Enhancement of artemisinin content in tetraploid *Artemisia annua* plants by modulating the expression of genes in artemisinin biosynthetic pathway

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Abstract.

Tetraploid *Artemisia annua* plants were successfully inducted by using colchicine, and their ploidy was confirmed by flow cytometry. Higher stomatal length but lower frequency in tetraploids were revealed and could be considered as indicators of polyploidy. The average level of artemisinin in tetraploids was increased from 39% to 56% than that of the diploids during vegetation period, as detected by high-performance liquid chromatography–evaporative light scattering detector. Gene expressions of 10 key enzymes related to artemisinin biosynthetic pathway in different ploidy level were analyzed by semiquantitative polymerase chain reaction and significant upregulation of *FPS*, *HMGR*, and artemisinin metabolite-specific *Aldh1* genes were revealed in tetraploids. Slight increased expression of *ADS* was also detected. Our results suggest that higher artemisinin content in tetraploid *A. annua* may result from the upregulated expression of some key enzyme genes related to artemisinin biosynthetic pathway.

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1. Introduction

Malaria is one of the most serious parasitic diseases worldwide [1]. Currently, the most effective antimalarial drug is artemisinin combinational therapy, the central component of which is the sesquiterpene lactone endoperoxide, artemisinin [2]. Recently, artemisinin has been demonstrated as a promising versatile weapon from nature against some other parasitic and viral diseases, schistosomiasis, and certain cancers [3–6]. Thus, the demand of artemisinin is vastly growing. However, as artemisinin is mainly isolated and purified from the traditional Chinese medicinal plant *Artemisia annua* L. ($2n = 18$) with low content (0.8%), currently the yield of artemisinin cannot meet the demand. Large-scale production of artemisinin through synthetic chemistry is possible, but due to high cost, it is not economically feasible [7].

To increase the production of artemisinin in the plant, synthetic autotetraploids may be adopted. Polyploidy is a widespread phenomenon in the evolution of flowering plants and is considered as a key element in plant speciation and diversification [8]. Polyploids often show novel phenotypes, such as broader leaves, good quality, high yield, and increased resistance to environment stress and diseases [9],[10]. To induce autopolyploids, colchicine treatments are widely used [11–15]. With the basic genetic material remain the same, chromosome doubling in autopolyploids is often accompanied by conspicuous changes in secondary metabolism. The data calculated by Lavania [16] revealed that polyploidy brought about an increase in the concentration of secondary metabolites in most cases including artemisinin. Tetraploid *A. annua* ($4n = 36$) was first induced in 1999 by using colchicine [17]. It was reported that averaged artemisinin level in tetraploids was 38% higher as compared with the diploid progenitor during the whole vegetation period. Similar result was also reported in the tetraploid hairy root [18], further supporting that synthetic autotetraploids could increase the artemisinin content. But owing to the lower biomass accumulation, the net yield of artemisinin per square meter of field-grown plants decreased by 25% [17]. Thus, the cultivation of the tetraploid *A. annua* plants with more biomass is still in large demand.

Abbreviations: ADS, Amorpha-4,11-diene synthase; BA, 6-Benzyladenine; CPR, Cytochrome P450 reductase; DMAPP, dimethylallyl pyrophosphate; DTT, Dithiothreitol; DXS, 1-Deoxyxylulose 5-phosphate synthase; FPP, Farnesyl diphosphate; FPS, Farnesyl diphosphate synthase; HEPES, 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid; HMGR, 3-Hydroxy-3-methyl-glutaryl coenzyme A reductase; HRP, Horseradish peroxidase; IBA, Indole-3-butyric acid; MVA, mevalonate; NADPH, nicotinamide adenine dinucleotide phosphate; RT-PCR, Reverse transcriptase-polymerase chain reaction.

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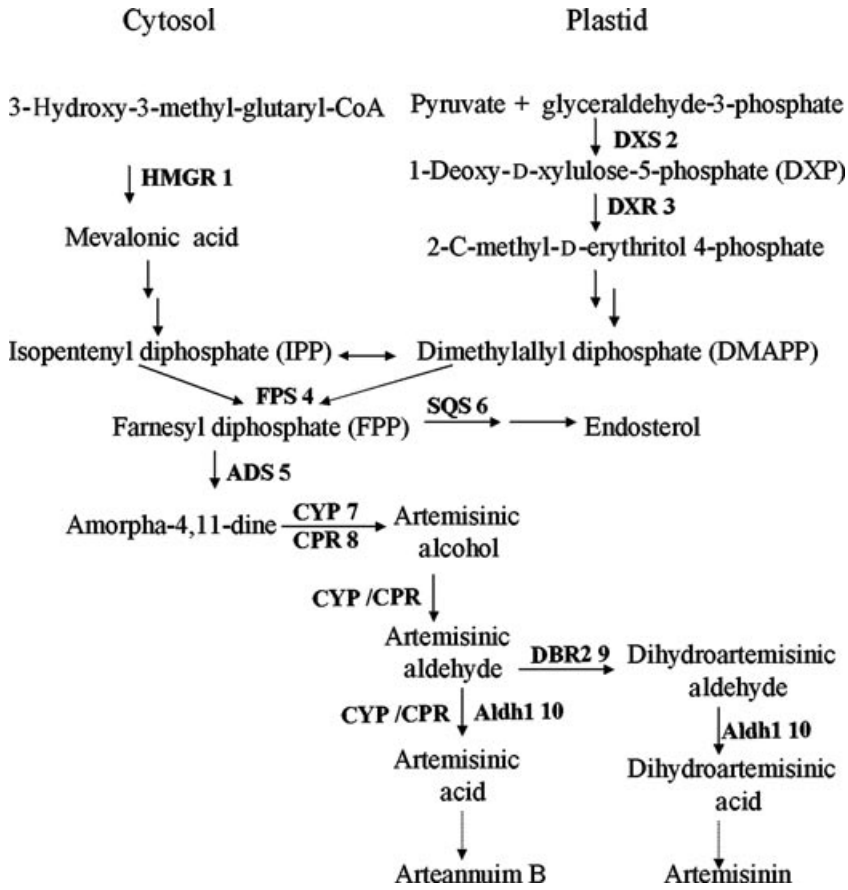

Fig. 1. Biosynthesis of artemisinin. (Modified from Ref. [28].)

Although the synthetic autotetraploids can increase the artemisinin content, the underlying molecular mechanism is still unknown. Because the biosynthesis of artemisinin has almost been completely clarified, it is possible to examine the expression level of the enzymes involved in the biosynthesis of artemisinin. As illustrated in the Fig. 1, nine genes involved in the biosynthesis of artemisinin and one gene (*SQS*) in the competitive pathway were exhaustively researched. 3-Hydroxy-3-methyl-glutaryl coenzyme A reductase (HMGR) catalyzes the conversion of HMG-CoA to mevalonate (MVA), and it is the first rate-limiting step in the cytosol-based MVA pathway [19]. The initial step of the other plastid-based non-MVA (MEP) pathway was the formation of 1-deoxy-D-xylulose 5-phosphate (DXP) catalyzed by 1-deoxyxylulose 5-phosphate synthase (DXS). DXP is then converted to DXS through the nicotinamide adenine dinucleotide phosphate (NADPH)-dependent reduction catalyzed by the key enzyme DXR. Both the MVA and the MEP pathways are involved in the production of inositol pentaphosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). Three of these C-5 isoprene molecules are then condensed to farnesyl diphosphate (FPP) through the activity of farnesyl diphosphate synthase (FPS). As a common precursor of the isoprenoid end products, FPP is then cyclized to amorpha-4,11-diene by amorpha-4,11-diene synthase (ADS) [20],[21] or converted to squalene and sterol production through the activity of *SQS* [22]. In artemisinin biosynthesis, amorpha-4,11-diene is regioselectively oxidized to artemisinic acid in three steps by a single cytochrome P450 monooxygenase, CYP71AV1, with cytochrome P450 reductase (CPR) as a redox partner [23],[24]. But so far, it is not sure how the artemisinin is finally synthesized. Brown and coworkers [25],[26] suggested that artemisinic acid may then be converted into either arteannuin B or potentially to dihydroartemisinic acid and finally to artemisinin. But the recently isolated *Dbr2* and *Aldh1* challenged this hypothesis. Both these enzymes catalyze the oxidation of artemisinic aldehyde to its dihydro form and then to the dihydro acid in the trichome-specific artemisinin biosynthesis, with Aldh1 also showing activity in the conversion of artemisinic aldehyde to artemisinic acid [27],[28].

In order to cultivate tetraploid *A. annua* plants with higher content and more biomass, we chose the high-production *A. annua* plants in the People's Republic of China as the starting material. Tetraploid *A. annua* plants were confirmed by flow cytometry (FCM). The tetraploid *A. annua* plants have larger leaves with bigger stomatas than the diploids. In agreement with the previous reports, those tetraploid plants also had higher artemisinin content. Reverse transcriptase-polymerase chain reaction (RT-PCR) results showed that the expression level of key enzymes, such as FPS, HMGR, ADS, and Aldh1, in artemisinin synthetic pathway was increased. Immunoblotting results further support the RT-PCR results. Thus, the tetraploid *A. annua* plants may produce more artemisinin than diploids by modulating the expression of key enzymes.

2. Materials and methods

2.1. Plant material and tetraploid induction

Artemisia annua seeds of high artemisinin content (0.9%) were collected from Chongqing municipality, People's Republic of China. After 1-Min surface sterilization in 75% (v/v) ethanol, 20-Min treatment in 20% (v/v) NaOCl, and three times wash in sterile distilled water, the seeds were sown in Murashige and Skoog (MS) medium. A plantlet with the best growth vigor was selected and a large number of shoots were proliferated on MS medium.

Uniform shoots were selected in this experiment. For tetraploid induction, these shoots were first soaked in 250 ppm colchicine for 48 H under 25°C and then transferred to MS + 6-Benzyladenine (BA) 0.3 mg/L + indole-3-butyric acid (IBA) 0.5 mg/L. After 7 weeks, shoots were transferred into the rooting medium, that is, 1/2 MS.

Fully developed plantlets were transplanted first to 10 × 10 × 5 cm plastic pots in a constant-temperature chamber (25°C) for 2 months and then to an unheated glasshouse. All plants received regular water with biweekly fertilizer treatments.

2.2. Flow cytometric ploidy measurement

A 50-mg sample of fully developed fresh leaves was collected and analyzed using flow cytometry against a known ploidy level (external standard). Nuclei suspension extractions were based on a modified protocol of Arumuganathan and Earle [29]. The sample was chopped with a sharp razor blade in 1 mL extraction solution, containing 5 mM $MgSO_4$, 25 mM KCl, 2.5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 0.125% Triton X-100, and 6.5 mM Dithiothreitol (DTT), at pH 8.0. The extraction liquid was then forced through a 30 μm nylon filter, followed by centrifugation at low speed (956 *g*) for 5 Min. The supernatant was discarded and the pellet formed in extraction solution was resuspended with 0.1 mg/mL propidium iodide and 1 mg/mL RNase, and then incubated for 15 Min at 37°C. The flow cytometric analyses were carried out using a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) with a 15 mW air-cooled 488 nm argon ion laser obtained from Becton Dickinson Immunocytometry Systems (San Jose, CA, USA), operated with CellQuest software (Becton Dickinson, San Jose, CA, USA).

2.3. Leaf morphological and anatomical characteristics

Major morphological and growth habit characteristics of confirmed tetraploidy (derived from 250 ppm colchicine treatments) were compared with the confirmed diploid status (also derived from 250 ppm colchicine treatments). Size and density of stomata on the adaxial surface of leaves were quantitatively measured. For leaf sampling, three tillers were randomly selected from each five 4-month-old tetraploid and diploid plants, and from each tiller, the fully developed basal leaves were collected.

Stomatal length was measured under 100 × magnification using a fluorescence microscope with an ocular scale, where each ocular unit of the scale measured 2.5 μm. Stomatal density was also measured by the fluorescence microscope under 40 × magnification. Stomata in 15 microscopic fields were counted for each leaf. Counts were taken twice per leaf at random locations across the surface in the unit of 0.15 mm^2. Each biological repeat was averaged and at least five leaves were measured per condition.

2.4. Extraction and analysis of artemisinin by high-performance liquid chromatography–evaporative light scattering detector

Leaves of both diploid and tetraploid *A. annua* were harvested every 3 weeks (*i.e.*, June 20, July 10, and August 1) after the plants were transferred to the glasshouse. After drying at 45°C for 48 H, 100 mg of dried-leaf powder extracted with 1 mL ethanol was driven by an ultrasonic treatment at 45 W for 30 Min, and then centrifuged for 10 Min at 2,655 *g* to remove the suspended particles. The final supernatant was filtered through a 0.25 μm pore size filter and analyzed using a Waters Alliance 2695 (Waters Corporation, Milford, MA, USA) high-performance liquid chromatography (HPLC) system coupled with Waters 2420 evaporative light scattering detector (ELSD) (Waters Corporation, Milford, MA, USA). The HPLC column was an endcapped Purosphere (Hitachi Technologies, Atlanta, GA, USA) C18-RP 250 × 4.0 mm ID (5.0 μm pore size) column was kept at room temperature. The mobile phase was water/methanol [40:60, (v/v)] at a flow rate of 1 mL/Min with a stop time of 10 Min. ELSD conditions were optimized in order to achieve maximum sensitivity, nebulizer gas pressure of 345 kPa (50 lbf/in^2), drift tube temperature of 45°C, and gain 7. The data were collected and processed by the Waters' chromatography data software (Empower). The measurements were repeated three times and averaged for analysis.

2.5. RNA isolation and semiquantitative RT-PCR procedure

The extraction of total messenger RNA from 100 mg leaf tissue was performed using the RNAprep pure Plant Kit (Tiangen Biotech, Beijing, China) according to the manufacturer's recommendations. Extracted RNA was quantified by spectrophotometry absorbance at 260 nm. One microgram of the treated RNA was reverse-transcribed with Prime Script™ Reverse Transcriptase Reagent Kit (Takara, Dalian, China) using an oligo(dT) primer following manufacturer's instructions. Complementary DNA (cDNA) template (0.5 μL) was used for the semiquantitative PCR with the following parameters: 94°C for 5 Min, followed by 28 cycles of amplification (94°C for 30 Sec, 51°C–56°C for 30 Sec, and 72°C for 40 Sec), and by 72°C for 10 Min. All the designed gene-specific primers were listed in Table 1, with product size and T_m (melting temperature) values. Expression of *RPS9* was used as an internal control to equalize cDNA quantity in each reaction.

2.6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunodetection

FPS-specific multiclonal antibody with amino acid sequence IMDESHTRRGQPC was purchased from CoWin Biotech

Table 1
Primers used in semiquantitative RT-PCR analysis

Gene name	Sequence (5′-3′)	Product size (base pair)	T_m (°C)
HMGR	F-TTGTGTGCGAGGCAGTAAT	183	55.41
	R-CCTGACCAGTGGCTATAAAGA		58.01
DXR	F-TTGCTGGCGGTCCCTTTGTTC	258	61.92
	R-AGTGTGGCAGAGTCAACCGTG		56.06
DXS	F-CGGGATTCACCAAACGCTCTG	260	61.92
	R-CAAGATTGGCAGTAGGTAAAG		56.06
FPS	F-TCATTGTCTATTCACCGCCG	435	57.80
	R-CACCGCTTGGACTGCTTTGCT		61.92
ADS	F-AATGGGCAAATGAGGGACAC	252	57.80
	R-TTTCAAGGCTCGATGAACTATG		56.35
SQS	F-ATCCCATCACAACCTCAC	450	61.92
	R-ACAACCCTATTCCAACAAGTC		61.94
CYP	F-CACCCTCCACTACCCTTG	234	59.58
	R-GACACATCCTTCTCCCAGC		59.72
CPR	F-AGCCTCTTTGCCACCTCCT	405	59.72
	R-GAACAGACTCCCTTGTGAACG		59.97
DBR2	F-GGTGGGTCATTAGAAAACCGCTG	356	61.92
	R-TCTAGTGTAACCACCGCAGCATA		61.94
Aldh1	F-GGTCATCAAGCCTGCCGAACATA	356	56.794
	R-ACACGAGACCCTGCCACACACAT		62.94
RPS9	F-GCGTTTGGATGCTGAGTTGAAG	260	60.07
	R-GGCGCTCAAGGAAGTTCTCTAC		61.94

Company (Beijing, People's Republic of China). About 100 mg of leaf tissue were crushed and homogenized in 100 μL 2 × sample buffer [containing 120 mM Tris–HCl, pH 6.8, 4% (w/v) sodium dodecyl sulfate (SDS), 5% β-mercaptoethanol, and 150 mM DTT]. Samples were boiled for 10 Min and after centrifugation at 5,000*g* for 10 Min, the supernatant fluid with soluble protein was saved. SDS-polyacrylamide gel electrophoresis (PAGE) was carried out on 12% polyacrylamide gels following a modified Laemmli system in 1970 [30]. Protein samples from both diploid and tetraploid plants were analyzed. The two replicated gels were either stained with Coomassie blue or electroblotted onto Immobilon™ membranes (Millipore, Bedford, MA, USA). Western blotting was performed for 25 Min using a Bio-Rad SemiDry apparatus (Bio-Rad, Hercules, CA, USA) with Immobilon™-P PVDF membrane (Millipore) at a constant voltage of 12 V and 10 A/10 cm². Membranes were first blocked in the blocking reagent with 5% (w/v) nonfat dried milk and 0.05% Tween-20, and then incubated with a rabbit serum as primary antibody at a concentration of 1:1,000. The Horseradish peroxidase (HRP)-conjugated second antibody was goat anti-rabbit antibody at the dilution of 1:5,000. Signals were detected by SuperSignal West Pico enhanced chemiluminescence substrate (Pierce, Rockford, IL, USA) as per recommendations of the manufacturer and visualized with LAS-3000 PLUS (Fuji Photo Film Company, Kanagawa, Japan).

3. Results

3.1. Ploidy level determination by flow cytometry

Thirty colchicine-treated plantlets were fully developed on the rooting medium, that is, 1/2 MS, with 83% rooting rate. These plantlets were then transferred to the soil pots and analyzed by FCM. Non-colchicine-treated wild-type *A. annua* ($2n = 18$) was used as a control and produced a peak at the position of channel 211 (Fig. 2a). Failure-induced plantlets with unchanged nuclear DNA ploidy level ($2n = 18$) showed the same peak as control. Tetraploids, that is, with four copies of DNA, showed histogram with a peak aligned at channel 423 (Fig. 2b). Seven autotetraploid plants were obtained, with an induction rate of 25%.

3.2. Leaf morphological and anatomical characteristics

Morphological variations, such as slower plant growth, darker green pigmentation, and thicker, wider, and larger leaves, were observed among the colchicine-treated populations of plants (Figs. 3a and 3b). Significant differences were also noted in stomatal size and frequency (Figs. 3c–3f). For quantitative traits, tetraploids had a greater mean stomatal length of 17.9 ± 1.12 μm than that of their diploid counterparts with the size of 12.1 ± 0.98 μm (mean ± SE) (Fig. 3e). This positive relationship between ploidy level and stomatal length in *A.*

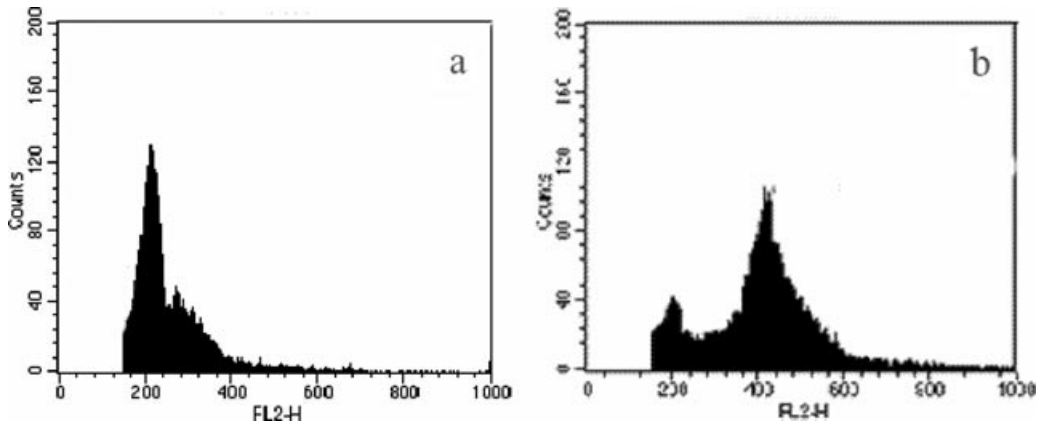

Fig. 2. Flow cytometric analysis of surviving individuals of *A. annua* L. (a) Diploid plant (control), (b) induced tetraploid plant.

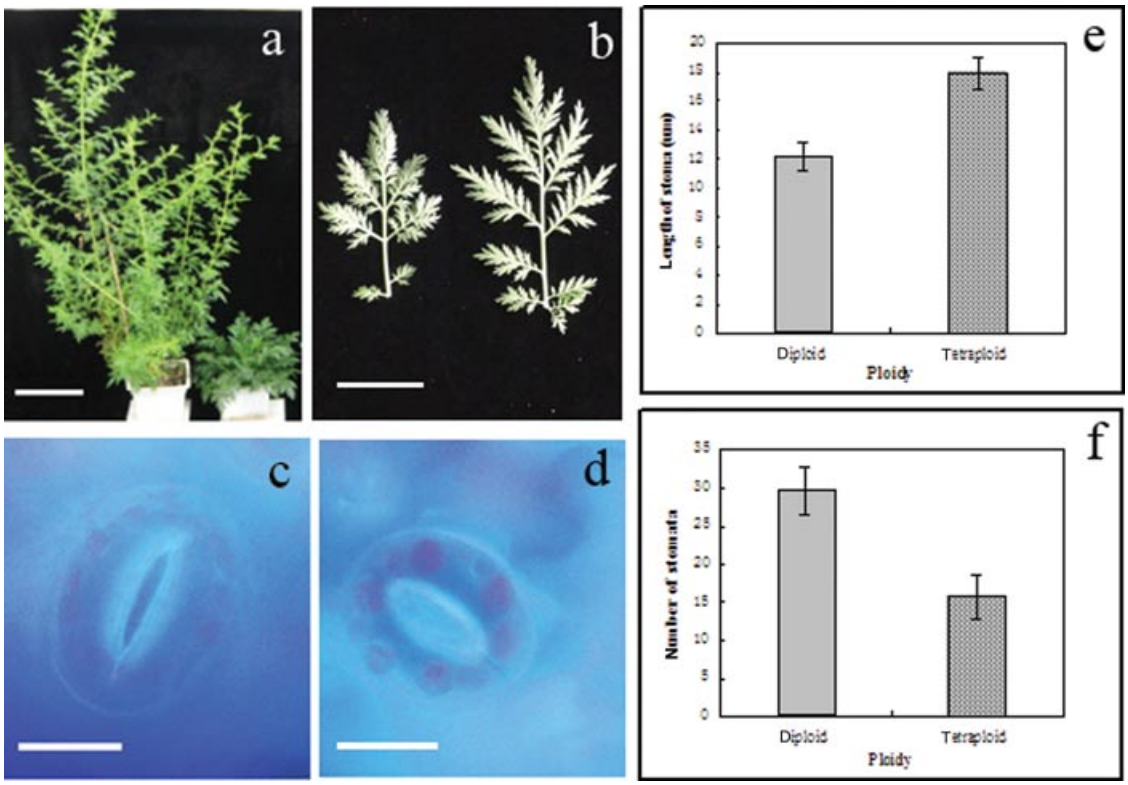

Fig. 3. Morphological characterization of diploid and tetraploid plants. (a and b) Two months after transplanting to a constant-temperature chamber. (a) Diploid plant (left) and tetraploid plant (right); (b) fully expanded leaves of diploid (left) and tetraploid (right). (c–f), Two months after transplanting to the glasshouse. (c and d) Stomata of the tetraploid (c) and diploid (d); (e and f) comparison of stomata characters of different ploidy levels of length of stomata (e) and number of stomata per unit area (0.15 mm^2) (f).

annua was also found in various plant species. Thus, stomatal length has become one of the most widely used indicators of polyploidy [31–34]. A negative relationship was found between the frequency of stomata and ploidy level (Fig. 3f). Mean stomatal number per unit leaf area (0.15 mm^2) in the abaxial surfaces of diploid accessions was 29.6 $\pm$ 3.18, whereas the number decreased to 15.8 $\pm$ 2.95 in the tetraploid plants.

3.3. Extraction and analysis of artemisinin by HPLC–ELSD

Polyploidization has been shown to be effective in increasing the average artemisinin content in leaf and hairy roots [17],[18]. In our experiment, HPLC showed a significant enhancement of artemisinin content in a range from 39% to 56% in tetraploid plants compared with their diploid parents (Fig. 4). Similar to the previous works, the dates were all collected during a vegetation period from June 20 to August 1, whereas the increase found here was higher than the data in the previous paper (~38%).

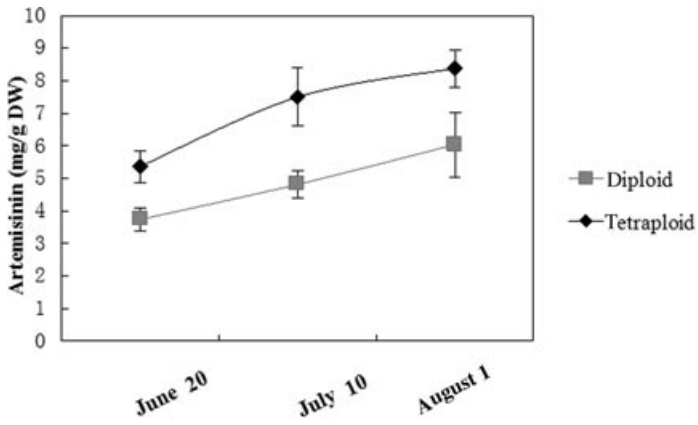

Fig. 4. Comparison of averaged artemisinin content in leaves of diploids and tetraploids of *A. annua* at three different time points during vegetation period, measured by HPLC. The measurement was repeated three times. Vertical bars represent standard deviation.

3.4. Gene expression profile

Expression analysis of 10 genes related to biosynthesis of artemisinin was carried out through semiquantitative RT-PCR. Figure 5 illustrated that the internal control *RPS9* showed a stable expression between different ploidy levels. Significant increase was observed in the expression of *HMGR*, *FPS*, and *Aldh1* in tetraploid plants as compared with their diploid counterparts. The *ADS* gene was also slightly upregulated. No significant difference was detected in the expression of the genes *DXS*, *DXR*, *CYP71AV1*, *CPR*, and *DBR2*.

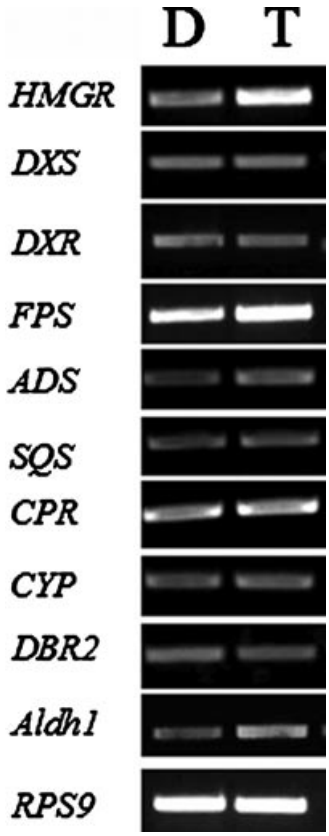

Fig. 5. Differential gene expression in diploids and tetraploids. D, diploid plants; T, colchicine-induced tetraploid plants. *RPS9* was used as an internal control. Experiments were repeated three times and a representative experiment is shown.

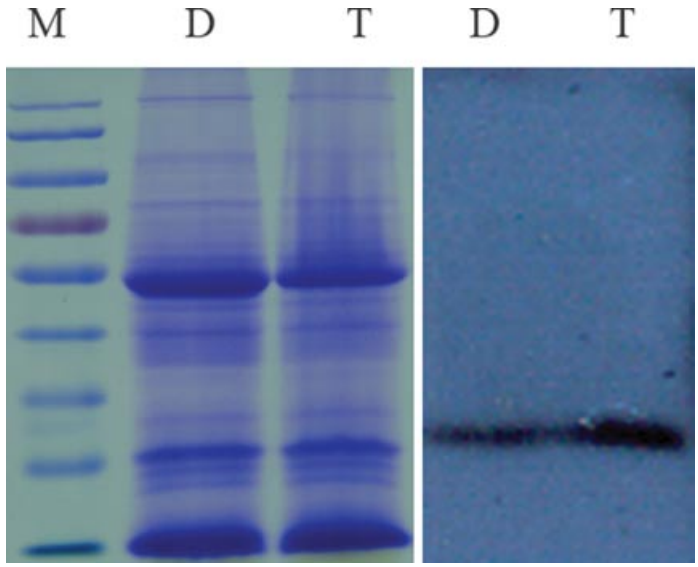

Fig. 6. Expression of FPS in diploid and tetraploid plants. (Right panel) Western blot result using FPS-specific primary antibody. (Left panel) A duplicated gel of left panel, stained by Coomassie blue. D, diploid plant; T, tetraploid plant; M, marker: PageRuler™ Prestained Protein Ladder (Fermentas, Canada).

FPS-specific multiclonal antibody was generated and then applied to detect the protein level in tetraploids and diploids. Comported well with the RT-PCR results, immunoblotting also showed that tetraploid plants had higher expression of *FPS* (Fig. 6).

4. Discussion

To understand the molecular mechanism underlying the increase of artemisinin content in polyploid *A. annua*, autotetraploid *A. annua* was successfully induced by colchicine. FCM, which is considered as a suitable method for rapid determination of ploidy levels, has been used widely in polyploid breeding [35–38]. In this study, FCM analysis was successfully carried out to screen tetraploids, and seven tetraploid plants were detected by this method.

Tetraploid plants always have larger stomata. Significant difference was also observed in stomatal size and frequency in the abaxial surfaces of leaves between diploids and tetraploids in our research. On an average, the tetraploids had a mean stomatal length of 17.9 $\pm$ 1.12 μm, which was found to be 48% longer than that of their diploid counterparts. On the contrary, the mean stomatal number per unit leaf area (0.15 mm^2) of diploid accessions was 29.6 $\pm$ 3.18, but the number decreased to 15.8 $\pm$ 2.95 in the tetraploid *A. annua* plants. Thus, stomatal analysis further supported the result of tetraploid determination by FCM.

New morphologic and growth phenotypes arose with polyploid formation, including slower plant growth, darker green pigmentation, and thicker, wider, and larger leaves. Despite its slower growth at early stage, no visible disadvantage in plant height was found in the later vegetation period (data not shown).

Interestingly, by using HPLC method, we found that artemisinin content was increased in a range of 39% to 56% in tetraploid *A. annua* plants. Possibly because of the different ecotypes used for producing tetraploids, our result of artemisinin content in tetraploids was a bit higher than that reported previously (around 38%) [17]. Bigger leaves with more artemisinin content indicate the feasibility of tetraploid *A. annua* to produce higher levels of artemisinin.

Recently, a similar study focusing on morphology of tetraploid *A. annua* was reported, with the tetraploidy of plants confirmed by both the flow cytometric measurement and chromosome number [39]. Similar to our results, the tetraploids had larger size of root system, stomata, and glandular secretory trichomes (GSTs), whereas the difference in artemisinin content of tetraploid was reported, which may have resulted from cultivar genotypes, geographical location, and climate of *A. annua* growing area.

In this study, as the enzymes involved in artemisinin synthesis have been explored, the expression levels of 10 genes related to biosynthesis of artemisinin were tested by semi-quantitative RT-PCR. Significant upregulation was observed in the expression of *HMGR*, *FPS*, and *Aldh1* in tetraploid plants.

It may be that with more chromatin coming into contact with the nuclear membrane due to the lower ratio of nuclear membrane to gene dosage after polyploidization, gene activity is elevated [40].

Hydroxymethylglutaryl coenzyme A reductase commits the first rate-limiting step in the synthesis of isoprenoid metabolism (Fig. 1). Overexpression of *HMGR* led to an increase of 22.5% artemisinin content in tetraploid *A. annua* plants compared with wild-type [41]. Recently, the limit of HMGR on artemisinin biosynthesis in *A. annua* was further investigated by a series of feeding experiments [42]. These results showed that the increasing exogenous supply of labeled HMG-CoA (substrate) got an enhanced incorporation of label (^{14}C) into artemisinin, whereas increasing exogenous supply of mevinolin (the competitive inhibitor) brought the decline of incorporation of label (^{14}C) into artemisinin. In our experiment, higher expression of *HMGR* in tetraploid *A. annua* plants suggested that the increase of artemisinin content in tetraploids may be partly due to the higher expression level of *HMGR* gene.

FPS catalyzes the sequential 1–4 condensation of IPP and DMAPP to form geranyl diphosphate (GDP), and of GDP with another IPP to form FPP (Fig. 1). In our study, the transcriptional expression level of *FPS* was examined by RT-PCR, and the translational expression level was tested by immunoblotting. Both results showed that *FPS* expressed more in tetraploid *A. annua*. Previous results showed that overexpression of either a cotton *FPS* or *A. annua FPS* had a 2–3-fold higher content of artemisinin in transgenic *A. annua* [43],[44]. Moreover, relatively high correlation ($R^2 = 0.78$) was further observed between level of expression of *FPS* and artemisinin content [44]. Studies on phytohomormone also provide evidence that abscisic acid and gibberellic acid could activate artemisinin biosynthesis in *A. annua*, with the modulation in *FPS* expression [45],[46].

Besides the upregulation of these two genes in common pathway of isoprenoid biosynthesis, two artemisinin-specific pathway genes (*ADS* and *Aldh1*) were also found to have higher expression level in the tetraploids. The ADS is a key enzyme in artemisinin biosynthesis, catalyzing the cyclization of the ubiquitous precursor FPP into the first specific precursor of artemisinin [20],[47]. Exogenous application of salicylic acid to *A. annua* leaves caused a temporary peak in *ADS* expression, followed by the increase in artemisinin concentration [48]. Aldh1 is found to catalyze the NADPH-dependent oxidation of the putative artemisinin precursors, artemisinic and dihydroartemsinic aldehydes [28]. Artemisinin was synthesized and stored in GSTs located on the surfaces of leaves, buds, and flowers [49]. The GST of *A. annua* is a 10-celled biseriate structure of two stalk cells, two basal cells, and three pairs of secretory cells [50]. Both *ADS* and *Aldh1* genes were expressed only in the two outer apical secretory cells where artemisinin was finally synthesized [49]. As the final step of the artemisinin biosynthesis was suggested a nonenzymatic, photo-oxidation reaction [25], the increasing artemisinin content in tetraploids might be a direct result of upregulation of these two genes, especially *Aldh1*, by offering a higher concentration of dihydroartemisinic acid as the late precursor of artemisinin.

With these results, we conclude that tetraploid *A. annua* plants increase the average level of artemisinin by modulating the expression of genes involved in artemisinin biosynthesis, especially *HMGR*, *FPS*, and *Aldh1*. As to the induced-polyploid breeding of *A. annua* in agriculture, we suggest that hybrid or transgenic *A. annua* with high artemisinin yield should be chosen as prior diploid ancestors.

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