GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

Overexpression of the cytochrome P450 monooxygenase (*cyp71av1*) and cytochrome P450 reductase (*cpr*) genes increased artemisinin content in *Artemisia annua* (Asteraceae)

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Genet. Mol. Res. 11 (3): 3298-3309 (2012)
Received March 3, 2012
Accepted July 31, 2012
Published September 12, 2012
DOI http://dx.doi.org/10.4238/2012.September.12.13

ABSTRACT. Finding an efficient and affordable treatment against malaria is still a challenge for medicine. Artemisinin is an effective anti-malarial drug isolated from *Artemisia annua*. However, the artemisinin content of *A. annua* is very low. We used transgenic technology to increase the artemisinin content of *A. annua* by overexpressing cytochrome P450 monooxygenase (*cyp71av1*) and cytochrome P450 reductase (*cpr*) genes. CYP71AV1 is a key enzyme in the artemisinin biosynthesis pathway, while CPR is a redox partner for CYP71AV1. Eight independent transgenic *A. annua* plants were obtained through *Agrobacterium tumefaciens*-mediated transformation, which was confirmed by PCR and Southern blot analyses. The real-time qPCR results showed that the gene *cyp71av1* was highly expressed at the transcriptional level in the transgenic *A. annua* plants. HPLC analysis

showed that the artemisinin content was increased in a number of the transgenic plants, in which both *cyp71av1* and *cpr* were overexpressed. In one of the transgenic *A. annua* plants, the artemisinin content was 38% higher than in the non-transgenic plants. We conclude that overexpressing key enzymes of the biosynthesis pathway is an effective means for increasing artemisinin content in plants.

Key words: *Artemisia annua* L.; *cyp71av1*; *cpr*; Transgenic plant; Artemisinin

INTRODUCTION

Malaria is a global health problem that affects more than 1 billion people living in areas with a high risk of disease transmission. Artemisinin-based combination therapies were recommended by the World Health Organization as the best choice for uncomplicated malaria caused by the *Plasmodium falciparum* parasite (Mutabingwa, 2005; Bhattarai et al., 2007). Artemisinin, an endoperoxide sesquiterpene lactone belonging to the terpenoids, is synthesized through the isoprenoid or mevalonate pathway, which occurs in the cytosolic and non-mevalonate pathway in plastids (Zeng et al., 2009; Maes et al., 2011). Like many other secondary metabolites, artemisinin accumulates in trace amounts in the *Artemisia annua* plant, about 0.01-0.8% dry weight (dwt), and its content varies depending on genotype and cultivation conditions (Duke et al., 1994; Abdin et al., 2003). The biosynthesis of artemisinin is almost completely elucidated, and the genes of most of the biosynthetic enzymes in the pathway have been cloned and characterized.

Up to now, a reasonably clear picture of artemisinin biosynthesis has emerged, as illustrated in Figure 1 (Maes et al., 2011; Liu et al., 2011). Overexpressing some of the artemisinin biosynthetic enzyme genes or suppressing the genes of competitive pathway enzymes has proven to be an effective way to increase the amount of artemisinin. A branch point enzyme, 3-hydroxy-3-methyl-glutaryl coenzyme reductase (HMGR), which shunts HMG-CoA to the isoprenoid pathway, is thought to be a key enzyme at the beginning of artemisinin biosynthesis. By increasing the concentration of HMG-CoA (3-^{14}C) from 2 to 16 μM, the incorporation of label (^{14}C) into artemisinin was enhanced from 7.5 to 17.3 nmol (up to 130%) (Ram et al., 2010). Transferring the *hmgr* gene from *Catharanthus roseus* L. into *A. annua* resulted in a significantly higher HMGR activity compared with wild-type controls in leaf tissue and an increase of 22.5% artemisinin content compared with wild-type *A. annua* (Aquil et al., 2009). Overexpression of the farnesyl pyrophosphate synthase gene (*fps*) exhibited significant enhancement of artemisinin yields of about 2.5 times in comparison to the wild-type plants (Banyai et al., 2010). Suppressing the expression of squalene synthase, a key enzyme of the sterol pathway, which is competitive with the amorpha-4,11-diene accumulation pathway, produced a 3.14-fold increase in artemisinin content in transformed plants compared with untransformed control plants (Zhang et al., 2009). Therefore, to improve artermisinin production, genetic transformation is a potentially useful strategy. Cytochrome P450 monooxygenase (CYP71AV1) belongs to the cytochrome P450 family and is a multifunctional sesquiterpene oxidase with a key role in the biosynthesis of sesquiterpene lactone artemisinin, which involves three steps, namely conversion of amorpha-4,11-diene to artemisinic acid via

artemisinic alcohol and artemisinic aldehyde middle metabolites (Teoh et al., 2006; Zeng et al., 2009; Arsenault et al., 2010; Maes et al., 2011). Cytochrome P450 oxidoreductase (CPR) has also been isolated from *A. annua*, and its biochemical function has been confirmed *in vitro*. CPR is a redox partner for CYP71AV1 and helps CYP71AV1 to catalyze the conversion of amorphadiene to more oxygenated products *in vivo* (Ro et al., 2006). Therefore, in this study, *cyp71av1* and *cpr* were co-overexpressed to increase artemisinin content in *A. annua*.

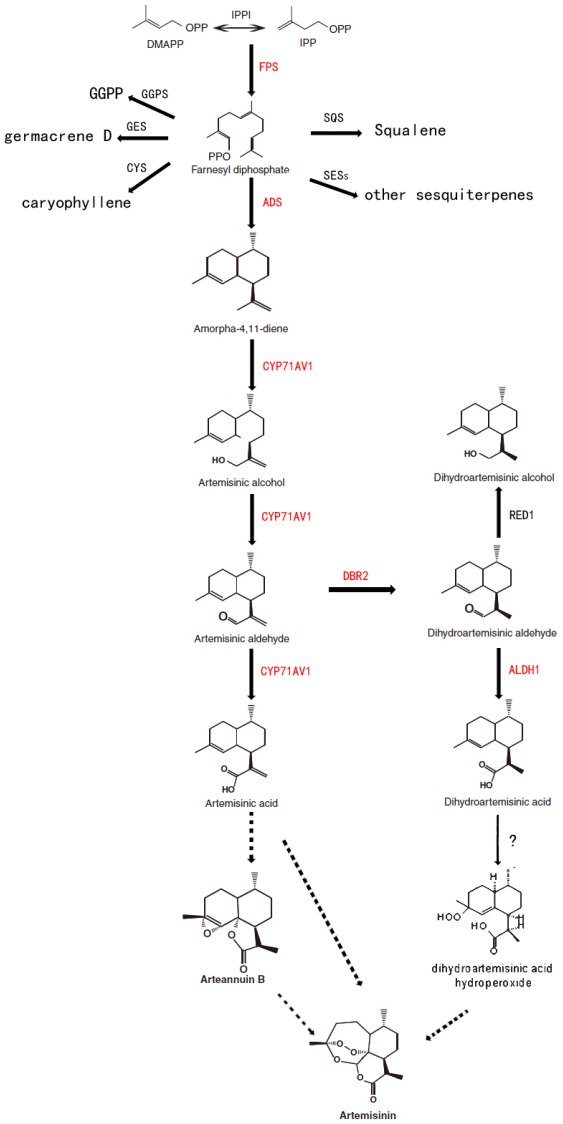

Figure 1. Biosynthetic pathways of some terpenoids and their relationship to artemisinin biosynthesis. Enzymes marked in red are key enzymes in artemisinin biosynthesis. Dashed arrows indicate steps for which no enzymes have been identified.

MATERIAL AND METHODS

Construction of transforming vector containing *cyp71av1* and *cpr* genes

Constructing the plasmid containing both *cyp71av1* (DQ268763) and *cpr* genes (DQ318192) was based on the intermediate plasmids pMDT18-p35S::cyp71av1::nos (with *Swa*I and *Pst*I restriction sites) and pMDT18-p35S::cpr::nos (with *Sma*I and *Pst*I restriction sites), which our laboratory already had. By cutting and ligating, we obtained the pMDT18-p35S::cyp71av1::nos::p35S::cpr::nos plasmid. The 4454-bp fragment that contains cyp71av1::nos::p35S::cpr was amplified by PCR by using the forward primer, CYPF1 (5'-GG**GGTACC**ATGAAGAGTATACTAAAAGCAATG-3' with *Kpn*I sites shown in bold and underlined) and reverse primer, CPRR1 (5'-GG**GGTACC**TTACCATACATC ACGGAGATATCTT-3' with *Kpn*I sites shown in bold and underlined). PCR was carried out under the following conditions: denaturation at 94°C for 4 min; 30 cycles of 30 s at 94°C, 30 s at 60°C, and 4 min at 72°C, and 72°C for 10 min. LA *Taq* (TaKaRa, Dalian) was used as the amplification enzyme. After confirmation by sequencing, the fragment was cloned into the plant expression vector named FSN, which was constructed by prior modification from pCAMBIA 2300 by our laboratory. The FSN vector has dual binary T-DNA borders, where one T-DNA border contains the neomycin phosphotransferase II (*npt*II) gene, which is driven by the cauliflower mosaic virus (CaMV) 35S promoter with nopaline opine synthase (NOS) as terminator, and the other T-DNA border is linked into our target fragment, which is driven by double CaMV 35S promoters with NOS as terminator. The constructed expression vector containing *cyp71av1* and *cpr* genes was named FSN-CYP71AV1-CPR (FYR). The FYR vector was then transferred into *Agrobacterium tumefaciens* strain EHA105 by a conventional freezing and thawing method, and the resulting strains were used in the transformation of *A. annua*.

Molecular analyses of transgenic plants

Transformation of A. annua

The *A. annua* seeds used in this research, which have been kept in our laboratory for years, had been collected from Chongqing, a municipality of China. First, the seeds were immersed in 75% (v/v) ethanol for 1 min and in 10% (v/v) NaOCl (sodium hypochlorite) for 10 min for surface-sterilization. The seeds were then washed 4 times with sterile distilled water. The germ-free seeds were placed on 0.5% Phytagel-solidified germination medium MS_0 [Murashige and Skoog (1962) basal medium with the addition of sucrose (30 g/L)] on 9-cm diameter Petri dishes and cultured in a chamber under the conditions of 25° ± 2°C with 16-h light/8-h dark photoperiod at 8000 lux. When reaching 4-5 cm in length, the seedlings were collected, and the leaves were cut into 0.5-cm diameter discs, co-cultivated with *A. tumefaciens* strain EHA105 at 25°C for 3 days using the protocol described by McCormick et al. (1986). The leaf discs were transferred to kanamycin selection medium MS_1 (MS_0 + 2.5 mg/L N_6-benzoyladenine + 0.3 mg/L naphthalene-1-acetic acid + 50 mg/L kanamycin + 250 mg/L carbenicillin), and the kanamycin-resistant plantlets were regenerated, subcultured every 10 days 3 times, and trans-

ferred to rooting medium MS_2 (half-strength MS_0 + 250 mg/L carbenicillin). After roots were formed (about 12-15 days), the rooted plantlets were transferred to soil pots in the growth chamber, with watering when necessary. After 2-3 months of growth, they were transplanted to the greenhouse for further growth.

PCR analysis of putative transgenic plants

Genomic DNA for PCR was isolated from 30-40 mg fresh leaves from 4-month-old plantlets grown in the greenhouse, by using the modified CTAB (cetyltrimethylammonium bromide) method (Doyle and Doyle, 1987; Stewart Jr. and Via, 1993; Chen and Ronald, 1999). For the presence of the introduced *cyp71av1* gene, we used the primers P35S (5'-TTCGTCAACATGGTGGAGCA-3') and CYPR2 (5'-GGAAGGCTTTTTTGGTGGATTTG-3') for PCR analysis (*cpr* was not analyzed by PCR because it is in the same T-DNA border with *cyp71av1*). The PCR was carried out in a 25-µL final reaction volume containing approximately 50 ng DNA template, using *Premix Taq*® Version 2.0 (TaKaRa) following manufacturer instructions. The thermal cycling conditions were: denaturation at 94°C for 4 min, followed by 30 cycles of amplification at 94°C for 30 s, at 54°C for 30 s and at 72°C for 45 s, and at 72°C for an additional 10 min. A 5-µL aliquot of PCR solution was electrophoresed on a 1.0% agarose gel (with 0.25 µg/mL ethidium bromide) at 140 V for 10 min and the bands visualized using a Gel Image System (Tanon 3500, Shanghai). The PCR analysis with right-length DNA fragments was considered to be positive for putative transgenic *A. annua* and then confirmed by Southern blotting.

Southern blot analysis

The genomic DNA of transgenic and wild-type control (from the rooting medium pot but not containing the inserted *cyp71av1* gene after PCR analysis) plants was isolated and purified by the modified CTAB method. Approximately 60 µg of each genomic DNA sample was digested by *Dra*I at 37°C for 16 h, separated by electrophoresis on a 0.8% agarose gel and then transferred onto a positively charged Hybond-N^+ nylon membrane (GE Healthcare, England). Using an alkaline-phosphatase-labeled *npt*II and partial cDNA sequence of *cyp71av1* as the probe, the probes were prepared by using Amersham AlkPhos Direct Labeling Reagents (GE Healthcare) according to manufacturer instructions. Hybridization of the probe with the membrane was carried out overnight in a hybridization oven at 55°C. Hybridization signals were detected by CDP-Star Detection Module (GE Healthcare) following manufacturer instructions. The hybridized signals were visualized by exposure to Kodak X-ray film at room temperature for 5 h.

RNA isolation and real-time qPCR analysis

RNA was extracted from leaf 9 (counting from the apical meristem) from 20-week-old transgenic *A. annua* and control plants by using the RNAprep pure Plant kit (TIANGEN Biotech, Beijing), following manufacturer instructions. The cDNAs were synthesized from the RNA samples using the Prime Script™ RT reagent kit (TaKaRa) according to manufac-

turer instructions, using oligo(dT) and random 6-mers as primers. The real-time qPCR was performed on a Peltier Thermal Cycler PTC200 (Bio-Rad, USA) by using the gene-specific primers (Table 1) RT-CYP F3 and RT-CYP R3, RT-CPR F2 and RT-CPR R2, and RT-actin F and RT-actin R for the analysis of genes *cyp71av1*, *cpr* and the reference gene *β-actin*, respectively. First-strand cDNAs were used as template in 25-µL reactions including 12.5 µL SYBR Green (SYBR Premix Ex *Taq*, TaKaRa), and 1 µL each primer. The real-time PCR cycling was performed at 95°C for 1 min, followed by 40 cycles at 95°C for 15 s, 55°C for 25 s, and 72°C for 25 s, and melting curves were performed from 68° to 95°C read every 0.5°C for 10 s. The melting curve was performed to determine the PCR product size and to detect possible primer dimers. Triplets of all samples were run. The cycle number at which the reaction crossed an arbitrarily placed threshold (C_T) was determined for each gene, and the relative expression of each gene was determined using the equation $2^{-\Delta\Delta CT}$, where $\Delta\Delta C_T = (C_{T,Target} - C_{T,Actin})_{sample} - (C_{T,Target} - C_{T,Actin})_{control}$ (Livak and Schmittgen, 2001).

Table 1. Nucleotide sequences of primers used in real-time qPCR.

Primer name	Forward primer sequence (5'-3')	Fragment size (bp)
RT-CYP F3	CGAGACTTTAACTGGTGAGATTGT	144
RT-CYP R3	CGAAGCGACTGAAATGACTTTACT	
RT-CPR F2	ACGTTTGCAGCCGAGATAC	166
RT-CPR R2	CTGGCTTTCTGTCATAGGCA	
RT-actin F	CCAGGCTGTTCAGTCTCTGTAT	180
RT-actin F	CGCTCGGTAAGGATCTTCATCA	

Artemisinin evaluation by HPLC

Artemisinin was determined by HPLC using approximately 200 mg fresh weight pooled samples of leaves 10, 15, and 20 (counting from the apical meristem) from 20-week-old greenhouse-grown plants. Samples were stored at -80°C until use for artemisinin analysis. Crude artemisinin was extracted according to the modified protocol from Graham et al. (2010). Samples were dipped into 3 mL chloroform and gently shaken for 5 min in 10-mL tubes. The solvent was evaporated in a fume hood at room temperature until absolutely dried prior to use as a crude extract. Exactly 3 mL methanol was added to the crude extract and mixed gently, and the solution was then treated with an ultrasonic processor (JYD-650, Shanghai Zhisun Instrument Co. Ltd., China) under the conditions of 37°C and 50 W for 30 min. The solution was filtered through a 0.45-µm Sartorious® membrane, and 200 µL filtrate was subjected to HPLC. The samples were analyzed using a Waters 2695 HPLC system coupled with a Waters 2420 ELSD detector. The HPLC conditions were as follows: column, Amethyst 5-µm C18-H 120A column (250 x 4.6 mm, Sepax Technologies Inc., USA); mobile phase, water/methanol (25:75, v/v); flow rate, 1 mL/min. The ELSD conditions were optimized at a nebulizer-gas pressure of 206.8 kPa (30 psi), drift-tube temperature was 40°C, and the gain was set at 9. Authentic artemisinin from Sigma was used as the standard in this experiment. For each sample, the injection

volume was 20 µL, and the results were analyzed using Empower (Waters' chromatography data software). The measurement was repeated three times.

RESULTS AND DISCUSSION

Construction of vector containing *cyp71av1* and *cpr*

By using the intermediate vectors that our laboratory already had, we cut and ligated the target genes together into one T-DNA border structure to generate the final overexpression vector, which was used for transformation (Figure 2). The target genes *cyp71av1* and *cpr* were in one T-DNA structure while the selectable marker gene *npt*II was located in another independent T-DNA structure.

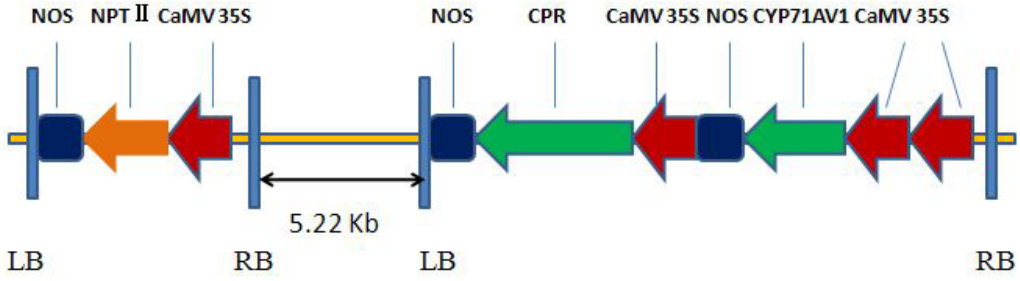

Figure 2. Schematic structure of the expression vector FSN-CYP71AV1-CPR (FYR). The vector has two independent T-DNA structures and the distance between these two T-DNA borders is 5.22 kb. *Cyp71av1* was driven by double 35S promoter and *cpr* was driven by single 35S promoter. The *npt*II is used as the selectable marker. NOS = nopaline opine synthase; NPTII = neomycin phosphotransferase II; CaMV = cauliflower mosaic virus; LB and RB = left and right borders, respectively.

Transformation of *A. annua*

The young seedlings were cut into small discs and used as explants in *A. tumefaciens*-mediated leaf disc transformation. After co-cultivation with EHA105 containing the FYR vector for 3 days, the explants were transferred to selection medium containing 50 mg/L kanamycin and subcultured 3 times at 10-day intervals. The regenerated shoots were transferred to rooting medium for rooting. Totally, more than 150 kanamycin-resistant plants were obtained (Figure 3). Using PCR and Southern blot analysis, 8 independent transgenic plants were confirmed (Figure 4). Southern blot results showed that there were 2 copies of *cyp71av1* genes in control, while there were more copies in the transgenic *A. annua* plants, indicating that our target genes were successfully integrated into transgenic *A. annua* genomic DNA (Figure 5A). For gene *npt*II, the Southern blot results demonstrated that the copy number of gene *npt*II was different with the *cyp71av1* gene, which means that they were integrated into genomic DNA independently (Figure 5B).

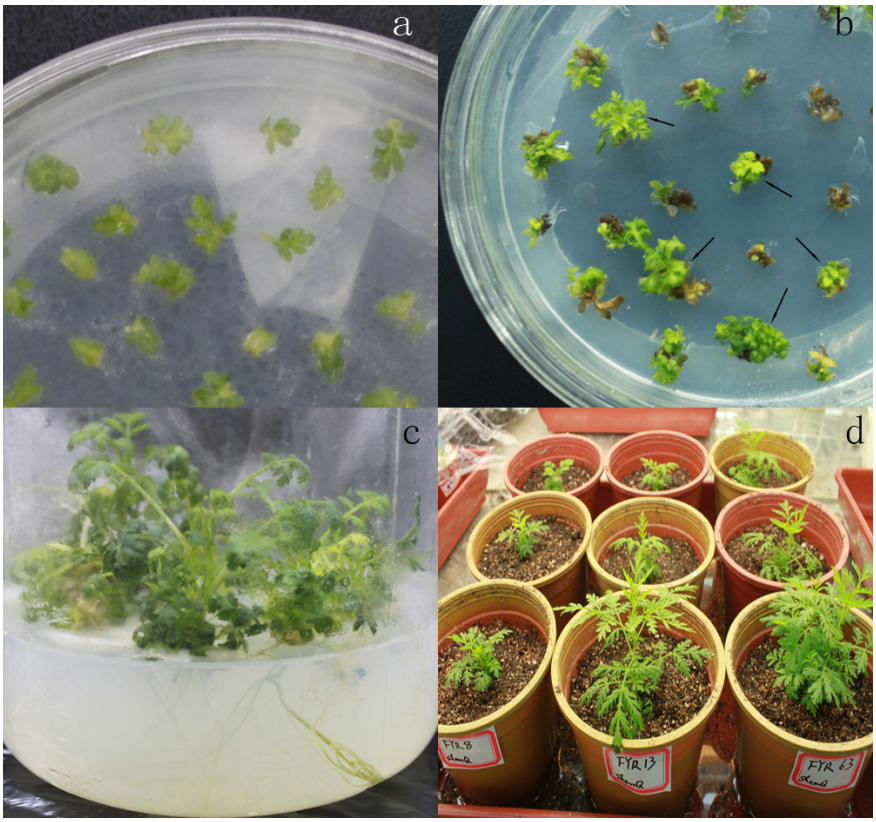

Figure 3. Regeneration of transformed plantlets from leaf discs of *Artemisia annua*. **a.** *A. annua* shoots co-cultivated with *Agrobacterium tumefaciens* for 72 h. **b.** Calli-derived shoot formation (indicated with arrow) obtained after transferring to regeneration medium. **c.** Plantlets of *A. annua* transferred into rooting medium. **d.** After roots were formed, the rooted plantlets were transferred to soil in pots in the growth chamber.

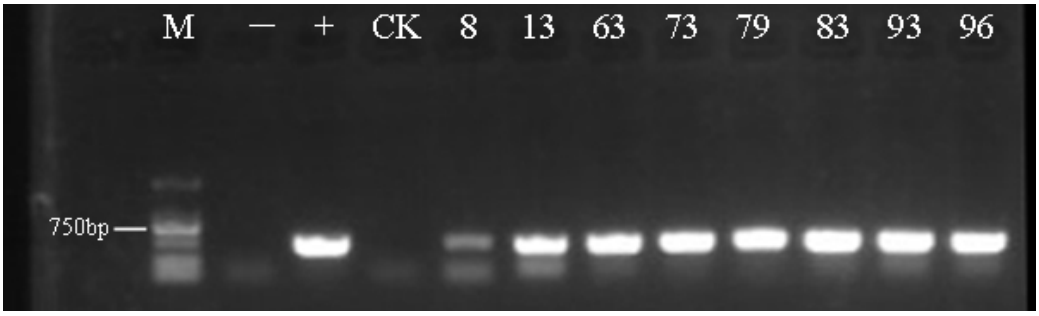

Figure 4. PCR analysis of kanamycin-resistant *Artemisia annua* plants by using the CaMV promoter-specific primer P35S and gene *cyp71av1*-specific primer CYPR2. *Lane M* = DL2000 DNA marker; *lane* (-) = negative control; *lane* (+) = positive control; *lane CK* = control plant; *lanes 8* to *96* = putative transgenic plants.

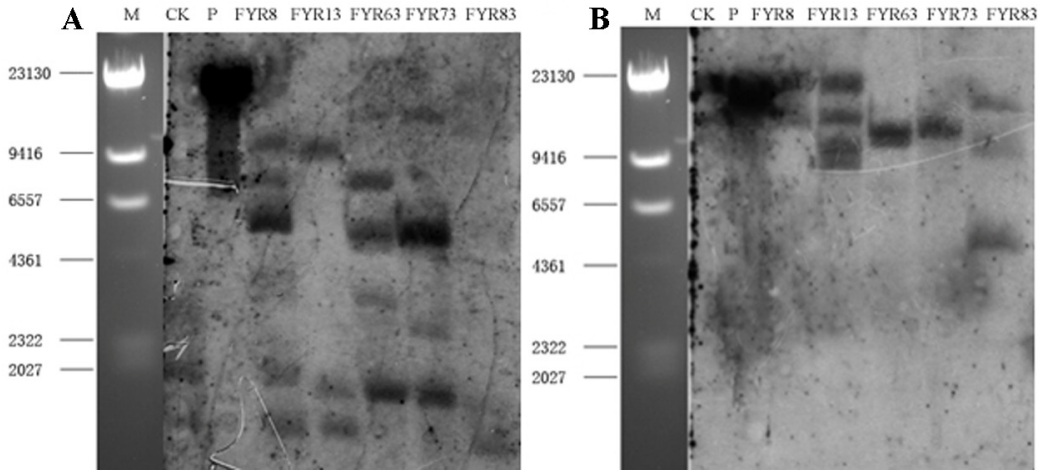

Figure 5. Southern blot hybridization of part PCR-positive transgenic plants. Genomic DNA of transgenic *Artemisia annua* and control plants were digested by *Dra*I in 6 samples while FYR plasmid (positive control) was digested by *Kpn*I. Hybridization was performed by the *cyp71av1* probe (**A**) and then reprobed by the *npt*II probe (**B**). (Code FYR is short for FSN-CYP71AV1-CPR). *Lane M* = DNA marker; *lane CK* = control plant; *lane P* = positive plasmid; *lanes FYR8-FYR83* = transgenic *A. annua* plants.

The expression of *cyp71av1* and *cpr*

To determine if the increase in artemisinin content was due to the overexpression of *cyp71av1* and *cpr*, we analyzed and compared *cyp71av1* and *cpr* transcripts between the transgenic plants of *A. annua* and the controls. Our results showed that there were significantly higher expressions of the *cyp71av1* gene in all transgenic plants compared with the controls. The highest one (FYR63) was 162-fold over the controls (Figure 6A). However, *cpr* overexpression seemed modest, where most of the transgenic plants showed transcripts that were 1.4- to 2.4-fold of control *A. annua* (the highest one was code FYR83; Figure 6B). However, the expression of gene *cpr* in FYR8, FYR13, FYR79, and FYR96 was lower than the controls. Although the results are difficult to reconcile, it should be mentioned that in our expression vector there were too many CaMV 35S promoters. For gene *cyp71av1*, there were two copies of CaMV 35S promoters ahead. It has been reported that multiple copies of CaMV 35S promoters may cause cosuppression (Napoli et al., 1990; McNellis et al., 1994; Ohashi et al., 2002), so that could be the reason why some of the transgenic plants displayed a lower expression of gene *cpr* compared to the controls. Likewise, other researchers have also reported a similar phenomenon, so cosuppression phenomenon occurs in overexpression transgenic plant regularity (Yang et al., 2008; Banyai et al., 2010).

Extraction and analysis of artemisinin content by HPLC-ELSD

Overexpression of the key enzyme genes of the artemisinin biosynthetic pathway has shown to be effective in increasing the artemisinin content in transgenic *A. annua* (Han et al., 2006; Aquil et al., 2009; Banyai et al., 2010; Nafis et al., 2011). We used leaves 10, 15 and 20

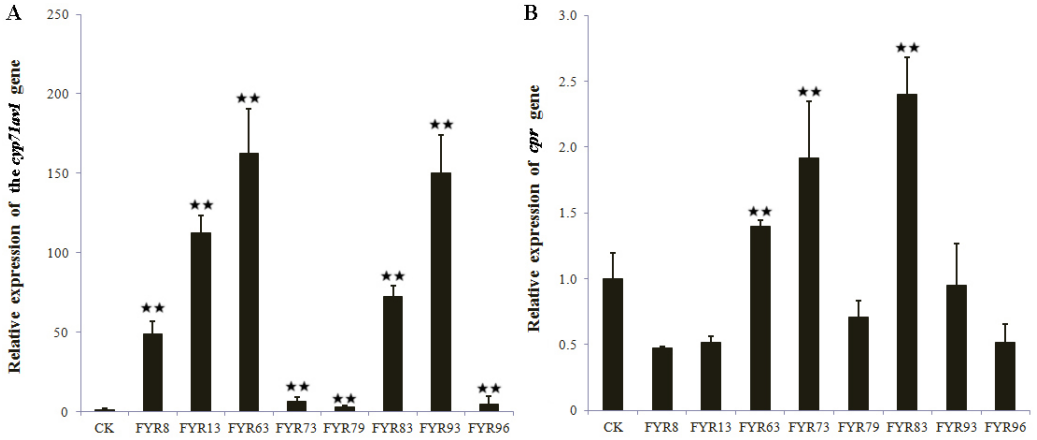

Figure 6. Expression of the *cyp71av1* gene (**A**) and the *cpr* gene (**B**) in control (CK) and transgenic plants of *Artemisia annua* (FYR8-FYR96). Triplets of all samples were run and the Student test was analyzed by the SAS9.1 software (**P < 0.01).

(counting from the apical meristem) from 20-week-old greenhouse-grown *A. annua* for HPLC analysis. In our experiment, a change in artemisinin content was observed in the FYR-derived transgenic *A. annua*. However, about half of the transgenic *A. annua* showed an increase in artemisinin levels compared with the controls. The highest artemisinin content was 980 ± 108 µg/g fresh weight, which was about 38% higher than the controls 708 ± 18 µg/g. There was a significant difference between the FYR63, FYR73, FYR83, and FYR93 transgenic plants and the controls after statistical analysis using the SAS9.1 software ($P < 0.01$, Student *t*-test) (Figure 7). However, the other half of the transgenic plants showed lower artemisinin content compared to the controls, which was consistent with previous studies. Banyai et al. (2010) transferred the *fps* gene into *A. annua* by *A. tumefaciens* strain EHA105 and about 25% of their transgenic lines showed a lower artemisinin content. This result indicated that potentially the T-DNA inserted position in the host genomic DNA and the copy number could have affected the artemisinin content in this research. Based on the results of artemisinin contents and the expression levels of genes *cpy71av1* and *cpr*, we found that co-expression of these two genes could enhance artemisinin biosynthesis in *A. annua*. The results indicated that the artemisinin content was increased and accompanied the increase in the *cyp71av1* and *cpr* mRNA expression levels, revealing that it was possible to increase the artemisinin content through genetic engineering.

In summary, our study proved that co-overexpressing genes *cyp71av1* and *cpr* could increase the artemisinin content in *A. annua* plant. This will facilitate genetic engineering of the artemisinin pathway in *A. annua*, as well as the dissection of gene functions related to various aspects of this medicinal plant species. Overexpressing biosynthetic genes such as *hmgr*, *fps*, *ads*, and *cyp71av1* could increase the artemisinin content in *A. annua*. However, overexpressing a single gene is not sufficient to increase artemisinin accumulation. This phenomenon is similar to that in other metabolic studies of plant secondary metabolites (Zhang et al., 2004). Based on these results, we conclude that genetic engineering is a useful method in plant metabolism research, but that there is more than one bottleneck in the artemisinin biosynthetic pathway.

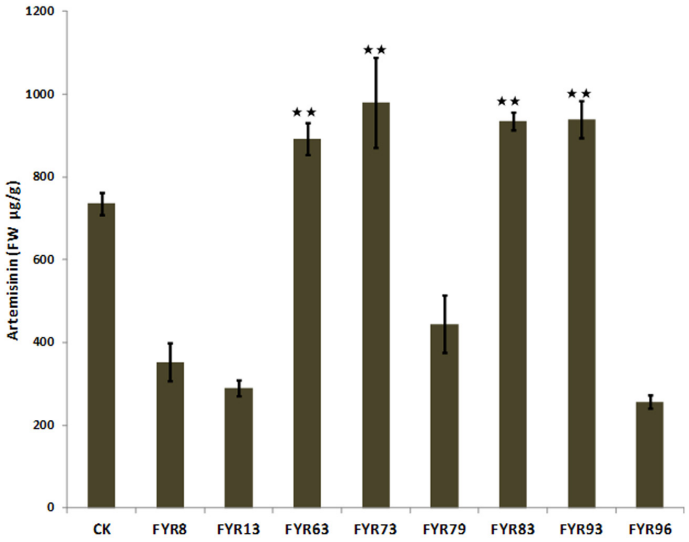

Figure 7. Artemisinin content of transgenic and control (CK) *Artemisia annua* plants (FYR8-FYR96) determined by HPLC-ELSD analysis. The measurement was repeated three times and the Student test was analyzed by the SAS9.1 software (**P < 0.01). FW = fresh weight.

According to the reasonable extent of the artemisinin biosynthetic pathway that is known, more and more results point to dihydroartemisinic acid as the precursor of artemisinin. Therefore, artemisinic aldehyde reductase (DBR2) (Zhang et al., 2008), which catalyzes the conversion of artemisinic aldehyde to dihydroartemisinic aldehyde, and aldehyde dehydrogenase homologue (ALDH1) (Teoh et al., 2009), which converts dihydroartemisinic aldehyde to dihydryoartemisinic acid, seem more important in artemisinin biosynthesis. To increase artemisinin in *A. annua*, we believe that in co-overexpressing the combination of artemisinin biosynthesis genes, *fps+dbr2*, *ads+dbr2*, *fps+aldh1*, and *ads+aldh1*, for example, should be good candidates.

ACKNOWLEDGMENTS

Research supported by the China "863" Program (Grant #2011AA100605), the Shanghai Science and Technology Committee (Grant #08391911800), and the Shanghai Leading Academic Discipline Project (#B209). We thank Pin Liu for assistance with the HPLC analysis.

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