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Research Article
Agrobacterium rhizogenes Mediated Hairy Root Induction in *Parasponia andersonii* Planch

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Abstract

Background and Objective: A comparative analysis of a unique Parasponia-rhizobium symbiotic system can help to define strategies for transferring nitrogen-fixing ability to non-legume crops. Hairy-root cultures and transgenic plants of *Parasponia andersonii* (*P. andersonii*) containing various target genes regenerated from them may become one of the model systems for the investigation of Parasponia-rhizobium symbiosis. Therefore, the purpose of this work was to generate Parasponia hairy roots carrying lectin pea *psl* gene and regenerate transgenic shoots from these roots. **Materials and Methods:** Stem segments of *P. andersonii* were transformed using *Agrobacterium rhizogenes* (*A. rhizogenes*) strain A4 containing the binary vector pCambia 1301, which is carrying the *GUS* (β-glucuronidase) reporter gene with catalase intron, a selective *hpt* gene (hygromycin) and pea lectin *psl* gene. Agrobacterium-mediated transformation was carried out using silicon carbide whiskers. Hairy roots cultures co-transformed with *rol*-genes and T-DNA of pCambia 1301 were selected using histochemical GUS and PCR analyze these roots. Student's t-test using the GraphPad Software(2.03) was also used to analyze the data. **Results:** *Parasponia andersonii* hairy roots showed vigorous growth and abundant lateral branching in Lloyd and McCown Woody Plant Medium (WPM) without phytohormones. Spontaneous shoot regeneration in cultures of co-transformed hairy roots growing on hormone-free medium was observed with an extremely low frequency, under both light and dark conditions. The frequency of shoot regeneration increased with the addition of thidiazuron in the medium. **Conclusion:** Silicon carbide whiskers increase the effectiveness of Agrobacterium-mediated transformation of Parasponia. The *P. andersonii* hairy roots expressing the pea *psl* gene can be used as a model system for studying the molecular bases of Parasponia-rhizobium symbiosis.

Key words: *Agrobacterium rhizogenes*, hairy roots, *Parasponia andersonii*, Parasponia-rhizobium symbiosis, *Pisum sativum* lectin, psl

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Tropical tree of *Parasponia andersonii* Planch. from *Cannabaceae* family is a unique plant which is the only known as non-legume woody plant that forms an effective nitrogen-fixing symbiosis with *Bradyrhizobium* and *Rhizobium*[1-3]. Other non-legume symbiosis are formed by actinomycete *Frankia*[4]. *P. andersonii* grows in mountainous regions along streams in exposed riverbeds between stones and gravel. The distribution of this species includes the Malay Archipelago and some Pacific Islands. Along with actinorhizal and legume–rhizobium symbiosis, *P. andersonii* is a key species for research of the accommodation of symbiotic nitrogen-fixing bacteria in plant cells[5]. Moreover, in addition to its high agronomic potential, nitrogen-fixing symbiosis offers scientists the unique opportunity of studying differences at the molecular level of both partners[3]. It is also known that high efficiency of nitrogen fixation in rhizobium-legume symbiosis is a reason of the long-desired goal of transferring this property to the non-legume crops[6]. A comparative analysis of three symbiotic systems: actinorhizal, legume-rhizobium and Parasponia-rhizobium symbioses can help define strategies for transferring nitrogen-fixing ability to non-legume crops[5].

One of the model systems for the investigation of Parasponia-rhizobium symbiosis can become hairy root cultures induced by *Agrobacterium rhizogenes* and transgenic plants regenerated from these roots. *Agrobacterium rhizogenes*-mediated genetic transformation methods have been developed for many plant species. Hairy-root cultures were induced on plants susceptible to infection with *A. rhizogenes*. This bacterium transferred the *rol*-genes from its Ri-plasmid into the genome of the host plant[7]. The expression of those *rolA*, *B*, *C*, *D* and other genes induces neoplastic root growth and the production of opines, which are sources of nitrogen and carbon for *A. rhizogenes*[8]. *Agrobacterium rhizogenes*-induced hairy-root cultures may be used to study the interactions between roots and nitrogen-fixing microorganisms, mycorrhizal fungi and nematodes[9]. However, *P. andersonii* hairy root culture induction methods have not been developed. Only Cao *et al.*[10] generated composite plants of *P. andersonii* by *A. rhizogenes*-mediated transformation. Their roots contained the red fluorescent protein DsRed1 reporter gene. However, the greatest interest is the transfer of target genes whose protein products are involved in the establishment of symbiosis between rhizobia and plant cells. For example, lectin *psl* gene of *Pisum sativum* may be used as a target gene. For this reason this lectin has been used in a number of works on changing the specificity of Rhizobium-legume symbiosis[11,12] and significant enhancing rhizobia colonization on rice, tobacco, tomato and rape[13,14].

In this study, a method for *A. rhizogenes*-mediated transformation of *P. andersonii* was developed. The possibility of induction Parasponia hairy root cultures containing the pea lectin *psl* gene was confirmed. The opportunity of shoot regeneration from Parasponia hairy root cultures was also demonstrated in this study.

MATERIALS AND METHODS

Plant material: This study was carried out in 2016. Seeds of *P. andersonii* were collected in Tahiti (Society Archipelago, French Polynesia) by research scientist Dr. Jean-Yves Meyer and kindly provided by Dr. Priscille Frogier. The aseptic *P. andersonii* plants were cultured *in vitro* on the WPM proliferation medium[15], supplemented with 0.5 mg kinetin L^{-1}. *In vitro* culture incubated in KBW 240 climatic chamber ("Binder". Tuttlingen, Germany) under 16 h light and 8 h darkness photoperiod at 28°C and under a photon flux density of 100 μM m^{-2} sec^{-1}. Plants were subcultured after every 2 months.

Bacterial strains: For agrobacterium-mediated transformation, used rifampicin resistant mutant of *A. rhizogenes* strain A4, harboring binary vector pCambia 1301/*psl*, containing reporter *GUS* gene with catalase intron fused to the 35S promoter. This vector also contained selective marker *hpt* gene, conferring hygromycin resistance, as well as the target lectin *psl* gene from *P. sativum* under control of the 35S promoter[14] (GenBank: X66368.1). The bacterial strain was cultured on Lysogeny broth (LB) medium[16]. The agrobacterium culture for transformation was grown in LB liquid medium supplemented with 100 mg rifampicin L^{-1} ("Sigma-Aldrich", Merck KGaA, Darmstadt, Germany) and 50 mg kanamycin L^{-1} ("Sigma-Aldrich", Merck KGaA, Darmstadt, Germany) on 120 rpm, 28°C, 24 h. Before inoculation of plant tissues, bacterial culture was centrifuged at 3500 rpm for 15 min, resuspended in liquid MS medium[17] supplemented with 100 μM acetosyringone ("Sigma-Aldrich", Merck KGaA, Darmstadt, Germany). Then agrobacteria were grown for 1 h at room temperature.

***Agrobacterium rhizogenes*-mediated transformation:** Stem sections of *in vitro P. andersonii* plant explants about 10-15 mm in length containing only one axillary bud without the leaf were used for transformation. These explants were dipped in a culture of *A. rhizogenes* for 1 h (OD = 0.6, 600 nm), blotted dry on sterile filter paper and incubated on agar-solidified (0.8% agar) EKM medium[2] for 3 days at a temperature 22°C in dark.

In some experiments, in order to increase the transformation efficiency during agrobacterium inoculation, silicon carbide (SiC) whiskers (Alfa Aesar, Thermo Fisher Scientific, Lancashire, United Kingdom) were used. Nodal explants were placed in a 50 mL Falcon centrifuge tube, containing 10 mL of liquid MS medium, 1 mL of agrobacterium culture (OD = 0.6, 600 nm), 2% SCW and shaken for 3 min on a vortex. Then explants transferred in Petri dishes with 10 mL liquid MS medium with the addition of 1 mL agrobacterium suspension and inoculated for 1 h. Subsequently, explants blotted dry on sterile filter paper and incubated on agar-solidified EKM medium for 3 days at 22°C in dark. As a control, MS liquid medium without bacteria was used to treat the explants.

After co-cultivation, the explants were transferred to the hormone-free WPM medium: Macro- and microsalts WPM, Gamborg's B5 vitamin, 2% sucrose, pH 6.0, supplemented with 300 mg cefotaxime L^{-1} ("Sigma-Aldrich", Merck KGaA, Darmstadt, Germany) and 300 mg carbenicillin L^{-1} ("Sigma-Aldrich", Merck KGaA, Darmstadt, Germany). Then the explants were cultured for 7 days at 22°C in dark. Further cultivation was carried out in KBW 240 climatic chamber ("Binder", Germany), equipped with fluorescent grow lamps Fluora ("Osram", Germany), at 28°C under 50 μM m^{-2} sec^{-1} photon flux density and photoperiod of 16/8 h (day/night). Explants were subcultured at 14 days intervals to eliminate the bacteria.

Hygromycin resistance test of GUS$^-$ hairy roots: GUS$^-$ hairy roots (without GUS expression) were cultured on selection WPM medium supplemented with different concentrations of hygromycin in the range of 0-15 mg L^{-1}. The percentage of roots survived after 20 days culture was determined.

GUS expression analysis: GUS activity of hairy roots was determined by removing laterals roots and their incubating in 5-Bromo-4-chloro-3-indolyl-β-D-glucuronide cyclohexyl ammonium salt (X-Gluc, "Fermentas", Waltham, USA) staining solution (15 mg X-Gluc dissolved in 20% (v/v) methanol supplemented with 0.1% (v/v) Triton X-100, 0.15% (w/v) potassium ferrocyanide, 8 mM EDTA and 80 mM Na_2HPO_4, pH 7.0) for 0.5-10 h at 37°C. After the incubation, dissected roots were soaked in 50% glycerol-water solution and then microscoped.

For *in situ* staining, X-Gluc-containing solution was added directly to the medium in the Petri dishes containing the explants with adventitious roots and the dishes were incubated overnight in the dark at 37°C.

PCR-analysis: Extraction of hairy roots total DNA was carried out using the cetyltrimethylammonium bromide (CTAB) method[18]. The polymerase chain reaction (Tercyk, "Dna-technology", Moscow, Russia) analysis was performed to verify the presence of *rol*-genes. 5'-TTCAGATTTACTATAGCAGGC-3' and 5'-GCAAGTACCTTGTTCATTCA-3' primers were used to identify *rolB* gene. The size of the amplicon was 266 bp, with optimal annealing temperature 54°C. 5'-GATGATGC GATGCTTTTATG-3' and 5'-CAGAGACTTTCCCTTTGTTGA-3' primers were used to identify *rolC* gene. The size of the amplicon was 250 bp, with optimal annealing temperature 55°C. For the PCR analysis of the pea lectin *psl* gene 5'-ATAATGGCTTCTCTTCAAACCC-3' and 5'-GCAAAAAAACTATG CATCTGCA-3' primers were used, the size of the amplicon was 840 bp, with optimal annealing temperature 53°C. To avoid the risk of agrobacterium contamination of hairy roots, we performed PCR analysis for the chromosomal gene of *A. rhizogenes* (WP_034523040) using primers 5'-CCCGCAC CCGATCCAAGACAAACTCA-3' and 5'-CGCCCGAAGCCTCACC CACGAAC-3'. The size of the amplicon was 476 bp, with optimal annealing temperature 62°C. The electrophoresis of the PCR products was performed on 1.0% agarose gel under a constant voltage of 80 V. The gel was subsequently stained with ethidium bromide solution and examined under UV light.

Plant regeneration from hairy roots: The segments of hairy roots (about 3-5 cm length) were placed on 1% agar-solidified MS medium containing 10 g sucrose L^{-1} and various concentrations of 6-Benzylaminopurine (BA) or thidiazuron (TDZ). The cultures were incubated at 28°C in KBW 240 climatic chamber under the conditions indicated above.

Statistical analysis: Student's t-tests were performed to determine the statistical difference between two treatments ($p = 0.05$) using the GraphPad Software InStat version 2.03 statistical package (GraphPad Software, San Diego, CA)[19].

RESULTS

Adventitious roots were observed at the cut surface of the stem segments after 25 days of inoculation with *A. rhizogenes* (Fig. 1a). Adventitious roots were formed mostly on the basal cut surface of the explants. The frequency of adventitious root formation on explants after 40 days of inoculation was 18.0±1.9% without use silicon carbide whiskers and 14.5±2.7% with SCW. There was no significant difference in the percentage of explants showing root formation and a number of roots/explant between the control (without agrobacterium, 15.8±2.2%) and explants inoculated with *A. rhizogenes*. The studies were carried out as two independent experiments with 4 replicates/treatment. Each replicate contains 10 explants (total 80 explants).

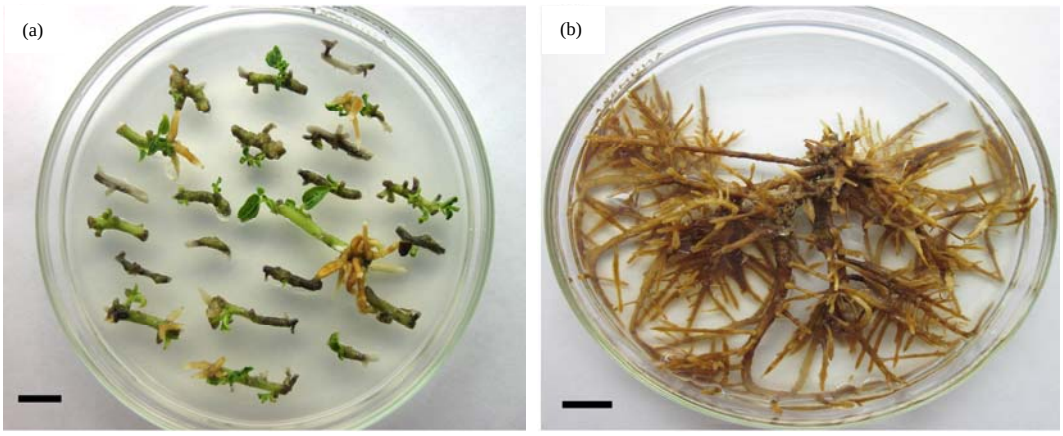

Fig. 1(a-b): Generation of *P. andersonii* hairy roots, (a) Adventitious roots formation from cut surface of stem segments inoculated with *A. rhizogenes* A4+pCambia 1301/*psl* (25 days after inoculation) and (b) Vigorous growth of *P. andersonii* hairy roots on WPM medium without phytohormones. Scale bar, 1 cm

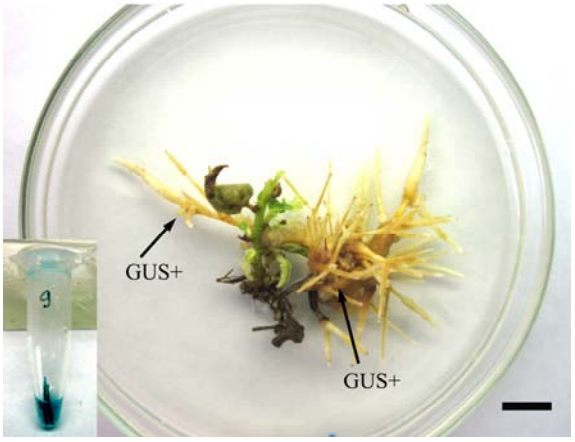

Fig. 2: Histochemical *GUS* analysis of *P. andersonii* hairy roots. Scale bar, 1 cm

It was difficult to distinguish the transformed roots from the non-transformed roots at the initial stage of root formation. Further, some of the roots from inoculated explants eventually showed the typical hairy root phenotype such as high branching on a medium without phytohormones, rapid growth and plagiotropism (Fig. 1b).

To facilitate identification of double-transformed hairy roots (with *rol*-genes and T-DNA of pCambia 1301 together) and thus to evaluate the efficiency of co-transformation, *GUS* analysis of lateral sections of well growing roots (after 6-7 weeks of *A. rhizogenes* infection) was used. For this, approximately 7-8 mm root tips were excised aseptically to encourage root growth and branching. GUS staining of the lateral root sections is a simple histochemical marker to identify jointly co-transformed roots (Fig. 2).

Table 1: Effect of inoculation with *A. rhizogenes* A4 strain with and without using SCW on generation of hairy roots clones

Variants	GUS+clones (%)	GUS^+/Hyg^+ clones (%)
Control (without agrobacterium)	0.0±0	0.0±0
Inoculation	26.7±2.8	87.5±9.5
Inoculation+SCW	43.3±4.1*	91.7±8.3

Two independent experiments with 4 replicates/treatment. Each replicate contains 10 explants (total 80 explants); data (Mean±SE). The symbol *indicates statistical significant difference at p = 0.05

There is little or no detectable β-glucuronidase activity in almost any higher plants. In the roots of *in vitro* growing *P. andersonii* plants, endogenous β-glucuronidase activity has never been detected. GUS analysis of the isolated hairy roots showed that the effectiveness of the co-transformation and the formation of GUS^+ hairy roots significantly increased (p = 0.05) when the explants were inoculated together with SCW (Table 1). This is probably due to the fact that silicon carbide whiskers wounded the nodal explants and improved the penetration of agrobacterium cells into plant tissues. It seems that SCW has caused wounds which resulted in good infection.

After that, all the selected GUS^+ hairy roots clones were tested for hygromycin resistance. Hygromycin is a highly toxic antibiotic for plant tissue. To determine the minimum concentration of hygromycin in the medium required to kill the untransformed roots, hairy roots clones with negative results of the histochemical analysis (GUS^-) were placed on WPM medium containing a range of hygromycin concentration to examine their resistance to the antibiotic. All actively growing GUS^- hairy roots survived at the medium without hygromycin. When the medium contains 1-7 mg L^{-1} hygromycin concentration the survival frequency significantly

decreased (p = 0.05). At 10 or 15 mg L^{-1} hygromycin concentration hairy roots did not survive (Fig. 3).

On this basis, the GUS^+ hairy roots were successfully subcultured at agar-solidified WPM medium supplemented with 8 mg L^{-1} hygromycin. Most isolated GUS^+ hairy roots clones (about 90%) were resistant to hygromycin (Table 1).

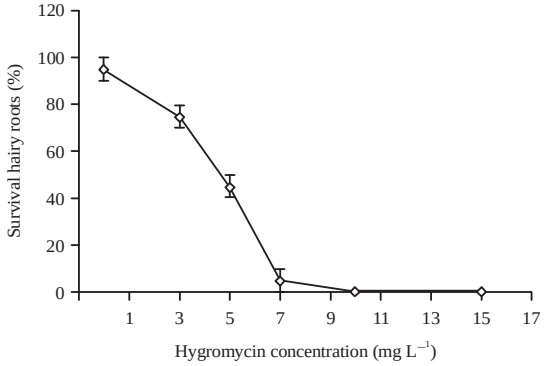

Fig. 3: Effect of hygromycin concentration on GUS^- (minus) hairy roots, bars indicate standard error (Mean±SE)

Then, we conducted PCR analysis of 5 clones of *P. andersonii* GUS^+/Hyg^+ hairy roots to detect *rolB* and *rolC* genes. All five root samples were positive for both *rol*-genes (Fig. 4a, b), which is another evidence of transformation success. No bacterial contamination was indicated, all the samples were negative for chromosomal *A. rhizogenes* gene WP_034523040. PCR analysis of five samples of co-transformed hairy roots, detecting expression of the reporter *GUS* gene and actively growing on a selective medium with the addition of 8 mg L^{-1} hygromycin, showed the presence of the pea lectin *psl* gene (Fig. 4c).

Transformed *P. andersonii* hairy roots showed spontaneous regeneration of adventitious shoots in media without the addition of plant grows regulators (cytokinins), although extremely low frequency (0-2 shoot clusters at Petri dish with hairy roots culture). Shoot regeneration was observed both when culturing of hairy roots in the light and in the dark (Fig. 5a, b). Thus, spontaneous regeneration of *P. andersonii* shoots from hairy roots does not depend on light. The frequency of adventitious shoots formation not

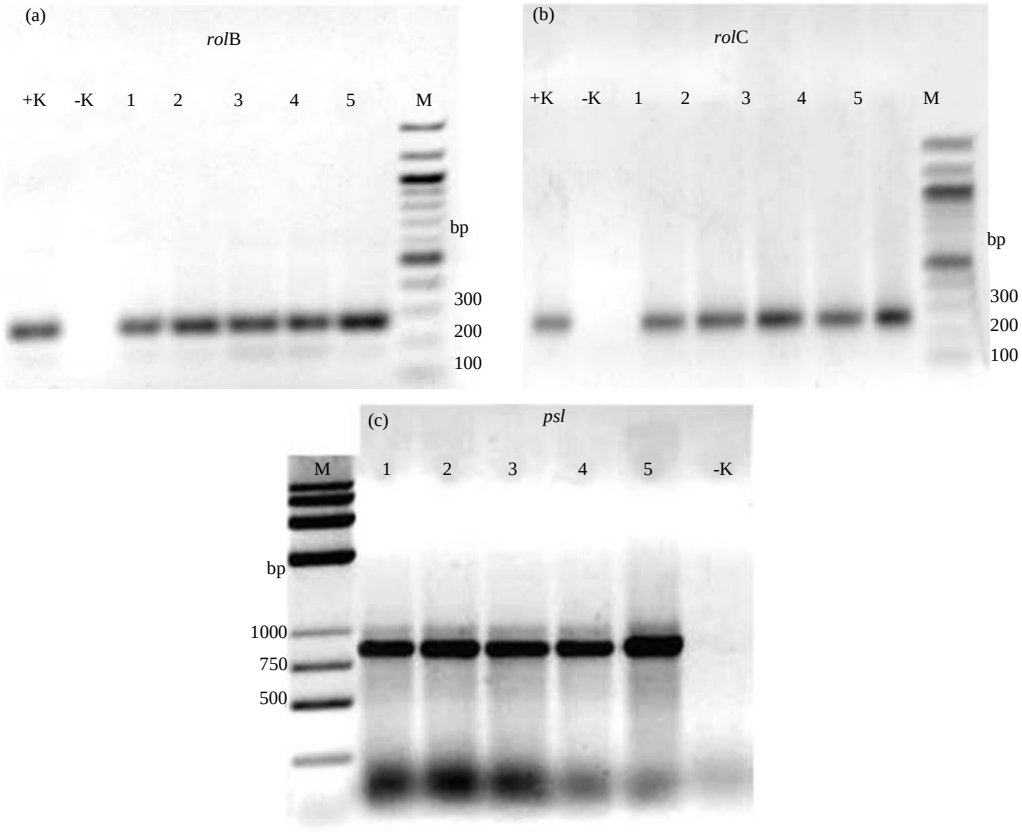

Fig. 4(a-c): PCR analysis of GUS^+/Hyg^+ hairy roots, (a) Detection of *rolB* gene in *P. andersonii* GUS^+/Hyg^+ hairy roots, (b) Detection of *rolC* gene in *P. andersonii* GUS^+/Hyg^+ hairy roots and (c) Detection of the pea lectin gene *psl* in *P. andersonii* GUS^+/Hyg^+ hairy roots

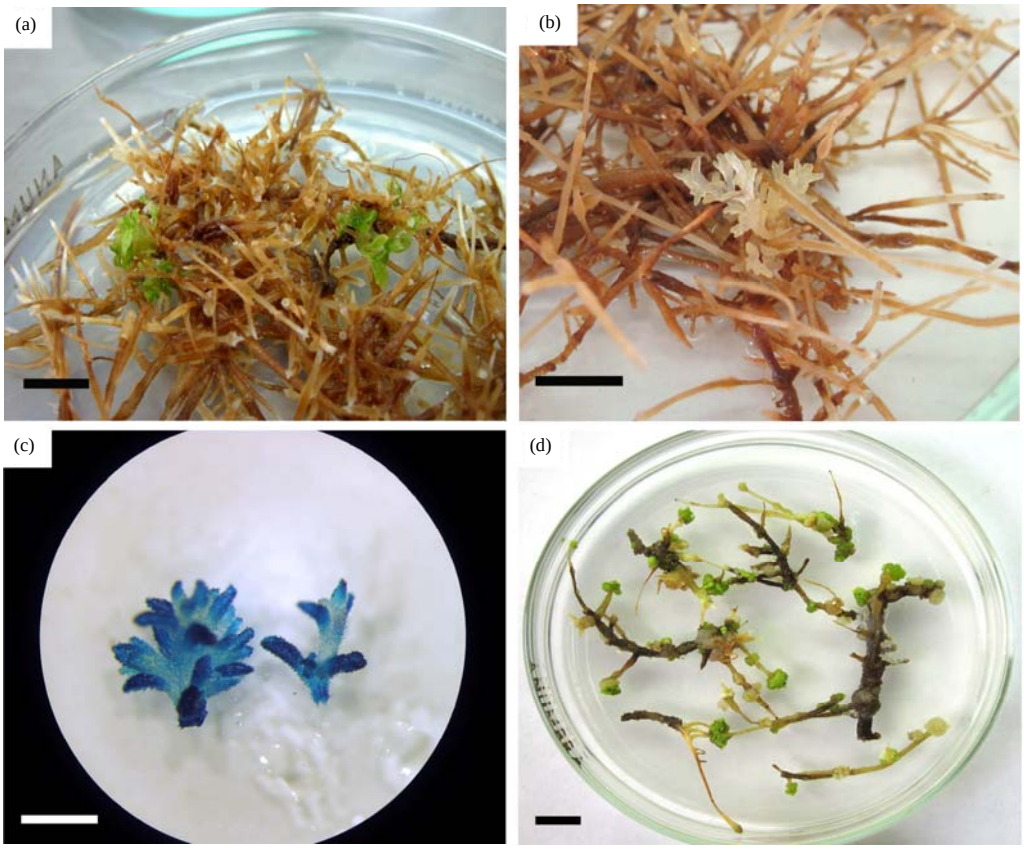

Fig. 5(a-d): Spontaneous induction of the shoots on the isolated hairy roots culture of *P. andersonii* on hormone-free MS medium, supplemented with 8 mg L^{-1} hygromycin. Spontaneous induction of the shoots on the isolated hairy roots culture of *P. andersonii* (a) In light; (b) In dark. (c) Histochemical *GUS* analysis of shoots regenerated from hairy roots in dark and (d) Green calli formed from hairy roots of *P. andersonii* cultured on WPM medium containing 0.1 mg L^{-1} TDZ. Scale bar, 1 cm

Table 2: Effects of concentrations of BA and TDZ on callus induction percentages from GUS^+/Hyg^+ hairy roots of *P. andersonii* after 4 weeks

Variations	Response (%)*	No. of calluses/root*
1.0 mg L^{-1} BA	-	-
0.5 mg L^{-1} BA	-	-
0.2 mg L^{-1} BA	-	-
0.5 mg L^{-1} TDZ	68.8±12.0	1.38±0.27
0.2 mg L^{-1} TDZ	76.3±10.3	1.64±0.24
0.1 mg L^{-1} TDZ	69.2±3.6	3.50±0.60
0.05 mg L^{-1} TDZ	63.8±3.8	2.43±0.42

*Two independent experiments with 4 replicates/treatment. Each replicate contains 5 explants (total 40 explants); data (Means±SE)

not increased by the application of 0.20-1.0 mg L^{-1} 6-Benzylaminopurine (BA) (Table 2). Only individual green calli began to form from the hairy roots with an extremely low frequency even after 6 weeks of cultivation on the medium containing 0.5 mg L^{-1} BA. But a lot of dark green calli nodules with shoots primordia began to form with the addition of TDZ into the medium after 4 weeks of cultivation. Often the green calli began to form from the hairy roots tips, which showed a specific swelling (Fig. 5c) and then shoots primordia appeared. The histochemical analysis of regenerated shoots on both hormone-free medium and medium supplemented with TDZ showed that they contained *GUS* gene, thus they were transgenic (Fig. 5d). Unfortunately all obtained shoots mostly failed to grow normally due to severe hyperhydricity and then eventually died. Reducing the sucrose concentration in the medium to 0.5-1% and increasing the agar concentration to 1% did not result in an observable reduction of hyperhydricity. Solving the problem of hyperhydricity requires further research.

DISCUSSION

The effective protocol for genetic transformation of *P. andersonii* using *A. rhizogenes* and for production of a large number of hairy roots clones, containing various target genes, was developed. Although a large number of plant

species have been tested for *A. rhizogenes* infection and hairy roots generation, there is only one report of successful transformation and induction of hairy roots *in vitro* in *P. andersonii.* The authors generated composite plants and only reporter gene encoding red fluorescent protein was transferred into roots[10].

In this study, the efficiency of *A. rhizogenes*-mediated co-transformation with different transgenes was improved by using silicon carbide whiskers. The transformed roots generated in this study and carried the gene of hygromycin resistance, pea lectin *psl* gene and reporter *GUS* gene showed typical hairy root phenotype. Previous study has shown that the presence of the selective agent in a medium may adversely affect the regeneration of new tissues[20]. For example the inhibition of transformed cells growth due to the death of non-transformed cells surrounding transformed ones. Therefore, hygromycin was not used as an early selection means.

A. rhizogenes-mediated transformation is widely used for transgenic shoot regeneration from hairy root cultures. Although shoot regeneration was achieved in hairy roots cultures of different plant species[21], morphogenesis is associated with some problems, since this process strongly depends on the plant genotype[22]. Highly species-specific methods are required to overcome the difficulty of inducing of *in vitro* shoot regeneration[23]. Regeneration of transgenic plants from hairy roots can be either spontaneous or can be induced with plant growth regulators. For shoot formation sometimes requires high cytokinin levels. Although shoots, obtained in this work, were spontaneously induced on a hormone-free medium, or they could be induced from calli by applying low concentrations of TDZ, they could not normally grow due to hyperhydrification. This phenomenon was observed for various species[24-25]. The researchers recommended some different procedures to overcome this problem, such as using high agar concentration, low humidity and optimal gas exchange in the medium[26]. In the present study, reducing the sucrose concentration and increasing the agar concentration did not solve this problem. Therefore the regenerated shoots did not show active growth and then died. Overcoming of this problem requires further investigation.

Generation of non-leguminous plants, capable of forming symbiosis with nitrogen-fixing rhizobia is an urgent problem of plant genetic engineering[14]. Due to the ability to form symbiosis with some rhizobia, Parasponia is a unique plant, though it does not belong to the *Fabaceae* family[3]. Therefore, Parasponia is considered as an important model object in the study of the molecular mechanisms of nitrogen-fixing plant-rhizobium symbiosis. The hairy roots of Parasponia containing the target pea lectin *psl* gene were generated. This lectin is involved in the interaction of the pea roots with rhizobia and is an important in the first step of the development of legume-rhizobium symbiosis[11, 12]. Obtained Parasponia hairy roots will be used in the further studies dedicated to atypical symbiotic reactions with rhizobium strains specific for pea.

CONCLUSION

An efficient protocol of generating hairy roots of unique endemic for Oceania plant species *P. andersonii* using *A. rhizogenes* and silicon carbide whiskers was developed. Using this protocol, the hairy roots of *P. andersonii* carrying the pea lectin *psl* gene was first generated. These hairy roots of Parasponia can be used as a model system for studying the molecular bases of plant-bacteria symbiosis. Moreover for the first time this study demonstrated the possibility of regeneration of Parasponia transgenic shoot from hairy roots. If the hyperhydration problem of transgenic *in vitro* shoot is solved, it will be possible to obtain transgenic Parasponia plants with target genes.

SIGNIFICANCE STATEMENT

This study discovers an increase in the efficiency of *A. rhizogenes*-mediated transformation of *P. andersonii*, when silicon carbide fibers were used. Silicon carbide fibers probably increase the number of wounds and improve the penetration of *A. rhizogenes* into plant tissue. The study will help the researchers to generate a large number of hairy root clones containing the target genes and subsequently to regenerate transgenic shoot of Parasponia. Thus, the new way of transforming this unique tropical plant of *P. andersonii* was developed in this study.

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