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# Construction of a plant expression vector of chalcone synthase gene of *Ginkgo biloba* L. and its genetic transformation into tobacco

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**Abstract** The chalcone synthase gene (*chs*) from *Ginkgo biloba* L. was cloned by PCR procedure. For constructing a plant expression vector of *Gbchs*, the gene was digested with *Xba*I and *Bam*HI and inserted into the pBI121 vector. *Gbchs* was transferred into tobacco mediated by *Agrobacterium tumefaciens* LBA4404. PCR and Southern blot were performed, and the results showed that *chs* had been transformed into the genomic DNA of tobacco. The total flavones in the transformed tobacco leaves was extracted by the methanol extraction method and tested by UV spectrophotometry. The results showed that, compared to the control tobacco, the content in the transgenic tobaccos,  $n = 6$ , was generally higher, and the difference reached a significant level ( $P < 0.05$ ) and highly significant level ( $P < 0.01$ ), except one sample. Moreover, the highest sample was nearly 7.7 times greater than that of the controls. All these results show that utilizing genetic manipulations to improve plants in order to regulate flavone content by gene engineering may be an effective and hopeful method.

**Keywords** chalcone synthase gene, *Ginkgo biloba* L., plant expression vector, flavones

## 1 Introduction

*Ginkgo biloba* L., a living fossil tree, is well studied for its

leaf extracts, which contains many active ingredients and have a long history in traditional medicine in many countries. Some of the important active ingredients are the ginkgo flavonoids, which have been shown to have many pharmaceutical properties beneficial to human health (Gibb et al., 1997; Pietta et al., 1997; Pang et al., 2005). Furthermore, flavonoids are the major flower pigments in plants and also play important roles in many other biologic functions, including protection from UV light (Schmelzer et al., 1988), plant-microbe interactions (Harborne and Turner 1986; Schmelzer et al., 1988), and male fertility (Taylor and Jorgensen, 1992).

Chalcone synthase (CHS, EC2.3.1.74) is the key enzyme in the flavonoid biosynthesis pathway, which catalyzes the condensation of three acetate residues from malonyl-coenzyme A (CoA) with 4-coumaroyl-CoA to form naringenin chalcone (Harborne and Turner 1986; Estabrook and Sengupta-Gopalan 1991), which is the first C15 flavonoid skeleton. Further substitutions following the isomerization of naringenin chalcone lead to the formation of flavone, flavonol, anthocyanin, UV absorbing flavonoid pigments, and the isoflavone, antimicrobial phytoalexin (Saslowky et al., 2000; Farzad et al., 2005; Xu et al., 2007).

Because of its key function in the flavonoid biosynthetic pathway, the genes encoding CHS and cDNA have been used to manipulate flowering plants, in order to change the petal colors. In all attempts, the sense and antisense constructs of CHS have been found to give a reduction in the CHS mRNA level and CHS activity, and consequently, fainter flower color or even white flowers have resulted (Fukusaki et al., 2004; Della Vedova et al., 2005; Koseki et al., 2005; Hanumappa et al., 2007; Schijlen et al., 2007). However, most reports about the exogenous gene from the herbaceous plants, such as *Arabidopsis thaliana* (van der Krol et al., 1988), *Petunia hybrida* Vilm (van der Leede-Plegt et al., 1997; Davies et al., 1998; Tanaka et al., 1998), *Gerbra hybrid* (Elomaa et al., 1993; Elomaa et al., 1996), *Dianthus caryophyllu* (Tanaka et al., 1998), Rosa (Zuker et

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al., 1998), and few from ligneous plant especially from this living fossil tree. In addition, few reports talk about the change of the flavonoid glycoside in the CHS transgenic plants. In this study, we cloned the *chalcone synthase* gene of *G. biloba* (*Gbchs*) and investigated its heterologous overexpression in tobacco. We show that the constitutive overexpression of the *Gbchs* gene in transgenic tobacco altered the accumulation of flavonoids.

## 2 Materials and methods

### 2.1 Plant materials and reagents

*G. biloba* 'Jiafoshou' 12-year old plant was used for the experiment. Leaves were obtained from plants growing in an open field of the Ginkgo Science Research Garden of Yangtze University, Jingzhou, Hubei Province, China.

The tobacco variety used for genetic transformation was NC89; the vector pBI121; bacterium *E. coli* TOP10; and *Agrobacterium tumefaciens* LBA4404.

*Taq* DNA polymerase, restriction enzyme, T4 DNA ligase, DNA marker, dNTPs were obtained from Promega (Madison, WI, USA), pMD18-T vector was purchased from Dalian TaKaRa, China. Kanamycin, Ampicil, Carbenicillin, Rifamycin, and high-purity PCR product purification kit were obtained from Dalian TaKaRa, China. Primed synthesis and DNA sequencing were constructed on commission by Shanghai Sangon Biotechnology Company, China. DIG High Prime DNA Labeling and Detection Starter Kit I were obtained from Roche (Mannheim, Germany).

### 2.2 Methods

#### 2.2.1 Isolation of *chs* gene from *G. biloba*

Genomic DNA was extracted from the fresh leaves of *G. biloba* using CTAB (cetyltrimethyl-ammonium bromide) method described by Jiang and Cai (Jiang and Cai, 2000). The quality and the concentration of genomic DNA were determined by agarose gel electrophoresis and spectrophotometer analysis.

According to the published *Gbchs* gene sequence information (GenBank, accession number: DQ054841), the special clone *Gbchs* primers *chs*UP (ATGGAAGACT-TGGAGGCATTC) and *chs* DOWN (TTACTTGTTC-CAGGGAACGCT) were custom designed. The PCR reaction mixture (50 L) contained 10–15 ng DNA, 2 L of 10 mol·L<sup>-1</sup> specific primers, 1 L of 10 mol·L<sup>-1</sup> each of dNTPs, 5 L of 10 × reaction buffer, and 5 U *Taq* polymerase. Moreover, the PCR reaction was performed at 95°C for 3 min, 35 cycle (95°C for 50 s, 61°C for 60 s, and 72°C for 90 s), and 72°C for 10 min. The amplified products were purified, ligated into pMD18-T vector, and

cloned into *E. coli* strain TOP10 followed by sequencing.

#### 2.2.2 Plasmid construction and tobacco transformation

Two restriction sites, *Xba*I and *Bam*HI, were added to forward and reverse primers, respectively, which were made as a pair of directed cloning primers: *chs*dxP1: 5'-TATTCTAGAATGGAAGACTTGGAGGCAT-3' (*Xba*I site is underlined, and the start codon is boxed) and *chs*dxP2: 5'-ATAGGATCCTTACTTGTTCAGGGAAC-3' (*Bam*HI site is underlined, and the stop codon is boxed). The gene with *Xba*I and *Bam*HI sites was obtained by PCR amplification. The PCR program was 95°C for 3 min, 35 cycles (94°C for 40 s, 62°C for 60 s, and 72°C for 90 s), and 72°C for 10 min.

After the sequence and pBI121 were respectively digested with *Xba*I and *Bam*HI, the digested sequence and linear vector were linked by T4 DNA ligase. Then, the *Gbchs* was directly 'in-frame' cloned into the binary plant transformation vector pBI121. This vector contained the NPTII gene for kanamycin selection of putative transgenic plants.

#### 2.2.3 Transformation of pBI121-*Gbchs* into *Agrobacterium tumefaciens* LBA4404

The recombinant plasmid pBI121-*Gbchs* was introduced into *Agrobacterium tumefaciens* by means of freezing-thawing. The main features of this process are as follows: 1 g pBI121-*Gbchs* plasmid was added into 200 L LBA4404 competent cells, which had been previously twice treated with 20 mmol·L<sup>-1</sup> CaCl<sub>2</sub>; the added sterile glycerin into the final concentration was 25%, and this was mixed gently. The mixture was ice cooled for 30 min and then immersed in liquid nitrogen for 1 min before incubating at 37°C for 5 min. Then, 1-mL YEP liquid culture medium, without antibiotics, was added to the mixture, which was agitated gently at 28°C for 3 to 4 h. 100 L of this new mixture was laid on the YEP plate with antibiotics (50 g·mL<sup>-1</sup> Kan and 40 g·mL<sup>-1</sup> Rif). The plate inversion was incubated at 28°C for 48 h.

#### 2.2.4 Transformation of tobacco mediated by *Agrobacterium tumefaciens* LBA4404

The leaves of aseptic tobacco seedlings were cut into segments about 0.5–0.8 cm × 0.5–0.8 cm and dipped in the suspension of *A. tumefaciens* LBA4404 with *Gbchs* (OD<sub>600nm</sub> = 0.5) for 6–9 min, and at the same time, some of the explants were treated with *A. tumefaciens* LBA4404 with only pBI121 vector as the negative control. Then, the segments were cultured on MS medium containing 0.5 mg·L<sup>-1</sup> 6-BA and 0.1 mg·L<sup>-1</sup> NAA at 28°C in the dark for 2 d before they were transferred onto the MS medium

containing  $0.5 \text{ mg} \cdot \text{L}^{-1}$  6-BA,  $0.1 \text{ mg} \cdot \text{L}^{-1}$  NAA,  $500 \text{ mg} \cdot \text{L}^{-1}$  Carbenicillin, and  $100 \text{ mg} \cdot \text{L}^{-1}$  Kanamycin for shoot induction at  $(24 \pm 2)^{\circ}\text{C}$  under 2000 lx. When the kanamycin-resistant shoots were about 1–2 cm in height, they were transferred onto the rooting medium, which was MS medium containing  $0.1 \text{ mg} \cdot \text{L}^{-1}$  NAA,  $500 \text{ mg} \cdot \text{L}^{-1}$  carbenicillin, and  $80 \text{ mg} \cdot \text{L}^{-1}$  kanamycin at  $(24 \pm 2)^{\circ}\text{C}$  under 2000 lx. When the roots were about 2 cm, the plants were transferred to a greenhouse. The transgenic plants were grown under standard conditions.

## 2.2.5 PCR and Southern blot analysis of transgenic tobacco

The genomic DNA of transgenic tobacco was extracted from young leaves by CTAB-based DNA isolation method (Jiang and Cai 2000). *Gbchs* was amplified by PCR using transgenic tobacco DNA as templates and the special clone *Gbchs* primers (*chs* UP and *chs* DOWN) as the primers. The PCR reaction volume was same to that described above. Moreover, the PCR reaction was performed at  $95^{\circ}\text{C}$  for 3 min, 35 cycle ( $95^{\circ}\text{C}$  for 50 s,  $61^{\circ}\text{C}$  for 60 s, and  $72^{\circ}\text{C}$  for 90 s), and  $72^{\circ}\text{C}$  for 10 min. The amplified products were purified, denatured, and labeled with digoxigenin (DIG) as probes for southern blot.

Southern blot was carried out according to standard methods. 25 g of genomic DNA from both transgenic and control leaves was extracted and completely digested with *Eco*RI, fractionated on a 0.8% agarose gel, and blotted onto Hybond N nylon membrane. Southern blot was carried out 9 h at  $42^{\circ}\text{C}$  by using DIG labeled *Gbchs* as probes. The hybridized filters were washed at  $25^{\circ}\text{C}$  with  $2 \times \text{SSC}$ , 0.1% SDS, 2 times for 5 min;  $68^{\circ}\text{C}$  with  $0.5 \times \text{SSC}$ , 0.1% SDS, 2 times for 15 min. In addition, the other details were followed according to the Roche DIG High Prime DNA Labeling and Detection Starter Kit I.

## 2.2.6 RT-PCR assay of *Gbchs* expression profiles

To investigate the *Gbchs* expression in positive transgenic tobacco at the transcription level, total RNA was extracted from leaves of transgenic and negative control tobacco by CTAB-based RNA isolation method (Xu et al., 2008), followed by incubation with RNase-free DNase I at  $37^{\circ}\text{C}$  for 30 min according to the manufacturer's instruction (Takara, Japan). Aliquots of 1 g total RNA was used as template in RT-PCR using 'One Step' RNA PCR Kit (TaKaRa, Japan) with *chs*UP and *chs* DOWN as the primers, which were designed to amplify the coding sequence of *Gbchs*. The template was reversely transcribed at  $50^{\circ}\text{C}$  for 30 min and denatured at  $94^{\circ}\text{C}$  for 2 min, followed by 25 cycles of amplification ( $94^{\circ}\text{C}$  for 50 s,  $61^{\circ}\text{C}$  for 60 s, and  $72^{\circ}\text{C}$  for 90 s) and by extension at  $72^{\circ}\text{C}$  for 10 min. The PT-PCR reaction for the housekeeping gene ( *$\beta$ -actin* gene), using specific primers *actin*F (5'-GTGACAATGGAAGTGG-3') and *actin*R

(5'-AGACGGAGGATAGCGTGAGG-3'), was performed, as described above as control. The PCR products were separated on 1% agarose gels stained with ethidium bromide.

## 2.2.7 The change of flavones content in transgenic tobacco

A methanol extraction method was used to extract the total flavones of the tobacco leaf, while UV spectrophotometry was used to measure the quantity of flavones.

To extract the flavones, the tobacco leaves, 0.5 g (net weight), were refluxed by 100 mL methanol for 4–5 h in Soxhlet's apparatus until there was no color presented and then extracted for 10 h by alcohol at 0.7 volume fraction. Filtrate and decompress to 50 mL as well as extract by petroleum ether at equal volumes.

To measure the quantity of flavones in the transgenic samples, we used dried rutin, a common phyto-flavone standard. We took rutin samples of 0.076 g constant weight, which were dissolved and then metered by alcohol at 0.3 volume fraction in a 250-mL measuring flask and mixed to the concentration of  $0.3040 \text{ g} \cdot \text{L}^{-1}$  and reserved for later use. Rutin solutions of 0.00, 0.04, 0.80, 1.20, 1.60, and 2.00 mL; each were placed for 6 min in a 25-mL conical flask containing 12.5 mL 30% alcohol and 0.8 mL 5%  $\text{NaNO}_2$  solution. Afterward, 0.8 mL 5%  $\text{Al}(\text{NO}_3)_3$  was added for another 6 min. Finally, 5 mL 4% NaOH solution was supplemented to dilute 30% alcohol to make the solution volume 25 mL, and then, the solution was placed for 12 min. Absorption at 510 nm was measured, and we drew the standard curve using the six data points.

0.5 mL extraction liquids from six randomly selected healthy transgenic and control tobacco leaves were treated as per the previous instructions. Absorption was measured at 510 nm to determine the total flavones in the leaf tissues. The data was expressed as percentage through transformation using arc sine prior to ANOVA and then converted back to the original scale (Compton 1994). Treatment means were compared by following Duncan's Multiple Range Test (Duncan 1955). All statistical calculations and analyses were done by SPSS 16.0 for Windows.

# 3 Results and analysis

## 3.1 Cloning the *Gbchs* gene

The genomic DNA sequence of *chs* gene was cloned from *G. biloba* by using a pair of specific primers. The results of DNA sequencing showed that it was 1238 bp long, encoding a 304 amino acid protein, and the DNA sequence was in accordance with the sequence in GenBank (accession number was DQ054841).

## 3.2 Testing of plant expression vector of *Gbchs* gene

Following PCR amplification with *Gbchs* primers and the

kanamycin-resistant *E. coli* as templates, the PCR results were observed on the agarose gel, and we found that the *Gbchs* gene had been successfully introduced into the expression vector pBI121 but not into the control (Fig. 1). The recombinant plasmid was named pBI121-*Gbchs*.

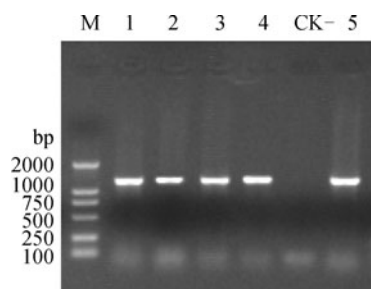


**Fig. 1** Colony PCR detection of pBI121-*Gbchs*

Note: 1–5: PCR product of positive colonial; CK-: PCR product of wild colonial; M: DL2000 marker.

According to the sequencing test report, the 3'-end of *Gbchs* was next to the *Bam*HI site, the 5'-end of *Gbchs* was next to the *Xba*I site of pBI121, and *Gbchs* was totally released from the vector pBI121-*Gbchs* when it was enzymatically digested by *Xba*I and *Bam*HI. Positive colony was tested by PCR amplification beforehand, extracted its plasmid, and then digested with *Xba*I and *Bam*HI to remove about 1.2 kb fragment. The result confirmed that the plant expression vector of *Gbchs* had been constructed successfully (Fig. 2).

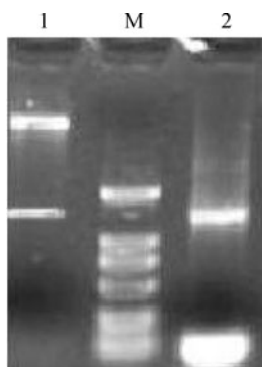


**Fig. 2** The restriction enzyme digestion analysis of pBI121-*Gbchs*

Note: M: DL2000 marker; 1: the restriction enzyme digestion of recombinant vector; 2: *Gbchs*.

### 3.3 Testing of transformation of *Gbchs* into LBA4404

To determine whether the pBI121-*Gbchs* was introduced into *A. tumefaciens* LBA4404, we picked out four single colonies from YEP plate laced with the antibiotics Kanamycin and Rifamycin. Then, PCR amplification was performed using the selected colonies as templates. The result showed that the target fragment from the colonies

could be amplified by PCR, indicating that the *Gbchs* had been transferred into *A. tumefaciens* LBA4404 (Fig. 3).

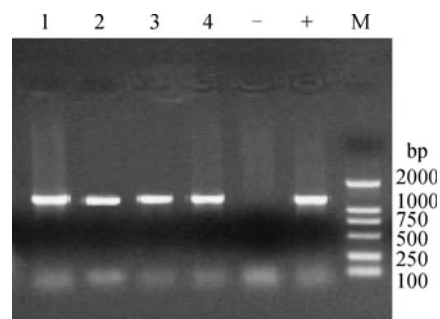


**Fig. 3** Colony PCR detection of pBI121-*Gbchs* in LBA4404

Note: + : PCR product of pBI121-*Gbchs*; -: PCR product of wild LBA4404 colonial; 1–4: PCR product of positive colonial; M: DL2000 marker.

### 3.4 Testing of putative transgenic tobacco

#### 3.4.1 The analysis of PCR

PCR was performed to amplify *Gbchs* from Kanamycin-resistant plants DNA used as a template. Additionally, the pBI121-*Gbchs* plasmid was treated as a positive template and the pBI121 vector as the negative control. The result showed that a DNA fragment about 1.2 kb could be amplified from the Kanamycin-resistant plants by PCR (Fig. 4).

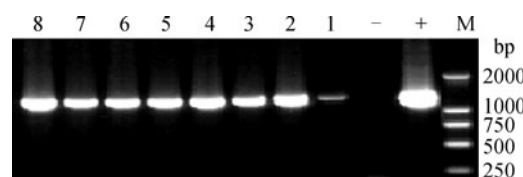


**Fig. 4** Identification of transgenic tobaccos by PCR

Note: + : PCR product of pBI121-*Gbchs*; -: PCR product of control tobacco; M: DL2000 marker; 1–8: PCR product of Kanamycin-resistant plants.

#### 3.4.2 Southern blot

We selected five plants from the Kanamycin-resistant ones, amplified the 1.2 kb gene fragment through extraction, digestion, and hybridization of the genomic DNA, and later visualized the result (Fig. 5). The results showed that transformed control tobacco (containing tDNA from the pBI121 vector only) had no hybridizing bands under high-stringency washing conditions. All of the identified transgenic plants (1, 2, 3, 4, and 5) had the foreign gene; the plants 1, 2, and 5 maybe had but one copy of the gene, and the plants 3 and 4 maybe had two copies. This result shows integration of the foreign gene but also indicates

low copy reliability of the *Agrobacterium*-mediated transgenic plant procedure.

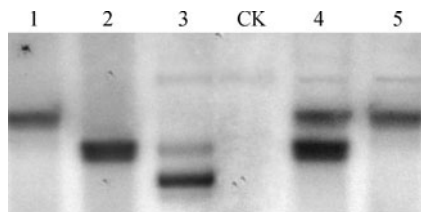


**Fig. 5** Southern blot analysis on putative primary transformants of tobaccos

Note: CK: control tobacco; 1–5: positive tobaccos by PCR analysis.

### 3.5 Transcript accumulation of the *Gbchs* and the change of flavone content in transgenic tobaccos

To investigate the transcription level of *Gbchs* in transgenic tobaccos, total RNA was isolated from leaves of transgenic and control tobaccos and then subjected to RT-PCR analysis. The result showed that the *Gbchs* transcripts could be detected in all selected transgenic tobaccos, but with varying levels, and no transcript accumulation was detected in control tobacco (Fig. 6). The highest level of transcribed RNA was found in the fourth sample.

The standard curve and regression equation of the rutin standards used to quantify the unknowns samples was established. Regression equation:

$A = 0.300 \times 10^{-7}C - 0.0017$  with a correlation coefficient,  $r = 0.9999$ .

The results of the absorption spectra of unknowns plotted to the standard curve (Fig. 7) showed that, compared to the control (containing tDNA from the pBI121 vector only), the content of flavones in the transgenic tobaccos was generally higher, and the difference reached a significant level in five of six samples. Two samples were significantly different ( $P < 0.05$ , marked with\*), and three were very significantly different from the control level ( $P < 0.01$ , marked with\*\*). Sample four was highest in total flavones containing 0.9593 percent in net weight, nearly to 7.7 times of the control.

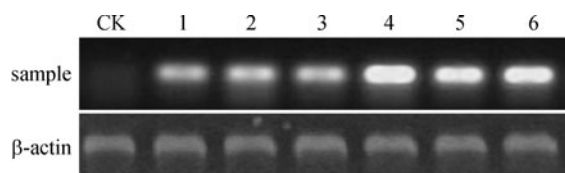


**Fig. 6** Transcript accumulation of the *Gbchs* in transgenic or control tobaccos by RT-PCR (upper panel)

Note: The  $\beta$ -actin gene was used as the control to show the normalization of the amount of templates in PCR reactions (lower panel). CK: control tobacco; sample 1–6: transgenic tobaccos.

## 4 Discussion

In this study, a *G. biloba chs* transgene was introduced into tobacco by *A. tumefaciens*, and the content of flavone in transgenic tobacco leaves sharply increased, which is similar to the research of Muir (Muir et al., 2001). Both of these results show that utilizing genetic manipulations to improve plants by regulating flavones content through gene engineering is an effective method. However, the transgenic tobaccos samples varied widely in flavones content, and the reason may be related to the site and copy differences in the target genome, tobacco DNA. The foreign *Gbchs* gene was inserted by *A. tumefaciens* into the tobacco leaves in a completely random fashion (Dorer and Henikoff, 1997). During our study, some transgenic plants exhibited abnormal plant morphology or grew slowly. There have been no previous reports of this kind of phenomena. The reasons may be inferred that CHS is the key enzyme in the flavonoid biosynthesis pathway (Harborne and Turner, 1986; Estabrook and Sengupta-Gopalan, 1991), and its expression and activity play very important roles in plants morphogenesis. However, whether the foreign gene in tobaccos can be transcribed and translated successfully, so further research, such as Northern blot and Western blot, needs to be done. Otherwise, it has been well documented that the introduction of additional copies of *chs* gene into plants, such as *Petunia* and *Arabidopsis*, frequently results in events of post transcriptional gene silencing or cosuppression, which leads to the sharp decrease in cyanidin content (Fukusaki et al., 2004; Della Vedova et al., 2005; Koseki et al., 2005; Hanumappa et al., 2007; Schijlen et al., 2007). In the anthocyanin biosynthetic pathway, cyanidin is biosynthesized from flavone, and the flavone biosynthesis is also a very complex process involving many enzymatic reactions and regulatory processes. Transforming a foreign gene into plants may lead to the accumulation of certain compounds in tissues, and many of these compounds belong to the chalcone groups, which are known to be toxic and inhibit cell growth (Lawrence et al., 2000; Iwashita et al., 2000; Samoszuk et al., 2005). Therefore, endogenous unknown enzyme(s) presumably catalyze parts of the accumulated chalcone compounds to other derivatives in these transgenic plants. There have recent researches about *chi*-suppression by RNA interference that showed reduced pigmentation and change of flavonoid components in flower petals (Nishihara et al., 2005). However, to the best of our knowledge, the change of flavone contents in these introductions of 'sense chimeric' *chs* into tobacco plants has not been reported.

In summary, the present study provides an effective approach to efficiently increase the end products of secondary metabolic pathways by gene engineering using *A. tumefaciens*. With the *chs* in particular, which encodes a key regulatory enzyme in the flavonoid synthesis pathway,

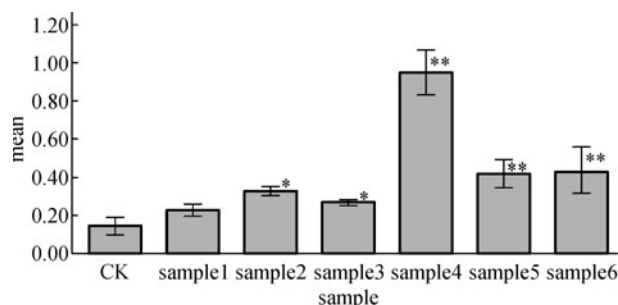


**Fig. 7** The content of flavone in the transgenic or control tobacco leaf in net weight/%

Note: CK: control tobacco; sample 1–6: transgenic tobaccos. The difference reached significant level ( $P < 0.05$ ) marked with \*, very significant level ( $P < 0.01$ ) marked with \*\*.

its overexpression in plants did lead to enhanced flavone biosynthesis.

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