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Regeneration and Transformation of a Roadside Tree *Pittosporum tobira* A.

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Abstract

The regeneration of multiple shoots from hypocotyl sections of *Pittosporum tobira* was studied on woody plant medium supplemented with differing concentrations of thidiazuron and naphthaleneacetic acid. Maximally 75% of hypocotyl sections formed multiple shoots when using the medium containing 10 μM thidiazuron and 3.2 μM naphthaleneacetic acid. Multiple shoots were transferred to a shoot-elongation medium followed by transfer to rooting medium. Plantlets formed were acclimatized, and complete plants were regenerated about 10 months after the initiation of the culture. A plasmid vector pANiR, which bears the cDNA of the nitrite reductase (NiR) gene from *Arabidopsis thaliana* under the control of cauliflower mosaic virus 35S promoter and nopaline synthase terminator, was introduced - together with pCH-bearing hygromycin resistance gene - into hypocotyl sections of *Pittosporum tobira* using a particle gun. A number of hygromycin-resistant calli were obtained, from which shoots were regenerated. The integration of Arabidopsis NiR cDNA into the genome of *Pittosporum tobira* was detected by the polymerase chain reaction method, confirming that these shoots were transgenic.

Keywords: nitrite reductase, particle bombardment, phytoremediation, *Pittosporum tobira* A., regeneration, tissue culture, transgenic, woody plant.

Abbreviations

GFP, Green fluorescent protein; *hph*, Hygromycin phosphotransferase gene; NAA, α-Naphthaleneacetic acid; *nii*, Nitrite reductase gene; NiR, Nitrite reductase; SEM, Shoot-elongation medium; SIM, Shoot-inducing medium; TDZ, Thidiazuron; WPM, Woody plant medium (Lloyd and McCown, 1980).

Introduction

Significant attention is now being directed at phytoremediation, an emerging technology that uses plants to remove pollutants from the environment. Phytoremediation could provide an affordable way to restore the economical value of contaminated sites. Woody plants are indispensable to phytoremediation. Although the regeneration of woody plants has been widely reported (walnut: Tulecke and Mcgranahan, 1985; locust: Han *et al.*, 1993; white spruce: Ellis *et al.*, 1993; grape: Perl *et al.*, 1996; eucalyptus: Mullins *et al.*, 1997; white birch: Mohri *et al.*, 1997; lime: Pena *et al.*, 1997; acacia: Xie and Hong, 2001), there are few reports on this being achieved in roadside trees.

Pittosporum tobira is an evergreen shrub that is widely used as a hedge, garden tree, and roadside tree. It is typically 2-3 m high, and is known to be salt tolerant (Hagiladi *et al.*, 1989). *Pittosporum tobira* was ranked 137th in its capability of assimilating nitrogen dioxide (NO_2) among 217 taxa of naturally occurring plants, in which a variation of greater than 600-fold was observed in the ability to assimilate NO_2 (Morikawa *et al.*, 1998). We also have reported that transgenic plants that overexpress the cDNA of the nitrite reductase gene (*nii*) had an increased capability to assimilate NO_2 (Takahashi *et al.*, 2001).

In the present study we first established the optimal conditions for regeneration of plants from explants of *Pittosporum tobira* cultured *in vitro*, and then produced transgenic plants of this shrub whose shoots bore the foreign nitrite reductase (NiR) cDNA.

Materials and Methods

Formation of multiple shoots and plant regeneration

Seeds of *Pittosporum tobira* were first washed with detergent using a toothbrush, and then sterilized by immersion for 1 h each in 70% ethanol and 2.5% (w/v) sodium hypochlorite solution, after which they were rinsed five times with sterile distilled water, and then kept in sterile water for 24 h. The seeds were cut in half and opened to expose embryos, which were removed using forceps and aseptically placed onto woody plant medium (WPM) solidified with 0.3% Gellan Gum (Wako Pure Chemical Industries, Osaka, Japan) and supplemented with 1% sucrose (pH 5.8). They were then kept for germination at 25 ℃ and a light/dark cycle of 15/9 h under fluorescent light (30-40 μmol m^{-2} s^{-1}) in a growth chamber (model TCR-5P; Nippon Medical and Chemical Instruments, Osaka, Japan). Seedlings (2-3 cm long) were formed after about 8 weeks.

Hypocotyls of the seedlings were cut into 1-mm sections, and placed onto a shoot-inducing medium (SIM) consisting of WPM solidified with 0.3% Gellan Gum and supplemented with differing concentrations of thidiazuron (TDZ; Wako Pure Chemical Industries) and naphthalene acetic acid (NAA; Wako Pure Chemical Industries). About 3 months later the multiple shoots that had formed were transferred to a shoot-elongation medium (SEM), which consisted of WPM solidified with 0.3% Gellan Gum and supplemented with differing concentrations TDZ and NAA, to allow elongation of the shoots. About 4 months after the culture, shoots approximately 2-3 cm long were cut, immersed into 4.9 μM indole-3-butyric acid (Wako Pure Chemical Industries) at the cut end, and placed onto plastic containers containing Florialite (Nisshinbo Industries, Tokyo, Japan) moistened with WPM to allow rooting. Six weeks later the plantlets that had formed were removed from the container for acclimatization and transferred into pots containing vermiculite and perlite (1:1, v/v). They were grown at 22 ℃ under natural light and 70% humidity in a growth chamber (model BTH-P1-5. 7S-TH; Nippon Medical and Chemical Instruments).

Transient expression of green fluorescent protein

Hypocotyls from eight-week-old seedlings cultured *in vitro* were cut into 1-mm sections and transferred to SIM. One to fifteen days after transfer to SIM, approximately 40 hypocotyl sections were placed on filter paper (No. 2, 5.5-cm diameter; Advantec Toyo Roshi Kaisha, Tokyo, Japan) so as to form a disk (35 mm diameter), and bombarded with pTH-2 (a gift from Dr. Y. Niwa, Shizuoka Prefectural University, Japan) using a particle gun. pTH-2 contains green fluorescent protein (GFP) from jellyfish (*Aequorea victoria*) gene under the control of the CaMV 35S promoter and NOS terminator. pTH-2 was shot five times at each sample; other bombardment conditions were essentially the same as described by Takahashi and Morikawa (1996). After bombardment, the filter paper with hypocotyl sections was placed onto SIM, and the hypocotyls were incubated for 24 h at 25 ℃. The hypocotyl sections were observed under a binocular fluorescence microscope (model MZ FL III; Leica, Germany) using an excitation filter (470/40 nm) and a barrier filter (525/50 nm) to count the number of regions containing cells expressing GFP.

Stable transformation

Approximately 40 hypocotyl sections that were precultured for 13 days on SIM were bombarded with an equimolar mixture of pANiR and pCH using a particle gun as described in Takahashi and Morikawa (1996). Plasmid vector pANiR bears the NiR cDNA from *Arabidopsis thaliana* under the control of CaMV 35S promoter and NOS terminator. pCH bears the hygromycin resistance gene under the control of CaMV 35S promoter and NOS terminator (Goto *et al.*, 1993). The following day, hypocotyl sections were transferred to SIM supplemented with 95 μM hygromycin (Wako Pure Chemical Industries) to select hygromycin-resistant transformants. Hygromycin-resistant calli were obtained three months after bombardment. The integration of the foreign NiR gene in the calli was confirmed by detection of a band specific to Arabidopsis NiR cDNA using the polymerase chain reaction (PCR) method (see below). After another 3 months shoots had regenerated from PCR positive-calli that had been transferred to an appropriate culture medium.

DNA isolation

The total DNA was extracted from putatively transgenic calli and shoots of *Pittosporum tobira* using the procedure described in Doyle and Doyle (1990), with slight modifications. Plant tissues were frozen in liquid nitrogen and homogenized in a mixer mill (model MM-2000; Mitamura Riken Kogyo, Tokyo, Japan). After homogenization, 250 μl of CTAB buffer (3% cetyltrimethylammonium bromide, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0) was added, incubated at 60 ℃ for 30 min, and centrifuged at 6000*g* for 5 min. The super-

natant was transferred to a 1.5-ml microtube (Greiner, Germany), to which 250 μl of chloroform was added. After vortex, the mixture was centrifuged at 6000*g* for 5 min. The supernatant was transferred to a 1.5-ml microtube. After the addition of 165 μl of 2-propanol, the mixture was stirred and centrifuged at 6000*g* for 5 min. The pellet was rinsed with 70% ethanol, and centrifuged at 6000*g* for 5 min. The resulting pellet was dried and redissolved in TE buffer (10 mM Tris-HCl, 0.2 μM EDTA, pH 8.0) for PCR analysis.

PCR analysis

The PCR reaction mixture (20 μl) contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each of four deoxynucleoside triphosphates (dATP, dCTP, dGTP and dTTP), 1.25 units of Taq polymerase (Takara Shuzo, Japan), 10 ng of total DNA, and 0.5 μl each of the following primers: for the Arabidopsis *nii* gene (Tanaka *et al.*, 1994), 5'-TGCTTGTGGGAGGATTCTTTAGTC-3' (forward) and 5'-TTGGCATTCTCTTCTCTA-CCTCAG-3' (reverse), which define a 218-bp fragment specific to the gene; and for the hygromycin phosphotransferase (*hph*) gene (Gritz and Davies, 1983), 5'-GTTATGTTTATCGGCACTTT-G-3' (forward) and 5'-GTCCATCACAGTTT-GCCAG-3' (reverse), which define a 320-bp fragment specific to the gene. The reaction mixture were then heated at 94 ℃ for 5 min and subjected to 35 cycles of 1 min at 94 ℃, 2 min at 60 ℃, and 1 min at 72 ℃ using an programmable temperature control system (model PC-800; Astec, Japan). PCR products were separated by electrophoresis on a 1.8% agarose gel and visualized with ethidium bromide.

Results and Discussion

Regeneration of plants from hypocotyl explants

Successful transformation of *Pittosporum tobira* requires the establishment of an *in vitro* culture system. We found the following experimental procedures to be vital for the success of such a culture for this shrub: (1) the surface of the seeds must be washed thoroughly with detergent using a toothbrush prior to sterilization treatment to remove oily substances that cover the seed surface, and (2) the embryos must be isolated from seeds to allow germination. Germination was observed in isolated embryos within 4 weeks, but germination did not occur until after 6 months when the embryos were not isolated.

When the hypocotyl sections were cultured on SIM, calli or multiple shoots (or both) formed within 6 weeks. Their formation varied depending on the concentrations of NAA and TDZ. **Table 1** shows the percentage of multiple shoot formation after the 6-week culture on SIM for TDZ concentrations of 0.1-1000 μM and NAA concentrations of 0-5.4 μM. High rates of multiple shoot formation were observed for 10 μM TDZ in the presence of NAA, with the rate being maximal for 3.2 μM NAA (**Table 2**). Moreover, the shoots formed under these conditions appeared to be greener than those formed under other conditions. Therefore, 3.2 μM NAA and 10 μM TDZ was used as the SIM in the subsequent part of this study.

Because no shoot elongation was observed even after prolonged culture (i.e., more than 6 months) on SIM, we tested various concentrations of NAA and

Table 1 The percentage of multiple shoot formation per 30 hypocotyl sections after 6-week culture on SIM where the concentration of TDZ was changed from 0.1 to 1000 μM and that of NAA from 0 to 5.4 μM.

Concentration of TDZ (μM)	Concentration of NAA (μM)	Percentage of multiple shoots formation (%)[1)]
0.1	0.0	0.0 ± 0.0
	1.1	0.0 ± 0.0
	2.2	0.0 ± 0.0
	3.2	0.0 ± 0.0
	4.3	0.0 ± 0.0
	5.4	0.0 ± 0.0
10	0.0	5.0 ± 0.0
	1.1	40.0 ± 3.5
	2.2	85.0 ± 21.0
	3.2	75.0 ± 7.0
	4.3	55.0 ± 0.0
	5.4	70.0 ± 7.0
100	0.0	10.0 ± 7.0
	1.1	35.0 ± 0.0
	2.2	35.0 ± 0.0
	3.2	60.0 ± 14.0
	4.3	35.0 ± 7.0
	5.4	65.0 ± 10.5
1000	0.0	0.0 ± 0.0
	1.1	0.0 ± 0.0
	2.2	0.0 ± 0.0
	3.2	0.0 ± 0.0
	4.3	0.0 ± 0.0
	5.4	0.0 ± 0.0

[1)] Results are the percentage of multiple shoots formation ± SD of two replicates. Each replicate contained 20 hypocotyl sections.

Table 2 The number of multiple shoots per section after 6-week culture on SIM where the concentration of NAA was changed from 0 to 5.4 μM and 10 μM TDZ.

TDZ (μM)	NAA (μM)	Number of multiple shoots per section[1)]
10	1.1	3.2 ± 1.7
	2.2	3.9 ± 2.1
	3.2	5.5 ± 2.1
	4.3	4.9 ± 2.5
	5.4	4.3 ± 2.2

[1)] Results are the mean ± SD of 20 hypocotyl sections.

TDZ in the SEM. We found that WPM solidified with 0.3% Gellan Gum and supplemented with 1 μM TDZ and 0.32 μM NAA gave the best result, where vigorous elongation of shoots was observed within 4 months of culture initiation. Thus, this was used as the SEM in this study. **Fig. 1(A-F)** shows representative samples of the regeneration of plants from *Pittosporum tobira* hypocotyl sections cut from seedlings cultured *in vitro*. From a hypocotyl section placed on SIM as shown in **Fig. 1A**, multiple shoots were formed after 4 weeks of the culture (**Fig. 1B**). The multiple shoots formed were elongated to a small extent on SIM (**Fig. 1C**), but when these shoots were placed onto SEM they elongated significantly (**Fig. 1D**). When the elongated shoots (about 2-3 cm long) were cut and, after being immersed in 4.9 μM indole-3-butyric acid, aseptically transferred into a plastic container containing Florialite moistened with WPM, roots were formed within a month (**Fig. 1E**). When elongated shoots were transferred to WPM solidified with agar, root formation was not observed but calli were formed (data not shown). The plants grown in the growth chamber for about 10 months from the plantlets that had been cultured for 6 weeks in Florialite appeared normal (**Fig. 1F**).

Transient expression of green fluorescent protein

Fig. 1G shows a typical hypocotyl section of *Pittosporum tobira* that contains approximately ten regions of fluorescing cells that are expressing the GFP gene. This particular section were cultured for 13 days, bombarded with pTH-2, and incubated for 24 h before the observation. The culture period of 13 days before bombardment appeared to give the best result in terms of transient expression of this gene.

Production of transgenic shoots

One millimeter sections were cut from 8-week-old seedlings of *Pittosporum tobira* cultured *in vitro*, and transferred to SIM. Thirteen days after the culture, hypocotyl sections were bombarded with a mixture of pANiR and pCH. pANiR bears the cDNA of NiR from *Arabidopsis thaliana* that is under the control of CaMV 35S promoter and NOS terminator. pCH bears the *hph* gene driven by CaMV 35S promoter and NOS terminator (Goto *et al.*, 1993).

Bombarded hypocotyl sections were cultured on SIM with or without 95 μM hygromycin. In the presence of hygromycin, only calli were formed from the sections, and most of these calli turned brown and eventually died. Hygromycin-resistant calli that survived were transferred onto hygromycin-free SIM. A part of the hygromycin-resistant calli was cut, and assayed for the Arabidopsis *nii* gene by the PCR method using a pair of primers specific to the transgene. Seventeen transgenic calli that were shown to bear the Arabidopsis *nii* gene were obtained from a total of 2000 bombarded explants. Although those calli showed vigorous growth, no shoot formation was observed even after 5 months of culture. Therefore, we changed the concentrations of plant hormones even though the SIM gave the highest yield of multiple shoots in nonbombarded and nonhygromycin-treated explants.

Interestingly, shoots were formed within 3 months when calli were cultured on a WPM supplemented

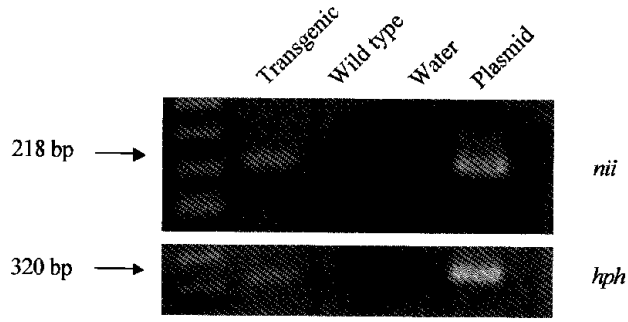

Fig. 2 Agarose gel electrophoresis of PCR-amplified DNA from the hygromycin-resistant regeneration shoot of *Pittosporum tobira* that developed from hypocotyl explants bombarded with pANiR and pCH. Size markers are shown in the leftmost lane. The primer sequences for *nii* are 5'-TGCTTGTGGGAGGATTCTTTAGTC-3' (forward) and 5'-TTGGCATTCTCTTCTCTA-CCTCAG-3' (reverse), which define a 218-bp fragment (upper), and for *hph* are 5'-GTTA-TGTTTATCGGCACTTTG-3' (forward) and 5'-GTCCATCACAGTTTGCCAG-3' (reverse), which define a 320-bp fragment (lower).

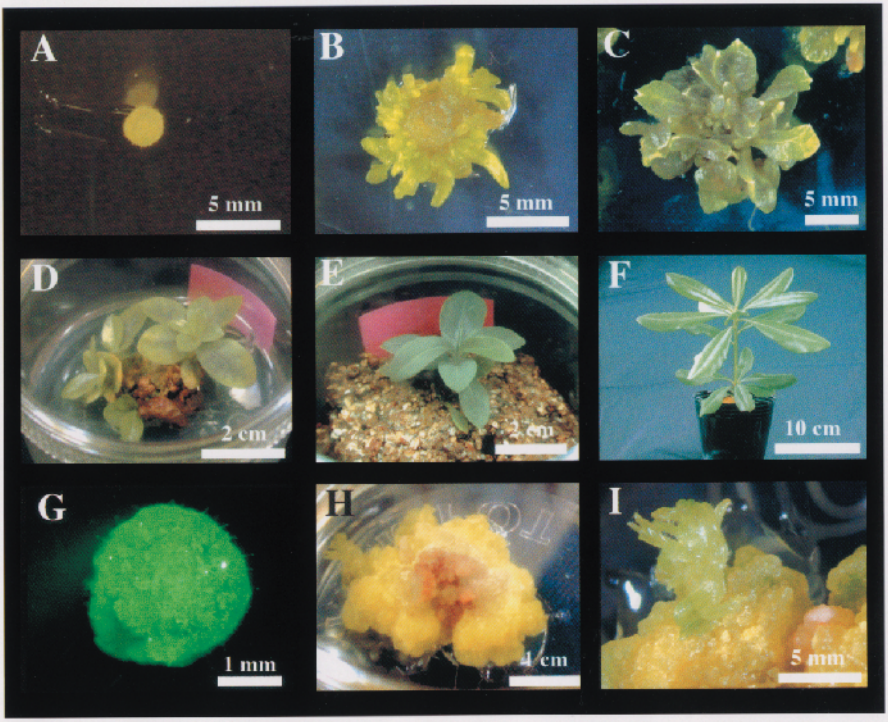

Fig. 1 Regeneration and transformation of *Pittosporum tobira* plants. (A) Hypocotyl section of *Pittosporum tobira* after a week on SIM. (B) Multiple shoots formed on a hypocotyl section after 4 weeks on SIM. (C) Multiple shoots after 10 weeks on SIM. (D) Elongated shoots after 12 weeks on SEM. (E) Plantlet developed from a shoot that was trasferred to a pot containing Florialite moistened with WPM and aseptically cultured for one month. (F) Regenerated plant after being transferred to a pot containing vermiculite and perlite (1:1, v/v) and cultured at 22°C under natural light and 70% humidity in a growth chamber. (G) A typical hypocotyl section of *Pittosporum tobira*, taken under a fluorescent microscope, showing about ten white-colored of GFP-expressing cells. The section had been cultured for 13 days, bombarded with pTH-2, and incubated for 24 h before the observation. (H) Calli regenerating multiple transgenic shoots. (I) Transgenic shoots regenerated from calli.

with 10 μM TDZ and 96 μM NAA, and solidified with 0.3% Gellan Gum as shown in **Fig. 1H** and **I**. A specimen of tissue from the regenerated shoots was analyzed by PCR for the presence of the transgene. As shown in **Fig. 2**, regenerated shoots exhibited 218-bp and 320-bp bands that are specific to the Arabidopsis *nii* and *hph* genes, respectively. These bands were entirely absent when the total DNA from nontransformed control calli was used as the template DNA in the PCR mixture. Taken together, these results show that the shoots thus obtained are transgenic. Regeneration of plants from those transformed shoots is currently being studied in our laboratory.

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