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Evaluation of the Nitrogen-fixing Ability of Endophytic Clostridia based on Acetylene Reduction and Reverse Transcription-PCR Targeting the *nifH* Transcript and Ribosomal RNA

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To examine whether plant-derived clostridia and their consortium fix nitrogen in plants, an inoculation system was developed using the grass *Miscanthus sinensis* under aseptic conditions. Among 13 clostridial strains previously isolated from *M. sinensis*, *Clostridium* sp. strain Kas107-1 was selected as the best colonizer in the plant with the non-diazotrophic bacterium *Entrobacter* sp. strain B901-2. Nitrogen-fixing (acetylene-reducing) activity was not observed in the plants inoculated with Kas107-1 without a carbon source. On the other hand, nitrogen-fixing activity was detected when carbon sources were supplied to the roots. To confirm the endophytic nitrogen-fixing activity, we cloned *nifH* genes and monitored their expression in strain Kas107-1. Although this bacterium possessed at least two copies of *nifH* (*nifH1* and *nifH2*), the *nifH1* transcript was exclusively detected in free-living cells and endophytic cells in the plants by reverse transcription (RT)-PCR analysis. RT-PCR analysis of ribosomal RNA suggested the endophytic colonization of the plants by Kas107-1. These results indicate that *Clostridium* sp. strain Kas107-1 can potentially fix nitrogen in plants. A RT-PCR analysis targeting both functional gene transcripts and the ribosomal RNA molecule is useful for researching endophyte ecology, because their function and colonization in plants can be examined simultaneously with a single preparation of RNA.

Key words: *Clostridium*, endophyte, reverse transcription-PCR, *nifH* expression, 16S rRNA

Biological nitrogen fixation is the conversion of atmospheric nitrogen into ammonia by symbiotic, associative and free-living bacteria, which is of tremendous importance to the environment and agriculture[6]. The availability of nitrogen is frequently the limiting factor for plant growth in terrestrial ecosystems[11]. Nitrogen fixation is unique to *Bacteria* and *Archaea*[11]. Animals and plants may profit from biologically fixed nitrogen directly when they are in association or symbiosis with nitrogen-fixing prokaryotes, or indirectly after the mineralization of these bacteria[11]. One of the best-studied interactions is the root nodule symbiosis between rhizobia and legumes. However, non-leguminous plants include some of the most important crops in the world, such as rice and wheat, and belong to the *Gramineae*, which do not naturally form these specialized symbiotic structures[11]. Endophytes are microorganisms that can colonize living plant tissues without harming the host[21]. It has been reported that *Herbaspirillum*[7,9,13,30], *Acetobacter*[12,24], and *Azoarcus*[10] were able to fix nitrogen inside plants. However, their contribution to the nitrogen economy in plants has remained to be elucidated.

Minamisawa *et al.*[15] have recently observed the existence of anaerobic nitrogen-fixing consortia (ANFICOs), consisting of nitrogen-fixing clostridia and various non-diazotrophic bacteria, in gramineous plants. Many ANFICOs consisting of clostridia and non-diazotrophs have been isolated from various gramineous plants[15]. The genus *Clostridium* belongs to the obligate anaerobic Gram-positive bacteria and is composed of heterogeneous phylogenetic groups[4]. Although some species in this genus are known to be nitrogen-fixing bacteria[26], little attention had been paid hitherto to the role of *Clostridium* species and their microbial consortium as endophytes inside plants. Thus, it is crucial

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to evaluate nitrogen fixation by this novel anaerobic endophyte in plants.

The aim of this study is to examine whether plant clostridia and their consortium fix nitrogen in plants. To this end, a grass, *Miscanthus sinensis*, was inoculated with clostridia isolated from plants. The colonization and nitrogen fixation of the inoculated plants were intensively evaluated using MPN counts, the acetylene reduction assay and detection of the *nifH* transcript/16S rRNA of the inoculated clostridia.

Materials and Methods

Bacterial strains, plasmid, plant materials and growth media

The bacterial strains and plasmid used in this study are listed in Table 1. Clostridial strains and non-diazotrophic bacteria were cultured separately in a modified Rennie (RMR) broth medium[7,22] under anaerobic and aerobic conditions, respectively, as described previously[15,29]. *Escherichia coli* DH5α was cultured in a nutrient broth (NB) medium. Seeds of *Miscanthus sinensis* collected at Sendai City in 2002 were used as described previously[29]. Semi-solid agar containing nutrients (plant growth medium) was used for the cultivation of the grass as described previously[29].

Plant cultivation and inoculation with clostridia and accompanying non-diazotrophs

Dehulled seeds of *Miscanthus sinensis* were sterilized with a 1% sodium hypochlorite (NaOCl) solution for 5 min, then rinsed a few times with sterile distilled water. The seeds were washed 2 times with sterile distilled water for 15–20 min each time. The sterilized seeds were sown on semi-solid nutrient agar in a plant box with a lid, and grown in a growth chamber as described previously[29]. Clostridia and non-diazotrophs were grown separately in 9 ml of RMR broth medium for 24 h at 30°C with shaking anaerobically and aerobically, respectively[15,29]. A mixed broth culture was used for the inoculation of plants. Plants approximately 5 cm in height at the 4-leaf stage (4 weeks after sowing) were inoculated with the mixed culture by vacuum infiltration as described previously[29].

Bacterial population in surface-sterilized grass assessed by MPN counts

To estimate the population of bacteria inside the plants, 7 days after inoculation (DAI), the plants of *M. sinensis* were surface-sterilized with a 1% NaOCl solution for 30 sec, and

Table 1. Bacterial strains and plasmid used in this study

Strains and plasmid	Description[a]			Source or reference
Clostridia				
Clostridium sp. strain U201	PG I,	*Miscanthus sinennsis* stem,	Unzen lahar, Japan	15
Clostridium sp. strain U101-b	PG I,	*M. sinennsis* root,	Unzen lahar, Japan	15
Clostridium sp. strain Sukash-1	PG I,	*M. sinennsis* stem,	Unzen lahar, Japan	15
Clostridium sp. strain Kas107-2	PG II,	*M. sinennsis* stem,	Kashimadai, Japan	15
Clostridium sp. strain Kas107-1	PG IV,	*M. sinennsis* shoot,	Kashimadai, Japan	15
Clostridium sp. strain Kas203-1	PG IV,	*M. sinennsis* root,	Kashimadai, Japan	15
Clostridium sp. strain Kas104-4	PG IV,	*M. sinennsis* leaf,	Kashimadai, Japan	15
Clostridium sp. strain Kas202-1	PG V,	*M. sinennsis* stem,	Kashimadai, Japan	15
Clostridium sp. strain UsS102-1	PG V,	*M. sinennsis* root,	Usu lahar, Japan	15
Clostridium sp. strain UsS101-2	PG V,	*M. sinennsis* shoot,	Usu lahar, Japan	15
Clostridium sp. strain Kas201-1	PG V,	*M. sinennsis* leaf,	Kashimadai, Japan	15
Clostridium sp. strain Kas106-4	PG V,	*M. sinennsis* root,	Kashimadai, Japan	15
Clostridium sp. strain UsS101-1	PG V,	*M. sinennsis* shoot,	Usu lahar, Japan	15
Non-diazotrophic bacteria[b]				
Enterobacter sp. strain B901-2	Isolate with *Clostridium* sp. strain B901-1b			15
Kas103-2	Isolate with *Clostridium* sp. strain Kas107-1			15
Kas105-5a	Isolate with *Clostridium* sp. strain Kas107-1			15
Kas105-6	Isolate with *Clostridium* sp. strain Kas107-1			15
Kas107-3	Isolate with *Clostridium* sp. strain Kas107-1			15
Kas107-4	Isolate with *Clostridium* sp. strain Kas107-1			15
Escherichia coli DH5 α	*recA*, cloning strain			Toyobo Co. Ltd.
pCR 2.1 Vector	Cloning vecter, Ap^r			Invitrogen Co.

[a] PG I ,Phylogenetic Group I; PG II, Phylogenetic Group II; PG IV, Phylogenetic Group IV; PG V, Phylogenetic Group V.[15]
[b] The genera of Kas103-2, Kas105-5a, Kas105-6, Kas107-3 and Kas107-4 were not determind. Ap^r, ampicillin resistant.

then rinsed three times with sterile distilled water. The plants were then shaken in 0.85% sterile saline for a few minutes, before being rinsed a several times with sterile distilled water. The plants were weighed and macerated in 0.85% sterile saline. The macerate was serially diluted and inoculated into a semi-solid RMR in test tubes. After incubation for 3 days at 30°C, the population of clostridia and non-diazotrophs was estimated by a most probable numbers (MPN) count based on acetylene reduction activity (ARA) and growth, respectively, as described previously[16].

Acetylene reduction assay

The nitrogenase activity of the plants with and without inoculation was evaluated by assaying acetylene-reducing activity (ARA). Ten days after the inoculation, the plants were washed with sterile distilled water, and introduced into a 27-ml test tube sealed with a double rubber stopper. The test tubes contained 9 ml of inorganic nutrient solution[29] with and without carbon sources (Sodium lactate 0.03% (w/v), Sucrose 0.5% (w/v), Mannitol 0.3% (w/v), and Sodium malate 0.2% (w/v)) (Fig. 2B). Pure acetylene was injected at a final concentration of 10% (v/v), and incubated for 41 h at 30°C. The ethylene concentration in a 1-ml gas sample from each tube was determined using a Simadzu GC-18A gas chromatograph equipped with a flame ionization detector and a Porapack N column as described previously[7].

Cloning and sequencing of the nifH gene

DNA manipulation, the isolation of plasmid DNA and the bacterial transformation of *E. coli* were performed as described by Sambrook and Russell[23]. *Clostridium* sp. strain Kas107-1 was cultured in RMR medium at 30°C overnight. The 10 ml (10^7 cells ml^{-1}) culture was centrifuged at 8,600×g for 10 min at 4°C. The bacterial pellet was suspended in 567 µl of TE buffer solution containing 0.25 M sucrose and 1 mg ml^{-1} of lysozyme. Total DNA was prepared as described by Ausubel *et al.*[1]. PCR was performed with an Ex *Taq* polymerase (Takara, Kyoto, Japan) according to the manufacturer's instructions. We used 100 ng of total DNA as the template in 50 µl of reaction mixture. The PCR primers CnifH-1F (5'-GCGATTTATGGAAAGGGTGG-3') and CnifH-2R (5'-AAGCCACCGCAAACAACGTC-3') were used to amplify a fragment (383-bp) of the *nifH* gene, and were designed by the modification of 19F and 407R[27]. The PCR cycling parameters were 94°C for 30 sec, and 28 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 40 sec. The amplified PCR products were electrophoresed on 1.5% TAE agarose gels (Nippon Gene, Toyama, Japan), and stained with ethidium bromide. The PCR product was purified with a QIAEX II Gel Extraction Kit (Qiagen, Hileden, Germany) according to the manufacturer's instructions, and cloned into the Vector pCR 2.1 (Invitrogen Co., Carlsbad, CA). DNA sequencing was performed using the M13 forward primer (5'-GTAAAACGACGGCCAGT-3') and M13 reverse primer (5'-CAGGAAACAGCATTGAC-3'), ABI PRISM Big Dye Terminator Cycle Sequencing Reaction Kit v3.0 (Applied Biosystems, Foster City, CA), and a ABI310 Genetic Analyzer (Applied Biosystems). The nucleotide sequence of DNA fragments of *nifH1* and *nifH2* in *Clostridium* sp. strain Kas107-1 appears in the DDBJ database under accession numbers AB236339 and AB236340.

RNA preparation

The reagents and instruments used for the preparation of the RNA were autoclaved at 121°C for 30 min. To prepare total RNA of the cultured cells, *Clostridium* sp. strain Kas107-1 was cultured in RMR media with and without 25 mM ammonium nitrate at 30°C overnight. A 60-ml of volume of culture (approximately 10^7 cells ml^{-1}) was centrifuged at 8,600×g for 10 min at 4°C. The bacterial pellets were suspended in 1 ml of Trizol Reagent (Invitrogen Life Technologies, Tokyo, Japan), and immediately transferred into the Lysing matrix A tube (Qbiogene, Carlsbad, CA) with two 1/4" cylindrical Beads. The cell suspension from Kas107-1 was homogenized with a MINI-BEAD-BEATER-8 bead beater (BioSpec, Bartlesville, Okla) for 3 min to break cells and extract RNA. The solutions were heated for 1 min at 60°C, and then incubated for 5 min at room temperature. After 0.2 ml of chloroform was added, the solutions were mixed thoroughly by vortexing and incubated for 3 min at room temperature. After centrifugation at 17,400×g for 15 min at 4°C, the supernatants were transferred to fresh tubes, and purified two times by phenol-chloroform extraction to eliminate scraped beads and proteins. The resulting supernatants were further purified by 2-propanol precipitation, and dissolved in 30 µl of sterile distilled water. To remove intact DNA completely, the resultant total RNA extract was treated with DNase I (Takara) for 2 h at 37°C, followed by phenol-chloroform extraction and ethanol precipitation. The resultant total RNA was resuspended in 30 µl of RNase-free water. Each sample was examined for RNA and purity as detected by absorbance at 260 and 280 nm with a Gene Quant II (Amersham Bioscience, Piscataway, NJ). The final RNA preparations were stored at −80°C until the RT-PCR analysis. The total RNA of *Enterobacter* sp. strain B901-2 was prepared the same as way as that of *Clostridium* sp. strain Kas107-1 except for the proce-

dures of homogenization by bead beater and purification by phenol-chloroform extraction.

In this study, we extracted bacterial RNA of the inoculated endophytes together with plant RNA to analyze the *nifH* transcript and ribosomal RNA of the endophytes in plants. After an acetylene-reducing assay (ARA) of the inoculated plants was performed, the plants were washed twice with sterile distilled water, and wiped. The plants were weighed, and macerated thoroughly in liquid nitrogen using a cooled pestle and mortar. The macerated powder (0.1 g) was immediately transferred into Lysing matrix A tubes (Qbiogene) with two 1/4" cylindrical Beads per tube. After 1 ml of Trizol Reagent (Invitrogen Life Technologies) was added, the plant macerate was homogenized with a MINI-BEADBEATER-8 (BioSpec) for 5 min at room temperature. The procedures of RNA purification were the same as those for the cultured cells described above.

Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR was performed with a One-Step RT-PCR Kit (Qiagen) according to the manufacturer's instructions. To check for DNA in the prepared RNA, the RT reaction was eliminated, and PCR was performed using only Hot StarTaq polymerase (Qiagen) in the One-Step RT-PCR Kit (Qiagen). The PCR primers CnifH-1F and CnifH-2R were used to amplify a 383-bp DNA fragment from the *nifH* transcript. We used 50 ng of total RNA of the cultured cells (free-living) as a template in a total reaction volume of 50 µl. RT-PCR was performed with a RT reaction at 50°C for 30 min, a hot start at 95°C for 15 min, and cycling parameters of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec for 25 cycles, followed by a final extension at 72°C for 10 min. On the other hand, we used 200 ng of total RNA of plants as a template in a total reaction volume of 50 µl. RT-PCR was performed with a RT reaction at 50°C for 30 min, a hot start at 95°C for 15 min, and cycling parameters of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec for 40 cycles, followed by a final extension at 72°C for 10 min.

PCR primers C142f (5'-AAABGRMKRYTAATACCGCATAA-3') and C796r (5'-CCCNACACCTAGTAYYCATC-3')[16] were used for amplifying the 16S rRNA gene of *Clostridium* sp. strain Kas107-1. Primers 460F (5'-AATAACCTTGTCGATTGACG-3') and 813R (5'-ACAACCTCCAAGTCGACATC-3') were used for amplifying the 16S rRNA gene of *Enterobacter* sp. strain B901-2. We used 50 ng of total RNA of the cultured cells (free-living) as a template in a total reaction volume of 50 µl. RT-PCR was performed with a RT reaction at 50°C for 30 min, a hot start at 95°C for 15 min, and cycling parameters of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec for 25 cycles, followed by a final extension at 72°C for 10 min.

As for endophytic cells, we used 200 ng of total RNA from the plants as a template in a total reaction volume of 50 µl. RT-PCR was performed with a RT reaction at 50°C for 30 min, a hot start at 95°C for 15 min, and cycling parameters of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec for 30 cycles, followed by a final extension at 72°C for 10 min. The amplified PCR products were electrophoresed on 1.5% TAE agarose gels (Nippon Gene), and stained with ethidium bromide.

Results

Endophytic population of clostridia in inoculated plants

To examine whether clostridia fix nitrogen in plants, we first developed an inoculation system using *Miscanthus sinensis* under aseptic conditions. We wanted to select a clostridial strain that well colonized plants in the inoculation system to address this issue. Plant-derived clostridia were associated with non-diazotriphic bacteria during the isolation steps and the bacterial consortia exclusively expressed nitrogenase activity in a semi-solid medium under an aerobic atmosphere[15]. Thus we basically inoculated *M. sinensis* plants with a mixture of the respective clostridial strain and non-diazotrophic bacterium. From many trials, we found that it was important for successful colonization of the clostridia that one-month-old plants after germination are inoculated by vacuum infiltration as described in Materials and Methods (data not shown).

To select a clostridial strain as a best colonizer, we tested 13 clostridial strains with non-diazotrophic *Enterobacter* sp. strain B901-2 (Table 1)[15]. B901-2 has been consistently used as representative of non-diazotrophic bacteria[15,29]. In addition, the 13 clostridial strains could fix nitrogen with strain B901-2 in RMR medium, respectively (data not shown). Surface-sterilized plants at 7 days after inoculation (DAI) were subjected to MPN counts for the clostridia and non-diazotropic B901-2 (Fig. 1). The endophytic populations of clostridia significantly depended on clostridial stains, which ranged from zero to 10^5 cells g^{-1} plant fresh weight (Fig. 1A). In particular, strains Kas107-1 (group IV) and Kas201-1 (group V) showed higher population levels in the plants. This was true when three independent experiments were conducted (Bars for standard error in Fig. 1A). The clostridial population levels were not likely correlated with phylogenetic groups I, II, IV and V based on 16S rRNA gene sequences (Fig. 1A)[15].

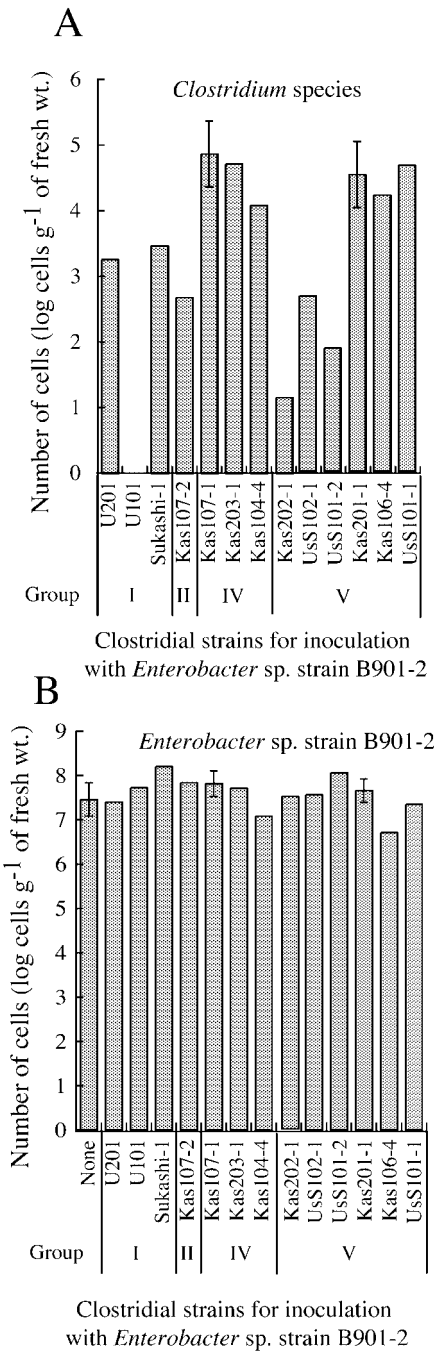

Fig. 1. Population of different clostridial strains (A) and non-diazotrophic *Enterobacter* sp. strain B901-2 (B) in the grass *Miscanthus sinensis* 7 days after inoculation. One-month-old plants inoculated with different clostridial strains and *Enterobacter* sp. strain B901-2 were surface-sterilized with 1% sodium hypochlorite for 30 sec. MPN counts of clostridia were as evaluated by an acetylene reduction assay while those of *Enterobacter* sp. strain B901-2 were as evaluated by cell turbidity in MPN tubes containing RMR medium. Bars indicate the standard error for three determinations.

On the other hand, endophytic levels of *Enterobacter* sp. strain B901-2 were consistently high regardless of the co-inoculated clostridial strains, and ranged from 10^6 to 10^8 cells g^{-1} plant fresh weight (Fig. 1B). Greater colonization by B901-2 was also observed in the plants inoculated with the strain in the absence of clostridia (Fig. 1B). To examine whether non-diazotrophic bacteria affect the colonization levels of *Clostridium* sp. strain Kas107-1 in the plants, inoculation experiments were carried out using additional non-diazotrophic bacteria (Kas103-2, Kas105-5a, Kas105-6, Kas107-3 and Kas107-4 in Table 1), which were co-isolates from the same plant materials as *Clostridium* sp. strain Kas107-1. However, strain Kas107-1 showed higher population levels with *Enterobacter* sp. strain B901-2 (Kas107-1 population, 1.0×10^4~4.6×10^6 cells g^{-1} plant fresh weight) than with the other non-diazotrophic bacteria (Kas107-1 population, 5.9×10^3~2.2×10^5 cells g^{-1} plant fresh weight).

Because the largest endophytic clostridial population (4.6×10^6 cells g^{-1} plant fresh weight) was detected in the plants inoculated with *Clostridium* sp. strain Kas107-1 and *Enterobacter* sp. strain B901-2, we selected these strains as inoculants for subsequent experiments on nitrogen fixation.

Nitrogen-fixing activity of clostridia in plants

To examine whether clostridia strains fix nitrogen in plants, the grass *Miscanthus sinensis* was inoculated singly or in pairs with *Clostridium* sp. strain Kas107-1 and the non-diazotrophic *Enterobacter* sp. strain B901-2. The growth of the plants singly inoculated with Kas107-1 was similar to that of uninoculated plants (Fig. 2A). On the other hand, the plants inoculated with B901-2 showed slight disease symptoms in terms of growth and browning at leaf tips (Fig. 2A), probably due to large populations of B901-2 in the plants (Fig. 1). These observations suggest that *Clostridium* sp. strain Kas107-1 did not damage the plants.

The plants of *Miscanthus sinensis* inoculated with *Clostridium* sp. strain Kas107-1 and *Enterobacter* sp. strain B901-2 showed no nitrogen fixation without a source of carbon (Table 2). Recently, carbon sources have been supplied to the roots of plants inoculated with diazotrophic endophytes to evaluate their potential to fix nitrogen in plants[2,8,9,13]. Thus, we supplied carbon sources to the root system of inoculated plants. The chemical species and concentrations of the carbon sources were set to be equivalent to those included in the RMR medium (Sodium lactate 0.03% (w/v), Sucrose 0.5% (w/v), Mannitol 0.3% (w/v), and Sodium malate 0.2% (w/v)). When the carbon sources were supplied to the plant roots, significant nitrogen-fixing activity was detected in clostridia-inoculated plants, which

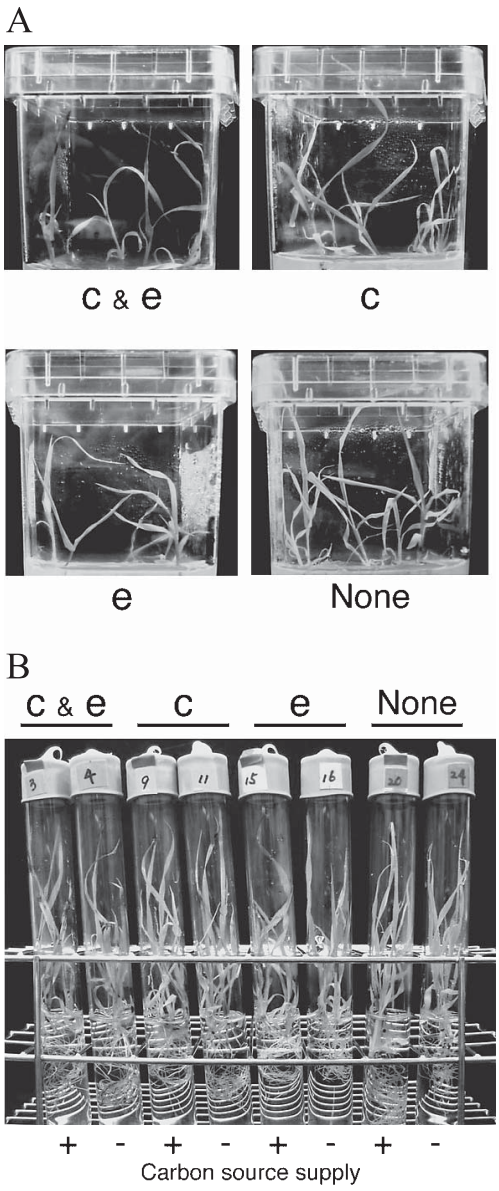

Fig. 2. The grass *Miscanthus sinensis* singly and doubly inoculated with *Clostridium* sp. strain Kas107-1 and *Enterobacter* sp. strain B901-2. A: Plants were inoculated singly or doubly with Kas107-1 and B901-2. B: Test tube systems for the acetylene reduction assay of *Miscanthus sinensis*. "c", "e" and "None" indicate inoculated with *Clostridium* sp. strain Kas107-1, inoculated with *Enterobacter* sp. strain B901-2, and the uninoculated control, respectively.

were inoculated with both *Clostridium* sp. strain Kas107-1 and *Enterobacter* sp. strain B901-2, and with just *Clostridium* sp. strain Kas107-1 (Table 2). On the other hand, no nitrogen-fixing activity was observed in the plants inoculated only with *Enterobacter* sp. strain B901-2 and uninoculated plants (Table 2). These results suggest that the addition of carbon sources induced nitrogen-fixing activity of clostridia in the plants.

nifH gene cloning from Clostridium *sp. strain Kas107-1*

The test tube system for the acetylene reduction assay contained plants and a nutrient solution (Fig. 2B). Thus, we could not rule out the possibility that the carbon source induced the fixation of nitrogen by clostridial cells exclusively in the plant nutrient solution outside the plants. In other words, the observed activity might be due to the cells growing in the solution supplemented with carbon sources.

The *nifH* gene encodes the Fe protein (dinitrogenase reductase) as one of the nitrogenase components[18,28]. Because *nif* gene transcription is strictly regulated by oxygen and nitrogen to minimize unnecessary energy consumption[6], there is a tight relationship between nitrogen-fixing activity and *nif* gene transcription[6,7,13]. Thus, we decided to monitor *nifH* expression in clostridia inside the plants to clarify the *in planta*-nitrogen fixation by clostridia.

Many clostridial species have often multiple copies of *nifH* genes[14,17,28,31]. When *nifH*-specific PCR amplification was carried out using total DNA of *Clostridium* sp. strain Kas107-1 as a template, a *nifH*-specific PCR product was observed at the position of approximately 340 bp (data not shown). DNA sequencing revealed that Kas107-1 had at least two copies of *nifH*, *nifH1* and *nifH2* (Fig. 3A). When the DNA sequences were translated into amino acid sequences, the deduced *nifH1* and *nifH2* proteins contained three functionally important cysteine residues (Cys-37, Cys-82, and Cys-94; *C. pasteurianum* AY603957 numbering) and one arginine residue (Arg-97; *C. pasteurianum* AY603957 numbering)[5,14] (Fig. 3B). Therefore, *nifH1* and *nifH2* were definitely *nifH* genes.

nifH expression of clostridia in culture

We first examined the correlation between *nifH* expression and nitrogen fixation (ARA) in free-living cells of *Clostridium* sp. strain Kas107-1. Nitrogen-fixing and non-fixing clostridial cells were prepared from cultures in RMR medium without and with ammonia nitrate, respectively. ARA in free-living cells grown in RMR medium was 241±20 n mol hr^{-1} ml^{-1}, while ARA in cells grown in RMR medium with 25 mM ammonia nitrate was less than 1.0 n mol hr^{-1} ml^{-1}. Thus, the addition of 25 mM ammonia nitrate into RMR medium completely inhibited the nitrogen-fixing activity (ARA) of *Clostridium* sp. strain Kas107-1. A band corresponding to the *nifH* transcript (343 bp) was detected exclusively in the nitrogen-fixing cells (Fig. 4A). No band corresponding to the clostridial *nifH* transcript was ob-

Table 2. Nitrogen-fixing activity (Acetylene-reducing activity) of *Miscanthus sinensis* singly and doubly inoculated with *Clostridium* sp. strain Kas107-1 and *Enterobacter* sp. strain B901-2

Inoculation[a]	Carbon[b] source	Acetylene-reducing activity (ARA)[c]	
		(nmol hr^{-1} [g fresh weight]$^{-1}$)	(nmol $hr^{-1}plant^{-1}$)
c & e	+	74 ± 24[d]	5.7 ± 2.2
c & e	-	<1	<0.1
c	+	96 ± 47	8.4 ± 4.5
c	-	<1	<0.1
e	+	<1	<0.1
e	-	<1	<0.1
None	+	<1	<0.1
None	-	<1	<0.1

[a] c: *Clostridium* sp. strain Kas107-1, e: *Enterobacter* sp. strain B901-2.
[b] Carbon source; 0.5% Sucrose, 0.3% Mannitol, 0.2% Sodium malate and 0.03% Sodium lactate.
[c] ARA values were calculated from the ethylene concentration between 20 h and 41 h after application of carbon sources in the presence of 10%(vol/vol) acetylene gas.
[d] Values are means with standard errors for three determinations.

served in free-living *Enterobacter* sp. strain B901-2 cells (Fig. 4B). The elimination of the RT reaction confirmed that the 343-bp band was derived from the *nifH* mRNA and not from the *nifH* DNA (Fig. 4A and 4B). Thus, there was a correlation between *nifH* expression and nitrogen fixation (ARA) in free-living cells of *Clostridium* sp. strain Kas 107-1.

Moreover, the DNA sequence of the *nifH* transcript from free-living *Clostridium* sp. strain Kas107-1 was identical to the *nifH1* sequence, and different from the *nifH2* sequence (Fig. 3A). These results indicate that the *nifH1* gene is exclusively expressed in the nitrogen-fixing cells of free-living *Clostridium* sp. strain Kas107-1.

nifH expression of clostridia in plants

After the ARA assay (Table 2, Fig. 2B), total RNA was extracted from the washed plants, and subjected to an analysis of the *nifH* transcript by means of RT-PCR. No band corresponding to the *nifH* transcript (approximately 340 bp in size) was observed in the inoculated plants without a source of carbon in the ARA assay (Fig. 4C). When carbon sources were supplied, the *nifH* transcript (343 bp) was detected in the plants inoculated with both *Clostridium* sp. strain Kas107-1 and *Enterobacter* sp. strain B901-2 (Lane marked with c&e and + in Fig. 4C), and with just strain Kas107-1 (Lane marked with c and + in Fig. 4C). The elimination of the RT reaction confirmed that the 343-bp band was derived from the *nifH* mRNA, not from the *nifH* DNA (Fig. 4C). Uninoculated plants and plants inoculated only with non-diazotrophic *Enterobacter* sp. strain B901-2 showed no background of RT-PCR amplification specific for the clostridial *nifH* transcript (Fig. 4C). The DNA sequence of the *nifH* transcript from endophytic *Clostridium* sp. strain Kas107-1 was identical to the *nifH1* sequence as well as that of the free-living cells (Fig. 3A).

Therefore, we concluded that the carbon source markedly induced *nifH1* gene expression in *Clostridium* sp. strain Kas107-1 even in the inoculated plants. Because of the close relationship between nitrogen-fixing activity and *nif* gene transcription[6,7,13], it is expected that the endophytic *Clostridium* sp. strain Kas107-1 will express nitrogen-fixing activity in plants as well.

RT-PCR analysis of ribosomal RNA of inoculated bacteria in culture

The population levels of living endophytes in plants have been enumerated by culture-based methods including plate counts and the MPN method[7,8,9,12,13,15,16,24,29]. In this study, we wanted to determine the endophytic population of *Clostridium* sp. strain Kas107-1 in inoculated plants during assays for ARA and the *nifH* transcript, because the population levels might affect nitrogen fixation. However, conventional culture-based methods require many samples, and are time-consuming.

The amount of ribosomal RNA reflects the active population for a specific bacterium of interest[25]. Since highly purified RNA preparations were available in this study, we tried

A

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nifH1           1 AATAGGTAAATCTACAACAACACAAAACTTAACATCAGGGCTTGCTACAATGGACAAGAA    60
Free-living     1 AATAGGTAAATCTACAACAACACAAAACTTAACATCAGGGCTTGCTACAATGGACAAGAA    60
Endophyte       1 AATAGGTAAATCTACAACAACACAAAACTTAACATCAGGGCTTGCTACAATGGACAAGAA    60
nifH2           1 AATAGGAAAATCCACTACTGTTTCTAATATATCTGCTGCTTTGGTGGCTATGGGTTATAA    60

nifH1          61 GATAATGGTAGTAGGATGTGATCCTAAGGCTGACTCAACAAGGTTATTACTAGGAGGACT   120
Free-living    61 GATAATGGTAGTAGGATGTGATCCTAAGGCTGACTCAACAAGGTTATTACTAGGAGGACT   120
Endophyte      61 GATAATGGTAGTAGGATGTGATCCTAAGGCTGACTCAACAAGGTTATTACTAGGAGGACT   120
nifH2          61 GGTTATGCAGATAGGGTGTGATCCTAAGGCGGATTCTACAAGAAACTTAACTAATGGGGA   120

nifH1         121 AGCACAAAAAAGTGTTCTTGATACATTAAGAGAAGAAGGAGATGACGTAGATTTAGATTC   180
Free-living   121 AGCACAAAAAAGTGTTCTTGATACATTAAGAGAAGAAGGAGATGACGTAGATTTAGATTC   180
Endophyte     121 AGCACAAAAAAGTGTTCTTGATACATTAAGAGAAGAAGGAGATGACGTAGATTTAGATTC   180
nifH2         121 GTCTATACCTACTGTTTTAGAGACTTTAAGAACATTAGGAAATAAACCCTTAACTTTGGA   180

nifH1         181 AATCTTAAAGCCAGGATT---TAGAGGTATAAAATGTGTTGAATCAGGCGGTCCAGAACC   237
Free-living   181 AATCTTAAAGCCAGGATT---TAGAGGTATAAAATGTGTTGAATCAGGCGGTCCAGAACC   237
Endophyte     181 AATCTTAAAGCCAGGATT---TAGAGGTATAAAATGTGTTGAATCAGGCGGTCCAGAACC   237
nifH2         181 AGATTTAGTTTTTGAAAGTAGTTCAGGGGTTTTATGTGTTGAAGCAGGTGGTCCAACTCC   240

nifH1         238 AGGAGTTGGATGTGCAGGAAGAGGTATAATAACTTCAATCAATATGCTAGAGCAATTAGG   297
Free-living   238 AGGAGTTGGATGTGCAGGAAGAGGTATAATAACTTCAATCAATATGCTAGAGCAATTAGG   297
Endophyte     238 AGGAGTTGGATGTGCAGGAAGAGGTATAATAACTTCAATCAATATGCTAGAGCAATTAGG   297
nifH2         241 TGGTATAGGTTGTGCTGGAAGAGGGATAATAACTGCCTTTGAAAAACTTAAGGAATTAAA   300

nifH1         298 TGCTTACGAATCAGATTTAGATTATGTTTTC---TATGATGTATTAGGT             343
Free-living   298 TGCTTACGAATCAGATTTAGATTATGTTTTC---TATGATGTATTAGGT             343
Endophyte     298 TGCTTACGAATCAGATTTAGATTATGTTTTC---TATGATGTATTAGGT             343
nifH2         301 AGCTTACGAAACTTATAATCCCGATGTTGTAATATATGATGTTTTAGGT             349
```

B

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                                                  *
NifH1                 IGKSTTTQNLTSGLATMDKKIMVVGCDPKADSTRLLLGGLAQKSVLDTLREEGDDVD-L
NifH2                 IGKSTTVSNISAALVAMGYKVMQIGCDPKADSTRNLTNGESIPTVLETLRTLGNKPLTL
Cp          MRQVAIYGKGGIGKSTTTQNLTSGLHAMGKTIMVVGCDPKADSTRLLLGGLAQKSVLDTLREEGEDVE-L
Ca          MRQVAIYGKGGIGKSTTTQNLTSGLAELGKKIMVVGCDPKADSTRLLLGGLAQKTVLDTLREEGEDVD-L
Cb          MRQVAIYGKGGIGKSTTTQNLTSALAEMGKNIMIVGCDPKADSTRLVLGGLAQKTVLDTLREEGDDIE-L

                      *           *  #
NifH1       DSILKPGFRGIKCVESGGPEPGVGCAGRGIITSINMLEQLGAYESDL-DYVFYDVLG
NifH2       EDLVFESSSGVLCVEAGGPTPGIGCAGRGIITAFEKLKELKAYETYNPDVVIYDVLG
Cp          DSILKEGYGGIRCVESGGPEPGVGCAGRGIITSINMLEQLGAYTDDL-DYVFYDVLGDVVCGGFAMP---
Ca          DTIMKTGFGNIKCVESGGPEPGVGCAGRGIITSINMLEQLGAYEDEL-DYVFYDVLGDVVCGGFAMP---
Cb          DAILKTGYGNIRCVESGGPEPGVGCAGRGIITSIGMLEQLGAYTPDL-DYVFYDVLGDVVCGGFAMP---
```

Fig. 3. Sequence alignment of DNA and putative amino acids of *nifH* genes in *Clostridium* sp. strain Kas107-1. A: DNA sequence comparison of *nifH* genes residing in Kas107-1 with those of *nifH* transcripts in free-living and endophytic cells. nifH1 and nifH2, DNA sequences of *nifH* gene fragments from strain Kas107-1 as amplified by PCR; Free-living, DNA sequence of the *nifH* transcript from free-living *Clostridium* sp. strain Kas107-1 as amplified by RT-PCR; endophyte, DNA sequence of the *nifH* transcript from endophytic *Clostridium* sp. strain Kas107-1 in the plant as amplified by RT-PCR. B: Alignments of the amino acid sequence deduced from DNA sequences of *nifH* genes in *Clostridium* sp. strain Kas107-1 and reference organisms. Cp, the *nifH* product fragment of *Clostridium pasteuriaum* (AY603957); Ca, the *nifH* product fragment of *Clostridium acetobutylicum* (AE007538); Cb, the *nifH* product fragment of *Clostridium beijerinckii* (AF266462). Three cysteine residues (*) and one arginine residue (#) of the *nifH* products are conserved.

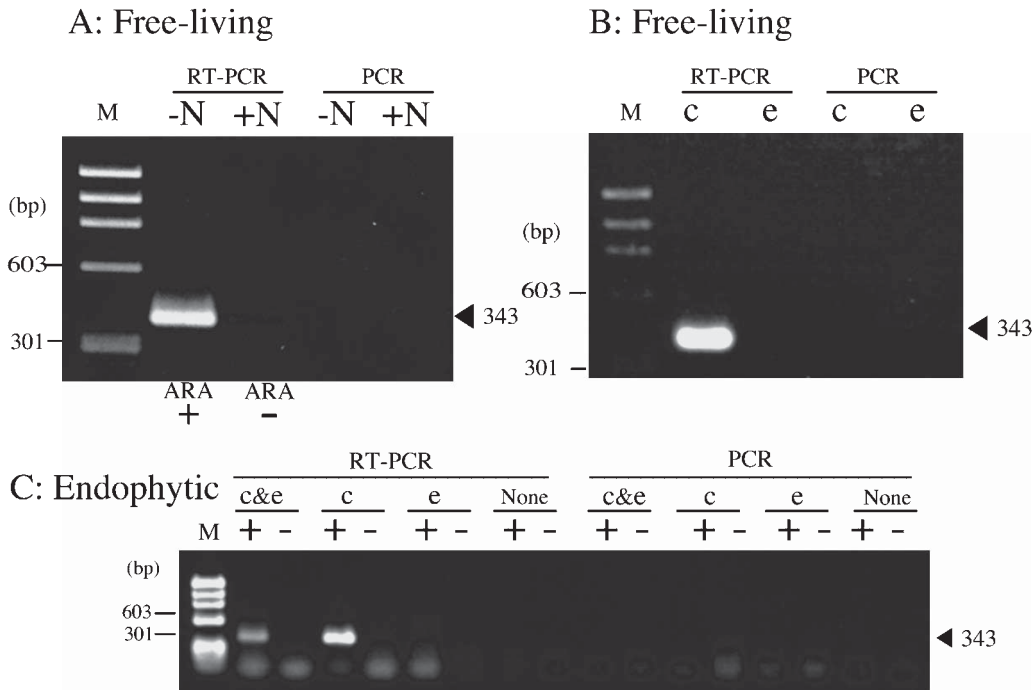

Fig. 4. RT-PCR analysis of the *nifH* transcript of *Clostridium* sp. strain Kas107-1 from total RNA extracted from various sources. A: Total RNA from free-living cells of *Clostridium* sp. strain Kas107-1 grown in the absence (–N) and presence (+N) of 25 mM ammonia nitrate. "+" and "–" indicates that nitrogen fixation (ARA) was detected or not detected. Lane M shows a DNA size marker. B: Total RNA from free-living cells of *Clostridium* sp. strain Kas107-1(c) and *Enterobacter* sp. strain B901-2 (e). C: Total RNA from the plants inoculated with *Clostridium* sp. strain Kas107-1(c) and *Enterobacter* sp. strain B901-2 (e) after acetylene reduction assay (Table 2). "c&e" and "None" indicate the plants doubly inoculated with Kas107-1(c) and B901-2 (e), and un-inoculated control plants, respectively. "+" means the addition of a carbon source to the plant nutrient solution (see text). RNA templates were adjusted to equal amounts within each panel. PCR amplification without a RT reaction was carried out to examine DNA contamination in prepared RNA throughout the RT-PCR analyses.

to evaluate the active populations of *Clostridium* sp. strain Kas107-1 and *Enterobacter* sp. strain B901-2 by analyzing of ribosomal RNA molecules of the inoculants instead of the conventional culture-based methodology.

We first examined the specificity of 16S rRNA primer sets using RNA preparations from free-living *Clostridium* sp. strain Kas107-1 cells and *Enterobacter* sp. strain B901-2 cells. The clostridial primers generated a band corresponding to the 16S rRNA of clostridia (654 bp) in free-living Kas107-1 cells, but not in free-living B901-2 cells (Fig. 5A). Similarly, primers specific for *Enterobacter* sp. strain B901-2 generated a band corresponding to the 16S rRNA of strain B901-2 (375 bp) in free-living *Enterobacter* sp. strain B901-2 cells, but not in free-living *Clostridium* sp. strain Kas107-1 (Fig. 5C). The elimination of the RT reaction produced no PCR band at all (Fig. 5A and 5C). Thus, we verified the primer specificity in the RT-PCR system targeting 16S rRNAs of both microbes.

RT-PCR analysis of ribosomal RNA of inoculated bacteria in plants

When we analyzed 16S rRNA molecules of endophytic clostridia and *Enterobacter* sp. strain B901-2 in the plant, a band corresponding to the 16S rRNA of clostridia (654 bp) was detected in the carbon supplied plants inoculated with both Kas107-1 and B901-2 and with just Kas107-1 (Fig. 5B). Without a carbon source, clostridial 16S rRNA was detected in the plants inoculated only with Kas107-1, but not in the co-inoculated plants. In these plants, the supply of carbon significantly increased the signal for clostridial 16S rRNA (Fig. 5B). The single inoculation produced a relatively larger population of *Clostridium* sp. strain Kas107-1 in the plants than the co-inoculation of *Enterobacter* sp. strain B901-2. Uninoculated plants and plants inoculated with just *Enterobacter* sp. strain B901-2 showed no background of RT-PCR products for clostridial 16S rRNA (Fig. 5B). These results suggest that the single inoculation and carbon source produced a larger active population of *Clostridium* sp. strain

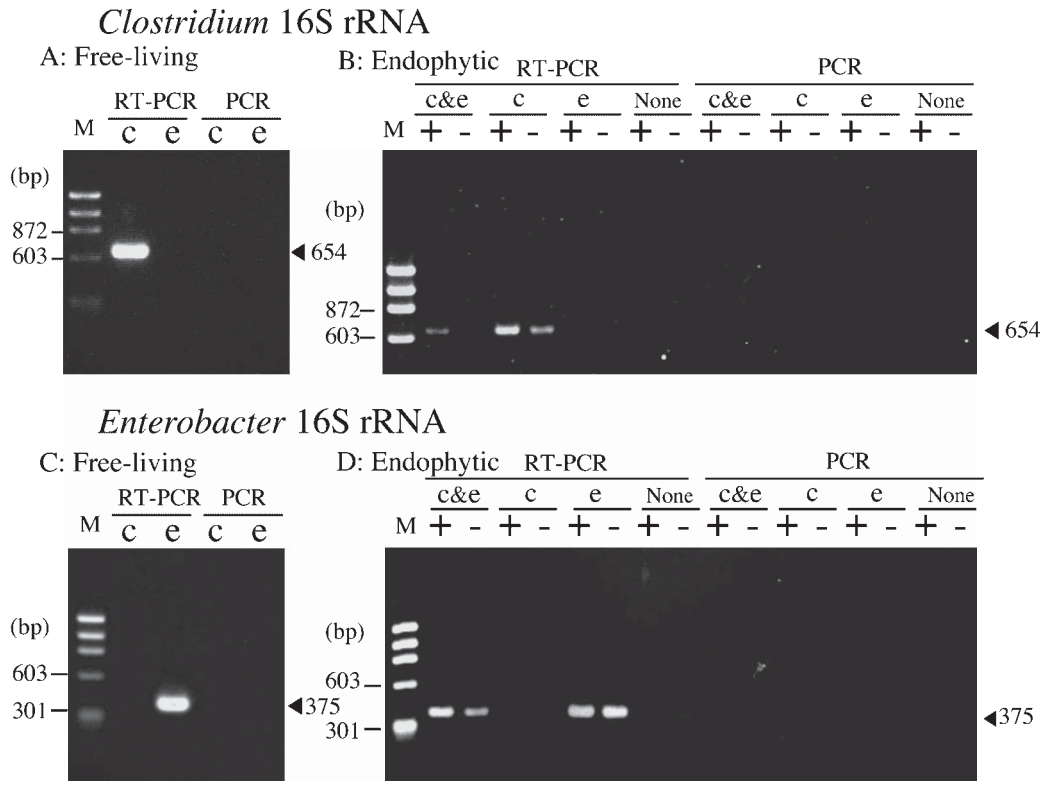

Fig. 5. RT-PCR analysis of ribosomal RNA molecules from total RNA extracted from various sources. A and C: Total RNA from free-living *Clostridium* sp. strain Kas107-1(c) and *Enterobacter* sp. strain B901-2 (e). B and D: Total RNA from the plants inoculated with *Clostridium* sp. strain Kas107-1(c) and *Enterobacter* sp. strain B901-2 (e). "c&e" and "None" indicate the plants inoculated with Kas107-1(c) and B901-2 (e), and uninoculated control plants, respectively. A and B, a primer set specific for the 16S rRNA sequence of *Clostridium* sp. strain Kas107-1 was used for (RT)-PCR; C and D, a primer set specific for the 16S rRNA sequence of *Enterobacter* sp. strain B901-2 used for (RT)-PCR. "+" means the addition of a carbon source to the plant nutrient solution (see text). Lane M indicates a DNA size marker. RNA templates were adjusted to equal amounts within each panel. RCR amplification without a RT reaction was carried out to examine DNA contamination in prepared RNA throughout the RT-PCR analyses.

Kas107-1 in the plants.

As for non-diazotrophic bacteria, a band corresponding to the 16S rRNA of *Enterobacter* sp. strain B901-2 (375 bp) was consistently detected in the plants co-inoculated with Kas107-1 and B901-2, and singly inoculated with B901-2 (Fig. 5D). Uninoculated plants and plants inoculated with only *Clostridium* sp. strain Kas107-1 showed no background either (Fig. 5D). Supplying a carbon source slightly increased the 16S rRNA signals of strain B901-2 (Fig. 5D) as compared with clostridial signals (Fig. 5B). The elimination of the RT reaction produced no PCR band (Fig. 5B and 5D).

RT-PCR signals of 16S rRNAs mean that viable cells of *Clostridium* sp. strain Kas107 and *Enterobacter* sp. strain B901-2 inhabited the plant. The addition of carbon sources enhanced the 16S rRNA signals of both strains (Fig. 5BD). This suggests two possibilities; (1) an enhancement of the respective bacterial growth in the plant, and/or (2) an increase of rRNA molecules in the bacteria, although we have not tested the possibilities in this study.

Discussion

The aim of this study is to examine whether plant clostridia and their consortium fix nitrogen in plants. First we developed an inoculation system using plants of *M. sinensis* under aseptic conditions (Fig. 2). Based on colonization tests, we selected a combination of *Clostridium* sp. strain Kas107-1 and *Enterobacter* sp. strain B901-2 as best colonizers in *M. sinensis* (Fig. 1). The *in planta*-nitrogen fixation was not detected in the absence of the carbon sources. When carbon sources were supplied to the plant roots, the *in planta*-nitrogen fixation of endophytic clostridia was shown by ARA assay (Table 2) and the *nifH* gene was ex-

pressed (Fig. 4C). After the carbon sources were supplied, 16S ribosomal RNA (Fig. 5B) also increased as well as the *nifH* transcript of clostridial strain Kas107-1 in the inoculated plant. These results suggested that if an ample source of carbon is supplied by plant photosynthesis, the endophytic clostridia might be activated and fix nitrogen in the plant.

Plant-derived clostridia were found to be associated with non-diazotriphic bacteria during their isolation, where the concept of an anaerobic nitrogen-fixing consortium (ANFICO) consisting of N_2-fixing clostridia and diverse non-diazotrophic bacteria was proposed[15]. A major feature of ANFICO is that nitrogen fixation by anaerobic clostridia is supported by the elimination of molecular oxygen by the non-diazotrophic bacteria in culture[15]. In group II clostridia, non-diazotrophic bacteria induced the fixation of N_2 by the clostridia in culture[15]. Thus, we want to know that these interactions extend to endophytic situations, although the selected clostridial strain belongs to group IV, not group II.

Unexpectedly, the results of this study show that the anaerobic *Clostridium* sp. strain Kas107-1 was able to colonize plants and express nitrogenase without the non-diazotrophic *Enterobacter* sp. strain B901-2 (Fig. 4C and Fig. 5B). When a source of carbon was supplied, the single inoculation tended to enhance the expression of *nifH* (Fig. 4C) and nitrogen fixation (Table 2) of *Clostridium* sp. strain Kas107-1 rather than the co-inoculation with *Enterobacter* sp. strain B901-2. This is probably due to the larger active population of Kas107-1 in the single inoculation experiment (Fig. 5B). In contrast, the population of Kas107-1 was likely repressed in the presence of B901-2.

This means that the anaerobic *Clostridium* sp. strain Kas107-1 can adapt to environments inside the plant without apparent oxygen-consuming microbes. Ye *et al.*[29] showed that plant clostridia endophytically inhabited the plant tissues of *M. sinensis*, where there is molecular oxygen in the air, and evolved by photosynthesis. Possible explanations for the clostridial colonization following a single inoculation are (1) vigorous plant respiration and/or (2) the presence of hidden non-diazotrophic bacteria in the plant that have not yet been cultivated[20,21].

The *nifH* gene is one of the nitrogenase structural genes[31]. The *nifH* sequence database is rapidly expanding and is currently composed of over 1500 sequences, most of which have been obtained from environmental samples[31]. Many microorganisms have multiple copies of nitrogenase genes or presumptive nitrogenase genes[19,31]. *Archaea* often possesses two *nifH* homologues[31]. *Rhizobium phaseoli* varieties possess three copies of *nifH* although *Rhizobium leguminosarum* and *R. trifolii* have single copies of *nifH*[19]. In the genus *Clostridium*, many species possess more than one copy of *nifH*. *Clostridium pasteurianum* possesses two copies[3,14]. *Clostridium acetobautylicum* possessed a *nifH* gene and two *nifHD* genes[17,31]. However, the function of multiple copies of nitrogenase genes has not yet been studied except in the cyanobacterium, *Anabena varuabilis*. *A. varuabilis* possesses two sets of *nif* genes, one expressed in the heterocysts when oxygen is abundant, the other expressed in the filaments when oxygen is sparse[19].

Clostridium sp. strain Kas107-1 possessed at least two copies of *nifH*: *nifH1* and *nifH2* (Fig. 3). The transcription of *nifH1* was exclusively detected in both free-living and endophytic cells by RT-PCR analysis (Fig. 3A), which suggests that the *nifH1* gene product functions as a dinitrogenase reductase for the fixation of N_2 even inside the plants. It is important for nitrogen fixation of endophytes possessing multiple copies of *nifH* genes to determine a functional *nifH* copy, because the analysis of a non-functional copy ought to lead to mistakes.

In this study, 16S rRNA molecules of *Clostridium* sp. strain Kas107 and *Enterobacter* sp. strain B901-2 were specifically detected by means of RT-PCR without interference from plant RNA (Fig. 5). In particular, the RT-PCR analysis of *nifH* expression was a powerful tool to evaluate *in planta* the fixation of nitrogen by the clostridial endophytes. Two papers have been reported for RT-PCR analyses of *nifH* expression of *Azoarcus* and *Herbaspirillum* endophytes[10,30]. However, this study is unique in that *nifH* expression was detected from an obligate anaerobic endophyte in plants.

In addition, the RT-PCR analysis of 16S ribosomal RNA molecules showed the fluctuations in the endophyte population (Fig. 5C), although the changes have not yet been characterized in terms of cell activity and numbers. A RT-PCR analysis targeting both functional gene transcripts and the ribosomal RNA molecule is useful for research of endophyte ecology, because endophyte function and colonization in plants can be examined simultaneously using a single RNA preparation.

The ability of aerobic endophytes such as *Herbaspirillum* to fix nitrogen *in planta* largely depends on the strain[7], host plant[7] and environment[30] irrespective of their colonization levels. In this study, we adopted a strategy to examine nitrogen fixation by the best colonizer, *Clostridium* sp. strain Kas107-1, which is classified as a group IV plant clostridium[15]. Because a RT-PCR analysis of clostridial endophytes targeting the *nifH* transcript and 16S rRNA molecules was established here, we can start to screen for nitrogen-fixing clostridia in plants under various conditions. As for the interactions in ANFICOs, group II clostridia are still

important targets, because specific non-diazotrophic bacteria induced the fixation of N_2 by the clostridia in culture[15].

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References

1) Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith and K. Struhl (ed.). 1994. Current protocols in molecular biology Vol. 2. John Wiley & Sons, Inc., USA.
2) Chelius, M.K. and E.W. Triplett. 2000. Immunolocalization of dinitrogenase reductase produced by *Klebsiella pneumoniae* in association with *Zea mays* L. Appl. Environ. Microbiol. **66**: 783–787.
3) Chen, J.S., J. Toth and M. Kasap. 2001. Nitrogen-fixation genes and nitrogenase activity in *Clostridium acetobutylicum* and *Clostridium beijerinckii*. J. Ind. Microbiol. Biotechnol. **27**: 281–286.
4) Collins, M.D., P.A. Lawson, A. Willems, J.J. Cordoba, J. Fernandez-garayzabal, P. Garcia, J. Cai, H. Hippe and J.A.E. Farrow. 1994. The phylogeny of the geneus *Clostridium*: proposal of five new genera and eleven new species combination. Int. J. Syst. Bacteriol. **44**: 812–826.
5) Dean, D.R. and M.R. Jacobson. 1992. Biological genetics of nitrogenase, pp. 763–834. *In* G. Stacy, R.H. Burris and H.J. Evans (ed.), Biological nitrogen fixation. Chapman and Hall, New York.
6) Dixon, R. and D. Kahn. 2004. Genetic regulation of biological nitrogen fixation. Nat. Rev. Microbiol. **2**: 621–631.
7) Elbeltagy, A., K. Nishioka, T. Sato, H. Suzuki, B. Ye, T. Hamada, T. Isawa, H. Mitsui and K. Minamisawa. 2001. Endophytic colonization and in planta nitrogen fixation by a *Herbaspirillum* sp. isolated from wild rice species. Appl. Environ. Microbiol. **67**: 5285–5293.
8) Gyaneshwar, P., E.K. James, N. Mathan, P.M. Reddy, B. Reinhold-Hurek and J.K. Ladha. 2001. Endophytic colonization of rice by a diazotrophic strain of *Serratia marcescens*. J. Bacteriol. **183**: 2634–2645.
9) Gyaneshwar, P., E.K. James, P.M. Reddy and J.K. Ladha. 2002. *Herbaspirillum* colonization increases growth and nitrogen accumulation in aluminium-tolerant rice varieties. New Phytol. **154**: 131–145.
10) Hurek, T., L.L. Handley, B. Reinhold-Hurek and Y. Piche. 2002. *Azoarcus* grass endophytes contribute fixed nitrogen to the plant in an unculturable state. Mol. Plant Microbe Interact. **15**: 233–242.
11) Hurek, T. and B. Reinhold-Hurek. 2003. *Azoarcus* sp. strain BH72 as a model for nitrogen-fixing grass endophytes. J. Biotechnol. **106**: 169–178.
12) James, E.K., V.M. Reis, F.L. Olivares, J.I. Baldani and J. Dobereiner. 1994. Infection of sugar cane by the nitrogen-fixing bacteria *Acetobacter diazotrophicus*. J. Exp. Bot. **45**: 757–766.
13) James, E.K., P. Gyaneshwar, N. Mathan, W.L. Barraquio, P.M. Reddy, P.P. Iannetta, F.L. Olivares and J.K. Ladha. 2002. Infection and colonization of rice seedlings by the plant growth-promoting bacterium *Herbaspirillum seropedicae* Z67. Mol. Plant Microbe Interact. **15**: 894–906.
14) Kasap, M. and J.S. Chen. 2005. *Clostridium pasteurianum* W5 synthesizes two NifH-related polypeptides under nitrogen-fixing conditions. Microbiology **151**: 2353–2362.
15) Minamisawa, K., K. Nishioka, T. Miyaki, B. Ye, T. Miyamoto, M. You, A. Saito, M. Saito, W.L. Barraquio, N. Teaumroong, T. Sein and T. Sato. 2004. Anaerobic nitrogen-fixing consortia consisting of clostridia isolated from gramineous plants. Appl. Environ. Microbiol. **70**: 3096–3102.
16) Miyamoto, T., M. Kawahara and K. Minamisawa. 2004. Novel endophytic nitrogen-fixing clostridia from the grass *Miscanthus sinensis* as revealed by terminal restriction fragment length polymorphism analysis. Appl. Environ. Microbiol. **70**: 6580–6586.
17) Nolling, J., G. Breton, M.V. Omelchenko, K.S. Makarova, Q. Zeng, R. Gibson, H.M. Lee, J. Dubois, D. Qiu, J. Hitti, Y.I. Wolf, R.L. Tatusov, F. Sabathe, L. Doucette-Stamm, P. Soucaille, M.J. Daly, G.N. Bennett, E.V. Koonin and D.R. Smith. 2001. Genome sequence and comparative analysis of the solvent-producing bacterium *Clostridium acetobutylicum*. J. Bacteriol. **183**: 4823–4838.
18) Peters, J.W., K. Fisher and D.R. Dean. 1995. Nitrogenase structure and function: a biochemical-genetic perspective. Annu. Rev. Microbiol. **49**: 335–366.
19) Postgate, J. 1998. Nitrogen fixation, 3rd ed. Cambridge university press, Cambridge, United Kingdom.
20) Rappe, M.S. and S.J. Giovannoni. 2003. The uncultured microbial majority. Annu. Rev. Microbiol. **57**: 369–394.
21) Reinhold-Hurek, B. and T. Hurek. 1998. Life in grasses: diazotrophic endophytes. Trends Microbiol. **6**: 139–144.
22) Rennie, R.J. 1981. A single medium for the isolation of acetylene-reducing (dinitrogen-fixing) bacteria from soils. Can. J. Microbiol. **27**: 8–14.
23) Sambrook, J. and D.W. Russell. 2001. Molecular cloning: a laboratory manual, vol. 1 and vlo. 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
24) Sevilla, M., R.H. Burris, N. Gunapala and C. Kennedy. 2001. Comparison of benefit to sugarcane plant growth and 15N2 incorporation following inoculation of sterile plants with *Acetobacter diazotrophicus* wild-type and Nif-mutants strains. Mol. Plant Microbe Interact. **14**: 358–366.
25) Sharma, S., M.K. Aneja, J. Mayer, J.C. Munch and M. Schloter. 2005. Characterization of bacterial community structure in rhizosphere soil of grain legumes. Microb. Ecol. **49**: 407–415.
26) Sneath, P.H.A., N.S. Mair and M.E. Sharpe. 1986. Genus *Clostridium*, pp. 1141–1200. *In* J.G. Holt (ed.), Bergey's manual of systematic bacteriology, Vol. 2. Williams and Wilkins, Baltimore, USA.
27) Ueda, T., Y. Suga, N. Yahiro and T. Matsuguchi. 1995. Remarkable N2-fixing bacterial diversity detected in rice roots by molecular evolutionary analysis of *nifH* gene sequences. J. Bacteriol. **177**: 1414–1417.
28) Wang, S.Z., J.S. Chen and J.L. Johnson. 1988. The presence of five nifH-like sequences in *Clostridium pasteurianum*: sequence divergence and transcription properties. Nucleic Acids Res. **16**: 439–454.
29) Ye, B., A. Saito and K. Minamisawa. 2005. Effect of inoculation

with Anaerobic nitrogen-fixing consortium on salt tolerance of *Miscanthus sinensis*. Soil Sci. Plant Nutr. **51**: 243–249.
30) You, M., T. Nishiguchi, A. Saito, T. Isawa, H. Mitsui and K. Minamisawa. Expression of the *nifH* gene of a *Herbaspirillum* endophyte in wild rice species: daily rhythm during the light-dark cycle. Appl. Environ. Microbiol. in press.
31) Zehr, J.P., B.D. Jenkins, S.M. Short and G.F. Steward. 2003. Nitrogenase gene diversity and microbial community structure: a cross-system comparison. Environ. Microbiol. **5**: 539–554.