Agrobacterium rhizogenes-mediated genetic transformation of *Psammosilene tunicoides* and identification of high saponin-yielding clones

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Abstract

Psammosilene tunicoides (WC Wu et CY Wu), a medicinal plant, was transformed with *Agrobacterium rhizogenes* strain ATCC15834, resulting in development of hairy roots from the cut surface of leaves. In total, 36 hairy root clones demonstrating prolific rooting and ageotropism were isolated. Hairy roots from all 36 clones were cultured in B_5 liquid medium. Each clone had a different saponin content. Among the 36 clones, highest saponin content of 0.7‰ was observed in A-23. PCR confirmed that *rolB* and *rolC* genes of the Ri plasmid of *A. rhizogenes* had been transferred and expressed successfully in the hairy roots of *P. tunicoides* A-23.

Key words: *Agrobacterium rhizogenes*, genetic transformation, hairy roots, *Psammosilene tunicoides*, saponins.
Abbreviations: BA, 6-benzyladenine; CK, untransformed mother plant; DW, dry weight; NAA, α-naphthaleneacetic acid.

Introduction

Psammosilene tunicoides (WC Wu et CY Wu) is a perennial supine herbaceous belonging to the Caryophyllaceae family (Wu 1995). It is a valuable species for studies on evolutionary systematics of the Caryophyllaceae (Oxelman, Liden 1995; Smissen et al. 2003; Zhang et al. 2012). *P. tunicoides* is an autogenus medicinal plant endemic to China showing pain-relieving, anti-inflammatory, haemostasia and immunomodulatory properties (Qu et al. 2010ab; Tian et al. 2010; 2012; Kaur et al. 2012; Zhang et al. 2012). Only one new carboline alkaloid isolated from the aerial parts of *P. tunicoides* has been shown to have weak cytotoxic activity against the HCT116 human tumour cell line (Wen et al. 2014).

Due to excessive destructive exploration, natural resources of *P. tunicoides* have dwindled and become threatened. It is listed in the China Plant Red Data Book as an endangered species (Fu 1992). Due to its long growth phase and low natural rate of regeneration, a new means to increase production of this medicinal resource is required. Hairy root culture using genetic transformation of plant tissues by *Agrobacterium rhizogenes* is a promising biotechnological tool to produce secondary metabolites, due to rapid tissue growth and the stability of secondary metabolites (Srivastava, Srivastava 2007; Nosov 2012). Hairy root culture has been used to produce secondary metabolites from a wide range of medicinal plants (Hu, Du 2006; Veena, Taylor 2007; Fukuyama et al. 2012; Sujatha et al. 2012; Wilczańska-Barska et al. 2012). For example, saponins have been produced by *Panax* (Kiselev et al. 2010) and *Maesa lanceolata* (Faizal, Geelen 2012) hairy root culture.

There are only two studies to date that have examined the use of *A. rhizogenes* to induce hairy roots in *P. tunicoides*. An initial, preliminary study found that *A. rhizogenes* strain ATCC15834 could induce hairy roots in leaves and stem segments of *P. tunicoides* (Li et al. 2011). In a more recent study, Zhang et al. (2014) used plant growth regulators to increase secondary metabolite production from hairy root-induced callus following infection with *A. rhizogenes* strain ACCC10060. In this study, another *A. rhizogenes* strain ATCC15834 was used to infect leaf explants of *P. tunicoides* and to establish a highly efficient hairy root culture system as well as a saponin detection system. This study lays a foundation for large-scale culture and industrialization of *P. tunicoides*.

Materials and methods

Plant cultivation

P. tunicoides plants were obtained from a greenhouse of the Biotechnological Institute of Guangdong Academy of Forestry where they had been grown since 2006. Healthy

and young shoots were surface sterilized in 70% (v/v) ethyl alcohol for 10 s and 0.1% (w/v) mercuric chloride for 10 min, rinsed with sterile distilled water three times, then cut into 1 to 2 cm long segments with buds and placed on agar-solidified (0.7%) MS (Murashige, Skoog 1962) basal media supplemented with 0.4 mg L^{-1} 6-benzyladenine (BA) and 0.2 mg L^{-1} α-naphthaleneacetic acid (NAA). The medium was also supplemented with 3% sucrose and pH was adjusted to 5.8 before autoclaving. The culture jars were maintained in a culture room at 25 ± 2 °C and a 16-h photoperiod with a 50 μmol m^{-2} s^{-1} photon flux density. The shoots were also subcultured on the same medium to propagate them (Fig. 1A). *A. rhizogenes* strain ATCC15834 (Shi et al. 2011) was a kind gift from Professor Heping Shi. This strain was inoculated in liquid YEB medium (0.1% yeast extract, 0.5% beef extract, 0.5% peptone and 0.5% sucrose, pH 7.0) and cultured at 28 °C, shaken at 120 rpm in the dark for 20 h to reach the logarithmic phase, then diluted to an OD_{600} of 0.8. This dilution was used to infect leaf explants.

Induction of hairy roots

The leaf explants were inoculated on agar-solidified (0.7%) MS medium and cultured at 25 °C in the dark. After preculture for 2 days, 15 to 20 explants were transferred into 5 mL of *A. rhizogenes* suspension for 10 min. The surfaces of explants were blotted dry with sterilized filter paper, then explants were cultured at 25 °C in the dark for 2 days. When the *A. rhizogenes* plaque grew out, explants were washed with sterilized water three times. Explants were transferred for one day to MS medium supplemented with 500 mg L^{-1} cefotaxime (Clafora®, Hoechst, Somerville, NJ, USA) at 25 °C and a 14-h photoperiod under 50 μmol m^{-2} s^{-1} photosynthetic photon flux density. Under these conditions, explants were subcultured every 5 days until *A. rhizogenes* colonies disappeared.

Hairy root amplification

All rapidly growing hairy roots 2 to 3 cm long were independently marked and then transferred to 50 mL of B_5 (Gamborg et al. 1968) liquid medium in 250-mL flasks to amplify the cultures. Three flasks were repeated for every hairy root clone. After culturing for 30 days, the hairy roots were gently removed from flasks, blot dried on filter paper and their fresh weight, dry weight (DW) and total saponin content was calculated. All hairy roots were dried at 60 °C in a convection oven for 2 days.

Standard curve and content of total saponins in transformed hairy roots and mother plant roots

Oleanolic acid was selected as the reference standard to determine total saponin content by vanillin-perchloric acid colorimetry (Liu 1995). Various volumes (0.10, 0.20, 0.40, 0.60, 0.80, 1.00, 1.20 mL) of oleanolic acid standard solution were precisely decanted into seven 10-mL volumetric flasks. Flasks were placed in a 70 °C water bath and supplemented with 0.5 ml of newly prepared 5% vanillin-glacial acetic acid solution and 1.5 mL of perchloric acid. Flasks were shaken slightly, placed in a 60 °C water bath for 15 min, then cooled in water for 3 min and made up with glacial acetic acid to constant volume. Blanks contained no sample and absorption was measured at 500 nm with a Shimadzu UV-3600 spectrophotometer (Kyoto, Japan). Every concentration was measured three times. A standard curve of average light absorption values against oleanolic acid concentration was derived. Sample solution (5.0 mL) was placed in 10-mL measuring flasks and the above procedures were repeated three times. Mean total saponin content (mg) was calculated from the standard curve and calculated as: total saponin (mg) / DW (g) × 1000‰. The materials included hairy roots from 36 transformed clones and roots from a control (untransformed) *in vitro* mother plant. Data was analyzed by one-way analysis of variance (ANOVA) and treatment means were considered to be significantly different from controls by the Least Significant Difference (LSD) test at $P \leq 0.05$ using SPSS version 17.0.

Confirmation of genetic transformation

The hairy roots (100 mg) of each clone were vortexed with 850 μL cetyltrimethyl ammonium bromide in 1.5-mL Eppendorf tubes filled with small steel balls at 65 °C for 20 min; then they were placed into a 60 °C water bath for 30 min (Edwards et al. 1991). During this period, the Eppendorf tube was tilted slightly to prevent precipitation and supplemented with an equal volume of chloroform and inverted to mix. The Eppendorf tube was centrifuged at 12 000 rpm for 20 min and the supernatant was supplemented with 1/10 (v/v) of 3 M sodium acetate and two to three volumes of ethanol, shaken slightly five times, and then placed at –20 °C for 30 min. Tubes were centrifuged at 12 000 rpm for 20 min. The supernatant was carefully removed and the pellet was air dried, dissolved in 100 μL of deionized water, and then stored at –20 °C until use.

The total genomic DNA of untransformed leaf explants was used as the negative control and total genomic DNA of *A. rhizogenes* ATCC15834 was used as the positive control (Georgiev et al. 2011). PCR primers based on Furner's (1986) sequences were designed to amplify the *rol*B and *rol*C gene fragments. The *rol*B-specific primers were 5'-GCTCTTGCAGTGCTAGATTT-3' (forward) and 5'-GAAGGTGCAAGCTACCTCTC-3' (reverse) while the rolC-specific primers were 5'-CTCCTGACATCAAACTCGTC-3' (forward) and 5'-TGCTTCGAGTTATGGGTACA-3' (reverse). In a silicon centrifuge tube, 5 ng of primer (*rolB* or *rolC*) and 25 ng of DNA were mixed with 2 μL of Taq DNA polymerase (Sigma) in a total volume of 25 μL. *rolB* and *rolC* amplification were performed separately by an initial hot start at 94 °C for 3 min, then 35 cycles of denaturation (94 °C, 60 s), annealing (55 °C, 60 s) and extension (72 °C, 60 s), and a final extension at 72 °C for 10 min. PCR amplification

products were analyzed by electrophoresis in 1% agarose gels containing 10 mg per mL ethidium bromide. PCR-positive clones were indicated by specific genomic DNA fragments, 540 bp for *rolB* and 770 bp for *rolC*.

Results and discussion

Induction of hairy roots

Shoots that were cultured and propagated in B_5 medium for 20 to 30 days developed some opposite leaves (Fig. 1A) with almost no petiole. After infection of leaf explants with *A. rhizogenes* strain ATCC15834 for 15 to 20 days, some hairy roots formed on the cut surfaces of leaves (Fig. 1B). When the culture period was extended to 30 days, a mass of white highly branched and geotropic hairy roots formed on the surface of the medium (Fig. 1C). Hairy roots inoculated on B_5 liquid medium for continuous culture formed a mass of white hairy roots extending throughout the flask within 30 days (Fig. 1D). Explants that did not develop hairy roots tended to oxidize and necrose on solid medium (Fig. 1B, 1C). In total, as many as 40% of leaf explants formed hairy roots. Li et al. (2011) co-cultured leaf and stem segments with *A. rhizogenes* strain ACCC10060 on B_5 liquid medium, inducing more kanamycin-resistant hairy roots from the former than from the latter, with a 4.1-fold increase in fresh weigh biomass after 35 days. Separately, Zhang et al. (2013) induced an *in vitro* system to induce adventitious roots in liquid B_5 medium in the presence of 0.05 mg L^{-1} BA and 0.05 mg L^{-1} indole-3-acetic acid.

Screening hairy roots for saponin

The standard curve of oleanolic acid was plotted as $y = 34.432x - 0.0139$ ($R^2 = 0.9992$) (Fig. 2). In total, 36 transformed hairy root clones and one untransformed mother plant root were identified, most of which could form more than 0.5 g DW per month (Fig. 3). Among them, the most prolific clone was A-33, DW reaching 0.676 g per month. The roots of the untransformed mother plant (CK) formed less saponin. Some other clones, such as A-6, also had low saponin content.

The DW, saponin yield and total saponin content of all clones were measured (Fig. 3). All clones performed

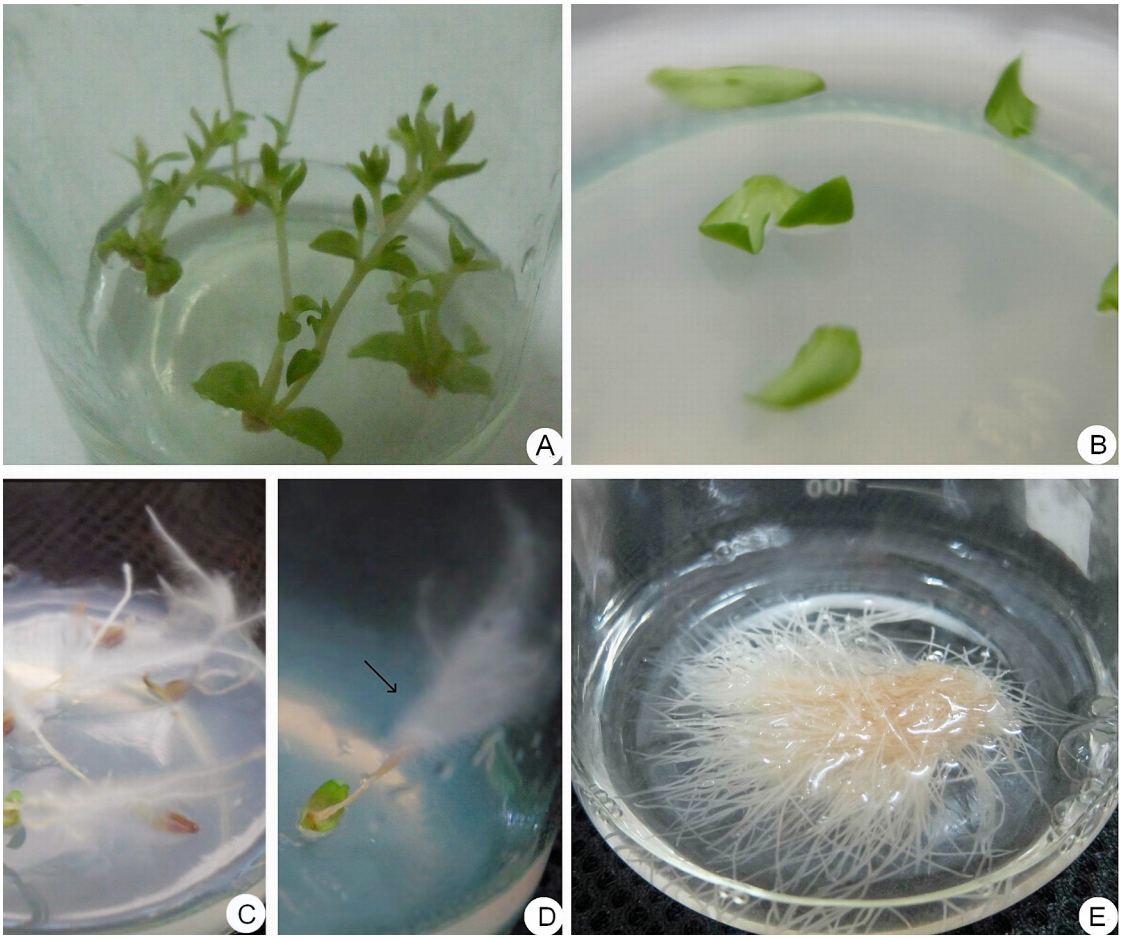

Fig. 1. Induction and culture of *P. tunicoides* hairy roots *in vitro*. A, shoots were mass propagated on MS solid medium supplemented with 0.4 mg L^{-1} BA and 0.2 mg L^{-1} NAA. B, *in vitro*-derived leaves were inoculated on the MS medium. C, the leaves were infected with *A. rhizogenes* ATCC15834 and then transferred to MS medium supplemented with 500 mg L^{-1} cefotaxime for 20 days. D, hairy roots (black arrow) were produced at the leaf cut surface after culture for 30 days. E, an extensive mass of hairy roots growing in a flask growing in liquid B_5 medium for 30 days

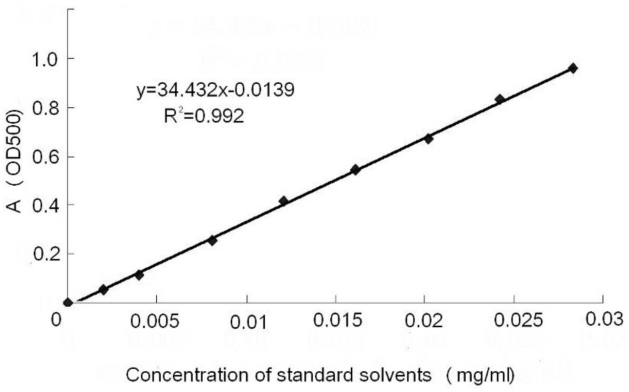

Fig. 2. The standard curve of oleanolic acid based on absorption at 500 nm.

differently, with saponin yield ranging between 0.97 and 4.6‰. The lowest saponin yield (0.97‰) was found in untransformed roots of the mother plant and the saponin yield of transformed roots was usually 1.2- to 4.6-fold higher than that of CK. The highest saponin content was

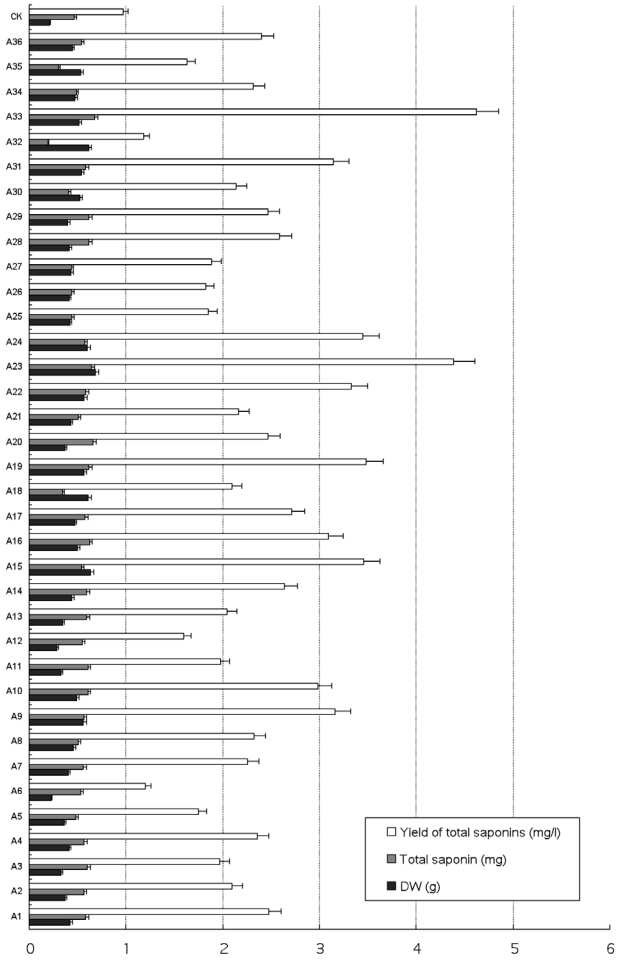

Fig. 3. Yield (dry weight and total saponins) in 36 transformed hairy root clones (A1–36) and untransformed mother plant root (CK) of *P. tunicoides*.

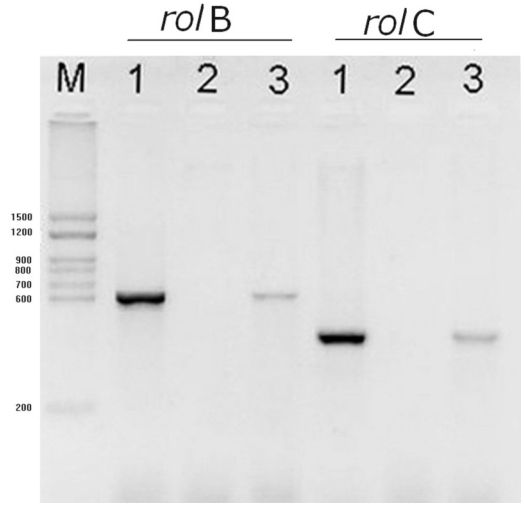

Fig. 4. PCR fragments of *rolB* and *rolC* genes amplified from the genomic DNA of hairy roots of *P. tunicoides* and total genomic DNA of *A. rhizogenes* ATCC15834 following gel electrophoresis. M: 2000-bp DNA markers; Lane 1: Fragments amplified from *A. rhizozgenes* ATCC15834 showing specific DNA fragments at 540 bp and 770 bp for *rolB* and *rolC* genes, respectively; Lane 2: Fragments amplified from untransformed roots showing no DNA fragment. Lane 3: Fragments amplified from hairy roots of A-23 showing specific DNA fragments of 540 bp and 770 bp, respectively.

observed in clone A-33 with a saponin yield of 4.6‰. The highest total saponin content was also observed for clone A-33: 4.619 mg after liquid culture for 30 days. All clones performed differently (growth and saponin production), most likely because different cells in different physiological states were infected. Integration of T-DNA on the Ri plasmid into plant tissue is random (Stanton 2003). Since the length and copy number of T-DNA inserts into a plant cell can differ, the hairy roots of the different clones would likely demonstrate different cell size, structure, genetics, physiology and biochemistry, i.e. different metabolic state and ability to synthesis secondary metabolites (Sujatha et al. 2012). Such differences in the ability to synthesize secondary metabolites are also species-specific (De Guzman et al. 2011; Wilczańska-Barska et al. 2012). Li et al. (2011) noted that the total saponin content of callus and seedlings was 0.388% and 0.217%, relative to hairy roots (0.857%) on a dry weight basis.

Identification of genetically transformed hairy roots

The expected specific genomic DNA fragments (540 bp and 770 bp for *rolB* and *rolC*, respectively) from *A. rhizogenes* strain ATCC15834-mediated transformed hairy roots of clone A-33 could be amplified by PCR (Fig. 4). No DNA fragments from untransformed plants were detected. The *rolB* and *rolC* genes from the *Ri* plasmid of *A. rhizogenes* were thus successfully transformed into the genomic DNA of *P. tunicoides* clone A-33 and showed integrant expression.

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