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Full Length Research Paper

Co-overexpression of the *HMGR* and *FPS* genes enhances artemisinin content in *Artemisia annua* L.

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Artemisinin, a sesquiterpene lactone endoperoxide derived from the plant *Artemisia annua* L., is considered to be the most effective antimalarial drug. To increase the production of artemisinin, the *hmgr* and *fps* genes which encode two key enzymes involved in artemisinin synthesis, were cloned from *A. annua,* respectively. The genes were inserted between the cauliflower mosaic virus 35S promoter and nopaline synthase terminator to construct the expression cassettes, and were then cloned into the plant expression vector pCAMBIA2300 to obtain the p2300-gfh vector containing both the *hmgr* and *fps* genes. An *Agrobacterium*-mediated method was used to stably transform *A. annua*, and 38 independent transgenic plants were obtained. Polymerase chain reaction and Southern blot analysis confirmed that the foreign genes were successfully introduced into the *A. annua* genome. The results obtained by high-performance liquid chromatography-evaporative light scattering detection showed that the artemisinin contents of most transgenic plants were higher than those in control plants. The highest artemisinin content in transgenic plants was about 1.8-fold that of the control plants. The results of real-time fluorescence quantitative analysis showed that the expression of *hmgr and fps* was enhanced in the transgenic plants analyzed. This study demonstrates that overexpression of the *hmgr* and *fps* genes are an effective approach to increase artemisinin content in *A. annua* L.

Key words: *Artemisia annua* L. (Asteraceae), artemisinin, genetic engineering, hmgr and fps genes.

INTRODUCTION

Artemisinin, a sesquiterpene lactone isolated from 10-celled biseriate glandular trichomes of the aerial parts of *Artemisia annua* (*A. annua* L.) plants, is universally acknowledged as the most effective drug against cerebral malarial. Compared with traditional drugs, such as chloroquine, mefloquine, and sulfadoxine-pyrimethamine, artemisinin has better efficiency against both chloroquinine-resistant and chloroquinine-sensitive strains of *Plasmodium falciparum* (Klayman, 1985). Artemisinin-based combination therapies (ACTs) have been recommended by the world health organization (WHO) as the preferred treatment method of drug-resistant malaria (WHO, 2006). Additionally, further research reveals that artemisinin and its derivatives have extra functions, such as antiinflammatory, antischistosomiasis, antitumor, and in immune regulation, making it a highly potential natural drug with a broad spectrum (Singh and Lai, 2001; 2006; Posner et al., 2004; Wang et al., 2007). With the increasing number of people suffering from malaria, the relatively low yield, 0.1 to 1.0% dry weight (DW), of artemisinin in *A. annua* plants greatly limits the commercialization of this drug, resulting to a shortage of supply in the international market.

Many researchers have been devoted into enhancing the production of artemisinin through various biological techniques, including organ culture, hormone medium, metabolic manipulation, and genetic engineering (He et

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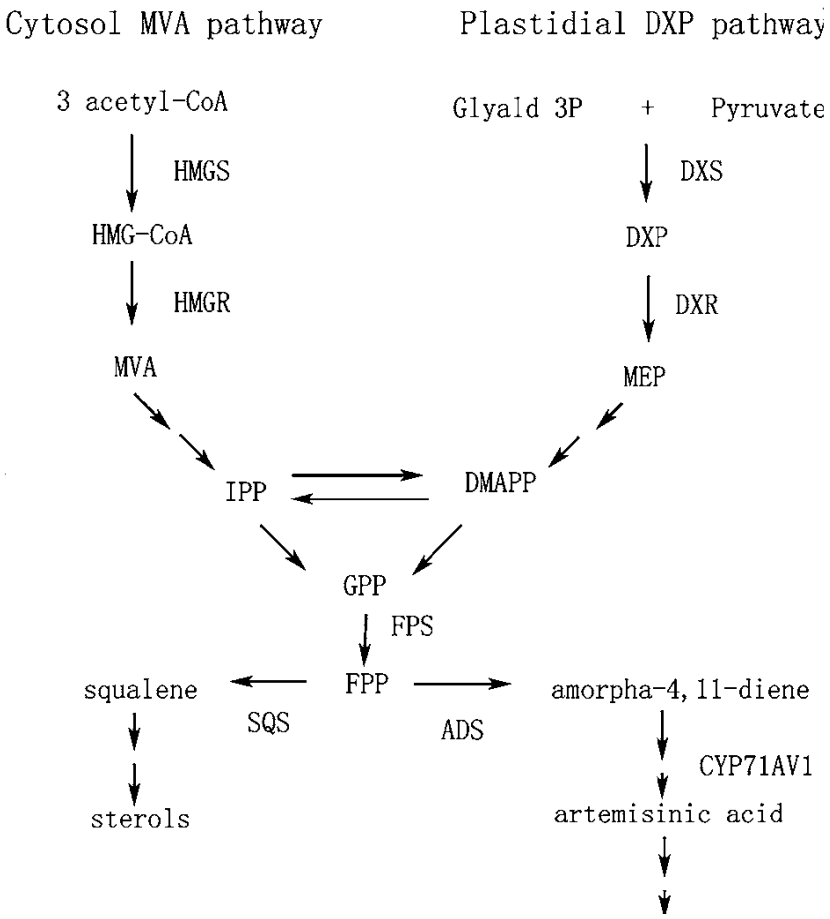

Figure 1. The presumed pathway of the artemisinin biosynthesis network (constructed from data taken from Liu et al., 2006; Bertea et al., 2005).

al.1983; Martinez and Staba, 1988; Simon et al., 1990; Woerdenbag et al., 1993; Fulzele et al., 1995; Kumar et al., 1997; Smith et al., 1997). In recent years, much progress have been made on the mechanism of artemisinin biosynthesis (Figure 1) (Liu et al., 2006; Bertea et al., 2005), especially after genes encoding key enzymes relevant to the biosynthesis of artemisinin were cloned. Precursors of artemisinin biosynthesis, namely isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP), have been shown to be derived from the mevalonate (MVA) pathway and 2-*C*-methyl-D-erthritol 4-phosphate (MEP) pathway (Liu et al., 2005), respectively. Geranyldiphosphate (GPP) is the product of the reaction between IPP with DMAPP, and it can further react with IPP to produce farnesyldiphosphate (FPP).

The reaction from 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) to 3-mevalonic acid (MVA), catalyzed by 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), is considered as the first rate-limiting step in MVA pathway (Zhang et al., 2010). The downstream branches of the MVA pathway lead to various isoprenoid derivatives, such as sitosterol, campesterol, stigmasterol, carotenoids, etc. Researches on many plants revealed that HMGR belongs to a multigene family, and its different isoforms determine the type of end-product sterols (Enjuto et al., 1993; Chappell et al., 1995; Chappell, 1995; Weissenborn et al., 1995). Lumbreras et al. reported that *Arabidopsis thaliana* contains 2 differentially expressed genes (*hmgr1* and *2*) encoding 3 isomers (HMGR1S, HMGR1L, HMGR2), whose expression levels vary in different plant tissues (Lumbreras et al., 1995). cDNA of HMGR has been also cloned from *A. annua*. Previous studies on subcellular localization of *Arabidopsis* HMGR carried out by Leivar et al. found that this enzyme is localized not only within the endoplasmic reticulum (ER) as expected, but also within vesicle-like structures of 0.2 to 0.6 µm diameter in the cytoplasm, which were believed to be derived from segments of HMGR-ER (Leivar et al., 2005). Taken together, these findings reveal that regulation of the *hmgr* gene plays a key role in the regulation of the MVA pathway in plants.

Farnesyl-diphosphate synthase (FPS) can catalyze the condensation of IPP and DMAPP into GPP, and the condensation of GPP and IPP into FPP, providing

substrates for sesquiterpene synthase to eventually form various sesquiterpenes, including artemisinin. In different plants, FPS is also encoded by a mutigene family. Cunillera et al. found that *A. thaliana* contains 2 differentially expressed gene encoding FPS (*fps1* and *2*), whose levels of mRNA accumulated vary from each other in different organs and during different developmental periods. *fps1* mRNA is widely expressed in all tissues, especially in roots and inflorescences, whereas *fps2* mRNA is specifically expressed in inflorescences during the early stage of bud development. It is inferred that *fps1* directs the synthesis of terpenes having essential functions in plant cells, while *fps2* is involved in the biosynthesis of terpenes with special functions (Cunillera et al., 1996).

Because of the complex metabolic regulation systems of *A. annua*, previous researches that focused on the overexpression of a single gene did not reach a significant efficacy (Chen et al., 1998). Thus, simultaneous overexpression of multiple genes is a promising approach. In this study, overexpression of the genes encoding HMGR and FPS, 2 key enzymes involved in artemisinin biosynthesis, were attempted to enhance the production of artemisinin from *A. annua* L.

MATERIALS AND METHODS

Gene clones of *hmgr* and *fps* from *A. annua*

Total RNA was isolated and purified from young leaves of *A. annua* plants, using Plant Total RNA extraction Kit (Tiangen, Beijing), and then reverse transcribed into cDNA by Reverse Transcriptase XL (AMV) (TaKaRa, Japan). Gene-specific primers were designed in accordance with the sequences of the *hmgr* gene (GenBank accession no. AF142473) and the *fps* gene (GenBank accession no. AF112881): HMGRF1 and HMGRR1, FPSF1, and FPSR1. The 50 µL PCR reaction system included the following mixture: 1 µL forward primer (10 µM), 1 µL reverse primer (10 µM), 3 µL dNTPs (2.5 mM each), 5 µL 10× exTaq PCR buffer, 2 µL DNA Template (100 ng/µL), 0.3 µL exTaq DNA Polymerase (Takara), and ddH_2O as the final solution added. PCR reaction was performed as follows: 94°C for 3 min, followed by 36 cycles of amplification (94°C degeneration for 30 s; 56°C annealing for 30 s, 72°C extension for 3 min), and finally 72°C for 8 min.

PCR products were gel-purified and cloned into vector pMD18-T (Takara, Japan) for sequencing (Sunny, Shanghai).

Construction of the plant expression vector pCAMBIA2300-*gfh* and transformation of *Agrobacterium tumefaciens*

The gene expression cassette of p35S-gfp-gus-nos was cloned from vector pCAMBIA1304 by using PCR, with the specific primers p35SFA and nosRA. The PCR reaction was performed as follows: 94°C for 3 min, followed by 36 cycles of amplification (94°C for 30 s; 54°C for 30 s, 72°C for 3 min), and 72°C for 8 min. After gel purification, the PCR product was cloned into vector pMD18-T for sequencing, after which the expression cassette of *gfp-gus* was digested with *Swa*I plus *Pst*I and inserted to pCAMBIA2300 vector to form the intermediate vector pCAMBIA2300::p35S::*gfp-gus*::nos. Simultaneously, the expression cassettes of *gfp-gus* from pMD18::p35S::*gfp-gus*::nos were respectively replaced by the *hmgr* or *fps* gene with *Spe*I plus *Bst*EII, to form vectors pMD18::p35S::*hmgr:*:nos and pMD18::p35S::*fps*::nos. Finally, the expression cassette of *fps* digested from pMD18::p35S::*fps*::nos with *Swa*I plus *Pst*I was cloned into vector pCAMBIA2300::*gfp-gus* predigested with *Sma*I plus *Pst*I, to construct vector pCAMBIA2300::*gfp-gus*::*fps*.

The final plant expression vector, pCAMBIA2300::p35S::*gfp-gus*::nos::p35S::*fps*::nos::p35S::*hmgr*::nos, was obtained by cloning the *hmgr* cassette into the newly constructed vector by using the same method as that used for the *fps* cassette (Figure 2).

Transformation of *A. annua*

The expression vector pCAMBIA2300-*gfh* containing both the *hmgr* and *fps* genes was introduced into *A. tumefaciens* EHA105 through the freeze-thaw method. Transformation of *A. annua* plants was performed according to the methods of Vergauwe et al. (Vergauwe et al., 1996), with some modifications: after immersing in 75% ethanol for 1 min, seeds of *A. annua* were sterilized by 20% NaOCl (v/v) for 20 min, rinsed with sterile water 3 to 4 times, germinated on hormone-free MS_0 (Murashige and Skoog) (Murashige et al., 1962) basal media, and finally cultivated under a photoperiod of 16 h light/8 h dark with light at 8,000 lux (metal halide source) and at a temperature of 25°C. When the aseptic seedlings reached 5 cm in length, leaves were cut, collected, and finally placed in cultivation medium MS_1 (half-strength MS with the addition of 100 µM acetosyringone) for *A. tumefaciens*–mediated leaf disc transformation.

After germination at 28°C and 24 h/day dark for 3 days, the *A. annual* explants were transferred into shooting medium MS_2 (MS_0 + 2.5 mg/L N6-benzoyladenine + 0.25 mg/L naphthalene-1-acetic acid + 50 mg/L kanamycin + 250 mg/L carbenicillin) and cultivated with a photoperiod of 16 h light/8 h dark at 25°C. After being regenerated every 2 weeks for 2 to 3 times, the kanamycin-resistant regenerated plantlets were then transferred into rooting medium MS_3 (half-strength MS_0 + 250 mg/L carbenicillin) to obtain kanamycin-resistant plants.

DNA isolation and PCR detection

The *fps* and *hmgr* transgenic plants were identified from regenerated *A. annua* plants by PCR analysis, with genomic DNA as templates, and with P35S and HMGRR2 as primers. The 25 µL PCR reaction system included the following mixture:

0.5 µL forward primer (10 µM), 0.5 µL reverse primer (10 µM), 1.5 µL dNTPs (2.5 mM each), 2.5 µL 10× PCR buffer (Mg^{2+} free), 1.5 µL $MgCl_2$ (25 mM), 1.5 µL DNA template (100 ng/µL), and 0.3 µL rTaq DNA Polymerase (Takara).

The PCR reaction was performed as follows: 94°C for 3 min, followed by 30 cycles of amplification (94°C for 30 s; 54°C for 30 s; 72°C for 1 min) and 72°C for 8 min. The 988-bp products were then gel detected.

Southern blot analysis

The genomic DNA (50 µg) of transgenic plants was digested with *Pst*I overnight and then transferred onto a positively charged Hybond-N+ nylon membrane (Amersham Biosciences, UK). The 535-bp probe designed according to the sequences encoding *hmgr* was amplified by PCR and was labeled using Amersham AlkPhos Direct Labeling Reagents Kit. Hybridization, blocking, washing membrane, and detection were all according to Amersham CDP-Star™ Detection Kit.

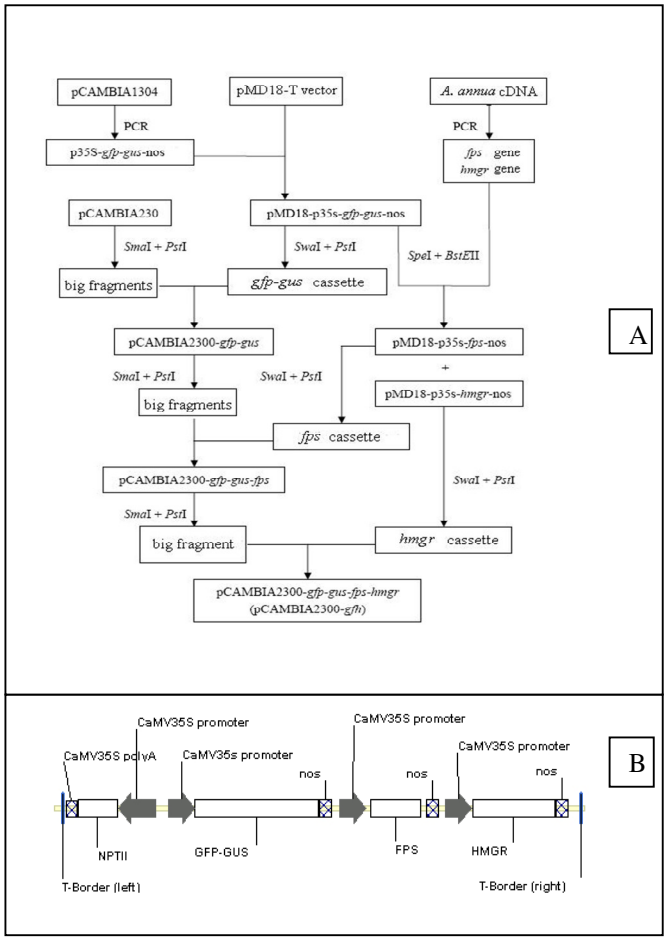

Figure 2. A) Scheme of the construction of pCAMBIA2300-*gfh*. B) The structure of plant expression vector pCAMBIA2300::p35S::*gfp-gus*::nos::p35S::*fps*::nos::p35S::*hmgr*::nos

Real-time fluorescence quantitative analysis

Total RNA was extracted from the leaves of *A. annua* using Plant Total RNA extraction Kit (Tiangen, Beijing) and converted to cDNA using SYBR PrimerScript™ RT-PCR Kit (Perfect Real Time) (Takara, Japan). Gene-specific primers were designed for the reaction:

HMGRF2 and HMGRR2 to detect the expression level of *hmgr*, FPSF2 and FPSR2 to detect the expression level of *fps*, and UBCF and UBCR to detect the expression level of *ubc* (ubiquitin-conjugating gene). SYBR Premix Ex Taq™ (Takara, Japan) was used to quantify the amount of dsDNA. Real-time RT-PCR analysis was performed in Mastercycler Ep Realplex™ (Eppendorf, Germany). The relative Ct (threshold cycle value) method was used to estimate the initial amount of template present in the reactions using ABIPRISM7700 Sequence Detection System and PerkinElmer/Applied Biosystems.

Quantification of artemisinin using HPLC

Leaves of transgenic *A. annua* plants were baked at 50°C and grinded into powder before extraction with ethanol (1.5 mL/sample). Then, samples were treated using an ultrasonic processor (twice, 15 min each), and centrifuged for 10 min at 5,000 rpm to remove suspended particles. The final supernatant was filtered in a 0.22-μm-pore filter. Samples (0.1 g each) were repeatedly analyzed 3 times using a Waters Alliance 2695 HPLC system and a Waters 2420 ELSD detector. ELSD condition was optimized at nebulizer-gas pressure of 5 bars and drift-tube temperature of 40°C, and the gain coefficient was set at 7. Standard artemisinin purchased from Sigma were used as control.

RESULTS

Using polymerase chain reaction (PCR), 1,201-bp fragments of the *fps* gene and 1,701-bp fragments of the *hmgr* gene were amplified from *A. annua*. Sequence identification confirmed that the fragments obtained are consistent with the sequences published in GenBank (*hmgr* gene: GenBank accession no. AF142473; *fps* gene: GenBank accession no. AF112881). For simultaneous overexpression in *A. annual* plants, the *hmgr* and *fps* genes were constructed into vector pCAMBIA2300 for transformation. Based on the intermediate vector pMD18-p35S-gfp-gus-nos, we inserted *hmgr* and *fps* expression cassettes into vector pCAMBIA2300 and finally obtained 1 binary expression vector, pCAMBIA 2300-*gfh*, which contained both the *hmgr* and *fps* genes. After kanamycin selection, 209 kanamycin-resistant plants were regenerated, which were then analyzed by PCR. Fragments of 988 bp from target DNA were successfully amplified from 38 independent plants, which were considered to be transgenic lines containing both the *hmgr* and *fps* genes. By contrast, these fragments did not occur in control plants (Figure 3A).

The results of the introduction of foreign target DNA fragments into *A. annua* genomes were investigated using Southern blot analysis (Figure 3B). Plants numbered 123, 138, and 177 did not show obvious hybridization bands, while other plants (numbered 13, 81, and 145) produced expected hybridization bands different from those of the control plants (CK). These results indicate that the T-DNA containing the *hmgr* and *fps* genes was introduced into the genomes of *A. annua* plants. The results of high-performance liquid chromatography (HPLC)-evaporative light scattering detection showed that the contents of artemisinin in transgenic plants were generally about 2 to 9 mg/g (DW) (Figure 4), significantly enhanced in partial plants compared with the controls, with the highest artemisinin content in transgenic plants (GFH138) being about 1.8-fold of those in control plants. All results indicated that simultaneous overexpression of the *hmgr* and *fps* genes enhanced the artemisinin content of *A. annual* plants.

Real-time RT-PCR was carried out to analyze the expression of the *hmgr* and *fps* genes in transgenic plants at the transcriptional level. The results showed that the expression of *hmgr* and *fps* was enhanced in the transgenic plants analyzed (Figure 5). Two independent

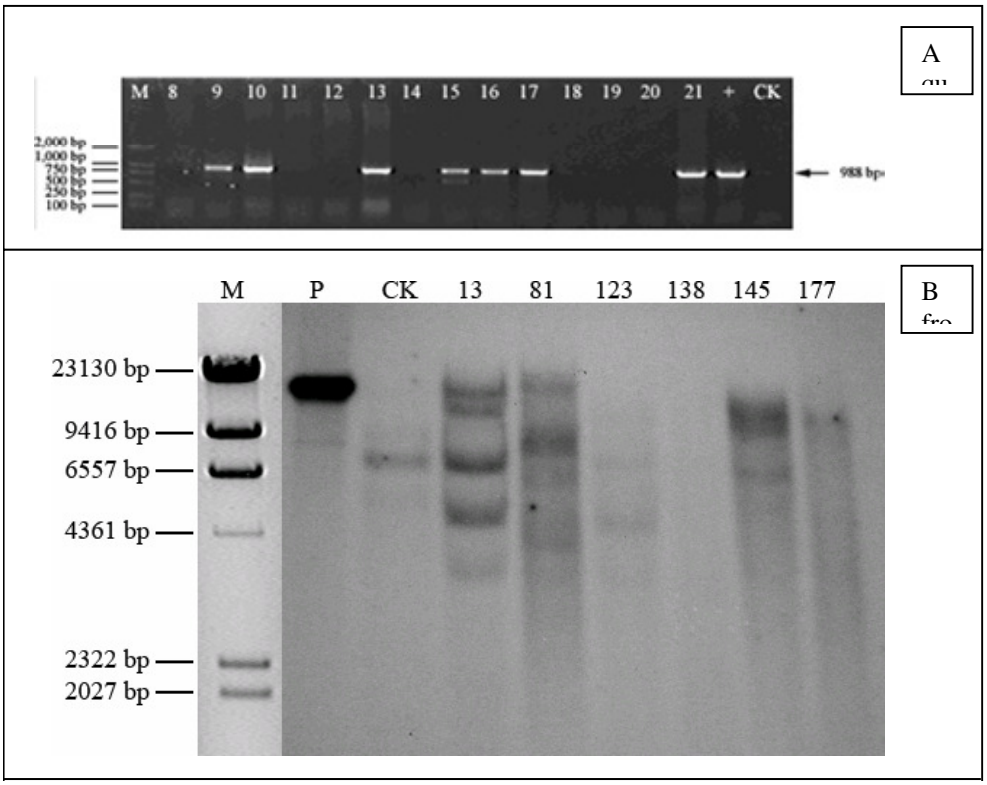

Figure 3. A) PCR detection results of regenerated *A. annua* plants. M: DNA Marker DL2000; 8 to 21: independent kanamycin-resistant *A. annua* plants; +: plasmid of pCAMBIA2300-*gfh*; CK: control (nontransgenic *A. annua* plant). B) Southern blot analysis of transgenic *A. annua* plants cotransformed with *fps* and *hmgr* (using a partial *hmgr* coding sequence as probe). M: λ/*Hin*dIII DNA molecular marker; P: plasmid of pCAMBIA2300-*gfh*; CK: control (nontransgenic *A. annua* plant); 13, 81, 123, 138, 145, and 177: independent transgenic *A. annua* plants.

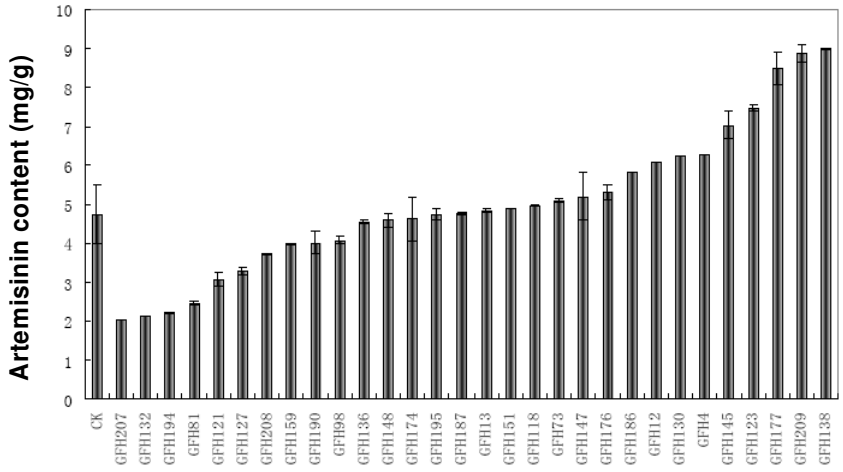

Figure 4. Artemisinin content of transgenic *A. annua* plants cotransformed with *fps* and *hmgr.*

transgenic lines were chosen:

Transgenic line 138 had the highest artemisinin content, and artemisinin content in line 13 was higher than that in the non-transgenic line (control group) but was lower than that in line 138. In transgenic *A. annua* L. line 138, the

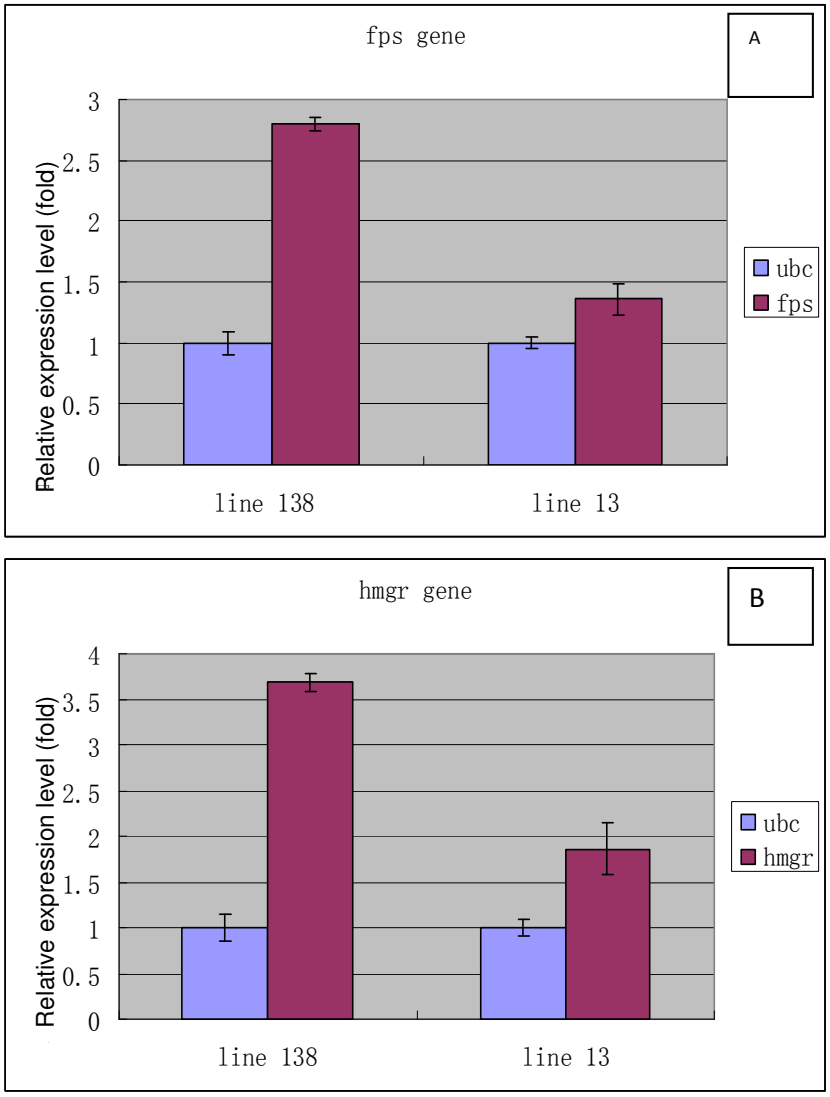

Figure 5. Real-time fluorescence quantitative PCR analysis of the expression of *hmgr* and *fps* in transgenic *A. annua* L. CK: control (nontransgenic *A. annua* plant); 138 and 13: independent transgenic *A. annua* plants

expression levels of the *hmgr* and *fps* genes were 2.80-fold and 3.68-fold when compared with nontransgenic *A. annua* L. In line 13, the expression levels of the *hmgr* and *fps* genes were 1.36-fold and 1.86-fold when compared with nontransgenic *A. annua* L.

DISCUSSION

With the construction of a transformation system in *A. annua* and a more in-depth study of the biosynthesis of artemisinin, enhancing the artemisinin content in *A. annua* through transgenic technology is showing large potential. Our study demonstrates that overexpression of the *hmgr* and *fps* genes, which encode key enzymes in the biosynthesis of artemisinin, can significantly increase the production of artemisinin in *A. annua*. The results of HPLC detection showed that transgenic plants can have as much as 9 mg/g (DW) artemisinin content, which is about 1.8-fold of the content in control plants. The expression of *hmgr* and *fps* at the transcription level in line 138 was enhanced more significantly than that in line 13, and the amount of artemisinin in line 138 was much more than that in line 13. Thus, the artemisinin content in line 138 was considerably more improved than it was in line 13. Although, genetic engineering can significantly produce higher contents of artemisinin in transgenic *A. annua* plants, our study revealed that several additional challenges still exist for further yield improvements.

Artemisinin contents varied greatly in different

Table 1. Primers used in this study.

HMGRF1 5′	GACTAGTCAACCCACGATGGATCTC	3′ (with SpeI site)
HMGRR1 5′	GGTAACCTTTCACACCTTTGACGCA	3′ (with BstII site)
FPSF1 5′	ACTAGTCCCAACACACACTCACAAACT	3′ (with SpeI site)
FPSR1 5′	GGTTACCAACATACAGACAACATCGCCT	3′ (with BstII site)
p35SFA 5′	ATTTAAATCATGGAGTCAAAGATTCAAA	3′ (with SwaI site)
nosRA 5′	GCTGCAG CCCCGGGGCCCGATCTAGTAACATAGAT	3′ (with SmaI and PstI sites)
P35S 5′	TTCGTCAACATGGTGGAGCA	3′
HMGRR2 5′	ACCTAAAGTCGCCTCTAACG	3′
HMGRF2 5′	TTGTGTGCGAGGCAGTAAT	3′
HMGRR2 5′	CCTGACCAGTGGCTATAAAGA	3′
FPSF2 5′	TCATTGTCTATTCACCGCCG	3′
FPSR2 5′	CACCGCTTGGACTGCTTTGCT	3′
UBCF 5′	CACACTTGAGGTTGAGTCCAG	3′
UBCR 5′	CATAACATTTGCGGCAGATAG	3′

transgenic *A. annua* lines, ranging from 2 to 9 mg/g DW. Compared with previous investigations in which a single enzyme (HMGR or FPS) was overexpressed, simultaneous overexpression of the *hmgr* and *fps* genes did not result to a significant improvement in artemisinin content as expected; some results are even lower than those of previous studies. The probable reasons for these phenomena are further discussed. HMGR is a key enzyme involved in MVA biosynthesis pathways (Liao et al., 2009), so its overexpression may cause increased mevalonate acid, which was then transformed into other end products instead of artemisinin (Table 1). FPP can enter two different pathways, to form sesquiterpene by either sesquiterpene synthase or to eventually form sterols and saponins by squalene synthase. Theoretically, as a result, utilizing inhibitors of squalene synthase, such as miconazole, inhibit the pathways competing for artemisinin biosynthesis. On the other hand, FPP is a transcriptional repressor for many mevalonate pathway genes, whose accumulation by overexpression of the *fps* gene may inhibit the upstream processes of artemisinin biosynthesis, eventually resulting to a bottleneck along the metabolism pathway.

To detect which points of these pathways form bottlenecks, a gas chromatography/mass spectrometry analysis is needed. Additionally, the promoter used in this study to catalyze the expression of target genes is the cauliflower mosaic virus promoter p35S, which may have an unknown influence on the normal metabolism of certain plant tissues. However, artemisinin biosynthesis specifically occurs in glandular hair cells (Duke and Paul, 1993), the specific promoter of which may be a better choice for later studies in artemisinin secondary metabolic engineering. Furthermore, the copy number of inserted exogenous DNA fragments may not be consistent with that of the integrated sites, which can lead to a discrepancy in artemisinin contents of different *A. annua* plants. To conduct further analysis of the expression status, detection of the expression levels of the enzymes HMGR and FPS by western blot or/and ELISA is required. Artemisinin contents vary greatly among different *A. annua* plants in nature. This variation is related to both their inherent differences and environmental factors such as illumination (Wang et al., 2001; Liu et al., 2002), temperature (Guo et al., 2004), salt stress (Qian et al., 2007), plant hormones (Jung et al., 1990; Whipkey et al., 1992; Weathers et al., 2005), and others.

It has been reported that the amount of artemisinic acid in *A. annua* plants is 10 times that of artemisinin (Jung et al., 1990), which suggests that further studies of the metabolic pathway and regulation system of artemisinin have great potential in enhancing the artemisinin content, especially after other downstream key enzymes, such as *cyp71av1* and *dbr2*, are cloned. It can be predicted that the transgenic technology used for enhancing artemisinin content will be more improved and efficient in the future.

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