

Stable Transformation of Suspension-Cultured *Glycyrrhiza inflata* Batalin Cells with *Agrobacterium tumefaciens*

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A protocol for the efficient genetic transformation of licorice (*Glycyrrhiza inflata* Batalin) cells in suspension culture using *Agrobacterium tumefaciens*-mediated T-DNA delivery is described. *G. inflata* cells in suspension culture were infected with *A. tumefaciens* strain LBA4404 harbouring the binary vector pCambia1303, which contains the β -glucuronidase (GUS) reporter gene and a hygromycin resistance gene (*hpt* II), respectively, under the transcriptional control of the CaMV35S promoter. Optimal transformation efficiency was achieved with an *A. tumefaciens* suspension having an OD_{600} of 0.4 and a period of 24 h of co-cultivation with 3-day-old cells in a medium supplemented with 200 μ M acetosyringone. The transgenic cell lines have been maintained in suspension subculture for 5 months. PCR and Southern blot analyses confirmed the stable integration of transgenes into the *G. inflata* genome. The introduced genes had no discernable effect on cell growth or accumulation of total licorice flavonoids in the transgenic cell lines. This study provides the basis for the development of transgenic *G. inflata* cells.

Key words: *Agrobacterium tumefaciens*, *Glycyrrhiza inflata*, Transformation, Flavonoids

Introduction

Licorice (*Glycyrrhiza inflata* Batalin) is one of the most popular medicinal plants in the world and a fundamental component of many traditional Chinese and Japanese medicines. Chemical investigations have revealed that licorice contains a wide variety of bioactive constituents (Fukai *et al.*, 2002; Zhang and Ye, 2009). For example, licorice flavonoids are pharmacologically active compounds that have antioxidant, antibacterial, anti-tumour, and HIV-inhibitory activities (Zhang *et al.*, 2009). These properties are responsible for the growing demand for *Glycyrrhiza* and its flavonoid extracts. Recent environmentally irresponsible overcollection of wild licorice has resulted in the decrease and extinction of wild licorice resources. As an alternative approach, cell suspension sys-

tems are generally considered the most suitable method for large-scale production. Many studies (Ayabe *et al.*, 1990; Mousa *et al.*, 2007; Shams-Ardakani *et al.*, 2007; Yang *et al.*, 2008, 2009) have reported the development of licorice callus and cell cultures. Since licorice flavonoids are present in insufficient amounts in suspension cells, a promising strategy is to develop high-yielding transgenic licorice cell lines by means of genetic engineering, but an efficient protocol for the stable genetic transformation of licorice cells is lacking.

Plant transformation mediated by *Agrobacterium tumefaciens* was first reported in the 1980s. Since then, *A. tumefaciens*-mediated transformation has become a standard method to genetically modify dicotyledonous plants due to the simplicity, low cost, and low transgene copy number integrated into the plant genome (Ding *et al.*, 2006; Mehrotra *et al.*, 2011; Qu and Dong, 2005; Wu *et al.*, 2008). However, to our knowledge, there has been no report on the successful transformation of licorice cell cultures by *A. tumefaciens*. A critical point in developing an efficient transforma-

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; NAA, naphthaleneacetic acid; 6-BA, 6-benzyladenine; AS, acetosyringone; GUS, β -glucuronidase; MS, Murashige and Skoog; LB, Luria Bertani; *hpt* II, hygromycin resistance gene; PCR, polymerase chain reaction; YEB, yeast extract broth.



tion system is to optimize the conditions affecting the transformation efficiency, such as the length of the pre-cultivation period, the density of *A. tumefaciens* cells, and the length of the co-cultivation period (Gmitter *et al.*, 2002). In the present study, a protocol for the efficient genetic transformation of *G. inflata* cells in suspension culture using *A. tumefaciens*-mediated T-DNA delivery is described, and the above-mentioned major parameters affecting the transformation efficiency were tested. This study provides the basis for the development of transgenic *G. inflata* cells.

Material and Methods

Chemicals and reagents

Naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), 6-benzyladenine (6-BA), and hygromycin B were purchased from Sigma-Aldrich (St. Louis, MO, USA). Water was obtained from a Milli-Q (Millipore, Bedford, MA, USA) water purification system. Other chemicals used in this study were Chinese analytical reagent grade products.

Plant cell cultures

The original *G. inflata* seeds were collected in Sinkiang, China. The calli formed from young *G. inflata* explants were subcultured on solid Murashige and Skoog (MS) medium, supplemented with 2,4-D (1.0 mg/l), NAA (0.5 mg/l), and 6-BA (0.5 mg/l), at 4-weeks intervals. Cell suspension cultures were initiated via the agitation of 6 g of calli in 250-ml Erlenmeyer flasks containing 100 ml of MS liquid medium, supplemented with NAA (0.5 mg/l), 6-BA (0.5 mg/l), and 3% (w/v) sucrose, at 120 rpm for 3 weeks under the same conditions as for the callus culture until a suspension of free cells had formed. In order to maintain the suspension culture, a 15-ml aliquot of a 2-week-old culture was transferred to 100 ml of fresh MS liquid medium (Yang *et al.*, 2008).

Hygromycin B resistance test

Solutions with incremental concentrations of hygromycin B (0, 10, 15, 20, 25, 30, 35, and 40 µg/ml) were added to pre-sterilized molten media for direct incubation of *G. inflata* cells at 28 °C for 3 weeks to test the selection concentration of hygromycin B for transformed *G. inflata* cells. Cell growth was observed within a culture cycle.

A. tumefaciens-mediated transformation

A. tumefaciens strain LBA4404 was cultured in yeast extract broth (YEB) medium supplemented with 50 µg/ml of streptomycin and 50 µg/ml of rifampicin at 28 °C. The strain harbouring the binary vector pCAMBIA1303, which contains the β -glucuronidase (GUS) reporter gene and the hygromycin phosphotransferase resistance gene (*hpt* II) under the control of the CaMV 35S promoter, was used for transformation of the *G. inflata* suspension cells. A single colony of *A. tumefaciens* strain LBA4404 was grown in 5 ml of Luria Bertani (LB) media supplemented with 50 µg/ml of kanamycin, 50 µg/ml of rifampicin, and 50 µg/ml of streptomycin for 2 d at 250 rpm and 28 °C. Two hundred µl of these cultures were transferred to 2 ml LB medium and incubated at 28 °C until a series of *Agrobacterium* densities was obtained.

A fresh *A. tumefaciens* culture was centrifuged at 5000 \times g for 10 min, and the resulting pellet was resuspended in modified MS liquid medium. This bacterial suspension and *G. inflata* cells from various incubation periods (0, 1, 2, 3, 4, 6, 8, and 10 d from subculture) were mixed for co-cultivation experiments at 25 °C in the dark. After co-cultivation (6, 12, 24, 48, and 72 h) with supplementation of different acetosyringone (AS) concentrations (50, 100, 150, 200, 250, and 300 µM), the cultures were filtered via a Buchner funnel and washed by gently aspirating the medium with a 5-ml pipette and replacing with fresh medium containing 300 mg/l of cefotaxime. Preliminary experiments had shown that cefotaxime at 300 mg/l eliminated *A. tumefaciens* with no effect on the growth of the *G. inflata* cells. The washed *G. inflata* cells were transferred to solid selection media containing 300 mg/l of cefotaxime and hygromycin B to select for transformants. The transformants were subcultured every month on selective media. Following sufficient growth on agar medium, cell suspensions were initiated from calli and grown with hygromycin B for 5 months and subcultured every 2 weeks. Transformation experiments were conducted three times, and putative transformed cell lines were compared with non-transformed wild-type cells.

Histochemical GUS assay

The histochemical GUS assay of transgenic *G. inflata* cells was carried out according to the methods described by Jefferson *et al.* (1987).

Staining solution was prepared by dissolving 2 mM X-Gluc (5-bromo-4-chloro-3-indolyl glucuronide) in 100 μ l of dimethyl formamide and adding the mixture to 10 ml of 100 mM sodium phosphate buffer. Triton X-100 (0.05%) was added to improve cell penetration by the staining solution. Putative transgenic cells were immersed in the staining solution, and a short vacuum filtration was applied for 5 min. The samples were incubated at 37 °C for 12 h, and the transformation efficiency was monitored through instantaneous GUS expression (Nakamura and Ishikawa, 2006).

Polymerase chain reaction (PCR) and Southern blot analyses

The putative transformed cell lines were subcultured in suspension for 5 months and then harvested. Fresh cells (1 g) were ground to a fine powder in liquid nitrogen, and genomic DNA was extracted using a plant genomic DNA kit (Tiangen, Beijing, China). Extracted genomic DNA (100 ng) was used as a template for PCR analysis. The primers *hpt*-F (5'-TTC-GATGTAGGAGGGCGTGGAT-3') and *hpt*-R (5'-CGCGTCTGCTGCT CCATACAAG-3') were used to identify the presence of the *hpt* II gene with the following parameters: 95 °C for 6 min, then 35 cycles at 93 °C for 40 s, 53 °C for 40 s, and 72 °C for 40 s, followed by 72 °C for 10 min. The genomic DNA from untransformed *G. inflata* cells was used as a negative control, while the plasmid pCAMBIA1303 was used as a positive control in PCR analysis.

For Southern blot analysis, 30 μ g of genomic DNA from *G. inflata* cells of 2 randomly selected transgenic lines and a non-transformed line were digested overnight with *Bam*HI and *Eco*RI restriction enzymes (Fermentas, Burlington, Ontario, Canada), respectively, and the pCAMBIA1303, linearized with *Bam*HI, was used as a positive control. Southern blot analysis was performed according to the procedure described by Zhang *et al.* (2011a). A 1026-bp PCR-amplified *hpt* II fragment was used as a probe.

Analysis of the growth and flavonoid accumulation of transformed G. inflata cells

The growth of transformed *G. inflata* cells and accumulation of licorice flavonoids were continuously monitored every month since establishment of the suspension cultures (Zhang *et al.*,

2011b). The cells were harvested by filtration via a Buchner funnel. Fresh cells were weighed and dried at 50 °C to a constant dry mass.

The licorice flavonoids were extracted from the *G. inflata* cells and their absorbance determined according to the methods of Wang and Qi (2009) and Yang *et al.* (2008) with minor modifications. The dried cells were ground to a fine powder and filtered through a 100-mesh sieve, and cell samples (0.10 g) were exactly weighed and put into a 10-ml flask. The flavonoids were extracted with 30 volumes of ethanol/water (70:30, v/v) by sonication for 1 h at room temperature. After centrifugation at 5500 \times g for 6 min, the supernatant was extracted three times with ethyl acetate (EtOAc), and the precipitation dissolved in 95% ethanol. A rutin solution (0.1 mg/ml) was used as a standard sample. A volume of 0.5 ml of potassium hydroxide solution (10%) was added to an 1-ml sample and the mixture shaken for 5 min for colour reaction; then the UV spectrum of both the extracted flavonoid sample and a standard sample were scanned between 190 and 600 nm. The peak analysis indicated that both exhibited a maximum absorbance at 410 nm. Therefore, the absorbance of all samples was measured at 410 nm.

Results and Discussion

Hygromycin B sensitivity test

In preliminary experiments, we found that *G. inflata* cells were not hypersensitive to rifampicin, kanamycin, and cefotaxime (data not shown), but were sensitive to hygromycin B. When *G. inflata* cells were grown on the modified MS agar medium containing various hygromycin B concentrations (0, 10, 15, 20, 25, 30, 35, and 40 μ g/ml) at 28 °C for 3 weeks, cellular growth was significantly affected, as they gradually turned brown and could not grow on the medium supplemented with hygromycin B at a concentration higher than 35 μ g/ml. Therefore, 35 μ g/ml hygromycin B was used for selection in later experiments.

Confirmation of transformation

Prominent GUS activity was detected in some transformed cells with stronger instantaneous expression, while the non-transformed cells did not show any GUS activity (Fig. 1). The genomic DNA of putative transformed suspensions, subcultured for 5 months, was extracted

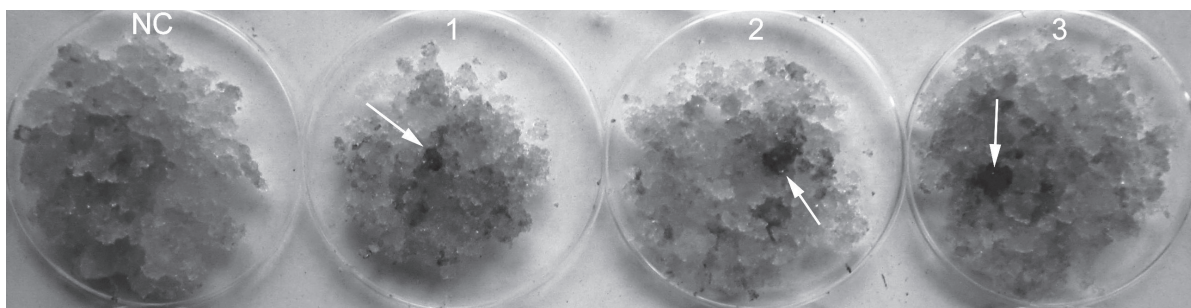


Fig. 1. Detection of GUS activities in the transgenic *G. inflata* cells: transformed (1, 2, 3) and non-transformed cells as negative control (NC).

for PCR analysis of *hpt II*. A single predicted band of ~1 kb was amplified from the selective transformants, and pCambia1303 was used as a positive control. However, no amplification was observed with untransformed *G. inflata* cells used as negative controls (Fig. 2B). These results indicated that transgenic *G. inflata* cells had been obtained by *A. tumefaciens*-mediated transformation.

Integration of the introduced gene into the genomic DNA of transgenic lines was further confirmed by Southern blot analysis. The results showed that the two selected transformants had a single-copy T-DNA insertion and a variety of hybridization patterns, which indicated that integration randomly occurred at different genome sites (Fig. 2C). Southern blot analysis further confirmed that the foreign gene, *hpt II*, in pCAM-

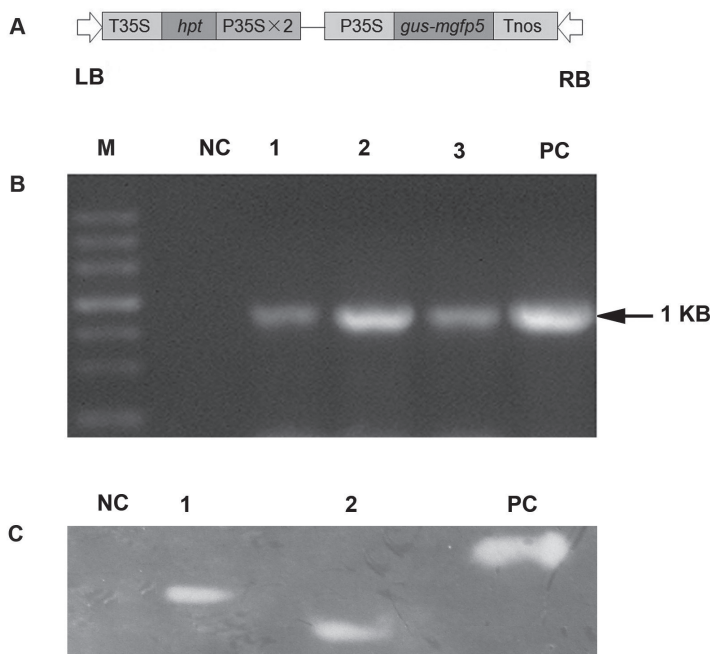


Fig. 2. PCR and Southern blot analysis of putative transformed *G. inflata* cells after suspension culture for 5 months. (A) Schematic representation of T-DNA of pCambia1303; (B) PCR analysis for *hpt II* of three randomly selected transgenic lines of *G. inflata* cells; (C) Southern blot analysis of two randomly selected transgenic lines of *G. inflata* cells. NC, the non-transformed *G. inflata* cell as negative control; PC, the plasmid pCambia1303 as positive control; M, 250 DNA marker plus.

BIA1303 had been successfully integrated into the chromosomal DNA of *G. inflata*.

Analysis of experimental parameters

To establish a more efficient method for *Agrobacterium*-mediated transformation of *G. inflata* suspended cells, several important parameters affecting the transformation efficiency were optimized. These factors included the length of the pre-cultivation period, the density of *A. tumefaciens* cells, the length of the co-cultivation period, and the AS concentration.

To determine the pre-conditioning effect on the transformation frequency, 0-, 1-, 2-, 3-, 4-, 6-, 8-, and 10-day-old cells were subjected to *Agrobacterium* co-cultivation and analysed for GUS expression. Fig. 3A shows that pre-cultivation had a marked effect on the transformation frequency, and the maximum transformation frequency was achieved after pre-conditioning for 3 days. Cells undergo a physiological and developmental shift for morphogenic competency during pre-conditioning. When the T-DNA is inserted following

this short period, the recipient cells have already entered the regeneration pathway (Ainsley *et al.*, 2001). This is consistent with the experience with tobacco cells, where the maximum transformation frequency was obtained with 3- or 4-day-old cells (An, 1985).

The effect of *A. tumefaciens* cell density on the transformation frequency was determined by transforming suspended *G. inflata* cells with *Agrobacterium* cell suspensions of an OD_{600} of 0.1, 0.2, 0.3, 0.4, 0.6, and 0.8. The histochemical GUS assay showed a positive correlation between the concentration of *Agrobacterium* cells in the co-cultivation mixture and the transformation frequency at $OD_{600} \leq 0.4$ at which the maximum transformation frequency was achieved (Fig. 3B). At higher concentrations the transformation efficiency decreased dramatically, and at OD_{600} 0.8 explants were completely colonized by bacteria, which were more difficult to eliminate during the subsequent selection and screening regimes.

The effect of the AS concentration on the transformation frequency was determined by adding AS at six concentrations (50, 100, 150,

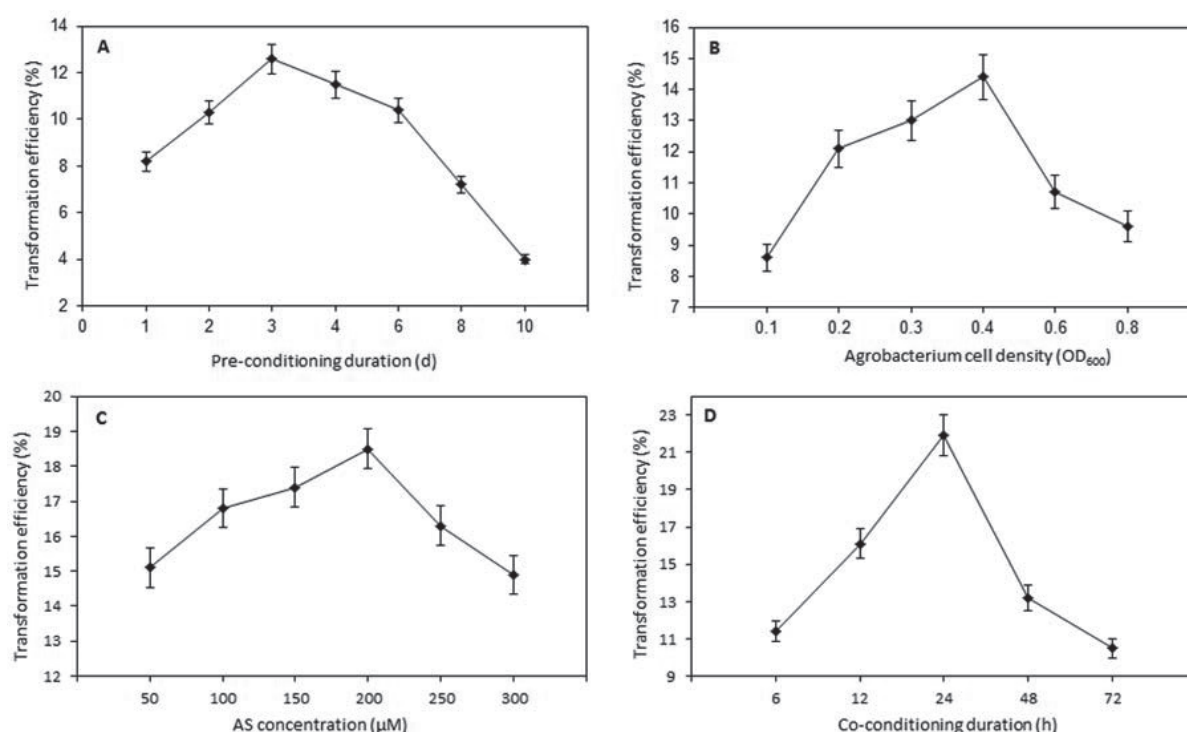


Fig. 3. Effect of some important parameters on the transformation efficiency. (A) Length of pre-cultivation period; (B) *Agrobacterium* cell density; (C) AS concentration; (D) co-cultivation period.

200, 250, and 300 μM) to the co-cultivation medium. The transformation frequency increased with increasing AS concentrations up to 200 μM , whereas a further increase to 300 μM resulted in a significant reduction in the transformation frequency (Fig. 3C). That the addition of AS to the co-cultivation medium markedly enhances the cell transformation frequency has been reported for many plant species (Mehrotra *et al.*, 2011; Mohri *et al.*, 1997; Pandey *et al.*, 2010; Zhang *et al.*, 2011). AS is a phenolic compound that is released by wounded cells of some dicotyledonous plants and plays an important role in the natural infection of plants by *A. tumefaciens* by activating the virulence genes of the Ti plasmid and thus initiating the transfer of the T-DNA region into the plant genome.

Three-day-old *G. inflata* cells were co-cultivated with *A. tumefaciens* for 6, 12, 24, 48, and 72 h to evaluate the effect of the duration of co-cultivation on the transformation efficiency. Co-cultivation for 24 h was found to be optimal, while longer co-cultivation resulted in superfluous proliferation of bacteria and consequently decreased transformation frequency (Fig. 3D), and some transformed cells turned brown and died on the selection medium soon thereafter. During co-cultivation, the genes encoded by the virulence (*vir*) region of the Ti plasmid are induced in the bacteria, resulting in the integration of T-DNA into the plant genome (Stachel *et al.*, 1986). In general, 2–3 days of co-cultivation is standard in most transformation protocols, as longer periods may result in bacterial overgrowth (Mourgues *et al.*, 1996; Nakamura *et al.*, 2006). However, in our experiment the optimum transformation frequency was achieved at 24 h of co-cultivation.

The growth of transgenic cells and total flavonoid accumulation

Transgenic *G. inflata* cells generated, following *Agrobacterium*-mediated transformation under optimized conditions, were continuously subcultured under hygromycin B selection for more than 5 months and their antibiotic resistance was monitored. During hygromycin B selection, yellow-coloured, resistant, transformed cells grew vigorously, whereas non-transformed cells gradually died. These results demonstrated that putative transgenic cells were stable with no loss in vigour.

If transgenic cell cultures are to be used for large-scale production of licorice flavonoids, it is essential that these cells grow as vigorously as the normal *G. inflata* cells and stably synthesize licorice flavonoids during repeated subculture (Ketchum *et al.*, 2007). In this study, growth and flavonoid production of the transgenic cells were continuously monitored, once the suspension cultures had been established. The transgenes introduced by the pCambia1303 vectors had no apparent effect on total flavonoid production in the transgenic cells (Fig. 4). During monthly monitoring, the flavonoid content was similar in both transgenic and non-transgenic *G. inflata* cells, respectively.

Conclusion

The low yield of flavonoids in suspension cells limits their industrial utility; but, the yield might be increased through genetic engineering by

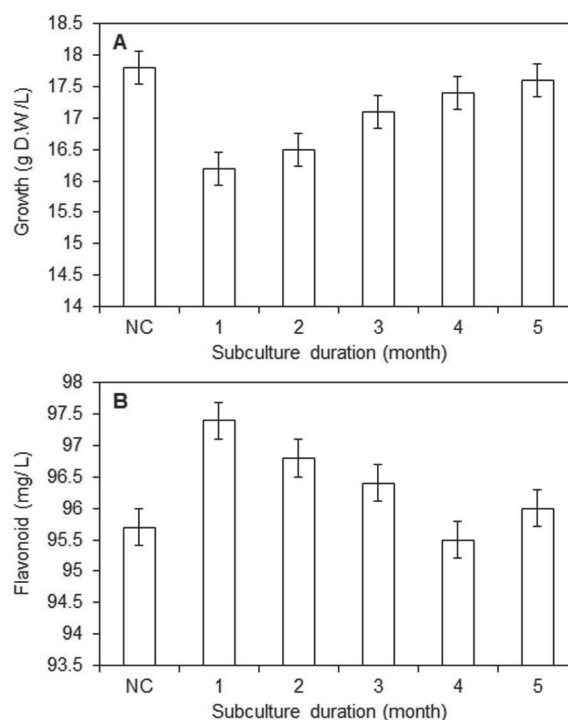


Fig. 4. (A) Growth and (B) flavonoid production in suspension cultures of transgenic *G. inflata* cells monitored continuously and non-transformed cells as negative control (NC). Bars represent the mean \pm SD of three independent measurements.

overexpressing genes that encode rate-limiting enzymes in the flavonoid biosynthesis (Zhang *et al.*, 2009b). This requires the availability of efficient methods for the transformation of the cells. In this study, we successfully developed a protocol for the *A. tumefaciens*-mediated transformation of *G. inflata* suspension cells by optimizing several important parameters of the transformation system.

Putative transgenic cell lines selected using hygromycin B resistance and GUS activities proved the integration of transgenes into the *G. inflata* genome as confirmed by PCR and Southern blot analyses. Additionally, we detected no obvious influence on cell growth and flavonoid production in the transgenic culture due to the introduced

genes. These results indicate that the integrated genes *per se* do not affect growth and productivity in comparison to the parent controls. Therefore this study provides a basis for the standardization of transformation protocols for reverse genetic approaches in *G. inflata* cells, and has provided a foundation for the genetic transformation and modification of *G. inflata* cells.

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