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ORIGINAL ARTICLE

AaWRKY4 upregulates artemisinin content through boosting the expressions of key enzymes in artemisinin biosynthetic pathway

Huizhen Huang[1] · Shihai Xing[2,3] · Kexuan Tang[2] · Weimin Jiang[1,2]

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Abstract

Artemisinin is widely used as an antimalarial drug, and the regulation of artemisinin metabolism is at the forefront of artemisinin research. A WRKY transcription factor, named as *AaWRKY4*, was cloned from high artemisinin-yielding *Artemisia annua*, which has similar expression pattern with the key enzymes in artemisinin biosynthetic pathway. *AaWRKY4* was preferentially expressed in glandular secretory trichomes (GSTs) of young leaves and flower buds, but weakly expressed in other tissues. To further study the function of *AaWRKY4*, plant expression vector pHB-AaWRKY4 containing *AaWRKY4* driven by CaMV 35S promoter was constructed and introduced into *A. annua* via *Agrobacterium tumafeciens*-mediated transformation. Expression analysis showed that the expression of *AaWRKY4* was increased in transgenic plants. Four independent transgenic plants overexpressing *AaWRKY4* were selected for further analysis. The expression levels of artemisinin biosynthetic pathway genes *ADS*, *CYP71AV1*, *DBR2* and *ALDH1* were dramatically increased in *AaWRKY4*-overexpressing *A. annua* plants. Furthermore, the artemisinin yield was increased by 35–50% in *AaWRKY4*-overexpressing *A. annua* plants. These results indicate AaWRKY4 can upregulate artemisinin content through regulating artemisinin metabolism.

Key message

A WRKY transcription factor, named as AaWRKY4, was cloned from high artemisinin-yielding*Artemisia annua*. AaWRKY4 was preferentially expressed in glandular secretory trichomes (GSTs)of young leaves and flower buds. The expression levels of artemisinin biosynthetic pathway genes*ADS, CYP71AV1, DBR2* and *ALDH1* were dramatically increased in AaWRKY4-overexpressing *A.annua* plants. Furthermore, the artemisinin yield was increased by 35–50% in AaWRKY4-overexpressing *A. annua* plants.

Keywords *Artemisia annua* L. · Artemisinin · Secondary metabolism · Transcription factor · AaWRKY4

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Huizhen Huang and Shihai Xing contributed equally to this work.

✉ Weimin Jiang
wmjiang@hynu.edu.cn

[1] Hunan Key Laboratory for Conservation and Utilization of Biological Resources in the Nanyue Mountainous Region, College of Life Sciences and Environment, Hengyang Normal University, Hengyang 421008, Hunan, China

[2] Joint International Research Laboratory of Metabolic and Developmental Sciences, Plant Biotechnology Research Center, Fudan-SJTU-Nottingham Plant Biotechnology R&D Center, School of Agriculture and Biology, Shanghai Jiao Tong University, Shanghai 200240, China

[3] Institute of Traditional Chinese Medicine Resources Protection and Development, College of Pharmacy, Anhui University of Chinese Medicine, Hefei 230012, China

Abbreviations

GSTs	Glandular secretory trichomes
DMAPP	Dimethylallyl diphosphate
IPP	Isopentenyl diphosphate
MVA pathway	Mevalonate pathway
MEP pathway	Nonmevalonate pathway
ADS	Amorpha-4, 11-diene synthase
CYP	Cytochrome P450 enzyme CYP71AV1
DBR2	Artemisinic aldehyde Δ11 (13) reductase
ALDH1	Aldehyde dehydrogenase

Introduction

Artemisia annua, also named Huang Hua Hao, is an important Chinese medicinal plant according to the Chinese materia medica (Hsu 2006; Miller et al. 2011). Artemisinin,

extracted from *A. annua*, was discovered by Youyou Tu and used in the treatment of malaria, which has saved millions of lives all over the world (Miller et al. 2011). In addition, some other functions of artemisinin such as inducing apoptosis in human cancer cells were reported (Singh et al. 2004; Romero et al. 2005). As the genome of *A. annua* was sequenced recently, the *A. annua* genome and transcriptome data are quite valuable for fundamental biological research and applied breeding programs (Shen et al. 2018).

The artemisinin biosynthetic pathway in *A. annua* was studied for many years and it was almost completed (Covello 2008). The precursors of artemisinin biosynthesis dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP) were formed from both mevalonate pathway (MVA pathway) and nonmevalonate pathway (MEP pathway). IPP and DMAPP form farnesyl diphosphate (FPP) by farnesyl diphosphate synthase, which is the common precursor of sesquiterpenes (Towler 2007). Amorpha-4, 11-diene synthase (ADS), the first key enzyme in the artemisinin biosynthetic pathway, can convert FPP to amorpha-4, 11-diene (Bouwmeester et al. 1999; Wallaart et al. 2001; Picaud et al. 2005). Through cytochrome P450 enzyme CYP71AV1 (CYP), amorpha-4, 11-diene is oxidized to artemisinic alcohol, and is further oxidized to artemisinic aldehyde (Teoh et al. 2006). Futhermore, through artemisinic aldehyde Δ11 (13) reductase (DBR2) encoding a member of the enoate reductase family with similarity to plant 2-oxophytodienoate reductases, artemisinic aldehyde is reduced to dihydroartemisinic aldehyde (Zhang et al. 2008). Through an aldehyde dehydrogenase (ALDH1), dihydroartemisinic acid (DHAA) is formed (Teoh et al. 2009). The process from DHAA to artemisinin is still not clear now. It is considered to be a spontaneous non-enzymatic reaction (Sy et al. 2002; Covello 2008). However, it was also reported that peroxidase enzymes or an alternative series of oxidations might catalyze the last reaction (Bryant et al. 2015).

Transcription factors play an important role in the regulation of plant secondary metabolism. Transcriptional regulation of key enzymes in plant secondary metabolism is an important regulatory process, and regulation of biosynthetic pathways is often achieved through specific transcription factors. These transcription factors include bHLHs, MYBs, WRKYs, ERFs and other transcription factor families, which are widely involved in many secondary metabolic processes, including the synthesis of pigments, terpenoids, and alkaloids (Broun 2004). Artemisinin is an important sesquiterpene extracted from *A. annua*, and several transcription factors have been cloned and proved to have functions on secondary metabolism, especially artemisinin metabolism in *A. annua*. Ma et al. cloned a transcription factor AaWRKY1, which was preferentially expressed in GSTs. Biochemical tests showed that AaWRKY1 could activate the expression of *ADS* (Ma et al. 2009). Han et al. transformed AaWRKY1 into *A. annua*, and the results showed that trichome-specific overexpression of AaWRKY1 can significantly increase content of the artemisinin in the transgenic plants, up to about 1.8 times (Han et al. 2014). A bHLH transcription factor AabHLH1 can regulate the expression of key enzyme genes in the artemisinin biosynthetic pathway of *A. annua*, thus regulating the synthesis of artemisinin (Ji et al. 2014). AaORA transcription factor belongs to the AP2/ERF transcription factor family. Overexpression of AaORA resulted in significantly increased expression of key enzyme genes in the artemisinin biosynthetic pathway, as well as the artemisinin content in transgenic plants, while artemisinin content decreased with RNAi interference of AaORA (Lu et al. 2013). Shen et al. cloned AaMYC2 in *A. annua*, an important transcription factor in Jasmonic acid signal transduction pathway. AaMYC2 can bind to the promoter of key enzymes *CYP71AV1* and *DBR2*. Excessive expression of AaMYC2 can increase the content of artemisinin (Shen et al. 2016).

AaMYB1 was also cloned by our group, which positively affects artemisinin biosynthesis and other related processes (Matias-Hernandez et al. 2017). The molecular mechanisms how AaMBY1 control artemisinin metabolism is still unclear. In this study, a transcription factor AaWRKY4 was isolated from AaMYB1 overexpressed transgenis plants, in which the expression level of AaWRKY4 was obviously increased compared with the control. The results show that AaWRKY4 regulates artemisinin metabolism by regulating the expression of key enzyme genes in the artemisinin biosynthesis pathway. It can aslo further reveal the molecular mechanisms how AaMBY1 control artemisinin biosynthesis.

Materials and methods

Plant materials and growth conditions

The seeds of *A. annua* were obtained from Southwest University, Chongqing, China. Seeds were sterilized and germinated on Murashige and Skoog (MS) medium under a 16 h light/8 h dark photoperiod, providing 40–60 μmol m^{-2} s^{-1} light intensity, at 23 ± 1 °C. The seedling was transferred to greenhouse in Shanghai Jiao Tong University after 10 days.

Reverse transcription PCR

Total RNA was extracted from different tissues of *A. annua* using RNAprep pure Plant Kit (Tiangen Biotech, China) according to instructions. The total RNA samples acquired above were employed in first-strand synthesis of cDNA by PrimeScript RT Master Mix (TaKaRa, Japan) according to the instructions. The expression of genes was carried out by quantitative reverse transcription PCR (qPCR) using the

fluorescent intercalating dye SYBR Green (TaKaRa, Japan). *AaActin1* was used as the control in the qPCR analysis.

Bioinformatics analysis

Nucleotide acid sequences and protein sequences in this study were analyzed using Vector NTI software (Invitrogen, USA). Bioinformatics analysis of *AaWRKY4* and other genes was performed online at the NCBI database (http://www.ncbi.nlm.nih.gov/) and EBI database (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

A. annua transformation and PCR analysis

pHB-AaWRKY4 was constructed by amplifying the gene with the specific primers AaWRKY4-up and AaWRKY4-down (Table 1), and then excising the amplified sequence with *Bam*H I and *Sac* I. pHB vector was digested with the same enzymes. The construct was introduced into *A. tumefaciens* strain EHA105. The *A. annua* plant transformation was carried out as previously described [21]. Hygromycin B-resistant plants were regenerated by *A. tumefaciens*-mediated transformation. After genomic DNAs were extracted by CTAB method, *AaWRKY4*-overexpressing *A. annua* plants were identified from regenerated *A. annua* plants by PCR analysis.

Protein subcellular localization

The *AaWRKY4* cDNAs were recombined into pEarly-Gateway104 vector (pEG104) to generate pEG104-*AaWRKY4*.

Table 1 Primers used in this study

Primers	Primer sequences
Actin-RT-up	CCAGGCTGTTCAGTCTCTGTAT
Actin-RT-down	CGCTCGGTAAGGATCTTCATCA
ADS-RT-up	GGGAGATCAGTTTCTCATCTATGAA
ADS-RT-down	CTTTTAGTAGTTGCCGCACTTCTT
CYP-RT-up	CGAGACTTTAACTGGTGAGATTGT
CYP-RT-down	CGAAGTGACTGAAATGACTTTACT
CPR-RT-up	GCTCGGAACAGCCATCTTATTCTT
CPR-RT-down	GAAGCCTTCTGAGTCATCTTGTGT
DBR2-RT-up	GCGGTGGTTACACTAGAGAACTT
DBR2-RT-down	ATAATCAAAACTAGAGGAGTGACCC
ALDH1-RT-up	CATCGGAGTAGTTGGTCACAT
ALDH1-RT-down	GGAGTATGTTCGGCAGGCTT
AaWRKY4-up	ATGGCCGTTGATCTCATCATGT
AaWRKY4-down	TTAAGATGATTCTAATATAAGTCC
rbc-48A	GCATTGAACTTGACGAACGTTGTCGA
AaWRKY4-RT-up	CAAGTACCGTTTGTTCCTCCAC
AaWRKY4-RT-down	TGTTTACCGTCAGAATCACCAG

The AaWRKY4-YFP fusion constructs were used for transient expression in tobacco as described previously [22]. After 2–5 days, the fluorescence was imaged by confocal microscope.

HPLC measurement

For each sample, 20 µL of filtrate was injected into the HPLC sample injector. The samples were determined using a Waters Alliance 2695 high-performance liquid chromatography system in series with a 2420 ELSD detector (Milford, USA). 60% methanol was used as a mobile phase. Filtrate passed through 5 µm C18 column at a flow rate of 1 mL/min. The content of the measured substance in the sample was calculated according to the concentration and peak area of the standard substance. Artemisinin content was indicated as mg/g dry weight. Three biological replicates were measured for each sample.

Results

Cloning of AaWRKY4 transcription factor

Using qPCR method to analyze the expression of transcription factors WRKYs, ERFs and bHLHs which were co-expressed with the key enzyme genes in the artemisinin synthetic pathway, a transcription factor *AaWRKY4* from the WRKY transcription factor family was obtained by analyzing *A. annua* genome and transcriptome data already acquired in the transgenic plants overexpressing *AaMYB1* [20].

By comparing the transcription factors AaWRKY4 and Arabidopsis WRKY transcription factor family, AaWRKY4 and AtWRKY15 were found to have the closest evolutionary relationship (Fig. 1). AtWRKY15 is a member of group IId subfamily of WRKY transcription factors. AaWRKY4 may also belong to the same group.

Sequence analysis of AaWRKY4

The protein structure of AaWRKY4 was analyzed. The ORF of AaWRKY4 contains 316 amino acids. AaWRKY4 contains only one WRKY structural domain, and the zinc finger structure is C2H2, belonging to the WRKY Group II subfamily. Through protein structure analysis, it was further confirmed that AaWRKY4 belongs to WRKY Group IId subfamily (Fig. 2).

Expression analysis of *AaWRKY4* in different tissues

The expression level of *AaWRKY4* was detected by qPCR. *AaWRKY4* was preferentially expressed in buds and young

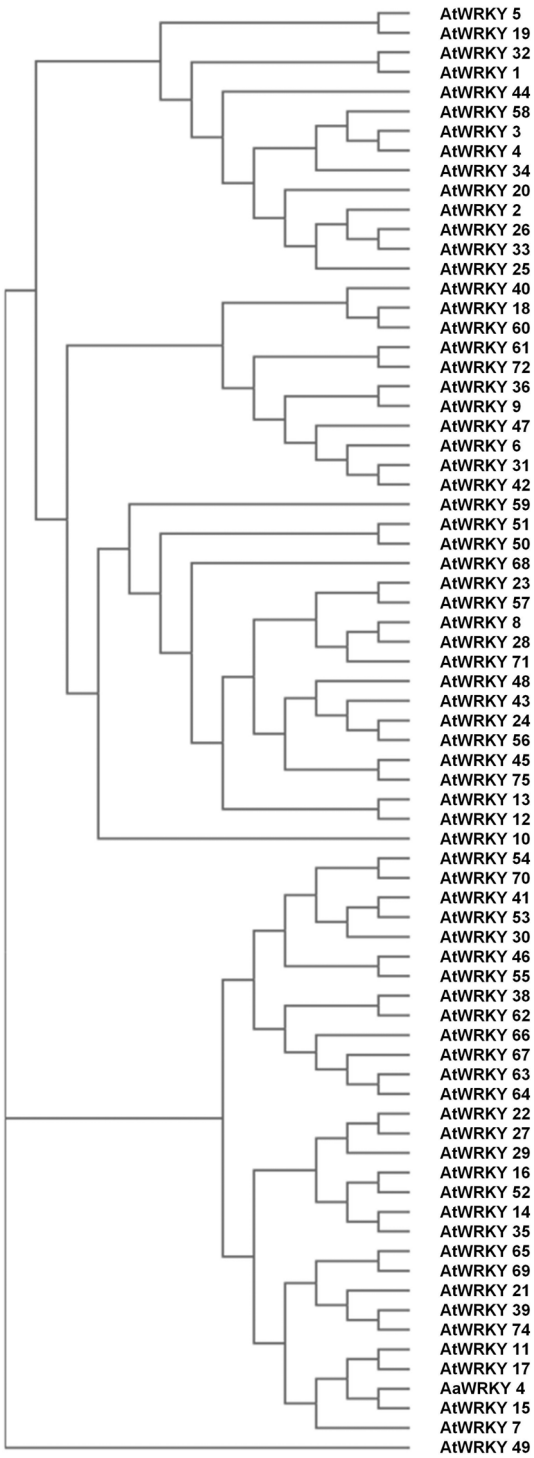

Fig. 1 Phylogenetic tree showing the relationship between AaWRKY4 and Arabidopsis WRKY proteins

leaves, weakly expressed in older leaves and fully developed flowers, and had a quite low expression in roots and stems. The expression level of *AaWRKY4* in the bud was about 15.4 times higher than that in the root. This expression pattern is similar to the expression of key enzymes including *ADS*, *CYP71AV1*, *DBR2* and *ALDH1* in the artemisinin biosynthetic pathway (Fig. 3). At the same time, some transcription factors regulating artemisinin synthesis, such as AaORA, are also highly expressed in flower buds. The expression pattern of *AaWRKY4* suggests that AaWRKY4 may be involved in the metabolic regulation of artemisinin in *A. annua*.

Subcellular localization

In this study, subcellular localization of AaWRKY4 was studied by transient transformation of tobacco. The C-terminal of YFP on the pEG104 vector was fused with AaWRKY4 to form YFP-AaWRKY4 fusion protein. The subcellular localization of AaWRKY4 in tobacco was observed by microscope after transient transformation of tobacco. The results showed that AaWRKY4 was located in the nucleus (Fig. 4).

Identification and qPCR analysis of transgenic *A. annua* plants overexpressing *AaWRKY4*

A. annua was transformed with *A. tumefaciens* containing *pHB-AaWRKY4* construct, and ninety-five plants were obtained by Hygromycin B screening. Using AaWRKY4-up as the upstream primer and rbc-48A as the downstream primer for PCR amplification, the band size of about 1 kb could be amplified. In this study, DNA was extracted from the leaves of transgenic plants 6 weeks after transferred to the soil. Through PCR identification, 47 plants were confirmed as positive transgenic plants. Four independent lines *AaWRKY4-6*, *AaWRKY4-22*, *AaWRKY4-24* and *AaWRKY4-28* were randomly selected from these positive plants for further analysis (Fig. 5).

The four independent transgenic lines were used for expression analysis. The leaves of the same part of transgenic plants were selected as materials for qPCR analysis. The expression analysis of these four independent transgenic lines showed that the expression level of *AaWRKY4* in these transgenic lines was significantly increased compared with that of the control. The expression level of *AaWRKY4* in these transgenic lines was increased by about 19-fold, 22-fold, 21-fold and 15-fold respectively (Fig. 6).

Overexpression of *AaWRKY4* resulted in increased expression of key enzymes in the artemisinin biosynthetic pathway

The expression levels of key enzyme genes in the artemisinin biosynthetic pathway was analyzed. Compared with control plants, the expression levels of *ADS* in transgenic *A. annua* plants is significantly improved, 6.0- to 9.0-fold higher. The expression levels of *CYP71AV1*, *DBR2* and *ALDH1* in transgenic *A. annua* plants were also

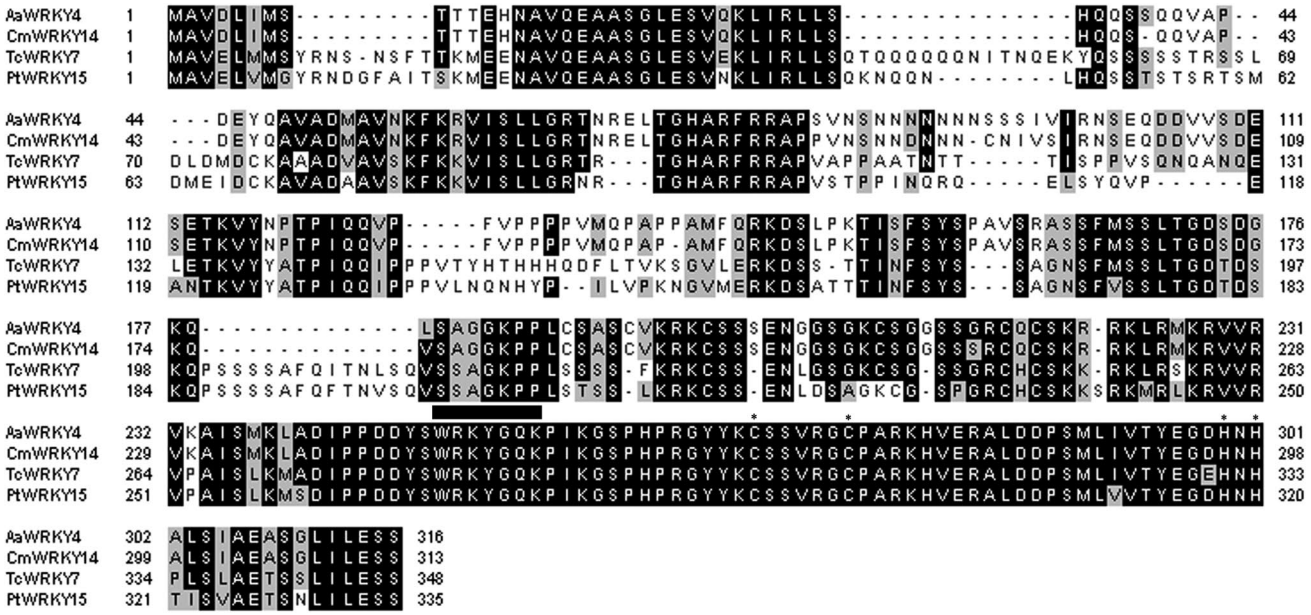

Fig. 2 The protein sequence alignment of AaWRKY4 with other similar WRKY transcription factors in other species. AaWRKY4 (from this research), CmWRKY14, TcWRKY7 and PtWRKY15. The sequences marked with thick black line are conserved WRKYGQK motif, and the sequences marked with * were the cystein and histidine amino acid residues characteristic of the zinc finger motif. The sequences with black background indicate the completely identical residues and the sequences with gray background indicate the similar residues

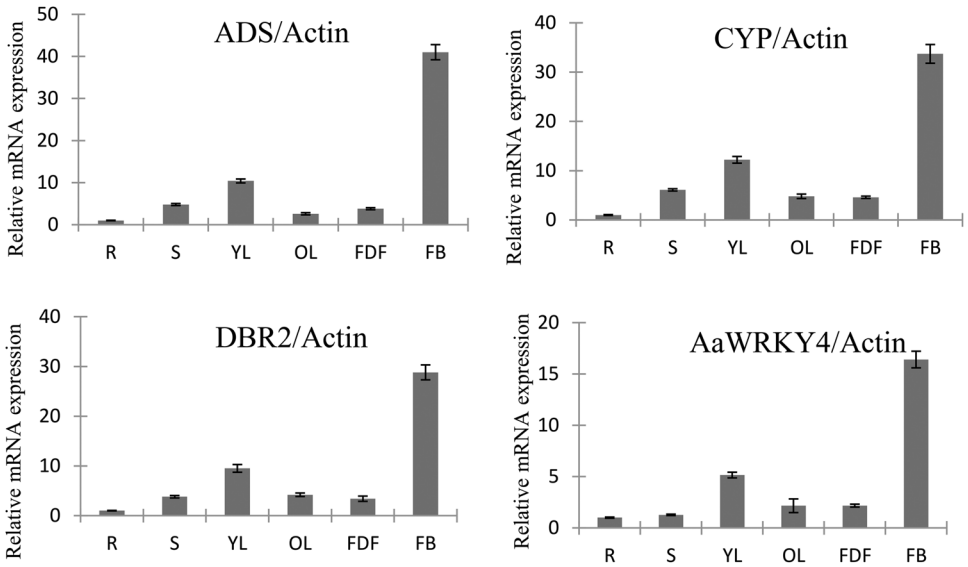

Fig. 3 Expression levels of *AaWRKY4* and key enzyme genes in artemisinin biosynthetic pathway in various tissues of *A. annua* Plants. Expression levels of AaWRKY4, ADS, CYP71AV1 and DBR2 by qPCR in roots (R), stems (S), young leaves (YL), old leaves (OL), flower buds (FB) and fully developed flowers (FDF). Values indicate the mean fold compared with sample R. Three biological repeats were measured for each sample. Error bars are SE (n = 3)

detected. Compared with that in the control plants, the expression of *CYP71AV1* was increased by 6.1- to 9.8-fold, the expression of *DBR2* was increased by 5.2- to 6.6-fold, and the expression of *ALDH1* was increased by 2.0- to 2.7-fold (Fig. 7). AaWRKY4 may regulate expression of key enzymes in the artemisinin biosynthetic pathway, thus regulating the content of artemisinin in plants.

Overexpression of *AaWRKY4* gene resulted in increased artemisinin content

In this study, artemisinin content of *A. annua* was measured by HPLC. Compared with non-transgenic *A. annua* control plants, the content of artemisinin in transgenic plants overexpressing *AaWRKY4* was significantly increased. The

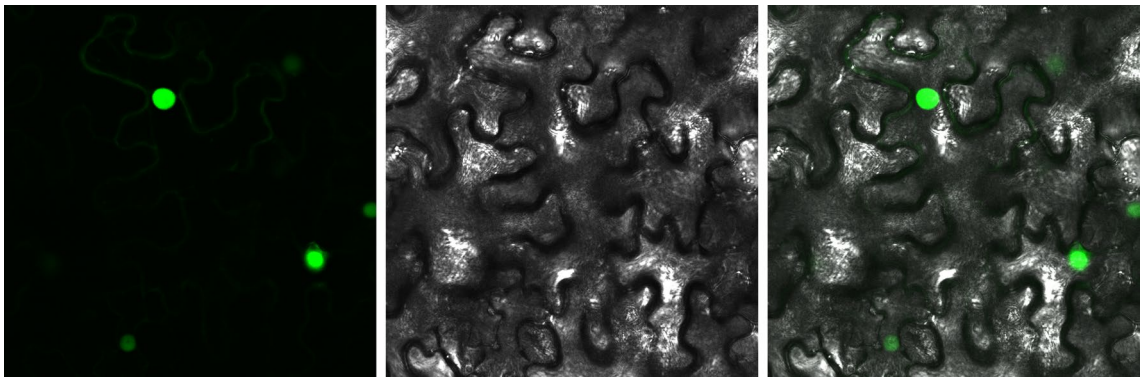

Fig. 4 Nuclear localization of AaWRKY4 in tobacco leaves. The left panel shows the fluorescence image; the middle panel shows the bright image; the right panel shows the merged image of the fluorescence image and the bright image

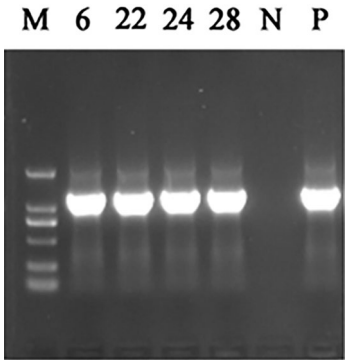

Fig. 5 PCR analysis of transgenic plants overexpressing *AaWRKY4*. Forward primer AaWRKY4-up and reverse primer rbc-48A were used in PCR analysis. M: DNA size marker DL2000; 6, 22, 24, and 28: independent lines of transgenic plants overexpressing *AaWRKY4*; N: non-transgenic *A. annua* control plant; P: positive plasmid control

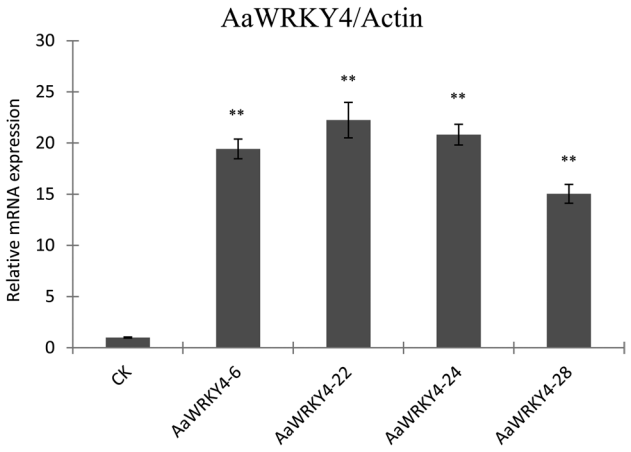

Fig. 6 Expression analysis of *AaWRKY4* in transgenic plants overexpressing *AaWRKY4*. Expression analysis of *AaWRKY4* in transgenic plants overexpressing *AaWRKY4*. AaWRKY4-6, AaWRKY4-22, AaWRKY4-24 and AaWRKY4-28: independent lines of transgenic plants overexpressing AaWRKY4. Three biological repeats were measured for each sample. Statistical significance was determined by Student's t-test (**P < 0.01)

content of artemisinin in the four independent transgenic lines AaWRKY4-6, AaWRKY4-22, AaWRKY4-24 and AaWRKY4-28 were increased by 48%, 50%, 48% and 35% respectively (Fig. 8).

Discussion

Transcription factors play an important role in the regulation of artemisinin metabolism. Transcription factors can regulate the expressions of key enzymes in artemisinin biosynthetic pathway (Ma et al. 2009; Yu et al. 2012; Lu et al. 2013; Ji et al. 2014; Zhang et al. 2015; Shen et al. 2016; Chen et al. 2016; Jiang et al. 2016). Transcription factors can also regulate trichome development (Tan et al. 2015; Yan et al. 2016, 2018; Shi et al. 2017; Wang et al. 2019). AaMYB1 can regulate the artemisinin biosynthetic pathway by regulating the expressions of key enzymes in artemisinin biosynthetic pathway, and also regulate trichome development [20]. Although AaMYB1 has been found to regulate the artemisinin biosynthesis, the mechanism is not well understood. In this study, based on transgenic plants overexpressing *AaMYB1*, a WRKY transcription factor, AaWRKY4, which has similar expression patterns to key enzyme genes in the artemisinin biosynthetic pathway, was identified. *AaWRKY4* was preferentially expressed in buds and young leaves, and had low or no expression in other tissues, which was the same as the expression pattern of key enzyme genes in the artemisinin biosynthetic pathway. According to previous studies, the key enzyme genes in the artemisinin biosynthetic pathway are mainly expressed in the GSTs of flower buds and young leaves. The results above indicate that AaWRKY4 may be involved in artemisinin metabolism regulation.

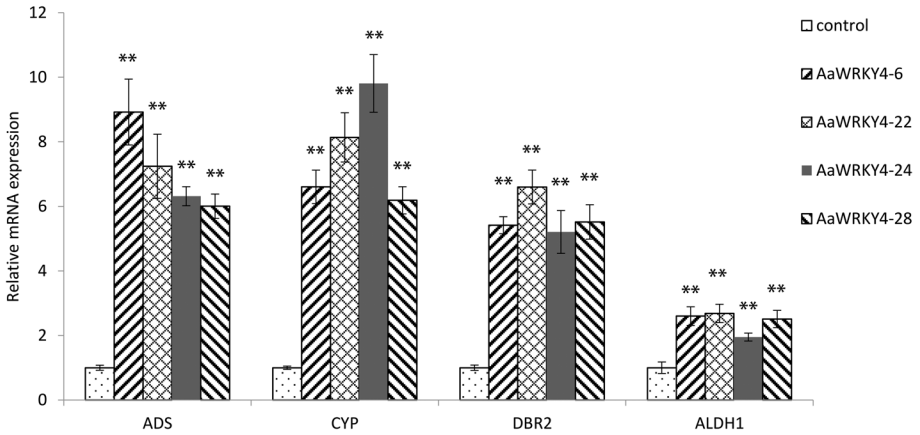

Fig. 7 Expression analysis of key enzyme genes in artemisinin biosynthetic pathway in transgenic plants overexpressing *AaWRKY4*. Expression analysis of the key enzyme genes in artemisinin biosynthetic pathway by qPCR in transgenic plants overexpressing *AaWRKY4*. AaWRKY4-6, AaWRKY4-22, AaWRKY4-24 and AaWRKY4-28: independent lines of transgenic plants overexpressing *AaWRKY4*. Three biological repeats were measured for each sample. Statistical significance was determined by Student's t-test (**P < 0.01)

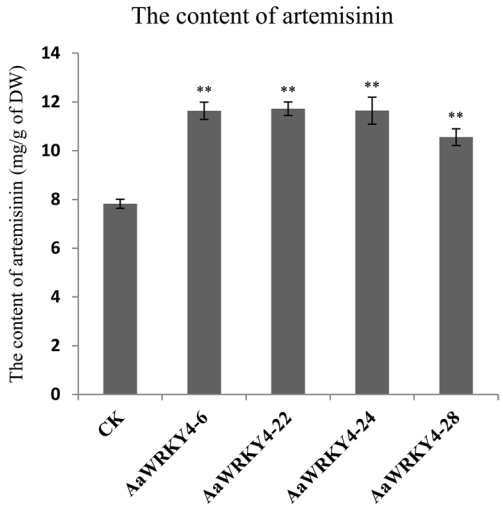

Fig. 8 The content of artemisinin measured in transgenic plants overexpressing *AaWRKY4.* The content of artemisinin measured by HPLC in transgenic plants overexpressing *AaWRKY4*. AaWRKY4-6, AaWRKY4-22, AaWRKY4-24 and AaWRKY4-28: independent lines of transgenic plants overexpressing *AaWRKY4*. Three biological repeats were measured for each sample. Statistical significance was determined by Student's t-test (**P < 0.01)

The results showed that the expression levels of key enzymes in the artemisinin biosynthetic pathway in transgenic plants overexpressing *AaWRKY4*, were significantly increased. At the same time. Compared with *A. annua* control plants, artemisinin content in transgenic plants *overexpressing AaWRKY4* was also significantly increased. Therefore, AaWRKY4 might be involved in the transcriptional regulation network of artemisinin metabolism in *A. annua*. However, the mechanism AaWRKY4 is involved in the regulation of artemisinin metabolism requires further investigation.

In conclusion, AaWRKY4 might be involved in the transcriptional regulation network of artemisinin metabolism in *A. annua*, which has important application value for artemisinin metabolic engineering.

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Author contributions WJ and KT conceived and designed the project. HH and XH conducted the experiments and analyzed the data. HH and WJ drafted the paper. WJ and KT reviewed the manuscript. All authors read and approved the final manuscript.

Data availability The Gene Bank number for AaWRKY4 is MW169031. Sequence data from this article can be found in the Arabidopsis Genome Initiative or the GenBank databases.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Consent to participate Written informed consent was obtained from individual or guardian participants.

Consent for publication Written informed consent for publication was obtained from all participants.

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