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# Effect of Antisense Squalene Synthase Gene Expression on the Increase of Artemisinin Content in *Artemisia annua*

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## 1. Introduction

Artemisinin, a sesquiterpene lactone endoperoxide, is a valuable and powerful antimalarial drug obtained from the aerial parts of a Chinese herb, *Artemisia annua* (Liu et al., 1979). Artemisinin and its derivatives show few or no side effects with the existing antimalarial drugs, it has consequently been regarded as the next generation of antimalarial drugs (Looaresuwan, 1994). Currently, commercial production of artemisinin mainly based on its extraction and purification from plant material, however, the endogenous production of artemisinin is very low (0.01%-0.8% dry weight) (Wallaart et al., 1999). In view of the limited availability of artemisinin and the increased demand, the synthetic preparation of artemisinin becomes an attractive proposition. However due to its complex structure, the complete chemical synthesis is very difficult (Schmid & Hofheinz, 1983). *Artemisia annua* as the only valid source, many research groups have directed their investigations toward the enhancement of artemisinin production in *A. annua* cell cultures or whole plants by biotechnological approaches. However these approaches were still proved to be not successful (Ghingra et al., 2000). Recently, several genes in artemisinin biosynthesis have been cloned, and important advances in artemisinin biosynthesis have been achieved, which makes it possible to regulate artemisinin biosynthesis in a direct way, for example, by metabolic engineering (Abdin et al., 2003).

Artemisinin is synthesized by the isoprenoid pathway. In the cytosol, isoprenoids are synthesized via the classical acetate/mevalonate pathway (Fig. 1). In this pathway, farnesyl diphosphate (FDP) occupies a central position and serves as a common substrate for the first committed reactions of sterols and sesquiterpenes, such as artemisinin. Therefore, this point represents a potentially important controlling point for balancing sterol synthesis and sesquiterpenes synthesis. From metabolic engineering point of view (Fig. 1), there are two

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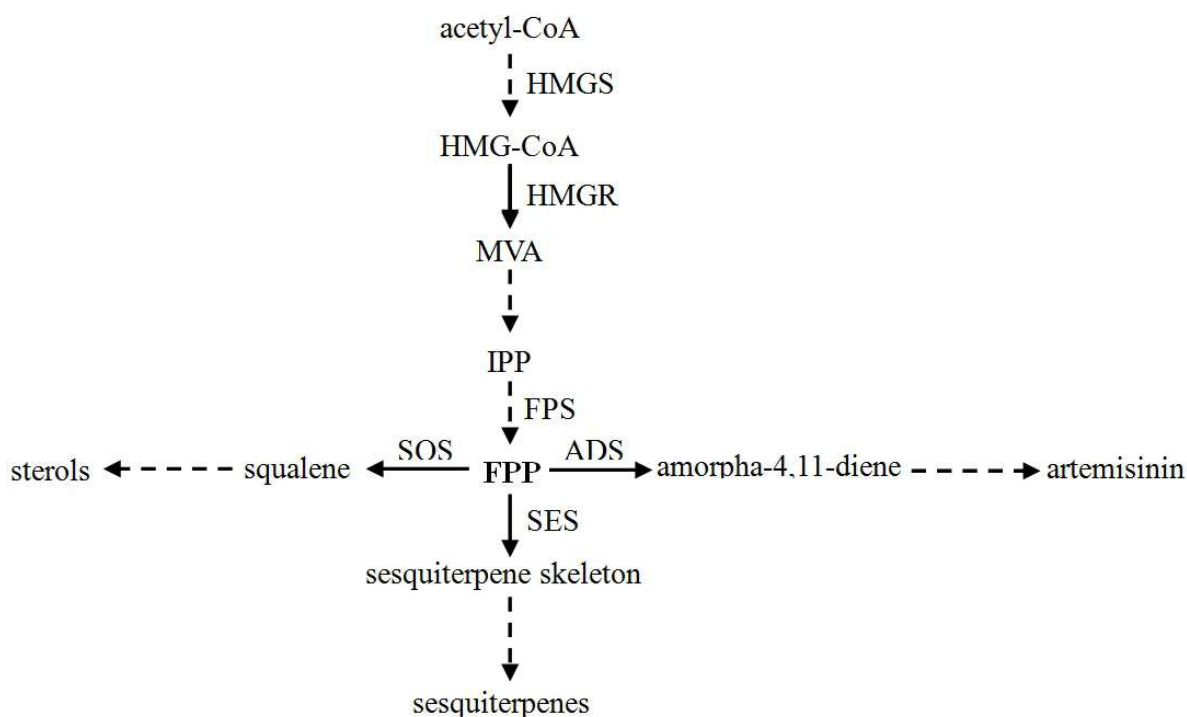
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ways to increase the flux to artemisinin biosynthesis, on the one hand, we can overexpress the key genes involved in the biosynthesis of artemisinin; on the other hand, we can inhibit the genes involved in other pathways competing for its precursors.



ADS: amorpha-4,11-diene synthase; FPP: farnesyl diphosphate; FPS: farnesyl diphosphate synthase; HMG-CoA: 3-hydroxy-3-methylglutaryl CoA; HMGR: HMG-CoA reductase; HMGs: HMG-CoA synthase; IPP: isopentenyl diphosphate; MVA: mevalonate; SES: sesquiterpene synthase; SQS: squalene synthase.

Fig. 1. Diagram of the mevalonate pathway leading to the biosynthesis of sesquiterpenes and sterols.

Squalene synthase (SQS) catalyzes the condensation of two molecules of farnesyl diphosphate (FDP) to form the linear 30 carbon compound squalene, the first committed precursor for sterol biosynthesis (Goldstein & Brown, 1990). SQS is generally described as a crucial branch point enzyme for synthesizing sterol intriguing as a potential regulatory point that controls carbon flux into either sterol or into non-sterol isoprenoids (such as sesquiterpenes). So if the SQS gene expression is inhibited by genetic manipulation, the carbon flux into sterol may be diverted to sesquiterpenes, and the biosynthesis of sesquiterpenes may be increased. With the purpose to increase artemisinin production, we have cloned squalene synthase cDNA (SQS) (Liu et al., 2003). In this chapter, we report the construction of the antisense SQS plant expression vector, and its effects on inhibition of SQS gene expression on squalene and artemisinin biosynthesis.

## 2. Materials and methods

### 2.1 Plant materials

A high artemisinin producing *Artemisia annua* L. strain 001 was collected from Sichuan Province of China. The seeds were surface sterilized and cultured on the Murashige &

Skoog (1962) basal medium with 0.7% agar and 3% sucrose in growth chamber at 26 °C and 16 h photoperiods. Leaves of 2-week-old seedlings were used for *Agrobacterium*-mediated transformation. After transformation, the transgenic plants and control plants were grown in green house with natural light and watered manually.

## 2.2 Construction of antisense plant gene expression vector

The original plasmid pSQF2 containing squalene synthase cDNA (SQS) gene, cloned from *A. annua* by our laboratory (Liu et al., 2003), was used as a template for the amplification of SQS fragment, then the amplified fragment was used for the construction of antisense plant expression vector. The PCR primers were designed according to the sequence at the 5'- and 3'-terminal region of SQS gene: 5'-GAC GGA TCC AAC AAA CAG TAC AAT TGG TG-3' (*Bam*H I restriction site underlined) and 5'-GCA GAG CTC GGA TTT GGA TCT TGA AGA AG-3' (*Sac* I restriction site underlined). The amplified SQS fragment was 1.5 kb in length, after digested with *Bam*H I and *Sac* I, the resulting large fragment was collected. The binary vector pBI121, containing both the *NPT II* gene controlled by the NOS promoter and the *GUS* gene controlled by the cauli-flower mosaic virus 35S promoter, was digested with *Bam*H I and *Sac* I, and the resulting large fragment was collected. The above two collected fragments were fused with T4 DNA ligase (Takara) (Fig. 2). The recombinant plasmid pBISQS was transformed into the competent *E. coli* DH5 $\alpha$  prepared using CaCl<sub>2</sub> method and pBISQS was extracted using alkaline lysis method then sequenced by Genecore Company.

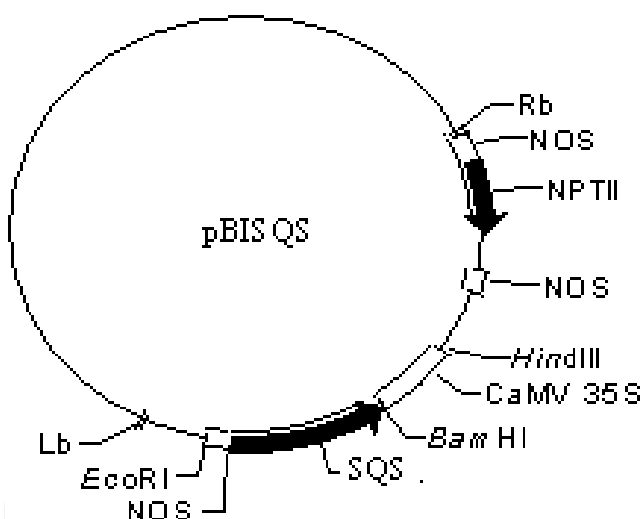


Fig. 2. Structure and diagram of recombinant plasmid pBISQS.

## 2.3 *Agrobacterium* preparation and genetic transformation

The binary vector pBISQS was introduced into *Agrobacterium tumefaciens* strain EHA105 by the freeze-thaw method and used for genetic transformation. Transformants were selected on LB medium supplemented with 50 mg/L kanamycin (Kan) and 50 mg/L rifampicin. The corresponding wild type strain was cultured in LB medium containing 50 mg/L rifampicin.

Plant transformation was basically performed according to the method of Han et al (2005). The leaves of 2-week-old aseptic seedlings were immersed in 50 mL *A. tumefaciens*, which was at the log phase of growth and was diluted 10-fold with MS medium. After 20 min, the

infected leaves were taken out, blotted with sterile paper and co-cultured on solid MS medium at 26 °C for 2 or 3 d. After co-cultivation, the leaves were transferred to shoot-inducing medium (MS medium supplemented 1.0 mg/L 6-BA and 0.05 mg/L NAA) containing 20 mg/L Kan to induce shoot and 500 mg/L cefotaxime (Cefo) to kill residual *Agrobacterium*. The medium without Kan was used as a control. Here the key difference with Han's method is that a sheet of sterile filter paper was placed on the shoot-inducing medium, since we found by such a simple action, the shoot induction frequency can be noticeably increased. One week later, the leaves were transferred to fresh MS selection medium with 400 mg/L Cefo. After 4-6 weeks selection Kan resistant shoots were obtained and transferred to 100 mL Erlenmeyer flask with 40 mL regeneration medium (MS basal medium supplemented with 0.05 mg/L NAA) to induce roots. All the rooted seedlings were kept in the greenhouse at 25 °C under the fluorescent lamps (with a light intensity of 3000 lx, 16h), with a relative humidity of 40%.

## 2.4 PCR detection

The integration of SQS gene into *A. annua* genome was confirmed by the polymerase chain reaction (PCR). The CTAB method was used to purify the genomic DNA from transgenic *A. annua* leaves. The forward primer was 5'-CCA CGT CTT CAA AGC AAG TGG ATT -3', designed according to the sequence of CaMV 35S promoter, and the reverse primer was 5'-GCA GAG CTC GGA TTT GGA TCT TGA AGA A G -3', designed according to the sequence at the 3'-terminal region of SQS gene. 30-cycle reactions, each consisted of heated denaturation (94 °C for 40 s), annealing (55 °C for 30 s) and extension (72 °C for 2 min), were carried out and the reaction mixtures were subjected to agarose gel electrophoresis.

## 2.5 RNA isolation and RT-PCR detection

For reverse transcription-polymerase chain reactions (RT-PCR), 1 g of total RNA isolated from the leaves of the transformed and non-transformed plants was used. The RNA extraction protocol was done as described in Sambrook et al (1989). The first strand cDNA was synthesized by using a first-strand cDNA synthesis kit (TaKaRa), according to the manufacturer's instructions. The resultant first-strand cDNA was used as a template, and the PCR primers, P1: 5'-GGA ACC ATG GGT AGT TTG AAA GCA GTA TTG-3, and P2: 5'-GCC TGG ATC CCT TGA CTC TCT CTT AAC TAT-3', were designed according to the SQS gene sequence of *A. annua*. The PCR was performed in the same way as described in the Section of PCR detection. At the same time, to normalize the amount of mRNA in each PCR reaction, a PCR product of actin in *A. annua* was amplified, and the primers were: 5'-AAC TGG GAT GAC ATG GAG AAG ATA T-3', and 5'-TCA CAC TTC ATG ATG GAG TTG TAG G-3'.

## 2.6 Squalene analysis

Squalene analysis was carried out according to the method described by Wentzinger et al (2002). For all the plants analyzed, young leaves was collected. Extraction and purification of the samples were in accordance with Wentzinger et al (2002), and the samples were analyzed by GC, the GC injection port was operated at 120 °C. The oven temperature programmed from 120 °C to 180 °C at 15 °C min<sup>-1</sup> and from 180 °C to 260 °C at rate of 25 °C min<sup>-1</sup>. The final temperature was maintained for 25 min. The results were compared to standards.

## 2.7 Determination of artemisinin

The detection of artemisinin was performed according to the method of Zhao & Zeng (1986). Fresh leaves of the transformed and non-transformed plants were collected and dried to constant weight in an oven at 50 °C. Then the dried leaves were ground to fine powder. Exactly 0.05 g powder was added to an extraction bottle containing 40 mL petroleum ether (30-60 °C) and treated in a supersonic bath for 2 min. The extraction mixture was filtered and the petroleum ether was evaporated. The residue was dissolved in 1 mL methanol and centrifuged at 12000 r/min to precipitate the undissolved components. The supernatant was used for detection of artemisinin by HPLC.

200 µL of the above prepared methanol solution was placed in a 10 mL tube and 800 µL methanol and 4 mL 0.2% sodium hydroxide were added, mixed and maintained in a 50 °C water bath for 30 min, then the reaction mixture was cooled to room temperature. 0.5 mL of the reaction mixture was placed in a 1.5 mL Eppendorf tube, and 100 µL methanol and 400 µL 0.05 M acetic acid were added, mixed and the sample purified by filtering on a NC filter (40 µm). The artemisinin standard (Sigma, MO) solutions with concentrations of 3, 6, 12, 24 and 48 µg/mL were prepared in the same way as the sample.

C18 reverse column was 4.6×250 mm, 5 µm. The mobile phase was 0.01 M phosphate buffer (pH 7.0) : methanol (55:45), with flow rate 1 mL/min. The wavelength of the UV detector was 288.6 nm and the injection volume was 20 µL. Artemisinin standard appeared at 4 min 30 s under the above mentioned conditions.

## 3. Results

### 3.1 Regeneration of transgenic *A. annua*

Leaf discs, which were infected with *A. tumefaciens* strain EHA105 harboring the binary vector pBISQS, were co-cultured for 36-48 h at 26 °C in dark, then transferred to shoot-inducing medium. Shoots usually start to appear within 2-3 weeks on this medium. In order to obtain higher frequency of shoot induction, a sheet of filter paper was placed on the shoot-inducing medium during the transformant selection step, which was proved to be a very effective means for shoot induction (Song et al., 2006). Regenerated shoots are rooted on MS medium with 0.05 mg/L NAA, followed by transferring to greenhouse (16 h light at 25 °C).

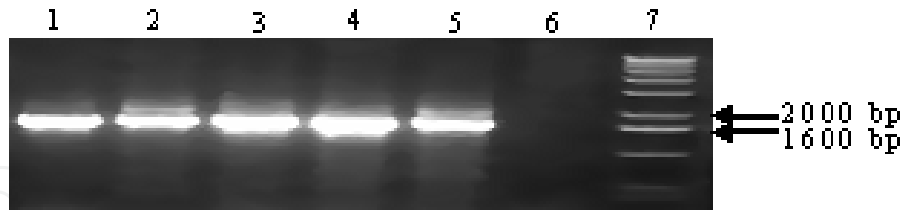
### 3.2 Molecular analysis of transgenic plants

To investigate the presence of antisense SQS gene in the putatively transformed plants, genomic DNA of 4 Kan-resistant plants regenerated from the leaves inoculated with EHA105 was isolated, and PCR analysis was performed. The antisense SQS gene was detected as 1660-bp fragments in all 4 analyzed plants (Fig. 3). The amplified fragments were of the same size as the predicted one. The fragment in the nontransformed plant was not amplified.

RT-PCR was performed using specifically designed primers according to the squalene synthase cDNA sequence in *Artemisia annua*. These primers allow specific amplification of *A. annua* squalene synthase cDNA. The results showed that the suppressed expression of the

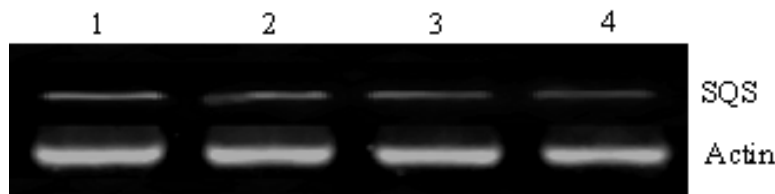


endogenous *A. annua* squalene synthase gene in lines SQS3 and SQS5, but the transcriptional level in line SQS2 had no noticeable difference to that of the control (Fig. 4).



1. positive control; 2-5. transformed lines; 6. negative control; 7. DNA ladder.

Fig. 3. PCR amplification of the transformed plants and the control.

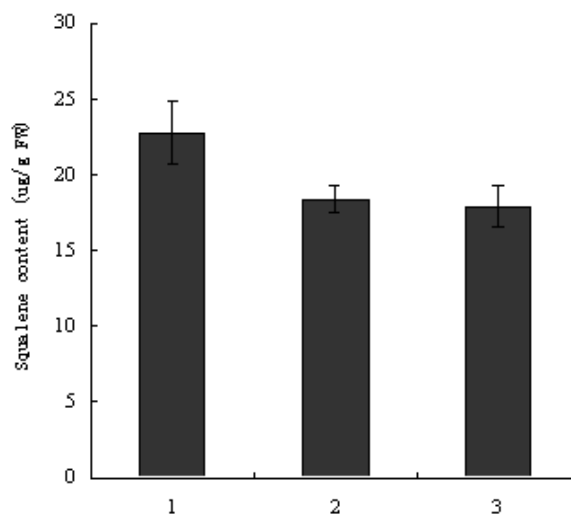


1. 001 line (non-transgenic control); 2. SQS2; 3. SQS3; 4. SQS5.

Fig. 4. RT-PCR analysis of the transformed plants and the control.

### 3.3 Detection of squalene content

In order to determine the effects of inhibiting squalene synthase gene expression on sterol biosynthesis, the leaves of transgenic lines SQS3, SQS5, and these of the control 001 were selected to detect squalene content. The results of GC-MS showed that in SQS3 and SQS5 transgenic lines, squalene content is decreased by 19.4% and 21.6% respectively in comparison with the control (Fig. 5).



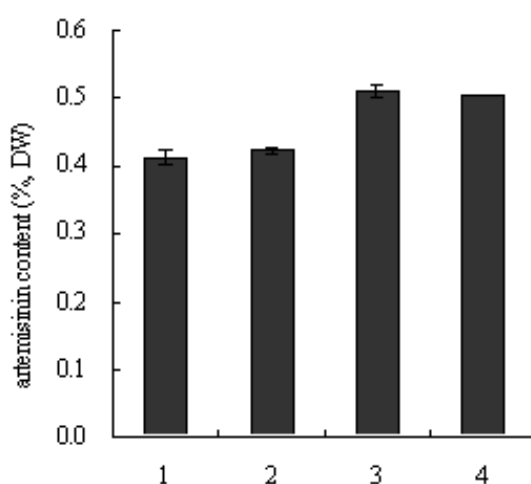
1. 001 line (control); 2. SQS3; 3. SQS5.

Fig. 5. Analysis of squalene content of the transgenic plants and the control.

### 3.4 Determination of artemisinin

Artemisinin was detected by HPLC. The results indicated that artemisinin content of SQS3 and SQS5 transgenic lines was increased by 23.2% and 21.5%, respectively compared with that of the control (Fig. 6) and in SQS2 transgenic line, the artemisinin content manifested no obvious variation compared with the control.

The above results demonstrated a clear negative correlation between squalene content and artemisinin content, which implies that the inhibiting of squalene synthase gene expression caused part of the flux for squalene biosynthesis diverting to artemisinin biosynthesis.



1. 001 line (control); 2. SQS2; 3. SQS3; 4. SQS5

Fig. 6. Analysis of artemisinin content of the transgenic plants and the control.

## 4. Discussion

Since squalene synthase is commonly depicted as the incipient and crucial branch point enzyme of the isoprenoid pathway to sterol biosynthesis, it has attracted considerable interest as a potential regulatory point that controls carbon flux into sterols. Several researchers reported the induction of sesquiterpene phytoalexins biosynthesis had been correlated with suppression of sterol biosynthesis in elicitor-treated tobacco cell cultures (Chappell et al., 1989; McGarvey & Croteau, 1995; Yin et al., 1997). The induction of one enzyme and suppression of the other are thought to be one mechanism that regulates the production of squalene and sesquiterpene (Devarenne et al., 1998). The biosynthesis of artemisinin belongs to the isoprenoid pathway, in this pathway, squalene synthase and amorpha-4,11-diene synthase are positioned at putative branch points in isoprenoid metabolism, these two enzymes catalyze the common farnesyl diphosphate to form squalene and amorpha-4,11-diene, respectively. Furthermore, amorpha-4,11-diene synthase is considered as a key enzyme in artemisinin biosynthesis (Bouwmeester et al., 1999), so squalene synthase can be considered as a competitive enzyme of artemisinin biosynthesis. Therefore, the inhibiting of SQS gene expression may increase the biosynthesis of artemisinin.



In order to increase artemisinin content, we introduced antisense squalene synthase gene into *A. annua* via *Agrobacterium*-mediated transformation and transgenic plants were obtained. It has been shown that the transgenic plants had an increase of artemisinin content in lines SQS3 and SQS5 and a reduction in squalene content. This may be due to the suppression of squalene synthase gene expression caused part of the carbon flux to squalene biosynthesis diverting to artemisinin biosynthesis. At the same time, in correspondence with the decline in squalene content, the endogenous squalene synthase transcript level of the SQS3 and SQS5 transgenic lines were reduced compared with the control. These results strongly supported that the overexpression of squalene synthase in antisense orientation had a relevant effect on endogenous squalene metabolism in transgenic tobacco plants (Zhang et al., 2005). Though the SQS gene expression of the transgenic plants was inhibited, both the growth and the phenotype of the transgenic plants showed no obvious difference to those of the control, and this was in consistent with the results of transgenic tobacco (Zhang et al., 2005).

Our results as well as other related reports all indicated that it is possible to increase artemisinin content of *A. annua* by inhibiting the expression of genes competing precursors with artemisinin in transgenic plants (Yang et al., 2008; Zhang et al., 2009; Chen et al., 2011). But it has caused only a limited increase in artemisinin content, perhaps this is because of the biosynthesis of artemisinin is controlled by multi-genes and the contribution of manipulating one gene to artemisinin biosynthesis is limited. Recently, the study of artemisinin has made great progress, and more and more genes of artemisinin biosynthesis, such as cytochrome P450 monooxygenase (CYP71AV1), double bond reductase 2 (DBR2) and aldehyde dehydrogenase 1 (ALDH1) (Teoh et al., 2006; Covello et al., 2007; Zhang et al., 2008; Teoh et al., 2009), have been cloned, this makes it possible to regulate multi-genes of artemisinin biosynthesis in the future.

## 5. Conclusion

The antisense squalene synthase (SQS) gene was transferred into *A. annua* via *Agrobacterium*-mediated transformation, and the artemisinin content of one of the transgenic lines showed an increase of 23.2% in comparison to the wild-type control. The results demonstrated that inhibiting pathway competing for precursor of artemisinin by anti-sense technology is an effective means of increasing the artemisinin content of *A. annua* plants.

## 6. Acknowledgements

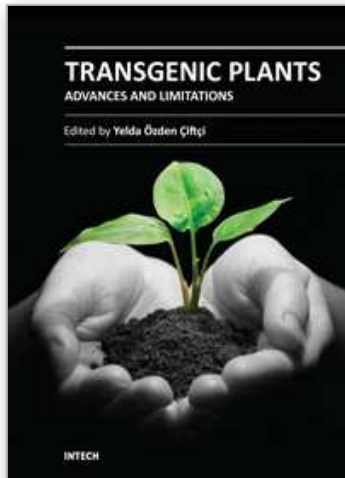
This work was supported by the National Natural Science Foundation of China (NSFC) (No. 61173098) and the Knowledge Innovation Program of the Chinese Academy of Sciences (KSCX2-EW-J-29).

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## **Transgenic Plants - Advances and Limitations**

Edited by PhD. Yelda Ozden Çiftçi

ISBN 978-953-51-0181-9

Hard cover, 478 pages

**Publisher** InTech

**Published online** 07, March, 2012

**Published in print edition** March, 2012

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Hong Wang, Yugang Song, Haiyan Shen, Yan Liu, Zhenqiu Li, Huahong Wang, Jianlin Chen, Benye Liu and Hechun Ye (2012). Effect of Antisense Squalene Synthase Gene Expression on the Increase of Artemisinin Content in *Artemisia annua*, *Transgenic Plants - Advances and Limitations*, PhD. Yelda Ozden Çiftçi (Ed.), ISBN: 978-953-51-0181-9, InTech, Available from: <http://www.intechopen.com/books/transgenic-plants-advances-and-limitations/effect-of-antisense-squalene-synthase-gene-expression-on-the-increase-of-artemisinin-content-in-arte>

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