



RESEARCH PAPER

Interaction of bZIP transcription factor TGA6 with salicylic acid signaling modulates artemisinin biosynthesis in *Artemisia annua*

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Received 10 October 2018; Editorial decision 22 March 2019; Accepted 22 March 2019

Editor: Nick Smirnov, University of Exeter, UK

Abstract

Artemisinin is a sesquiterpene lactone produced by the Chinese traditional herb *Artemisia annua* and is used for the treatment of malaria. It is known that salicylic acid (SA) can enhance artemisinin content but the mechanism by which it does so is not known. In this study, we systematically investigated a basic leucine zipper family transcription factor, AaTGA6, involved in SA signaling to regulate artemisinin biosynthesis. We found specific *in vivo* and *in vitro* binding of the AaTGA6 protein to a 'TGACG' element in the *AaERF1* promoter. Moreover, we demonstrated that AaNPR1 can interact with AaTGA6 and enhance its DNA-binding activity to its cognate promoter element 'TGACG' in the promoter of *AaERF1*, thus enhancing artemisinin biosynthesis. The artemisinin contents in AaTGA6-overexpressing and RNAi transgenic plants were increased by 90–120% and decreased by 20–60%, respectively, indicating that AaTGA6 plays a positive role in artemisinin biosynthesis. Importantly, heterodimerization with AaTGA3 significantly inhibits the DNA-binding activity of AaTGA6 and plays a negative role in target gene activation. In conclusion, we demonstrate that binding of AaTGA6 to the promoter of the artemisinin-regulatory gene *AaERF1* is enhanced by AaNPR1 and inhibited by AaTGA3. Based on these findings, AaTGA6 has potential value in the genetic engineering of artemisinin production.

Keywords: Artemisinin, *Artemisia annua*, salicylic acid, transcription factor.

Introduction

Malaria is a serious disease that threatens the health of more than 212 million people (World Health Organization, 2016). The sesquiterpene artemisinin is produced in the glandular trichomes of the Chinese medicinal plant *Artemisia annua*

(Duke *et al.*, 1994) and in recent decades artemisinin combination therapy (ACT) has been recommended by the World Health Organization (WHO) to treat malaria caused by the *Plasmodium falciparum* parasite. Artemisinin has also been shown to have additional potential roles in the treatment of several human cancers and viral diseases (Ma *et al.*, 2014; O'Brien *et al.*, 2015). It may also inhibit *Mycobacterium tuberculosis* and has great potential to cure diabetes (Li *et al.*, 2016; Zheng *et al.*, 2017).

The artemisinin biosynthetic pathway has been determined (Bouwmeester *et al.*, 1999; Mercke *et al.*, 2000; Teoh *et al.*, 2006, 2009; Zhang *et al.*, 2008) (Supplementary Fig. S1 at JXB online) and the small-scale production of an artemisinin precursor in engineered *Saccharomyces cerevisiae* has been developed; however, this is only enough to supplement, rather than replace, the agricultural production of artemisinin (Westfall *et al.*, 2012). The price of artemisinin remains relatively high in developing countries, and increasing its yield is an effective way to reduce the cost of production. In this context, the genetic engineering of artemisinin production is a promising method to improve the availability of this compound (Tang *et al.*, 2014).

Salicylic acid (SA), a phenolic compound, plays an important role in plant defense responses against pathogens (Raskin *et al.*, 1990). Many studies have indicated that NON-EXPRESSOR OF PATHOGENESIS-RELATED GENES 1 (NPR1) is an important modulator of plant immunity in SA signal transduction (Després *et al.*, 2000; Kinkema *et al.*, 2000; Zhou *et al.*, 2000; Mukhtarv *et al.*, 2009; Spoel *et al.*, 2009; Wu *et al.*, 2012). NPR1 contains ankyrin repeats and a BTB/POZ (broad-complex, tramtrack, and bric-a-brac/pox virus and zinc finger) domain (Rochon *et al.*, 2006; Boyle *et al.*, 2009). The ankyrin repeat domain is thought to be involved in interactions with TGA transcription factors (Després *et al.*, 2003) and the BTB/POZ domain is necessary for the DNA binding activity of the TGA transcription factors. Under the stimulation of SA, TGA2 can be incorporated into a transactivating complex with the BTB/POZ domain (residues 80–91) of NPR1 and form an enhanceosome (Fan and Dong, 2002; Rochon *et al.*, 2006). There are 10 TGA transcription factors in Arabidopsis, and three TGAs (TGA2/5/6, termed class II TGAs) interact with NPR1 via strong binding (Zhang *et al.*, 1999; Després *et al.*, 2000; Zhou *et al.*, 2000).

TGA factors are the basic region/leucine zipper motif (bZIP) transcription factors involved in the regulation of pathogen defense, stress signaling, flower development, and seed maturation by binding to the *as-1* element (Lam and Lam, 1995; Xiang *et al.*, 1997; Zhang *et al.*, 1999; Després *et al.*, 2000; Zhou *et al.*, 2000). The class II TGA proteins are the essential molecular linkers of the SA and jasmonic acid/ethylene signaling networks for defense responses (Zander *et al.*, 2010; Van der Does *et al.*, 2013). Moreover, class II TGA factors can interact with NPR1 to form an enhanceosome complex at the *PR-1* promoter, indicating that they have redundant roles in modulating gene expression in the process of defense (Rochon *et al.*, 2006). In addition, *in vivo* assays indicate that some homodimers and heterodimers are formed between TGA factors (Foster *et al.*, 1994; Lam and Lam, 1995).

Much research has shown that SA regulates the biosynthesis of secondary metabolites (Pu *et al.*, 2009; Okada *et al.*, 2009). SA can improve the artemisinin content (Pu *et al.*, 2009; Yu *et al.*, 2012), but the mechanism of the process is still unknown. In general, AP2/ERF transcription factors are targets of SA (Van der Does *et al.*, 2013; Zander *et al.*, 2014). AP2/ERF transcription factors have a conserved AP2 DNA-binding domain of ~60 amino acid residues. In plants, the AP2/ERF family plays important roles in the modulation of secondary metabolite biosynthesis. The AP2/ERF proteins AaERF1 and AaERF2 are involved in enhancing transcription levels of the artemisinin biosynthetic pathway genes *ADS* and *CYP71AV1*. Artemisinin levels are increased by 19–67% and 24–51% in *AaERF1*- and *AaERF2*-overexpressing plants, respectively, suggesting that *AaERF1/AaERF2* play important roles in sesquiterpenoid biosynthesis (Yu *et al.*, 2012). Several other AP2/ERF transcription factors have also been shown to regulate artemisinin production, including AaORA (Lu *et al.*, 2013) and AaTAR1 (Tan *et al.*, 2015).

In this study, we examined the SA-mediated molecular mechanisms that modulate artemisinin biosynthesis and found that a bZIP transcription factor AaTGA6 specifically targets the promoter of *AaERF1*. In addition, we demonstrated that AaNPR1 acts as a transcriptional co-activator and interacts with AaTGA6, forming an enhanceosome at the *AaERF1* promoter. AaTGA3 interacts with AaTGA6 to form a heterodimer and this exerts a negative effect on the DNA-binding activity of AaTGA6. Finally, we provided evidence of AaTGA6-mediated artemisinin synthesis by the AaNPR1–AaTGA6–AaTGA3 complex, which modulates *AaERF1* to influence the accumulation of artemisinin. This network may provide a reference for other medicinal plants.

Materials and methods

Plant material

Seeds of *Artemisia annua* L. (Qinghao) 'Huhao 1' from Chongqing, China, were surface-sterilized with 75% ethanol for 1 min and then sterilized using 20% (v/v) NaOCl (sodium hypochlorite) for 20 min (Shen *et al.*, 2016). All seeds were washed three times for 5 min in sterilized water, planted on MS medium (Murashige and Skoog, 1962), and incubated with a photoperiod of 16/8 h light/dark with 7500 lux at 26 °C.

Seeds of *Nicotiana benthamiana* were sown in a soil mixture (vermiculite:perlite:peat moss, 7:0.5:2) and incubated under the same conditions. The tobacco leaves were used for infiltration experiments after 5 weeks.

Plant hormone treatments

For plant hormonal treatment, 1-month-old *A. annua* seedlings were sprayed with 1 mM SA (Sigma-Aldrich; adjusted to pH 7.0 with NaOH) (Pu *et al.*, 2009) and then sampled at 0, 0.5, 1, 3, 6, 12, and 24 h for RNA isolation.

RNA isolation and qPCR analysis

Total RNA was extracted using an RNAPrep Pure Plant Kit (Tiangen Biotech) (Lv *et al.*, 2016). Samples of 500 ng of total RNA were used for first-strand cDNA synthesis using a cDNA synthesis kit (TaKaRa Biotech) according to the manufacturer's instructions. For qPCR, the diluted cDNA was used as a template, and SYBR Green (Kapa Biosystems)

was used to detect transcript levels. The β -*ACTIN* gene of *A. annua* was used as an internal control (Zhang, et al., 2015; Lv et al., 2016; Shen, et al., 2016). The primers used for qPCR are listed in Supplementary Table S1.

Dual-LUC assay

AaTGA6, *AaTGA3*, *AaNPR1*, *N-AaNPR1*, *C-AaNPR1*, *N-AaTGA6*, and *C-AaTGA6* were individually subcloned into the PHB vector to generate the effector. The promoters of *AaERF1* and *ADS* were fused to the vector pGreenII0800 to generate a reporter. The reporter and effector constructs were then separately transformed into *Agrobacterium tumefaciens* strain GV3101. The bacterial cells were resuspended in MS medium with 10 mM methylester sulfonate and 150 μ M acetosyringone to $OD_{600}=0.6$ and then incubated at room temperature for 3 h. The bacteria-harboring constructs were infiltrated into tobacco leaves according to Zhang, et al. (2015). The leaves were collected after 48 h for dual-LUC assays using a Dual-Luciferase Reporter Assay System according to the manufacturer's instructions (Promega). Three independent biological replicates were measured for each sample.

Y2H, BiFC, and pull-down analysis

For yeast two-hybrid (Y2H) assays, the ORF of *AaTGA6* was cloned into the yeast GAL4 DNA-binding domain vector GBKT7 (Clontech) and used as bait. The ORFs of *AaTGA3* and *AaNPR1* were fused to the vector GADT7 as prey. The plasmids of bait and prey were co-transformed into the yeast strain AH109 (Clontech) by the LiAC-polyethylene glycol method (Gietz and Schiestl, 2007). The transformants were reconfirmed on Leu- and Trp-deficient synthetic dropout (SD) medium plates. The interactions of *AaTGA6* with *AaNPR1* and *AaTGA3* were tested on SD-Leu-Trp-His or SD-Ade-Leu-Trp-His plates with 3-amino-1,2,4-triazole. At least five individual clones were analysed.

For the bimolecular fluorescence complementation (BiFC) assays, *AaNPR1* and *AaTGA3* were subcloned into the vector pEG202 to generate *AaNPR1*-pEG202 and *AaTGA3*-pEG202; *AaTGA6* was subcloned into the vector pEG201 to generate *AaTGA6*-pEG201. Plasmids of *AaNPR1*-pEG202, *AaTGA3*-pEG202, and *AaTGA6*-pEG201 were transformed into *A. tumefaciens* strain GV3101. The *A. tumefaciens*-harboring sets of constructs were resuspended in MS liquid medium buffer with 10 mM methylester sulfonate and 150 μ M acetosyringone at $OD_{600}=0.6$ and then incubated at room temperature for 3 h. Tobacco leaves were infiltrated with the *A. tumefaciens* suspensions. Yellow fluorescent protein (YFP) was observed by confocal laser microscopy.

For pull-down assays, His, *AaTGA6*-His, GST, *AaTGA3*-GST, and *AaNPR1*-GST were expressed *E. coli* Rosetta (DE3) (Novagen). The recombinant proteins were purified using glutathione sepharose beads (Amersham Biosciences) and Ni-NTA agarose beads (QIAGEN). The pull-down assays were conducted according to Bratzel et al. (2010).

Y1H assays

To detect whether *AaTGA6* could interact with the 'TGACG' box in yeast, yeast one-hybrid (Y1H) assay were used and examined according to Zhang et al. (2015).

EMSA analysis

To test whether *AaTGA6* could interact with the 'TGACG' box, an electrophoretic mobility shift assay (EMSA) was performed. The ORF regions of *AaTGA6*, *AaNPR1*, and *AaTGA3* were cloned into the PET28A vector (Novagen) and the resulting constructs (*AaTGA6*-PET28, *AaNPR1*-PET28, and *AaTGA3*-PET28) were introduced into *E. coli* strain BL21(DE3). The purification of the *AaTGA6*-PET28, *AaNPR1*-PET28, and *AaTGA3*-PET28 fusion proteins was performed by Ni-NTA agarose, according to the manufacturer's instructions (Qiagen). The 40-bp oligonucleotides were the recognition sites of *AaTGA6*, labeled by digoxin as probes for the EMSA studies, and the sequences are shown in Supplementary Table S1. The EMSA reactions were performed using a DIG Gel Shift Kit, 2nd Generation, according to the manufacturer's

instructions (Roche). After the EMSA reaction, the products were loaded on a 5% polyacrylamide gel (acrylamide:bisacrylamide, 37.5:1; Bio-Rad) by applying a voltage of 80 V for electrophoresis. The DNA-protein complex was transferred to a nylon membrane for 120 min at 30 V. The membrane was then cross-linked at 120 MJ. The detection was performed according to instructions from Roche.

RNA-seq analysis

total RNA samples were isolated from 1-month-old plants of *A. annua* and sequenced on a HiSeq 4000 (Illumina Inc.). RNA-seq analysis was performed according to Shi et al. (2018).

Quantification of artemisinin using HPLC-ELSD

Artemisinin was quantified using HPLC-ELSD (evaporative light scattering detection). Young leaves at the upper levels of secondary branches of *A. annua* were collected and dried at 50 °C for 24 h and ground into powder (Nair et al., 2013). The powdered samples (0.1 g) were used for artemisinin detection with a Waters Alliance 2695 HPLC system (Zhang et al., 2009).

Accession Numbers

Sequence data from this article are deposited in the GenBank databases under the following accession numbers: *AaNPR1* (MH201457), *AaNPR2* (MH201458), *AaNPR3* (MH201459), *AaNPR4* (MH201460), *AaNPR5* (MH201461), *AaTGA1* (MH201462), *AaTGA2* (MH201463), *AaTGA3* (MH201464), *AaTGA4* (MH201465), *AaTGA5* (MH201466), *AaTGA6* (MH201467). The accession number of Raw RNA-seq data is available from the NCBI Sequence Read Archive (PRJNA524494).

Results

Characterization of *AaTGA6*

SA regulation of artemisinin content has previously been shown to increase the transcript level of *ADS* in the artemisinin biosynthetic pathway in *A. annua* (Supplementary Fig. S2) (Pu et al., 2009; Yu et al., 2012). We hypothesized that the TGA transcription factors might modulate the expression level of *ADS*, either directly or indirectly. According to transcriptome sequencing, we first analysed the data and obtained the gene sequences of NPRs and TGAs in *A. annua*. There were six basic leucine zipper (bZIP) transcription factors of TGA (*AaTGA1*, *AaTGA2*, *AaTGA3*, *AaTGA4*, *AaTGA5*, and *AaTGA6*) and five NPR genes (*AaNPR1*, *AaNPR2*, *AaNPR3*, *AaNPR4*, and *AaNPR5*) in the transcriptome database of *A. annua*. We constructed a phylogenetic tree to identify TGA transcription factors in *A. annua*, which showed that *AaTGA6* clustered with *AtTGA2/AtTGA5/AtTGA6* (Fig. 1A). Previous studies have indicated that monocot and dicot plants share a conserved sequence and function in NPR1 (Kuai and Despré, 2016). *AaNPR1* had a close phylogenetic relationship with *AtNPR1* (Fig. 1B), indicating that *AaNPR1* may play a co-activator role in *A. annua*. To investigate which TGAs had the potential to affect the accumulation of artemisinin, we used qPCR to monitor mRNA levels of *AaTGA1–6* in the different tissues (roots, stems, leaves, and buds) and different leaves (leaf 0–leaf 7; Ma et al., 2018). Most of TGA factors were mainly expressed in the roots, buds, and in leaves 4–7 (Supplementary Fig. S3).

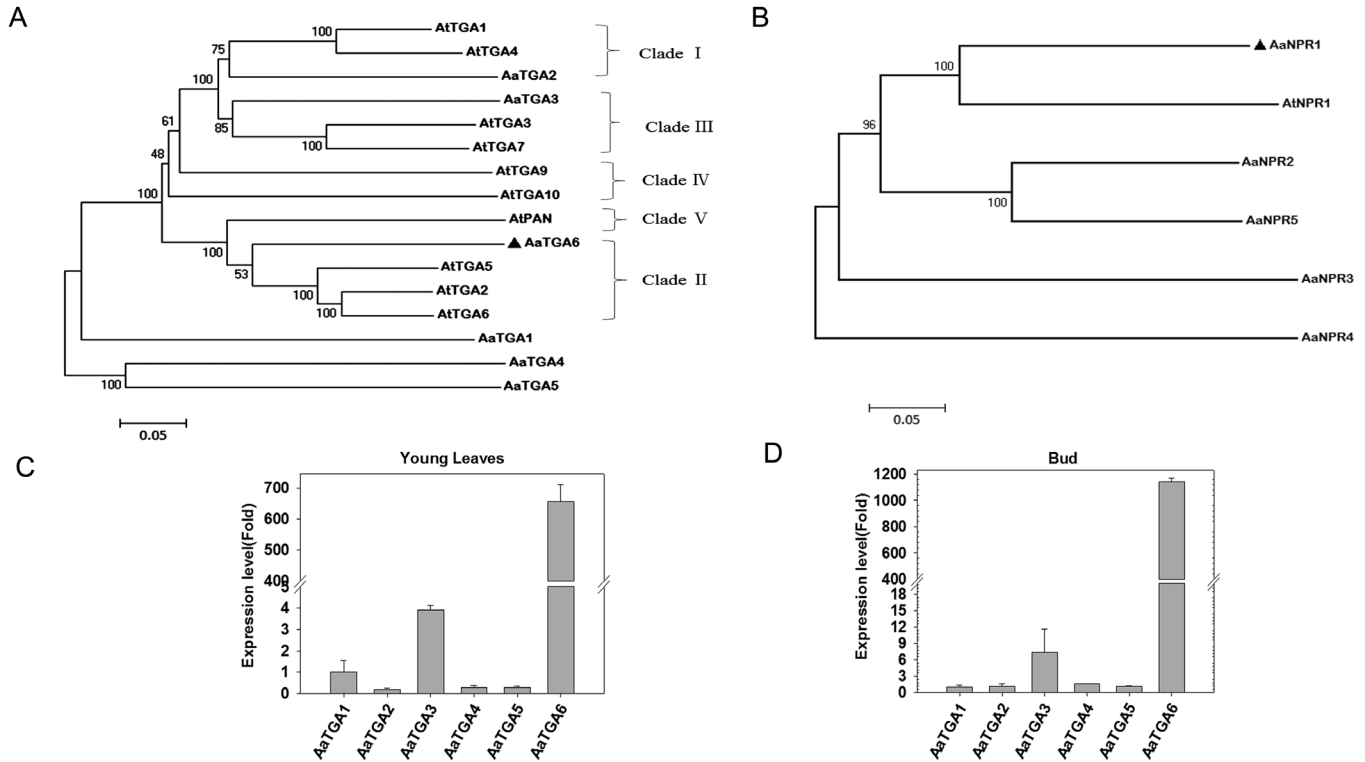


Fig. 1. Characterizations of NPRs and TGAs in *Artemisia annua*. Phylogenetic trees of (A) TGAs and (B) NPRs in *A. annua* (Aa) and *Arabidopsis* (At). The trees were constructed using MEGA 5 with the neighbor-joining method (1000 bootstrap replicates). The scale bars indicate the evolutionary distance of the sequences examined. *AaTGA6* and *AaNPR1* are indicated with black triangles. Expression of TGAs in (C) young leaves and (D) buds. Expression values are relative to those of TGA1. Data are means (\pm SE) of $n=3$ biologically independent samples.

The artemisinin biosynthetic pathway genes *ADS*, *CYP71AV1*, *DBR2*, and *ALDH1* are mainly expressed in young leaves and buds (Lommen et al., 2006; Lu et al., 2013), and our qPCR assays showed a significantly higher expression level of *AaTGA6* in these tissues (Fig. 1C, D), indicating that *AaTGA6* may be the principle TGA that regulates artemisinin biosynthesis. In addition, *AaTGA3* had similar expression patterns to *AaTGA6*, although at much lower levels.

AaTGA6 was localized to the nucleus in *A. annua* (Supplementary Fig. S4A) and may have been involved in artemisinin biosynthesis (Fig. 1C). We performed dual-LUC assays and these indicated that *AaTGA6* was active in the transcription of *ADS* (Supplementary Fig. S4C).

AaTGA6 activates the transcription of *AaERF1* in vivo

Previous studies have indicated that the AP2/ERF transcription factor *ORA59* is the direct target of class II TGA transcription factors (Zander et al., 2014), and we hypothesized that *AaTGA6* could directly modulate AP2/ERF transcription factor expression levels. Three AP2/ERF transcription factors (*AaERF1*, *AaERF2*, and *AaORA*) involved in artemisinin biosynthesis have been identified (Yu et al., 2012; Lu et al., 2013). We scanned the promoter region of *AaERF1/AaERF2/AaORA* and found ‘TGACG’ elements existing in the promoter of *AaERF1* (Supplementary Fig. S5).

To survey the target transcription factors of *AaTGA6*, we performed deep RNA sequencing. One-month-old *A. annua*

seedlings were treated with 1 mM SA, sampled at 0, 1, 5, and 7 h, and sequenced using the Illumina platform. According to the resulting heat map the expression levels of 101 genes were significantly enhanced under SA treatment, ($P<0.01$; Supplementary Fig. S6). Thus, all the available evidence indicated that *AaERF1* may be a candidate target for modulation by *AaTGA6*. Further studies were therefore focused on the relationship between *AaTGA6* and *AaERF1*. Using a dual-LUC assay, we examined whether *AaTGA6* activated the transcription of *AaERF1* in vivo. The results showed that *AaTGA6* activated the expression of *AaERF1* promoters, as evidenced by a higher value of LUC/REN than the control (Fig. 2B). *AaTGA6* may therefore have great potential for increasing the artemisinin content in *A. annua*.

A Y1H assay was performed and demonstrated that *AaTGA6* bound directly to the *AaERF1* promoter in vivo (Fig. 2D). In addition, an EMSA analysis was performed to demonstrate the DNA-binding activity of *AaTGA6*. A retarded band of *AaTGA6* was observed when *AaTGA6* was added to the binding mixture (Fig. 2E, lane 2). To further test the binding specificity, the competition probe concentration was increased and this increased the number of retarded bands (Fig. 2E, lanes 3–6). Certain sequences that flank the conserved region ‘TGACG’ also played key roles in *AaTGA6* binding. When the flanking sequences were mutated, the DNA-binding effects of *AaTGA6* were changed (Supplementary Fig. S7). These results indicated that *AaTGA6* specifically recognized the ‘TGACG’ sequence within the *AaERF1* promoter.

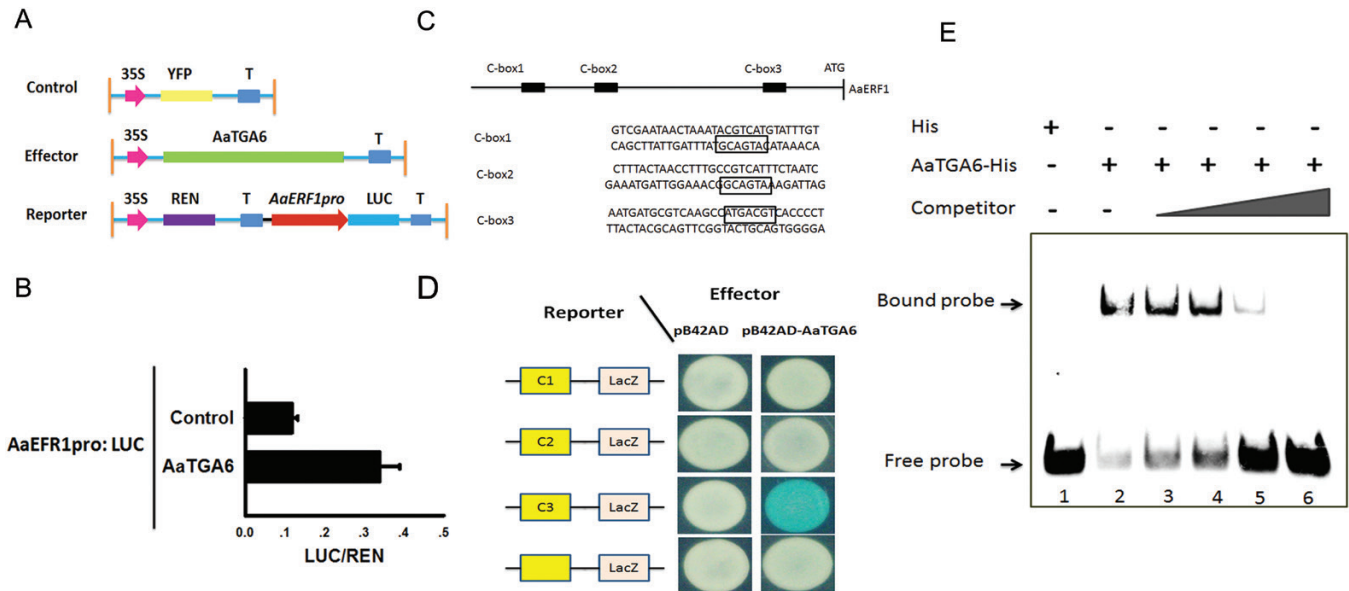


Fig. 2. AaTGA6 is a transactivator that binds to the C-‘TGACG’ box in the promoter of *AaERF1*. (A) Schematic diagram of the reporter and effector constructs used in the transient dual-LUC assays. (B) Transient dual-LUC analysis showing AaTGA6 activation of the transcription of *AaERF1* in *N. benthamiana* leaves. LUC/REN represents the luciferase/Renilla ratio of $n=3$ independent experiments; $P<0.05$ as determined using Student’s *t*-test. (C) Schematic diagram of the *cis*-element in the sequences of the *AaERF1* promoter. C-box1, C-box2, and C-box3 were used for the yeast one-hybrid (Y1h) assays. The ‘TGACG’ boxes are indicated. (D) Y1h assays showing the interaction between AaTGA6 and ‘TGACG’ boxes in the promoter of *AaERF1*. Yeast transformants were diluted and plated on synthetic dropout medium without Ura and Trp plus 20 mM X-gal. (E) EMSA analysis to test the binding of AaTGA6-His to the ‘TGACG’ motifs in the promoter of *AaERF1*. Unlabeled ‘TGACG’ was used as competitor DNA at molar ratios of 1:1; 1:6; 1:50; and 1:100. (This figure is available in colour at JXB online.)

AaTGA6 interacts with AaNPR1

The class II TGA transcription factors (AtTGA2/5/6) can interact with AtNPR1, which is known as a mediator protein between TGA2/5/6 and the *PR1* gene in *Arabidopsis* (Fan and Dong, 2002). We hypothesized that AaNPR1 might interact with the class II TGA transcription factor AaTGA6 in *A. annua*, and therefore performed a yeast two-hybrid (Y2H) assay. As shown in Fig. 3A, the interaction between AaNPR1 and AaTGA6 was confirmed. In addition, we performed YFP BiFC assays and pull-down assays, which also confirmed the interaction (Fig. 3B, C).

AaNPR1 enhances the binding of AaTGA6 to the ‘TGACG’ sequence

Previous studies have shown that NPR1 increases the binding of TGA2 to its cognate promoter element, *as-1* (Després *et al.*, 2000). The transcription factor ABF2 substantially enhances the binding of ANAC096 to the *NACRS* elements at the RD29A promoter (Xu *et al.*, 2013). To determine whether it behaved in a similar way, we investigated the effect of AaNPR1 on the binding of AaTGA6 factors to the ‘TGACG’ sequence within the *AaERF1* promoters. Dual-LUC assays were performed in the presence or absence of *in vitro*-translated AaNPR1, AaTGA6, or both. We found that the relative reporter activity of LUC/REN was low in the presence of AaNPR1 or AaTGA6, but in the presence of both the activation of LUC/REN significantly increased (Fig. 4B). In addition, AaNPR1 also activated the promoter of *AaERF1*.

Surprisingly, the amino-terminal regions of AaNPR1 alone (N-AaNPR1, 1–351 residues) seemed to have a high level of activation of the *AaERF1* promoters (Supplementary Fig. S8). Since full-length AaNPR1 could activate the transcription of *AaERF1*, we hypothesized that N-AaNPR1 or C-AaNPR1 might also activate it. A dual-LUC assay showed that N-AaNPR1 exhibited high transcriptional activities at the *AaERF1* promoters (Supplementary Fig. S8).

To gain further insights into the role of AaNPR1, EMSA assays were performed in the presence or absence of AaTGA6, AaNPR1, or both. No migrating band was observed when AaNPR1 was present in the binding mixture (Fig. 4C, lane 2) and a retarded band was detected when AaTGA6 was present (Fig. 4C, lane 3). However, the addition of AaNPR1 to the AaTGA6 binding mixture substantially increased the number of migrating bands (Fig. 4C, lane 4). These results confirmed that AaNPR1 enhanced the binding of AaTGA6 to the ‘TGACG’ sequence within the *AaERF1* promoters.

AaTGA3 interacts with AaTGA6 and inhibits its binding to the ‘TGACG’ sequence

Previous studies have indicated that heterodimers are preferentially formed in bZIP transcription factors (Ehlert *et al.*, 2006). We had found that AaTGA3 had similar expression patterns to AaTGA6 in young leaves and buds (Fig. 1C, D) and we hypothesized that AaTGA6 might form heterodimers with other TGAs. Y2H, BiFC, and pull-down assays demonstrated that AaTGA3 could interact with AaTGA6 (Fig. 5A–C). To verify the biological significance of the interaction, dual-LUC assays were performed and indicated

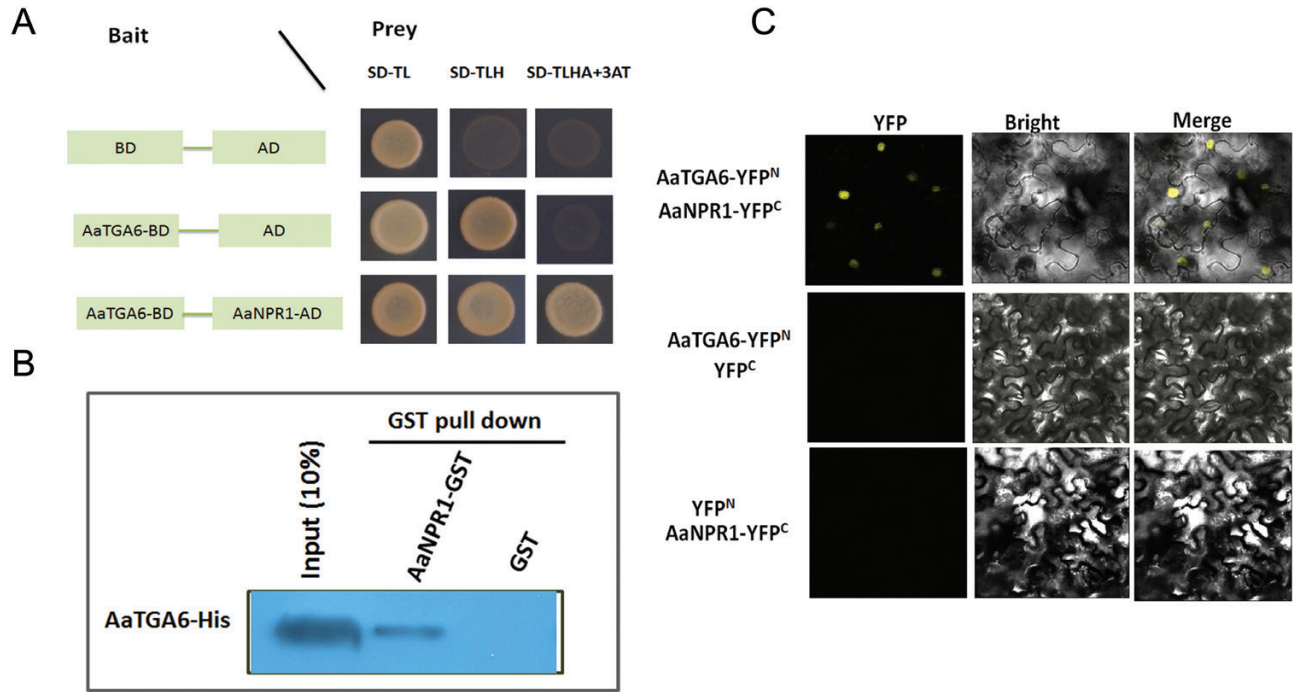


Fig. 3. Interactions between AaNPR1 and AaTGA6. (A) Yeast two-hybrid assays. Yeast cells were plated on synthetic dropout media either without Leu and Trp (SD-TL), without Leu, Trp, and His (SD-TLH), or without Leu, Trp, His, and Ade but containing 80 mM 3-AT (SD-TLHA+3AT). (B) Interactions between AaNPR1 and AaTGA6 examined by BiFC in *N. benthamiana* leaves. *AaNPR1* and *AaTGA6* were fused in the vectors pEarleyagte202 and pEarleyagte201, respectively. (C) Pull-down assays showing the interaction of AaTGA6 and AaNPR1. Prokaryotic expression of AaTGA6 and AaNPR1 proteins fused to His or a GST tag were subjected to GST pull-down, and the proteins were detected by immunoblotting using a GST antibody. (This figure is available in colour at JXB online.)

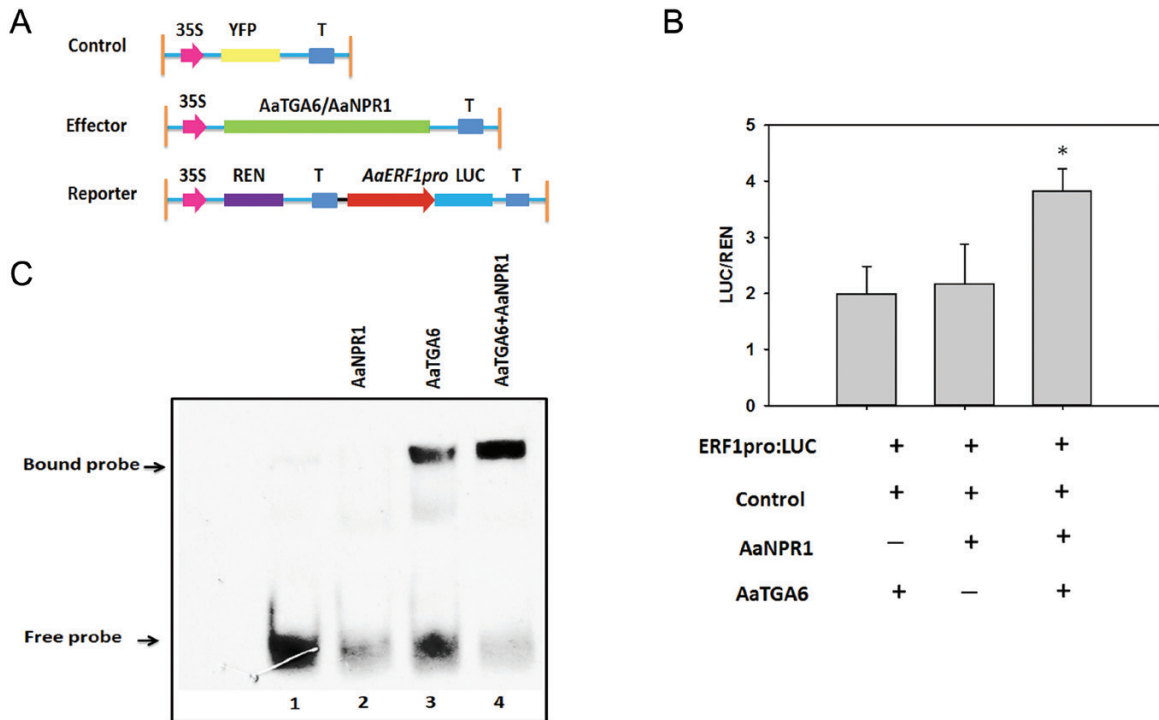


Fig. 4. AaNPR1 enhances the DNA-binding activity of AaTGA6. (A) Schematic diagram of the reporter and effector constructs used in the transient dual-LUC assays. (B) Transient dual-LUC analysis showing that AaNPR1 increases AaTGA6 activation of the transcription of *AaERF1*. LUC/REN represents the luciferase/Renilla ratio of $n=3$ independent experiments; Significant differences were determined using Student's *t*-test: * $P<0.05$. (C) EMSA analysis showing that AaNPR1 enhances the DNA-binding activity of AaTGA6. (This figure is available in colour at JXB online.)

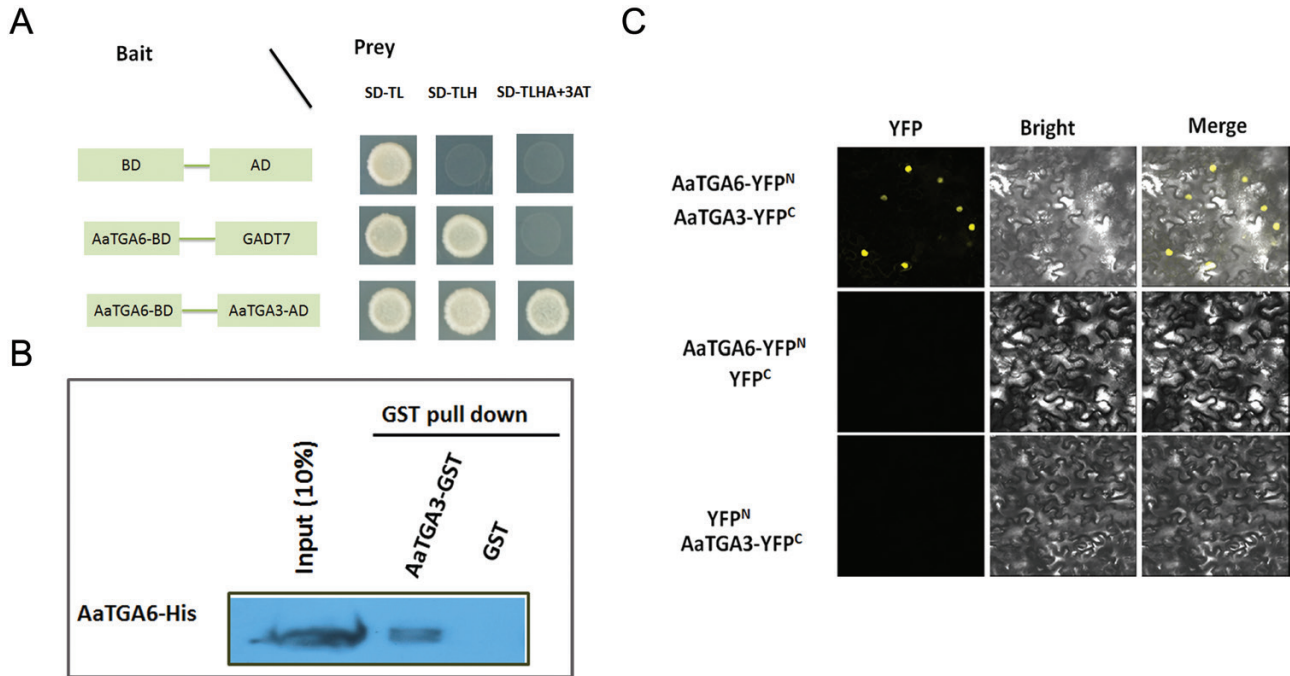


Fig. 5. Interactions between AaTGA3 and AaTGA6. (A) Yeast two-hybrid assays with AaTGA3 and AaTGA6 fused to GAL4 activation domain (AD) or GAL4-binding domain (BD). Interactions were examined on synthetic dropout media either without Leu and Trp (SD-TL), without His, Leu, and Trp (SD-TLH), or without Ade, Leu, Trp, His but containing 80 mM 3 AT (SD-TLHA). 3 AT inhibited the self-transcription activity of AaTGA6-BD. (B) Interactions between AaTGA3 and AaTGA6 were examined by BiFC in *N. benthamiana* leaves. AaTGA3 and AaTGA6 were fused in the vectors pEarleyagte201 and pEarleyagte202, respectively. (C) Pull-down assay showing the interaction of AaTGA6 and AaTGA3. Prokaryotic expression of AaTGA6 and AaTGA3 proteins fused to His or a GST tag were subjected to a GST pull-down, and the proteins were detected by immunoblotting using a GST antibody. (This figure is available in colour at JXB online.)

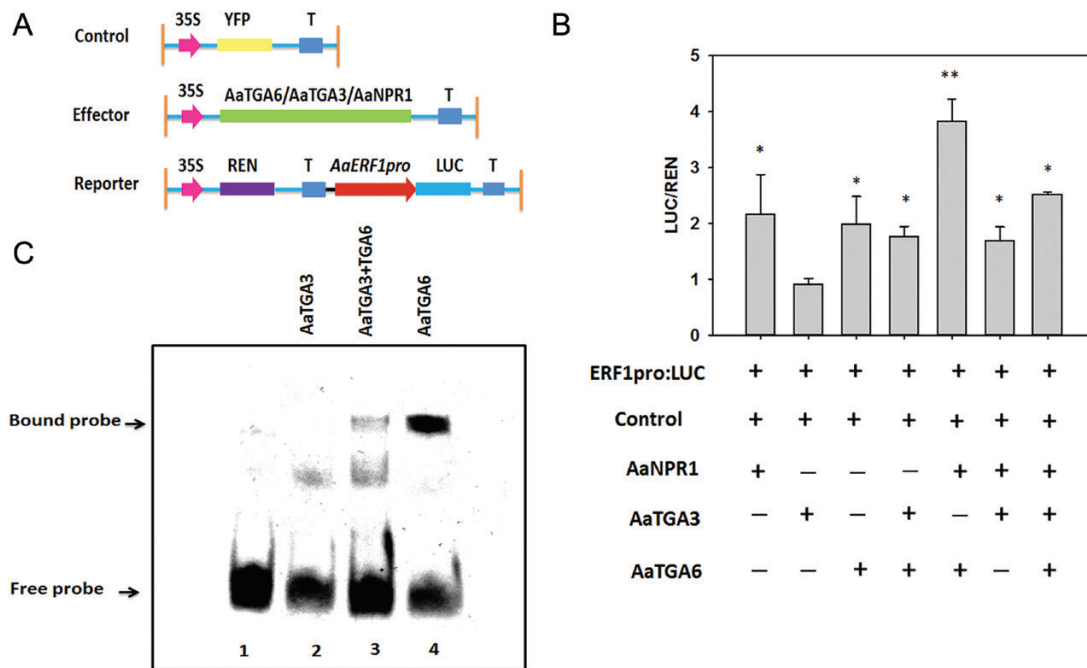


Fig. 6. AaTGA3 represses the DNA-binding activity of AaTGA6. (A) Schematic diagram of the reporter and effector constructs in the transient dual-LUC assays. (B) Transient dual-LUC analysis showing that AaTGA3 inhibits AaTGA6 activation of the transcription of *AaERF1*. Luc/Ren represents the luciferase/Renilla ratio of $n=3$ independent experiments; Significant differences were determined using Student's *t*-test: * $P<0.05$; ** $P<0.01$. (C) EMSA analysis showing that AaTGA3 inhibits the DNA-binding activity of AaTGA6. (This figure is available in colour at JXB online.)

that AaTGA3 substantially repressed the binding of AaTGA6 to its cognate promoter element 'TGACG' in the promoter of *AaERF1* (Fig. 6B). We also confirmed the inhibition using EMSA analysis,

which showed that co-incubation of AaTGA3 with AaTGA6 resulted in a weak retarded band, indicating that AaTGA3 inhibition was functional in the EMSA assay (Fig. 6C, lane 3).

Molecular and chemical characterization of *AaTGA6*-overexpression and -RNAi knockdown transgenic lines

The data presented above indicated that *AaTGA6* was predominantly modulated at the mRNA level of *AaERF1*, the positive regulator of artemisinin. We therefore overexpressed *AaTGA6* in *A. annua*. Driven by the double-35S promoter, the *AaTGA6* was infused into the plasmid PHB to generate the overexpression vector *AaTGA6*-PHB. There was no significant difference in the phenotype between the transgenic lines and the wild-type under normal growth conditions (Supplementary Fig. S9).

Three overexpression and three RNAi lines of transgenic plants were selected for further study and qPCR was employed to determine the expression levels of *AaTGA6*. The results showed that the relative mRNA levels were increased by 11- to 15-fold in the overexpression plants and decreased by 70–90% in the RNAi plants (Fig. 7A, B). As key catalysis

genes involved in the artemisinin biosynthetic pathway, the expression levels of *ADS*, *CYP71AV1*, *DBR2*, and *ALDH1* were also evaluated and were found to be increased in the *AaTGA6* overexpression lines by between 1- to 7-fold (Fig. 7A). The artemisinin content in the overexpression plants was increased by 90–120% compared with the wild-type (Fig. 7C). In the *AaTGA6*-RNAi plants, the expression levels of *ADS*, *CYP71AV1*, *DBR2*, and *ALDH1* were reduced by between 20–70% (Fig. 7B) and the artemisinin content was decreased by 20–60% compared with the wild-type (Fig. 7D).

Discussion

AaTGA6 modulates artemisinin content by directly binding to the promoter of *AaERF1*

The AP2/ERF transcription factors are often targeted by TGA factors, such as TGA2/5/6, which directly bind to the

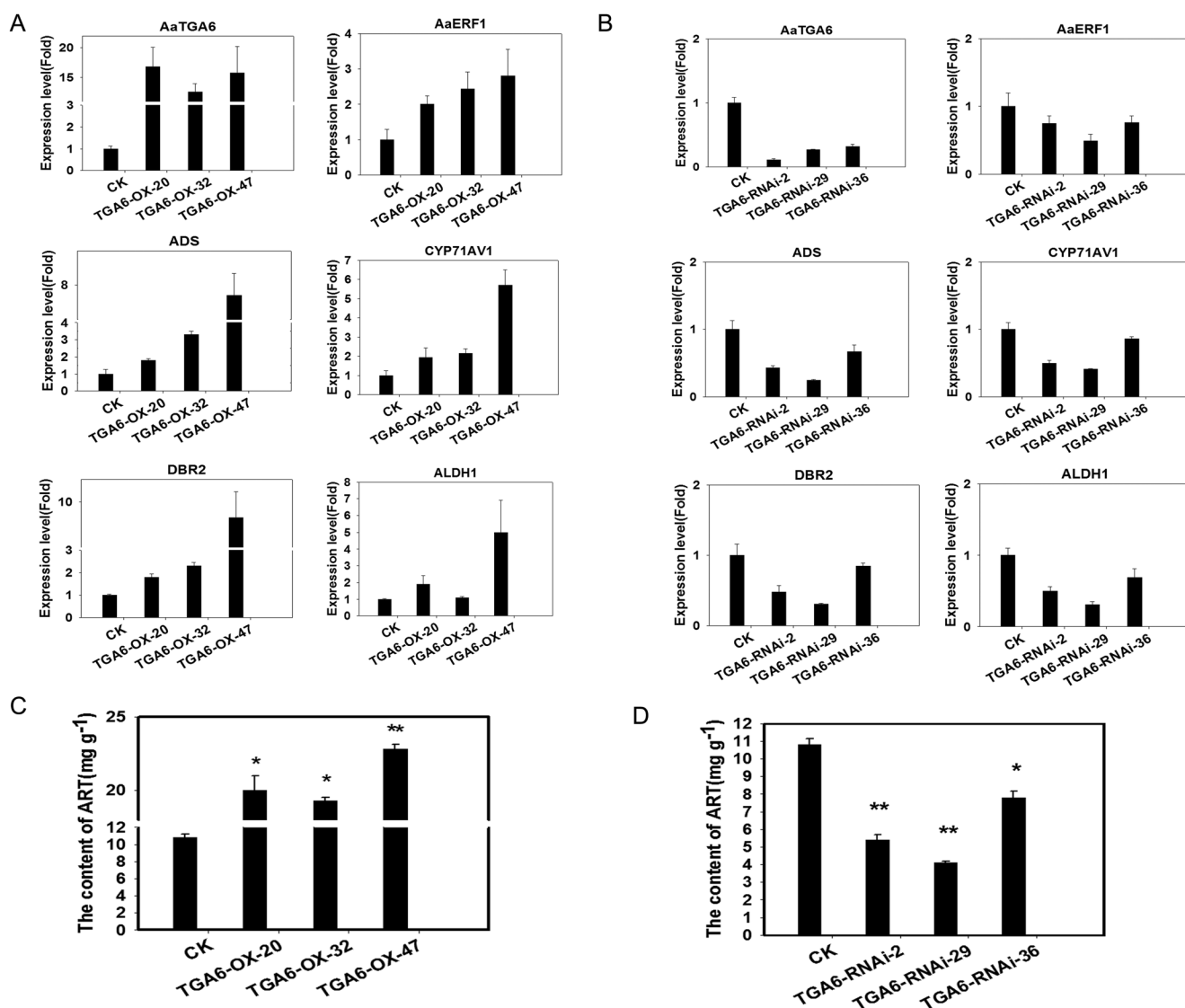


Fig. 7. *AaTGA6* is a positive modulator of artemisinin biosynthesis. (A, B) Expression levels of *AaTGA3*, *AaERF1*, *ADS*, *CYP71AV1*, *DBR2*, and *ALDH1* in (A) *AaTGA6*-overexpression (OX) and (B) *AaTGA6*-RNAi plants, as determined by qPCR analysis. β -ACTIN was used as the endogenous reference gene. Data are means (\pm SE) of $n=3$ biologically independent samples. (C, D) Artemisinin (ART) contents of (C) OX and (D) RNAi plants, as detected by HPLC. CK, wild-type control.

cis-element ‘TGACG’ of the promoter of *ORA59* (Zander *et al.*, 2014). Previous studies have indicated that three AP2/ERF transcription factors, namely, AaERF1, AaERF2 and AaORA, play important roles in the regulation of artemisinin biosynthesis (Yu *et al.*, 2012; Lu *et al.*, 2013). Our promoter sequence analysis indicated that only the that of *AaERF1* harbored the ‘TGACG’ *cis*-element. Since we found that AaTGA6 was an important modulator for the regulation of artemisinin content in *A. annua* (Fig. 7C), we first established its target gene by transcriptome sequencing. Six highly significantly different transcription factors were found in the transcriptome, including AaERF1 (Supplementary Fig. S6). In *AaTGA6*-RNAi transgenic plants, the levels of *AaERF1* mRNA decreased, which coincided with decreased artemisinin content (Fig. 7D), so we postulated that *AaERF1* was the direct target of AaTGA6, and Fig. 2 shows that AaTGA6 directly activated the promoter of *AaERF1*. Therefore, the results are consistent with previous studies showing that AP2/ERF transcription factors are direct targets of class II TGA factors.

N-AaNPR1 activates the promoter of AaERF1

Previous studies have indicated that NPR1 may act with a receptor for SA and play important roles in immune responses by interacting with NPR3/ NPR4 (Fu *et al.*, 2012; Wu *et al.*, 2012; Ding *et al.*, 2018), and thus NPR1 plays an important role in SA signaling. There were five NPR genes in *A. annua*. NPR1 contains two domains: an ankyrin repeat domain and a BTB/POZ domain, which was first cloned by Cao *et al.* (1997). Ankyrin repeats play important roles in the interaction with TGA transcription factors, and the BTB/POZ domain is the enhancer for the transactivation function of the TGA2–NPR1 compound (Boyle *et al.*, 2009). To investigate the functions of the different domains of AaNPR1 in *A. annua*, we analysed the activation of N-AaNPR1 (1–351 residues) and C-AaNPR1 (352–570 residues) using dual-LUC assays. N-AaNPR1 showed a high level of *AaERF1* transactivation activity, while C-AaNPR1 had a low level of activity (Supplementary Fig. S8).

The N-terminus of AaNPR1 had a SKP1/BTB/POZ domain, which might be involved in the transactivation activity of *AaERF1* (Sedgwick and Smerdon, 1999). Previous studies have indicated that the BTB/POZ domain may play a specific role in protein–protein interactions (Bardwell and Treisman, 1994). We assume that N-AaNPR1, which acted as a modulator of *AaERF1* and enhanced its transactivation activity, may interact with other proteins. The reason for this may be that the BTB/POZ domain of N-AaNPR1 represses the C-terminal transactivation domain (Wu *et al.*, 2012).

AaNPR1 increases the DNA-binding activity of *AaTGA6*

TGA factors act as regulatory components of the SA signaling pathway. The class II TGA transcription factors, such as *TGA2*, *TGA5*, and *TGA6* in Arabidopsis, have been demonstrated to regulate the *as-1* element of *PR-1* (Zhang *et al.*, 1999; Després *et al.*, 2000). AP2/ERF transcription factors are potential targets

of class II TGA transcription factors (Van der Does *et al.*, 2013; Zander *et al.*, 2014). We determined that AaTGA6 belongs to the class II TGA transcription factors (Fig. 1A) and can activate the promoter of *AaERF1* (Fig. 4D). It has previously been shown that *AaERF1* is an important modulator of artemisinin biosynthesis (Yu *et al.*, 2012). We postulated that AaTGA6 may bind to the promoter of *AaERF1* directly, and this was supported by the results of the Y1H assays and EMSAs (Fig. 2D, E). Hence, AaTGA6 enhances artemisinin content by directly binding to the promoter of *AaERF1*.

Y2H and BiFC assays indicated that AaTGA6 appeared to interact with AaNPR1 (Fig. 3A–C), which is consistent with previous results in Arabidopsis (Zhang *et al.*, 1999; Zhou *et al.*, 2000). However, it was unclear whether AaNPR1 enhanced the DNA-binding activity of AaTGA6. Dual-LUC assays and EMSAs were employed and clearly demonstrated that AaNPR1 was required for more efficient binding of AaTGA6 to the ‘TGACG’ element in the *AaERF1* promoter (Fig. 4). These results are consistent with previous findings that NPR1 causes an increase in DNA-binding activity in Arabidopsis (Zhang *et al.*, 1999; Després *et al.*, 2000).

AaTGA3 plays a negative role in artemisinin biosynthesis

There are many kinds of highly concentrated secondary metabolites in glandular trichomes with pharmaceutical, pesticidal, flavor- and fragrance-related activities (Duke, 1994). Artemisinin is an important product of the glandular trichome of *A. annua*, and it has a phytotoxic effect on most plants, including itself (Duke, 1994). To avoid autotoxicity hazards, plants may limit the production of artemisinin by a putative feedback mechanism (Arsenault *et al.*, 2010).

AaERF1 has previously been shown to be a positive regulator of artemisinin biosynthesis (Yu *et al.*, 2012). We found that AaTGA6 activated the promoter of *AaERF1*, while AaTGA3 behaved in the opposite manner (Fig. 6B). The interaction of AaTGA3 with AaTGA6 (Fig. 5A–C) reflected the fact that heterodimerization between them occurs (Llorca *et al.*, 2014). Thus, AaTGA3 inhibits the binding of AaTGA6 to its cognate promoter element ‘TGACG’ in the promoter of *AaERF1* and participates in a negative feedback loop of artemisinin biosynthesis. This may be a mechanism by which *A. annua* protects itself from autotoxicity.

Mode of regulation of artemisinin by the AaNPR1-AaTGA6-AaTGA3 complex

Our results showed that AaTGA6 can increase artemisinin content by binding to the promoter of *AaERF1*, which encodes a transcription factor that plays an important role in the modulation of artemisinin content by directly binding to the promoters of *ADS* and *CYP71AV1* (Yu *et al.*, 2012). In addition, we found that AaNPR1 interacts with AaTGA6 to enhance its DNA-binding activity. However, there may be feedback inhibition of artemisinin biosynthesis in *A. annua*, as we showed that another transcription factor, AaTGA3, suppresses the transcription activity of *AaERF1* by interacting with AaTGA6. Thus,

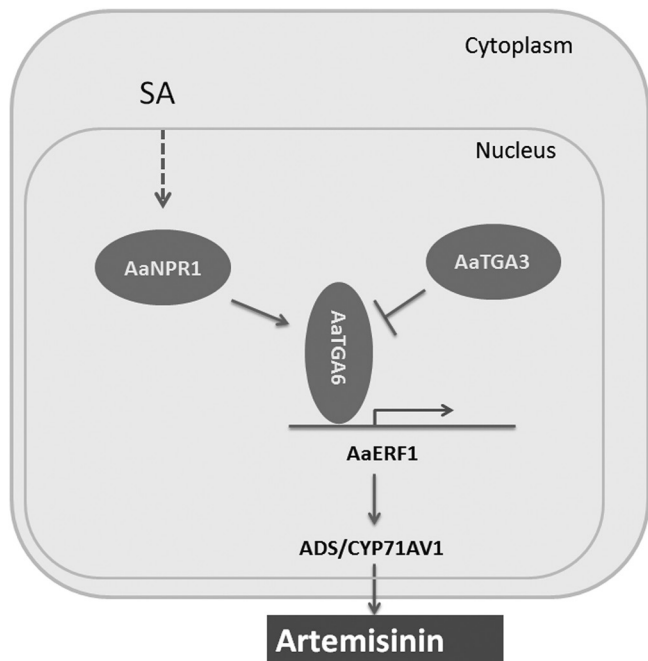


Fig. 8. Proposed molecular regulatory network for modulation of artemisinin biosynthesis by AaTGA6 in *Artemisia annua*. AaERF1 directly regulates artemisinin biosynthesis by activating transcription of artemisinin-related genes such as *ADS* (Yu et al., 2012). AaNPR1 interacts with AaTGA6 and activates transcription of artemisinin-related genes such as *AaERF1*. AaTGA3 repression of the binding of AaTGA6 to the promoter of *AaERF1* may play a feedback-inhibition role in artemisinin biosynthesis. Arrows indicate positive regulation and blocked lines indicate negative regulation; the dashed line indicates an unidentified pathway.

SA modulation of artemisinin content may be mediated by the AaNPR1–AaTGA6–AaTGA3 complex (Fig. 8).

Supplementary data

Supplementary data are available at *JXB* online.

Fig. S1. The artemisinin biosynthesis pathway in *A. annua*.

Fig. S2. Expression levels of *ADS*, *CYP71AV1*, *DBR2*, and *ALDH1* in *A. annua* under SA treatment.

Fig. S3. Expression levels of *AaTGA1–6* in different organs in *A. annua*.

Fig. S4. Subcellular location and transient dual-LUC assays of AaTGA6 expressed in tobacco leaves.

Fig. S5. Promoter sequences of *AaERF1*, *AaERF2*, and *AaORA*.

Fig. S6. Heat map of expression of SA-related genes in *A. annua*.

Fig. S7. Results of EMSAs to test the binding of AaTGA6–His to mutant ‘TGACG’ motifs in the promoter of *AaERF1*.

Fig. S8. Dual-LUC assays showing that N-AaNPR1 can activate the promoter of *AaERF1*.

Fig. S9. Phenotypes of transgenic *A. annua* plants with either overexpression or knockdown of AaTGA6, compared with the wild-type.

Table S1. List of primers used in this study.

Acknowledgements

This work was funded by the China Transgenic Research Program (grant no. 2016ZX08002001-006), the National Science and Technology Major Project (2017ZX09101002-003-002), the Shanghai Leading Academic Discipline Project (Horticulture), and the State Key Laboratory of Subtropical Silviculture, Zhejiang A & F University (Grant no. 2018FR003, ZY20180206). The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Author contributions: ZL, KT, WC, and Le.Z conceived and designed the research; ZL, ZG, FZ, WJ, QS, XF, and TY performed most of the experiments; PS, LL, XH, YM, MC, and WC provided technical assistance; ZL, KT, WC, and Li.Z wrote the manuscript; and LL, KT, WC, and Le.Z helped with the organization and editing.

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