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Research

GLANDULAR TRICHOME-SPECIFIC WRKY 1 promotes artemisinin biosynthesis in *Artemisia annua*

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Summary

- Artemisinin is a type of sesquiterpene lactone well known as an antimalarial drug, and is specifically produced in glandular trichomes of *Artemisia annua*. However, the regulatory network for the artemisinin biosynthetic pathway remains poorly understood. Exploration of trichome-specific transcription factors would facilitate the elucidation of regulatory mechanism of artemisinin biosynthesis.
- The WRKY transcription factor *GLANDULAR TRICHOME-SPECIFIC WRKY 1* (*AaGSW1*) was cloned and analysed in *A. annua*. *AaGSW1* exhibited similar expression patterns to the trichome-specific genes of the artemisinin biosynthetic pathway and AP2/ERF transcription factor *AaORA*. A β-glucuronidase (GUS) staining assay further demonstrated that AaGSW1 is a glandular trichome-specific transcription factor.
- AaGSW1 positively regulates *CYP71AV1* and *AaORA* expression by directly binding to the W-box motifs in their promoters. Overexpression of *AaGSW1* in *A. annua* significantly improves artemisinin and dihydroartemisinic acid contents; moreover, AaGSW1 can be directly regulated by AaMYC2 and AabZIP1, which are positive regulators of jasmonate (JA)- and abscisic acid (ABA)-mediated artemisinin biosynthetic pathways, respectively.
- These results demonstrate that AaGSW1 is a glandular trichome-specific WRKY transcription factor and a positive regulator in the artemisinin biosynthetic pathway. Moreover, we propose that two trifurcate feed-forward pathways involving *AaGSW1*, *CYP71AV1* and *AaMYC2/AabZIP1* function in the JA/ABA response in *A. annua*.

Introduction

Malaria is a global health problem with more than three billion people at high risk (World Health Organization, 2015). Artemisinin-based combination therapies (ACTs) are the recommended treatment for uncomplicated *Plasmodium falciparum* malaria (World Health Organization, 2015). Although semi-synthetic production of artemisinin in yeast has been successfully developed at high production levels (Ro *et al.*, 2006; Paddon *et al.*, 2013), the *Artemisia annua* plant remains as the main source for artemisinin production (Peplow, 2016). Artemisinin is the key component that gives the whole plant effectiveness against malaria, although production of artemisinin is quite low (0.1–1.0% DW). Boosting production of artemisinin in the plant could improve the antimalarial properties of whole plant preparations, which are known to reduce rates of drug resistance vs pure artemisinin (Elfawal *et al.*, 2012, 2015; Weathers *et al.*, 2014). Therefore, dissection of the regulatory mechanism of artemisinin biosynthesis may provide clues for activating the pathway and for producing artemisinin of high yield and quality.

The glandular trichomes (GSTs) of *A. annua* where artemisinin biosynthesis and accumulation take place are composed of 10 cells (Olofsson *et al.*, 2012). The biosynthetic pathway has been almost completely elucidated in the last decade (Fig. 1; Tang *et al.*, 2014). In brief, two molecules of isopentenyl diphosphate (IPP) from the mevalonate (MVA) pathway and one molecule of dimethylallyl diphosphate (DMAPP) from the 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway are converted to form farnesyl diphosphate (FPP) by farnesyl diphosphate synthase (FPS) (Schramek *et al.*, 2010). The first committed step of the artemisinin biosynthetic pathway is the cyclization of FPP to the amorpha-4, 11-diene by amorpha-4,11-diene synthase (ADS) (Bouwmeester *et al.*, 1999; Mercke *et al.*, 2000). Subsequently, amorpha-4, 11-diene is oxidized to artemisinic acid (AA) via artemisinic alcohol and artemisinic aldehyde intermediates catalysed by a cytochrome P450 monooxygenase CYP71AV1

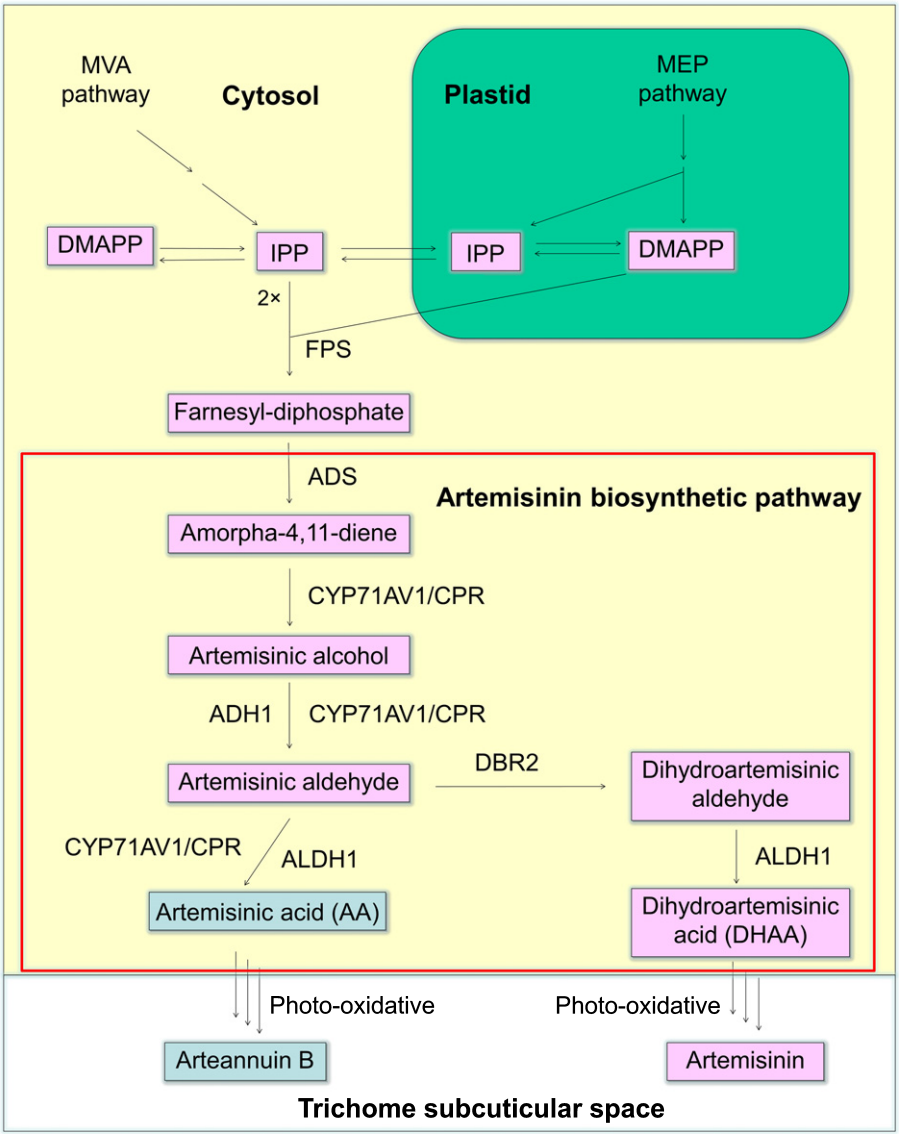

Fig. 1 The artemisinin biosynthetic pathway in *Artemisia annua*. MVA, mevalonate; MEP, 2-*C*-methyl-D-erythritol 4-phosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; FPS, farnesyl diphosphatesynthase; ADS, amorpha-4,11-diene synthase; CYP71AV1, cytochrome P450 monooxygenase;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

(Ro *et al.*, 2006; Teoh *et al.*, 2006). AA can also be converted to artemisinic aldehyde by alcohol dehydrogenase 1 (ADH1) (Paddon *et al.*, 2013). Then, artemisinic aldehyde Δ-11(13) reductase (DBR2) catalyses artemisinic aldehyde to form dihydroartemisinic aldehyde (Zhang *et al.*, 2008), which is further converted to dihydroartemisinic acid (DHAA) by aldehyde dehydrogenase (ALDH1) (Teoh *et al.*, 2009). DHAA is predicted to be transported to the trichome subcuticular space, and converted there to artemisinin by an enzyme-independent reaction (Brown & Sy, 2004).

Several transcription factors have been reported to regulate artemisinin biosynthesis. The WRKY transcription factor AaWRKY1 was reported to promote the transcription of *ADS*, *CYP71AV1* and *DBR2*, positively regulating artemisinin biosynthesis (Ma *et al.*, 2009; Han *et al.*, 2014; Jiang *et al.*, 2016). AaERF1, AaERF2, AaORA and AaTAR1, all belonging to the AP2/ERF transcription factor family, improve artemisinin biosynthesis through promoting *ADS*, *CYP71AV1* and *DBR2* expression (Yu *et al.*, 2011; Lu *et al.*, 2013; Tan *et al.*, 2015). The basic helix–loop–helix (bHLH) transcription factor AaMYC2 was reported to be involved in the promotion of artemisinin biosynthesis by jasmonate (JA) (Shen *et al.*, 2016a). The other bHLH transcription factor AabHLH1 also acts as a positive regulator of artemisinin biosynthesis (Ji *et al.*, 2014). AabZIP1, a bZIP transcription factor, was identified to be involved in the promotion of artemisinin biosynthesis by abscisic acid (ABA) (Zhang *et al.*, 2015). Given that artemisinin is especially biosynthesized in GSTs and all four key enzymes in artemisinin biosynthesis show trichome-specific expression patterns (Wang *et al.*, 2011, 2012, 2016; Olofsson *et al.*, 2012; Jiang *et al.*, 2014), the exploration of trichome-specific transcription factors would facilitate the elucidation of the regulatory mechanism of artemisinin biosynthesis. However, only the AP2/ERF transcription factor AaORA has been identified to be trichome-specific among the transcription factors cloned so far (Lu *et al.*, 2013). Consequently, it is of interest to determine whether there are other

trichome-specific transcription factors involved in artemisinin biosynthesis.

The WRKY transcription factor family is unique to plants, and is one of the largest transcription factor families in plants (Eulgem *et al.*, 2000; Rushton *et al.*, 2010; Phukan *et al.*, 2016). The WRKY transcription factors are well known for their involvement in plant abiotic and biotic stress responses, and in regulating plant-specialized secondary metabolism through recognizing and binding the *cis*-acting element W-box (TTGACC/T) (Schluttenhofer & Yuan, 2015; Phukan *et al.*, 2016; Zhou & Memelink, 2016). It is unknown if other WRKY transcription factors might also be involved in the regulation of artemisinin biosynthesis in *A. annua*. In this article, we identified a glandular trichome-specific WRKY transcription factor, GLANDULAR TRICHOME-SPECIFIC WRKY 1 (AaGSW1), which promotes artemisinin biosynthesis by positively regulating *CYP71AV1* and *AaORA* expression. Moreover, we propose a trifurcate feed-forward regulatory model involving *AaGSW1*, *CYP71AV1* and *AaMYC2*/*AabZIP1* in *A. annua*.

Materials and Methods

Plant materials

Artemisia annua L. high artemisinin cultivar 'Huhao 1' (artemisinin, 0.8–1.0% DW) which has been developed in Shanghai for several years was used in all *A. annua* related assays (Shen *et al.*, 2016a). *Nicotiana benthamiana* Domin (tobacco) was sown directly on soil and grown under a 16 h light photoperiod at 24 ± 2°C.

Bioinformatic analysis

The bioinformatic analysis was performed as described previously (Zhang *et al.*, 2015). In brief, the hidden Markov model (HMM) profiles of the WRKY (PF03106) domain were obtained from the Pfam database (Finn *et al.*, 2016). The WRKY homologues were identified in our *A. annua* transcriptome sequences by the HMM search with *E* value $< 10^{-5}$ (Eddy, 2011; Zhang *et al.*, 2015). The RNA-seq data of trichomes of different tissues (SRR019254 for meristem; SRR019549 for cotyledon; SRR019546 for young leaf trichome; SRR019548 for bud trichome; SRR019547 for mature leaf trichome) were downloaded from the NCBI. WRKY transcription factors which are expressed in trichomes were identified by a BLASTN search against the five corresponding databases with *E* value $< 10^{-6}$. The read counts of each WRKY transcription factor in the five different tissues were normalized by computing the value of reads per kilobase per million (RPKM). The expression heatmap of these *A. annua* WRKY transcription factors in the five tissues was performed with the MultiExperiment VIEWER 4.9.0 (Dana-Farber Cancer Institute, Boston, MA, USA). The most similar sequences from *Arabidopsis* were obtained by using a BLASTP search against the reference proteins database at NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The unrooted phylogenetic tree was constructed with MEGA v.5 (http://www.megasoftware.net) via the neighbour-joining method based on amino acid sequence alignment, and bootstrap analysis was performed using 5000 replicates.

Relative expression analysis by quantitative real time PCR (qRT-PCR)

The different tissues (root, stem, old leaf, young leaf, bud0 (flower bud gathered 5 d after budding), bud1 (flower bud gathered 10 d after budding) and flower (flower gathered 20 d after budding)) and different position leaf (leaf0, leaf1, leaf2, leaf3, leaf5 and leaf9) samples were gathered as described previously (Lu *et al.*, 2013). The trichome samples were collected according to the previous protocol with some modifications (Gershenzon *et al.*, 1992; Lange *et al.*, 2000). In brief, trichomes were removed from flower buds as follows: 20 g of bud1 was fixed at 4°C in 10 volumes of freshly prepared fixative solution (3 : 1, ethanol : acetic acid) for 4–12 h. Following fixation, buds were washed three times in ice-cold deionized water for 10 min to remove fixative. The buds were then transferred to the 350 ml bevelled flask of the beadbeater (Biospec Products, Inc., Bartlesville, OK, USA) along with 300 ml of ice-cold trichome isolation buffer (200 mM D-sorbitol, 50 mM Tris-HCl, 20 mM sucrose, 10 mM KCl, 14 mM β-mercaptoethanol, 5 mM $MgCl_2$, 5 mM succinic acid, 0.5 mM K_2PO_4, 1 mM EGTA, 1% (w/v) polyvinylpyrrolidone, and 0.6% (w/v) methylcellulose) and 40 g of glass beads. Trichomes were removed from the buds with three pulses of 1 min at 100 V, with a 1 min rest between pulses. After passing the resulting mixture through 350, 105 and 20 μm mesh cloth, the trichomes were collected on the 20 μm mesh cloth. Collected trichomes were washed 10 times on the mesh cloth with ice-cold deionized water, transferred to a 1.5 ml centrifuge tube, and placed in liquid nitrogen for RNA extraction.

qRT-PCR was used to analyse the expression patterns of genes in *A. annua* as described previously (Shen *et al.*, 2016a). All the primers used in this article are listed in Supporting Information Table S1.

Plasmid construction

For yeast one-hybrid assays, the triple tandem copies of the seven W-box (TTGACC) motifs in the promoters of *ADS*, *CYP71AV1*, *DBR2* and *AaORA* and their mutants (TTAGCC) were inserted into pLacZ between *EcoR*I and *Xho*I as reporters, respectively. The sequences of all fragments are listed in Table S2. Five G-box motifs and four ABRE motifs in the promoter of AaGSW1 were gathered together and inserted into pLacZ between *EcoR*I and *Xho*I as reporters, respectively. The open reading frame (ORF) fragments of *AaGSW1*, *AaWRKY75b*, *AaMYC2* and *AabZIP1* were cloned into pB42AD as effectors.

For the electrophoretic mobility shift assay (EMSA), the ORF fragment of *AaGSW1* was inserted into PET-30a prokaryotic expression vector, and the ORF fragment of *AaWRKY75b* was inserted into PET-32a prokaryotic expression vector.

For the dual-luciferase (Dual-LUC) assay, the promoters of *ADS*, *CYP71AV1*, *DBR2* and *AaGSW1* were inserted into pGREEN 0800 (Hellens *et al.*, 2005), respectively, to drive the luciferase reporters. The ORF fragments of *AaGSW1*, *AaWRKY75b*, *AaMYC2* and *AabZIP1* were cloned into pHB vector as effectors (Mao *et al.*, 2005).

For *A. annua* transformation, the ORF fragment of *AaGSW1* was inserted into the modified pCAMBIA2300+ vector with *CYP71AV1* promoter replacing the CaMV 35S promoter, to generate the glandular trichome-specific overexpressing vector *pPCYP-AaGSW1*. The 2074 bp promoter fragment of *AaGSW1* was cloned into pCAMBIA 1391Z to generate the vector pAaGSW1 containing *GUS* driven by the *AaGSW1* promoter to investigate *AaGSW1* tissue distribution.

For subcellular localization, the ORF fragment of *AaGSW1* was first cloned into PENTR-Topo to get Topo-AaGSW1, which was then inserted into pEarleyGate 101 via the LR recombination reaction to get CaMV 35S promoter-driven AaGSW1-YFP vector. All the primers used in plasmid construction are listed in Table S1.

Yeast one-hybrid assays

The yeast one-hybrid assays were operated as described previously (Zhang *et al.*, 2015). The pB42AD-related plasmids were co-transformed into yeast strain EGY48a with pLacZ-related plasmids. The transformants were cultivated on SD/-Ura/-Leu medium for 48 h and tested on SD/-Ura/-Leu medium with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) for 24 h. The empty pB42AD and pLacZ plasmids were used as negative controls.

EMSA

For protein expression and purification, PET-30a-AaGSW1 and PET-32a-AaWRKY75b plasmids were transferred into *Escherichia coli* strain BL21. The fusion proteins were expressed in BL21 cells by adding 10 μM isopropyl β-D-1-thiogalactopyranoside (IPTG) to culture medium for 16 h at 25°C (for AaGSW1) or 16°C (for AaWRKY75b) and purified using Ni-NTA agarose (Invitrogen).

The EMSA was performed as described previously (Zhang *et al.*, 2015). The oligonucleotides for the W-box elements were synthesized by Sangon (Shanghai, China). The assay was performed using the DIG Gel Shift Kit, 2nd Generation (Roche) according to the manufacturer's instructions. The probes used in the EMSA assay are listed in Table S2.

Dual-LUC assay

The pHB-AaGSW1, pHB-AaWRKY75b, pHB-AaMYC2, pHB-AaBZIP1 and pHB empty plasmid were each transferred into *Agrobacterium tumefaciens* strain GV3101. The pGREEN-pADS, pGREEN-pCYP71AV1, pGREEN-pDBR2 and pGREEN-pAaGSW1 were each co-transformed with pSoup19 into GV3101. Subsequently, the assay was performed as reported previously (Zhang *et al.*, 2015). The pHB empty plasmid was used as a negative control. The 35S promoter-driven *Renilla* was used as an internal control. Three biological repeats were measured for each sample.

Artemisia annua transformation

pCYP-AaGSW1 and pCAMBIA 1391Z-pAaGSW1 were each transferred into *A. tumefaciens* strain EHA105, and the latter were used to transform *A. annua* via *Agrobacterium*-mediated transformation as described previously (Zhang *et al.*, 2009).

Artemisinin, DHAA and AA content measurement

Leaves of 4-month-old *A. annua* plants grown in the glasshouse were collected and dried at 50°C, and then used for the measurement of artemisinin, DHAA and AA contents by high-performance liquid chromatography (HPLC) as described previously (Shen *et al.*, 2016a,b).

Methyl jasmonate and ABA treatments

For methyl jasmonate (MeJA) and ABA treatments, 100 μM MeJA or 10 μM ABA (both from Sigma-Aldrich) was used, and water was used as mock treatment. *A. annua* plants grown on MS_0 medium plates for 15 d were sprayed with 10 ml each of solutions. Young leaves were gathered at 0, 1, 3, 6, 9, 12, 24 and 48 h after spaying. The entire samples described earlier were collected from five individual plants, and a pool was constructed for RNA isolation. To analyse artemisinin biosynthesis-related gene expression in pCYP-AaGSW1 plants after MeJA and ABA treatments, the 2-month-old cutting propagation of pCYP-AaGSW1 and control plants were treated with 100 μM MeJA or 10 μM ABA, and sampled at 6 h for RNA isolation and at 3 d for artemisinin extraction.

Statistics

All experiments were performed using at least three biological replicates. Data are given as means ± SD. To compare group differences, paired, two-tailed Student's *t*-tests were used. *P*-values < 0.05 were regarded as statistically significant.

Results

Coexpression analysis identifies WRKY transcription factors predicted to be expressed in a trichome-specific manner in *A. annua*

In *A. annua*, two or three W-box motifs exist in the promoters of *ADS*, *CYP71AV1* and *DBR2* (Wang *et al.*, 2011, 2012; Yang *et al.*, 2015), which are the potential binding sites of WRKY transcription factors. WRKY1 was identified for binding to the W-box of the *ADS* promoter to regulate artemisinin biosynthesis (Ma *et al.*, 2009), while other WRKYs involved in the regulation

of this pathway remain unknown. To investigate more artemisinin biosynthesis-related WRKY transcription factors, we searched for *A. annua* putative WRKY proteins that contain conserved WRKY domains in our transcriptome database of *A. annua*, and, subsequently, 122 WRKY transcription factors were identified, among which only 42 were found to be expressed in trichomes based on Graham's RNA-seq data (Graham *et al.*, 2010). To further identify whether these WRKY transcription factors are trichome-specific in *A. annua*, four glandular trichome-specific key enzyme genes (*ADS*, *CYP71AV1*, *DBR2* and *ALDH1*) in the artemisinin biosynthetic pathway and the trichome-specific AP2/ERF transcription factor *AaORA* were used as baits to retrieve coexpressed WRKY transcription factors in five tissues based on Graham's research (Fig. 2a). Subsequently, *AaGSW1* and *AaWRKY75b* were found to be clustered with the five trichome-specific genes in the heatmap, which showed that they were coexpressed with the five genes with high expression in young leaf trichome and bud trichome, moderate expression in meristem and low expression in mature leaf trichome and cotyledon. According to the phylogenetic analysis, both AaGSW1 and AaWRKY75b are clustered with *Arabidopsis* AtWRKY75 (Fig. 2b), which is reported to play an important role in root hair development, leaf senescence, phosphate acquisition and disease defence (Devaiah *et al.*, 2007; Encinas-Villarejo *et al.*, 2009; Li *et al.*, 2012; Chen *et al.*, 2013; Lei *et al.*, 2014; Rishmawi *et al.*, 2014).

Expression patterns of *AaGSW1* and *AaWRKY75b*

AaGSW1 and *AaWRKY75b* expressions were re-evaluated by qRT-PCR. The results revealed that the two genes showed very similar expression patterns with the trichome-specific genes *ADS* and *AaORA* (Fig. 3). They were all expressed highly in young leaf, bud0 and bud1, compared with the low levels found in root, stem and old leaf (Fig. 3a–d). Furthermore, their expression was highest in the youngest leaves (leaf0), and decreased rapidly with leaf age (Fig. 3e–h). Moreover, their expression levels in trichomes were > 10-fold higher than in bud1 covered with the highest density of trichomes (Figs 3i–l, S1). Taken together, these results strongly suggest that AaGSW1 and AaWRKY75b are trichome-specific WRKY transcription factors that may be involved in the regulation of artemisinin biosynthesis.

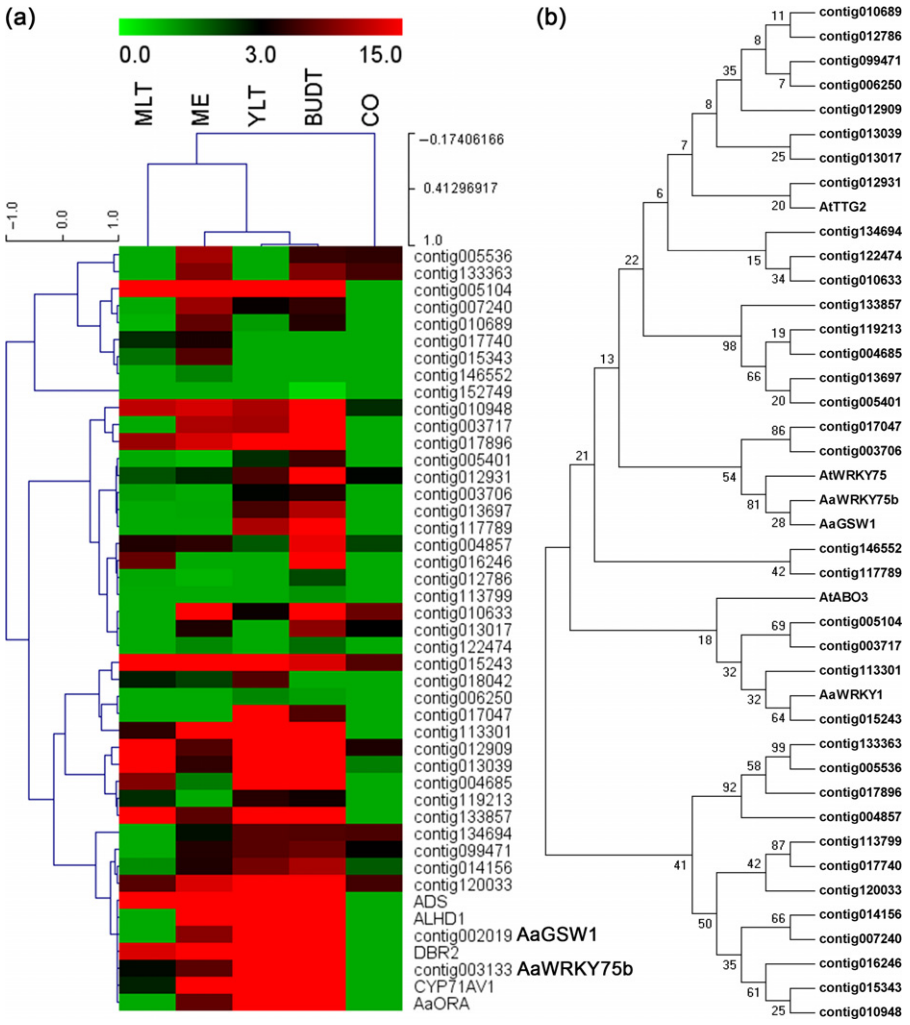

Fig. 2 Global expression profile and phylogenetic analysis of WRKY transcription factors in *Artemisia annua*. (a) Hierarchical cluster analysis of WRKY transcription factors. MLT, mature leaf trichome; ME, meristem; YLT, young leaf trichome; BUDT, flower bud trichome; CO, cotyledon. The colour scale at the top represents the value of transformed reads per kilobase per million mapped reads. (b) Phylogenetic tree showing the relationship between WRKY transcription factors expressed in trichomes of *A. annua* and some WRKY transcription factors from *Arabidopsis*. The tree presented here is a neighbour-joining tree based on amino acid sequence alignment. The tree was constructed using the program MEGA.

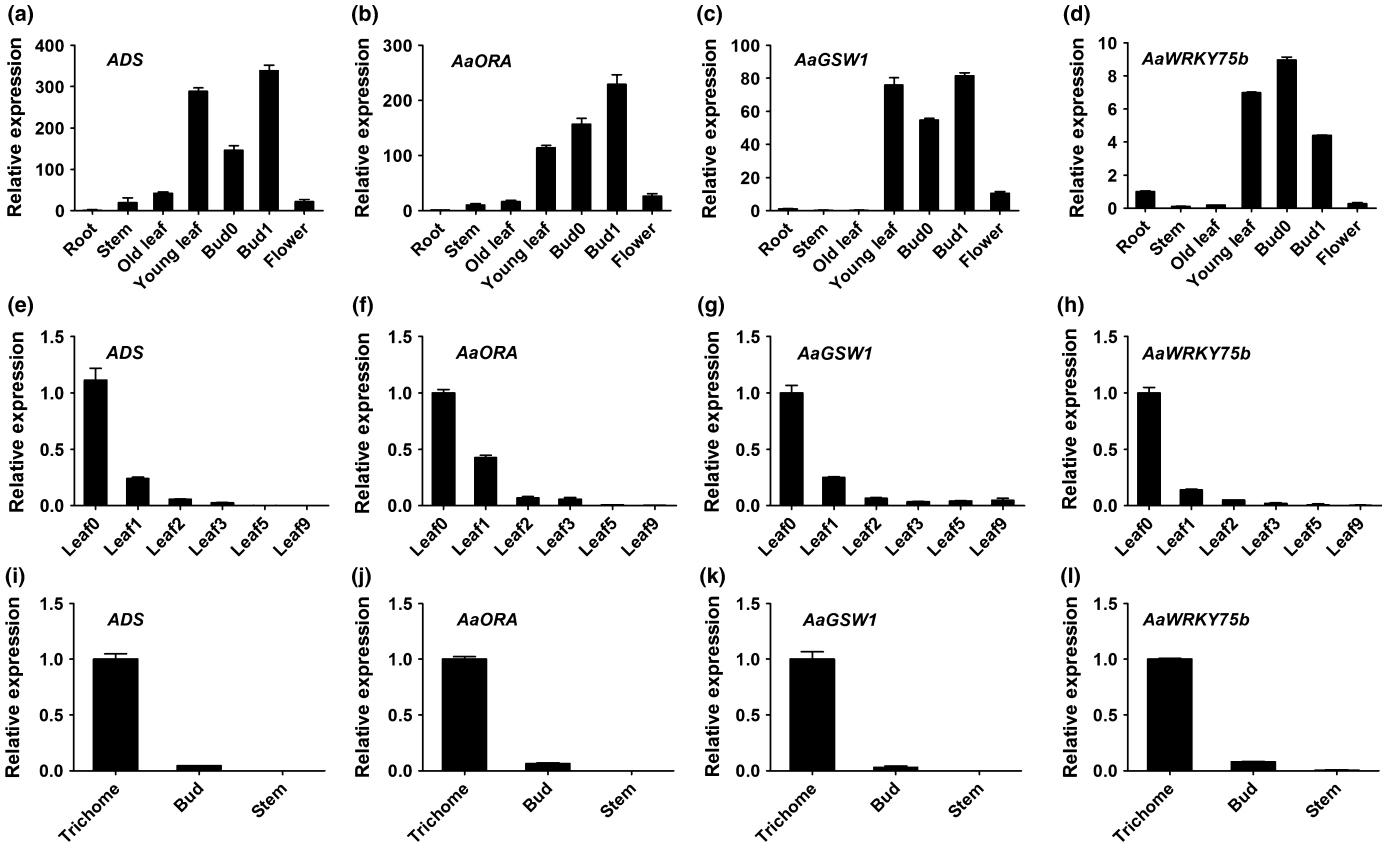

Fig. 3 Expression patterns of *ADS*, *AaORA*, *AaGSW1* and *AaWRKY75b* in *Artemisia annua*. (a–d) Expression levels of *ADS*, *AaORA*, *AaGSW1* and *AaWRKY75b* in different tissues. The expression levels of *ADS*, *AaORA*, *AaGSW1* and *AaWRKY75b* in root, stem, leaf, bud0, bud1 and flower of *A. annua* were measured by quantitative real-time PCR (qRT-PCR). *Actin* was used as an internal reference. Error bars indicate SD ($n = 3$). (e–h) Expression levels of *ADS*, *AaORA*, *AaGSW1* and *AaWRKY75b* at different positions of the leaves in *A. annua*. The expression levels of *ADS*, *AaORA*, *AaGSW1* and *AaWRKY75b* of meristem (leaf0), leaf1, 2, 3, 5 and 9 were measured by qRT-PCR. *Actin* was used as an internal reference. Error bars indicate SD ($n = 3$). (i–l) Expression levels of *ADS*, *AaORA*, *AaGSW1* and *AaWRKY75b* in trichome of *A. annua*. The expression levels of *ADS*, *AaORA*, *AaGSW1* and *AaWRKY75b* of trichome, bud and stem were measured by qRT-PCR. *Actin* was used as an internal reference. Error bars indicate SD ($n = 3$).

AaGSW1 binds to the W-box in the *CYP71AV1* promoter *in vitro* and activates the transcription of *CYP71AV1 in vivo*

To further demonstrate if *AaGSW1* and *AaWRKY75b* are regulators of artemisinin biosynthesis, we tested whether they could regulate the expression of *ADS*, *CYP71AV1*, *DBR2* and *ALDH1 in vitro* and *in vivo*. Among the four genes, only *ADS*, *CYP71AV1* and *DBR2* have W-box motifs in their promoters (Fig. S2a; Wang *et al.*, 2011, 2012, 2016; Jiang *et al.*, 2014; Yang *et al.*, 2015). The yeast one-hybrid assay showed that *AaGSW1* only bound to one of the W-box motifs in the promoter of CYP71AV1 (pCYP71AV1), while AaWRKY75b bound to none of them (Figs 4a, S2b). EMSA also proved that AaGSW1 directly bound to the W-box in the CYP71AV1 promoter *in vitro*, while AaWRKY75b did not (Fig. 4b). These results were further supported by Dual-LUC assays, in which only AaGSW1 activated the *CYP71AV1* promoter, whereas AaWRKY75b did not (Fig. 4c). Furthermore, *ADS* and *DBR2* promoters were activated by neither AaGSW1 nor AaWRKY75b (Fig. S2c,d). The results reveal that AaGSW1, not AaWRKY75b, may be a positive regulator of artemisinin biosynthesis. Therefore, AaGSW1 was chosen for further investigation.

AaGSW1 is a glandular trichome-specific transcription factor

To obtain more precise expression patterns of *AaGSW1*, a 2077 bp promoter sequence of *AaGSW1* was cloned. Putative *cis*-elements in the *AaGSW1* promoter were predicted using PLANTPAN (http://plantpan2.itps.ncku.edu.tw/) software and PLANTCARE software (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). Some common binding sites, such as W-box, G-box and ABRE motifs, were marked in the promoter sequence (Fig. S3). Transgenic *A. annua* lines transformed with *GUS* driven by the *AaGSW1* promoter were generated. Two types of trichomes exist on the epidermis of *A. annua* aerial organs, one being T-shaped nonglandular (TSTs) and the other GSTs (Duke & Paul, 1993). In this study, β-glucuronidase (GUS) staining was only observed in GSTs of young leaves and stems (Fig. 5a–d). No GUS activity was observed in mature leaves (Fig. S4). The results demonstrate that AaGSW1 is a glandular trichome-specific WRKY transcription factor of *A. annua*.

To investigate the subcellular localization of AaGSW1 *in vivo*, AaGSW1–YFP fusion protein was transiently expressed in tobacco leaves. Yellow fluorescent protein (YFP) fluorescence was

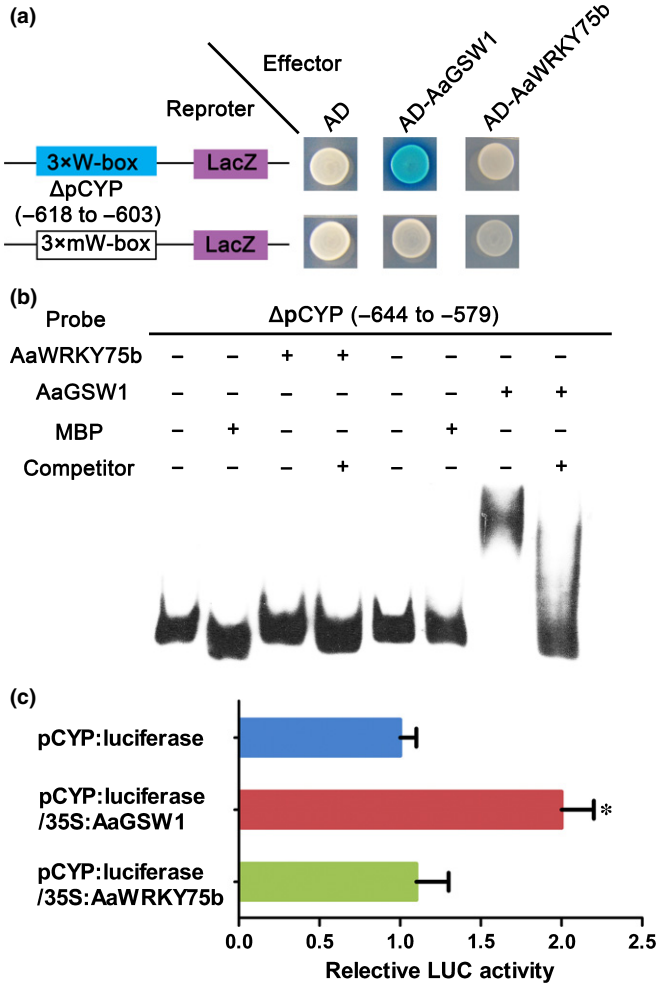

Fig. 4 AaGSW1 binds to the W-box in the *CYP71AV1* promoter *in vitro* and activates the transcription of *CYP71AV1* in tobacco. (a) Yeast one-hybrid assay indicating AaGSW1 binds to the W-box (−614 to −609) in the *CYP71AV1* promoter, but cannot bind to the W-box mutants. AaWRKY75b binds to neither of them. Mutant W-box (mW-box) was used as a control. (b) Electrophoretic mobility shift assay (EMSA) showing AaGSW1 binding to the W-box (−614 to −609) of the *CYP71AV1* promoter. The His–AaGSW1 or His–AaWRKY75b fusion protein and labelled ΔpCYP (−644 to −579) probe (W-box derived from the *CYP71AV1* promoter region, −644 to −579) were used. Nonlabelled ΔpCYP (−644 to −579) served as cold competitor (200×). MBP protein was included as a negative control. MBP protein alone was included as a negative control. (c) Effects of AaGSW1 and AaWRKY75b on *CYP71AV1* promoter activation. *CYP71AV1* promoter was fused to the luciferase (LUC) reporter and the promoter activity was determined by a transient dual-LUC assay in tobacco. The relative LUC activity was normalized to the reference Renilla (REN) luciferase. Error bars indicate SD ($n = 3$). Student's *t*-test: *, $P < 0.05$.

detected in the nucleus (Fig. 5e–h), which is consistent with AaGSW1's role as a transcription factor.

Overexpression of *AaGSW1* in *A. annua* increases artemisinin biosynthesis

Since AaGSW1 was able to bind to and activate the *CYP71AV1* promoter, we examined whether overexpression of *AaGSW1* could enhance artemisinin biosynthesis in *A. annua*. It was reported that a transcription factor driven by the glandular trichome-specific *CYP71AV1* promoter improved artemisinin biosynthesis much more effectively than that driven by the constitutive CaMV 35S promoter (Han *et al.*, 2014). Consequently, in this study, the vector was constructed in which the *CYP71AV1* promoter was used to drive *AaGSW1* (*pCYP:: AaGSW1*) and used to transform *A. annua*.

Twenty independent transgenic plants were first confirmed by genomic PCR using the *CYP71AV1* promoter forward primer and *AaGSW1* reverse primer, and qRT-PCR was then used to analyse *AaGSW1* transcript level in the transgenic lines. Three independent lines with high *AaGSW1* overexpression were subsequently chosen for further analysis. Compared with the control, the transcript level of *AaGSW1* increased by over five-fold, whereas the expressions of *ADS*, *CYP71AV1*, *DBR2* and *ALDH1* increased by about three- to five-, three- to six-, two- to three- and four- to seven-fold, respectively, in *AaGSW1*-overexpressing transgenic lines (Fig. 6a). The result was not consistent with the previous results that AaGSW1 only activates *CYP71AV1* promoter *in vivo*. Thus, it is suspected that AaGSW1 may promote *ADS*, *DBR2* and *ALDH1* expression indirectly. Interestingly, we found the transcript level of trichome-specific transcription factor *AaORA* was increased by four-fold in transgenic lines (Fig. 6a). Therefore, the increase of *ADS* and *DBR2* expression may be due to the increase of *AaORA* transcript level in transgenic lines. To investigate whether *AaORA* expression was directly regulated by AaGSW1, a yeast one-hybrid assay was performed. The result showed that AaGSW1 directly bound to the W-box of the *AaORA* promoter in yeast (Fig. 6b). The artemisinin, DHAA and AA contents of 4-month-old AaGSW1-overexpressing transgenic *A. annua* lines were measured by HPLC. In AaGSW1-overexpressing lines, the artemisinin and DHAA contents were significantly increased by 55–100 and 50–60% (Fig. 6c), respectively, while the AA content was significantly decreased by 30–90% (Fig. S5) ($P < 0.05$, Student's *t*-test). Fluorescence micrographs of mature leaves showed that there was no significant difference of GST density between the transgenic and control plants (Fig. S6a,b). These results reveal that AaGSW1 is a positive regulator of artemisinin biosynthesis.

Both AaMYC2 and AabZIP1 form a feed-forward loop with AaGSW1 to regulate *CYP71AV1*

Previous reports showed that artemisinin biosynthesis is promoted by JA and ABA treatments (Zhang *et al.*, 2015; Shen *et al.*, 2016a). To examine the role of AaGSW1 in the promotion of artemisinin accumulation by JA or ABA, expression of *AaGSW1* under JA and ABA treatments was analysed by qRT-PCR. The results showed that expression of *AaGSW1* was significantly increased after both JA and ABA treatments (Fig. 7a). Analysing the *AaGSW1* promoter sequence revealed five G-boxes and four ABRE motifs (Fig. 7b). The G-box was reported to be recognized and bound by AaMYC2, which was the direct target of JA (Shen *et al.*, 2016a). Meanwhile, AabZIP1, which was directly regulated by ABA, was found to bind to the ABRE motif

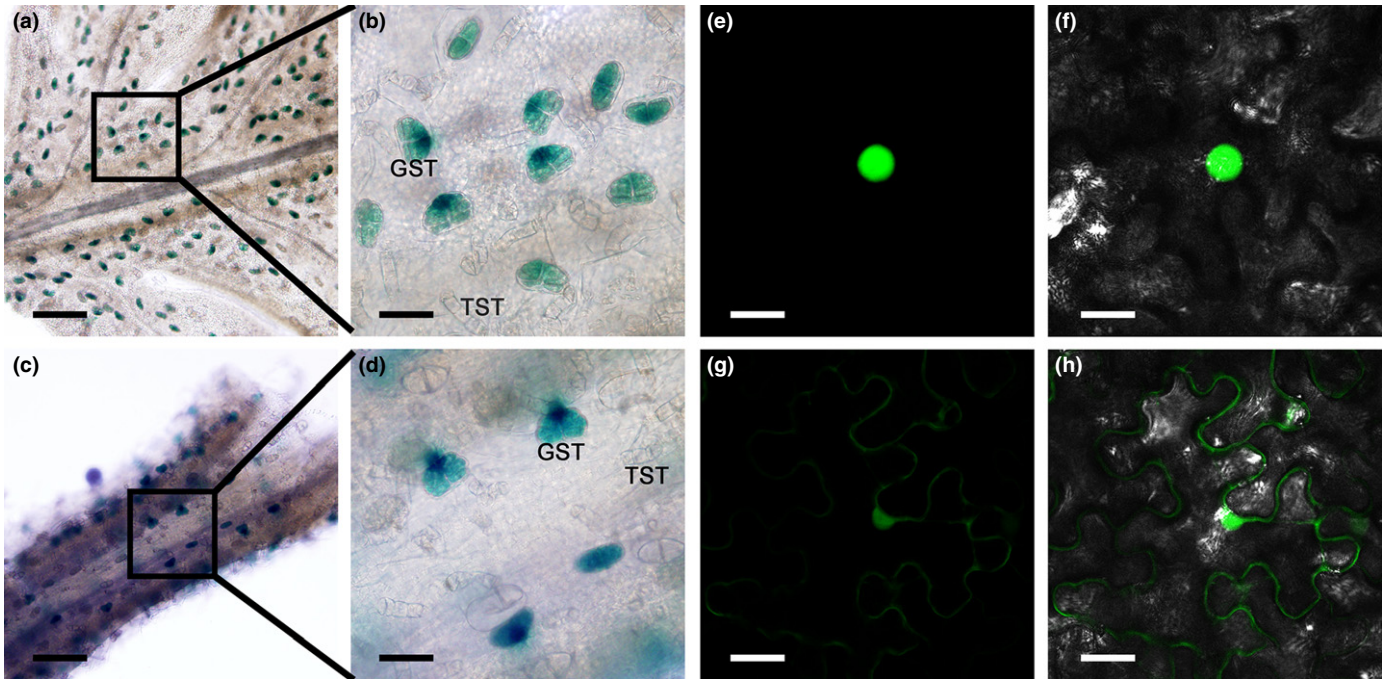

Fig. 5 β-Glucuronidase (GUS) staining of transgenic *Artemisia annua* transformed using the pAaGSW1-GUS plasmid and subcellular localization of AaGSW1. (a, b) *AaGSW1* promoter–GUS (pAaGSW1-GUS) expression on the young leaf. GST, glandular trichome; TST, T-shaped nonglandular trichome. (c, d) pAaGSW1-GUS expression on young stem. (e–h) Subcellular localization of (e, f) 35S : AaGSW1-GFP and (g, h) 35S : GFP in tobacco leaf epidermal cells. Bars: (a, c) 200 μm; (b, d) 40 μm; (e, f) 30 μm; (g, h) 60 μm.

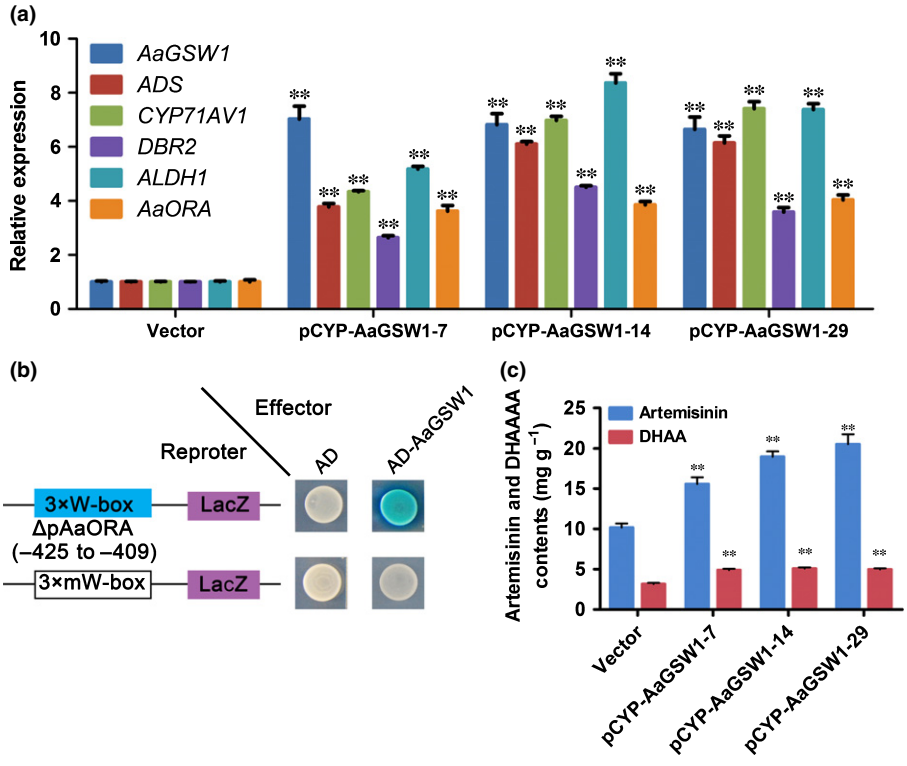

Fig. 6 Analysis of transgenic plants overexpressing AaGSW1, and AaGSW1 binds to the W-box in AaORA promoter in yeast. (a) Expression levels of *AaGSW1*, *ADS*, *CYP71AV1*, *DBR2*, *ALDH1* and *AaORA* in pCYP-AaGSW1 (glandular trichome-specific *CYP71AV1* promoter-driven AaGSW1 overexpression) transgenic plants were measured by quantitative real-time PCR (qRT-PCR). Vector indicates plant transformed with empty pCAMBIA 2300 vector. *Actin* was used as an internal reference. Error bars indicate SD ($n = 3$). Student's *t*-test: **, $P < 0.01$. Asterisks indicate the difference between overexpression transgenic plants and control plants. (b) AaGSW1 binds to W-box (−420 to −415) in the *AaORA* promoter; W-box mutant (mW-box) was used as a control. (c) Artemisinin and DHAA contents in pCYP-AaGSW1 plants were measured by high-performance liquid chromatography (HPLC).

(Zhang *et al.*, 2015). We therefore investigated whether JA and ABA regulate *AaGSW1* expression via AaMYC2 and AabZIP1, respectively. Consequently, a yeast one-hybrid assay was performed. The result showed that AaMYC2 bound to the G-boxes and AabZIP1 bound to the ABRE motifs in the *AaGSW1* promoter (Fig. 7c,d). These results were further supported by Dual-LUC assays, in which both AaMYC2 and AabZIP1 activated activity of the *AaGSW1* promoter significantly (Fig. 7e).

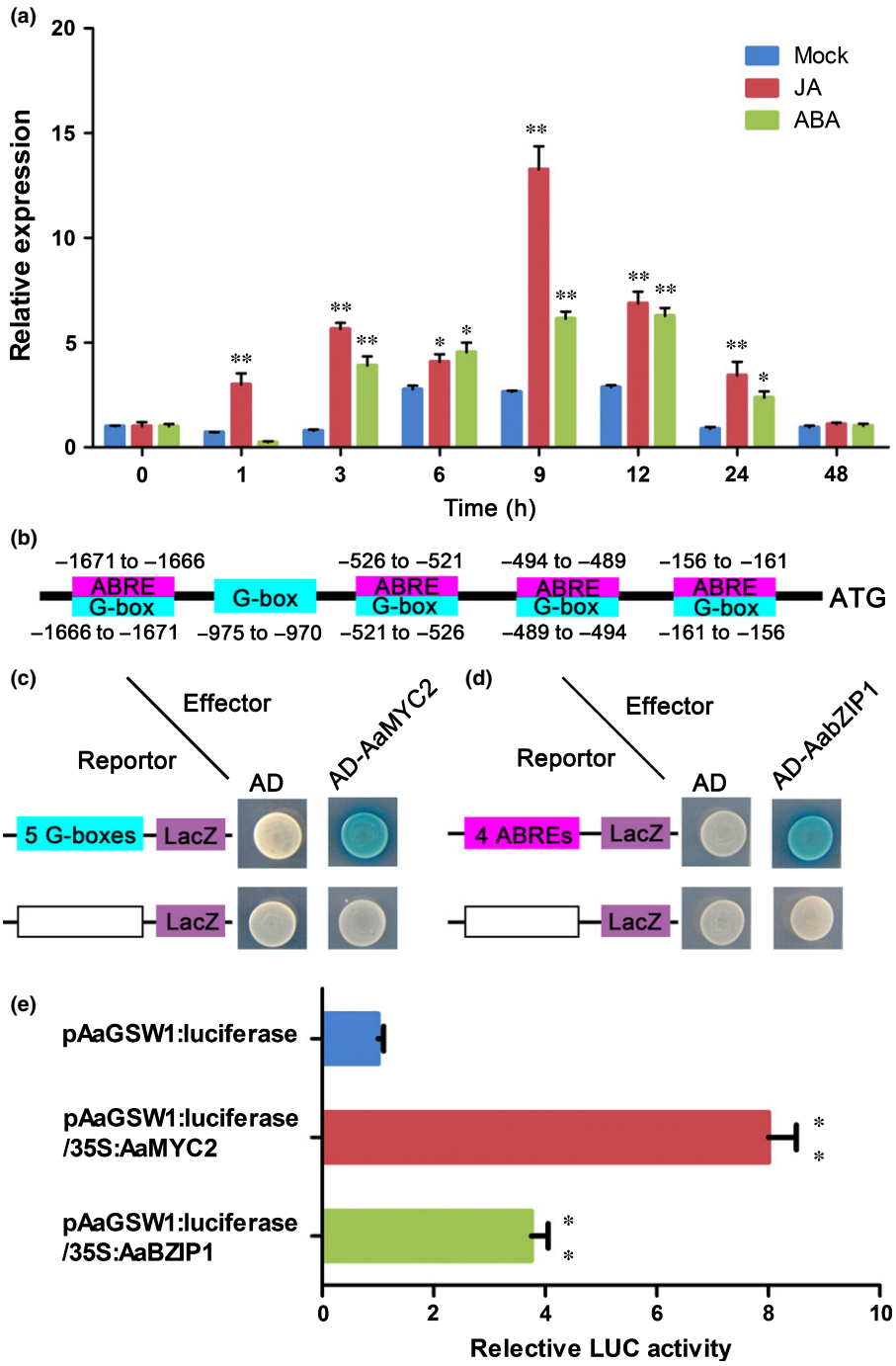

Fig. 7 Regulation of jasmonate (JA) and abscisic acid (ABA) on *AaGSW1* expression. (a) The expression of *AaGSW1* was induced by JA and ABA treatments determined by quantitative real-time PCR (qRT-PCR). Water was used as mock. Actin served as an internal reference. (b) Schematic structure of G-boxes and ABREs in the *AaGSW1* promoters. G-boxes are marked in aquamarine frames, and ABREs are marked in rose red frames. The numbers of scale plates indicate the location of these motifs upstream from the transcription start site. (c) AaMYC2 binds to the combined five G-boxes of the *AaGSW1* promoter. Empty vectors were used as a control. (d) AabZIP1 binds to the combined four ABREs of the *AaGSW1* promoter. Empty vectors were used as a control. (e) The *AaGSW1* promoter was fused to the luciferase (LUC) reporter and the promoter activity was determined by a transient dual-LUC assay in tobacco. The relative LUC activity was normalized to the reference Renilla (REN) luciferase. Effects of AaMYC2 and AabZIP1 on activities of the *AaGSW1* promoter. Error bars indicate SD ($n = 3$). Student's *t*-test: *, $P < 0.05$; **, $P < 0.01$.

Furthermore, expression of *AaGSW1* was significantly increased in the AaMYC2 (Fig. S7a) and AabZIP1 (Fig. S7b) overexpression lines, respectively. These results reveal that JA promotes AaGSW1 expression via AaMYC2, and ABA promotes AaGSW1 expression via AabZIP1.

To further investigate the role of AaGSW1 in the JA- and ABA-mediated regulation of artemisinin biosynthesis, we treated the *AaGSW1*-overexpressing transgenic lines with solutions of MeJA and ABA. Upon MeJA treatment, the expression of *AaGSW1* was increased by *c.* 14- to 16-fold in *AaGSW1*-overexpressing plants in comparison with the control plant (Fig. S8a). Expression of *ADS*, *CYP71AV1*, *DBR2*, *ALDH1* and *AaORA* was also strongly increased by about four- to seven-fold in *AaGSW1*-overexpressing plants when compared with the control plant (Fig. S8b–f). The content of artemisinin in control *A. annua* was increased by 18%, upon MeJA treatment, while that in AaGSW1-overexpressing *A. annua* was increased by 37–50% (Fig. S9); Upon ABA treatment, the expression level of *AaGSW1* was increased by *c.* 15- to 18-fold in *AaGSW1*-overexpressing plants in comparison with the control plant (Fig. S8a). The expression levels of *ADS*, *CYP71AV1*, *DBR2*, *ALDH1* and AaORA were also strongly induced by about

four- to six-fold in *AaGSW1*-overexpressing plants when compared with control plant (Fig. S8b–f). The content of artemisinin in control *A. annua* was increased by 16%, upon ABA treatment, while that in *AaGSW1*-overexpressing *A. annua* was increased by 39–43% (Fig. S9). Thus, the *AaGSW1*-overexpressing lines were more sensitive to JA or ABA treatments than control plants.

Discussion

Artemisinin is biosynthesized in *A. annua* via a glandular trichome-specific metabolic pathway (Olofsson *et al.*, 2012). The four key genes *ADS*, *CYP71AV1*, *DBR2* and *ALDH1* of the artemisinin biosynthetic pathway were identified to have the trichome-specific expression patterns as reported by the promoter–GUS fusion assays (Wang *et al.*, 2011, 2012, 2016; Jiang *et al.*, 2014). Although a few transcription factors that regulate artemisinin biosynthesis have been identified, knowledge about how this regulatory network functions is far from complete (Shen *et al.*, 2016b). Therefore, the exploration of new transcription factors (especially glandular trichome-specific ones) would facilitate enrichment of the regulatory network of artemisinin biosynthesis. In an earlier report, the trichome-specific AP2/ERF transcription factor *AaORA* was selected as exhibiting similar expression patterns to *ADS*, *CYP71AV1* and *DBR2* in different tissues and at different positions of the leaves of *A. annua*, and further identified by promoter–GUS fusion assay (Lu *et al.*, 2013). Based on this, we found that the WRKY transcription factor AaGSW1 was also coexpressed with *ADS* and *AaORA* in different tissues, at different positions of the leaves and trichomes of *A. annua* (Fig. 3a–l). Subsequently, GUS staining of *AaGSW1* promoter–GUS transgenic plants shows that AaGSW1 is a glandular trichome-specific transcription factor (Fig. 5a–d), explaining why *AaGSW1* shows a similar expression pattern to *ADS* and *AaORA*. As artemisinin is specifically synthesized in GSTs (Olofsson *et al.*, 2012), AaGSW1 may play an important role in regulating artemisinin biosynthesis.

Our results demonstrate that AaGSW1 binds to and activates the *CYP71AV1* promoter both *in vitro* and *in vivo* (Fig. 4a–c). Subsequently, the *AaGSW1*-overexpressing transgenic lines were generated and analysed. Expression of *AaGSW1* was significantly increased in *AaGSW1*-overexpressing transgenic lines, as well as the transcript levels of *ADS*, *CYP71AV1*, *DBR2* and *ALDH1* (Fig. 6a). As expected, in *AaGSW1*-overexpressing transgenic lines, artemisinin and DHAA contents were increased by 55–100 and 50–58%, respectively (Fig. 6c). As AaGSW1 cannot bind to or activate *ADS*, *DBR2* and *ALDH1* promoters *in vitro* and *in vivo* (Fig. S2a–d), AaGSW1 may activate their expressions via an indirect means. We then found that the expression level of AaORA, a trichome-specific positive regulator of artemisinin biosynthesis (Lu *et al.*, 2013), was significantly increased in *AaGSW1*-overexpressing transgenic lines (Fig. 6a), and was directly regulated by AaGSW1 (Fig. 6b). Therefore, AaGSW1 could activate *ADS* and *DBR2* expressions through AaORA. Furthermore, the density of GSTs showed no significant difference between AaGSW1-overexpressing and control plants (Fig. S6a,b). Hence we identified that AaGSW1 activates artemisinin

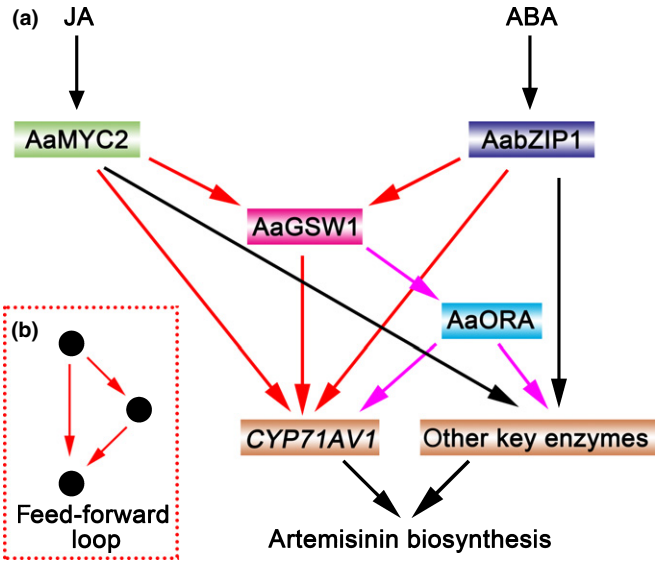

Fig. 8 Regulation of artemisinin biosynthesis genes by jasmonate (JA) and abscisic acid (ABA) signalling pathways. (a) Under JA treatment, AaMYC2 activates the expression of *CYP71AV1* by forming a coherent feed-forward loop with the glandular trichome-specific WRKY transcription factor AaGSW1. Under ABA treatment, AabZIP1 activates the expression of *CYP71AV1* by forming a coherent feed-forward loop with AaGSW1. The red line indicates the two coherent feed-forward loops. After induction by JA or ABA, AaGSW1 activates expression of the trichome-specific AP2/ERF transcription factor AaORA, and AaORA then promotes the expressions of CYP71AV1 and other key artemisinin biosynthetic enzymes. Therefore, the biosynthesis of artemisinin is improved. (b) A sample coherent feed-forward loop.

biosynthesis through a direct CYP71AV1-dependent pathway and an indirect AaORA-dependent pathway (Fig. 8a).

Although we have determined how AaGSW1 promotes *ADS*, *CYP71AV1* and *DBR2* expressions, why ALDH1 expression increased in *AaGSW1*-overexpressing transgenic lines remains unknown, as AaORA is reported to have no influence on *ALDH1* expression (Lu *et al.*, 2013). Therefore, there must be other transcription factors regulated by AaGSW1 to promote *ALDH1* expression, which needs further exploration.

AaGSW1 is a small WRKY transcription factor composed of 183 amino acids, and clusters with the 145 amino acid *Arabidopsis* AtWRKY75, which suppresses root hair development (Rishmawi *et al.*, 2014), positively regulates leaf senescence (Li *et al.*, 2012), and acts as a modulator of phosphate acquisition and a positive regulator of disease defence (Devaiah *et al.*, 2007; Encinas-Villarejo *et al.*, 2009; Chen *et al.*, 2013; Lei *et al.*, 2014). Consistent with its function, *AtWRKY75* is expressed highly in root and old leaves of *Arabidopsis*. However, AaGSW1 is highly expressed in young leaves but relatively low in root and old leaves of *A. annua*, indicating that AaGSW1 and AtWRKY75 may perform distinct roles in the two plant species. Here we find that AaGSW1 is a glandular trichome-specific transcription factor and plays a positive role in regulating artemisinin biosynthesis, thereby indicating the functional diversity among *AaGSW1* (*AtWRKY75*) genes in different plant species. We found that the AaWRKY75b also clusters with AtWRKY75, and may be a

trichome-specific WRKY transcription factor, too. However, no connection is found between AaWRKY75b and the artemisinin biosynthetic pathway (Figs 4a–c, S2b–d). Therefore, the function of AaWRKY75b in trichomes is worthy of further investigation.

JA and ABA are two phytohormones that can be used efficiently to improve artemisinin content in *A. annua* (Zhang *et al.*, 2015; Shen *et al.*, 2016a). They positively regulate artemisinin biosynthesis via AaMYC2 or AabZIP1, respectively. However, the connection between JA and ABA signalling in regulating artemisinin biosynthetic pathways remains unknown. Here we find that the promoter of *AaGSW1* can be bound and activated by both AaMYC2 and AabZIP1 (Fig. 7c–e), and its expression can be induced by both JA and ABA treatments (Fig. 7a). Furthermore, AaGSW1, AaMYC2 and AabZIP1 can all directly bind to and activate the promoter of *CYP71AV1*, thus forming two coherent feed-forward loops in regulating *CYP71AV1* expression (Fig. 8a,b). This kind of coherent feed-forward loop is the commonest coherent configuration in transcription network databases and may improve the robustness of biological signalling processes (Mangan & Alon, 2003). Consequently, AaGSW1 is involved in the crosstalk between JA and ABA signalling in regulating the artemisinin biosynthetic pathway. Considering there are many *cis*-elements that may be recognized by transcription factors regulated by other phytohormones in the promoter of *AaGSW1* (Fig. S3), AaGSW1 may regulate the crosstalk between JA/ABA and other phytohormones in the regulation of artemisinin biosynthesis and form more feed-forward loops with other transcription factors.

Our results showed that artemisinin content in *AaGSW1* overexpression plants was increased much more than that in control plants after MeJA or ABA treatments (Fig. S9). This may be because, in our *AaGSW1* overexpression plants, *AaGSW1* was driven by the *CYP71AV1* promoter, which was highly expressed in GSTs and strongly induced by MeJA or ABA. Our results demonstrate that AaGSW1 binds to and activates the *CYP71AV1* promoter both *in vitro* and *in vivo* (Fig. 4a–c). Consequently, the *CYP71AV1* promoter-driven *AaGSW1* forms a positive feedback loop which significantly promotes the transcript level of *AaGSW1* (Fig. S8a), and then the expression levels of *AaORA*, *ADS*, *CYP71AV1*, *DBR2* and *ALDH1* are increased (Fig. S8b,c). This positive feedback is greatly enhanced under MeJA or ABA treatments. These results demonstrate that *AaGSW1*, especially the CYP71AV1 promoter-driven *AaGSW1*, is useful for engineering artemisinin biosynthesis for enhanced artemisinin yield in *A. annua*.

Data deposition

Sequence data in this article are available as follows: AaGSW1 (KX465128), AaWRKY75b (KX465129), pAaGSW1 (KX465140), AaMYC2 (KP119607), AaORA (JQ797 708).

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Author contributions

K.T. and M.C. planned and designed the research. M.C., T.Y., Q.S., Y.H., X.F., M.L. and W.J. performed experiments. M.C. and T.Y. conducted fieldwork. M.C., T.Y., Q.S., X.L., Q.P., Y.T., Z.L., P.S., Y-n.M., X.H., L.Z. and L.L. analysed data. M.C. and T.Y. wrote the manuscript.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 Trichomes isolated from 10-d-old flower buds.

Fig. S2 Regulation of AaGSW1 and AaWRKY75b on the *ADS* and *DBR2* promoter.

Fig. S3 Nucleotide sequence of the cloned *AaGSW1* promoter with putative *cis*-acting elements shown.

Fig. S4 GUS staining of mature leaves.

Fig. S5 DHAA content in *pCYP-AaGSW1* plants.

Fig. S6 The trichome density comparison between *pCYP-AaGSW1* and control plants.

Fig. S7 *AaGSW1* expression in *AaMYC2-* or *AabZIP1-*overexpressing plants.

Fig. S8 Relative expression of *AaGSW1*, *ADS*, *CYP71AV1*, *DBR2*, *ALDH1* and *AaORA* in *AaGSW1*-overexpressing *A. annua* plants after MeJA and ABA treatments.

Fig. S9 Artemisinin contents in *AaGSW1*-overexpressing *A. annua* plants after MeJA and ABA treatments.

Table S1 Primers used in this study

Table S2 Fragments used to construct yeast one-hybrid vectors and as probe in EMSA in this study

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