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Symbiotic *Bradyrhizobium japonicum* Reduces N_2O Surrounding the Soybean Root System via Nitrous Oxide Reductase

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N_2O reductase activity in soybean nodules formed with *Bradyrhizobium japonicum* was evaluated from N_2O uptake and conversion of ^{15}N-N_2O into ^{15}N-N_2. Free-living cells of USDA110 showed N_2O reductase activity, whereas a *nosZ* mutant did not. Complementation of the *nosZ* mutant with two cosmids containing the *nosRZDFYLX* genes of *B. japonicum* USDA110 restored the N_2O reductase activity. When detached soybean nodules formed with USDA110 were fed with ^{15}N-N_2O, they rapidly emitted ^{15}N-N_2 outside the nodules at a ratio of 98.5% of ^{15}N-N_2O uptake, but nodules inoculated with the *nosZ* mutant did not. Surprisingly, N_2O uptake by soybean roots nodulated with USDA110 was observed even in ambient air containing a low concentration of N_2O (0.34 ppm). These results indicate that the conversion of N_2O to N_2 depends exclusively on the respiratory N_2O reductase and that soybean roots nodulated with *B. japonicum* carrying the *nos* genes are able to remove very low concentrations of N_2O.

Nitrous oxide (N_2O) is a key atmospheric greenhouse gas that contributes to global climate change through radiative warming and depletion of stratospheric ozone (1, 24). Agricultural land is a major source through denitrification and nitrification (14, 35) and contributes significantly to the net increase in atmospheric N_2O (1, 34). Several attempts have been made to reduce the emission of N_2O from agricultural systems (34, 35).

The complete denitrification of nitrate by bacteria to dinitrogen (N_2) is generally an anaerobic respiratory process, where the last step is mediated by N_2O reductase (54). The corresponding structural gene is *nosZ* and is assembled in the *nosRZDFYL* gene operon (54). Several species capable of denitrification are also nitrogen-fixing bacteria, including rhizobia such as *Bradyrhizobium japonicum* (4, 6, 44, 49) and *Sinorhizobium meliloti* (7, 20). Indeed, genes responsible for denitrification have been found in rhizobial genomes (15, 23).

Earlier studies (19, 37) reported the evolution of ^{15}N-N_2 from ^{15}N-N_2O from sliced or detached soybean nodules. Recently, Velasco et al. (50) reported that *nosZ* and *nosR* insertion mutants of *B. japonicum* USDA110 accumulate N_2O when cultured microaerobically in the presence of nitrate. The *nosZ* gene was also expressed in soybean nodules (29). However, it has not yet been fully proved that N_2 evolution from N_2O by soybean nodules is mediated by N_2O reductase encoded by the *nosZ* gene in *B. japonicum* (4, 9, 38). The aims of this work were to confirm whether the *nos* gene cluster of *B. japonicum* is responsible for respiratory N_2O reduction to N_2 in nodules and to evaluate the capability of the nodulated roots to transform N_2O into N_2.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used are listed in Table 1. *Bradyrhizobium* cells were grown at 30°C in HM salt medium (8) supplemented with 0.1% arabinose and 0.025% (wt/vol) yeast extract (Difco, Detroit, MI), which is termed HM medium here. HM medium was further supplemented with 0.55 μM $Na_2MoO_4 \cdot 2H_2O$, 1 μM $FeCl_3$, and 1 μM $CuSO_4 \cdot 5H_2O$ (HMM medium) for the denitrification assay (44). *Escherichia coli* cells were grown at 37°C in Luria-Bertani medium (42). Antibiotics were added to the media at the following concentrations: for *B. japonicum*, 100 μg of tetracycline (Tc)/ml, 100 μg of spectinomycin (Sp)/ml, 100 μg of streptomycin (Sm)/ml, 100 μg of kanamycin (Km)/ml, and 50 μg of polymyxin B/ml; for *E. coli*, 15 μg of Tc/ml, 50 μg of Sp/ml, 50 μg of Sm/ml, 50 μg of Km/ml, and 100 μg of ampicillin/ml.

DNA manipulations. Isolation of plasmids, DNA ligation, and transformation of *E. coli* were performed as described by Sambrook et al. (42). DNA preparation and Southern hybridization were carried out as described previously (22, 30, 43).

Construction of a *B. japonicum* USDA110 *nosZ* mutant. A 4-kb BamHI DNA fragment identified from the genome sequence of *B. japonicum* USDA110 (23) was excised from BamHI-digested total DNA and inserted into the BamHI site of pTZ18R (Fig. 1A). The plasmid containing the fragment was selected by PCR amplification specific for the *nosZ* gene of *B. japonicum* USDA110 with primer 3 (5′-GAGGATCGTTTCGCATGAGCGACAGCGACAACAT-3′) and primer 4 (5′-CGCTCCCGATCAGACCGATTT-3′), in which the underlined nucleotides form the USDA110 *nosZ* sequence. From the cloned 4-kb DNA fragment, pK18mob-*nosZ*::Ω was constructed (Fig. 1A). Biparental mating was conducted on HM agar plates using *E. coli* S17-1 (47). Double crossover was verified by Southern hybridization with the 4-kb *nosRZD* fragment as a probe (Fig. 1A).

Genetic complementation by *nos* genes. Two cosmids, brc02856 and brc01733, carrying the *nos* gene cluster (*nosRZDFYLX*) were selected from the pKS800 cosmid library, which was constructed for the sequencing of *B. japonicum* USDA110 (23). The cosmids were introduced into *B. japonicum* strains by triparental mating via pRK2013 (39, 47, 52).

Gas chromatography. To determine N_2O concentrations, we used two ^{63}Ni electron capture detector gas chromatographs. In the quick N_2O analysis, 0.2 ml of sample gas was injected into a Shimadzu (Kyoto, Japan) GC-17A gas chromatograph with a CP-PoraBOND Q capillary column (internal diameter, 0.32 mm, length, 25 m; Varian, Palo Alto, CA). The temperatures of injection, the column, and the detector were 250, 65, and 300°C, respectively. In the sensitive N_2O analysis, 0.8 ml of sample gas was injected into a Shimadzu GC-14BPsE gas chromatograph equipped with tandem packed columns of a Porapak N (80/100 mesh; diameter, 0.3 mm; length, 1 m) and a Porapak Q (80/100 mesh; diameter, 0.3 mm; length, 3 m). The temperatures of injection, the column, and the detector were 80, 80, and 340°C, respectively. The carrier gas used for both N_2O analyses was composed of 5% (vol/vol) CH_4 in Ar. ^{15}N-N_2 was determined with a thermal conductivity detector gas chromatograph as described previously (44).

TABLE 1. Bacterial strains and cosmids used in this study

Strain or plasmid	Characteristics[a]	Source or reference
Strains		
Bradyrhizobium japonicum		
USDA110	Wild type; *nosZ*$^+$	23
T7	Field isolate in Tokachi, Hokkaido, Japan; *nosZ* natural mutant	32, 43
USDA110ΔnosZ	USDA110 *nosZ*::del/ins Ω cassette; Sm^r Sp^r	This study
Escherichia coli		
DH5α	*recA*; cloning strain	Toyobo Inc.
JM109	*recA*; cloning strain	Toyobo Inc.
HB101	*recA hsdR hsdM pro* Sm^r	5
S17-1	*hsdR* RP4-2(kan::Tn*7*)(tet::Mu); Sm^r Sp^r	47
Plasmids		
pRK2013	ColE1 replicon carrying RK2 transfer genes; Km^r	13
pTZ18R	Cloning vector; pMB1*ori*; Ap^r	46
pTZ18RnosZ	pTZ18R carrying 4.0-kb *nosZ* fragment; Ap^r	This study
pTZ18rRΔSacI-nosZ	*Sac*I-deleted pTZ18R carrying 4.0-kb *nosZ* fragment; Ap^r	This study
pHP45Ω	Plasmid carrying 2.1-kb Ω cassette; Sp^r Sm^r Ap^r	40
pK18mob	Cloning vector; pMB1*ori oriT*; Km^r	46
pK18mob-nosZ::Ω	pK18mob carrying 4.0-kb *nosZ* fragment; Km^r Sm^r Sp^r	This study
Cosmids		
pKS800	Derivative of broad-host-range cosmid pLAFR1; IncP Tc^r	17, 23
brc02856	pKS800 carrying *nos* gene cluster[b]; Tc^r	23
brc01733	pKS800 carrying *nos* gene cluster[b]; Tc^r	23

[a] Ap^r, ampicillin resistant; Tc^r, tetracycline resistant; Km^r, kanamycin resistant; Sm^r, streptomycin resistant; Sp^r, spectinomycin resistant.
[b] See Fig. 1.

For the acetylene reduction assay, ethylene was measured by a flame ionization detection gas chromatograph, as described previously (12).

N_2O uptake by free-living cells. *B. japonicum* cells were washed with HMM medium by centrifugation (5,000 × *g*, 15 min, 4°C), and the cell density was adjusted to 6×10^9 cells/ml (33). The cell suspension (4.6 ml, 6×10^9 cells/ml) was introduced into a 123-ml airtight vial (V-100; Nichiden-Rika Glass, Kobe, Japan). After the gas phase was replaced with N_2, N_2O gas was introduced into the vial at a final concentration of approximately 0.1% (vol/vol).

^{15}N-N_2 emission from ^{15}N-labeled N_2O by free-living cells. *B. japonicum* cells (7 ml; 6×10^9 cells/ml) were introduced into a 14-ml airtight specimen vial. After the N_2 gas replacement, ^{15}N-N_2O (^{15}N, >98 atom%; Cambridge Isotope Laboratories Inc., Andover, MA) was introduced. After incubation at 30°C for 67 h, the concentration of ^{15}N-N_2 in the gas phase was determined by TCD gas chromatography (44).

Real-time PCR. Beacon Designer software (Premier Biosoft International, Palo Alto, CA) was used to design the *nosZ* primer set (nosZ2F, 5′-GTCGTC ATCTTCAACCTCAAG; nosZ2R, 5′-TATTCATGCCATGCGGATTG). Total RNA was prepared as described previously (53). Quantitative reverse transcription-PCR analysis was carried out by the i-Cycler optical system (Bio-Rad Laboratories, Inc., Tokyo, Japan) as described previously (53).

Plant cultivation and inoculation. The surface-sterilized soybean seeds (*Glycine max* cv. Enrei) were germinated and transplanted to a Leonard jar (25, 48), which contained sterile vermiculite and nitrogen-free sterilized nutrient solution (31, 39). The cell suspension of *B. japonicum* (1 ml) was used for inoculation at 1×10^7 cells per seed. For N_2O-feeding experiments, plants were grown in a growth chamber (LH200; Nippon Medical & Chemical Industries, Tokyo, Japan). For the nitrogen fixation experiment, soybeans were grown in pots (1 liter) filled with vermiculite in a phytotron (Koitotron type KC; Koito Industries, Tokyo, Japan). A nitrogen-free sterilized nutrient solution was periodically supplied to the pots (31, 39).

N_2O uptake, ^{15}N-N_2 emission, and ^{15}N incorporation by nodules. After the nodulated roots of 45-day-old soybean plants were washed with water, the nodules were detached from the roots and weighed. The nodules were introduced into a 15-ml airtight vial. After the vials were sealed, ^{15}N-N_2O was injected. After the gas measurements, the nodules were dried at 80°C for 3 days and then powdered with a mortar and pestle. The nodule samples were subjected to tracer ^{15}N and total-N analyses by an automatic gas chromatograph-mass spectrometer (EA1110-DELTA^{plus} Advantage ConFloIII system, Thermo Electron Co., Bremen, Germany).

The whole root system of 32-day-old soybean plants was inserted into a 51-ml test tube (19.4 mm in diameter by 176 mm in height) containing 20 ml nitrogen-free nutrient solution (31, 39). The test tube was sealed with a silicon rubber stopper with a gas sampling port and a hole in the center. This system enclosed the whole root system of intact soybeans in the test tube, leaving the aerial parts in air. Then N_2O gas was injected into the closed test tube.

Nitrogen fixation and plant growth. The nitrogen-fixing activity of nodules was examined by acetylene reduction assays in an incubation jar (300-ml capacity) containing the excised root system. Acetylene gas was injected into the jar at 15% (vol/vol), and the roots were incubated for 60 min at 25°C. Separated shoot, roots, and nodules were weighed.

Bacteroid antibiotic test. Nodules were removed from soybeans 18 days after inoculation with T7 or T7 with brc01733. Homogenates of surface-sterilized nodules were plated on HM agar medium with and without tetracycline.

RESULTS

Construction of the *nosZ* deletion mutant. To examine whether the *nos* gene cluster of *B. japonicum* was responsible for N_2O reductase activity, we constructed a *nosZ* deletion mutant of *B. japonicum* USDA110 (Table 1). After deletion of most of the *nosZ* gene from the cloned DNA fragment containing *nosRZD*, the Ω cassette was inserted into the position (Fig. 1). When free-living cells of USDA110 and USDA110ΔnosZ were anaerobically incubated in gas containing 0.1% (vol/vol) N_2O, the parent strain consumed N_2O, whereas the mutant did not show N_2O uptake (Fig. 2A). To further examine the N_2O reductase reaction, ^{15}N-N_2O was supplied to cultures of *B. japonicum* USDA110 and USD A110ΔnosZ. Wild-type USDA110 stoichiometrically produced ^{15}N-N_2 from ^{15}N-N_2O, whereas USDA110ΔnosZ did not (data

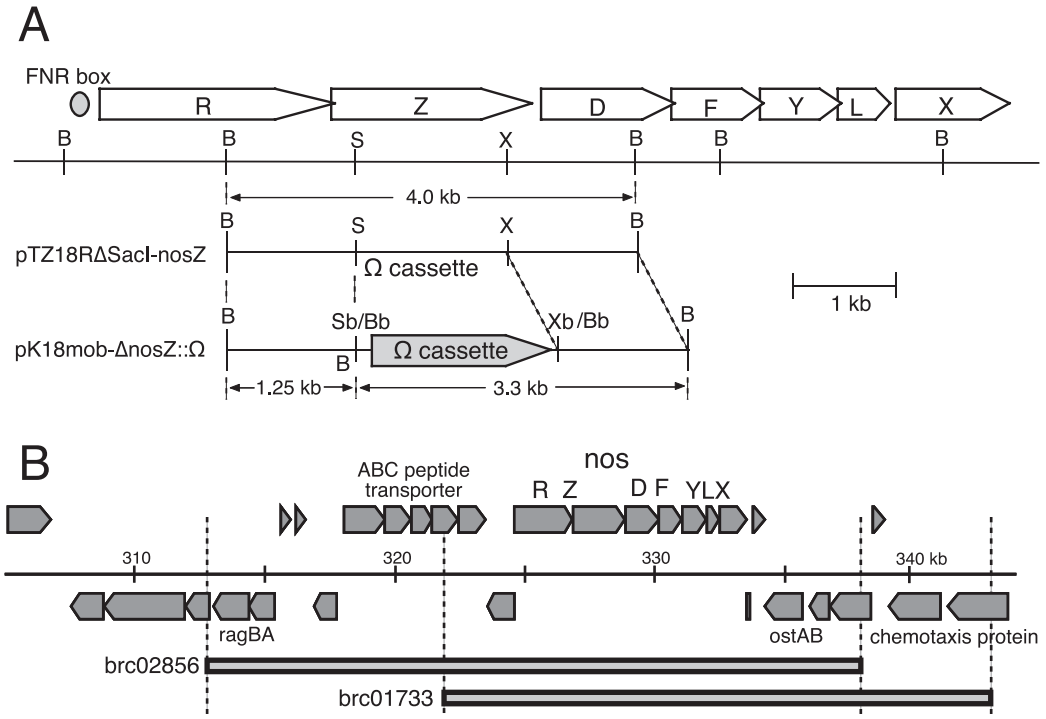

FIG. 1. Construction of a *nosZ* mutant (A) and complementation cosmid inserts (B) of *Bradyrhizobium japonicum* USDA110. (A) Cloned fragments in pTZ18RΔSacI-nosZ and pK18mob-ΔnosZ::Ω are shown alongside the physical map of the *nos* gene cluster of *B. japonicum* USDA110. B, BamHI; S, SacI; X, XhoI; Sb, SacI blunt end; Bb, BamHI blunt end; Xb, XhoI blunt end. During blunt-end ligation, the Sb/Bb site produced a BamHI site in pK18mob-ΔnosZ::Ω. Upstream of *nosR* lies a consensus sequence of an FNR box (TTGAT-N_4-ATCAA) (50). The cloned 4-kb DNA fragment containing the *nosZ* gene in pTZ18R-nosZ was reconnected to pTZ18RΔSacI, which was produced by blunting of the SacI site of pTZ18R, and pTZ18RΔSacI-nosZ was generated. The Ω cassette, which was excised from pHP45Ω (40), digested with BamHI, and blunt ended, was inserted into the blunt-ended SacI and XhoI sites of pTZ18RΔSacI-nosZ to generate pTZ18RΔSacI-nosZ::Ω. Finally, the fragment containing the *nosZ* and Ω cassette fusion was inserted between the SmaI and XbaI sites of pK18mob to generate pK18mob-nosZ::Ω. (B) Positions covered by two cosmid inserts, brc02856 and brc01733 (Table 1), in the genome of *B. japonicum* USDA110 (http://www.kazusa.or.jp/rhizobase/) (23).

not shown). These results suggest that *nosZ* of *B. japonicum* USDA110 is responsible for the N_2O reductase activity that converts N_2O to N_2.

Complementation of USDA110ΔnosZ with *nos* genes. To further examine the validity of *nosZ*-dependent N_2O reductase activity, two cosmids containing the *nos* gene cluster were introduced into USDA110ΔnosZ (Fig. 1B). The transconjugants were anaerobically incubated in the presence of 0.1% (vol/vol) N_2O and tetracycline, and USDA110 and USDA110ΔnosZ carrying the pKS800 vector were used as controls (Fig. 2B). USDA110 and USDA110ΔnosZ carrying pKS800 (Fig. 2B) showed N_2O uptake profiles similar to those of wild-type USDA110 and USDA110ΔnosZ, respectively (Fig. 2A). However, the two cosmids in Fig. 1B restored strong N_2O uptake (Fig. 2B). The amino acid sequences of *B. japonicum* *nosRZDFYLX* (23, 50) have a high degree of similarity to those

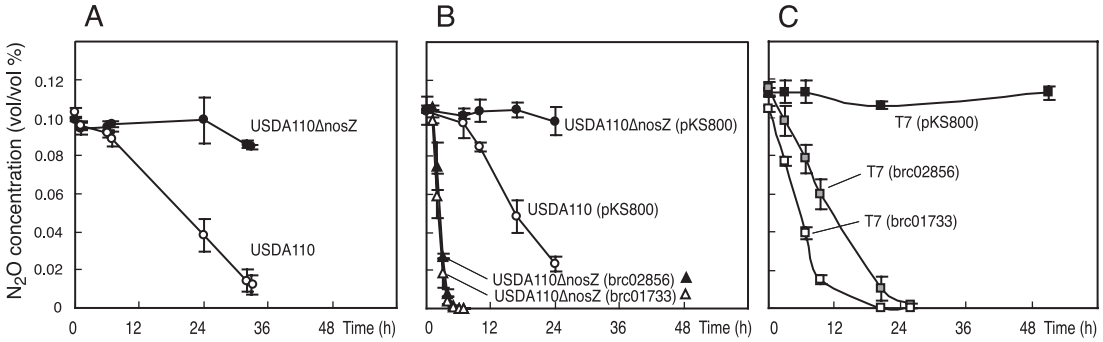

FIG. 2. N_2O uptake by free-living cells of the *nosZ* mutant (A) and *nos* complementation of USDA110ΔnosZ (B) and T7 (C). pKS800 is the cosmid vector used for complementation (17). Two cosmid clones, brc02856 and brc01733, have inserts containing *nos* genes of USDA110 (Table 1; Fig. 1). N_2O reductase activities (means ± SDs) estimated from maximum rates of N_2O uptake were 4.6 ± 0.2 [USDA110(pKS800)], 22.9 ± 0.7 [USDA110ΔnosZ(brc02856)], and 23.6 ± 1.9 N_2O [USDA110ΔnosZ(brc01733)] μmol h^{-1} 10^7 $cells^{-1}$.

TABLE 2. Growth and N_2-fixing activity of soybean plants inoculated with *Bradyrhizobium japonicum* USDA110 or the *nosZ* mutant[a]

Strain	Growth (g [fresh wt] plant^{-1})			N_2-fixing activity (C_2H_2-reducing activity) (μmol h^{-1} g of nodule [fresh wt]$^{-1}$)
	Shoot	Root	Nodule	
USDA110	10.8 ± 0.7	4.8 ± 0.4	1.0 ± 0.1	10.3 ± 1.3
USDA110ΔnosZ	10.4 ± 0.9	4.5 ± 0.4	0.9 ± 0.1	9.0 ± 2.0
Uninoculated	6.9 ± 0.2	5.5 ± 0.2	0	0

[a] Values for growth and N_2-fixing activity are shown as means ± SDs, with six replications.

of other N_2O reductases from *Pseudomonas stutzeri* (21). Both *B. japonicum* cosmids contain *nosRZDFYLX* and adjacent genes (Fig. 1B). Their addition to USDA110ΔnosZ produced similar activities of N_2O reductase, as indicated by N_2O uptake (Fig. 2B). Moreover, the introduction of a cosmid (brc02856 or brc01733) into USDA110ΔnosZ restored the strain's ability to convert ^{15}N-N_2O into ^{15}N-N_2 (data not shown). Thus, the complementation experiments confirmed that the *nos* gene cluster per se is responsible for N_2O reductase activity in *B. japonicum* USDA110.

Expression of *nosZ*. Strain USDA110ΔnosZ with the two cosmids containing the *nos* cluster rapidly and strongly absorbed N_2O, much more than the parent strain USDA110 containing the pKS800 vector (Fig. 2B). When the N_2O reductase activity was estimated from the maximum rate of N_2O uptake in Fig. 2B, the activities of USDA110ΔnosZ containing brc02856 and brc01733 (22.9 ± 0.7 and 23.6 ± 1.9 N_2O μmol h^{-1} 10^7 cells^{-1}) were five times that of wild-type USDA110 (4.6 ± 0.2 N_2O μmol h^{-1} 10^7 cells^{-1}). In addition, the lag time for the induction of N_2O reductase activity in the wild-type USDA110(pKS800) disappeared in the cosmid-complemented USDA110ΔnosZ.

Because we used pKS800, a derivative of cosmid pLAFR1 (RK2), as a vector for the *nos* complementation (17), it is possible that the cosmid introduction increases the copy number and thus results in higher expression of *nos* genes. USDA110ΔnosZ carrying brc01733 expressed the *nosZ* gene at 5-times-higher levels on the basis of total RNA than USDA110ΔnosZ carrying the pKS800 vector 1 h after anaerobic incubation in the presence of 0.1% (vol/vol) N_2O. This result suggests that the higher expression of the *nos* genes enhanced N_2O reductase activity and shortened the lag time for induction in USDA110ΔnosZ with the *nos* cosmids.

N_2O uptake and N_2 emission by nodules. Denitrification genes of *B. japonicum* are expressed in a microaerobic state in both legume nodules and free-living bacteria via the *fixLJ* and *fixK$_2$* regulatory cascades (3, 28). Mesa et al. (29) reported that the *nir*, *nor*, and *nos* denitrification genes in *B. japonicum* were expressed in soybean root nodules. We thus examined the nodulation phenotypes of the *nosZ* mutant. The nodule weight and nitrogen-fixing activity of USDAΔnosZ showed no significant differences from those of USDA110 (Table 2), indicating that *nosZ* mutation does not alter symbiotic nitrogen fixation. This agrees with the results of a report on the nodulation kinetics (29) of a *nosZ* insertion mutant of *B. japonicum* (50).

To evaluate the extent of N_2O uptake and N_2 emission by N_2O reductase in soybean nodules, ^{15}N-N_2O was supplied to detached nodules formed with the *nosZ* mutant or the parent strain (Fig. 3). Soybean nodules formed with USDA110 rapidly absorbed ^{15}N-N_2O and emitted ^{15}N-N_2 outside the nodules, whereas nodules formed with the *nosZ* mutant did not mediate the conversion of ^{15}N-N_2O to ^{15}N-N_2 at all (Fig. 3).

Because the transformation of ^{15}N-N_2O into ^{15}N-N_2 was likely stoichiometric (Fig. 3), we determined the amount of fixed ^{15}N in the nodules and made a ^{15}N balance sheet for ^{15}N-N_2O exposure. ^{15}N atoms of ^{15}N-N_2O were distributed 98.5% ± 0.1% (mean ± standard deviation [SD]) outside the nodules as ^{15}N-N_2 and 1.5% ± 0.1% (mean ± SD) fixed in the nodules. Thus, most N_2 molecules produced by N_2O reductase in bacteroids were released outside the nodules, and nitrogenase was not able to utilize most of them as a substrate for nitrogen fixation. This is probably due to the diffusion of N_2 into various compartments for N_2O reductase and nitrogenase. The apparent rate of N_2O uptake by USA110 nodules under about 3% N_2O (Fig. 3) was estimated to be 7.8 μmol h^{-1} per g (fresh weight) of nodule; this was comparable with the acetylene-reducing activity (Table 2). These results indicate that the conversion of N_2O into N_2 in soybean nodules depends on the function of the *nos* gene cluster in *B. japonicum*.

N_2O uptake by intact soybeans nodulated with USDA110. To examine the extent of N_2O concentrations that the nodu-

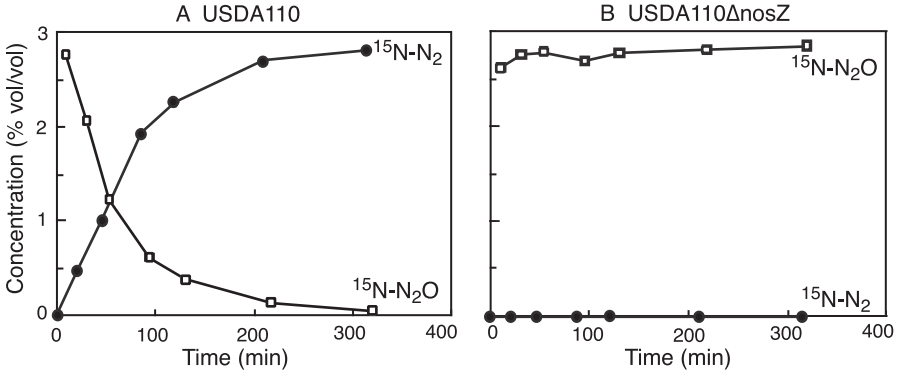

FIG. 3. ^{15}N-N_2O uptake and ^{15}N-N_2 emission by detached soybean nodules formed with *Bradyrhizobium japonicum* USDA110 (A) and the *nosZ* mutant USDA110ΔnosZ (B). ^{15}N-labeled N_2O was supplied to vials (15 ml) containing soybean nodules (1.6 to 1.8 g). Five independent experiments gave similar results.

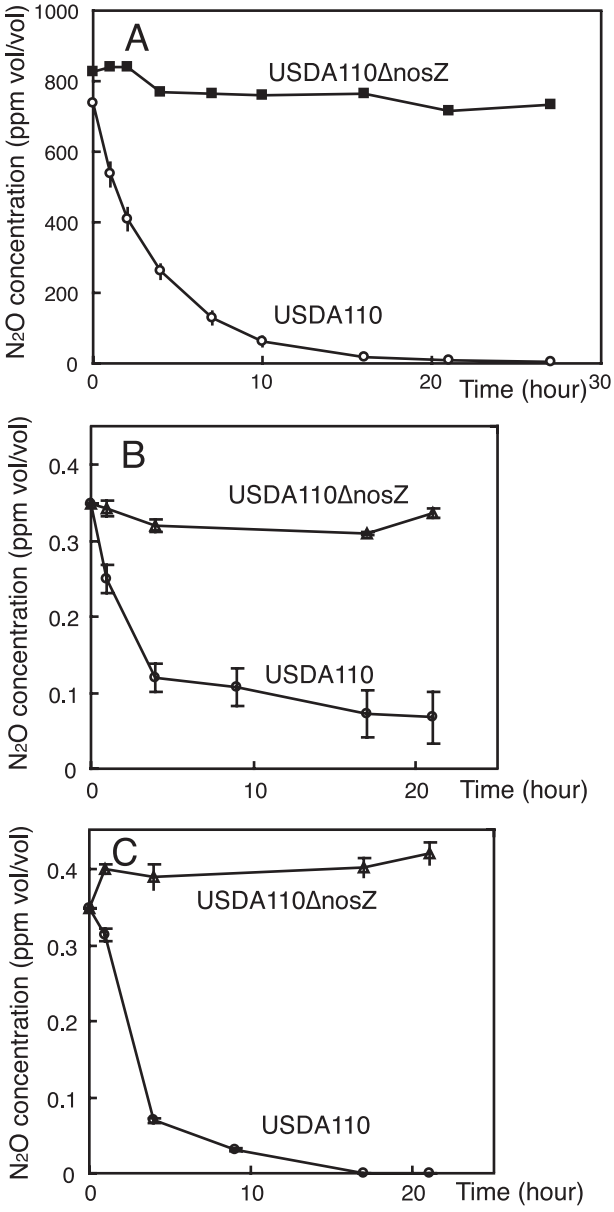

FIG. 4. N_2O uptake from root systems of intact plants (A and B) and decapitated root systems (C) inoculated with the wild-type strain or the *nosZ* mutant of *Bradyrhizobium japonicum* USDA110. Initial gas phases were adjusted to approximately 800 ppm (A) (intact plant) and 0.34 ppm (B [intact plant] and C [decapitated root system]) of N_2O in air. Soybean plants that harbored 0.26 to 0.30 g (A), 0.15 to 0.22 g (B), or 0.18 to 0.36 g (C) of nodules (fresh weight per plant) were examined by a test tube system (see the text). Triplicate determinations were carried out, except that data for USDA110ΔnosZ inoculation in panel A show means of duplicate determinations. Error bars, SDs.

lated roots can take up, the whole root systems of intact plants nodulated by USDA110 or the *nosZ* mutant were enclosed. After the introduction of N_2O gas, N_2O concentrations around the whole root system were monitored (Fig. 4A and B). At an initial N_2O concentration of 800 ppm (Fig. 4A), the root systems nodulated by USDA110 absorbed N_2O, whereas the *nosZ* mutant-inoculated roots did not take up N_2O at all. After a 21-h incubation, the N_2O concentration reached 4 to 8 ppm around the roots nodulated with USDA110 (Fig. 4A). Thus, the initial concentrations of N_2O were gradually decreased to 50, 5, 1, and 0.34 ppm. Surprisingly, the root systems nodulated by USDA110 were able to take up 0.34 ppm N_2O, reducing the concentration to 0.11 ± 0.02 ppm (mean ± SD) 22 h after the start of incubation (Fig. 4B). When a shoot-decapitated root system was completely enclosed in the test tube with a rubber stopper to avoid gas leakage, the N_2O concentration around the root system nodulated by USDA110 decreased to below the detection limit (<0.02 ppm) by 17 h (Fig. 4C).

Generally, the N_2O concentration in soils (0.36 ppm to 8,300 ppm) is higher than that in ambient air (0.31 ppm) (1, 26, 34, 45). Therefore, these results strongly suggest that soybean nodules formed with *B. japonicum* carrying *nos* genes potentially scavenge N_2O gas in soil.

Introduction of *nos* genes into a naturally occurring *nosZ*-deficient strain. Strains of *B. japonicum* can apparently be classified into three denitrification types: full denitrifiers (up to N_2), truncated denitrifiers (up to N_2O), and nondenitrifiers (44, 49). Their incidence depends on the field site and the phylogeny (44). We tested whether introduction of the *nos* gene cluster into the naturally occurring *nosRZD*-deficient strain T7 would induce N_2O reductase activity. Introduction of brc02856 or brc01733 into strain T7 induced N_2O uptake, but T7 carrying the cosmid vector did not show such activity (Fig. 2C).

To test whether strain T7 carrying brc01733 exhibited N_2O reductase activity in nodules, this strain was inoculated into soybean plants. However, the instability of cosmid brc01733 interfered with this experiment: Bacteroid cells from the nodules lost cosmid brc01733 during nodule development, as suggested by plate counts of tetracycline-resistant colonies ($<10^{-6}$). Because similar instability of pLAFR1 derivatives has been reported (10), other strategies are needed for the symbiotic testing of strain T7 carrying *nos* genes.

DISCUSSION

The *nosZ* mutant of USDA110 was not able to convert $^{15}N\text{-}N_2O$ to $^{15}N\text{-}N_2$ in nodules (Fig. 3) or as free-living cells (Fig. 2) and continued to produce nitrogen-fixing nodules (Table 2). Thus, the evolution of $^{15}N\text{-}N_2$ from $^{15}N\text{-}N_2O$ in soybean nodules is due exclusively to N_2O reductase, which is encoded by *nosZ*.

An important finding of this work is that N_2O reductase expressed in *B. japonicum* can take up very low concentrations of N_2O from outside of the nodules, equivalent to the natural concentration of N_2O in air (approximately 0.31 ppm). Probably this indicates that N_2O reductase has a high affinity for N_2O as a substrate (54), and the gas movement through the tissues of the nodules does not severely interfere with the enzyme property.

Agricultural soil is a major source of the N_2O emissions that contribute to global warming (1, 34). The N_2O concentration in the soil gas phase fluctuates with water, organic matter, and nitrogen fertilizer but is generally higher than that of ambient air (1, 14, 18, 24, 26, 45). The results of N_2O uptake by soybean nodules (Fig. 3 and 4) suggest that soybean nodules formed with *B. japonicum* carrying *nos* genes scavenge N_2O in soil, thus lessening N_2O emission to the atmosphere from soybean fields.

However, several groups have reported that cultivation of legume crops often enhances N_2O emission from fields of alfalfa (11), soybean (51), white clover (36), and Bengal gram (16). Thus, we want to discuss the N_2O paradox of legume root nodules for those results and ours. One possible explanation for the N_2O paradox is that the nitrate absorbed via legume roots and nodule surfaces from the soil solution might be transported in nodules (27, 41), which would emit N_2O from nitrate in bacteroids that lack *nos* genes. Another explanation is that N_2O is produced by rhizosphere organisms from degrading nodules (51), where fixed nitrogen would become a source of N_2O emission irrespective of the *nos* genes in the bacteroids. If either assumption is correct, the presence of *nos* genes in rhizobia ought still to decrease N_2O levels in the rhizosphere and N_2O emission from field soils.

Rhizobial inoculation technology focuses on increasing legume yields. The *nos* gene-dependent transformation of N_2O into N_2 in soybean nodules prompted us to enhance this activity in bradyrhizobial inoculants—a technology that could be environmentally friendly. The introduction of cosmids carrying *nos* genes enhanced *nosZ* transcription and N_2O reductase activity (Fig. 2B), suggesting that up-regulation of *nos* gene expression would result in the enhancement of N_2O reductase activity in *B. japonicum*.

Natural populations of *B. japonicum* that lacks N_2O reductase activity were often dominant in soybean fields (44). To generate bradyrhizobial inoculants with high N_2O reductase activity in soybean nodules, one can adopt several strategies: (i) introduction of *nos* genes by using stable vectors or chromosome integration with strong promoters, (ii) selection of natural isolates with high N_2O reductase activity, and (iii) simple use of USDA110 and other wild-type strains carrying *nos* genes as inoculants. Denitrification steps, including N_2O reduction, are anaerobic respiration processes. It is thus important to examine the allocations of electrons to O_2 and N_2O in soybean nodules, because energy metabolism in bacteroids is supported by cbb_3 terminal oxidase, which has a high affinity for O_2 and adapts to a microoxic environment in root nodules (2).

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