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# Branch Pathway Blocking in *Artemisia annua* is a Useful Method for Obtaining High Yield Artemisinin

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**There are many biosynthetic pathways competing for the metabolic flux with the artemisinin biosynthetic pathway in *Artemisia annua* L. To study the relationship between genes encoding enzymes at branching points and the artemisinin biosynthetic pathway, β-caryophyllene, β-farnesene and squalene were sprayed on young seedlings of *A. annua*. Transient expression assays indicated that the transcription levels of β-caryophyllene synthase (*CPS*), β-farnesene synthase (*BFS*) and squalene synthase (*SQS*) were inhibited by β-caryophyllene, β-farnesene and squalene, respectively, while expression of some artemisinin biosynthetic pathway genes increased. Thus, inhibition of these genes encoding enzymes at branching points may be helpful to improve the artemisinin content. For further study, the expression levels of four branch pathway genes *CPS*, *BFS*, germacrene A synthase (*GAS*) and *SQS* were down-regulated by the antisense method in *A. annua*. In anti-CPS transgenic plants, mRNA levels of *BFS* and *ADS* were increased, and the contents of β-farnesene, artemisinin and dihydroartemisinic acid (DHAA) were increased by 212, 77 and 132%, respectively. The expression levels of *CPS*, *SQS*, *GAS*, amorpha-4,11-diene synthase (*ADS*), amorphadiene 12-hydroxylase (*CYP71AV1*) and aldehyde dehydrogenase 1 (*ALDH1*) were increased in anti-BFS transgenic plants and, at the same time, the contents of artemisinin and DHAA were increased by 77% and 54%, respectively, and the content of squalene was increased by 235%. In anti-GAS transgenic plants, mRNA levels of *CPS*, *BFS*, *ADS* and *ALDH1* were increased. The contents of artemisinin and DHAA were enhanced by 103% and 130%, respectively. In anti-SQS transgenic plants, the transcription levels of *BFS*, *GAS*, *CPS*, *ADS*, *CYP71AV1* and *ALDH1* were all increased. Contents of artemisinin and DHAA were enhanced by 71% and 223%, respectively, while β-farnesene was raised to 123%. The mRNA level of artemisinic aldehyde Δ11(13) reductase (*DBR2*) had changed little in almost all transgenic plants.**

**Keywords:** Antisense • *Artemisia annua* L • Artemisinin biosynthesis • β-Caryophyllene synthase (*CPS*) • β-farnesene synthase (*BFS*) • Germacrene A synthase (*GAS*) • Squalene synthase (*SQS*).

**Abbreviations:** ADS, amorpha-4,11-diene synthase; ALDH1, aldehyde dehydrogenase 1; BFS, β-farnesene synthase; CPS, β-caryophyllene synthase; CYP71AV1, amorphadiene 12-hydroxylase; DBR2, artemisinic aldehyde Δ11(13) reductase; DHAA, dihydroartemisinic acid; DMAPP, dimethylallyl diphosphate; ECS, *epi*-cedrol synthase; FDS, farnesyl diphosphate synthase; FPP, farnesyl diphosphate; GAS, germacrene A synthase; HMGR, 3-hydroxy-3-methyl-glutaryl coenzyme A reductase; MS, Murashige and Skoog; qRT-PCR, quantitative real-time PCR; SQS, squalene synthase.

## Introduction

In the world, more than 1 billion people are under threat from the risk of malaria. The antimalarial drug artemisinin is extracted from the trichomes of leaves or buds of the Chinese traditional herb *Artemisia annua* L. However, the artemisinin content in *A. annua* is low and varies from 0.01% to 1%. It is reported that the artemisinin content in some cultivated varieties reaches up to 1.4% but in wild types it is usually about 0.4%. Recently, malarial parasite resistance to artemisinin has been found, which may be devastating for many poor Africa and South East Asia countries (Brown 2010, Graham et al. 2010).

In an attempt to reduce the risk of resistance of the malarial parasite, Artemisinin Combination Therapy (ACT) was developed, which was made up of an artemisinin derivate and another antimalarial drug to eliminate the development of resistance. Artemisinin and its derivatives have also been reported to repress cancer and tumor activity (Tran et al. 2014). Although artemisinin can be obtained by chemical synthesis, the method is uneconomical and difficult to obtain for the poorer African and South East Asian countries. One of the promising approaches is to produce its precursor in *Saccharomyces cerevisiae* (Westfall et al. 2012) or *Escherichia coli* (Anthony et al. 2009) by recombinant microbial pathway for semi-synthetic production of artemisinin. Another method is plant genetic engineering. This method is likely to lower the cost of artemisinin by increasing the biomass of *A. annua* plants and the content of artemisinin, which has aroused great interest in this field (Covello 2008; Tang et al. 2014).

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In the process of artemisinin biosynthesis, one molecule of isopentenyl diphosphate (IPP) and one molecule of dimethylallyl diphosphate (DMAPP) are condensed to geranyl diphosphate (GPP). GPP condenses with one unit of IPP and generates farnesyl diphosphate (FPP). There is a cross-talk between the mevalonate (MVA) pathway and the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway, which was confirmed by $^{13}C$ labeling studies (Towler and Weathers 2007, Schramek et al. 2010). FPP is an important product at the branch point of terpenoid metabolism. It is converted into β-farnesene, β-caryophyllene, germacrene A, *epi*-cedrol, squalene and amorphadiene by β-farnesene synthase (BFS), β-caryophyllene synthase (CPS), germacrene A synthase (GAS), *epi*-cedrol synthase (ECS), squalene synthase (SQS) and amorpha-4,11-diene synthase (ADS) (Bouwmeester et al. 1999, Mercke et al. 1999, Mercke et al. 2000, Cai et al. 2002, Picaud et al. 2005, Bertea et al. 2006), respectively. Amorphadiene is converted into dihydroartemisinic acid (DHAA) by a series of enzymes, such as amorphadiene 12-hydroxylase (CYP71AV1), artemisinic aldehyde Δ11(13) reductase DBR2) and aldehyde dehydrogenase 1 (ALDH1) (Teoh et al. 2006, Zhang et al. 2008, Teoh et al. 2009, Rydén et al. 2010) (**Fig. 1**).The conversion of DHAA to artemisinin is suggested to be an enzyme-independent reaction (Brown 2010).

In recent years, many genes at branching points have been widely studied in *A. annua*. In the MVA pathway, mevinolin specifically inhibits the 3-hydroxy-3-methyl-glutaryl coenzyme A reductase (HMGR) activity (Gunde-Cimerman et al. 1993). Under treatment with mevinolin, the artemisinin concentration can decrease by 80% (Towler and Weathers 2007). Fosmidomycin (FOS) inhibits the activity of 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR) (Kuzuyama et al. 1998) of the MEP pathway. When *A. annua* is exposed to FOS, the artemisinin content can also decrease by 70% (Towler and Weathers 2007). Miconazole, naphtiphine or terbinafine are used as inhibitors for blocking sterol biosynthesis (Towler and Weathers 2007, Caretto et al. 2011). When naphtiphine is added to the shoot culture medium, artemisinin content may increase a lot (Towler and Weathers 2007, Caretto et al. 2011). Upon down-regulation of expression of *ERG9* (which encodes SQS) in *S. cerevisiae*, amorphadiene production increases 2-fold (Ro et al. 2006). β-Caryophyllene, a type of volatile compound, is essential for attracting nematodes and defense against pathogen attack. Another sesquiterpene volatile compound, β-farnesene, is a well-known aphid alarm pheromone, which blocks other aphids feeding. Germacrene A is also regarded as an alarm pheromone in aphids in many reports (Bowers et al. 1977). Squalene is involved in plant defense reactions (Beale et al. 2006, Köllner et al. 2008, Manavalan et al. 2012). When the branch pathway gene *SQS* is silenced by employing RNA interference (RNAi) techniques, the content of artemisinin increases to 31.4 mg $g^{-1}$ DW (Zhang et al. 2009). *CPS*, another gene competing for FPP with *ADS*, was down-regulated by the antisense method, which increases the content of artemisinin to 55% (Chen et al. 2011).

The present study reports the effects of blocking the competitive pathways of artemisinin biosynthesis by silencing the branch pathway genes including *BFS*, *CPS*, *SQS* and *GAS*, and analyzes the metabolic flux and gene expression levels to obtain high yields of artemisinin in transgenic plants. These findings demonstrate for the first time that inhibition of the mRNA levels of *SQS* and *BFS* can direct the carbon flow mainly through FPP to the artemisinin biosynthetic pathway and increase the content of artemisinin. Moreover, as the first comprehensive study on inhibition of encoding enzymes at branching points, we analyzed the mRNA levels of branching pathway genes and artemisinin biosynthetic pathway genes, and provide the first evidence that inhibition of *BFS* and *SQS* can increase the expression of genes in the artemisinin biosynthetic pathway and the content of artemisinin.

## Results

### Gene expression in response to terpenoid treatment

The relationship between the artemisinin biosynthetic pathway and the sesquiterpene branch pathway is complex (Towler and Weathers 2007, Schramek et al. 2010, Olofsson et al. 2011). To understand the relationship, the expression of key genes of the artemisinin biosynthetic pahway and competing pathways was measured and analyzed under treatment with the terpenoids β-caryophyllene, β-farnesene and squalene. By spraying 3-week-old young seedlings with β-caryophyllene, the transcription level of *CPS* was inhibited and the mRNA levels of *BFS*, *GAS*, *CYP71AV1*, *DBR2* and *ALDH1* were increased (**Fig. 2**). When young seedlings were treated with β-farnesene, the mRNA level of *BFS* decreased, while transcript levels of *CPS* and *CYP71AV1* were increased 3- and 5-fold, repectively (**Fig. 3**). The transcription level of *SQS* was inhibited when seedlings of *A. annua* were exposed to squalene. All the studied genes, such as *BFS*, *CPS*, *GAS*, *ADS*, *CYP71AV1*, *DBR2* and *ALDH1*, were increased 2- to 5-fold (**Fig. 4**). Therefore, down-regulation of genes at branching points can increase gene expression levels of the artemisinin biosynthetic pathway, and may have a great potential for increasing the artemisinin content. For further studies, the antisense method has been employed.

### Expression patterns of the *CPS*, *BFS*, *GAS* and *SQS*

Five-month-old *A. annua* plants grown on substrate were used for gene expression pattern analyses. The total RNAs of *A. annua* were extracted from roots, stems, mature leaves, young leaves and buds. The meristem and the first, second, third, fourth, fifth, sixth and seventh leaves of *A. annua* counted from the apex of the plant (leaf0, leaf1, leaf2, leaf3, leaf4, leaf5, leaf6 and leaf7) (Zhang et al. 2012) were collected for RNA extraction and quantitative real-time PCR (qRT-PCR).

The expression patterns of *CPS*, *BFS*, *GAS* and *SQS* were studied in different tissues (roots, stems, mature leaves and buds) and in leaves from different positions (leaf0, leaf1, leaf2, leaf3, leaf4, leaf5, leaf6 and leaf7). *CPS* was expressed at a higher level in roots and stems relative to other tissues (**Fig. 5A**). This expression pattern indicated that *CPS* had a close connection with its role in defending against pathogen and worm attack (Köllner et al. 2008). In addition, the transcription level of *CPS*

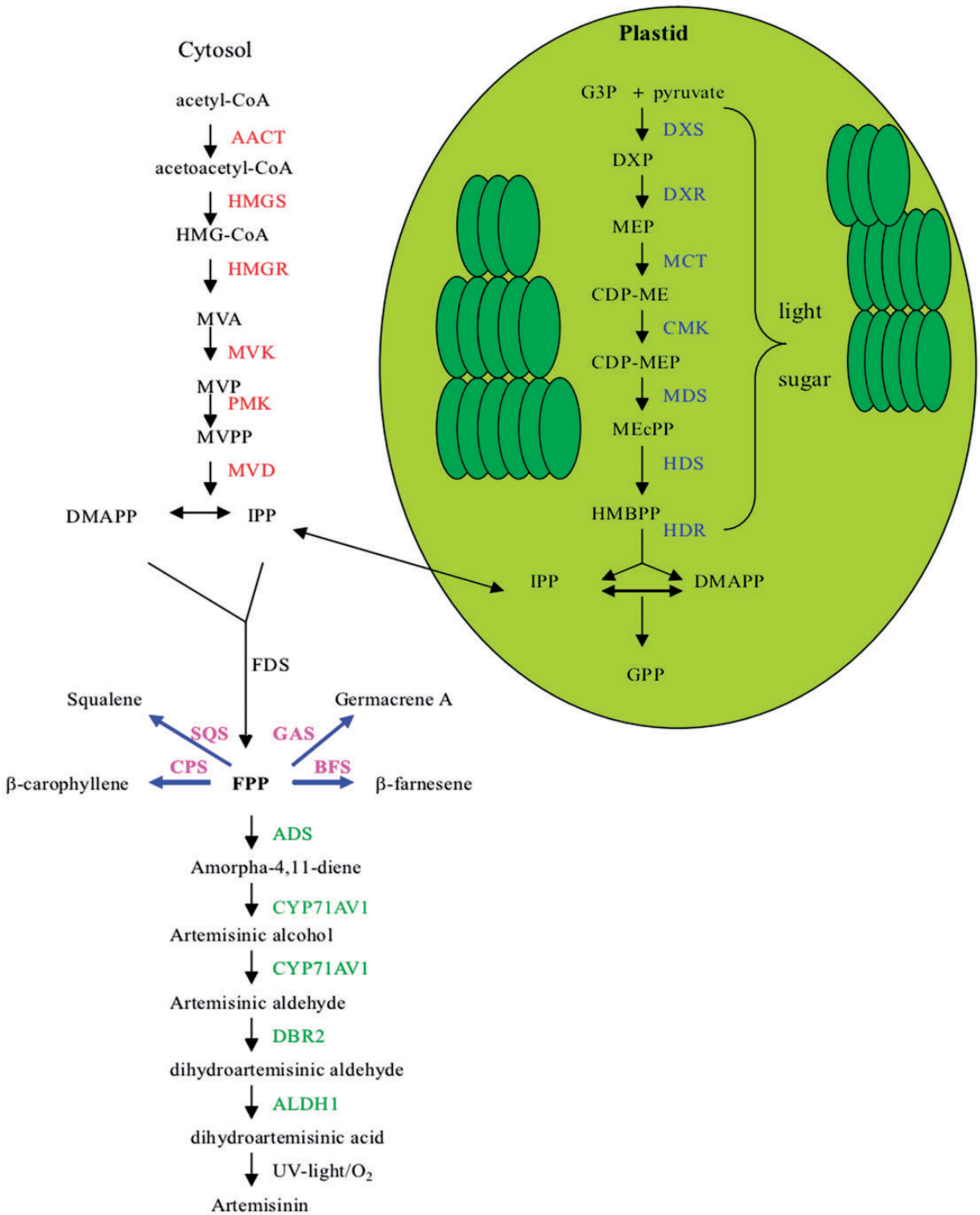


**Fig. 1** Isoprenoid biosynthetic pathways in *Artemisia annua*. The mevalonate (MVA) pathway enzymes are shown in red. The 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway enzymes are shown in blue.The enzymes from the artemisinin biosynthetic pathway are shown in green. There is a cross-talk between the MVA pathway and the MEP pathway. GPP comes from the plastid compartment and generates farnesyl diphosphate (FPP) using IPP from the mevalonate pathway. FPP is the intermediate product at the branch point of terpenoid metabolism and then is transformed into amorpha-4,11-diene and artemisinin. The GenBank accession numbers of the cloned enzymes are: ADS, amorpha-4,11-diene synthase (AF138959); ALDH1, aldehyde dehydrogenase 1 (FJ809784); BFS, β-farnesene synthase (AY835398); CPS, β-caryophyllene synthase (AF472361); CYP71AV1, amorphadiene-12-hydroxylase (DQ453967); DBR2, artemisinic aldehyde Δ11(13) reductase (EU704257); FDS, farnesyl diphosphate synthase (U36376); GAS, germacrene A synthase (DQ447636); HMGR, 3-hydroxy-3-methyl-glutaryl coenzyme A reductase (AF142473); HMGS, 3-hydroxy-3-methyl-glutaryl coenzyme A synthase (GQ468550); SQS, squalene synthase (AY445505); DXR, 1-deoxy-D-xylulose-5-phosphate reductoisomerase (AF182287); DXS, 1-deoxy-D-xylulose-5-phosphate synthase (AF182286).

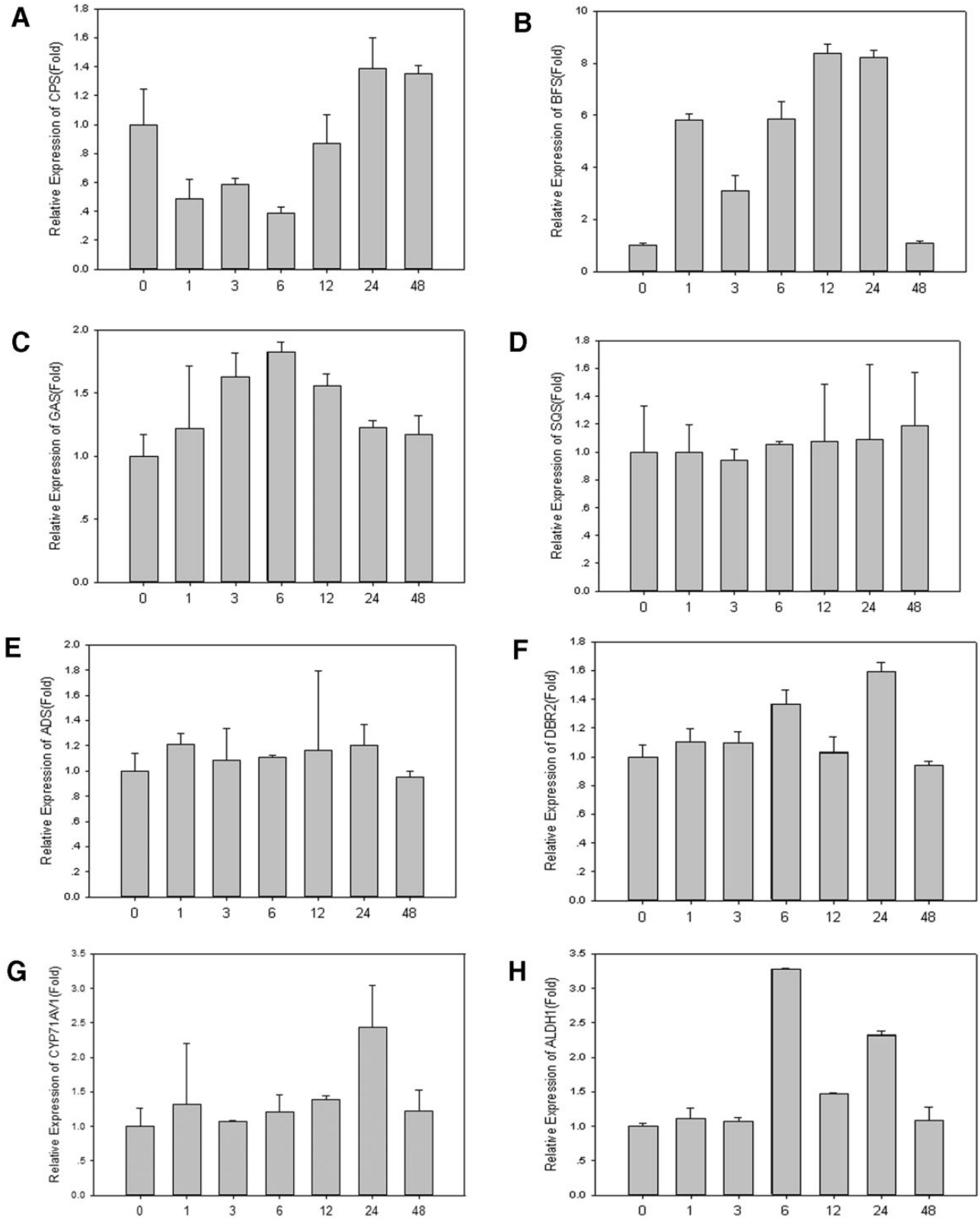


**Fig. 2** Changes in gene expression by spraying with β-caryophyllene. Young seedlings were collected at 0, 1, 3, 6, 12, 24 and 48 h after treatment with β-caryophyllene. Expression of β-caryophyllene synthase (*CPS*) (A), β-farnesene synthase (*BFS*) (B), germacrene A synthase (*GAS*) (C), squalene synthase (*SQS*) (D), amorpha-4,11-diene synthase (*ADS*) (E), artemisinic aldehyde Δ11(13) reductase (*DBR2*) (F), amorphadiene 12-hydroxylase (*CYP71AV1*) (G) and aldehyde dehydrogenase 1 (*ALDH1*) (H) by qRT-PCR analyses. Error bars are the SE ($n = 3$).

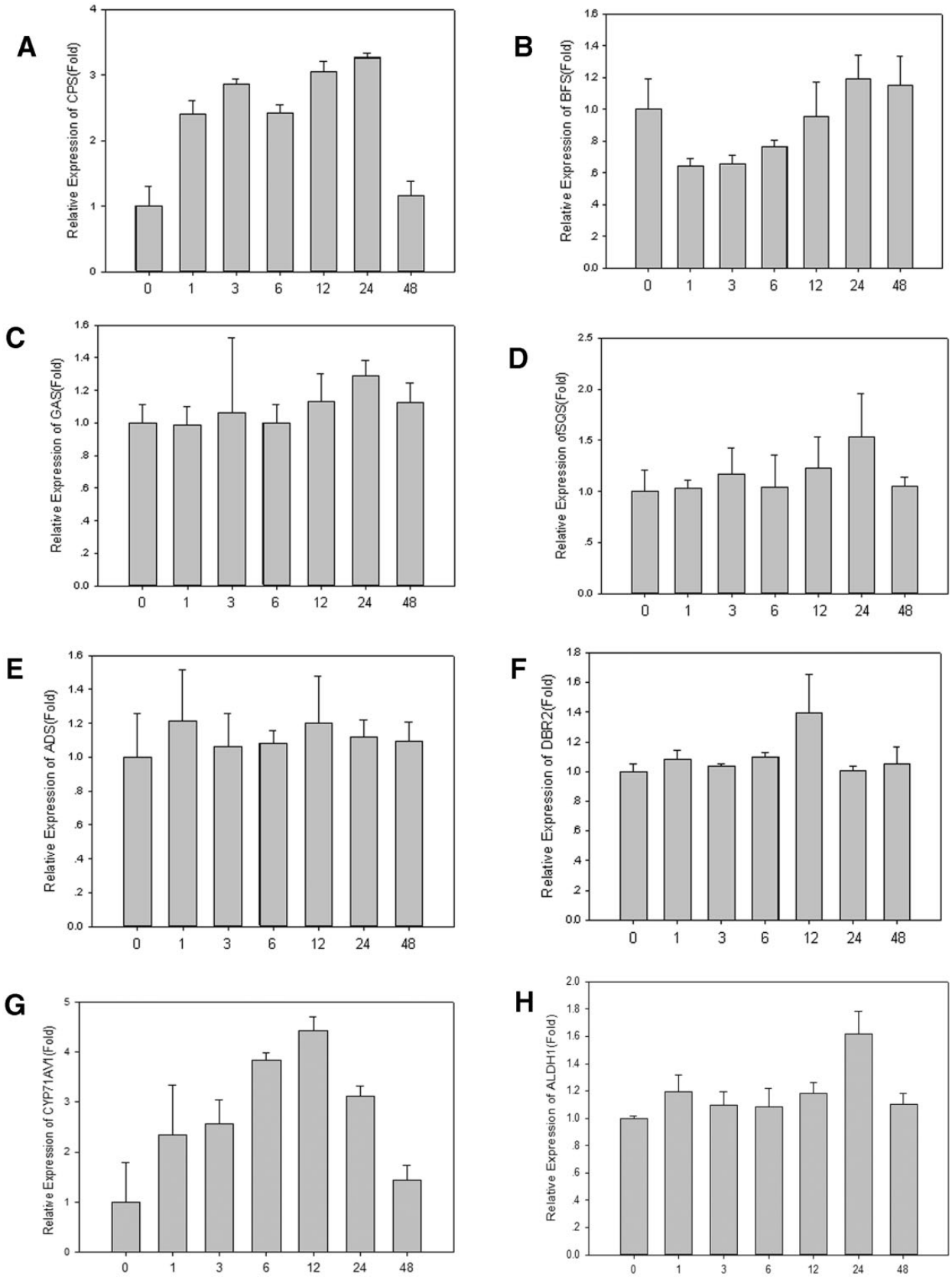


**Fig. 3** Changes in gene expression by spraying with β-farnesene. Young seedlings were collected at 0, 1, 3, 6, 12, 24 and 48 h after treatment with β-farnesene. Expression of β-caryophyllene synthase (*CPS*) (A), β-farnesene synthase (*BFS*) (B), germacrene A synthase (*GAS*) (C), squalene synthase (*SQS*) (D), amorpha-4,11-diene synthase (*ADS*) (E), artemisinic aldehyde Δ11(13) reductase (*DBR2*) (F), amorphadiene 12-hydroxylase (*CYP71AV1*) (G) and aldehyde dehydrogenase 1 (*ALDH1*) (H) byqRT-PCR analyses. Error bars are the SE ($n = 3$).

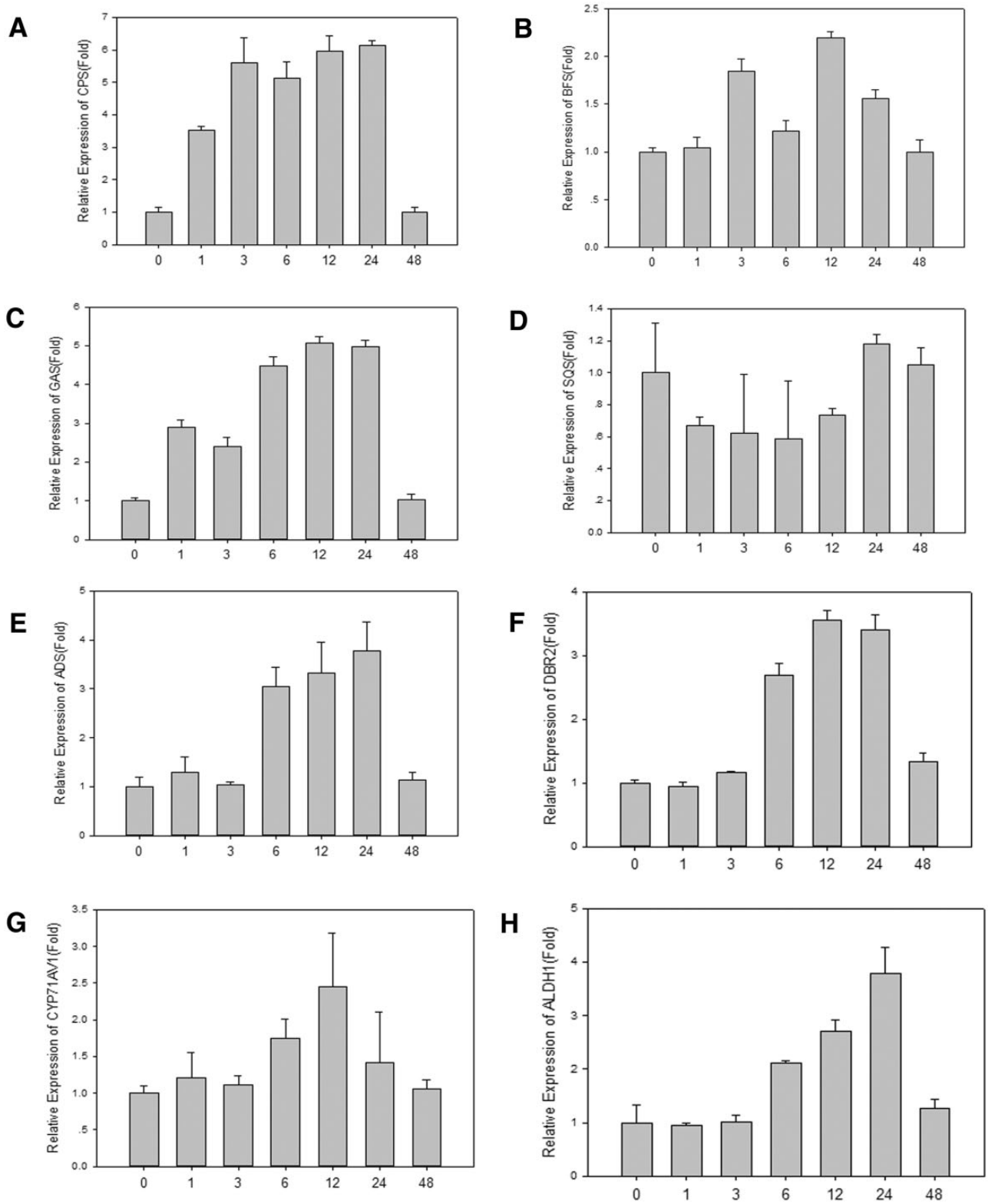


**Fig. 4** Changes in gene expression by spraying with squalene. Young seedlings were collected at 0, 1, 3, 6, 12, 24 and 48 h after treatment with squalene. Expression of β-caryophyllene synthase (*CPS*) (A), β-farnesene synthase (*BFS*) (B), germacrene A synthase (*GAS*) (C), squalene synthase (*SQS*) (D), amorpha-4,11-diene synthase (*ADS*) (E), artemisinic aldehyde Δ11(13) reductase (*DBR2*) (F), amorphadiene 12-hydroxylase (*CYP71AV1*) (G) and aldehyde dehydrogenase 1 (*ALDH1*) (H) by qRT-PCR analyses. Error bars are the SE ($n = 3$).

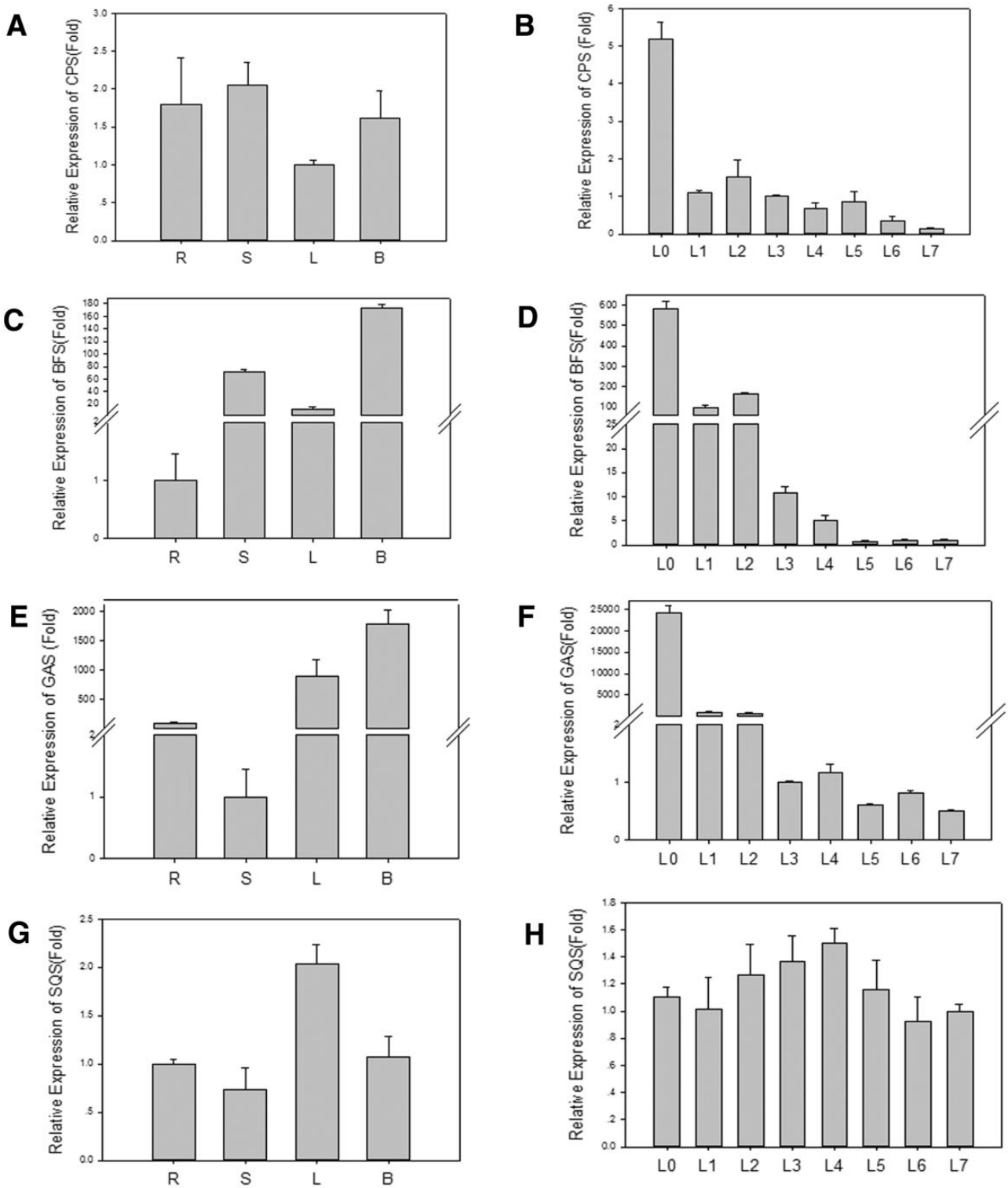


**Fig. 5** Expression patterns of *CPS* (A, B), *BFS* (C, D), *GAS* (E, F) and *SQS* (G, H) in R (roots), S (stems), L (mature leaves), B (buds), L0 (leaf0), L1 (leaf1), L2 (leaf2), L3 (leaf3), L4 (leaf4), L5 (leaf5), L6 (leaf6) and L7 (leaf7). qRT-PCR was used for determination of the expression level. Error bars are the SE ($n = 3$).

was 5-fold higher in leaf0 than in leaf1 to leaf7 (**Fig. 5B**). Young leaves are fragile and often attacked by worms. Therefore, it is necessary to produce a lot of β-caryophyllene for self-protection. *BFS* and *GAS* were mainly expressed in buds and young leaves (**Fig. 5C–F**). Both *BFS* and *GAS* were expressed at the highest level in leaf0, leaf1 and leaf2. In leaf6 and leaf7, their mRNA levels were hardly detected. The expression patterns of *GAS* and *BFS* were consistent with that of *ADS*. There was no

significant difference in the expression level of *SQS* among the roots, stems, leaves, buds and different leaves (leaf0, leaf1, leaf2, leaf3, leaf4, leaf5, leaf6 and leaf7) (**Fig. 5G, H**).

## The content of artemisinin and DHAA in transgenic plants

In order to obtain transgenic plants, young leaves of *A. annua* were co-cultivated with *Agrobacterium* EHA105 according to previous methods (Zhang et al. 2009). Transgenic plants were selected in hygromycin-containing medium and genomic PCR was used to confirm the transformation. The transgenic plants showed normal growth in terms of phenotype and yield of seeds.

In anti-CPS transgenic plants, the artemisinin and DHAA concentrations were increased by 77% and 132%, respectively (**Fig. 6A**). The contents of artemisinin and DHAA in anti-BFS transgenics were increased by 77% and 54%, respectively (**Fig. 6B**). In anti-GAS plants, the artemisinin and DHAA contents were enhanced by 103% and 130%, respectively, while in anti-SQS transgenic plants they were increased by 71% and 223% compared with non-transgenic plants, respectively (**Fig. 6C, D**).

## Effect on the terpenoid pathway in the transgenic plants

The levels of gene expression and artemisinic metabolites are dynamic (Arsenault et al. 2010). Thus if the level of gene expression is increased, the metabolic flux will be significantly affected. When the expression level of *CPS* was inhibited, the concentration of β-farnesene was increased by 212%. By inhibiting the transcription activity of *BFS*, the squalene content was increased by 235%. In anti-GAS plants, the contents of β-farnesene, β-caryophyllene and squalene were little changed, while the content of artemisinin was increased. The content of β-farnesene was increased by 123% by reducing the mRNA level of *SQS* (**Fig. 7**).

## Gene expression in the anti-CPS plants

In anti-CPS plants, the expression level of *CPS* was decreased by 74% (**Fig. 8A**). The transcript levels of *GAS* and *SQS* were slightly enhanced, while the transcript level of *BFS* was enhanced 3- to 7-fold. The *ADS* transcript level was increased 3- to 4-fold compared with the control (**Fig. 9A**). The expression levels of other genes of the artemisinin biosynthetic pathway, such as *CYP71AV1*, *DBR2* and *ALDH1*, were only slightly changed.

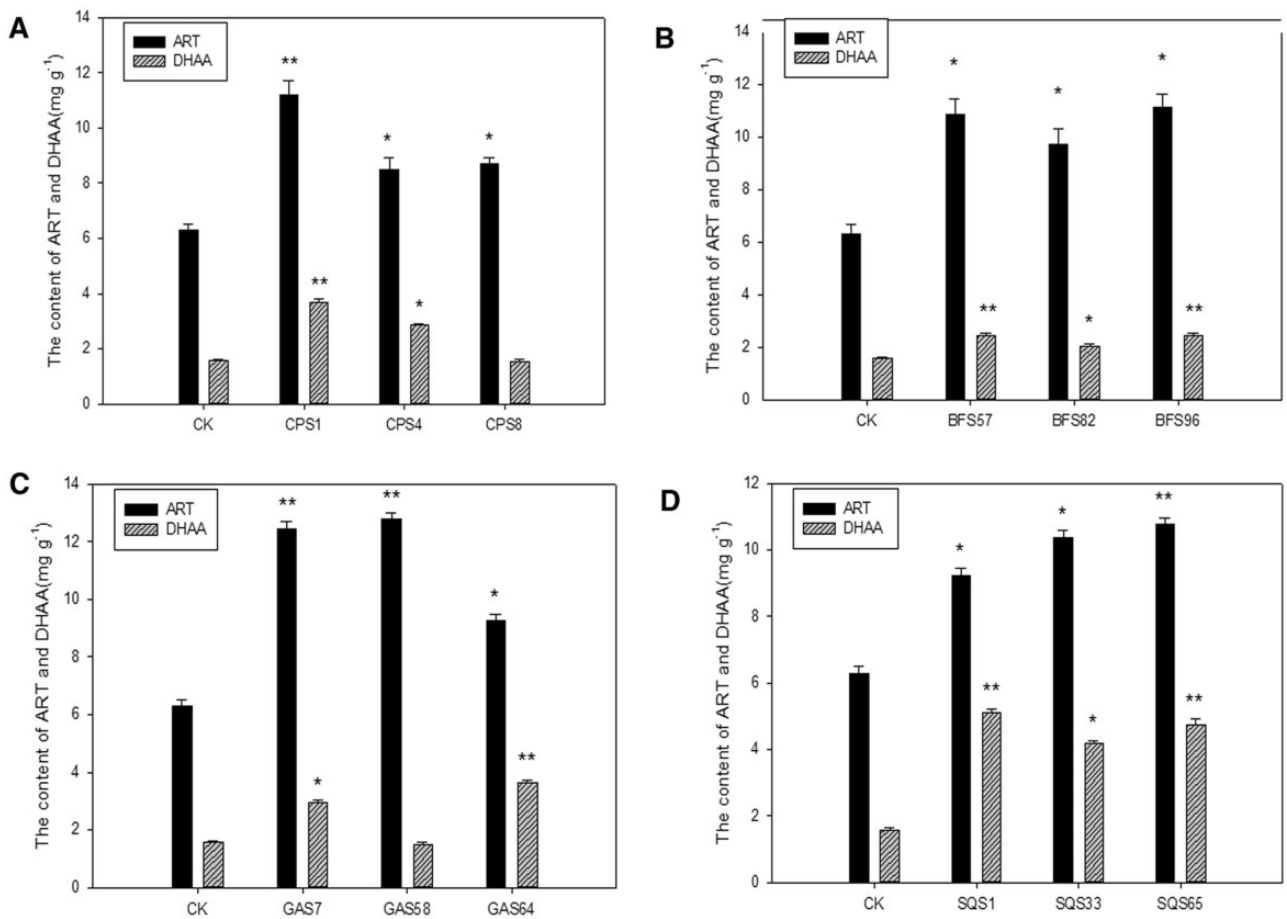


**Fig. 6** The contents of artemisinin (ART) and DHAA in anti-CPS (A), anti-BFS (B), anti-GAS (C) and anti-SQS (D) transgenic plants and controls (CK). Each data point represents the means ± SE from three replicates. Statistical significance was determined by Student's *t*-test (**$P < 0.01$; *$P < 0.05$). Asterisks indicate the difference between CK and transgenic plants of *A. annua*.

## Gene expression in the anti-BFS plants

The transcript level of *BFS* in the anti-BFS transgenic plants was decreased by 13–40% when compared with the control (**Fig. 8B**). Owing to down-regulation of the *BFS* transcript, the transcriptional activity of *SQS* was raised (**Fig. 8B**) and the carbon flux from FPP to squalene was increased (**Fig. 7**). At the same time, the expression levels of *ADS*, *CYP71AV1* and *ALDH1* were increased to different degrees (**Fig. 9B**). Consequently, the contents of artemisinin and DHAA were detected, with an increasing trend (**Fig. 6B**). However, the transcript level of *DBR2* did not change in the transgenic plants.

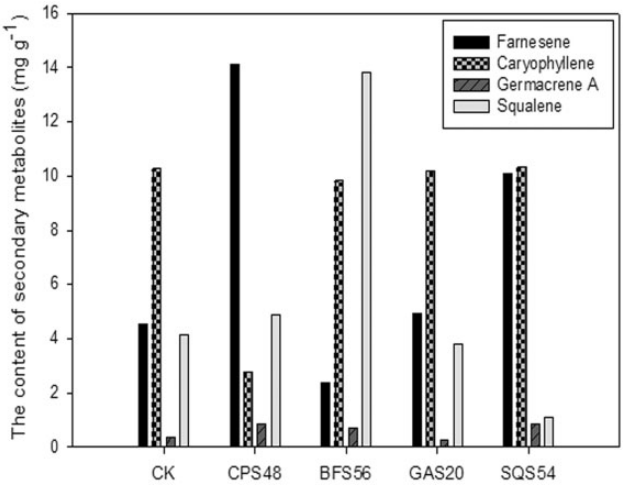


**Fig. 7** The contents of caryophyllene (β-caryophyllene), farnesene (β-farnesene), germacrene A (identified as β-elemene because the Cope rearrangement occurred easily at room temperature) and squalene in the transgenic anti-CPS, anti-BFS, anti-GAS, anti-SQS and control (CK) plants.

## Gene expression in the anti-GAS plants

To verify whether down-regulation of the expression of *GAS* can redirect carbon flux in the artemisinin biosynthetic pathway, the expression levels of *BFS*, *CPS*, *SQS*, *GAS* and artemisinin biosynthetic pathway genes were analyzed. The transcript level of *GAS* was decreased by 11–26% compared with the control (**Fig. 8C**). On the other hand, the transcript levels of *BFS* and *CPS* were increased 3- to 6-fold in some transgenic plants. The expression level of *SQS* was not changed. In addition, the expression level of *ADS* was increased about 6-fold when the other genes (*CYP71AV1*, *DBR2* and *ALDH1*) were changed by about 1- to 3-fold (**Fig. 9C**).

## Gene expression in the anti-SQS plants

To investigate the effect of inhibiting *SQS*, its expression was detected by qRT-PCR. The transcript level of *SQS* was decreased

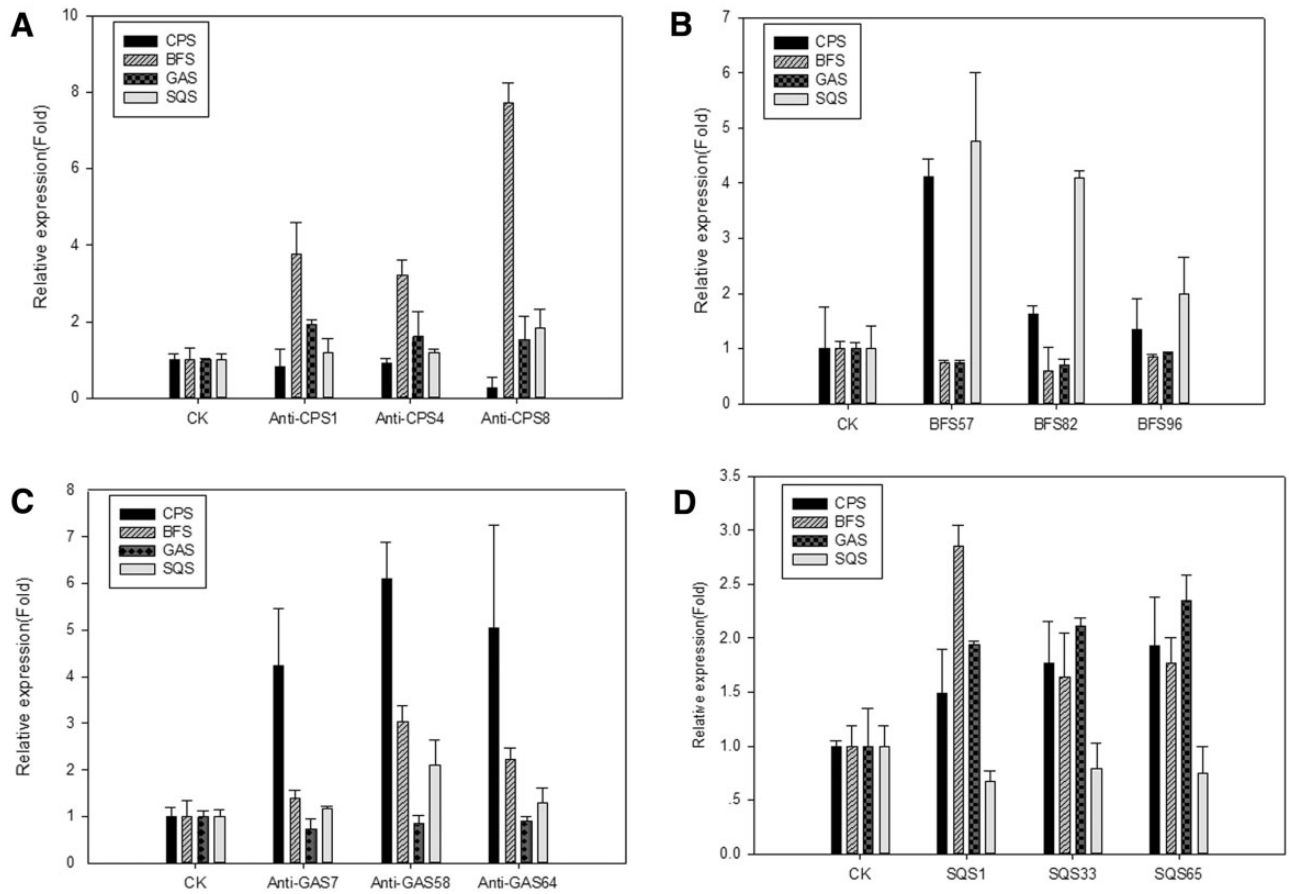


**Fig. 8** The expression levels of *CPS*, *BFS*, *GAS* and *SQS* in the transgenic anti-CPS (A), anti-BFS (B), anti-GAS (C) and anti-SQS (D) plants. qRT-PCR was used for determination of the expression level. Error bars are the SE ($n = 3$).

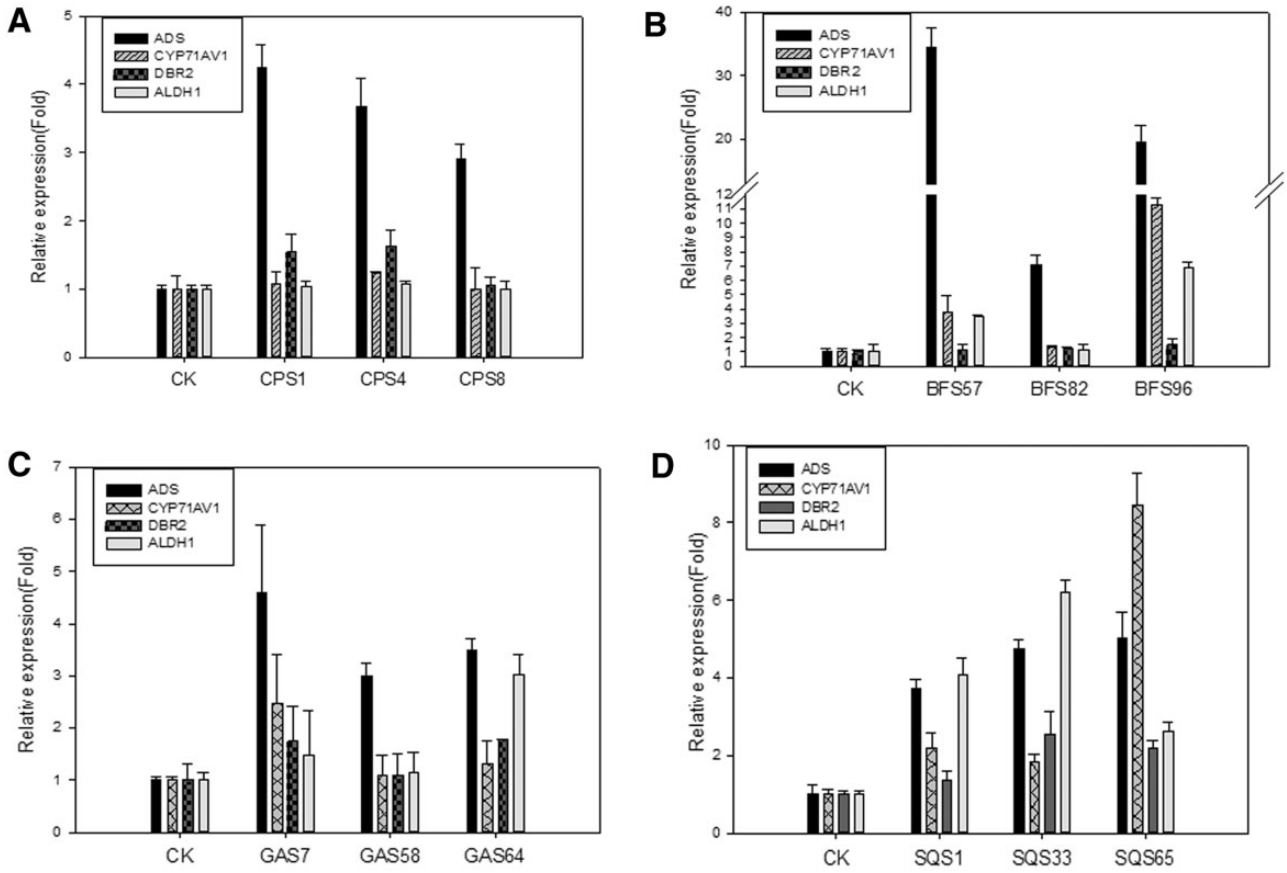


**Fig. 9** The expression level of *ADS*, *CYP71AV1*, *DBR2* and *ALDH1* in the transgenic anti-CPS (A), anti-BFS (B), anti-GAS (C) and anti-SQS (D) plants. qRT-PCR was used for determination of the expression level. Error bars are the SE ($n = 3$).

in the transgenic plants (**Fig. 8D**). The expression levels of other genes (*BFS*, *CPS* and *GAS*) were increased about 1- to 3-fold at the same time. On the other hand, the four genes of the artemisinin biosynthetic pathway were enhanced to different degrees (**Fig. 9D**). In fact, the *ADS* mRNA level always remained higher in the transgenic lines.

## Discussion

The Chinese traditional herbal medicinal plant *A. annua* is a miracle drug for curing malaria. The key component of *A. annua* is artemisinin, which is a 1,2,4-trioxane ring structure and highly oxygenated sesquiterpene. The enzymes BFS, CPS, GAS, SQS and ECS are in competition with the artemisinin pathway enzyme ADS for carbon flux coming from FPP. The promoters of *ECS* and *CPS* are active in the same part (T-shaped trichomes) (Wang et al. 2013a), but the content of *epi*-cedrol is very low (Wang et al. 2009) and β-caryophyllene can be found at levels of up to 5–10% of the total essential oil (Brown 2010). Therefore, we chose inhibition of the expression of *CPS* in this study. Based on our previous findings that using RNAi technology to inhibit a branch pathway gene such as SQS might result in early flowering and influence the biomass of transgenic plants, we chose the antisense method in this study. Inhibition of *BFS*, *CPS*, *GAS* and *SQS* at branching points by antisense technology may be helpful to improve the artemisinin content. In this study, we investigated both the expression levels of important genes and their products in transgenic plants, indicating that inhibition of branch pathway genes is a useful method for improving the content of secondary metabolism.

### The relationship between genes of branching points and artemisinin biosynthetic pathway

When a gene is highly expressed in roots, it may have some roles in defending against pathogens and worms (Wang et al. 2004). *CPS* shows high expression in roots and stems (**Fig. 5A**) (Olofsson et al. 2011). In addition, the product of CPS is β-caryophyllene, which is a volatile metabolite located in non-glandular trichomes (Wang et al. 2013a). Therefore, *CPS* may play an important role in defending against pathogens.

When the expression level of *CPS* was inhibited in *A. annua* by antisense technology, the transcription level of *BFS* was increased and, consequently, the content of β-farnesene was increased (**Fig. 7**). β-Farnesene has been shown to play an important role as an alarm pheromone (Francis et al. 2005, Köllner et al. 2008). *CPS* is located in non-glandular trichomes while *BFS* is located in both glandular trichomes and non-glandular trichomes (Wang et al. 2013a). Although down-regulation of the

expression of *CPS* can improve the artemisinin content (Chen et al. 2011), the relationships among *CPS*, *BFS*, *GAS* and *SQS* are not clear yet. We focused on the changed expression levels of *CPS*, *BFS*, *GAS* and *SQS*, and analyzed the product of metabolism. The results indicated that down-regulation of *CPS* may redirect the metabolic flux mainly towards the β-farnesene and artemisinin biosynthetic pathways, resulting in increased artemisinin content. Moreover, we showed that the expression of *ADS* was increased 3- to 4-fold in anti-CPS plants, but other artemisinin biosynthetic pathway genes did not change much. These genes downstream of *ADS* with poor expression levels may become the bottleneck for improving artemisinin yields. Therefore, although down-regulating *CPS* can improve the artemisinin content to some degree (Chen et al. 2011), it may not influence the biosynthesis of artemisinin to a great extent.

In this study, the *BFS* gene was shown to exhibit high expression in buds (**Fig. 5C**) and its expression patterns were similar to those of *ADS* and *CYP71AV1* (**Supplementary Fig. S1A, E**). *BFS* exhibited a higher transcription level in young leaves (leaf0, leaf1 and leaf2) and it was also the same for *ADS* and *CYP71AV1* (**Supplementary Fig. S1B, F**). In previous studies, the *BFS* promoter was found to be active in glandular trichomes and the *ADS* promoter was glandular trichome specific (Wang et al. 2011, Wang et al. 2013a). These results indicated that amorpha-4,11-diene and β-farnesene are accumulated in the same tissues. The enzymes BFS and ADS are in competition with the substrate FPP and the two genes are expressed in the same sites in *A. annua*. Therefore, inhibition of the expression of *BFS* may decrease the competition relationship between the enzymes BFS and ADS. When inhibiting the mRNA level of *BFS*, the expression level of *SQS* was increased and, consequently, the content of squalene was increased (**Fig. 7**). The expression levels of *ADS*, *CYP71AV1* and *ALDH1* were increased several fold, and the artemisinin content was enhanced in anti-BFS transgenic plants. Inhibition of the expression of *BFS* may have a potential application in the future for improving artemisinin content.

The expression level of *GAS* is the highest in buds and mature leaves, and it exhibits strong activity in young leaves such as leaf0, leaf1 and leaf2 (**Fig. 5E, F**). These results are similar to those of a previous study (Olofsson et al. 2011). *GAS* is most probably located in the glandular trichome since it was cloned from a glandular trichomes expessed sequence tag (EST) library (Bertea et al. 2006). *GAS* has a similar expression pattern to *ADS* (**Supplementary Fig. S1A, B**). By inhibiting the expression of *GAS*, the mRNA levels of *CPS* and *BFS* were increased. The metabolic flux may be redirected towards β-caryophyllene and β-farnesene. In fact, there was little increase in the contents of β-caryophyllene and β-farnesene (**Fig. 7**). Therefore, the flux was mainly changed into artemisinin (**Fig. 6C**). However, the content of germacrene A is low in the wild type (**Fig. 7**) (Tellez et al. 1999); consequently, down-regulation of the expression of *GAS* may not have any significant effect on the biosynthesis of artemisinin.

*SQS* is the key enzyme of sterol and triterpene biosynthesis in terpene metabolism, and there are many sterols located downstream of it; therefore, it may be worth studying it for improving artemisinin content. A previous study indicated that SQS activity in *Nicotiana bethamiana* was localized to the shoot apical meristem (Devarenne et al. 2002). In Arabidopsis, the *AtSQS1* gene is widely expressed in all tissues, whereas *AtSQS2* is expressed in the vascular tissue of leaf and cotyledon petioles. *SQS* may never be expressed particularly in the trichomes and is mainly expressed in leaves (**Fig. 5G**). There is no significant difference in expression levels in leaf0 to leaf7 (**Fig. 5H**). Therefore, by blocking the flux of squalene, the flow will be distributed in all tissues.

Down-regulation of the expression of *SQS* in transgenic plants could significantly increase the expression levels of *BFS*, *ADS*, *CYP71AV1*, *DBR2* and *ALDH1*. The concentrations of the metabolic products β-farnesene (**Fig. 7**), DHAA and artemisinin (**Fig. 6D**) were significantly increased. Due to the change in expression level of *SQS*, the carbon flow was changed and the yield of artemisinin was increased. Preliminary data from our lab showed that inhibition of *SQS* can improve the yield of artemisinin up to 314% (Zhang et al. 2009), but the relationships of gene expression changes among *SQS*, *CPS*, *BFS*, *GAS*, *ADS*, *CYP71AV1*, *DBR2* and *ALDH1* are not clear yet. We confirm the findings of Zhang et al. (2009) that inhibition of *SQS* is a useful method. Moreover, we show that down-regulation of the expression of *SQS* may redirect the metabolic flux mainly towards the artemisinin biosynthetic pathways, and the expression levels of *ADS* (5-fold), *CYP71AV1* (8-fold) and *ALDH1* (6-fold) are increased. At the same time, the content of β-farnesene was greatly increased. Therefore, inhibition of the expression of *SQS* is a very promising method for improving artemisinin content.

## The relationship between artemisinin biosynthetic pathway genes and artemisinin

The expression patterns of artemisinin biosynthetic pathway genes have been described and studied in many reports (Olofsson et al. 2011, Lu et al. 2013). ADS is the first and the key enzyme of the artemisinin biosynthetic pathway in *A. annua* (Bouwmeester et al. 1999, Kim et al. 2008, Wang et al. 2011, Wang et al. 2013b, Zhu et al. 2013). *ADS* exhibits high expression in young leaves and buds but low activity in other tissues (**Supplementary Fig. S1A, B**), and it is highly correlated to the concentration of artemisinin (Lommen et al. 2007, Arsenault et al. 2010). Many studies indicated that improving the mRNA levels of *ADS* may increase artemisinin production (Lu et al. 2013, Zhang et al. 2015).When overexpressing *ADS* in *A. annua*, the contents of artemisinin, artemisinic acid and DHAA were increased by 82, 65 and 59%, respectively (Ma et al. 2009). Thus, the content of artemisinin is proportional to the expression of *ADS*. However, when the expression level of *ADS* is increased >6-fold in the transgenic plants (**Fig. 9A–D**), other genes in the artemisinin biosynthetic pathway may become a bottle neck of artemisinin biosynthesis and will never increase the artemisinin concentration.

*CYP71AV1* is another important gene in the artemisinin biosynthetic pathway. Similar expression patterns were observed for both *CYP71AV1* and *ADS* (**Supplementary Fig. S1A, B, E, F**). By spraying plants with artemisinic acid or artemisinin, the

expression of *CYP71AV1* was decreased. It is suggest that some feedback inhibition is effective in reducing the expression level of *CYP71AV1* and influencing the production of artemisinin (Arsenault et al. 2010). Another study shows that CYP71AV1 is embedded in the endoplasmic reticulum and may limit the carbon flow (Olofsson et al. 2011). Therefore, increasing the expression of *CYP71AV1* may increase carbon flow and divert flux to artemisinin. However, overexpression of *CYP71AV1* only slightly increased the artemisinin content (by 38%) (Shen et al. 2012). The reason may be that the expression levels of other artemisinin biosynthetic pathway genes are low and limit the carbon flow towards artemisinin biosynthesis. For example, the expression level of *CYP71AV1* in transgenic anti-BFS96 plants is higher than in anti-BFS57, but anti-BFS96 and anti-BFS57 have the same concentration of artemisinin. However, the enzyme activity of CYP71AV1 is also very important for obtaining a high yield artemisinin. A previous report showed that different chemotypes of CYP71AV1 (high artemisinin production and low artemisinin production chemotypes) have different enzyme activities for catalyzing the substrate artemisinic alcohol (Ting et al. 2013). In fact, the highly active CYP71AV1 is decived by an amino acid residue (Ser479) (Komori et al. 2013). So, to obtain transgenic plants with a high yield of artemisinin, the CYP71AV1 with high enzyme activity should be chosen.

Previous studies indicate that *DBR2* is specifically expressed in glandular trichomes of *A. annua* (Olsson et al. 2009, Jiang et al. 2014) and it has a high expression level in the apical cells of glandular trichomes. Thus, it is reasonable that *DBR2* is highly expressed in buds. (**Supplementary Figs. S1C**). In addition, *DBR2* shows a remarkably higher expression (between 4- to 9-fold) in leaf0 and leaf1 compared with other leaves. However, the expression of *DBR2* changed little (a 1- to 2-fold change) in high yield artemisinin or low yield artemisinin transgenic plants (**Fig. 9A–D**). The explanation may be that the enzyme DBR2 is excessive for artemisinin biosynthesis. Therefore, increasing the expression of *DBR2* may not play an important role in improving artemisinin content.

*ALDH1* is mainly expressed in buds and young leaves, which is similar to *ADS* expression according to **Supplementary Fig. S1G** and **H** (Olofsson et al. 2011). With the same expression of *ADS*, *ALDH1* plays a decisive role in the content of DHAA in the anti-GAS transgenic plants anti-GAS58 and anti-GAS64 (**Fig. 6C**). In addition, DHAA is the direct precursor of artemisinin (Olofsson et al. 2011). The content of artemisinin could be increased to about 300% when *ALDH1* is overexpressed in *A. annua* (Tang et al. 2014). It competes with dihydroartemisinic aldehyde reductase for the substrate dihydroartemisinic aldehyde to direct the carbon flow mainly through dihydroartemisinic aldehyde to DHAA.

## Gene combination for obtaining high yield artemisinin plants

In the anti-CPS transgenic plants, *ADS* expression was increased by 4-fold, while expression of other genes in the artemisinin biosynthetic pathway was little changed. Thus, inhibition of the expression of *CPS* is not a good choice. By down-regulation of the expression of the *BFS* gene, the expression levels of most artemisinin biosynthetic pathway genes were increased. In some anti-BFS transgenic plants, the content of artemisinin can increase to 300%. Thus, by inhibiting the expression of *BFS*, the metabolic flux was diverted to the artemisinin biosynthetic pathway. In the anti-GAS transgenic plants, most of the artemisinin biosynthetic pathway genes were increased, but the content of germacrene A was so low in *A. annua* that it may not play a large role in artemisinin biosynthesis. When *SQS* was inhibited by antisense methodology, the expression of artemisinin biosynthetic pathway genes was also significantly increased, with expression levels of *ADS*, *CYP71AV1*, *DBR2* and *ALDH1* all increased. The artemisinin content was, subsequently, increased by 71%. Taken together, these results indicate that blocking of *BFS* or *SQS* is important for increasing gene expression in the artemisinin biosynthetic pathway. Therefore, inhibition of *BFS* or *SQS* at branching points could be useful for improving artemisinin content.

ADS is the first key enzyme in the artemisinin biosynthetic pathway and it supplies CYP71AV1, DBR2 and ALDH1 with amorpha-4,11-diene as substrate. Hence, the combination of suppression of the *BFS* or *SQS* genes and simultaneously overexpressing the *ADS* gene can be a useful method for significantly improving artemisinin content in *A. annua*.

# Materials and Methods

## Plant material and culture conditions

*Artemisia annua* seeds were collected from Chongqing, China. They were surface-sterilized by 75% ethanol for 1 min and then sterilized using 20% (v/v) NaOCl (sodium hypochlorite) for 20 min. Then all the seeds were washed three times with sterilized water. Subsequently, the seeds were planted on MS (Murashige and Skoog, 1962) medium with 3.0% sucrose and 0.8% agar and cultured with a photoperiod of 16 h light/8 h dark and light at 7,500 lux at 26°C. After 4 weeks, the leaves of germinated seedlings were cut and used as explants for transformation with the *Agrobacterium tumefaciens*-mediated leaf disc transformation method (Zhang et al. 2009).

## Sesquiterpenes treatment

*Artemisia annua* seeds were sown in soil mixture (vermiculite : perlite : peat moss = 7 : 0.5 : 2) with a photoperiod of 16 h light/8 h dark and light at 7,500 lux at 26°C. Three weeks later, seedlings were sprayed with 100 μg $ml^{-1}$ β-caryophyllene, β-farnesene or squalene, respectively. After 0, 1, 3, 6, 12, 24 and 48 h, the aerial parts of the seedlings were harvested for RNA isolation and qRT-PCR.

## Construction of the antisense vector

The genes *BFS* (GenBank accession No. AY835398), *CPS* (GenBank accession No. AF472361), *GAS* (GenBank accession No. DQ447636) and *SQS* (GenBank accession No. AF405310) were cloned from *A. annua* cDNA by KOD Plus (Toyobo) and then cloned into the pMD18 vector and sequenced. The first 312 bp of the genes *GAS*, *SQS*, *BFS* and *CPS* were amplified by PCR using KOD Plus, and primers carrying restriction sites are listed in **Supplementary Table S1**. The fragments obtained and the transformation vector pCAMBIA1305 were digested with the appropriate FastDigest restriction enzymes (Fermentas). Ligation of fragments and vector was carried out using T4 DNA ligase (Fermentas) to generate the recombinant plasmid. The recombinant plasmids were sequenced and transformed into EHA105 for transformation of *A. annua*.

## Transformation of *A. annua*.

The explants were co-cultivated with *Agrobacterium* harboring the recombinant vector (OD = 0.8) on half-strength MS medium in the dark at 28°C for 3 d. Then all the explants were transferred into inducing medium MS1 [MS + 0.5 mg $l^{-1}$ 6-benzylaminopurine (6-BA) + 0.05 mg $l^{-1}$ naphthaleneacetic acid (NAA) + 50 mg $l^{-1}$ kanamycin + 250 mg $l^{-1}$ carbenicillin] with a photoperiod of 16 h light/8 h dark and light at 7,500 lux at 26°C. After cultivation two or three times (each time 3-week) in the inducing medium, the explants generated shoots and all the shoots were transferred into rooting medium MS2 (1/2 MS + 250 mg $l^{-1}$ carbenicillin). After 3 or 4 weeks, the rooted plantlets were planted into chambers with a soil mixture (vermiculite : perlite : peat moss = 7 : 0.5 : 2).

## Isolation of genomic DNA and PCR analysis

The transgenic plants were identified by PCR and genomic DNA was obtained from the young leaves by the cetyltrimethylammonium bromide (CTAB) method (Allen et al. 2006). The sequence of the *Cauliflower mosaic virus* (CaMV) 35S promoter was used for designing the forward primer to detect the transgenic plants. The reverse primers were designed according to the sequence of the four genes constructed in the vector. The 20 µl PCR was performed using 10 pmol forward primer, 10 pmol reverse primer, 50 ng of plant genomic DNA, 7 µl of sterilized water and 10 µl of 10× *Taq* DNA polymerase mix (TAKARA).

## RNA isolation and real-time PCR analysis

Young leaves of the transgenic plant were used for extracting total RNA with the RNA prep pure Plant Kit (Tiangen Biotech.) according to the manufacturer's instructions. Then total RNA was transcribed by a TAKARA cDNA synthesis kit for synthesis of the first strand of cDNA.

qRT-PCR was performed with SYBR Green (Kapa Biosystems) according to the manufacturer's instructions. The comparative cycle threshold (CT) method (Applied Biosystems) was used for determination of the relative mRNA levels of samples. The β-actin gene of *A. annua* was used to quantify relative transcript levels. The primers for qRT-PCR are listed in **Supplementary Table S1**.

## Quantification of artemisinin using HPLC-ELSD (HPLC-evaporative ligh scattering detection)

Leaves of *A. annua* were collected and dried at 50°C for 24 h, then ground into powder. The powder (0.1 g) of each sample was weighed and suspended in ethanol (1 ml) for ultrasonication. After 10 min, the samples were centrifuged and the supernatant was filtered through a 0.25 µm pore size filter. A Waters Alliance 2695 HPLC system was used to analyze the samples (Zhang et al. 2009).

## Quantification of secondary metabolites by GC-MS

Fresh leaf samples were ground in liquid nitrogen and freeze-dried at −50°C for 48 h. Samples (5 mg) were weighed and suspended in chloroform (4 ml) for ultrasonication. The supernatants were filtered through a 0.25 µm pore size filter then dried by $N_2$ and redissolved in 200 µl of chloroform. The samples were used for detection. An elaborate protocol of GC-MS was employed according to Zhang et al. (2009).

## Supplementary data

**Supplementary data** are available at PCP online.

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## Disclosures

The authors have no conflicts of interest to declare.

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