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ARTICLE *in* PLANT MOLECULAR BIOLOGY · JANUARY 2013

Impact Factor: 4.26 · DOI: 10.1007/s11103-012-9986-y · Source: PubMed

Plant Mol Biol (2013) 81:119–138
DOI 10.1007/s11103-012-9986-y

Trichome-specific expression of the amorpha-4,11-diene 12-hydroxylase (*cyp71av1*) gene, encoding a key enzyme of artemisinin biosynthesis in *Artemisia annua*, as reported by a promoter-GUS fusion

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Received: 1 March 2012 / Accepted: 30 October 2012 / Published online: 19 November 2012

Abstract Artemisinin derivatives are effective anti-malarial drugs. In order to design transgenic plants of *Artemisia annua* with enhanced biosynthesis of artemisinin, we are studying the promoters of genes encoding enzymes involved in artemisinin biosynthesis. A 1,151 bp promoter region of the *cyp71av1* gene, encoding amorpha-4,11-diene 12-hydroxylase, was cloned. Alignment of the cloned promoter and other *cyp71av1* promoter sequences indicated that the *cyp71av1* promoter may be different in different *A. annua* varieties. Comparison to the promoter of amorpha-4,11-diene synthase gene showed a number of putative *cis*-acting regulatory elements in common, suggesting a co-regulation of the two genes. The *cyp71av1* promoter sequence was fused to the β-glucuronidase (*GUS*) reporter gene and two varieties of *A. annua* and *Nicotiana tabacum* were transformed. In *A. annua*, *GUS* expression was exclusively localized to glandular secretory trichomes (GSTs) of leaf primordia and top expanded leaves. In older leaves, there is a shift of expression to T-shaped trichomes (TSTs). Only TSTs showed GUS staining in lower leaves and there is no GUS staining in old leaves. *GUS* expression in flower buds was specifically localized to GSTs. The recombinant promoter carries the *cis*-acting regulatory elements required for GST-specific expression. The *cyp71av1* promoter shows activity in young tissues. The recombinant promoter was up to 200 times more active than the wild type promoter. *GUS* expression in transgenic *N. tabacum* was localized to glandular heads. Transcript levels were up-regulated by MeJA. Wound responsiveness experiment showed that the *cyp71av1* promoter does not appear to play any role in the response of *A. annua* to mechanical stress.

Keywords *Agrobacterium tumefaciens* · Amorpha-4,11-diene 12-hydroxylase · *Artemisia annua* · Artemisinin biosynthesis · β-glucuronidase · Gene regulation · Promoter activity · Stable transformation

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Introduction

Artemisinin is an effective anti-malarial drug, which is an important component of artemisinin-based combination therapies (ACTs) (Rathore et al. 2005). Today, ACTs is the first line antimalarial drugs in most malaria endemic countries. Artemisinin is produced by *Artemisia annua* L. The biosynthesis of artemisinin is located to glandular secretory trichomes (GSTs) predominantly on leaves and inflorescences. Production of artemisinin in leaves is limited because the total production of artemisinin precursors per unit leaf (dry weight) is low, and the conversion of precursors to artemisinin is partial and slow (Lommen et al. 2007, 2008). Artemisinin content in *A. annua* plants ranges from 0.01 to 1 % of dry weight. In some hybrid varieties of *A. annua*, the content can reach 1.4 %. Hence, it is urgent to find effective methods to improve the artemisinin production.

The biosynthesis of artemisinin is fairly well understood as far as the enzymology is concerned (Fig. 1). Cyclization of farnesyl diphosphate (FDP) to amorpha-4,11-diene by amorpha-4,11-diene synthase (ADS) is the initial step of the artemisinin biosynthetic pathway (Mercke et al. 2000) and amorpha-4,11-diene is the committed precursor (Bouwmeester et al. 1999). This first committed step is rate

limiting for the artemisinin biosynthesis. In the following step, amorpha-4,11-diene is hydroxylated to yield artemisinic alcohol. This reaction is catalyzed by a cytochrome P450 dependent amorpha-4,11-diene 12-hydroxylase (CYP71AV1) (Teoh et al. 2006). This enzyme can covert the amorpha-4,11-diene all the way to artemisinic acid, which is the final enzymatic intermediate and precursor of arteannuin B (Brown and Sy 2004). Alternatively, the artemisinic alcohol can be oxidized to the corresponding aldehyde by alcohol dehydrogenase 1 (ADH1) (Polichuk et al. 2010). In the next step, the aldehyde is reduced to dihydroartemisinic aldehyde by artemisinic aldehyde Δ11(13) reductase (DBR2) (Zhang et al. 2008). The final enzymatic step in artemisinin biosynthesis is catalyzed by aldehyde dehydrogenase 1 (ALDH1) (Teoh et al. 2009). The intermediates artemisinic acid and dihydroartemisinic acid are converted to arteannuin B and artemisinin, respectively, in a non-enzymatic reaction. All the genes encoding these enzymes have been cloned and the recombinant enzymes at least partly characterized. Recently, two chemotypes of *A. annua* have been characterized. One is high in artemisinic acid and arteannuin B and the other is high in dihydroartemisinic acid and artemisinin (Brown and Sy 2004, 2007). Glandular trichome specific expression of biosynthetic genes (Teoh et al. 2006; Olsson et al. 2009; Olofsson et al. 2012) strongly supported the notion that artemisinin is sequestered and localized to GSTs of *A. annua* (Duke et al. 1994; Covello et al. 2007).

Many studies have been carried out to improve the yield of artemisinin in *A. annua*. Different biotic elicitors have been used to improve artemisinin content in plants and cell cultures (Putalun et al. 2007; Baldi and Dixit 2008; Jing et al. 2009; Maes et al. 2011; Lei et al. 2011). Using the antisense technique, squalene synthase (Li et al. 2009) and β-caryophyllene synthase (Chen et al. 2011) were down-regulated in transgenic *A. annua* plants leading to an improved yield of artemisinin. Over-expression of the endogenous farnesyl diphosphate synthase (FDS) (Han et al. 2006) or hydroxy-methyl-glutaryl-CoA reductase (HMGR) (Nafis et al. 2011) resulted in higher artemisinin yields. All these attempts to increase the yield of artemisinin have only partly been successful with relatively limited increases in yields.

In order to design transgenic plants of *A. annua* with enhanced biosynthesis of artemisinin by metabolic engineering, further studies on the regulation of the biosynthetic pathway are needed. We have initiated studies on the activity of promoters of biosynthetic genes. We use expression of promoters fused to the *GUS* reporter gene in transgenic *A. annua*. Recently, we reported our results on the *ads* promoter (Wang et al. 2011b). We have now extended our studies and here we report on the activity of the *cyp71av1* promoter fused to *GUS* and on its tissue-specific expression in transgenic *A. annua*.

Materials and methods

Plant materials

Two varieties of *A. annua* (Chongqing and Anamed) were used for these experiments. Anamed (A-3) was obtained from Anamed, Germany and Chongqing seeds from the Southwest University in Chongqing, China.

Wild-type and transgenic plants of *A. annua* (varieties Anamed and Chongqing) were grown under 16 h days and 8 h nights at 22 °C to a height of approximately 1 m

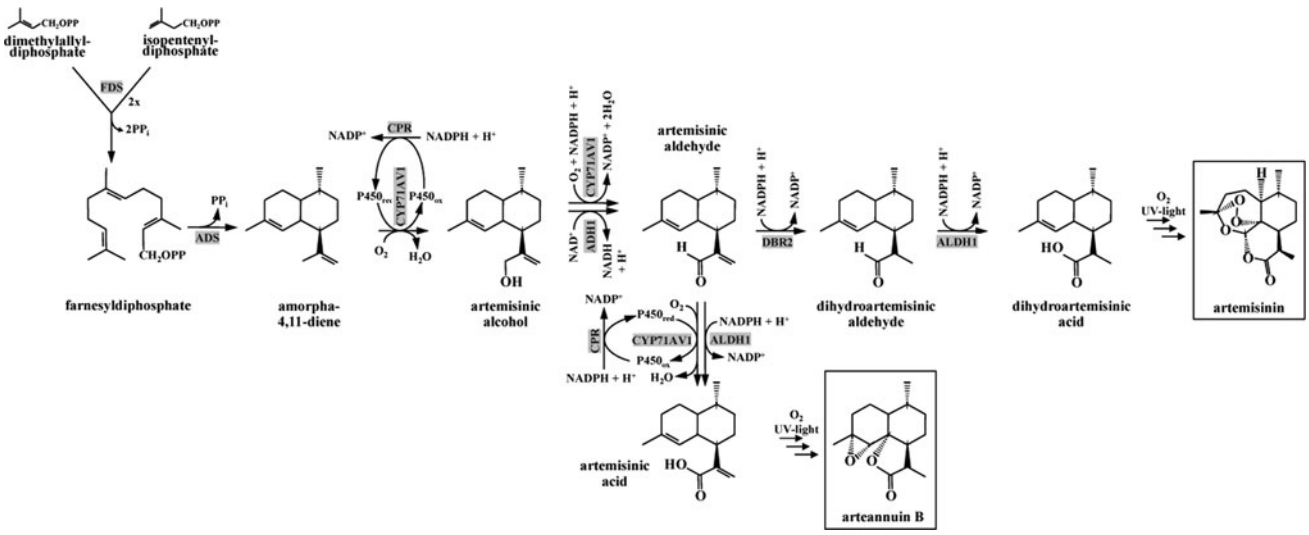


Fig. 1 Biosynthetic pathway for artemisinin in *Artemisia annua*. Enzyme abbreviations are in *gray boxes*. *FDS* farnesyldiphosphate synthase, *ADS* amorpha-4,11-diene synthase; *CYP71AV1* amorpha-4,11-diene 12-hydroxylase, *CPR* cytochrome P450 reductase, *ADH1* alcohol dehydrogenase 1, *DBR2* artemisinic aldehyde Δ11(13) reductase, *ALDH1* aldehyde dehydrogenase 1

followed by flower buds induction at 8 h days and 16 h nights at 22 °C. Flower buds, young leaves, old leaves, stems and roots were collected for GUS staining. Samples of these tissues were frozen in liquid nitrogen, grounded and then used for RNA extraction. Plants of *Nicotiana tabacum* were grown under the same conditions (16 h days and 8 h nights at 22 C).

Promoter cloning

Genomic DNA was extracted from fresh young leaves of *A. annua* using the CTAB method (Fütterer et al. 1995). The GenomeWalker Universal kit (Clontech) was used to amplify the promoter region of *cyp71av1* by PCR as instructed by the manufacturer. Two adaptor primers (2 and 4; Table 1) and two gene specific primers (1 and 3; Table 1) were used in the PCR reactions (Table 1). The nucleotide sequence obtained was used to design primers for PCR amplification (primers 5 and 6; Table 1) of a 1,151 bp fragment of the *cyp71av1* promoter using *Pfu* polymerase (Fermentas, St Leo-Roth, Germany). Primers 5 and 6 carried *Eco*RI and *Nco*I restriction site, respectively, used for cloning the fragment into the plant transformation vector as outlined below.

Transformation vector construction

The pCAMBIA 1381Z vector (CambiaLabs, Brisbane, Australia) carrying the *GUS*-gene was used for the transformation of *A. annua*. However, the vector had been previously modified in that the plant resistance gene was changed from hygromycin to kanamycin (Wang et al. 2011b).

Promoter region was double-digested with *Nco*I/*Eco*I and inserted into the modified pCAMBIA 1381Z vector digested with the same restriction enzymes. The plant transformation vector obtained (pCAMBIA1381Z-*pCYP71AV1-GUS*) was introduced into *E. coli* Nova Blue, which was grown on LB medium containing kanamycin (50 mg/l). The vector was purified using the GeneJet Plasmid Miniprep Kit (Fermentas) and used for transformation of *Agrobacterium tumefaciens* EHA105. The plant transformation vector was introduced into *A. tumefaciens* EHA105 by the freeze and thaw method. *A. tumefaciens* EHA105 carrying the plant transformation vector was grown on YEP medium containing kanamycin (100 mg/l), rifampicin (40 mg/l) and streptomycin (25 mg/l) at 28 °C to an $OD_{600} = 0.8–1$. The cells were collected by centrifugation and resuspended in MSMO liquid medium to an $OD_{600} = 0.3–0.5$.

An empty control vector was constructed by filling in the overhangs of the double-digested modified pCAMBIA 1381Z vector. Subsequently the vector was closed by ligase treatment. This control vector was prepared for transformation in the same way as described above for the pCAMBIA1381Z-*pCYP71AV1-GUS*.

Plant transformation

Seed sterilization and germination as well as plant transformation was carried out as previously described (Wang et al. 2011b).

Table 1 Nucleotide sequence of primers used

Primer no	Name	Application	Primer sequence
1	CYP1	GenomeWalker	5′-GCTTTTTTTGGTGGATTTGGAACGAGTAG-3′
2	AP1	GenomeWalker	5′-GTAATACGACTCACTATAGGGC-3′
3	CYP2	GenomeWalker	5′-CAATGGAAGTGGTCAGTGAGAGTGC-3′
4	AP2	GenomeWalker	5′-ACTATAGGGCACGCGCGTGGT-3’
5	CYP_F	Cloning	5′-GGAATTCAATGGGTCAATTTCGG G-3′
6	CYP_R	Cloning	5′-CATGCCATGGTGCTTTTAGTATACTCTTCA-3′
7	β-$actin_F$	qPCR	5′-CCAGGCTGTTCAGTCTCTGTAT-3′
8	β-$actin_R$	qPCR	5′-CGCTCGGTAAGGATCTTCATCA-3′
9	$CYP71AV1_F$	qPCR	5′-CGAGACTTTAACTGGTGAGATTGT-3′
10	$CYP71AV1_R$	qPCR	5′-GTAGATAGTGTTGGGTTGGTGTGA-3′
11	GUS_F	qPCR	5′-AACCGTTCTACTTTACTGGCTTTGG-3′
12	GUS_R	qPCR	5′-GCATCTCTTCAGCGTAAGGGTAAT-3′
13	GUS_F	probe	5′-AACCGTTCTACTTTACTGGCTTTGG-3′
14	GUS_R	probe	5′-CGAAGTTCATGCCAGTCCAG-3′

Restriction sites are underlined

F forward, *R* reverse

Scanning electron microscopy

Scanning electron microscopy (SEM) was carried out on unfixed tissues from wild-type and transgenic *A. annua* plants using a relatively low energy beam (5 kV).

GUS assay

Leaf primordia at the apex and expanded leaves at different nodes, stems, roots from shoots and flowering plants, and flower buds were sampled from transgenic *A. annua* plants to perform GUS analysis. GUS histochemical staining of the various plant tissues was carried out as previously described (Jefferson et al. 1987). GUS stained tissues were studied under a microscope (Nikon ECLIPSE E400) and photographs were taken using a digital camera (Nikon DP11).

qPCR

Different tissues of transgenic *A. annua* plants were sampled to analyze expression pattern using *β-actin* as reference gene. RNA was extracted using Purelink™ Plant RNA Reagent (Invitrogen, Carlsbad, CA, USA) 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-MuLV reverse transcriptase (Fermentas) primed with 0.5 μg oligo$(dT)_{18}$ primer. RNA was removed from the cDNA obtained by treatment with RNase H (Fermentas).

The qPCR was performed on a 7500 qPCR thermocycler (Applied Biosystems, Foster City, USA) using primers listed in Table 1 for β-actin (primers 7 and 8), CYP71AV1 (primers 9 and 10) or GUS (primers 11 and 12). First single-stranded cDNA was used as template in a 20 μl reaction mixture containing 10 μl Power SYBR® Green PCR Master Mix (Applied Biosystems) and 2 pmol of each primer. qPCR thermal cycling was performed at 50 °C (2 min), 95 °C (10 min), 40 cycles at 95 °C (15 s), 60 °C (1 min) and finally 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.

Wounding and hormonal treatments

Upper leaves at 1st and 2nd node and lower leaves of transgenic *A. annua* plants at vegetative stage (1 month after roots appeared) and at reproductive stage were wounded by cutting along the midrib with a razor blade. For each variety, five transgenic lines were tested and five plants of the same variety transformed with the control vector were used as controls. Responsiveness to wounding was checked by GUS staining and qPCR. Samples were GUS-stained after 0 (immediately after wounding), 2, 4, 8, 16, 24 and 48 h.

Propagation of transgenic lines was carried out by shoot cuttings. Plants were used for hormone experiments 1 month after roots appeared. The plants were sprayed with 300 μM methyljasmonate (MeJA). Control plants of the same transgenic line were sprayed with water. Treated plants were kept in open air for 3 h until there was no liquid on leaf surfaces. Subsequently, the plants were returned to the standard growth conditions for 7–45 h resulting in total incubation times of 10–48 h. Upper leaves (leaf primordia and leaves at the 1st and 2nd node) were sampled for transcripts expression analysis. qPCR was used to check the levels of transcripts of CYP71AV1 and GUS after the hormonal treatments. For each variety, two lines were checked. For each line, three technical replicates were carried out.

Southern blot

Genomic DNA was digested completely by *Hind*III or *Bam*HI/*Eco*RI, and electrophoretically separated on an agarose gel (0.7 % w/v), then transferred to a positively charged nylon membrane (Boheringer Mannheim GmbH, Mannheim, Germany). The 923 bp GUS probe was labeled with digoxigenin by PCR using primers 13 and 14 (Table 1). Hybridization was carried out overnight at 42 °C. Disodium 3-(4-methoxyspiro {1,2-dioxetane-3,2'-(5'-chloro)tricyclo $[3.3.1.1^{3,7}]$decan}-4-yl)phenyl phosphate (CSPD) (Roche Molecular Biochemicals) was used as substrate for chemiluminescent detection, after high stringency washes. The membrane was exposed to X-ray film for 15–60 min to obtain the desired image strength.

Statistic methods

Grubbs test ($G = |\text{Suspect value} - x_{mean}| / s$) was used to test for outliers. The outliers were rejected if $G_{calculated} > G_{critical}$ at $P = 0.05$. The critical value of G is 1.155 when the sample size is three (Miller and Miller 2010).

Results and discussion

Analysis of promoter nucleotide sequence

A 1,148 bp promoter region upstream of the *cyp71av1* start codon (ATG) was isolated from genomic DNA from a high artemisinin chemotype of *A. annua* (Anamed) by genome walking using primers as listed in Table 1. We have also isolated the *cyp71av1* promoter from a high arteannuin B chemotype of *A. annua* (Iran 254) by PCR (data not shown). The two cloned promoters are identical. The obtained sequence (GenBank entry FJ870128) is shown in Fig. 2. Alignment of this sequence to other published

cyp71av1 promoter sequences (GenBank entries HM48927 and EF015297 and in Wang et al. 2011a) showed that a relatively high sequence homology is observed close to the start codon, *i.e.* around 100 bp upstream of the start codon (Fig. 3). From this alignment it is evident that the nucleotide sequences of the *cyp71av1* promoters are different in different *A. annua* varieties. The regulation of these promoters may differ. Figure 3 also shows that the open reading frame corresponding to the different promoters encode one and the same protein. Consequently, the different *cyp71av1* promoters appear to regulate one and the same *cyp71av1* gene. However, there may be more than one *cyp71av1* gene in the genome of *A. annua*. When we aligned the open reading frame (1,467 bp) of eight *cyp71av1* sequences available in the GenBank (DQ26 8763, DQ315671, DQ453967, DQ667171, DQ872632, EF197889, EU684540, HQ315834), seven of these sequences showed only up to 6 base substitutions while one sequence (DQ667171) showed substitution of 58 bases compared to the consensus sequence. This corresponds to a similarity of 96 % between DQ667171 and DQ453967 (the open reading frame corresponding to the promoter studied here). The corresponding amino acid sequences showed 0 to 3 substitutions except for DQ667171 that contained 30 substituted amino acids including 23 conserved substitutions. The identity and similarity between the amino acid sequences of DQ667171 and DQ453967 were 94 and 97 %, respectively. Based on these alignments, it may be suggested that there are at least two members in the *cyp71av1* gene family in *A. annua*.

The four promoters aligned in Fig. 3 have a common ATG start codon at position +1 but two of the promoters also have a putative start codon in frame at position −21. If this latter ATG is the actual start codon, these two proteins will have a 7 amino acids N-terminal extension compared to the other two proteins. At present, we cannot conclude if the four *cyp71av1* genes encode proteins of different length.

Attempts to predict the transcription start site (TSS) of the *cyp71av1* promoters using the TSSP software (http://linux1.softberry.com/berry.phtml) failed. However, we may assign a putative TSS of these promoters to an adenine base in the sequence TGACACCATGAA, which fits into the consensus sequence CAN(A/C)(A/C)(C/A)C(C/A)NNA(C/A) (underlined sequences are identical) determined for the TSS of highly expressed plant genes (Sawant et al. 1999). If this putative TSS located 24 bp upstream of the ATG codon common for all four genes is correct it is highly likely that the ATG-codon at position +1 is the start codon for all four genes (Figs. 2, 3).

The predicted core promoter elements TATA-box and CAAT-box were found at positions −27 to −22 (TATAAA) and −54 to −50 (CAATT), respectively, upstream of the putative TSS using the PlantCARE software (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (Fig. 2). These boxes were within conserved regions of all four *cyp71av1* promoter sequences (Fig. 3). The PlantCARE and PLACE (http://www.dna.affrc.go.jp/PLACE/) software were used to identify putative *cis*-acting regulatory elements in the *cyp71av1* promoters.

For *A. annua*, there are 514 putative transcription factors (TFs) classified in 48 families listed in the Plant Transcription Factor Database (http://planttfdb.cbi.edu.cn/index.php?sp=Aan). Some of these TFs may be involved in the regulation of the artemisinin biosynthesis. During recent years, some studies on the effects of a few TFs on the biosynthesis of artemisinin in *A. annua* have been reported. These include two jasmonate (JA)-responsive *APETALA2*/ethylene response factor (AP2/ERF) TFs, i.e. AaERF1 and AaERF2 (Yu et al. 2012) and the AaWRKY1 TF involved in the activation of defense-related genes (Ma et al. 2009).

Transient co-expression of *AaERF1* and a *pCYP71AV1-GUS* construct in tobacco induced the *pCYP71AV1* activity around six-fold while transient co-expression of *AaERF2* and *pADS-GUS* resulted in a three-fold increased activity of the *ads* promoter. Over-expression of AaERF1 or AaERF2 in transgenic *A. annua* plants resulted in elevated transcript levels of ADS (2- to eightfold) and CYP71AV1 (1.2- to fivefold) and in an increased accumulation of artemisinin and artemisinic acid (Yu et al. 2012). These results demonstrate that AaERF1 and AaERF2 are two positive regulators of artemisinin biosynthesis. The AP2/ERF TFs bind to CBF2 ([G/a][T/c]CGAC) and RAA (CAACA) motifs. One modified CBF2 motif (CTCGAC) was found at positions −106 to −101 and four RAA motifs (−33 to −29; −43 to −47; −436 to −432; −861 to −857) as shown in Fig. 2. In addition to these motifs, we have also found three CGTCA/TGACG JA-responsive *cis*-acting regulatory elements at positions −309 to 305, −308 to −312 and −894 to −898 (Fig. 2).

Basic/helix-loop-helix (bHLH) TFs may also be involved in the JA responsiveness of plants (Miyamoto et al. 2012). There are 35 such proteins listed in the *A. annua* TF database. Most bHLH TFs bind to the E-box sequence (CANNTG). The E-box sequence is also recognized by MYB and bZIP TFs. Five E-boxes are present in the *cyp71av1* promoter at positions −8 to −3, −74 to −69, −689 to −684, −763 to −758 and −1,021 to −1,016 (Fig. 2). Thus, E-box(es) may also be involved in the response of *A. annua* to JA.

Thus, the *cyp71av1* promoter carries a number of JA responsive *cis*-acting elements, which may be involved in the response of *A. annua* plants to methyljasmonate (MeJA). In fact, treatment of plants of *A. annua* with MeJA results in the induction of CYP71AV1 and other enzymes

of artemisinin biosynthesis such as FDS, ADS, DBR2 and ALDH1 leading to increased production of artemisinin (Guom et al. 2010; Maes et al. 2011; Wu et al. 2011; Caretto et al. 2011; Yu et al. 2012).

The WRKY TFs constitute a large family of TFs in plants (Rushton et al. 2010). They function via interaction with other proteins such as MAP kinases, 14-3-3 proteins or calmodulin and they bind to W1-boxes ([C/T]TGAC[C/T]). WRKY TFs may be involved in the simultaneous regulation of different processes.

For *A. annua,* there are 29 WRKYs listed in the plant transcription factor database. One of these has been cloned and its effect on artemisinin biosynthesis investigated (Ma et al. 2009). Transient expression of the AaWRKY1 TF in leaves of *A. annua* resulted in increased levels of HMGR (5x), ADS (7x), CYP71AV1 (3x) and DBR2 (4x) showing that several of the genes involved in artemisinin biosynthesis are induced by binding of the AaWRKY1 TF to W-boxes. Two such elicitor-responsive boxes have been identified at position −204 to −209 and −585 to −590 (Fig. 2). We have previously reported that the ads promoter also carries two W1-boxes (Wang et al. 2011b).

The effects of biotic elicitors (e.g. chitosan) involving WRKY TFs on artemisinin biosynthesis in plants (Lei et al. 2011), hairy root cultures (Putalun et al. 2007) and suspension cultures (Baldi and Dixit 2008) of *A. annua* have been reported. Foliar application of chitosan on *A. annua* resulted in increase of dihydroartemisinic acid and artemisinin by 72 and 53 %, respectively (Lei et al. 2011). Furthermore, semi-quantitative PCR showed increased levels of ADS, CYP71AV1 and DBR2 transcripts 2, 6 and 4 h, respectively, after application of chitosan.

Fig. 3 Alignment of nucleotide sequences of *cyp71av1* promoters from *Artemisia annua*. Conserved nucleotides are in *black boxes*. The putative transcription start codon ATG at position +1 is *underlined*; putative TATA- and CAAT-boxes are shown in *gray boxes*; putative transcription start site is indicated by a *dot* (·). The nucleotide sequence of the 5′-end of the open reading frames is included in *lower case* letters ▶

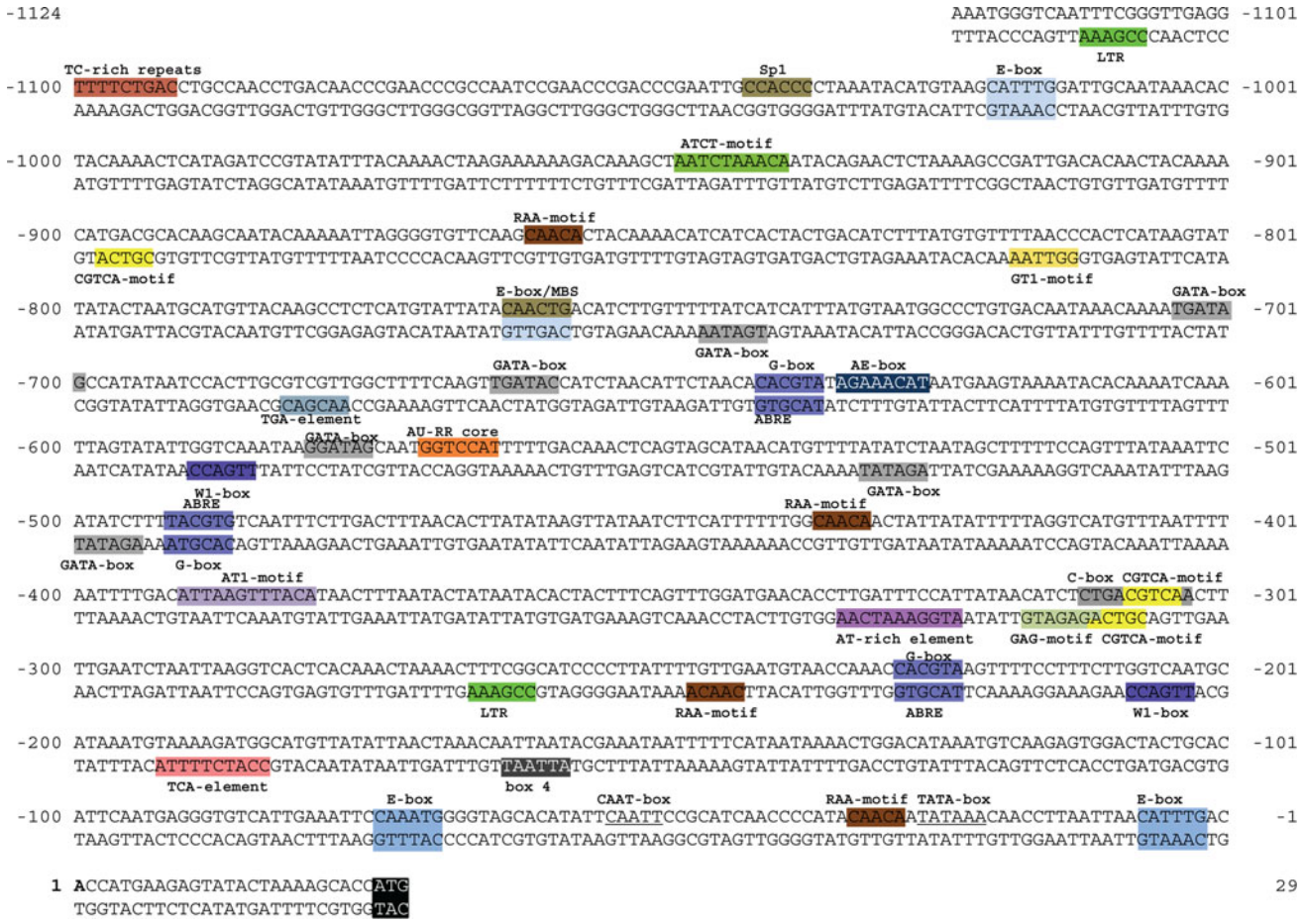
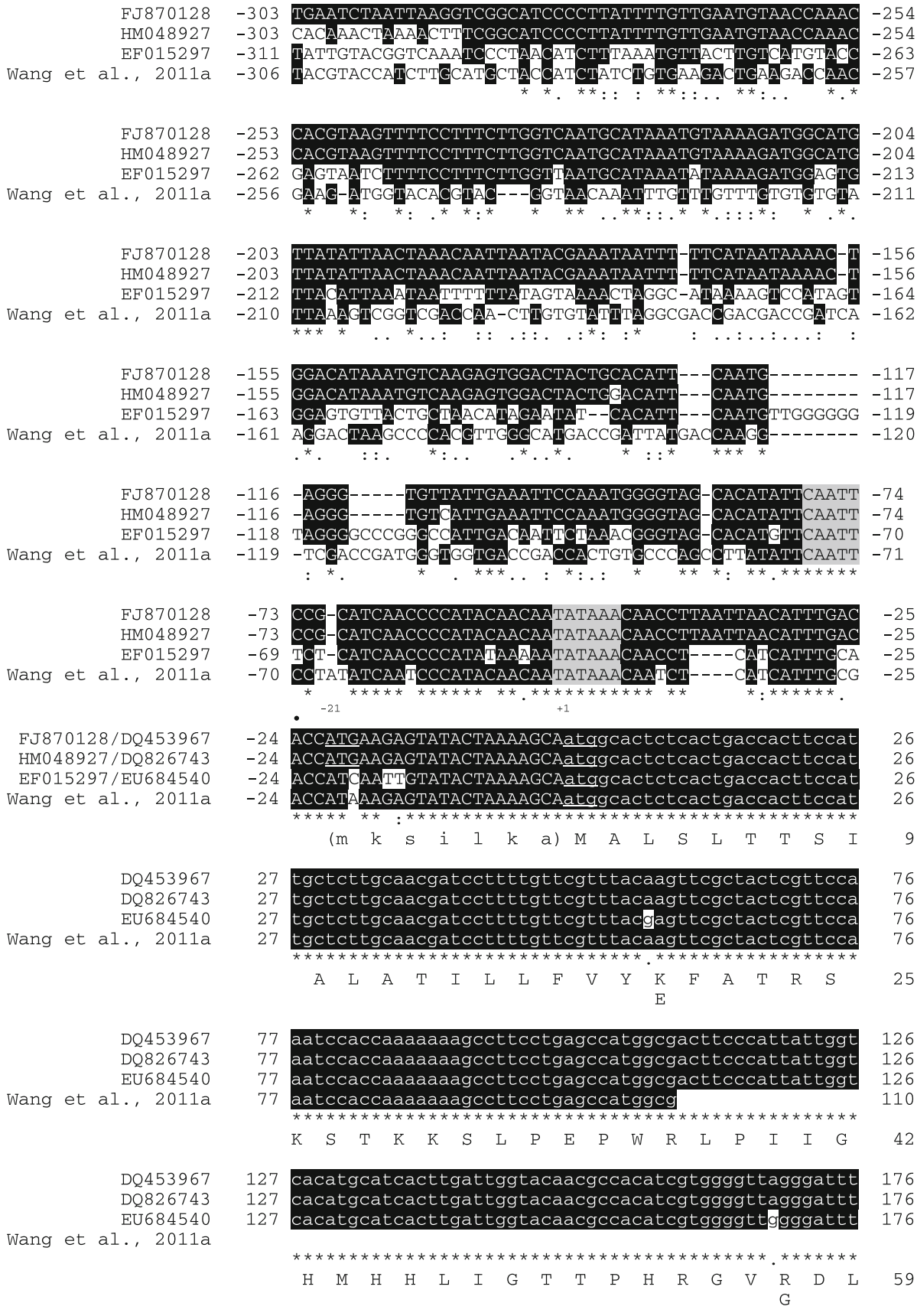


Fig. 2 Nucleotide sequence of the cloned *cyp71av1* promoter with putative *cis*-acting regulatory elements shown. The putative transcription start site is in bold. The TATA- and CAAT-box sequences are *underlined*. The first 200 bases of the open reading frames are included. The ATG start codon is *underlined*


FJ870128 -303 TGAATCTAATTAAGGTCGGCATCCCCTTATTTTGTTGAATGTAACCAAAC -254
HM048927 -303 CACAAACTAAAACTTTCGGCATCCCCTTATTTTGTTGAATGTAACCAAAC -254
EF015297 -311 TATTGTACGGTCAAATCCCTAACATCTTTAAATGTTACTTGTCATGTACC -263
Wang et al., 2011a -306 TACGTACCATCTTGCATGCTACCATCTATCTGTGAAGACTGAAGACCAAC -257
FJ870128 -253 CACGTAAGTTTTCCTTTCTTGGTCAATGCATAAATGTAAAAGATGGCATG -204
HM048927 -253 CACGTAAGTTTTCCTTTCTTGGTCAATGCATAAATGTAAAAGATGGCATG -204
EF015297 -262 GAGTAATCTTTTCCTTTCTTGGTTAATGCATAAATATAAAAGATGGAGTG -213
Wang et al., 2011a -256 GAAG-ATGGTACACGTAC---GGTAACAAATTTGTTTGTTTGTGTGTGTA -211
FJ870128 -203 TTATATTAACTAAACAATTAATACGAAATAATTT-TTCATAATAAAAC-T -156
HM048927 -203 TTATATTAACTAAACAATTAATACGAAATAATTT-TTCATAATAAAAC-T -156
EF015297 -212 TTACATTAAATAATTTTTTATAGTAAAACTAGGC-ATAAAAGTCCATAGT -164
Wang et al., 2011a -210 TTAAAGTCGGTCGACCAA-CTTGTGTATTTAGGCGACCGACGACCGATCA -162
FJ870128 -155 GGACATAAATGTCAAGAGTGGACTACTGCACATT---CAATG-------- -117
HM048927 -155 GGACATAAATGTCAAGAGTGGACTACTGGACATT---CAATG-------- -117
EF015297 -163 GGAGTGTTACTGCTAACATAGAATAT--CACATT---CAATGTTGGGGGG -119
Wang et al., 2011a -161 AGGACTAAGCCCCACGTTGGGCATGACCGATTATGACCAAGG-------- -120
FJ870128 -116 -AGGG-----TGTTATTGAAATTCCAAATGGGGTAG-CACATATTCAATT -74
HM048927 -116 -AGGG-----TGTCATTGAAATTCCAAATGGGGTAG-CACATATTCAATT -74
EF015297 -118 TAGGGGCCCGGGCCATTGACAATTCTAAACGGGTAG-CACATGTTCAATT -70
Wang et al., 2011a -119 -TCGACCGATGGGTGGTGACCGACCACTGTGCCCAGCCTTATATTCAATT -71
FJ870128 -73 CCG-CATCAACCCCATACAACAATATAAACAACCTTAATTAACATTTGAC -25
HM048927 -73 CCG-CATCAACCCCATACAACAATATAAACAACCTTAATTAACATTTGAC -25
EF015297 -69 TCT-CATCAACCCCATATAAAAATATAAACAACCT----CATCATTTGCA -25
Wang et al., 2011a -70 CCTATATCAATCCCATACAACAATATAAACAATCT----CATCATTTGCG -25
-21
+1
FJ870128/DQ453967 -24 ACCATGAAGAGTATACTAAAAGCAatggcactctcactgaccacttccat 26
HM048927/DQ826743 -24 ACCATGAAGAGTATACTAAAAGCAatggcactctcactgaccacttccat 26
EF015297/EU684540 -24 ACCATCAATTGTATACTAAAAGCAatggcactctcactgaccacttccat 26
Wang et al., 2011a -24 ACCATAAAGAGTATACTAAAAGCAatggcactctcactgaccacttccat 26
(m k s i l k a) M A L S L T T S I 9
DQ453967 27 tgctcttgcaacgatccttttgttcgtttacaagttcgctactcgttcca 76
DQ826743 27 tgctcttgcaacgatccttttgttcgtttacaagttcgctactcgttcca 76
EU684540 27 tgctcttgcaacgatccttttgttcgtttacgagttcgctactcgttcca 76
Wang et al., 2011a 27 tgctcttgcaacgatccttttgttcgtttacaagttcgctactcgttcca 76
A L A T I L L F V Y K F A T R S 25
E
DQ453967 77 aatccaccaaaaaaagccttcctgagccatggcgacttcccattattggt 126
DQ826743 77 aatccaccaaaaaaagccttcctgagccatggcgacttcccattattggt 126
EU684540 77 aatccaccaaaaaaagccttcctgagccatggcgacttcccattattggt 126
Wang et al., 2011a 77 aatccaccaaaaaaagccttcctgagccatggcg 110
K S T K K S L P E P W R L P I I G 42
DQ453967 127 cacatgcatcacttgattggtacaacgccacatcgtggggttagggattt 176
DQ826743 127 cacatgcatcacttgattggtacaacgccacatcgtggggttagggattt 176
EU684540 127 cacatgcatcacttgattggtacaacgccacatcgtggggttggggattt 176
Wang et al., 2011a
H M H H L I G T T P H R G V R D L 59
G

ABA-responsive element (ABRE) is a major *cis*-acting element (PyACGTGGC) in ABA-responsive gene expression. Three such ABA-responsive elements (TACGTG) were found at the positions −224 to −229, −492 to −487 and −636 to −641 (Fig. 2). A single copy of the ABRE is not sufficient to mediate ABA regulation unless a coupling *cis*-element such as coupling element 1 (CE1) (TGCCACCGG) (Shen and Ho 1995), CE3 (ACGCGTGTCCTC) (Shen et al. 1996) or dehydration responsive element (DRE) (TACCGACAT) (Narusaka et al. 2003) is present. We have not found any such element in this promoter except for a putative CE1 element (TGCCACCCC) at position −1,044 to −1,036 (Fig. 2). This CE1 element may be involved in a response of this promoter to ABA. However, a response to ABA can be seen when the promoter caries multiple copies of the ABRE element (Hobo et al. 1999) and therefore we may expect a response of the *cyp71av1* gene to ABA. It is well established that bHLH TFs are involved in the response of plants to ABA (Kim and Kim 2006). As discussed above these proteins bind to the E-box sequence. There are five E-boxes in the *cyp71av1* promoter (Fig. 2).

The ABRE and E-box *cis*-elements may both be involved in the response to ABA. It has recently been shown that the expression of *CYP71AV1* is induced in *A. annua* by ABA (Jing et al. 2009).

Salicylic acid (SA) is involved in the defense response of plants leading to the biosynthesis of secondary products such as phytoalexins (Angelova et al. 2006). Treatment of *A. annua* plants with 1 mM SA resulted in a slight increase (around 35 %) in the levels of CYP71AV1 transcripts 4–8 h after the treatment (Pu et al. 2009). HMGR and ADS transcripts were strongly induced by SA. Increased levels of artemisinic acid, dihydroartemisinic acid and artemisinin were observed 2–4 days after treatment. One SA-responsive TCA-element (CCATCTTTTA) was found at positions −184 to −193 (Fig. 2). Similarly, we found one TCA-element in the *ads* promoter (Wang et al. 2011b).

Treatment of *A. annua* with gibberellic acid 3 (GA_3) results in an increased formation of artemisinin (Zhang et al. 2005; Aftab et al. 2010; Banyai et al. 2011; Maes et al. 2011). In one study, it was shown with qPCR that the levels of transcripts of FDS, ADS and CYP71AV1 were increased by treatment with 20 mg/l GA_3 by soil drenching (Banyai et al. 2011). The GARE motif (AAACAGA) is involved in the GA-responsiveness. While such an element is present in the *ads* promoter (Wang et al. 2011b), we could not find any GARE-motif in the present promoter. However, in the *cyp71av1* promoter described by Wang et al. (2011a), two GARE motifs are present. This indicates that the different *cyp71av1* promoters are differently regulated and possibly involved in different responses of the plant to external stimuli.

A number of other putative *cis*-acting regulatory elements are present in the *cyp71av1* promoter (Fig. 2). These include a number of light responsive elements (one AE-box (AGAAACAT) −634 to −627; one AT1-motif (ATTA-ATTTTACA) −382 to −391; one ATCT-motif (AATC-TAATCT) −948 to −939; one C-box (CTGACGTCAG) −113 to −304; three G-boxes (CACGTA) −229 to −224, −487 to −492 and −641 to −636; one GAG-motif (AG-AGATG) −312 to −318; two GATA boxes ([A/T]GATA[A/G]) −705 to −700 and −746 to −751; one GT1-motif (GGTTAA) −814 to −819; one Sp1 (CC[G/A]CCC) −1,042 to −1,037), temperature responsive elements (two LTR (CCGAAA) −261 to −266 and −1,108 to −1,113), auxin responsive elements (one AuxRR-core (GGTCCAT) −570 to −564; one TGA-element (AAC-GAC) −677 to −682), drought-inducibility (one MBS (CAACTG) −763 to −758 and elements involved in endosperm expression (one GCN4-motif (TGTGTCA −910 to −916; three Skn-1 motifs (GTCAT) −87 to −83, −415 to −411 and −895 to −899).

Further analysis of the different promoter sequences showed that some homologous sequences could be found far apart in the promoters. For instance, the sequences AAATGTCAAGAGTGGACT (−126 to −109), GCACA (−105 to −101) and TTCAATGAG (−CTG100 to −92) of the promoter cloned by us (Fig. 2) could be found further upstream in a mixed order at positions −704 to −687 (AAAAGTCAAGAGTGGAC), −157 to −153 (GCACA) and −612 to −604 (TTCAATGAG) in the promoter from Wang et al. (2011a). No *cis*-acting regulatory elements could be predicted in these sequences using the PlantCare and PLACE softwares. However, it may be assumed that these conserved sequences may be involved in the regulation of the two promoters.

Studies on the activity of the cyp71av1 *promoter of in transgenic* Artemisia annua

We have studied the activity of the *cyp71av1* promoter cloned by us (FJ870128) using a promoter-*GUS* fusion in transgenic *A. annua*. The binary transformation vector was prepared as described for the construction of the *ads* promoter *GUS* fusion in a modified pCAMBIA 1381Z vector (Wang et al 2011b) (Fig. 4).

We have used two different varieties of *A. annua* for these studies, *i.e.* Chongqing and Anamed. Both these varieties are of the high artemisinin type. However, the glandular trichome density is somewhat higher in Anamed as compared to Chongqing as shown by scanning electron microscopy (Fig. 5). Figure 5 also shows the presence of TSTs on both varieties.

Sixty-one transgenic lines of the Chongqing variety and fifteen lines of the Anamed variety transformed with the

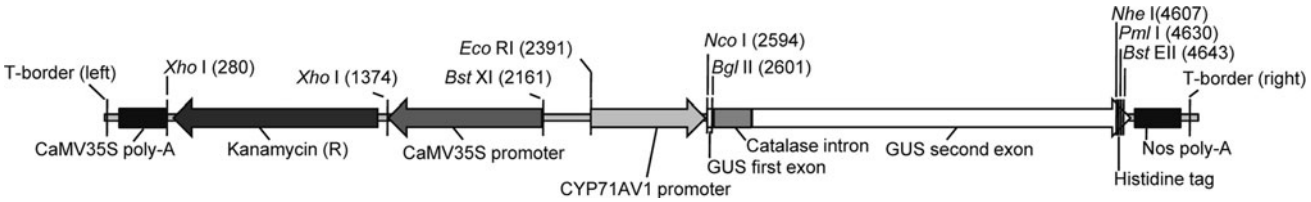


Fig. 4 Schematic diagram of T-DNA region of the modified pCAMBIA 1381Z used to transform *Artemisia annua*

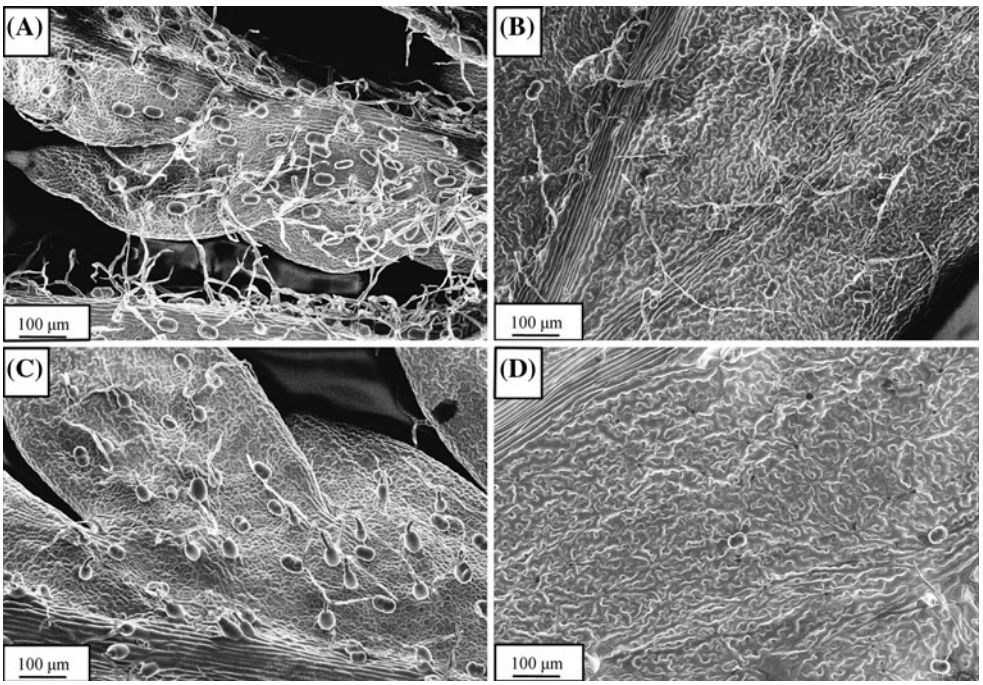


Fig. 5 Scanning electron micrographs of leaves from *Artemisia annua*. **a** and **b** young and old leaf from the Anamed variety, respectively; **c** and **d** young and old leaf from the Chongqing variety, respectively

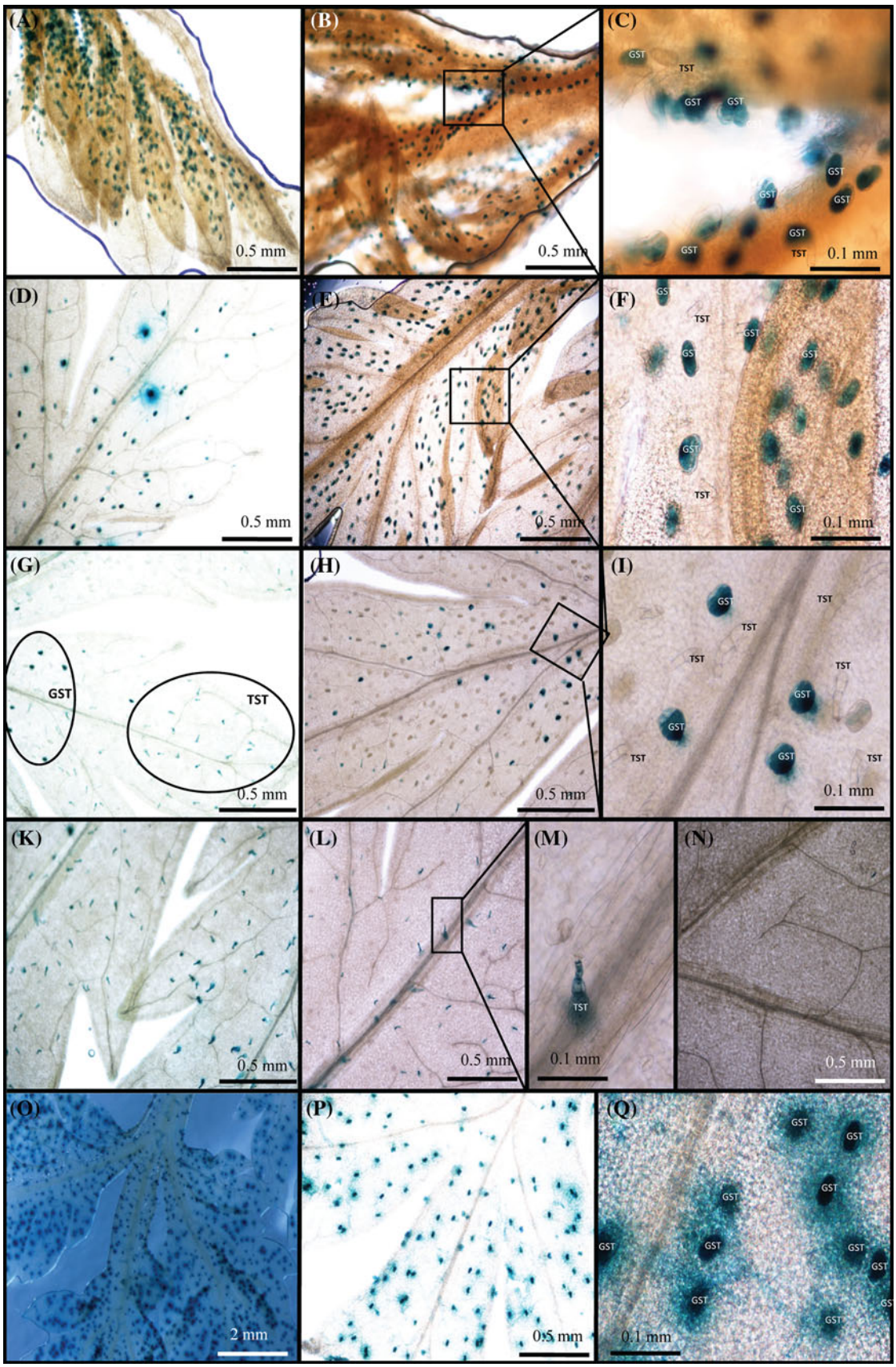
pCYP71AV1-GUS construct were obtained by *Agrobacterium*-mediated transformation. At the same time, 24 and 15 transgenic plants transformed with the control transformation vector were obtained for the Anamed and Chongqing variety, respectively, which were used as control plants. All transgenic lines showed *GUS* expression pattern while control plants showed as expected no GUS staining.

For the Chongqing variety, we observed that *GUS* expression in leaves at different nodes is different. *GUS* expression is exclusively located to glandular trichomes of leaf primordia at the apex, and become stronger as the plant grows older (Fig. 6a–c). In expanded leaves at node 1–2, *GUS* expression is still specifically localized to glandular trichomes, but near the leaf tip, a few TSTs showed *GUS* expression (Fig. 6d–f). In the leaves of the 3rd to 4th node, *GUS* expression showed a shift from glandular trichomes to TSTs, i.e. more TSTs and less GSTs showed GUS staining (Fig. 6g–m). Finally, in old leaves no *GUS* expression was observed (Fig. 6n).

GUS expression pattern is basically the same in the Anamed variety (Fig. 6o–q). *GUS* expression was exclusively located to GSTs of leaf primordia (Fig. 6o) and top expanded leaf (Fig. 6p). As the leaf becomes older there is a shift to TSTs. Only TSTs showed GUS staining in lower leaf and there is no GUS staining in old leaves at bottom of the plant. Some transgenic lines showed very strong *GUS* expression in leaf primordial and top leaf leading to leakage of GUS and staining of the leaf surface around the GSTs (Fig. 6q).

The results obtained showed similar expression pattern in the two varieties tested and they confirmed that the cloned promoter carries the main *cis*-acting regulatory elements involved in GST specific expression. A possible explanation for the observed differences may be that the number of GSTs and the developmental events are different in the two varieties.

GUS expression in flower buds at earlier stage of development of the Chongqing variety is specifically


(A)
0.5 mm
(B)
0.5 mm
(C)
GST
TST
GST
GST
GST
GST
GST
GST
TST
0.1 mm
(D)
0.5 mm
(E)
0.5 mm
(F)
GST
TST
GST
GST
GST
TST
GST
0.1 mm
(G)
GST
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0.5 mm
(H)
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GST
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0.1 mm
(K)
0.5 mm
(L)
0.5 mm
(M)
TST
0.1 mm
(N)
0.5 mm
(O)
2 mm
(P)
0.5 mm
(Q)
GST
GST
GST
GST
GST
GST
GST
GST
GST
GST
0.1 mm

◀**Fig. 6** GUS staining of leaves of different ages from two varieties. *Panels* **a**–**n** Chongqing variety; **a** bract at apex from 15 cm plantlet; **b** bract at apex from 50 cm plant; **c** magnification (×5) of selected are in *panel* **b**; **d** expanded leaf at node 1–2 from 15 cm plantlet; **e** expanded leaf at node 1–2 from 50 cm plant; **f** magnification (×5) of selected are in *panel* **e**; **g** expanded leaf at node 3–4 from 15 cm plantlet; **h** expanded leaf at node 3–4 from 50 cm plant; **i** magnification (×5) of selected are in *panel* **h**; **k** expanded leaf at node 5–6 from 15 cm plantlet; **l** expanded leaf at node 5–6 from 50 cm plant; **m** magnification (×5) of selected are in *panel* **l**; **n** expanded leaf at bottom from 50 cm plant. *Panels* **o**–**q**: Anamed variety. **o**: expanded leaf at node 1–2 from 15 cm plantlet; **p**: expanded leaf at node 1–2 from 50 cm plant; **q**: magnification (×5) of expanded leaf at node 1–2 from 50 cm plant

located to glandular trichomes (Fig. 7a–c, f). No TSTs showed *GUS* expression at this stage. However, flower buds at later stages showed *GUS* expression in GST as well as in TSTs on basal bracts and pedicles (Fig. 7d–e). However, the density of GSTs (90 %) is much higher than that of TSTs in flower buds (Teoh et al. 2006). In the whole flower bud stage, GUS showed specific expression in GSTs, which indicated that *cyp71av1* expression is specifically located to GSTs in flower buds.

We may conclude that the expression of *cyp71av1* is highest in relatively young tissues with GSTs in early developmental stages. This is also true for the *ads* gene (Wang et al. 2011b) These findings support the idea that the total quantity of artemisinin precursors per leaf (i.e. dihydroartemisinic acid and other upstream precursors) is highest in early leaf cycle when the leaf is still expanding. It has been shown that the concentration of dihydroartemisinic acid (cf. Fig. 1) is declining during leaf development (Lommen et al. 2006).

GUS expression was observed exclusively in glandular secretory trichomes in young tissues. This shows that the cloned promoter carries *cis*-elements that are related to specific expression in GSTs. Expression in TSTs occurred in leaves at node 1 to 5 but as the leaves grow older it decreased and almost no *GUS* expression was observed in the old leaf at the bottom of the plant. A possible reason for this may be that the trichomes of old leaves have collapsed (Lommen et al. 2006). This is in line with the finding that the molar quantity of dihydroartemisinic acid is higher than that of artemisinin in green leaf, but only 19–27 % of that of artemisinin in dead leaves (Lommen et al. 2007). The observed pattern of *GUS* expression in glandular trichomes and TSTs indicated that the *cyp71av1* gene is only expressed at special stages of trichome development. At present, we cannot say if the wild-type *cyp71av1* promoter is active in TSTs in the same way as the cloned promoter. It is possible that expression of GUS in TSTs is due to the removal of one or more *cis*-acting elements during cloning.

Studies on the activity of the cyp71av1 *promoter of in transgenic* Nicotiana tabacum

In order to investigate if the *cyp71av1* promoter exhibits trichome-specific expression in other plant species, we transformed *Nicotiana tabacum* with the *pCYP71AV1-GUS* construct. Transgenic plants were verified by PCR. GUS-staining of transgenic *N. tabacum* showed that the *cyp71av1* promoter is active in this plant and that the expression is trichome-specific (Fig. 8). *GUS* expression is seen in glandular heads, which is the site for exudate production. No staining could be detected in stalk cells.

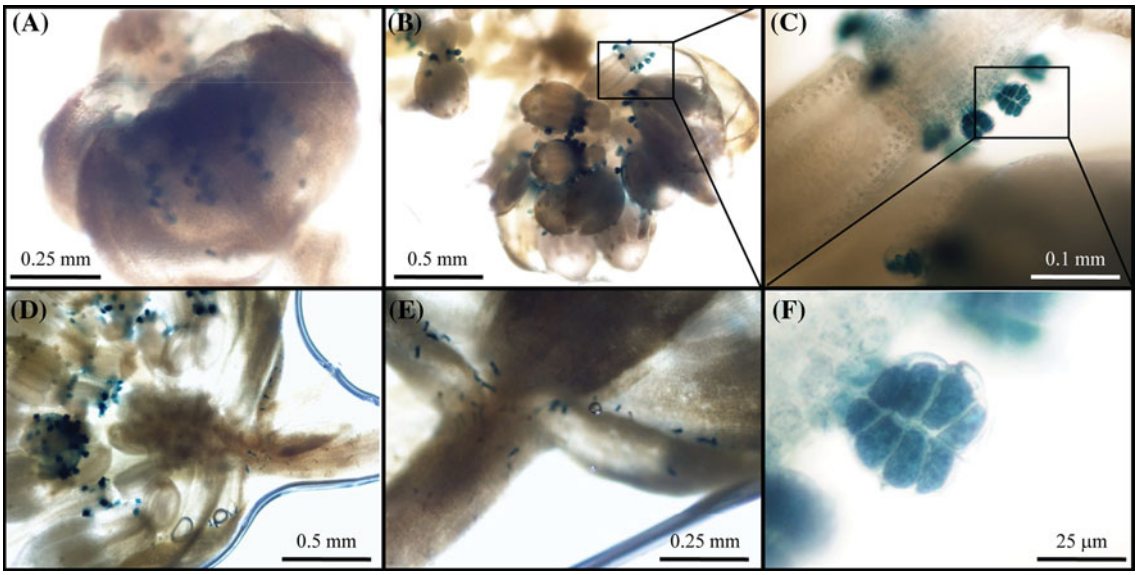

Fig. 7 GUS staining of flower buds and flowers of transgenic *Artemisia annua* (Chongqing variety). **a** Intact flower buds; **b** section of flower buds; **c** magnification (×5) of selected are in *panel* **b**; **d** flower; **e** flower; **f** magnification (×4) of selected are in *panel* **c**

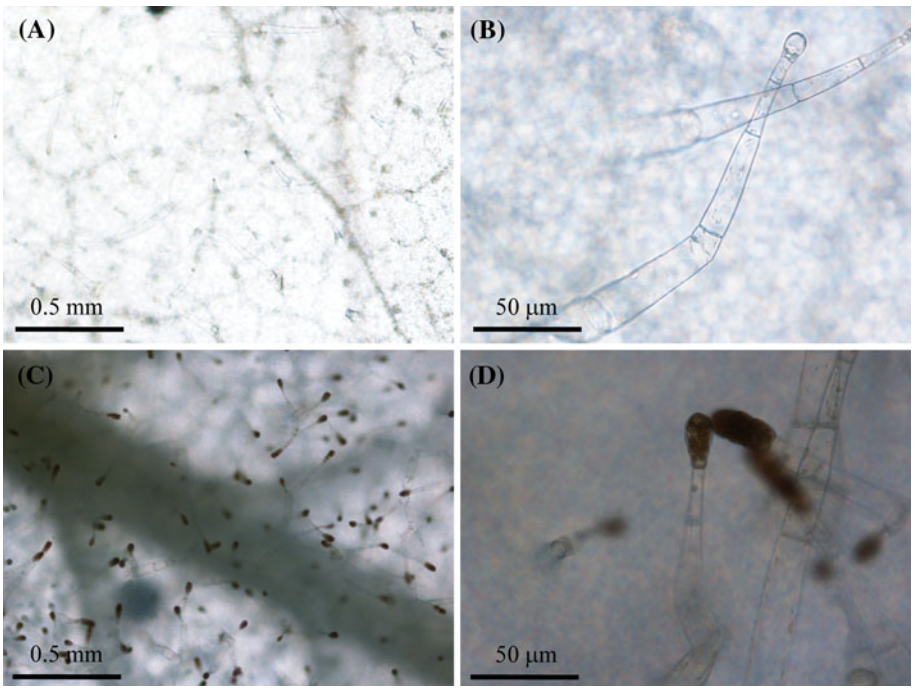

Fig. 8 GUS staining of leaves of *Nicotiana tabacum*. **a** Leaf from a non-transformed plant (control); **b** magnification (×5) of leaf from a non-transformed plant; **c** leaf from a transgenic plant expressing the *pCYP71AV1-GUS* construct; **d** magnification (×5) of leaf from a transgenic plant

Thus, the cloned *cyp71av1* promoter from *A. annua* may offer a tool for the expression of transgenes in glandular trichomes of other plants.

Expression pattern of pCYP71AV1-CYP71AV1 and pCYP71AV1-GUS in Artemisia annua

The relative activity of the wild-type and the recombinant *cyp71av1* promoters has been quantified by qPCR using primers as listed in Table 1. β-Actin was used as reference gene. Relative expression levels of *pCYP71AV1-GUS* and *pCYP71AV1-CYP71AV1* were estimated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Softeware REST 2009 (http://www.REST.de.com) was used to analysis the data. The highest relative activity of the wild-type *cyp71av1* promoter was seen in leaf primordia at apex and leaves at nodes 1–2 (Fig. 9a) as compared to leaves at nodes 5–6 (relative expression set to 1.0). In leaves at lower nodes the activity of the wild-type promoter drops rapidly showing that the *cyp71av1* gene is specifically expressed in glandular trichomes of upper leaves (Teoh et al. 2006; Olsson et al. 2009). The observed expression of *pCYP71AV1-GUS* shows a similar but not identical pattern (Fig. 9b). It indicates that the cloned promoter carries the main *cis*-acting elements that are related to the specific expression of *cyp71av1* in GSTs, even if some remote *cis*-acting elements are missing. These may include *cis*-elements involved in the down-regulation of *cyp71av1* expression in TSTs in lower leaves. These results were in line with GUS staining results obtained.

Expression levels of *pCYP71AV1-CYP71AV1* and *pCYP71AV1-GUS* in different tissues of transgenic *A. annua* were also investigated by qPCR (Fig. 10). The relative expression in roots was set to 1.0 in these experiments. The wild type *cyp71av1* promoter showed lower expression levels in flower buds than leaf and stem (Fig. 10a) while the cloned promoter showed higher relative expression levels in flower buds and leaf, compared to stem and root (Fig. 10b). This indicates that the activity of the cloned promoter has been altered during cloning and that the expression in GSTs is enhanced as discussed below. The low activity of the wild-type promoter in transgenic plants (Fig. 10a) may be due to the presence of the highly active recombinant promoter (Fig. 10b) and the competition for transcription factors. The limited availability of transcription factors may result in a decrease activity of the wild-type *cyp71av1* promoter to a level even lower that that observed in leaves and stem. We have previously shown that the overall activity of the *cyp71av1* promoter is around 200 times higher in flower buds and young leaves as compared to old leaves of wild-type plants (Olofsson et al. 2012).

We may also compare the relative activity of the two promoters in one and the same tissue using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). The cloned promoter showed a considerably higher activity in flower buds than the wild-type promoter. The activity of the cloned promoter was around 250 and 500 times higher in flower buds at early and late stage of development, respectively.

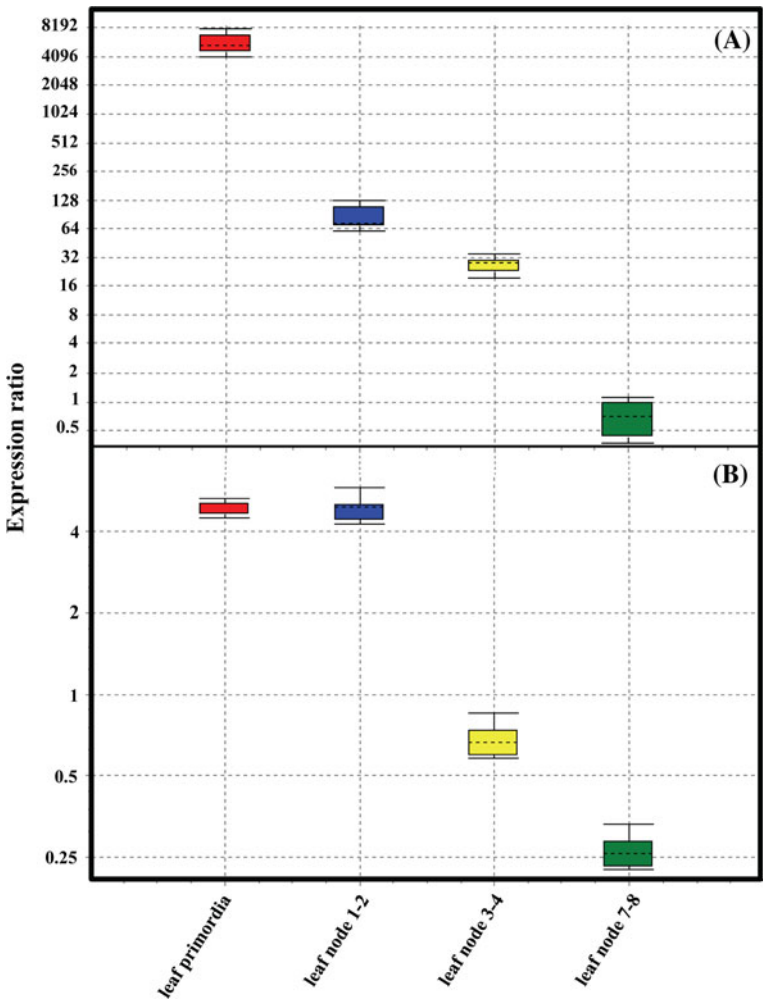

Fig. 9 Relative expression of *pCYP71AV1-CYP71AV1* (**a**) and *pCYP71AV1-GUS* (**b**) in leaves of *Artemisia annua* at different stages of development. All activities are relative to the activity in leaf at nodes 5–6, which was set to 1.0

Apparently, one or more silencing *cis*-acting regulatory elements are not included in the cloned *cyp71av1* promoter. The lack of this silencing element may be the reason for the observed expression of GUS in TSTs. It is possible that such silencers are located downstream of the *cyp71av1* start codon, *e.g.* in an intron as has been reported for other plant genes (Mascarenhas et al. 1990; Deyholos and Sieburth 2000). There is no genomic sequence of the coding region available for the two varieties used in this study and therefore we cannot look for *cis*-acting regulatory elements in any intron.

The cloned *cyp71av1* promoter appears to be an excellent promoter to use for metabolic engineering of *A. annua* and other plants producing valuable compounds in their trichomes. This promoter shows trichome specific expression and it is deregulated, which will lead to high expression of any transgene. We have initiated studies on the use of the recombinant *cyp71av1* promoter for metabolic engineering of *A. annua* with the aim to increase the yield of artemisinin.

Response of the cyp71av1 *promoter to wounding*

Upper expanded leaf at the 1st and 2nd nodes and lower leaves at the 3rd to 6th node of five transgenic plants (two varieties) 1 month old after rooting and at reproductive stage were wounded by cutting along the midrib with a razor blade. Samples were GUS stained after different incubation times (0–48 h). No GUS staining was observed at the wounded location in any of the samples. Transcript levels of *pCYP71AV1-CYP71AV1* and *pCYP71AV1-GUS* were essentially the same before and after wounding. It showed that *CYP71AV1* appears not play any role in *A. annua* responsiveness to wounding. However, in another study, artemisinin content was increased 50 % four hours after wounding (Liu et al. 2010). The transcript level of *cyp71av1* was also increased. The differences observed may be due to the use of different varieties of *A. annua*. Some sesquiterpene synthases, such as caryophyllene and *epi*-cedrol synthases, play a direct role in the wounding responsiveness of *A. annua* (Wang, unpublished).

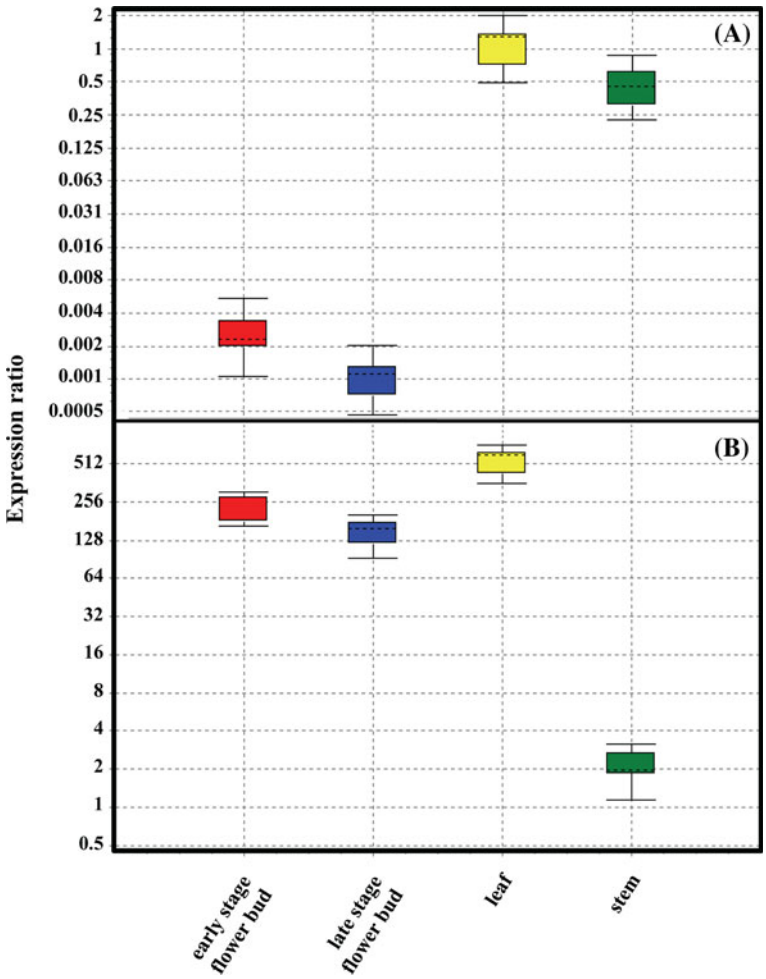

Fig. 10 Relative expression of *pCYP71AV1-CYP71AV1* (**a**) and *pCYP71AV1-GUS* (**b**) in different tissues of *Artemisia annua*. All activities are relative to the activity in roots, which was set to 1.0

Response to hormones

Cuttings of two transgenic lines of each variety of *A. annua* were used for hormone responsiveness experiments. The plants were about 1 month old after rooting. MeJA responsiveness experiments were carried out by spraying the hormone on the plants. Plants treated with water were used as control. Upper leaves were sampled at 10, 24 and 48 h after treatment. The expression levels of the *cyp71av1* and *GUS* transcripts were determined by qPCR. The two promoters showed similar response pattern to MeJA treatment in the two transgenic lines of each variety. It indicated that the main *cis*-acting elements that control responsiveness to MeJA are present in the recombinant promoter. In the Chongqing variety, both promoters showed the highest level of expression at 24 h after spraying with MeJA (Fig. 11a). Transcripts of *pCYP71AV1-CYP71AV1* and *pCYP71AV1-GUS* were induced 23 and 75 times respectively. In the Anamed variety, both promoters reached the highest expression levels 48 h after spraying (Fig. 11b). At this point, the transcript levels of *pCYP71AV1-CYP71AV1* and *pCYP71AV1-GUS* were 51 and 8 times higher than in control plants, respectively. These results verify that transcripts of *pCYP71AV1-CYP71AV1* show a chemotype–dependent expression in different varieties of *A. annua* (Wu et al. 2011). Furthermore, we may conclude that the *cis*-elements that control the expression differences in the chemotypes are maintained in the recombinant promoter. From above, we can see that the main *cis*-acting elements that control inducible expression to MeJA are included in the cloned promoter.

Southern blot

Genomic DNA from wild-type and transgenic plants were digested completely by *Hind*III or *Bam*H1/*Eco*RI, and the fragments obtained were analyzed by Southern blotting using a hybridization probe binding to the *GUS* gene. Four transgenic lines of the Anamed variety were included in the analysis and the copy number of the transgene was at least three, two, three and three for the lines cyp8, cyp1–3, cyp15 and cyp16, respectively. Cyp15 and cyp16 are included in the Southern blot shown in Fig. 12. For two transgenic lines of the Chongqing variety (cypB12 and

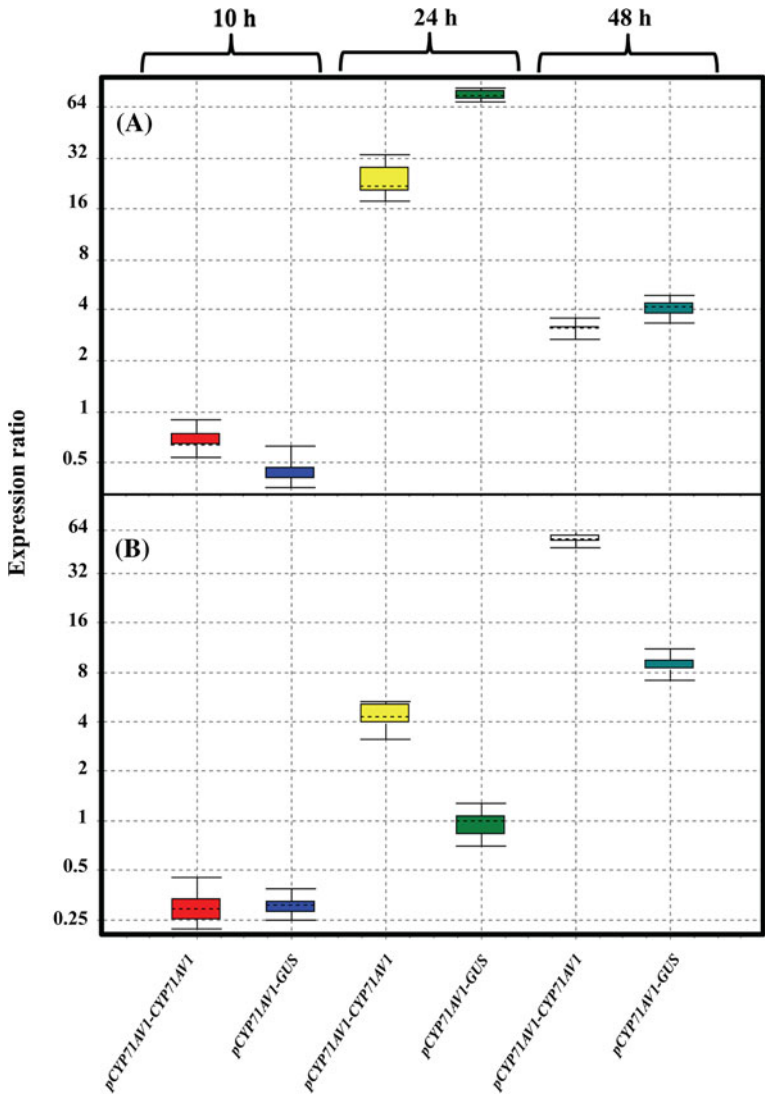

Fig. 11 Relative expression of *pCYP71AV1-CYP71AV1* and *pCYP71AV1-GUS* after treatment with MeJA compared to untreated transgenic *Artemisia annua* plants. The levels of transcripts were determined by qPCR. **a** Chongqing variety, **b** Anamed variety

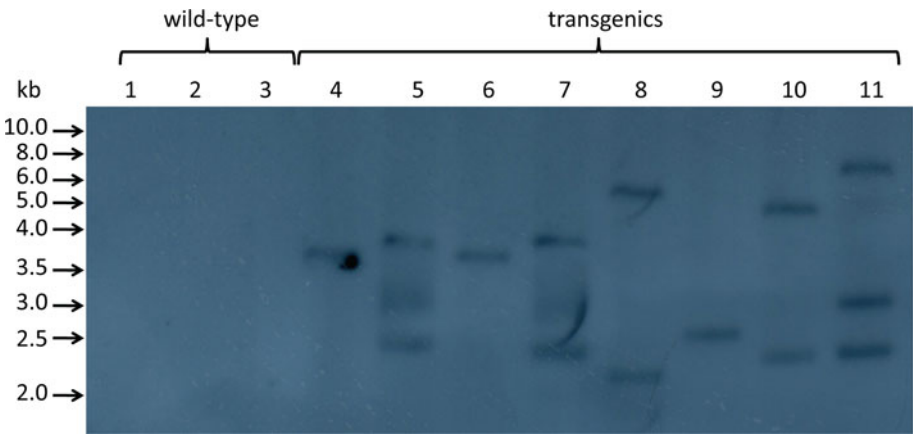

Fig. 12 Southern blot of genomic DNA isolated from wild-type (*lane 1–3*) and transgenic (*lane 4–11*) *Artemisia annua* using a digoxigenin-labeled GUS probe. *Lane 1* Wild-type Anamed digested with *Eco*R1 and *Bam*H1, *lane 2* wild-type Chongqing digested with *Eco*R1 and *Bam*H1, *lane 3* wild-type tobacco digested with *Eco*R1 and *Bam*H1, *lane 4* transgenic Anamed cyp15 digested with *Eco*R1 and *Bam*H1, *lane 5* Transgenic Anamed cyp15 digested with *Hind*III, *lane 6* transgenic Anamed cyp16 digested with *Eco*R1 and *Bam*H1, *lane 7* transgenic Anamed cyp16 digested with *Hind*III, *lane 8* transgenic Chongqing cypB12 digested with *Eco*R1 and *Bam*H1, *lane 9* transgenic Chongqing cypB12 digested with *Hind*III, *lane 10* transgenic tobacco cyp221 digested with *Eco*R1 and *Bam*H1 *lane 11* transgenic tobacco cyp221 digested with *Hind*III

Table 2 Selected *Cis*-acting regulatory elements in promoters of genes encoding enzymes involved in artemisinin biosynthesis in *Artemisia annua*

Motif	Number of motifs				Nucleotide sequence	Description
	ADS	CYP71AV1 FJ870128	CYP71AV1 EF015297	CYP71AV1 Wang et al. (2011a)		
ABRE	*2*	*3*	*5*	*4*	*TACGTG*	Cis-*acting element involved in the abscisic acid responsiveness*
ACE	1	0	6	1	AAA[C/A]CG[T/G]TTA/GCGACGTACC	*Cis*-acting element involved in light responsiveness
ATC-motif	0	1	1	0	GCCAATCC/AGTAATCT	Part of a conserved DNA module involved in light responsiveness
AE-box	2	1	0	1	AGAAACA(A/T)	Part of a module for light response
ARE	4	0	0	2	TGGTTT	*Cis*-acting regulatory element essential for unaerobic induction
AT1-motif	0	1	0	0	ATTAATTTTACA	Part of a light responsive module
ACTC-motif	0	1	0	0	AATCTAATCT	Part of a conserved DNA module involved in light responsiveness
As1	0	1	0	0	TGACGTCA	*Cis*-acting regulatory element involved in the root-specific expression
AT-rich element	0	1	0	0	ATAGAAATCAA	Binding site of AT-rich DNA binding protein (ATBP-1)
AU-RR core	1	1	0	0	GGTCCAT	*Cis*-acting regulatory element involved in auxin responsiveness
Box 1	2	0	2	1	TTTCAAA	Light responsive element
Box II	0	0	2	0	ACACGTTGT	Part of light responsive element
Box 4	2	1	0	0	ATTAAT	Part of a conserved DNA module involved in light responsiveness
Box-W1	*2*	*3*	*1*	*1*	*TTGACC*	*Fungal elicitor responsive element*
CAT-box	0	0	0	1	GCCACT	*Cis*-acting regulatory element related to meristem expression
CATT-motif	0	0	0	1	GCATTC	Part of a light responsive element
CBFHV	2	0	0	8	RYCGAC	*Cis*-acting elements related to cold acclimation
C-box	0	1	0	0	CTGACGTCAG	*Cis*-acting regulatory element involved in light responsiveness
CCGTCC-box	0	0	0	1	CCGTCC	*Cis*-acting regulatory element related to meristem specific activation
CGTCA-motif	0	3	0	1	CGTCA	*Cis*-acting regulatory element involved in the MeJA-responsiveness
Circadian	2	0	1	2	CAANNNNATC	*Cis*-acting regulatory element involved in circadian control
E-box	*12*	*5*	*5*	*6*	*CANNTG*	*JA-, BR-, and ABA-responsiveness; recognized by MYB, bZIP and bHLH TFs*
ERE	1	0	0	0	ATTTCAAA	Ethylene-responsive element
GAG-motif	1	1	0	0	(A/G)GAGATG	Part of a light responsive element
GARE-motif	1	0	2	2	AAACAGA	Gibberellin-responsive element
GATA-box	13	6	1	13	GATA	Part of light responsive element
G-box	*3*	*4*	*11*	*4*	*CACG(A/T)(C/T)*	Cis-*acting regulatory element involved in MeJA and light responsiveness*
GCN4	2	1	0	2	TG(A/T)GTCA	*Cis*-regulatory element involved in endosperm expression
GT1-motif	3	1	1	2	AATCCACA/GGTTAAT	Light responsive element
HSE	2	0	0	1	AAAAAATTTC	*Cis*-acting element involved in heat stress responsiveness

Table 2 continued

Motif	Number of motifs				Nucleotide sequence	Description
	ADS	CYP71AV1 FJ870128	CYP71AV1 EF015297	CYP71AV1 Wang et al. (2011a)		
LTR	0	2	0	1	CCGAAA	*Cis*-acting element involved in low-temperature responsiveness
MBS	*2*	*1*	*1*	*3*	*TAACGG/CGGTCA*	*MYB binding site*
MNF1	0	0	0	1	GTGCCC(A/T)(A/T)	Light responsive element
O2-site	0	2	0	2	GATGAT(A/G)TGG	*Cis*-acting regulatory element involved in zein metabolism regulation
P-box	0	0	0	1	CCTTTTG	Gibberellin-responsive element
Py-rich streach	1	0	0	0	TTTCTTCTCT	*Cis*-acting element conferring high transcription levels
RAA-motif	*4*	*4*	*1*	*5*	*CAACA*	*Binding site for AP2/ERF transcription factors*
RY-element	0	0	0	3	CATGCATG	*Cis*-acting regulatory element involved in seed-specific regulation
Skn-1	8	3	2	5	GTCAT	*Cis*-acting regulatory element required for endosperm expression
Sp1	2	1	1	1	CC(G/A)CCC	Light responsive element
TCA-element	*1*	*1*	*2*	*0*	*GAGAAGAATA/CCATCTTTTT*	Cis-*acting element involved in salicylic acid responsiveness*
TC-rich repeats	*1*	*1*	*3*	*4*	*(A/G)TTTTCTT(A/C)(C/A)*	Cis-*acting element involved in defense and stress responsiveness*
TCT-motif	1	0	3	0	TCTTAC	Part of a light responsive element
TGA-element	*1*	*1*	*3*	*1*	*AACGAC*	*Auxin-responsive element*

The italicized *Cis*-acting elements are discussed in more detail

cypD25), the copy number of the transgene was two for both lines. CypB12 is shown in Fig. 12. Finally, two lines of transgenic tobacco (cyp221 and cyp222) were analyzed by Southern blot. There are at least 3 copies of the transgene inserted in genomic DNA of these two tobacco lines. Cyp221 is included in the blot shown in Fig. 12.

The results from Southern blotting showed that transgenic lines, which exhibit similar GUS expression may be originating from the same transformation event. For example, Anamed cyp15 and cyp16 show the same fragments in the Southern blots (Fig. 12). One transgenic control plant of each variety was tested. The copy number was two and one for Anamed and Chongqing, respectively (data not shown).

Comparison of the ads *and* cyp71av1 *promoters*

The activity of the *cyp71av1* promoter has been investigated in this study. We have previously studied the activity of the *ads* promoter (Wang et al. 2011b). We have shown that these promoters are specifically active in GSTs of young tissues of *A. annua*. This suggests that the two promoters are co-regulated. A number of putative *cis*-acting regulatory elements are common for the two promoters as summarized in Table 2. We have also included an analysis of the two other *cyp71av1* promoters mentioned above (Fig. 3) in Table 2. At least two of these three *cyp71av1* promoters appear to be active in the plant and give trichome-specific expression of *cyp71av1* (this paper; Wang et al. 2011a). All four promoters carry a number of putative *cis*-acting regulatory elements in common which suggest that they are co-regulated. It has been shown that both ADS and CYP71AV1 are induced by treatment with various agents (Jing et al. 2009; Guom et al. 2010; Lei et al. 2011; Banyai et al. 2011). Some of the *cis*-acting elements involved in the response to these external stimuli are present in the four promoters. Examples are:

- ABRE (TACGTG) (2–5 copies) *cis*-acting element involved in the abscisic acid responsiveness.
- All four promoters carry at least one Box-W1 (TTGACC) that is recognized by WRKY TFs. These TFs are unique to plants and involved in pathogen defense, senescence and trichome development.
- Between 5 and 12 E-boxes, which are involved in the response to MeJA, brassinosteroids, and ABA, are present in the four promoters. The E-box sequence (CANNTG) is recognized by MYB, bZIP and bHLH transcription factors that control light-responsive and

tissue-specific activation of secondary metabolism, oxidative stress, dehydration- and wound-responsive genes.

- G-boxes ([C/T]ACGT[A/G]) are primary involved in light responsiveness but it has also been shown that G-boxes may be involved in MeJA-responsiveness. The binding of bHLH TFs, such as MYC2, to G-box elements within the promoters of various genes to activate MeJA-induced transcriptional activation is well documented (Boter et al. 2004). A number of G-box sequences (3–11) are found in the four promoters.
- MBS sequences (CAACTG) are recognized by MYB transcription factors, which are key factors in regulatory networks controlling development, metabolism and responses to biotic and abiotic stresses in plants.
- RAA-motifs (CAACA) are recognized by AP2/ERF transcription factors that are positive regulators of artemisinin biosynthesis (Yu et al. 2012). Both ADS and CYP71AV1 is increased in *A. annua* after over-expression of ERF TFs.
- The TCA-element is involved in responsiveness to SA. At least one TCA-element was found in three of the four promoters.
- Up to four TC-rich repeats ([A/G]TTTTCTT[A/C][C/A]) can be found in the four promoters. This element is involved in defense and stress responsiveness.
- The TGA-element (AACGAC) present in the four promoters is an auxin-responsive element.

Conclusion

The study of recombinant promoters may be somewhat problematic since all *cis*-acting regulatory elements may not be present in the cloned sequence. The fact that the cloned *cyp71av1* promoter shows an enhanced activity compared to the wild-type promoter may be due to the fact that some silencing regulatory elements are not present in the recombinant promoter. The absence of these elements may also be the reason why we see activity of this promoter in TSTs. However, at this stage, we cannot exclude that the *cyp71av1* promoter actually is active in TSTs. Studies on isolated TSTs may resolve this issue. We will use laser microdissection to isolate TSTs and study the activity of wild-type and recombinant promoters by qPCR (Olofsson et al. 2012).

We have shown that the *cyp71av1* promoter is active in GSTs of young tissues of *A. annua* in a way similar to that shown for the *ads* promoter (Wang et al. 2011b). This indicates a co-regulation of these two genes encoding the first enzymes of artemisinin biosynthesis. Further studies on the *ads* and *cyp71av1* promoters are in progress in our laboratory to study the trichome-specificity and to study the binding of various transcription factors to these promoters. A deeper knowledge about the transcriptional regulation of the artemisinin biosynthesis in *A. annua* will give us tools to improve the yield of the valuable antimalarial compound by metabolic engineering.

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