



Establishment of in vitro culture system for *Codonopsis pilosula* transgenic hairy roots

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Abstract

The aim of the study was to establish a reliable system of transgenic hairy roots in *Codonopsis pilosula* through *Agrobacterium*-mediated genetic transformation. For this, we optimized several steps in the process of *A. rhizogenes* strain C58C1 mediated hairy root induction, including the most appropriate medium, explant type, time for infection and co-cultivation. We achieved an induction rate of up to 100% when the roots of *C. pilosula* seedlings were used as explants, infected with *A. rhizogenes* C58C1 harboring pCAMBIA1305 for 5 min, followed by induction on 1/2MS supplemented with 0.2 mg/L naphthylacetic acid and 200 mg/L cefotaxime sodium. The co-transformed hairy roots were confirmed by PCR amplification of hygromycin phosphotransferase II gene and histochemical GUS assay, and the efficiency of transformation was 70% and 68.3%, respectively, when no hygromycin selection pressure was exerted. To increase biomass production, we excised and self-propagated the transformed hairy roots, which produce saponins. Our successful establishment of an in vitro culture system of transgenic hairy root for this species lays the foundation not only for assessing gene expression and function but also for obtaining high levels of secondary metabolites through genetic engineering technology.

Keywords *Agrobacterium rhizogenes* · *Codonopsis pilosula* · Gene transformation · Hairy root · Saponins

Introduction

Agrobacterium rhizogenes is a Gram-negative soil bacterium that induces plants to produce hairy roots upon infection at the wounding sites. Its root-inducing (Ri) plasmid, containing transfer DNA encoding root locus (rol) gene loci (*rolA*, *rolB*, and *rolC*), is responsible for the stable introduction of genetic material into host cells (Chilton et al. 1982). The hairy roots have some common features, such as a high rate of proliferation in phytohormone-free media, ageotropic growth, an elevated rate of lateral branching, and genetic stability, the latter being a feature that renders this system appropriate for producing valuable secondary metabolites

through in vitro culture (Hosseini et al. 2017; Kochan et al. 2018; Contreras et al. 2019). Furthermore, researchers have exploited the capacity of *A. rhizogenes* to transfer the disarmed T-DNA region from the *A. tumefaciens*-based binary vector along with T-DNA from the Ri plasmid to develop co-transformed transgenic hairy root cultures (Hamill et al. 1987). These co-transformed hairy roots are an extremely powerful tool for elucidating gene functions (Limpens et al. 2004; DeBoer et al. 2009; Kajikawa et al. 2009; Ron et al. 2014), analyzing promoter activity (Preisner et al. 2001), rapidly and efficiently visualizing gene expression (Wiśniewska et al. 2013; Ron et al. 2014), enhancing heterogenous protein expression (Menzel et al. 2003), producing antibodies (Sharp and Doran 2001), modifying plant metabolic pathways (Sommer et al. 1999; Mitra et al. 2002), and studying functional genomics (Seki et al. 2008).

The root of *Codonopsis pilosula*, known as “Dangshen”, is a famous traditional Chinese medicine that has been widely used to replenish qi (vital energy), strengthen the immune system, improve appetite, and cure gastric ulcers. Dangshen is also regarded as a health-care food in China, especially as a dietary supplement when brewing wine, making tea, cooking porridge, or making soup. Chemical and

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pharmacological studies have shown that its active ingredients are mainly composed of alkaloids, polysaccharides, and terpenoids, which participate in anti-gastric ulcer, anti-tumor, anti-aging, and anti-inflammatory activities; as well as aiding in improved respiratory function and memory; and treatments to cure diabetes, increase hemoglobin, and regulate immunity (Sun 2009; Xin et al. 2012; Barnes 2014; Chu et al. 2016; Li et al. 2017a). Because of its extensive pharmacological effects, the demand for Dangshen has greatly increased in domestic and foreign markets. This has stimulated the large-scale cultivation of *C. pilosula*, especially in Gansu Province of China, a region that accounts for 90% of the entire area planted with this species in that country (Deng et al. 2011).

Although some genes have been identified from *C. pilosula* (Gao et al. 2015; Ji et al. 2019), none has been verified by experiments. To test the functions of these candidate genes, a transformation system is necessary. Although hairy roots have been induced (Li et al. 2017b), no reports have been made about a highly effective transformation system for that species. The advantage associated with *Agrobacterium*-mediated transformation is that any foreign genes of interest placed in a binary vector can be simultaneously transferred to the transformed hairy root clones (Hamill et al. 1987). Here, we present a reproducible protocol for developing a transgenic hairy root system in *C. pilosula* through *A. rhizogenes*-mediated co-transformation of a recombinant binary vector with the beta-glucuronidase (*GUS*) reporter gene. To the best of our knowledge, this is the first description of a transgenic hairy root system for *C. pilosula* that facilitates the expression of a gene of interest, i.e., *GUS*. The adoption of our transformation techniques will greatly advance functional genomics studies in this species.

Materials and methods

Plant materials

Seeds of *Codonopsis pilosula* were collected from Gansu Province, China. They were disinfected with 15% NaClO for 13 min and rinsed with sterile water four or five times. Afterwards, the sterile seeds were placed on an MS medium (with 3% sucrose and 0.6% agar; pH 5.8 ± 0.2) and incubated under a 12-h photoperiod in a 24 ± 2 °C incubator. Aseptic seedlings were obtained after 4 weeks of such treatment.

Bacterial strain and recombinant DNA

Agrobacterium rhizogenes strain C58C1 was purchased from Huayueyang Biotechnology Co. Ltd. (Beijing, China). The *Agrobacterium* binary vector pCAMBIA1305, with hygromycin- and kanamycin- resistance and GUSPlus genes

driven by the Cauliflower Mosaic Virus 35S (CaMV35S) promoter, was also purchased from Huayueyang. It was transformed into *A. rhizogenes* C58C1 by the freeze–thaw method (Weigel and Glazebrook 2006). The *A. rhizogenes* C58C1 harboring pCAMBIA1305 was conserved in our laboratory.

Bacterial culture

The native C58C1 *Agrobacterium* strain (negative control) and recombinant C58C1 strain carrying pCAMBIA1305 were streaked on a solid TY medium containing either 50 mg/L rifamycin (Rif) or 50 mg/L Rif plus 50 mg/L kanamycin (Kan). They were then incubated at 27 °C for 2–3 days. A single colony was inoculated into the TY liquid medium containing the specified antibiotics and incubated overnight at 27 °C (shaking at 180 rpm) until the OD₆₀₀ value of the bacterial solution was 1.2–1.3. This solution was centrifuged for 8 min (5000 rpm). Afterward, the precipitated cells were suspended in a 1/2 MS liquid medium to achieve an OD₆₀₀ value of 0.6–0.8. This then served as our infection solution.

Optimization of protocol for inducing hairy roots

The leaves of 4-week-old aseptic seedlings were cut into small pieces (approx. 0.5 cm²), and the stems and roots were trimmed to an average length of 0.5 cm. The surface of each explant type was gently scratched with the back of a scalpel, then placed on the MS medium. The tissues (stem, leaf, or root) were pre-cultured at 24 ± 2 °C for 2 days in the dark. Afterwards, they were immersed in the infection solution for 5 min and placed on a 1/2 MS solid medium for 2 days of co-cultivation. Following this inoculation period, the explants were transferred to a 1/2 MS solid medium supplemented with 200 mg/L cefotaxime sodium (Cef) plus different concentrations of naphthylacetic acid (NAA) to induce hairy roots. The effects on induction efficiency were investigated according to explant type and NAA concentration. Then, we optimized infection time and co-cultivation period with roots explants on the 1/2 MS solid medium supplemented with 0.2 mg/L NAA and 200 mg/L Cef. The single root about 2–3 cm in length was excised and sub-cultured every 14 days on the 1/2MS solid medium containing 0.2 mg/L NAA and 200 mg/L Cef. The induction rate was calculated after the explants had been cultured for 7 days on hygromycin-free induction medium.

Ten explants were placed on each plate (two to three plates per replicate) and all experiments were conducted with three replicates. All data were expressed as means ± standard errors and compared using Duncan's multiple range tests at a 5% level of significance.

PCR identification of transgenic hairy roots

Genomic DNA was extracted from the proliferated transgenic hairy roots with a DNA Extraction Kit according to the manufacturer's instructions (Huayueyang). Genomic DNA from the roots of wild-type (WT) seedlings was used as the negative control. Each PCR-amplification was conducted in a 50- μ L volume containing 40 ng of genomic DNA, 0.4 μ M of each primer, and 25 μ L of 2 \times EasyTaq PCR SuperMix (TransGen, Beijing, China). The forward and reverse gene-specific primers for the Ri plasmid-derived *RolA* were *RolA-F* (5'-CATGTTTTCAGAATGGAATTA-3') and *RolA-R* (5'-AGCCACGTGCGTAT TAATCC-3'), while those for the pCAMBIA1305 derived hygromycin phosphotransferase II gene (*hptII*) were *hptII-F* (5'-CTATTTCTTTGCCCT CGGAC-3') and *hptII-R* (5'-CACTGGCAAACGTGTGATG GA-3'). The PCR reactions were performed as follows: initial denaturation at 95 °C for 3 min; then 34 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 45 s; with a final extension at 72 °C for 5 min. After this amplification, we performed a 1% agarose gel electrophoresis analysis.

Histochemical GUS assay

Histochemical GUS assays were conducted as described previously (Chattopadhyay et al. 2011).

Growth of transgenic hairy roots in liquid culture

Fresh samples of our proliferated transgenic hairy roots (0.5 g each) were transferred to 250-mL flasks (total of 15 flasks), each containing 50 mL of a liquid medium (1/2MS + 200 mg/L Cef) and cultured at 24 °C (120 rpm). The fresh weights of these transgenic hairy root cultures were recorded at 10-day intervals (three flasks per event) to create a growth curve for the liquid culture. To do so, the sampled roots were oven-dried for 24 h at 50 °C to a constant weight.

Determination of total saponins content

The previous method was used to extract saponin and determine the saponin content (Dou et al. 2015). Standard substance of ginsenoside Re was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The dried and powdered hairy roots (0.1 g) were extracted with methanol (3 mL) in an ultrasonic bath (Kunshan Instrument Co., Ltd., China) in a designed ultrasonic power (50–150 W) for 90 min. After filtration, the solvent was removed. Then it was dissolved in 1 mL water, followed by extraction with petroleum ether for three times and butyl alcohol extraction three times. The total saponins were obtained

by removing butyl alcohol and dissolved in 1 mL methanol, which was used as samples for content determination. Samples of 0.1 mL were transferred to vials and dried at 80 °C, followed by 0.2 mL 5% (w/v) vanillin solution in acetic acid and 0.8 mL perchloric acid. A control sample using methanol was prepared. Samples were vortexed and heated at 60 °C for 30 min. Vials were cooled and added 5 mL acetic acid before absorbance was measured at 536 nm using a spectrophotometer (BioSpectrometer, Eppendorf, Germany) against the control sample containing methanol. The total saponins content was obtained from a standard curve of ginsenoside Re.

Results

Induction of hairy roots in *Codonopsis pilosula*

The results from our investigation of explant type and NAA concentration indicated that the induction rate for hairy roots was optimal when we selected an infection time of 5 min and a 2-day co-cultivation period. Specifically, hairy roots were observed from the *C. pilosula* samples at 7–9 days after exposure to *A. rhizogenes* C58C1 carrying pCAMBIA1305 (Fig. 1). The root explants proved to be much more susceptible when compared with the stem and leaf explants. The optimal medium for initiating hairy roots combined 1/2 MS + 0.2 mg/L NAA (Table 1). Based on the data shown in Table 1, induction rates on that particular medium were highest for root explants, i.e., $77.78 \pm 8.31\%$, followed by only $3.33 \pm 2.36\%$ for the stems. No hairy roots were induced from leaf explants when tested on that medium.

Further experiments were conducted to determine the optimum infection time and co-cultivation period for inducing hairy roots from root explants. During a co-cultivation period of 2 days, we found that the induction rate was $73.33 \pm 4.71\%$, $48.33 \pm 2.88\%$, $55.00 \pm 13.29\%$, and $35.00 \pm 5.00\%$ when the infection time was set as 5 min, 10 min, 15 min, and 20 min, respectively (Table 2). We also tested the effects of different lengths of co-cultivation on the induction rate, based on an infection time of 5 min. That rate reached 100% when infected root explants did not undergo co-culturing. In contrast, induction rates were $88.33 \pm 2.88\%$, $73.33 \pm 4.71\%$, and $46.67 \pm 2.89\%$ when the infected root explants were co-cultured for 1, 2, and 3 days, respectively. This demonstrated that the success of induction was gradually reduced as co-culturing became prolonged. From these data we determined that the optimized protocol for inducing hairy roots, i.e., at a rate of up to 100%, involved the use of root explants that were first immersed in an *Agrobacterium* solution for 5 min and then transferred to an *Agrobacterium*-elimination medium.

Fig. 1 Hairy roots of *Codonopsis pilosula* induced from root explants at 3 days (a), 7 days (b), 15 days (c), and 30 days (d) after infection by *Agrobacterium rhizogenes* C58C1 harboring pCAMBIA1305

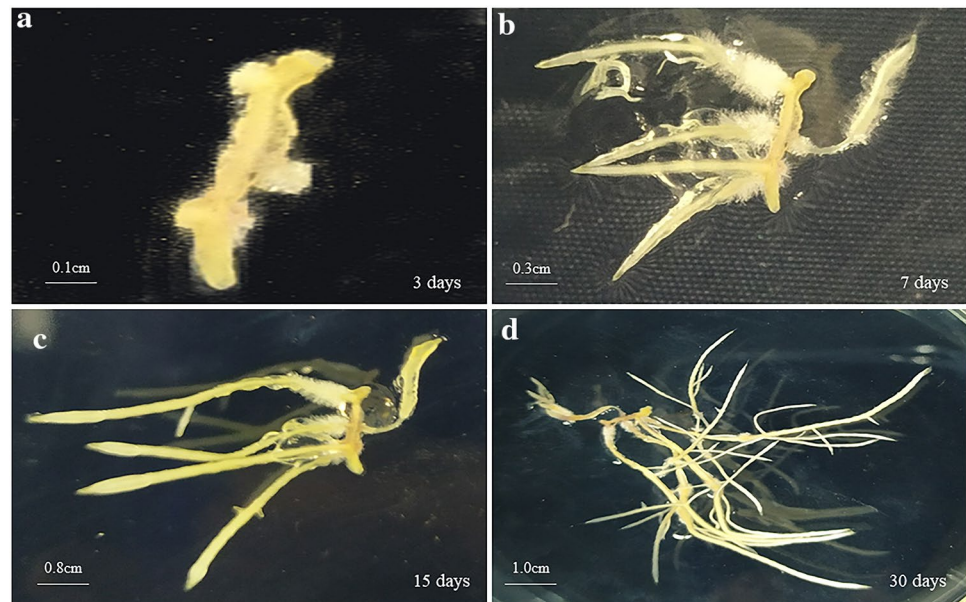


Table 1 Effects of explant type and NAA concentration on the rate of hairy root induction

NAA concentration (mg/L)	Explant type	Number of explants	Number of explants with hairy roots	Induction rate of hairy roots (%)*
0.00	Root	30	16.00 ± 1.00	53.33 ± 2.72 ^b
0.05	Root	30	18.67 ± 0.58	62.22 ± 1.57 ^{bc}
0.10	Root	30	19.67 ± 4.73	65.56 ± 12.86 ^c
0.20	Root	30	23.33 ± 3.06	77.78 ± 8.31 ^d
0.00	Stem	20	0.00 ± 0.00	0.00 ± 0.00 ^a
0.05	Stem	20	0.33 ± 0.58	1.67 ± 2.36 ^a
0.10	Stem	20	0.67 ± 1.15	3.33 ± 4.71 ^a
0.20	Stem	20	0.67 ± 0.58	3.33 ± 2.36 ^a
0.00	Leaf	20	0.00 ± 0.00	0.00 ± 0.00 ^a
0.05	Leaf	20	0.00 ± 0.00	0.00 ± 0.00 ^a
0.10	Leaf	20	0.00 ± 0.00	0.00 ± 0.00 ^a
0.20	Leaf	20	0.00 ± 0.00	0.00 ± 0.00 ^a

*Rates followed by same letter are not significantly different ($P=0.05$), based on Duncan's multiple range tests

Detection of co-transformed transgenic hairy roots by PCR

The *Rol* genes are important Ri plasmid genes of *A. rhizogenes* and play an important role in hairy root induction (Lima et al. 2009). Using PCR-amplification with *RolA*-F/R and *hptII*-F/R gene-specific primers, we detected the Ri-derived *rolA* as well as the pCAMBIA1305-derived *hptII* in our co-transformed hairy root lines. An expected band of 360 bp was amplified in all 30 co-transformed hairy root lines, whereas no detectable band was observed in the WT control (Fig. 2a), indicating the successful presence of the *RolA* in all tested hairy root lines genome. Among those 30 lines, a desired 546-bp amplicon was found in 21 of them

(Fig. 2b). This demonstrated that 70% of the co-transformed hairy roots contained pCAMBIA1305-derived T-DNA in the plant genome.

Detection of transgenic hairy roots by GUS-staining

Because the vector pCAMBIA1305 carries the *gusA* reporter gene, we further examined the protein expression level of the pCAMBIA1305-derived *gusA* in transgenic hairy roots. Results from our histochemical GUS assays revealed that 28 out of the 41 transgenic lines tested here showed the characteristic intense blue color, i.e., 68.3% co-transformation efficiency, while no blue coloration was observed in the control hairy roots (Fig. 3a, b).

Table 2 Effects of infection time and co-cultivation period on induction of hairy roots from root explants

Infection time (min)	Co-cultivation time (d)	Number of explants	Number of explants with hairy roots	Induction rate of hairy roots (%) ^a
5	2	20	14.67 ± 0.67	73.33 ± 4.71 ^c
10	2	20	9.67 ± 0.57	48.33 ± 2.88 ^b
15	2	20	11.00 ± 2.64	55.00 ± 13.29 ^b
20	2	20	7.00 ± 1.00	35.00 ± 5.00 ^a
5	0	20	20.00 ± 0.00	100.00 ± 0.00 ^e
5	1	20	17.67 ± 0.57	88.33 ± 2.88 ^d
5	2	20	14.67 ± 0.67	73.33 ± 4.71 ^c
5	3	20	9.33 ± 0.57	46.67 ± 2.89 ^b

^aRates followed by same letter are not significantly different ($P=0.05$), based on Duncan's multiple range tests

We further monitored GUS activity in the proliferated transgenic hairy roots, which were confirmed by PCR to contain pCAMBIA1305-derived T-DNA and cultured in the 1/2MS liquid medium for 4 weeks. GUS activity was observed in all the transgenic hairy root lines tested, whereas no such activity was detected in control lines (Fig. 3c, d).

Growth of transgenic hairy roots

Hairy root growth was observed in the 1/2MS liquid medium. As culture time became prolonged, the hairy roots gradually changed from white to pale-yellow and dark-yellow (Fig. 4a). Their fresh weights increased to 0.70, 1.04,

1.12, 1.58, and 1.99 g when the hairy roots were cultured in the liquid medium for 10, 20, 30, 40, and 50 days, respectively (Fig. 4b). The fresh weight on Day 50 was 3.98 times greater than that recorded at the start of the inoculation period. Hairy root dry weights were 0.07, 0.10, 0.11, 0.16, and 0.19 g after being cultured in the liquid medium for 10, 20, 30, 40, and 50 days, respectively (Fig. 4c).

Content of total saponins in transgenic hairy roots

Dangshen extract saponins clearly demonstrated the protective effects on kidney ischemia–reperfusion injury after kidney transplantation (He et al. 2011). We determined the content of total saponins in transgenic hairy roots. Our result showed that the content of total saponins was 3.58, 4.01, 3.05, 3.29, 3.47 and 1.79 mg/g when the hairy roots were cultured in the 1/2MS liquid medium for 0, 10, 20, 30, 40 and 50 days, respectively (Fig. 4d).

Discussion

Hairy roots can be utilized as a biological reactor for the production of valuable compounds from medicinal plants (Guillon et al. 2006). Some of those species can quickly produce hairy roots, including *Salvia miltiorrhiza* (Huang et al. 2012), *Panax ginseng* (Sathiyamoorthy et al. 2010), *P. quinquefolius* (Kochan et al. 2013), *Tetrastigma hemsleyanum* (Du et al. 2015), *Stevia rebaudiana* (Fu et al. 2015), and *Anisodus luridus* (Qin et al. 2014). Here, we established a rapid

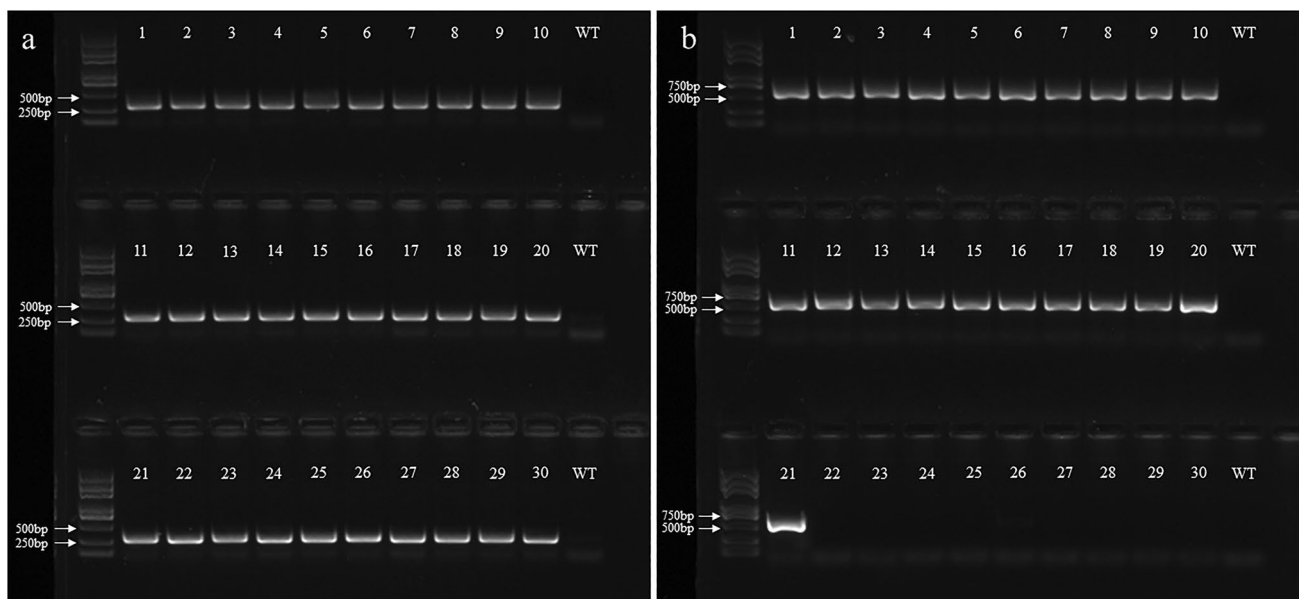


Fig. 2 PCR-amplification of 360-bp *rolA* DNA fragment (a) and 546-bp hygromycin phosphotransferase II gene (b) in different co-transformed hairy root lines of *Codonopsis pilosula* and wild-type (WT) control

Fig. 3 Histochemical GUS assay of *Codonopsis pilosula* transgenic hairy roots and untransformed control. **a** GUS activity in emerging hairy roots after 15 days of infection by *Agrobacterium rhizogenes* C58C1. **b** GUS activity in co-transformed transgenic hairy roots after 15 days of infection by strain C58C1 harboring pCAMBIA1305. **c** GUS activity in hairy roots after 28 days in liquid culture. **d** GUS activity in co-transformed transgenic hairy roots after 28 days in liquid culture

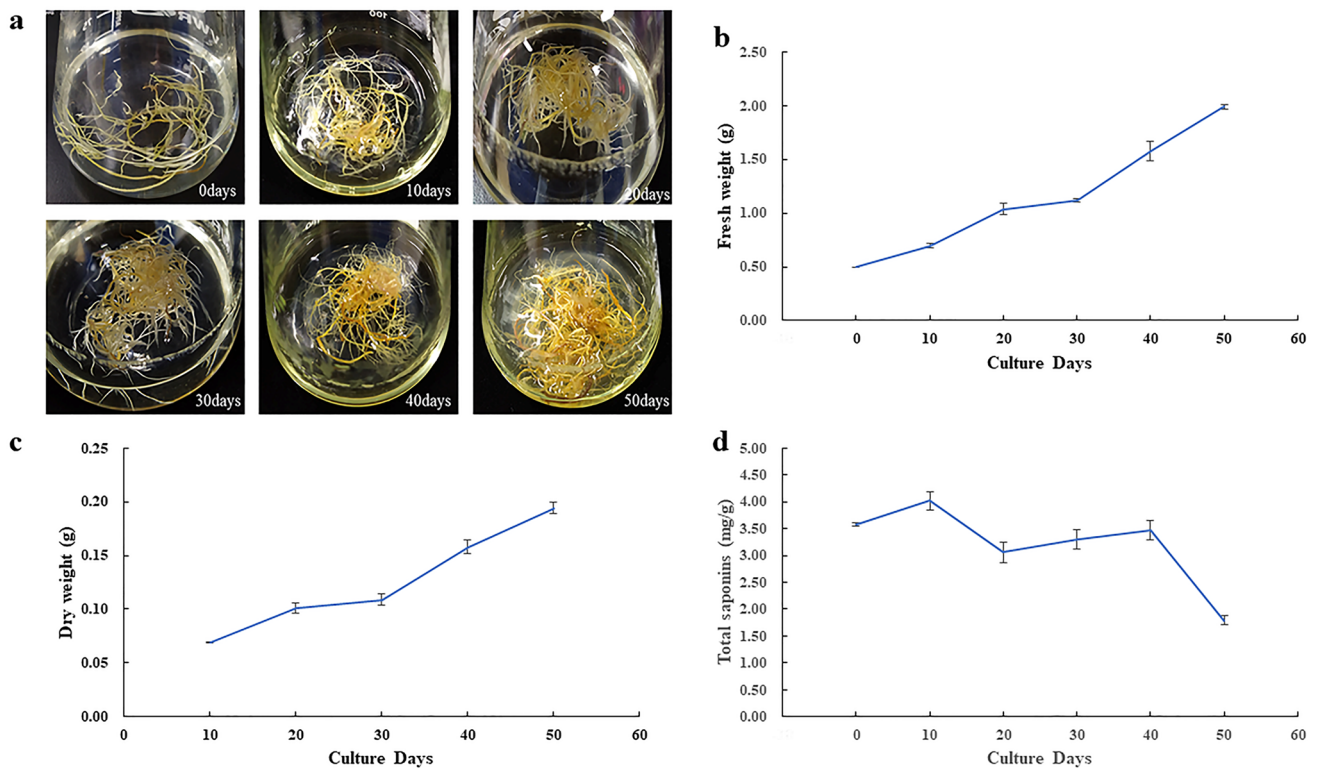
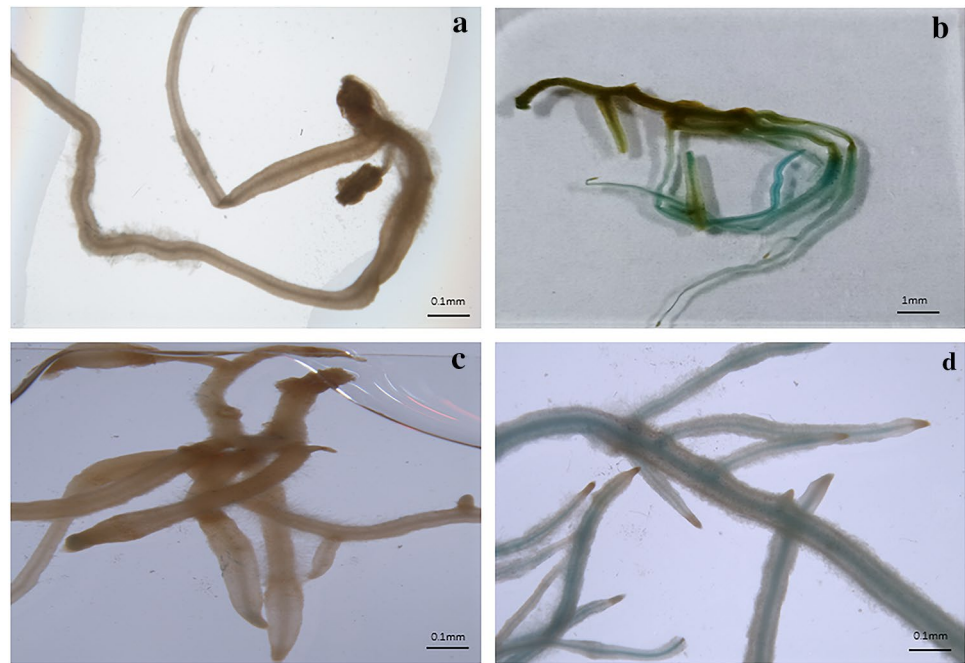


Fig. 4 Growth of *Codonopsis pilosula* hairy roots in phytohormone-free 1/2 strength liquid MS medium. **a** Growth of transgenic hairy roots (initial fresh weight of 0.5 g per sample) after 10, 20, 30, 40,

and 50 days. **b** Growth curve for fresh weights of hairy root. **c** Growth curve for dry weights of hairy root biomass. **d** Total saponins content in transgenic hairy roots

in vitro system for the culturing of *C. pilosula* transgenic hairy roots. This initial effort provides a promising alternative for the accelerated production of bioactive components in that species.

Several factors influence the induction of hairy roots, including explant type, choice of *Agrobacterium* strain, infection time, and period of co-cultivation. The part of the plant that is most susceptible to *A. rhizogenes* infection for hairy root formation differs among species. For example, leaf explants from *Salvia miltiorrhiza* show the highest induction rate for hairy roots (Huang et al. 2012) while the bud is the most suitable explant to use when inducing hairy roots in *Semecarpus anacardium* (Panda et al. 2017). For *Chenopodium murale*, the best target explant is the root (Mitic et al. 2012). For *C. pilosula* hairy root, the stem is better than either the leaf or petiole, and the induction rate is maximized, at 48%, when those stem explants are infected with *A. rhizogenes* A4 rather than with strain C58C1 or A1476 (Li et al. 2017b). In the present study, we found that root explants were highly susceptible to *A. rhizogenes* C58C1. Furthermore, our optimized protocol led to an induction rate of up to 100%.

The *hptII* is widely used as a selectable marker in plant transformation systems. Its expression allows transformed hairy roots to grow on media supplemented with that antibiotic. When selecting for highly sensitive transgenic hairy roots, the induction medium is often supplemented with 5 mg/L hygromycin (Chattopadhyay et al. 2011; Rizvi et al. 2015). However, we noted in the experiments described here that hairy roots of *C. pilosula* could be induced on a hygromycin-free medium, at a transformation efficiency of 68.3%, when we used the T-DNA from pCAMBIA1305. This indicated that co-transformation was very successful for *C. pilosula* even in the absence of antibiotic selective pressure. In fact, we found *C. pilosula* were highly sensitive to hygromycin and the rate of induction for co-transformed hairy roots was only 28.5% on the selection medium with 2 mg/l hygromycin (data not shown).

As a well-known traditional Chinese medicine, Dangshen has the effect of strengthening spleen and tonifying lung, nourishing blood and engendering liquid. Modern research showed that saponins extracted from Dangshen had protective effects on kidney ischemia–reperfusion injury after kidney transplantation (He et al. 2011). We observed accumulation of saponins in the co-transformed hairy roots, which indicated that transgenic hairy roots of *C. pilosula* could be used to produce secondary metabolites.

Conclusion

We have developed an efficient *A. rhizogenes*-mediated transformation approach for *Codonopsis pilosula* transgenic hairy roots. The rate of induction for hairy roots reached

100% when root explants were used. This method was most successful when the samples were infected for 5 min with *A. rhizogenes* C58C1 harboring pCAMBIA1305, followed by induction on 1/2MS + 0.2 mg/L NAA + 200 mg/L Cef. Overall, 70% of the co-transformed hairy roots contained pCAMBIA1305-derived *hptII* in the plant genome and 68.3% of those hairy roots showed GUS activity, even when no selection pressure was exerted. These findings indicated that T-DNA from the exogenous plasmid pCAMBIA1305 could be efficiently integrated into the *C. pilosula* genome and then expressed stably. Our protocol helps lay the foundation for genetic engineering of that species.

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Author contributions XC designed the study and wrote the manuscript. JY performed experiments and wrote the manuscript. XY analyzed the data. BL, XL and JK performed experiments.

Compliance with ethical standards

Conflict of interest All the authors declare that they have no conflicts of interest.

Ethical statements This article does not contain any studies with human participants or animals performed by any of the authors.

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