Plant Mol Biol
DOI 10.1007/s11103-015-0284-3

The activity of the artemisinic aldehyde Δ11(13) reductase promoter is important for artemisinin yield in different chemotypes of *Artemisia annua* L.

Ke Yang · Rashidi Sajad Monafared · Hongzhen Wang · Anneli Lundgren · Peter E. Brodelius

Received: 7 October 2014 / Accepted: 12 January 2015

Abstract The artemisinic aldehyde double bond reductase (DBR2) plays an important role in the biosynthesis of the antimalarial artemisinin in *Artemisia annua*. Artemisinic aldehyde is reduced into dihydroartemisinic aldehyde by DBR2. Artemisinic aldehyde can also be oxidized by amorpha-4,11-diene 12-hydroxylase and/or aldehyde dehydrogenase 1 to artemisinic acid, a precursor of arteannuin B. In order to better understand the effects of DBR2 expression on the flow of artemisinic aldehyde into either artemisinin or arteannuin B, we determined the content of dihydroartemisinic aldehyde, artemisinin, artemisinic acid and arteannuin B content of *A. annua* varieties sorted into two chemotypes. The high artemisinin producers (HAPs), which includes the '2/39', 'Chongqing' and 'Anamed' varieties, produce more artemisinin than arteannuin B; the low artemisinin producers (LAPs), which include the 'Meise', 'Iran#8', 'Iran#14', 'Iran#24' and 'Iran#47' varieties, produce more arteannuin B than artemisinin. Quantitative PCR showed that the relative expression of DBR2 was significantly higher in the HAP varieties. We cloned and sequenced the promoter of the *DBR2* gene from varieties of both the LAP and the HAP groups. There were deletions/insertions in the region just upstream of the ATG start codon in the LAP varities, which might be the reason for the different promoter activities of the HAP and LAP varieties. The relevance of promoter variation, DBR2 expression levels and artemisinin biosynthesis capabilities are discussed and a selection method for HAP varieties with a DNA marker is suggested. Furthermore, putative *cis*-acting regulatory elements differ between the HAP and LAP varieties.

Keywords Artemisinin · *Artemisia annua* · *Cis*-acting regulatory elements · *DBR2* · GC–MS · qPCR · Promoter cloning

Abbreviations

AA	Artemisinic acid
AAld	Artemisinic aldehyde
AB	Arteannuin B
ABA	Abscisic acid
ADH1	Alcohol dehydrogenase 1
ADS	Amorpha-4,11-diene synthase
bHLH	Basic/helix-loop-helix
ALDH1	Aldehyde dehydrogenase 1
AP2	APETALA2
ART	Artemisinin
CaMV	Cauliflower mosaic virus
CPR	Cytochrome P450 reductase
CYP71AV1	Amorpha-4,11-diene 12-hydroxylase
DBR2	Artemisinic aldehyde Δ11(13) reductase
ERF	Ethylene response factor

Electronic supplementary material The online version of this article (doi:10.1007/s11103-015-0284-3) contains supplementary material, which is available to authorized users.

K. Yang · R. S. Monafared · H. Wang · A. Lundgren · P. E. Brodelius (✉)
Department of Chemistry and Biomedical Sciences, Linnaeus University, Kalmar, Sweden
e-mail: peter.brodelius@lnu.se

Present Address:
R. S. Monafared
Department of Plant Breeding and Biotechnology, Faculty of Agriculture, Tarbiat Modares University, 14115-336 Tehran, Iran

Present Address:
H. Wang
Zhejiang Agriculture and Forestry University, Linan 311300, Zhejiang, People's Republic of China

Published online: 24 January 2015

DHAA	Dihydroartemisinic acid
DHAAld	Dihydroartemisinic aldehyde
FAR	β-Farnesene
FDS	Farnesyl diphosphate synthase
GA	Gibberellinic acid
GSP	Gene specific primer
GST	Glandular secretory trichome
HAP	High artemisinin producer
HMGR	3-Hydroxy-3-methyl-glutaryl-CoA reductase
IDI	Isopentenyldiphosphate isomerase
JA	Jasmonate
LAP	Low artemisinin producer
MeJA	Methyl jasmonate
MYB	MYB transcription factor
OPR	12-Oxophytodienoate reductase
SA	Salicylic acid
TSS	Transcription start site
WRKY	WRKY transcription factor

Introduction

Malaria is still one of the most serious infectious diseases that caused an estimated 655,000 deaths in 2010. With the wide-spread occurrence of parasites resistant to chloroquine, the artemisinin combination therapy (ACT) has been strongly recommended by the WHO. Every year, the number of ACT courses is increasing. During 2013, 392 million ACTs courses were produced (WHO 2014). Artemisinin and its derivatives, which are the essential components of the ACT formulations, are the most rapid-acting drugs against *Plasmodium falciparum* malaria (White 1997). The industrial production of artemisinin is mainly based on plant extraction, which can hardly meet the needs of the world procurement (RBM/UNITAID/WHO 2011). Recently, a yeast-based production of artemisinic acid, which can be synthetically converted to artemisinin in relatively good yields (40–45 %), has been developed (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/pressroom/422/). The yearly production will be around 50–60 tons of artemisinin in 2014, which, however, is not sufficient to cover the demand of this drug. Consequently, the plant will remain an important source for artemisinin. The low content of artemisinin (0.1–10 mg/g dry weight) in the plant is the reason for the world shortage and high cost of artemisinin (Zhang et al. 2009).

Great progress has been made during the last decade to understand the enzymology of the artemisinin biosynthetic pathway in detail. Such knowledge is a prerequisite for increasing the artemisinin concentration in *Artemisia annua* plants by biotechnological methods. It is well established that the enzymes of artemisinin biosynthesis are specifically expressed in glandular secretory trichomes (GSTs) and that artemisinin biosynthesis take place in GSTs (Wang et al. 2011, 2013; Jiang et al. 2014). From the synthesis of the universal sesquiterpene precursor farnesyl diphosphate (FDP) from precursors formed by both the mevalonate and MEP pathways in the cytosol and plastids, respectively, to the final enzymatic step of the artemisinin biosynthetic pathway, i.e. the oxidation of dihydroartemisinic aldehyde to dihydroartemisinic acid (catalyzed by aldehyde dehydrogenase 1, ALDH1), the pathway has become clearer. During recent years researchers have been focusing on the last few steps before the final endoperoxide sesquiterpene lactone artemisinin is produced (McGarvey and Croteau 1995; Wallaart et al. 2000). It has become clear that the biosynthetic pathway is branched and that two chemotypes of *A. annua* can be identified (Fig. 1). One chemotype is high in dihydroartemisinic acid and artemisinin (high artemisinin producer; HAP) and the other chemotype is high in artemisinic acid and arteannuin B (low artemisinin producer; LAP) (Brown and Sy 2004, 2006). Artemisinic acid and dihydroartemisinic acid are transformed in vivo or in vitro into arteannuin B and artemisinin, respectively, by photosensitized oxygenation through a transient mediate tertiary allylic hydroperoxide (Brown 2010). These conversions are considered to be non-enzymatic reactions (Brown 2010; Covello 2008).

Artemisinic aldehyde (AAld) is the last common intermediate of the two pathways leading to artemisinin (ART) or arteannuin B (AB). Consequently, the relative levels of the enzymes converting this intermediate into artemisinic acid (AA) or dihydroartemisinic aldehyde (DHAAld) may play important roles in the determination of the chemotypes of *A. annua*. In the artemisinin biosynthesis, AAld is reduced to dihydroartemisinic acid (DHAA) by an artemisinic aldehyde Δ11(13) reductase, i.e. a double bond reductase (DBR2) (Zhang et al. 2008). In the next step, DHAA, the direct precursor of ART, is formed by oxidation of DHAAld by ALDH1 (Teoh et al. 2009). While DBR2 catalyzes the conversion of the common precursor into artemisinin production, ALDH1 and amorpha-4,11-diene 12-hydroxylase (CYP71AV1), an enzyme of the cytochrome P450 super family, may work together to catalyze the conversion of AAld into AA, which eventually turns into the non anti-malarial product AB (Fig. 1; Teoh et al. 2006).

In order to obtain high levels of artemisinin, AAld should be efficiently reduced by DBR2 to DHAAld. This has been shown for transgenic *A. annua* plants overexpressing DBR2 (Yuan et al. 2014). The amount of DHAA and ART was doubled in the transgenic plants. It may be assumed that the catalytic efficiency of DBR2 in different *A. annua* varieties will be correlated with the ratios of

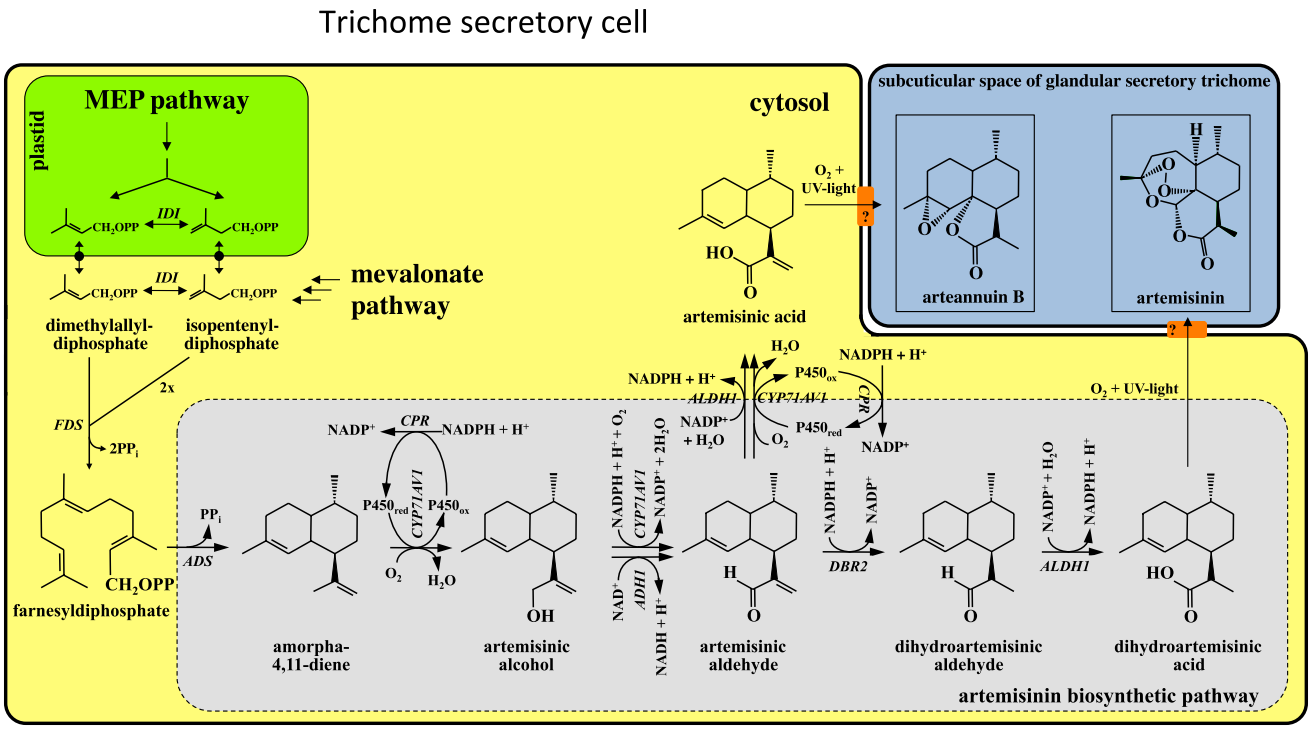

Fig. 1 Biosynthetic pathways for the formation of artemisinin and arteannuin B in a glandular trichome secretory cell of *A. annua* from FDP produced from isopentenyldiphosphate from the cytosolic mevalonate and plastidic MEP pathways. The subcuticular space storing the produced artemisinin and arteannuin B is depicted. ADH1: alcohol dehydrogenase 1; ADS: amorpha-4,11-diene synthase; ALDH1: dihydroartemisinic aldehyde dehydrogenase; CPR: cytochrome P450 reductase; CYP71AV1: amorphadiene-12-hydroxylase; DBR2: artemisinic aldehyde Δ11(13) reductase; FDS: farnesyldiphosphate synthase; IDI: isopentenyldiphosphate isomerase

ART/AB and DHAA/AA, which is related to ART yield. To test this hypothesis we have collected seeds from different *A. annua* varieties of both the HAP group including the '2/39', 'Chongqing' and 'Anamed' varieties and the LAP group including the 'Meise', 'Iran#8', 'Iran#14', 'Iran#24' and 'Iran#47' varieties. At the flowering stage, the content of DHAA, ART, AA and AB in flower buds of all the varieties were measured by gas chromatography–mass spectrometry (GC–MS). Assuming that the enzyme levels are proportional to gene expression, we also used qPCR to determine transcript levels of several genes encoding enzymes of ART and AB biosynthesis, i.e. *amorpha-4,11-diene synthase* (*ADS*), *CYP71AV1, DBR2* and *ALDH1* in the different varieties. The results suggested that the promoter of the *DBR2* gene plays an important role in the variation of *DBR2* gene expression and ART content in the different chemotypes. Furthermore, the promoters of these genes were cloned from both HAP and LAP varieties. The results from characterization of promoters from different *A. annua* varieties suggested that some putative *cis*-acting elements are important for promoter activity and a DNA marker sequence for identification of high yielding artemisinin varieties has been identified.

Materials and methods

Plant materials

Eight *A. annua* L. varieties were used in the experiments. Seeds of 'Iran#8', 'Iran#14', 'Iran#24' and 'Iran#47' were harvested in Iran. Seeds of 'Meise' and '2/39', originally provided by the National Botanic Garden of Belgium and State University of Campinas, Brazil, respectively, were supplied by Dr. Sandra Soetaert, University of Ghent, Belgium. Seeds of 'Anamed' were obtained from Anamed, Germany (www.anamed.net). Seeds of 'Chongqing' were provided by Professor K. Tang, Shanghai Jiao Tong University. In addition, six more *A. annua* varieties collected in Iran, i.e. 'Iran#3', 'Iran#11', 'Iran#20', 'Iran#27', 'Iran#41', and 'Iran#44' were used in some experiments. Seeds of all the varieties were grown in greenhouse under controlled conditions (16 h days, 8 h nights, and 22 °C). For inducing flowering, 8 h day and 16 h night at 22 °C condition was used. For drying, the plant samples were placed in a forced-air oven set at 30 °C for 24 h and were kept dark and dry until analyzed.

GC–MS analysis

Ground dry flower buds (100 mg) or dry leaves (100 mg) were extracted with 10 ml hexane for approximately 15 h. The mixture was filtered to remove solids and then evaporated to dryness in *vacuo* at 30 °C. The residues were dissolved in hexane (1.0 mL). Silylation was carried out by adding N,O-bis-(trimethylsilyl) acetamide:pyridine (1:1 v/v; Sigma-Aldrich, St. Louis, MO, US) solution (20 µl) to an aliquot of each sample (20 µl) before analysis. The samples were analyzed by capillary GC on an Agilent 6890 gas chromatograph (Agilent Technologies, Inc, DE) equipped with a HP-5M5 5 % phenyl methyl silo hexane capillary column (0.25 µm film thicknesses, 30 m × 0.25 mm i.d.) and an Agilent 5973 Network Mass Selective Detector. The oven temperature was programmed 1 min at 60 °C, 60–190 °C at 3 °C per min, 190–235 °C at 20 °C per min, 235–300 °C at 30 °C per min and a final time of 4 min. Helium was used as a carrier gas. Sample size was 1 µl in the splitless mode. The sesquiterpenoids were identified by comparing the mass spectra and retention indices with those of standards.

AA, DHAA, ART and AB quantification were performed by using calibration curves. The calibration curves were constructed based on eight different concentrations of (+)-valencene (Fluka) i.e. 0.1, 0.2, 0.4, 0.6, 0.8, 1.00, 1.2 and 1.4 mg/ml and ART (Sigma-Aldrich) i.e. 0.4, 0.6, 0.8, 1.00, 1.2, 1.4, 1.6 and 1.8 mg/ml with the following regression equation: 1: $Y = 6e + 08X - 6e + 06$ and 2: $Y = 2e + 08X - 3e + 07$ for valencene and ART, respectively. Where Y is the area under the peak and X is the concentration (µg/µl). Equation 1 was used to calculate the DHAA, AA and AB content; Eq. 2 was used to calculate the content of ART. The correlation coefficient (R^2) for the two data sets was 0.993 and 0.983 for the (+)-valencene and ART standards, respectively.

Quantitative polymerase chain reaction

Flower buds of the different varieties were collected to analyze the correlation between the expression levels of genes encoding biosynthetic enzymes and the ART content. RNA was extracted using Purelink™ Plant RNA Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instruction. Single strand cDNA was synthesized by RevertAid™ H Minus-MuLV reverse transcriptase (Fermentas) using 0.5 µg oligo $(dT)_{18}$-primer. A 7500 qPCR thermocycler (Applied Biosystems, USA) was used to carry out the amplifications. The reaction for each sample was set up with 10 µl SYBR Green PCR Master Mix (Applied Biosystem), 1 µl forward primer, 1 µl reverse primer, 7 µl dH_2O and 1 µl cDNA as template. The thermal cycling program was: 50 °C 2 min, 1 cycle; 95 °C 10 min, 1 cycle; 95 °C 15 s, 60 °C 1 min, 40 cycles; 95 °C 15 s, 60 °C 1 min, 95 °C 15 s, 1 cycle. The primers for each gene are listed in Table 1 (Primers 1–10).

Promoter cloning

Young leaves of different varieties of *A. annua* were used to extract genomic DNA using the CTAB method. The promoters of the *CYP71AV1* and *ADS* genes of the "Anamed" variety have previously been cloned (Wang et al. 2011, 2013) (GenBank accession nos. ADS: DQ448294 and CYP71AV1: FJ870128). For cloning of the DBR2 and ALDH1 promoters of the "Anamed" variety, we used the GenomeWalker™ Universal Kit (Clontech) to get the 5' upstream sequence of the translation start site of the two genes. Genomic DNA was initially digested by *Dra*I, *Eco*RV, *Pvu*II or *Stu*I and then the GenomeWalker Adaptor was ligated to create DNA libraries. Two pairs of gene specific primers (GSPs) were designed according to the user manual and available cDNA sequences for DBR2 (primers 13 and 14 in Table 1) and ALDH1 (primers 17 and 18 in Table 1).

The cycling program for the nested PCR was: for primary PCR, 94 °C 25 s, 72 °C 3 min, 7 cycles; 94 °C 25 s, 67 °C 3 min, 32 cycles; 67 °C 7 min, 1 cycle; for secondary PCR, 94 °C 25 s, 72 °C 3 min, 5 cycles; 94 °C 25 s, 67 °C 3 min, 20 cycles; 67 °C 7 min, 1 cycle. The final PCR products were purified and cloned into the pJET vector (Fermentas) for sequencing. For cloning of the promoters of ADS, CYP71AV1, DBR2 and ALDH1 from the other varieties, primers were designed based on the nucleotide sequence of the promoters of the "Anamed" variety. The primers used are listed in Table 1 (primers 19 to 26).

DBR2 gene cloning

The *DBR2* gene was amplified by PCR using genomic DNA as template and primers 11 and 12 (Table 1). The PCR system included 10 pmol forward primer and 10 pmol reverse primer, 0.1 µg of genomic DNA, 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, 5 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.

Table 1 Primers used in qPCR, gene cloning, promoter cloning and promoter analysis

Primer no.	Primer name	Use	Primer sequence
1	$ADS_{forward}$	qPCR	5′-GGGAGATCAGGTTCTCATCTATGAA-3′
2	$ADS_{reverse}$	qPCR	5′-CTTTTAGTAGTTGCCGCACTTCTT-3′
3	$CYP71AV1_{forward}$	qPCR	5′-CGAGACTTTAACTGGTGAGATTGT-3′
4	$CYP71AV1_{reverse}$	qPCR	5′-CGAAGCGACTGAAATGACTTTACT-3′
5	$DBR2_{forward}$	qPCR	5′-GCGGTGGTTACACTAGAGAACTT-3′
6	$DBR2_{reverse}$	qPCR	5′-ATAATCAAAACTAGAGGAGTGACCC-3′
7	$ALDH1_{forward}$	qPCR	5′-CATCGGAGTAGTTGGTCACATCAT-3′
8	$ALDH1_{reverse}$	qPCR	5′-GGAGTATGTTCGGCAGGCTTG-3′
9	$ACTIN_{forward}$	qPCR	5′-CCAGGCTGTTCAGTCTCTGTAT-3′
10	$ACTIN_{reverse}$	qPCR	5′-CGCTCGGTAAGGATCTTCATCA-3′
11	$DBR2_{forward}$	Gene cloning	5′-TCTGCCTACAAGATGGGCAAGTTCAA-3′
12	$DBR2_{reverse}$	Gene cloning	5′-TTGTCAAGAGGAGGGTAATCCGTGTA-3′
13	DBR2 GSP1	Genome walking	5′-TCGGTAATAAGAAAGCCACCAGCAGTT-3′
14	DBR2 GSP2	Genome walking	5′-TAACACCACCCTGTGAGAAAGATTGA-3′
15	DBR2 GSP3	Genome walking	5′-TTTTCATCAGTGACAGCGATTAGCAT-3′
16	DBR2 GSP4	Genome walking/deletion part	5′-GGAACAACGTAATACCTGTGAGAAAG-3′
17	ALDH1 GSP1	Genome walking	5′-AAGAGGTTTACTTGACCAGGTTGTTTG-3′
18	ALDH1 GSP2	Genome walking	5′-GAATGATGACTTAAGGCTTTGCATAGGAC-3′
19	$pADS_{forward}$	Promoter cloning	5′-TCCCCCGGGAAAGTTAATTGCACATAT-3′
20	$pADS_{reverse}$	Promoter cloning	5′-AACTGCAGGATTTTCAAAACTTGAA-3′
21	$pALDH1_{forward}$	Promoter cloning	5′-CGCGGATCCATGAACCATTAGAAGGGAAGGA-3′
22	$pALDH1_{reverse}$	Promoter cloning	5′-CCATGGCTTTGTTTTTTATGAAATTTTTATTC-3′
23	$pCYP71AV1_{forward}$	Promoter cloning	5′-GGAATTCAAATGGGTCAATTTGGGG-3′
24	$pCYP71AV1_{reverse}$	Promoter cloning	5′-CCCAAGCTTTGCTTTTAGTATACTCTT-3′
25	$pDBR2_{forward}$	Promoter cloning	5′-GGATCCGAGATAGGGAACTAACAAGATCCACAC-3′
26	$pDBR2_{reverse}$	Promoter cloning	5′-CCATGGTATTGAGTTTGATGTTGACCAGGATC-3′
27	$pDBR2_{forward}$	Deletion part	5′-GTCAACATGGTGCCACTGATACATCTA-3′

Results and discussion

GC–MS analysis of different *Artemisia annua* varieties

The amounts of four compounds, i.e. AA, DHAA, ART and AB, were determined by GC–MS using the two standard curves described in the method section. It should be pointed out that ART is thermolabile and three peaks are obtained when a standard sample is analyzed by GC–MS using the conditions described in the method section. We used the major peak (R_f = 47.2 min) for quantification of ART (Figure 1S). GC–MS has been used to quantitate ART by a number of investigators (Woerdenbag et al. 1991; Sipahimalani et al. 1991). We calculated the molar ratios of ART/AB and DHAA/AA in samples from flower buds and young leaves from different varieties (Fig. 2; Table 2). According to previous results, the 'Meise' variety is classified as a LAP variety and the '2/39' variety as a HAP variety (Maes et al. 2011). Indeed, in our test the DHAA/AA and ART/AB molar ratios of 'Meise' young leaves were 0.04 and 0.9, respectively, while these ratios were 14.2 and 58.3, respectively, in young leaves of the '2/39' variety belonging to the HAP group. In flower buds, the DHAA/AA ratio was 0.69 and 3.7 and the ART/AB ratio 5.9 and 13.3 in the 'Meise' and '2/39' varieties, respectively.

According to Zhang et al. (2009) the 'Chongqing' variety is a good candidate to use for breeding transgenic *A. annua* lines. The DHAA/AA and ART/AB ratios of young leaves from the 'Chongqing' variety were 1.6 and 8.1, respectively, which were higher than those of young leaves from the 'Meise' variety, but lower than those of the '2/39' variety. In flower buds, the DHAA/AA and ART/AB ratios of the 'Chongqing' variety were 2.9 and 3.7, respectively, which were lower than those of the '2/39' variety, which were 3.7 and 13.3, respectively. The relatively low ART/AB ratio of the 'Chongqing' variety suggested that either this variety could not convert DHAA into ART efficiently or could convert more AA into AB in the flowering stage. The genetically unmodified 'Anamed' variety is considered to be a good artemisinin producer. The DHAA/AA and ART/

AB ratios of young leaves from the 'Anamed' variety were 2.1 and 38.2, respectively, the latter of which was inferior to that of the '2/39' variety, which were 14.2 and 58.3, respectively. In addition, four *A. annua* LAP varieties collected in Iran ('Iran#8', 'Iran#14', 'Iran#24'and 'Iran#47') were also included in this study. For the 'Iran#24' variety, the DHAA/AA and ART/AB ratios were 0.17 and 0.40, respectively, in young leaves. The DHAA/AA ratio was 0.04 to 0.34 and the ART/AB ratio 0.05 to 0.35 in flower buds of the four Iranian varities. All these values are much lower than the corresponding values of the HAP varieties. Our results showed that in the HAP varieties like '2/39' and 'Anamed', the DHAA/AA ratio is much higher than that of the LAP varieties like 'Meise' and 'Iran#24'. In addition, within the HAP varieties the capability to convert DHAA and AA into ART and AB, respectively, differed and had an impact on these ratios.

From our results, we may conclude that in young leaves of all the varieties studied, accumulation of more DHAA than AA always leads to the accumulation of more ART than AB and vice versa. In flower buds, the results were similar except for the 'Meise' variety. This variety accumulated more ART than AB (5.9-fold) but slightly more AA than DHAA (1.4-fold) in flower buds. However, in most cases, the ART/AB and DHAA/AA ratios were consistent with the classification of chemotypes. Consequently, we can confirm that the '2/39' and 'Anamed' varieties are HAP, while the 'Meise' 'Iran#8', 'Iran#14', 'Iran#24'and 'Iran#47' varieties are LAP varieties (Table 2; Fig. 2). The 'Chongqing' variety exhibited intermediate DHAA/ART and AA/AB ratios and may be classified as a weak HAP chemotype.

These findings of the relations between the DHAA/AA and ART/AB ratios were consistent with the suggestion that DHAA is the direct precursor of artemisinin (Brown and Sy 2004). Thus, we suggest that the main differences between the HAP and LAP groups are the efficient reduction of AAld to DHAAld (precursor of DHAA) in HAPs

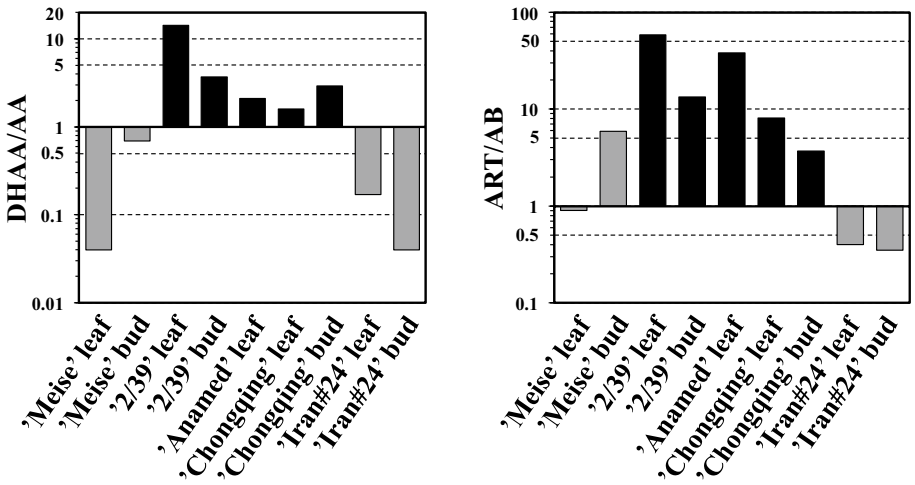

Fig. 2 The molar ratios of DHAA/AA and ART/AB in young leaves and flower buds from HAP (*black bars*) and LAP (*gray bars*) varieties of *A. annua*. The correlated data are shown in Table 2

Table 2 Amount of metabolites in leaves and flower buds from different varieties of *A. annua*

Material	DHAA (mg/g DW)	ART (mg/g DW)	AA (mg/g DW)	AB (mg/g DW)	DHAA/AA (molar ratio)	ART/AB (molar ratio)
Meise leaf	0.65	4	16.8	4.3	0.04	0.9
Meise bud	0.38	1.6	0.55	0.27	0.69	5.9
Chongqing leaf	*0.35*	*1.7*	*0.22*	*0.21*	*1.6*	*8.1*
Chongqing bud	*1.3*	*3.5*	*0.45*	*0.95*	*2.9*	*3.7*
Anamed leaf	*0.35*	*14.5*	*0.17*	*0.38*	*2.1*	*38.2*
2/39 Leaf	*15.6*	*7.0*	*1.1*	*0.12*	*14.2*	*58.3*
2/39 Bud	*0.37*	*1.6*	*0.01*	*0.12*	*3.7*	*13.3*
Iran#24 Leaf	7.4	8.0	43.0	20.0	0.17	0.40
Iran#24 Bud	1.4	5.2	35.0	15	0.04	0.35
Iran#8 Bud	1.1	0.11	3.2	2.4	0.34	0.05
Iran#14 Bud	0.83	0.21	4.6	2.6	0.18	0.08
Iran#47 Bud	2.5	0.38	14	5.0	0.18	0.08

The data from HAP varities are in italics

or the preferential oxidation to AA in LAPs. In fact, the flux of intermediates through the two branches of the pathway may provide a molecular basis for the HAP and LAP varieties. For example, in the 'Anamed' variety, ART is the major product, whereas the biosynthetic machinery of the 'Iran#24' variety clearly promotes the flux towards AB.

Quantitative polymerase chain reaction

GST-specific expression of biosynthetic genes (Teoh et al. 2006; Wang et al. 2011, 2013; Maes et al. 2011; Olofsson et al. 2012; Jiang et al. 2014) shows that artemisinin is sequestered and localized to GSTs of *A. annua*, which are distributed mainly on leaves and floral parts of *A. annua* (Duke et al. 1994; Covello et al. 2007). These trichomes possess all the enzymes involved in the artemisinin biosynthetic pathway. Transcriptional co-regulation of genes involved in secondary metabolite pathways is an important mechanism to regulate such pathways in general. For understanding the relationship between the expression pattern of different genes and the content of terpenoid metabolites involved in artemisinin biosynthesis, the qPCR method was used.

We have previously shown that expression of enzymes of ART biosynthesis are expressed at high rates in tissues carrying GSTs at an early stage of development, such as flower buds and young leaves. The transcript levels of the *ADS*, *CYP71AV1*, *DBR2* and *ALDH1* genes were 40- to 500-fold higher in flower buds than in old leaves of the 'Anamed' variety (Olofsson et al. 2011). In order to study the level of enzymes involved in ART/AB biosynthesis in HAP and LAP varities of *A. annua*, we determined the transcript levels of seven genes, i.e. *HMGR, FDS, ADS, CYP71AV1, CPR, DBR2* and *ALDH1* in flower buds of different varieties of *A. annua* by qPCR. The transcript levels of all genes were normalized to that of *β-ACTIN*. The transcript levels of the *HMGR*, *FDS*, *ADS* and *CPR* genes were essentially the same in flower buds from the HAP 'Anamed' variety and the LAP varieties 'Iran#8', 'Iran#14', and 'Iran#47' (Fig. 3), while the transcript levels of the *CYP71AV1*, *DBR2* and *ALDH1* genes were higher in the HAP variety. In fact, the transcript level of the DBR2 gene was over 100 times higher in the 'Anamed' variety than in the 'Iran#8', 'Iran#14' or 'Iran#47' varities. These results indicate that DBR2 plays an important role in *A. annua* HAP chemotype and low expression of DBR2 may limit ART biosynthesis in the LAP varities.

A similar experiment was carried out with samples from young leaves of five varieties ('Anamed', '2/39', 'Chongqing', 'Meise' and 'Iran#24'). In this case, we normalized the transcript levels of four biosynthetic genes (*ADS*, *CYP71AV1*, *DBR2* and *ALDH1*) in four varieties ('Anamed', '2/39', 'Chongqing' and 'Meise') to those

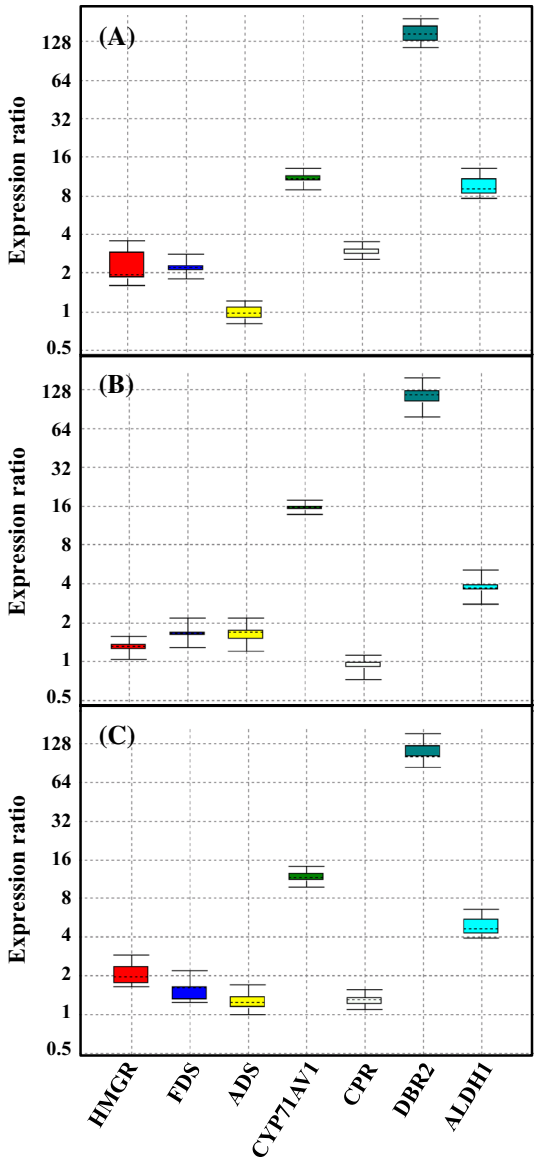

Fig. 3 Relative levels of *HMGR, FDS, ADS, CYP71AV1, CPR, DBR2* and *ALDH1* transcripts in flower buds of 'Anamed'. All transcript levels are relative to the corresponding transcript levels of 'Iran#8' (**a**), 'Iran#14' (**b**) and 'Iran#47' (**c**), which were all set to 1.0. The standard errors were calculated using the REST 2009 software

of the 'Iran#24' variety using the REST 2009 software (Fig. 4). We found that the transcript levels are to some extent correlated to the amounts of metabolites (Fig. 2).

ADS transcript levels of the '2/39', 'Chongqing' and 'Meise' varieties were 1.6-, 2.5- and 4.5-fold higher than that of 'Iran#24', respectively, while for the 'Anamed' variety the transcript level of *ADS* was only 67 % of that of the 'Iran#24' variety (Fig. 4). For the *CYP71AV1* gene, the transcript levels of the 'Anamed', '2/39', 'Chongqing' and 'Meise' varieties were 2.8-, 2.2-, 3.7- and 3.5-fold higher than that of the 'Iran#24' variety, respectively. Compared

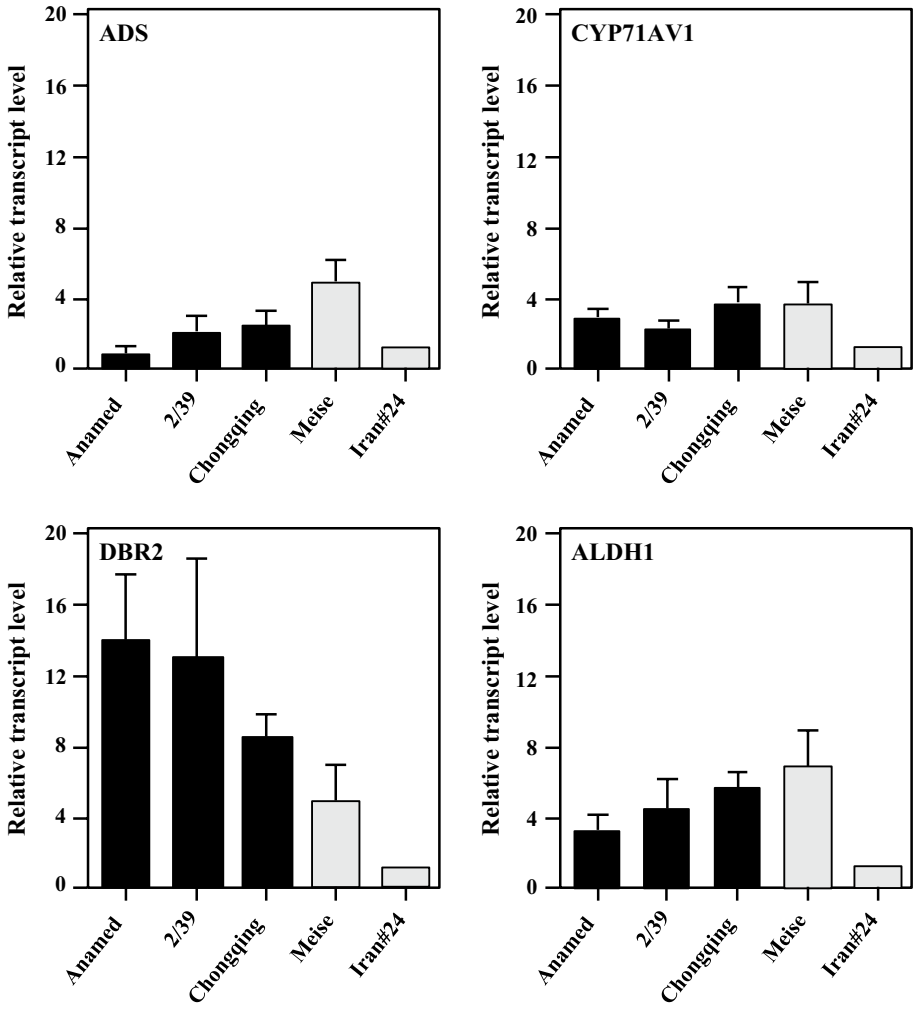

Fig. 4 Relative levels of *ADS, CYP71AV1, DBR2* and *ALDH1* transcripts in leaves of 'Anamed', '2/39', 'Chongqing' and 'Meise' varieties. All transcript levels are relative to the transcript levels of 'Iran#24', which were set to 1.0. The standard errors were calculated using the REST 2009 software

to the 'Iran#24' variety, the transcript levels of DBR2 in the 'Anamed', '2/39', 'Chongqing' and 'Meise' varieties were 14.0-, 13.2-, 8.6- and 5.0-fold higher, respectively. Finally for *ALDH1* the transcript levels of 'Anamed', '2/39', 'Chongqing' and 'Meise' varieties were 3.2-, 4.7-, 5.0- and 6.7-fold higher, respectively, than that of the 'Iran#24' variety.

Within the LAP group the expression levels of all four genes were 4- to sevenfold higher in the 'Meise' variety than in the 'Iran#24' variety (Fig. 4). Apparently, the 'Meise' variety exhibited a higher biosynthetic capability, which, however, was not reflected in the amounts of metabolites (Table 2). The *ADS* transcript levels of the HAP group were lower than that of the 'Meise' variety of the LAP group. When comparing the transcript level of the *CYP71AV1* gene between the two groups, no significant differences were found. In the HAP group the transcript levels of *CYP71AV1* were around threefold higher than that of the 'Iran#24' variety, while the transcript level of the 'Meise' variety from the LAP group was at a similar level as the HAP group. The expression pattern of the *ALDH1* gene was similar to that of the *ADS* gene, which increased slightly from the HAP group to 'Meise' variety in the LAP group. However, as seen for flower buds (Fig. 3) the transcript level of the *DBR2* gene in young leaves decreased significantly from the HAP group to the LAP group (Fig. 4). The *DBR2* gene exhibited considerably higher expression levels in the HAP group as compared to the LAP group including the 'Meise' variety. All these results indicate that a high DBR2 activity appears to be of great importance for the classification of the HAP chemotype.

It has been shown that *ADS* is the rate-limiting enzyme of artemisinin biosynthesis (Bertea et al. 1999; Alam and Abdin 2011). Furthermore, it has been shown that the ART content of transgenic plants overexpressing the *ADS* gene is higher than that of non-transgenic plants (Ma et al. 2009b). It has been demonstrated that the transcript levels of the four biosynthetic genes, were up to 500-fold higher in flower buds of the 'Anamed' variety when compared to old leaves (Olofsson et al. 2011). Maes et al. (2011) reported similar results and in this case the transcript levels of *ADS*, *CYP71AV1*, *DBR2* and *ALDH1* genes were

around 50-, 13-, 160- and 55-fold higher, respectively, in isolated GSTs (from flower buds) compared to leaves All these results showed that the biosynthetic genes were expressed at very high levels in artemisinin producing tissues such as flower buds. It is interesting to note that the ADS transcript level is higher in leaves of the LAP variety 'Meise' compared to leaves of the three HAP varieties (Fig. 4). This observation demonstrates that the LAP variety exhibited a higher capacity to produce AA and to accumulate AB due to the relatively low *DBR2* gene expression. It is possible that over-expressing the *CYP71AV1* gene may help the plant to synthesize more precursors of ART such as artemisinic alcohol and AAld. However, the increased AAld is also likely to be oxidized to AA as a result of the over-expression of the *CYP71AV1* gene, which may result in less accumulation of the target compound, i.e. ART. Similar results can be expected when the *ALDH1* gene is over-expressed since *ALDH1* converts dihydroartemisinic aldehyde to DHAA but also converts AAld to AA. Even though the ALDH1 transcript level was relatively higher in leaves of the 'Meise' variety than in the three HAP varieties, it could not support the accumulation of ART. From our GC–MS analyses and qPCR results, we may conclude that high transcript levels of *ADS, CYP71AV1* and *ALDH1* genes are not sufficient for high ART levels. An appropriate expression level of the *DBR2* gene is necessary for a high ART yield in HAP varieties of *A. annua*.

Promoter cloning and alignment of *ADS*, *CYP71AV1*, *DBR2* and *ALDH1*

The activity of genes involved in the biosynthesis of secondary metabolites in plants is regulated by transcription factors, which bind to the *cis*-acting regulatory elements of the promoters. The qPCR results showed that the *ADS*, *ALDH1* and *CYP71AV1* transcript levels exhibit no significant differences between the HAP and LAP groups (Fig. 4). However, the *DBR2* transcript level was much higher in the HAP group than in the LAP group. If we assume that the biosynthetic genes are regulated by the relevant transcription factors under the same conditions in different varieties, it should be differences in the promoter sequences that are responsible for the observed differences in expression levels in different *A. annua* varieties. The promoters of the *ADS* and *CYP71AV1* genes from the 'Anamed' variety were cloned and characterized previously in our laboratory (Wang et al. 2011, 2013). Using the genome walking method, we now have cloned the promoters of the *DBR2* and *ALDH1* genes from the 'Anamed' variety and also all four promoters from the 'Iran#24' variety. The promoters of the *ADS* (GenBank accession no. KC118528), *CYP71AV1* (GenBank accession no. KC118527), *DBR2* (GenBank accession no. KC118529) and *ALDH1* (GenBank accession no. KC118522) genes from the 'Iran#24' variety were 1,184, 1,181, 1,657 and 1,614 bp, respectively. The DBR2 (GenBank accession no. KC118521) and ALDH1 (GenBank accession no. KC118525) promoters from the 'Anamed' variety were 2,205 and 1,620 bp, respectively.

Here we may point out that the cloning of the DBR2 promoter from the 'Anamed' variety was somewhat complicated due to the fact that the DBR2 cDNA (EU704257) shares a high nucleotide sequence identity with a 12-oxophytodienoate reductase-like protein (OPR3-like) cDNA (EU848577) from *A. annua* (97 %) (Figure 2S). We expected that the two DBR2 GSPs (GSP1 and GSP2; primers 13 and 14 in Table 1) would bind to the corresponding coding sequences giving fragments of both the DBR2 and OPR3-like promoters. In the open reading frame of the expected fragments one base is different and by cloning and sequencing a number of fragments, we expected to isolate the upstream region of the DBR2 open reading frame. However, the cloned and sequenced amplicons could all be aligned to the OPR3 sequence. A possible explanation for this observation is that one of the sequences corresponding to the sequences of the two DBR2 GSPs is "destroyed" by an intron. To answer this question, we PCR cloned the DBR2 gene using primers 11 and 12 (Table 1). A fragment of 5.1 kb was obtained. The nucleotide sequence of this cloned fragment confirmed that the cloned gene was DBR2 and revealed that the open reading frame carried 4 introns (Figure 3S). The positions of the primers used are shown. It was confirmed that the GSP2 primer (primer 13 in Table 1) was located at the first exon/intron junction of the *DBR2* gene. Two new GSP primers (primers 15 and 16 in Table 1) were designed for cloning of the DBR2 promoter by genome walking. In a recent report on the cloning of two putative DBR2 promoters by genome walking by Jiang et al. (2014) (GenBank accession numbers KC347592 and KC347593), two gene specific primers were used that would bind to both the DBR2 and OPR3 genomic sequences (Figure 2S). Consequently, it cannot with certainty be concluded that the two reported promoters are involved in the regulation of the expression of the DBR2 in glandular trichomes of *A. annua*. A 1,014 bp OPR3 promoter has been cloned and sequenced (GenData bank KC118531). Sarvestani et al. (2014) reported on a putative DBR2 promoter from an Iranian *A. annua* cultivar (JX413513). However, this sequence does not show any similarity to the DBR2/OPR3 promoters discussed above. A possible explanation for this is that the sequences of the gene specific primers used are reverse but not complementary to the DBR2/OPR3 gene sequences (underlined in Figure 2S).

The nucleotide sequence of the DBR2 promoter from the 'Anamed' variety was used to design primers 25 and 26 (Table 1) for PCR cloning of the DBR2 promoter from

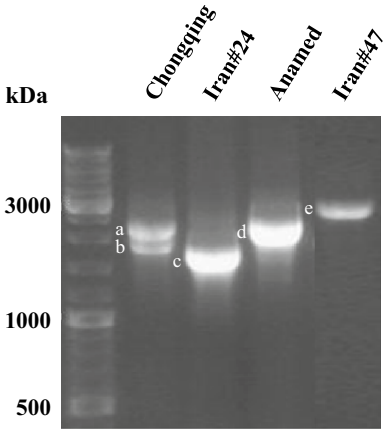

Fig. 5 Agarose gel electrophoresis of PCR amplified DBR2 promoters from different cultivars of *A. annua* using primers 25 and 26. The fragments were cloned and sequenced. (1) Molecular size markers; (2) 'Chongqing'; (3) 'Iran#24'; (4) 'Anamed'; (5) 'Iran#47'

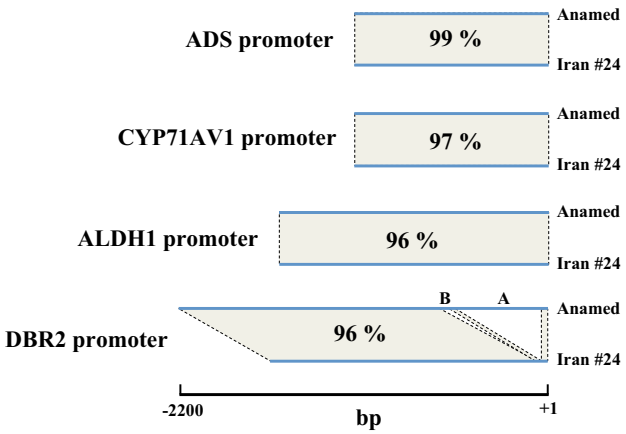

Fig. 6 Schematic alignments of the promoters of the *ADS*, *CYP71AV1*, *ALDH1* and *DBR2* genes in 'Anamed' and 'Iran#24' varieties. (1) Alignment of the *ADS* promoters of the 'Anamed' and 'Iran#24' varieties; (2) alignment of the *CYP71AV1* promoters of the 'Anamed' and 'Iran#24' varieties; (3) alignment of the *ALDH1* promoters of the 'Anamed' and 'Iran#24' varieties; (4) alignment of the *DBR2* promoters of the 'Anamed' and 'Iran#24' varieties; *a* deletion part A from −44 to −427 upstream of the ATG start codon; *b* deletion part B from −468 to −659 upstream of the ATG start codon

the 'Chongqing', 'Iran#24' and 'Iran#47' varieties. These promoters were of different sizes as shown by agarose gel electrophoresis (Fig. 5). After sequencing, an alignment of the nucleotide sequences showed that the differences in size is due to a number of deletions/insertions in the area just upstream of the ATG start codon (Figure 4S). We have also included the sequences of the two reported DBR2 promoters (KC347592 and KC347593) as well as the OPR3-like promoter sequence (KC118531) in this alignment. It is clear from this alignment that the DBR2 promoters cloned in this paper is different from the two DBR2 promoters reported by Jiang et al. (2014). The latter two sequences show high homology to the OPR3 promoter.

We used NCBI BLAST to align the promoter sequences of each gene from the 'Anamed' and 'Iran#24' varieties (Fig. 6). The NCBI GenBank accession number for the various promoters are listed in Table 1S. The *ADS* and *CYP71AV1* promoters, which are both trichome specific promoters as shown in our previous work (Wang et al. 2011, 2013), showed very high similarities of 99 % and 97 % between the two varieties, respectively. The *ALDH1* promoter also showed a high similarity (96 %) between the two varieties with length of approximately 1,600 bp. The *DBR2* promoter from the two varieties were of different sizes as discussed above (Fig. 5) and sequencing showed that large parts of the approximately first 650 bp upstream of the ATG start codon were deleted in the 'Iran#24' variety (Fig. 6).

As schematically shown in Fig. 6, the major differences between the *DBR2* promoter of the 'Anamed' and 'Iran#24' varieties, were that the latter promoter shows two deletions (deletion A from −44 to −427 and deletion B from −468 to −659 upstream of the ATG start codon). The rest of the two promoter sequences shared a high similarity (96 %) (Fig. 6). The DBR2 promoter may be divided into a variable region (−1 to −660; 'Anamed' promoter numbering) and a conserved region (from −661 to the 3′-end; 'Anamed' promoter numbering). We assumed that the different expression levels of the *DBR2* gene observed may be linked to the deletions, which may play important roles in promoter function.

To test our hypothesis, we used PCR (primers 16 and 27 in Table 1) to clone the 3′-end of the DBR2 promoter from all varieties collected including 'Anamed', 'Iran#24', 'Meise', '2/39', 'Chongqing' and a number of other Iranian varieties, which are all LAP varieties. The results showed that this region is highly varying in size (Fig. 7). We cloned and sequenced the PCR products from the varieties 'Meise' (fragments **a** and **b**), '2/39' (fragment **c**), 'Chongqing' (fragments **d** and **e**), 'Iran#24' (fragments **f,** **g** and **h**), 'Anamed' (fragment **i**) and 'Iran#47' (fragment **k**). An alignment of the ten sequences (fragments **a** to **k**; Fig. 7) is shown in Figure 5S. The '2/39' and 'Chongqing' varieties (fragments **c** and **d**) exhibited essentially the same sequence as the 'Anamed' variety (fragment **i**). The LAP varieties 'Meise', 'Iran#47'and 'Iran#24' varieties carried a highly conserved inserted sequence of 335 bases (−420 to −85) close to the 3′-end (fragments **a**, **f** and **k**). In addition, around 160 bases were deleted (between bases −559 and −560) in the 'Iran #47'(fragment **k**) close to the 5′-end. The 'Meise' and 'Iran#24' varieties carried additional fragments (fragments **b** and **g**) somewhat larger than the fragments from the HAP varities. Some regions of the fragments **b** and **g** show a relatively low homology to the other

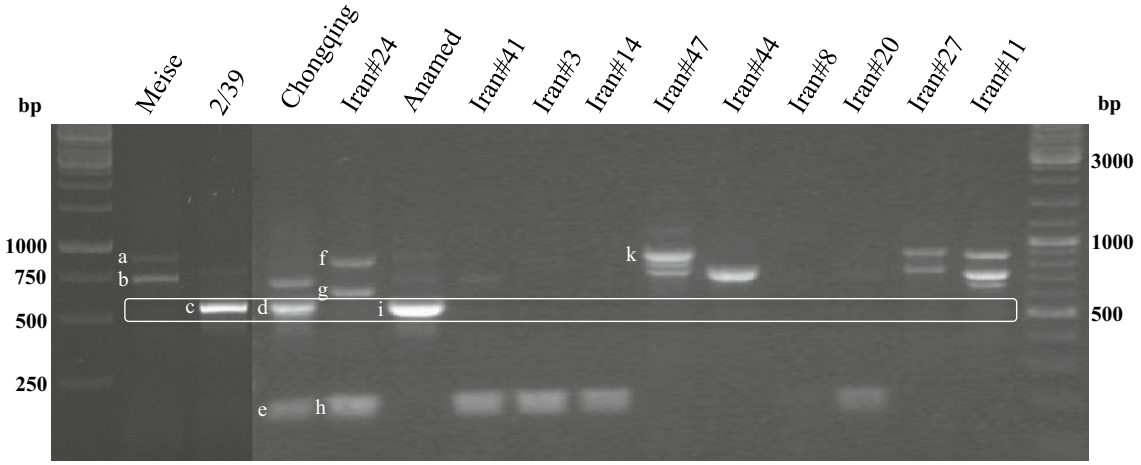

Fig. 7 Agarose gel electrophoresis of PCR amplified 3′-end of DBR2 promoters from different cultivars of *A. annua* as indicated using primers 27 and 16. The labeled fragments (*a*–*k*) were cloned and sequenced

sequences and a number of inserts are present in these fragments. The sample from the 'Chongqing' variety also carried a second fragment, which is only 96 bases long (fragment **e**). This fragment is identical to the small fragments of the LAP variety 'Iran #24' (fragment **h**). This small fragment appears also to be present in the 'Iran#41', 'Iran#3', 'Iran#14' and 'Iran#20' varieties (Fig. 7).

These complex results indicate the presence of more than one *DBR2* promoter type in most varieties (Figs. 6, 7, 5S). We may conclude that HAP varieties such as the '2/39' and 'Anamed' varieties give one fragment of 580 bp while none of the LAP varieties give this fragment using primers 16 and 27 (Fig. 7). Furthermore, the combination of one HAP DBR2 promoter (580 bp) and one LAP DBR2 promoter (96 bp) in the 'Chongqing' variety may be the reason why this variety may be classified as a weak HAP variety. However, it was noticed that the LAP varieties 'Meise', 'Iran#24'and 'Iran #47' showed addition bands larger than 580 bp using the specific primers. These fragments carried the deletion part A but they were interrupted by a few insertions. Thus, we assumed that even with parts A and B present, once they have been interrupted by insertions, the DBR2 promoter does not function normally.

In our study, the results of the GC–MS, qPCR and promoter sequence analyses make it reasonable to assume that the specific deletion part sequence of the DBR2 promoter is highly linked to the artemisinin content of each variety. When performing PCR with appropriate primers, high artemisinin producers will show a fragment of 580 bp, while low producers will not. We have tested more than 14 varieties of *A. annua* by PCR using genomic DNA as template and primers 16 and 27 (Table 1). The PCR products were analyzed by agarose gel electrophoresis and if a fragment of 580 bp was observed, the tested variety belonged to the HAP group (Fig. 7). This is a fast way to screen for HAP varieties within unknown *A. annua* varieties.

Analysis of the DBR2 promoter

Attempts to predict the transcription start site (TSS) of the DBR2 promoters using the TSSP software (http://linux1.softberry.com/berry.phtml) failed. In order to get some information on the TSS of the DBR2 gene we screened available DNA-libraries (GenBank NT-, EST- and TSA-libraries) using a 68 bp sequence around the ATG start codon as indicated in Figure 5S. From this screening a number of EST-sequences were obtained as shown in Figure 6S. From the alignment we may conclude that the 3′-end of the various promoters are highly conserved. Furthermore, based on this alignment we may suggest that the TSS of the various DBR2 and OPR3 promoters is located up to 90 bp upstream of the ATG start codon. No TATA-box (TATAAA) can be found 25–35 bp upstream of these putative TSS.

A comparison of the putative DBR2 and OPR3 promoters (Figure 6S) indicate that there are some apparently consistent differences. For instance, there is a TC and a C insertion between positions −70/−71 and −27/−28, respectively, of the DBR2 promoter. Furthermore, there are two G to C substitutions at positions 28 and 45 in the first exon of the DBR2 open reading frame. Based on these observations we may suggest that most of the ESTs retrieved appear to be OPR3-like sequences. In addition, in line with this reasoning, the two promoters cloned by Jiang et al. (2014) and reported as putative DBR2 promoters may be promotors of the 12-oxophytodienoate reductase-like (OPR3-like) gene.

Using the PLACE (http://www.dna.affrc.go.jp/PLACE/) and PlantCARE (http://bioinformatics.psb.ugent.be/webtools/

plantcare/html/) (Lescot et al. 2002) databases, we identified a number of putative *cis*-acting regulatory elements of the DBR2 promoters as shown in Figs. 5S and 7S. We have divided the nucleotide sequence in two regions; one variable region (corresponding to bases −1 to −460 of the 'Anamed' variety; Figure 5S) and one conserved region (corresponding to bases −460 to −2200 of the 'Anamed' variety; Figure 7S). A summary of the identified putative *cis*-acting regulatory elements may be found in Table 2S.

We have previously cloned and analyzed the ADS and CYP71AV1 promoters (Wang et al. 2011; 2013). Some putative regulatory elements are common for the three promoters but some elements are unique for the DBR2 promoter. It has been shown that the ADS, CYP71AV1 and the putative DBR2 promoters are specifically active in glandular trichomes of *A. annua* (Wang et al. 2011; 2013, Jiang et al. 2014). 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) for *A. annua*. Some of these TFs are involved in the regulation of the promoters studied by binding to various *cis*-acting regulatory elements.

Some studies on the effects of some TFs on the production of artemisinin in *A. annua* have been published during recent years. These include two jasmonate (JA)-responsive APETALA2/ethylene response factors (AP2/ERF) TFs, i.e. AaERF1 and AaERF2 (Yu et al. 2012) and an AaORA (Lu et al. 2013) as well as the AaWRKY1 TF involved in the activation of defense-related genes (Ma et al. 2009a; Han et al. 2014). Recently, a study on a basic/helix-loop-helix (bHLH) transcription factor (AabHLH1) from *A. annua* was reported (Ji et al. 2014).

Transient expression of AaERF1 in *A. annua* resulted in the up-regulation of ADS (6- to sevenfold), CYP71AV1 (3- to fourfold) and DBR2 (fourfold) as well as AaWRKY1 (5- to sixfold) (Ma et al. 2009a). 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. Transgenic *A. annua* overexpressing AaORA, a trichome specific AP2/ERF transcription factor, shows enhanced DHAA (22–35 %) and ART (40–53 %) production (Lu et al. 2013). The increased content of DHAA and ART observed was accompanied by enhanced transcript levels of ERF1 and the biosynthetic enzymes ADS, CYP71AV1 and DBR2, while the level of ALDH1 was unaffected.

The AP2/ERF TFs bind to CBF2 ([G/a][T/c]CGAC) and RAA (CAACA) motifs. Five modified CBF2 motifs (CACGAC −1950 to −1945; TTCGAC −1940 to −1935; TACGAC −1464 to −1459; TTCGAC −831 to −826; AACGAC −494 to −489) and five RAA motifs (−1404 to −1400; −1212 to −1216; −464 to −460; −63 to −59; −17 to −13) were found in the DBR2 promoter from the 'Anamed' variety as shown in Figs. 5S and 7S. In addition to these motifs, we have also found two putative CGTCA/TGACG JA-responsive *cis*-acting regulatory elements at positions −468 to −472 and −1742 to −1746 (Figure 7S). Essentially all these elements are conserved in the various DBR2 promoters (Figures 5S and 7S). There is also a putative T/G-box (AACGTG), another *cis*-acting regulatory element involved in MeJA-responsiveness, present in the variable region of the promoter of the three HAP varieties (7S). The T/G box binds the MYC TF in tomato and Arabidopsis involved in JA signaling (Boter et al. 2004).

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. Ten putative E-boxes are present in the DBR2 promoter from the 'Anamed' variety at positions −340 to −335, −470 to −465, −983 to −978, −1338 to −1333, −1390 to −1385, −1456 to −1451, −1471 to −1466, −1534 to −1529, −1764 to −1759 and −2027 to −2022 (Figure 7S). The other DBR2 promoters carry 9–10 E-boxes (Figs. 5S and 7S). Transgenic *A. annua* overexpressing AabHLH1 showed increased levels of ADS, CYP71AV1 and DBR2 and an increased artemisinin production (Ji et al. 2014). Mutation of the six E-boxes of the CYP71AV1 promoter abolished the activation of the *CYP71AV1* gene by the AabHLH1. It is likely that the AabHLH1 exhibits a similar E-box dependent activation of the ADS and DBR2 promoters, which carry 14 and 10 E-boxes, respectively.

In *Arabidopsis thaliana*, six signal-responsive genes (AtSR1 to 6) have been identified, which are related to the tobacco ethylene-responsive gene NtER1 encoding a calmodulin-binding protein (Yang and Poovaiah 2001). The AtSR genes are rapidly and differentially induced by environmental signals such as extreme temperatures, UV-B, salt, wounding, hormones such as ethylene and abscisic acid (ABA), and signal molecules such as MeJA, H_2O_2, and salicylic acid (SA) (Yang and Poovaiah 2001). AtSR1 targets the nucleus and specifically recognizes a 6-bp CGCG-box ([A/C/G]CGCG[G/T/C]). One or more CGCG *cis*-acting elements are found in promoters of genes involved in ethylene, ABA and SA signaling, and light signal perception. One putative CGCG-box was found in the conserved region of the DBR2 promoters (−1676 to −1671; Figure 7S) while two CGCG-boxes were located to the large insert of fragments **a**, **f** and **k** (−399 to −394 and −111 to −106; Figure 5S).

Thus, the DBR2 promoters carry a number of JA responsive *cis*-acting regulatory 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 DBR2 and other enzymes of artemisinin biosynthesis such as FDS, ADS, CYP71AV1 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).

ABA-responsive element (ABRE) is a major *cis*-acting element (PyACGTG) in ABA-responsive gene expression. One such ABA-responsive element (TACGTG) was found at the positions −293 to −288 (Figure 5S). However, 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 modified putative CE1 element (TGCCACGG) at position −1,437 to −1,430 (Figure 7S). This CE1 element may be involved in a response of this promoter to ABA. It is well established that bHLH transcription factors 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 ten E-boxes in the DBR2 promoter as discussed above.

Four putative ethylene responsiveness ERE-elements (ATTTCAAA) are localized at −1046 to −1053, −1011 to −1018, −725 to −718 and −545 to −538. Furthermore, a putative gibberellin (GA)-responsive element (the GARE-motif AAACAGA) is present at position −1404 to −1398. Five TC-rich repeats (ATTTT(C/T)T(C/T)(T/C)A), which are involved in defense and stress responses, were localized to position −2035 to −2026, −1132 to −1141, −558 to −566, −504 to −495 and −71 to −62 of the DBR2 promoter from the 'Anamed' variety. The other DBR2 promoters carry 4–5 putative TC-rich repeats. Two TCA-elements, involved in SA responsiveness, are present in the conserved region of the DBR2 promoters. Finally, two TGA-elements (AACGAC; −479 to −474 and −1142 to −1137), which are involved in auxin responsiveness, were found in the conserved region of each DBR2 promoter (Figure 7S).

The elements described above may be involved in the response of *A. annua* to plant hormones (ABA, SA, ethylene, MeJA and GA). In fact, treatment of *A. annua* plants with ABA (Jing et al. 2009), SA (Baldi and Dixit 2008; Pu et al. 2009), JA (Baldi and Dixit 2008) or GA (Zhang et al. 2005; Banyai et al. 2011) resulted in the induction of a number of genes encoding enzymes involved in artemisinin biosynthesis and increased yields of artemisinin. However, no information on the effect of these plant hormones on the expression of DBR2 has so far been reported.

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]), which are fungal elicitor responsive elements. WRKY TFs may be involved in the simultaneous regulation of different processes. In cotton, the GaWRKY1 is regulating the activity of cadinene synthase 1 carrying two W1-boxes, which is involved in the biosynthesis of sesquiterpene phytoalexins (Xu et al. 2004). 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. 2009a). 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 W1-boxes. Three W1-boxes, defined as elicitor-responsive elements, have been identified at positions −15 to −20, −478 to 473 and −968 to −963 in the DBR2 promoter from the 'Anamed' variety (Figs. 5S and 7S). The other varieties carried two to four W1-boxes (Figs. 5S and 7S). We have previously reported that the ADS and CYP71AV1 promoters each carries two W1-boxes (Wang et al. 2011, 2013). Transgenic *A. annua* overexpressing the AaWRKY1 from the 'Chongqing' variety under the control of a trichome-specific promoter exhibited an increased artemisinin production but only a slight induction of DBR2 was observed (Han et al. 2014). In addition to the W1-boxes, the DBR2 promoters carry one conserved EIRE sequence (TTC-GACC) (Figure 7S), which is a *cis*-acting regulatory element essential for elicitor responsiveness (Rushton et al. 2010).

Studies on the effects of biotic elicitors (e.g. chitosan) 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 carried out. Foliar application of chitosan on *A. annua* resulted in increased production of DHAA and ART (Lei et al. 2011). Furthermore, semi-quantitative PCR showed increased levels of ADS, CYP71AV1 and DBR2 transcripts 2, 6 and 4 h after application of chitosan, respectively.

Four putative MYB binding sites (MBS) (TAACNG) were found at positions −1765 to −1760, −1534 to −1529, −1333 to −1338 and −962 to −967. In *Arabidopsis*, they are the binding site for the myb proteins ATMYB1 and ATMYB2 involved in regulation of genes that are responsive to water stress (Urao et al. 1993) and in *Petunia hybrida* for MYB.Ph3 involved in regulation of flavonoid biosynthesis (Solano et al. 1995). Twenty-eight MYB transcription factors have been identified in *A. annua* according to the plant transcription factor database (http://planttfdb.cbi.pku.edu.cn/index.php?sp=Aan). To our knowledge, no study on the effects of MYB transcription factors on

metabolism in *A. annua* has been reported. Preliminary results from our ongoing study on the effects of MYB TFs suggests that overexpressing a trichome specific AaMYB1 in the "Anamed" variety results in increased artemisinin content in young leaves and flower buds (K. Yang et al. unpublished).

GATA factors, involved in light-mediated regulation, are a class of transcriptional regulators present in plants that normally recognize the consensus sequence (T/A) GATA(G/A) (Reyes et al. 2004). Six putative GATA-boxes (−2187 to −2181, −2017 to −2012, −1963 to −1958, −1588 to −1593, −1536 to −1541 and −1524 to −1519) were found in the conserved promoter region of DBR2 (Figure 7S). Additional GATA-boxes are located to the variable fragments **b** and **g** (Figure 5S). Other elements putatively involved in light-mediated regulation are one ACE-motif (AAACCGGTTA; position −896 to −887), one AE-box (AGAAACAA; position −1511 to −1504), nine Box 1 motifs (TTTCAAA; between positions −106 and −2154), three Box 4 motifs (ATTAAT; positions −1322 to −1327, −1544 to −1549 and −1852 to −1457), three G-box motifs (CACGT(T/C); positions −1950 to −1945, −388 to −393 and −288 to −293), two GT1-motifs (AATCCACA; positions −978 to −983 and −1555 to −1560), and one Sp1-motif (CC(G/A)CCC; position −1488 to −1483).

The DBR2 promoter carries two CAAnnnnATC motifs (−476 to −485 and −45 to −36) transmitting circadian rhythmicity in plants. In fact, after treatment of *A. annua* with MeJA, the level of DBR2 transcripts showed maxima after 48 h and 168 h and a minimum at 72 h indicating a circadian rhythmicity (Zare Mehrjerdi et al. 2013).

One LTR motif (CCGAAA; −918 to −913), which is a *cis*-acting regulatory element required in cold inducible gene expression, is present in the DBR2 promoter. Finally, thirteen HSE elements (AAAAAATTTC), which are involved in response to heat, are localized to the conserved region of the DBR2 promoter (Figure 7S).

Concluding remarks

The result of the qPCR experiments showed that the DBR2 transcript level was much higher in the HAP varieties than in the LAP varieties indicating its role in the determination of chemotype (Figs. 3, 4). The GC–MS results suggested that the low transcript level of the *DBR2* gene is the most likely reason for the channeling of AAld to AA and AB in the LAP varieties. DBR2 appears to be a key enzyme in the competition for AAld between the two branches of the pathway, which lead to the accumulation of the two related final sesquiterpenoids, ART or AB. The accumulation of DHAA is associated with the production of ART, whereas that of AA is associated with the production of AB. These results confirm the finding that the precursor of ART in vivo is DHAA (Brown and Sy 2004).

From Figs. 5S and 7S it is evident that the different DBR2 promoters carry many putative *cis*-acting regulatory elements in common. However, some differences are observed. In addition, there may be a number of elements present in the promoters, which have not been identified in our analysis. The different DBR2 transcript levels observed may be due to the activity of a limited number of *cis*-acting regulatory elements. More extensive studies are required to determine which elements are of particular importance for the expression of the DBR2 gene. Anyhow, we ascribe the different DBR2 transcript levels in HAP and LAP varities of *A. annua* to the activity of different *cis*-acting regulatory elements present in the DBR2 promoters. Furthermore, we may suggest that the classification of *A. annua* varities into HAP and LAP is linked to the DBR2 transcript level.

In a previous attempt to establish the metabolic differences between HAP and LAP varities, the *CYP71AV1*, *DBR2* and *ALDH1* genes were isolated from the two chemotypes and characterized using transient expression in *Nicotiana benthamiana* (Ting et al. 2013). No difference in the enzyme activity of DBR2 and ALDH1 from the two chemotypes could be observed but structural differences in CYP71AV1 from the LAP and HAP chemotypes did result in altered enzyme activity. From these results it was concluded that the structural difference in the CYP71AV1 protein (a N-terminal addition of 7 amino acids (MKSILKA) in the enzyme from LAP varieties, resulting in higher specific enzyme activity, was determining the chemotype.

Thus, there are two putative mechanisms reported for the metabolic differences between HAP and LAP varities; one based on CYP71AV1 activity (Ting et al. 2013) and one based on the activity of the DBR2 promoter (this study). It is possible that a combination of these two mechanisms is operative in *A. annua* resulting in HAP and LAP varieties.

Acknowledgments We would like to thank Professor Kexuan Tang of Shanghai Jiao Tong University for technical advice and assistance. We also thank Tehran University and Ghent University for providing *A. annua* seeds. This work was supported by the Faculty of Life and Health Sciences, Linnaeus University.

References

Alam P, Abdin MZ (2011) Over-expression of HMG-CoA reductase and amorpha-4,11-diene synthase genes in *Artemisia annua* L. and its influence on artemisinin. Plant Cell Rep 30:1919–1928

Baldi A, Dixit VK (2008) Yield enhancement strategies for artemisinin production by suspension cultures of *Artemisia annua*. Biores Technol 99:4609–4614

Banyai W, Mii M, Supaibulwatana K (2011) Enhancement of artemisinin content and biomass in *Artemisia annua* by exogenous GA3 treatment. Plant Growth Reg 63:45–54

Bertea CM, Freije JR, van der Woude H, Verstappen FW, Perk L, Marquez V, De Kraker JW, Posthumus MA, Jansen BJ, de Groot A, Franssen MC, Bouwmeester HJ (2005) Identification of intermediates and enzymes involved in the early steps of artemisinin biosynthesis in *Artemisia annua*. Planta Med 71:40–47

Boter M, Ruiz-Rivero O, Abdeen A, Prat S (2004) Conserved MYC transcription factors play a key role in jasmonate signaling both in tomato and Arabidopsis. Genes Dev 18:1577–1591

Brown GD (2010) The biosynthesis of artemisinin (Qinghaosu) and the phytochemistry of *Artemisia annua* L. (Qinghao). Molecules 15:7603–7698

Brown GD, Sy L-K (2004) In vivo transformations of dihydroartemisinic acid in *Artemisia annua* plants. Tetrahedron 60:1139–1159

Brown GD, Sy L-K (2006) In vivo transformations of artemisinic acid in *Artemisia annua* plants. Tetrahedron 60:1139–1159

Caretto S, Quarta A, Durante M, Nisi R, De Paolis A, Blando F, Mita G (2011) Methyl jasmonate and miconazole differently affect arteminisin production and gene expression in *Artemisia annua* suspension cultures. Plant Biol 13:51–58

Covello PS (2008) Making artemisinin. Phytochemistry 69:2881–2885

Covello PS, Teoh KH, Polichuk DR, Reed DW, Nowak G (2007) Functional genomics and the biosynthesis of artemisinin. Phytochemistry 68:1864–1871

Duke MV, Paul RN, Elsohly HN, Sturtz G, Duke SO (1994) Localization of artemisinin and artemisitene in foliar tissues of glanded and glandless biotypes of *Artemisia annua* L. Int J Plant Sci 155:365–372

Guom X-X, Yang X-Q, Yang R-Y, Zeng Q-P (2010) Salicylic acid and methyl jasmonate but not Rose Bengal enhance artemisinin production through invoking burst of endogenous singlet oxygen. Plant Sci 178:390–397

Han J, Wang H, Lundgren A, Brodelius PE (2014) Effects of overexpression of AaWRKY1 on artemisinin biosynthesis in transgenic *Artemisia annua* plants. Phytochemistry 102:89–96

Ji J Xiao, Shen Y, Ma D, Li Z, Pu G, Li X, Huang L, Liu B, Ye H, Wang H (2014) Cloning and characterization of AabHLH1, a bHLH transcription factor that positively regulates artemisinin biosynthesis in *Artemisia annua*. Plant Cell Physiol 55:1592–1604

Jiang W, Lu X, Qiu B, Zhang F, Shen Q, Lv Z, Fu X, Yan T, Gao E, Zhu M, Chen L, Zhang L, Wang G, Sun X, Tang K (2014) Molecular cloning and characterization of a trichome-specific promoter of artemisinic aldehyde Δ11(13) reductase (DBR2) in *Artemisia annua*. Plant Mol Biol Rep 32:82–91

Jing F, Zhang L, Li M, Tang Y, Wang Y, Wang Y, Wang Q, Pan Q, Wang G, Tang K (2009) Abscisic acid (ABA) treatment increases artemisinin content in *Artemisia annua* by enhancing the expression of genes in artemisinin biosynthetic pathway. Biologia 64:319–323

Kim J, Kim HY (2006) Molecular characterization of a bHLH transcription factor involved in Arabidopsis abscisic acid-mediated response. Biochim Biophys Acta 1759:191–194

Lei C, Ma D, Pu G, Qiu X, Du Z, Wang H, Li G, Ye H, Liu B (2011) Foliar application of chitosan activates artemisinin biosynthesis in *Artemisia annua* L. Ind Crops Prod 33:176–182

Lescot M, Déhais P, Moreau Y, De Moor B, Rouzé P, Rombauts S (2002) PlantCARE: a database of plant *cis*-acting regulatory elements and a portal to tools for in silico analysis of promoter sequences. Nucleic Acids Res 30:325–327

Lu X, Zhang L, Zhang F, Jiang W, Shen Q, Zhang L, Lv Z, Wang G, Tang K (2013) AaORA, a trichome-specific AP2/ERF transcription factor of *Artemisia annua*, is a positive regulator in the artemisinin biosynthetic pathway and in disease resistance to *Botrytis cinerea*. New Phytol 198:1191–1202

Ma D, Pu G, Lei C, Ma L, Wang H, Guo Y, Chen J, Du Z, Wang H, Li G, Ye H, Liu B (2009a) Isolation and characterization of AaWRKY1, an *Artemisia annua* transcription factor that regulates the amorpha-4,11-diene synthase gene, a key gene of artemisinin biosynthesis. Plant Cell Physiol 50:2146–2161

Ma C, Wang H, Lu X, Wang H, Xu G, Liu B (2009b) Terpenoid metabolic profiling analysis of transgenic *Artemisia annua* L. by comprehensive two-dimensional gas chromatography time-of-flight mass spectrometry. Metabolomics 5:497–506

Maes L, van Nieuwerburgh FCW, Zhang Y, Reed DW, Pollier J, Vande Casteele SRF, Inzé D, Covello PS, Deforce DLD, Goossens A (2011) Dissection of the phytohormonal regulation of trichome formation and biosynthesis of the antimalarial compound artemisinin in *Artemisia annua* plants. New Phytol 189:176–189

McGarvey DJ, Croteau R (1995) Terpenoid metabolism. Plant Cell 7:1015–1026

Miyamoto K, Shimizu T, Lin FQ, Sainsbury F, Thuenemann E, Lomonossoff G, Nojiri H, Yamane H, Okada K (2012) Identification of an E-box motif responsible for the expression of jasmonic acid-induced chitinase gene OsChia4a in rice. J Plant Physiol 169:621–627

Narusaka Y, Nakashima K, Shinwari ZK, Sakuma Y, Furihata T et al (2003) Interaction between two *cis*-acting elements, ABRE and DRE, in ABA-dependent expression of Arabidopsis rd29A gene in response to dehydration and high-salinity stresses. Plant J 34:137–148

Olofsson L, Engström A, Lundgren A, Brodelius EP (2011) Relative epression of genes of terpene metabolism in different tissues of *Artemisia annua* L. BMC Plant Biol 11:45

Olofsson L, Lundgren A, Brodelius PE (2012) Trichome isolation with and without fixation using laser microdissection and pressure catapulting followed by RNA amplification: expression of genes of terpene metabolism in apical and sub-apical trichome cells of *Artemisia annua* L. Plant Sci 183:9–13

Paddon CJ, Westfall PJ, Pitera DJ, Benjamin K, Fisher K, McPhee D, Leavell MD, Tai A, Main D, Eng D, Polichuk DR, Teoh KH, Reed DW, Treynor T, Lenihan J, Jiang H, Fleck M, Bajad S, Dang G, Dengrove D, Diola D, Ellens KW, Fickes S, Galazzo J, Gaucher SP, Geistlinger T, Henry R, Hepp M, Horning T, Iqbal T, Jiang H, Kizer L, Lieu B, Melis D, Moss N, Regentin R, Secrest S, Tsuruta H, Vazquez R, Westblade LF, Xu L, Yu M, Zhang Y, Zhao L, Lievense J, Covello PS, Keasling JD, Reiling KK, Renninger NS, Newman JD (2013) High-level semi-synthetic production of the potent antimalarial artemisinin. Nature 496:528–532

Pu GB, Ma DM, Chen JL, Ma LQ, Wang H, Li GF, Ye HC, Liu BY (2009) Salicylic acid activates artemisinin biosynthesis in *Artemisia annua* L. Plant Cell Rep 28:1127–1135

Putalun W, Luealon W, De-Eknamkul W, Tanaka H, Shoyama Y (2007) Improvement of artemisinin production by chitosan in hairy root cultures of *Artemisia annua* L. Biotechnol Lett 29:1143–1146

RBM/UNITAID/WHO (2011) Artemisinin Conference, Hanoi, Viet Nam, November 2–3, 2011. http://www.mmv.org/sites/default/files/uploads/docs/events/2011/2011_Artemisinin_Conference_Report.pdf

Reyes JC, Muro-Pastor MI, Florencio FJ (2004) The GATA family of transcription factors in Arabidopsis and rice. Plant Physiol 134:1718–1732

Rushton PJ, Somssich IE, Ringler P, Shen QJ (2010) WRKY transcription factors. Trends Plant Sci 15:247–258

Sarvestani R, Peyghambary SA, Abbasi A (2014) Isolation and characterization of *DBR2* gene promoter from Iranian *Artemisia annua*. J Agr Sci Tech 16:191–202

Shen QX, Ho THD (1995) Functional dissection of an abscisic-acid (ABA)-inducible gene reveals 2 independent ABA-responsive

complexes each containing a G-box and a novel *cis*-acting element. Plant Cell 7:295–307

Shen QX, Zhang PN, Ho THD (1996) Modular nature of abscisic acid (ABA) response complexes: composite promoter units that are necessary and sufficient for ABA induction of gene expression in barley. Plant Cell 8:1107–1119

Sipahimalani AT, Fulzele DP, Heble MR (1991) Rapid method for the detection and determination of artemisinin by gas chromatography. J Chromatogr A 538:452–455

Solano R, Nieto C, Avila J, Cañas L, Diaz I, Paz-Ares J (1995) Dual DNA binding specificity of a petal epidermis-specific MYB transcription factor (MYB. Ph3) from *Petunia hybrid*. EMBO J 14:1773–1784

Teoh KH, Polichuk DR, Reed DW, Nowak G, Covello PS (2006) Specific cDNAs reveal CYP71AV1, a cytochrome P450 with a key role in the biosynthesis of the antimalarial sesquiterpene lactone artemisinin. FEBS Lett 580:1411–1416

Teoh KH, Polichuk DR, Reed DW, Covello PS (2009) Molecular cloning of an aldehyde dehydrogenase implicated in artemisinin biosynthesis in *Artemisia annua*. Botany 87:635–642

Ting H-M, Wang B, Ryden A-M, Woittiez L, van Herpen T, Verstappen FWA, Ruyter-Spira C, Jules Beekwilder J, Bouwmeester HJ, van der Krol A (2013) The metabolite chemotype of Nicotiana benthamiana transiently expressing artemisinin biosynthetic pathway genes is a function of CYP71AV1 type and relative gene dosage. New Phytol 199:352–366

Urao T, Yamaguchi-Shinozaki K, Urao S, Shinozaki K (1993) An Arabidopsis myb homolog is induced by dehydration stress and its gene product binds to the conserved MYB recognition sequence. Plant Cell 5:1529–1539

Wallaart TE, Bouwmeester HJ, Hille J, Poppinga L, Maijers NC (2000) Amorpha-4,11-diene synthase: cloning and functional expression of a key enzyme in the biosynthetic pathway of the novel antimalarial drug artemisinin. Planta 212:460–465

Wang H, Olofsson L, Lundgren A, Brodelius PE (2011) Trichome-specific expression of amorpha-4,11-diene synthase, a key enzyme of artemisinin biosynthesis in *Artemisia annua* L., as reported by a promoter-GUS fusion. Am J Plant Sci 2:619–628

Wang H, Han J, Kanagarajan S, Lundgren A, Brodelius PE (2013) 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. Plant Mol Biol 81:119–138

White NJ (1997) Assessment of the pharmacodynamic properties of antimalarial drugs *in vivo*. Antimicrob Agents Chemother 47:1413–1422

Woerdenbag HJ, Pras N, Bos R, Visser JF, Hendriks H, Malingré TM (1991) Analysis of artemisinin and related sesquiterpenoids from *Artemisia annua* L. by combined gas chromatography/mass spectrometry. Phytochem Anal 2:215–219

World Health Organization (2014) World Malaria Report 2014 http://www.who.int/malaria/publications/world_malaria_report_2014/en/

Wu W, Yuan M, Zhang Q, Zhu YM, Yong L et al (2011) Chemotype-dependent metabolic response to methyl jasmonate elicitation in *Artemisia annua*. Planta Med 77:1048–1053

Xu Y-H, Wang J-W, Wang S, Wang J-Y, Chen X-Y (2004) Characterization of GaWRKY1, a cotton transcription factor that regulates the sesquiterpene synthase gene (+)-γ-cadinene synthase-A. Plant Physiol 135:507–515

Yang TB, Poovaiah BW (2001) A calmodulin-binding/CGCG box DNA- binding protein family involved in multiple signaling pathways in plants. J Biol Chem 277:45049–45058

Yu ZX, Li JX, Yang CQ, Hu WL, Wang LJ et al (2012) The jasmonate-responsive AP2/ERF transcription factors AaERF1 and AaERF2 positively regulate artemisinin biosynthesis in *Artemisia annua* L. Mol Plant 5:353–365

Yuan Y, Liu W, Zhang Q, Xiang L, Liu X, Chen M, Lin Z, Wang Q, Liao Z (2014) Overexpression of artemisinic aldehyde Δ11(13) reductase gene-enhanced artemisinin and its relative metabolite biosynthesis in transgenic *Artemisia annua* L. Biotechnol Appl Biochem. doi:10.1002/bab.1234

Zare Mehrjerdi M, Bihamta M-R, Omidi M, Naghavi M-R, Soltanloo H, Ranjbar M (2013) Effects of exogenous methyl jasmonate and 2-isopentenyladenine on artemisinin production and gene expression in *Artemisia annua*. Turk J Bot 37:499–505

Zhang YS, Ye HC, Liu BY, Wangand H, Li GF (2005) Exogenous GA3 and flowering induce the conversion of artemisinic acid to artemisinin in *Artemisia annua* plants Russ. J Plant Physiol 52:58–62

Zhang Y, Teoh KH, Reed DW, Maes L, Goossens A, Olson DJH, Ross ARS, Covello PS (2008) The molecular cloning of artemisinic aldehyde Δ11(13) reductase and its role in glandular trichome-dependent biosynthesis of artemisinin in *Artemisia annua*. J Biol Chem 283:21501–21508

Zhang L, Jing F, Li F, Li M, Wang Y, Wang G, Sun X, Tang K (2009) Development of transgenic *Artemisia annua* (Chinese wormwood) plants with an enhanced content of artemisinin, an effective anti-malarial drug, by hairpin-RNA-mediated gene silencing. Biotechnol Appl Biochem 52:199–207