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Genetic transformation of *Pueraria phaseoloides* with *Agrobacterium rhizogenes* and puerarin production in hairy roots

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Abstract An efficient transformation system for the medicinal plant *Pueraria phaseoloides* was established by using agropine-type *Agrobacterium rhizogenes* ATCC15834. Hairy roots could be obtained directly from the cut edges of petioles of leaf explants or via callus 10 days after inoculation with the bacteria. The highest frequency of explant transformation by *A. rhizogenes* ATCC15834 was about 70% after infection for 30 days. Hairy roots could grow rapidly on solid, growth regulator-free Murashige and Skoog medium and had characteristics of transformed roots such as fast growth and high lateral branching. Paper electrophoresis revealed that bacteria-free hairy roots of *P. phaseoloides* could synthesize agropine and mannopine. The polymerase chain reaction amplification of rooting locus genes showed that left-hand transferred DNA of the root inducing plasmid of *A. rhizogenes* was inserted into the genome of transformed *P. phaseoloides* hairy roots. The content of puerarin in hairy roots reached a level of 1.190 mg/g dry weight and was 1.067 times the content in the roots of untransformed plants.

Keywords *Agrobacterium rhizogenes* · Hairy roots · *Pueraria phaseoloides* · Puerarin

Abbreviations *HPLC*: High performance liquid chromatography · *MS*: Murashige and Skoog medium · *PCR*: Polymerase chain reaction · *Ri*: Root inducing · *rolB*: Rooting locus B · *rolC*: Rooting locus C · *YEB*: Yeast extract broth

Introduction

The soil-borne bacterium *Agrobacterium rhizogenes* carries root-inducing (Ri) transferred DNA that induces the formation of hairy roots after its integration into the plant genome (White and Nester 1980; Tepfer 1984; Tepfer and Casse-Delbart 1987). Since the genetic transformation does not impair the natural root's synthetic capacities, hairy roots, which can often grow vigorously and produce high levels of secondary metabolites, have been recognized as a potential way of producing important pharmaceuticals as secondary compounds from medicinal plants (Hamill et al. 1987; Yoshikawa and Furuya 1987).

Pueraria roots prepared from *Pueraria phaseoloides* (Roxb.) Benth are one of the most important crude drugs in the pharmaceutical industry, especially in Chinese traditional medicine. Studies on the main active constituents of *Pueraria* roots, puerarin, showed the presence of hypothermic, spasmolytic, hypotensive and anti-arrhythmic activities (Takaya and Itokawa 1982; Chai et al. 1985; Song et al. 1988). To date, however, puerarin is still mainly extracted from roots of wild plants. Evidently, this practice cannot meet the increasing demands for puerarin from the pharmaceutical industry, due to the limited resources of wild plants, and will also lead to considerable reduction of the natural medicinal plant population and soil erosion. So establishment of *Pueraria* hairy root cultures will be an effective alternative way towards large-scale production of puerarin instead of using wild plants. However, there have been no reports of hairy root induction with *A. rhizogenes* in *P. phaseoloides*. In this paper we describe an efficient system for genetic transformation of *P. phaseoloides* with *A. rhizogenes* and production of puerarin by hairy root cultures.

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Materials and methods

Plant material

Seeds of *Pueraria phaseoloides* (Roxb.) Benth were collected from the biological garden of the Institute of Life Science, South China Normal University, China. Seeds were surface-sterilized in 0.1% (w/v) aqueous HgCl₂ solution containing 0.1% Tween 20 for 25 min, followed by washing 3 times with sterile water. They were then placed on water-wetted cotton in 150 ml-flasks to germinate at 25°C under a 14-h photoperiod with a light intensity of 35 μmol m⁻² s⁻¹. After 4 weeks, stems and petioles excised from the germinated seedlings were rapidly propagated at 25°C with the same photoperiod according to previously described methods (Shi 2000).

Agrobacterium culture

A single clone of *A. rhizogenes*, ATCC15834, harboring agropine-type plasmid pRiA4b, was selected and cultured into 40 ml yeast extract broth (YEB, yeast extract 1.0 g l⁻¹, beef extract 5.0 g l⁻¹, peptone 5.0 g l⁻¹, sucrose 5.0 g l⁻¹, MgSO₄·7H₂O 0.49 g l⁻¹, pH 7.2) in the dark at 28°C on a rotary shaker at 160 rev/min. When OD₆₀₀ was approximately 1.0, the bacterial solution was collected and centrifuged at 4,000 rev/min for 5 min, followed by re-suspension in growth regulator-free liquid MS medium (Murashige and Skoog 1962) for inoculation.

Induction and culture of *P. phaseoloides* hairy roots

Young leaves (second and third leaves from the apex) from the 2-month-old seedlings were cut into leaf explants with or without petioles and pre-cultured on solid, growth regulator-free MS medium for 24 h. The leaf explants were then infected by dipping them into an *Agrobacterium* suspension in MS medium for 20 min. Following infection, the leaf explants were washed once with sterile double distilled H₂O and blotted with filter paper to remove excess *Agrobacteria*. After 2 days of co-cultivation at 28°C in the dark, the explants were transferred onto MS medium containing 500 mg l⁻¹ carbenicillin (filter-sterile, added to the medium) and kept in an air-conditioned chamber at 25°C, under 14 h/day light (35 μE m⁻² s⁻¹) to induce hairy roots. Control explants were given the same treatment but were dipped in sterile YEB medium. Each treatment consisted of 30 explants (6 explants per 100 ml-Erlenmayer flask) and the infection experiments were repeated twice.

Thirty days after infection, hairy roots were excised from infected *Pueraria* leaf explants and cultured on growth regulator-free MS agar (1%) medium with 500 mg/l carbenicillin to eliminate the agrobacterium; after several days of culture, the elongating root tips were cut off and transferred to growth regulator-free MS agar medium without carbenicillin. This procedure was repeated 3–4 times until no colony of bacteria appeared. Sterile hairy root cultures were maintained at 25°C in the dark on growth regulator-free MS medium without antibiotic for confirmation.

Confirmation of hairy roots by opine detection

Opine synthesis is a firm indication that hairy roots are transformed (Hamill et al. 1987). Extraction and detection of opiens (agropine and mannopine) in bacteria-free hairy roots were carried out according to the method of Petit et al. (1983). High voltage paper electrophoresis was performed at 400 V for 1.5 h.

Identification of transformed *Pueraria* hairy roots by polymerase chain reaction

For confirmation, isolation of total DNA from bacterium-free *Pueraria* hairy roots and natural (non-transformed) roots was

conducted using established methods (Edwards et al. 1991). Polymerase chain reaction (PCR) identification of the rooting locus genes *rolB* and *rolC* was performed using DNAs from the hairy roots as template and the non-transformed roots as control, respectively. The following primers, which were designed totally according to Furner et al. (1986), were used for amplification of *rolB* and *rolC* sequences: *rolB*-1 (5'-GCTCTTGCAAGTGTAG-ATTT-3'); *rolB*-2 (5'-GAAGGTGCAAGCTACCTCTC-3'); *rolC*-1 (5'-CTCCTGACATCAAACCTCGTC-3'); *rolC*-2 (5'-TGCTTCGA-GTTATGGGTACA-3'). For amplification, the PCR parameters consisted of a denaturation step of 3 min at 94°C and 35 cycles (each consisting of 1 min at 94°C, 1 min at 53.5°C and 1 min at 72°C), followed by a final extension at 72°C for 6 min. The PCR products were fractionated by electrophoresis on a 0.8% agarose gel using Tris-acetate-EDTA buffer and photographed under a UV lamp at a wavelength of 260 nm.

Puerarin extraction and analysis by high performance liquid chromatography

The hairy roots subcultured on growth-regulator-free MS medium for 10 days were harvested and dried to constant weight; 200 mg dry weight of hairy roots were ground and extracted with 10 ml methanol at 4°C for 24 h. The extract was then filtered through a filter paper. The extraction procedure was repeated three times. Next, a total of 30 ml extracted solution was concentrated to dryness. Methanol was added to dissolve the residue to a final volume of 2.0 ml before analysis. Roots excised from untransformed plants were extracted as control by the same procedure.

The puerarin content was analysed by high performance liquid chromatography (HPLC) using a Beckman System Gold HPLC (Beckman Instruments, Fullerton, Calif., USA) equipped with a UV detector (System Gold 168) and with a System Gold 125 Solvent module. Sample solution (20 μl in methanol) was injected into an Elite Hypersil BDS C18 (Dalian Elite Scientific Instruments, Dalian, China) column (particle size 5 μm, 25 cm×4.6 mm) and eluted with a mixture of methanol and water (85/15, v/v). The flow rate was 1.0 ml/min throughout the analysis. The puerarin content was detected by UV absorbance at 248 nm. For quantitative analysis, the system was calibrated with authentic puerarin purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Standard curves were fitted by linear regression.

Results

Transformation and establishment of hairy root cultures

During initial experiments, cotyledon explants of *P. phaseoloides* were infected with *A. rhizogenes* ATCC15834. However, no hairy roots were obtained from cotyledon explants 2 months after inoculation. Consequently, further infection experiments were only performed with leaf explants with or without petioles. When leaf explants with petioles were inoculated with freshly grown *A. rhizogenes* suspensions in MS medium, hairy roots were induced from micro-calli of leaf petioles or directly from the cut edges of leaf petiole (Fig. 1), but the frequency of root induction (mean number of roots per explant) was higher from micro-calli than directly from the cut edges of leaf petioles. The first roots were visible 10 days after inoculation. With increasing incubation periods, the percentages of rooted leaf explants also increased. Thirty days after inoculation, the percentage of rooted leaf explants with petioles transformed by *A. rhizogenes* ATCC15834 was about 70%. When leaf

explants without petioles were infected, hairy roots were only induced via callus from the leaf vein of cut edges of leaf explants 6–8 weeks after infection; the proportion of rooted leaf explants was about 40%. No roots were observed in non-infected leaf explants after approximately 8 weeks but a small callus was formed at the cut edges of leaf veins.

The induced hairy roots were excised and put onto fresh, growth regulator-free MS medium containing 500 mg/l carbenicillin to eliminate agrobacteria. After several days of culture, elongating root tips were cut off and transferred to growth regulator-free MS agar medium without carbenicillin. This procedure was repeated 3–4 times until no colony of bacteria appeared. When axenic hairy roots were transferred onto MS agar (1%) medium without antibiotic, they grew vigorously in the dark at 25°C and had characteristics of transformed roots, such as fast growth and high lateral branching (Fig. 2A). During incubation, hairy roots changed gradually from white to red-brown and some red-brown substance secreted from hairy roots after 3–4 weeks could be observed. In comparison with roots in solid culture, hairy roots cultured in liquid, growth regulator-free MS medium grew rapidly and had high lateral branching, but with increasing incubation time, the initial parts of the hairy roots and the culture medium turned brown (Fig. 2B). As a control, adventitious roots excised from *P. phaseoloides* sterile seedlings were cultured on solid, growth regulator-free MS medium, but these roots grew very slowly, did not branch and perished after 2–3 subcultures.

Evidence for transformation by opine detection

A. rhizogenes ATCC15834 is of the agropine-type. Both opines (agropine and mannopine) were detected by paper electrophoresis in extracts derived from *Pueraria* hairy roots but not in untransformed control roots (Fig. 3). The production of opines, along with rapid growth on growth regulator-free medium, was considered a proof of transformation.

Confirmation of *P. phaseoloides* hairy roots by PCR analysis

By using DNAs from the hairy roots as template and the non-transformed roots as control, PCR products amplified with *rolB* primers and *rolC* primers, respectively, could be detected (Fig. 4). It was demonstrated that two fragments, with lengths of 540 bp and 770 bp and corresponding to *rolB* and *rolC*, respectively, were amplified only from hairy root cultures but not from untransformed roots. These results indicated that the *rolB* and *rolC* genes from the Ri plasmid of *A. rhizogenes* ATCC15834 were integrated into the genome of *P. phaseoloides* hairy roots.

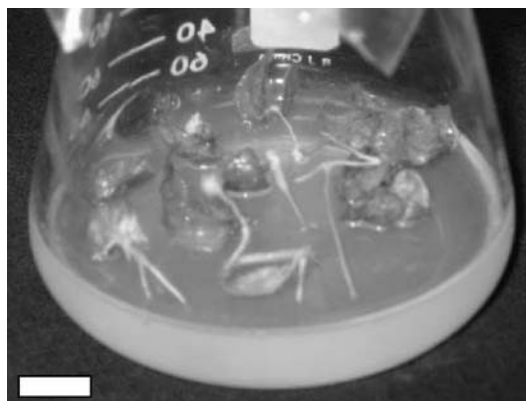


Fig. 1 Hairy roots induced from the leaf explants with petioles on growth regulator-free Murashige and Skoog medium (MS) 20 days after infection by *Agrobacterium rhizogenes* ATCC15834 (scale bar 1.5 cm)

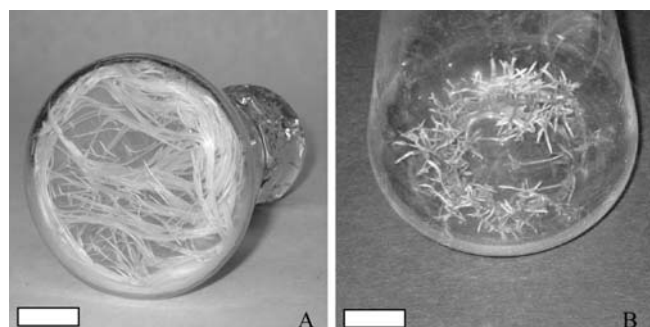


Fig. 2 **A** Sterile hairy roots of *Pueraria phaseoloides* cultured on solid, growth regulator-free MS medium for 18 days (scale bar 2.5 cm). **B** Sterile hairy roots in liquid MS medium, cultured for 10 days (scale bar 2.2 cm)

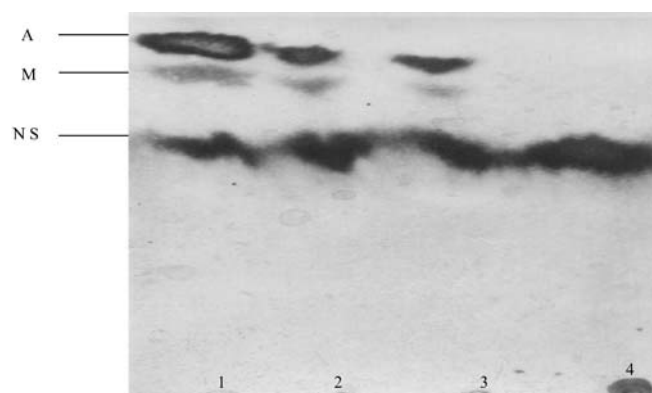


Fig. 3 Detection of agropine(A) and mannopine (M) by paper electrophoresis in extract of two hairy root cultures. Lane 1 agropine and mannopine standards, lanes 2 and 3 hairy root cultures, lane 4 non-transformed control roots of sterile plants, NS neutral sugars

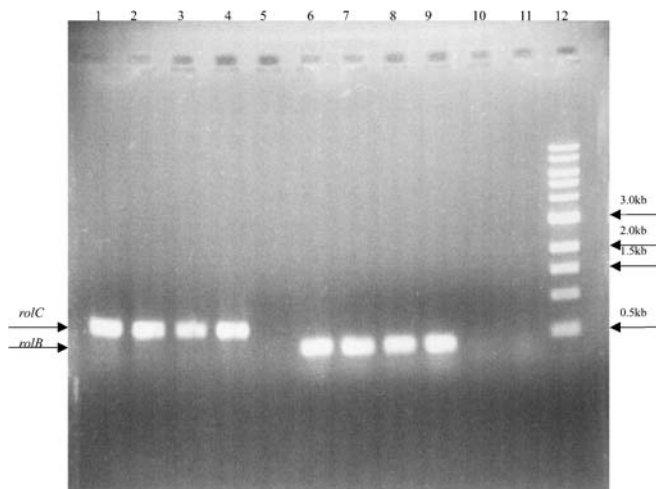


Fig. 4 PCR analysis of hairy roots. PCR was performed with primers for the rooting locus genes *roIC* (lanes 1–5) and *roIB* (lanes 6–11). Lanes 1–4 and lanes 6–9 fragments from hairy roots, lanes 5 and 10 fragments from control roots, lane 11 control without template DNA, lane 12 marker (1 kb DNA ladder)

Production of puerarin in hairy roots

Hairy roots cultured onto growth regulator-free MS medium for 10 days were harvested and used for determination of puerarin contents with HPLC. As shown in Table 1 and Fig. 5, hairy roots could also produce puerarin, and the content of puerarin in *P. phaseoloides* hairy roots reached a level of 1.190 mg/g dry weight and

was 1.067 times the puerarin content of the roots of untransformed plants.

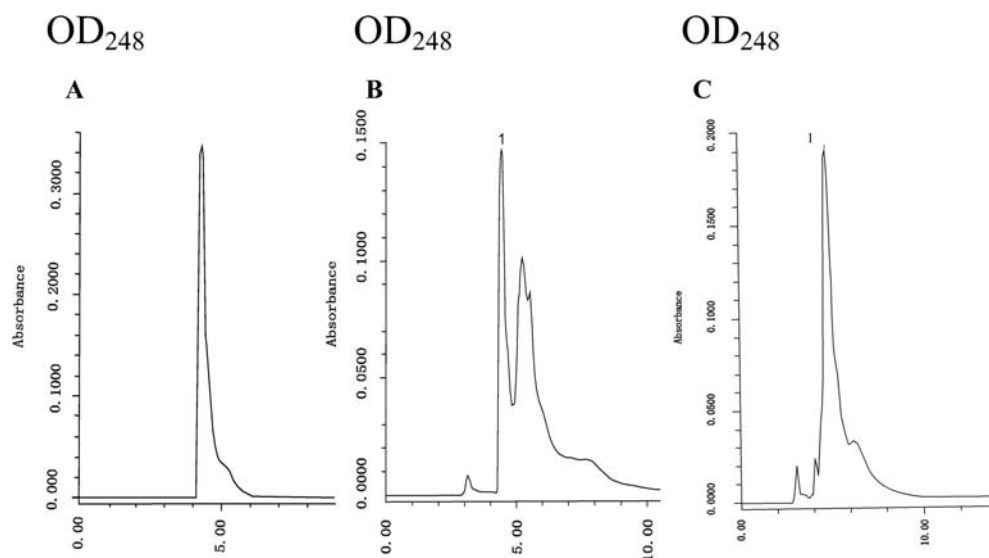
Discussion

Hairy roots induced by the Ri plasmid of *A. rhizogenes* are widely used for the production of important pharmaceutical chemicals from many medicinal plants, such as *Catharanthus roseus* (Parr et al. 1988), *Panax ginseng* (Yoshikawa and Furuya 1987), *Datura candida* (Christen et al. 1989), *Salvia miltiorrhiza* (Hu and Alfermann 1993) and *Ocimum basilicum* (Tada et al. 1996). So far, no protocols on the efficient transformation of *P. phaseoloides* with *A. rhizogenes* have been reported. In our study, we established an efficient transformation system for *P. phaseoloides* by using *A. rhizogenes* ATCC15834. Our results showed that *P. phaseoloides* is susceptible to *A. rhizogenes* ATCC15834 infection at a 70% frequency for explant transformation. This increased hairy root induction from petioles, as compared with cotyledons and leaf explants, may be due to the fact that petioles are more competent for transformation than cotyledons or leaf explants. Bercetche et al. (1987) reported that hairy roots could only be induced from calli of carrot tuber explants and stem segments of *Nicotiana tabacum*. After inoculation with *A. rhizogenes*, hairy roots could be induced directly from infected leaf and tuber explants, but only induced via callus from stem explants (Ottani et al. 1990). This suggests that differences in morphological patterns of hairy roots could be dependent upon different species and different plant organs or infected sites.

Table 1 Contents of puerarin in *Pueraria phaseoloides* hairy roots detected by high performance liquid chromatography

Root type	Puerarin content (mg/g dry weight), mean of three measurements
Untransformed roots	1.115±0.024
Hairy roots induced by ATCC15834	1.190±0.016

Fig. 5A–C HPLC analysis of puerarin in hairy roots of *P. phaseoloides*. **A** Authentic puerarin. **B** Roots of sterile untransformed *P. phaseoloides* plants. **C** Sterile hairy roots of *P. phaseoloides* induced by *Agrobacterium rhizogenes* ATCC15834



In our study, we obtained *P. phaseoloides* hairy roots with rapid growth on solid medium. Hairy roots could also be cultured in liquid medium but older parts of such roots become dark-brown, eventually darkening the culture medium. This might have been related to the effect of some metabolites released into the medium. Efficient culture of rapidly growing hairy roots in liquid medium is a key prerequisite for large-scale production of puerarin from *P. phaseoloides*. Some reports described effects of the medium composition and culture conditions on the growth rate of hairy roots and the production rate of secondary roots (Sauerwein et al. 1991). We have recently also succeeded in culturing *Pueraria* hairy roots in a 1.5 l airlift bioreactor, where root browning was not observed (manuscript under preparation).

The results presented here showed that *P. phaseoloides* hairy roots could produce puerarin, whose concentration is slightly higher than those in roots of untransformed plants and may be used as a useful system for large-scale production of puerarin. We are currently focusing our research on defining the optimal medium and culture conditions for the scale-up liquid culture of *P. phaseoloides* and puerarin production, as well as investigation various biochemical parameters (such as key metabolic enzymes, mitochondrial function and cell membrane potential) possibly related to both hairy root growth and puerarin production.

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