Original Paper

Genetic engineering of nitrite reductase gene improves uptake and assimilation of nitrogen dioxide by *Rhaphiolepis umbellata* (Thunb.) Makino

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Abstract   Hypocotyl sections of *Rhaphiolepis umbellata* (Thunb.) Makino were transformed by *Agrobacterium tumefaciens* bearing a binary vector pIG121-AtNiR, which contains cDNA of the nitrite reductase gene from *Arabidopsis thaliana* (*Atni*) under the control of a modified cauliflower mosaic virus 35S promoter and chimeric hygromycin phosphotransferase gene (*hph*). A 4% of the hypocotyl explants transfected with *Agrobacterium* formed hygromycin resistant adventitious shoots, and most of them rooted upon root induction treatment. The presence and expression of the introduced transgene in putative transgenic plants (12 months after transfection) were respectively confirmed by polymerase chain reaction (PCR) analysis using primers specific to *Atni* and by western blot analysis using anti NiR antibody. A total of 37 transgenic plant lines were obtained. Plants 33 months after the transfection were fumigated with 200 ± 50 ppb $^{15}NO_2$ under the natural light for one week in the fumigation chamber in a confined glasshouse. The amount of total nitrogen derived from NO_2 (reflecting uptake of NO_2) and that of Kjeldahl nitrogen derived from NO_2 (reflecting assimilation of NO_2) were determined using a mass spectrometer. One of the 9 transgenic plants tested was 1.6–2.0 times higher both in the uptake and assimilation of NO_2 than non-transformed wild-type ones.

Key words: Nitrite reductase, nitrogen dioxide, phytoremediation, *Rhaphiolepis umbellata* (Thunb.) Makino, transgenic plant

Nitrogen dioxide (NO_2) is a major pollutant in the urban air. It produces photooxidants, including ozone, by a photochemical reaction with volatile organic compounds in the atmosphere (Stockwell et al. 1997). Plants can take up NO_2 through stoma, and convert its nitrogen to organic nitrogen through the primary nitrate assimilation pathway (Yoneyama and Sasakawa 1979; Morikawa et al. 2003). Ferredoxin-dependent nitrite reductase (NiR) is the second enzyme in the primary nitrogen assimilation pathway, which catalyzes the six-electron reduction of nitrite to ammonia in the chloroplast. Takahashi et al. (2001) showed that enrichment of the NiR enzyme by the introduction and expression of foreign NiR gene (*nii*) improves the ability *Arabidopsis thaliana* by 1.4 times to assimilate NO_2. In order to invent novel phytoremediation materials, we studied transformation of woody species such as *Pittosporum tobira* (Kondo et al.

2002) and *R. umbellata* (Erkin et al. 2003), which are a typical and important roadside tree in Japan, by a chimeric *nii* vectors using particle bombardment. However, no regenerants of *P. tobira* were obtained (Kondo et al. 2002), and all regenrants of *R. umbellata* died during acclimatization for unknown reason (Erkin et al. 2003). Therefore, we here investigated *Agrobacterium*-mediated transformation of *R. umbellata*, and determination of uptake and assimilate NO_2 by transgenic plants.

Materials and methods

Construction of plasmids

A cDNA (accession no. AB006032) of NiR gene from *A. thaliana* (*Atnii*) was PCR-cloned based on the genomic sequence (Tanaka et al. 1994). The cDNA was fused to a
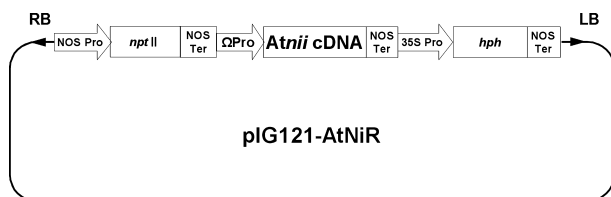

Figure 1. Schematic representation of the plasmid pIG121-AtNiR. *Atnii* cDNA, cDNA of *A. thaliana* nitrite reductase gene; NOS Pro, nopaline synthase promoter; *npt*II, neomycin phosphotransferase gene; NOS Ter, nopaline synthase terminator; 35S Pro, cauliflower mosaic virus 35S promoter; Ω Pro, 35S promoter with the omega sequence; *hph*, hygromycin phosphotransferase gene; RB, right border; and LB, left border.

modified cauliflower mosaic virus (CaMV) 35S promoter containing the Ω sequence of tobacco mosaic virus (omega promoter) from pE2113-GUS (a gift from Dr. Y. Ohashi, National Institute of Agrobiological Sciences; Mitsuhara et al. 1996) and to nopaline synthase (NOS) terminator as described previously (Erkin et al. 2003). This chimeric expression cassette was inserted into a binary vector pIG121-Hm (a gift from Dr. K. Nakamura, Nagoya Univ.) that contains chimeric hygromycin phosphotransferase gene (*hph*) and neomycin phosphotranseferase gene (*npt*II) to give pIG121-AtNiR as depicted in Figure 1.

Plant materials
Seeds of *R. umbellata* harvested at the Hiroshima University campus at Higashi Hiroshima city (Hiroshima, Japan) were washed with detergent, sterilized for 1 h with 5% (w/v) sodium hypochlorite and rinsed three times with sterilized pure water (18.0 MΩ) as described by Erkin et al. (2003). The sterilized seeds were then placed onto woody plant medium (WPM; Lloyd and McCoen 1980) with 2% sucrose solidified with 0.3% (v/w) Gellan Gum (Wako Pure Chemical Industries, Osaka, Japan) in plastic Petri dishes (Atect Co., Osaka, Japan). Throughout this study, WPM contained 2% sucrose and its pH was adjusted to 5.8 with 1 N KOH. Petri dishes were then placed in a growth chamber (model TCR-5P; Nippon Medical & Chemical Instruments, Osaka, Japan), and cultured at $25 \pm 1°C$ and $70 \pm 10\%$ relative humidity (RH) under 15-h light with fluorescent light (30–40 μmol photons $m^{-2} s^{-1}$) and 9-h dark conditions. After germination (ca. 10 days after sowing), hypocotyls (2–3 mm long) were cut, placed on solidified WPM supplemented with 2.0 mg l^{-1} benzyladenine (BA) and 0.2 mg l^{-1} α-naphthalene acetic acid (NAA) and cultured for 10 days prior to transformation. Culture of *A. thaliana* ecotype C24 was carried out as described previously (Takahashi et al. 2001).

Transformation
Agrobacterium tumefaciens strain LBA4404 was grown

at 28°C in LB medium supplemented with 50 mg l^{-1} hygromycin B (Wako) and 100 mg l^{-1} rifampicin (Sigma). Bacterial cells were pelleted by centrifugation at 3000 g for 3 min and resuspended in 10 mM MgSO$_4$ to an optical density of ca. 0.1 at 600 nm. Apexes of hypocotyls of *R. umbellata* that had been precultured for 10 days were pricked with a scalpel, after which hypocotyls were submerged in the bacterial suspension for a few seconds and blotted on sterilized paper. Hypocotyls were then placed on solidified WPM supplemented with 2.0 mg l^{-1} BA, 1.1 mg l^{-1} NAA and 19.6 mg l^{-1} (100 μM) acetosyringone (Sigma) in Petri dishes. They were then cultured for 7 days under the conditions described above, after which the explants were transferred to Petri dishes containing solidified WPM supplemented with 2.0 mg l^{-1} BA, 1.1 mg l^{-1} NAA, 500 mg l^{-1} carbenicillin (Wako) and 10 mg l^{-1} hygromycin. They were subcultured every 2 weeks in the same medium for up to total of 6 weeks, and then for 6 more weeks in the same medium lacking hygromycin.

Regeneration of transgenic plants
Hygromycin resistant adventitious shoots (0.5–0.8 cm) of *R. umbellata* emerged (about 13 weeks after *Agrobacterium* transfection) directly from the hypocotyls sections were cut, and their cut ends were dipped into 1 mg l^{-1} indole-3-butyric acid (IBA) for a few seconds, after which they were placed on solidified WPM in Peri dishes and cultured for 5–8 weeks more to allow root formation. Rooted plantlets were pulled out from the culture medium, and the roots were washed thoroughly with sterilized water. Plants were then transferred onto Florialite (Nisshinbo Industries, Tokyo, Japan) moistened with WPM in sterilized plastic containers (Agripot, Kirin), and cultured for 7 months more. Putative transgenic plants (ca. 12 months after *Agrobacterium* transfection) were transferred to plastic pots containing vermiculite and perlite (1 : 1, v/v). Pots with plants were placed in the other growth chamber (NMCI model ER-20-A) for acclimatization. Plans were irrigated every 4 days with 0.1% Hyponex (Hyponex Japan, Osaka, Japan), and grown at $22 \pm 1°C$ and $70 \pm 10\%$ RH under continuous fluorescent light (100 μmol photons $m^{-2} s^{-1}$) for 20 months more. Fully acclimatized plants (32 months after *Agrobacterium* transfection) were then transferred into a confined glasshouse (NMCI model BTH-P1-TH). Plants were grown under the natural light and irrigation with 0.1% Hyponex at $22 \pm 1°C$, $70 \pm 10\%$ RH and 340 ± 80 ppm CO$_2$ for one month, and then subjected to fumigation with NO$_2$ to determine NO$_2$ uptake and assimilation.

Culture of wild type plants
    To prepare wild type plants whose age was "the same" as that of transgenic ones, culture of wild type *R.*

umbellata was initiated as the transformation and carried out under the same conditions as for the transgenics. Seeds were germinated and aseptically cultured on solidified WPM in Petri dishes for about 5 months followed by the aseptic culture in plastic containers for 7 months. Plants were then transferred into plastic pots containing vermiculite and perlite (1 : 1, v/v) and grown non-aseptically for 20 months more, after which they were transferred into the confined glasshouse, and grown for one month more prior to fumigation with NO_2 to determine NO_2 uptake and assimilation.

DNA extraction and PCR analysis

Total DNA was extracted from leaves of *R. umbellata*, and subjected to PCR analysis as reported previously (Erkin et al. 2003). PCR reaction mixture (20 μl) contained 500 ng total DNA, 10 mM tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each of dNTPs (Takara Bio INC, Shiga, Japan), 1.25 units of rTaq (Takara) and 0.5 μM each of the primers for *Atnii* gene. Primers used were 5′-TGCTTGTGGGAGGATTCTTTAGTC-3′ (forward) and 5′-TTGGCATTCTCTTCTCTACCTCAG-3′ (reverse), for the amplification of a 218 bp. After denaturation at 94°C (5 min), amplification was achieved by 35 cycles of 94°C (1 min), 60°C (2 min), and 72°C (1 min) plus a final segment of 72°C for 3 min. Using 2 μl of the PCR products as a template, the second PCR was performed as the first one.

Protein extraction and two-dimensional gel electrophoresis

Leaves of 1-year-old wild type (approx. 1 g) and transgenic (1g) *R. umbellata*, and those (0.3 g) of 4 week-old *A. thaliana* were ground to a fine powder with liquid nitrogen by using a mortar and a pestle. A buffer (9 μl 100 mg^{-1} leaves) containing 40 mM tris-HCl (pH 8.0), 2% (w/v) SDS, and 5% (v/v) β-mercaptoethanol was added and further homogenized. The homogenate was centrifuged twice at 20,000 g for 20 min, and the supernatant was recovered and sonicated for 30 sec followed by ultrafiltration using a 50 kD Omega membrane in Microsep Centrifugal Devices (Pall co., East Hills, NY, USA). Acetone was added to the filtrate to final concentration of 80% (v/v), and the mixture was stood for 4 h at −20°C. After centrifugation at 20,000 g for 10 min, the resulting pellet (extracted protein) was air dried, after which proteins were dissolved in a buffer consisted of 8M urea, 2% (w/v) CHAPS, 2% (v/v) IPG buffer (pH 4–7; Amersham Biosciences), and 0.001% (w/v) bromophenol blue. The protein content was determined using Bio-Rad DC Protein Assay Kit 2 (Bio-Rad) according to Bradford (1976).

Two-dimensional PAGE was done as described by Takahashi et al. (2001). Briefly, an approximate 100 μg of extracted protein was loaded on the first-dimension gel (Immobiline Dry Stip, pH 4–7, Amersham Biosciences). Isoelectric focusing was done horizontally with a Multiphor II apparatus (Amersham Biosciences) by linear increase from 300 V to 3,500 V during 3 h and holding at 3,500 V for 4.5 h . The isoelectric focusing gel was equilibrated for 30 min in SDS sample buffer containing 50 mM tris-HCl pH 6.8, 6 M urea, 30% glycerol, 2% SDS, and bromophenol blue, and was then mounted on a 10% (w/v) polyacrylamide-SDS slab gel and was electrophoresed for 1.5 h at 20 mA. Proteins that separated in the two-dimensional gels were transferred to a membrane (Immobilon-P, Millipore, Bedford, MA) by an electroblotter (Trans-Blot SD, Bio-Rad). Immunodetection was done with polyclonal antibody raised against recombinant tobacco NiR (see below).

Antibody

A PCR-cloned sequence of the full-length NiR1 cDNA of tobacco (Vaucheret et al. 1992) was subcloned into pDEST^{TM}17 vector (Invitrogen) according to the Gateway protocols provided by the manufacture. Polyhistidine-tagged tobacco NiR1 protein was produced in *Escherichia coli* BL21-SI cells, and purified using a TALON column (Clontech). Rabbits were immunized with purified recombinant NiR at Tanpaku Seisei Kohgyo Co. (Maebasi, Gumma, Japan), and the antibody was purified from the serum by using a HiTrap affinity column (Amersham Biosciences).

Western blot analysis

Western blot was done as described by Takahashi et al. (2001) using the polyclonal anti tobacco NiR antibody. Briefly, PVDF membranes were blocked for 8 h using 2% (w/v) nonfat milk dissolved in PBS plus 0.1% (v/v) Tween-20 (PBST). Membranes then were blotted with the antibody. The membranes then were washed two times in PBST, and then were probed with a goat anti-rabbit IgG horseradish peroxidase conjugate (VECTOR Lab., CA, USA). After washing the membrane two times in wash buffer, the immunopositive spots were visualized using a Western Blot Chemiluminescence Reagent Plus (NEN Life Science, MA, USA) and VersaDoc 5000 imaging system (Bio-Rad).

NO_2 uptake and assimilation analysis

Transgenic (ca. 33 months after *Agrobacterium* transfection) and wild type plants (ca. 33 months after germination) were fumigated with 200±50 ppb $^{15}NO_2$ (55.6 atom% ^{15}N) for one week under natural light in November, 2005 at 22±0.3°C and 70±4% RH and 340±80 ppm CO_2 in Nox-fumigation chambers in a confined glasshouse as described elsewhere (Takahashi et al. 2005). Leaves were harvested from the upper (1st to 4th leaves from the shoot apex) and middle (5th to 8th

leaves) part of each plant. They were then washed with distilled water, freeze-dried and stored in a desiccator. Total nitrogen derived from NO_2 (designated TNNO2) and reduced or Kjeldahl nitrogen derived from NO_2 (designated RNNO2) were determined using a mass spectrometer as described elsewhere (Morikawa et al. 2004).

Results and discussion

Transformation

Figure 2 shows the typical photographs of (A) a hypocotyl section of *R. umbellata* used for the transformation, (B) hygromycin-resistant adventitious shoots formed on the hypocotyl section 13 weeks after transfection, and (C) rooted (putatively transformed) plantlet (21 weeks after transfection), (D) fully acclimatized transgenic plantlets (12 months after transfection), and transgenic plants in the confined glasshouse (34 months after transfection). About 4% of hypocotyl explants transfected with *Agrobacterium* formed hygromycin-resistant adventitious shoots (Figure 2B). More than 70% of the shoots rooted after treatment with IBA (Figure 2C). They were then tested for the presence and expression of the transgene (see below). Plants positive for the tests were allowed to be acclimatized, and the culture was continued further.

PCR and western blot analyses

Putatively transformed plantlets (ca. 21 weeks after transfection) were subjected to PCR analysis. As shown in Figure 2F, all of the tested plants were found to show a 218 bp band corresponding to the coding region of the *Atnii* gene, confirming the presence of the transgene in *R. umbellata* genome. A total of 37 PCR-positive transgenic plant lines were obtained.

Western blot

PCR-positive transgenic lines were subjected to 2D-western blot analysis using a polyclonal antibody raised against recombinant tobacco NiR as the probe. All of the lines tested were found to be positive for the western blot analysis, confirming that the introduced At*nii* is expressed in transgenic plants. The typical results are depicted in Figure 3. Also depicted are the results of wild type *R. umbellata* and wild type *A. thaliana*. Transgenic *R. umbellata* appeared to give about eight immunopositive spots of molecular mass approx. 63 kD and pI 5 to 6. On the other hand, wild-type *R. umbellata* gave three immunopositive spots of very similar molecular mass at the basic side (pI>6). *A. thaliana* gave four immunopositive spots of similar molecular mass at acidic side (pI<6). It is therefore concluded that the introduced *Arabidopsis* NiR cDNA was successfully expressed in the transgenic *R. umbellata*. NiR proteins of
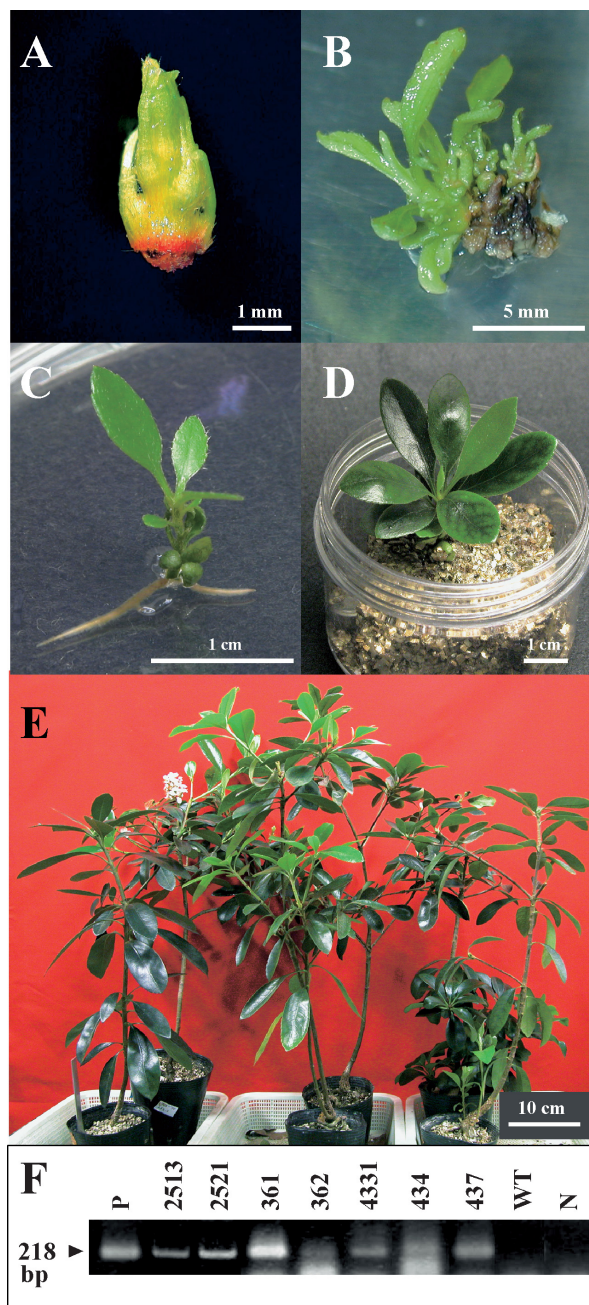

Figure 2.   Photographs of transgenic shoots or plants of *R. umbellata* and PCR results. (A) Hypocotyl 10 days after germination, (B) adventitious shoots, (C) a rooted shoot, (D) transgenic plant growing on Florialite in plastic container, and (E) transgenic plants growing in a growth chamber. The age of shoots/plants was 13 weeks (B), 21 weeks (C), 12 months (D) and 34 months (E) after *Agrobacterium* transfection. (F) PCR analysis of wild type (WT) and transgenic lines (2513, 2521, 361, 362, 4331, 434, 437). Arrow indicates the 218 bp band specific to At*nii*. M, 100 bp ladder marker; N, negative control (no template DNA); and P, positive control (0.95 μg pIG121-AtNiR).

A. thaliana (Tanaka et al. 1994), spinach (Back et al. 1988), tobacco (Neininger et al. 1994a), tomato (Migge et al. 1998), birch (Friemann et al. 1992), and Scots pine (Neininger et al. 1994b) reportedly have a molecular mass of 63 kD. These are in line with the western blot
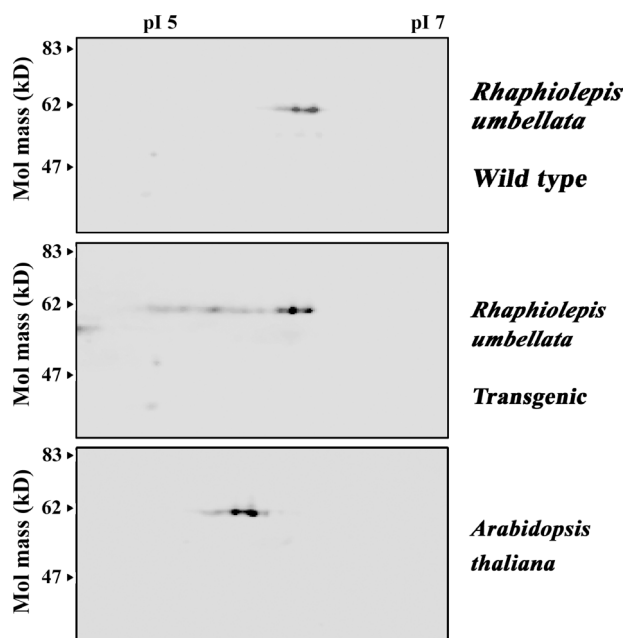
Figure 3. Western blot analysis of NiR proteins separated by 2-D gel electrophoresis. Proteins (20–100 μg) extracted from wild type and transgenic *R. umbellata* (line 2513) and *A. thaliana* (20 μg) were separated by two-dimensional gel electrophoresis. Gels were blotted with a polyclonal rabbit antibody raised against tobacco NiR. NiR signals were visualized by using a goat anti-rabbit IgG antibody conjugated with horseradish peroxidase and chemiluminescence reagent.

results obtained in the present study.

NO$_2$ uptake and assimilation

PCR- and western blot-positive transgenic plants were further cultured for 20 months under the fluorescent light followed by culture under the natural light in the confined glasshouse for one month. Plants (ca. 33 months after *Agrobacterium* transfection) were then subjected to fumigation with 200 ± 50 ppb $^{15}NO_2$ for one week under the natural light in November, 2005, after which leaves were harvested from the upper (1st to 4th from the shoot apex) and middle (5th to 8th) part of each plant (designated U and M, respectively), and analyzed separately for TNNO2 (reflecting uptake of NO$_2$) and RNNO2 (reflecting assimilation of NO$_2$).

The typical results of 9 transgenic lines (2512, 2513, 2514, 2521, 2523, 2524, 4331, 4332 and 4333) are shown in Figure 4. Also shown are results of wild type of "the same age" (ca. 33 months after germination). The value for the wild type represents the mean (with SD) of 3 individuals, while that for the transgenic line represents the mean of 3 replicates from a plant of each line (see legend for Figure 4) because to date only a single plant is available for each transgenic line. Transgenic lines are currently propagated by the cutting, and results will be published elsewhere.

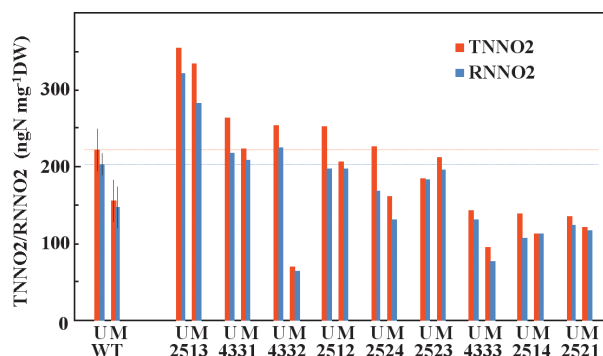A transgenic line (2513) was 1.6–2.0 times higher both in TNNO2 and RNNO2 than the wild type. This

Figure 4. TNNO2 and RNNO2 of non-transformed wild type and 9 transgenic lines (2512, 2513, 2514, 2521, 2523, 2524, 4331, 4332 and 4333). Wild type was ca. 33 months after germination and transgenic lines were ca. 33 months after *Agrobacterium* transfection (see Materials and Methods). Leaf samples were cut from the upper (1st to 4th leaves from the shoot apex) and middle (5th to 8th leaves) part of each plant (designated U and M, respectively). Data of wild type represent mean of 3 different trees (3 replicates for each plant) with SD. Data of transgenic lines represent mean of three replicates of an individual plant. Relative variation among replicates was less than 10%.

was true for the upper and middle part of the plants (see Figure 4). It is therefore concluded that the introduction and expression of chimeric At*nii* cDNA improves uptake and assimilation of NO$_2$ by *R. umbellata*. This result is in line with our previous report that the capability of *A. thaliana* to assimilate NO$_2$ is improved by introduction and expression of chimeric NiR cDNA from spinach. The remaining eight transgenic lines showed TNNO2 and RNNO2 similar to or even lower than the respective value of the wild type (see Figure 4). The reason for this is unclear. It is to be noted that leaves from upper part of the plant appeared to be higher in TNNO2 and RNNO2 in both wild type and transgenic lines except for the line 2523.

Our attempt to determine NiR enzyme activity of matured plants as used for the analysis of uptake and assimilation of NO$_2$ was not successful because the extraction of undenatured proteins from those plants was not successful, although we did succeed the extraction of denatured proteins from matured plants (as for the western blotting).

As reported previously (Erkin et al. 2003), all transgenic regenerants of *R. umbellata* obtained by particle bombardment-mediated transformation bearing a chimeric *nii* vector died during the acclimatization. However, no regenerants obtained in the present study died during acclimatization or other treatment except for hygromycin selection. The reason why regenerants obtained in our previous study died still remains unclear.

The present finding that the capability of *R. umbellata* to uptake and assimilate NO$_2$ was improved by 1.6–2.0 times strongly suggests that genetic modification is vital to improve the quality of *R. umbellata*. However, since this road side tree is ranked 151st among 217 taxa of

naturally occurring plants in the capability to assimilate NO_2 (Morikawa et al. 1998), much higher improvement of the capability of this species may be needed. Innovation of new promoters for this woody species will be a plausible approach to address such issue. In our previous study, overexpression of genes of nitrate reductase and glutamine synthase, that are respectively the first and third enzyme in the primary nitrate assimilation pathway, resulted in no appreciable improvement of the uptake and assimilation of NO_2 by plants (Takahashi et al. 2001). Metabolic engineering with Dof transcription factors (Yanagisawa et al. 2004) and enzymes involved in the metabolism of reactive nitrogen species (Sakamoto et al. 2002, 2003, 2004) will be another plausible approach to improve the capability of *R. umbellate* to mitigate atmospheric NO_2. We are currently investigating along this line. Results will be reported elsewhere.

Acknowledgements

References

Back E, Burkhart W, Moyer M, Privalle L, Rothstein S (1988) Isolation of cDNA clones coding for spinach nitrite reductase: Complete sequence and nitrate induction. *Mol Gen Genet* 212: 20–26

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248–254

Erkin OC, Takahashi M, Sakamoto A, Morikawa H (2003) Development of regeneration systems for *Rhaphiolepis umbellata* L. plants using particle bombardment. *Plant Biotech* 20: 145–152

Friemann A, Brinkmann K, Hachtel W (1992) Sequence of a cDNA encoding nitrite reductase from the tree *Betula pendula* and identification of conserved protein regions. *Mol Gen Genet* 231: 411–416

Kondo K, Takahashi M, Morikawa H (2002) Regeneration and transformation of a roadside tree *Pittosporum tobira* A. *Plant Biotechnol* 19: 135–139

Lloyd GB McCown BH (1980) Commercially feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot tip culture. *Ptoc Int Ptant Propagators Soc* 30: 421–427

Migge A, Carrayol E, Hirel B, Lohmann M, Meya G, Becker TW (1998) Two nitrite reductase isoforms are present in tomato cotyledons and regulated differently by UV-A or UV-B Iight and during plant development. *Planta* 207: 229–234

Mitsuhara I, Ugaki M, Hirochika H, Ohshima M, Murakami T, Gotoh Y, Katayose Y, Nakamura S, Honkura R, Nishiyama S, Ueno K, Mochizuki A, Tanimoto H, Tsugawa H, Otsuki Y, Ohashi Y (1996) Efficient promoter cassettes for enhanced expression of foreign genes in dicotyledonous and monocotyledonous plants. *Plant Cell Physiol* 37: 49–59

Morikawa H, Higaki A, Nohno M, Takahashi M., Kamada M, Nakata M, Toyohara G, Okamura Y, Matsui K, Kitani S, Fujita K, Irifune K, Goshima N (1998). More than a 600-fold variation in nitrogen dioxide assimilation among 217 plant taxa. *Plant Cell Environ* 21: 180–190

Morikawa H, Takahashi M, Kawamura Y (2003) Air Pollution Clean up Using Pollutant-Philic Plants– Metabolism of nitrogen dioxide and genetic manipulation of related genes. In: McCutcheon SC, Schnoor JL (eds) *Phytoremediation: Transformation and Control of Contaminants*. John Wiley and Sons, Hokoben, New Jersey, pp 765–786

Morikawa H, Takahashi M, Sakamoto A, Matsubara T, Arimura G, Kawamura Y, Fukunaga K, Fujita K, Sakurai N, Hirata T, Ide H, Nonoyama N, Suzuki H (2004) Formation of unidentified nitrogen in plants: an implication for a novel nitrogen metabolism. *Planta* 219: 14–22

Neininger A, Kronenberger J, Mohr H (1994a) Coaction of light, nitrate and a plastidic factor in controlling nitrite-reductase gene expression in tobacco. *Planta* 187: 381–387

Neininger A, Seith B, Hoch B, Mohr H (1994b) Gene expression of nitrite reductase in Scots pine (*Pinus sylvestris* L.) as affected by light and nitrate. *Plant Mol Biol* 25: 449–457

Sakamoto A, Sakurao S, Fukunaga K, Matsubara T, Ueda-Hashimoto M, Tsukamoto S, Takahashi M, Morikawa H (2004) Three distinct *Arabidopsis* hemoglobins exhibit peroxidase-like activity and differentially mediate nitrite-dependent protein nitration. *FEBS Lett* 572: 27–32

Sakamoto A, Tsukamoto S, Yamamoto H, Ueda-Hashimoto M, Takahashi M, Suzuki H, Morikawa H (2003) Functional complementation in yeast reveals a protective role of chloroplast 2-Cys peroxiredoxin against reactive nitrogen species. *Plant J.* 33: 841–851

Sakamoto A, Ueda M, Morikawa H (2002) *Arabidopsis* glutathione-dependent formaldehyde dehydrogenase is an *S*-nitrosoglutathione reductase. *FEBS Lett* 515: 20–24

Stockwell WR, Kramm G., Scheel HE, Mohnen VA, Seiler W (1997) Ozone formation, destruction and exposure in Europe and United States. In: Sandermann H, Wellburn AR, Heath RL (eds) *Forest Decline and Ozone: A Comparison of Controlled Chamber and Field Experiments*. Springer-Verlag, Berlin, pp 1–38

Takahashi M, Sasaki Y, Ida S, Morikawa H (2001) Nitrite reductase gene enrichment improves assimilation of NO_2 in Arabidopsis. *Plant Physiol* 126: 731–741

Takahashi M, Nakagawa M, Sakamoto A, Ohsumi C, Matsubara T, Morikawa H (2005) Atmospheric nitrogen dioxide gas is a plant vitalization signal to increase plant size and the contents of cell constituents. *New Phytol* 168: 149–154

Tanaka T, Ida S, lrifune K, Oeda K, Morikawa H (1994) Nucleotide sequence of a gene for nitrite reductase from *Arabidopsis thaliana*. *DNA Seq* 5: 57–61

Vaucheret H, Kronenberger J, Lepingle A, Vilaine F, Boutin JP, Caboche M (1992) Inhibition of tobacco nitrite reductase activity by expression of antisense RNA. *Plant J* 2: 559–569

Yanagisawa S, Akiyama A, Kisaka H, Uchimiya H, Miwa T. (2004) Metabolic engineering with Dof1 transcription factor in plants: Improvednitrogen assimilation and growth under low nitrogen conditions. *Proc Nat Acad Sci USA* 101: 7833–7838

Yoneyama T, Sasakawa H (1979) Transformation of atmospheric NO_2 absorbed in spinach leaves. *Ptant Cell Physiol* 20: 263–266