

Quantitative Transcript Profiling Reveals Down-Regulation of A Sterol Pathway Relevant Gene and Overexpression of Artemisinin Biogenetic Genes in Transgenic *Artemisia annua* Plants

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Key words

- ◉ *Artemisia annua*
- ◉ Asteraceae
- ◉ expression profiling
- ◉ quantification
- ◉ artemisinin
- ◉ transgenic plant

Abstract



To investigate the dynamic fluctuation of terpenoid relevant transcriptomics in transgenic *Artemisia annua* plants that express the genomic integrated antisense squalene synthase gene (*asSS*), we have quantified the transcript levels of the sterol anabolic *SS* gene as well as artemisinin biogenetic amorphaadiene synthase (*ADS*), cytochrome P450 monooxygenase (*CYP71AV1*) and cytochrome P450 reductase (*CPR*) genes by real-time fluorescent quantitative polymerase chain reaction (RFQ-PCR). The *SS* mRNA level in transgenic plants sharply dropped to 7.4% – 55.3% (i.e., 44.7 – 92.6% reduction as the wild-type control), strongly implying that the expression of endogenous *SS* gene is significantly suppressed by the exogenous *asSS* gene. In a synchronous fashion, *ADS*, *CYP71AV1* and *CPR* mRNA levels elevated with the decline of *SS* mRNA level in transgenic plants, and the maximal *ADS*, *CYP71AV1* and *CPR* mRNA levels in transgenic plants were 3.0-, 4.4- and 2.5-fold, respectively, higher than those in the control. Without a lethal effect but with a distinguishable impact on the organogenesis and

morphology of transgenic plants, the down-regulation of *SS* gene has also led to the coordinated overexpression of *ADS*, *CYP71AV1* and *CPR* genes together with the overproduction of artemisinin although no fully perfect correlation among the available experimental data has been shown.

Abbreviations



ADS:	amorphaadiene synthase
<i>asSS</i> :	anti-sense squalene synthase gene
CPR:	cytochrome P450 reductase
<i>CYP71AV1</i> :	cytochrome P450 monooxygenase
FPP:	farnesyl diphosphate
HPLC:	high-performance liquid chromatography
MVA:	mevalonate
RFQ-PCR:	real-time fluorescent quantitative polymerase chain reaction
SS:	squalene synthase

Supporting information available online at <http://www.thieme-connect.de/ejournals/toc/plantamedica>

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Introduction



Artemisinin, as a naturally derived antimalarial sesquiterpene lactone with a unique endoperoxide architecture, is accumulated in a trace amount in *Artemisia annua* (annual or sweet wormwood, Asteraceae) [1]. Efforts aiming at enhanced artemisinin production by conventional breeding, somatic propagation, and genetic modification have been pursued since the last decade [2]. Much progress on artemisinin overproduction by the metabolic engineering strategy has been made in most recent years [3], [4], relying on our knowledge regarding the artemisinin biogenetic pathway [5], [6]. Akhila first proposed ar-

temisinin as being originated via the classical MVA pathway [7], which was supported by other authors [8], [9], [10]. In *A. annua*, the committed step specific to artemisinin biosynthesis is the cyclization of FPP, which is catalyzed by a sesquiterpene cyclase, *ADS*, to generate amorpha-4,11-diene [11]. Subsequently, amorpha-4,11-diene is subjected to the sequential redox reactions catalyzed by *CYP71AV1* and consequently gives rise to a series of artemisinin-like sesquiterpene intermediates, in which artemisinic alcohol, artemisinic aldehyde and artemisinic acid have been experimentally confirmed in the engineered yeast (*Saccharomyces cerevisiae*) that co-expresses the *ADS* gene with the *CYP71AV1* and *CPR* genes of *A. annua* [12].

On the MVA pathway, FPP is a crucial intermediate to other terpenoids with versatile structures and functions. In *A. annua*, FPP serves as a common precursor of artemisinin and steroids that is diverted by ADS and SS through two distinct and competitive pathways [13]. Therefore, the conversion step catalyzed by either of these two enzymes represents a metabolic shunting point at which the cellular carbon flux is directed towards steroids or artemisinin, depending on their competition with the available FPP pool. Indeed, SS has been considered as a regulatory enzyme for the redirection of carbon flux [14]. Kudakasseril et al. [15] found that the inhibition of sterol biosynthesis by a squalene monooxygenase inhibitor, naphthipine, and a sterol demethylase inhibitor, miconazol, leads to increased production of artemisinin in *A. annua* shoot cultures.

In our present work, we have attempted to introduce *asSS* cDNA into the genome of *A. annua* by an *Agrobacterium*-mediated transformation approach. To quantitatively evaluate the transcriptomics upon the expression of *asSS* transgene in regard to the production of sterols and artemisinin *en route*, we report here the comparative quantification profiles of SS mRNA as well as of *ADS*, *CYP71AV1* and *CPR* mRNAs, and provide direct evidence for the *asSS*-mediated down-regulation of sterol anabolic SS mRNA and the overexpression of artemisinin biogenetic *ADS*, *CYP71AV1* and *CPR* mRNAs. The present data may reflect the dynamic variations of holistic carbon flux and the redirection of metabolic precursor flow upon the functional expression of *asSS* gene in *A. annua*.

Materials and Methods

Leaf-disk transformation of *A. annua*

A. annua plants, germinated from the seed of a cultivar (Fengshun No.1) grown in Guangdong of China, were cultured on the phytohormone-free 1/2 MS medium in a luminescent chamber at 25 °C and with a photoperiod of 8 h light/16 h dark. The *Agrobacterium*-mediated transformation of *A. annua* was performed as described [16] with minor modifications [17]. Briefly, leaves were cut into pieces (0.5 cm × 0.5 cm) and pre-cultured in the dark for two days on solid MS medium supplemented with 0.5 µg/mL of benzyladenine (BA) and 0.5 µg/mL of naphthalene-acetic acid (NAA). The engineered *A. tumefaciens* LB4404 strain harboring the binary plasmids with *asSS* gene was grown at 28 °C on solid YEB plates supplemented with 25 µg/mL of kanamycin and 50 µg/mL of rifampicin. A single colony of the engineered strain was inoculated into the liquid YEB medium containing 25 µg/mL of kanamycin and 50 µg/mL of rifampicin and cultured overnight at 28 °C with agitation. A diluted strain culture was mixed with the pre-cultured leaf disks for 30 s and incubated at 25 °C on the 1/2 MS plates for two days. After having been transferred onto solid MS medium with 5 µg/mL of BA, 0.5 µg/mL of NAA and 500 µg/mL of carbenicillin, the vessels with infected leaf disks were incubated in an illuminated chamber with the 8 h light/16 h dark photoperiod. After six weeks, shoots were maintained on 1/2 MS medium by sub-culturing at a two-week intervals until rooting. The wild-type and transgenic plants were sub-cultured on the same medium every four weeks for further identification.

Isolation of total RNAs and reverse transcription

Leaves were collected from the wild-type and transgenic *A. annua* plants cultured on the 1/2 MS medium for total RNA isolation by a commercially available Plant RNA Kit (Omega). Re-

verse transcription was performed with the random hexamer primers (RHPs) and by RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer's instructions. Briefly, 1 µg of total RNAs and 0.2 µg of RHPs were mixed and DEPC water was supplemented to 12 µL total volume. The mixture was incubated at 70 °C for 5 min and placed on ice for 2 min. Then, 4 µL of 5 × reaction buffer, 2 µL of 10 mmol/L dNTPs, and 20 units of ribonuclease inhibitor were added to each sample and incubated at 25 °C for 5 min. After adding 1 µL of RevertAid H Minus M-MuLV (200 U), the reaction mixture with 20 µL total volume was centrifuged and incubated first at 25 °C for 10 min, then at 42 °C for 60 min, and finally at 70 °C for 10 min. The cDNA preparation was stored at -20 °C until use.

Quantification of transcripts by RFQ-PCR

In the present quantitative analysis, 18S rDNA was chosen as the inner reference gene. The standard curves of 18S rDNA and SS, *ADS*, *CYP71AV1* and *CPR* cDNAs were plotted with corresponding recombinant plasmids constructed in this laboratory, pROK-SS, pET-ADS, pMD-CYP71AV1, pMD-CPR and pMD-B5. The plasmid DNAs were extracted and digested by *Hind* III (only for pMD-B5) or *Bam*H I (for other plasmids), all digested fragments with a series of concentrations, 19, 37, 30, 14 and 3.5 µg/mL for SS, *ADS*, *CYP71AV1*, *CPR* cDNAs and 18S rDNA, were then diluted into 10⁷, 10⁶, 10⁵, 10⁴, 10³, and 10² copies/µL based on the following formula: copy numbers = (concentration/molecular weight) × 6.023 × 10²³, from which the regression equations and coefficients were calculated.

The RFQ-PCR assay was carried out with the specific primers designed for each gene (Table 1) and by the commercially available SYBR Green Real Time PCR Master Mix (TOYOBO). For performing the amplification reaction, 12.5 µL of SYBR Green Real Time PCR Master mixture, 10 µM of primer pairs each, and 2.5 µL of 10-fold diluted cDNA were mixed, and the sterile distilled water was finally supplemented to 25 µL total volume. The quantitative assay was evaluated in an ABI 7300 Fluorescent Quantitative PCR instrument under the following condition: 95 °C for 60 s, 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 45 s, 40 cycles in total.

Determination of artemisinin content by HPLC

Artemisinin was determined by HPLC as previously described [18]. Briefly, leaves were toasted in a 50 °C oven and ground into fine powder. Approximately 30 mL of acetone were mixed with 300 mg of the sample powder for artemisinin extraction by the sonication method. After filtration and drying, the extract was transferred into a 25-mL flask and diluted by acetone to 10 mL total volume. For artemisinin quantification, 20 µL of the sample solution were injected into the sampling outlet of the HPLC instrument (Dionex-100). The tests were carried out in a Phenomenex BDS C18 column (250 mm × 4.6 mm, 5 µm particle size). The acetonitrile-water mixture (48:52, V/V) was used as a mobile phase with 1.00 mL/min flow rate, 30 °C column temperature, and 216 nm wave length for detection. Artemisinin content (mg/g DW) was calculated from artemisinin amount (µg)/sampling volume (20 µL) × total volume (10 mL)/sample weight (300 mg) × 1000.

To prepare the positive control sample, 10 mg of artemisinin (Sigma) was dissolved in methanol, then the concentration of artemisinin was calibrated to 0.4 mg/µL. For plotting a standard curve of the artemisinin content, aliquots of 2, 4, 8, 12, 16, and

Table 1 The primers designed for amplification of *A. annua* *SS*, *ADS*, *CYP71AV1*, and *CPR* gene by RFQ-PCR

Tested genes	Primers		Amplicon length (bp)
Name	GenBank accession number	Name	Sequence
<i>SS</i>	AY445506	SS-3	5'-cggactaaaacaatggctgatg-3'
		SS-4	5'-gtgatggtcgttgggcatt-3'
<i>ADS</i>	AF241826	ADS-5	5'-gtcgaatgggtgtctctgc-3'
		ADS-6	5'-ttcttcttgctcggccttg-3'
<i>CYP71AV1</i>	DQ872632	CYP71AV1-3	5'-tggttctgccaagagatgc-3'
		CYP71AV1-4	5'-ggctcctattatcgcaagacg-3'
<i>CPR</i>	DQ984181	CPR-3	5'-gataacgaagacgggacacca-3'
		CPR-4	5'-gctcaaacatcgcatagga-3'
18S rRNA	To be accessed	18S-3	5'-gcaacaacccccgacttctg-3'
		18S-4	5'-tgcatccgtcgagttatca-3'

20 μ L of artemisinin solution were individually injected into the sampling outlet of a Dionex-100 for quantification as above procedure.

Statistical analysis

The quantification of all transcripts were represented in terms of the relative mRNA level (abbreviated simply by the mRNA level), which was defined as the specific mRNA copy numbers/18S rRNA copy numbers)_{transgenicplant} / (the specific mRNA copy numbers/18S rRNA copy numbers)_{wild-typeplant} $\times 100\%$. All data were expressed as the means $\pm$ standard deviations (SD) from three biological replicates with three samples each, and statistical analysis of the independent sample tests including *t*-test for equality of means was performed with the SPSS 11.5 for Windows software package (Statistica). The statistically significant difference values ($P < 0.05$) are labeled with a single asterisk (*), and the statistically very significant difference values ($P < 0.01$) are labeled with double asterisks (**).

Supporting information

The morphological comparison of transgenic plants with the wild-type control and the melting curves of strand dissociation for 18S rRNA, *SS* mRNA, *ADS* mRNA, *CYP71AV1* mRNA and *CPR*

mRNA are available as Supporting Information or at http://i.cn.yahoo.com/qpzeng03/blog/p_1/.

Results and Discussion

▼ In the present study, none of the transgenic plants died during subculture. Nevertheless, T36 and T47 still demonstrated some growth retardation in plant height and root length, implying the affected phenotypic traits being transgene-dependent and *SS* gene-involved. In particular, the young seedling of T47 grew slowly with shortened height and sporadic roots or even without roots (see the black-white figure, **Fig. 1**, in the main text and the color figure, **Fig. 1S**, in the Supporting information). The partial suppression of the *SS* gene may, therefore, delay the development of transgenic plants in the early growth stage although their differentiation and organogenesis into adult plants are unlikely to be severely inhibited.

It is quite certain that the expression of the *SS* gene should be only partially attenuated rather than completely blocked, because the *SS* enzyme that catalyzes the generation of squalene and finally leads to the production of sterols that are essential components of biomembranes is implicated in cell division and

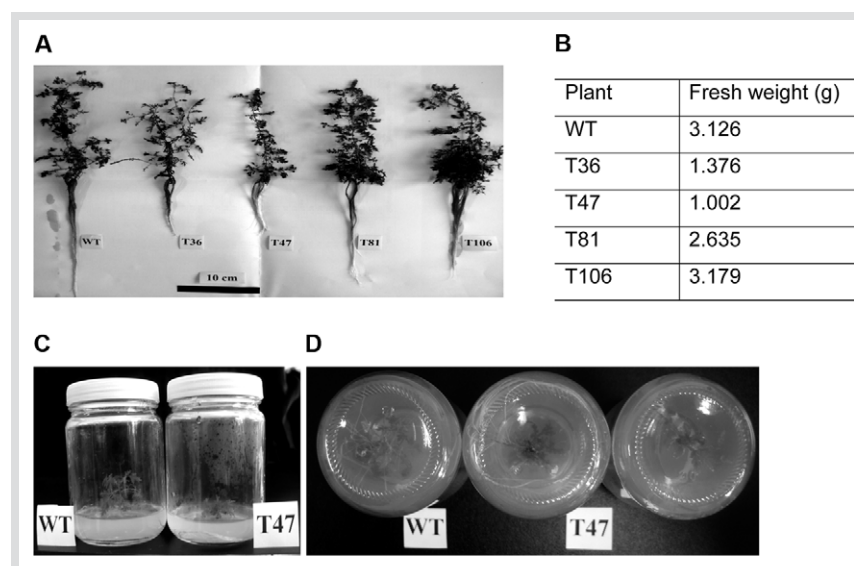


Fig. 1 Morphological comparison of transgenic plants with the wild-type control. **(A)** Whole plants cultured on the phytohormone-free 1/2 MS medium for two months. **(B)** The fresh weight of whole plants illustrated in **(A)**. **(C)** Seedlings cultured on the phytohormone-free 1/2 MS medium for three weeks. **(D)** The bottom view of three week-old seedlings of the control (WT, left) and transgenic plants (T47) with roots (middle) or without roots (right).

propagation [19]. Otherwise, deleterious embryonic lethality may occur in the plant cell whose *SS* gene has been deleted and the sterol constituents are no longer available as described in our previous report [20], where the endogenous *SS* gene had been replaced by a composite *SS1-GFP-SS2* gene cassette for the targeting gene knockout.

To define the *SS* mRNA level *in planta*, the simultaneous amplification of reference 18S rRNA and target *SS* mRNA were conducted for the wild-type and transgenic plants. In practice, the melting curves of 18S rRNA and *SS* mRNA were drawn prior to quantification to monitor the specificity of the amplification reactions. A singular peak was formed by a group of melting curves that represented the strand dissociation mode for each amplification, as illustrated in Fig. 2S through Fig. 6S in the Supporting Information. These singlet curve peaks imply a high amplification specificity that warrants accurate quantification of transcript copy numbers.

All transgenic plants showed a significantly lower *SS* mRNA level than the wild-type control, as shown in Fig. 2. Among them, T81, T47 and T106 demonstrated a significantly declined *SS* mRNA level that corresponded to 92.6%, 85.1% and 86.5% reduction of the control, whereas T36 only exhibited a moderately declined *SS* mRNA level equal to a 44.7% decrease of the control. In other words, there were only 7.4%, 13.5%, 14.9% and 55.3% of *SS* mRNA in T81, T47, T106 and T36, respectively. This means that T81, T47 and T106 may have higher efficiencies of antisense suppression than T36 with the difference being approximately 4–8-fold. Although the transcript length, copy numbers and some other factors have been proposed to modulate the degree of gene silencing [21], the *bona fide* reason for such a discrepancy in the down-regulation of intrinsic *SS* gene by the introduced *asSS* gene needs more detailed analysis among the transgenic plants with the same integrated *asSS* gene.

To probe the possible relevance of *SS* gene to three artemisinin biosynthetic genes, *ADS*, *CYP71AV1* and *CPR*, the copy numbers of mRNA transcribed from these genes were quantified in the wild-type and transgenic plants. Consequently, an appreciably marked elevation of *ADS* mRNA level up to 3.38-, 3.18- and 1.9-fold of the control was estimated in T47, T106 and T81 (Fig. 3A). There is nearly a perfect synchrony between the elevation of *ADS* mRNA level and the decline of *SS* mRNA level in T47, T81, and T106. As to T36, its *ADS* mRNA level is almost equal to that of the control, which is well consistent with the slight drop of *SS* mRNA level in this transgenic plant (Fig. 2).

As shown in Fig. 3B, the maximal and minimal *CYP71AV1* mRNA levels were observed in T106 (4.4-fold as the control) and T47 (1.02-fold as the control), while a moderate level was seen in T81 (2.4-fold) and T36 (1.9-fold). There is a complicated situation regarding the correlation of declined *SS* mRNA level with the elevated *CYP71AV1* mRNA level. For example, T81 and T106 have a lower *SS* mRNA level coupled with a higher *CYP71AV1* mRNA level, whereas T47 has a lower *SS* mRNA level combined with a lower *CYP71AV1* mRNA level. The reason for such inconsistent fluctuation between *SS* mRNA and *CYP71AV1* mRNA in T47 awaits further explanation.

With regard to *CPR* mRNA, the elevation of transcript level was observed in T81 (2.5-fold), T47 (2.3-fold) and T36 (1.9-fold), but a surprising decline of *CPR* mRNA, even slightly less than the control, was seen in T106 (Fig. 3C). Interestingly, except for T106, all other transgenic plants exhibited good correlation of a low *SS* mRNA level with a high *CPR* mRNA level.

We suggest here, therefore, that the down-regulation of the *SS* gene may simultaneously be followed by, but does not equally result in, the simultaneously enhanced expression of *ADS*, *CYP71AV1* and *CPR* genes, which represents a perplexing transcript profile that is likely complicated by the downstream metabolic channels toward the diverged terpenoids and other metabolites.

On the other hand, as illustrated in Fig. 4, an increased artemisinin content was determined in two transgenic plants, T47 and T81, with a lower *SS* mRNA level and a decreased artemisinin content was measured in one transgenic plant, T36, with a higher *SS* mRNA level. However, it is not the case for T106, which demonstrated a relatively lower *SS* mRNA level but also a surprisingly lower artemisinin content.

Furthermore, no reasonable correlations of the enhanced artemisinin production can be established with the elevated *ADS*, *CYP71AV1* and *CPR* mRNA levels, thereby reflecting a complicated consequence in redirecting the cellular carbon flux by suppressing only one competitive branch pathway. Nevertheless, if T106 was omitted due to its abnormal mRNA level and unexpectedly lowest artemisinin content, we feel safe to conclude that the “saved” carbon resource originally pooled in sterols via squalene has already “driven” the transcript level being higher and “added” it to artemisinin that locates on another competitive metabolite chain in *A. annua*. Table 2 lists the quantitative data of *SS*, *ADS*, *CYP71AV1* and *CPR* mRNA and artemisinin in the wild-type and transgenic plants.

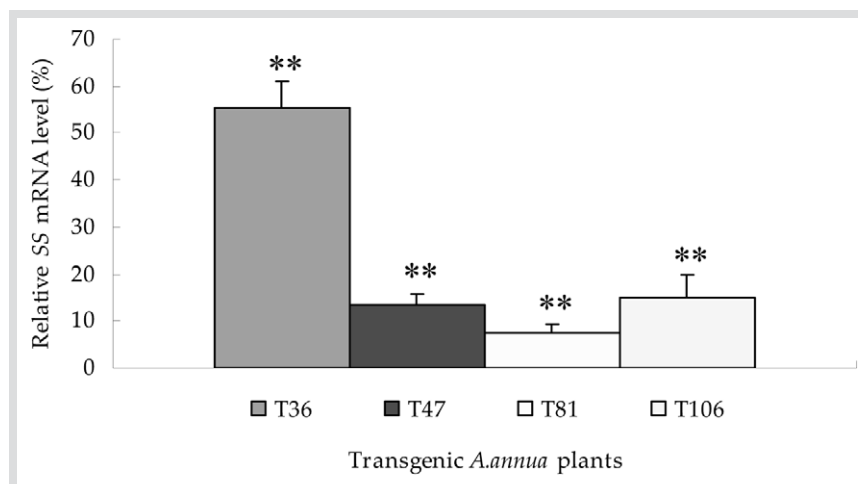


Fig. 2 Down-regulation of the *SS* gene by the introduced *asSS* gene in transgenic *A. annua* plants. All data were expressed as the means $\pm$ standard deviations (SD) from three biological replicates with three samples each. The quantitative results are calculated from the standard curves of copy numbers with $Y = -3.3458X + 37.2536$, $R^2 = 0.999050$ for *SS* gene and $Y = -3.2968X + 38.1508$, $R^2 = 0.999887$ for 18S rRNA gene. The double asterisks (**) represent statistically very significant difference ($P < 0.01$) of transgenic plants from the control.

As shown in **Table 2**, the average content of artemisinin in transgenic plants did not increase proportionally with the down-regulation of *SS* mRNA although the transgenic plant with an extraordinarily lower *SS* mRNA level, T47, gave rise to a maximal content reaching 12.8 mg/g dry weight in one single test or 9.9 mg/g dry weight on average, suggesting that the suppressed *SS* mRNA may only “narrow” the route that directs the carbon flux toward squalene, but not certainly guide it to artemi-

sinin. Therefore, more powerful pulling force is needed to facilitate the accumulation of more amounts of artemisinin.

Previous studies have shown that, when challenged with fungal elicitors, the suspension cultured cells of *Nicotiana tabacum* displayed significant reduction of *SS* mRNA levels [21] and the coordinated induction of enzymes for sesquiterpene phytoalexin biosynthesis [22], [23], [24]. Interestingly, we have recently found that at transient exposure to low temperature (4 °C for 30 min),

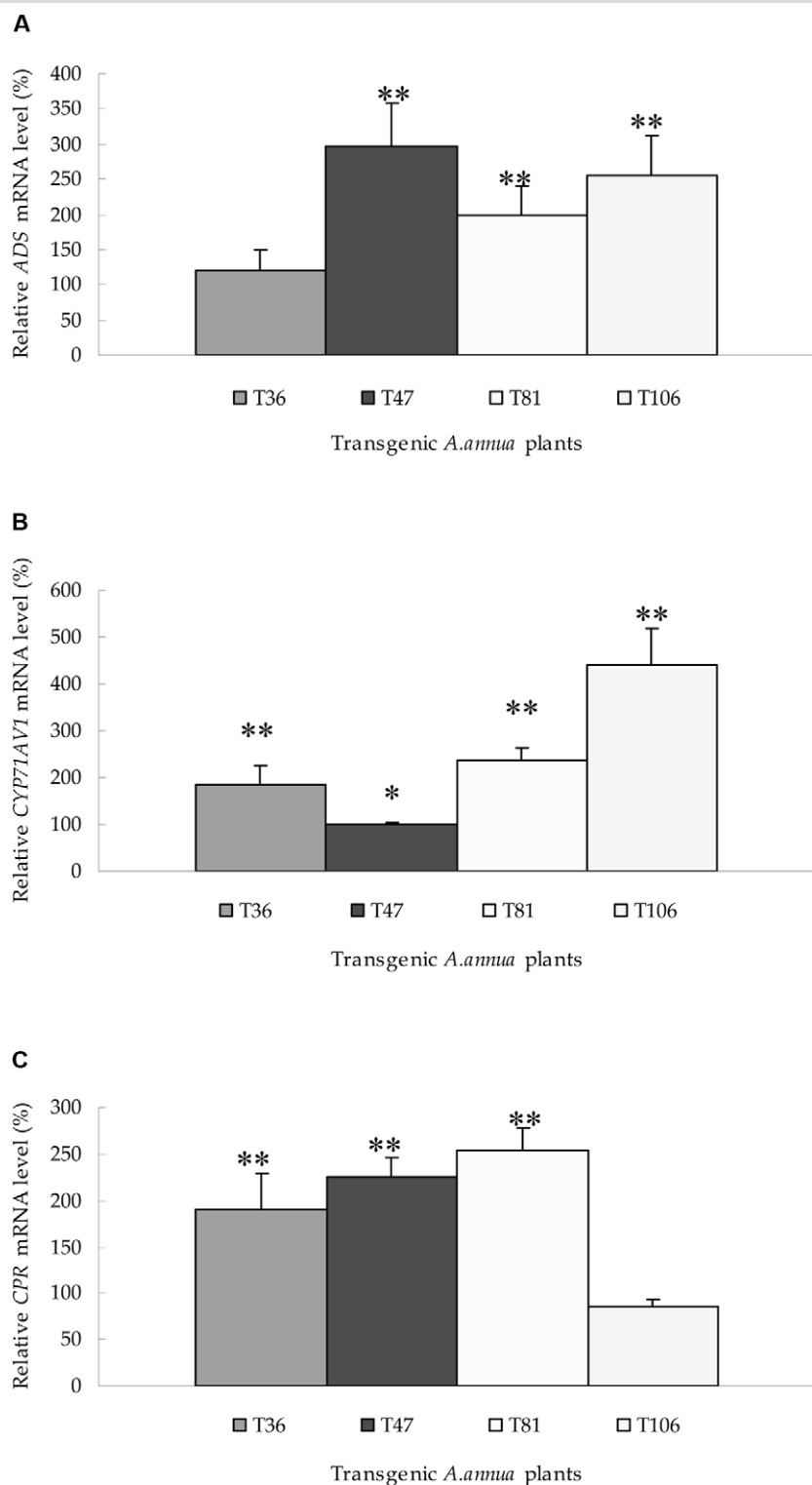


Fig. 3 Overexpression of *ADS* (A), *CYP71AV1* (B) and *CPR* (C) genes in transgenic *A. annua* plants. All data were expressed as means $\pm$ standard deviations (SD) from three biological replicates with three samples each. The quantitative data were calculated from the standard curves of copy numbers by the regression equation, $Y = -3.2152X + 36.3365$ with the coefficient of $R^2 = 0.997308$ for *ADS* gene, $Y = -3.2826X + 32.7893$, $R^2 = 0.999833$ for *CYP71AV1* gene, $Y = -3.397X + 36.6638$, $R^2 = 0.999091$ for *CPR* gene, and $Y = -3.2968X + 38.1508$, $R^2 = 0.999887$ for 18S rRNA gene. The single asterisk (*) represents statistically significant difference ($P < 0.05$) of transgenic plants from the control, and the double asterisks (**) represent statistically very significant difference ($P < 0.01$) of transgenic plants from the control.

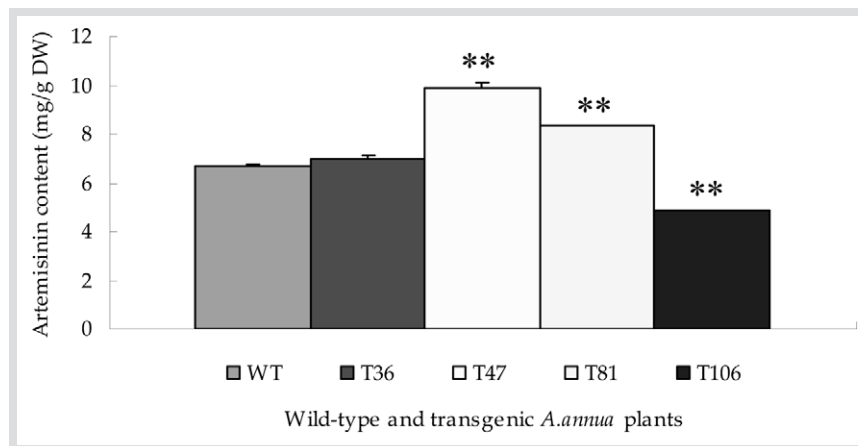


Fig. 4 Determination of artemisinin content in the wild-type and transgenic plants. The quantitative data were calculated from the standard curves of artemisinin content by the regression equation, $Y = 0.4612X + 0.0236$, $R^2 = 0.9995$. DW represents the dry weight of leaf tissues; WT is the wild-type control; T36, T47 and T81 are transgenic plants. The double asterisks (**) label the statistical implication with very significant difference ($P < 0.01$).

Table 2 Comparison of the transcript level with the artemisinin content in transgenic *A. annua* plants and the wild-type control

Plant line	Relative transcript level ($\times 10^{-5}$)				Artemisinin (mg/g)
	SS	ADS	CYP71AV1	CPR	
WT	4.227 ± 0.059	1.523 ± 0.500	1.257 ± 1.147	1.993 ± 0.322	6.713 ± 0.075
T36	2.337 ± 0.221*	1.555 ± 0.589	2.053 ± 1.679**	3.626 ± 0.919**	7.008 ± 0.101
T47	0.571 ± 0.099**	5.143 ± 2.646**	1.280 ± 0.876*	4.390 ± 0.912**	9.909 ± 0.222**
T81	0.315 ± 0.080**	2.890 ± 1.748**	3.987 ± 3.857**	3.670 ± 1.967**	8.337 ± 0.012**
T106	0.628 ± 0.198**	4.850 ± 3.386**	8.560 ± 8.220**	1.177 ± 0.168	4.843 ± 0.012**

Note: The relative transcript level is defined as the copy numbers of specific mRNA/the copy numbers of 18S rRNA. The statistical difference from the control (WT) is indicated by the single asterisk (*, $P < 0.05$) or double asterisks (**, $P < 0.01$).

A. annua plants really displayed incremental elevation of ADS and CYP71AV1 mRNA levels up to eleven- and seven-fold, respectively. Accordingly, the chilling stress-exposed plants generated more artemisinin than the control line in the magnitude of content as 66.7 – 95.6% [18]. These results seem to imply that the chilling stress-induced overexpression of ADS and CYP71AV1 genes is crucial for artemisinin overproduction, and also demonstrate a possibility of pulling the carbon flow toward the artemisinin biogenic pathway by chilling stress exposure or other extreme environmental stimuli [25].

We propose here that the multiple cascades for genetic and environmental modulation in *A. annua* may be naturally operated for coordinated carbon flux distribution throughout the entire metabolic network-regulated biogenetic pathways for artemisinin accumulation. Moreover, we are confident to predict that upon exposure to stresses, the transcript levels of ADS and CYP71AV1 should be exceedingly up-regulated and would accelerate artemisinin accumulation in transgenic *A. annua* plants, which is the next goal of our ongoing investigation for further elucidating the homeostatic and regulatory mechanisms of such putative stress-inducible patterns.

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