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Research

The jasmonate-responsive AaMYC2 transcription factor positively regulates artemisinin biosynthesis in *Artemisia annua*

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Summary

- The plant *Artemisia annua* is well known due to the production of artemisinin, a sesquiterpene lactone that is widely used in malaria treatment. Phytohormones play important roles in plant secondary metabolism, such as jasmonic acid (JA), which can induce artemisinin biosynthesis in *A. annua*. Nevertheless, the JA-inducing mechanism remains poorly understood.
- The expression of gene *AaMYC2* was rapidly induced by JA and AaMYC2 binds the G-box-like motifs within the promoters of gene *CYP71AV1* and *DBR2*, which are key structural genes in the artemisinin biosynthetic pathway.
- Overexpression of *AaMYC2* in *A. annua* significantly activated the transcript levels of *CYP71AV1* and *DBR2*, which resulted in an increased artemisinin content. By contrast, artemisinin content was reduced in the RNAi transgenic *A. annua* plants in which the expression of *AaMYC2* was suppressed. Meanwhile, the RNAi transgenic *A. annua* plants showed lower sensitivity to methyl jasmonate treatment than the wild-type plants.
- These results demonstrate that AaMYC2 is a positive regulator of artemisinin biosynthesis and is of great value in genetic engineering of *A. annua* for increased artemisinin production.

Introduction

Artemisinin is a sesquiterpene lactone endoperoxide isolated from *Artemisia annua* of the Asteraceae family, and its derivatives are widely used antimalarial drugs (Mutabingwa, 2005; Duffy & Mutabingwa, 2006; WHO, 2014). Although semisynthesis of artemisinin via artemisinic acid in yeast is feasible at present (Ro *et al.*, 2006; Paddon *et al.*, 2013), *A. annua* plants are still the main and only natural commercial source of artemisinin. However, the artemisinin content in plant leaves is relatively low (0.1–0.8% by DW) (Abdin *et al.*, 2003), resulting in short supply and high costs of this effective drug (Kumar *et al.*, 2004).

Because of the great medical value of artemisinin, many investigations have been devoted to clarifying its biosynthetic pathway and the pathway is almost completely elucidated (Fig. 1) (Tang *et al.*, 2014). The first committed step is the conversion of farnesyl diphosphate (FPP) to amorpha-4,11-diene catalyzed by amorpha-4,11-diene synthase (ADS) (Mercke *et al.*, 2000; Kim *et al.*, 2008). Subsequently, amorpha-4,11-diene is hydroxylated to artemisinic alcohol and further oxidized to artemisinic aldehyde and artemisinic acid by a multifunction cytochrome P450 monooxygenase (CYP71AV1) (Ro *et al.*, 2006; Teoh *et al.*, 2006). Recent experiments have shown that in the plant *A. annua* dihydroartemisinic acid is the precursor of artemisinin. Dihydroartemisinic acid is formed from artemisinic aldehyde in a reaction catalyzed by artemisinic aldehyde Δ11 (13) reductase (DBR2) (Zhang *et al.*, 2008) to generate dihydroartemisinic aldehyde and then oxidized to dihydroartemisinic acid by aldehyde dehydrogenase 1 (ALDH1) (Teoh *et al.*, 2009). Thus far, no enzyme has been found to catalyze the conversion from dihydroartemisinic acid to artemisinin. Rather, the reaction appears to be nonenzymatic photo-oxidized in the cell-free cuticular space outside the glandular trichome secretory cells (Brown & Sy, 2004).

However, to date, although there are several transcription factors that have been identified to be involved in artemisinin biosynthesis, the transcriptional regulation of the artemisinin biosynthetic pathway remains unclear. Meanwhile, plants regulating secondary metabolites against herbivores and pathogens and accumulation of these metabolites are often affected directly or indirectly by phytohormones (Pozo *et al.*, 2008; Hong *et al.*, 2012; Lu *et al.*, 2013; Nagel *et al.*, 2014). Among these, jasmonic acid (JA) has been shown to regulate secondary metabolism in many plant species. It upregulated nicotine biosynthesis in tobacco (De Boer *et al.*, 2011), terpenoid indole alkaloid biosynthesis in *Catharanthus roseus* (van der Fits & Memelink, 2000) and artemisinin biosynthesis in *A. annua* (Wang *et al.*, 2010). However, there are only few transcription factors that have been reported to be involved in JA regulation of artemisinin biosynthesis. The MYC2 transcription factor, which belongs to the bHLH

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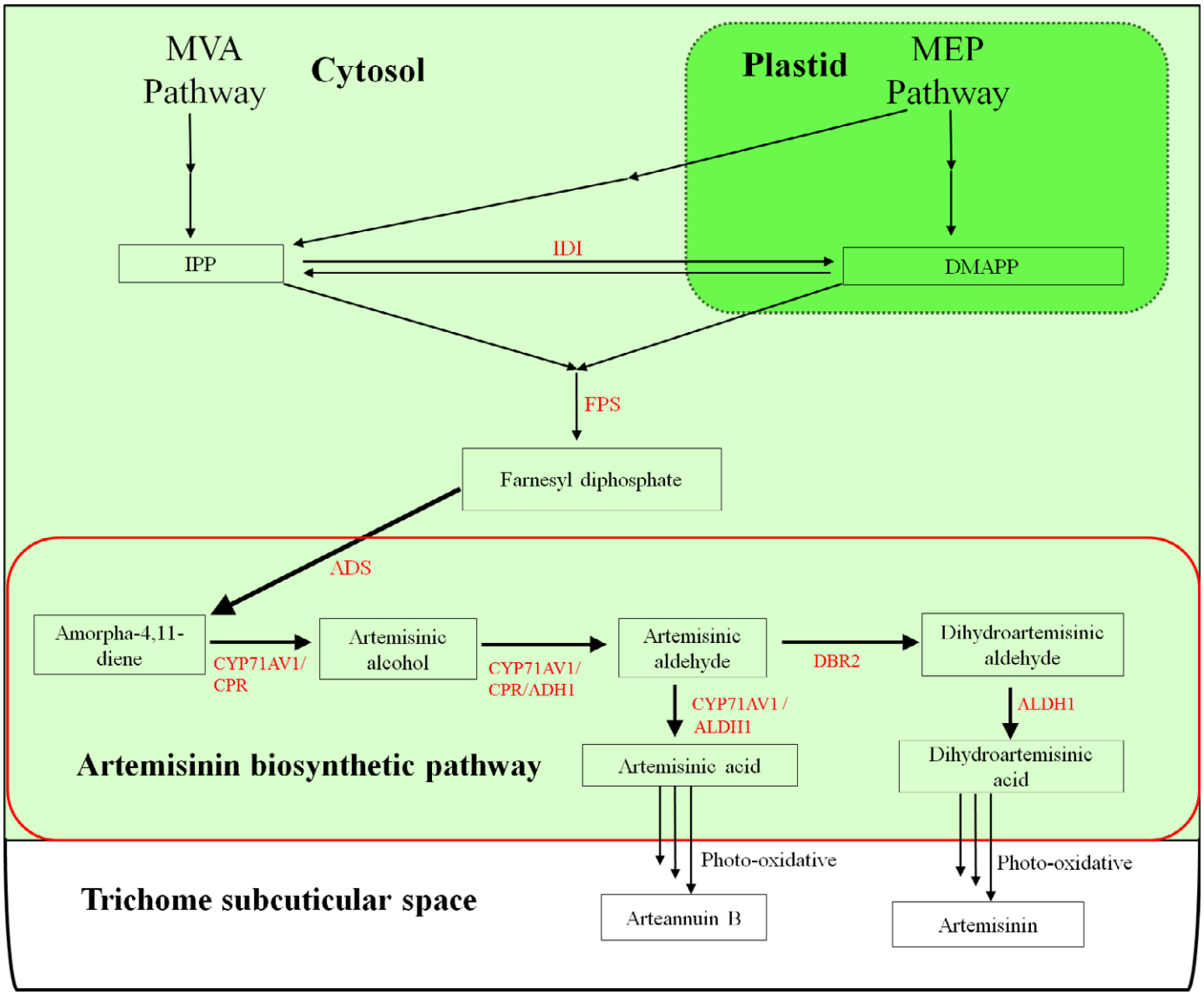

Fig. 1 Biosynthetic pathways of artemisinin in *Artemisia annua*. MVA, mevalonate; MEP, 2-C-methyl-D-erythritol 4-phosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; IDI, isopentenyl diphosphate isomerase; FPS, farnesyl diphosphatesynthase; ADS, amorpha-4,11-diene synthase; CYP71AV1, cytochrome P450 monooxygenase; CPR, cytochrome P450 reductase; ADH1, alcohol dehydrogenase 1; DBR2, artemisinic aldehyde Δ11(13) reductase; ALDH1, aldehyde dehydrogenase 1.

transcription factor family with the ability to bind G-box or G-box-like motifs in gene promoters, is considered as the core factor in JA signaling (Dombrecht *et al.*, 2007; Zhang *et al.*, 2012). In tobacco, it was reported that NtMYC2 specifically activated JA-inducible *NtPMT1a* expression by binding a G-box motif within the *NtPMT1a* promoter during *in vivo* and *in vitro* assays, and then regulated nicotine biosynthesis in tobacco (Zhang *et al.*, 2012). It was also shown that methyl jasmonate (MeJA)-responsive expression of alkaloid biosynthesis genes in *C. roseus* is controlled by CrMYC2 regulating ORCA2 and ORCA3, which in turn regulate a subset of alkaloid biosynthesis genes (Zhang *et al.*, 2011). The COI1 F-box and additional SCF modulators have long been known to play a crucial role in the JA-signaling pathway in *Arabidopsis thaliana*. It has been confirmed that jasmonate zim-domain transcription factor (JAZ) proteins were direct targets of COI1 and were degraded by the 26S proteasome in response to the hormone. Meanwhile, JAZ proteins also directly interact with MYC2, repressing its activity as repressors of the JA pathway (Thines *et al.*, 2007; Chini *et al.*, 2009). More recently, a new role for MYC2 as an integrator of signals from both the JA and GA pathways has been reported. During the regulation of sesquiterpene biosynthesis, the GA signal was transduced to the JA signal pathway through a DELLA-MYC2 interaction (Hong *et al.*, 2012).

Considering the important role of the MYC2 factor in JA signaling and plant secondary metabolism, it is interesting to clarify whether a MYC2 transcription factor is involved in the regulation of artemisinin biosynthesis in *A. annua*. In this study, we have first cloned and characterized a homolog MYC2 in *A. annua*, named AaMYC2. It shows that the AaMYC2 transcription factor

can partly rescue the MYC2 mutant in *A. thaliana*, and it has similarly functions with AtMYC2. AaMYC2 is able to bind the G-box-like *cis*-elements of *CYP71AV1* and *DBR2* promoters and activate their expression; overexpression of *AaMYC2* gene results in increased accumulation of artemisinin in transgenic *A. annua* plants, whereas RNAi downregulates *AaMYC2* expression, thus decreasing the content of artemisinin.

Materials and Methods

Plant materials

High artemisinin producing *Artemisia annua* L. cv named as 'Huhao 1', was developed in Shanghai after selection for several years, having originated in Chongqing, China. The seeds were first surface-sterilized in 70% ethanol for 1 min, followed by 10% sodium hypochlorite solution for 10 min, rinsed four times with sterile water. After that the seeds were plated on Murashige and Skoog (MS) medium (Sigma-Aldrich) with 88 mM sucrose and 0.7% agar (pH 5.8).

Seeds of *Arabidopsis thaliana* (L.) wild-type (WT) Col-0 and the mutant *jin1-9* (SALK_017005) along with transgenic lines (all in Col-0 background) were sown in soil matrix. After 2 wk, seedlings were transferred to pots and further cultivated in a growth chamber with 16 h : 8 h, light : dark cycle at 22°C with 65% relative humidity. For experiments with *in vitro* grown plants, germ-free seedlings were grown on plates containing MS_0 medium, pH 5.7, supplemented with 88 mM sucrose and 0.8% agar.

Different tissues of *A. annua* were collected for RNA extraction by using a RNAprep pure Plant kit (Tiangen Biotech, Beijing, China) following the manufacturer's instructions. Tissue samples (roots, stems, leaves) were gathered from 5-month-old plants in the glasshouse, whereas young and mature flower buds were collected 7 and 14 d after budding, respectively.

For hormone treatments, 100 μM MeJA (Sigma-Aldrich), 100 μM abscisic acid (ABA; Sigma-Aldrich) and 100 μM gibberellic acid (GA_3; Sigma-Aldrich) were used, whereas water with 1‰ concentration of DMSO was used as a mock treatment. Ten-day-old *A. annua* seedlings from MS_0 medium plates were placed on a moist filter paper and sprayed with different hormone solutions. Seedling samples were collected at 0, 1, 3, 6, 12, 24, 36 and 48 h after spraying. For each group, five individual seedlings were used as biological repeats. Pooled seedling samples were frozen in liquid nitrogen and stored at −80°C for further analysis.

Isolation and characterization of *AaMYC2*, *AaJAZs* and *AaDELLAs*

A cDNA library of *A. annua* leaves previously constructed in our laboratory was used (Lu *et al.*, 2013). Sequencing of the randomly picked clones was conducted by Beijing Genomic Institute Co. (BGI, Shenzhen, China). Computational analysis shows that there were five partial length cDNA sequences that belong to the MYC2 transcription factor family. After quantitative (q)PCR analysis, we found that one of the fragments showed the same expression profile pattern as the MYC2 transcription factors that were cloned from MeJA treated (50 μM) *A. thaliana* and *Nicotiana tabacum* plants. Two gene-specific primers MYC2-3′ RACE and MYC2-5′RACE (Supporting Information Table S1) were designed for 3′RACE and 5′RACE, respectively. Rapid amplification of cDNA ends method was used to clone the full-length *MYC2* gene from *A. annua*, by using a SMART-RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA), according to the manufacturer's instructions.

Based on cDNA library sequencing information, we found four full-length cDNAs of AaJAZs –*AaJAZ1*, *AaJAZ2*, *AaJAZ3* and *AaJAZ4* – and three full-length cDNAs of AaDELLAs – *AaDELLA1*, *AaDELLA2* and *AaDELLA3*. These four *AaJAZs* and three *AaDELLAs* were obtained from the *A. annua* leaf cDNA by using gene-specific primers as listed in Table S1.

Relative expression analysis by qPCR

All the relative expression levels of genes in *A. annua* were analyzed using qPCR. Aliquots of 0.5 μg of total RNA were used in the reverse transcriptase reaction using random hexamer primers for the synthesis of first-strand cDNA. The relative expression levels of genes were normalized to the expression of *A. annua* β-Actin. Real-time qPCR was performed in a Roche LightCycler96 (Roche) Real-Time PCR Machine. Amplification was carried out using SYBR Green qPCR MasterMix (Tiangen Biotech) according to the manufacturer's instructions. The thermal profile for SYBR Green qPCR was 95°C for 2 min, followed by 40 cycles of 95°C for 20 s, 54°C for 20 s and 72°C for 20 s. All of the primers used in qPCR are listed in Table S1.

Construction and transformation of *A. annua*

AaMYC2-F and AaMYC2-R (Table S1) were designed to amplify the ORF of AaMYC2 with *A. annua* leaf cDNA as template. The full-length ORF of AaMYC2 was cloned into the *Pst*I and *Xba*I sites of the pHB^+ vector under the control of the CaMV35S promoter to generate *pHB-CaMV35S:: sGFP-AaMYC2::NOS* with the sGFP fused to the N-terminal of AaMYC2. For the RNAi interference experiments, the primers attB1-iAaMYC2-F and attB2-iAaMYC2-R were designed to amplify a 456-bp fragment that carried part of the 5′UTR of the AaMYC2 gene. By using the gateway cloning system, the fragment was first cloned into pDONR vector via the BP reaction (Invitrogen, USA) and then cloned into the pHELLSGATE12 vector via the LR reaction (Invitrogen) to generate the final RNAi construction, *pHELLSGATE-CaMV35S:: iMYC2::NOS*.

The resulting constructs (pHB-sGFP-AaMYC2 and pHELLS GATE-iMYC2) were introduced into *Agrobacterium tumefaciens* strain EHA105 and *A. annua* transgenic plants were generated as described previously (Shen *et al.*, 2012). Generally, seeds were first placed on germination medium MS_0 and then cultured in a chamber at a temperature of 25 ± 1°C with 16 h : 8 h, light : dark photoperiod at 8000 lux. After 2 wk, the germinated seedlings were collected and the leaves were cut into 0.5-cm-diameter discs and

used as the explants that were co-cultivated with *A. tumefaciens* strain EHA105 at 25°C for 3 d. After hygromycin (pHB-GFP-AaMYC2) or kanamycin (pHellsgate-iMYC2) selection in the selective medium MS_1 (MS_0 + 2.5 mg l^{-1} N_6-benzoyladenine + 0.3 mg l^{-1} naphthalene-1-acetic acid + 50 mg l^{-1} hygromycin or 50 mg l^{-1} kanamycin + 250 mg l^{-1} carbenicillin), the antibiotic-resistant plantlets were regenerated, subcultured twice and then transferred into rooting medium MS_2 (½ MS_0 + 250 mg l^{-1} carbenicillin). After 1 month, the rooted plantlets were transferred to soil pots in the growth chamber.

Yeast two-hybrid assays

Interaction assays in yeast were performed following the Invitrogen ProQuest Two-Hybrid System instructions with modifications (Invitrogen). All of the *AaJAZ* and *AaDELLA* cDNAs were cloned into gateway pDEST22 vector by LR recombination reaction to form the prey plasmids, whereas *AaMYC2* cDNA was cloned into gateway pDEST32 vector by LR recombination reaction to form the bait plasmid. All of the constructs were verified by sequencing. The indicated construct pairs were transformed into yeast stain AH109 using the LiAc method following the Invitrogen Yeast Transformation instructions (Invitrogen). After transformation, the transformed yeasts were selected on synthetic defined SD/-Leu/-Trp agar medium plates and cultured at 30°C. Six individual transformants were randomly picked, dropped in 10× dilutions on SD/-Leu/-Trp/-His with 10 mM 3-AT and SD/-Leu/-Trp/-His/-Ade and allowed to grow for 2–3 d at 30°C. The experiments were repeated twice.

Bimolecular fluorescence complementation analysis for interaction between proteins

For bimolecular fluorescence complementation (BiFC) analysis experiments, the Gateway-compatible BiFC vectors pEarleyagte201-YN and pEarleygate202-YC, kindly supplied by Dr. Yuhai Cui, were used (Lu *et al.*, 2010). The full-length coding regions of *AaJAZs* and *AaDELLAs* were fused in-frame with the nYFP-tag of the pEarleygate 201 vector whereas *AaMYC2* was fused in-frame with the cYFP-tag of pEarleygate 202 vector. The fusion constructs were transformed into *A. tumefaciens* strain EHA 105. Then, the positive-transformed cells were cultured at 28°C in LB medium with kanamycin (50 μM), rifampicin (50 μM) and streptomycin (50 μM) for 24 h. The cell cultures were then infiltrated into tobacco leaves using a syringe according to the agroinfiltration procedure (Voinnet *et al.*, 2003). After 3 d, a piece of infiltrated leaf was cut and the images were obtained under LAS AF Lite laser scanning confocal microscopy (Leica, Wetzlar, Germany), with argon laser excitation at 488 nm and a 505–550 nm emission filter set.

Yeast one-hybrid assays

A protein–DNA interaction assay in yeast was concluded by the MATCHMAKER Gold Yeast One-Hybrid system according to the manual (Clontech). The yeast reporter plasmid was constructed by inserting the triple tandem copies of the G-box-like element (CACGTT or CACGTA) into the pAbAi vector. The G-box fragment and the G-box mutant fragment were used as positive and negative controls, respectively (Table S2). We also amplified several 150–200-bp fragments from the promoters of the *CYP71AV1* and *DBR2* genes, which contain the G-box-like elements. The fragments obtained were inserted into pAbAi vector. The primers used for amplification of the G-box-like fragments are listed in Table S1. All of the bait constructs were linearized and integrated into the genome of the Y1H Gold yeast stain, selected on synthetic defined SD/-Ura agar medium plate and cultured at 30°C for 2 d. The prey construct was the ORF of AaMYC2 fused in-frame with the yeast GAL4 transcription activation domain (GAL4 AD) in pGADT7 between *Eco*RI and *Bam*HI restriction sites. The prey construct was then introduced into yeast cells previously transformed with the bait constructs, with blank pGADT7 plasmid as control. The positively transformed clones were cultured at 30°C on SD/-Leu medium with different concentrations of aureobasidin A (AbA).

HPLC analysis of artemisinin and dihydroartemisinic acid

Samples for HPLC analysis were prepared as described previously (Lu *et al.*, 2013). *Aretemis annua* leaves were dried in a drying oven at 45°C and then ground to powder. Samples of dried leaf powder (0.1 g) were extracted twice with 2 ml methanol in an ultrasonic processor (JYD-650; Shanghai Zhisun Instrument Co. Ltd, Shanghai, China) under the conditions of 25°C and 50 W for 30 min. The samples were centrifuged for 10 min at 10 000 ***g*** and then the supernatants were passed through a 0.25-μm membrane.

The filtrates were analyzed by the Waters Alliance 2695 HPLC system coupled with a Waters 2420 ELSD detector (Milford, MA, USA). The HPLC conditions were set as described previously (Lu *et al.*, 2013). Standard of artemisinin was purchased from Sigma and standards of dihydroartemisinic acid were from Guangzhou Honsea Sunshine Bio Science and Technology Co. Ltd (Guangzhou, Guangdong, China).

Root growth response to methyl jasmonate

Different genotypes of *A. thaliana* seeds (Col-0, *jin1-9* and *jin1-9*-AaMYC2) were first sowed on 0.5× MS_0 plates for germination, then after 7 d the seedlings were plated on 0.5× MS_0 with 50 μM methyl jasmonate (Sigma) on square plates and were finally transferred to a growth room in a vertical position. Root growth was monitored regularly, plates were photographed and root length was measured after 7 d vertically cultivation.

Anthocyanin measurements

Anthocyanin quantification was performed as described before (Shan *et al.*, 2009). Weighed 0.1 g FW samples of *A. annua* stems or leaves were placed into 1 ml extraction buffer (18% propanol, 1% HCl and 81% water), boiled for 3 min and then

incubated in darkness overnight at room temperature. The quantity of anthocyanin was determined by spectrophotometric measurements at two wavelengths (A535 and A650). The amount of anthocyanins was reported as (A535–A650) g^{-1} FW.

Accession numbers

Sequence data from this article can be found in the *A. thaliana* Genome Initiative or GenBank databases under the following accession numbers: *AaMYC2* (KP119607), *jin1-9* (SALK_017005), *AaDELLA1* (KJ651996), *AaDELLA2* (KJ651997), *AaDELLA3* (KJ651998), *AaJAZ1* (KJ651999), *AaJAZ2* (KJ652000), *AaJAZ3* (KJ652001) and *AaJAZ4* (KJ652002).

Results

Isolation and characterization of *AaMYC2*

In order to isolate the putative MYC family transcription factors, we searched a cDNA library of *A. annua* available in our lab using conserved MYC domain sequences, and we retrieved five cDNA fragments. The expression of the selected candidate was induced by MeJA in a similar manner to that of *AtMYC2* (Boter *et al.*, 2004), *NtMYC2a* and *NtMYC2b* (Zhang *et al.*, 2012) (Fig. 2), which increased quickly during the 3–6 h period after MeJA treatment and then decreased.

The full-length cDNA, named *AaMYC2*, was isolated By 5′-rapid amplification of cDNA ends (5′-RACE) and 3′-rapid amplification of cDNA ends (3′-RACE). The open reading frame of the *AaMYC2* gene contained 1878 bp and encoded a protein of 625 amino acids (GenBank accession no. KP119607). To test whether genomic *AaMYC2* contained intron(s), the primers used for amplifying full-length cDNA were used to amplify the gene from genomic DNA. A specific DNA fragment was obtained, whose sequence was identical to that of the full-length cDNA, suggesting that there was no intron in *AaMYC2*, which was consistent with the findings in *A. thaliana* or rubber tree (Dombrecht *et al.*, 2007; Zhao *et al.*, 2011).

The result of the BLAST-Protein (BLASTP) online (http://www.ncbi.nlm.gov/blast) showed that AaMYC2 shared a highly

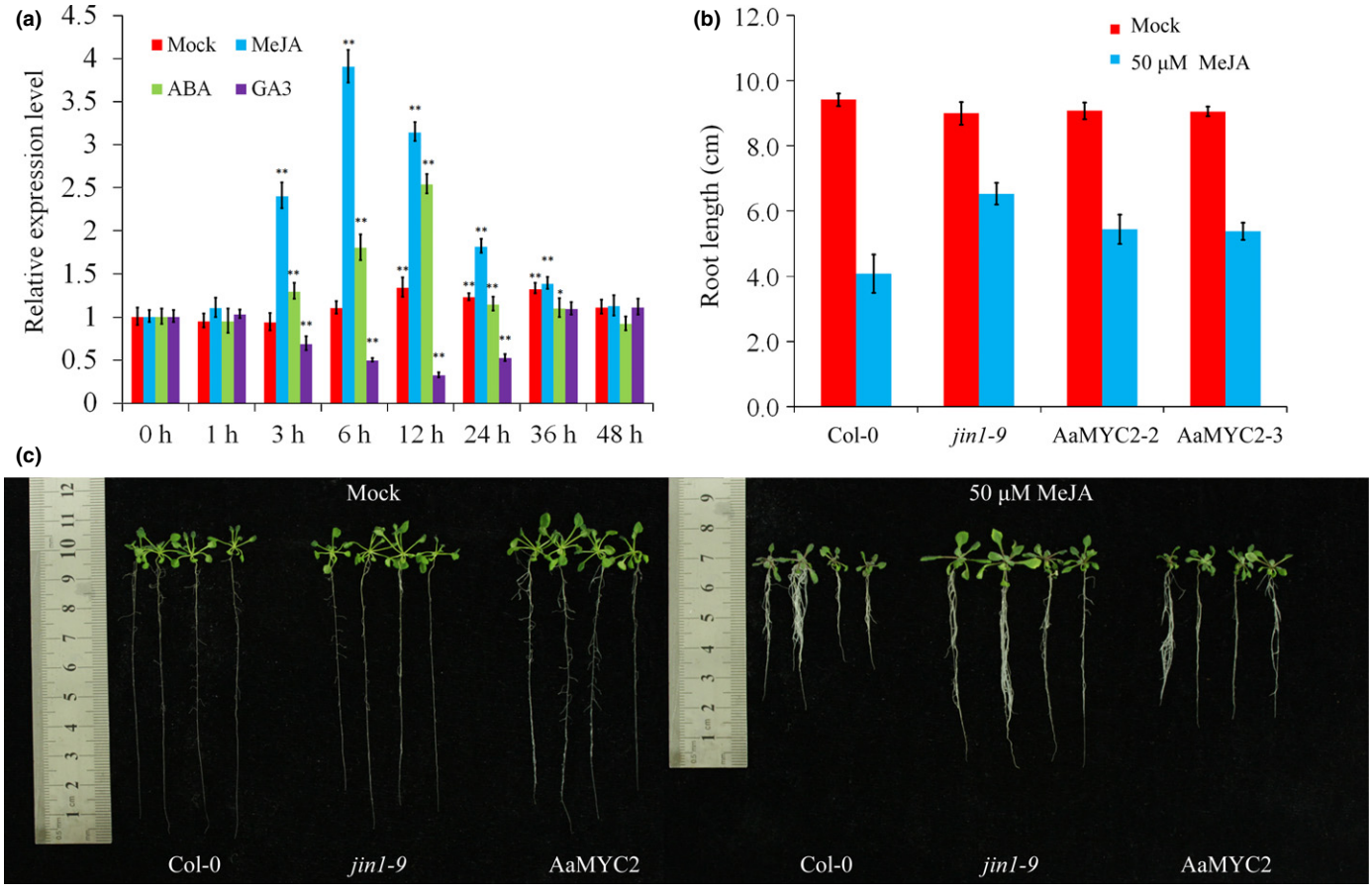

Fig. 2 AaMYC2 is an homologous transcription factor of AtMYC2. (a) Relative expressions of *AaMYC2* in response to methyl jasmonate (MeJA, 100 μM), abscisic acid (ABA, 100 μM) and gibberellic acid (GA_3, 100 μM) treatments after 48 h. Water was used as mock. Statistical significance was determined by Student's *t*-test (**, $P < 0.01$; *, $P < 0.05$). Asterisks indicate the difference between treated and nontreated plants. (b) AaMYC2 partly rescues the *Arabidopsis thaliana* MYC2 mutant *jin1-9* phenotype. Root lengths of different genotypes of *A. thaliana* seedlings between MeJA-treated (50 μM) and Mock-treated. Col-0, wild-type; *jin1-9*, MYC2 mutant type; AaMYC2, overexpressing *AaMYC2* in *jin1-9* type. Data represent means ± SE from 10 independent plants. Statistical significance was determined by Student's *t*-test (**, $P < 0.01$; *, $P < 0.05$). Asterisks indicate the difference between *jin1-9* and AaMYC2 plants. (c) Photograph of root lengths of different genotypes of *A. thaliana* seedlings after 7 d vertically cultivated on 1/2 Murashige and Skoog plates with or without MeJA.

conserved bHLH-MYC superfamily domain at the N-terminal region and a helix-loop-helix DNA binding domain at the C-terminal region with other MYC2 proteins (Fig. S1). It shared *c.* 55% identity of amino acid sequence with the MYC2 proteins from other plant species including *A. thaliana*, *N. tabacum*, *C. roseus* and *Solanum lycopersicum*. The bHLH family is a big transcription factor family, which has > 200 members in plants and is classified in four groups (A–D) based on Atchley's classification (Atchley & Fitch, 1997). AaMYC2 belongs to Group B, which has the ability to bind the CACGTG box. As shown from the phylogenetic tree, constructed by MEGA 5 using the neighbor-joining method, AaMYC2 has the closest evolutionary relationship to *Chrysanthemum boreale* MYC2 (Genbank accession: AIT39751) (Fig. S2).

Expression and induction patterns of *AaMYC2*

Studies on the expression pattern of *AaMYC2* in different tissues showed that it is widely expressed in plants, with the highest level of transcripts in seeds and the lowest level in the roots (Fig. S3). Phytohormones such as MeJA, ABA and GA_3 are essential signaling molecules that, in addition to plant secondary metabolic regulation processes, coordinate plant response to biotic and abiotic challenges (Zhu, 2002; Chini *et al.*, 2009; Hong *et al.*, 2012). Previous investigations have shown that exogenous MeJA, ABA or GA_3 treatments on *A. annua* could increase the artemisinin content (Jing *et al.*, 2009; Wang *et al.*, 2010; Banyai *et al.*, 2011; Caretto *et al.*, 2011). To dissect whether *AaMYC2* responded to phytohormones, the expression of *AaMYC2* after exogenous hormone treatment was analyzed by qPCR at different time points. It was found that MeJA and ABA were effective in inducing *AaMYC2* expression whereas GA_3 downregulated *AaMYC2* expression (Fig. 2a). The *AaMYC2* was more sensitive to MeJA than ABA. The transcript level of *AaMYC2* increased rapidly after 3 h MeJA treatment, and peaked at 6 h after MeJA treatment, and then slowly declined until 48 h (Fig. 2a). The *AaMYC2* response to ABA treatment was relatively slow; it peaked at 12 h after the treatment and then quickly declined (Fig. 2a). These results were consistent with previously reported results for other plant species (Lorenzo *et al.*, 2004; Dombrecht *et al.*, 2007; Chini *et al.*, 2009; Woldemariam *et al.*, 2013). GA_3 treatment downregulated the *AaMYC2* transcript level, with the lowest point occurring 6 h after the treatment. A low transcription level was maintained until 12 h after treatment and slowly recovered after 36 h (Fig. 2a). Sequence alignment information and hormone treatment expression patterns suggest that AaMYC2 may act as a transcription factor that has similar functions to other MYC2 transcription factors. This means that AaMYC2 may also act as a core regulator in the JA signal pathway and in artemisinin biosynthesis.

AaMYC2 partly rescues the *A. thaliana MYC2* mutant phenotype

In order to further confirm that the gene cloned from *A. annua* is a MYC2 homologous gene and that it exhibits similar functions, transgenic plants constitutively expressing the full-ORF-length *AaMYC2* (35S:AaMYC2) were obtained in *A. thaliana* mutant background *jin1-9* (homologous gene of *AaMYC2*) (Lorenzo *et al.*, 2004). These transgenic plants can be used to elucidate whether *AaMYC2* is sufficient to activate the JA pathway or at least partly rescue the mutant phenotype. *jin1-9* has shown a jasmonate-insensitive phenotype. Its root growth was not inhibited in media supplemented with MeJA. The root growth test has shown that overexpression of *AaMYC2* in the *jin1-9* background partially rescued the jasmonate-insensitive phenotypes, as shown in Fig. 2(b,c). Hence, it exhibited that *AaMYC2* was an *AtMYC2* homologous gene, and it had similar functions to MYC2 transcription factors of other plant species.

AaMYC2 protein interacts with AaJAZs and AaDELLAs in *A. annua*

Because both MeJA and GA_3 treatment could increase the contents of artemisinin (Wang *et al.*, 2010; Banyai *et al.*, 2011), which also belongs to the sesquiterpenes, it is speculated that these hormone signals might also be transduced via the AaMYC2 protein. To determine whether AaMYC2 interacts with JAZ proteins and DELLA proteins, we cloned four *AaJAZ* genes and three *AaDELLA* genes from the *A. annua* cDNA library. AaJAZs and AaDELLAs have shown highly conserved domains compared with the counterparts from other plant species. Subsequently, yeast two-hybrid assays were performed. From the yeast two-hybrid assays we found that when co-transformed with the AaMYC2 and AaJAZs / AaDELLAs vectors, the yeast could grow on the selective synthetic medium, which means that AaMYC2 could interact with all the AaJAZ proteins and all the AaDELLA proteins in yeast cells (Fig. 3a). Furthermore, we performed a BiFC assay to examine the AaMYC2-AaJAZ and AaMYC2-AaDELLA interactions in tobacco plants. The N-terminus (amino acids 1–174) and C-terminus (amino acids 175–239) of yellow fluorescent protein (YFP) were subcloned into pEarleyhgate201 and pEarleygate202, respectively. As shown in Fig. 3(b), a robust fluorescence was observed in the nuclei and some of the membranes of the tobacco cells expressing AaMYC2-cYFP and AaJAZs-nYFP or AaMYC2-cYFP and AaDELLAs-nYFP, whereas no fluorescent signal was observed in the cells expressing AaMYC2-cYFP and the empty vector control with only nYFP fragment. The current consensus is that JAZ is core regulator in JA signal whereas DELLA plays a core role in the GA signal. Together, these data demonstrate that the JA and GA hormone signal transduction pathways in *A. annua* are conserved with *A. thaliana* and *N. tabacum* plants. These two signal pathways are linked via MYC2 transcription factors in plants.

AaMYC2 binds the G-box-like motifs in *CYP71AV1* and *DBR2* promoters

In order to test whether AaMYC2 can interact with the G-box-like sequence that corresponds to the qualitative sequence within the promoter of the *CYP71AV1* and *DBR2* genes, yeast one-hybrid (Y1H) assays were performed. First, we designed triple

repeated G-box, G-box-like and G-box mutant motifs, and integrated them into the genome of yeast cells to test the AaMYC2 protein's binding ability to the G-box. After introducing AaMYC2 into the yeast strains, it was found that AaMYC2 was indeed able to bind the artificial triple G-box and G-box-like motifs. Certainly, AaMYC2 appears to have a higher affinity binding to the G-box than to the G-box-like motifs (Fig. 4a,c). There are two G-box-like motifs in both *CYP71AV1* and *DBR2* promoters (Fig. 4b). In the *CYP71AV1* promoter (FJ870128; Wang *et al.*, 2012), the two boxes are a long way apart, whereas in the *DBR2* promoter (KC347592 and KC347593, Jiang *et al.*, 2014), they are close to each other (Fig. 4b). Then, PCR was used for amplification, and *c.* 250–350-bp fragments were amplified from the promoters that contained the G-box-like motifs, and the fragments were subsequently integrated into the yeast genome for yeast one-hybrid assays. Yeast one-hybrid assays showed that AaMYC2 exhibited binding activities to the G-box-like motifs of the *CYP71AV1* and *DBR2* promoter fragments. From the selection media SD/-L added with 300 nmol l^{-1} Aureobasidin A (AbA), the *DBR2* promoter fragments have shown higher binding affinity than the *CYP71AV1* promoter fragments (Fig. 4d). The flanking nucleotides of the G-boxes have an effect on the binding activity of MYC2 (Dombrecht *et al.*, 2007), and thus it is likely that the flanking nucleotides of the G-box 1 are not

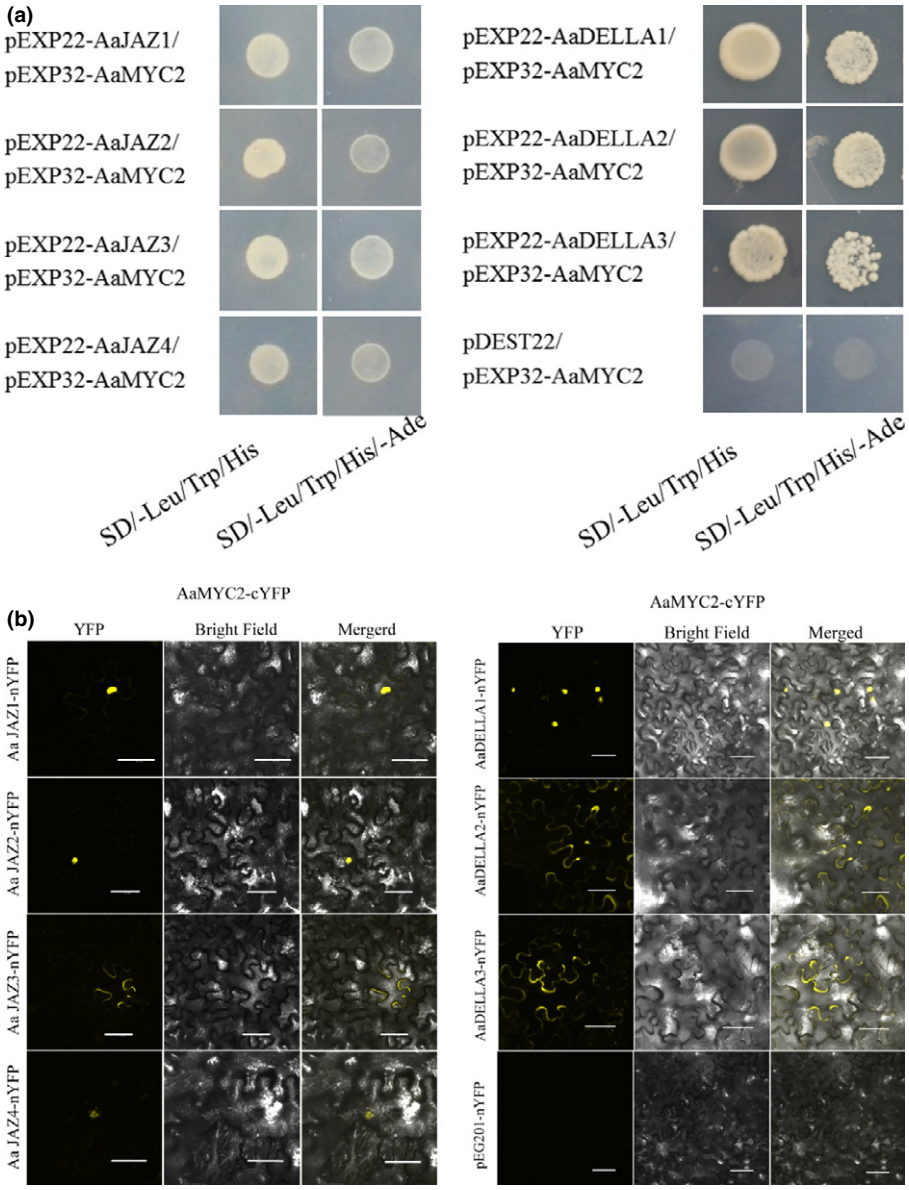

Fig. 3 AaMYC2 transcription factor interacts with AaJAZs and AaDELLAs transcription factors in yeast and tobacco. (a) AaMYC2 proteins interact with AaJAZs and AaDELLAs. Yeast cells were co-transformed with the prey and bait vectors, and were grown on agar plates containing the synthetic medium lacking trytophan, leucine, histidine or lacking trytophan, leucine, histidine and adenine to test interaction. pDEST 22 was used as a negative control for prey plasmid. (b) Bimolecular fluorescence complementation assay to detect the interactions of AaMYC2 (fused with C-terminal fragment of yellow fluorescent protein (YFP) in pEarleyagte202-YC) with AaJAZs and AaDELLAs (fused with N-terminal fragment of YFP in vector pEarleyagte201-YN). Construct pairs were coinfiltrated into leaves of *Nicotiana benthamiana*. The pEarleyagte202-YC-AaMYC2 and empty pEarleyagte201-YN construct pairs were used as negative control. YFP fluorescence was detected 3 d after infiltration. Bars, 40 μm.

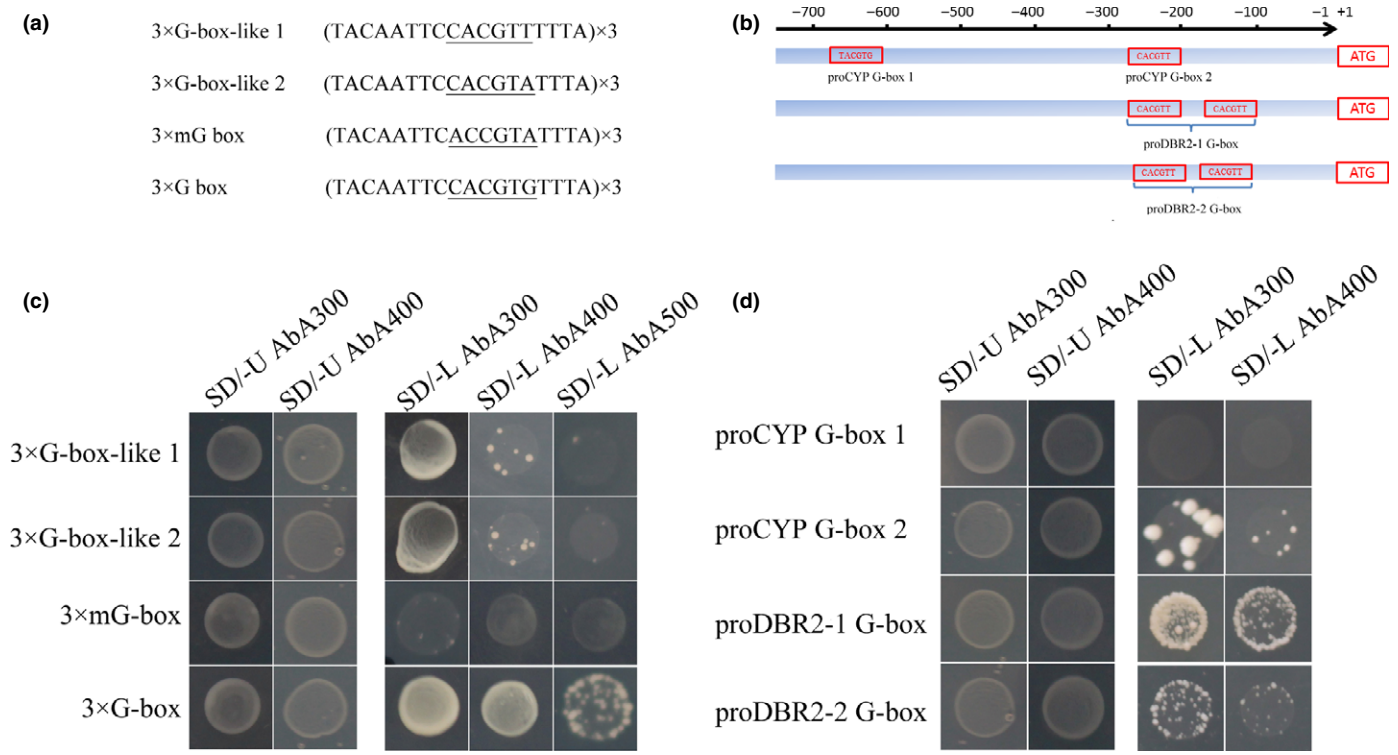

Fig. 4 AaMYC2 binding with the G-box motifs of the *CYP71AV1* and *DBR2* promoters in yeast cells. (a) Artificial triple repeated G-box or G-box-like motif sequences, the G-box or G-box-like elements are underlined. (b) Schematic structure of G-box-like motifs in the *CYP71AV1* and *DBR2* promoters. G-box-like motifs are marked in red frames, and the numbers of scale plates indicate the location of these G-box-like motifs upstream from the ATG start codon. (c) AaMYC2 can successfully bind the artificial triple repeated G-box and G-box-like motifs in yeast cells whereas it fails to bind the mutant mG-box under 300 ng l^{-1} concentration of aureobasidin A (AbA). Left columns, the Y1HGold yeast reporter strains containing the artificial G-box or G-box-like motifs in the AbA bait reporter are unable to grow on synthetic medium lacking uracil under 300 or 400 ng l^{-1} concentration of AbA. Right columns, above Y1HGold reporter strains transformed with AaMYC2 prey plasmid can grow on synthetic medium lacking leucine under 300 or 400 ng l^{-1} concentration of AbA. (d) AaMYC2 can successfully bind the G-box-like motifs in ProCYP G-box2, ProDBR2-1 G-box and ProDBR2-2 G-box in yeast cells under 300 ng l^{-1} concentration of AbA. Left columns, the Y1HGold yeast reporter strains containing the G-box-like motifs in the AbA bait reporter are unable to grow on synthetic medium lacking uracil under 300 or 400 ng l^{-1} concentration of AbA. Right columns, above Y1HGold reporter strains transformed with AaMYC2 prey plasmid can grow on synthetic medium lacking leucine under 300 or 400 ng l^{-1} concentration of AbA. ProCYP G-box1 and ProCYP G-box2, two G-box-like motifs in *CYP7AV1* promoter, ProDBR2-1 G-box (KC347592) and ProDBR2-2 G-box (KC347593), G-box-like motifs in two *DBR2* promoters, respectively.

optimal in the promoter of *CYP71AV1*, and therefore the AaMYC2 could not bind it. There are two *DBR2* promoters reported before, and we found that AaMYC2 could bind both of them. The *DBR2* promoter 1 (KC347592) showed a slightly higher affinity than the *DBR2* promoter 2 (KC347593) (Fig. 4d).

Thus, the yeast one-hybrid assay showed that AaMYC2 recognizes and interacts with the G-box-like motifs present in the *CYP71AV1* and *DBR2* promoters, and that there is an extremely high probability that AaMYC2 regulates artemisinin biosynthesis via regulating the enzymes involved in the artemisinin biosynthetic pathway.

AaMYC2 promotes artemisinin biosynthesis

Because AaMYC2 could bind the *CYP71AV1* and *DBR2* promoters, it was interesting to further analyze the role of *AaMYC2* in regulating artemisinin biosynthesis in *A. annua*. We overexpressed *AaMYC2* in *A. annua* which was under the control of the CaMV35S promoter. The transgenic plants were first confirmed by genomic DNA-based PCR using the GFP forward and reverse primers (Fig. S4a), and then qPCR was used to analyze the transcript levels in the transgenic lines. In the overexpression plants examined, the transcripts of *AaMYC2* were increased by two- to five-fold compared with the WT plants (Fig. 5a).

Consequently, we further analyzed the genes encoding enzymes of the artemisinin biosynthetic pathway including *ADS*, *CYP71AV1*, *DBR2* and *ALDH1* in *A. annua* overexpressing *AaMYC2*. As predicted, the transcript levels of all the enzyme genes were increased significantly. The expression levels of *ADS*, *CYP71AV1*, *DBR2* and *ALDH1* were increased by 1.7- to 8.4-fold, 2.0- to 5.9-fold, 1.2- to 2.9-fold and 1.2- to 2.5-fold, respectively, in *AaMYC2* overexpressing lines (Fig. 5b). The artemisinin, dihydroartemisinic acid and artemisinic acid contents in 5-month-old *A. annua* plants were analyzed by HPLC. Compared with the WT, the artemisinin and dihydroartemisinic acid contents were increased by 23–55% and 17–217%, respectively, in *AaMYC2* overexpressing lines (Fig. 5c). Meanwhile, there was a significant reduction of artemisinic acid levels in *AaMYC2* overexpressing lines, which nicely lined up with the

increase in dihydroartemisinic acid and artemisinin contents. It suggested that the increase in the expression of *DBR2* in *AaMYC2* overexpressing lines resulted in an increase in the flux from amorpha-4,11-diene towards dihydroartemisinic acid and artemisinin and a reduction in flux towards artemisinic acid. Because MYC2 transcription factor is JA-inducible, it is speculated that exogenous MeJA treatment with *AaMYC2* overexpressing transgenic plants may further increase artemisinin content. However, after treatment, we did not see significant difference of artemisinin increments between WT and transgenic *A. annua* plants (Fig. S5).

In order to further prove the function of AaMYC2 in regulation of artemisinin biosynthesis in *A. annua*, we downregulated the *AaMYC2* expression by the RNA interference (RNAi) approach. The transgenic plants were first confirmed by PCR using the 35S forward primer and iAaMYC2 reverse primer (Fig. S4b). In the RNAi transgenic lines, the transcript levels of *AaMYC2* were suppressed to 27–46% of the control level (Fig. 5d), whereas the expressions of *ADS*, *CYP71AV1*, *DBR2* and *ALDH1* were reduced to 10–30%, 10–15%, 10–32% and 19–24%, respectively, of the control levels (Fig. 5e). As a result of reduced *ADS*, *CYP71AV1*, *DBR2* and *ALDH1* expression, the contents of artemisinin and dihydroartemisinic acid in iAaMYC2 transgenic plants were decreased to 54–81% and 17–95% of the control level (Fig. 5f). Meanwhile, we analyzed the expressions of *AaMYC2*, *ADS*, *CYP71AV1* and *DBR2* in the *AaMYC2* RNAi transgenic plants after 3 h MeJA (100 μM) treatment. It was found that the *AaMYC2* RNAi transgenic plants showed less sensitivity to methyl jasmonate than the control plants (Fig. S6). We did not observe any morphological difference between *AaMYC2*-overexpressing or RNAi transgenic lines and the WT plants. These data demonstrated that AaMYC2 was a positive transcription factor regulator in artemisinin biosynthesis, and it upregulated the transcript levels of *CYP71AV1* and *DBR2* genes, resulting in an increased production of artemisinin in *A. annua*.

AaMYC2 promotes anthocyanin biosynthesis in *A. annua* stem

Anthocyanins are secondary metabolites found widely in plant species and responsible for the purple, blue and pink coloration of plant parts. Previous investigations have demonstrated that MYC2 is a positive regulator of the JA-mediated anthocyanin biosynthesis in *A. thaliana*, although the molecular mechanism behind this phenomenon is still not completely clear (Lorenzo *et al.*, 2004; Dombrecht *et al.*, 2007). In our studies, we found that all of the plants overexpressing *AaMYC2* showed deeper purple stems than the nontransgenic plants and the *AaMYC2* RNAi transgenic plants (Fig. S7). To test whether *AaMYC2* is involved in the regulation of anthocyanin biosynthesis in *A. annua*, we analyzed the anthocyanin content of extracts of stems and leaves of WT, *AaMYC2* overexpressing and *AaMYC2* RNAi plants, respectively, by measuring the absorbance of the extracts at 535 and 650 nm. It was demonstrated that there was a significant increase of anthocyanins in the stems of plants overexpressing *AaMYC2* compared with the WT. In the same way, a significant decrease of anthocyanins was shown in the *AaMYC2* RNAi plants. Meanwhile, we found that there were no significant changes of the anthocyanin content in the leaf tissues of the three plant types (Fig. 6). From these results – although the molecular mechanisms behind them are still unknown – we suggest that *AaMYC2* may regulate genes of the anthocyanin biosynthetic pathway or, especially, regulate genes in the *A. annua* stem.

Discussion

The phytohormone jasmonic acid (JA) plays an important role in many aspects of plant development and particularly in plant responses to biotic and abiotic stresses that are partly achieved by enhancing biosynthesis of secondary metabolites, including terpenoids (Dombrecht *et al.*, 2007; Hong *et al.*, 2012; Yu *et al.*, 2012). The MYC2 protein is considered to be a core regulator of JA signaling (De Geyter *et al.*, 2012; Kazan & Manners, 2013), regulating diverse jasmonate-dependent functions in *Arabidopsis thaliana*, *Nicotiana tabacum* and *Catharanthus roseus* (Dombrecht *et al.*, 2007; Zhang *et al.*, 2011, 2012; Woldemariam *et al.*, 2013). Here we identified a bHLH transcription factor AaMYC2 from *A. annua*, which is a close homolog of AtMYC2, a well-known transcription factor mediating plant responsiveness to JAZs. Previous reports indicate that MYC2 can bind the G-box-related hexamers 5′-CACNTG-3′, 5′-CACATG-3′, and 5′-(T/C)ACGTG-3′(Yadav *et al.*, 2005; Dombrecht *et al.*, 2007). In our investigation, we found that AaMYC2 showed higher affinity to the G-box motif (5′-CACGTG-3′) than to the G-box-like motifs (5′-CACGTT-3′ and 5′-CACGTA-3′). When the G-box was mutated the activation of AaMYC2 was abolished (Fig. 4c). These results are consistent with previous studies on *A. thaliana* (Dombrecht *et al.*, 2007).

Another notable function of MYC2 is the regulation of crosstalk between the signaling pathways of JA and those of other phytohormones such as abscisic acid (ABA) (Abe *et al.*, 2003), salicylic acid (SA) (Laurie-Berry *et al.*, 2006), gibberellins (GAs) (Wild *et al.*, 2012) and ethylene (ET) (Song *et al.*, 2014). Furthermore, MYC2 also regulates interactions between JA signaling and light (Yadav *et al.*, 2005). In our studies, we found that despite the difference among plant species, their responses to phytohormones were conservative. The AaMYC2 protein could interact with JAZs to transduce the JA signaling response, and the interaction between AaMYC2 and DELLAs modulates antagonism between jasmonate and gibberellins (Fig. 3). Together, our data demonstrate that the transcription factor MYC2 regulates the expression of artemisinin biosynthetic genes at the transcriptional level. MeJA and GA jointly regulate the biosynthesis of artemisinin through AaMYC2 (Fig. 7).

Because of the tremendous medicinal value of artemisinin, the biosynthetic pathway and its regulation are very attractive research topics worldwide. To date, all genes encoding the enzymes involved in the artemisinin biosynthesis have been cloned and characterized, including ADS (Mercke *et al.*, 2000; Kim *et al.*, 2008), CYP71AV1 (Teoh *et al.*, 2006; Wang *et al.*, 2012), DBR2 (Zhang *et al.*, 2008; Jiang *et al.*, 2014) and ALDH1 (Teoh *et al.*, 2009). Moreover, most of them have been

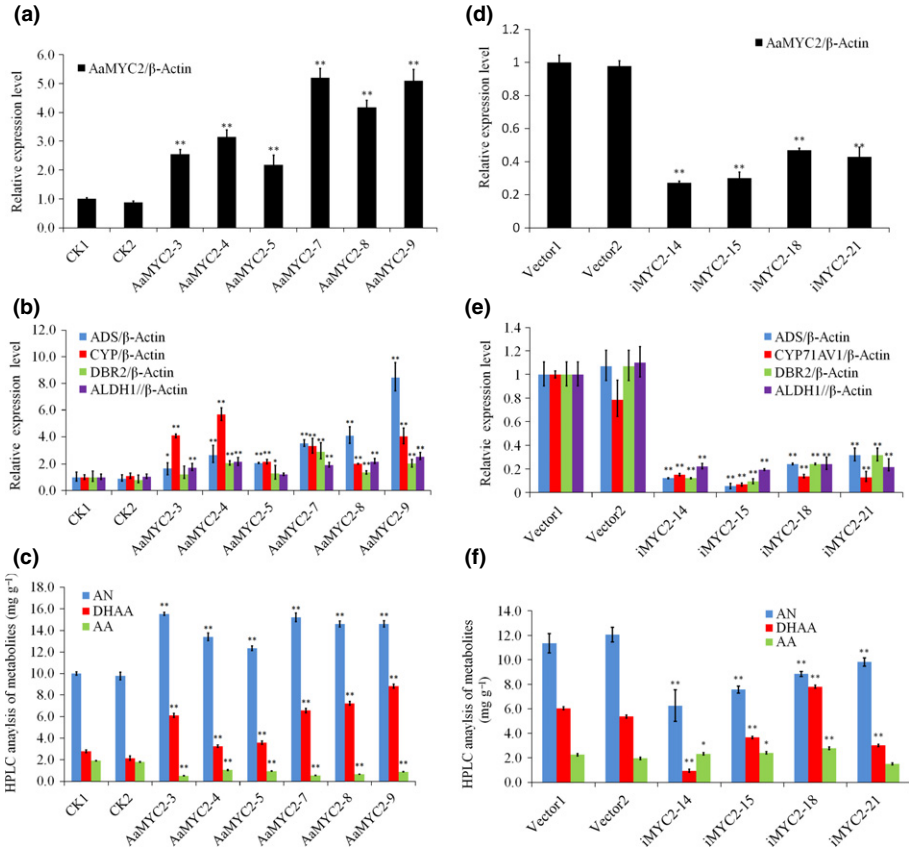

Fig. 5 Relative expression of genes and HPLC analysis of metabolites in transgenic plants overexpressing or silencing *AaMYC2*. (a) Expression of *AaMYC2* in leaves of overexpressed plants. β-actin was used as the internal standard. Error bars indicate ± SD of three technical replicates. (b) Expression of *ADS*, *CYP71AV1*, *DBR2* and *ALDH1* in leaves of overexpressed plants. β-actin was used as the internal standard for each gene. (c) Contents of artemisinin (AN), dihyroartemisinic acid (DHAA) and artemisinic acid (AA) in the leaves of overexpressed plants, determined by HPLC. The contents in transgenic plants leaves were compared to those in the control plants. Statistical significance was determined by Student's *t*-test (**, $P < 0.01$; *, $P < 0.05$). Asterisks indicate the difference between overexpressing transgenic plants and control plants. (d) Expression of *AaMYC2* in the leaves of RNAi plants. β-actin was used as the internal standard. (e) Expression of *ADS*, *CYP71AV1*, *DBR2* and *ALDH1* in the leaves of RNAi plants. β-actin was used as the internal standard for each gene. (f) Contents of AN, DHAA and AA in the leaves of RNAi plants, determined by HPLC. The contents in transgenic plants leaves were compared to those in the control plants. Statistical significance was determined by Student's *t*-test (**, $P < 0.01$; *, $P < 0.05$). Asterisks indicate the difference between RNAi transgenic plants and control plants. CK, wild-type control plants; AaMYC2, plants overexpressing the AaMYC2 gene. Vector, control plants transformed with pHellsgate empty vector; iMYC2, RNAi transgenic plants.

shown to be specifically expressed in glandular trichomes where artemisinin is produced. In this study, we found that AaMYC2 can bind to both of the G-box-like motifs present in the *CYP71AV1* and *DBR2* promoters (Fig. 4d), and as a result, when *AaMYC2* is overexpressed in *A. annua*, the artemisinin content is increased (Fig. 5c). An investigation showed that the relative expression of DBR2 was significantly higher in the high artemisinin producing *A. annua* varieties compared with the low artemisinin producing *A. annua* varieties (Yang *et al.*, 2015). This supports our result that enhancing the expression of *DBR2* will increase artemisinin contents in transgenic plants. Because artemisinin was synthesized and stored in the trichome of *A. annua*, whereas JA was known to control trichome initiation, we compared the trichome density between *AaMYC2* overexpressed, silenced transgenic and wild-type (WT) plants. Fluorescent microscope photographs of leaves showed that there was no significant difference of trichome density between the transgenic and WT plants (Fig. S8).

Recently, several transcription factors have been reported to regulate artemisinin biosynthesis in *A. annua*, including AaERF1 and AaERF2 (Yu *et al.*, 2012), AaORA (Lu *et al.*, 2013), AaWRKY1 (Ma *et al.*, 2009; Han *et al.*, 2014), and AabHLH1 (Ji *et al.*, 2014), all of them showed binding affinity to the *ADS* promoter or to the *CYP71AV1* promoter or to both. In our investigation, we found that artemisinin biosynthesis in *A. annua* is also promoted by a bHLH family transcription factor AaMYC2, which directly binds to the G-box-like regions of the *CYP71AV1* and *DBR2* promoters (Fig. 4d). Considering that JA exhibits various functions of plant secondary metabolism and induces the artemisinin production in *A. annua*, studies on how such a regulatory mechanism is established and maintained during evolution are an interesting subject for further investigation. Previous studies have reported that the homologous CrMYC2 protein in *C. roseus* acts as the major activator of MeJA-responsive ORCA3 gene (AP2/ERF transcription factor) expression by binding to the promoter region (Zhang *et al.*, 2011). Similarly,

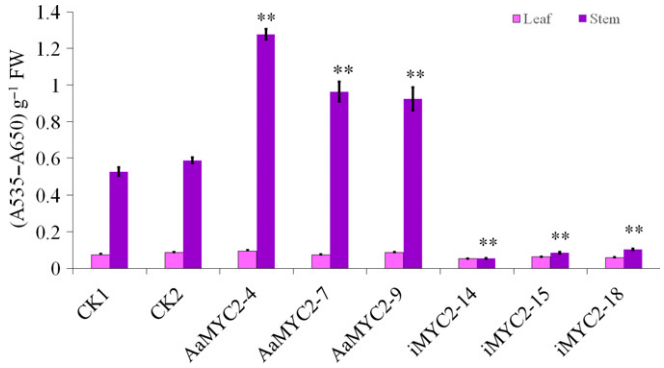

Fig. 6 AaMYC2 regulates the biosynthesis of anthocyanins in *Artemisia annua*. Contents of anthocyanins in leaves and stems of 5-month-old plants grown in a glasshouse. CK, nontransgenic control plants; AaMYC2, plants overexpressing the *AaMYC2* gene; iMYC2, *AaMYC2* RNAi transgenic plants. The contents in transgenic plants leaves were compared to those in the control plants. Error bars indicate ± SD of three technical replicates. Statistical significance was determined by Student's *t*-test (**, $P < 0.01$). Asterisks indicate the difference among overexpressed and RNAi transgenic plants with control plants.

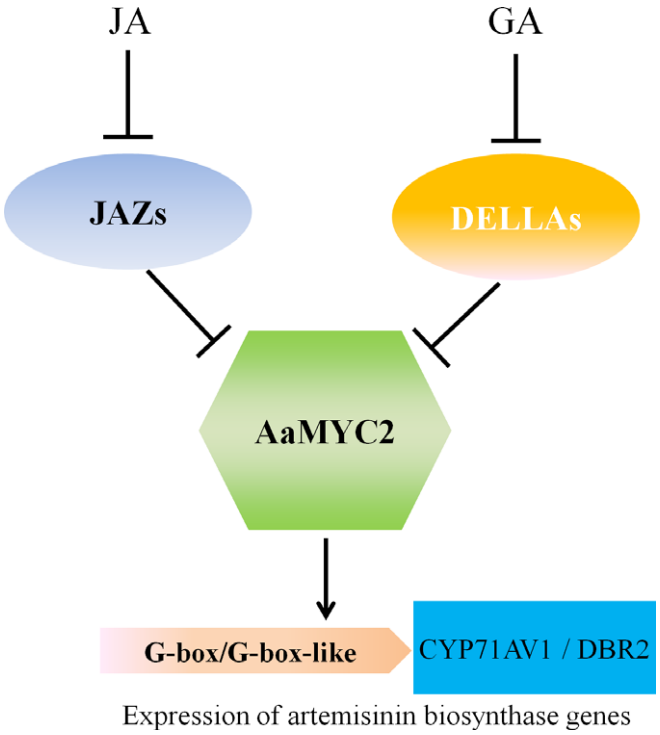

Fig. 7 Regulation of artemisinin biosynthase genes by jasmonate (JA) and gibberellin (GA) signaling pathways. JAZs, jasmonate zim-domain transcription factors; DELLAs, DELLA transcription factor; AaMYC2, *Artemisia annua* MYC2 transcription factor; G-box/G-box-like, *cis*-element in gene promoter region; CYP71AV1, cytochrome P450 monooxygenase; DBR2, artemisinic aldehyde Δ11(13) reductase.

in tobacco, NtMYC2 regulates jasmonate-inducible genes of nicotine biosynthesis directly and by way of interacting with NIC2-locus ERF genes (Shoji & Hashimoto, 2011). It will be interesting to investigate whether there is an AP2/ERF-domain transcription factor regulated by AaMYC2 in artemisinin biosynthesis in *A. annua*. Isolation and characterization of *cis*-elements in promoter regions in *AaEFRs* will facilitate our understanding of the regulation network of artemisinin and the biosynthesis of other terpenes in *A. annua*. Our results showed that exogenous MeJA treatment did not bring significant difference of artemisinin content between the WT and *AaMYC2* overexpressing transgenic plants. It is speculated that the transcript level of *AaMYC2* was enough after MeJA treatment and that AaMYC2 might have a similarly fine regulation as *AtMYC2* (Dombrecht *et al.*, 2007), that MYC2 negatively regulates its own transcription. Interestingly, the *AaMYC2* overexpressing transgenic *A. annua* plants showed purple stems and contained more anthocyanin than WT plants in stems. Although the underlying mechanism of how AaMYC2 is involved in this regulation was unclear, the MYC2 transcription factor that regulates anthocyanin biosynthesis pathways in other plant species may give some suggestion for further studies. In *A. thaliana*, MYC2 was required for anthocyanin accumulation (Lorenzo *et al.*, 2004; Dombrecht *et al.*, 2007), and it was speculated that MYC2 regulates the anthocyanin pathway by interacting with MYB proteins (de Pater *et al.*, 1997).

In conclusion, transcription factors show great potential in regulating plant secondary metabolites (van der Fits & Memelink, 2000; Zhang *et al.*, 2011; Yu *et al.*, 2012). Biosynthesis of plant secondary metabolites usually involves multiple enzymatic steps and, as a result, increasing target metabolite production in plants by overexpressing one or two key enzymes is often proven to be inefficient. Transcription factors often regulate key steps or even the whole biosynthetic pathway (van der Fits & Memelink, 2000; De Boer *et al.*, 2011; Yu *et al.*, 2012). Therefore, for the engineering of the regulation of plant secondary metabolism, transcription factors have become great candidates. AaMYC2 is potentially a valuable tool in regulating artemisinin biosynthesis, as it occupies a core position in JA signaling and directly regulates the expressions of the *CYP71AV1* and *DBR2* genes, and possibly of other terpene synthase genes in *A. annua*.

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Author contributions

K.T., Q.S. and X.L. planned and designed the research. Q.S., X.L., T.Y., X.F. and G.W. performed experiments. Z.L. and F.Z. conducted fieldwork. Q.S., X.L., Q.P. and X.S. analyzed data. Q.S., X.L. and K.T. wrote the manuscript.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Amino acid alignment of AaMYC2 with other MYC2/MYC-like proteins.

Fig. S2 Phylogenetic analysis of AaMYC2 with other MYC2/MYC-like proteins.

Fig. S3 Relative expression of *AaMYC2* in different *Artemisia annua* tissues.

Fig. S4 PCR analysis of *AaMYC2*-overexpressing and RNAi transgenic *Artemisia annua* plants.

Fig. S5 HPLC analysis of artemisinin content in transgenic plants overexpressing *AaMYC2* after MeJA (100 μM) treatment.

Fig. S6 Relative expression of *AaMYC2*, *ADS*, *CYP71AV1* and *DBR2* genes in *AaMYC2* silenced *Artemisia annua* plants after MeJA treatment.

Fig. S7 The whole plant morphologies and stem phenotypes of 5-month-old wild-type, AaMYC2 and iMYC2 *Artemisia annua* plants.

Fig. S8 The trichome density comparison among wild-type, AaMYC2 and iMYC2 *Artemisia annua* plants.

Table S1 Primers used in this investigation

Table S2 Artificial G-box motifs used in Y1H analysis

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