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The Jasmonate-Responsive AP2/ERF Transcription Factors AaERF1 and AaERF2 Positively Regulate Artemisinin Biosynthesis in *Artemisia annua* L.

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ABSTRACT Plants of *Artemisia annua* produce artemisinin, a sesquiterpene lactone widely used in malaria treatment. Amorpha-4,11-diene synthase (ADS), a sesquiterpene synthase, and CYP71AV1, a P450 monooxygenase, are two key enzymes of the artemisinin biosynthesis pathway. Accumulation of artemisinin can be induced by the phytohormone jasmonate (JA). Here, we report the characterization of two JA-responsive AP2 family transcription factors—AaERF1 and AaERF2—from *A. annua* L. Both genes were highly expressed in inflorescences and strongly induced by JA. Yeast one-hybrid and electrophoretic mobility shift assay (EMSA) showed that they were able to bind to the CRTDREHVCBF2 (CBF2) and RAV1AAT (RAA) motifs present in both *ADS* and *CYP71AV1* promoters. Transient expression of either *AaERF1* or *AaERF2* in tobacco induced the promoter activities of *ADS* or *CYP71AV1*, and the transgenic *A. annua* plants overexpressing either transcription factor showed elevated transcript levels of both *ADS* and *CYP71AV1*, resulting in increased accumulation of artemisinin and artemisinic acid. By contrast, the contents of these two metabolites were reduced in the RNAi transgenic lines in which expression of *AaERF1* or *AaERF2* was suppressed. These results demonstrate that AaERF1 and AaERF2 are two positive regulators of artemisinin biosynthesis and are of great value in genetic engineering of artemisinin production.

Key words: Artemisinin; *Artemisia annua*; sesquiterpene; amorpha-4, 11-diene synthase; CYP71AV1; jasmonate; AP2/ERF transcription factor.

INTRODUCTION

Artemisinin (*Qinghaosu* in Chinese), a sesquiterpene lactone with an endoperoxide bridge, was first isolated from *Artemisia annua* over 30 years ago (Liu et al., 1979; Tu et al., 1981). Artemisinin and its semi-synthetic derivatives are extensively used in the treatment of malaria, mostly in artemisinin-based combination therapies (ACTs) so as to reduce resistance (Duffy and Mutabingwa, 2006; Haynes, 2006; White, 2008). *A. annua* is the only natural source of artemisinin; however, its content in plants is relatively low (0.1–0.8% by dry weight) (Abdin et al., 2003). Engineering metabolic pathways of *A. annua* plants holds a great potential for increasing artemisinin production (Ro et al., 2006), but the success relies highly on deep understanding of the artemisinin biosynthesis pathway and the regulation of the enzyme genes.

Plants of *A. annua* produce a rich amount of monoterpenes and sesquiterpenes (Cai et al., 2002; Lu et al., 2002). Because of the great medical value of artemisinin, many investigations have been devoted to clarifying its biosynthetic pathway (Figure 1). The first committed step—cyclization of farnesyl diphosphate (FDP) into the sesquiterpene intermediate—is catalyzed by the sesquiterpene synthase, amorpha-4,11-diene synthase (ADS) (Bouwmeester et al., 1999; Chang et al., 2000; Wallaart et al., 2001). CYP71AV1, a cytochrome P450 monooxygenase, catalyzes the oxidation of amorpha-4,11-diene to artemisinic acid, via artemisinic alcohol and artemisinic aldehyde intermediates (Ro et al., 2006; Teoh et al., 2006). Recent reports show that a double bond reductase, DBR2,

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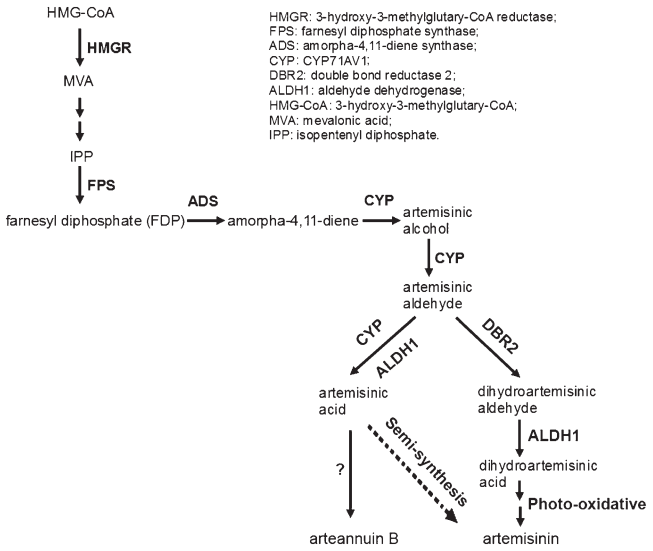

Figure 1. The Artemisinin and Arteannuin B Biosynthetic Pathway.

catalyzes the reduction of the Δ11(13) double bond of artemisinic aldehyde (Zhang et al., 2008), and an aldehyde dehydrogenase, ALDH1, catalyzes the oxidation of both artemisinic and dihydroartemisinic aldehydes (Teoh et al., 2009). Most of these enzymes are localized in the two apical cells of glandular trichomes, indicating that at least the initial steps of artemisinin biosynthesis occur in these cells (Olsson et al., 2009).

In comparison with the great progress made in cloning enzymes of the artemisinin pathway and engineering the pathways in microorganisms (Bouwmeester et al., 1999; Ro et al., 2006; Teoh et al., 2006; Zhang et al., 2008; Dietrich et al., 2009; Teoh et al., 2009), much less is known about the regulatory mechanisms of artemisinin biosynthesis in plants. A WRKY transcription factor, *AaWRKY1*, was reported to positively regulate the transcription of *ADS* by binding to the W-box in its promoter (Ma et al., 2009). In addition, we found that overexpression of *AtCRY1*, a blue light receptor of *Arabidopsis*, led to increased accumulation of artemisinin (Hong et al., 2009), implying a correlation between light signaling and artemisinin biosynthesis.

Plants have developed structurally diverse and tightly regulated secondary metabolites to battle against herbivores and pathogens (Bednarek and Osbourn, 2009), and accumulation of these metabolites in plants is often regulated directly or indirectly by phytohormones. It is well known that the phytohormones of jasmonate (JA), salicylic acid (SA), and abscisic acid (ABA) aid plants in survival from the biotic and abiotic stresses by triggering a *de novo* synthesis of protective metabolites and proteins (Wolucka et al., 2005; Tuteja, 2007; Yuan and Lin, 2008; Browse, 2009). Of these, JA has been shown to regulate secondary metabolism in many plant species. It up-regulates alkaloid biosynthesis in tobacco (De Boer et al., 2011), terpenoid indole alkaloid biosynthesis in *Catharanthus roseus* (van der Fits and Memelink, 2000), and it also stimulates the *de novo* biosynthesis of vitamin C in plant cell suspensions (Wolucka et al., 2005). Recent reports showed that JA treatment of *A. annua* plants resulted in enhanced artemisinin production (Baldi and Dixit, 2008; Wang et al., 2010), possibly by stimulating both the glandular trichome formation and the sesquiterpene accumulation in glandular trichome (Maes et al., 2011). However, transcription factors involved in JA regulation of artemisinin biosynthesis have not been reported.

Transcription factors of the APETALA2/ethylene response factor (AP2/ERF) family are characterized by the presence of DNA binding AP2 domain, which consists of ~60 conserved amino acid residues (Jofuku et al., 1994; Ohme-Takagi and Shinshi, 1995). Based on the number of AP2 domains and the existence of other domains, such as B3 DNA binding domain, this family can be divided into five subfamilies: AP2, DREB, ERF, RAV, and the fifth (comprising members not assigned to other four groups), of which the ERF subfamily constitutes the largest group (Sakuma et al., 2002; Feng et al., 2005). Members of the AP2/ERF family function as key regulators in both plant development and stress responses. For example, in *Arabidopsis*, the AP2 subfamily members targeted by miR172 are important in phase transition (Yant et al., 2010), the DREB members are predominantly involved in the regulation of abiotic stress response (Liu et al., 1998), and most ERF factors participate in biotic stress response and hormone (ethylene, JA, and SA) signaling (Gu et al., 2000; Brown et al., 2003; Lorenzo et al., 2003). There have been reports that AP2/ERF family transcriptional factors play an important role in plant secondary metabolism. For example, overexpression of the JA-inducible AP2/ERF-domain transcription factor *ORCA3* of *C. roseus* led to increased expression of several metabolic biosynthetic genes and consequently increased accumulation of terpenoid indole alkanoids in suspension cells (van der Fits and Memelink, 2000). Further investigation revealed that ORCA3 activated *strictosidine synthase* (*Str*) expression by directly interacting with a JA- and elicitor-responsive element (JERE) in its promoter regions (van der Fits and Memelink, 2001).

Considering the important role of AP2/ERF factors in JA signaling and plant secondary metabolism, as well as the positive role of JA on artemisinin biosynthesis, it is interesting to ask whether these transcription factors are involved in the regulation of artemisinin biosynthesis. By characterization the genes of two JA-responsive AP2/ERF subfamily transcriptional factors (*AaERF1* and *AaERF2*) isolated from an *A. annua* glandular trichome cDNA library, we show that both of them are able to bind to the *cis*-elements of *ADS* and *CYP71AV1* promoters and activate their expression; overexpression of the two AP2/ERF genes results in increased accumulation of artemisinin and artemisinic acid in transgenic *A. annua* plants.

RESULTS

Induction of *ADS* and *CYP71AV1* Expression by MeJA

To investigate the effects of JA on artemisinin biosynthesis, genes encoding the general mevalonate (MVA) pathway enzymes of 3-hydroxy-3-methylglutaryl coenzyme A reductase

(HMGR) and farnesyl diphosphate synthase (FPS), as well as the artemisinin pathway enzymes of ADS and CYP71AV1 were analyzed for their spatial patterns of expression and responses to methyl jasmonate (MeJA). Quantitative RT–PCR (qRT–PCR) showed that the transcript levels of *ADS* and *CYP71AV1* were high in inflorescences, moderate in young leaves, and low in mature leaves and stems (Figure 2A)—a pattern that was consistent with the report that artemisinin content was 4–11-fold higher in inflorescences than in leaves of *A. annua* L. (Ferreira et al., 1995). When young plants were treated with MeJA, expression level of *ADS* was increased rapidly and peaked within 1 h post treatment, followed by a quick decline (Figure 2B). The response of *CYP71AV1* to MeJA treatment was relatively slow but lasted longer, with the maximum transcript level occurring at 9 h post treatment (Figure 2B). The expression of *HMGR* and *FPS* genes was, however, neither expressed highly in inflorescences nor responded to MeJA treatment (Figure 2A and Supplemental Figure 1). These results indicate that JA promotes expression of key enzyme genes of the artemisinin biosynthesis pathway.

To find out *cis*-elements responsible for the MeJA induction of *ADS* and *CYP71AV1* expression, promoter regions of the two genes were scanned with PLACE software (www.dna.affrc.go.jp/htdocs/PLACE/signalscan.html) for putative transcription factor binding sites. We found that both promoters contained CRTDREHVCBF2 (CBF2) and RAV1AAT (RAA) motifs, which were reported to be the binding sites of AP2/ERF transcription factors (Kagaya et al., 1999; Xue, 2003), implying that transcription factors of this family might participate in transcriptional regulation of *ADS* and *CYP71AV1*.

Isolation and Characterization of AaERF1 and AaERF2

To isolate the candidate AP2/ERF family transcription factors, we searched expressed sequence tags (ESTs) from a glandular trichome cDNA library of *A. annua* for conserved AP2 domain sequences, which retrieved seven cDNA fragments. Subsequent qRT–PCR analyses showed that two of them were highly expressed in inflorescence (Figure 2A) and responsive to MeJA treatment (Figure 2C).

By 5′-rapid amplification of cDNA ends (5′-RACE), full-length cDNAs of the two genes were isolated and named *AaERF1* and *AaERF2*, respectively. *AaERF1* contains an open reading frame (ORF) encoding a protein of 253 amino acids, whereas the protein encoded by *AaERF2* contains 204 amino acids. Both AaERF1 and AaERF2 contain only one conserved DNA-binding domain (AP2/ERF domain), which shows a high sequence identity (79.7%) between each other. In the AP2/ERF domain, the 14th alanine and the 19th aspartic acid along with a WLG motif are highly conserved (Sakuma et al., 2002) (Supplemental Figure 2A). AaERF1 and AaERF2 share a very low sequence identity (~10.6%) except for the conserved AP2/ERF domain. Based on the sequence information and domain structure, AaERF1 and AaERF2 both belong to the ERF subfamily (Sakuma et al., 2002). Members of this subfamily have been reported to function as either activators or suppressors of defense

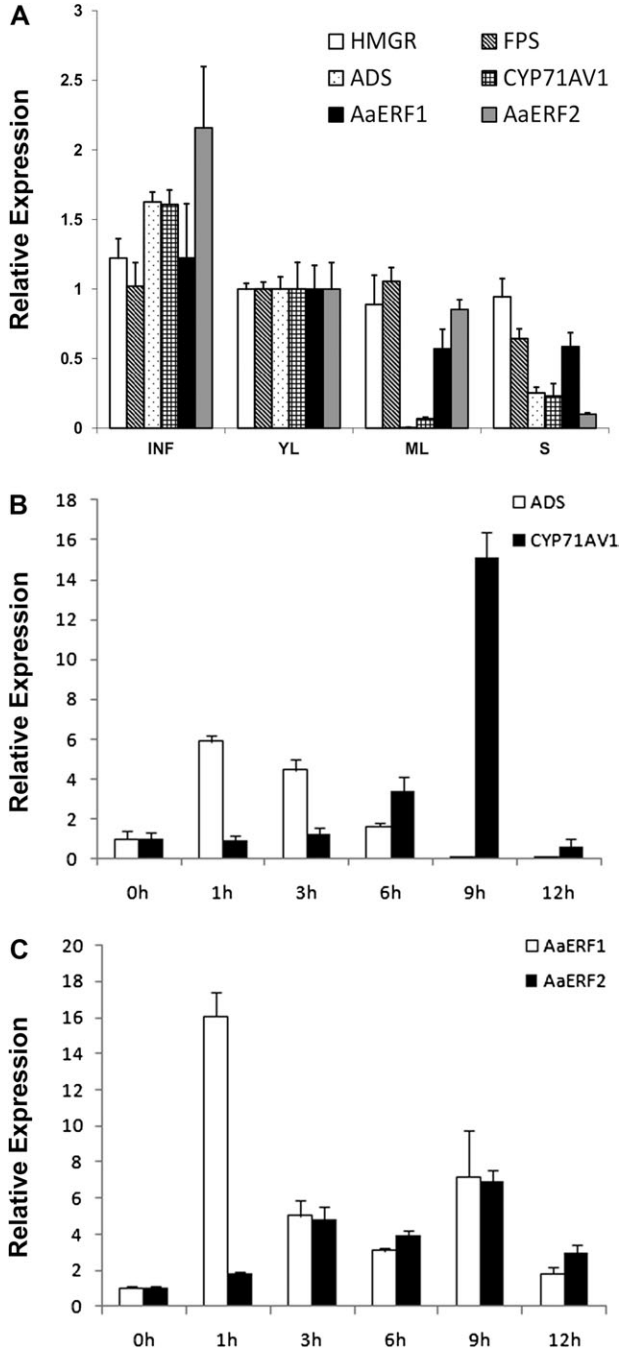

Figure 2. Expressions of *ADS*, *CYP71AV1*, *AaERF1*, and *AaERF2* in Plants of *A. annua* and Their Response to MeJA Elicitation.

(A) Expression levels of *HMGR*, *FPS*, *ADS*, *CYP71AV1*, *AaERF1*, and *AaERF2* in inflorescence (INF), young leaves (YL, from the 1/3 upper stem), mature leaves (ML, from the 1/3 lower stem), and stem (S). *ACTIN* was used as the internal standard.

(B) *ADS* and *CYP71AV1* expression in response to MeJA treatment. The 4-week-old plants were dipped in DMSO or MeJA solution (50 μM). Young rosette leaves were collected at 0, 1, 3, 6, 9, and 12 h of the treatment for qRT–PCR analysis.

(C) *AaERF1* and *AaERF2* expression in response to MeJA treatment. Plant materials used were the same as described in (B). *ACTIN* was used as an internal control. Error bars indicate SD of three technical replicates, and the results were consistent in three biological replicates.

genes, and play important roles in cross-talk of ethylene and JA signaling pathways (Lorenzo et al., 2003; Anderson et al., 2004; Gutterson and Reuber, 2004; McGrath et al., 2005). The ERF subfamily can be further divided into six groups (B1–B6) according to the conserved amino acid residues and the existence of other motifs. B1 group members share a conserved EAR repression domain and act as transcription repressors (Sakuma et al., 2002; Kazan, 2006; Dong and Liu, 2010), whereas B3 group is consisted of transcription activators, such as AtERF1, AtERF2, AtERF5, of which AtERF2 functions positively in plant defense and JA-dependent responses (Fujimoto et al., 2000; McGrath et al., 2005). Sequence comparison and phylogenetic analysis place both AaERF1 and AaERF2 in the B3 group (Supplemental Figure 2B). Moreover, AaERF1 shows 63% protein sequence identity to tomato LeERF1 whereas AaERF2 is 51% identical to *Nicotiana sylvestris* NsERF4; both LeERF1 and NsERF4 are involved in ethylene-mediated wounding responses (Kitajima et al., 2000; Tournier et al., 2003).

Expression and Induction Patterns of *AaERF1* and *AaERF2*

The expression patterns of both *AaERF*s in *A. annua* plants were then compared to those of the artemisinin pathway genes. According to qRT–PCR, both *AaERF1* and *AaERF2* exhibited a spatial expression pattern similar to that of *ADS* and *CYP71AV1*, with the highest level of transcripts in inflorescence, and a moderate level in young leaves (Figure 2A).

It has been reported that artemisinin accumulation in plants and cultured cells was increased after treatment with MeJA (Baldi and Dixit, 2008; Wang et al., 2010). We then treated the 4-week-old *A. annua* plants with various phytohormones, including MeJA, SA, auxin (3-indole acetic acid, IAA), gibberellic acid (GA_3), 1-aminocyclopropane-1-carboxylate (ACC, a precursor of ethylene). We found that, among the five hormones tested, MeJA was most effective in inducing *ADS* and *CYP71AV1* expressions (Supplemental Figure 1). Importantly, expression of both *AaERF*s also responded to MeJA treatment. The transcript level of *AaERF1* was increased rapidly and peaked within 1 h after MeJA treatment, followed by a quick decline (Figure 2C), whereas the *AaERF2* response to MeJA treatment was relatively slow but lasted longer, with the highly elevated transcript level occurring between 3 and 9 h post treatment (Figure 2C). Together, the expression patterns and sequence information suggested that both AaERF1 and AaERF2 may serve as transcription factors in mediating the MeJA regulation of artemisinin pathway genes.

Activation of *ADS* and *CYP71AV1* Promoters by AaERF1 and AaERF2

To determine the subcellular localization of AaERF1 and AaERF2, the ORFs of *AaERF1* and *AaERF2* were in-frame fused to the yellow fluorescent protein (YFP) derivative VENUS, respectively. After transferring the constructs into onion epidermal cells, the florescence was observed exclusively in nuclei; by contrast, cells transformed with 35S:VENUS showed florescence throughout the cell (Figure 3). These data indicate that both AaERF1 and AaERF2 proteins are nuclear-localized, consistently with their putative role as transcription factors.

To test whether AaERF1 and AaERF2 could bind to promoters of *ADS* and *CYP71AV1*, yeast one-hybrid assays were performed. Triple repeated RAA and CBF2 motifs were integrated into the genome of yeast cells, respectively. After introducing *AaERF1* and *AaERF2* into the yeast strains, we found that the two ERF factors indeed were able to bind to both the CBF2 and the RAA motifs, respectively, and AaERF2 appeared to have a higher affinity than AaERF1 to these two DNA motifs (Figure 4A).

To further examine the binding activities of AaERF1 and AaERF2 to the *ADS* and *CYP71AV1* promoters, an electrophoretic mobility shift assay (EMSA) was performed. The recombinant

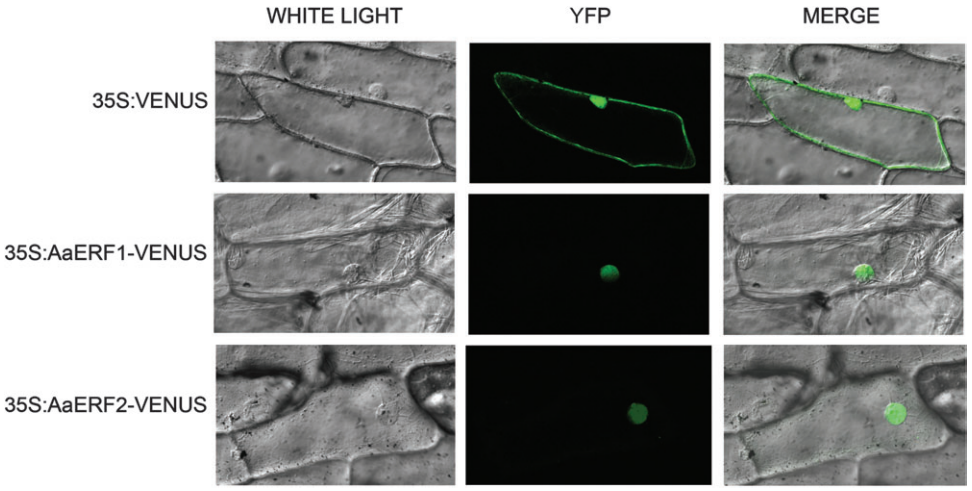

Figure 3. Nuclear Localization of AaERF1 and AaERF2 Proteins in Onion Epidermal Cells.

Onion epidermal cells were transiently transformed with constructs harboring AaERF1–VENUS, AaERF2–VENUS, or a control VENUS, respectively, under the control of 35S promoter. Bright-field (left), fluorescence (middle), and the corresponding overlay (right) images of cells were shown.

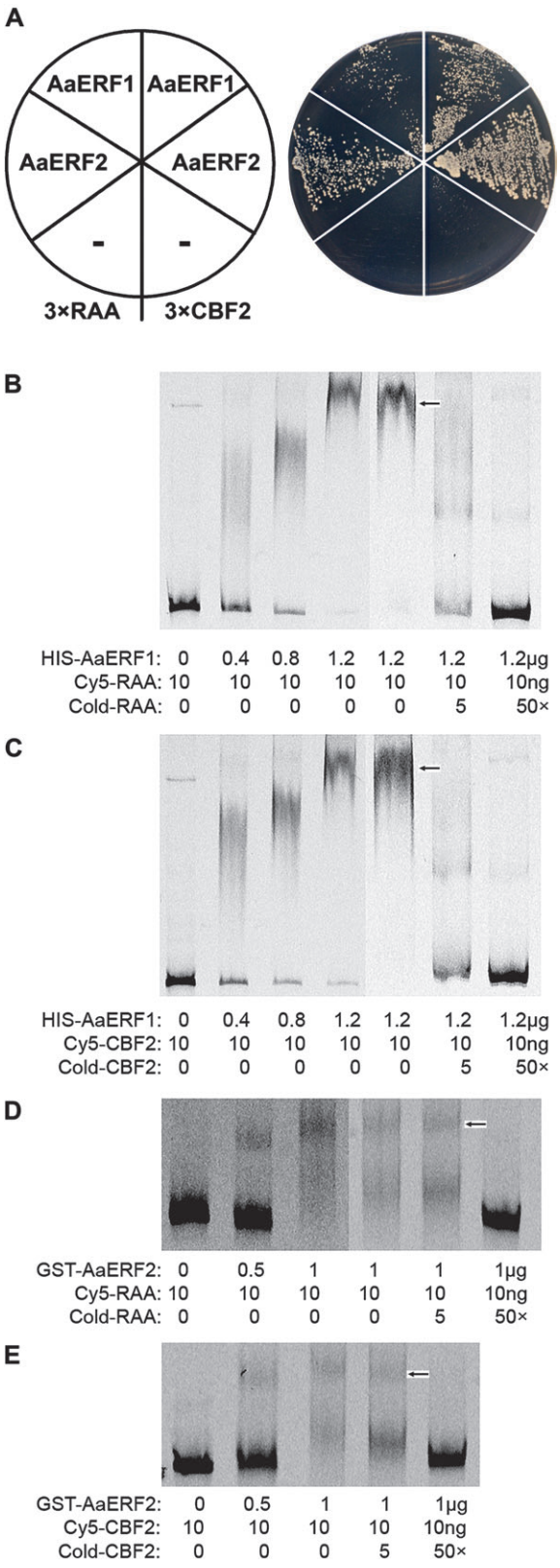

Figure 4. Binding Assay of AaERF1 and AaERF2 to RAA and CBF2 *cis*-Elements.

(A) Diagram and the result of yeast one-hybrid assay for the interaction between each AaERF protein with DNA motifs. Triple RAV1-AAT (RAA) motif and CRTDREHVCBF2 (CBF2) motif were used as bait, respectively. Yeast cells carrying pGAD424, pGAD–AaERF1, or pGAD–AaERF2 were grown for 3 d at 30°C on SD/-His/-Leu base with 10 mM 3-amino-1,2,4-triazole (3-AT).

(B, C) EMSA analysis of binding of AaERF1 protein to the RAA and CBF2 *cis*-elements. Ten micrograms of Cy5-labeled RAA (B) or CBF2 (C) were incubated with different concentrations HIS–AaERF1 fusion protein (0, 0.4, 0.8, and 1.2 μg) at 25°C for 30 min to compete with cold probes (0, 5, 50 X) and then analyzed by electrophoresis.

(D, E) EMSA analysis of binding of AaERF2 protein to the RAA and CBF2 *cis*-elements. Ten micrograms of Cy5-labeled RAA (D) or CBF2 (E) were incubated with different concentrations of the GST–AaERF2 fusion protein (0, 0.5, and 1 μg) at 25°C for 30 min to compete with cold probes (0, 5, 50 X) and then analyzed by electrophoresis. The bands were clarified with a solid black arrow.

protein of HIS–AaERF1 showed binding activities to both the 3×RAA and the 3×CBF2 fragments (Figure 4B and 4C). The DNA-binding specificity was then confirmed in a competition experiment with excess amounts of the unlabeled probes of 3×RAA and 3×CBF2, respectively (Figure 4B and 4C). Similarly, the recombinant protein of GST–AaERF2 with improved solubility also interacted with the 3×RAA and 3×CBF2 DNA fragments (Figure 4D and 4E). Thus, data gained from both yeast one-hybrid assay and *in vitro* EMSA support that AaERF1 and AaERF2 recognize and interact with the RAA and CBF2 palindromes present in *ADS* and *CYP71AV1* promoters.

To provide more evidence that *AaERF*s regulate *ADS* and *CYP71AV1* transcription, an *Agrobacterium*-mediated transient expression assay was performed. The promoter fragments of *ADS* (~2 kb) and *CYP71AV1* (~1.3 kb) were used to drive the *GUS* reporter gene, respectively, and the ORF of either *AaERF*s was overexpressed under the control of the 35S promoter (Figure 5A). Each promoter–*GUS* construct was then co-transferred with either 35S:*AaERF1* or 35S:*AaERF2*, respectively, into tobacco (*Nicotiana benthamiana*) leaves. We found that both AaERF1 and AaERF2 increased the *GUS* reporter gene expression. Based on *GUS* transcript abundance, up to threefold increase in the *ADS* promoter activity was achieved by AaERF2, and that of *CYP71AV1* promoter was increased about sixfold by AaERF1 (Figure 5B).

AaERF1 and AaERF2 Promote Artemisinin Biosynthesis

Since AaERF1 and AaERF2 could bind to the *ADS* and *CYP71AV1* promoters and enhance gene transcription, it was then interesting to further analyze the role of the two transcription factors in regulating artemisinin biosynthesis in plants of *A. annua*. Both *AaERF1* and *AaERF2* were overexpressed in *A. annua* under the control of the 35S promoter, respectively. The transgenic plants were first confirmed by semi-qRT–PCR and genomic DNA-based PCR on kanamycin-resistant gene (Supplemental Figure 3), and then three transgenic lines for each gene were chosen for further analysis.

In the overexpression (ox) plants examined, transcripts of *AaERF1* and *AaERF2* were increased by about twofold, respectively (Figure 6A and 6B). Consequently, expression levels of

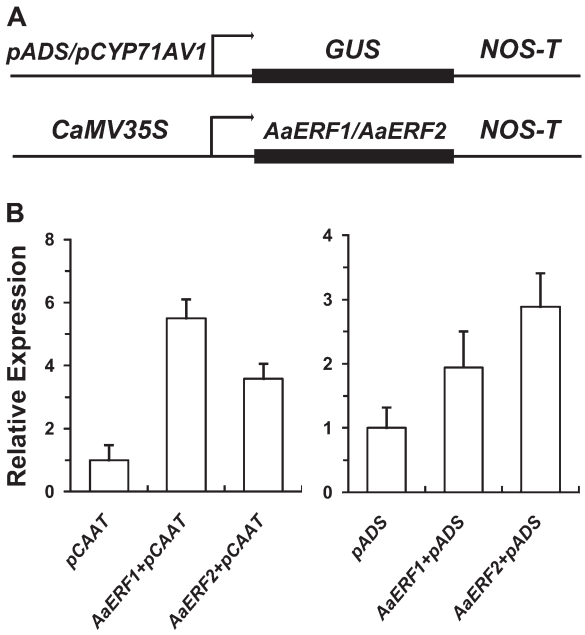

Figure 5. Enhancing *ADS* and *CAAT* (*CYP71AV1*) Promoter Activities by AaERF1 and AaERF2.

(A) Schematic diagram of the reporter (pADS/pCAAT:GUS) and effector (35S:*AaERF1*/*AaERF2*) constructs used in the co-expression experiment. The constructs were co-transformed into leaves of 1-month-old tobacco (*N. benthamiana*). After growing in a greenhouse for 3 d, leaves were harvested for qRT–PCR analysis.

(B) qRT–RCR analysis of the *GUS* gene expression in transiently transformed *N. benthamiana* leaves. AaERF1 and AaERF2-activated *GUS* gene expression in pCAAT:*GUS* (left) and pADS:*GUS* (right) reporters, respectively. *NbACTIN1* (*ACTIN1* of *N. benthamiana*) was used as the internal standard. Error bars indicate SD of three technical replicates, and the results were consistent in three biological replicates.

ADS and *CYP71AV1* were also elevated drastically, ranging from 2- to 8- and 1.2- to 5-fold, respectively (Figure 6A and 6B). The expression of *DBR2* was also moderately increased, whereas the expression levels of the upstream MVA pathway enzyme genes of *HMGR* and *FPS* were barely or only slightly changed (Supplemental Figure 4). The artemisinin and artemisinic acid contents in mature leaves of the 3-month-old *A. annua* plants were analyzed by high-performance liquid chromatography (HPLC). Compared to the wild-type control, artemisinin and artemisinic acid levels were increased by 19–67% and 11–76% in *AaERF1-ox* transgenic plants, and by 24–51% and 17–121% in *AaERF2-ox* plants, respectively (Figure 6C and 6D).

To further prove the function of *AaERF1* and *AaERF2* in regulation of artemisinin biosynthesis *in planta*, we down-regulated their expression by an RNA interference (RNAi) approach. In the dsRNA transgenic lines, the expression of *AaERF1* and *AaERF2* was suppressed to 27–62% and 19–55% of the wild-type level, respectively. Consequently, *ADS* and *CYP71AV1* transcript levels were reduced to 35–13% and 38–7% of the wild-type level in *AaERF1-ds* plants, and to 9–4% and 12–3% in *AaERF2-ds* plants, respectively (Figure 7A and 7B). As a result of reduced *ADS* and *CYP71AV1* gene expression, the contents of artemisinin and artemisinic acid were decreased to 76–58% and 55–30% of the wild-type level in *AaERF1-ds* plants. In *AaERF2-ds* plants the reduction was 70–51% for artemisinin and 69–37% for artemisinic acid, respectively (Figure 7C and 7D). We did not observe any morphological difference between AaERF overexpression or RNAi transgenic lines and the wild-type plants (QT). These data demonstrate clearly that *AaERF*s are positive regulators of *ADS* and *CYP71AV1* and contribute to the production of artemisinin and artemisinic acid in plants.

DISCUSSION

The phytohormone JA plays an important role in many aspects of plant life, including flower development (Xie et al., 1998), root growth (Staswick et al., 1992), leaf senescence (Kong et al., 2006), and particularly plant response to biotic and abiotic stresses that are partly achieved by enhancing biosynthesis of secondary metabolites, including terpenoids (Farmer et al., 2003; Buseman et al., 2006). For example, our previous investigation showed that, in rice, JA induced the expression of *OsTPS3*, a sesquiterpene synthase gene, leading to increased release of (E)-β-caryophyllene (Cheng et al., 2007). In suspension-cultured cells of cotton (*Gossypium arboreum*), accumulation of the sesquiterpene aldehyde gossypol and expression of the sesquiterpene synthase ((+)-δ-cadinene synthase) gene were strongly induced by treatments of JA and a fungal elicitor (Xu et al., 2004). In *C. roseus*, regulation of biosynthesis of terpenoid indole alkaloids biosynthesis was mediated by *ORCA3*, an AP2/ERF subfamily transcriptional factor (van der Fits and Memelink, 2000). In this investigation, we found that stimulation of artemisinin biosynthesis in *A. annua* by JA was also mediated by the AP2/ERF subfamily members AaERF1 and AaERF2, which directly bound to promoter regions of *ADS* and *CYP71AV1*. These suggest that, despite diverse chemical structures and biological functions of plant terpenoids, similar regulatory mechanisms exist in plants of different taxa. Considering the signaling functions of JA and defense functions of plant secondary metabolites, how such regulatory mechanism is established and maintained during evolution is an interesting subject of further investigation.

The basic helix-loop-helix transcription factors play important roles in plant organ development, light signaling, stress response, and plant secondary metabolism (Gonzalez et al., 2008; Leivar et al., 2009; Groszmann et al., 2010; Fernandez-Calvo et al., 2011). The *Arabidopsis* bHLH transcription factor AtMYC2 has long been considered a central regulator of JA signaling pathway, mediating both developmental and stress responses (Yadav et al., 2005; Dombrecht et al., 2007). Recently, AtMYC3 and AtMYC4 were reported to act additively with AtMYC2 to regulate different subsets of JA responses (Cheng et al., 2011; Fernandez-Calvo et al., 2011). It was also reported that the homologous protein in *C. roseus*, CrMYC2, mediated the JA signaling and terpenoid indole alkaloid biosynthesis by binding to the promoter region of the AP2/ERF transcription

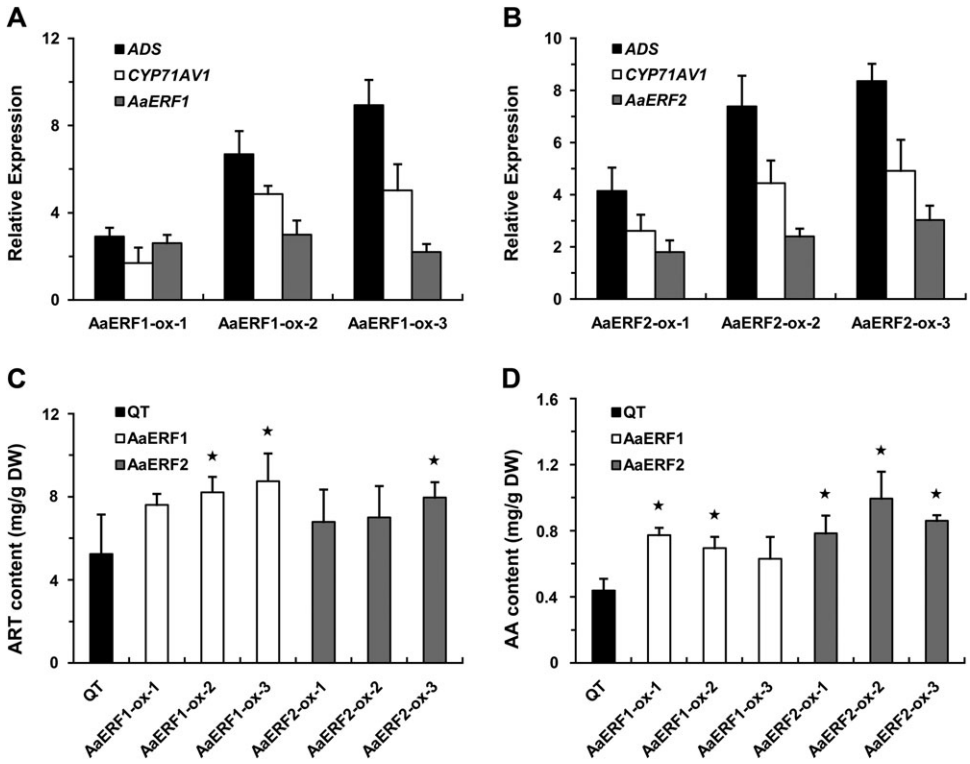

Figure 6. Elevated Expression of *ADS* and *CYP71AV1*, and Artemisinin (ART) and Artemisinic acid (AA) Contents in *A. annua* Overexpressing *AaERF1* or *AaERF2*. Untransformed plants of *A. annua* cv. QiuTe (QT) were used as control.

(A) Expression of *AaERF1*, *ADS*, and *CYP71AV1* in leaves of the 3-month-old plants transformed with 35S:*AaERF1* (AaERF1-ox). *ACTIN* was used as the internal standard; for each gene, the expression level relative to *ACTIN* in the control plant (QT) was taken as 1. Error bars indicate SD of three technical replicates, and the results were consistent in three biological replicates.

(B) Expression of *AaERF2*, *ADS*, and *CYP71AV1* in leaves of the 3-month-old plants transformed with 35S:*AaERF2* (AaERF2-ox).

(C) Contents of artemisinin (ART) in leaves of AaERF1-ox and AaERF2-ox transgenic plants, determined by HPLC. The content of ART was compared to dry weight of sample. Error bars indicate SD of three biological replicates. Asterisks denote Student's *t*-test significance compared with QT (* $P < 0.05$).

(D) Contents of artemisinic acid (AA) in leaves of AaERF1-ox and AaERF2-ox transgenic plants, determined by HPLC. The content of AA was compared to dry weight of sample.

factor gene *ORCA3* (Zhang et al., 2011a). Coincidently, in tobacco, NtMYC2 positively regulated nicotine biosynthesis not only by binding to the G-box in the biosynthetic genes' promoters, but also via activating *NIC2*-locus *ERF* genes that functioned redundantly with NtMYC2 in up-regulating nicotine biosynthesis (Shoji and Hashimoto, 2011; Zhang et al., 2011b). It should be interesting to investigate whether both types of transcription factors are also involved in artemisinin biosynthesis in *A. annua*. Isolation and characterization of *cis*-elements in promoter regions of *AaERFs* will facilitate our understanding of the signaling network in regulating sesquiterpene biosynthesis in *A. annua*.

Artemisinin is an effective ingredient for malaria treatment; however, its utilization has been limited due to low yield in plants of *A. annua*. Despite the great potential of engineering microbes to produce artemisinin, until now, only artemisinic acid has been produced in large quantities (Ro et al., 2006). In tobacco, heterologous expression of *ADS* and *CYP71AV1* led to the production of amorpha-4,11-diene and artemisinic alcohol, and additional expression of *DBR2* and *ALDH1* led to the accumulation of dihydroartemisinic alcohol (Zhang et al., 2011c). However, no artemisinin was detected, largely due to unclarified remaining steps in the artemisinin biosynthesis pathway. Therefore, further elucidation of the artemisinin biosynthetic pathway and the regulatory mechanism is of great importance. Biosynthesis of plant secondary metabolites usually involves multiple enzymes, increasing the target metabolite production in the host plant by overexpressing one or two enzymes, or engineering one organism to produce specialized secondary metabolites of another organism by heterologous expression of enzymes, and is often inefficient. Regulatory components often target the key steps or even the whole biosynthetic pathway. We have shown that overexpression of the two AP2/ERF-type transcription factors, AaERF1 and AaERF2, resulted in increased artemisinin accumulation in transgenic *A. annua* plants. Therefore, engineering the regulatory mechanism, especially transcription factors, is of great potential in engineering plant secondary metabolism. In addition, blocking

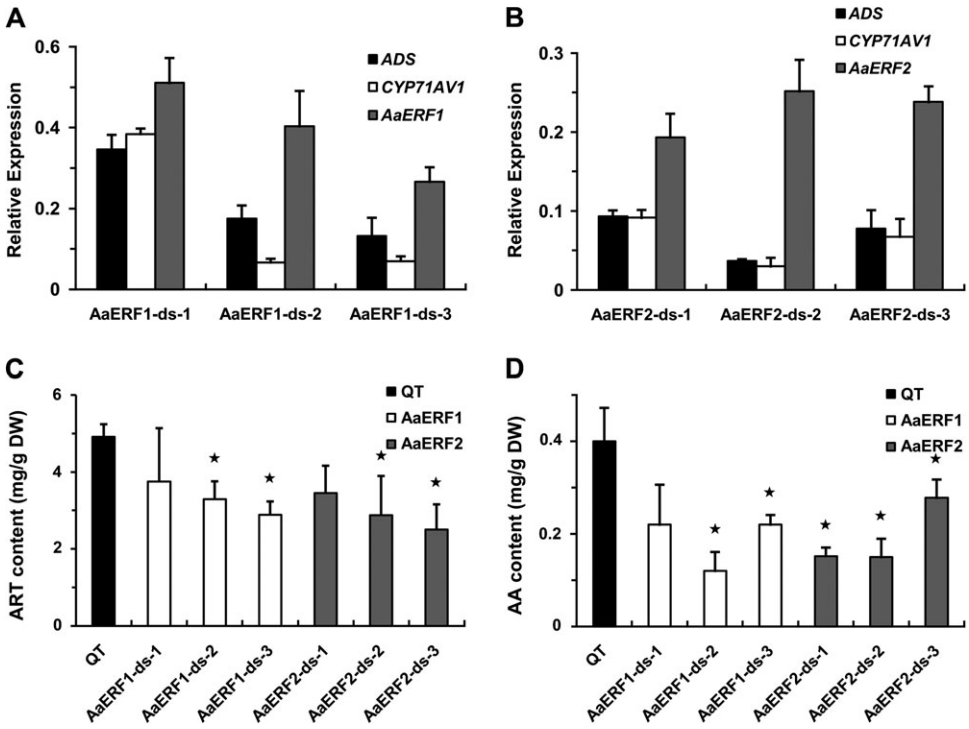

Figure 7. Reduced Expression of *ADS* and *CYP71AV1*, and ART and AA Contents in *A. annua* Expressing the dsRNA Targeting *AaERF1* or *AaERF2*. Untransformed plants of *A. annua* cv. QiuTe (QT) were used as control.

(A) Expression of *AaERF1*, *ADS*, and *CYP71AV1* in the leaves of the 3-month-old AaERF1-ds plants. *ACTIN* was used as the internal standard; for each gene, the expression level relative to *ACTIN* in the control plant (QT) was taken as 1. Error bars indicate SD of three technical replicates, and the results were consistent in three biological replicates.

(B) Expression of *AaERF2*, *ADS*, and *CYP71AV1* in leaves of the 3-month-old AaERF2-ds plants.

(C) Contents of artemisinin (ART) in leaves of AaERF1-ds and AaERF2-ds transgenic plants, determined by HPLC. The content of ART was compared to that of the sample. Error bars indicate SD of three biological replicates. Asterisks denote Student's *t*-test significance compared with QT (* $P < 0.05$).

(D) Contents of artemisinic acid (AA) in leaves of AaERF1-ds and AaERF2-ds transgenic plants, determined by HPLC. The content of AA was compared to that of the sample.

other competing pathways, such as sterol and β-caryophyllene biosynthesis, could enrich precursors for artemisinin formation (Yang et al., 2008; Chen et al., 2011). Combining these two strategies will shed new light on increasing artemisinin production. As artemisinin content increases obviously in the full-flower stage and correlates closely to trichome changes (Arsenault et al., 2010), the control of *A. annua* phase transition and trichome density is worthy of further investigation.

METHODS

Plant Materials and Phytohormone Treatments

A. annua L. cv. QT was used in this investigation, and the seeds were obtained from Sichuan Province, China. Plants of *A. annua* L. and *Nicotiana benthamiana* were grown in a greenhouse with 14/10-h light/dark photoperiod at 26°C. For MeJA treatment, plants of *A. annua* were dipped in MeJA solution (50 μM) for 1, 3, 6, 9, and 12 h, as indicated, and the DMSO solution was used as mock. For other phytohormone (MeJA, SA, IAA, GA_3, ACC), the leaves of 4-week-old *A. annua* plants were collected at 4-h post treatments. For tissue expression pattern analysis, the 2-month-old plants were transferred to 12/12-h photoperiod for flowering. After another month, different parts were collected; leaves on the 1/3 upper stem were defined as young leaves and those on the 1/3 lower stem as mature leaves.

Isolation of cDNAs

Based on expressed sequence tags (ESTs), full-length cDNAs of *AaERF1* and *AaERF2* were obtained by 5′-rapid amplification of cDNA ends (5′-RACE), using the GeneRacer Kit (CLONTECH Laboratories, Inc.). An adaptor primer (forward) and two gene-specific primers (reverse) were used for isolation. All the primers used in this investigation are listed in Supplemental Table 1.

Transgenic Plants Confirmation and Expression Analysis

The genomic DNA of kan-resistant and control plants was isolated as follows: 1-month-old plant material of 0.2 g was ground

to powder and 0.6 ml buffer (100 mM Tris, 50 mM EDTA, 0.5 M NaCl, 10 mM β-mercaptoethanol) added, and it was boiled for 10 min before centrifugation at 13 000 rpm for 10 min. The supernatant was mixed with isopropanol and placed at –20°C for 30 min, centrifuged at 13 000 rpm under 4°C, and the precipitate was dissolved as templates for PCR analysis.

Total RNAs were prepared with Trizol reagent (Invitrogen). One microgram total RNA was used as a template for reverse transcription with oligo (dT) as primer in a 20-μl reaction system, using the RNA PCR (AMV) kit (Promega). Quantitative real-time RT–PCR (qRT–PCR) was performed with the SYBR-Green PCR Mastermix kit (Takara) on a Mastercycler ep RealPlex2 (Eppendorf) monitor. The relative expression level of each gene was determined as described previously (Yu et al., 2010) and the transcript level of *A. annua ACTIN* (EU531837) was used as a control.

Plant Transformation

The full-length coding regions of *AaERF1* and *AaERF2* were amplified by PCR with sticky *Pst*I and *BamH*I ends, and inserted into the binary vector pCAMBIA2300 under the control of the CaMV 35S promoter. Each construct was introduced into *Agrobacterium tumefaciens* strain EHA105 for plant transformation, which was performed as previously described (Hong et al., 2009). The defined ORF fragments of *AaERF1* (183–517 bp downstream of ATG codon) and *AaERF2* (39–359 bp downstream of ATG codon) were PCR-amplified for RNAi constructs (for primers, see Supplemental Table 1), followed by inserting into the modified pCAMBIA2300 under the control of the 35S promoter. Plants of the wild-type *A. annua* L. cv. QT were used for transformation. The rest of the plants that were not transformed were used as control for both gene expression and HPLC analysis. At least three independent control lines (QT) were tested in these experiments and the mean value was shown as the control.

Protein Subcellular Localization

The full-length coding regions of *AaERF1* and *AaERF2* were in-frame fused to the N-terminus of the *VENUS* gene with a glycine linker under the control of the 35S promoter. The AaERF1-linker–VENUS and AaERF2-linker–VENUS fusions as well as the VENUS control vectors were bombarded into onion epidermal cells by particle bombardment (Bio-Rad). Transformed cells were cultured on MS medium for 24 h in dark and images were obtained with an LSM510 laser scanning confocal microscope (Zeiss), with argon laser excitation at 488 nm and a 505–550-nm emission filter set.

Yeast One-Hybrid Assay

The binding assay utilizing MATCHMAKER yeast one-hybrid system (Clontech) was performed according to the manual. The triple tandem copies of the CRTDREHVCBF2 motif (5′-AGGTCGACCGATAGGTCGACCGATAG GTCGACCGAT-3′) and the RAV1AAT motif (5′-CTCAACATTGTCTCAACATT GTCTCAACATTGT-3′) were inserted into pHISi-1 between *Sac*I and *Xba*I as baits, respectively. These two bait constructs were linearized and integrated into the genome of yeast strain *YM4271*. The preys were the ORFs of *AaERF1* and *AaERF2* in-frame fused with the GAL4 activation domain in pGAD424 between *BamH*I and *Pst*I. The preys were then introduced into yeast cells previously transformed with baits, with blank pGAD424 plasmid as control. The positively transformed clones were analyzed on SD/-His/-Leu medium with different concentrations of 3-amino-1,2,4-triazole (3-AT).

Electrophoretic Mobility Shift Assay

The ORF of *AaERF1* was inserted into the expression vector pET32a (Novagen) between the *Sac*I and *Sal*I sites, and the ORF of *AaERF2* was inserted into the expression vector pGEX–4T-1 (Amersham Pharmacia Biotech) between the *Bam*HI and *Sal*I sites so as to increase protein solubility. The recombinant AaERF1 and AaERF2 proteins were purified according to the instruction manual. The probe sequences were the same as those used in yeast one-hybrid assay except for labeled with Cy5 on both ends. The DNA fragment and purified proteins were incubated at 25°C for 30 min and then separated with native polyacrylamide gel electrophoresis (PAGE). The fluorescence was detected with a FUJIFILM FLA-9000 image scanner.

Transient Expression Assay

With an *Xba*I sticky end (5′) and a *Sma*I blunt end (3′), the 2 037-bp fragment of *ADS* promoter (DQ448297) and a 1 274-bp fragment of *CYP71AV1* promoter (CN 200910045041) were fused with *β-glucuronidase* (*GUS*) reporter gene and cloned into the binary vector pBI101, respectively (Kim et al., 2008; Tang et al., 2009). The ORFs of *AaERF1* and *AaERF2* were inserted into the binary vector pCAMBIA1300 between *BamH*I and *Sal*I sites under the control of 35S promoter. These constructs (including modified pCAMBIA1300 as a control) were then introduced into *A. tumefaciens* strain GV3101, respectively. For transient transformation, the *Agrobacterium* clones were inoculated in 2 ml LB culture (containing appropriate selection antibiotics) and grew overnight at 28°C with rotation at 200 rpm, followed by transferring into 50 ml LB containing 20 μM acetosyringone and 10 mM MES (pH 5.7) for additional culture for 16–20 h. Cells were collect by centrifugation at 5000 rpm for 5 min at 4°C, re-suspended in a transformation solution (10 mM $MgCl_2$, 10 mM MES, pH 5.7, 150 μM acetosyringone) and kept for at least 3 h at room temperature. The *Agrobacterium* suspensions of reporter and effecter were mixed and infiltrated into tobacco leaves using a syringe. The infiltrated plants were grown in a greenhouse for an additional 3 d and total RNAs in the infiltrated leaves were used for qRT–PCR. Quantitative analysis was performed at least three times throughout this investigation.

Chemical Analysis

Contents of artemisinin and artemisinic acid were determined by high-performance liquid chromatography (HPLC) as described (Zhao and Zeng, 1986), with slight modifications. Leaves of

~1 g from the 3-month-old *A. annua* plants were collected and dried overnight at 50°C. The material was pestled into fine powder and extracted with 60 ml petroleum ether (boiling range 30–60°C) for 3 h at 55°C and then evaporated till dryness. The residue was dissolved in 1 ml ethanol with supersonic for 2 min. The suspension was centrifuged at 12 000 rpm for 5 min and the supernatant was mixed with 640 μl 0.2% (w/v) NaOH and incubated at 50°C for 30 min. The solution was cooled to room temperature before 800 μl of 0.05 M acetic acid and 200 μl of an internal standard (ethyl-4-hydroxybenzate) were added. After filtering with a nitrocellulose filter (0.45 μm), the samples were used for HPLC (Agilent, Eclipse plus C18 analytical column 4.6 × 150 mm, 5 μm) analysis with a mobile phase of 0.01 M sodium phosphate buffer (pH 7.0):methanol (50:50) at a flow rate of 1 ml min^{-1}, and artemisinin was detected at 260 nm.

For artemisinic acid analysis, plant material was extracted in 50 ml methanol for 2 h, centrifuged at 12 000 rpm for 5 min, and the supernatant was evaporated until dry. The residue was dissolved in 6 ml of ethyl ether and 10 ml water, and the aqueous layer was re-extracted with 20 ml ethyl ether. The extracts were washed with 15 ml 5% sodium hydroxide solution, centrifuged at 12 000 rpm for 5 min, and the under layer was adjusted with 12 M HCl to pH 1.0. After stirring the mixture at room temperature for 30 min and extraction three times with 5 ml ethyl ether, the extracts were evaporated to dryness and dissolved with 1 ml methanol for HPLC analysis. The artemisinic acid was detected at 210 nm with a mobile phase of acetonitrile: 0.5% acetic acid (55:45, v/v) at a flow rate of 1 ml min^{-1}.

Accession Numbers

Genes mentioned in this article can be accessed as follows: *AaERF1* (JN162091), *AaERF2* (JN162092), *HMGR* (AF142473), *FPS* (GQ420346), *ADS* (AJ251751), *CYP71AV1* (EU684540), *DBR2* (EU704257), *ACTIN* (EU531837), *NbACTIN1* (AY594294), *LeERF1* (Q84XB3), *NsERF4* (Q9LW48), *Pti4* (ACF57857), *ORCA3* (ABW77571), *AtERF1* (AAM63284), *OPBP1* (AAB38748), *ERF1* (NP_188965), *AtEBP* (NP_188299), *RAP2.6* (NP_175008), *RAP2.11* (NP_197480), *AtERF-3* (O80339), AL035394.

SUPPLEMENTARY DATA

Supplementary Data are available at *Molecular Plant* online.

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