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Isolation and Characterization of AaWRKY1, an *Artemisia annua* Transcription Factor that Regulates the Amorpha-4,11-diene Synthase Gene, a Key Gene of Artemisinin Biosynthesis

Regular Paper

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Amorpha-4,11-diene synthase (ADS) of *Artemisia annua* catalyzes the conversion of farnesyl diphosphate into amorpha-4,11-diene, the first committed step in the biosynthesis of the antimalarial drug artemisinin. The promoters of *ADS* contain two reverse-oriented TTGACC W-box *cis*-acting elements, which are the proposed binding sites of WRKY transcription factors. A full-length cDNA (*AaWRKY1*) was isolated from a cDNA library of the glandular secretory trichomes (GSTs) in which artemisinin is synthesized and sequestered. *AaWRKY1* encodes a 311 amino acid protein containing a single WRKY domain. *AaWRKY1* and *ADS* genes were highly expressed in GSTs and both were strongly induced by methyl jasmonate and chitosan. Transient expression analysis of the *AaWRKY1–GFP* (green fluorescent protein) reporter revealed that AaWRKY1 was targeted to nuclei. Biochemical analysis demonstrated that the AaWRKY1 protein was capable of binding to the W-box *cis*-acting elements of the *ADS* promoters, and it demonstrated transactivation activity in yeast. Co-expression of the effector construct *35S::AaWRKY1* with a reporter construct *ADSpro1::GUS* greatly activated expression of the *GUS* (β-glucuronidase) gene in stably transformed tobacco. Furthermore, transient expression experiments in agroinfiltrated *Nicotiana benthamiana* and *A. annua* leaves showed that AaWRKY1 protein transactivated the *ADSpro2* promoter activity by binding to the W-box of the promoter; disruption of the W-box abolished the activation. Transient expression of *AaWRKY1* cDNA in *A. annua* leaves clearly activated the expression of the majority of artemisinin biosynthetic genes. These results strongly suggest the involvement of the AaWRKY1 transcription factor in the regulation of artemisinin biosynthesis, and indicate that *ADS* is a target gene of AaWRKY1 in *A. annua*.

Keywords: Amorpha-4,11-diene synthase • *Artemisia annua* • Artemisinin • W-box • WRKY transcription factor.

Abbreviations: ACTs, artemisinin-based combination therapies; ADS, amorpha-4,11-diene synthase; 3-AT, 3-amino-1,2,4-triazole; BSA, bovine serum albumin; CaMV, cauliflower mosaic virus; CPR, cytochrome P450 reductase; CYP71AV1, cytochrome P450 enzyme; DBR2, artemisinic aldehyde Δ11(13) reductase; DMAPP, dimethylallyl diphosphate; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; EST, expressed sequence tag; FPP, farnesyl diphosphate; FPS, farnesyl diphosphate synthase; GFP, green fluorescent protein; GST, glandular secretory trichome; GUS, β-glucuronidase; HMGR, 3-hydroxy-3-methylglutaryl coenzyme A reductase; IPP, isopentenyl diphosphate; MJ, methyl jasmonate; MOPSO, 3-(*N*-morpholino)-2-hydroxypropanesulfonic acid; NLS, nuclear localization signal; ORF, open reading frame; RACE, rapid amplification of cDNA ends; RT–PCR, reverse transcription–PCR.

The nucleotide sequence reported in this paper has been submitted to GenBank under the accession number FJ390842.

Introduction

Artemisinin, isolated from traditional Chinese herb *Artemisia annua* L. by Chinese scientists in the 1970s, is a sesquiterpene lactone endoperoxide that provides the basis for effective treatments of malaria, especially for the cerebral and the chloroquine-resistant forms of this disease. Artemisinin-based combination therapies (ACTs) have now been

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recommended since 2001 by the World Health Organization (WHO) to reduce the odds of drug resistance. Currently, artemisinin derivatives are the only available drugs that are globally effective against the malaria parasite. Besides their antimalarial activity, artemisinins have also recently been reported to possess antiviral (Romero et al. 2006), anticancer (Efferth 2006) and antischistosomal activities (Utzinger et al. 2007). At present, the *A. annua* plant is still the only commercial source of artemisinins. The low artemisinin content in *A. annua*, ranging from 0.1 to 0.8% of the plant dry weight, makes artemisinin a relatively expensive drug, especially for economically disadvantaged people in developing countries where most malaria occurs. One of the most promising approaches to reduce the price of artemisinin-based antimalarial drugs is metabolic engineering of the plant in an attempt to obtain a higher artemisinin content. However, more knowledge about the biosynthetic pathway is required for this purpose.

The first committed step of artemisinin biosynthesis in *A. annua* is the cyclization of farnesyl diphosphate catalyzed by amorpha-4,11-diene synthase (ADS), which has been cloned independently by several groups (Chang et al. 2000, Mercke et al. 2000, Wallaart et al. 2001). Amorpha-4,11-diene is then consecutively transformed into artemisinic alcohol, artemisinic aldehyde, dihydroartemisinic aldehyde and dihydroartemisinic acid by a sequence of enzymatic reactions (Bertea et al. 2005). Genes encoding trichome-specific enzymes that are believed to be involved in this sequence of reactions have also been cloned recently (Teoh et al. 2006, Zhang et al. 2008, Teoh 2009). Based on these data, a hypothetical pathway of artemisinin biosynthesis has been summarized in **Supplementary Fig. S1** (Pu et al. 2009). Thus far, there have been no reports on the regulation of artemisinin biosynthesis at the transcriptional level.

Coordinate transcriptional control of biosynthetic genes has emerged as a major mechanism dictating the final levels of secondary metabolites in plant cells. This regulation of biosynthetic pathways is achieved by specific transcription factors (Vom Endt et al. 2002). Transcription factors have been isolated and characterized for several plant secondary metabolic pathways, including those leading to biosynthesis of flavonoids, terpenoid indole alkaloids, benzylisoquinoline alkaloids and sesquiterpene phytoalexins. Transcription factors can often activate several genes of the same pathway, e.g. overexpression of the MYB transcription factor PAP1 from Arabidopsis resulted in strongly enhanced expression of phenylpropanoid biosynthesis genes as well as enhanced accumulation of lignin, hydroxycinnamic acid esters and flavonoids, including various anthocyanins that are responsible for the purple color (Borevitz et al. 2000). In the plant species *Catharanthus roseus*, a jasmonate-responsive transcriptional regulator of primary and secondary metabolism, ORCA3, has been shown to regulate the jasmonate-responsive activation of several terpenoid indole alkaloid biosynthesis genes (van der Fits and Memelink 2000). A WRKY-type transcription factor, GaWRKY1, from *Gossypium arboreum* has been suggested to participate in regulation of sesquiterpene biosynthesis in cotton (Xu et al. 2004). Recently, a *Coptis japonica* transcription factor CjWRKY1 has been isolated, and transient expression of cDNA in *C. japonica* protoplasts was shown to increase the level of transcripts of benzylisoquinoline alkaloid berberine biosynthetic genes (Kato et al. 2007). Therefore, overexpression of transcription factor genes has been proposed as a promising approach to induce entire secondary metabolic pathways (Verpoorte and Memelink 2002, Petersen 2007).

The WRKY proteins are one of the major groups of plant-specific transcriptional factors. They are encoded by a multigene family, with at least 72 and 100 members in Arabidopsis (Dong et al. 2003) and rice (*Oryza sativa*) (Wu et al. 2005), respectively. WRKY transcriptional factors share different numbers of WRKY domains, a 60 amino acid stretch containing a conserved amino acid sequence WRKYGQK together with a zinc finger-like motif (Eulgem et al. 2000). Numerous in vitro and in vivo experiments have demonstrated that WRKY proteins specifically bind to the W-box (T)TGAC(C/T), a *cis*-acting DNA element found frequently in the promoter of defense-related genes (Rushton et al. 1996, Yu et al. 2001, Zheng et al. 2006). The WRKY transcriptional factors can either up- or down-regulate the expression of a given gene (Oh et al. 2008). The WRKY proteins have been implicated in a variety of developmental and physiological roles in plants. Most WRKY proteins are involved in defense and disease responses (Eulgem et al. 2000, Turck et al. 2004, Park et al. 2006, Zheng et al. 2007). WRKY genes can also regulate embryogenesis (Lagace and Matton 2004), seed coat and trichome development (Johnson et al. 2002), senescence (Hinderhofer and Zentgraf 2001, Robatzek and Somssich 2001, Miao et al. 2004) and the biosynthesis of secondary metabolites (Xu et al. 2004).

In *A. annua*, the glandular secretory trichomes (GSTs) are thought to be the site of biosynthesis and storage of artemisinin (Duke and Paul 1993, Duke et al. 1994, Li et al. 2006, Covello et al. 2007). In order to identify new genes of artemisinin biosynthesis, we generated expressed sequence tags (ESTs) using isolated GSTs of *A. annua* as starting material. In addition to ESTs representing the known genes involved in artemisinin biosynthesis, including *FPS* (farnesyl diphosphate synthase), *ADS* and *CPR* (cytochrome P450 reductase), we identified two ESTs among the 1,985 ESTs we sequenced that contained the consensus WRKY sequence. A full-length WRKY cDNA, designated *AaWRKY1*, was isolated and characterized.

In the present study, we report that two W-box elements are present in the *ADS* promoter. *AaWRKY1* codes for a leucine zipper-containing WRKY protein, which interacted with

the 3×W-box elements derived from the *ADS* promoter in a yeast (*Saccharomyces cerevisiae*) one-hybrid system and in an electrophoretic mobility shift assay (EMSA). Overexpression of *AaWRKY1* greatly activated the *ADS* promoters in stably transformed tobacco and in transiently transformed *Nicotiana benthamiana* and *A. annua*, and disruption of the W-box abolished this activation. Transient expression of *AaWRKY1* cDNA in *A. annua* leaves clearly increased the level of transcripts of artemisinin biosynthetic genes. These data demonstrate that AaWRKY1 is a transcriptional activator of *ADS*, and participates in the regulation of artemisinin biosynthesis.

Results

Isolation of AaWRKY1 cDNA

To study the transcriptional regulation of ADS, a key enzyme in the biosynthesis pathway of artemisinin, the promoter of *ADS* (AY528931, cloned in our laboratory in 2004) was scanned with PLACE (Higo et al. 1999) (http://www.dna.affrc.go.jp/htdocs/PLACE/signalscan.html). The promoter was found to contain two reverse-oriented TTGACC W-box *cis*-acting elements (**Fig. 1**). Since, as mentioned above, W-box *cis*-acting elements are frequently found to be the binding sites of WRKY transcription factors (Eulgem et al. 2000), it is reasonable to assume that WRKY proteins are involved in the transcriptional regulation of the *ADS* gene.

The 10-celled biseriate GSTs were isolated by a method based on the mechanized abrasion of plant leaves, followed by filtration (**Supplementary Fig. S2**). A cDNA library of *A. annua* was then constructed using GSTs as the source of mRNA. In total, 1,985 randomly picked clones were sequenced. Two EST fragments that contained the consensus WRKY sequence and two EST fragments of the *ADS* gene were found in the cDNA library. The EST fragments that contained the consensus WRKY sequence were subjected to SMART-RACE-PCR. The full-length cDNA of *AaWRKY1* (accession No. FJ390842) was found to be 1,478 bp long and contained a 936 bp open reading frame (ORF). The ORF encoded a protein of 311 amino acids (ACJ12926) with a calculated mol. wt. of 34.70 kDa and an isoelectric point of 5.31.

The AaWRKY1 protein contained one putative WRKY domain, together with one zinc finger-like motif in the C-terminal region (C-X7-CX23-H-X1-C) (**Fig. 2**). Based on the criteria described earlier (Eulgem et al. 2000), the AaWRKY1 protein appears to be a member of the group III WRKY family of plant transcription factors. Alignment of AaWRKY1 with some of the WRKY proteins that are involved in regulating plant secondary metabolism was performed (**Fig. 2**). AaWRKY1 exhibited 32.5% sequence identity with Arabidopsis AtWRKY53 protein, 13.2% sequence identity with *C. japonica* CjWRKY1 protein, and 15.9% identity with *G. arboretum* GaWRKY1 protein. However, other than for the conserved WRKY domain and the zinc finger-like motif, there was little sequence conservation among the WRKY proteins.

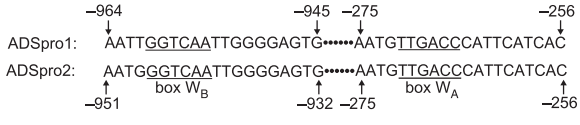

Fig. 1 Analysis of the W-box elements of two *ADS* promoters. *ADSpro1* and *ADSpro2* represent the promoters derived from GenBank accession Nos. AY528931 and DQ448294, respectively.

AaWRKY1 and ADS are highly expressed in GSTs and are induced by defense signals

To determine if *AaWRKY1* expression coincides with *ADS* expression in *A. annua*, reverse transcription–PCR (RT–PCR) was carried out to analyze its expression patterns in roots, stems, leaves, flowers and GSTs. *AaWRKY1* was highly expressed in GSTs and a moderate expression was observed in leaves and flowers. *ADS* showed exactly the same expression pattern (**Fig. 3A**). These results indicate that AaWRKY1 might target the *ADS* gene.

Methyl jasmonate (MJ) and chitosan are proven effective elicitors of artemisinin biosynthesis in cell cultures and hairy root cultures of *A. annua* (Putalun et al. 2007, Baldi and Dixit 2008). Because ADS is a key enzyme of artemisinin biosynthesis, it is reasonable to assume that *ADS* transcription might be activated by these two elicitors. Also, if AaWRKY1 was a regulator of *ADS*, we would also expect an inducible expression of *AaWRKY1*. Thus, the expression of the *ADS* and the *AaWRKY1* gene under MJ and chitosan treatments was investigated using semi-quantitative RT–PCR. Expression of *AaWRKY1* was induced rapidly and robustly following treatment with 0.3 mM MJ or 150 mg l^{-1} chitosan. In MJ-treated leaves, the transcript level of *AaWRKY1* increased rapidly and peaked within 30 min; this activation persisted up to 1 h post-treatment and then declined to the original level by 2 h (**Fig. 3B**). *ADS* showed a slower rate of induction, with the transcript level increasing slightly from 0.5 to 2 h and reaching a maximum at 4 h. In chitosan-treated leaves, the transcript level of *AaWRKY1* showed similar kinetics to those seen for MJ induction, whereas the level of *ADS* transcripts increased more rapidly compared with MJ induction, reaching its maximum at 1.5 and 2.0 h (**Fig. 3C**). Thus, the transcript level of *AaWRKY1* increased rapidly and transiently upon elicitation, and preceded that of *ADS*.

DNA binding activity of AaWRKY1

As shown in **Fig. 1**, the promoter of the *ADS* gene cloned by our laboratory (AY528931, 2004) was designated *ADSpro1*, while the promoter of the *ADS* gene cloned

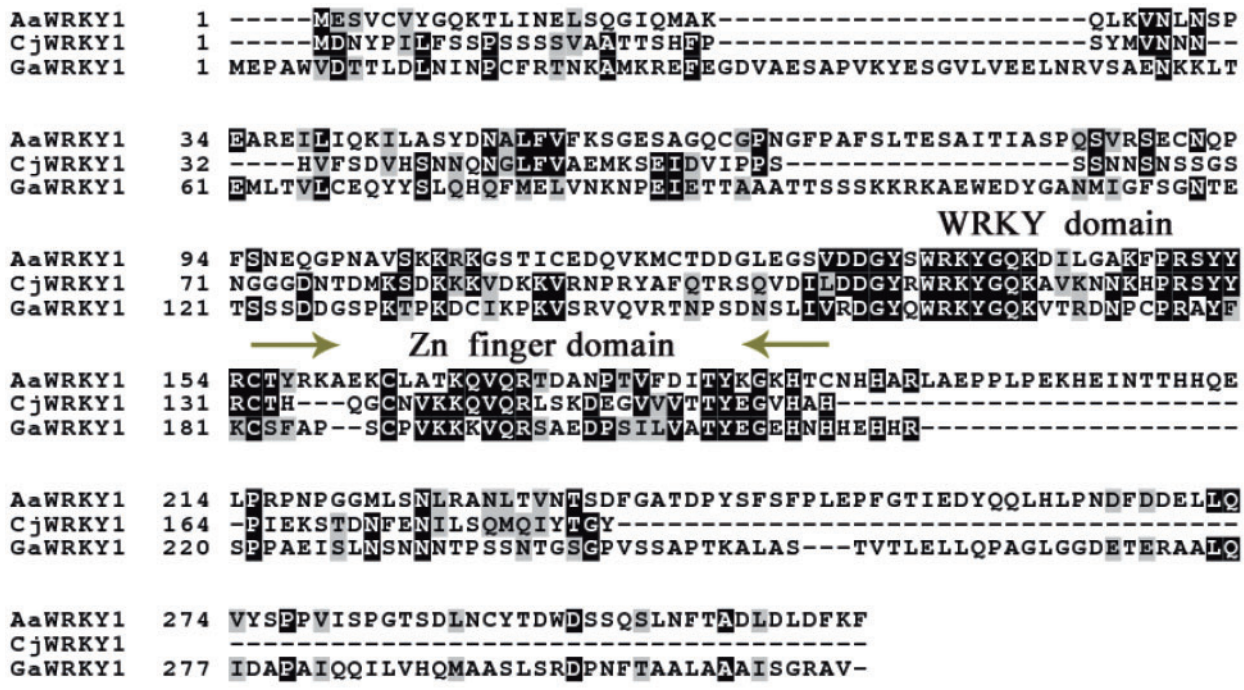

Fig. 2 Alignment of AaWRKY1 with some WRKY proteins. The deduced amino acid sequence of AaWRKY1 was aligned with Arabidopsis AtWRKY53 (NP_194112), *Coptis japonica* WRKY1 (accession No. BAF41990) and *Gossypium arboretum* WRKY1 (accession No. AAR98818) using Clustal W (Thompson et al. 1994) with default parameters through EMBnet (http://www.ch.embnet.org/software/ClustalW.html). Black and gray shadings, done with BOXSHADE 3.21 (http://www.ch.embnet.org/software/BOX_form.html), indicate conserved amino acid residues. Predicted domains are indicated above the sequences.

by Kim et al. (2008) from a Korean *A. annua* (DQ448294) was designated *ADSpro2*. Both promoters contain two W-box elements, which are putative recognition sites for WRKY transcription factors. The position and sequence of Box W_A in the two promoters is well conserved, with the W-box *cis*-acting element (TTGACC) located in the region between −271 and −266 bp. Box W_B in the two promoters is also conserved, except that W_B in *ADSpro1* is located in the region between −960 and −955 bp, while W_B in *ADSpro2* is located in the region between −947 and −942 bp.

In order to test AaWRKY1 protein–W-box DNA interactions, an EMSA was performed. Production of recombinant AaWRKY1 protein in native conditions in a bacterial system (*Escherichia coli*) proved unsuccessful. Therefore, the protein was isolated in denatured form and dialyzed for refolding. The refolded protein band of AaWRKY1 was detected by SDS–PAGE (**Supplementary Fig. S5**). The DNA fragments containing the 3×W-box and mutated 3×W-box elements were triple tandem repeats of the W-box from the promoter of the *ADS* gene, and the complementary oligonucleotides could anneal to form double-stranded structures. Two shifted bands, W_A and W_B, were observed with the recombinant AaWRKY1 protein incubated with the 3×W-box probe (**Fig. 4A**, C: lanes 2–6). No binding signal was detected with the mutated 3×W-box elements as probes (**Supplementary Fig. S6**), and no binding signal was detected in the negative control, where the W_A-box×3 was incubated with 0.5 μg of his-tagged AaIPPI (DQ666334) protein (**Fig. 4A**, lane 8) and the W_B-box×3 was incubated with 2 μg of bovine serum albumin (BSA) (**Fig. 4C**, lane 8). Therefore, the observed probe retardation was assigned to the DNA binding activity of AaWRKY1. To verify AaWRKY1 binding capacity, the same amounts of W-box DNA were incubated with higher amounts of AaWRKY1: 1.5-, 2-, 2.5- and 3-fold that of lane 2 in lanes 3, 4, 5 and 6, respectively. The numbers of shifted protein–DNA complexes showed a tendency to increase gradually, and free DNA levels decreased accordingly. Thus, AaWRKY1 was able to recognize and interact with the W-box elements of the *ADS* promoter in vitro.

In the EMSA, two shifted bands were observed with AaWRKY1 protein–3×W-box DNA interactions (**Fig. 4**). It is possible that the large retardant band is an AaWRKY1 protein dimer, because AaWRKY1 in native-PAGE does not give a single band (**Supplementary Fig. S5**). Sequence analysis of the AaWRKY1 protein indicated that there was a leucine zipper-like motif ($L_{X6}A_{X6}L_{X6}E_{X6}L_{X6}L$) from amino acid

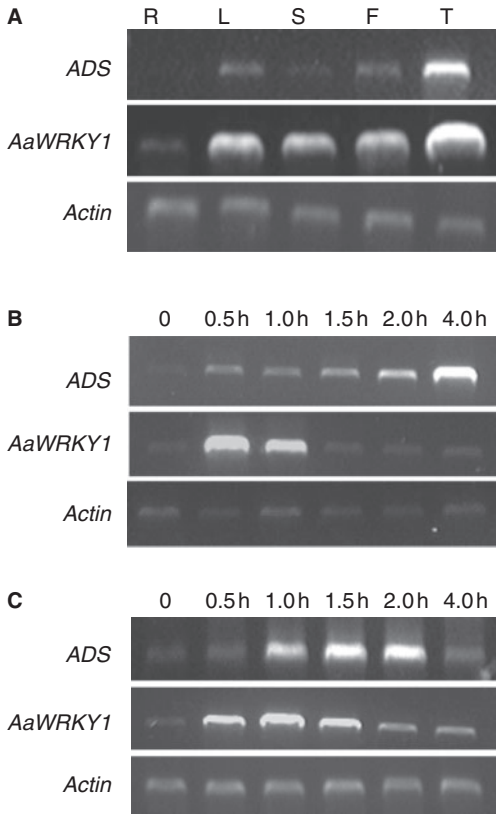

Fig. 3 The expression pattern of *AaWRKY1* and *ADS*. (A) *AaWRKY1* and *ADS* are highly expressed in glandular secretory trichomes (GSTs). Ethidium bromide-stained agarose gels showing RT–PCR results for roots (R), leaves (L), stems (S), flowers (F) and GSTs (T). (B) *AaWRKY1* and *ADS* expression in response to MJ. (C) *AaWRKY1* and *ADS* expression in response to chitosan. Leaves were sprayed with methyl jasmonate (MJ; 0.3 mM) or chitosan (150 mg l^{-1}). Leaves were harvested at the indicated times after treatment for total RNA preparation. Accumulation of *AaWRKY1* and *ADS* transcripts was monitored by semi-quantitative RT–PCR. The *Actin* gene was used as an internal control.

position 16 to position 51 (**Fig. 2**); this motif may play a significant role in the formation of a homodimeric WRKY protein.

To determine if AaWRKY1 binds to the W-box of the *ADS* promoter in vivo, a yeast one-hybrid assay was performed. The W_A-box×3 fragment was fused to a *HIS* reporter gene under the control of the *GAL1* minimal promoter and introduced into yeast strain yWAM2. The ORF of *AaWRKY1* was inserted into the pPC86 vector to generate pPC86-AaWRKY1. The pPC86-AaWRKY1 and the empty pPC86 vector were introduced into yeast strain yWAM2 containing a plasmid harboring a W_A-box×3 fragment. On SD-Trp-Leu-His selective medium containing 15 mM 3-amino-1,2,4-triazole (3-AT), only the yeast clones harboring pPC86-AaWRKY1 could grow (**Fig. 5**). The result indicated that AaWRKY1 bound to the W_A-box and activated transcription in vivo.

AaWRKY1 activated ADSpro1 in transgenic tobacco

The transactivation of the *ADS* promoter by AaWRKY1 was confirmed in stably double-transformed tobacco plants harboring the reporter pCAMBIA-*ADSpro1::GUS* and the effector pBI-*AaWRKY1* (**Fig. 6A**). Tobacco plants were first transformed with a binary vector pCAMBIA1301 harboring the *ADSpro1::GUS* reporter gene under the selective pressure of hygromycin, then the effector construct *CaMV35S-AaWRKY1* was transformed into the primary transformant under the selective pressure of kanamycin and hygromycin. In the double transgenic tobacco plants, constitutive expression of AaWRKY1 activated the *ADSpro1* promoter (**Fig. 6B**). Compared with the leaves of primary transformants, the GUS (β-glucuronidase) activities in leaves of secondary transformants were increased about 4- to 5-fold (**Fig. 6C**). These results demonstrated that AaWRKY1 strongly activated the *ADSpro1* promoter in transgenic tobacco.

AaWRKY1 activated ADSpro2 in a transient expression system

Kim et al. (2008) reported another *ADS* promoter, *ADSpro2* (DQ448294) from a Korean *A. annua*. In order to confirm that AaWRKY1 could also activate *ADSpro2*, we amplified the *ADSpro2* promoter from our *A. annua* strain and generated three mutants: *ADSpro2*: mW_A*ADSpro2*, mW_B*ADSpro2* and mW_{AB}*ADSpro2* (see Materials and Methods). We then tested the interactions of AaWRKY1 with the wild-type and mutant *ADS* promoters in an *Agrobacterium*-mediated transient expression system in *N. benthamiana* and *A. annua* leaves. When only the reporter plasmid was introduced into *N. benthamiana*, the GUS expression directed by the wild type and mutant *ADSpro2* was similar (**Fig. 7a, c, e, g**). When both reporter and effector plasmids were introduced into *N. benthamiana*, the *ADSpro2*-directed GUS expression increased approximately 4-fold (**Fig. 7b**). Compared with the wild-type *ADSpro2*, mW_A*ADSpro2*-. mW_B*ADSpro2*- and mW_{AB}*ADSpro2*-directed GUS expression decreased approximately 40–50, 60–70 and 100% (**Fig. 7d, f, h**), respectively. Similar results have also obtained in the transient expression system in *A. annua* (**Fig. 8**). These results indicated that AaWRKY1 protein transactivated the *ADS* promoter in a W-box-dependent way.

AaWRKY1 regulates the expression of the tobacco sesquiterpene synthase gene

When *AaWRKY1* was ectopically expressed in tobacco, the transcript level of the 5-epi-aristolochene synthase gene (*EAS4*) was up-regulated, and the transcript level of *EAS4* declined when anti-*AaWRKY1* was expressed. The transcript

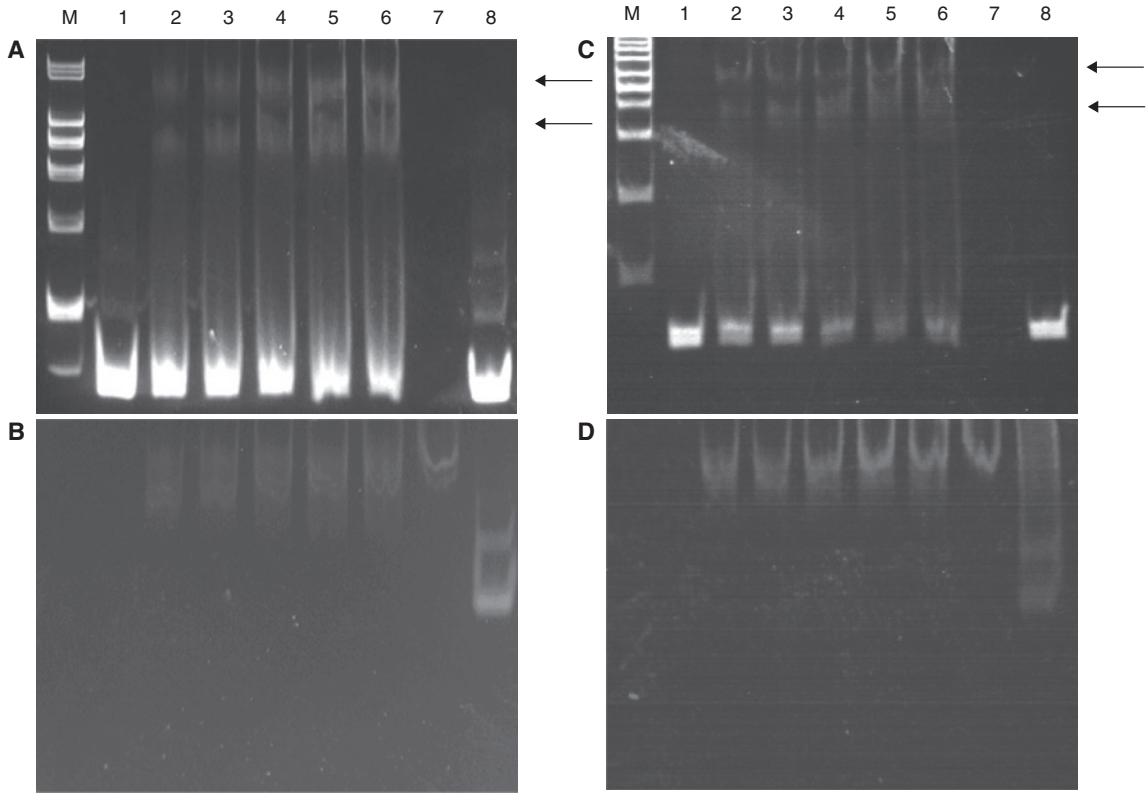

Fig. 4 The ability of the AaWRKY1 protein to bind to the W-box *cis*-acting elements, as analyzed by EMSA. (A) Titration of W_A-box×3 DNA with AaWRKY1 protein stained with SYBR Green EMA for visualizing DNA. (B) The same gel as in A stained with SYPRO Ruby EMSA for visualizing protein. (C) Titration of W_B-box×3 with AaWRKY1 protein stained with SYBR Green EMSA for visualizing DNA. (D) The same gel as in C stained with SYPRO Ruby EMSA for visualizing protein. Increasing amounts of AaWRKY1 protein were added to W_A-box×3 DNA or W_B-box×3 in a final 15 µl volume. M, DNA maker (DL2000). Lane 1, *cis*-acting element DNA only. Lanes 2–6, *cis*-acting element DNA with increasing amounts of AaWRKY1 protein (46, 69, 92, 115 and 138 ng). Lane 7, AaWRKY1 protein only (138 ng). Lane 8, *cis*-acting element DNA with 2 µg of BSA (A, B) and 0.5 µg of pET-*AaIPPI* (C, D), respectively. The arrows indicated the AaWRKY1–*cis*-acting DNA element complex.

level of the squalene synthase gene (*SQS*) was almost invariable (**Fig. 9**). Since the promoter region of *EAS4* reportedly contains a W-box (Yin et al. 1997), AaWRKY1 might regulate *EAS4* expression through interaction with the W-box in the gene promoter. The fact that transcription factors often have at least partially conserved functions between different species has been reported (Lloyd et al. 1992, Borevitz et al. 2000).

AaWRKY1 activated genes of the artemisinin biosynthetic pathway in A. annua

Since overexpression of *AaWRKY1* greatly activated the *ADS* promoters in stably transformed *Nicotiana tabacum* and in transiently transformed *N. benthamiana* and *A. annua*, it was of interest to know whether AaWRKY1 would also activate other genes of the artemisinin biosynthetic pathway.

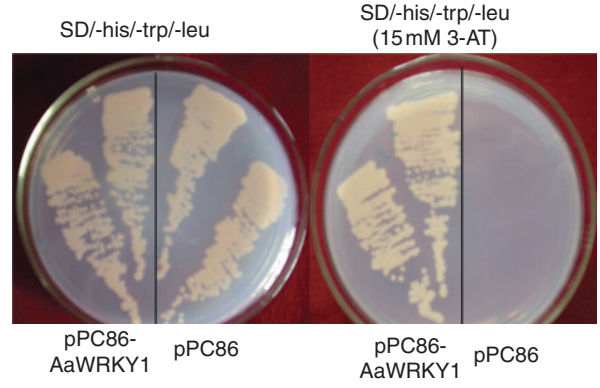

Fig. 5 Yeast one-hybrid assay using the W_A-box×3 as bait. Yeast cells carrying pPC86 or pPC86-*AaWRKY1* were grown for 3 d at 30°C in SD/−his/−trp/−leu or SD/−his/−trp/−leu plus 15 mM 3-amino-1,2,4-triazole (3-AT), respectively.

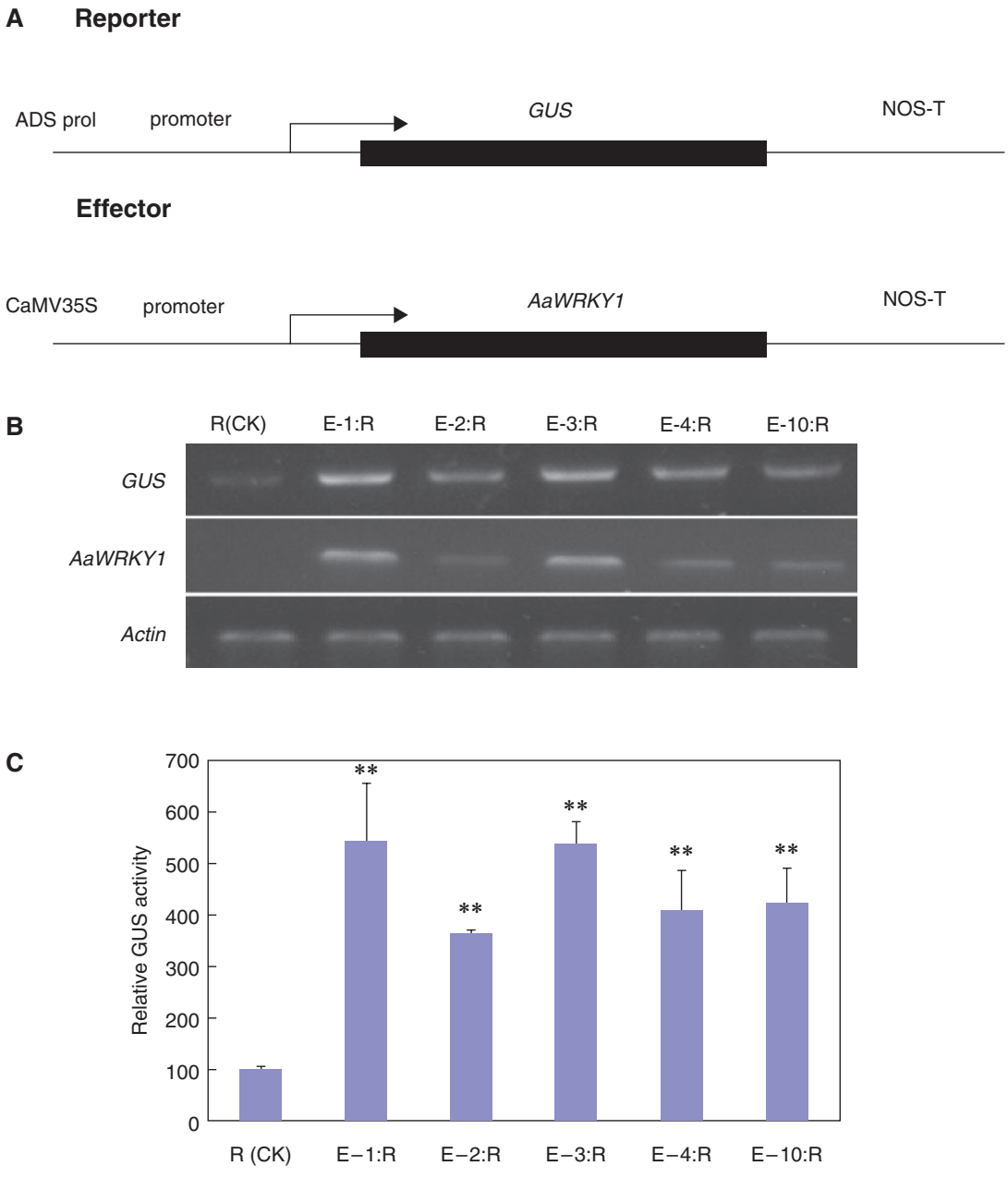

Fig. 6 Activation of the *ADSpro1* promoter by co-expression of the effector construct *CaMV35S-AaWRKY1* with the reporter construct *ADSpro1::GUS* in transgenic tobacco plants. (A) Schematic diagram of the reporter and effector constructs used in the co-expression experiment. (B) RT–RCR analysis of the *GUS* and *AaWRKY1* transcripts in transgenic tobacco. *Actin* amplification was used as the constitutive control. R (CK) represents the transgenic tobacco line harboring the reporter construct as the control. The plasmid harboring the effector construct (E) was transformed into the transgenic tobaccos harboring the reporter construct. E-1:R, E-2:R, E-3:R, E-4:R and E-10:R represent five transgenic tobacco lines carrying the effector construct (E) and the reporter construct (R) at the same time. (C) Measurement of GUS activities in the different transgenic tobacco lines. GUS activity was normalized in every double transformant relative to the transgenic tobacco line harboring the reporter construct R (CK). Data were statistically analyzed by SPSS, and asterisks indicate values that are significantly different (**$P < 0.01$) from the reporter construct R (CK).

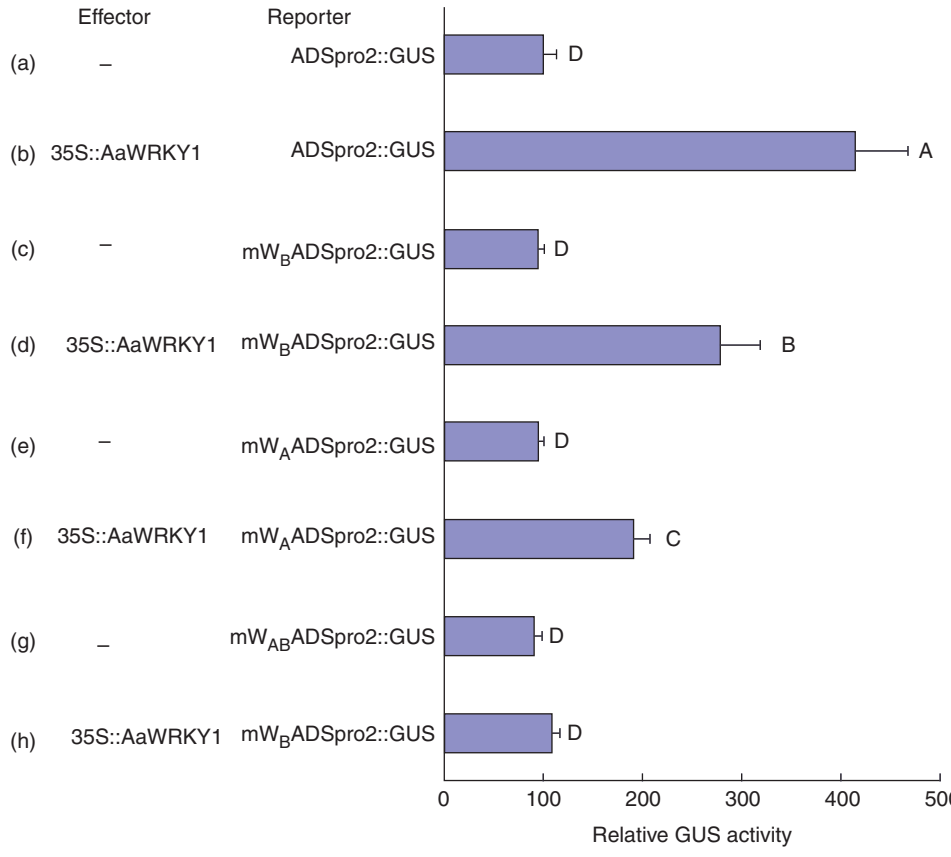

Fig. 7 Transactivation of *ADSpro2::GUS* gene expression by AaWRKY1 in agroinfiltrated *Nicotiana benthamiana* leaves. A transient assay of GUS activities was carried out in *N. benthamiana* leaves 2 d after infiltration with *Agrobacterium tumefaciens* cells harboring (a) *ADSpro2::GUS*; (b) *ADSpro2::GUS/35S::AaWRKY1*; (c) *mW$_B$ ADSpro2::GUS*; (d) *mW$_B$ ADSpro2::GUS/35S:: AaWRKY1*; (e) *mW$_A$ADSpro2::GUS*; (f) *mW$_A$ADSpro2/35S:: AaWRKY1*; (g) *mW$_{AB}$ADSpro2::GUS*; and (h) *mW$_{AB}$ADSpro2::GUS/35S:: AaWRKY1*. GUS activity was normalized in treatments (b–h) relative to that of *N. benthamiana* leaves agroinfiltrated with *ADSpro2::GUS* (a). Duncan multiple comparison tests were done, A, B, C and D indicate values that are significantly different (**$P < 0.01$) from each other.

Therefore, the pBI-*AaWRKY1* construct and the empty vector pBI121 were introduced into leaves of *A. annua* by vacuum-assisted agroinfiltration (see Materials and Methods). The success of the transformation was confirmed by GUS staining (**Supplementary Fig. S7**). When the PBI-*AaWRKY1* construct was introduced into *A. annua* leaves, transcript levels for *AaWRKY1* were 5-fold higher than those of the control after 72 h of co-cultivation (**Fig. 10A, B**). Semi-quantitative RT–PCR results showed that the expression levels of known genes of the artemisinin biosynthesis pathway were significantly increased with the overexpression of *AaWRKY1*, except for that of *FPS* (**Fig. 10A, B**). The transcripts of *HMGR*, *ADS*, *CYP71AV1* and *DBR2* increased 5.18±0.75-, 6.56±0.65-, 3.01±0.57- and 3.84±0.80-fold, respectively (**Fig. 10B**). Thus, overexpression of *AaWRKY1* could strongly activate the expression of the majority of artemisinin biosynthetic genes.

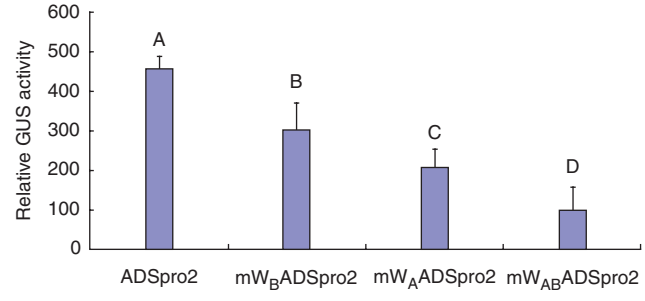

Fig. 8 Transactivation of *ADSpro2::GUS* gene expression by AaWRKY1 in *Artemesia annua* leaves. A transient assay of GUS activities was carried out in *A. annua* leaves 3 d after co-cultivation with *Agrobacterium tumefaciens* cells harboring *ADSpro2::GUS*, *mW$_B$ ADSpro2::GUS*, *mW$_A$ADSpro2::GUS* or *mW$_{AB}$ADSpro2::GUS*. GUS activity from *mW$_{AB}$ADSpro2::GUS* was used for normalization. Duncan multiple comparison tests were done, A, B, C and D indicate values that are significantly different (**$P < 0.01$) from each other.

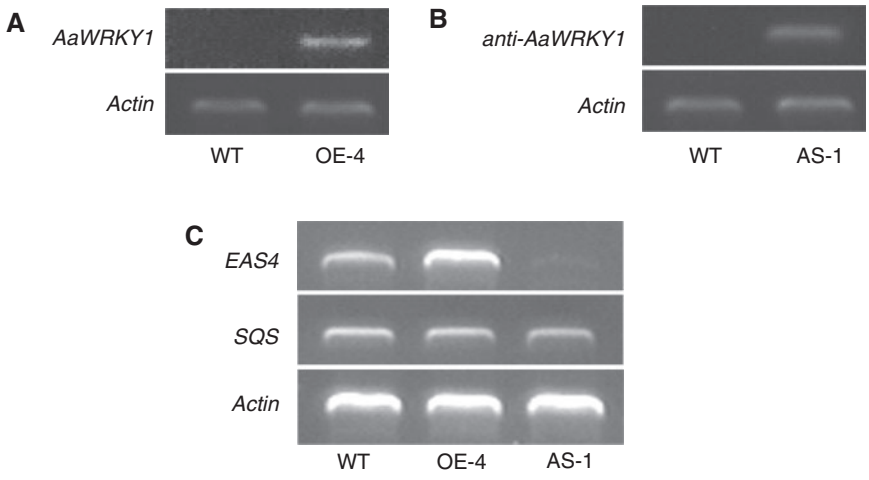

Fig. 9 AaWRKY1 regulates the expression of the tobacco 5-epi-aristolochene synthase gene (*EAS4*). (A and B) Expression levels of the transgene in the *AaWRKY1*-overexpressing tobacco line-4 (OE-4) and antisense *AaWRKY1* line-1 (AS-1) were estimated by RT–PCR. *Actin* amplification was used as constitutive control. WT indicates the wild-type tobacco line. (C) RT–RCR analysis of the *EAS4* and *SQS* transcripts in *AaWRKY1*-overexpressing line 4 (OE-4), antisense-1 line (AS-1) and the WT tobacco line. *SQS*, squalene synthase.

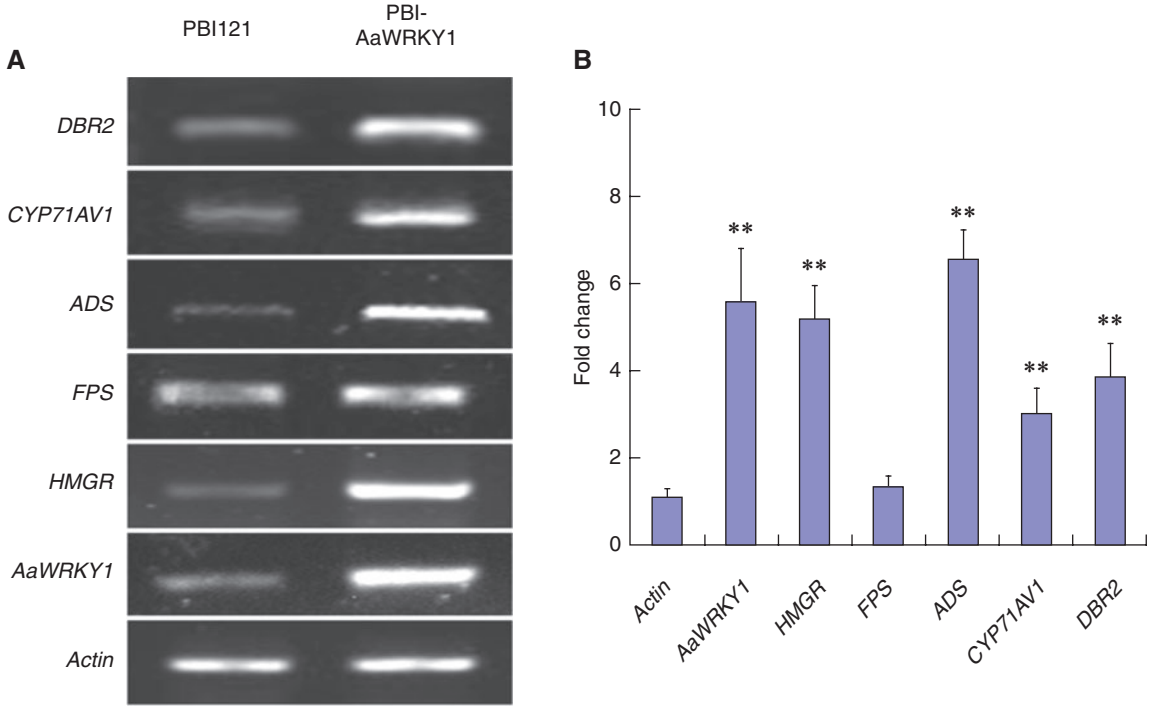

Fig. 10 AaWRKY1 activated the known genes involved in the artemisinin biosynthesis pathway. (A) RT–PCR analysis of gene expression levels in empty vector PBI121 (control) and PBI-*AaWRKY1* transiently transformed *A. annua*. (B) Comparison of gene transcript levels in PBI121 (control) and PBI-*AaWRKY1* transiently transformed *Artemesia annua*. The gene transcript levels of PBI121 transiently transformed *A. annua* were set to 1 and compared with the transcript levels in PBI-*AaWRKY1* transiently transformed *A. annua*. Signals obtained from *Actin* were used for normalizations. Data were statistically analyzed by SPSS, and asterisks indicate values that are significantly different (**$P < 0.01$).

Discussion

Because of the tremendous medicinal significance of artemisinin, its biosynthesis and metabolic engineering are presently very attractive research topics worldwide. To date, several genes encoding enzymes of the artemisinin biosynthetic pathway have been cloned and characterized (Bouwmeester et al. 1999, Chang et al. 2000, Wallaart et al. 2001, Teoh et al. 2006, Zhang et al. 2008, Teoh 2009). However, the transcriptional regulation of this important pathway is still not yet understood. In the present study, we isolated and characterized the first transcription factor, AaWRKY1, from *A. annua*. The other EST containing the consensus WRKY sequence was also identified from the cDNA library and isolated by RACE (rapid amplification of cDNA ends), and was designated *AaWRKY2*. The AaWRKY2 protein contained one putative WRKY domain, together with one zinc finger-like motif in the C-terminal region (C-X5-C-X23-H-X1-H), which was clustered with the group II WRKY protein. *AaWRKY1* was highly expressed in the GSTs on the leaves and flowers of *A. annua* (**Fig. 3A**). As has been reported for other genes related to artemisinin biosynthesis, such as *ADS*, *CYP71AV1* and *DBR2* (Teoh et al. 2006, Zhang et al. 2008), the expression of *AaWRKY1* was fast, transient and preceded that of *ADS* upon induction by either MJ (**Fig. 3B**) or chitosan (**Fig. 3C**). *AaWRKY1* showed a strong induction by MJ and chitosan; however, *AaWRKY2* showed insignificant variation in response to the same treatments (**Supplementary Fig. S4**). Because *AaWRKY1* and *ADS* in GSTs were found to have a similar expression pattern, this enabled us to suggest that AaWRKY1 is a transcription factor regulating the *ADS* promoter.

AaWRKY1 is a group III WRKY transcription factor, based on its sequence analysis. It contains a putative nuclear localization signal (NLS) sequence ($_{105}$KKRK$_{108}$) (**Fig. 2**) and, indeed, the targeting experiment showed that AaWRKY1 was localized to the nucleus (**Supplementary Fig. S3**). The results indicate that AaWRKY1 is a nuclear protein, which is consistent with its function as a transcriptional regulator. AaWRKY1 contains one WRKY domain with a WRKYGQK core sequence that is the major WRKY core sequence (Eulgem et al. 2000). The EMSA and yeast one-hybrid results indicated that AaWRKY1 interacts with the *cis*-acting elements (TTGACC) of the W-box in the promoter region of the *ADS* gene (**Figs. 4**, **5**). Furthermore, the in vivo transactivation by AaWRKY1 of the *ADS* promoter was confirmed with stably transformed tobacco plants (**Fig. 6**). The activation of the *ADS* promoter by AaWRKY1 required functional W-box elements in the *ADS* promoter, as was also confirmed experimentally (**Figs. 7**, **8**). These results strongly suggest that *ADS* is a target gene of AaWRKY1.

Transcription factors can often activate several genes of the same pathway. The results presented here demonstrated that AaWRKY1 has a similar propensity. Transient expression of AaWRKY1 activated the expression of *HMGR*, *ADS*, *CYP71AV1* and *DBR2* of the artemisinin biosynthesis pathway (**Fig. 10**, **Supplementary Fig. S1**). It is possible that the W-box also existed in the promoters of *CYP71AV1*, *HMGR* and *DBR2* in *A. annua*. Indeed, the W-box has been found in the promoters of cytochrome P450 genes of many other plants such as Arabidopsis (Narusaka et al. 2004), *C. japonica* (Kato et al. 2007) and cotton (Xu et al. 2004), while two W-box elements were also found in the *HMGR1* promoter of *Camptotheca acuminate* (Burnett et al. 1993). Isolation and characterization of the promoters of *HMGR*, *CYP71AV1*, *DBR2* and other genes in the artemisinin biosynthetic pathway would be beneficial for further evaluation of the function of WRKY transcription factors in artemisinin metabolism. Generation of transgenic *A. annua* plants that overexpress or silence the *AaWRKY1* gene is currently under way in our laboratory. Detailed analysis of these transgenic plants will provide a more complete picture of how AaWRKY1 regulates artemisinin biosynthesis, and will also address whether overexpression of the AaWRKY1 gene could be a new strategy for increasing artemisinin production in transgenic *A. annua*.

FPS is considered to be a regulatory enzyme in plant isoprenoid biosynthesis (Dudley et al. 1986, Hugueney et al. 1996, Ramos-Valdivia et al. 1997, Chen et al. 2000). In the present study, overexpression of *AaWRKY1* did not increase *FPS* expression. In a salicylic acid-induced *A. annua* plant, *FPS* expression was also not induced, although artemisinin content increased (Pu et al. 2009). Overexpression of the *FPS* gene in *A. annua* has also been shown not to increase the content of artemisinin significantly (Han et al. 2006). These results suggest that FPS might not be a rate-limiting factor in artemisinin biosynthesis. This would be similar to the observation that overexpression of *Arabidopsis FPS1S* did not significantly increase the content of sterols (Cunillera et al. 2000, Masferrer et al. 2002).

ADS showed a slower rate of induction in MJ-treated leaves than in chitosan-treated leaves following *AaWRKY1* expression (**Fig. 3B, C**). It is possible that the induction of the transcript of *AaWRKY1* was stronger and longer (0.5–1.5 h) in chitosan-treated leaves than in MJ-treated leaves (0.5–1.0 h). It might also be that other transcription factors are involved in the regulation of *ADS* expression, because other *cis*-acting elements involved in salicylic acid responsiveness, defense and stress responsiveness, ethylene responsiveness, auxin responsiveness and gibberellin responsiveness are also present in the *ADS* promoter (**Supplementary Table S1**). More than one transcription factor can coordinately regulate the expression of pathway genes; for example, in maize, the majority of the structural genes encoding enzymes committed to anthocyanin biosynthesis are coordinately regulated by the bHLH protein-encoding gene *R* and the

MYB gene *C1* in the aleurone layer of maize kernels (Mol et al. 1998). The bHLH transcription factor (three ESTs), bZIP protein (two ESTs), zinc finger protein (two ESTs including one C_2H_2 type and one CCCH type), AP2 domain transcription factor (one EST), trihelix transcription factor (one EST) and MYC transcription factor (one EST) were all found in the cDNA library (data not shown). Characterization of these transcription factors will aid in finding activator proteins that regulate the artemisinin biosynthesis pathway. In turn, further elucidation of the regulatory machinery controlling artemisinin biosynthetic genes will allow greater advancement of artemisinin biotechnology.

Materials and Methods

Plant materials

The high artemisinin-yielding strain 001 of *A. annua* L. from Sichuan Province, China (Chen et al. 2000, Zhang et al., 2006, Wang et al. 2007) was used in this study. *Artemisia annua* seedlings were micropropagated as described by Zhang et al. (2006). The rooted in vitro plantlets were transported into pots and grown in a greenhouse with 16 h/25°C day and 8 h/20°C night. *Nicotiana tabacum* cv. Wisconsin 38 (W38) was used for transformation, and *N. benthamiana* was used for agroinfiltration.

Isolation of glandular secretory trichomes and cDNA library construction

Leaves from 4-month-old (pre-flowering) *A. annua* were used for the isolation of GSTs. The collected leaves were chilled in ice-cold water for 10 min, then abraded using a cell disrupter (Bead Beater, Biospec Products, Bartlesville, OK, USA). The extraction mixture contained 20–30 g of leaves, 80–100 g of glass beads (0.5 mm diameter), 20–30 g of XAD-4 resin and 100 ml of isolation buffer [25 mM MOPSO, pH 6.6, 200 mM sorbitol, 10 mM sucrose, 5 mM thiourea, 2 mM dithiothreitol (DTT), 5 mM $MgCl_2$, 0.5 mM sodium phosphate, 0.6% (w/v) methylcellulose and 1% (w/v) polyvinylpyrrolidone (M_r 40,000)]. GSTs were isolated as described by Teoh et al. (2006). Total RNA from GSTs was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany). The cDNA library was constructed with the Creator SMART cDNA Library Construction Kit (Clontech, Mountain View, CA, USA) following the protocols suggested by the manufacturer.

AaWRKY1 cDNA isolation

Randomly picked clones were sequenced with an ABI 3700 DNA sequencer using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Sequences were assembled and edited using PHRED/PHRAP (Ewing and Green 1998), with a minimatch score of 35, and all contigs and singlets were imported into a gap4 database for viewing (Bonfield et al. 1995). The non-redundant unigenes (contigs and singlets) were subjected to similarity searches against the GenBank non-redundant (nr) protein databases using the BLASTX algorithm with default parameters. The EST fragment which contains the consensus WRKY sequence was identified and subjected to SMART-RACE-PCR (nested PCR) with first-strand cDNA as temple using a primer binding to the pDNR-LIB vector arm (5′-AAG CAG TGG TAT CAA CGC AGA GT-3′) (forward) and two gene-specific primers (reverse). The gene-specific primers for *AaWRKY1* are GSP1 (5′-CGC TAC CCT CTA ACC CAT CAT CAG T-3′) and GSP2 (5′-ACT CAG ATC GTA CGC TTT GTG GGC T-3′).

Subcellular localization of AaWRKY1

The ORF of *AaWRKY1* was amplified by a sticky-end PCR method (Zeng 1998) to introduce an *Xba*I sticky end (CTAG) upstream of the start codon and a *Sma*I blunt end (CCC) downstream of the ORF. The sticky-end PCR product (*Xba*I upstream and *Sma*I downstream) was ligated into pBI221 digested with *Xba*I and *Sma*I to generate the *CaMV35S::AaWRKY1-GFP* construct, which was verified by sequencing. The *CaMV35S::GFP* construct was used as a control. Onion inner epidermal cells were stretched out inside-up on MS medium, and bombarded by using the PDS-1000/He system (Bio-Rad, Hercules, CA, USA) at 1100 p.s.i. with DNA-coated gold particles. The transformed cells were cultured on MS medium at 25°C for 24 h, and then observed under a confocal microscope (Leica TCS SP2, Germany). Green fluorescent protein (GFP) was excited at 488 nm with an argon laser, and fluorescence was detected at 500–540 nm and imaged with a 40.0×/1.25 HCX PL APO objective lens.

Heterologous expression and purification of AaWRKY1

The ORF of *AaWRKY1* was amplified with forward primer 5′-GAT GGA TCC ATG GAA AGT GTT TGT GTT TAT-3′ (with the *Bam*HI site underlined) and reverse primer 5′-GTA CTC GAG AAA TTT GAA ATC AAG GTC TAA-3′ (with the *Xho*I site underlined), and then fused to the *Bam*HI–*Xho*I sites in the pET-30a (+) vector (Novagen, Madison, WI, USA). After sequence confirmation, the pET-*AaWRKY1* plasmid was transformed into *E. coli* strain BL21 (DE3) competent cells for protein expression. *Escherichia coli* cells containing pET-*AaWRKY1* were cultured in LB medium supplied with 50 mg l^{-1} ampicillin at 37°C. When the OD_{600} of the cultures reached 0.8, 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added and the cultures were incubated for another 4 h at 25°C. The cells were harvested by centrifugation and resuspended in 10 ml of 0.05 M sodium phosphate buffer (pH 7.5) containing 20 mM imidazole, and sonified on

ice for 5 min at 50% pulses using a Ultrasonic Crasher (Ningbo Scientz Biotechnology Co. Ltd., China). The homogenate was centrifuged for 10 min at 10,000×*g* and 4°C. Since most of the expressed AaWRKY1 proteins existed in the inclusion body, the pellet was resolved in 8 mol l^{-1} urea, then the proteins were isolated as denatured forms through a pre-packed 5 ml Hi-trap Ni column (Pharmacia) following the manufacturer's protocol. For refolding, the purified proteins were dialyzed in turn against 50 mmol/l phosphate-buffered saline buffers containing 6, 4, 3, 2, 1 and 0 mol l^{-1} urea (pH 7.4) at 4°C, for at least 4 h for each urea concentration. After removing the precipitates by centrifugation, the dialyzed protein solution was lyophilized, and stored at −80°C before use.

Electrophoretic mobility shift assay

The DNA binding ability of AaWRKY1 was analyzed by EMSA. Two oligonucleotides were synthesized, including the sense strand sequence of W_A-box×3 (5′-AAT GTT GAC CCA TTC ATC ACA ATG TTG ACC CAT TCA TCA CAA TGT TGA CCC ATT CAT CAC-3′) and the corresponding antisense strand sequence, the two oligonucleotides were incubated for 5 min at 95°C, and thereafter 5 min at 65°C to renature the products. The oligonucleotides of W_B-box×3 (5′-AAT GGG TCA ATT GGG GAG TGA ATG GGT CAA TTG GGG AGT GAA TGG GTC AAT TGG GGA GTG-3′) and the corresponding antisense strand sequence were synthesized and annealed to form double-stranded DNA using the same method. The core sequence of the W_A-box TTGACC was mutated into TTGAAA and the core sequence of the W_B-box GGTCAA was mutated into AATCAA. The mutated W_A-box and W_B-box probes were formed by the same way.

The EMSA was performed with the Electrophoretic Mobility Shift Assay kit (Invitrogen, USA) following the manufacturer's instruction. The DNA–protein binding reaction was performed by incubating double-stranded oligonucleotides with purified protein at room temperature for 30 min in a total volume of 15 µl. The reaction system contained 20 mmol Tris–HCl (pH 7.6), 30 mmol KCl, 0.2% (w/v) Tween-20, 1 mmol DTT and 10 mmol $(NH_4)_2SO_4$. The binding mixture was resolved on a 6% non-denaturing polyacrylamide gel in 0.5×TBE buffer. The gel was first stained with SYBR Green EMSA stain for visualizing DNA, and photographed; the same gel was stained with SYPRO Ruby EMSA stain for monitoring protein, and photographed.

Binding assay in yeast one-hybrid system

All yeast transformation was carried out according to the transformation protocol of Gietz et al. (1995) using the yeast strain yWAM2 (*MATα Δgal4 Δgal80 URA3::GAL1-lacZlys2801*^{amber} *his3-Δ200trp1-Δ63leu2ade2-101*^{ochre} *CYH2*). The yeast reporter plasmid was constructed by inserting annealed complementary oligonucleotides containing the triple tandem copies of the W_A-box fragment from the *ADS* promoter (annealed to yield *Xba*I- and *Bam*HI-compatible ends) into pRS315HIS. The sequence of the W_A-box×3 fragment is 5′-CTT GAC CCA TTT TGA CCC ATT TTG ACC CAT TT-3′. Yeast cells transformed with pRS315HIS::W_A-box×3 plasmid were grown on supplemented synthetic dextrose medium (SD) without leucine. The full-length *AaWRKY1* fragment was reamplified from cDNA by PCR. The ORF was ligated into the pPC86 vector to generate pPC86-*AaWRKY1* using the restriction sites *Eco*RI (forward) and *Spe*I (reverse). The pPC86-*AaWRKY1* and the empty pPC86 vector were introduced into the yeast containing the pRS315HIS::W_A-box×3 plasmid. The yeast strains transformed with the above constructs were grown on SD-Trp-Leu-His selective medium containing different concentrations of 3-AT at 30°C for 3 d.

Transactivation experiments in stably transformed tobacco and agroinfiltrated N. benthamiana

For the reporter construct, the *ADSpro1* promoter (AY528931, cloned in our laboratory in 2004) was amplified from the *A. annua* genome by PCR with the primer pair ADSpro1F (5′-CGT ACT GCA GGC ATA AGA ACA TAC AAA GCA-3′) and ADSpro1R (5′-GCT ACC ATG GGA TTT TCA AAA CTT TGA ATA TAT G-3′). The 2,385 bp long *ADSpro1* promoter was inserted into the pCAMBIA1301 vector, generating pCAMBIA-*ADSpro1::GUS*. For the effector construct, the coding region of *AaWRKY1* was inserted into the reconstructed pBI121 vector with *Bam*HI and *Xho*I restriction sites (PCR-based mutagenesis was applied to create an *Xho*I site immediately after the GUS TGA site) under the control of the cauliflower mosaic virus (CaMV) *35S* promoter. The recombinant plasmid pCAMBIA-*ADSpro1::GUS* was introduced into the *Agrobacterium tumefaciens* EHA105 strain; this strain was then used to transform *N. tabacum* cv. Wisconsin 38 (W38). Transformants were identified through selection in MS medium containing 15 mg l^{-1} hygromycin. The transgenic lines were confirmed by PCR using primers ADSpro1F and ADSpro1R with genomic DNA extracted from leaves of primary transformants as template. One positive primary transformant line was used as recipient for transformation of the effector gene. The effector construct pBI-*AaWRKY1* was directly transformed into the transgenic *GUS* reporter plant, and double transformants were identified through screening for antibiotic resistance (kanamycin and hygromycin) and confirmed by PCR using AaWRKY1F and AaWRKY1R with genomic DNA extracted from leaves of secondary transformants as template.

For transient expression experiments, the 1,176 bp *ADSpro2* promoter sequence (Kim et al. 2008) was isolated using primers ADSpro2F (5′-ACT GGT ACC CAT GGC TAA AAC CGA ATT CAA-3′) and ADSpro2R (5′-AGT CCA TGG CTT TTC ATA GAT GAG AAA CT-3′). The W_B-box (5′-AAT

GGG TCA ATT GGG GAG TG-3′) in *ADSpro2* was mutated into 5′-AAT TAA TCA ATT GGG GAG TG-3′ by the primer extension method with the primers mW_BboxF (5′-TTG GGA AAA ATT AAA TTA ATC AAT TGG GGA-3′) and mW_BboxR (5′-TTA ATT TAA TTT TTC CCA AGC CCA AGT CCA-3′); the resulting promoter was named *mW_BADSpro1*. The W_A-box (5′-AAT GTT GAC CCA TTC ATC AC-3′) in ADSpro2 was mutated into 5′-AAT GTT GAA ACA TTC ATC AC-3′ with the primer mW_AboxF (5′-TGT TTT TGA AAT GTT GAA ACA TTC ATC ACG-3′) and mW_AboxR (5′-TTT CAA CAT TTC AAA AAC ATA TTA GTC TTA-3′); the resulting promoter was named *mW_AADSpro2*. Double mutation *mW_{AB}ADSpro2* was carried out in the same way. *ADSpro2*, *mW_BADSpro2*, *mW_AADSpro2* and *mW_{AB}ADSpro2* were inserted into the pCAMBIA 1301 vector, replacing the 35S promoter. The four above-mentioned *ADSpro2::GUS*, *mW_BADSpro2::GUS*,*mW_AADSpro2::GUS*,*mW_{AB}ADSpro2::GUS* plasmids and *35S::AaWRKY1* were introduced into *A. tumefaciens* strain EHA105. In order to verify the effects of AaWRKY1 overexpression on W-boxes of the *ADS* promoter, a transient GUS activation was performed in tobacco leaves after infiltration with *A. tumefaciens* cells harboring (i) *ADSpro2::GUS*; (ii) *ADSpro2::GUS/35S::AaWRKY1*; (iii) *mW_B ADSpro2::GUS*; (iv) *mW_B ADSpro2::GUS/35S::AaWRKY1*; (v) *mW_AADSpro2::GUS*; (vi) *mW_AADSpro2/35S::AaWRKY1*; (vii) *mW_{AB}ADSpro2::GUS*; and (viii) *mW_{AB}ADSpro2::GUS/ 35S::AaWRKY1*. The ratio of *35S::AaWRKY1* to *ADSpro2::GUS*, *mW_B ADSpro1::GUS*, *mW_A ADSpro2::GUS*, *mW_{AB} ADSpro2::GUS* was adjusted to 1 : 1 for co-infiltration. Transient assay using *N. benthamiana* leaves was performed as described in the literature (Yang et al. 2000). Fully expanded leaves of 8-week-old *N. benthamiana* plants were used for agroinfiltration. After agroinfiltration, the *N. benthamiana* was covered with transparent plastic bags and maintained in a growth chamber at 22°C under 16 h light for 2 d. Experiments were done in triplicate; in each experiment six 12 mm discs from infiltrated areas of three independently infiltrated plants were transferred to Eppendorf tubes, frozen in liquid nitrogen and stored at −80°C.

Transient transformation of A. annua

Glycerol stocks of *A. tumefaciens* strain EHA105 containing *35S::GUS* and *A. tumefaciens* strain EHA105 containing *35S::AaWRKY1* constructs were thawed and then streaked onto solid LB medium (pH 7.0, containing 100 mg l^{-1} rifampicin and 100 mg l^{-1} kanamycin), then individual single colonies were used to start 5 ml of liquid YEB medium (5 g l^{-1} beef extract, 1 g l^{-1} yeast extract, 5 g l^{-1} peptone, 5 g l^{-1} sucrose, 0.5 g l^{-1} $MgCl_2$). These cultures were grown overnight with shaking at 28°C/200 r.p.m. The subsequent 100 ml cultures were started with 1 ml of the preceding 5 ml overnight culture and again incubated with shaking at 28°C/200 r.p.m. until OD_{600} = 0.5. Bacterial cells were harvested by centrifugation at 6,000 × *g* for 5 min and washed once with 10 ml of washing solution containing 10 mM $MgCl_2$ and 100 μM acetosyringone. After centrifugation at 6,000 × *g* for another 5 min, the pellet of bacterial cells was resuspended in 50 ml of liquid MS medium.

Artemisia annua leaves from 4-week-old aseptic seedlings were cut and soaked with 50 ml of MS liquid medium containing *A. tumefaciens* cells supplemented with 0.005% (v/v; i.e. 50 μl l^{-1}) surfactant Silwet L-77 in 100 ml Erlenmeyer flasks, then the Erlenmeyer flasks were placed in the desiccator and a vacuum was applied at 30 inHg for 10 min under the control of a vacuum pump (Barnant Co., Barrington, IL, USA).The vacuum pump was then switched off and air was slowly let into the desiccator, so as to minimize the damage to the tissues. Liquid medium was removed and the explants were plated on MS solid medium; co-cultivation was in the dark at 25°C for 72 h.

The *A. tumefaciens* EHA105 strain containing either *ADSpro2::GUS*, *mW_BADSpro2::GUS*, *mW_AADSpro2::GUS* or *mW_{AB}ADSpro2::GUS* was introduced into *A. annua* leaves by the above transient transformation method. After 72 h co-cultivation, the *A. annua* leaves were transferred to Eppendorf tubes, frozen in liquid nitrogen and stored at −80°C.

GUS activity assay

For stable transformation, three leaf discs of each transgenic plant were separately assayed for GUS activity, with each data point being the average of three transgenic plants. For transient GUS expression measurements in *N. benthamiana*, homogenates were made of six 12 mm discs from infiltrated areas of three independently infiltrated plants. For transient GUS expression measurements in *A. annua*, an equal amount of leaves was used. GUS activity was determined using an F-4500 Fluorescence spectrophotometer (Hitachi, Tokyo, Japan) as described in the literature (Jefferson et al., 1987) using 4-umbelliferyl-D-glucuronide as a substrate and 4-methylumbelliferone to calibrate the fluorometer. Protein content was determined according to the Bradford protein assay using BSA as a standard. GUS activity, normalized against protein concentration, was determined from the average of duplicate measurements per sample. Data were subjected to analysis of variance (ANOVA) and Duncan multiple comparison tests.

Gene expression analysis by semi-quantitative RT–PCR

For tissues expression analysis, total RNA was isolated from roots, leaves, stems, flowers and isolated trichomes of *A. annua* with an RNeasy Mini Kit (Qiagen). For elicitor treatments, 4-week-old plants were sprayed with 0.3 mM MJ or 150 mg l^{-1} chitosan which was prepared according to a reported method (Young et al. 1982). Leaves from three

plants were pooled for each time point, frozen in liquid nitrogen, and stored at −80°C before RNA extraction. Following co-cultivation, *A. annua* leaves were washed twice in aseptic distilled water and total RNA was isolated from explants with an RNeasy Mini Kit (Qiagen). First-strand cDNA synthesis was carried out with 1.0 μg of total RNA treated with DNase I (Roche) and Powerscript reverse transcriptase (Clontech). The resultant first-strand cDNA was used as a template for PCR. The conserved tobacoo *Actin* gene was used as an internal control.

The primers used were: actinF (5′-AAC TGG GAT GAC ATG GAG AAG ATA T-3′) and actinR (5′-TCA CAC TTC ATG ATG GAG TTG TAG G-3′) for *Actin*; AaWRKY1F (5′-ATG GAA AGT GTT TGT GTT TAT-3′) and AaWRKY1R (5′-AAA TTT GAA ATC AAG GTC TAA-3′) for *AaWRKY1*; ADSF (5′-ATG TCA CTT ACA GAA GAA AAA-3′) and ADSR (5′-TAT ACT CAT AGG ATA AAC GAG-3′) for *ADS*; HMGRF (5′-ATG ACT AGA GCG CCT GTT GTT-3′) and HMGRR (5′-GCA GAC ATC AAC GAC AAT TCA-3′) for *HMGR*; FPSF (5′-CTA TGT GGA TCT TGT GGA CCT-3′) and FPSR (5′-CCT GAA GAT TGA GAG TGT GGT-3′) for *FPS*; ADSF (5′-ATG TCA CTT ACA GAA GAA AAA-3′) and ADSR (5′-TAT ACT CAT AGG ATA AAC GAG-3′) for *ADS*; CYP71AV1F (5′-CGA AAG CAA TGG AGA AAG TAC-3′) and CYP71AV1R (5′-CTC AGT CTT TCT TTG CAT CGT-3′) for *CYP71AV1*; and DBR2F (5′-ATA AAT GAC AGA ACG GAC GAA-3′) and DBR2R (5′-GTC AAG AGA AGG GTA ATC CGT-3′) for *DBR2*. PCR products were electrophoresed on a 1% agarose gel and visualized after ethidium bromide staining. Pictures of gels were taken using a GelDoc EQ imager (Bio-Rad).

For transient gene expression analysis, band intensities were quantified using Quantity One software (Bio-Rad). The expression levels of genes involved in artemisinin biosynthesis were normalized using the *Actin* intensity as a loading control (set at 1.0). An average of three ethidium bromide-stained gels was determined and the standard deviation was calculated. Measurement data were expressed as mean ± SD. The analyses were performed using the statistical package for the Social Sciences (SPSS) statistical software for Windows, Version 9.0 (SPSS Inc., Chicago, IL, USA).

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