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Expression of the *nifH* Gene of a *Herbaspirillum* Endophyte in Wild Rice Species: Daily Rhythm during the Light-Dark Cycle

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The expression of nitrogenase genes of *Herbaspirillum* sp. B501 associated in shoot (leaf and stem) of wild rice, *Oryza officinalis*, was studied by means of reverse transcription-PCR (RT-PCR) targeted at the *nifH* gene. RT-PCR analyses indicate that *nifH* transcript was detected exclusively from nitrogen-fixing cells of *gfp*-tagged strain B501*gfp*1 in both free-living and endophytic states by using a constitutive *gfp* gene transcript as a positive control. Transcription of *nifH* and nitrogen fixation in free-living cells were induced maximally at a 2% O_2 concentration and repressed in free air (21% O_2). *nifH* transcription was monitored in the endophytic cells by using total RNA extracted from B501*gfp*1-inoculated wild rice plants during daily light-dark cycles. The level of *nifH* transcription in planta varied dramatically, with a maximum during the light period. Moreover, the light radiation enhanced *nifH* expression even in free-living cells grown in culture. These results suggest that in planta nitrogen fixation by the endophyte shows a daily rhythm determined by the plant's light environment.

The availability of fixed nitrogen limits primary productivity in plant ecosystems (6). During their evolution, legumes have developed a symbiotic partnership with rhizobia that can fix atmospheric nitrogen and can thus attain the necessary fixed nitrogen. Among nonleguminous plants, several genera of diazotrophs have been isolated and characterized as nitrogen-fixing endophytes, including *Gluconacetobacter* (2, 4, 32), *Azoarcus* (7, 8, 13), *Klebsiella* (14), *Herbaspirillum* (2, 4, 9, 18), *Azospirillum* (9, 10), and *Clostridium* (23, 24).

To determine the contribution of fixed nitrogen to the host plants by these species (17), the long-term amount of endophyte-dependent nitrogen fixation has been studied by means of the incorporation of $^{15}N_2$ gas (9, 13, 32) and the ^{15}N isotope dilution method (14). Recently, expression of the *nifH* gene has been studied by means of reverse transcription-PCR (RT-PCR) to evaluate the nitrogen-fixing activity of endophytic *Azoarcus* sp. (13) and those of nitrogen-fixing microbial communities in the termite gut (25), soil (5), and marine water (37). The *nifH* gene encodes the Fe protein (dinitrogenase reductase) and is one of the nitrogenase structural genes (*nifHDK*) (27). Transcription of *nif* genes is strictly regulated by levels of molecular oxygen and fixed nitrogen to minimize unnecessary energy consumption (6). Thus, there are tight relationships between nitrogen-fixing activity and *nif* gene transcription (6, 7, 13).

In performing transcription analysis, the Northern and dot blot hybridization methods are insufficiently sensitive to detect low copy numbers of transcripts (11). Indeed, the densities of endophytic bacteria are usually $<10^7$ cells per gram (fresh weight) of the host plant (9, 18). In contrast, RT-PCR is highly sensitive and thus is probably suitable for transcription analysis of endophytes residing at low densities in plant tissues. In addition, real-time PCR systems using a fluorescent dye (SYBR Green I) that binds with double-stranded DNA are becoming popular methods to quantify mRNA by RT reaction (12).

Because endophytic microorganisms depend on an energy supply from their host plants, their metabolic functions should be affected by variations in the physiological properties of their hosts, such as rates of photosynthesis. However, plant photosynthesis might increase O_2 concentrations around the endophyte and thereby repress expression of the endophyte's *nif* structural genes during the light period.

Shoot-associated *Herbaspirillum* sp. B501 is a diazotrophic endophyte that is capable of fixing nitrogen in leaves and stems of wild rice, *Oryza officinalis* W0012 (9). Thus, this bacterium represents a suitable system in which to examine whether nitrogen fixation by endophytes is subject to variations in the physiological status of the host plant. The aim of the present study was to understand the lifestyle of an endophyte, *Herbaspirillum* sp. B501*gfp*1, within its host plant in terms of the host's physiological functions and environments. To accomplish this, we developed a method for monitoring *nifH* transcription of the endophytic bacterium by means of RT-PCR.

MATERIALS AND METHODS

Bacterial strains, plasmids, plant material, and growth media. The bacterial strains and plasmids used in our study are listed in Table 1. *Herbaspirillum* sp. B501 was previously isolated from wild rice species as a nitrogen-fixing endophyte and tagged with the *gfp* gene for observation of bacterial colonization in rice plants (9). *Herbaspirillum* sp. strains were grown in nutrient broth (NB) medium or modified Rennie (MR) medium, as described previously (9, 29). For growth under defined oxygen concentrations, strain B501*gfp*1 (a *gfp*-tagged strain of *Herbaspirillum* sp. B501) was grown in MR medium to an optical density at 660 nm of 0.05, and the culture (1 ml) was added to 300 ml of fresh MR medium in a 500-ml flask. The medium was incubated at 30°C while being bubbled with gas filtered through a membrane filter with a pore size of 0.2 μm (Toyo Roshi Kaisha, Ltd., Tokyo, Japan): the gas was either free air (21.0% O_2) or a mixture of O_2 and N_2 with O_2 concentrations of 0.0, 0.2, 0.4, 0.6, 1.0, 2.0, 5.0, 8.0, or 12.0% (vol/vol; Tomoe Shokai Co., Ltd., Tokyo, Japan). For all gases, the flow rate was 100 ml/min. A species of wild rice, *Oryza officinalis* W0012, was used, as

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TABLE 1. Bacterial strains and plasmids used in this work

Strain or plasmid	Description[a]	Reference or source
Strains		
Herbaspirillum sp. strain B501	Wild type	9
Herbaspirillum sp. strain B501*gfp*1	*Herbaspirillum* sp. strain B501, *gfp*; Km^r	9
E. coli DH5	*recA*; cloning strain	Toyobo Co., Ltd.
E. coli S17-1	*recA*; Sm^r	33
Plasmids		
pBluescript II SK(+)	Cloning vector; Ap^r	Takara Bio, Inc.
pB501nifH1	pBluescript II SK(+) carrying a 2.9-kb EcoRI/PstI fragment of *nifHD*; Ap^r	This study

[a] Ap^r, ampicillin resistant; Km^r, kanamycin resistant; Sm^r, streptomycin resistant.

described previously (9). Semisolid agar containing nitrogen-free nutrients was used for rice growth as described previously (21). Cell numbers of strain B501*gfp*1 in culture were counted with a bacterial counting chamber by microscopy (9, 24). To estimate the cell-based nitrogen fixation and *nif* transcript concentration of endophytic strain B501*gfp*1, the cell number in plant macerate was determined by plate counts of green colonies by using selective NB medium containing kanamycin and a fluorescence stereomicroscope (9).

Cloning and sequencing of *nifH*. DNA manipulations, including plasmid isolation, digestion, and transformation, were performed as described by Sambrook et al. (30). Total DNA was prepared from *Herbaspirillum* sp. B501 cells grown in NB (15). Southern hybridization analysis of B501 DNA was carried out as described previously (15). For the probe, a 0.4-kb DNA fragment was amplified from the B501 DNA by means of PCR using primers 19F and 407R, whose sequences are highly conserved in *nifH* genes from various organisms (34); these primers are described in Table 2. We then purified a 2.9-kb DNA fragment, which corresponded to the signal detected in the Southern hybridization, from the EcoRI/PstI-digested B501 DNA using the QIAEX II DNA extraction kit (QIAGEN GmbH, Hilden, Germany); it was then ligated to the EcoRI/PstI-digested pBluescript II SK(+) and transformed into *Escherichia coli* DH5α. The resulting colonies were lifted onto nylon membranes (Amersham Biosciences Corp., Piscataway, NJ), and screened by hybridization using the 0.4-kb DNA fragment of the *nifH* gene. As a result, we obtained a positive clone and designated the contained plasmid pB501nif1. The 2.9-kb insert was sequenced on both strands by using primers −21 M13, M13 Rev, nif370, nif370-1, nif370-2, nif370-3, nif370-4, nif270, nif270-1, nif270-2, nif270-3, and nif270-5 (Table 2). DNA sequencing was performed using the ABI PRISM Big Dye Terminator Cycle Sequencing kit and a 370 DNA sequencer (Applied Biosystems, Inc., Foster City, CA). The nucleotide sequence of the 2.9-kb DNA fragment appears in the DDBJ database under accession number AB196476.

TABLE 2. Primers used in the present study

Application	Primer and sequence[a]	Reference or source
Cloning		
19F	5′-GCIWTYTAYGGIAARGGIGG-3′	34
407R	5′-AAICCRCCRCAIACIACRTC-3′	34
Sequencing		
−21 M13	5′-TGTAAAACGACGGCCAGT-3′	Applied Biosystems
M13 Rev	5′-CAGGAAACAGCTATGACC-3′	Applied Biosystems
nif370	5′-TGTAACACCAGAAAGAAGTG-3′	Present study
nif370-1	5′-GGACACCATCCTGTCGCTGG-3′	Present study
nif370-2	5′-ACAAGGAACTGGAACTGGCC-3′	Present study
nif370-3	5′-TAGCGCAGGTTTGTTTACC-3′	Present study
nif370-4	5′-ACATCAGCCATGGTCCGGT-3′	Present study
nif270	5′-CTCGACCCAGGGAAATGCC-3′	Present study
nif270-1	5′-CTCGTCGACGATCTTTTCCA-3′	Present study
nif270-2	5′-TTTCTTCAACTGTGAGGCTC-3′	Present study
nif270-3	5′-AGTTGGCGTACTTCAAAATG-3′	Present study
nif270-5	5′-TTATTGGTCTTCGTCTCA-3′	Present study
RT-PCR		
nif270-3	5′-AGTTGGCGTACTTCAAAATG-3′	Present study
nif370-1	5′-GGACACCATCCTGTCGCTGG-3′	Present study
gfp553	5′-TCAACTAGCAGACCATTATCAACA-3′	Present study
gfp681	5′-ACCATGTGGTCTCTCTTTTCG-3′	Present study
Real-time RT-PCR		
nifH238	5′-ATCGGCTACCAGAACATC-3′	Present study
nifH366	5′-GTAATCGGTGTCATCATAGG-3′	Present study

[a] I represents inosine, R represents A or G, W represents A or T, and Y represents C or T.

Rice cultivation and inoculation with *Herbaspirillum*. Dehulled seeds of *Oryza officinalis* W0012 were surface sterilized with 70% ethanol for 1 min and then washed five times with sterile distilled water. Then seeds were shaken in 1% NaOCl solution for 1 min and then washed again five times with sterile distilled water.

For seed inoculation, *Herbaspirillum* sp. B501*gfp*1 was cultured in NB medium, the cells were harvested when the culture reached an optical density of 1.0 at 660 nm by means of centrifugation at 17,742 × *g* for 5 min at 4°C. The cells were washed twice with sterile distilled water and resuspended in sterilized 0.85% (wt/vol) NaCl solution. Sterilized rice seeds were placed on semisolid medium in a 350-ml plant box (Cul-JAR300; Iwaki, Tokyo, Japan), and a suspension of *Herbaspirillum* sp. B501*gfp*1 was added at a concentration of 10^6 cells per seed. The seeds were incubated in a plant growth cabinet (LH300; NK System Co., Ltd., Osaka, Japan) that provided 65 μmol photons m^{-2} s^{-1} of photosynthetically active radiation (400 to 700 nm) under a daily cycle of 16 h of light and 8 h of dark at 25°C.

Acetylene reduction assay. The nitrogen-fixing activity of the endophyte was examined by means of acetylene reduction activity. To determine the activity of free-living bacteria, *Herbaspirillum* sp. B501*gfp*1 was grown in NB or MR medium at 30°C for 24 h, and then 9 ml of the culture was transferred into a 27-ml test tube sealed with a rubber stopper. A total of 0.9 ml of acetylene gas (purity of 99.9999% [vol/vol]; Toho Acetylene Co., Tokyo, Japan) was injected to provide a concentration of 5% (vol/vol), and the test tube was incubated for 12 or 24 h at 30°C.

To determine the nitrogenase activity under defined O_2 concentrations, 5 ml of B501*gfp*1 culture grown under the flow of different O_2/N_2 gas mixtures was transferred into a 29-ml L-type tube sealed with a double rubber stopper in the presence of the O_2/N_2 gas mixtures. After acetylene gas was injected to a concentration of 5% (vol/vol), the tube was shaken with 100 stroke min^{-1} at 30°C for 2 min by a L-tube shaker (Monod-mini; Taitec Co., Ltd., Koshigaya, Japan).

To determine the in planta nitrogenase activity, plants inoculated with B501*gfp*1 were washed with sterile distilled water and then transferred into a 33-ml bottle sealed with a rubber septum. Acetylene gas (1.65 ml) was injected to provide a concentration of 5% (vol/vol), and the bottle was incubated for 12 or 24 h at 30°C in the dark.

Gas samples (each, 1 ml) from each tube and bottle were analyzed for the presence of ethylene with a Shimadzu GC-18A gas chromatograph equipped with a flame ionization detector and a Porapack R column as described previously (9).

Light and dark cultivation of free-living cells. *Herbaspirillum* sp. B501*gfp*1 was grown in MR medium to an optical density of 0.05 at 660 nm, and the culture (3 ml) was added to 300 ml of fresh MR medium in a 500-ml flask. The flask was bubbled with a gas, a mixture of 2.0% (vol/vol) O_2, under light and dark conditions at 25°C. A light-treated flask was illuminated by 65 μmol photons m^{-2} s^{-1} of photosynthetically active radiation (400 to 700 nm) using fluorescence lamps. A dark-treated flask was completely wrapped with black aluminum foil. The light and temperature were adjusted to the conditions where rice plants were culti-

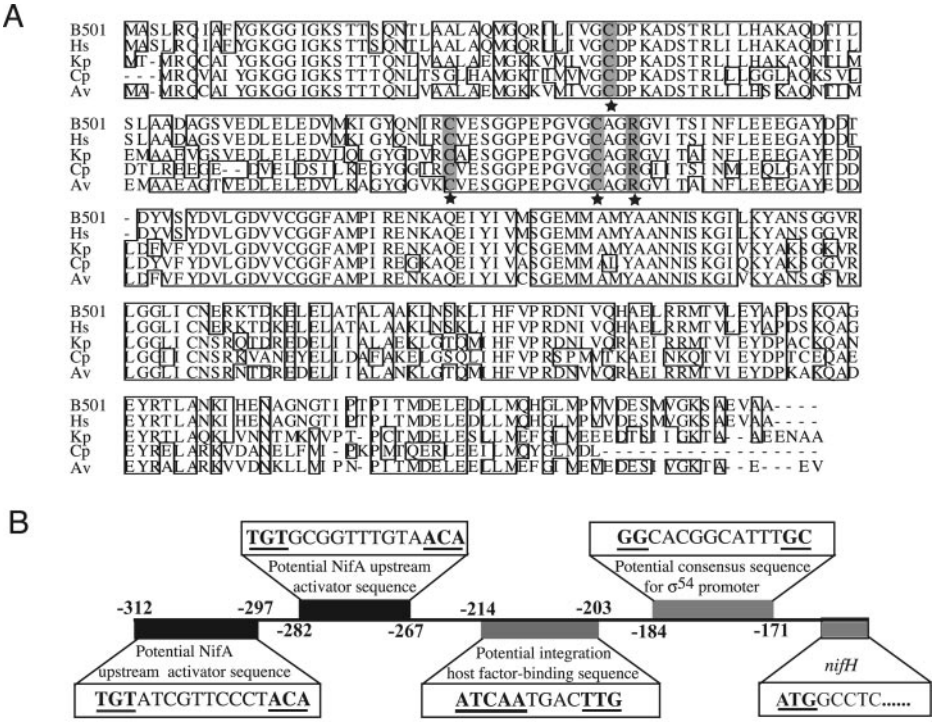

FIG. 1. (A) Alignment of the amino acid sequence of the *nifH* product in our study with other homologues. B501, the *nifH* product of *Herbaspirillum* sp. strain B501 (AB196476); Hs, the *nifH* product of *Herbaspirillum seropedicae* (Z54207) (20); Kp, the *nifH* product of *Klebsiella pneumoniae* (V00631) (31); Cp, the *nifH* product of *Clostridium pasteurianum* (X07472) (36); and Av, the *nifH* product of *Azotobacter vinelandii* (M20568) (16). Three cysteine and one arginine residue (★) of the *nifH* product are conserved. (B) Structural organization of the *nifH* promoter region of the *Herbaspirillum* sp. B501 strain. Consensus sequences are underlined and in boldface type. Numbers are nucleotide positions from *nifH* gene of strain B501.

vated. Culture was sampled 14 to 16 h after inoculation at an optical density at 660 nm of approximately 0.15 for RNA extraction.

RNA preparation. To prepare total RNA of the cultured cells, 10 ml of culture was centrifuged at 17,742 × *g* for 10 min at 4°C. The bacterial pellet was homogenized by being vortexed in 1 ml of TRIzol reagent (Invitrogen Corp., Carlsbad, CA) for 1 min, and the solution was incubated at 60°C for 15 min. After 0.2 ml of chloroform was added, the mixture was centrifuged at 17,742 × *g* for 10 min. We collected the upper phase, added 0.2 ml of chloroform, and repeated the phase separation. The resulting upper phase was mixed with 0.5 ml of 2-propanol and the solution was centrifuged at 12,000 × *g* for 10 min at 4°C. The pellet was washed twice with 99.5% (vol/vol) ethanol, air dried, and then dissolved in 40 μl of sterile distilled water. We then added 5 μl of DNaseI (5 units/μl) and 5 μl of 10× buffer (Takara Bio, Inc., Otsu, Japan). The mixture was incubated at 37°C for 2 h, extracted with 50 μl of chloroform, and centrifuged at 14,700 × *g* for 10 min. RNA was recovered from the upper phase by 2-propanol precipitation, as described above. Each sample was examined for RNA concentration and purity using Gene Quant II (Amersham Bioscience, Piscataway, NJ) at 260, 280, and 320 nm and adjusted to the same RNA concentration for all samples.

To prepare the total RNA of an inoculated plant, we froze 0.03 to 0.10 g (fresh weight) of *O. officinalis* W0012 seedlings 7 days after inoculation with B501*gfp*1 in liquid nitrogen and ground the sample finely with a mortar and pestle. For time course experiments of *nifH* expression during light-dark cycles, we used the seedlings that were older than 7 days after inoculation. After 10 volumes of TRIzol reagent (Invitrogen Corp.) was added to the samples at room temperature, the solution was homogenized well using the mortar and pestle. The content was transferred into a fresh tube and incubated at 60°C for 15 min. The other procedures for RNA extraction and the DNaseI treatment were carried out as described above, except that we performed the chloroform extraction twice.

RT-PCR. The RT reaction was carried out in a 20-μl solution containing 0.12 μg of total RNA with an Omniscript RT Kit (QIAGEN) according to the manufacturer's instructions. We then incorporated 2 μl of the resulting RT

TABLE 3. Nitrogen-fixing activity (estimated by means of acetylene reduction) in free-living and endophytic *Herbaspirillum* sp. strain B501*gfp*1

Condition	Acetylene	Ethylene evolution[d]	Acetylene reduction activity	
			Total activity[e]	Sp act, nmol h^{-1} $(10^8$ cells$)^{-1}$
Culture[a]				
Modified Rennie (MR)	+	65.5 ± 4.7	65.5 ± 4.7	6.4 ± 0.5
Nutrient broth (NB)	+	ND[b]	ND[b]	
Plant				
(*Oryza officinalis* W0012)[c]				
	+	15.1 ± 3.0	14.8	7.6
	−	0.3 ± 0.1		

[a] Ethylene evolution from free-living cells grown in semisolid MR medium at 2% (vol/vol) O_2 and in NB medium for 24 h. Values given are the means ± standard deviation for three determinations.

[b] ND, not detected (<0.2 nmol h^{-1} $tube^{-1}$).

[c] Ethylene evolution from 7-day-old rice seedlings inoculated with *Herbaspirillum* sp. strain B501*gfp*1 in the presence and absence of 5% (vol/vol) acetylene gas for 24 h. Uninoculated plants emitted ethylene at a rate of 0.2 to 0.5 nmol h^{-1} g (fresh wt)$^{-1}$. Values of ethylene evolution in the dark are the means ± standard deviation for three determinations. Acetylene reduction activity was calculated from the difference in ethylene evolution between rice seedlings in the presence and absence of 5% (vol/vol) acetylene gas.

[d] Values for ethylene evolution for cultures are in nanomoles per hour per tube; for plants, values are in nanomoles per hour per gram (fresh weight).

[e] Values for total acetylene activity are in nanomoles per hour per tube for cultures; for plants, values are nanomoles per hour per gram (fresh weight).

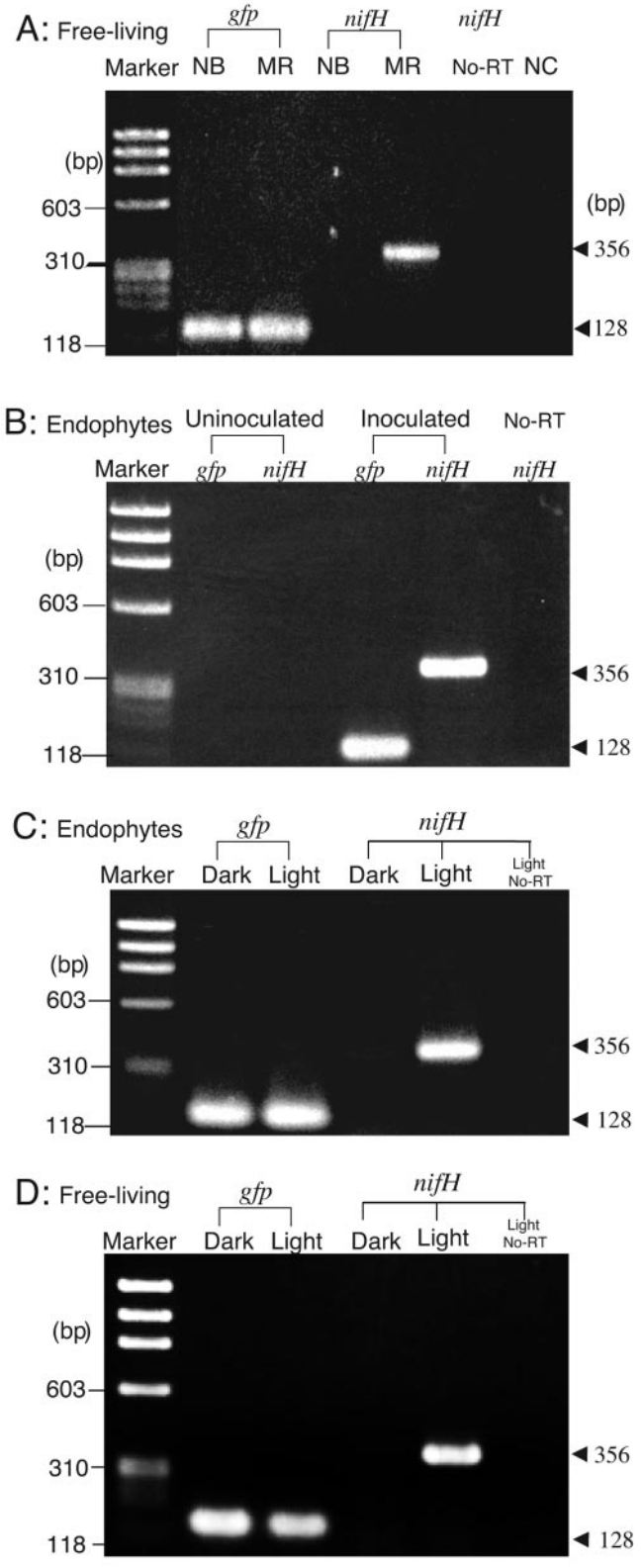

FIG. 2. Products amplified by means of RT-PCR from total RNA extracted from various sources. (A) Free-living *Herbaspirillum* sp. B501*gfp*1. (B) Endophytic *Herbaspirillum* sp. B501*gfp*1; 7-day-old plants inoculated with B501*gfp*1 were sampled during the light period and then subjected to acetylene reduction (Table 3) and this RT-PCR assay. (C) *nifH* and *gfp* transcripts from endophytic *Herbaspirillum* sp. B501*gfp*1 in the wild rice plants between the dark and light periods. (D) *nifH* and *gfp* transcripts from free-living cells of *Herbaspirillum* sp. B501*gfp*1 grown in light and dark environments (see Materials and Methods). *gfp*, a *gfp* primer used for PCR; *nif*, a *nifH* primer used for PCR; NB, template from cells cultured in nutrient broth medium; MR, template from cells cultured in modified Rennie medium; No-RT, PCR amplification without reverse transcription; NC, negative control reaction without PCR template; Uninoculated, template from plants not inoculated with B501*gfp*1; Inoculated, template from plants inoculated with B501*gfp*1. Total RNA for RT-PCR was adjusted to the same amount within each panel.

reaction mixture into the PCR. PCR was performed in a 50-μl solution with 30 to 40 cycles, eacho consisting of of 30 s at 94°C, 30 s at 60°C, and 30 s at 72°C using the HotStart*Taq* PCR kit (QIAGEN), following the manufacturer's instructions. Primer sets gfp533/gfp681 and nif370-1/nif270-3 (Table 2) were used to target the *gfp* and *nifH* genes, respectively. PCR products were analyzed by means of electrophoresis on Tris-borate-EDTA–agarose (2%) gels (Nippon Gene Co., Ltd., Toyama, Japan) stained with ethidium bromide (30).

Real-time PCR. The primer set nifH238/nifH366 (Table 2) was designed to have the best performance in the real-time RT-PCR for amplifying the *nifH* gene with the Beacon Designer software (Premier Biosoft International, Palo Alto, CA.). We performed the real-time PCR with a QuantiTect SYBR Green RT-PCR kit (QIAGEN) and the i-Cycler optical system (Bio-Rad Laboratories, Inc., Tokyo, Japan), following the manufacturers' instructions. The 50-μl reaction mixture, containing 0.05 to 0.10 μg of total RNA, was incubated first at 50°C for 30 min to allow reverse transcription and then at 95°C for 15 min for initial activation of *Taq* polymerase, followed by 50 cycles, each consisting of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C. At the final step, a melting-curve analysis of the PCR products was carried out to verify specific PCR amplification. The concentration of each *nifH* transcript was determined by plotting the threshold cycle value of its amplification against the standard curve, which we created by calibrating the values obtained from the EcoRI-digested pB501nif1 DNA used as templates at amounts ranging between 2.82×10^{-11} and 2.82×10^{-15} μmol.

RESULTS AND DISCUSSION

***nifH* sequence of *Herbaspirillum* sp. B501*gfp*1.** The 2.9-kb DNA fragment in pB501nifH1 (Table 1) contained *nifH* and truncated *nifD* genes with a *nifH* promoter region (accession number AB196476). The deduced amino acid sequence of *nifH* (292 amino acid residues) from *Herbaspirillum* sp. B501 was identical to that of *Herbaspirillum seropedicae* Z78 from sugarcane (Fig. 1A) (20). Functionally important residues were conserved (34): cysteine residues (Cys-40, Cys-87, and Cys-99) and one arginine residue (Arg-102) (Fig. 1A). The *nifH* promoter region of B501 had two potential NifA-binding sites, a potential integration host factor (IHF)-binding site, and a σ^{-54}-like promoter (Fig. 1B), which are similar to those of *H. seropedicae* Z78 (20). Thus, regulation of the *nif* genes is probably mediated by *nifA* expression and NifA activation, which respond to the level of molecular oxygen and fixed nitrogen, as is the case with most other members of the *Proteobacteria* (6). Southern hybridization showed that the *nifH* gene is a single copy in strain B501 (data not shown). This indicates that *nifH* is a target gene suitable for monitoring *nif* transcription in the bacterium, because the expression of a single *nifH* gene is straightforwardly involved in functional nitrogenase activity.

***nifH* transcription in free-living cells.** We first examined the correlation between *nifH* transcription and nitrogen fixation in free-living *Herbaspirillum* sp. B501*gfp*1. Nitrogen-fixing and non-nitrogen-fixing cells were prepared from cells cultured in MR or NB medium, respectively (Table 3). Because strain

B501*gfp*1 harbored *gfp* genes with a constitutive promoter, *PpsbA* (9, 35), the *gfp* transcript was used as a positive control throughout this work. A band corresponding to the *gfp* transcript (128 bp) was observed in nitrogen-fixing and non-nitrogen-fixing cells grown in NB and MR media (Fig. 2A). In contrast, a band corresponding to the *nifH* transcript (356 bp) was detected exclusively in nitrogen-fixing cells cultured in MR medium (Fig. 2A). Exclusion of the RT reaction produced no PCR band for the *nifH* gene (Fig. 2A). These results indicate that *nifH* transcription was successfully detected using total RNA extracted from nitrogen-fixing cells by means of RT-PCR.

Effects of oxygen concentrations on *nifH* transcription. The *nif* promoter sequence of *Herbaspirillum* sp. B501 suggested that the *nif* genes are subjected to transcriptional activation by NifA (Fig. 1), which responds to the concentration of molecular oxygen (5). Thus, we examined the effect of O_2 concentration on *nifH* transcription and nitrogenase activity in free-living cells of B501*gfp*1.

Although the *gfp* transcript showed a constant level regardless of the O_2 regime and thus served as a positive control, the levels of *nifH* transcription changed drastically in response to changing O_2 concentrations (Fig. 3A and B). At a 2% O_2 concentration, nitrogenase activity (Fig. 3C) and *nifH* expression (Fig. 3A and B) reached their maximum levels in the bacterium. In contrast, the activity of the *nifH* gene and nitrogenase were strongly repressed under aerobic conditions (21% O_2) (Fig. 3A to C). These results indicate that *Herbaspirillum* sp. B501 requires a microaerobic environment to be capable of expressing nitrogenase activity, as is the case for other diazotrophic endophytes (1, 8, 26) and nitrogen-fixing microbes (6, 28). RT-PCR analysis of *nifH* expression was an efficient method for monitoring the potential nitrogen-fixing activity of *Herbaspirillum* sp. B501*gfp*1.

***nifH* transcription in endophytic cells.** The 7-day-old seedlings of *O. officinalis* W0012 inoculated with B501*gfp*1 showed nitrogen-fixing activity (Table 3), which is similar to previously reported results (9). After the assay, total RNA was extracted from the surface-washed seedlings and subjected to *nifH* transcription analysis by means of RT-PCR. Bands corresponding to the *gfp* and *nifH* genes were observed in B501*gfp*1-inoculated plants at expected positions of 128 and 356 bp, respectively (Fig. 2B). Elimination of the RT reaction confirmed that the 356-bp band was derived from *nifH* mRNA but not from *nifH* DNA (Fig. 2B). Uninoculated plants showed no background of PCR amplification products for *gfp* and *nifH* transcripts (Fig. 2B). These results indicate that the *gfp* and *nifH* transcripts of endophytic *Herbaspirillum* sp. B501*gfp*1 in the plant tissues of wild rice could be detected by means of RT-PCR without interference from the plant RNA.

Photosynthesis by the host plant supplies photosynthate to the endophytes as their energy source (4), but during the light period, it also generates O_2 that might inhibit nitrogen fixation by the endophytes (9, 22). Indeed, the activity of the *nifH* gene and nitrogenase in free-living B501 cells were strongly repressed under aerobic conditions (21% O_2) (Fig. 3). Thus, we compared *nifH* expression by endophytic B501*gfp*1 in 7-day-old seedlings sampled at midnight (dark period) and at noon (light period). Surprisingly, we detected *nifH* transcription exclusively in the noon sample but observed similar levels of *gfp* transcription both at midnight and at noon (Fig. 2C). This result suggests that light and/or presumably photosynthesis in response to that light enhances rather than represses *nifH* transcription.

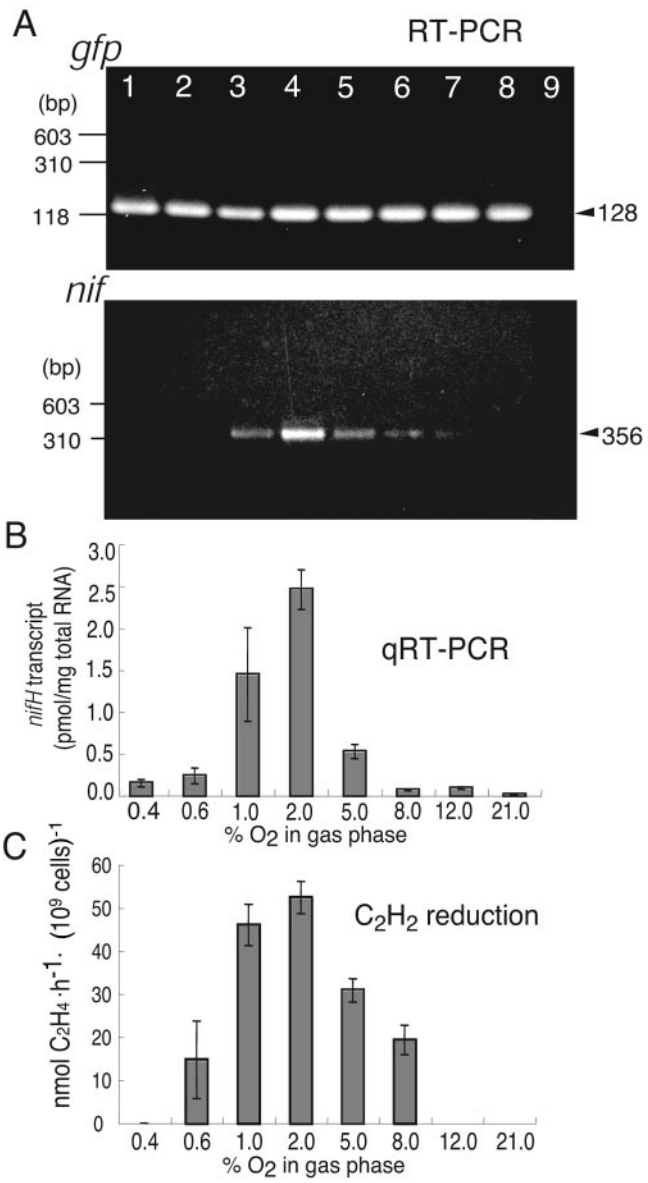

FIG. 3. (A) Products of *nifH* amplified by means of RT-PCR using total RNA extracted from free-living *Herbaspirillum* sp. B501*gfp*1 cultured under different oxygen concentrations. Lanes 1 to 8, RNA was extracted from cells cultured under 0.4, 0.6, 1.0, 2.0, 5.0, 8.0, 12.0, and 21.0% (vol/vol) oxygen concentrations, respectively; lane 9, negative control reaction without PCR template. *gfp*, a pair of *gfp* primers was used to amplify the *gfp* transcripts; *nif*, a pair of *nifH* primers was used to amplify the *nifH* transcripts. Total RNA was adjusted to the same amount for the respective RT-PCRs. (B) Quantification of the *nifH* transcripts of *Herbaspirillum* sp. B501*gfp*1, cultured under different oxygen concentrations, by means of real time RT-PCR. (C) Acetylene reduction activity of *Herbaspirillum* sp. B501*gfp*1 cultured under different oxygen concentrations.

Daily rhythm of *nifH* transcription in endophytic cells. Preliminary RT-PCR results (i.e., the detection of *nifH* transcrip-

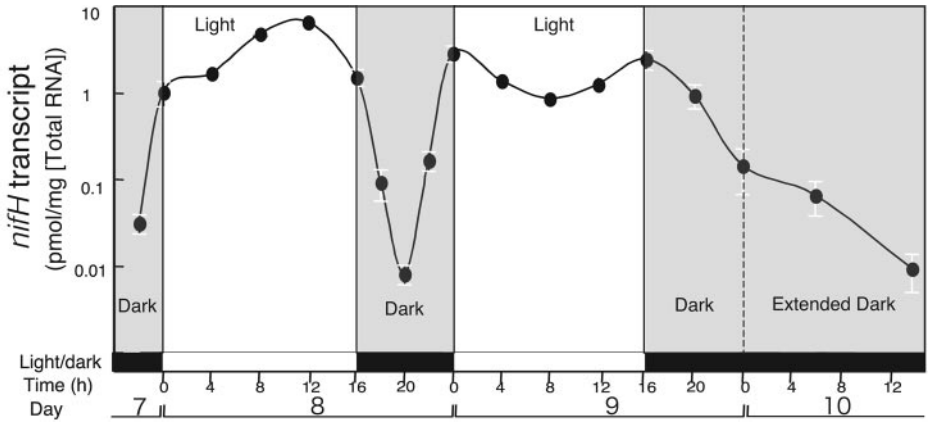

FIG. 4. Fluctuations in *nifH* transcription by endophytic *Herbaspirillum* sp. B501*gfp*1 during light and dark cycles. The *nifH* transcript of B501*gfp*1 was determined with three replications by means of real-time RT-PCR. Shaded zones represent the dark period, whereas white zones represent the light period; Time, time (in hours) during the course of one light-dark cycle; Day, days of growth of the *O. officinalis* W0012 seedlings after inoculation with *Herbaspirillum* sp. B501*gfp*1. Each point indicates the mean of three measurements using single RNA preparation extracted from three inoculated plants.

tion in the light period) (Fig. 2C) prompted us to follow the time course of *nifH* expression between the light and dark periods. We quantified *nifH* transcription in endophytic B501*gfp*1 by means of real-time RT-PCR using 7- to 10-day-old *O. officinalis* W0012 plants inoculated with the endophyte.

Significant fluctuations in the *nifH* transcription level were observed during light and dark cycles (Fig. 4). The level of *nifH* transcription during the light period was consistently higher than during the dark period. Based on the amount of total RNA extracted from the plants, transcription during the light period reached a maximum of 100 times the transcription level during the dark period (Fig. 4). From the curve for levels of *nifH* transcripts from 22:00 h on day 7 to 16:00 h on day 9, we observed a daily rhythm in *nifH* transcription: the amount of *nifH* transcription decreased to its minimum level by around midnight and reached its maximum level during the light period (Fig. 4).

Since *gfp* transcription was detected during the night and day in the inoculated plants (Fig. 2C), the daily rhythm of *nifH* transcription was also not due to proliferation and death of the endophyte within the rice plants. Tight relationships exist between nitrogen-fixing activity and *nif* expression, as shown in Fig. 3 and as reported by other researchers (6, 7, 13). Thus, our results strongly suggest that nitrogen fixation by the endophytes fluctuated markedly in response to the daily rhythm of *nifH* expression during light and dark periods. The presence of a daily rhythm of *nifH* expression also indicated how to measure the nitrogen-fixing activity of endophytes in plants by the $^{15}N_2$ and acetylene methods. The plant's light environment, the time of sampling, and the incubation duration probably all affect the endophyte's activity and should thus be accounted for by the sampling design.

To examine whether this daily rhythm represented a circadian rhythm (19), we preliminarily extended the dark period from days 9 and 10. This extension on day 10 showed no apparent increase in the level of *nifH* transcription; however, *nifH* mRNA began to accumulate prior to illumination in the experiment. Thus, we were not able to conclude that the daily rhythm was brought about by a circadian rhythm or light/dark stimulation so far.

Comparison of *nifH* expression between endophytic and free-living cells. In the present study, we determined the amounts of *nifH* transcription in free-living and endophytic cells of *Herbaspirillum* sp. B501 by means of real-time RT-PCR. Thus, it is appropriate to compare transcription levels, although we assumed that the efficiencies of RNA extraction, the RT reaction, and the PCR were equal between both cell types. Expressed as cell numbers, the concentration of *nifH* transcripts in endophytic cells averaged 670 pmol/10^{10} cells during the light period and 5 pmol/10^{10} cells during the dark period (Table 4) versus only 10 pmol/10^{10} cells in free-living

TABLE 4. Quantification of *nifH* transcription in the total RNA of free-living and endophytic *Herbaspirillum* sp. B501*gfp*1[a]

Condition	*nifH* transcription	
	pmol (mg total RNA)$^{-1}$ (A)	pmol (10^{10} cells)$^{-1}$ (B)[b,c]
Culture[a]		
Modified Rennie (2% O_2)	2.5 ± 0.4	10 ± 2
Nutrient broth	ND[d]	
Plant (*Oriza officinalis* W0012)[a]		
Inoculated (light period)	2.3 ± 0.6	670 ± 160
Inoculated (dark period)	0.03 ± 0.009	5 ± 2
Uninoculated	ND[d]	

[a] Culture, the amount of *nifH* transcription in free-living B501*gfp*1 cells grown in semisolid modified Rennie (MR) medium at 2% (vol/vol) O_2 and in nutrient broth (NB) medium for 24 h. Values given are the means ± standard deviation for three determinations. Plant, the amount of *nifH* transcription in endophytic B501*gfp*1 cells during the light and dark periods (Fig. 4). Values given are the means ± standard deviation for 10 data points at 0:00, 4:00, 8:00, 12:00, and 16:00 h on days 8 and 9 for the light period, and four data points at 18:00, 20:00, and 22:00 h on days 7 and 8 for the dark period (Fig. 4).

[b] B = A × (μg of total RNA)/[(total cells or CFU)/10^{10}].

[c] Cell numbers were estimated by direct counting with a microscope for cultures and by plate counting for endophytic populations.

[d] ND, not detected (<1.4×10^{-3} pmol (mg total RNA)$^{-1}$).

cells of B501*gfp*1 grown at an optimal O_2 concentration (2%) in MR medium. If our assumption of comparability is correct, then the level of *nifH* transcription by endophytic cells during the light period was markedly higher (by a factor of 67) than that of free-living cells at the optimal O_2 concentration.

***nifH* expression of free-living cells under light and dark conditions.** RT-PCR analyses demonstrated that the level of *nifH* transcription in *Herbaspirillum* sp. B501 during the light period was higher than that during the dark period (Fig. 2C and 4; Table 3). The light period-dependent *nifH* expression occurred in a complex biological system with the plant and endophyte. To address which organisms perceive light, we simply examined the *nifH* expression of free-living cells grown under light and dark conditions. *Herbaspirillum* sp. B501*gfp*1 was grown in MR broth medium in a flask at 2% (vol/vol) O_2 under light and dark conditions: the light intensity and temperature were adjusted to the levels used for cultivation of the inoculated rice plants.

Similar levels of *gfp* transcription were observed in cells grown in both the light and the dark (Fig. 2D). Nevertheless, *nifH* transcription was detected exclusively in the cells grown in the light but not detected in the cells grown in the dark (Fig. 2D), which is similar to results obtained with endophytic cells (Fig. 2C). This result implies that at least the endophyte is able to percept light and up-regulates *nifH* gene expression, although we cannot completely exclude the involvement of the plant into the light period-dependent *nifH* expression.

To our knowledge, our study represents the first observation of a daily rhythm of *nif* gene expression in endophytic microbes within plant tissues during the light-dark cycles. In addition, it is a preliminary but fascinating discovery that light radiation induces *nifH* gene expression in free-living *Herbaspirillum* sp. strain 501. So far, we do not yet know exactly how the daily rhythm of *nifH* expression in the endophyte was generated during the light-dark cycles and how light induces *nifH* gene expression even in the free-living bacterium.

Photosynthetic bacteria, such as cyanobacteria, have evolved the ability to sense light and show phototaxis to choose a more favorable position for optimal growth (3). Thus, we want to discuss the physiological and ecological implications of our findings: light period-dependent *nifH* expression in the endophytes. The plant probably will need more fixed nitrogen when it is photosynthetically active, while the bacterium would take advantage of the photosynthate that is produced during the light period. Therefore, one would expect the bacterium to have evolved a mechanism to up-regulate *nif* gene expression during the light reaction. The photosynthate-mediated interactions between plant and endophyte might be more important rather than the so-called oxygen paradox for nitrogen fixation (22).

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ADDENDUM IN PROOF

Additional experiments were performed after this paper was accepted for publication. In these experiments, time courses of *nifH* and *gfp* transcripts in free-living cells of *Herbaspirillum* sp. strain B51*gfp1* grown in modified Rennie broth in light and dark environments were monitored. The cells exposed to the light radiation showed slightly higher levels of growth (Fig. A1A) and *nifH* gene expression (compared with *gfp* signal) than those in the dark (Fig. A1B). However, the *nifH* gene was expressed even in the free-living cells grown in the dark (Fig. A1B, lanes D3 and D4). In our experimental system, the expression of the *nifH* gene started around growth points L2 and D2 (Fig. A1). Because the previous result (Fig. 2D) was obtained at this growth stage, we erroneously concluded that the *nifH* gene was not expressed in the cells in the dark at all (Fig. 2D). The new finding (Fig. A1) does not affect the significance

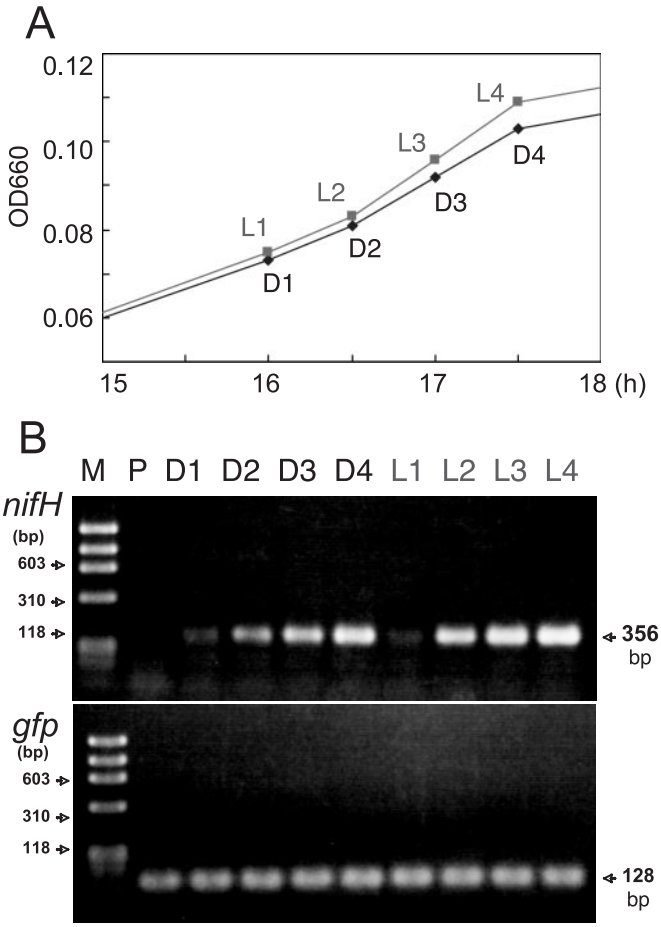

FIG. A1. Growth of and gene expression by free-living cells of *Herbaspirillum* sp. strain B501*gfp1* grown in light and dark environments. (A) Tiem courses of optical density at 660 nm (OD660) for *Herbaspirillum* sp. strain B501*gfp1* cells grown in light (L1, L2, L3, and L4) and dark (D1, D2, D3, and D4) environments (see text). (B) RT-PCR analysis targeted to *nifH* and *gfp* genes from total RNA from cultured cells of *Herbaspirillum* sp. B501*gfp1*. Lane M, size marker; lane P, preculture for inoculation. Cells of lanes D1, D2, D3, D4, L1, L2, L3, and L4 were sampled as indicated in the growth curves in panel A. Total RNA was adjusted to the same amount for each reaction in the RT-PCR analysis.

of this work, because the levels of in planta *nifH* transcription varied drastically, with a maximum during the light period (Fig. 2C and 4). However, this finding further clarifies the contribution of plants to light period-dependent *nifH* expression in endophytes.

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