Plant Biotechnology 39, 251–257 (2022)
DOI: 10.5511/plantbiotechnology.22.0520a

Original Paper

Establishment of a genetic transformation system for *Codonopsis pilosula* callus

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Received March 4, 2022; accepted May 20, 2022 (Edited by A. Iwase)

Abstract *Codonopsis pilosula*, a traditional Chinese medicinal and edible plant, contains several bioactive components. However, the biosynthetic mechanism is unclear because of the difficulties associated with functional gene analysis. Therefore, it is important to establish an efficient genetic transformation system for gene function analysis. In this study, we established a highly efficient *Agrobacterium*-mediated callus genetic transformation system for *C. pilosula* using stems as explants. After being pre-cultured for 3 days, the explants were infected with *Agrobacterium tumefaciens* strain GV3101 harboring pCAMBIA1381-35S::*GUS* at an OD_{600} value of 0.3 for 15 min, followed by co-cultivation on MS induction medium for 1 day and delayed cultivation on medium supplemented with 250 mg l^{-1} cefotaxime sodium for 12 days. The transformed calli were selected on screening medium supplemented with 250 mg l^{-1} cefotaxime sodium and 2.0 mg l^{-1} hygromycin and further confirmed by PCR amplification of the *GUS* gene and histochemical GUS assay. Based on the optimal protocol, the induction and transformation efficiency of calli reached a maximum of 91.07%. The establishment of a genetic transformation system for *C. pilosula* calli lays the foundation for the functional analysis of genes related to bioactive components through genetic engineering technology.

Key words: callus, *Codonopsis pilosula*, genetic transformation.

Introduction

Codonopsis pilosula (Franch.) Nannf. (*C. pilosula*) is a perennial herb. The root, known as "Dangshen", is a commonly used traditional Chinese medicine renowned for its remarkable effect on improving gastrointestinal function, nourishing the spleen and lungs, enhancing immunity, and delaying senescence (Bai et al. 2020). It is also a popular health food and is included in the daily diet (He et al. 2015). The pharmacological and health care effects of *C. pilosula* are attributed to its bioactive compounds, including secondary metabolites, such as polysaccharides, lobetyolin, syringin, angelicin, and atractylenolide III (Gao et al. 2019).

Several genes related to the biosynthesis of bioactive compounds have been identified in *C. pilosula* (Gao et al. 2015). For example, Ji et al. (2019) found that *CpUGPase* and *CpPMK* may participate in bioactive metabolite accumulation under methyl jasmonate (MeJA) induction (Ji et al. 2019). However, none has been verified by experiments due to the lack of a genetic transformation system.

An *Agrobacterium*-mediated genetic transformation is a suitable option. The Ti plasmid in *Agrobacterium tumefaciens* can stably transfer genetic material through plant wounds into the host cells (Chilton et al. 1982). In addition, the binary vector pCAMBIA1381 carries the hygromycin phosphotransferase gene. Therefore, successfully transformed tissues or plants can be rapidly screened for hygromycin resistance (Idnurm et al. 2017). Genetic transformation systems have been established for many plants. Most genetic transformation systems in medicinal plants use hairy roots. Miao et al. optimized the hairy root transformation conditions for *Rubia yunnanensis* (Miao et al. 2021). The hairy roots of *Scutellaria* spp. *(Lamiaceae)* were induced using wild-type *Agrobacterium rhizogenes* strain A4 with cotyledons as explants (Stepanova et al. 2021). In *C. pilosula*, the hairy root genetic transformation system has been established (Yang et al. 2020). Overexpression of the functional genes *CpSPL2* and *CpSPL10* could promote the growth of hairy roots and the accumulation of secondary metabolites, such as total saponins, in *C. pilosula* (Yang et al. 2021). However, hairy root systems have certain limitations. For example, plant traits cannot be observed in hairy roots compared with regenerated transgenic plants. Callus induction is an

Abbreviations: Cef, cefotaxime sodium; Hyg, hygromycin; Kan, kanamycin; MS, callus-inducing medium; Rif, rifampicin; 6-BA, 6-Benzylaminopurine; 2,4-D, 2,4-Dichlorophenoxyacetic acid.
Published online August 4, 2022

important process for the regeneration of intact plants (Li N et al. 2020). In addition, bioactive components, such as polysaccharides (Wang et al. 2012), can also be synthesized in the callus of *C. pilosula*.

In this study, the *C. pilosula* callus genetic transformation system was established by transforming a recombinant vector with a beta-glucuronidase (*GUS*) reporter gene. These results provide a technological basis for studying functional genomics and metabolic pathways of active compounds.

Materials and methods

Plant materials

Seeds were collected from Pingshun County, Shanxi Province, China, and identified as *C. pilosula* seeds by Professor Jian-Ping Gao. After soaking in warm water for 1 h, the seeds were disinfected with 75% ethanol for 15 s and washed three times with sterile water. The seeds were then sterilized in 1% NaClO for 20 min and rinsed five times with sterile water. Finally, the sterile seeds were placed on 1/2 MS medium (with 30 g l^{-1} sucrose and 7 g l^{-1} agar, pH 6.5±0.2) and cultivated for 7 days at 25±1°C in the dark. After germination, the sterile seedlings were cultured at 25±1°C with a 16 h light/8 h dark cycle for 3 weeks.

Bacterial strain and recombinant DNA

The plant expression vectors pCAMBIA1381-35S and pCAMBIA1381-35S::GUS were transformed into *A. tumefaciens* GV3101 using the freeze-thaw method (Weigel and Glazebrook 2006).

Bacteria culture

The native GV3101 *A. tumefaciens* strain (negative control) and the recombinant GV3101 strain harboring pCAMBIA1381-35S and pCAMBIA1381-35S::*GUS* were streaked on solid LB medium containing either 50 mg l^{-1} rifampicin (Rif) or 50 mg l^{-1} rifampicin (Rif) plus 50 mg l^{-1} kanamycin (Kan) and incubated at 28°C in the dark for 2–3 days. After PCR identification using the *GUS* reporter gene, a positive single colony was inoculated into LB liquid medium containing the specified antibiotics and incubated overnight at 28°C (shaking at 180 rpm) until the OD_{600} value of the bacterial solution was 1.0–1.2. The culture was then diluted at a ratio of 1 : 30, and incubated at 28°C (shaking at 180 rpm) until the OD_{600} value of the bacterial solution was 0.3, 0.6, 0.9, and 1.2. The cultures were centrifuged for 8 min at 5,000 rpm, and the precipitated cells were resuspended in the same volume of MS liquid medium. This served as the infection solution.

Hygromycin resistance test of C. pilosula

The stems of 4-week-old *C. pilosula* seedlings without axillary buds were sterilized and trimmed to an average length of 0.5 cm for use as explants. The explants were placed on MS medium containing 30 g l^{-1} sucrose, 7 g l^{-1} agar, 0.5 mg l^{-1} 6-Benzylaminopurine (6-BA), 0.2 mg l^{-1} 2,4-Dichlorophenoxyacetic acid (2,4-D), and different concentrations of hygromycin (Hyg) (0, 0.5, 1.0, 1.5, and 2.0 mg l^{-1}) to induce callus. The most suitable screening concentration was selected according to the induction rate of calli after being cultured for 20 days (Olhoft et al. 2003). Each treatment was repeated in triplicate.

Optimization of protocol for inducing transgenic callus

The explants were pre-cultured on the MS medium containing 30 g l^{-1} sucrose and 7 g l^{-1} agar supplemented with 0.5 mg l^{-1} 6-BA and 0.2 mg l^{-1} 2,4-D. Then the pre-cultured explants were immersed in the infection solution and placed on the MS medium supplemented with 0.5 mg l^{-1} 6-BA and 0.2 mg l^{-1} 2,4-D in the dark for co-cultivation. Following the inoculation period, the explants were transferred to the MS medium supplemented with 0.5 mg l^{-1} 6-BA, 0.2 mg l^{-1} 2,4-D, and 250 mg l^{-1} cefotaxime sodium (Cef) for delayed cultivation at 25°C in the light. Then, the explants were transferred to the MS medium supplemented with 0.5 mg l^{-1} 6-BA, 0.2 mg l^{-1} 2,4-D, and 250 mg l^{-1} Cef plus Hyg at incubated at 25°C in the light to screen the positive callus. The effect of Hyg concentration on the induction efficiency was investigated. We then optimized the pre-cultivation time, infection time, co-cultivation time, delayed cultivation time, and bacterial concentration with stem explants on the MS medium (Table 1) using an orthogonal experimental design ($L_{16}(4^5)$). The induction rate was calculated after explants were cultured for 30 days.

Ten explants were placed on each plate, and all experiments were conducted in triplicate. All data are expressed as mean± standard error and were compared using Duncan's multiple range tests at a 5% significance level.

Molecular analysis of callus

Genomic DNA was extracted from transgenic and control calli using the cetyltrimethylammonium bromide (CTAB) method (Ghosh et al. 2009). Each PCR-amplification was conducted in a 25 μl reaction volume containing 2 μl of template, 17 μl sterile water (ddH_2O), 2.5 μl 10× buffer, 2 μl dNTPs (2.5 mM), 0.5 μl of each primer, and 0.5 μl of Easy Taq DNA Polymerase (Takara Bio, Beijing, China). Primers were designed for the *GUS* reporter gene. The sequences of the forward and reverse gene-specific primers were *GUS*-F (5′-GTCAGTGGCAGT GAAGGG-3′) and *GUS*-R (5′-CGAGGTACGGTAGGAGTT-3′), respectively. The PCR reactions were performed as follows: initial denaturation at 94°C for 5 min; then 34 cycles of 94°C for 30 s, 53°C for 30 s, and 72°C for 1 min; and a final extension at 72°C for 10 min. PCR products were detected using 1% agarose gel electrophoresis. In addition, specific primer pairs (pc1381-F/R 5′-CAAAAGCACAAACACGCTAAGT-3′ and 5′-AGA GCATCGGAACGAAAAA-3′) were designed according to the partial DNA fragment (656 bp) outside the T-DNA region in the pCAMBIA1381-35S::*GUS* binary vector to exclude the possibility of *Agrobacterium* contamination of regenerated

Table 1. Orthogonal test design of the protocol of *C. pilosula* callus genetic transformation system ($L_{16}(4^5)$).

Group	Pre-incubation (d)	Infection (min)	Co-cultivation (d)	Delayed incubation (d)	Bacteria concentration	Number of calli	Callus induction rate (%)*
1	0	5	0	0	1	4.67±0.58	57.41±8.49^{c}
2	0	10	1	4	2	2.50±0.71	18.46±0.40^{e}
3	0	15	2	8	3	0.67±0.58	5.34±4.64^{fg}
4	0	20	3	12	4	0.00±0.00	0.00±0.00^{g}
5	1	5	1	8	4	1.33±0.58	11.49±3.40^{ef}
6	1	10	0	12	3	7.00±0.00	70.00±10.00^{b}
7	1	15	3	0	2	0.00±0.00	0.00±0.00^{g}
8	1	20	2	4	1	1.33±0.58	13.70±5.48^{ef}
9	2	5	2	12	2	3.67±0.58	37.78±3.85^{d}
10	2	10	3	8	1	6.00±1.00	56.11±5.36^{c}
11	2	15	0	4	4	0.00±0.00	0.00±0.00^{g}
12	2	20	1	0	3	0.00±0.00	0.00±0.00^{g}
13	3	5	3	4	3	2.00±0.00	19.39±1.05^{e}
14	3	10	2	0	4	1.67±0.58	17.04±5.13^{e}
15	3	15	1	12	1	14.00±1.41	90.55±3.27^{a}
16	3	20	0	8	2	7.67±1.15	65.50±3.23^{b}

The bacterial concentration 1, 2, 3, and 4 represent OD_{600} values of 0.3, 0.6, 0.9, 1.2. *Different letters indicated the significant differences ($p<0.05$).

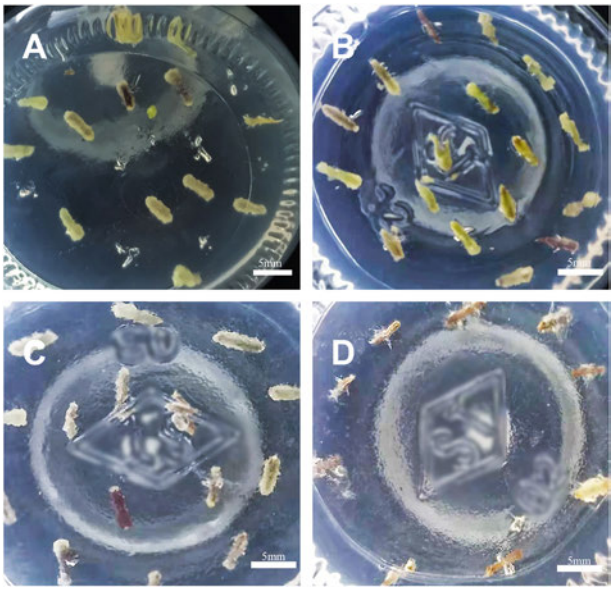

Figure 1. Effect of different hygromycin concentrations on the induction of callus in *C. pilosula*. (A) 0 mg l^{-1}. (B) 1.0 mg l^{-1}. (C) 1.5 mg l^{-1}. (D) 2.0 mg l^{-1}.

plants.

GUS staining of callus

GUS assays were conducted using the GUS Blue KIT (Huayueyang Biotech Co., Ltd., Beijing, China) according to the instructions. Tissues-stained blue were regarded as positive transgenic explants (Jefferson et al. 1987; Liu et al. 2020).

Results

Effect of different hygromycin concentrations on the differentiation of C. pilosula callus

The effect of hygromycin on *C. pilosula* calli was analyzed to ascertain the suitable screening pressure for hygromycin. When a hygromycin concentration of 2.0 mg l^{-1} was used, most of the explants did not expand (Figure 1), and the rate of callus induction decreased distinctly by 27.98% (Table 2). However, the callus induction rate was greater than 75% when the hygromycin concentration was lower than 1.5 mg l^{-1}. Therefore, the adequate concentration was 2.0 mg l^{-1} to screen for positive transgenic calli.

Table 2. Effect of hygromycin concentration on the induction of callus in *C. pilosula*.

Hygromycin concentration (mg l^{-1})	Number of samples	Number of calli	Callus induction rate (%)*
0	33	30	91.39±7.47^{a}
0.5	33	31	92.22±6.94^{a}
1	45	34	74.97±6.71^{b}
1.5	44	36	81.75±4.32^{ab}
2	36	10	27.98±1.78^{c}

*Different letters indicated the significant differences ($p<0.05$).

Callus induction in C. pilosula

The orthogonal test design indicated that the induction rate for transgenic callus was optimal with a 3-day pre-cultivation period, a bacterial concentration of OD_{600} value of 0.3, an infection time of 15 min, a 1-day co-cultivation period, and a 12-day delayed cultivation period. Based on the data shown in Table 1, induction rates with the optimal protocol were highest, i.e., 90.55±3.27%, followed by only 70.00±10.00% for the protocol with a 1-day pre-cultivation period, an OD_{600} value of 0.9 for bacterial concentration, an infection time of 10 min and a 12-day delayed cultivation period. No calli were induced in groups 4, 7, 11, and 12, which might be due to the high bacterial concentration, short pre-cultivation duration and delayed cultivation, or long duration of

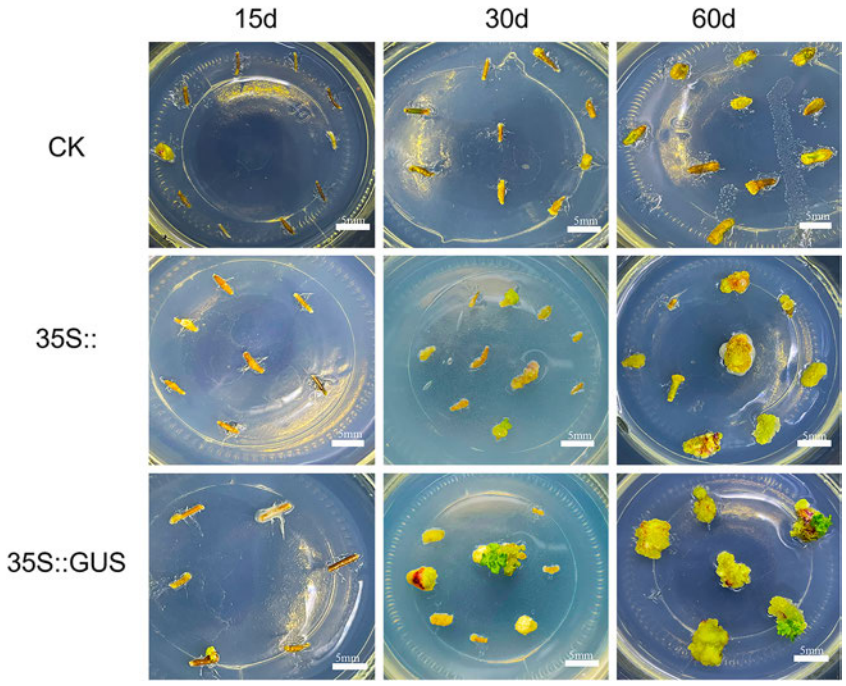

Figure 2. Callus of *C. pilosula* induced from stem explants at 15 days, 30 days, 60 days after infection by *Agrobacterium tumefaciens* GV3101 harboring pCAMBIA1381-35S and pCAMBIA1381-35S::*GUS*. The native GV3101 strain was used as control (CK).

infection and co-cultivation.

For group 15, explants expanded and white calli were observed 30 days after exposure to *A. tumefaciens* GV3101 carrying pCAMBIA1381-35S and pCAMBIA1381-35S::*GUS*, both containing a hygromycin resistance gene. In contrast, the explants exposed to native GV3101 did not differentiate into calli until 60 days after infection due to the lack of the hygromycin resistance gene (Figure 2). After transformed with the strain GV3101 harboring the expression vectors for 60 days, the white calli differentiated into green calli. And the green ones further differentiated into green buds. These results indicated that the optimal genetic transformation medium system with 2.0 mg l^{-1} hygromycin in the screening medium could screen out the positive calli. The *GUS* gene in the vector did not affect the induction rate and growth of calli of *C. pilosula*.

Molecular detection of transformed transgenic callus using PCR

The *GUS* reporter gene was detected by PCR amplification using *GUS*-F and *GUS*-R gene-specific primers to confirm the successful transformation of the callus lines with the heterologous gene. The expected 150 bp band was amplified in the 10 callus lines transformed with pCAMBIA1381-35S::*GUS*, whereas no band was observed in the callus lines transformed with pCAMBIA1381-35S (Figure 3A). Meanwhile, no fragments from recombinant vector pCAMBIA1381-35S::*GUS* were amplified in the callus cells (Lane 1–10), while a 656-bp fragment was amplified in the sample

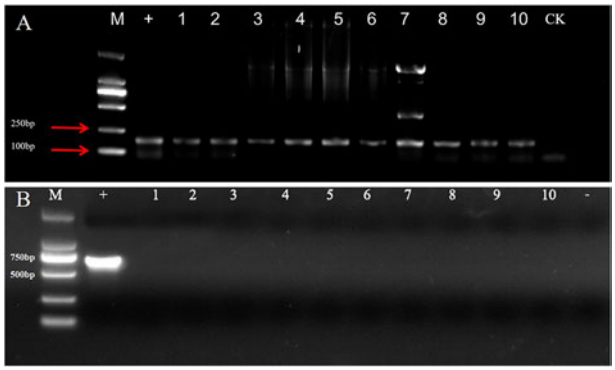

Figure 3. Molecular analysis of *GUS* gene (A) and the sequence outside the T-DNA region of the recombinant vector (B) in different transformed calli of *C. pilosula*. M, 2,000 bp DNA Marker; −, negative control; +, bacteria harboring plasmid pCAMBIA1381-35S::*GUS*; CK, callus lines transformed with pCAMBIA1381-35S; 1–10, callus lines transformed with pCAMBIA1381-35S::*GUS*.

with bacterial harboring plasmid pCAMBIA1381-35S::*GUS* as template (+) (Figure 3B), excluding the possibility of *Agrobacterium* cell contamination. All these indicated that the transgenic callus cells no longer contain bacteria and would be able to claim that the signal comes from stably transformed *GUS* genes.

GUS staining method to detect callus induction rate

Because the pCAMBIA1381-35S::*GUS* vector carried the *GUS* reporter gene, we further examined the protein expression level of the *GUS* reporter gene in transgenic calli. Among the 21 randomly tested samples, 19 were

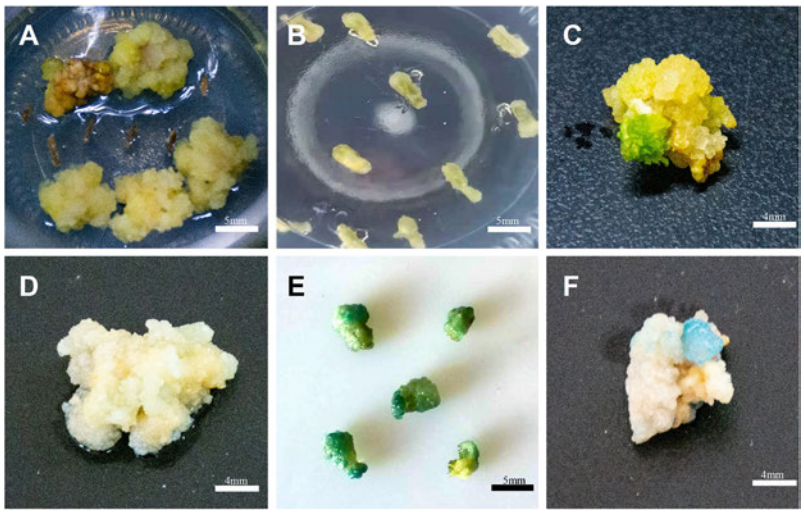

Figure 4. Histochemical GUS assay of *C. pilosula* transgenic callus infected by pCAMBIA1381-35S and pCAMBIA1381-35S::*GUS*. (A) Transformed transgenic callus infected by *A. tumefaciens* GV3101 strain harboring pCAMBIA1381-35S. (B, C) Transformed transgenic callus after 15 days (B) and 60 days (C) of infection by GV3101 strain harboring pCAMBIA1381-35S::*GUS*. (D) GUS activity in transformed transgenic callus infected by GV3101 strain harboring pCAMBIA1381-35S. (E, F) GUS activity in transformed transgenic calli after 15 days (E) and 60 days (F) of infection by GV3101 strain harboring pCAMBIA1381-35S::*GUS*.

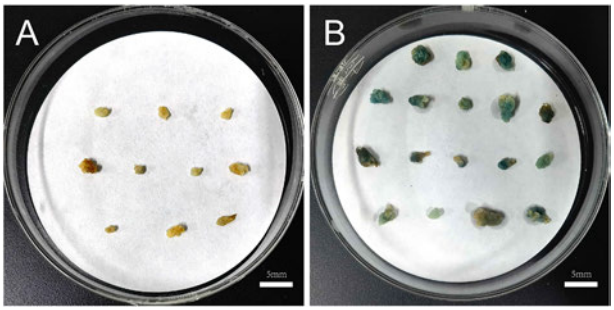

Figure 5. Validation of the transformation efficiency of the optimal callus genetic transformation system. (A) GUS activity in transformed calli infected by strain GV3101 harboring pCAMBIA1381-35S. (B) GUS activity in transformed calli infected by strain GV3101 harboring pCAMBIA1381-35S::*GUS*.

positive. The results show that the conversion rate was as high as 90.47% (Figure 4E). Blue staining was not observed in the negative control group (Figure 4D).

We analyzed GUS activity in proliferated transgenic calli successfully infected by strain GV3101 harboring pCAMBIA1381-35S::*GUS* confirmed by PCR and cultured for 60 days. GUS activity was observed in all transgenic callus lines tested, whereas no such activity was detected in the negative control lines.

We verified the transformation rate of the calli using GUS staining. According to the optimal protocol of the callus genetic transformation system, calli were successfully induced at an induction rate that reached 91.07%. Additionally, 92.13% of calli showed the characteristic intense blue color. No blue coloration was observed in the control group. (Figure 5).

Discussion

In traditional Chinese medicine, *C. pilosula* strengthens the spleen and lungs, nourishes the blood, and promotes fluid (Gao et al. 2018). Polysaccharides are some of the main ingredients. The fructans were separated into CP-A and CP-B (Li JK et al. 2018; Li J 2020), and CP-A was found to have an anti-gastric ulcer effect (Li JK et al. 2017). However, most studies have focused on the chemical composition and pharmacological action of *C. pilosula*. Reports on the function of genes related to polysaccharide synthesis are severely limited because of the lack of a genetic transformation system (Cao et al. 2020; Wang et al. 2018).

Callus can be utilized for plant regeneration and as a biological reactor for producing bioactive compounds from medicinal plants (Guillon et al. 2006; Wang et al. 2012). Establishing a genetic transformation system lays the foundation for studying plant functional genes. Targeted genes can be introduced into plants by *Agrobacterium* transformation for functional analysis. The establishment of the *C. pilosula* callus genetic transformation system will lay the foundation for functional analysis of genes related to bioactive ingredient biosynthesis.

Several factors, including the pre-incubation time, bacterial concentration, infection time, period of co-cultivation, and delayed cultivation, influence the genetic transformation rate. The highest transformation frequency was found with stems of *C. pilosula* pre-cultured for three days, infected for 15 min with 0.3 OD_{600}, and followed by co-cultivation for 1 day and delayed cultivation for 12 days. Pre-cultivation of the explants before infection can reduce the stress damage of

Agrobacterium during the infection period and promote cell division. This makes the explant cells more sensitive to *Agrobacterium* to facilitate its adsorption, thereby improving the transformation efficiency of explants (Zhu et al. 2013). In this study, when the infection time was 15 min, the same as the that in optimal protocol, the transformation rate was decreased along with the decreasing of the pre-cultivation time, less than 10%. The callus transformation rate increased with prolonged pre-cultivation time, consistent with the findings of Zhang et al. (2020). The concentration of *Agrobacterium* and the infection time can affect the transformation rate (Wang et al. 2002). A too high bacterial concentration or too long infection time can easily lead to *Agrobacterium* contamination and browning or necrosis of explants (Sun and Wang 2007). We found that the transformation rate was less than 15% in the group with a long infection period (20 min) and short pre-cultivation period (less than 2 days). All groups with high bacterial concentrations showed a low transformation rate of less than 20%. If the concentration of the bacterial solution is too low or the infection time is too short, cell adsorption is insufficient, which will result in a low transformation rate. However, we found that a lower bacterial concentration led to a higher transformation rate in the optimal group, which might be due to the long delayed-cultivation period. The delayed cultivation medium was supplemented with cephalosporin to inhibit the growth of *Agrobacterium*. However, the lack of antibiotics for screening positive transgenic plants in a short time could enhance the adaptability to screening pressure, which could reduce the browning of the explants (Li et al. 2003). Wu et al. (2020) found that within a certain range, the induction and differentiation rates of resistant calli showed an increasing trend with increasing delays in cultivation time (Wu et al. 2020). In this study, we found that when the delayed cultivation period was less than 4 days, the transformation rate was lower.

The expression of *hptII*, widely used as a selectable marker in plant transformation systems, allows transformed plants to grow on media supplemented with antibiotics (Chattopadhyay et al. 2011; Rizvi et al. 2015). *C. pilosula* was highly sensitive to hygromycin, and the induction rate of transformed hairy roots was only 28.5% on the selection medium with 2 mg l^{-1} hygromycin (Yang et al. 2020), consistent with the results of the experiments described here.

In conclusion, we successfully established a genetic transformation system for *C. pilosula* calli with a transformation rate higher than 90%. In addition, calli can be subcultured or further induced into regenerated plants (not shown in the text). It provides a new method for studying the function of genes in *C. pilosula*, and lays the foundation for the establishment of a genetic transformation system for *C. pilosula* plant regeneration.

Acknowledgements

This research was funded by the National Key Research and Development Program of China (2018YFC1706301, 2018YFC1706304, 2019YFC1710800), the Key Projects of Key Research and Development Program of Shanxi Province (201603D3111005). We would like to thank Editage (www.editage.cn) for English language editing.

Author contributions

Z-YL and J-JJ designed the study and wrote the manuscript. Z-YL performed experiments and analyzed the data. X-RT and FJ prepared the cefotaxime sodium and hygromycin. J-KL and J-PG supervised the analysis and critically revised the manuscript.

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