Genetic transformation of the endangered Tibetan medicinal plant *Przewalskia tangutica* Maxim and alkaloid production profiling revealed by HPLC

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Abstract

To achieve a high yield of tropane alkaloids (TA) and exploit the alpine plant sustainably, an optimized protocol for induction and establishment of hairy roots culture of *Przewalskia tangutica* Maxim was developed through selection of appropriate *Agrobacterium* strain and the explant type. The hypocotyl is more readily facile to induce the HR than the cotyledon is when infected with the three different agrobacterium strains. MUS440 has an efficiency (of up to 20%), whereas the ATCC10060 (A4) can induce HR on both types of explants with the highest frequency (33.33%), root length (21.17 ± 2.84 cm), and root number (10.83 ± 1.43) per explant than the other strains. The highest HR production resulted from using hypocotyl as explants. Independent transformed HR was able to grow vigorously and to propagate on a no-hormone 1/2MS liquid medium. The presence of pRi *ro/B* gene in transformation of HR was confirmed by PCR amplification. In the liquid medium, the HR growth curve appeared to be “S” shaped, and ADB had increased to 4.633 g/l. Moreover, HPLC analysis showed that HR lines have an extraordinary ability to produce atropine (229.88 mg/100 g), anisodine (4.09 mg/100 g), anisodamine (12.85 mg/100 g), and scopolamine (10.69 mg/100 g), which were all more significant than the control roots. In conclusion, our study optimized the culture condition and established a feasible genetics reactor for *P. tangutica* green exploration and biological study in the alpine region.

Keywords *Przewalskia tangutica* Maxim · Hairy root · Tropane alkaloid · HPLC

Introduction

Tropane alkaloids are important secondary metabolites in many Solanaceae species, such as *Hyoscyamus niger* and *Datura metel* L., which include anisodine, anisodamine, scopolamine, and atropine, and are now widely used in anti-asthmatic activity, curing epilepsy, and as an anti-inflammatory (Spinks et al. 2007; Gaire and Subedi 2013). Particularly, scopolamine is treated as an anticholinergic agent; it acts on the parasympathetic nervous system and has fewer side-effects than the other three. Its market demand increased more than tenfold over the others (Gryniewicz and Gadzikowska 2008). The study of species with high levels of tropane alkaloids is, therefore, an important way to meet this great market demand.

Przewalskia tangutica Maxim (*P. tangutica*), which is a well-known traditional Tibetan medicinal herb generally called “Ai Langdang” or “Ma Niaopao,” belongs to the Solanaceae perennial species. Historically, the whole plant was used to treat various conditions such as

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gastrointestinal spasm pain and diphtheria (Yang et al. 1991). It has been reported that the total tropane alkaloids and hyoscyamine reached levels of 2.06–4.01% and 1.67–3.82% in wild *P. tangutica*, respectively (Xiao and He 1982). These levels are much higher than those in other Solanaceae species in China (Hsiao et al. 1973; Wang et al. 2002a). In the TA biosynthesis pathway, hyoscyamine can turn into scopolamine catalyzed by 6 β -hydroxylase (Zhang et al. 2004). Therefore, *P. tangutica* holds the most promising for producing tropane alkaloids, especially scopolamine (Xiao and He 1982). However, *P. tangutica* is naturally distributed in the glacier region on the Qinghai-Tibet Plateau (QTP), at elevations ranging from 3000 to 4000 m (Liu et al. 1996; Wan et al. 2008). The ecosystem of QTP is very weak, and the over-exploitation of this wild resource could trigger species extinction and destroy the fragile environment (Lei et al. 2015). Moreover, the seeds are poorly germinated and species grow very slowly and take several years to produce quality roots. Therefore, the commercial application of *P. tangutica* is not only limited by its natural occurrence, but also is hindered by the lack of biotechnological studies.

Recently, hairy root (HR) culture has developed as a promising biotechnology system for producing the vital secondary metabolites of medicinal plants (Sevón and Oksman-Calden 2002; Häkkinen et al. 2014; Tian 2015). HR culture results from transfer and integration of the pRi gene from agrobacterium into the plant genome, which can then direct the auxins triggered hairy root growth (White et al. 1985). These plants can produce many roots in a stable and rapid manner and, simultaneously, synthesize pharmaceutical chemicals (Sevón and Oksman-Calden 2002; Häkkinen et al. 2014; Tian 2015). Since this system was developed, it has been applied successfully to many medicinal plants such as *Panax ginseng* (Yoshikawa and Furuya 1987), *Datura candida* (Christen et al. 1989), and *Gentiana macrophylla* (Zhang et al. 2010), to produce active compounds. Thus, HR has the promising to enhance the amount and type of secondary products produced by plants.

To exploit this endemic, endangered species of the traditional Tibetan herb on an industrial scale in the future, new strategies should be developed to increase its scopolamine content. The most promising approach to this goal would be in vitro production. Therefore, in the present study, three *A. rhizogenes* (MSU440, LBA9402, and A4) were used to induce HR production and increase the TAs content in *P. tangutica*. The type of explants and bacterium strains were optimized; moreover, the growth state in liquid medium was studied to improve HR efficiency firstly. This study would develop the model to replace the traditional approaches for TA utilization of this species in the alpine region.

Materials and methods

Plant materials

The seeds of *P. tangutica* were collected from the region of Guoluo (elevation is 4200 m; N 34.7°E 99°) in Qinghai province. Mature seeds were treated with 400 μ M gibberellic acid (GA) for 24 h and then sterilized with 10% NaClO for 20 min. The seeds were washed with sterilized water 4–5 times and inoculated in the petri dishes with Murashige and Skoog (MS) medium to obtain aseptic seedlings (Lei et al. 2015). One-week seedlings were transferred to conical flask with MS medium and the seedlings were grown for 2 weeks in the growth chamber under the conditions of 25 °C, 300 μ mol/m² (white fluorescent tubes), a 12-h photoperiod, and 50% humidity. Samples were cut from cotyledon and hypocotyl of seedlings for inoculation.

A. rhizogenes strains

A. rhizogenes strains, ATCC10060 (A4), and MSU440, were obtained from the China General Microbiological Culture Collection Center (Beijing, China). The wild A4 strain harboured an agropine-type Ri plasmid pRiA4, and the MSU440 strain, harbouring an agropine-type Ri plasmid had the streptomycin resistance. LBA9402 was purchased from the Guangdong Microbiology Culture Centre. This strain had the rifampicin-resistant derivative of NCPPB1855 and possessed the agropine-type plasmid pRi1855. A4 and LBA9402 strains were incubated in the YEB medium (1.0 g/l yeast extract, 5.0 g/l beef extraction, 5.0 g/l peptone, 5.0 g/l sucrose, 0.49 g/l MgSO₄·7H₂O, pH 7.2) with 50 mg/l kanamycin. The MUS440 strain was growing in liquid Luria–Bertani medium containing 50 mg/l rifampicin, 10 g/l tryptone, 5 mg/l yeast extract, and 10 mg/l NaCl, pH 7.2. The solution was shaken at 250 rpm at 28 °C overnight, until the O.D₆₀₀ reached 0.8–1.0. Then, 200 μ l of the fresh bacterial solution was mixed with 200 ml of the YEB culture medium supplement and with 100 mg/l AS (3,5-methoxy-4-hydroxyacetophenone) (Sigma-Aldrich, Germany). The concentration of the medium was monitored until the OD₆₀₀ arrived at 0.6–0.8. The culture solution was centrifuged at 3000 rpm and the pellet was suspended in an MS medium containing 100 μ M AS; the final concentration was adjusted to 0.6 prior to inoculation.

Infection and culture of HR from *P. tangutica*

The cotyledons and hypocotyls were cut into 0.5 cm lengths from the 2-week seedlings. Specimens were then individually immersed in one of the three *A. rhizogenes* solutions

(OD₆₀₀ of 0.6), respectively. The explants were immersed in a solution for 10 min while being shaken at 70 rpm. After that, the explants were washed with sterilized water and cleaned with autoclaved paper (Whatman No. 1), and then transferred to a hormone-free MS medium cultured in the dark at room conditions. 2 days later, the explants were transferred to MS agar (0.8% w/v) plates with 500 mg/l cefotaxime without hormones, and cultured under 16 h of daylight of 300 µmol/m² illumination at 25 °C. This was repeated every 2–4 days until no agrobacterium could be detected. The control explants were cultured in the same condition except for inoculation. After bacterial inoculation for 25 days, the root lengths and the number of HR data were collected, and the HR inducing frequency was evaluated on each explant according to formula [HR/the number of infected explants] × 100. Each experiment was replicated three times; each iteration contained at least 20 explants.

DNA isolation and PCR identification of HR

The HR were cut from the explants, and their genomic DNA was extracted with a plant DNA mini-preparation kit (Omega Com, USA), according to the instructions in the manual. The roots from the sterile seedlings were used as the negative control. Plasmid DNA of Ri was used as a positive control.

PCR was performed with the following procedure: 95 °C for 4 min, followed by 35 cycles of desaturating at 95 °C for 40 s, annealing at 55 °C for 40 s, extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min. The PCR production was fractionated on 0.8% agar gel with a small amount of ethidium bromide and photographed under UV light at 260 nm. The 20 µl reaction mixture contained 20 ng of DNA. The primers of *rolB* were designed as follows: 5'-TCTCGCGAGAAGATGCAGAA-3' (Forward primer); 5'-TAAAGGTTGCCTGCAGGGG-3' (Reversed primer).

Cultivation of hairy roots from *P. tangutica*

One week later, the 4–5 cm HR was excised from the explant and cultured on hormone-free 1/2MS solution containing 500 mg/l cefotaxime. The process was repeated in seven rounds with an interval of 1 week to selected clean well-grown hairy roots. Subsequently, HRs were propagated on 1/2MS medium in 250 ml flasks and shaken at 110 rpm, in the dark at 25 °C. The fresh weight and dry weight of the HR were monitored every 4 days and the test lasted for 35 days. Accumulated dry biomass (ADB) and growth index were determined to monitor the HR growth. ADB = final dry biomass-initial dry biomass (g/l); GIDW = (final dry biomass-initial dry biomass)/initial dry biomass.

Tropane alkaloids determination in HR

HRs was dried at 65 °C for 3 days. The dry material was ground to a fine powder and soaked in a 1.5 ml ammonia solution, and extracted with 10 ml chloroform overnight. The extracted solution was vaporized on a water bath; the residue was dissolved in 0.25 M of H₂SO₄. All extractions were filtered through a 0.45 µm nylon filter and analyzed using an HPLC chromatograph (Waters 515E) (Water Com., USA) equipped with an ultraviolet detector (Waters2996), chromatograph workstation (Waters Empower pro), and Kromasil C18 5 µm (4.6 × 150 mm) column. Standards of four tropane alkaloids (atropine, anisodine, anisodamine, and scopolamine) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products. The absorbance peak wavelengths were 245 nm. In this instance, the peaks were symmetrical and the baselines were smooth. The injection volume was 10 µl, and the flow rate was 1.0 ml/min. Quantification was made by a comparison of standards (Wang et al. 2002a, b). For comparison, the sterile seedlings of *P. tangutica* were also grown for 3 months and analyzed as the positive control; the tropane alkaloids were extracted using the same methods.

Statistical analysis

Statistical analysis of the data was performed using SPSS 16.0 software package for Windows. All the experiments repeated three times and the results were expressed as the mean ± standard error. The data were analyzed with LSD and Independent Samples *t* test method, respectively. A *p* value less than 0.05 was considered statistically significant.

Results

Formation of HR of *P. tangutica*

The explants of cotyledon and hypocotyl were cut from the sterile seedlings (Lei et al. 2015) and infected with the three different *A. rhizogenes* strains A4, MSU440, and LBA9402. After 10 days, the small white hairy roots began to protrude around the basic cut ends of the veins on the cotyledon or the hypocotyl (Fig. 1a). The HR grew fast and branched highly, with no geotropism. They had the characteristic of HR, which appeared white in the dark of the culture environment (Fig. 1b). In the same culture conditions, the control explants died after 10 days.

Induction frequency was affected by the *A. rhizogenes* strain and plant tissue

Although both the cotyledon and hypocotyl of *P. tangutica* both had the potential to generate HR, the frequency,

Fig. 1 Morphogenesis of hairy root on *P. tangutica*. **a** Cotyledon explants initiated hair root after 3 weeks. **b** HRs produced many new HRs after transferred to new MS medium without antibiotic. **c** HRs

grew on the MS solid medium in the dark. **d** HRs grew in the MS liquid medium in the dark. **e** Calli formed on the HR on the MS solid

HR number, and length in each case differed significantly (Table 1a, b). The three *A. rhizogenes* strains protruded more readily from the edge of the hypocotyl explants than from the cotyledon. The hypocotyl frequency was approximately twice that of the cotyledon. In the cotyledon, A4 strains caused only 13.33% of the explants to produce HR, while in the case of the hypocotyl, the A4 and MSU440 strains resulted in a production frequency of 33.3 and 20%, respectively. The induction frequency was significantly higher on the hypocotyl than it was on the cotyledon ($p < 0.05$) (Table 1a, b). Regarding HR number and average lengths, the hypocotyl induced by A4 was also 3.93- and 2.35-fold

higher than that of the cotyledon, respectively (Table 1a, b). Moreover, the different agrobacterium strains had distinct effects. As shown in Table 1a and b, the three *A. rhizogenes* strains had varying impacts on HR development. The A4 strain-induced HR in both tissues, while the MSU440 produced HR only on the hypocotyl (Table 1b). In the hypocotyl, the induction frequency of A4 was 65% higher than that of MSU440. Furthermore, statistical analysis showed that the MSU440 and A4 had a significant difference on the hypocotyl ($p < 0.05$). Similarly, root length and root number of HR induced by A4 were also significantly higher than those of MSU440 (Table 1b). However, both were better

Table 1 Influence of (a) cotyledon, (b) hypocotyl and different *A. rhizogenes* on inducing hairy root of *P. tangutica*

Explants of <i>P. tangutica</i>	Strains	Inductivity ($X \pm SE$)	Number of hairy root per explant ($X \pm SE$)	Length of hairy root ($X \pm SE$ cm)	Days of occurrence of hairy root ($X \pm SE$ d)
(a) Cotyledon	LBA9402	0 ^b	0 ^b	0 ^b	—
	MSU440	0 ^b	0 ^b	0 ^b	—
	A4	13.33 $\pm$ 3.33 ^a	2.75 $\pm$ 0.55 ^a	9 $\pm$ 2.00 ^a	28 $\pm$ 0
(b) Hypocotyl	LBA9402	0 ^c	0 ^c	0 ^c	—
	MSU440	20 $\pm$ 0 ^b	1.60 $\pm$ 0.32 ^b	4.40 $\pm$ 0.66 ^b	29.67 $\pm$ 0.88 ^a
	A4	33.33 $\pm$ 3.33 ^a	10.83 $\pm$ 1.43 ^a	21.17 $\pm$ 2.84 ^a	32 $\pm$ 1.15 ^a

Value represents mean $\pm$ SE values of three independent measurements made 25 days after bacterium inoculation. Mean comparisons using LSD at the 5% level were considered; the different little letters represented the significant difference between groups ($p < 0.05$)

than those of LBA9402, which could not induce HR generation on both explants.

Identification of HR from the *P. tangutica*

To identify the HRs generated from the explant of hypocotyl, DNA was extracted from them using the kit from the Omega Company. The DNA purification and concentration were also checked with the Nanodrop (Thermo Fisher Scientific, USA) and 0.8% agar gel. After amplification, the result showed that all HR and Ri plasmid had one band of approximately 500 bp; however, the negative control had no such bands (Fig. 2). Thus, the *rol B* gene had been already integrated into the genome of *P. tangutica*.

Culture of HR from *P. tangutica*

The HR from the explants of the hypocotyl were cut and grown on the 1/2MS plate without any hormones for 10 days, resulting in the typical hairy root phenotype with excessive branching on the 1/2MS medium plates (Fig. 1c). However, after seven culture iterations, some HRs induced by MSU40 generated some calli during the growth period (Fig. 1d). While the HRs induced by A4 had no such phenotype, they produced excessive branching on hormone-free plates. According to these HR growth rates, we chose the HRs that proliferated quickly and branched vigorously on hormone-free plates as good lines. The best growing hairy roots were selected for further analyses.

The HR induced by A4 was submerged for cultivation in shake-flasks (Fig. 1e). The fresh weight and dry weight of this HR were monitored every 4 days. As shown in Fig. 3, the growth curve of both appeared to be “S”-shaped during the period of culturing. Over the first 2 weeks, the HR grew slowly. In the third and fourth weeks, the biomass increased rapidly, while in the fifth week, both the fresh weight and the dry weight accumulation rates decreased, and the biomass became the highest. After a culture period of 5 weeks, the ADB had increased to 4.63 g/l and the calculated GIDW value was 84.2. The hairy roots were further selected for tropane alkaloids analyses.

Fig. 3 Growth curve of HR in 1/2 MS medium. The gray dotted line indicated the dry weight changes; the dark curve indicated the fresh weight changes

Four tropane alkaloids determination

The four tropane alkaloids (anisidine, anisodamine, scopolamine, and atropine) were measured with HPLC using the four tropane alkaloids standards (Wang et al. 2002a, b). According to Fig. 4, both the HR and the sterile seedling contained all four tropane alkaloids; however, the content varied. In HR, the atropine was 220 mg/g DW, which was the highest constituent of all. The anisodamine content was the lowest of the four, at only 4.09 mg/g DW. The anisidine and scopolamine are 12.85 mg/g DW and 10.69 mg/g DW, respectively. In the sterile seedlings, the four tropane alkaloids have a similar rank (Fig. 4). However, in each case, the content was significantly lower than that of the HR (Table 2).

Discussion

P. tangutica is an endemic endangered species on the Qinghai-Tibetan Plateau. It has a higher content of tropane alkaloids than other Solanaceae species, particularly in the Qinghai province (Hsiao et al. 1973). Therefore, to exploit this species continually, it is urgent to develop new ways to replace the classical approaches at present. Although a

Fig. 2 PCR identification of hairy root culture. Lane 1 is DNA ladder. Lanes 2, 3, and 4 represent the *rol B* gene amplified from *P. tangutica* genome DNA of HR. Lane 5 *rol B* gene amplified from the pRiA4b-

KanR plasmid from *A. rhizogenes* ATCC10060 (positive control). Lane 6 PCR amplified with DNA from sterilized seedling of *P. tangutica* (negative control)

Fig. 4 Content of four tropane alkaloids in hairy root and seedlings of *P. tangutica*

Table 2 Compared content of four tropane alkaloids in hairy roots with roots of seedling of *P. tangutica*

Tissue	Content of tropane alkaloids (mg/100 g DW)			
	Anisodamine	Anisodine	Atropine	Scopolamine
Root of seedlings	2.63 ± 0.01 ^b	0.29 ± 0.02 ^b	8.03 ± 0.01 ^b	3.18 ± 0.02 ^b
Hairy root	12.85 ± 0.05 ^{a**}	4.09 ± 0.02 ^{a**}	229.88 ± 0.14 ^{a**}	10.69 ± 0.05 ^{a**}

Mean comparisons using independent samples *t* test at the 5% level were considered, the different little letters represented the significant difference between groups ($p < 0.05$). * means that the difference was significant at the 0.05 level and ** means that the difference was significant at the 0.01 level

rapid propagation system (Xu et al. 2009) and a sterilizing seedling culture method (Lei et al. 2015) would help in the ex situ conservation of this rare species, its growth remains slowly and limiting the supply of valuable medicinal plants. In this study, we set up a hairy root system for this species for the first time. The three agrobacterium strains were tested; and A4 and MSU440 were proven to be the ideal strains for hairy roots generation on this species, wherein the former is better than the latter on HR inductivity, length, and number, while the LBA9402 did not produce hairy roots

(Table 1a, b). However, in *H. nigor*, A4 and LBA9402 both induced the explants generated hairy root (Lu et al. 2005). This finding suggested that agrobacterium strains have a distinct sensitivity to different species. Previously, it documented that the agrobacterium strain had a different virulence that would affect the transformation efficiency (Verma et al. 2007; Chandran et al. 2011; Skala et al. 2015). When the agrobacterium entered the wounded part, it needed some secondary metabolites or hormone to help the Ri plasmid integrate into the genome of the plants. Different species

might vary in secondary metabolites or content, such as polyphenic (Skala et al. 2015). *P. tangutica* also produces some polyphonic compounds on the wounded part, and thus, it may lead to differences in the hairy root generation frequency among the three agrobacterium strains.

Explants were an important factor that affecting the transformation efficiency. In the present study, the data also demonstrated that the hypocotyl was the better tissue for the generation of hairy roots on *P. tangutica*. Although the cotyledon could also be induced to generate the HR, the frequency was very low, only 13.33% or 0 (Table 1a). Similarly, the HR number and length were significantly lower than those of hypocotyl, respectively (Table 1b). Different parts were reported to have different ability (Zhang et al. 2010). The results showed that the hypocotyl was superior to the cotyledon in HR induction, which might be related to the differences in the production of polyphonic compounds or hormones. The detail reason should be studied further.

Inducing hairy roots on the hypocotyl showed white slim and branched roots in the 1/2MS agar medium or solution (Fig. 1). They propagated vigorously under the 1/2MS medium without any hormones. The PCR results showed that the *rol B* gene was integrated into the genome of *P. tangutica* (Georgiev et al. 2015). This ability maintained the genetic stability after seven generations of subculture.

According to growing state (branching, growth rate, etc.), several HR lines were selected and cultured in the 1/2MS liquid medium. The observation results showed that the HR grew stably and branched well in the flask. The growth curve was similar to that of *V. eriophorum* (Marchev et al. 2016). The dry biomass reached 4.63 g l⁻¹ and the GIDW values were 84.2. These values were higher than those of *V. eriophorum* (6.5–8.2) (Marchev et al. 2016). Therefore, HR liquid culture of *P. tangutica* has a potential application in the future.

As a useful reactor, the capability of producing active components in the hairy root is a key parameter. HPLC analysis data demonstrated that the HR has a high content of tropane alkaloids than the part of sterilized seedling grown for more than 2 months (Fig. 4). In a previous study, the tropane alkaloids were shown to have a high content in the wild species (Hsiao et al. 1973; Xiao and He 1982; Wang et al. 2002a). However, the tropane alkaloids content of HR is lower than that of wild species. This result corroborates the conclusion of *Gentiana macrophylla* (Zhang et al. 2010). *P. tangutica* grew at high altitudes of above 3000 m, with frequent exposure to cold, high UV radiation, high wind, or other stress factors, which could affect its growth and metabolites content (Wei et al. 2000; Shi SB 2016). In addition, the wild species which they studied grew several years (Hsiao et al. 1973; Xiao and He 1982; Wang et al. 2002a), while the HR in this study was cultured for only 1 month. Thus, this may account for the difference between the HR

and wild species of *P. tangutica*. This phenomenon was also observed in *G. macrophylla* (Zhang et al. 2010). However, HR has short growth periods and produces much biomass (Fig. 3), independent of the environment and easily applied in the factory. Moreover, the TA content of HR is higher than that of sterilized seedlings (Fig. 4). Therefore, optimizing the culture condition and developing genetics reactor of *P. tangutica* would yield higher tropane alkaloids than that of wild species.

In conclusion, this is the first study comparing hairy root lines obtained from the rare Tibetan herb *P. tangutica*. The generated hairy roots contained higher TAs than those of control roots of seedlings. In the 1/2MS liquid culture medium, HR could produce a high dry mass and a high content of scopolamine and other TAs and proved to be of potential application value. Explants and bacterial strains are important factors to consider. A4 and MSU440 are better bacterial strains, and the hypocotyl is demonstrated as the better explant for the generation of hairy roots. Thus, pretreating agrobacterium with acetosyringone (500 mg/L) under 300 μmol/m² and co-cultivation on MS media led to a high level of root generation efficiency from the hypocotyl in *P. tangutica*.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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