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

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Artemisinin is an effective anti-malarial drug isolated from *Artemisia annua* L. (Chinese wormwood), but the content of artemisinin in *A. annua* is low. In the present study we explored the possibility of using genetic engineering to increase the artemisinin content of *A. annua* by suppressing the expression of SQS (squalene synthase), a key enzyme of sterol pathway (a pathway competitive with that of artemisinin biosynthesis) by means of a hairpin-RNA-mediated RNAi (RNA interference) technique. A total of 23 independent transgenic *A. annua* plants were obtained through *Agrobacterium tumefaciens*-mediated transformation, which was confirmed by PCR and Southern-blot analyses. HPLC-evaporative light-scattering detection analysis showed that the artemisinin content of some transgenic plants was significantly increased, with the highest values reaching 31.4 mg/g dry weight, which is about 3.14-fold the content observed in untransformed control plants. Real-time reverse transcription–PCR analysis demonstrated that the expression of SQS was suppressed significantly, and GC–MS analysis showed that sterol was efficiently decreased in the transgenic plants. The present study demonstrated that genetic-engineering strategy of RNAi is an effective means of increasing artemisinin content in plants.

Introduction

Artemisinin, a sesquiterpene isolated from *Artemisia annua* L. (Chinese wormwood), is an increasingly popular drug against malaria, a disease which causes at least 500 million cases globally every year and results in more than one million deaths [1,2]. Artemisinin-based combination therapy is recommended by the World Health Organization as the most effective way to combat drug-resistant malaria [3–5]. Moreover, there is increasing evidence that artemisinin and its derivatives have many other properties, including antitumour activity [6,7], making it a potential to be used as a multifunctional natural drug.

As the demand for artemisinin is increasing and artemisinin content of *A. annua* is very low [usually 0.1–10 mg/g DW (dry weight)], great efforts have been made to try to increase artemisinin production. As the total synthesis of artemisinin is difficult and costly [8,9], many other approaches have been attempted to improve artemisinin production, such as environmental-factor manipulation for *A. annua* plants [4] and genetic engineering [2,10– 12].

The mechanism of artemisinin biosynthesis has recently become much clearer ([1,13]; Scheme 1). It has been shown that artemisinin belongs to the isoprenoid group of compounds, which are derived from two common precursors, namely IPP (isopentenyl diphosphate) and its isomer DMAPP (dimethylallyl diphosphate). GPP (geranyl diphosphate) is formed by chain elongation from IPP and

Key words: anti-malarial drug, *Artemisia annua* L. (Chinese wormwood), artemisinin, genetic engineering, RNA interference (RNAi), squalene synthase hairpin RNA (SQS hpRNA).

Abbreviations used: ADS, amorpha-4,11-diene synthase; CTAB, cetyltrimethylammonium bromide; DMAPP, dimethylallyl diphosphate; DW, dry weight; DXP, 1-deoxy-D-xylulose 5-phosphate; DXR, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; DXS, 1-deoxy-D-xylulose 5-phosphate synthase; ELSD, evaporative light-scattering detection; FPP, farnesyl diphosphate; FPS, farnesyl diphosphate synthase; GPP, geranyl diphosphate; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; HMGR, HMG-CoA reductase; HMGS, HMG-CoA synthase; hp, hairpin; hpRNA, hairpin RNA; IPP, isopentenyl diphosphate; MEP, 2-C-methyl-D-erythritol 4-phosphate; MVA, mevalonic acid; *nptII*, neomycin phosphotransferase gene conferring resistance to kanamycin; ocs terminator, octopine synthase terminator; p2300, plasmid pCAMBIA2300; PDK, pyruvate orthophosphate dikinase; RNAi, RNA interference; RT–PCR, reverse transcriptase–PCR; SQS, squalene synthase; UBC, ubiquitin-conjugating enzyme.

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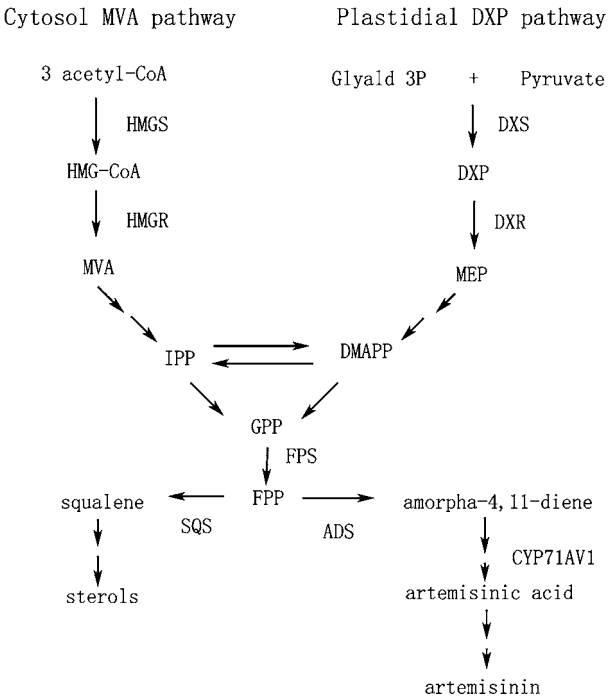

Scheme 1 Presumed pathway of artemisinin biosynthesis network (constructed from data taken from Liu [1] and Bertea [13])

DMAPP when they react with a carbonium ion, and GPP can then further react with IPP to produce FPP (farnesyl diphosphate). FPP can be converted by enzymic catalysis to produce various isoprenoid final products such as artemisinin and sterol.

ADS (amorpha-4,11-diene synthase) can catalyse the first step from FPP to artemisinin, and its product, amorpha-4,11-diene, can be oxidized by CYP71AV1 (a member of cytochrome P450 subfamily) and finally produce artemisinin. The previous strategies of genetic engineering to improve artemisinin production mainly focus on the overexpression of genes involved in the artemisinin biosynthesis, such as those coding for FPS (farnesyl diphosphate synthase) [11], ADS [12] and CYP71AV1 [2].

On the other hand, SQS (squalene synthase) is the key enzyme catalysing the first step of the sterol biosynthetic pathway, a pathway in competition with that artemisinin biosynthesis (Scheme 1). It has already been cloned from *A. annua* [14]. Previous studies showed that the inhibition of the sterol biosynthetic pathway by chemical methods could improve the artemisinin content of *A. annua* [15,16]. Woerdenbag et al. [15] proved that artemisinin production was enhanced by the addition of naphtiphine, an inhibitor of the enzyme squalene epoxidase, to the medium. Kudakasseril et al. [16] demonstrated that the application of many sterol inhibitors, including miconazole or chlorocholine, resulted in an increase in artemisinin in shoot cultures of *A. annua*. Fairly recently, Ro et al. [2] proved that down-regulation of *ERG9* (ergosterol-biosynthesis-pathway gene 9), a gene that encodes SQS in yeast, using a methionine-repressible promoter (PMET3), increased amorpha-4,11-diene production twofold in a yeast strain into which FPP-synthetic-pathway genes and the ADS (amorpha-4,11-diene synthase) gene from *A. annua* had been incorporated. These studies imply that suppression of the sterol-biosynthetic pathway by genetic engineering may be an effective way to improve artemisinin content.

RNAi (RNA interference)-mediated by hpRNA (hairpin RNA) has been used in gene silencing in many species of plants. Liu et al. [17] reported that hpRNA mediated down-regulation of ghSAD-1 and ghFAD2-1, two key enzymes in the fatty-acid-biosynthesis pathway in cotton (*Gossypium hirsutum*), elevated the stearic acid content (44% compared with a normal level of 2%) and oleic acid content (77% compared with a normal level of 15%) in cotton seeds. It was also reported that suppression of one key enzyme, CaMXMT1, involved in the caffeine-biosynthetic pathway through hpRNA-mediated interference in coffee (*Coffea* spp.), decreased obromine and caffeine accumulation efficiently (30–50% of that normally found in the species) [18]. In a study by Allen et al. [19], a hpRNA construct containing sequences from multiple cDNAs of genes in the codeine reductase gene family in the opium poppy (*Papaver somniferum*) was used to silence these enzymes in the pathway. In the transgenic plants, the non-narcotic alkaloid (*S*)-reticuline, which occurs upstream of codeine in the pathway, accumulated at the expense of morphine, codeine, opium and thebaine. There was another example in tomato [*Solanum lycopersicum* (formerly *Lycopersicon esculentum*] [20], the hp (hairpin) construct was used to suppress an endogenous photomorphogenesis regulatory gene, DET1, driven by a fruit-specific promoter. DET1 was degraded and the carotenoid and flavonoid content were increased while all other traits for fruit quality remained unchanged in transgenic plants compared with that in wild-type tomato. Furthermore, suppression of an arsenic reductase gene *ACR2* in *Arabidopsis* (thale cress) using hp constructs could improve arsenic content significantly in transgenic shoots (10–16-fold compared with that in the wild-type) [21].

In the present study we attempted, for the first time, to use hpRNA-mediated RNAi technology by suppressing the expression of SQS (which catalyses the first step of the sterol-biosynthetic pathway, a pathway in competition with that of artemisinin biosynthesis), to increase the artemisinin content in *A. annua*. Our study has demonstrated that RNAi is an effective means of increasing the artemisinin content of plants.

Materials and methods

Construction of hpRNA vector targeting the *SQS* gene

The 327 bp fragment of *SQS* (GenBank® accession no. AF405310), corresponding to *SQS* cDNA from nts 1080–1406, was cloned from *A. annua* by RT–PCR (reverse transcriptase–PCR) using RNA extracted from leaves of germinated *A. annua* seedlings with Plant (Leaves) Total RNA Isolation Kit (Watson Biotechnologies, Inc, Shanghai, China). In RT–PCR, SQSF1 (5′-CC**CTCGAG**CG**GGATCC**CGCCCAAACGACCATCACTAG-3′, with *Xho*I and *Bam*HI sites shown bold and underlined) and SQSR1 (5′-CC**ATCGAT**GG**GGTACC**CCTACCACCAACCATACCAACC-3′, with KpnI and ClaI sites shown bold and underlined) were used as the forward and reverse primers respectively, and the RT–PCR was carried out under the following condition: 94 °C for 5 min, followed by five cycles of first-round amplification (94 °C for 20 s, 58 °C for 20 s, 72 °C for 20 s), 5 cycles of second-round amplification (94 °C for 20 s, 56 °C for 20 s, 72 °C for 20 s), 20 cycles of third-round amplification (94 °C for 20 s, 54 °C for 20 s, 72 °C for 20 s) and by 72 °C for 8 min. The amplified fragment was cloned into pMD18 vector and sequenced.

After confirmation by sequencing, the fragment was forwardly and reversely placed on the two end sides of the PDK (pyruvate orthophosphate dikinase) intron of pHANNBAL to construct the hp structure, which was driven by the 35S promoter and terminated by the ocs (octopine synthase) terminator. Then the expression cassette was excised with SacI and PstI from pHANNBAL containing the *SQS* hp structure and ligated into the expression vector p2300 (pCAMBIA2300) to get the final *hpSQSi*-containing vector pCAMBIA2300::p35s-hairpinSQS-ocs. p2300 containing only *nptII* (neomycin phosphotransferase gene conferring resistance to kanamycin) was used as the control vector in transformation. The pCAMBIA2300::p35s-hairpinSQS-ocs and p2300 vectors were then transferred into *Agrobacterium tumefaciens* strain EHA105 by a conventional freezing-and-melting method, and the resulting strains were used in the transformation of *A. annua*.

Transformation of *A. annua*

Seeds of *A. annua* were collected from Chongqing, a municipality within the Sichuan Province of China. The seeds were surface-sterilized by immersing them in 75 % (v/v) ethanol for 1 min, followed by treatment with 20 % (v/v) NaOCl (sodium hypochlorite) for 20 min. They were then washed three to four times with sterile distilled water and finally sown in germination medium MS_0 [Murashige and Skoog (MS) [22] basal medium with the addition of sucrose (30 g/l) and Phytagel® (Sigma) (2.6 g/litre)] in 9-cm-diameter Petri dishes in a growth chamber with a photoperiod of 16 h light/8 h dark and light at 8000 lux (metal halide source) at 25 °C. When they had reached 5 cm in length, the germinated seedlings were collected and the leaves were cut into 0.5-cm-diameter discs and used as the explants in *Ag. tumefaciens*-mediated leaf disc transformation [23]. After kanamycin selection in the selective shooting medium MS_1 (MS_0+2.5 mg/l N^6-benzoyladenine+0.25 mg/l naphthalene-1-acetic acid+50 mg/l kanamycin+250 mg/l carbenicillin), the kanamycin-resistant plantlets were regenerated and then transferred into rooting medium MS_2 (half-strength MS_0+250 mg/l carbenicillin). After roots were formed (in about 10–14 days), the rooted plantlets were transferred into soil into pots in the growth chamber. After 3–4 months of growth they were transplanted into the field, covered by plastic film, for further growth.

PCR analysis of *hpSQS* and *nptII* genes in transgenic plants

The *hpSQS* and p2300 transgenic plants were identified from regenerated *A. annua* plants by PCR analysis, using genomic DNA isolated by the CTAB (cetyltrimethylammonium bromide) method [24] as template, for the presence of introduced *hpSQS* gene and *nptII* genes using primers p35s (5′-TTCGTCAACATGGTGGAGCA-3′) and SQSR1 (5′-TACCACCAACCATACCAACC-3′), and primers p35sF3 (5′-ATGACGCACAATCCCACT-3′) and nptIIR2 (5′-CCCTGATGCTCTTCGTCCA-3′) respectively. The PCR reaction was carried out using the following mixture [1 μl of forward primer (10 μM), 1μl of reverse primer (10 μM), 1.5 μl of dNTPs (2.5 mM), 2.5 μl of 10 $\times$ PCR buffer (Shanghai Sangon Biological Engineering Technology & Services Co. Ltd.; 1.5 μl of $MgCl_2$ (25 mM), 2 μl of DNA template (100 ng/μl), 0.3 μl of Taq polymerase (5 units/μl); doubly distilled water was added to bring the volume up to 25 μl] under the following conditions: 94 °C for 5 min, followed by 35 cycles of amplification (94 °C for 45 s, 54 °C for 45 s and 72 °C for 45 s) and by 72 °C for 8 min, resulting in 768 bp and 635 bp DNA fragments for the *hpSQS* gene and *nptII* gene respectively.

DNA isolation and Southern-blot analysis

The genomic DNA of transgenic and control plants was isolated and purified by the CTAB method [24]. Approx. 40 μg of DNA per sample was digested with HindIII, fractionated by 1.0%-agarose-gel electrophoresis, transferred on to a positively charged Hybond-N^+ nylon membrane (GE Healthcare) and hybridized with an alkaline-phosphatase-labelled partial cDNA sequence of *SQS* as the probe. The probe (327 bp) was generated by PCR with the cDNA sequence of *SQS* as the template, and with primers SQSF1 and SQSR1. Probe labelling (alkaline phosphatase), hybridization and signal detection were performed using Amersham AlkPhos Direct Labeling Reagents (GE Healthcare) and CDP-Star

Detection Module following the manufacturer's instructions. The hybridized signals were visualized by exposure to Fuji X-ray film at room temperature (25 °C for 12 h.

RNA isolation and real-time RT–PCR analysis

RNA was extracted from upper leaves of control and transgenic *A. annua* plants with the Plant (Leaves) Total RNA Isolation Kit (Watson Biotechnologies). DNA contamination was removed using DNase I following the protocol provided by the manufacturer (TaKaRa). The cDNAs were synthesized from the RNA samples using Prime Script™ Reverse Transcriptase Reagent according to the manufacturer's (TaKaRa) instructions, using oligo(dT) as primer.

The real-time RT–PCR analysis was performed in a Peltier Thermal Cycler PTC200 (Bio-Rad), using the cDNAs as templates and gene-specific primers for the genes analysed [SQSF4 (5′-ATCCCATCACAACCTCAC-3′) and SQSR4 (5′-ACAACCCTATTCCAACAAGTC-3′) for *SQS* gene (corresponding to *SQS* cDNA from nt 114 to nt 564); UBC-F (5′-CACACTTGAGGTTGAGTCCAG-3′) and UBC-R (5′-CATAACATTTGCGGCAGATAG-3′) for the UBC (ubiquitin conjugating enzyme) gene]. SYBR Green (SYBR Premix Ex Taq; Takara) was used in the PCR reactions to quantify the amount of dsDNA. The relative C_t (threshold cycle value) method (User Bulletin 2, ABIPRISM7700 Sequence Detection System, update 2001; PerkinElmer/Applied Biosystems) was used to estimate the initial amount of template present in the reactions.

Quantification of artemisinin using HPLC-ELSD (HPLC–evaporative light-scattering detection)

Leaves of *A. annua* were dried at 45 °C and, when ground, the dried-leaf powder (0.1 g/sample) was extracted with ethanol (1 ml) using a Shanghai Zhisun Instrument Co. Ltd model JYD-650 ultrasonic processor (two bursts of 15 min each), then centrifuged for 10 min at 3910 ***g*** to remove the suspended particles. The final supernatant was filtered through a 0.25-μm-pore-size filter.

The samples were analysed using a Waters Alliance 2695 HPLC system coupled with a Waters 2420 ELSD detector. The HPLC conditions were as follows: column, Waters C_{18}; mobile phase, water/methanol (40:60, v/v); flow rate, 1 ml/min. The ELSD conditions were optimized at a nebulizer-gas pressure of 345 kPa (50 lbf/in^2) and drift-tube temperature of 45 °C, and the gain was set at 7. Authentic artemisinin from Sigma was used as the standard. For each sample, the injection volume was 20 μl, and the results were analysed using Empower (Waters' chromatography data software). The measurement was repeated three times.

Quantification of sterols by GC–MS

Quantitative analysis of four major sterols (campesterol, stigmasterol, β-sitosterol and ergosterol) in the sterol-biosynthetic pathway in the transgenic and the control *A. annua* plants was performed by GC–MS. Leaves of two independent transgenic *A. annua* plants (S212A and S168B) and a control plant were dried at 45 °C and the ground dried leaf powder (0.5 g/sample) was extracted with dichloromethane (20 ml) using an ultrasonic processor (two bursts, 30 min each). The solvent was treated with anhydrous Na_2SO_4 for 12 h and the supernatant liquor was then moved to another container and evaporated. The residue was dissolved in methanol (3 ml) and then centrifuged for 10 min at 3910 ***g*** to remove the suspended particles. The final supernatant was filtered through a 0.25-μm-pore-size filter.

A 1 ml portion of the supernantant was analysed by GC–MS (Auto System XL GC/Turbo Mass MS; PerkinElmer) equipped with a DB-5MS capillary column (30 m long × 0.25 mm internal diameter; 0.25 μm particle size) under the following conditions: injection port temperature, 290 °C; carrier gas, He (99.999 %) 1.0 ml/min; split ratio, 2:1; initial column temperature, 110 °C (raised at 30 °C/min to 290 °C and then maintained for 54 min at 290 °C); ionization voltage, 70 eV. The sterols were analysed according to the National Institute of Standards NIST98 and the Wiley Registry of Mass Spectral Data (7th edition) mass spectrum databases, and relative sterol contents were calculated from the peak areas of the relevant compounds.

Results and discussion

By RT–PCR, the 327 bp fragment of the *SQS* gene was amplified from *A. annua* and its primary structure was subsequently confirmed by sequencing. CAMBIA2300::p35s-hairpinSQS-ocs was then constructed (Figure 1) and its primary structure was also confirmed by sequencing.

The young leaves of *A. annua* were transformed with EHA105 (pCAMBIA2300::p35s-hairpinSQS-ocs) and EHA105 (p2300) respectively by *A. tumefaciens*-mediated transformation. After two rounds of kanamycin (50 mg/l) selection, more than 200 kanamycin-resistant plants were regenerated (Figures 2A and 2B), among which

Figure 1 Schematic structure of suppressing transforming vector pCAMBIA2300::p35s-hpSQS-ocs constructed to produce hpRNA and initiate silencing of SQS used in *Ag. tumefaciens*-mediated *A. annua* transformation

The hairpin structure including the SQS fragment (SQSanti) and pyruvate orthophosphate dikinase (PDK) intron is driven by the 35 S promoter (35S P) and terminated by the ocs terminator (ocs T). *NPTII*, *nptII* as the selectable marker. LB and RB, the left and right borders respectively.

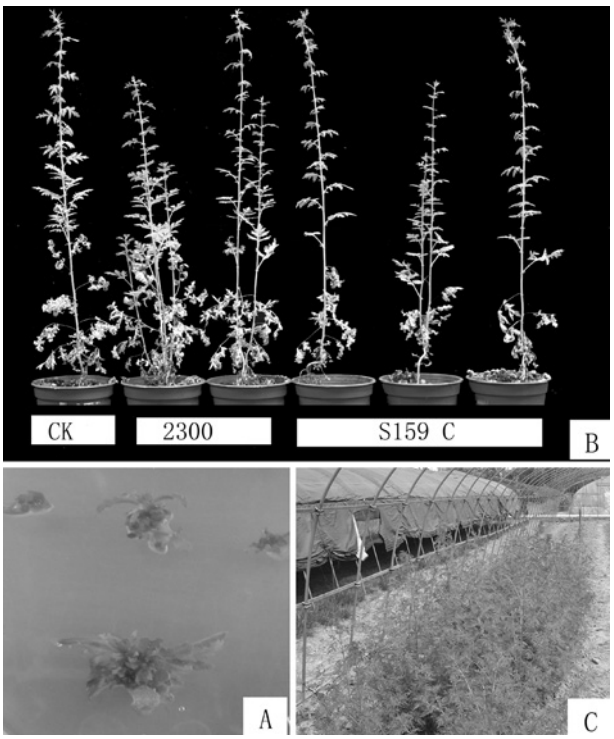

Figure 2 Regeneration of transgenic *A. annua* plants

(**A**) Shoot regeneration; (**B**) transgenic and control plants in pots; (**C**) transgenic plants in the field.

23 independent transgenic plants transformed with EHA105 (pCAMBIA2300::p35s-hairpinSQS-ocs) and 13 independent transgenic plants transformed with EHA105 (p2300) had their transgenic status subsequently confirmed by PCR and/or Southern-blot analyses (Figure 3). The copy numbers varied among the different transgenic plants.

Compared with the control plants, the growth of the hpSQS and p2300 transgenic plants was not changed significantly (Figure 2B). In the growth chamber, all the plants in pots were slender and weak (Figure 2B), but after they had been transplanted into the field for a period of time, the plants appeared healthier (Figure 2C).

Artemisinin contents in all the hpSQS and p2300 transgenic plants, as well as the untransformed control plants, were analysed at two growth stages (after 3 months of growth in the growth chamber and at the beginning of florescence in the field) using HPLC–ELSD. The results showed that, after they have been grown in pots in the growth chamber for 3 months, some hpSQS transgenic plants contained significantly higher artemisinin contents than untransformed control plants (10 mg/g DW), with the highest up to 31.4 mg/g DW (Figure 4A).

After they had been transplanted and grown in the field for 2 months, the plants reached the flowering stage. At the beginning of florescence, the difference of artemisinin contents between hpSQS plants and control plants was still significant (Figure 4B). However, the artemisinin content of these plants was not as high as that seen in the growth chamber. It had been reported previously that the highest content of artemisinin was detected before plant flowering, and the artemisinin content decreased rapidly after flowering [14]. This may explain the present finding, namely that the artemisinin content in *A. annua* plants was lower at the beginning of florescence than when the plants were cultured in the growth chamber.

Earlier studies had shown that the artemisinin content of *A. annua* could be increased by the process of transformation. Vergauwe et al. [25] found the artemisinin content of the leaves of regenerated *A. annua* plants transformed with *A. tumefaciens* was 1.7 mg/g DW, a value that is a little higher than that found in the leaves of plants cultured normally (1.1 mg/g DW).

To demonstrate that the significant enhancement of artemisinin in the hpSQS transgenic plants is mainly caused by the suppression of *SQS*, a control experiment involving the transfer of empty plasmid p2300 to *A. annua* was also conducted. An analysis of the artemisinin content of p2300 and hpSQS transgenic plants after they had been grown in a growth chamber for 4 months showed that the p2300 transgenic plants contained amounts of artemisinin similar to those found in the untransformed control plants, and the artemisinin contents in hpSQS transgenic plants were significantly higher than those in p2300 transgenic and control plants (Figure 4C). The present study demonstrated that the influence of transformation process itself on artemisinin accumulation was not very significant, and the increase in the artemisinin content in hpSQS transgenic plants would seem to be attributable largely to the introduction of the hpSQS gene.

To investigate whether the improvement in the flux of the artemisinin pathway is due to the suppression of the metabolic flux of the sterol pathway by the hpRNA-mediated RNAi, the contents of four major sterols in sterol pathway, namely campesterol, stigmasterol, β-sitosterol and ergosterol, in the hpSQS transgenic plants was measured by GC–MS. The result showed that the campesterol, stigmasterol, β-sitosterol and ergosterol contents were indeed decreased in the hpSQS transgenic plants (s212A and s168B) (Figure 5) in which the artemisinin contents were significantly increased. All the four sterols in the hpSQS transgenic plant s212A were reduced significantly (37–58% of the control-plant values), and in the plant s168B, although the campesterol and stigmasterol content was not noticeably altered, the ergosterol and β-sitosterol content was much lower than that in the control (49 and 58% of the control value respectively). It seems possible, therefore, that the suppression of the sterol-biosynthetic pathway could

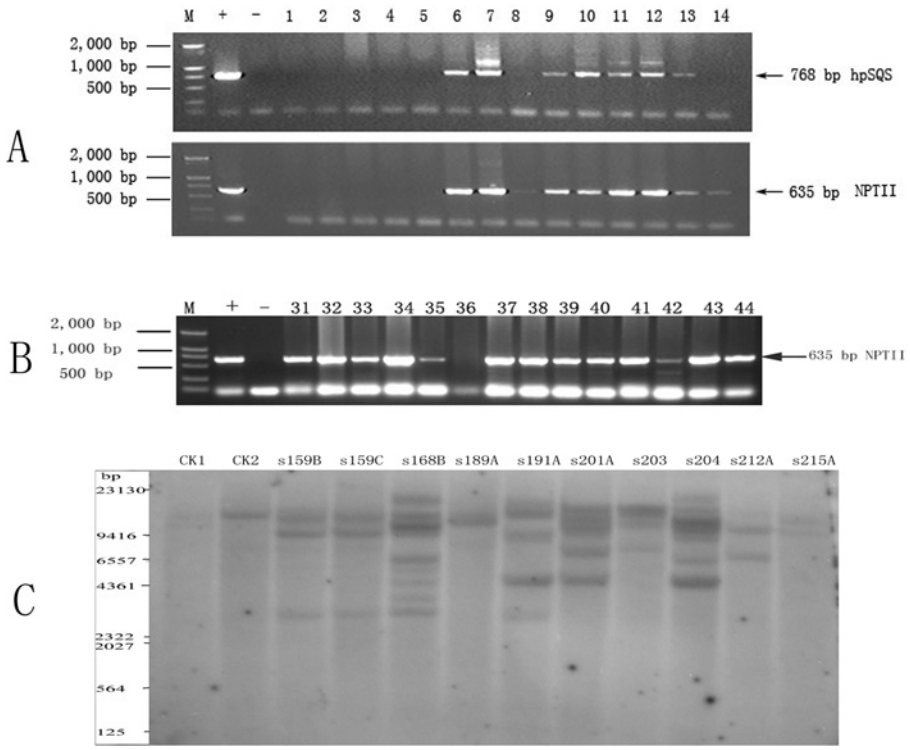

Figure 3 Representative PCR and Southern-blot analyses of independent transgenic *A. annua* plants

(**A**) PCR analysis for the presence of *hpSQS* and *npt*II genes in kanamycin-resistant *A. annua* plants regenerated by transformation with pCAMBIA2300::p35s-hpSQS-ocs. M, DL 2000 DNA ladder (a marker from TaKaRa); +, positive control (pCAMBIA2300::p35s-hpSQS-ocs); −, water; 1–5, untransformed control plants; 6–14, regenerated *hpSQS* plants. (**B**) PCR analysis for the presence of *nptII* genes in kanamycin-resistant *A. annua* plants regenerated by transformation with pCAMBIA2300. M, DL 2000; +, positive control (pCAMBIA2300); −, water; 31–44, regenerated p2300 plants. (**C**) Southern-blot analysis of transgenic *A. annua* plants. CK1 and CK2, untransformed control plants; s159B–s215A, hpSQS transgenic plants; s159B and s159C, clonal plants derived from the same plant.

be due to the suppression of SQS expression, resulting in a deviation of metabolic flux in the direction of artemisinin biosynthesis. To examine this hypothesis, real-time RT–PCR was carried out to analyse the expression of *SQS* in hpSQS transgenic plants. The results showed that the expression of *SQS* was suppressed in most of the hpSQS transgenic plants analysed (Figure 6). In some transgenic plants the expression of *SQS* was suppressed by 60% (s159C, s203 and s212A), whereas in some others the expression of *SQS* was suppressed by 20–40% (s189A, s191A and s168B). This finding was consistent with the sterol-analysis results. The expression of *SQS* at the transcript level in s212A was suppressed more significantly than that in s168B, and the amount of sterol in s212A was much less than that in s168B (Figure 5). As a result, the artemisinin content in the s212A was improved considerably more than it was in s168B (Figure 4B). All these results demonstrate again that the *hpSQS* gene could indeed suppress the expression of *SQS*, resulting in a movement of metabolic flux from FPP sterol towards FPP artemisinin, and the flux was positively correlated with the extent to which *SQS* gene was repressed.

Chen et al. [11] transferred the cotton *FPS* gene placed under the CaMV (cauliflower mosaic virus) 35 S promoter into *A. annua* via *Ag. tumefaciens*. In the transgenic plants, the artemisinin content was approx. 8–10 mg/g DW, which is 2–3-fold higher than that in the control. In the present study, in transgenic *A. annua* plants in which the competitive pathway of artemisinin synthesis was suppressed by RNAi, the artemisinin content was also 2–3-fold higher than in the control plants. Since the artemisinin content in our control plants is higher than that in the control plants used by Chen et al., the abundance of artemisinin in our transgenic plants (up to 31.4 mg/g DW) is therefore much higher than that reported by them. Qian et al. [4] increased artemisinin contents in *A. annua* plants by exposing them to salinity stress. The artemisinin content was improved up to 20–30 mg/g DW in plants treated with 4–6 g/l NaCl, but these plants grew much more slowly and weakly than the control plants.

Sterols are very important in the development of plants, having functions in regulating membrane fluidity and permeability [26]. The sterol content in *A. annua* might

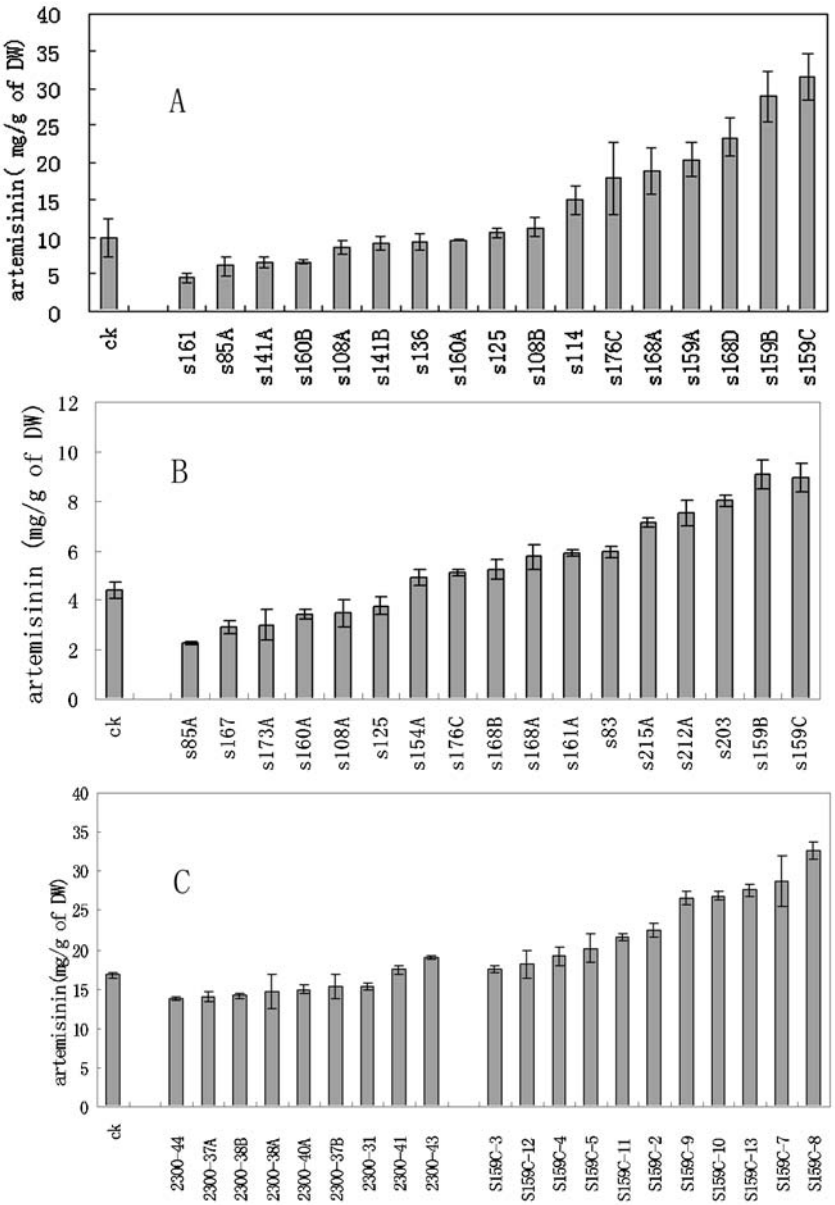

Figure 4 Artemisinin content of transgenic and untransformed *A. annua* plants determined by HPLC–ELSD analysis

(**A**) The artemisinin content of the control and hpSQS transgenic plants cultured in a growth chamber for 3 months. ck, average content for 15 control plants; s161–s159C, hpSQS transgenic plants. (**B**) Artemisinin content of control and hpSQS transgenic plants at the beginning of florescence after the plants had been transferred to the field. Ck, average content for 20 control plants; s85A–s159C, hpSQS transgenic plants. (**C**) Artemisinin content of control, p2300 and hpSQS transgenic plants cultured in a growth chamber for 4 months. ck, control plants; 2300-44–2300-43, p2300 transgenic plants; s159C-3–s159C-8, micropropagated plants of s159C.

therefore need to be maintained to a certain level and the decrease in sterols may also influence the growth of transgenic plants.

However, in the present study, in most hpSQS transgenic plants analysed, the expression of *SQS* was not suppressed entirely, at most by 60%. We showed that suppressing SQS by 60% in the transgenic plants, which resulted in the sterol content being reduced to 37–58% of the control value, still did not alter plant growth significantly.

The present study demonstrates that the genetic-engineering strategy of suppressing sterol pathway using RNAi is an effective and suitable means of increasing the artemisinin content of plants. Further studies will be

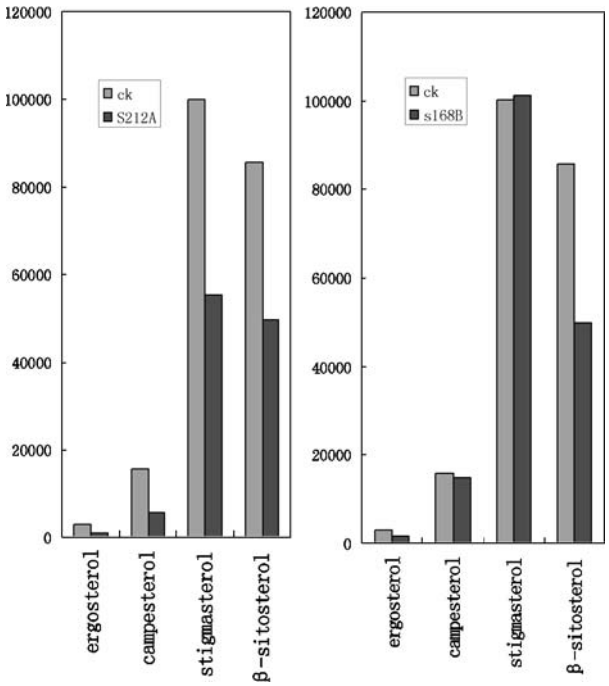

Figure 5 Relative contents of sterols in the control and hpSQS transgenic plants determined by GC–MS analysis

Abbreviations: ck, untransformed control plant; s212A and s168B, hpSQS transgenic plants.

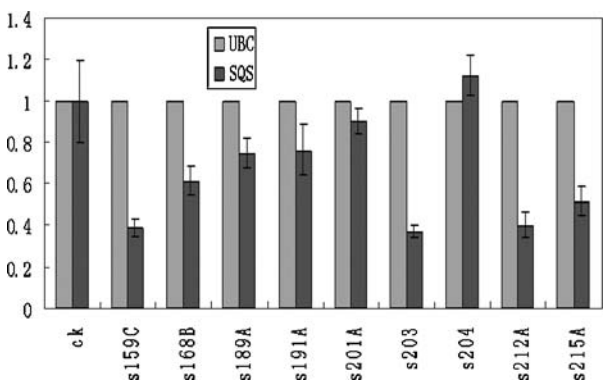

Figure 6 SQS mRNA accumulation in transgenic plants determined by real-time RT–PCR

Abscissa: Ck, untransformed control plant; s159C–S215A, independent hpSQS transgenic plants. Ordinate: relative amount of mRNA; UBC, UBC-gene mRNA; SQS, *SQS* mRNA.

required that use other strategies, combined with the RNAi strategy, such as manipulating the transcription factors involved in the artemisinin-biosynthetic pathway, the enzymes involved in its catabolic pathway and transporters involved in the transport of artemisinin, in order to achieve a maximum increase in artemisinin in transgenic plants.

Acknowledgment

We thank the Instrumental Analysis Center of the Shanghai Jiao Tong University for assistance with the GC–MS analysis.

Funding

This work was funded by the China '973' Program [grant no. 2007CB108805]; the China '863' Program [grant no. 2007AA10Z189]; the Ministry of Education [grant no. C04N]; the Shanghai Science and Technology Committee [grant no. 08391911800]; and the Shanghai Leading Academic Discipline Project [grant no. B209].

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Received 9 May 2008/4 June 2008; accepted 13 June 2008
Published as Immediate Publication 13 June 2008, doi:10.1042/BA20080068