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ORIGINAL ARTICLE

The stacked over-expression of *FPS*, *CYP71AV1* and *CPR* genes leads to the increase of artemisinin level in *Artemisia annua* L.

Yunfei Chen · Qian Shen · Yueyue Wang · Tao Wang · Shaoyan Wu · Ling Zhang · Xu Lu · Fangyuan Zhang · Weimin Jiang · Bo Qiu · Erdi Gao · Xiaofen Sun · Kexuan Tang

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Abstract Artemisinin is an endoperoxide sesquiterpene lactone isolated from the aerial parts of *Artemisia annua* L., and is presently the most potent anti-malarial drug. Owing to the low yield of artemisinin from *A. annua* as well as the widespread application of artemisinin-based combination therapy recommended by the World Health Organization, the global demand for artemisinin is substantially increasing and is therefore rendering artemisinin in short supply. An economical way to increase artemisinin production is to increase the content of artemisinin in *A. annua*. In this study, three key genes in the artemisinin biosynthesis pathway, encoding farnesyl diphosphate synthase, amorpha-4, 11-diene C-12 oxidase and its redox partner cytochrome P450 reductase, were over-expressed in *A. annua* through *Agrobacterium*-mediated transformation. The transgenic lines were confirmed by Southern blotting and the over-expressions of the genes were demonstrated by real-time PCR assays. The HPLC analysis showed that the artemisinin contents in transgenic lines were increased significantly, with the highest one found to be 3.6-fold higher (2.9 mg/g FW) than that of the control. These results demonstrate that multigene engineering is an effective way to enhance artemisinin content in *A. annua*.

Keywords *Artemisia annua* L. · Artemisinin · Genetic engineering · CYP71AV1 · CPR · FPS

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Introduction

Malaria, one of the most serious health problems in many tropical countries, is responsible for nearly 1 million deaths every year (Barnes et al. 2009). As present research on malaria vaccines is not proceeding as smoothly as expected, the most effective way to control malaria has turned to relying heavily on anti-malaria drugs. Artemisinin (AN), an endoperoxide sesquiterpene lactone initially isolated from the aerial part of a Chinese herb *Artemisia annua* L. (Liu et al. 1979), has been proved to be the most effective anti-malaria drug, particularly in terms of the treatment of drug-resistant malaria. And, recently, it has also been reported that AN as well as its derivatives has antitumor activity, i.e. against melanoma, breast, ovarian, prostate, and renal cancer cell lines (Nam et al. 2007; Lee 2007). Therefore, AN has been considered to be a promising multi-functional natural product (Posner et al. 2004; Singh and Lai 2004; Efferth 2006).

AN is mainly extracted from the leaves of *A. annua*, which is now the only commercial source. However, the percentage of AN in *A. annua* is only 0.1–0.8 % DW (dry weight) (Lin et al. 2011; Zhang et al. 2009). It follows that there is an insufficient supply of AN which is failing to meet the fast-growing demand and thus leads to a comparatively high price that patients in the under-developed countries cannot afford. Since the chemical synthesis of AN is expensive and hence not commercially feasible (Xu et al. 1986; Avery et al. 1992), a great deal of effort has been made to increase the concentration of AN in plants. Using colchicines, Lin et al. (2011) successfully induced tetraploid *A. annua* plants, and detected a significant enhancement of AN through HPLC, Qian et al. (2007) reported that the AN content in *A. annua*, if treated with 4–6 g/L NaCl, could be significantly enhanced up

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threefold. Wang et al. (2010) documented a 49 % increase of AN content in *A. annua* on the 8th day after treatment with methyl jasmonate. Salicylic acid and gibberellic acid have also been proved as activators for AN accumulation (Banyai et al. 2011; Pu et al. 2009). Aftab et al. (2011) revealed that the irradiated sodium alginate (ISA) significantly helped to accumulate artemisinin in *A. annua*.

Besides those mentioned above, metabolic engineering is another frequently-used strategy for increasing AN content in *A. annua*. Recent studies have already made evident progress in revealing the biosynthetic pathway of AN (Fig. 1). Numerous key enzyme genes in the pathway have been cloned and functionally elucidated. AN is derived from two precursors: isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). Both the two C-5 molecules can be generated from both the cytosol-based MVA pathway and the plastid-based MEP pathway. IPP and DMAPP are then condensed to farnesyl diphosphate (FPP) catalyzed by farnesyl diphosphate synthase (FPS), this being the first committed step in sesquiterpene biosynthesis. Amorpha-4,11-diene synthase is responsible

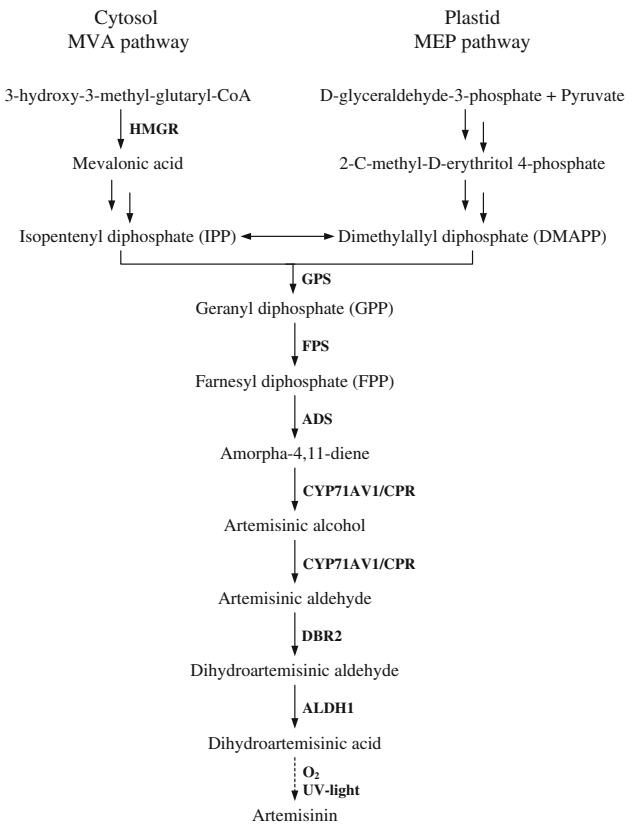

Fig. 1 Biosynthetic pathway of artemisinin in *A. annua*. *HMGR* 3-hydroxy-3-methyl-glutaryl coenzyme A reductase, *GPS* geranyl diphosphate synthase, *FPS* farnesyl diphosphate synthase, *ADS* amorpha-4,11-diene synthase, *CYP71AV1* amorpha-4,11-diene C-12 oxidase, *CPR* cytochrome P450 reductase, *DBR2* artemisinic aldehyde Δ11(13) double bond reductase, *ALDH1* aldehyde dehydrogenase

for cyclizing FPP into amorpha-4,11-diene. After that, amorpha-4,11-diene is oxidized to form artemisinic alcohol and then artemisinic aldehyde by cytochrome P450 monooxygenase (CYP71AV1) and its redox partner cytochrome P450 reductase (CPR). In 2008, artemisinic aldehyde double bond reductase (DBR2) deoxidizing artemisinic aldehyde to dihydroartemisinic aldehyde was cloned. And it was reported in 2009 that a novel enzyme, aldehyde dehydrogenase (ALDH1), catalyzes the conversion from dihydroartemisinic aldehyde to dihydroartemisinic acid. What is still unknown is how dihydroartemisinic acid is converted to AN. However, an increasing amount of evidence implies that this step is probably a spontaneous photo-oxidation reaction, rather than an enzymatic one (Brown and Sy 2007; Matsushita et al. 1996; Mercke et al. 2000; Nguyen et al. 2011; Sy and Brown 2002; Teoh et al. 2006; Wallaart et al. 2001; Zhang et al. 2008; Teoh et al. 2009).

Based on these, numerous attempts have been made to increase AN content through metabolic engineering. Banyai et al. (2010) over-expressed FPS in *A. annua* and gained a 2.5-fold increase of AN content at best. Nafis et al. (2011) over-expressed the 3-hydroxy-3-methyl-glutaryl coenzyme A reductase (HMGR), which shunted HMG-CoA into the isoprenoid pathway, and enhanced AN concentration to a maximum of 38.9 % higher than that in non-transgenic plants. Jing et al. (2008) over-expressed CYP71AV1 and CPR in *A. annua* and the AN content was increased by as much as 2.4-fold the content in control plant. To the best of our knowledge, however, more than one rate-limiting step probably exists in AN biosynthesis pathway. Therefore, the over-expression of a single gene may be insufficient to modify the metabolic pathway to reach the most desirable result. In other words, the over-expression of a single gene has not yet fulfilled the potential of metabolic engineering, while the simultaneous transfers of multiple genes into *A. annua* may be more effective for researchers to get to understand and then manipulate the entire metabolic pathway. As a matter of fact, multigene engineering has already been applied to other species in the past decade. This concept has gained prevalence since its successful application in "Golden rice" (Paine et al. 2005; Ye et al. 2000). Then, there were further great improvements and it was applied in crops ranging from soybean and maize to potato and canola (Naqvi et al. 2010). Recently, researchers have also become interested in simultaneously over-expressing two key enzymes in *A. annua.* Alam and Abdin (2011) constructed a vector with *ADS* and *HMGR* gene expression cassettes within one T-DNA region and then transformed it into *A. annua.* And it was reported that, as an optimistic result, one of the transgenic lines was found to have an AN content of 1.73 mg/g (DW), 7.65-fold higher than that in the non-transgenic plant. This trial indicated that co-over-expressing genes

would be much more effective and more efficient than over-expressing a single gene.

Our present study tries an over-expression of a three-gene combination in *A. annua*: a combination of *FPS*, *CYP71AV1* and *CPR*, each driven by a constitutive cauliflower mosaic virus 35S promoter, and investigates the relationships between the expression levels of the genes in research and production of AN.

Materials and methods

Construction of the plant expression vector

A fragment containing *gfp*:*gus*A expression cassette was cloned (Forward primer: ATTTAAATCATGGAGTCAAAGATTCAAA, with *Swa*I site shown in bold and underlined; Reverse primer: GCTGCAGCCCCGGGGCCCGATCTAGTAACATAGAT, with *Pst*I and *Sma*I sites shown in bold and underlined) from pCAMBIA1304 (Fig. 2a). *gfp*:*gus*A was then replaced by *FPS* (AF112881), *CYP71AV1* (DQ268763) and *CPR* (DQ318192) genes with the help of the restriction enzymes *Spe*I and *Bst*EII to generate *FPS* expression cassettes, *CYP71AV1* expression cassettes and *CPR* expression cassettes. The *CYP71AV1* expression cassette was firstly digested by *Swa*I and *Pst*I, and then cloned into pCAMBIA2300 (Fig. 2b) digested by *Sma*I and *Pst*I. This intermediate vector was digested by *Sma*I and *Pst*I again, allowing the insertion of *CPR* expression cassette excised by *Swa*I and *Pst*I. The last one, *FPS* expression cassette, was inserted following the same procedure. The *NPTII* gene contained in the recombinant vector was used as selection marker (Fig. 2c).

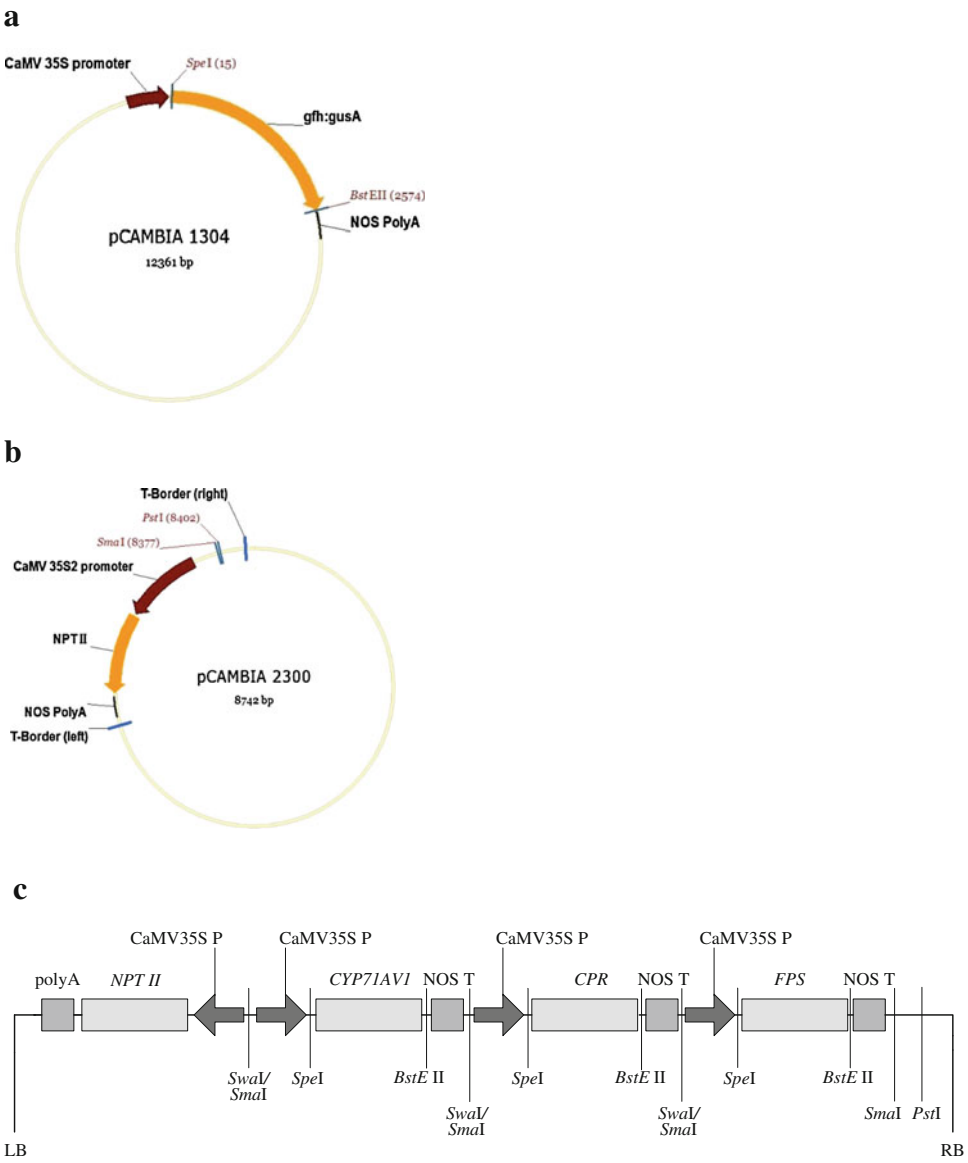

Fig. 2 Construction of the plant expression vector. **a** Profile of pCAMBIA1304, **b** profile of pCAMBIA2300, **c** T-DNA structure of the recombinant pCAMBIA2300 vector. The *CYP71AV1*, *CPR* and *FPS* genes are tagged with the CaMV35S promoters (*CaMV35S P*) and terminated by the NOS terminators (*NOS T*) in the pCAMBIA2300 binary vector, respectively. Neomycin phosphotransferase II gene (*NPTII*) is used as the selection marker. *LB* and *RB*, the left and right borders, respectively

Table 1 Primers used in real-time PCR analysis

Tested genes	GenBank accession number	Primers	Fragment size (bp)
β-actin	EU531837.1	Forward: 5′-CCAGGCTGTTCAGTCTCTGTAT-3′	180
		Reverse: 5′-CGCTCGGTAAGGATCTTCATCA-3′	
FPS	AF112881	Forward: 5′-TGAGAACTATGGGAAAAAGGAC-3′	166
		Reverse: 5′-TTTCAACACCGCTTGGACTG-3′	
CYP71AV1	DQ268763	Forward: 5′-CGAGACTTTAACTGGTGAGATTGT-3′	144
		Reverse: 5′-CGAAGCGACTGAAATGACTTTACT-3′	
CPR	DQ318192	Forward: 5′-ACGTTTGCAGCCGAGATAC-3′	166
		Reverse: 5′-CTGGCTTTCTGTCATAGGCA-3′	

Plant material and transformation

The seeds of *A. annua* were collected from Youyang, Chongqing, China. They were first surface-sterilized in 75 % (v/v) ethanol for 1 min, immersed in NaOCl (sodium hypochlorite) for 20 min, and then washed three times with MilliQ water. Finally, the seeds were germinated in Murashige and Skoog basal medium (Murashige and Skoog 1962) at an unchanged temperature of 25 °C, with a photoperiod of 16/8 h (light/dark). Young leaves from 3-week-old seedlings were cut into discs as a source of explants and *Agrobacterium tumefaciens* EHA105 harboring the recombinant expression vector p2300-*CYP71AV1-CPR-FPS* was put to use for transformation. Then, the infected leaves were transferred onto cocultivation medium (1/2 MS medium + 100 μM acetosyringone) and cultured in the dark for 3 days. Afterwards, the leaves were transferred onto shoot-inducing medium (MS medium + 0.5 mg/L 6-benzylaminopurine + 0.05 mg/L α-naphthaleneacetic acid + 50 mg/L kanamycin +500 mg/L carbenicillin) and subcultured every 2 weeks to select kanamycin-resistant plantlets. Two months later, the selected plantlets were transferred onto root-inducing medium (1/2 MS medium + 125 mg/L carbenicillin) to induce roots.

PCR analysis on transformed plantlets

DNA isolated from putatively transformed plantlets by the cetyltrimethyl ammonium bromide (CTAB) method was used as the template. The forward primer F35S (5′-TTC GTCAACATGGTGGAGCA-3′) and reverse primer RFPS (5′-CACCTCGGGAGCACCAAA-3′) were designed in accordance with the sequences of CaMV35S promote and *FPS* respectively. Each PCR reaction was performed in the following 25 μl reaction mixture: 2.5 μl of 10× PCR buffer, 1.5 μl of $MgCl_2$ (25 mM), 1.5 μl dNTPs (2.5 mM), 1 μl of forward primer (10 μM), 1 μl of reverse primer (10 μM), 2 μl of DNA template (100 ng/μl), and 0.3 μl r*Taq* DNA polymerase (5U/μl); double-distilled water was added to bring the volume up to 25 μl. The reaction was heated at 94 °C for 5 min, and then followed by 27 cycles of 94 °C for 30 s, 54 °C for 30 s, and 72 °C for 1 min, with an additional final extension step at 72 °C for 10 min.

RNA extraction and real-time RT-PCR analysis

Leaves from the 10th–13th lateral branches (counted from the apical meristem) of 12-week-old plants were harvested as samples. Total RNA was isolated from these leaves with the help of the RNAprep pure Plant Kit (Tiangen Biotech, Beijing, China) and was reversely transcribed into cDNA using the PrimeScript II 1st Strand cDNA Synthesis Kit (Takara Biotechnology, Dalian, China), according to the manufacturer's instructions. The real-time PCR was performed on a Thermal Cycler PTC200 qPCR equipment (Bio-Rad Laboratories, Hercules, USA) with the specific primers designed for the genes in research (Table 1). First strand cDNA was used as the templates in 25 μl reactions including 12.5 μl SYBR® Premix Ex Taq™ II (Takara Biotechnology) and 10 pmol of each primer. The real-time PCR was performed first at 95 °C (30 s), and then in 40 cycles of the sequence of 95 °C (5 s) and 55 °C (1 min), and finally in a dissociation stage of 95 °C (15 s), 55 °C (1 min) and 95 °C (15 s). All the samples were run 3 times and a negative control was performed in all the real-time PCR runs.

Southern blotting

The genomic DNA of putatively transgenic plants and non-transgenic plants were isolated from fresh leaves. The plasmid DNA containing the *NPTII* gene was used as the positive control and DNA from non-transgenic plants (WT) as negative control. In Southern blotting analysis, 60 μg genomic DNA per sample was digested by *Dra*I at 37 °C for 20 h, resolved in 0.8 % agarose gel, transferred on to a positively charged hybond-N^+ nylon membrane (GE Healthcare, UK), and then hybridized with an alkaline-phosphatase-labeled partial cDNA sequence of *NPTII* as the probe. Hybridization, probe-labeling and detection

were performed by using an AlkPhos Direct Labeling and Detection Kit from Amersham Pharmacia Biotech (Buckinghamshire, UK) according to the manufacturer's instructions. The signals were visualized in exposition to Fuji X-ray film at room temperature for 4 h.

Determination of artemisinin by high-performance liquid chromatography

Leaves from the 10th–13th lateral branches (counted from the apical meristem) of 12-week-old plants were harvested as samples. One gram of each sample was immersed in 10 ml chloroform and immediately vortexed for 1 min. Having discarded the extracted leaves, the solution was evaporated at room temperature for 36 h until it was absolutely dry and then dissolved in 3 ml methanol to get the crude extract. The crude extract was treated through sonication at 45 W at 30 °C for 30 min before filtered through a filter (pore size 0.22 μm).

The filtered liquid (20 μL) was analyzed by using Waters Alliance 2695 high performance liquid chromatography (HPLC) coupled with Waters 2420 evaporative light scattering detector (Waters, Milford, USA). Metabolites were separated on a 250 × 4.6 mm Amethyst C18-H column (Sepax Technologies, Delaware, USA). The mobile phase used was methanol/water (75:25, v/v), and the flow rate was set at 1 ml/min. The ELSD conditions were optimized at a nebulizer-gas pressure of 345 kPa and at a drift-tube temperature of 45 °C. The standard curve was prepared by using chromatography-grade AN from Sigma dissolved in methanol to make a series of stock solutions. The AN content of the samples was quantified against the standard curve. The measurement was repeated three times.

Results

The *Agrobacterium tumefaciens* strain EHA105 was transformed with pCAMBIA2300 harboring *FPS*, *CYP71AV1* and *CPR* gene expression cassettes, and co-cultivated with young leaves of *A. annua*. After 3 rounds of subculturing on the selective shoot-inducing medium, 189 kanamycin-resistant regenerated plantlets were acquired. Since *FPS*, *CYP71AV1* and *CPR* gene expression cassettes were constructed in the same T-DNA, a pair of primers, F35S and RFPS, was used to detect the presence of target genes. Eight independent transgenic plants were identified. They showed the predicted 1.1-kb band, whose size is the same as that of the positive control, while no amplification was detected in the non-transgenic line (Fig. 3).

PCR-positive transgenic plants were selected for a further confirmation of the integration of the foreign DNA fragment into genomic DNA and of its copy number by

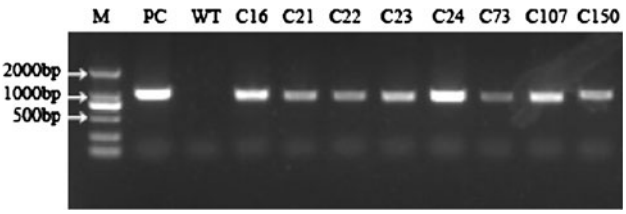

Fig. 3 PCR analysis for the presence of the *NPTII* gene in regenerated *A. annua*. *M* DL 2000 DNA ladder (Takara Biotechnology, Dalian, China); *PC* positive control recombinant pCAMBIA2300; *WT* non-transgenic control plant; *C16*, *C21*, *C22*, *C23*, *C24*, *C73*, *C107*, *C150*, kanamycin-resistant *A. annua* plants

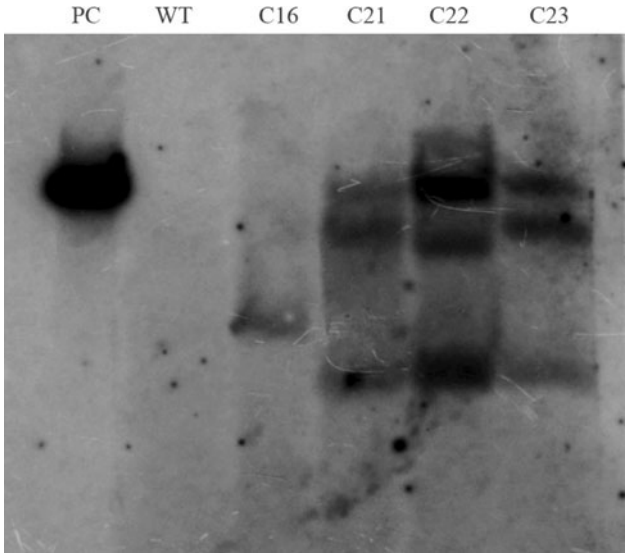

Fig. 4 Southern blotting analysis of transgenic plants using *NPTII* probe. *PC* positive control recombinant pCAMBIA2300, *WT* non-transgenic control plant, *C16*, *C21*, *C22*, *C23* transgenic plants

Southern blot. The specific primers for probe used in Southern blot were designed according to the sequence of *NPTII*, as *NPTII* is a bacterial gene not existing in non-transgenic *A. annua*. All the PCR-positive plants under test showed hybridized bands while the untransformed plant showed an absence of any band, as expected. This demonstrated that the foreign genes had been integrated into the genome of *A. annua*. C16 showed a presence of a single copy of the transgenes, while C21, C22, and C23 were found to contain three copies, respectively (Fig. 4).

The transgenic plants were transferred into a medium of perlite, and 1 month later they were transplanted into pots. No distinct phenotypic difference was detected between the transgenic plants and the untransformed control plants in terms of leaf shape, branch number, etc. (data not shown). In a short period after they had been transplanted into pots, some slight differences were observed in plant height; 2 weeks later, however, the discrepancy disappeared and no more growth retardation was found.

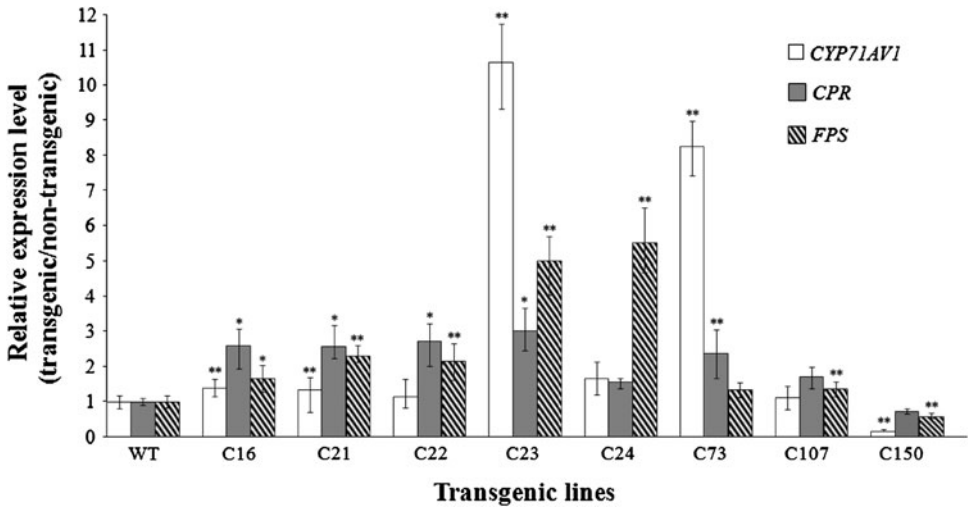

Fig. 5 Real-time PCR analysis of the expressions of *FPS*, *CYP71AV1* and *CPR* in transgenic *A. annua*. *WT* non-transgenic control plant, *C16*, *C21*, *C22*, *C23*, *C24*, *C73*, *C107*, *C150* transgenic plants. The relative transcript level is calculated from three independent experiments. The *standard bars* were obtained from standard deviation (SD) of three replicates. Statistical significance was determined by Student's *t* test (**$P < 0.01$, *$P < 0.05$)

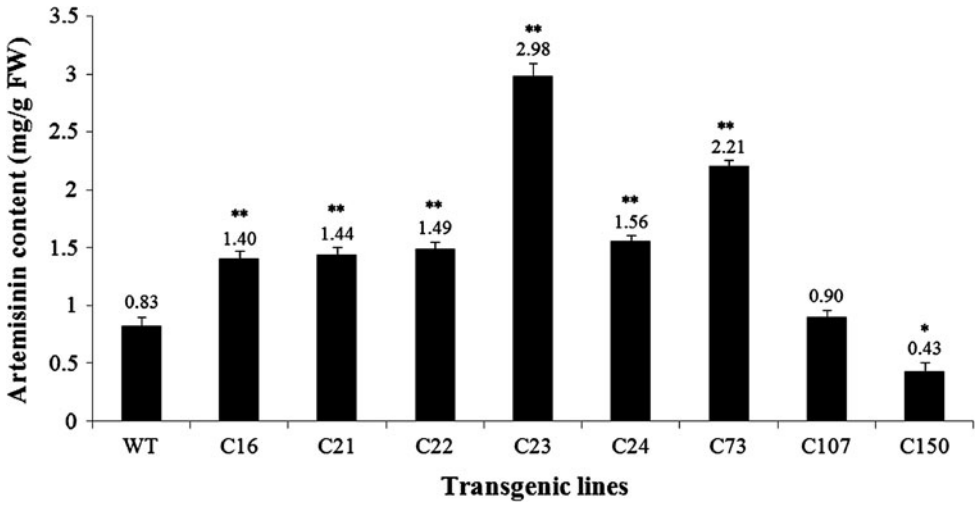

Fig. 6 Artemisinin content of transgenic *A. annua* and non-transgenic *A. annua*. *WT* non-transgenic control plant, *C16*, *C21*, *C22*, *C23*, *C24*, *C73*, *C107*, *C150* transgenic plants, *FW* fresh weight. The artemisinin levels were a mean value calculated from three biological replicates. The *standard bars* were obtained from standard deviation (SD) of three replicates. Statistical significance was determined by Student's *t* test (**$P < 0.01$, *$P < 0.05$)

In order to demonstrate that the *FPS*, *CYP71AV1* and *CPR* genes were over-expressed in the transgenic plants at the mRNA level, real-time PCR was carried out by using the total RNA extracted from the leaves of the transgenic plants and those of the non-transformed plants. It was shown thatn in the majority of the transgenic lines, the expression levels of the three genes were differently elevated. Specifically, most significant changes were observed in C23, where the expression level of *CYP71AV1* was increased to 10.65-fold that in non-transgenic plants, while the level of *FPS* was 5 times higher and that of *CPR* 3 times higher. In C73, the expression level of *CYP71AV1, CPR* and *FPS* were 8.2-, 2.3-, 1.4-fold, respectively, in comparison with that of the non-transgenic *A. annua* (Fig. 5). However, C150 showed declines in expression levels of the three genes, presumably caused by transcriptional gene silencing.

The AN content was determined by HPLC, and the different enhancements in 87.5 % transgenic plants were recorded. Compared with the non-transgenic *A. annua,* the maximum AN content was observed in C23 (3.6-fold, 2.9 mg/g FW), and the AN content in C73 followed as the second (2.7-fold, 2.2 mg/g FW) (Fig. 6).

Discussion

The biosynthetic pathway for AN is intricate, with many likely rate-limiting steps that are regulated by key enzymes. Our previous study in *Hyoscyamus niger*, whose metabolic pathway features a similar complexity, implied that the over-expression of multiple genes controlling the different steps in the pathway appeared more efficient than that of a single gene, as the latter might render subsequent reactions more rate-limiting (Zhang et al. 2004). Actually, research on the so-called multigene engineering is in the ascendant at present. In 2009, Fujisawa et al. (2009) introduced seven key enzyme genes from three species

related to ketocarotenoid biosynthesis into *Brassica napus*. The maximum carotenoid content was elevated to 657 mg/g fresh weight, a 30-fold increase over the content in the wild-type, and at the same time, 190 mg/g fresh weight ketocarotenoids was detected.

Previous studies showed that *FPS*, *CYP71AV1* and *CPR* had played essential roles in AN biosynthesis in *A. annua*. The over-expression of the endogenous *FPS* gene led to 34.4 and 150 % increases in AN concentration, respectively, according to Han et al. (2006) and Banyai et al. (2010), and over-expressing *CYP71AV1* and *CPR* led to a 2.4-fold increase (Jing et al. 2008). However, our study introduced a combination of the three genes into *A. annua*, with each gene surrounded by an appropriate promoter and terminator. In comparison, plants over-expressing the combination of the three genes exhibited greater amounts of AN content, 3.6-fold higher than that of the control group, demonstrating a larger potential and hence proving the multigene engineering strategy as a feasible and more effective way to increase AN content.

The reason for transgene silencing is mechanically diversified. In our study, there are two possible reasons contributing to the assumed transgene silencing in C150. The first one is position effects; in other words, that chromosomal insertion sites (e.g., the heterochromatin regions) and flanking DNA sequences of transgene loci can hinder the expression of foreign genes (Kooter et al. 1999). Iglesias et al. (1997) found that, in transgenic tobaccos, the stably expressed loci were flanked by plant DNA containing AT-rich regions that were bound to nuclear matrices in vitro, whereas the unstably expressed loci were located in the heterochromatin domains or near the centromeres. However, a more likely explanation is homology-dependent gene silencing (HDGS). HDGS could be caused both by multiple homologous transgenes and by transgenes, and by homologous endogenous genes. As for two homologous transgenes, HDGS is often associated with the interactions between the homologous promoter regions of transgenes, leading to the methylation on the promoters (Kooter et al. 1999; Stam et al. 1997). Vauchere reported that 90 bp of homology in the promoter sequence would cause inactivation of CaMV 35S promoters (Vaucheret 1993). Accordingly, there is a possibility that HDGS occurred in transgenic plants containing multiple CaMV 35S promoters. In our study, four or more CaMV 35S promoters were transformed into *A. annua*. Probably, this is the reason for a down-regulation of expression levels of the studied genes in C150. In addition, the content of AN as well as the expression levels of three genes in C150 are all lower than those in the control plant. That implies that the endogenous genes were also suppressed and that the cosuppression occurred. However, this still needs further confirmation.

To reveal the possible relevance of the over-expressions of the three genes in research on the enhancement of AN content, real-time PCR and HPLC were conducted. Seven transgenic plants showed increases in transcriptional expression level of the genes, which exhibited a good synchrony with the enhancement of AN: C23 expressed the highest level of *CYP71AV1* and *CPR*, as well as the second highest level of *FPS*, resulting in the highest AN concentration; the transcriptional expression level of the three genes showed merely a slight elevation in C107, leading to the correspondingly smallest increase in AN concentration; and C150, whose mRNA expression levels of the three genes were lower than that of the non-transgenic *A. annua,* produced only 52 % of the AN in the control plant. However, we found that it was still difficult to establish a satisfactory correlation between the expression level of each gene and the accumulation of AN. In a previous study, Yang et al. (2008) suppressed the expression of squalene synthase (SQS) leading to the over-expression of *ADS*, *CYP71AV1* and *CPR* genes and enhanced AN, but they also found that no reasonable correlations of the enhanced AN production can be established with the elevated mRNA levels of each gene. This reflects the complexity of the metabolic network in *A. annua.*

In our study, the expression level of *CPR* in 7 artemisinin-increasing transgenic plants showed only a slight variation, in comparison with the significant variation in AN content between them. Olofsson et al. (2011) reported that the expression level of *CPR* was essentially the same in different tissues, flower buds, young leaves, old leaves stems, roots, and hairy roots, in wihch AN concentrations varied significantly. These imply that CPR might not be as important as other key enzymes in the AN biosynthetic pathway.

This study shows that over-expressing *FPS*, *CYP71AV1* and *CPR* genes is an effective way to breed a new variety of *A. annua* with a high AN concentration. And in practical use it might be a potentially better way than over-expressing each gene separately, which may therefore lead to a reduction of the cost of this anti-malaria drug.

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