

The establishment of transient expression systems and their application for gene function analysis of flavonoid biosynthesis in *Carthamus tinctorius* L. (safflower)

Bin Xian

Chengdu University of Traditional Chinese Medicine

Ziqing Xi

Chengdu University of Traditional Chinese Medicine

Chaoxing Ren

Chengdu University of Traditional Chinese Medicine

Jie Yan

Chengdu University of Traditional Chinese Medicine

Jiang Chen (✉ janshen1986@163.com)

Chengdu University of Traditional Chinese Medicine

Jin Pei

Chengdu University of Traditional Chinese Medicine

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Abstract

Background: Safflower (*Carthamus tinctorius* L.) is an important economic crop and traditional medicine rich in flavonoids, which can significantly improve cardiovascular and cerebrovascular diseases. Thus, many candidate genes for safflower flavonoid biosynthesis were cloned. However, due to the lack of homologous gene expression system, gene function research can only be carried out in model plants. It is necessary to establish gene function identification protocol in safflower.

Results: In this study, using safflower callus as experimental material, *Agrobacterium* transient expression system and bilolistic transient expression system were established. In the *Agrobacterium* transient expression system, the highest transformation rate were obtained at original *Agrobacterium* density of OD₆₀₀ 0.4, concentration of OD₆₀₀ 0.6, infection for 20 min, co-culture for 3 d, and AS concentration at 100 µmol·L⁻¹. In biolistic transient expression system, the highest transformation efficiency were observed at 1350 psi helium pressure, -0.8 bar vacuum degree, 6.5 cm flight distance, the bombardment once, 3 µg·shot⁻¹ of plasmid amount, and 100 µg·shot⁻¹ of gold particles amount. These two transient expression systems were used for the gene function analysis of *CtCHS1* as an example. After overexpression, the relative expression of *CtCHS1* was increased, especially in the *Agrobacterium*-transformed callus. The content of some flavonoids also changed, naringenin and genistein were significantly increased in *Agrobacterium*-transformed callus, and luteolin, luteolin-7-O-rutinoside and apigenin derivative were significantly decreased in biolistic-transformed callus.

Conclusion: Using safflower callus as the experimental material, *Agrobacterium* transient expression system and bilolistic transient expression system with high expression efficiency were successfully established, and demonstrating that both transient expression systems can be used to verify gene function. The safflower callus transient expression system will be a useful methods for further analysis in gene function analysis of flavonoid biosynthesis in safflower.

Background

Safflower (*Carthamus tinctorius* L.) is an annual herb belonging to genus Asteraceae, which has been cultivated in many countries because of the great medicinal and oil value. The flowers of safflower are rich in flavonoids, especially hydroxysafflor yellow A (HSYA), which have activities of antithrombotic, inhibiting the activation and release of inflammatory factors, and attenuating oxidative stress, so these compounds can protect ischemic and/or hypoxic cardiomyocytes and brain cells, which have been widely demanded for the treatment of stroke, myocardial infarction and other cardiovascular and/or cerebrovascular diseases [1, 2]. Because of the significant medical value, the flavonoids biosynthesis pathway in safflower attracts much attention, many genes related to flavonoids synthesis have been cloned. But due to the lack of verification system, the real functions of most of the genes are still not fully understood. Most gene are identified only by heterologous expression, such as in *Arabidopsis*. For example, the function identification of CHI (chalcone isomerase) [3], CYP (cytochrome P450) [4], CP1 (cysteine protease 1) [5] of safflower were carried out in *Arabidopsis*. But many medicinal plants, such as

safflower, contain their own unique secondary metabolites; in addition, *Arabidopsis* and safflower have significant differences in evolutionary pathways and genetic backgrounds from Cruciferae to Compositae, and from archichlamydeae to gamopetalae, which may cause a misleading or incomplete recognized for gene function. Therefore, it is very necessary to establish an effective homologous expression system in safflower.

Transient expression system is a widely used method for elucidating gene function in plants. Transient expression has the advantages of less affected by gene position and silencing effects, no heritable offspring for high biosafety, stable and high expression efficiency, simple operation and time saving [6]. Callus is one of the materials for transient expression, because the loose texture is beneficial to obtain high transformation efficiency and the potential to further development into complete plants.

Agrobacterium and biolistic are commonly methods for transient expression. The *Agrobacterium*-mediated transformation has the advantages of high gene transfer efficiency, ability to carry longer gene fragments, and easy to perform RNA and protein analysis [7]. biolistic-mediated transformation has the advantage of being able to transform a variety of plant tissues and without host limitations [6]. The efficiency of transient expression mediated by *Agrobacterium* will be affected by concentration, infiltration time, and AS concentration and etc.; while biolistic-mediated transient expression will be influenced by amount of gold particle, bombardment distance, and helium pressure and etc. [6]. In recent years, the transient expression system is no longer exclusive to model plants such as *Arabidopsis* [8] and *Nicotiana benthamiana* [9], many economic plants such as cotton[10], ricinus [11], citrus [12], *Chenopodium quinoa* [13], and *Vigna unguiculata* L. [14] have also established their own transient expression systems. However, there are still few medicinal plants possessing their own transient expression system.

In this study, cotyledons obtained from sterile seedling were used as explants to induce callus. Then, the optimal conditions for transient transformation of safflower callus which mediated by *Agrobacterium* and biolistic were screened, respectively. Finally, a *CtCHS1* (chalcone synthase) gene [15] was overexpressed in safflower callus by two transient expression methods to verify these transient expression systems. This study can lay a foundation for further analysis of the biosynthesis pathway of safflower special structure flavonoids, and also hoping to provide ideas for other gene function research in safflower.

Results

Callus induction of safflower

The sterilized mature safflower seeds were germinated in MS medium under a 16/8 h (light/dark) photo-period at 25±2°C. After 7 days of growth, sterile seedlings were obtained Fig. 1A . The cotyledons of sterile seedlings were used as explants, which were inoculated in medium containing different hormones to induce callus. After culturing for 40 days under the conditions of light/dark (16/8h) at 25±2°C, a large number of callus were developed on the cotyledons. The callus with the light yellow, loose structure were

grown in medium (0.1 mg·L⁻¹ NAA 2 mg·L⁻¹ 6-BA 15 mg·L⁻¹ KT-30) Fig. 1B , and at the same time, this medium also owned a high callus induction rate Fig. 1C .

***Agrobacterium*-mediated transient expression in safflower callus**

This experiment investigated the influence of *Agrobacterium* original density, concentration, treatment time, co-cultivation time, and AS concentration on the transient transformation efficiency of safflower callus by single-factor experiment Fig. 2 . The highest transformation efficiency was obtained when the original density of *Agrobacterium* was at OD₆₀₀ of 0.4, reached 79.54% (Fig. 2B). when treated with different concentrations of *Agrobacterium*, the highest transformation efficiency of callus, 72.52% ((Fig. 2C), was recorded when the concentration of OD600 was 0.6. With the increase of infection time, the transformation rate showed a slow increase trend, the highest transformation efficiency of 75.09% was obtained at 20 min (Fig. 2D), while decreased when exceeded 20 min. co-cultivation for 3 days after infection result in the largest number of positive callus, which was 72.76% (Fig. 2E). But with the further increase of the co-cultivation time, the excessive propagation of *Agrobacterium* caused the death of callus, which means the decline of transformation efficiency. AS can promote the expression of virulence genes of *Agrobacterium* and the transfer of T-DNA (Transfer DNA) region, which can significantly improve the transformation efficiency, and the best effect was observed when the concentration was 100 µmol·L⁻¹ in this study (Fig. 2F). However, an excessively high concentration of AS would lead to cell death in plants, so a significant decrease in the transformation efficiency was observed when the concentration of AS was 250 µmol·L⁻¹.

Biolistic-mediated transient expression in safflower callus

When studied the biolistic-mediated transient transformation of safflower callus, in order to obtain the highest transformation efficiency (represented by the content of 4-methylumbelliferone), six parameters, including the amount of gold particles, the amount of plasmids, helium pressure, vacuum, flight distance, and number of bombardments were investigated (Fig. 3). In all experiments, the highest transformation efficiency was obtained at a vacuum of -0.8 bar with a 4-MU (4-methylumbelliferone) content of 33.13 nM (Fig. 3E). Among the helium pressure experiment, the efficiency was higher at 1350 psi (Fig. 3D). When transforming callus at different flight distances, the highest transformation efficiency was obtained at a flight distance of 6.5 cm (Fig. 3F). Increasing the amount of plasmid can increase the expression of GUS, when the amount of plasmid was 3 ng·shot⁻¹, the highest level of 4-MU detected, which was 22.31 nM (Fig. 3C). However, when the amount of plasmid is further increased, it will cause a difficulty in dispersing of gold particles, resulting in a decrease in the transformation efficiency. The callus transformation efficiency decreased with the increase of the amount of gold particles. Experiment shown that the content of 4-MU decreased from 25.30 nM to 8.32 nM when the amount of gold particles increased from 100 µg·shot⁻¹ to 400 µg·shot⁻¹ (Fig. 3B). However, the number of bombardment times did not significantly affect the expression efficiency of GUS (Fig. 3G).

***Agrobacterium*- and biolistic-mediated overexpression of *CtCHS1* in safflower callus**

After optimizing the experimental parameters of the two transient transformation systems, the *CtCHS1* gene was overexpressed in callus to verify whether these transient expression systems could be used for the verification of gene function of safflower. First, *CtCHS1* was successfully cloned from safflower's cDNA (Fig. 4A). Then the expression vectors of *pRI201-CtCHS1* and *pBI221-CtCHS1* were constructed (Fig. 4B, 4C). Finally, the two transient expression methods were used to transform *CtCHS1* gene into safflower callus for overexpression, and the empty vectors were used as control. The transformation efficiency between the control groups and the experimental groups were detected (Fig. 5A, 5B), and no significant differences were found, which indicated that callus obtained by the same transformation method possessed similar condition.

The RNA (Fig. 5C, 5D) of the transformed callus was extracted and reverse transcribed for quantitative PCR analysis. Compared with the control group, the results shown that the expression of *CtCHS1* gene in transformed callus was significantly increased (Fig. 5E, 5F), indicating that *CtCHS1* was successfully overexpressed in safflower callus by *Agrobacterium* and biolistic-mediated methods. The total flavonoids in callus were analyzed by LC-MS. Six flavonoids were detected from the callus, they were luteolin, luteolin-7-O-rutin, naringenin, apigenin derivatives, genistein and dihydroquercetin. In *Agrobacterium*-transformed safflower callus, the contents of naringenin and genistein were significantly increased in overexpressed group (Fig. 5G). In biolistic-transformed safflower callus, luteolin, luteolin-7-O-rutinoside (LR) and apigenin derivative (AD) were significantly decreased in overexpressed group (Fig. 5H).

Discussion

The transient expression system is an efficient method for excavating gene functions, and this advantage allows it to be used in some medicinal plants, such as *Artemisia annua* L. [16], *Salviae Miltiorrhizae* Radix et Rhizoma [17], and *Taraxacum officinale* [18]. Transient expression materials are diverse, including callus, protoplasts, leaves, flowers, and fruits, and different explants have their own features. As a member of the Compositae family, safflower's features such as achenes, leathery leaves and tiny tubular flowers are not conducive to directly application for transient transformation. Protoplast is one of an approach, and the protoplast transient expression system of safflower has been used for gene function analysis [19]. However, the preparation of safflower protoplasts is affected by the flowering period, and requires high experimental operations. The callus has the advantages of not being limited by seasons, stable genotype, loose texture making easy to produce more positive samples. And it has been used in many plants, such as *Mesembryanthemum crystallinum* L. [20], *Pinus tabulaeformis* [21], *Sophora fragrans* [22]. Therefore, in this experiment, callus were induced from cotyledons of safflower sterile seedlings. The callus has a consistent genetic background, and can obtain a high level of expression efficiency, which is suitable for transient expression.

Agrobacterium is commonly employed for transient expression, but there are many factors can affect the transformation efficiency. *Agrobacterium* with strain EHA105 has been reported to have high infectivity to various types of plants, such as *Gossypium hirsutum* [23], *Aloe barbadensis* Miller [24], and *Artemisia annua* L. [16]. In this experiment, the EHA 105 *Agrobacterium* was used to investigate the influence of

several factors, including the density of the original *Agrobacterium*, *Agrobacterium* concentration, infection time, co-cultivation time and the concentration of AS. The experiment shown that the transformation rate was the highest when the original density *Agrobacterium* was OD600 0.4, the growth activity of *Agrobacterium* at this concentration was highest, which achieved the highest transformation efficiency. When examining different concentrations of *Agrobacterium*, we observed the most positive samples at OD600 of 0.6. The low concentration of *Agrobacterium* could lead to insufficient contact between *Agrobacterium* and callus, resulting low transformation efficiency; however, excessively high concentrations can cause browning or death of explants and lower the transformation rate too. The infection time is to influence the fully contact between *Agrobacterium* and callus. But, if the infection time is too long, callus will be damaged. In this experiment, the highest conversion rate was reached at 3 days of co-cultivation, which was similar to the experimental results of Song Y et al [25]. Sufficient co-cultivation time is favorable for the transfer of T-DNA to callus, but since the medium does not contain antibiotics, too long culturing will lead to the overgrowth of *Agrobacterium*. Since acetosyringone (AS) can activate the vir region of *Agrobacterium*, it can improve the transformation efficiency of T-DNA. The sensitivities to AS of different *Agrobacterium* strains and explants are changed [26]. For example, when the leaves of *Maesa lanceolata* were transformed, the transformation efficiency was the highest when the AS concentration was $100 \mu\text{mol}\cdot\text{L}^{-1}$ [27]; in *Nicotiana benthamiana*, $500 \mu\text{mol}\cdot\text{L}^{-1}$ AS was proven to have the best effect in tobacco leave [28]. In this experiment, the best effect was obtained when the concentration of AS was $100 \mu\text{mol}\cdot\text{L}^{-1}$. When exceeded the concentration, the transformation rate decreased, this could be caused by toxic effects of AS on the explants.

Biolistic is also a commonly used transient expression method. In the process of transforming, many parameters affect the transformation efficiency, such as helium pressure, bombardment distance, vacuum, bombardment times, gold particles amount, and plasmid amount [29]. The helium pressure determines the speed of the gold particles, enough speed is help to penetrate the cell wall and deliver of DNA. The experiment obtained a higher transformation efficiency at 1350 psi pressure, which was consistent with the experimental results of *Phoenix dactylifera* L. cv. 'Estamaran' [30] and peanuts [31]. The vacuum can affect the speed of the particles. The greater the degree of vacuum, the less power loss of the particles during flight; however, if the degree of vacuum is too large, water loss and viability reduction would occur in explants, resulting in lower transformation efficiency [32]. The bombardment distance affects the final velocity and the distribution of gold particles. The longer the bombardment distance, the slower the speed of gold particals and the more discrete the distribution. Therefore, this experiment found that with the increase of the bombardment distance, the expression of GUS in safflower callus decreased, which was consistent with the study by Narra M et al [33]. Increasing the times of bombardment can increase the probability of plasmids entering the explant, but there is no difference in the expression of GUS in the safflower callus bombarded with different times in this experiment. This is consistent with reports in *Tripterygium wilfordii* [34] and wheat [35]. Increasing the amount of gold particles may decrease the expression of GUS [36]. This may be because the limited plasmid cannot adequately encapsulate the gold particles; in addition, the density of gold powder particles is too high, the cell wall and cell membrane of cells are easily damaged; and the experiment found that too much gold

powder is easy to aggregate during processing, the agglomerated gold powder will kill the recipient cells[37]. Within a certain range, the expression efficiency will increase with the increasing amount of plasmid, but if the plasmid content is too high, the gold powder will agglomerate, which will reduce the transformation efficiency.

The transient expression system could be used for gene function analyze [38, 39], protein interaction, and subcellular localization [40, 41]. For example, after transient expression of *MdMYBPA1* gene in apple callus, the significant increase of proanthocyanidin content and expression level of several flavonoid-related synthetic genes were found, which demonstrated that MdMYBPA1 could activate proanthocyanidin synthesis in apple [42]. There are many plants that have established transient expression systems for these motivations, but it is not known whether these systems ultimately achieve their original purpose. Therefore, after initially establishing systems, overexpression of *CtCHS1* were carried out in safflower callus by these methods to verify the feasibility. After transient expression, the transformation efficiency between the control and experimental groups was detected, respectively. The results showed that there was no significant difference in the transformation efficiency between the control and experimental groups of the two methods, indicating that the experimental conditions are relatively consistent. q-PCR analysis showed that the expression levels of *CtCHS1* in the experimental group were significantly higher than control group, indicating that both transient expression methods could successfully mediate gene overexpression. The *CtCHS1* gene selected in the experiment can change the content of some flavonoids in safflower, and the changes of flavonoids were indeed detected in the callus transformed by the two methods. The contents of naringenin and genistein in *Agrobacterium*-transformed safflower callus were significantly increased, and luteolin, luteolin-7-O-rutinoside and apigenin derivatives in biolistic-transformed safflower callus were significantly decreased, which indicating that both transient expression methods can be used for the study of gene function.

Conclusions

Safflower is an international medicinal plant, and its flavonoid metabolites have attracted many researches. In recent years, the whole genome data of safflower has been published, and many genes related to flavonoid synthesis have been identified[43], but there is still a lot of work to be done in the future to fill in the gaps in gene function research. The two transient expression systems established in this experiment have the characteristics of high transformation efficiency, and can be practically used for gene function analyze, and the callus-based experiments have laid the foundation for the subsequent transgenic research in safflower.

Materials And Methods

Plant materials and treatments

The seeds were obtained from *Carthamus tinctorius* L. cultivated in the medicinal botanical garden of Chengdu University of Traditional Chinese Medicine, Chengdu City, Sichuan Province, China, and stored at

4°C. The safflower seeds were rinsed under running water for 4 h, disinfected with 0.1% HgCl₂ for 12 min, and rinsed 3 times with sterile water. The sterilized safflower seeds were sowed in MS medium (Table 1), under a 16/8 h (light/dark) photo-period at 25±2°C. After 7 days, sterile safflower seedlings were obtained.

callus was induced from the cotyledons of safflower sterile seedlings. In order to induce safflower callus, 6 induction medium containing different hormones were prepared in the study (Table 2). In addition to different hormones, these medias contained 4.43 g/L MS, 30 g/L sucrose and 8 g/L agar (Table 1). The medium was adjusted to pH 5.8-6.0 and sterilized at 121 °C for 20 minutes before being dispensed into petri dishes. Remove margins and midribs, wounds were made on the back, and then divided into 0.5 cm², cotyledons were inoculated the back side down in the prepared medium. The inoculated cotyledons were cultured in the dark at 20 °C for 40d.

***Agrobacterium*-mediated transient transformation**

The binary vector pRI201 plasmid, harboring GUS and CaMV 35S promoter was selected in the experiment. The plasmids were induced into *A. tumefaciens* EHA105 competent (Tsingke, China) via the freeze/thaw method. The *Agrobacterium* spread on LB plates supplemented with 50 mg·L⁻¹ Kana and 20 mg·L⁻¹ Rif, and culture at 28 °C for 2-3 days in the dark.

Single clone of *Agrobacterium* was pick into LB liquid medium supplemented with the same antibiotics, and incubate at 28 °C for 3-4h at 180-220 rpm in dark. The bacterial liquid was inoculated into several medium containing antibiotics, and *Agrobacterium* cells were harvested when the density reached OD₆₀₀ 0.3, 0.4, 0.5, 0.6, and 0.7, respectively. The *Agrobacterium* were collected by centrifuging at 5000 rpm for 12 min, and then rinsed twice with suspension solution (Table 1). Finally, the *Agrobacterium* cells were suspended to reach the needed OD₆₀₀.

The safflower callus were infiltrated with *Agrobacterium* solution, and the steps are as follows (The text in parentheses indicates the experimental parameters): (1) Put the safflower callus into a sterile centrifuge tube, and pour the *Agrobacterium* solution with original density of OD₆₀₀ 0.6 (OD₆₀₀: 0.3, 0.4, 0.5, 0.6, 0.7) and concentration of OD₆₀₀ 0.4 (OD₆₀₀: 0.2, 0.4, 0.6, 0.8, 1.0) into the tube to submerged the callus. (2) Gently upside down the centrifuge tube to make the callus fully contact with *Agrobacterium*. (3) 15 min (5, 10, 15, 20, 25 min) later, *Agrobacterium* liquid was removed. (4) The callus was moved in into the co-cultivation medium (Table 1), and then cultured at 28 °C for 2 days (1, 2, 3, 4, 5 d) in the dark. When studying the effect of AS concentration on the infecting efficiency, AS was added to make AS concentration reach 50, 100, 150, 200 and 250 mM·L⁻¹.

The transformed callus were submerged in GUS staining solution (Coolaber. China) for 24h in the dark at 37 °C. After removing the staining solution, samples were washed three times with 75% ethanol. The callus with the stained area more than 1/3 was regarded as positive.

Biolistics-mediated transient transformation

Binary vector pBI221 was used to optimize parameters for biolistic-mediated transient transformation. The vector contains a GUS reporter gene and the promoter is CaMV 35S. This plasmids were transformed into *E.coli*-DH5 α competent (Tsingke, China) to obtain sufficient plasmids for experiments. The transformed *E. coli* were propagated in dark at 37 °C and the plasmids were extracted.

The gold particle was prepared as follow[44]: (1) Take 50 mg of gold particles (1.0 μm , Bio-Rad) and wash them twice in 75% ethanol, then twice with sterile water, and finally 50% sterile glycerol was added to make the final concentration of gold particles at 50 $\text{mg}\cdot\text{mL}^{-1}$. (2) Vortex the gold particle solution to distribute the gold particles evenly in the solution, and quickly pipette 4 μL solution (when optimization the amount of gold particles, 2, 4, 6, and 8 μL solution were pipetted, respectively) into a sterile 1.5 ml centrifuge tube. (3) Keeping vortexing, 2 μL of plasmid (1000 $\text{ng}\cdot\mu\text{L}^{-1}$) (when optimization the amount of plasmid, 1, 2, 3, and 4 μL of the plasmid were pipetted respectively), 25 μL of 2.5 $\text{mol}\cdot\text{L}^{-1}$ CaCl_2 solution, and 10 μL of 0.1 $\text{mol}\cdot\text{L}^{-1}$ spermidine solution were added into the centrifuge tube in sequence. (4) Centrifugation at 5000 rpm for 5 min to collect gold particles. And then, the gold particles were washed twice with 200 μL of 75% and 100% ethanol. (5) The microparticles which have been coated with plasmids were resuspended in 20 μL of 100 % ethanol, and temporarily place on ice. The microcarriers prepared according to the above steps were used for one bombardment.

The Bio-Rad 1000/He PDS particle delivery system (Bio-Rad, USA) were used, and all items used in the experiment were sterilized. Safflower callus should be pre-cultured on hypertonic medium (Table 1) for 4 hours before bombardment. The safflower callus were transformed under different parameters, including the amount of gold particles (100, 200, 300, and 400 $\mu\text{g}\cdot\text{shot}^{-1}$), the amount of plasmids 1, 2, 3, and 4 $\mu\text{g}\cdot\text{shot}^{-1}$), helium pressure (1100psi and 1350psi), vacuum (-0.2, -0.4, -0.6, -0.8, and -1.0 bar), flight distance (6.5, 8.0, 9.5, and 11.0 cm) and number of bombardments (1, 2 and 3 times). The transformed callus was cultured in the dark at 28 °C in hypertonic medium for 24 h.

The transformation efficiency of callus was represented by GUS enzymatic activity. Successfully transformed callus can produce GUS protease, which can catalyze 4-methylumbelliferone glucuronide (4-MUG) into fluorescent 4-methylumbelliferone (4-MU). By measuring the fluorescence, the GUS (β -glucuronidase) enzymatic activity can be quantitatively detected. According to the method of the kit (Coolaber, China), transformation efficiencies under different experimental variables were detected.

***Agrobacterium*- and biolistic-mediated overexpression of CtCHS1 in safflower callus**

The flower of safflower was gathered from medical botanical garden of Chengdu University of Traditional Chinese Medicine too. RNA was extracted from flowers and reverse transcribed into cDNA. The sequence of *CtCHS1* is derived from a reported study, and the study showed that *CtCHS1* has a significant effect on the accumulation of some flavonoids [15]. The specific primer, *CtCHS1-F* and *CtCHS1-R* (Table 3), was designed with Primer Premier 5.0. PCR was carried out as follows: initial denaturation at 98 °C for 3 min;

34 cycles of 98 °C for 30 s, 56 °C for 30 s, and 72 °C for 2 min; and a final extension at 72 °C for 8 min. The PCR products were cloned into a pMD19-T vector (Tiangen, China) for sequencing (Tsingke, China).

The pRI201 plasmid was used for *Agrobacterium*-mediated transient expression, and the restriction enzyme site, Nde I (Takara, Japan), was selected in the experiment. The pBI221 plasmid was used for biolistic-mediated transient expression, and the restriction enzyme site was BamH I (Takara, Japan). On the basis of the ORF of the *CtCHS1* gene and restriction enzyme sites, gene-specific primers were designed (Table 2). The *CtCHS1* fragments were cloned into pRI201 and pBI221 respectively, and constructed plasmids verified by sequencing (Tsingke, China).

The constructed pRI201-CtCHS1 plasmid was extracted from *E. coli*, and then transformed into EHA105 *Agrobacterium*-competent, finally, the safflower callus was infiltrated under the optimized experimental condition. After multiplying in *E. coli*, constructed pRI201-CtCHS1 plasmid was extracted. And then, plasmid was loaded on gold particles, and delivered into the safflower callus under optimized conditions.

The transformed safflower callus were divided into three parts. One was used to measure the transformation efficiency. One was used to extract RNA which would be reverse transcribed for real time-PCR (RT-PCR) (Bio-Red, USA). The RT-PCR was employed to detect the expression level of CtCHS1 gene. The 25S rRNA gene, which obtained from *Carthamus tinctorius* L., was used as the internal reference to identify differences. The specific primers were designed with Primer Premier 5. software too. The RT-PCR cycling were carried out as follows: 95°C for 3 min, followed by 40 cycles of 95 °C for 10 s and 58.5 °C for 30 s [45]. The remaining samples was freeze-dried, and then were ultrasonically extracted in water twice to get total flavonoids. The extracts were finally dissolved in methanol, and analyzed by LC-MS system (Synapt, Waters, USA). The experimental methods referred to reference [19]. Safflower callus transformed with empty vector was used as control.

Statistical analysis

Statistical analysis of the callus induction rate, GUS strain frequency of *Agrobacterium*-transformed callus, GUS enzymatic activity in biolistic-transformed callus, the expression level of *CtCHS1* gene, and relative flavonoid content were performed by Student's t-test ($p < 0.01$). The callus induction rate (%) (number of successfully induced callus/total number of unstained explants $\times 100\%$), *Agrobacterium*-transformation efficiency (%) (number of positive callus/total number of infected callus $\times 100\%$), biolistic-transformation GUS enzymatic activity (calculated from established standard curve) and relative flavonoid content (the peak areas of experimental group/ the peak areas of control group) were calculated. Each experiment was repeated three times independently.

Abbreviations

AD: apigenin derivative; AS: acetosyringone; CHI: chalcone isomerase; *CHS1*: chalcone synthase; CP1: cysteine protease 1; CYP: cytochrome P450; HSYA: hydroxysafflor yellow A; GUS: β -glucuronidase; LR: luteolin-7-O-rutinoside; T-DNA: Transfer DNA; 4-MU: methylumbelliferone.

Declarations

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Authors' contributions

J. C. and J. P. conceived the project. B. X. performed the experiments and wrote the manuscript. All authors (Z.X., C.R., and J.Y.) discussed the results and commented on the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

All experimental studies on plants were complied with relevant institutional, national, and international guidelines and legislation.

Consent for publication

Not applicable.

Competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author details

¹ State Key Laboratory of Southwestern Chinese Medicine Resources, Chengdu University of Traditional Chinese Medicine, Chengdu 611137, Sichuan, China. ² College of Pharmacy, Chengdu University of Traditional Chinese Medicine, Chengdu 611137, Sichuan, China. ³ The State Bank of Chinese Drug Germplasm Resources, Chengdu University of Traditional Chinese Medicine, Chengdu 611137, China.

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Tables

Table 1 The composition of different media used in the study

Media	Composition
MS medium	1/2 MS, 30 g·L ⁻¹ source, 6 g·L ⁻¹ agar
Induction medium	MS, hormones, 30 g·L ⁻¹ source, 6 g·L ⁻¹ agar
Suspension solution	1/2 MS, 30 g·L ⁻¹ source
Co-cultivation medium	MS, 0.1 mg·L ⁻¹ NAA 2 mg·L ⁻¹ 6-BA 15 mg·L ⁻¹ KT-30, 30 g·L ⁻¹ source, 6 g·L ⁻¹ agar
Hypertonic medium	MS, 0.4 M·L ⁻¹ mannitol, 30 g·L ⁻¹ source, 6 g·L ⁻¹ agar

Table 2 The hormone of Safflower callus induction (mg·L⁻¹)

NO.	NAA	6-BA	KT-30
	0.1	2	10
	0.1	2	15
	0.1	2	20
	0.1	2	0
	0.5	2	0
	1.0	2	0

Table 3 Primers for gene cloning and vector construction

Primer	Sequence
CtCHS1-F	ATGGCATCCTTAACCGATATTG
CtCHS1-R	TTAAGCGGCAATGGGGGTGG
pRI201-CHS1-F	TCTACAGGACGTAACATATGATGGCATCCTTAACCGATAT
pRI201-CHS1-R	CTTCACTGTTGATACATATGTTAAGCGGCAATGGGGGTGG
pBI221-CHS1-F	TGTTGATAGTCGACGGATCCATGGCATCCTTAACCGATAT
pBI221-CHS1-R	ACCACCCGGGGATCCTTAAGCGGCAATGGG
qCtCHS1-F	CTGCCACAAAAGCCATTA
qCtCHS1-F	ACGGAAGGTGACCGCGGTGATCTCG
25S-F	GGAGGTTGAGGGAAAAGGAG
25S-R	GTGACCTCGTCACCCGTAGT

Figures

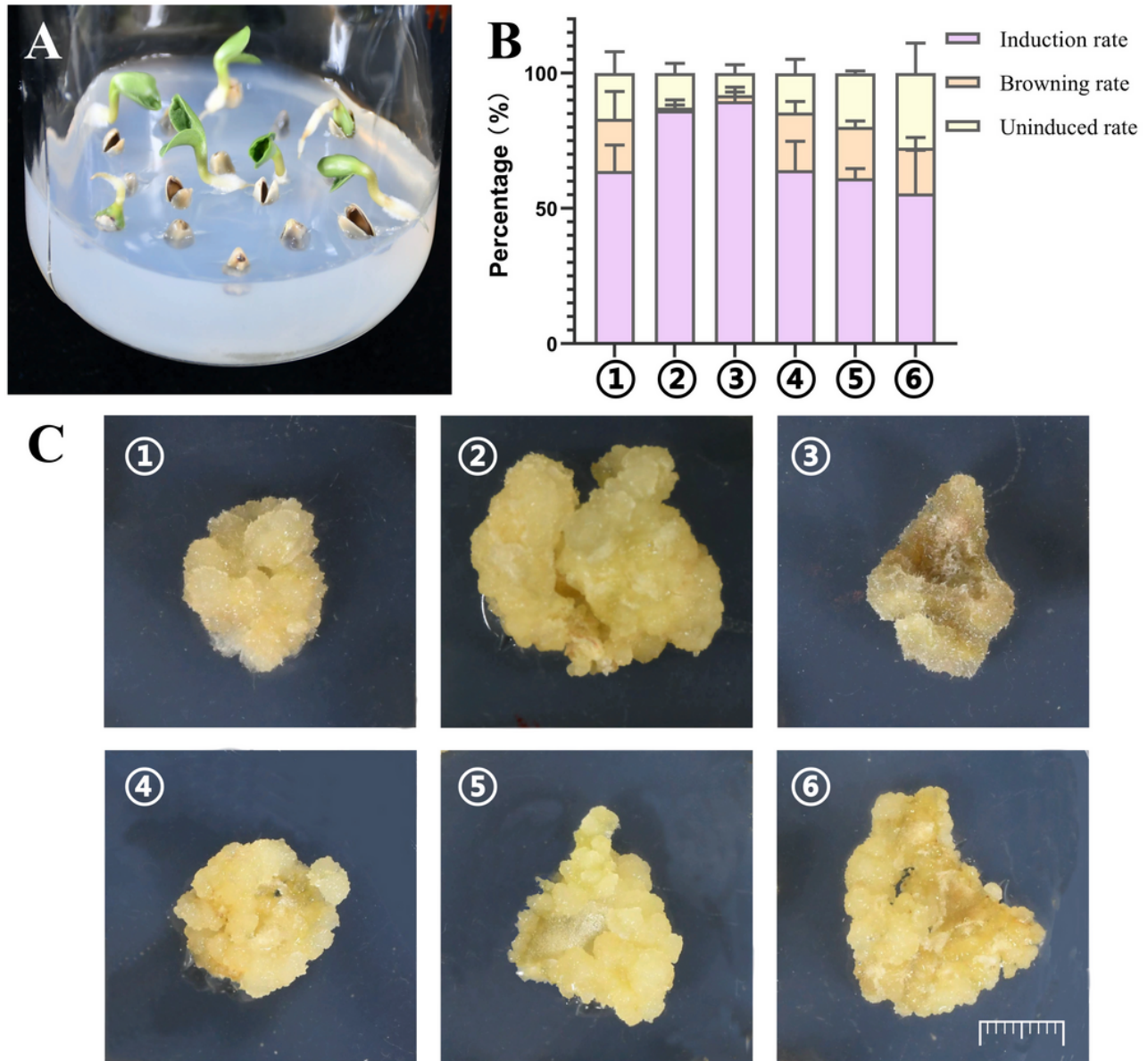


Figure 1

Callus induction of safflower. A The sterile seedlings of safflower. B Callus induction rate of different groups. C Safflower callus induced by 6 kinds of media.

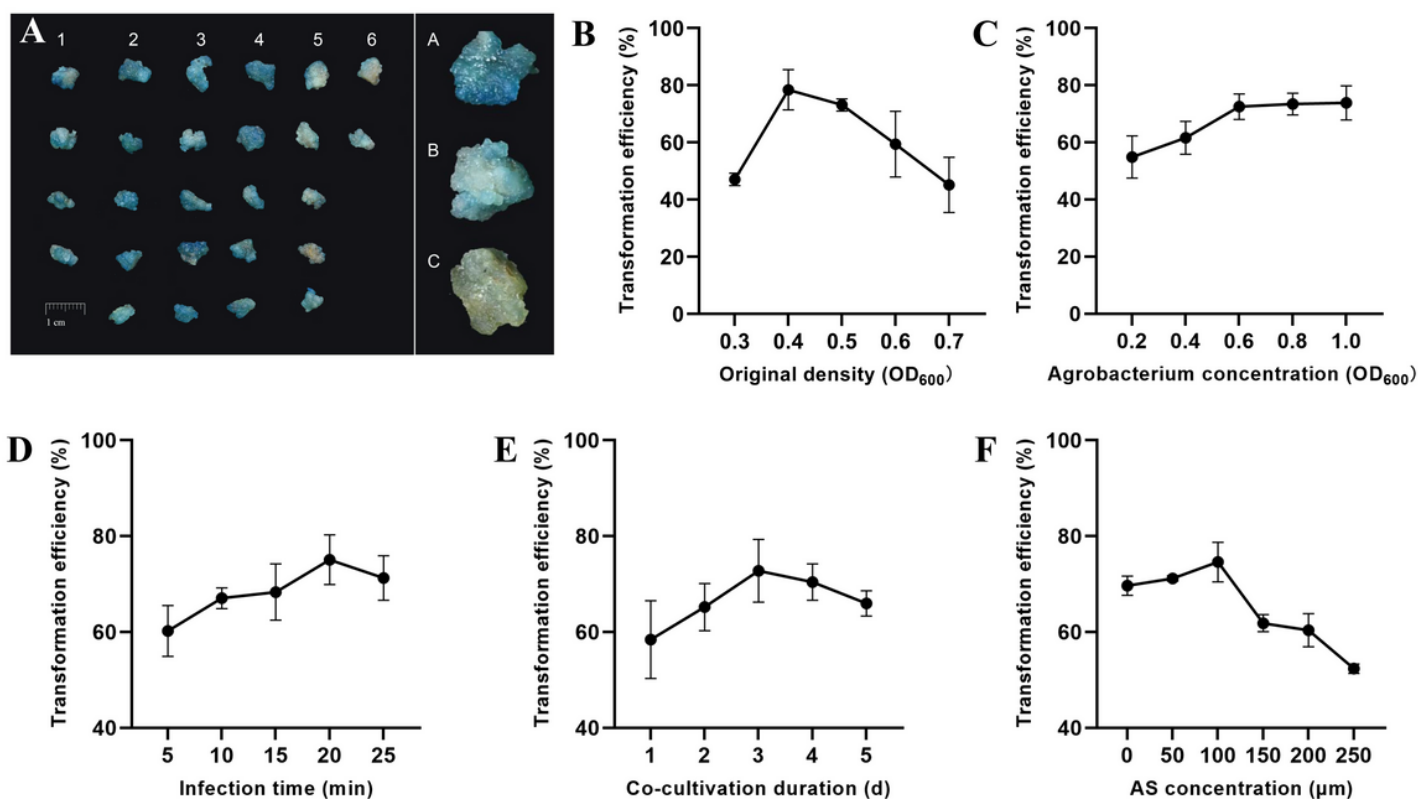


Figure 2

GUS expression analysis of safflower callus transformed by *Agrobacterium*. A GUS expression analysis of *Agrobacterium*-transformed safflower callus. 1, 2, 3, and 4 are considered as positive, and 5 and 6 are considered as negative; in the stained callus, A is positive, B is false positive, and C is negative control. B Effect of original density of *Agrobacterium* on transient transformation (OD_{600} values of 0.3, 0.4, 0.5, 0.6, and 0.7). C Effect of *Agrobacterium* concentration on transient transformation (OD_{600} values of 0.2, 0.4, 0.6, 0.8, and 1.0). D Effect of infection time on transient transformation (5, 10, 15, 20 and 25 min). E Effect of co-cultivation duration on transient transformation (1, 2, 3, 4 and 5 days). F Effect of AS concentration on transient transformation (50, 100, 150, 200 and 250 $\mu\text{M}\cdot\text{L}^{-1}$).

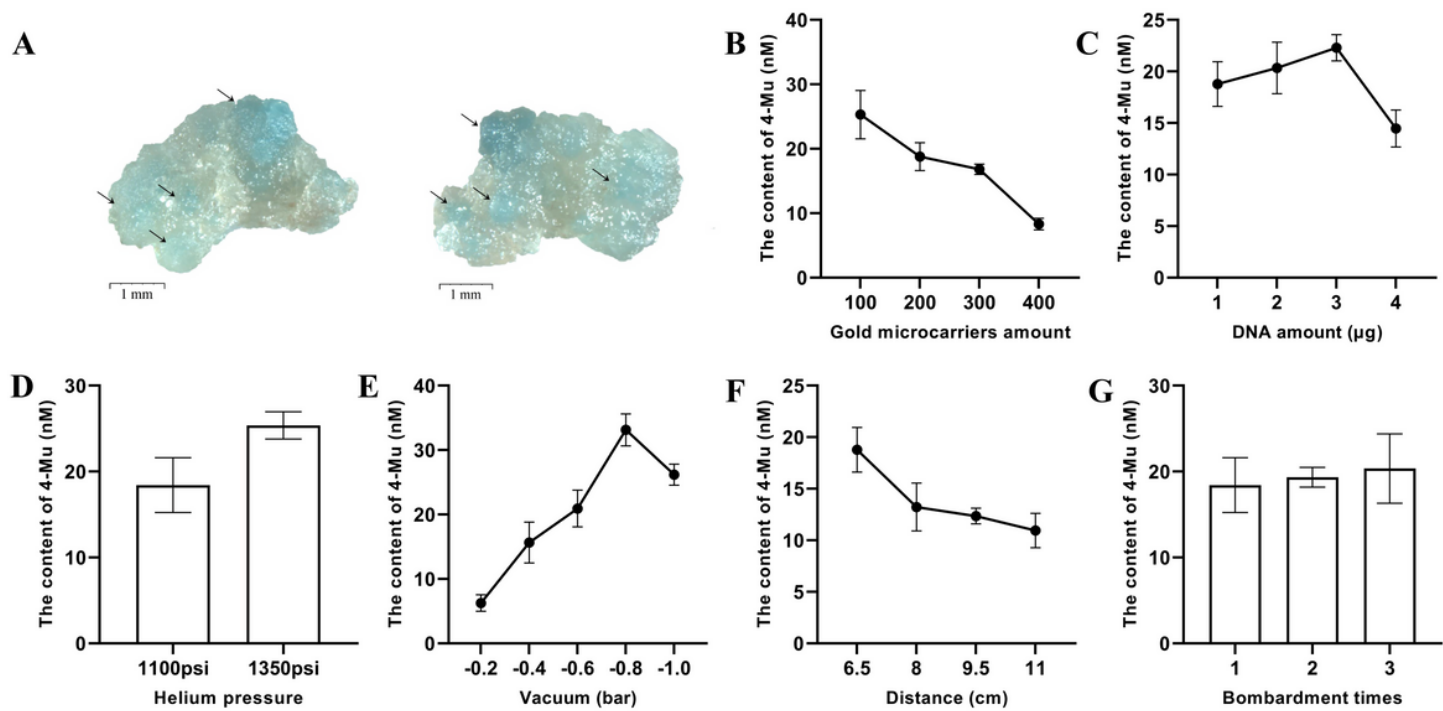


Figure 3

GUS enzyme activity assay of safflower callus transformed by biolistic. A GUS expression analysis of biolistic-transformed safflower callus. B Effect of gold particles amount on transient efficiency (100, 200, 300 and 400 $\mu\text{g}\cdot\text{shot}^{-1}$). C Effect of plasmids amount on transient efficiency (1, 2, 3 and $\mu\text{g}\cdot\text{shot}^{-1}$). D Effect of helium pressure on transient efficiency (1100 and 1350 psi). E Effect of vacuum on transient efficiency (-0.2, -0.4, -0.6, -0.8 and -1.0 bar). F Effect of flight distance on transient efficiency (6.5, 8, 9.5 and 11 cm). G Effect of bombardment times on transient efficiency (1, 2 and 3 times).

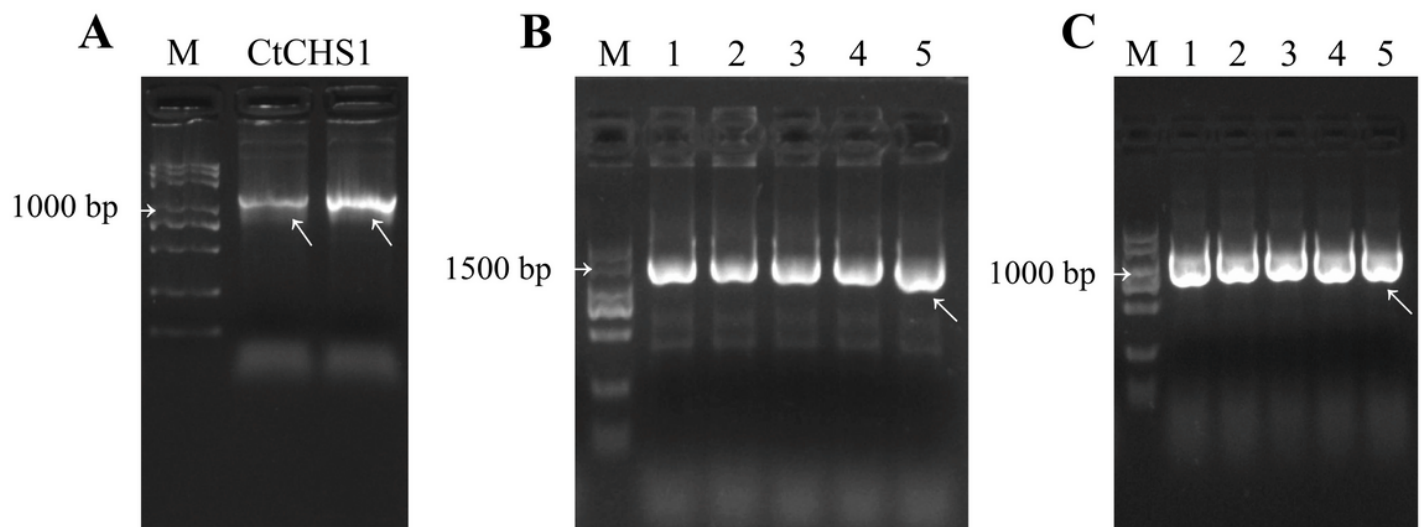


Figure 4

CtCHS1 gene cloning and vector construction. A PCR amplification results of *CtCHS1* gene. B Vector construction of *pRI201-CtCHS1*. C Vector construction of *pBI221-CtCHS1*.

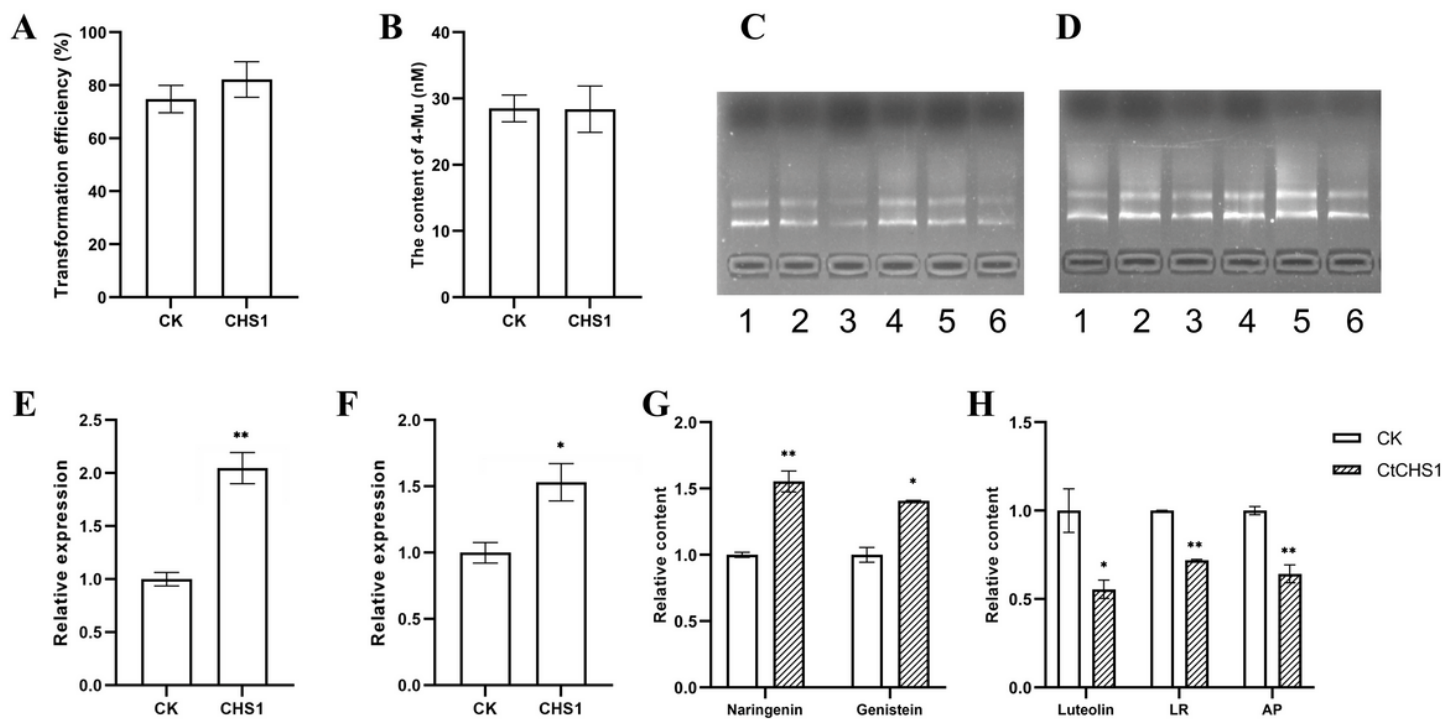


Figure 5

Results of *Agrobacterium* and biolistic-mediated overexpression of *CtCHS1* in safflower callus. A, B The transform efficiency of *Agrobacterium* and biolistic-mediated overexpression. C, D The RNA extracted from *Agrobacterium* and biolistic transformed callus. E, F The relative expression of *CtCHS1* in *Agrobacterium* and biolistic transformed and control callus. G Naringenin and genistein were significantly increased in *Agrobacterium*-transformed callus. H Luteolin, luteolin-7-O-rutinoside and apigenin derivative were significantly decreased in biolistic-transformed callus. The data are mean $\pm$ SEM (n=3), * indicates statistical significance at p<0.05 and ** indicates statistical significance at p<0.01

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