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Effects of overexpression of *AaWRKY1* on artemisinin biosynthesis in transgenic *Artemisia annua* plants

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ABSTRACT

The effective anti-malarial medicine artemisinin is costly because of the low content in *Artemisia annua*. Genetic engineering of *A. annua* is one of the most promising approaches to improve the yield of artemisinin. In this work, the transcription factor *AaWRKY1*, which is thought to be involved in the regulation of artemisinin biosynthesis, was cloned from *A. annua* var. Chongqing and overexpressed using the CaMV35S promoter or the trichome-specific CYP71AV1 promoter in stably transformed *A. annua* plants. The transcript level of *AaWRKY1* was increased more than one hundred times under the CaMV35S promoter and about 40 times under the CYP71AV1 promoter. The overexpressed *AaWRKY1* activated the transcription of *CYP71AV1* and moreover the trichome-specific overexpression of *AaWRKY1* improved the transcription of *CYP71AV1* much more effectively than the constitutive overexpression of *AaWRKY1*, i.e. up to 33 times as compared to the wild-type plant. However the transcription levels of FDS, ADS, and DBR2 did not change significantly in transgenic plants. The significantly up-regulated *CYP71AV1* promoted artemisinin biosynthesis, i.e. up to about 1.8 times as compared to the wild-type plant. It is demonstrated that trichome-specific overexpression of *AaWRKY1* can significantly activate the transcription of *CYP71AV1* and the up-regulated CYP71AV1 promotes artemisinin biosynthesis.

Introduction

Malaria, caused by the genus *Plasmodium*, has been a life-threatening disease for thousands of years. There are more than 380 million cases of malaria each year and more than 1 million deaths, according to the report from the World Health Organization (WHO, 2006). The traditional antimalarial medicines have the severe problem of drug resistance. In the 1970s, Chinese scientists found a new effective antimalarial medicine, i.e. artemisinin, which is extracted from the Chinese traditional medicinal herb *Artemisia annua* L. (Collaboration research group for Qinghaosu, 1979). Artemisinin is active against all *Plasmodium* species. Artemisinin and its derivatives, delivered in the form of artemisinin-based combination therapies (ACTs), are currently the best treatment option for malaria (WHO, 2006). Besides the antimalarial activity, artemisinin has antiviral, anticancer, and antischistosomal activity (Arsenault et al., 2008). Unfortunately the artemisinin content in *A. annua* is low, ranging from 0.01 to 1.0% dry wt., which results in a relatively high cost of artemisinin-based treatments. It is a serious problem for economically disadvantaged people in developing countries where malaria frequently occurs. Therefore a number of efforts have been made to enhance the production of artemisinin, including chemosynthesis, cell and tissue culture, plant genetic engineering, synthetic biology, and breeding of high artemisinin yielding plants (Covello, 2008; Graham et al., 2010; Liu et al., 2011). A yeast-based production of artemisinic acid, which can be converted to artemisinin in relatively good yields (40–45%), has been developed recently (Paddon et al., 2013). This process has been scaled up and the commercial production of artemisinin has been announced by Sanofi Aventis (http://www.path.org/news/press-room/422/). The yearly production will be around 50–60 tons of artemisinin, which, however, is not sufficient to cover the demand of this drug. Consequently, the plant will remain an important source for artemisinin.

In the last decade, there has been significant progress in elucidating the pathway of artemisinin biosynthesis (Fig. 1). Artemisinin is synthesized through the isoprenoid metabolic pathway.

Abbreviations: ADS, amorpha-4,11-diene synthase; ALDH1, aldehyde dehydrogenase 1; CaMV, cauliflower mosaic virus; CPR, cytochrome P450 reductase; CYP71AV1, amorpha-4,11-diene 12-hydroxylase; DBR2, artemisinic aldehyde Δ11(13) reductase; FDS, farnesyl diphosphate synthase; GST, glandular secretory trichome; JA, jasmonate; SA, salicylic acid; WRKY, WRKY transcription factor.

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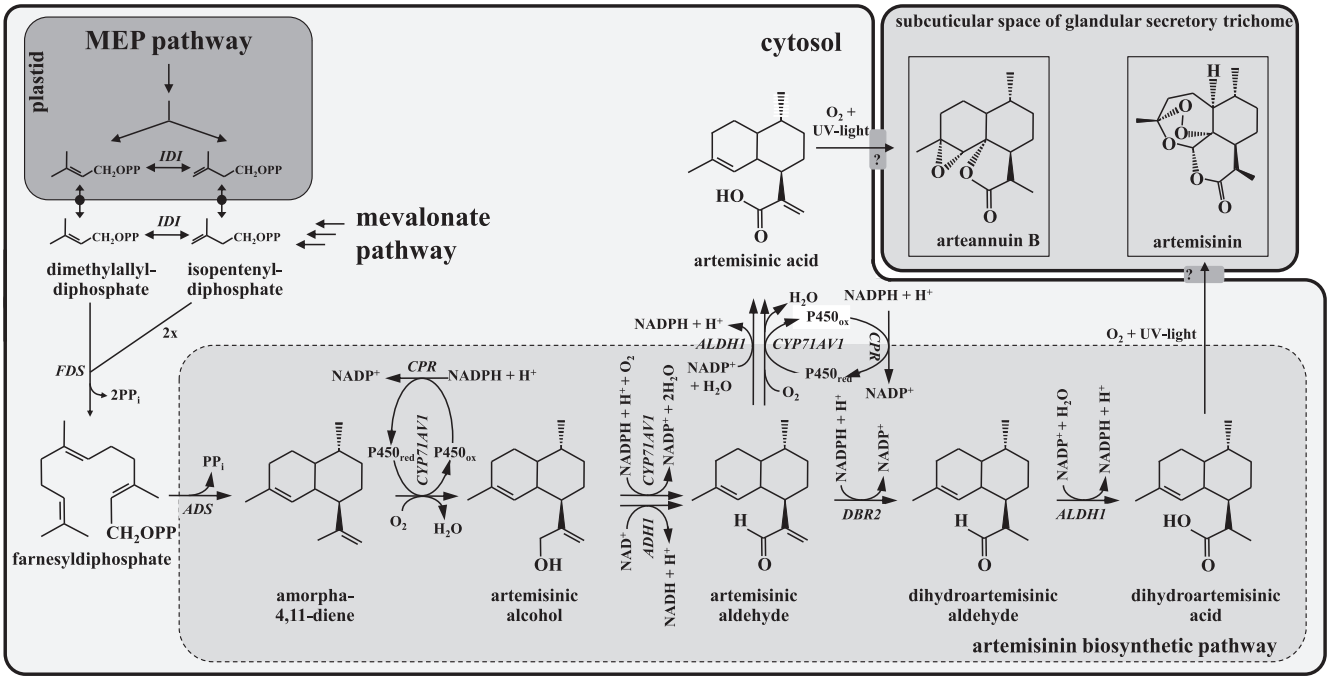

Fig. 1. Artemisinin biosynthetic pathway. FDS: farnesyl diphosphate synthase; ADS: amorpha-4,11-diene synthase; CYP71AV1: amorpha-4,11-diene 12-hydroxylase; CPR: cytochrome P450 reductase; ADH1: alcohol dehydrogenase 1; DBR2: artemisinic aldehyde Δ11(13) reductase; ALDH1: aldehyde dehydrogenase 1.

Cyclization of farnesyl diphosphate to amorpha-4,11-diene by amorpha-4,11-diene synthase (ADS) is the initial step of the artemisinin biosynthesis (Bouwmeester et al., 1999; Mercke et al., 2000; Wallaart et al., 2001). In the following step, a cytochrome P450 dependent amorpha-4,11-diene 12-hydroxylase (CYP71AV1) catalyzes the oxidation of amorpha-4,11-diene to produce artemisinic alcohol, which is oxidized to artemisinic aldehyde by CYP71AV1 and/or alcohol dehydrogenase 1 (ADH1) (Paddon et al., 2013; Teoh et al., 2006). Next, the artemisinic aldehyde is reduced to dihydroartemisinic aldehyde by artemisinic aldehyde Δ11(13) reductase (DBR2) and subsequently oxidized to dihydroartemisinic acid by aldehyde dehydrogenase 1 (ALDH1) (Teoh et al., 2009; Zhang et al., 2008). The artemisinin biosynthetic pathway is branched and the intermediate artemisinic aldehyde may be oxidized to artemisinic acid by CYP71AV1 and/or ALDH1 (Teoh et al., 2006, 2009). The intermediates dihydroartemisinic acid and artemisinic acid are converted to artemisinin and arteannuin B, respectively, in a non-enzymatic reaction (Brown, 2010).

All the genes encoding the enzymes mentioned above have been cloned and genetic engineering of *A. annua* is becoming one of the most promising approaches to improve artemisinin yield. Overexpression of *Gossypium arboretum* or *A. annua* farnesyl diphosphate synthase (FDS) in *A. annua* plants increased the artemisinin content (from 0.3 to 0.9–1.0% dry wt.) (Chen et al., 2000; Han et al., 2006). Overexpression of ADS in *A. annua* plants increased the amounts of artemisinin, dihydroartemisinic acid, and artemisinic acid around 100%, 59%, 65%, respectively, and the absolute content of artemisinin was 0.9–1.0% dry wt. (Ma et al., 2009a). In transgenic *A. annua* plants in which HMGR was overexpressed alone or together with ADS, the artemisinin content was increased 1.22- and 6.65-fold, respectively, compared to the non-transgenic line (artemisinin content 0.02% dry wt.) (Alam and Abdin, 2011; Aquil et al., 2009). In transgenic *A. annua* plants in which the *CYP71AV1* and cytochrome P450 reductase (*CPR*) genes were overexpressed, the artemisinin content was increased 38%, as compared to the non-transgenic line of *A. annua* (700 μg/g fresh weight) (Shen et al., 2012). Since multiple enzymes are involved in artemisinin biosynthesis, it is more appropriate to up-regulate the activities of artemisinin biosynthetic enzymes simultaneously to effectively improve artemisinin synthesis.

Artemisinin synthesis was promoted by jasmonate (JA) and salicylic acid (SA) (Maes et al., 2011; Pu et al., 2009). The transcriptional level of FDS, ADS, CYP71AV1, DBR2, and CPR were increased when *A. annua* plants were treated with JA (Maes et al., 2011). It indicates that a transcriptional control network is involved in artemisinin biosynthesis. Transcription factors play an important role in controlling the transcription of biosynthetic genes and it is a major mechanism regulating secondary metabolite production in plants (Endt et al., 2002). It has been reported that WRKY transcription factor and AP2/ERF transcription factors are involved in artemisinin synthesis (Lu et al., 2013; Ma et al., 2009b; Yu et al., 2011). The WRKY family is among the ten largest families of transcription factors in higher plants. These transcription factors carry the WRKY domain which is a 60-amino acid stretch containing a conserved amino acid sequence of WRKYGQK together with a zinc-finger like motif. WRKY proteins specifically bind to a *cis-acting* DNA element, i.e. the W-box (TTGACC/T) to realize their biological function. The WRKY proteins are involved in regulating defense responses and developmental and physiological processes of plants, such as trichome initiation, senescence, and metabolism (Rushton et al., 2010). In *Arabidopsis*, MPK3/MPK6 signaling leads directly to phosphorylation of AtWRKY33 and then the phosphorylated WRKY protein binds to the promoter of *PAD3*, which encodes a P450 enzyme (CYP71B15) that carries out the last step of camalexin biosynthesis, a major phytoalexin in *A. thaliana* (Mao et al., 2011). CjWRKY1, which was cloned from *Coptis japonica*, plays a specific and comprehensive role in the pathway of benzylisoquinoline alkaloid biosynthesis (Kato et al., 2007). CrWRKY1 positively regulates the terpenoid indole alkaloid biosynthesis in *Catharanthus roseus* (Suttipanta et al., 2011). In cotton plants, the transcription factor GaWRKY1 activates the transcription of the sesquiterpene synthase gene (+)-δ-cadinene synthase-A (CAD1-A) by means of interacting with the W-box element of the CAD1-A promoter (Xu et al., 2004). *AaWRKY1* was isolated from a cDNA library of the glandular secretory trichomes of *A. annua*; AaWRKY1 protein was capable of binding to the

W-box element; transient expression of *AaWRKY1* cDNA in *A. annua* leaves activated the expression of *ADS*, *CYP71AV1* and *DBR2* (Ma et al., 2009b). Recent studies on promoter nucleotide sequences show that there are two putative W-boxes in each of the ADS (Ma et al., 2009b; Wang et al., 2011), CYP71AV1 (Wang et al., 2013), and DBR2 promoters (unpublished). Therefore, it is very interesting to overexpress *AaWRKY1* in stably transformed *A. annua* plants to further explore the function of AaWRKY1 and to improve artemisinin biosynthesis. The biosynthesis of artemisinin has been proposed to be located to glandular secretory trichomes (GSTs) of *A. annua* since the enzymes of artemisinin biosynthesis, i.e. ADS, CYP71AV1, DBR2 and ALDH1, are highly expressed in GSTs (Olsson et al., 2009; Teoh et al., 2006; Teoh et al., 2009; Zhang et al., 2008; Wang et al., 2011, 2013). Our group has recently cloned the CYP71AV1 promoter, which is highly active specifically in GSTs (Wang et al., 2013). It appears to be an excellent promoter for the metabolic engineering of artemisinin biosynthesis in GSTs. In this study, *AaWRKY1* was cloned from *A. annua* var. Chongqing and was stably overexpressed in transgenic *A. annua* plants. The CaMV35S and CYP71AV1 promoters were respectively used to overexpress the *AaWRKY1* gene constitutively or trichome-specifically. The effects of overexpression of AaWRKY1 on artemisinin biosynthesis were investigated to explore the function of AaWRKY1 *in vivo* and the regulatory mechanism of artemisinin biosynthesis.

Results and discussion

Analysis of the cloned AaWRKY1 gene

AaWRKY1 was cloned from the high artemisinin-yielding strain 001 of *A. annua* (Ma et al., 2009b). In this work, one *WRKY* gene was cloned from *A. annua* var. Chongqing, using primers that were designed according to the nucleotide sequence of *AaWRKY1*. The open reading frame of the *WRKY* gene contained 936 bp and encoded a protein of 311 amino acids (GeneBank Accession No. KC118517). Alignment of the nucleotide sequences of the *AaWRKY1* (FJ390842) and the cloned *WRKY* gene showed that there are 21 sites of difference between them. The protein alignment showed that 9 amino acids were different between the two proteins; the putative WRKY motif and the amino acid residues that form the zinc finger were conserved (Fig. 2).

Although there were 9 amino acid substitutions between the deduced amino acid sequences of the newly cloned *WRKY* gene and *AaWRKY1*, the homology between the two genes was more than 97% and the WRKY motif and the amino acid residues forming the zinc finger were identical. We therefore conclude that they are alleles of the same gene from different ecotypes of *A. annua*.

Molecular analysis of transgenic A. annua

Eight lines of transgenic *A. annua* were analyzed by PCR and Southern blot. The PCR amplification fragment was 686 bp in length when the NPT II primers were used. The results of PCR analysis showed that there was one band of the right size in each lane of transgenic plants and positive control, while there was no amplification product in the lane of wild-type plant (Fig. 3). Using the WRKY cloning primers, the length of the amplified *AaWRKY1* gene was 950 bp. The results of PCR analysis showed that there was one strong band of the right size in each lane of transgenic plants and positive control, while there was no amplification product in the lane of wild-type plant (Fig. 3). However, another weak band that was about 1200 bp, was observed in all plant samples including the wild-type plant (Fig. 3). This band was most likely amplified from the endogenous *AaWRKY1* gene carrying some intron(s).

Southern blot analysis was performed using a 686-bp probe for the *NPT II* gene. As shown in Fig. 4, there is one strong hybridization band of the *NPT II* gene fragment in the lane of the positive control while there is no signal in the lane of wild-type plant. There is one hybridization band in the lanes of transgenic lines 35S-1, 35S-3, CYP-1, CYP-2, CYP-3 and CYP-4, while there are multiple bands in the lanes of transgenic lines of 35S-2 and 35S-4. The results of PCR and Southern blot showed that the exogenous genes (i.e. the *AaWRKY1* and the *NPTII* genes) were integrated into the genome of *A. annua*. *EcoRI* was used to digest the genomic DNA since there is one unique restriction site of *EcoRI* in the T-DNA region of the pCAMBIA vectors, which were used in this work. In this case, the numbers of hybridization band were to a large extent

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WRKY1           1 MESVCVYGQKTLINELSQGIQMAKQLKVNLNSPEAREILIQKILASYDNA  50
                  ||||||||||||||||||||||||||||||||||||||||||||||||||
Cloned WKRY     1 MESVCVYGQKTLINELSQGIQMAKQLKVNLNSPEAREILIQKILASYDNA  50

WRKY1          51 LFVFKSGESAGQCGPNGFPAFSLTESAITIASPQSVRSECNQPFSNEQGP 100
                  |||  |||||||||||||||| | ||||||||||||||||||||||||||||
Cloned WKRY    51 LFVLKSGESAGQCGPNGFPASSPTESAITIASPQSVRSECNQPFSNEQGP 100

WRKY1         101 NAVSKKRKGSTICEDQVKMCTDDGLEGSVDDGYSWRKYGQKDILGAKFPR 150
                  |||||||||| ||||||||||||||||||||||||||||||||||||||||
Cloned WKRY   101 NAVSKKRKGSIICEDQVKMCTDDGLEGSVDDGYSWRKYGQKDILGAKFPR 150

WRKY1         151 SYYRCTYRKAEKCLATKQVQRTDANPTVFDITYKGKHTCNHHARLAEPPL 200
                  |||| |||||||| |||||||||||||||||| ||||||| |||||||||||
Cloned WKRY   151 SYYRCTYRKAEKCLATKQVQRTDANPTVFEITYKGKHTCNHHARLAEPPL 200

WRKY1         201 PEKHEINTTHHQELPRPNPGGMLSNLRANLTVNTSDFGATDPYSFSFPLE 250
                  |||||||||||| |  |||| ||||||||||||||||||||||||||||||
Cloned WKRY   201 PEKHEINTTHHQQLSQPNPGEMLSNLRANLTVNTSDFGATDPYSFSFPLE 250

WRKY1         251 PFGTIEDYQQLHLPNDFDDELLQVYSPPVISPGTSDLNCYTDWDSSQSLN 300
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||
Cloned WKRY   251 PFGTIEDYQQLHLPNDFDDELLQVYSPPVISPGTSDLNCYTDWDSSQSLN 300

WRKY1         301 FTADLDLDFKF 311
                  ||||||||||||
Cloned WKRY   301 FTADLDLDFKF 311
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Fig. 2. Alignment of amino acid sequences of *AaWRKY1* (FJ390842) and the *AaWRKY1* cloned in this work. The amino acid residues in the grey box constitute the WRKY motif and the amino acid in black boxes form the zinc finger.

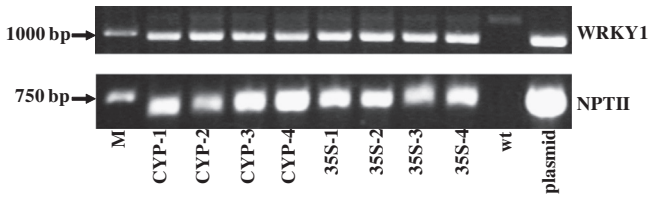

Fig. 3. PCR analysis of transgenic plants using NPT II primers and *AaWRKY1* primers. The target fragments of the *NPT II* and *AaWRKY1* genes are 686 and 950 bp, respectively. M: DNA marker; CYP-1, CYP-2, CYP-3, CYP-4: four transgenic lines transformed using *pCAMBIA-1381Z-M::pCYP::AaWRKY1*; 35S-1, 35S-2, 35S-3, 35S-4: four transgenic lines transformed using *pCAMBIA-1381Z-M::p35S::AaWRKY1*; wt: wild-type *A. annua*; plasmid: positive control in which the PCR template was the vector *pCAMBIA-1381Z-M::p35S::AaWRKY1*.

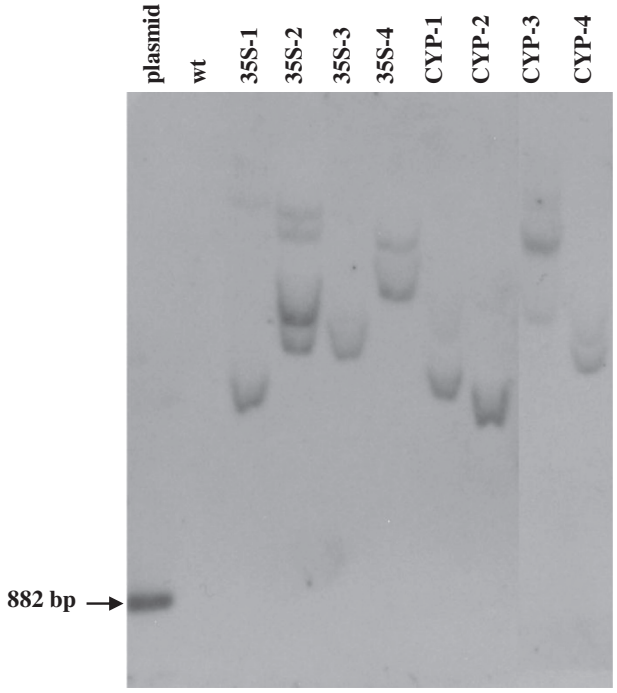

Fig. 4. Southern blot analysis of transgenic plants using a NPT II probe. Genomic DNA (15 μg) was digested with *EcoRI*. Plasmid: the plasmid *pCAMBIA-1381Z-M* was digested by *XhoI* to obtain the 882 bp NPT II gene fragment; wt: wild-type *A. annua*; 35S-1, 35S-2, 35S-3, 35S-4: four transgenic lines transformed using *pCAMBIA-1381Z-M::p35S::AaWRKY1*; CYP-1, CYP-2, CYP-3, CYP-4: four transgenic lines transformed using *pCAMBIA-1381Z-M::pCYP::AaWRKY1*.

identical with the copy number of exogenous gene. As far as plant transformation via the *Agrobacterium tumefaciens*-mediated method is concerned, it is likely to get single-copy transgenic plants. Among the analyzed transgenic plants, there were 6 single-copy lines and 2 multi-copy lines (Fig. 4). In the lane of 35S-2, it seemed that there were four restriction fragments, but the intensity of the hybridization signals were obviously different, namely two bands with strong signal and two bands with weak signal. The two weak bands were likely from partly digested fragments and could therefore most likely not represent copies of the transgene.

Effects of overexpression of AaWRKY1 on transcript levels of the enzymes involved in artemisinin biosynthesis

The transcript levels of AaWRKY1, FDS, ADS, CYP71AV1 and DBR2 were analyzed by qPCR. ALDH1 was also analyzed but the C_t value was lower than 35 or undetected. In transgenic plants, the transcript level of AaWRKY1 was increased dramatically, i.e. more than one hundred times under the control of the CaMV35S promoter and about 40 times under the control of the CYP71AV1 promoter, compared to the transcript level of AaWRKY1 in wild-type plant (Fig. 5). In transgenic plants, the transcript level of CYP71AV1 was increased, especially when the *AaWRKY1* gene was specifically overexpressed in glandular trichomes but the transcription levels of FDS, ADS, and DBR2 did not change significantly (Fig. 5).

The cloned CYP71AV1 promoter was capable of activating gene transcription specifically in GSTs and moreover it was more active than the wild-type CYP71AV1 promoter (Wang et al., 2013). Our results demonstrated that the cloned CYP71AV1 promoter was an effective promoter for metabolic engineering of artemisinin biosynthesis. The highest transcript level of AaWRKY1 under the control of the CYP71AV1 promoter was 40 times higher that of the wild-type plant (Fig. 5). Furthermore, GST-specific overexpression of *AaWRKY1* enhanced the transcription levels of CYP71AV1 up to 32 times, while it is just up to 4 times in the case of constitutive overexpression of *AaWRKY1*. There are two GGTCAA W-boxes in the CYP71AV1 promoter (Wang et al., 2013). The AaWRKY1 protein was able to bind the GGTCAA W-box *cis*-acting element as analyzed by the electrophoretic mobility shift assay and transient expression of the *AaWRKY1* cDNA in *A. annua* leaves activated the expression of CYP71AV1 (about 3-fold) (Ma et al., 2009b). It is very interesting to note that the GST-specific overexpression of AaWRKY1 was much more effective than the constitutive overexpression of AaWRKY1. From Fig. 5, it is clear that the extract from plants constitutively overexpressing *AaWRKY1* contains 3–4 times more AaWRKY1 transcripts than that from plants specifically

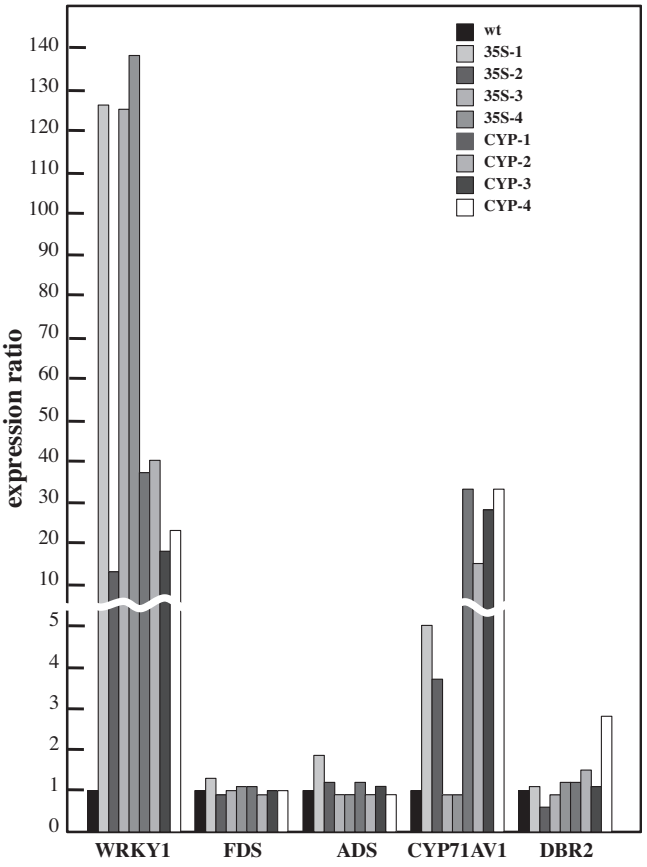

Fig. 5. Relative expression of the target genes in young leaves of transgenic *A. annua* plants compared to the correspondent genes in young leaves of wild-type plant. wt: wild-type *A. annua*; Transgenic lines: 35S-1, 35S-2, 35S-3, 35S-4 (*AaWRKY1* was overexpressed using the CaMV35S promoter); CYP-1, CYP-2, CYP-3, CYP-4 (*AaWRKY1* was overexpressed using the CYP71AV1 promoter).

overexpressing *AaWRKY1* in GSTs. However, the number of trichome cells is just a fraction of the total number of cells in a leaf. Consequently the cellular concentration of AaWRKY1 transcripts is much higher in the GSTs of plants specifically overexpressing *AaWRKY1* in trichomes than in the GSTs of plants constitutively overexpressing *AaWRKY1*. This concentration difference may be the reason for the higher level of CYP71AV1 transcipts observed when the *AaWRKY1* gene was specifically overexpressed in GSTs. Obviously the transcription of the *CYP71AV1* gene could not be effectively induced in other cell types by the overexpressed *AaWRKY1*. In addition, it is highly likely that other GST-specific proteins cooperate with AaWRKY1 in the regulation of the *CYP71AV1* gene. It is well established that WRKY transcription factors regulate the expression of genes through coordination with other DNA-binding or non-DNA-binding interacting proteins (Rushton et al., 2010). In a recent report, it was shown that AaORA, a GST-specific AP2/ERF transcription factor cloned from *A. annua*, up-regulated artemisinin biosynthesis. In transgenic *A. annua* plants in which *AaORA* was overexpressed, the level of CYP71AV1 transcipts was increased 5–17 times (Lu et al., 2013). The GST-specific AaORA is likely to cooperate with AaWRKY1 to enhance the expression of CYP71AV1.

Overexpression of *AaWRKY1* does not increase the transcript level of FDS in *A. annua*. This result is in agreement with another report, which showed that the transcript level of FDS was not increased when *AaWRKY1* was transiently overexpressed in *A. annua* leaves (Ma et al., 2009b). FDS catalyzes the biosynthesis of farnesyl diphosphate, which is the precursor for various terpenoids such as sesquiterpenes and sterols (Fig. 1). A small gene family encodes FDS isozymes in plants. In *Arabidopsis thaliana*, FDS1 is a house-keeping gene expressed in all tissues at all developmental stages and it is thought to be involved in the synthesis of isoprenoids serving basic plant cell functions such as production of sterols for various membranes (Cunillera et al., 2000). FDS2 is often inducible and found in special tissues at particular stages of development and it is thought to be involved in the synthesis of particular isoprenoids with specialized functions (Cunillera et al., 2000). The homology between FDS1 and FDS2 in plants of the Asteraceae family is 82.0% to 89.7% (Table 1S). The *FDS1* and *FDS2* genes cloned from *Artemisia tridentata* ssp. *spiciformis* showed 83% amino acid sequence identity. FDS1 had a higher specific activity than FDS2 and the transcript level of FDS1 was 20-fold higher than that of FDS2 in shoots (Hemmerlin et al., 2003). The nucleotide sequences of two *FDS* genes from *A. annua* designated as FDS1 and FDS2 may be found in the GenBank. However, the coding region of these two genes showed 96.6% nucleotide sequence identity. A phylogenetic analysis of FDS genes of the Asteraceae family indicated that the two *AaFDS* genes actually both represent the house-keeping *FDS1* as suggested by Hemmerlin et al. (2003) (Fig. 1S). The qPCR primers used in this work may detect the total level of transcripts of the *AaFDS1* gene and a putative inducible *AaFDS2* gene. AaWRKY1 may regulate the transcription of inducible *FDS2* but since this gene has not yet been cloned from *A. annua* we cannot selectively study its expression.

There are two putative W-boxes in the ADS (Wang et al., 2011) and DBR2 (unpublished) promoters. However, there was no significant difference of transcript levels of ADS and DBR2 between the transgenic and wild-type plants. The WRKY family is one of the ten largest families of transcription factors in higher plants. It is estimated that there are more than 70 WRKY proteins in *Arabidopsis* and 100 WRKY proteins in *Oryza sativa*. The WRKY family is divided into three groups based on the number of WRKY domains (two domains in group I proteins and one in the other two groups) and the structure of their zinc fingers (C2HC in Group III and C2H2 in others) (Rushton et al., 2010). The W-box (TTGAC(C/T)) is the minimal consensus required for WRKY protein binding but the nucleotide sequences adjacent to the W-box determine the strength and specificity of DNA binding (Ciolkowski et al., 2008). It is likely that the flanking nucleotides of the W-boxes are not optimal in the promoter of ADS and DBR2, and therefore the AaWRKY1 could not activate them effectively. The amino acid sequence of AaWRKY1 exhibits 15.9% identity with the GaWRKY1, which activates the transcription of the sesquiterpene synthase gene (+)-δ-cadinene synthase-A (*CAD1-A*) by means of interacting with the W-box of the CAD1-A promoter (Ma et al., 2009b; Xu et al., 2004). AaWRKY1 belongs to Group III of the WRKY family while GaWRKY1 belongs to Group II. We have recently cloned another *AaWRKY* gene, which belongs to WRKY family Group II and shows 47.5% homology with GaWRKY1. The plant transformation of this *WRKY* gene is underway in our laboratory.

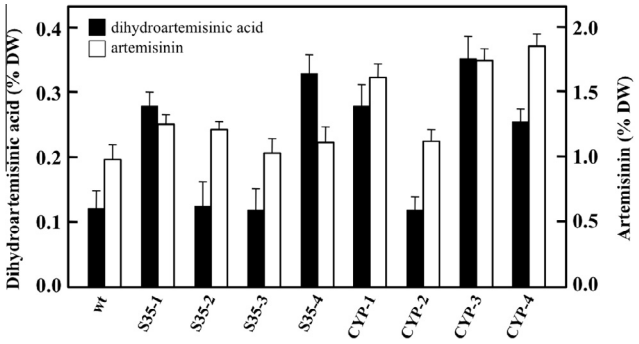

Fig. 6. Artemisinin and dihydroartemisinic acid content in transgenic and wild-type plants of *A. annua*. wt: wild-type *A. annua*; Transgenic lines: 35S-1, 35S-2, 35S-3, 35S-4 (*AaWRKY1* was overexpressed using the CaMV35S promoter); CYP-1, CYP-2, CYP-3, CYP-4 (*AaWRKY1* was overexpressed using the CYP71AV1 promoter).

The content of artemisinin and dihydroartemisinic acid in transgenic A. annua plants

The transgenic *A. annua* plants showed identical morphological characters with wild-type plants and there was no significant difference between transgenic plants and wild-type plants in terms of the density and size of GSTs on leaves (Fig. 2S). The artemisinin content in high artemisinin-yielding *A. annua* var. Chongqing was approximately 1.0% dry wt. according to the results of GC–MS analysis. The artemisinin content was increased by a large margin in transgenic lines CYP-1, CYP-3, CYP-4 (Fig. 6). In transgenic line CYP-4, in which the transcript levels of CYP71AV1 and DBR2 were increased 32 times and 1.8 times, respectively, the artemisinin content was about 1.9 times compared to the wild-type plant. The content of dihydroartemisinic acid, which is the direct precursor of artemisinin, is also shown in Fig. 6. Our results indicated that greatly up-regulated CYP71AV1 promoted artemisinin biosynthesis.

Conclusions

In this study, the regulatory function of AaWRKY1 was investigated by means of stable overexpression in *A. annua*. A comparison between constitutive (35S promoter) and trichome specifically (CYP71AV1 promoter) expression showed that overexpression of AaWRKY1 in GSTs is much more effective than that of overexpressing AaWRKY1 in whole plants. The trichome-specifically overexpressed AaWRKY significantly increased the transcription levels of CYP71AV1, up to 32 times as compared to the wild-type plant. The up-regulated CYP71AV1 promotes artemisinin biosynthesis.

Experimental

Plant materials

The seeds of high artemisinin-yielding *A. annua* L. var. Chongqing were surface sterilized by immersing in 75% ethanol for 2 min and subsequently soaking in 5% NaOCl for 10 min. The seeds were rinsed twice with sterile distilled water and then germinated in MS medium. Germination started within 2–3 days. After one month, the seedlings were used in plant transformation experiments.

AaWRKY1 gene cloning and construction of plant expression vectors

For PCR cloning of the *AaWRKY1* gene, primers were designed according to the sequences of the cloned *AaWRKY1* (FJ390842). The sequence of the forward primer was 5-CCGCCATGG AAAGTGTTTGTGTTTATG-3 (the underlined sequence is the restriction site of *NcoI*) and the sequence of the reverse primer was 5-CCGCACGTGTTAAAATTTGAAATCAAGG-3 (the underlined sequence is the restriction site of *PmlI*). RNA was extracted from the flower buds of *A. annua* var. Chongqing using Purelink Plant RNA Reagent (Invitrogen) according to the manufacture's instruction. Genomic DNA was removed by treatment with DNase I (Fermentas). RNA (1 μg) was reverse transcribed using RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas) according to the manufacture's instruction. The volume of reverse transcription reaction was 20 μl. The PCR system included 10 pmol forward primer and 10 pmol reverse primer, 2 μL of the reverse transcription reaction product, 0.25 mmol/L dNTPs, 5 μL of 10× Pfu DNA polymerase buffer, 2.5 U Pfu DNA polymerase (Fermentas) in a total volume of 50 μL. Amplification was performed in a thermal cycler (Biorad) as follows: 3 min at 95 °C; 30 cycles of 30 s at 95 °C, 30 s at 56 °C, 2 min at 72 °C; followed by 10 min at 72 °C. The amplification fragment was purified using Gene JET Gel Extraction kit (Fermentas) according to the manufacture's instruction. The purified DNA fragment was cloned into pJET vector (CloneJET PCR Cloning Kit, Fermentas) and sequenced.

The modified binary vector *pCAMBIA 1381Z* in which the plant resistance gene *HPTII* (hygromycin resistance) was replaced by *NPTII* (kanamycin resistance) (Wang et al., 2011), was used in this work and was named *pCAMBIA 1381Z-M* (Fig. 3SA). *pCAMBIA 1302* was digested with *EcoRI* and *NcoI* and the small fragment (813 bp), namely the CaMV35S promoter, was recovered and purified using Gene JET Gel Extraction kit (Fermentas). *pCAMBIA-1381Z-M* was digested with the same enzymes and the large fragment was recovered and purified. The purified fragments of CaMV35S promoter and the digested *pCAMBIA-1381Z-M* vector was ligated using T4 DNA Ligase (Fermentas) and then the ligation product was transformed into the competent cells of *E. coli* NovaBlue. The recombinant vector was named *pCAMBIA-1381Z-M::p35S* (Fig. 3SB). The CYP71AV1 promoter was recombined into *pCAMBIA-1381Z-M* using the same method and same restriction enzymes (Wang et al., 2013). The recombinant vector was named *pCAMBIA-1381Z-M::pCYP* (Fig. 3SC).

The recombinant pJET vector carrying the cloned *AaWRKY1* gene was purified using Gene JET Plasmid DNA Purification Kit (Fermentas). The purified vector was digested with *NcoI* and *PmlI* and the *AaWRKY1* fragment was recovered and purified. The recombinant vectors *pCAMBIA-1381Z-M::p35S* and *pCAMBIA-1381Z-M::pCYP* were respectively digested with *NcoI* and *PmlI* and then the digested vectors were recovered and purified. The purified *AaWRKY1* gene was ligated into the opened vectors to obtain the recombinant vectors which were named *pCAMBIA-1381Z-M::p35S::AaWRKY1* and *pCAMBIA-1381Z-M::pCYP::AaWRKY1*, respectively (Fig. 3SB and C). These vectors were separately transformed into *Agrobacterium tumefaciens* EHA 105 using the freeze–thaw method. The transformed *A. tumefaciens* was cultured on LB medium, which contained 50 mg/L rifampicin and 50 mg/L kanamycin.

Transformation and regeneration of A. annua

Genetic transformation of *A. annua* was performed according to our previous method (Han et al., 2005). The *A. tumefaciens* EHA105 strains harboring the binary vector *pCAMBIA-1381Z-M::p35S::AaWRKY1* or *pCAMBIA-1381Z-M::pCYP::AaWRKY1* were used for plant transformation. Clones of transformed *A. tumefaciens* were picked from the culture plates and incubated in 2 mL of LB liquid medium, which contained 50 mg/L rifampicin and 50 mg/L kanamycin, at 28 °C for 36 h. After PCR identification, 1 mL suspension of the positive bacteria was diluted to 50 mL LB medium containing 50 mg/L kanamycin and incubated to OD_{600} = 0.7–0.8. The bacterial suspension was centrifuged at 4 °C, 2860*g* for 10 min and then the bacterial precipitation was resuspended in 40 mL MS medium. After incubation for 3 h at 28 °C, the bacterial suspension was used for plant transformation. Leaves that were cut from 30 days-old seedlings were immerged into infection suspension for 10 min and then co-cultivated on MS medium in dark for 2 or 3 days. After co-cultivation, the leaves were transferred to shoot-inducing MS medium which contained 0.5 mg/L 6-BA, 0.05 mg/L NAA, 20 mg/L kanamycin, and 400 mg/L carbenicillin. The medium was changed every two weeks with gradually decreased concentrations of carbenicillin. Four weeks later, the induced shoots were transferred to MS medium, which contained 20 mg/L kanamycin, and 100 mg/L carbenicillin. Two weeks later, the shoots were cut and cultured in root-inducing MS medium which contained 20 mg/L kanamycin. The shoots rooted after one week. The newly-growing branches were cut from the 40-days-old regenerated plants and sub-cultured in MS medium containing 20 mg/l kanamycin. After that, the 40-days-old regenerated plants were cultured in a greenhouse.

PCR and Southern blot analysis of transgenic A. annua

A simplified CTAB method of DNA extraction was used to rapidly identify transgenic plants on a large scale. Four young leaves were cut from the tissue-cultured transgenic plant and ground to a fine paste in 800 μL 2% CTAB extraction buffer. The ground mixture was transferred to a microcentrifuge tube and incubated for 30 min at 60 °C in a water bath. After incubation, an equal volume of chloroform:iso-amyl alcohol (24:1) was added into the solution and mixed by inversion. Subsequently the mixture was centrifuged at 11 000*g* for 8 min. The upper aqueous phase was transferred to a new microcentrifuge tube and mixed with double volume of ice cold absolute ethanol. The precipitated DNA was washed with 75% ethanol and then it was dissolved in ultra-pure H_2O and used as template in PCR. The primers used were designed according to the *NPTII* gene sequence. The sequence of the forward primer was 5-TGGGCACAACAGACAATCGGCTGC-3 and the sequence of the reverse primer was 5-TGCGAATCGGGAGCGGCGATACCG-3. PCR was performed with PCR Beads (puReTaq™ Ready-To-Go™, GE Healthcare).

For Southern blot analysis, genomic DNA of high quality was prepared according to the standard CTAB method. DNA (20 μg) was digested with *EcoRI* over-night and then separated on a 0.7% (w/v) agarose gel. The DNA was transferred onto a positively charged nylon membrane (Boheringer Mannhein GmbH, Mannheim, Germany) by capillary blotting (Sambrook et al., 1989). The NPTII probe was labeled with digoxigenin by PCR. The PCR reaction included 10 pmol forward primer and 10 pmol reverse primer, 10 pg of plasmid DNA, 10 nmol dATP, 10 nmol dGTP, 10 nmol dCTP,

6.5 nmol dTTP, 3.5 nmol DIG-11-dUTP (Roche), 5 μL of 10× Taq DNA polymerase buffer, 2 mmol/L $MgCl_2$, and 2 U Taq DNA Polymerase (Thermo Scientific) in a total volume of 50 μL. Amplification was performed in a thermal cycler (Biorad) as follows: 2 min at 95 °C; 30 cycles of 30 s at 95 °C, 30 s at 56 °C, 40 s at 72 °C; followed by 7 min at 72 °C. Hybridization was performed according to the standard procedures (Sambrook et al., 1989). Detection of hybridization probes was performed by chemiluminescent method according to the manufacture's instruction. Anti-digoxigenin-AP (Roche) was used as antibody and CSPD (Roche) was used as chemiluminescent substrate. The membrane was exposed to X-ray film for 1 h.

Gene expression analysis by quantitative PCR (qPCR)

Transgenic plants were cultivated in the experimental greenhouse under 16 h light and 8 h dark at 22 °C to a height of approximately 1 m. Young leaves were collected and immediately stored in liquid nitrogen. RNA extraction was performed using Purelink Plant RNA Reagent (Invitrogen) according to the manufacture's instruction. DNA was removed by treatment with DNase I (Fermentas). RNA (1 μg) was reverse transcribed using RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas) according to the manufacture's instruction. The volume of reverse transcription reaction was 20 μL. qPCR was performed using specific primers on a 7500 qPCR equipment (Applied Biosystems). The sequences of primers of β-actin, FDS, ADS, CYP71AV1, DBR2, and ALDH1 are the same as those previously used (Olofsson et al., 2011). The sequences of WRKY primers were designed according to the 3' terminal sequences of *AaWRKY1* and the amplification fragment was 107 bp. The sequence of forward *AaWRKY1* primer was 5-GCAAGTCTATTCGCCACCTGTTATC-3 and the sequence of reverse *AaWRKY1* primer was 5-CAAGGTCTAAATCTGCCGTGAAATT-3. cDNA (1 μL) was used as template in 20 μL reactions including 10 μL Power SYBR Green PCR Master Mix 2× (Applied Biosystems) and 2 pmol of each primer. qPCR was performed at 50 °C (2 min), 95 °C (10 min), 40 cycles at 95 °C (15 s), 60 °C (1 min), and followed by a dissociation state at 95 °C (15 s), 60 °C (1 min), and 95 °C (15 s). Triplet samples were run for each cDNA sample. The relative expression of target genes in transgenic plants was analyzed using the software REST 2009 software v.2.0.13 (Qiagen, Hilden, Germany). *β-Actin* was used as reference gene and the transcript levels of the target genes in the wild-type plant were set to 1.0.

GC–MS analysis of artemisinin and dihydroartemisinic acid

Leaves were collected from the plants that were cultivated in the greenhouse under 16 h light and 8 h dark at 22 °C to a height of approximately 1 m and were dried for 24 h in an oven at 40 °C. The dried leaves were ground to a fine powder. The powder (100 mg) was extracted with hexane (10 ml) under ultrasonic treatment for 30 min. The mixture was filtered to remove solids and then evaporated to dryness in *vacuo* at 30 °C. The residues were dissolved in hexane (1 mL). As for dihydroartemisinic acid, the extraction sample (50 μL) was mixed with 1:1 mixture of pyridine and N, O-bis(trimethylsilyl)acetamide (Sigma) followed by GC–MS analysis. Samples were analyzed by capillary GC on an Agilent 6890 series gas chromatograph equipped with a HP-5MS 5% phenyl methyl siloxane capillary column (30 × 0.25 mm) and an Agilent 5937 Network Mass Selective Detector. The GC injection port was operated with a carrier gas of helium at a constant flow of 0.7 mL/min and a sample size of 2 μL in the splitless injection mode. GC was performed with an injector temperature of 40 °C. To identify and quantify artemisinin and dihydroartemisinic acid, the oven temperature was programmed from 60 to 190 at 3 °C/min, from 190 to 235 at 20 °C/min, from 235 to 300 at 30 °C/min. Mass spectra were measured in electron impact ionization mode at 70 eV with scanning from m/z 34 to 400 at 3.93 scans s^{-1}. Solvent delay of 10 min was allowed prior to the acquisition of MS data. The peaks were quantified by integration of peak areas using Enhanced Chemstation version E.02.00.493 (Agilent Technologies). For the quantification of artemisinin, the calibration curve was obtained using artemisinin standard (Sigma). The quantification of dihydroartemisinic acid was performed according to the calibration curve of farnesol.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2014.02.011.

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