Molecular Plant
Research Article

CellPress
PARTNER JOURNAL

A Basic Leucine Zipper Transcription Factor, AabZIP1, Connects Abscisic Acid Signaling with Artemisinin Biosynthesis in *Artemisia annua*

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http://dx.doi.org/10.1016/j.molp.2014.12.004

ABSTRACT

Artemisinin is a sesquiterpenoid especially synthesized in the Chinese herbal plant, *Artemisia annua*, which is widely used in the treatment of malaria. Artemisinin accumulation can be enhanced by exogenous abscisic acid (ABA) treatment. However, it is not known how ABA signaling regulates artemisinin biosynthesis. A global expression profile and phylogenetic analysis as well as the dual-LUC screening revealed that a basic leucine zipper family transcription factor from *A. annua* (namely AabZIP1) was involved in ABA signaling to regulate artemisinin biosynthesis. *AabZIP1* had a higher expression level in the inflorescences than in other tissues; ABA treatment, drought, and salt stress strongly induced the expression of *AabZIP1*. Yeast one-hybrid assay and electrophoretic mobility shift assay (EMSA) showed that AabZIP1 bound to the ABA-responsive elements (ABRE) in the promoter regions of the amorpha-4,11-diene synthase (ADS) gene and *CYP71AV1*, which are two key structural genes of the artemisinin biosynthetic pathway. A mutagenesis assay showed that the C1 domain in the N-terminus of AabZIP1 was important for its transactivation activity. Furthermore, the activation of *ADS* and *CYP71AV1* promoters by AabZIP1 was enhanced by ABA treatment in transient dual-LUC analysis. The AabZIP1 variant with C1 domain deletion lost the ability to activate *ADS* and *CYP71AV1* promoters regardless of ABA treatment. Notably, overexpression of AabZIP1 in *A. annua* resulted in significantly increased accumulation of artemisinin. Our results indicate that ABA promotes artemisinin biosynthesis, likely through 1 activation of *ADS* and *CYP71AV1* expression by AabZIP in *A. annua*. Meanwhile, our findings reveal the potential value of AabZIP1 in genetic engineering of artemisinin production.

Key words: artemisinin, *Artemisia annua*, sesquiterpene, abscisic acid (ABA), bZIP transcription factor

Zhang F., Fu X., Lv Z., Lu X., Shen Q., Zhang L., Zhu M., Wang G., Sun X., Liao Z., and Tang K. (2015). A Basic Leucine Zipper Transcription Factor, AabZIP1, Connects Abscisic Acid Signaling with Artemisinin Biosynthesis in *Artemisia annua*. Mol. Plant. **8**, 163–175.

INTRODUCTION

Malaria is a global health problem especially in tropical zones, with more than 1 billion people living in areas at a high risk of the disease (Graham et al., 2010). In the 1960s Chinese scientists isolated artemisinin (qinghao su), a sesquiterpene lactone endoperoxide, from a traditional Chinese herbal plant, *Artemisia annua* (qinghao) (Tu, 2011). Artemisinin-based combination therapies (ACTs) are recommended by the World Health Organization as the preferred treatment method for cerebral and the chloroquine-resistant malaria because of its high efficacy, rapid action, and absence of serious side effects (White, 2008). Besides the antimalarial activity, artemisinin has also been reported to have antiviral (Romero et al., 2006), anticancer (Efferth, 2006), and antischistosomal activities (Utzinger et al., 2007). The plants of *A. annua* are the only natural source of artemisinin. Unfortunately, the supply of artemisinin is limited because of the low content of artemisinin in *A. annua*,

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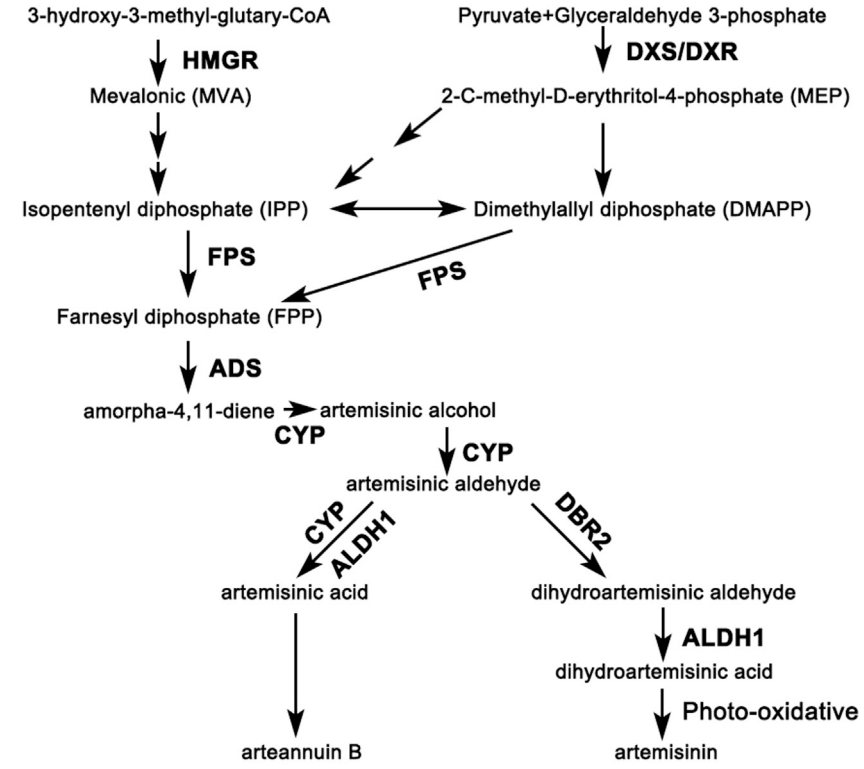

HMGR: 3-hydroxy-3-methylglutary-CoA reductase
DXS: 1-deoxyxylulose 5-phosphate synthase
DXR: 1-deoxyxylulose 5-phosphate reductoisomerase
FPS: farnesyl diphosphate synthase
ADS: amorpha-4,11-diene synthase
CYP: CYP71AV1, cytochrome P450-dependent hydroxylase
DBR2: double bond reductase 2
ALDH1: aldehyde dehydrogenase 1

Figure 1. The Artemisinin Biosynthetic Pathway.

precursor isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) are produced from cytosolic mevalonic acid (MVA) pathway and the plastidial methylerythritol phosphate (MEP) pathway. Subsequently, two molecules of IPP and one molecule of DMAPP as substrates are condensed to form farnesyl diphosphate (FPP) by farnesyl diphosphate synthase (FPS). The first committed step in the artemisinin-specific biosynthetic pathway is the cyclization of FPP to amorpha-4,11-diene by the amorpha-4,11-diene synthase (ADS) (Bouwmeester et al., 1999; Chang et al., 2000). In the second step, amorpha-4,11-diene is hydroxylated to yield artemisinic alcohol, which is subsequently oxidized to form artemisinic aldehyde. The two steps are catalyzed by a bifunctional cytochrome P450-dependent hydroxylase (CYP71AV1) together with the NADPH: cytochrome P450 oxidoreductase (CPR) (Ro et al., 2006; Teoh et al., 2006; Teoh et al., 2009; Jiang et al., 2013). A double bond reductase (DBR2) that catalyzes the reduction of the Δ11(13) double bond of artemisinin aldehyde to form dihydroartemisinin aldehyde (Zhang et al., 2008), and an aldehyde dehydrogenase (ALDH1) that catalyzes the oxidation of dihydroartemisinic aldehyde to form the direct precursor of artemisinin, dihydroartemisinic acid (Teoh et al., 2009), were reported. The conversion of dihydroartemisinic acid to artemisinin is suggested to be an enzyme-independent reaction (Brown and Sy, 2004).

and this makes the price of artemisinin too high for the poorer population of malarial victims to afford. Therefore, it is necessary and urgent to improve the artemisinin yield *in planta* by developing new varieties of *A. annua* with higher levels of artemisinin, or finding alternative sources of artemisinin. Genetic modification of the artemisinin biosynthetic pathway is a promising approach to increase artemisinin production in transgenic *A. annua*. Previous reports showed that overexpressing artemisinin biosynthetic genes (Banyai et al., 2010; Lu et al., 2013a) led to successful development of new varieties of *A. annua* with higher levels of artemisinin. An alternative method to increase artemisinin production is to spray plant growth regulators such as abscisic acid (ABA), which can immediately enhance the production of artemisinin (Jing et al., 2009). Nevertheless, the molecular mechanism underlying how ABA improves artemisinin biosynthesis is still unknown.

Artemisinin biosynthesis occurs in the glandular trichomes of *A. annua* (Duke and Paul, 1993; Olsson et al., 2009). Recently, the artemisinin biosynthetic pathway has been almost completely revealed by several groups (Figure 1). In brief, the

Although great progress has been made in dissecting artemisinin biosynthesis and reconstruction of artemisinin biosynthesis in microorganisms (Ro et al., 2006; Dietrich et al., 2009), the regulatory mechanisms of artemisinin biosynthesis *in planta* are still poorly understood. AaWRKY1, a WRKY transcription factor, was reported to function as a positive regulator of artemisinin biosynthesis by binding to the W-box in the *ADS* promoter (Ma et al., 2009a); AaERF1 and AaERF2, belonging to the AP2/ERF transcription factors family, positively regulate the transcription of *ADS* and *CYP71AV1* by binding to the RAA and CBF2 motif (Yu et al., 2012); AaORA, a trichome-specific AP2/ERF transcription factor, enhances biosynthesis of artemisinin when overexpressed (Lu et al., 2013b). In addition, overexpression of AaPYL9, an ABA receptor ortholog, increases the artemisinin content, implying a correlation between ABA signaling and artemisinin biosynthesis. The phytohormone ABA is a key regulator of plant development and stress responses, including drought, cold, and osmotic stress (Zhu, 2002; Cutler et al.,

2010). In addition to its critical roles in modulating stress responses, ABA also regulates secondary metabolism in many plant species, such as proanthocyanidin in *Persimmon* (Akagi et al., 2012), rubber in *Taraxacum brevicorniculatum* (Fricke et al., 2013), and anthocyanin in grape (Ban et al., 2003). The ABA receptor PYL4 has been reported to be involved in regulating metabolic reprogramming in *Arabidopsis* and tobacco (Lackman et al., 2011). In *A. annua*, ABA treatment enhances the artemisinin content by stimulating the expression of artemisinin biosynthetic genes (Jing et al., 2009, Supplemental Figure 1). However, the transcription factors (TFs) involved in ABA regulation of artemisinin biosynthesis have not been reported.

According to the findings in the model plant *Arabidopsis*, ABA acts through a complicated signaling cascade to regulate gene expression (Yoshida et al., 2010). To date, a large number of TFs in the ABA signaling pathway have been identified (Kobayashi et al., 2005; Kulik et al., 2011; Okamoto et al., 2013). Among them, one of the major TFs involved in ABA signaling belongs to the basic leucine zipper (bZIP) TF family (Jakoby et al., 2002). Based on the common domain, the bZIP family in *Arabidopsis* can be subdivided into 10 groups. Group A bZIP TFs bind to ACGTG-containing motifs, which are known as ABA-responsive elements (ABREs) (Choi et al., 2000; Wasilewska et al., 2008). This group is also named the ABI-ABF-AREB subfamily in *Arabidopsis*, because of some well-investigated members of this group such as ABF1, ABF2, ABF3, and ABI5 (Finkelstein and Lynch, 2000; Fujita et al., 2005; Furihata et al., 2006; Yoshida et al., 2010). Phosphorylation plays an important role in regulating the activity of ABI-ABF-AREB protein (Choi et al., 2000; Kulik et al., 2011). Proteins of this subfamily contain target sites for protein kinases such as casein kinase II (CKII), sucrose nonfermenting-1-related protein kinase 2 (SnRK2), and calmodulin-dependent protein kinases (CDPKs) (Choi et al., 2000; Hrabak et al., 2003; Kobayashi et al., 2005; Nakashima et al., 2009; Kulik et al., 2011). In addition to being involved in abiotic stress responses, bZIP TFs are also reported to play important roles in regulating plant secondary metabolism. For example, DkbZIP5 regulates proanthocyanidin biosynthesis in *Persimmon* by binding to the ABRE motifs in the promoter of *DkMYB4* (Akagi et al., 2012). TbbZIP1 regulates small rubber particle biosynthetic gene expression in *T. brevicorniculatum* in an ABA-dependent manner (Fricke et al., 2013).

In consideration of ABA promoting artemisinin biosynthesis and the important roles of group A bZIP TFs in ABA signaling, it is of interest to identify bZIP TFs involved in regulating artemisinin biosynthesis. In this study, based on global expression and phylogenetic analysis as well as dual-LUC screening, AabZIP1 was identified from more than 100 bZIP TFs in *A. annua*. The results gained from yeast one-hybrid assay, dual-LUC analysis, and gel-shift assay revealed that AabZIP1 activated *ADS* and *CYP71AV1* promoters by binding to the ABRE motifs. Transient expression in tobacco leaves and stable transgenic *A. annua* assays confirmed that AabZIP1 could activate *ADS* and *CYP71AV1* promoters *in planta*. Moreover, we showed that overexpression of *AabZIP1* increased the expression levels of *ADS* and *CYP71AV1* and thus enhanced artemisinin biosynthesis in transgenic *A. annua*.

RESULTS

Global Expression Analysis of bZIP TFs from *A. annua*

Previously, we cloned and characterized AaPYL9, an ABA receptor ortholog in *A. annua* that plays an important role in the ABA-induced overproduction of artemisinin (Zhang et al., 2013). Considering that bZIP TFs play critical roles in ABA signaling by directly binding to promoters of ABA-responsive genes, we searched for genes encoding putative bZIP proteins in *A. annua* using conserved bZIP domains as an input against publicly available EST sequences as well as the transcriptomic sequencing data (Fangyuan Zhang, Xueqing Fu, Zongyou LV, Lida Zhang, and Kexuan Tang, unpublished results) using the HMM search program. According to HMM search results, 145 unique genes encoding proteins with the bZIP domain were identified (Supplemental Data 1). Because artemisinin is specifically biosynthesized in glandular trichomes of *A. annua* (Olsson et al., 2009), the bZIP TFs expressed in glandular trichomes are most likely to participate in regulating artemisinin biosynthesis. Therefore, expression pattern analysis was performed based on published sequence data (Graham et al., 2010) to screen the specific bZIP TFs with expression in glandular trichomes. As shown in Figure 2A and Supplemental Figure 2, 64 bZIP TFs were found to be expressed in glandular trichomes. Subsequently, a phylogenetic tree was built using the 64 bZIP TFs as well as some typical bZIP TFs from *Arabidopsis* based on amino acid sequences. According to the phylogenetic analysis, six bZIP TFs belonging to group A that presumably play important roles in ABA signaling and are named AabZIP1 to AabZIP6, were selected for further study (Figure 2B).

AabZIP1 Activates the Transcription of *ADS* and *CYP71AV1 In Vivo*

To identify which of the six bZIP TFs might activate *ADS* and *CYP71AV1* expression, a dual luciferase (dual-LUC) assay screening was performed in tobacco leaf. Based on the EST search, we obtained the full-length cDNAs of these six bZIPs and inserted them into effector constructs (Figure 3A). Two reporter constructs (*ADSpro:LUC* and *CYP71AV1pro:LUC*) were produced by inserting the *ADS* promoter and *CYP71AV1* promoter into the pGreenII 0800-LUC vector, respectively. Sets of effector and reporter constructs were coinfiltrated into tobacco leaves. For each set of effector and reporter constructs, a yellow fluorescent protein (YFP) construct driven by the *35S* promoter (35S:YFP) was also coinfiltrated with the reporter constructs into the opposite position of the same tobacco leaf as the negative control. Among six bZIPs, only AabZIP1 activated the expression of *ADS* and *CYP71AV1* promoters by showing a higher value of LUC/REN than the YFP control (2.7-fold, $P < 0.01$), whereas AabZIP2 to AabZIP6 did not (Figure 3B and 3C). Together, these results show that AabZIP1 is a strong candidate with ability to transactivate the *ADS* and *CYP71AV1* promoters *in vivo*.

Characterization of AabZIP1

The full-length cDNA of AabZIP1 encodes a polypeptide of 414 amino acid residues with a calculated mass of 45 kDa and a p*I* of 9.64. Based on the sequence information and domain structure, AbZIP1 contains a conserved basic leucine zipper domain with 40%–46% amino acid sequence identity with those of *Arabidopsis* ABFs (ABA-responsive element binding factors) and TbbZIP1 from *T. brevicorniculatum* (Fricke et al., 2013),

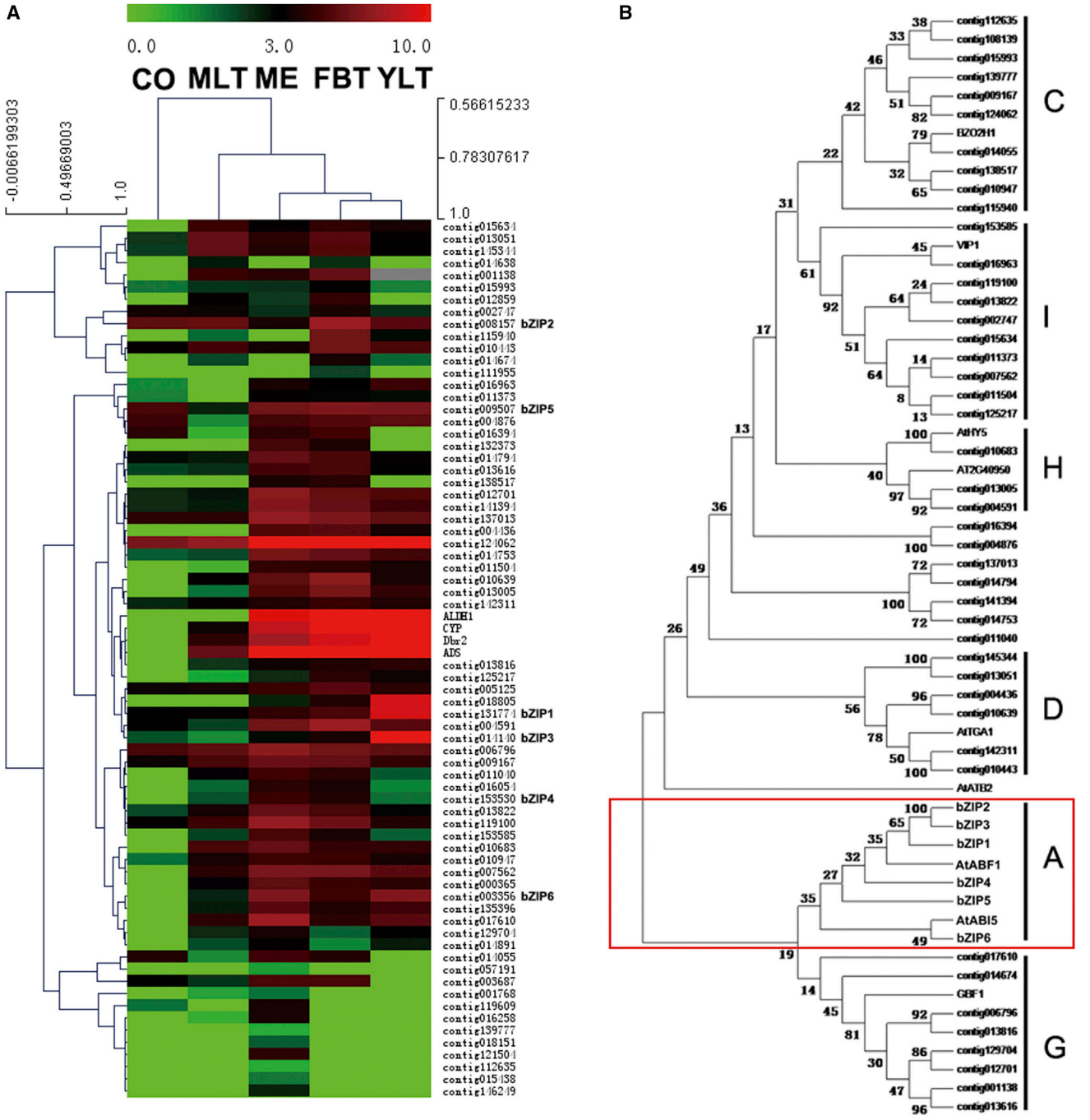

Figure 2. Global Expression Profile and Phylogenetic Analysis of bZIP Transcription Factors (TFs) in *A. annua*.

(A) Hierarchical cluster analysis of bZIP TFs. CO, cotyledon; FBT, flower bud trichome; ME, meristem; MLT, mature leaf trichome; YLT, young leaf trichome. The color scale at the top represents the value of log-transformed reads per kilobase per million of mapped reads.

(B) Phylogenetic tree showing the relationship between bZIP TFs expressed in trichomes of *A. annua* and some bZIP TFs from *Arabidopsis*. The tree presented here is a neighbor-joining tree based on amino acid sequence alignment. Protein sequences for alignments were derived from the following sources: bZIP TFs expressed in trichomes of *A. annua* as shown in **(A)**, and those from *Arabidopsis* BZO2H1, VP1, HY5, AT2G40950, TGA1, ATB2, ABF1, ABI5, and GBF1.

belonging to the group A/ABI5-ABF-AREB subfamily. Further sequence analysis indicated that the bZIP domain of AabZIP1 had a putative nuclear localization signal (NLS) and a zipper domain comprising three leucine repeats, a typical feature of group A bZIPs (Figure 4A). Three conserved N-terminal domains (C1, C2 and C3) and a single C-terminal domain on group A bZIPs were also present in AabZIP1, suggesting that AabZIP1 might be involved in the ABA-dependent biological process (Choi et al., 2000; Jakoby et al., 2002; Furihata et al., 2006; Okamoto et al., 2013).

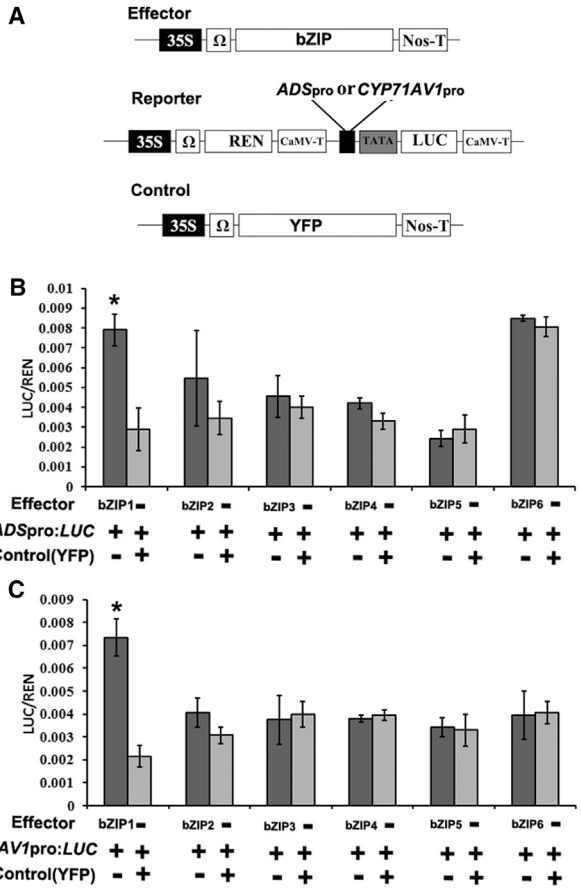

Figure 3. Transient Dual-LUC Screening of Six bZIP TFs.

(A) Schematic representation of effector and reporter constructs used in the transient dual-LUC assays. Effector constructs contain the *bZIP*s coding sequence driven by the CaMV35S promoter. *ADSpro:LUC* and *CYP71AV1pro:LUC* were used as reporter constructs. The expression of *REN* under the 35S promoter was used as internal control. The *YFP* driven by 35S promoter was used as negative control.

(B) Transient dual-LUC analysis showing bZIP1 activation of *ADS* in *N. benthamiana* leaves. The values were reached by calculating the ratio of LUC activities to REN activities (LUC/REN). Error bars represent ±SD ($n = 3$). *$P < 0.01$.

(C) Dual-LUC analysis similar to that of **(B)**, substituting *ADSpro:LUC* with *CYP71AV1pro:LUC*. Error bars represent ±SD ($n = 3$). *$P < 0.01$.

Expression and Induction Patterns of AabZIP1

It has been reported that artemisinin accumulation was increased by ABA treatment (Jing et al., 2009 and Figure 7D and 7E), and that overexpression of the ABA receptor in *A. annua* also enhanced the content of artemisinin in transgenic plants (Zhang et al., 2013). To examine whether AabZIP1 is involved in ABA-dependent artemisinin accumulation, the expression of *AabZIP1* under ABA, drought, and salt treatment was analyzed by quantitative RT–PCR. We found that the transcript level of *AabZIP1* rapidly increased upon these treatments, especially with ABA treatment (Figure 4B). To confirm that *AabZIP1* was expressed in tissues where artemisinin was biosynthesized, we investigated the expression profile of *AabZIP1* in various tissues of *A. annua* by quantitative real-time PCR. As shown in Figure 4C, expression of *AabZIP1* was found at different levels in all tissues examined, with the highest expression found in flower buds. The tissue expression profile of *AabZIP1* was similar to those of *ADS* and *CYP71AV1* and some other TFs such as AaERF1 and AaERF2 which have been reported to regulate artemisinin biosynthesis (Yu et al., 2012). The similarity of the expression pattern of *AabZIP1*, *ADS*, and *CYP71AV1* suggests that *AabZIP1* may regulate the expression of *ADS* and *CYP71AV1*.

AabZIP1 Is Localized to the Nucleus and Directly Binds to ABA Response Elements within the Promoters of *ADS* and *CYP71AV1*

Sequence analysis showed that AabZIP1 contains a putative NLS. To determine the subcellular localization of AzbZIP1 in plant cells, an *in vivo* targeting experiment was performed in tobacco leaf (*Nicotiana benthamiana*). To this end, the coding sequence of *AabZIP1* was fused in-frame with the YFP, and the *AabZIP1-YFP* fusion gene was driven by the *35S* promoter and delivered into leaf cells of tobacco by *Agrobacterium tumefaciens* infiltration. In the case of AabZIP1-YFP fusion, strong YFP fluorescence was observed in the nucleus of tobacco cells, whereas YFP alone was found to be localized in both the nucleus and the cytosol (Figure 4D). The results of the *in vivo* targeting experiment were consistent with sequence analysis, suggesting that AabZIP1 is localized in the nucleus.

The findings that AabZIP1 activated *ADS* and *CYP71AV1* promoters in the dual-LUC assay raised the possibility that AabZIP1 might be a transcriptional activator. To test this, we used a yeast assay system (Zhang et al., 2010). The GAL4 DNA-binding domain was fused to AabZIP1 to generate the GAL4BD-AabZIP1 construct. This construct was then cotransferred with reporter construct (pGADT7) into yeast cells, and the growth of the transformants was examined on selective medium. Only transformants harboring GAL4BD-AabZIP1 grew well in selective medium, indicating that AabZIP1 is a transcriptional activator (Figure 5B). To determine the regions of AabZIP1 responsible for transcriptional activation, we generated different deletions of AabZIP1 and fused them with the GAL4 DNA-binding domain. Given that AabZIP1 has three highly conserved N-terminal domains (C1, C2, and C3) and a signal variable C-terminal domain (C4), we made four constructs in which one of four domains (C1, C2, C3, and C4) of AabZIP was deleted. In combination with the reporter construct, the constructs that harbored AabZIP1 with the C1 domain deletion did not activate the reporter genes, whereas the constructs harboring AabZIP1 with the C1 domain did activate the reporter gene. This result mapped the C1 domain (1-77) of AabZIP1 as transcriptional activation domain (Figure 5B). In agreement with this result, the GLA4BD-AabZIP$_{1\text{-}77}$ grew well in selective medium. It is known that group A bZIP TFs recognized the consensus *cis*-acting elements, namely ABREs (Choi et al., 2000). Sequence analysis revealed that the promoters of *ADS* and *CYP71AV1* contained one and three ABREs, respectively (Figure 5A). To examine whether AabZIP1 bound to ABRE, we performed a yeast one-hybrid assay. A 200 bp DNA fragment containing ABRE of the *ADS* promoter, a 273 bp DNA fragment containing two ABREs of the *CYP71AV1* promoter, and a 263 bp DNA fragment containing the remaining one ABRE of *CYP71AV1* promoter (Figure 5A), were cloned and named E1, E2, and E3, respectively.

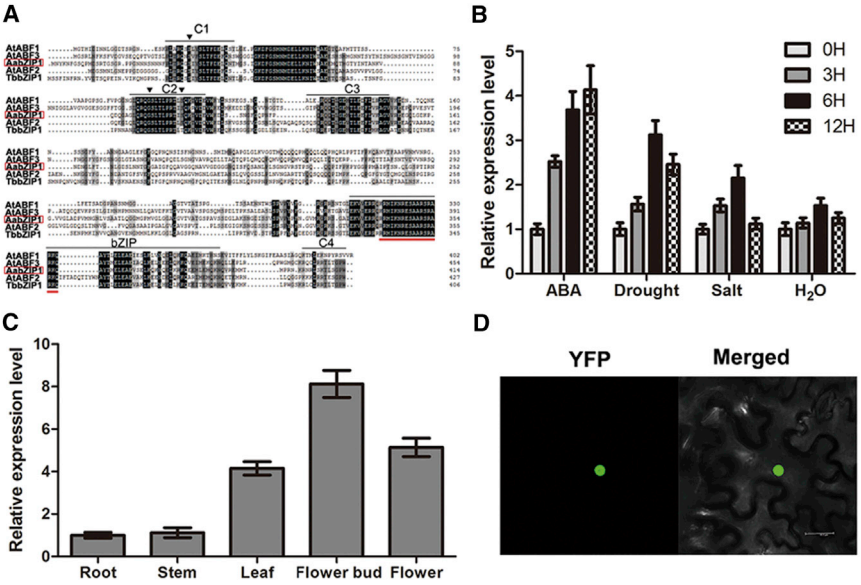

Figure 4. Characterization of AabZIP1.

(A) Alignment of the deduced protein sequence of AabZIP1 with some members of group A bZIP TFs from *Arabidopsis* or *T. brevicorniculatum* TbbZIP1. The bZIP domain and C1–C4 domain are represented by a line above the sequence. The putative NLS is highlighted by a red line below the protein sequence. The putative phosphorylation sites (R/K-X-X-S/T) by SnRK2are marked by arrowheads.

(B) The expression of *AabZIP1* was induced by ABA treatment and abiotic stress. *ACTIN* was used as internal control. Error bars indicate SD (n = 3).

(C) The expression levels of *AabZIP1* in root, stem, leaf, bud, and flower, respectively, of *A. annua*. *ACTIN* was used as internal control. Error bars indicate SD (n = 3).

(D) Nuclear localization of AabZIP1 in tobacco epidermal cells. The fluorescence (left) and bright-field merged with fluorescence (right) images of cells are shown.

Subsequently, these three DNA fragments were inserted into the LexA yeast one-hybrid vector p178 containing the *lacZ* reporter gene (Zhu et al., 2003) to generate E1:lacZ, E2:lacZ, and E3:lacZ reporter constructs. The coding sequence of AabZIP was fused in-frame with the B42 activation domain to generate the AD-AabZIP1 effector construct, which was cotransferred into yeast strain EGY48 with respective reporter constructs. As shown in Figure 5C, AabZIP1 was able to bind to the ABREs from the promoters of ADS and *CYP71AV1*, respectively.

To further confirm AabZIP1 binding to the *ADS* and *CYP71AV1* promoters, we performed an electrophoretic mobility shift assay (EMSA) with 6-His tagged AabZIP1 proteins purified from *Escherichia coli*. A slowly migrating band was detected for His-AabZIP1, but not for MBP (maltose-binding protein) protein (Figure 5D and 5E). The DNA-binding specificity was then confirmed in a competition experiment with excess amounts of the unlabeled competitors. Together, these results indicated that AabZIP1 directly binds to the ABRE motifs of the *ADS* and *CYP71AV1* promoters.

Activation of *ADS* and *CYP71AV1* Promoters by AabZIP1 Is Enhanced by ABA

In plants, ABA induces gene expression through *cis* elements including ABRE. Given that AabZIP1 directly bound to ABREs in *ADS* and *CYP71AV1* promoters, we tested whether the activation of *ADS* and *CYP71AV1* promoters by AabZIP1 was regulated by ABA. To this end, we investigated the transcriptional activation of *ADS* and *CYP71AV1* promoters when AabZIP1 was constitutively expressed with or without the application of 100 μM exogenous ABA through the dual-LUC assay. As described above, the reporter constructs *ADSpro:LUC* and *CYP71AV1pro:LUC* were coinfiltrated with the *35S:YFP-AabZIP1* construct into the tobacco leaf, respectively. ABA treatment dramatically increased the activities of *ADS* and *CYP71AV1* promoters when AabZIP1 was expressed (Figure 6A and 6B). However, the activities of *ADS* and *CYP71AV1* promoters were not significantly changed when AabZIP1 with deletion of the C1 domain was expressed upon ABA or mock treatment (Figure 6A and 6B).

We then tested whether activation of the *ADS* promoter by AabZIP1 was enhanced by ABA in genetically modified plants. Previously, we reported that the *ADS* promoter was predominantly expressed in trichomes of *Arabidopsis* (Zhu et al., 2013). Thus, *AabZIP1* was introduced into and overexpressed in the *pADS:GUS* transgenic *Arabidopsis* plants. As shown in Figure 6C, the expression pattern of *GUS* was not changed (still in trichomes) when *AabZIP1* was overexpressed in *pADS:GUS*. In contrast, the GUS activities were significantly increased when *AabZIP1* was overexpressed or ABA was applied, in accordance with dual-LUC assay by transient transformation in tobacco leaf. The GUS activities were increased even more drastically when *AabZIP1*-overexpressing plants were treated with ABA. Together, these results indicated that activation of *ADS* and *CYP71AV1* promoters by AabZIP1 was enhanced by ABA (Figure 6D).

Overexpression of *AabZIP1* in *A. annua* Increases Artemisinin Biosynthesis

Since AabZIP1 was able to bind to and activate *ADS* and *CYP71AV1* promoters, we analyzed whether overexpression of *AabZIP1* could promote artemisinin biosynthesis in *A. annua*. The *35S:YFP-AabZIP1* construct was transformed into *A. annua* (Zhang et al., 2009). The transgenic plants of *A. annua* growing well on hygromycin-selective medium were confirmed to be authentic transgenic plants by Western blot (Figure 7A). Four independent transgenic lines with high YFP-AabZIP1 protein levels were used for further analysis.

In the *AabZIP1*-overexpressing (OX) plants, transcripts of AabZIP1 were dramatically increased by approximately 20- to 35-fold compared with those in wild-type (Figure 7B). The transcript levels of *ADS* and *CYP71AV1* were indeed elevated remarkably, by about six- to eight-fold, in AabZIP1-ovexpressing plants compared with that in wild-type (Figure 7C). The transcript levels of *DBR2* and *ALDH1* were also increased in transgenic plants,

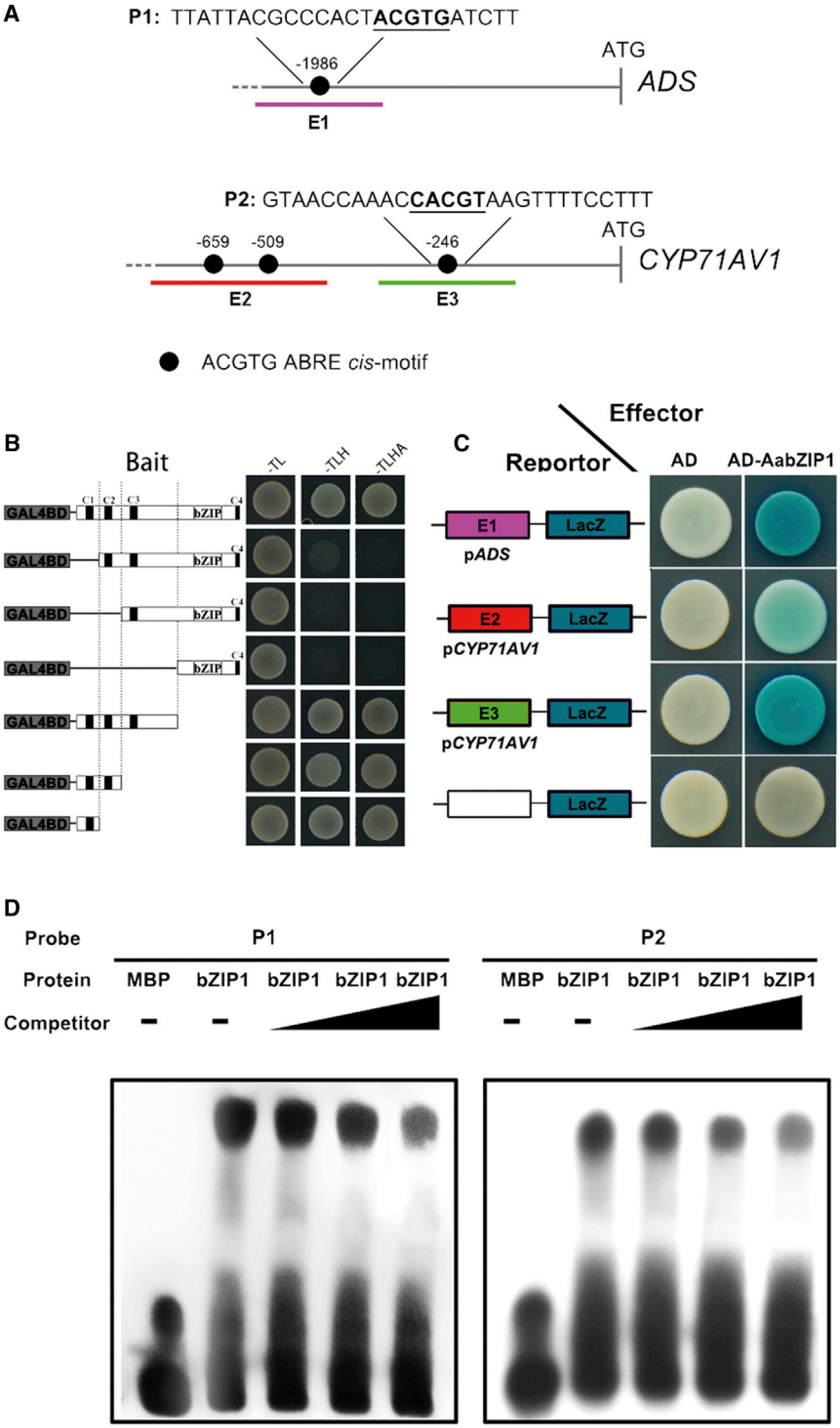

Figure 5. AabZIP1 Is a Transactivator Binding to the ABRE Motif in the Promoter Regions of *ADS* and *CYP71AV1*.

(A) Schematic representation of the positions of the ABA-responsive elements (circle) in the sequences of the *ADS* and *CYP71AV1* promoters are indicated, as well as the fragments (E1, E2, and E3) used for yeast one-hybrid assay. Sequences of oligonucleotides used in EMSA are presented. P1 and P2 indicate the probe derived from *ADS* and *CYP71AV1* promoter, respectively. ABRE motif is in boldface and underlined.

(B) AabZIP1 is a transcriptional activator in yeast cells. The left column depicts the constructs used in this assay. The right column represents the yeast cells grown on selective medium. -TL indicates lack of Trp and Leu; -TLH indicates lack of Trp, Leu, and His; -TLHA indicates lack of Trp, Leu, His, and Ade.

(C) Yeast one-hybrid assay indicating the interaction between AabZIP1 and ABRE motif in promoters of *ADS* and *CYP71AV1*, respectively. Left panel represents the reporter constructs used in the yeast one-hybrid assay. Right panel represents the yeast cells grown on X-gal plates.

(D) EMSA showing AabZIP1 binding to ABRE motif of *ADS* and *CYP71AV1* promoter, respectively. The His–AabZIP1 fusion protein and ABRE probe as shown in **(A)**, derived from the *ADS* (P1) or *CYP71AV1* (P2) promoter region, were used. Nonlabeled P1 or P2 served as cold competitors (0, 5×, 50×, 200×). MBP protein was included as a negative control. MBP protein alone was included as a negative control.

but to a lesser extent than *ADS* and *CYP71AV1*. As expected, the artemisinin and dihydroartemisinic acid (the direct precursor of artemisinin) contents were significantly higher in transgenic lines than in wild-type. Compared with the wild-type, the artemisinin and dihydroartemisinic acid content was increased by 0.7- to 1.5-fold and 0.3- to 0.8-fold in *AabZIP1*-OX lines, respectively (Figure 7E and 7F). These results indicated that AabZIP1 is a positive regulator of the artemisinin biosynthetic pathway. Dual-LUC analysis performed in tobacco leaf indicated that the activation of *ADS* and *CYP71AV1* promoters by AabZIP1 was enhanced by ABA. Consistently, upon ABA treatment the expression levels of *ADS* and *CYP71AV1* were increased by about 9- to 13-fold in *AabZIP1*-OX plants in comparison with wild-type; the expression of *DBR2* and *ALDH1* was also strongly induced by about five- to eight-fold in *AabZIP1*-OX plants when compared with wild-type (Figure 7D). The contents of artemisinin and dihydroartemisinic acid in wild-type *A. annua* were increased by 37% and 33%, respectively, upon ABA treatment, while those in *AabZIP1*-OX *A. annua* were increased by 40%–60% and 41%–53%, respectively (Figure 7E and 7F). These results indicated that AabZIP1 is a positive regulator of the artemisinin biosynthesis and that ABA promotes artemisinin biosynthesis, likely through AabZIP1.

DISCUSSION

AabZIP1 Creates a Potential Link between ABA Signaling and Artemisinin Biosynthesis

The plant growth regulator ABA plays important roles in many aspects of developmental and physiologic processes *in planta*

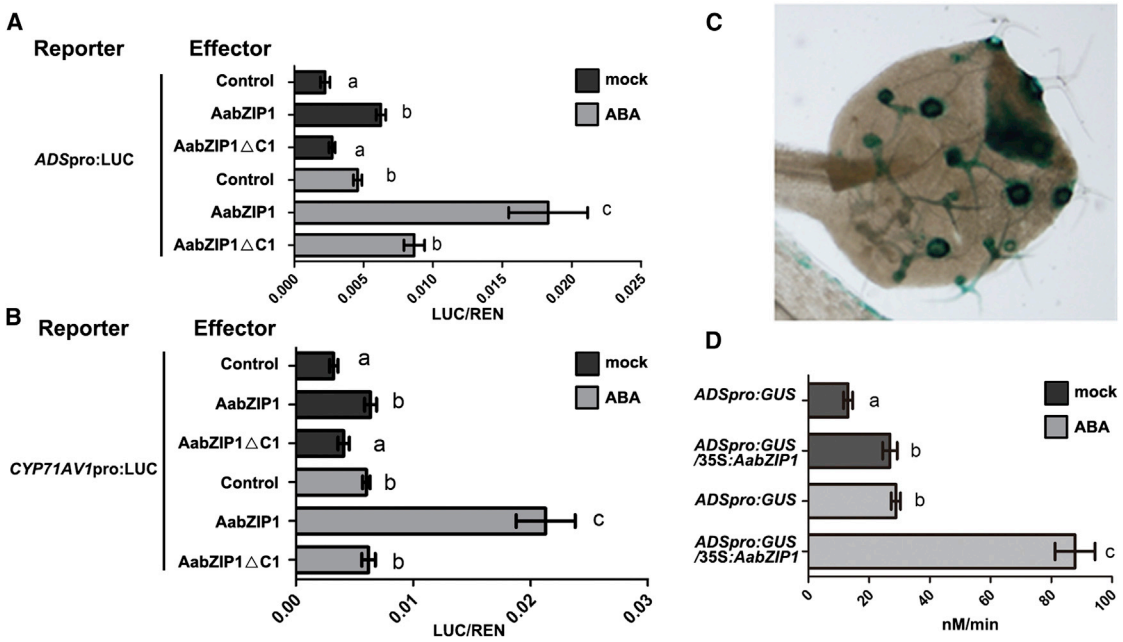

Figure 6. ABA-promoted Transactivation of *ADS* and *CYP71AV1* by AabZIP1.

(A) Transient dual-LUC assay in tobacco leaf. The leaves were treated with or without 100 μM ABA for 6 h by infiltration. The ratio of LUC activities to REN activities (LUC/REN) was calculated. The reporter constructs *ADSpro:LUC* and effector constructs 35S:AabZIP1 are described in Figure 3, AabZIP1ΔC1 indicates the C1 domain deletion mutant of AabZIP1. 35S:YFP was used as a negative control.

(B) Transient dual-LUC assay using *CYP71AV1pro:LUC* as reporter construct. The ratio of LUC activities to REN activities (LUC/REN) was measured with or without 100 μM ABA treatment for 6 h. 35S:YFP was used as a negative control.

(C) Overexpression of AabZIP1 in transgenic *Arabidopsis* harboring *pADS:GUS* did not change the expression pattern of *ADS* promoter. GUS staining of mature leaf is shown.

(D) GUS activity assay showing ABA/AabZIP1 activation of *ADS* promoter activity. Mature leaves were treated with or without 10 μM ABA for 16 h before GUS activity was measured. Error bars indicate SD (n = 3). The different letters after the bars represent the significant difference given by Duncan's multiple range tests ($P < 0.01$)

(Yoshida et al., 2002; Adie et al., 2007; Yoshida et al., 2010; Okamoto et al., 2013). Previously we found that the expression of *ADS* and *CYP71AV1* as well as artemisinin accumulation could be induced by exogenous ABA treatment (Jing et al., 2009). Subsequently, an ABA receptor, AaPYL9, was isolated from *A. annua*, overexpression of which upregulates expression of the artemisinin biosynthetic pathway genes (such as *ADS* and *CYP71AV1*) and consequently increases the artemisinin content. These studies indicated that ABA signaling is involved in artemisinin biosynthesis (Zhang et al., 2013). To investigate the mechanism of how ABA regulates artemisinin biosynthesis in more detail, a bZIP transcription factor, AabZIP1, was isolated in this study by global expression profile, phylogenetic analysis, and dual-LUC screening. AabZIP1 was structurally similar to members of the ABI5-ABF-AREB subfamily, which regulate gene transcription in response to ABA and/or abiotic stress by binding to the ABRE motif (Jakoby et al., 2002). The expression of *AabZIP1* was induced by exogenous ABA, suggesting that the AabZIP1 might be involved in ABA-regulated artemisinin biosynthesis. The yeast one-hybrid and EMSA assays demonstrated that AabZIP1 binds to the ABRE elements of *ADS* and *CYP71AV1* promoters. Transient dual-LUC analysis showed that AabZIP1 or ABA alone was able to transactivate the *ADS* and *CYP71AV1* promoters in tobacco leaves. This might be due to ABA also being present in tobacco leaf and the conserved ABA signaling pathway in the plant kingdom. Strikingly, *AabZIP1* overexpression in *A. annua* together with ABA treatment even more dramatically activates *ADS* and *CYP71AV1*. These results suggest that AabZIP1 requires ABA for full activity. The phosphorylation of TFs is essential in ABA signaling (Furihata et al, 2006). In *Arabidopsis*, the activities of ABF2 and ABF4 were modulated by the phosphorylation of serine and threonine residues in their conserved N-terminal domain through protein kinases such as SnRK2s (Kobayashi et al., 2005; Kulik et al., 2011). The conserved serine/threonine residues were crucial for the activities of ABFs (Kobayashi et al., 2005). The conserved serine/threonine residues were also present in the N-terminal C1 domain of AabZIP1 (Figure 4). Overexpression of the deletion mutant of AabZIP1 (AabZIP1ΔC1) in dual-LUC analysis exerted no influence on reporter activity regardless of ABA treatment, presumably because of loss of phosphorylation in AabZIP1ΔC1. It would be intriguing to explore in a future study whether the phosphorylation of AabZIP1 is required for ABA-regulated artemisinin biosynthesis (Zhang et al., 2013).

In addition to ABA, jasmonic acid (JA) also plays an important role in regulating the biosynthesis of artemisinin (Lu et al., 2013b). Some regulators involved in mediating JA stimulation of artemisinin biosynthesis have been reported, such as AaERF1, AaERF2 (Yu et al., 2012), AaORA (Lu et al., 2013b), and AaWRKY1 (Ma et al., 2009a). AaERF1, AaEFR2, and AaWRKY1

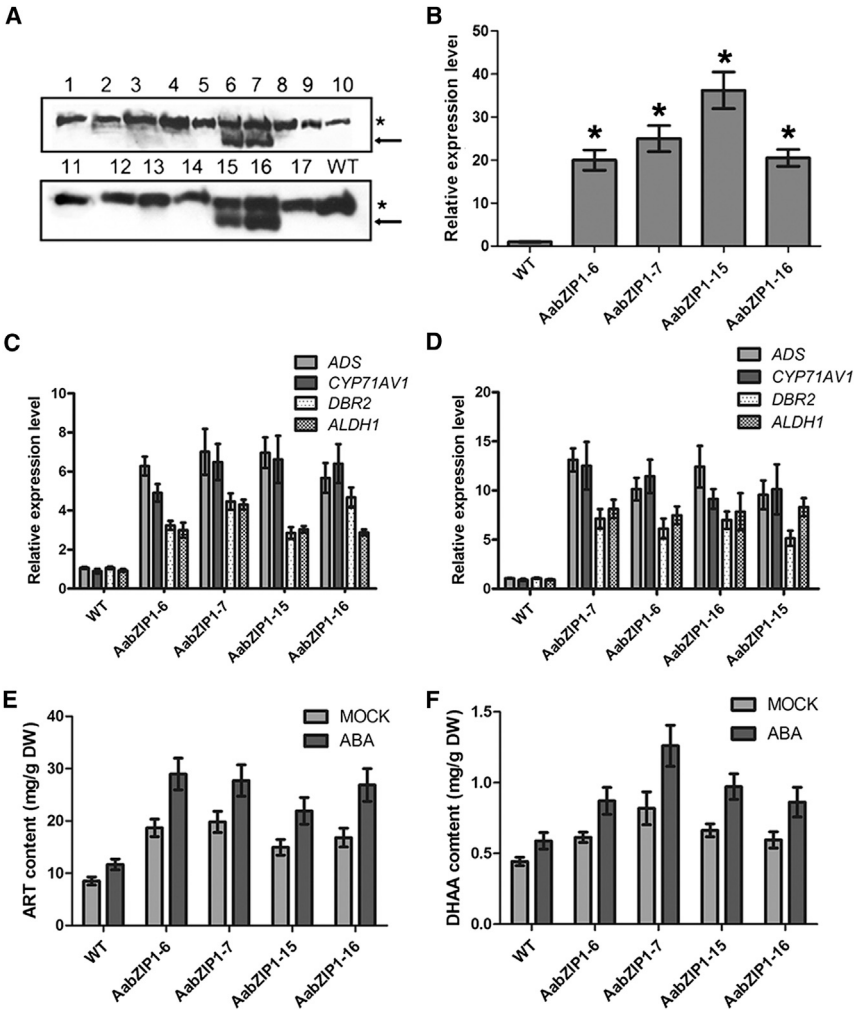

Figure 7. Overexpression of AabZIP1 Upregulated Expression Levels of Artemisinin Biosynthetic Genes and Increased the Artemisinin Content.

(A) Immunoblot analysis of AabZIP1-YFP protein levels in transgenic *35S:AabZIP1-YFPA. annua* lines. AabZIP1-YFP protein levels were determined using GFP antibody. Wild-type (WT) was used as negative control. The asterisks show nonspecific bands and arrows show the AabZIP1-YFP bands.

(B) Real-time quantitative PCR analysis of *AabZIP1* in leaves of 2-month-old transgenic*35S:AabZIP1-YFP* lines. *ACTIN* was used as the internal control. Error bars indicate SD (n = 3). *P < 0.01 (Student's t-test).

(C and D) Real-time quantitative PCR analysis of *ADS*, *CYP71AV1*, *DBR2*, and *ALDH1* in leaves of 2-month-old transgenic*35S:AabZIP1-YFP* lines without **(C)** and with ABA treatment **(D)**. *ACTIN* was used as the internal control. Error bars indicate SD (n = 3).

(E and F) Contents of dihydroartemisinic acid (DHAA, **F**) and artemisinin (ART, **E**) in leaves of AabZIP1-overexpressed transgenic plants were determined by HPLC. The light-gray bars represent artemisinin and dihydroartemisinic acid contents treated with mock; the dark-gray bars represent artemisinin and dihydroartemisinic acid contents treated with ABA. Error bars indicate SD (n = 3).

have been reported to regulate artemisinin biosynthesis by binding to promoter regions of artemisinin biosynthetic genes directly. Although overexpression of AaORA in *A. annua* enhanced the artemisinin content, it has not been reported that AaORA can bind to promoters of artemisinin biosynthetic genes. Lu et al. (2013b) predicted that AaORA might activate artemisinin biosynthesis through AaERF based on similar expression pattern. ABA-JA crosstalk plays an important role in many biological processes, especially in resistance to biotic and abiotic stress (Adie et al., 2007; Chen et al., 2012). Some components in JA signaling have also been reported to take part in ABA signaling in *Arabidopsis*. AtMYC2 is an important regulator in JA signaling and the convergence point of JA crosstalk with other signaling pathways. Interestingly, AtMYC2 is also involved in ABA signaling (Abe et al., 2003). Recently, the *Arabidopsis* mediator subunit MED25 was found to interact with AtMYC2 and ABI5 to regulate JA and ABA signaling, respectively (Chen et al., 2012). In *Catharanthus roseus*, CrMYC2 mediates JA signaling and terpenoid indole alkaloid biosynthesis by binding to the promoter region of the AP2/ERF transcription factor gene *ORCA3* (Zhang et al., 2011). Lackman et al. (2011) reported that JA signaling and ABA might coordinately regulate metabolic reprogramming in *Arabidopsis* and tobacco. In *A. annua*, the crosstalk between JA and ABA signaling has not been reported. To address this question, we performed quantitative real-time PCR analysis. The results showed that ABA treatment upregulated the expression of *AaERF1* and *AaERF2*, which were also found to be induced by JA treatment (Supplemental Figure 3; Yu et al., 2012). However, another two JA-induced genes, *AaORA* and *AaWRKY*, were either unaltered or downregulated upon ABA treatment (Supplemental Figure 3; Ma et al., 2009a; Lu et al., 2013b). These results indicate that JA and ABA might regulate artemisinin biosynthesis, likely in similar yet distinct manners. More genetic and biochemical data are needed to reveal their relationship.

AabZIP1 Serves as a Positive Regulator to Engineer Artemisinin Biosynthesis

Despite of the great progress in semisynthetic artemisinin in microbes, *A. annua* is still the main resource for artemisinin. However, as the artemisinin content in *A. annua* is very low, further elucidation of artemisinin biosynthesis and its regulatory mechanism is essential for enhancing artemisinin accumulation through transgenic technology. Because biosynthesis of artemisinin involves multiple enzymes, overexpression of one or two enzymes is not necessarily efficient in promoting artemisinin biosynthesis. TFs often target the key steps in the biosynthetic pathways. For example, we showed that overexpression of AaORA, an AP2/ERF transcription factor, increases the expression of multiple artemisinin biosynthetic genes and thus improves

artemisinin production (Lu et al., 2013b). Overexpression of AaERF1 and AaERF2 also increases the expression of *ADS* and *CYP71AV1* as well as artemisinin content (Yu et al., 2012). AaWRKY1, a WRKY transcription factor, upregulates the expression levels of *ADS* and some other artemisinin biosynthetic genes in transgenic *A. annua* (Ma et al., 2009a). The TFs are therefore very valuable in improving artemisinin accumulation. Our results demonstrated that the AabZIP1 binds to and transactivates *ADS* and *CYP71AV1* promoters both *in vitro* and *in vivo*. As the transcript and protein levels of AabZIP1 were increased in *AabZIP1*-OX transgenic lines (Figure 7A and 7B), the expression levels of *ADS*, *CYP71AV1*, *DBR2*, and *ALDH1* were also increased significantly (Figure 7C). As expected, overexpression of *AabZIP1* in *A. annua* increased the artemisinin content by 0.7- to 1.5-fold that in wild-type (Figure 7D). These results demonstrate that AabZIP1 is an important positive regulator that could be used for engineering artemisinin biosynthesis in *A. annua*.

METHODS

Plant Materials and Chemicals

A. annua used in this study was obtained from Chongqing, China. Plants were grown in pots in a growth chamber at 24°C under a 16-h light/8-h dark photoperiod at 80–100 mE m^{-2} s^{-2}. For *in vitro* culture, seeds of *A. annua* were surface sterilized in 10% sodium hypochlorite solution and 0.01% Triton X-100 for 5 min, and washed three times in sterile distilled water. After 3 days at 4°C, the seeds were sown on plates containing MS solid medium composed of MS basal salts and 20% sucrose, and solidified with 0.6% agar (pH 5.7). Plates were sealed and incubated in an environment-controlled growth chamber. Tobacco seeds (*N. benthamiana*) were sown directly on soil, and grown in pots under the same conditions as for *A. annua*.

ABA was purchased from Sigma-Aldrich (St Louis, MO, USA); the other chemicals were purchased from China National Medicines Corporation (Beijing, China).

Stress Treatments

For abiotic stresses and ABA treatment, 1-month-old *A. annua* plants were treated with 300 mM NaCl and 10 μM ABA and then sampled at 0, 3, 6, and 12 h. Drought stress was performed by leaving the intact 1-month-old *A. annua* plants in air and removing the soil without water supply, followed by sampling at 0, 3, 6, and 12 h. To analyze the artemisinin biosynthetic gene expression and the artemisinin and dihydroartemisinic acid contents under ABA treatment, the 2-month-old AabZIP1-OX and wild-type *A. annua* were treated with 10 μM ABA, and sampled at 6 h for RNA isolation and at 24 h for high-performance liquid chromatography (HPLC) detection.

Bioinformatics Analysis

To conduct the search for bZIP TFs in *A. annua*, the nucleotide sequence data, including 95 606 EST sequences downloaded from the National Center for Biotechnology Information (NCBI) EST database, 150 282 sequences published by Soetaert et al. (2013), 42 678 sequences published by Wang et al. (2009), and transcriptome sequences generated by our laboratory (Fangyuan Zhang, Xueqing Fu, Zongyou LV, Lida Zhang, and Kexuan Tang, unpublished results) were translated into protein sequences by six frames. The hidden Markov model profiles of bZIP (PF00170) domain were obtained from the Pfam database (Punta et al., 2012). The bZIP homologs were identified by the HMM search against the *A. annua* protein sequence database (Eddy, 2011) with *E* value $<10^{-5}$. The redundant sequences were then manually removed. Differential gene expression and tissue specificity were performed as described by Singh et al. (2013). The RNA-seq data (SRR019254 for meristem; SRR019546 for young leaf trichome; SRR019547 for mature leaf trichome; SRR019548 for flower bud trichome; SRR019549 for cotyledon) were downloaded from the NCBI Sequence Read Archive (SRA) database. The fastaq format data were converted to fasta format using the SRA toolkit (http://www.ncbi.nlm.nih.gov/Traces/sra/?view=software), subsequently transformed to blast-ready database. The reads of each bZIP TFs in meristem, young leaf trichome, mature leaf trichome, flower bud trichome, and cotyledon were obtained by BLASTN search against the corresponding database with *E* value $<10^{-6}$, and read counts were normalized by calculating the value of reads per kilobase per million for each transcript in different tissue samples. Hierarchical cluster analysis was performed with a MultiExperiment Viewer (MeV, v. 4.9.0). The molecular weight and the theoretical isoelectric point were calculated online (http://www.expasy.ch/tools/pi_tool.html). The most similar sequences from other species were obtained by using a BLASTP search against the nonredundant database at the website (http://www.ncbi.nlm.nih.gov/BLAST/). The protein sequences of the *Arabidopsis* and *A. annua* were aligned with ClustalW. The unrooted phylogenetic tree was constructed using the neighbor-joining method of MEGA software version 5 (Tamura et al., 2011). Bootstrap analysis was performed using 1000 replicates to evaluate the accuracy of our phylogenetic construction.

Cloning of the *AabZIP1* Gene

The first-stranded cDNA was synthesized with 2 μg total RNAs isolated from young leaves of *A. annua* and used as the reverse transcriptase PCR templates. Gene-specific primers, AabZIP1-F and AabZIP1-R, were designated according to EST sequences. PCR was performed according to the manufacturer's instructions for KOD DNA polymerase (Toyobo, Osaka, Japan). The PCR program was carried out under the following conditions: 94°C for 3 min, followed by 30 cycles of amplification (30 s denaturation at 94°C, 30 s annealing at 54°C, 30 s of extension at 68 °C), and finally by extension at 68°C for 10 min. The PCR products were subcloned into the pJET2.1 vector (Thermo Fisher Scientific, Waltham, MA, USA) and sequenced. All the primers used in the present study are listed in Supplemental Table 1.

Plasmid Construction

For dual-LUC assay, PCR-amplified fragments encoding AabZIP1 to AabZIP6 were cloned into gateway cloning vector pENTR (Invitrogen, Carlsbad, CA, USA), and subsequently transferred to the destination vector pEarleyGate104 by Gateway LR recombination reaction (Invitrogen) to generate pEarleyGate104-YFP-AabZIP1 to pEarleyGate104-YFP-AabZIP6 constructs, respectively. To make reporter constructs for transient expression in tobacco leaves, the 2800 bp *ADS* promoter and 1800 bp *CYP71AV1* promoter were inserted into pGreenII 0800-LUC plasmid through KpnI and BamHI sites to generate *ADSpro:LUC* and *CYP71AV1pro:LUC*, respectively (Hellens et al., 2005). For protein recombination in *E. coli*, the coding sequence of AabZIP1 was cloned into the BamHI and HindIII sites of pET28aplasmid to generate pET28-AabZIP1.

For transcription activation analysis, the PCR-amplified intact *AabZIP1* and deletion forms of *AabZIP1* were cloned into EcoRI and BamHI sites of pGBKT7 vector, respectively. For yeast one-hybrid assay, the intact coding sequence of *AabZIP1* was cloned into the EcoRI and XhoI sites of pJG4-5 vector in-frame with B42 activation domain to generate AD-AabZIP1. The fragments containing ABRE elements from the *ADS* and *CYP71AV1* promoters were cloned into the SalI site of p178 vector to generate the constructs of 178-ADSpro/E1, 178-CYP71AV1pro/E2, and 178-CYP71AV1pro/E3, respectively.

Dual-LUC Assay

The reporter and effector constructs are described above. Infiltration and detection were performed according to the protocols described previously (Luo et al., 2014) with minor modifications. All overnight *Agrobacterium* cultures were collected by centrifugation, then

resuspended in MS medium to OD_{600} = 0.6, and incubated at room temperature for 3 h. The reporter strain harboring *ADSpro:LUC* or *CYP71AV1pro:LUC* was mixed with the effector strain harboring 35Spro:bZIP1-6 at the ratio 1:1. The 35Spro:YFP construct was used as negative control. The mixture of *Agrobacterium* suspensions was infiltrated into tobacco leaves; the negative control was infiltrated with any pair of reporter and effects into the opposition position on the same leaves. After 48 h, the leaf samples were collected for dual-LUC assay using commercial dual-LUC reaction reagents according to the manufacturer (Promega, Durham, NC, USA). Three biological repeats were measured for each sample.

Transactivation Assay

The intact AabZIP1 and a series of deletion of AabZIP1 were cloned into pGBKT7 in-frame with the GAL4 DAN binding domain used as bait construct, with the empty pGADT7 vector used as prey construct. Sets of bait and prey constructs were cotransformed into AH109 yeast strain. The transformed yeast cells were selected on synthetic minimal double dropout medium deficient in Trp and Leu. AabZIP1 transactivation ability tests were assessed on triple dropout medium deficient in Trp, Leu, and His, or quadruple dropout medium deficient in Trp, Leu, His, and adenine, at 30°C for 3 days. At least five clones were analyzed, and experiments were repeated three times with similar results.

Yeast One-Hybrid Assay

The yeast one-hybrid assay was performed as described previously (Zhu et al., 2003). In brief, the AD-AabZIP1 plasmid was cotransformed into yeast strain EGY48 with 178-ADSpro/E1, 178-CYP71AV1pro/E2, or 178-CYP71AV1pro/E3. The yeast-harboring set of constructs was selected on synthetic minimal double dropout medium deficient in Trp and Ura. Five independent yeast cells grown on select medium plates were transferred into liquid select medium and cultured overnight. Subsequently, yeast cells were collected by centrifuge and resuspended with sterile water, then dropped on an X-gal medium plate at 30°C for 12–24 h. The empty p178 and pJG4-5 plasmids were used as negative controls.

Protein Recombination and Purification

The pET28a-AabZIP1 plasmid was introduced into *E. coli* strain BL21 for expression. Fusion proteins were expressed in BL21 cells by adding 0.1 mM IPTG into culture medium for 7 h at 28°C and purified using Ni-NTA agarose (Invitrogen). Purified recombinant protein was quantified using the Bradford assay (2-D Quant Kit; Amersham Biosciences, San Francisco, CA, USA).

EMSA

EMSA was performed as described by Zhang et al. (2010). In brief, the 3′ end biotin-labeled oligonucleotides for the ABRE elements were synthesized (Sangon, Shanghai, China) and equimolar pairs were annealed using the protocol provided by Sigma. EMSA was performed using the LightShift Chemiluminescent EMSA Kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. One microgram of recombinant fusion protein and 100 fmol biotin-labeled DNA with binding reaction buffer were kept for 30 min at room temperature before loading buffer was added. Gel electrophoresis was performed on a 10% native polyacrylamide gel with 6% glycerol. After blotting on a positively charged nylon membrane (Amersham Pharmacia, Piscataway, NJ, USA), the DNA was linked using a UV-crosslinker. The biotin-labeled DNA was detected by chemiluminescence and exposed to X-ray film (Kodak). The probes and primers used in the EMSA assay are listed in Supplemental Table 1.

Subcellular Localization of AabZIP1

For transient expression, *A. tumefaciens* strain GV3101 harboring construct pEG104:YFP:AabZIP1 was employed along with the P19 strain, which was used to inhibit transgenic silencing coinfiltrated into 6-week-old *N. benthamiana* leaves. YFP signal was observed at 48–60 h after infiltration by confocal laser microscopy (Leica TCS SP5-II). Results were verified by three repeats.

Plant Transformation

The overexpression construct pHB-YFP-AabZIP1 was introduced into *A. tumefaciens* strain EHA105 and subsequently used to genetically transform *A. annua* and *Arabidopsis* as described previously (Zhang et al., 2013).

Real-Time Quantitative PCR

Real-time quantitative PCR analysis was performed according to the previously described methods (Zhang et al., 2013). The different organs (roots, stems, leaves, flower buds, and flowers) of plants were collected from 5-month-old *A. annua* and frozen in liquid nitrogen immediately. The leaves of *A. annua* treated with ABA, NaCl, and drought as well as mock treatment were collected and frozen in liquid nitrogen immediately. For analysis of the expression levels of AabZIP1 in transgenic *A. annua*, the leaves of 2-month-old transgenic and wild-type *A. annua* were harvested. The total RNA exact from these samples was prepared as described by the manufacturer of the total plant RNA Extract Kit (Tiangen, China).The relative expression levels of genes were normalized to the expression of *A. annua ACTIN*. Real-time PCR was performed in a Bio-Rad MJ (Bio-Rad, Hercules, CA, USA). Amplification was carried out using Brilliant SYBR Green QPCR MasterMix (Takara, Tokyo, Japan) according to the manufacturer's instructions. The thermal profile for SYBR Green real-time PCR was 95°C for 2 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min.

Quantification of Artemisinin by HPLC

Leaves from the 2-month-old *A. annua* plants were collected and dried overnight at 45°C. The leaf materials were ground into powder and 1 g of leaf powder was extracted with 4 ml methanol under ultrasound for 30 min. The samples were centrifuged at 12 000 rpm for 5 min and the supernatant was filtered through a nitrocellulose filter (0.45 μm); the samples were used for HPLC. The method of HPLC analysis was as previously described (Lu et al., 2013b).

Histochemical GUS Staining, GUS Activity Analysis, and Western Blot

Histochemical GUS staining and GUS activity analysis were performed as previously described (Zhu et al., 2013). Approximately 200 mg of young leaves were ground to fine powder in liquid nitrogen. Subsequently 2× loading buffer was added, boiled for 5 min, and centrifuged for 1 min at 12 000 *g*. Protein samples were fractionated in 10% SDS–PAGE Mini Gel and blotted to a PVDF membrane (Amersham Pharmacia). The primary antibody was anti-GFP and the secondary antibody (Amersham Pharmacia) was goat antimouse antibody. The detection of proteins was conducted using the ECL PLUS kit (Pierce).

SUPPLEMENTAL INFORMATION

Supplemental Information is available at *Molecular Plant Online*.

FUNDING

This work was funded by China National High-Tech 863 Program (2011AA100605), the Shanghai Leading Academic Discipline Project (Horticulture), the Program for New Century Excellent Talents in University (NCET-12-0930), and the Fundamental Research Funds for the Central Universities (XDJK2013A024).

ACKNOWLEDGMENTS

We are very grateful to Professor Hongquan Yang (ShanghaiJiaoTong University) for providing help with the experiments. We appreciate Dr Fei Zhang (Oklahoma State University) for fruitful discussions and kind suggestions. No conflict of interest declared.

Received: July 7, 2014
Revised: November 1, 2014
Accepted: December 8, 2014
Published: December 15, 2014

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