Development of Regeneration and Transformation Systems for *Raphiolepis umbellata* L. Plants Using Particle Bombardment

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Abstract

When hypocotyl sections of *Raphiolepis umbellata* L. were cultured in a woody plant medium containing 8.9 μM benzylaminopurine and 1.1 μM α-naphthaleneacetic, adventitous shoots were formed. Fully developed plants were obtained in 3 months of the culture. A chimeric plasmid pENiR harboring cDNA of *Arabidopsis thaliana* nitrite reductase gene (*nii*) under the control of an enhanced promoter consisting of sequences from both cauliflower mosaic virus 35S promoter and tobacco mosaic virus omega regions and a nopaline synthase terminator was constructed and introduced to the hypocotyl sections by particle bombardment. A plasmid pCH bearing hygromycin phosphotransferase gene (*hph*) was cointroduced. Transformed shoots were selected for hygromycin resistance. Polymerase chain reaction analysis showed the presence of *hph* gene in 16 out of 2572 bombarded hypocotyl sections, and two of which were found to bear *Arabidopsis nii* gene. Translation of this gene in transgenic plants was confirmed by Western blot analysis.

Key words: nitrite reductase, particle bombardment, phytoremediation, *Raphiolepis umbellata* L., regeneration, tissue culture, woody plant.

Abbreviations

BAP, benzylaminopurine; CaMV, cauliflower mosaic virus; CTAB, cetyltrimethylammonium bromide; GFP, green fluorescent protein; *hph*, hygromycin phosphotransferase gene; IBA, indole-3-butyric acid; NAA, α-naphthaleneacetic acid; *nii*, nitrite reductase gene; NiR, nitrite reductase; NOS, nopaline synthase; RuR medium, *Raphiolepis umbellata* L. regeneration medium; WPM, woody plant medium.

Introduction

Phytoremediation - the use of vegetation to mitigate pollutants - is an innovative new technology for solving environmental problems. For example, reliable *in vitro* regeneration and genetic transformation systems can yield metabolically enhanced vegetation along roadsides or in urban areas that can provide *in situ* removal of environmental pollutants.

Stable transformations have been reported in a wide range of woody plants, including walnut (McGranahan *et al.*, 1988), white spruce (Ellis *et al.*, 1993), papaya (Fitch *et al.*, 1990), poplar (Fillatti *et al.*, 1987), cranberry (Serres *et al.*, 1992) and apricot (Singh and Sansavini, 1998). Studies on the improvements of these plant species using genetic engineering are concentrated mainly on reduction of the generation time, development of disease resistance, alteration in hormone biosynthetic pathways, and lignin and cellulose modification of plants for paper industries (Pena and Seguin, 2001). In contrast, utilization of gene-engineered woody plants in phytoremediation has been limited to only a few species such as yellow poplar (Bizily *et al.*, 2000) and *Pittosporum tobira* (Kondo *et al.*, 2002). Consequently, the potential for the use of genetically enhanced roadside woody plants as remediating agents *in situ* in polluted areas still remains to be explored.

As an inherent by-product of mainly road transportation, nitrogen dioxide (NO_2) together with the

other nitrogen oxides reacts with volatile organic compounds to form photooxidant ozone in the troposphere (Hill, 1967). Nitrogen oxides and tropospheric ozone are toxic to both animals (including humans) and plants (Pryor and Lightsey, 1981). The nitrogen in NO_2 taken up by plants is reportedly metabolized mainly through a primary nitrate assimilation pathway (Yoneyama and Sasakawa, 1979; Ramge et al., 1993; Morikawa et al., 1998a). Ferredoxin-dependent nitrite reductase (NiR) is one of the key enzymes in this pathway, which catalyzes the six-electron reduction of nitrite to ammonia in chloroplasts. Enrichment of the NiR enzyme was reported to improve the ability to assimilate NO_2 in Arabidopsis plants (Takahashi et al., 2001). We hypothesized that overexpression of the NiR gene and the corresponding increase in the amount of NiR enzyme produced by genetic engineering in roadside woody plants would improve the ability of these plants to assimilate NO_2, and that this vegetation could eventually be used to clean up NO_2 pollution in situ in urban areas.

Raphiolepis umbellata L. (*R. umbellata*) is an evergreen shrub species that is widely used for vegetation on roadsides and for ornamental purposes such as in hedgegrows and gardens in Northern China, Korea, and Japan. *R. umbellata* was ranked 151st among 217 taxa of naturally occurring plants in which a variation of more than 600-fold was observed in the capability to assimilate NO_2 (Morikawa et al., 1998b). Therefore, it is important to consider using genetic engineering of *R. umbellata* to improve its capability to decontaminate or metabolize pollutants in the environment.

Here we studied the establishment of a reproducible regeneration system and a transformation system using *R. umbellata* plants for the first time. We tried to produce transgenic lines of *R. umbellata* carrying chimeric Arabidopsis NiR cDNA under the control of a strong promoter by particle bombardment. The integration of the foreign gene was characterized by polymerase chain reaction (PCR) analysis. We also studied the expression of the transgene in the host plants by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis.

Materials and Methods

Plant materials and adventitious shoot formation

Seeds of *R. umbellata* were collected from roadsides in Hiroshima University campus on November of each year from 1999 to 2001 and stored at 4 ℃ until use. Surface sterilization was achieved by continuously stirring the seeds in 5% (w/v) sodium hypochlorite solution for 1 h followed by rinsing five times with sterile water. Seeds were then imbibed for 24 h in sterile water and aseptically placed onto woody plant medium (WPM; Lloyd and McCown, 1980) solidified with 0.3% (w/v) Gellan Gum (Wako Pure Chemical Industries, Osaka, JAPAN) and supplemented with 2% (w/v) sucrose (pH 5.8). The seeds were 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 culture room (model TCR-5P; Nippon Medical and Chemical Industries, Osaka, JAPAN). All plant growth regulators and hormones were purchased from Wako Pure Chemical Industries.

After 10 days of growth, cotyledonary tissues and the leaf and root parts of the small seedlings were removed by cutting with a razor blade, and the remaining young hypocotyls (0.3-0.5 cm long) were placed onto *R. umbellata* regeneration (RuR) medium, comprising WPM solidified with 0.3% Gellan Gum and supplemented with different concentration combinations of benzylaminopurine (BAP) and α-naphthaleneacetic acid (NAA) to allow adventitious shoot formation. Four to six weeks after, adventitious shoots (0.5-0.8 cm long) that had been formed directly on the surface of hypocotyl sections were removed and transferred into test tubes containing root-induction medium supported by Florialite (Nisshinbo Industries, Tokyo, JAPAN) and moistened with WPM containing 0.25 μM indole-3-butyric acid (IBA). After 1 month, fully developed plantlets were transferred into pots containing vermiculate and perlite (1:1, v/v) for acclimatization, and grown at 22 ℃ and 70% relative humidity with a light/dark cycle of 16/8 h under fluorescent light (30-40 μmol m^{-2} s^{-1}) in a growth chamber (model ER-20-A; Nippon Medical and Chemical Industries).

Plasmids

The chimeric plasmid constructs pTH-2 (a gift from Dr. Y. Niwa, Shizuoka Prefectural University, Japan) and pCH (Goto et al., 1993) were used. pTH-2 contains green fluorescent protein (GFP) from jelly fish (*Aequorea victoria*) whereas pCH bears the hygromycin phosphotransferase gene (*hph*). Both of the constructs are under the control of cauliflower mosaic virus (CaMV) 35S promoter and nopaline synthase (NOS) terminator. The chimeric plasmid pENiR (**Fig. 1**) was constructed as follows: a 2.25-kb *EcoRI/BamHI* fragment carrying a cDNA of the NiR gene (*nii*) from *Arabidopsis thaliana* and NOS terminator was excised from the plant expression vector pANiR (Tanaka et al., 1994; unpublished results) and replaced the corresponding
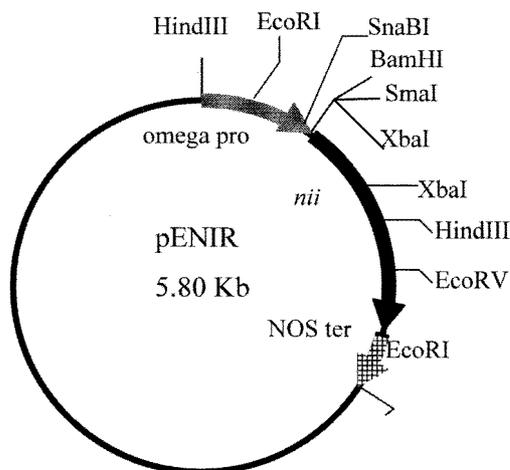
Fig. 1 Schematic illustration of the plasmid pENiR. omega pro, omega promoter; *nii*, nitrite reductase cDNA from *A. thaliana*; NOS ter, nopaline synthase terminator.

*Eco*RI/*Bam*HI restriction fragment of the chimeric gene construct pE2113-GUS that harbors the GUS (β -glucuronidase) gene (Mitsuhara *et al.*, 1996). The resulting chimeric plasmid pENiR carries an enhanced promoter region (omega promoter) consisting of the omega translation enhancer sequence of tobacco mosaic virus (Sleat *et al.*, 1987) that has been fused to the CaMV 35S core promoter sequence.

Transient expression and stable transformation

Plasmid DNAs were precipitated, adsorbed to gold particles (Morikawa *et al.*, 1989), and delivered to target tissues using an automatic pneumatic particle-gun device as reported elsewhere (Takahashi and Morikawa, 1996). For transient expression of GFP, hypocotyl sections from 10-day-old seedlings were cut and cultured on RuR medium for 1-15 days. Approximately 25 explants were fixed at their bottom part to form a disk (20 mm in diameter) in the center of a 6-cm diameter petri dish containing fresh RuR medium. The plasmid pTH-2 was delivered four times to each sample. After 24 h explants were viewed for GFP fluorescence under a binocular fluorescence microscope (model MZ FL III; Leica, Solms, GERMANY) using an excitation filter (470/40 nm) and a barrier filter (525/50 nm).

For stable transformations, an equimolar mixture of the chimeric plasmids pENiR and pCH was coated on gold particles and delivered by particle bombardment as described above to approximately 25 hypocotyl explants of *R. umbellata* that were cultured for 10 days on RuR medium. Explants were then transferred to fresh RuR medium and cultured for 3 days under the aforementioned culture conditions. After 3 days, explants were transferred to

RuR medium containing 38 μM hygromycin (Wako Pure Chemical Industries) for later selection. The putative hygromycin-resistant adventitious shoots were selected after 1 month and allowed to grow further in hygromycin-free RuR medium. Regenerating shoots were analyzed by the PCR method (see the next section).

DNA isolation and PCR analysis

The total DNA was extracted from the leaf of putatively transformed shoots of *R. umbellata* by cetyltrimethylammonium bromide (CTAB)-based method as described previously (Doyle and Doyle, 1990) but with slight modifications. Approximately 5-10 mg of plant leaf tissues were frozen in liquid nitrogen and homogenized using a mixer mill (model MM-2000; Mitamura Riken Kogyo, Tokyo, JAPAN). Then, 250 μl of CTAB buffer (3% w/v CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0) was added, and the homogenate was incubated for 30 min at 60 °C. The DNA from the homogenate was isolated by the addition of 250 μl of chloroform and 165 μl 2-propanol twice and between which the sample was centrifuged for 5 min at 6000 g and the supernatant was recovered. A final pellet was isolated, rinsed with 70% ethanol and, centrifuged. Finally TE buffer (10 mM Tris-HCl, 0.2 mM EDTA, pH 8.0) was added to resolve the pellet for subsequent analysis.

The PCR reaction was carried out in 20 μl of a mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM each of four dNTPs, 1.25 units of Taq polymerase (Takara Shuzo, Kyoto, Japan), and approximately 10 ng of total DNA. The following sets of primers were used: a 218 bp fragment specific to Arabidopsis *nii* gene (Tanaka *et al.*, 1994) was amplified by 24 nt each of a forward 5'-TGCTTGTGGGAGGATTCTTT-AGTC-3' and a reverse 5'-TTGGCATTCTCT-TCTCTACCTCAG-3' primer sequences; whereas a 803 bp fragment specific to *hph* gene (Gritz and Davies, 1983) was amplified by 18 nt each of a forward 5'-CGATGTAGGAGGGCGTGG-3' and a reverse 5'-TTCTGCGGGCGATTTGTG-3' primer sequences. The reaction mixtures were heated for 5 min at 94 °C and the subsequent denaturing, annealing, and extending steps were performed by 35 cycles of 94 °C for 1 min, 60 °C for 2 min, and 72 °C for 1 min respectively in a programmable temperature-control system (model PC-800; Astec, Tokyo, JAPAN). The amplified products were then electrophoresed in a 1% agarose gel and stained with ethidium bromide for UV visualization.

SDS-PAGE and Western blot analysis

Crude protein extracts from wild-type and transgenic plants of *R. umbellata* were prepared according to He *et al.* (1994) with slight modifications. Leaf samples from 1-month-old wild-type and transgenic plants were frozen in liquid nitrogen and then ground in a mortar with a pestle. A 100 μl aliquot of extraction buffer containing 40 mM Tris-HCl (pH 8.0), 2% (w/v) SDS, and 5% (v/v) β-mercaptoethanol was added to 100 mg of ground sample and homogenized. The homogenate was centrifuged at 7500 g and 4 °C for 5 min, and the resulting supernatant (crude protein extract) was used for Western blot analysis. Crude protein extracts from spinach and *A. thaliana* that were used as control plants were prepared as reported elsewhere (Takahashi *et al.*, 2001). The protein contents in the crude extracts were measured by the method of Bradford (1976) with bovine serum albumin as the standard. A sample extract (approximately 50 μg) was loaded and electrophoresed for 2 h at 25 mA on a 10% (w/v) polyacrylamide gel containing 0.1% SDS. Resolved proteins were electroblotted onto an Immobilon™-P transfer membrane (Millipore, Bedford, MA, USA). Detection of NiR was achieved using a polyclonal antibody raised against spinach NiR (Ida, 1987) and a Western Lightning Chemiluminescence Reagent *Plus* (Perkin-Elmer Life Sciences Co., Ltd., Yokohama, JAPAN) kit according to the manufacturer's instructions. Visualization of Western blots was performed on an imaging workstation (Versadoc 5000; BioRad, Tokyo, JAPAN) using an imaging software (Quantity One; BioRad).

Results and Discussion

Regeneration of adventitious shoots

One of the crucial steps in plant *in vitro* regeneration is the establishment of the aseptic conditions during seed germination. We observed that *R. umbellata* fruits easily became contaminated and lost their ability to germinate at room temperature. For this reason they were kept until use in sealed containers at 4 °C in the presence of silica gel for preservation. When required for use, the seeds were removed from the containers and surface sterilized in 5% sodium hypochlorite solution for 30 min. This has increased the germination efficiency to almost 100% by dropping seed contamination to acceptable levels.

The proper choice of a plant organ as the source of tissue for initiating cultures has been reported to influence the efficiency of the subsequent regeneration (Pierik, 1987). Our early attempts showed
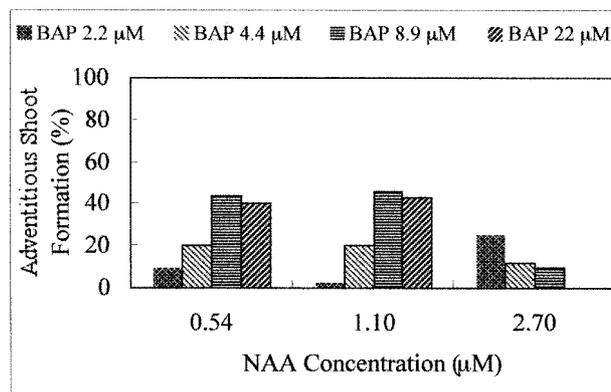
Fig. 2 Effects of BAP (2.2–22 μM) in combination with NAA (0.54–2.70 μM) on the adventitious shoot formation from *R. umbellata* hypocotyls after 4 weeks of culture on WPM. Results are the mean of 3 independent experiments with 30 explants.

that the best choice of explants is the young hypocotyls of *R. umbellata* that can evolve directly into adventitious shoots in the culture media (data not shown).

Fig. 2 shows the effects of BAP in combination with NAA on the adventitious shoot formation from hypocotyls of *R. umbellata* after 4 weeks of culture. When 10-day-old hypocotyl sections (**Fig. 3A**) were cultured on RuR medium, depending on the concentration of the growth regulators BAP and NAA, adventitious shoots associated with or without calli (or only calli) were formed within 1 month. Although it was possible to regenerate adventitious shoots using BAP and NAA in concentration ranges of 2.2–22 μM and 0.54–2.7 μM, respectively, the highest level of direct adventitious shoot formation was observed with 8.9 μM BAP in combination with 1.1 μM NAA after 4 weeks of culture. It is highly desirable to establish direct organogenesis or somatic embryogenesis with no callus formation in order to minimize the generation of somaclonal variants commonly associated with callus-mediated regeneration. For this reason we chose a combination of 8.9 μM BAP with 1.1 μM NAA, which had also produced the lowest standard deviation in the formation of adventitious shoots (**Table 1**). This combination of plant growth regulators was used in RuR for the formation of adventitious shoots in the subsequent part of the study. When adventitious shoots were isolated and subcultured into the same RuR medium, shoots were well elongated (0.5–1.0 cm long) within the following 2 weeks. Adventitious roots were regenerated from these shoots when they were subcultured into root-induction medium containing Florialite moistened with WPM that was supplemented with 0.25 μM IBA. Al-
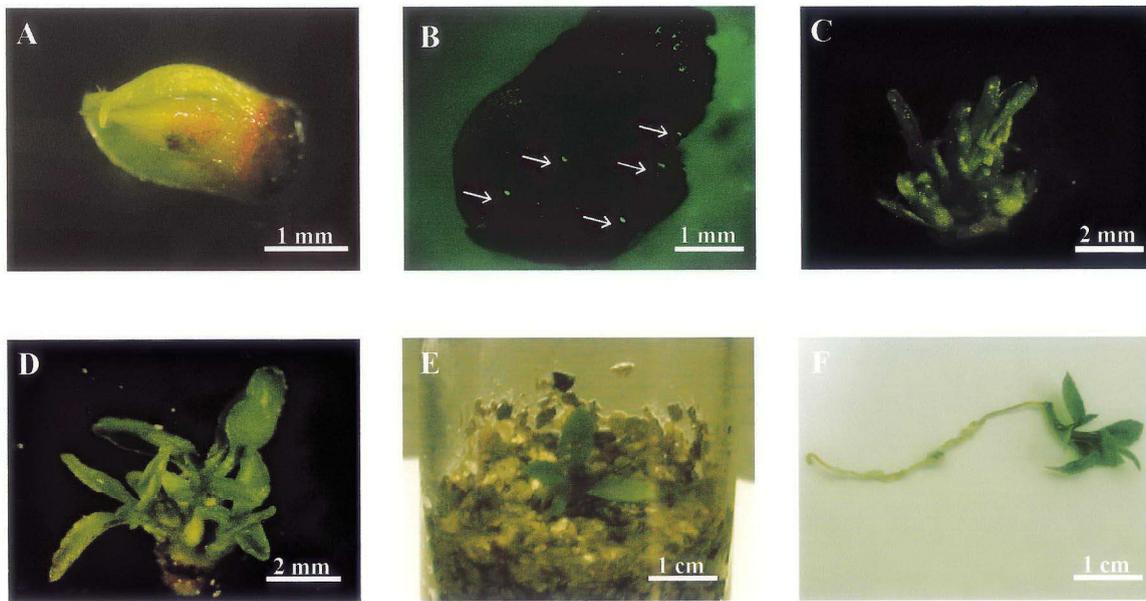
Fig. 3 Plant regeneration from transformed hypocotyl sections of *R. umbellata*. (A) A10-day-old hypocotyl section used for the initiation of the cultures. (B) Transient GFP expression in a 10-day-old hypocotyl section that was cultured for 10 days on RuR and bombarded with pTH-2. Arrows indicate the fluorescent cell clusters expressing the GFP gene 24 h after bombardment. (C) Adventitious shoot formation from a successfully transformed explant 4 weeks after bombardment. (D) Elongating adventitious shoots 6 weeks after bombardment. (E) A fully grown transformed shoot after being transferred to a tube containing Florialite watered with WPM that was supplemented with 0.25 μM IBA for root induction 10 weeks after bombardment. (F) Root formation from an adventitious shoot 15 weeks after bombardment. RuR supplemented with 38 μM hygromycin was used as the selection medium (B, C) and hygromycin was removed from the media when shoots were transferred for root induction (D).

Table 1 The number of adventitious shoots formed on hypocotyl explants from *R. umbellata* after 4 weeks on WPM supplemented with differing concentrations of NAA and BAP.

NAA(μ M)	BAP(μ M)	Number of adventitious shoots per explants[1]
0.54	2.2	4.00 ± 2.67
	4.4	2.30 ± 0.78
	8.9	2.54 ± 0.44
	22.0	2.21 ± 1.24
1.10	2.2	3.00 ± 1.40
	4.4	2.83 ± 0.92
	8.9	2.93 ± 0.27
	22.0	2.42 ± 1.54
2.70	2.2	2.87 ± 0.96
	4.4	2.00 ± 1.28
	8.9	2.00 ± 1.97
	22.0	0.00 ± 0.00

[1] Results are the mean of 3 independent experiments with 30 explants including SD

though other types of auxins such as NAA and indoleaceticacid were used, the formation of successive roots was not achieved and calli were formed in almost 80% of the adventitious shoots (data not shown). Acclimatization of regenerated plantlets in the aforementioned conditions was successfully achieved in 2–3 weeks, and the resultant plantlets were normal in appearance.

The somaclonal variations in woody plant tissue culture are also attributed to the relatively long culture time required for the regeneration of plants (McGranahan *et al.*, 1988; Trick and Finer, 1999; Wenck *et al.*, 1999). However, our results have demonstrated that fully developed *R. umbellata* plants can be regenerated from young hypocotyls within 3 months, which is a comparatively short regeneration time for a woody plant species.

### Transient expression and stable transformation

Transient assays for the activity of foreign genes with a biolistic system are useful tools for optimizing bombardment conditions. The GFP is used as a reporter in both animals and plants without the need for destructive analysis of the experimental material (Chalfie *et al.*, 1994). **Fig. 3B** shows the typical appearance of the fluorescent cells of a *R. umbellata* hypocotyl section that were expressing the GFP gene 24 h after bombardment with plasmid pTH–2. Note that unmarked illiminous parts on the right corner of explant are reflections of light from

the tissue under photographic conditions. We obtained our best results for the transient expression of GFP when the explants were cultured on RuR for 10 days and when using four repeated bombardments (data not shown).

Literally, stable transformations are known as the events leading to integration of foreign genes into the genome of a host cell in order to create stable transgenic phenotypes (Yang and Christou, 1994). In order to obtain stable transformants of *R. umbellata*, we cut and cultured hypocotyl explants from 10-day old seedlings on RuR medium for 10 days and bombarded them four times with an equimolar mixture of the chimeric plasmid constructs pENiR and pCH. After 24 h, the bombarded sections were transferred to RuR with or without 38 μM hygromycin. In our control experiments, explants became necrotic in the presence of 38 μM hygromycin and died after 1 week. However, the hygromycin-resistant explants survived the selection for 1 month (**Fig. 3C**), grew small shoots (**Fig. 3D**), and therefore were analyzed further for gene integration. A small part of leaf was excised from such explants, and DNA was isolated and assayed for the presence of *hph* and Arabidopsis *nii* genes by the PCR method using specific primers as described in Materials and Methods section. A total of 16 transgenic shoots were found to bear the chimeric *hph* gene among 2572 bombarded hypocotyls explants, but only two of them appeared to bear the Arabidopsis *nii* gene. The reason for this discrepancy between the transformation frequencies is unknown. Eventually, a total of 10 adventitious shoots regenerated into plantlets from these two transgenic shoots of *R. umbellata* bearing the Arabidopsis *nii* gene (**Fig. 3D, E**), and were used in the subsequent part of the study.

Stable plant transformation events in particle bombardment techniques depend on the successful passage of DNA-coated particles through tough cell walls so that the DNA reaches to the nuclei of susceptible competent cells in highly organized plant tissue (Yamashita *et al.*, 1991). However, the degree of tissue damage suffered during bombardment has been shown to affect the frequency of transformation events (Birch and Frank, 1991). We observed that *R. umbellata* hypocotyls easily become dehydrated during and after bombardment. The low penetration efficiency of particles and the high tissue damage may be two reasons for the observed low transformation frequency in our experiments.

The presence of specific 218 bp Arabidopsis *nii* and 803 bp *hph* PCR amplification products in bombarded *R. umbellata* plants is demonstrated in

150

M   1   2   3   4

A

218 bp ▶   *nii*

B

803 bp ▶   *hph*
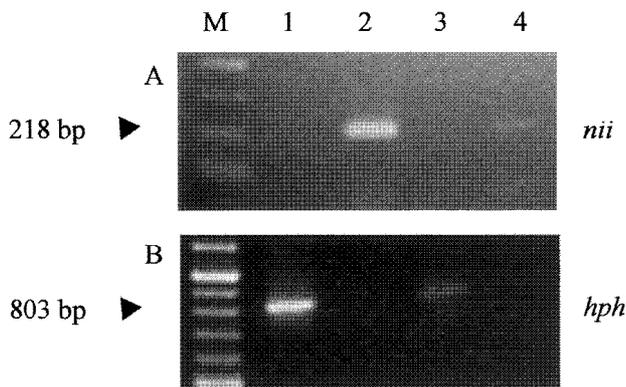
Fig. 4 PCR analysis of genomic DNA samples from hygromycin-resistant adventitious shoots of *R. umbellata* that developed after bombardment with the plasmids pENiR and pCH. Lane M shows a 100 bp marker. Lane 1, water; lane 2, plasmid pENiR; lane 3, wild-type line; lane 4, a transformed adventitious shoot. Arrow indicates the 218 bp PCR product specific for the nii gene (A). Lane 1, plasmid pCH; lane 2, wild-type line, a transformed adventitious shoot; lane 4, water. Arrow indicates the 803 bp PCR product specific for *hph* gene (B).

Fig. 4. The absence of corresponding bands in the PCR-amplified product of total DNA from wild-type control explants indicates the transgenic nature of these *R. umbellata* lines regenerated from bombarded explants.

SDS-PAGE Western blot analysis

Transgene expression and the subsequent translation in the host plant can be demonstrated by Western blot analysis. Western blots were probed with polyclonal antibodies raised against spinach NiR and reported to react also against Arabidopsis NiR protein (Takahashi *et al.*, 2001). Crude protein extracts from both spinach and Arabidopsis were used as internal controls in this study. It has been reported that NiR protein has an estimated molecular mass of 63 kDa in several plant species, including spinach (Back *et al.*, 1988), Arabidopsis (Tanaka *et al.*, 1994), tobacco (Neininger *et al.*, 1994a), tomato (Migge *et al.*, 1998) birch (Friemann *et al.*, 1992), and Scots pine (Neininger *et al.*, 1994b). Our results demonstrate the presence of a corresponding 63 kDa NiR protein signal in transgenic lines of *R. umbellata* as well as in spinach and Arabidopsis (**Fig. 5**), indicating that the introduced Arabidopsis *nii* gene is successfully expressed and properly translated in these transgenic *R. umbellata* plants. Moreover, the molecular mass coincidence of the immuno-reactive proteins from spinach and Arabidopsis control plants and two *R. umbellata*

63 kD ▶

LANE   A   B   C   D   E

Peak Intensity   9760   9520   4283   7330   6334
(pixels)
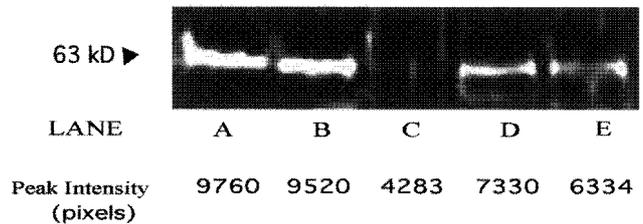
Fig. 5 SDS-PAGE Western blot analysis of NiR protein in transformed and wild-type *R. umbellata*. Fifty micrograms of crude protein extracts from *A. thaliana* (lane A), spinach (lane B), wild-type *R. umbellata* (lane C), and transformed lines (lanes D, E) were electrophoresed on 10% SDS-PAGE. Western blot analysis was performed with a polyclonal antibody raised against spinach NiR protein and a chemiluminescence assay. The intensities of the chemiluminescent bands signals were determined using an imaging workstation and imaging software.

transgenic plants indicates that the expressed precursor NiR with a transit-peptide had been processed correctly and might have been targeted to the chloroplasts of the transgenic plants. Note that initially there was no 63 kDa signal detected on the blotted membranes for the wild-type *R. umbellata* plants under normal visualization conditions. However, a detailed analysis of the peak intensities of chemiluminescence signals detected on the membranes revealed a faint band around 63 kDa that reacted with the antibody in the wild-type plants. The difference between the light peak intensities of the corresponding signals in wild-type and transgenic lines also confirms the proper translation of the Arabidopsis *nii* gene in transgenic *R. umbellata* plants.

We are currently investigating how to improve the transformation efficiency in order to produce sufficient transgenic material from *R. umbellata* hypocotyls for future purposes. The NiR activity and NO_2 assimilation capability of the transgenic plant lines obtained in this study are being determined, and the results will be published in due course.

Acknowledgements

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