

Full Length Research Paper

Hairy root culture of *Przewalskia tangutica* for enhanced production of pharmaceutical tropane alkaloids

Xiaozhong Lan* and Hong Quan

Tibet Agricultural and Animal Husbandry College, Nyingchi, Tibet 860000, People's Republic of China.

Accepted 26 April, 2010

The bacteria-free seedlings of *Przewalskia tangutica* were germinated from seeds. The leaves were used as initial explants for genetic transformation mediated by *Agrobacterium rhizogenes* strain A4, with the induction of Acetosyringone. The hairy roots were induced from the wounded sites of the leaves at the frequency of 100%. The independently transformed and fast growing hairy roots were subcultured in MS medium to establish the hairy root culture lines. Genomic DNA PCR analysis was applied to detect the presence of the rooting genes, including *rolB* and *rolC* that had integrated into the genome of *Przewalskia tangutica*. After 5-week culture in MS liquid medium, the hairy roots produced scopolamine and hyoscyamine at higher levels by comparison to the wild-type roots. The best scopolamine-production line produced 0.68 mg/g DW scopolamine, and the best hyoscyamine-production line produced 1.13 mg/g DW hyoscyamine. It was the first time that hairy root cultures of *P. tangutica* were established to produce tropane alkaloids.

Key words: *Przewalskia tangutica*, hairy root, scopolamine, hyoscyamine.

INTRODUCTION

Tropane alkaloids are usually used as anticholinergic agent, ataractic and narcotic, but the contents of these useful natural products are very low in Solanaceae plants (Yamada and Tabata, 1997). *Przewalskia tangutica*, belonging to plant family Solanaceae, is an important and rare medicinal plant in Tibet Plateau of China, which contains tropane alkaloids including hyoscyamine and scopolamine in root (Xiao et al., 1973), which are widely used as parasympatholytics that competitively antagonize acetylcholine with great commercial demands (Zhang et al., 2007). Now the desire for *P. tangutica* is increasing that has caused serious destructions of the wild resource. Plant biotechnology is an alternative way to solve the problem. Plant cell culture is a normal method used to produce natural products, but it is prone to genetically and biochemically unstable and the capability to synthesize useful secondary metabolites is low (Krolicka et al.,

2001). *Agrobacterium rhizogenes*-mediated hairy root cultures is the other feasible method, as these grow fast, are genetically stable and capable of synthesizing much more secondary metabolites than normal roots and other organs (Flores and Curtis, 1992; Wysokinska and Chmiel, 1997).

In the present study, the high-efficient genetic transformation system of *P. tangutica* mediated by *A. rhizogenes* was established. Moreover, the morphology and alkaloid production capacity of these hairy roots were compared with the normal roots used as control. This work provides a good method to produce pharmaceutical tropane alkaloids, and is also a fundamental work for metabolic engineering of tropane alkaloids in *P. tangutica* by over expressing the committed-enzymes in plant.

MATERIALS AND METHODS

Plant material

The mature seeds of *P. tangutica* were obtained from the botanical garden in Tibet Agricultural and Animal Husbandry College. The

*Corresponding author. E-mail: lanxiaozhong@163.com. Tel: 86-894-5886042. Fax: 86-894-5886042.



Figure 1. Hairy roots induced from the wound sites of leaf (A); callus-like hairy root line (B); typical hairy root line (C) and hairy roots cultured in MS liquid medium (D).

plump seeds were marinated in warm water at 45°C for 24 h. After being surface-disinfected with 70% (v/v) ethanol and 0.1% (w/v) HgCl₂ and five-time sterile water rinses, the seeds were cultured in 150 ml flasks containing 40 ml of MS solid medium (Murashige and Skoog, 1962), which is supplemented with 30 g/l sugar, solidified with 8 g/l agar and adjusted pH to 5.8, in an automatic incubator at 25 ± 0.5°C in the dark. The seeds germinated after 2 weeks. The 5-week-old fresh leaves can be used as initial explants for genetic transformation.

Bacteria preparation

A. rhizogenes agropine-type strain A4 were stored at -70°C, and was confirmed the presence of *rolB* and *rolC* gene by PCR analysis. Then bacteria stored at -70°C were inoculated into YEB liquid medium (containing 50 mg/l kanamycin) and be activated twice. During the second activation, acetosyringone (AS) was added into the medium at the final concentration of 100 µmol/l at the optical density value of 0.3 (OD₆₀₀). When the optical density reached 0.5 ~ 0.6 (OD₆₀₀), 50 ml mixture was centrifuged (4000g) at 25°C for 10 min to collect bacteria; and bacteria were suspended in 50ml MS liquid medium containing 100 µmol/l AS and continued culturing in orbital shaker (180 rpm) at 28°C for 30 min; then bacteria could be used for transformation.

Genetic transformation

The 5-week-old true leaves of *P. tangutica* were cut into 1.5 × 1.5 cm discs and dipped into the suspension of the activated bacteria for 5 min, then dried on sterile filter papers. All infected leaves were transferred onto MS solid medium containing 100 µmol/l AS and co-cultured at 25 ± 0.5°C in the dark for 48 h; the uninfected leaves used as control were cultured in the same conditions. After 48 h co-culture, the infected and the uninfected leaves were transferred onto MS solid medium containing 250 mg/l cefotaxime to inhibit the growth of bacteria.

Hairy root culturing

The induced roots grew from the cut edges 3 weeks later. They were excised and cultured on phytohormone-free MS medium supplemented with 30 g/l sucrose, 3.0 g/l phytigel and 250 mg/l cefotaxime. The induced roots were cultured at an automatic incubator at 25 ± 0.5°C in the dark, and routinely subcultured every 4 - 5 weeks. After 3 times subculture, the roots were transferred onto MS solid medium without phytohormones and antibiotics. The fast-growing, frequently-branching and bacteria-free root lines were used to establish hairy root lines. About 1g fresh roots were inoculated into 250 ml flasks containing 100 ml MS liquid medium,

cultured in orbital shaker (110 rpm) at 25 ± 1°C for 5 weeks.

Detecting the rooting genes

Total DNA was isolated from different hairy root lines and wild-type roots, by using sodium dodecyl sulfate (SDS) method described by Sambrook et al. (2002). Primers for *rolB* gene were *f-rolB* (5'-GCTCTTGAGTGTCTAGATTT-3') and *r-rolB* (5'-GAAGGTG CAAG-CTACCTCTC-3'); and the primers for *rolC* gene was *f-rolC* (5'-CTCCTGACATCAAACCTCGTC-3') and *r-rolC* (5'-TGCTTCG AGTT-ATGGGTACA-3') (Furner et al., 1986). The expected amplified fragment sizes were 423 bp for *rolB* gene and 626 bp for *rolC* gene. PCR reaction was carried out in total volume of 25 µl reaction mixtures: 50 ng genomic DNA, 200 µM dNTPs, 2.5 µl 10 × PCR buffer (Mg²⁺ plus), 1.25 units of Taq DNA polymerase (TaKaRa) and 25 pmol of each primer. For PCR reaction of *rolB* and *rolC*, DNA was denatured at 94°C for 5 min, followed with 34 amplification cycles (94°C for 45 s, 55°C for 40 s, 72°C for 1 min), and with final extension at 72°C for 7 min.

Analysis of hyoscyamine and scopolamine

5-week-old hairy roots were collected respectively and dried at 60°C until the weight did not reduce. Alkaloids extraction was carried out as essentially described by Hashimoto et al. (1993) and the content of hyoscyamine and scopolamine was analyzed by HPLC, according to the method described by Scragg et al. (1986). Each hairy root line was detected the content of both hyoscyamine and scopolamine.

RESULTS AND DISCUSSION

Initiation of hairy roots of *P. tangutica*

The hairy roots of *P. tangutica* commonly emerged from the wound sites after about 7 days of incubation on phytohormone-free MS medium (Figure 1A) because the wounded plant cells secreted some low-molecular phenolic signals that facilitated the plasmid to enter cells (Stachel et al., 1985); while the leaves were not infected by *A. rhizogenes* strain A4 were never observed root formation. The young leaves of plantlets showed high sensitivity to A4 for the effectiveness of the induction of hairy roots reached 100%. Each hairy root is an independently transformed root (monoclonal) because it

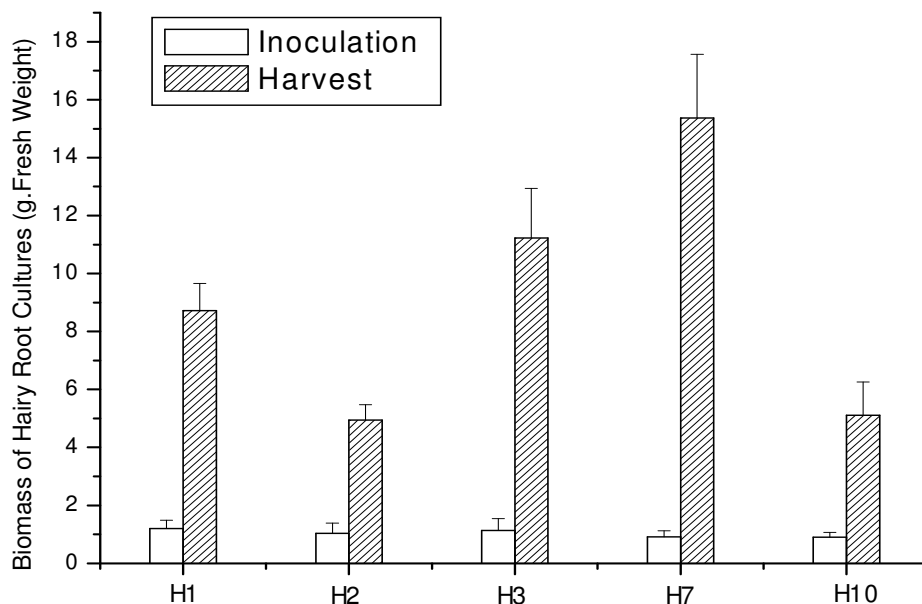


Figure 2. Biomass of fresh weight of 5 hairy root lines harvested after 35-day liquid culture.

develops from a single plant cell, of which genome the rooting genes had integrated into (Chilton et al., 1982). When the hairy roots grew to 2 - 3 cm in length, they were excised and subcultured in plant-hormone-free MS media.

Maintenance and liquid culture of hairy roots

In the present study, most of excised roots of *P. tangutica* grew well on phytohormone-free MS medium in which 250 mg/l cefotaxime could kill *Agrobacterium* bacteria well. After 3-time subculture, most hairy roots were free of bacteria and then cultured on antibiotics-free medium. The hairy roots showed two different morphologies. A few hairy roots showed callus-like morphology (Figure 1B) like others' discovery (Moyano et al., 1999); most hairy roots showed a typical morphology of hairy roots characterized by plagiotropic growth, high incidence of lateral branching and much faster growth (Figure 1C). In the next experiment, the typical hairy roots were selected for subculture for producing tropane alkaloids because they usually contained higher level of secondary metabolites (Moyano et al., 1999). Based on the typical morphologies, it might be concluded that the subcultured roots were the hairy roots genetically transformed by Ri plasmid, but that should have to be confirmed at the molecular level.

Then the selected monoclonal hairy root lines of *P. tangutica* confirmed by genomic PCR were respectively transferred into basic liquid MS medium for culture (Figure 1D). All the selected lines showed rapid proliferation in liquid culture but at different levels. After 5-week culture, biomass of Line 2 (H2) increased 4.8 times,

Line 10 (H10) increased 5.7 times, Line 1 (H1) increased 7.3 times, Line 3 (H3) increased 9.9 times and Line 7 (H7) increased 16.9 times (Figure 2). The rapid growth feature of cultured hairy roots made them the feasible materials for producing pharmaceutical tropane alkaloids in bioreactor.

The rooting genes integrated into plant genome confirmed by genomic PCR

Integration of the T-DNA, which was the cause of hairy root formation, into *P. tangutica* genome was confirmed at the molecular level by PCR with primers constructed on the sequences of *rolB* and *rolC* genes in *A. rhizogenes* as described before (Zhang et al., 2004). To be sure that the *rolB* and *rolC* genes were not amplified from A4, the following procedure was performed. Hairy root cultures were maintained on a medium with antibiotics for several subcultures, and then they were grown on a medium without antibiotics for several subcultures. Latterly, hairy root cultures were homogenized and suspensions obtained were plated on YEB agar medium. Plates were incubated for 7 days. And *A. rhizogenes* was never found in the plates. So, we could make sure that there were no any *A. rhizogenes* in the plant tissue. The target sequence of *rolC* with 626 bp at length were amplified from the positive control (plasmid A4 used as the template for PCR), from three independent transformed hairy root clones, but never found in the untransformed tissue of *P. tangutica*; and at the same time, the targeted sequence of *rolB* with 423 bp at length were amplified from the positive control and the hairy roots, but never amplified from the untransformed

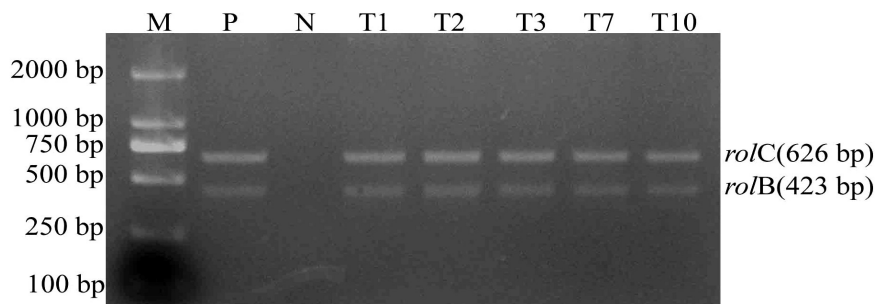


Figure 3. PCR analysis of *rolB* and *rolC* gene in transformed hairy roots. M: DL2000 maker (2000, 1000, 750, 500, 250 and 100 bp); P: positive control of *A. rhizogenes* strain A4; N: negative control of normal roots; T_n: different hairy root tested lines; *rolB/rolC*: *rolB* and *rolC* genes fragments

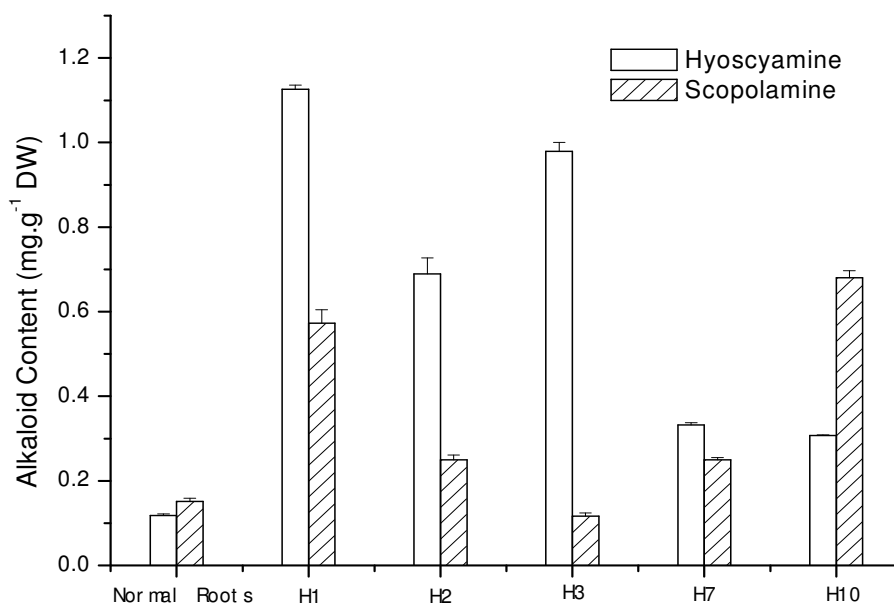


Figure 4. Alkaloids content of 5 hairy root lines and normal roots.

tissue (Figure 3). The PCR analysis results indicated that the *rolB* and *rolC* genes did integrate into *P. tangutica* genome that caused the hairy root formation (Ambros et al., 1986). Transformation of plant tissues by *A. rhizogenes* was earlier confirmed by biochemical detection of the production of opine in plant tissue (Petit et al., 1983) or some marker gene such as NPT II (Henzi et al., 1999). And primers here for *rolB* and *rolC* genes used in this work enabled molecular confirmation of the transformation with the wild type strain of *A. rhizogenes* A4 just like others' report (Ambros et al., 1986).

Analysis of the production of hyoscyamine and scopolamine in root

The content of hyoscyamine and scopolamine was

detected by HPLC in hairy roots and normal roots. Both the hairy roots and the normal roots could produce hyoscyamine and scopolamine but at different levels. All the selected hairy root clones produce much more tropane alkaloids than the normal roots.

The highest level of scopolamine production clone (Line 10) produced 0.68 mg/g DW scopolamine, which was 4.5 times compared to the content of scopolamine in the normal roots; and the highest level of hyoscyamine production line (Line 1) produced 1.13 mg/g DW hyoscyamine, which was 9.5 times compared to the content of hyoscyamine in the normal roots (Figure 4). The results suggested that the transformed hairy roots had the intact biosynthetic pathway of tropane alkaloids and higher capabilities of tropane alkaloid biosynthesis than the raw roots.

DISCUSSION

Hairy roots usually grow rapidly, show plagiotropic growth, and are highly branched on phytohormone-free medium and this makes it convenient for culture and proliferation (Hu and Du, 2006). The greatest advantage of hairy roots is that hairy root cultures often exhibit about the same or greater biosynthetic capacity for secondary metabolite production compared to their mother plants (Kim et al., 2002). So, many plant species have been genetically transformed by *A. rhizogenes* to establish hairy root cultures for producing the target secondary metabolites (Giri and Narasu, 2000). In the present study, *in vitro* culture system and hairy roots cultures of *P. tangutica* have been established in the present study that provides an alternative way to produce the pharmaceutical tropane alkaloids; and makes it possible for metabolic engineering of tropane alkaloid in *P. tangutica*.

ACKNOWLEDGEMENTS

This research was financially supported by key projects of Tibet Science and Technology Bureau (2009AG15 and 2010KJGX01-36).

REFERENCES

- Ambros PF, Matzke AJM, Matzyke MA (1986). Localization of *Agrobacterium rhizogenes* T-DNA in plant chromosomes by in situ hybridization. *The EMBO J.*, 5: 2073-2077.
- Chilton MD, Tepfer DA, Petit A, David C, Casse-Delbart F, Tempe J (1982). *Agrobacterium rhizogenes* inserts T-DNA into the genomes of the host plant root cells. *Nature*, 295: 432-434.
- Flores HE, Curtis WR (1992). Approaches to Understanding and Manipulating the Biosynthetic Potential of Plant Roots. *Ann. New York Acad. Sci.*, 665: 188-209.
- Furner IJ, Huffman GA, Amasino RM, Garfinkel DJ, Gordon MP, Nester EW (1986). An *Agrobacterium* transformation in the evolution of the genus *Nicotiana*. *Nature*, 319: 422-427.
- Giri A, Narasu ML (2000). Transgenic hairy roots: recent trends and applications. *Biotechnol. Advances*, 18: 1-22.
- Hashimoto T, Dae JY, Yamada Y (1993). Production of tropane alkaloids in genetically engineered root cultures. *Phytochemistry*, 32: 713-718.
- Henzi MX, Christey MC, McNeil DL, Davies KM (1999). *Agrobacterium rhizogenes*-mediated transformation of broccoli (*Brassica oleracea* L. var. *italica*) with an antisense 1-aminocyclopropane-1-carboxylic acid oxidase gene. *Plant. Sci.*, 143: 55-62.
- Hu ZB, Du M (2006). Hairy Root and Its Application in Plant Genetic Engineering. *J. Integrative. Plant. Biol.*, 48: 121-127.
- Kim YJ, Wyslouzili BE, Weathers PJ (2002). Invited review: Secondary metabolism of hairy root cultures in bioreactors. *Vitro. Cell. Dev. Biol. Plant.*, 38: 1-10.
- Krolicka A, Staniszevska I, Bielawski K, Malinsky E, Szafranek J, Lojkowska E (2001). Establishment of hairy root cultures of *Ammi majus*. *Plant. Sci.*, 160: 259-264.
- Moyano E, Fornalé S, Palazón J, Cusidó RM, Bonfill M, Morales C, Piñol MT (1999). Effect of *Agrobacterium rhizogenes* T-DNA on alkaloid production in Solanaceae plants. *Phytochemistry*, 52: 1287-1292.
- Murashige T, Skoog F (1962). A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. *Physiol. Plantarum*, 15: 473-497.
- Petit A, David C, Dahl GA, Ellis JG, Guyon P, Casse-Delbart F, Tempé J (1983). Further Extension of the Opine Concept: Plasmids in *Agrobacterium rhizogenes* Cooperate for Opine Degradation. *Mol. Gen. Genet.*, 190: 204-214.
- Sambrook J, Russell D, Maniatis T (2002). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Scragg AH, Allen EJ, Bond PA, Smart NJ (1986). In: Morris P, Scragg A, Fowler M (eds) *Secondary metabolism in plant cell*, Cambridge University Press, Cambridge, p. 82.
- Stachel SE, Messens E, Montagu MV, Zambryski P (1985). Identification of the signal molecules produced by wounded plant cells that active T-DNA transfer in *Agrobacterium tumefaciens*. *Nature*, 318: 624-629.
- Wysokinska H, Chmiel A (1997). Transformed root cultures for biotechnology. *Acta Biotechnologica*, 17: 131-159.
- Xiao PG, Xia GC, He LY (1973). The occurrence of some important tropane alkaloids in Chinese solanaceous plants. *J. Integ. Plant. Biol.*, 15: 187-194.
- Yamada Y, Tabata M (1997). Plant biotechnology of tropane alkaloids. *Plant biotechnology*, 14: 1-10.
- Zhang L, Ding RX, Chai YR, Bonfill M, Moyano E, Oksman-Caldentey KM, Xu TF, Pi Y, Wang ZN, Zhang HM, Kai GY, Liao ZH, Sun XF, Tang KX (2004). Engineering tropane biosynthetic pathway in *Hyoscyamus niger* hairy root cultures. *Proc Natl. Acad. Sci.*, 101: 6786-6791.
- Zhang L, Yang B, Lu BB, Kai GY, Wang ZN, Xia YY, Ding RX, Zhang HM, Sun XF, Chen WS, Tang KX (2007). Tropane alkaloids production in transgenic *Hyoscyamus niger* hairy root cultures over-expressing Putrescine N-methyltransferase is methyl jasmonate-dependent. *Planta*, 225: 887-896.