification (15 s at 95 °C, 15 s at 58 °C, and 25 s at 72 °C) and a final extension for 10 min at 72 °C. Each sample was applied in triplicate. The reference gene was *A. annua β-actin*. All the primers involved in qRT-PCR are listed in Table 1.

2.6 Transformation vector construction

Transformation vectors were constructed using a Gateway® cloning kit (Invitrogen, Waltham, MA, USA). The pHB vector was used for overexpression of the *AaICS1* gene, and the pHELLSGATE 1.2 vector was used for the interference *AaICS1* gene.

2.7 Plant transformation

After immersing the *A. annua* seeds in 75% ethanol for 1 min and in 10% NaClO for 10 min, they were washed with sterile distilled water more than four times. These germ-free seeds were germinated on 4.4 g/L Phytagel-solidified MS_0 medium (Murashige and Skoog, 1962) and cultured in a chamber under controlled conditions. Once the seedlings had grown to 4–5 cm tall, the leaves were cut into 0.5 cm diameter discs that were co-cultivated for 3 d at 25 °C with *Agrobacterium tumefaciens* strain EHA105 (McCormick *et al*., 1986). After two to three months of growth, the seedlings were transplanted into the greenhouse.

2.8 Determination of artemisinin content by HPLC

Twenty-week-old greenhouse-grown plants were used to quantify artemisinin content using fresh weight pooled leaf samples (counted from the apical meristem) by HPLC. The treatment was carried out three times. Until used for artemisinin analysis, samples were stored at −80 °C. Crude artemisinin extract was isolated in accordance with the modified protocol from Graham *et al*. (2010). Samples were put in 10-ml tubes, immersed in 3 ml of chloroform, and then shaken gently for 5 min. The solvent was evaporated at room temperature in a fume hood until the sample was absolutely dry. After adding 3 ml of methanol, the solution was mixed gently and treated with an ultrasonic processor. After filtering the solution through a 0.45-μm Sartorious® membrane, 200 μl of sample was loaded into the HPLC. A Waters 2695 HPLC system coupled with a Waters 2420 evaporative light scattering detector (ELSD) was used to analyze the samples. The HPLC conditions were as follows: amethyst 5-μm C18-H 120A column, water/methanol (25:75, v/v) mobile phase, 1 ml/min flow rate. The ELSD was carried out under the optimized conditions of a drift-tube temperature of 40 °C and a nebulizer-gas pressure of 206.8 kPa (30 psi). The standard was authentic artemisinin (Sigma). We injected a volume of 20 μl of each sample and analyzed the results using Empower.

3 Results

3.1 Cloning of the *AaICS1* gene

The *AaICS1* cDNA sequence was authenticated. Its opening reading-frame (ORF) was 1710 bp and encoded a 570-amino acid peptide (Fig. 1). The molecular weight was calculated as 63.81 kDa and the isoelectric point (pI) was predicted to be 6.88. Alignment revealed that *AaICS1* had 57.7% homology with the *A. thaliana ICS1* gene, suggesting that it could be the *A. annua ICS1* gene. The *AaICS1* sequence was deposited in the GenBank database under accession number KP100111.

Multialignment with other plant *ICS* polypeptides revealed that the deduced AaICS1 protein showed considerably high sequence homology (Fig. 2). The *AaICS1* gene showed 63.7% sequence similarity to *Populus tremuloides* (ACX46384), 63.3% to *Vitis vinifera* (XP_002267681), 61.6% to *Solanum lycopersicum* (NP_001234794), 61.4% to *Capsicum annuum* (AAW66457), 61.1% to *Glycine max* (XP_003522193), and 61.0% to *Fragaria vesca* subsp. vesca (XP_004301164). Among *AaICS1* polypeptide and *ICS* polypeptides from other species, there were different N-terminal domains among the first 30 amino acids, while the subsequent downstream residues in all *ICS* polypeptides were highly conserved.

To investigate the evolutionary relationships of *AaICS1*, several common species were chosen for species tree analysis. Based on the level of amino acids, an *ICS* phylogenic tree was constructed and calculated using MEGA 5.2 software. The *AaICS1* gene was clustered with high probability and high bootstrap values with *AtICS1*, *AtICS2*, *VvICS*, *SlICS*, *PtICS*, and *CrICS*. The inferred phylogenic tree (Fig. 3) displayed conserved *ICS* orthologs with the relative genetic distance (0.05 amino acid substitution per site).

The secondary structure of the *AaICS1* polypeptide was determined by SOPMA analysis. *AaICS1* consists predominantly of random coils (40.07%), and α helices (39.54%) together with β turns (4.75%) and extended strands (15.64%).

3.2 Prediction of *AaICS1* promoter region

A 1470-bp DNA fragment upstream from the *AaICS1* gene initiation codon (ATG) in *A. annua* was amplified from the genomic DNA. The sequence contained a predicted transcription start site (TSS, +1), which was found to be located 39 bp upstream of the start codon. At position −26, a TATA box was located with respect to TSS.

Several *cis*-acting elements were found (Fig. 1, Table 2). The *AaICS1* promoter contained many conserved *cis*-acting fragments in the 5' flanking regions related to chorismic acid biosynthesis of angiosperms.

A typical *cis*-acting element WRKY (TGAC) proven to be capable of activating the early genes in plant defence signaling (Eulgem *et al*., 1999), was found at the position −322, −457 of the *AaICS1* promoter. Analysis of the promoter boxes led to the finding of several *cis*-elements related to meristem-specific, stress, hormone, and numerous light response genes (Fig. 1, Table 2). Bioinformatic analysis

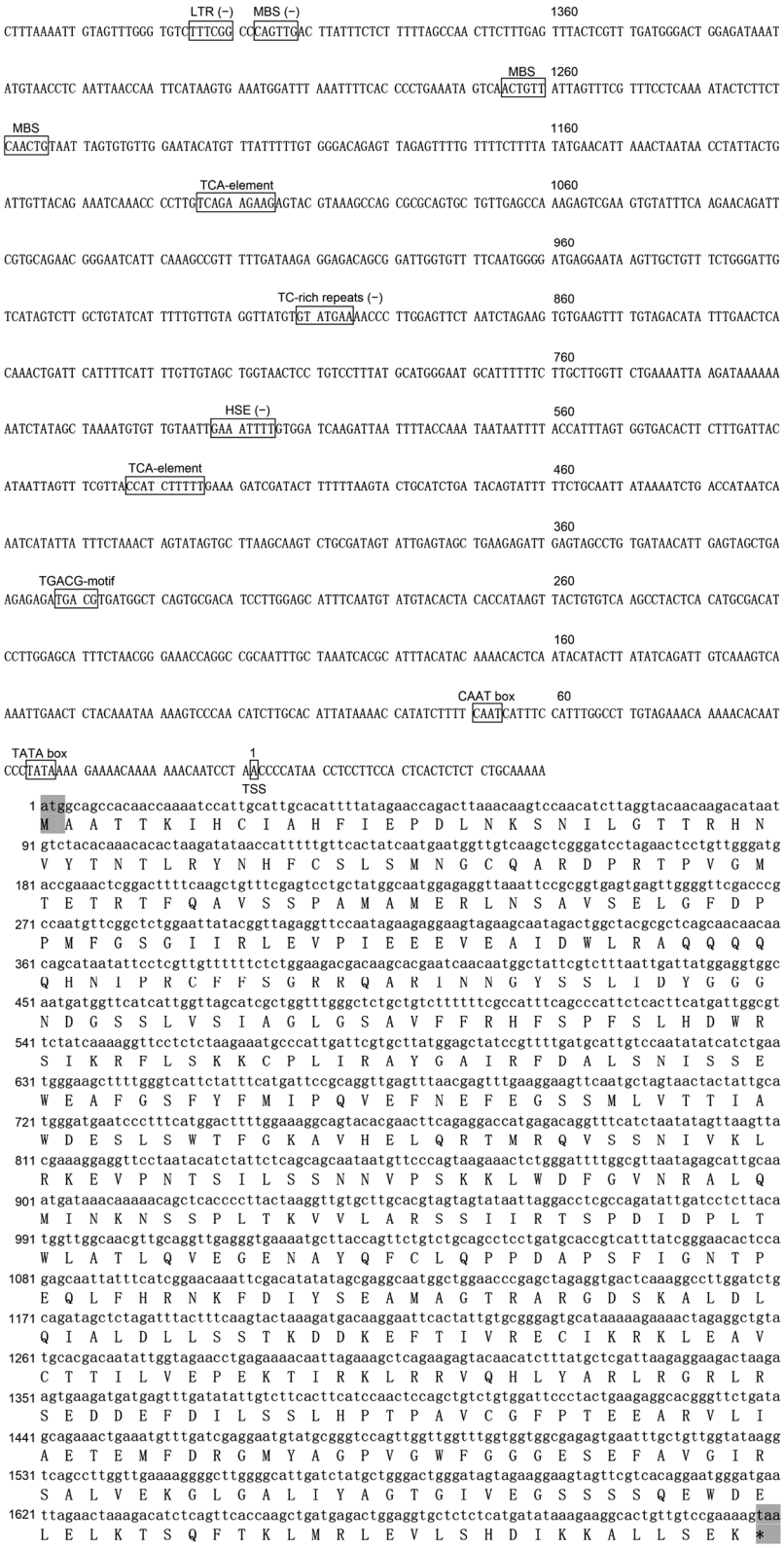

Fig. 1 *AaICS1* promoter, the nucleotide sequence and the deduced amino acid sequence of the *AaICS1* gene

Motifs similar to the putative core promoter consensus sequences and the previously predicted *cis*-acting elements are boxed and their names given. Numbering starts from the potential transcription start sites. Stop and start codons are shown in grey

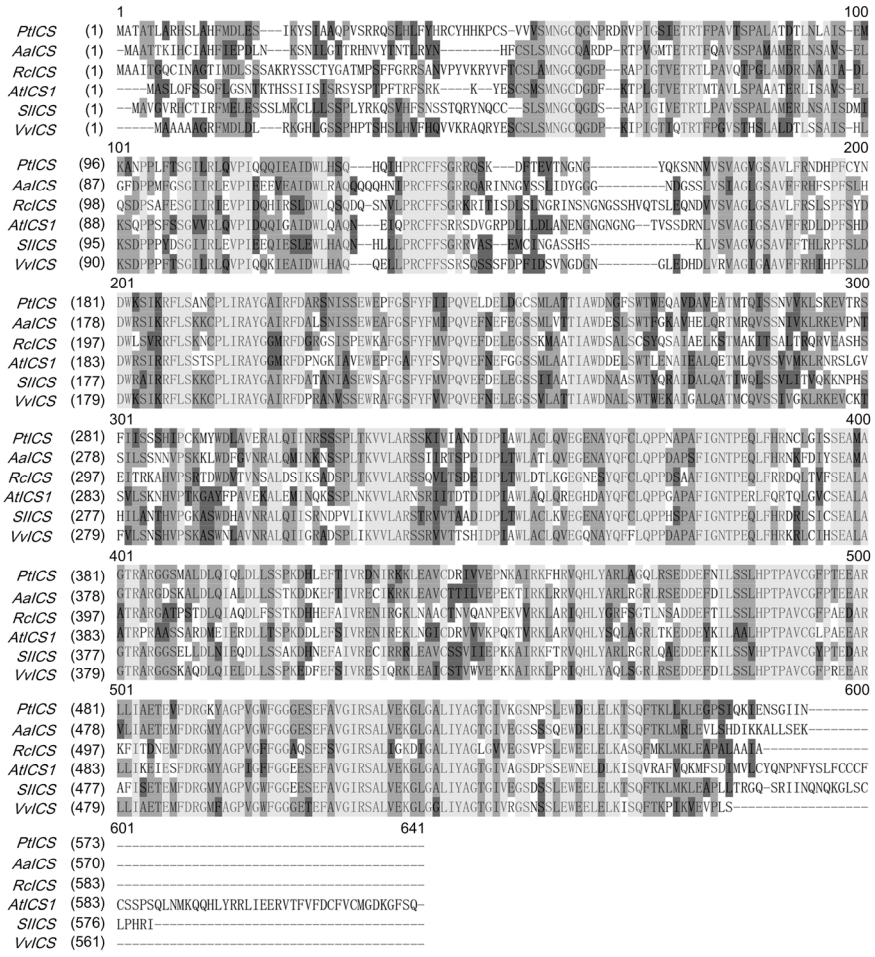

Fig. 2 Multiple alignment of amino acids translated from several isochorismate synthase gene cDNAs

The *ICS* sequences are as follows: *Populus tremuloides* (ACX46384), *Rubia cordifolia* (ABK79678), *Solanum lycopersicum* (NP_001234794), *Arabidopsis thaliana* (AT1G74710), *Vitis vinifera* (XP_002267681), and *Artemisia annua* (KP100111). Matching amino acids are shown in capital letters against a light grey background. Less similar amino acids are shown in capital letters against a grey or dark grey background. Different amino acids are shown in black capital letters against a white background

showed that several motifs were essential for some putative *cis*-acting motifs and tissue specific expression involved in MYB-like transcription factor binding sites.

3.3 Expression analysis of *AaICS1* in different tissues

It was considered important to determine the expression patterns of *AaICS1*, since it is the first and rate-limiting enzyme in the isochorismate biosynthesis of SA. Southern blot analysis suggested that *AaICS1* might be a single-copy gene (Fig. 4). RNAs extracted from leaves, stems, roots, flowers, and buds were used to examine expression levels of *AaICS1* by quantitative PCR. The results showed that *AaICS1* was most highly expressed in old leaves, followed by the buds and young leaves. The lowest expression was found in roots (Fig. 5).

3.4 Expression analysis of *AaICS1* under different treatments

Recent advances in studies of the signaling pathways of plants have indicated that the variation in plant metabolites induced by elicitors can be useful as a prediction tool for prospective pathway regulation and engineering. The expression of genes in the SA biosynthesis pathway can be induced by different environmental stresses, such as SA treatment (Hunter

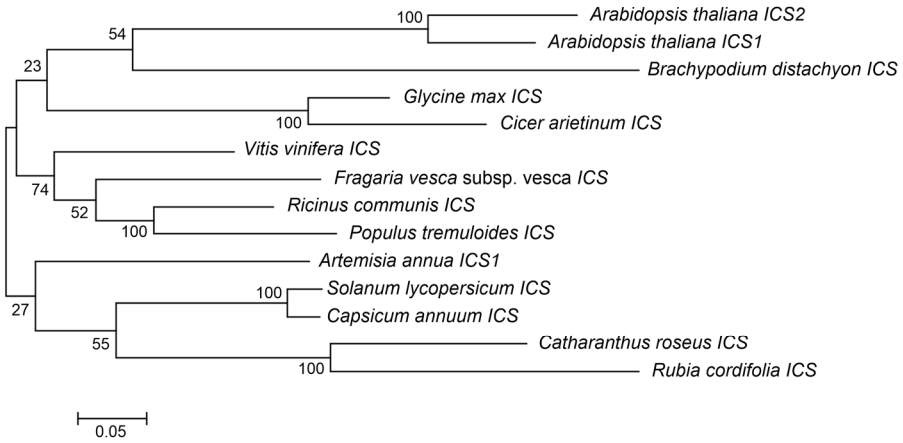

Fig. 3 Phylogenetic tree of the AaICS1 protein together with *ICS*s from other species

The tree was constructed using *AaICS1* and the following sequences: *Arabidopsis thaliana* (AT1G74710), *Arabidopsis thaliana* (AT1G18870), *Brachypodium distachyon* (XP_003578021), *Glycine max* (XP_003522193), *Cicer arietinum* (XP_004514127), *Ricinus communis* (XP_002511526), *Artemisia annua* (KP100111), *Vitis vinifera* (XP_002267681), *Solanum lycopersicum* (NP_001234794), *Capsicum annuum* (AAW66457), *Fragaria vesca* subsp. vesca (XP_004301164), *Catharanthus roseus* (Q9ZPC0), *Populus tremuloides* (ACX46384), and *Rubia cordifolia* (ABK79678)

Table 2 Putative *cis*-acting regulatory elements identified in the promoter

cis-Element	Position	Signal sequence	Putative function
TATA-box	26 (+)	TATA	Core promoter element around −30 of transcription start
TGACG-motif	321 (+)	TGACG	TGACG-motifs are involved in transcription of several genes by auxin and/or salicylic acid
HES	598 (−)	AAAAAATTTC	*cis*-Acting element involved in heat stress responsiveness
TCA-element	507 (+)	TCAGAAGAGG	*cis*-Acting element involved in salicylic acid responsiveness
	1098 (+)	CCATCTTTTT	
TC-rich repeats	888 (−)	GTTTTCTTAC	*cis*-Acting element involved in defense and stress responsiveness
MBS	1227 (+)	CAACTG	MYB binding site
	1263 (+)	TAACTG	
	1395 (−)	CAACTG	
LTR	1343 (+)	CCGAAA	*cis*-Acting element involved in low-temperature responsiveness

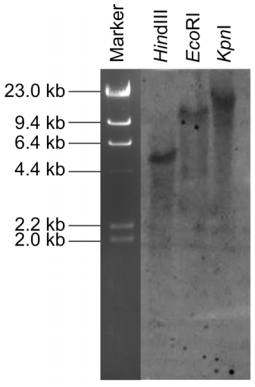

Fig. 4 Southern blot of *A. annua*

Molecular size markers are shown on the left

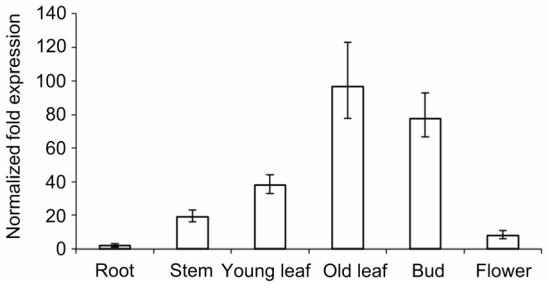

Fig. 5 Expression profiling of *AaICS1* in different tissues of *A. annua*

The ratio of *AaICS1* transcripts against *β-actin* was given as "1.0". The *y*-axis indicates the relative expression levels of *AaICS1*/β-actin in different tissues. Data are expressed as mean±standard deviation (SD) (*n*=3)

et al., 2013; Sadeghi *et al*., 2013). The predicted *cis*-acting elements in the *AaICS1* 5' flanking region suggested that *AaICS1* might respond to SA, MeJA, heat, cold, wounding, drought, and other stresses. In this study, the expression patterns of *AaICS1* under different treatments were obtained by quantitative PCR.

To investigate the reliance of *AaICS1* expression on SA, two different concentrations of SA (0.1 and 1.0 mmol/L) were applied to *A*. *annua* plantlets. *AaICS1* transcription levels were monitored at different time points. *AaICS1* was more effectively induced by 0.1 mmol/L SA. At 4 h, *AaICS1* was noticeably induced and reached its highest level 8–12 h after treatment, followed by a decline in expression. The higher concentration of SA had a more rapid effect on *AaICS1* expression, peaking at 2 h then decreasing slightly (Fig. 6a). The results suggested a crucial role of SA in the defense response against abiotic stimuli and pathogens. The *AaICS1* gene could be induced by exogenous SA, resulting in a greater supply of key precursors for defense compounds, like propanoids and flavonoids.

To investigate the salinity-dependence of *AaICS1*, various salt concentrations (100, 200, and 300 mmol/L NaCl) were applied to *A*. *annua* plantlets. *AaICS1* was markedly induced at 2 h with the 100 and 200 mmol/L salt treatments. The 200 mmol/L concentration led to a high expression of *AaICS1* after 2 h, which then declined sharply. The 300 mmol/L treatment caused a low level of expression similar to that of the controls at all sampling times (Fig. 6b).

To examine the activity of *ICS* in *A*. *annua* plants affected by wounding, we checked the expression patterns after leaf injury in a 48-h timeframe. *AaICS1* was markedly induced at 2 h and then declined, but after 4 h expression was still significantly higher than that of the controls (Fig. 6c). The result indicated that *AaICS1*, a key enzyme of the isochorismate pathway, had a great effect on the wounding response in *A*. *annua*.

Quantitative PCR was used to analyze the expression level of *AaICS1* under drought treatment. The transcript level of *ICS* was maximal after 24 h (Fig. 6d). The results indicated that all these stresses

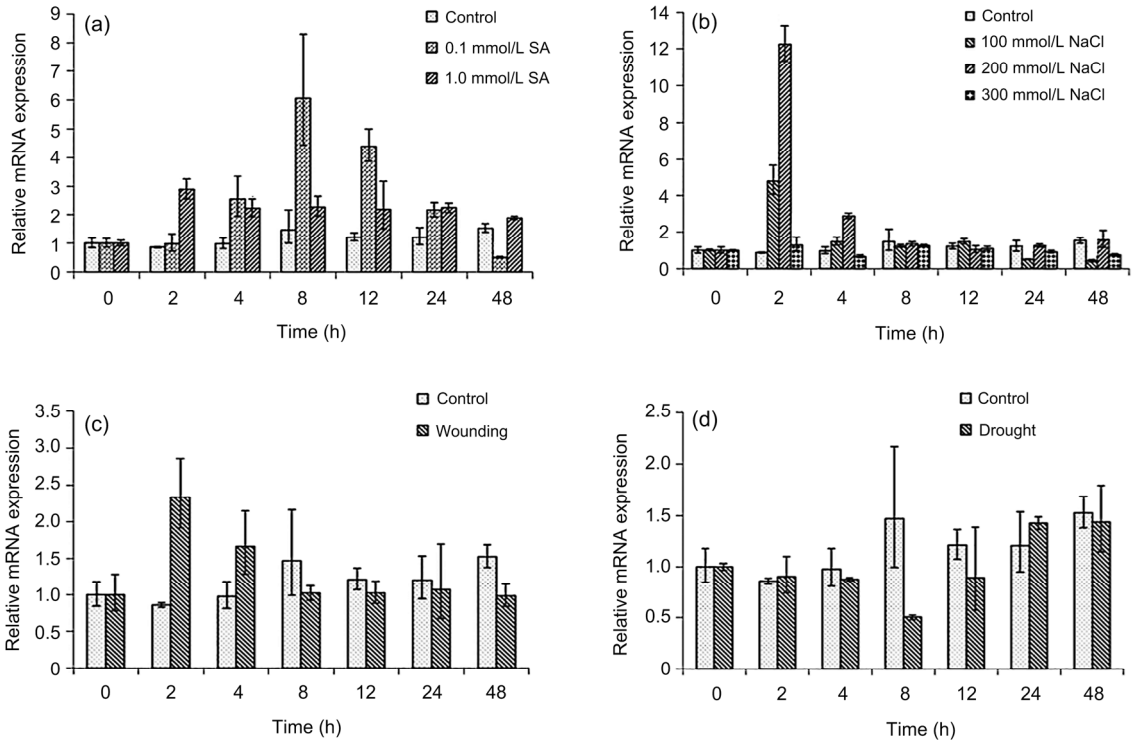

Fig. 6 Expression profiling of *AaICS1* under various treatments

(a) Salicylic acid (SA); (b) Salinity; (c) Wounding; (d) Drought. Data are expressed as mean±SD (n=3 replicates)

played a vital role in response to an internal or external stimulus.

3.5 Expression of *AaICS1* in transgenic plants

To determine whether overexpression of *AaICS1* increases the artemisinin content, we analyzed the expression of *AaICS1* in overexpressing and RNA interference transgenic plants of *A. annua*. The results showed that the expression of the *AaICS1* gene in overexpressing transgenic plants was significantly higher than that of the controls (Fig. 7b). Expression levels in RNA interference transgenic plants were quite low compared with those of the controls (Fig. 7a). However, the expression of the *AaICS1* gene in pHB-*AaICS1*-6 and pHELLSGATE-*AaICS1*-14 was not significantly different from that of the controls (Fig. 7). Researchers have reported that a co-suppression phenomenon can often occur in overexpressing transgenic plants (Yang *et al*., 2008; Banyai *et al*., 2010).

3.6 Estimation of artemisinin content by HPLC-ELSD

In the SA biosynthetic pathway of the transgenic *A. annua*, overexpression of the key enzyme genes could be an effective way to increase the artemisinin content (Han *et al*., 2006; Aquil *et al*., 2009; Banyai *et al*., 2010; Nafis *et al*., 2011). As SA treatment can improve the artemisinin content, the overexpression of a key gene in the SA biosynthesis pathway might increase endogenous SA, leading to improved artemisinin content (Fig. 8).

The present experiments were conducted to observe a change of artemisinin content following the

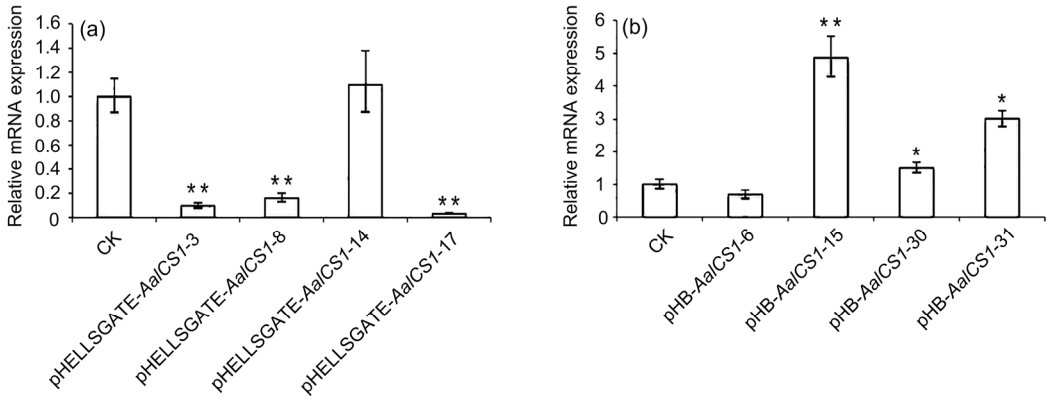

Fig. 7 Real-time PCR results from regenerated *A. annua* plants

(a) *A. annua* transformed with pHELLSGATE 1.2 *AaICS1*; (b) *A. annua* transformed with pHB-*AaICS1*. CK represents non-transgenic plants (control). Data are expressed as mean±SD (n=3). $^{*}P<0.05$, $^{**}P<0.01$, vs. CK

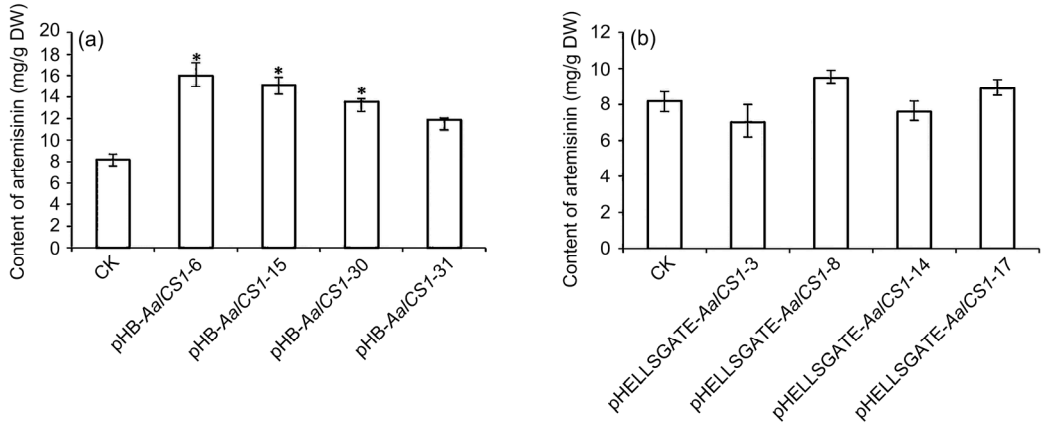

Fig. 8 Content of artemisinin in regenerated *A. annua* plants

(a) *A. annua* transformed with pHB-*AaICS1*; (b) *A. annua* transformed with pHELLSGATE 1.2 *AaICS1*. CK represents the non-transgenic plants (control). DW represents dry weight. Data are expressed as mean±SD (n=3). $^{*}P<0.05$, vs. CK

overexpression of *AaICS1* in transgenic *A. annua*. The transgenic *A. annua* accumulated more artemisinin than the controls. In particular, the highest content of artemisinin was 1.9-fold greater than that of the controls. The *AaICS1* RNA interference transgenic *A. annua* showed no significant difference in artemisinin content compared with the controls.

4 Discussion

Artemisia annua L. is the sole commercial source of artemisinin. The current low yield of artemisinin means that market demand cannot be met, and it is necessary to improve the production of artemisinin. As SA can raise the artemisinin content in *A. annua*, identifying the genes involved in the biosynthesis pathway of SA could be beneficial.

We isolated and characterized the *AaICS1* gene and its promoter. Bioinformatic analysis showed that the *AaICS1* gene belongs to *ICS1* among plants (Fig. 2). The MYB binding site (MBS) and light responsive elements involved in fungal elicitor response were identified and located in the upstream putative *cis*-elements of *AaICS1* (Fig. 1). Based on the present study, wounding, SA treatment, salt, and drought stress can induce the isochorismate pathway in *A. annua*. This implies that the *AaICS1* gene might play a role in the regulation of isochorismate biosynthesis, and could increase the SA content, and thereby improve the artemisinin content.

It has been recognized that accumulated secondary metabolites play crucial roles in stress-resistance of *A. annua*. *ICS*, as a vital gene in the isochorismate pathway, may stimulate the output of secondary metabolism products. Under different stress conditions, including salt (100, 200, and 300 mmol/L), SA (0.1 and 1.0 mmol/L), wounding, and drought, *AaICS1* expression was induced in the leaves of *A. annua* (Fig. 6). We conclude that these elements had a great effect on stimulation in response to the internal or external environment. All these results imply that *AaICS1* expression in *A. annua* is coordinated with environmental stresses.

To conclude, we identified the promoter and the full-length cDNA of *AaICS1* from *A. annua*. Bioinformatic analysis suggested that AaICS1 might have capabilities similar to those of other ICS polypeptides. Quantitative PCR analysis showed that *AaICS1* mRNA accumulated mostly in buds and young leaves. Wounding, salt stress, drought, and SA greatly induced *AaICS1* expression. *AaICS1* might be a significant step and a potential target in the artemisinin pathway of *A. annua*. The cloning and identification of the *AaICS1* gene will assist further transgenic studies aiming to promote SA and artemisinin content in *A. annua*.

In summary, our study proved that overexpressing *AaICS1* can improve artemisinin accumulation in *A. annua* plants. Not all transgenic plants had expression levels that were significantly different from those of the controls, and overexpressing a single gene might not be sufficient for raising the content of artemisinin. Similar results have been found in other studies of plant secondary metabolites (Zhang *et al*., 2004). We conclude that genetic engineering may represent a useful approach for further plant metabolism studies.

Contributors

Lu-yao WANG participated in the design of the study, performed the experiments, analyzed the data, and drafted the manuscript. Ying ZHANG contributed to the sample collection and helped finish figures. Xue-qing FU, Ting-ting ZHANG, and Jia-wei MA helped analyze the data and the results. Li-da ZHANG, Hong-mei QIAN, Ke-xuan TANG, and Shan LI participated in discussion of the results and helped revise the manuscript. Jing-ya ZHAO initiated the project, helped conceive the study, and revised the manuscript.

Compliance with ethics guidelines

Lu-yao WANG, Ying ZHANG, Xue-qing FU, Ting-ting ZHANG, Jia-wei MA, Li-da ZHANG, Hong-mei QIAN, Ke-xuan TANG, Shan LI, and Jing-ya ZHAO declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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中文概要

题　目：青蒿异分支酸合酶基因的克隆及功能分析

目　的：研究青蒿异分支酸合酶的表达模式，评价其对青蒿素含量的影响。

创新点：该研究首次克隆了青蒿异分支酸合酶基因（*AaICS1*），并发现 *AaICS1* 影响青蒿素的合成，为更有效地开发利用青蒿提供了新思路。

方　法：根据青蒿转录组数据，利用聚合酶链式反应（PCR）克隆 *AaICS1* 基因和启动子，并进行多重序列分析和启动子作用元件预测。通过实时定量 PCR（qRT-PCR）对 *AaICS1* 进行表达分析，用 Southern 杂交分析 *AaICS1* 的拷贝数。构建 *AaICS1* 过表达载体和干扰表达载体，转化青蒿获得转基因植株，用高效液相色谱法（HPLC）分析青蒿素含量。

结　论：*AaICS1* 含一个总长为 1710 bp 的完整阅读框，编码 570 个氨基酸，与其它植物的 *ICS* 基因具有较高的相似性。Southern 杂交结果表明 *AaICS1* 为单拷贝（图 4），qRT-PCR 结果显示该基因能够响应伤害、干旱、盐胁迫和水杨酸的处理，处理后基因表达量提高（图 6），和启动子作用元件预测相符。qRT-PCR 结果显示过表达转基因青蒿中 *AaICS1* 表达量提高，干扰转基因青蒿中该基因表达量降低（图 7）。HPLC 显示过表达 *AaICS1* 转基因植株中青蒿素含量提升，最高可达对照的 1.9 倍（图 8）。

关键词：水杨酸；青蒿；实时定量聚合酶链式反应；异分支酸合酶