Research Note

Endophytic Expression of *nif* Genes of *Azoarcus* sp. Strain BH72 in Rice Roots

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Transcriptional fusions of *gusA* and *gfp* to the *nifH* gene as well as immunogold labeling with antibodies against the iron protein of nitrogenase revealed high nitrogenase gene expression levels of the endophyte *Azoarcus* sp. strain BH72 inside infected rice roots (*Oryza sativa*) in the laboratory. Thus, environmental conditions inside rice roots are permissive for endophytic nitrogen fixation in bacterial microcolonies in the aerenchyma.

Additional keywords: ammonium, oxygen tolerance, repression.

Endophytic microorganisms multiply and spread inside plants without causing rapid damage of the host tissue or conferring an ecological threat to the plant (Quispel 1992). Several nitrogen-fixing, Gram-negative bacteria have now been recognized to be endophytes of gramineous plants, such as *Acetobacter diazotrophicus* in sugar cane (Gillis et al. 1989; James et al. 1994), *Herbaspirillum seropedicae* in sugar cane (James and Olivares 1998) and sorghum (James et al. 1997), and an *Azoarcus* sp. in Kallar grass and rice (Hurek et al. 1994b). In contrast to other root-colonizing or symbiotic bacteria such as *Azospirillum* spp. or rhizobia, they are tightly associated with plants, since they do not survive well in root-free soil or cannot be isolated from it (Boddey 1995; Reinhold-Hurek and Hurek 1998b; Reinhold-Hurek et al. 1993).

Grass-associated endophytic diazotrophs share pathways of invasion and sites of colonization (Reinhold-Hurek and Hurek 1998b), which have also been reported for rhizobia in non-legumes (Gough et al. 1997; Yanni et al. 1997). Primary ingress into roots has been observed at emergence points of lateral roots and at the elongation and differentiation zone above root tips. The outer cell layers and the cortex (aerenchyma in flooded roots) as well as the stele and xylem vessels are colonized inter- and intracellularly (Hurek et al. 1994b; James and Olivares 1998; James et al. 1997, 1994). There is no evidence for endosymbiosis inside living plant cells (Hurek et al. 1994b; James and Olivares 1998; Reinhold-Hurek and Hurek 1998a). A good record of colonization capacities of endophytes is now available; however, not much is known about their functions. Certain sugar cane cultivars in Brazil have

been reported to have high natural nitrogen fixation rates (Lima et al. 1987; Urquiaga et al. 1992), but which diazotroph contributes significant amounts of fixed nitrogen is not yet clear. In order to assess the metabolic capacities of diazotrophs in host plants and to improve endophytic, putatively beneficial associations for a more sustainable agriculture, a primary step is to localize the expression of bacterial genes of interest in the host plant. Suitable reporter genes for transcriptional fusions include genes encoding β -glucuronidase (GUS) of *Escherichia coli* (Jefferson et al. 1987), and the jellyfish green fluorescent protein (GFP) (Chalfie et al. 1994). The latter has the advantage of detection of autofluorescence without involvement of diffusible substrates or dyes. GFP has been used for tagging bacteria (Bloemberg et al. 1997; Gage et al. 1996; Tombolini et al. 1997) and for detection of bacterial gene expression in association with plants (Egner et al. 1998).

Azoarcus spp. are endophytes of Kallar grass (Reinhold et al. 1986; Reinhold-Hurek et al. 1993); however, they are capable of colonizing rice seedlings endophytically in the laboratory (Hurek et al. 1994b). In this report, the influence of environmental factors on *nif* gene expression and the expression inside roots was studied with a transcriptional *nifH::gusA* fusion in strain BH72 and the analogous *nifH::gfp* reporter strain. The results demonstrate that, in the cortex of rice roots, concentrations of O₂, combined nitrogen, and carbon sources are suitable to support highly active expression of *nif* genes in *Azoarcus* sp. strain BH72.

A cassette for the creation of transcriptional fusions of target genes with the GUS gene was constructed from pRAJ294 (Wilson et al. 1995) with primers that introduced translational stops in all three reading frames (bold) and the *Azoarcus* spp. *nifH* RBS (ribosomal binding site, underlined) to the 5' end of the *gusA* gene, in order to avoid translational fusions of *gusA* with the target genes. *EcoRI* restriction sites were added to both primer sequences (double underlined), which were designed to bind to the 5' end (5'-GGAATTCTGATTGATTGAGGAGAGGAGTTTCAACATGTTACGTCCTGTAGAAAC-3') and the 3' end (5'-GGAATTCAGTTGTTGATTGATTGTTG-3') of *gusA*. PCR (polymerase chain reaction) was carried out according to standard procedures (Ausubel et al. 1987) with an annealing temperature of 55°C and 30 cycles. For a transcriptional *nifH::gusA* fusion, the PCR fragment was inserted into pEN332, which carries 800 bp of the *Azoarcus* sp. strain BH72 *nifH* gene and the upstream regulatory region

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(sequence not shown) on a 1.45-kb *EcoRI/HindIII* fragment (Egener et al. 1998). The resulting construct pEGN3.1 was integrated into the chromosome of *Azoarcus* sp. strain BH72 by homologous recombination after electroporation, resulting in the mutant *Azoarcus* sp. strain BHGN3.1 (Fig. 1). A stable integration of a single copy into the chromosome enabled us to do long-term *in vivo* experiments without application of selective antibiotics.

To study the correlation of reporter protein and nitrogenase activity, quantitative GUS assays were compared with *in vivo* nitrogenase activities measured by the acetylene reduction method in batch cultures (Karg and Reinhold-Hurek 1996), with a Konik Gaschromatograph HRGC-4000A fitted with a Porapak N column (1 m by 2 mm inner diameter) at 50°C. O₂ in the headspace was determined with the same gas chromatograph fitted with a molar sieve column (5 Å, 80/100 mesh, 2 m by 2 mm inner diameter) at a pressure of 1.5 bar (2×10^5 Pa) and a column temperature of 80°C with helium as a carrier gas at an injector temperature of 112°C and detector temperature of 110°C. GUS activity was assayed as described by Jefferson et al. (1987), and expressed in Miller units defined as $E_{420} \times 1,000/t \text{ (min)} \times OD_{600}$. The given amount of oxygen in this type of batch culture enables the bacteria to derepress nitrogenase and grow on N₂ until the O₂ in the headspace has been

depleted to approximately 0.7 to 0.8% by bacterial respiration, when specific nitrogenase activities decrease due to oxygen and energy limitations of the bacteria (Hurek et al. 1994a). The GUS activity correlated well with the specific nitrogenase activity (Fig. 1B); a slight time delay was observed in the maximum activity of the GusA protein, because the activity of the nitrogenase enzyme might respond more quickly than gene expression to energy limitation at depleting O₂ concentrations.

Dependent on a strictly respiratory metabolism, but equipped with the oxygen-sensitive enzyme complex of nitrogenase, bacteria strictly regulate the expression of *nif* genes according to O₂ concentrations. To study the effect of O₂ on the expression of the *nifH::gusA* fusion of strain BHGN3.1, a small volume of culture was incubated under a large volume of gas phase with different O₂ concentrations. The pre-cultures were grown in SM medium (Reinhold et al. 1985) supplemented with ammonium (10 mM) and yeast extract (0.01%) to OD 0.5, then washed and resuspended in nitrogen-free medium and incubated for 4 h under different oxygen regimes in 10 ml of SM medium in 1-liter Erlenmeyer flasks with 1% acetylene in N₂ at 37°C with rotary shaking at 70 rpm (TR 50, Infors, Munich). The bacterial respiration did not alter the oxygen concentration in the flask within the 4 h of incubation (data not shown). Expression was found to be highest at 0.7 to 1.1% of oxygen in the gas phase (Fig. 2A) (corresponding to approximately 10 µM dissolved oxygen at 37°C), a sharp peak of acetylene reduction activity was found at 0.9% O₂ (not shown). No expression occurred at and above 4% (45 µM dissolved oxygen). This correlated with former experiments, showing that *Azoarcus* sp. strain BH72 can reach steady states in chemostat

Fig. 1. Expression of a *nifH::gusA* fusion in N₂-fixing *Azoarcus* sp. strain BHGN3.1. **A**, Physical map of the chromosomal integration of a pUC18 derived plasmid (pEGN3.1) carrying the *nifH::gusA* fusion. The fusion and the uninterrupted *nifHDK* operon are controlled by the original σ^{54} type promoter (arrow). E, *EcoRI* restriction sites. **B**, Specific acetylene reduction rate (open squares) and β -glucuronidase activity (filled circles) of an N₂-fixing batch culture under reduced-oxygen atmosphere starting with 2% O₂ in a 1-liter Erlenmeyer flask supplemented with 50 ml of N-free SM (Reinhold et al. 1985) medium.

Fig. 2. Effects of O₂ and combined nitrogen on *nifH::gusA*-expression. β -Glucuronidase activity determined after **(A)** 4 h of incubation under different O₂ concentrations in the headspace, or **(B)** 2 h of incubation with different concentrations of NH₄⁺ under 1% O₂.

Fig. 3. Localization of bacterial *nifH::gusA* and *nifH::gfp* expression in rice roots, 11 to 14 days after inoculation with *Azoarcus* sp. strain BH72 reporter strains. **A**, Histochemical β -glucuronidase (GUS) staining of rice roots inoculated with *Azoarcus* sp. strain BHGN3.1, showing weak (left) or strong (right) staining (w or ++, respectively). **B**, *nifH::gusA*-expressing bacteria are localized at emergence points (arrow) and on the elongation zone (arrowhead) of lateral roots. **C**, Interference contrast image of KOH-treated rice root intercellularly colonized with *nif* gene expressing *Azoarcus* sp. strain BHGN3.1., focused on second plant cell layer beyond root surface. **D**, Dark-field or (**E**–**G**) bright-field images of successive semithin sections of resin-embedded rice roots pre-stained for expression of the *nifH::gusA* fusion. Subsequent sections were immunogold-silver stained with (**D**, **E**) antiserum against *Azoarcus* sp. strain BH72 or (**F**) the iron protein of nitrogenase, or (**G**) inspected for blue GUS staining. Subsequent sections (**E**–**G**) of the highly colonized region in **D** shown at higher magnification. Roots were incubated for 30 to 90 min for GUS staining. **H**–**J**, *nifH::gfp* expression detected by fluorescence microscopy with digital confocal imaging of whole rice roots inoculated with *Azoarcus* sp. strain BHFN35. Images were derived from optical sections at 2- μ m intervals. **H**, Single optical section from root surface. **I**, Single optical section taken 30 μ m below root surface. **J**, Three-dimensional reconstruction of 35 merged optical sections, showing blue-green green fluorescent protein (GFP) fluorescence deep in root cortex layer; orientation of the view into the roots is depicted by arrows also shown in **H**; white line in **H** indicates approximate orientation of the z axis in which the root was optically sectioned for **J**. **K**, Bright-field image of transversal semithin section of resin-embedded root piece shown in **H**–**J**; **L**, Transmission electron micrograph of a successive section. Bacterial microcolonies (b) are visible in the aerenchyma (a) below outer cell layers of the root (o). Bars: **C**, **H**, and **I**, 100 μ m; **J**, 50 μ m; **K**, 30 μ m; **E** and **F**, 20 μ m; **G**, 10 μ m; or **L**, 5 μ m.

culture at oxygen concentrations of 0.5 to 25 μM with a maximum N_2 fixation activity below 10 μM O_2 (Hurek et al. 1987b). While the N_2 fixation increased with O_2 concentrations lower than 10 μM in the chemostat, the *nifH* gene expression was found to drop in the present study in batch cultures below 0.7% O_2 in the headspace. This discrepancy may be due to the experimental setup. At low O_2 concentrations in the gas phase, the diffusion rate into the liquid phase may be too low to maintain the corresponding dissolved oxygen tension in an actively respiring culture, resulting in a lower dissolved O_2 concentration than estimated and thus in energy limitation of the cells. In contrast, the sensitivity of nitrogen fixation (Hurek et al. 1987b) and *nif* gene expression to higher O_2 concentrations were in good agreement: Both showed a sharp drop at 27 and 23 μM O_2 , respectively. Within the group of grass-associated, nitrogen-fixing bacteria, this narrow range is very consistent, as in the endophytes *A. diazotrophicus* or *H. seropedicae* (Vande Broek et al. 1996), or in the mostly epiphytic *A. brasilense*, which is slightly more sensitive to O_2 (Hurek et al. 1987a; Vande Broek et al. 1996). An almost constitutive *nifH::gusA* expression in response to O_2 was reported in *Azoarcus indigenus* (Vande Broek et al. 1996), a microaerobically N_2 -fixing species of the same genus as *Azoarcus* sp. strain BH72 (Reinhold-Hurek et al. 1993). Since this apparently high O_2 tolerance of gene expression was obtained with a heterologous *nifH* promoter from *A. brasilense* (Vande Broek et al. 1996), the data have to be interpreted with caution.

The presence of combined nitrogen sources such as ammonia is also known to have a strong impact on *nif* gene expression in most diazotrophs (Merrick and Edwards 1995). An approach similar to that for O_2 was used to study the regulation of the *nifH* gene by NH_4^+ . Washed pre-cultures were aerobically incubated in N-free medium for 1 h to increase their response to nitrogen. No *nifH::gusA* expression occurred during this starvation phase. Then cells were transferred to different concentrations of NH_4^+ under 1% O_2 , and expression of the *nifH::gusA* fusion was determined after 2 h. The expression of nitrogenase genes was repressed at N concentrations of and above 0.5 mM NH_4^+ in the medium (Fig. 2B).

nif gene expression was also affected by some amino acids when added to semisolid SM medium. While the addition of 10 mM L-alanine only slowed down the growth and *nifH* expression of *Azoarcus* sp. strain BHGN3.1, L-glutamine, L-arginine, L-cysteine, L-histidine, or L-asparagine repressed the *nifH* gene and allowed growth on those nitrogen sources. L-tyrosine, L-lysine, and L-glycine inhibited *nif* gene expression as well as growth.

For plant experiments, surface sterilization of rice seeds (*Oryza sativa* L. *nihonbare*) and germination on agar plates containing 0.3% of yeast extract were carried out as described previously (Hurek et al. 1994b). The sterile seedlings were transferred to glass tubes containing 8 g of sterilized quartz sand (Merck) and 3 ml of plant medium containing 0.2 g of $\text{MgCl}_2 \times 7\text{H}_2\text{O}$, 0.1 g of NaCl, 0.026 g of $\text{CaCl}_2 \times 2\text{H}_2\text{O}$, 0.012 g of $\text{MnSO}_4 \times \text{H}_2\text{O}$, 4 mg of $\text{Na}_2\text{MoO}_4 \times 2\text{H}_2\text{O}$, 3 mg of H_3BO_3 , 0.2 mg of $\text{ZnSO}_4 \times 7\text{H}_2\text{O}$, 0.1 mg of $\text{CuSO}_4 \times 5\text{H}_2\text{O}$, 13 mg of Fe-citrate, 5 mg of DL-malic acid neutralized to pH 7.0 with KOH, 1.5 g each of K_2HPO_4 and KH_2PO_4 , adjusted to pH 6.8. The tubes were inoculated with 10^7 bacterial cells per g of quartz sand from a mid-logarithmic pre-culture (see above), washed in plant inoculation medium without DL-malic

acid. Plants were grown in the greenhouse with 12 h of day-time-light in addition to the natural day-night cycle. The roots were kept in the dark at 32°C in a water bath supplemented with black ink. For acetylene reduction assays, the tubes were tightly stoppered and acetylene was injected to a final concentration of 10% (vol/vol). Ethylene was quantified gas chromatographically after 16 h. For quantification of GUS activity in planta, the roots were homogenized with mortar and pestle in 2 ml of GUS extraction buffer with quartz sand. The suspension was centrifuged for 5 min at 1,000 rpm (centrifuge 5417R, Eppendorf, Hamburg, Germany) to pellet the root debris, while the supernatant was used for the quantitative assay (see above), protein determination, and CFU counts. For the histochemical detection of GUS activity, the roots were incubated for 0.5 to 2 h in 50 mM sodium cacodylate buffer (pH 7.5) with 0.5 mg of 5-bromo-4-chloro-3-indoyl- β -D-glucuronic acid (X-Gluc) per ml at 45°C (Hurek et al. 1994b). To increase the transparency of plant cells, the roots were KOH-treated by boiling the roots in 10% (wt/vol) KOH for 10 min and inspected for internal GUS expression.

In order to monitor the establishment of nitrogen-fixing bacteria on or in rice roots, GUS expression of roots and acetylene reduction rates were determined at various days after inoculation. Specific *nifH* expression by *Azoarcus* sp. strain BHGN3.1 was detected histochemically on roots (Fig. 3A,B) or seeds, whereas no GUS staining or acetylene reduction was observed in noninoculated roots or seeds (not shown). Well-colonized root systems showed intense staining (+++, more than 50% of roots stained, Fig. 3A) within a short time (0.5 to 2 h of incubation), indicating strong GUS activity. The emergence points of lateral roots were favored sites of bacterial microcolonies expressing the reporter gene strongly (Fig. 3B). In such root systems, approximately 3 in 4 achsils of lateral roots showed *nifH::gusA* expression. This pattern of colonization and infection is also common for *Acetobacter diazotrophicus* (James et al. 1994) and *H. seropedicae* (James and Olivares 1998; James et al. 1997) in other gramineous plants; however, no *nif* gene expression has been observed there as yet. The second major site of expression was the elongation and differentiation zone of lateral roots or large spots on the mature main roots (Fig. 3B). In contrast to observations for *Azospirillum* spp. (Vande Broek et al. 1993), GUS staining of *Azoarcus* sp.-colonized roots was not restricted only to the lowest part of the root system, even when plants were only weakly stained (w, left plant in Fig. 3A). One possible explanation may be the higher oxygen tolerance of nitrogen fixation in *Azoarcus* sp. strain BH72, compared with *Azospirillum* spp. (Hurek et al. 1987a).

However, root-associated *nifH::gusA* expression was variable (data from 7 to 10 replicates in three independent experiments). Highest root colonization rates with *nifH::gusA*-expressing bacteria were seen at 10 or 14 days after inoculation (42 or 78% of plants stained). Not all the root systems were equally well stained (days 10 or 14, respectively): few lateral roots and lateral root achsils stained (w), $13.4 \pm 9.4\%$ or $61.6 \pm 31.4\%$ of plants; several lateral roots and their achsils as well as 1 to 3 main roots stained (+/-), $20.0 \pm 18.0\%$ or $12.5 \pm 12.0\%$; up to 50% of roots stained (+), $6.3 \pm 7.3\%$ or $4.1 \pm 7.2\%$; more than 50% of roots stained (++), $2.7\% \pm 5.5\%$ or 0%. No GUS staining was observed on roots at day 6, although acetylene reduction rates were almost equally high as

at days 10 to 14 (ranging from 0.81 ± 0.67 to 2.6 ± 2.5 nmol C_2H_4 per h per plant). Most likely, at this stage the bacteria utilized carbon sources leaking from the seeds, and establishment of *nifH*-expressing bacteria on roots took place later. Acetylene reduction (0.3 ± 0.49 nmol C_2H_4 per h per plant) and histochemical GUS staining (75% of plants) decreased slightly at day 17, probably due to exhaustion of nutrients.

As observed previously (Hurek et al. 1994b), addition of carbon sources to the plant medium increased bacterial root colonization, which appeared to correlate with *nif* gene expression. When no malic acid was added in comparison to 5 mg/liter (standard medium used for experiments above), *nifH-gusA* staining was delayed and weaker (at day 10: w, $5.5 \pm 5.1\%$ of plants; at day 14: w, $37.5 \pm 12.5\%$; +/-, $12.5 \pm 12.0\%$ of plants). This effect cannot be due to vigorous growth of the bacteria, since the amount of carbon source was too low. It might help the bacteria to survive after inoculation into an otherwise nutrient-free environment, which is unlikely to exist in nature, where degradation processes of organic compounds in soil will deliver traces of soluble carbon sources. When a significant amount of carbon source is added (400 mg of malic acid per liter), the number of inoculated cells increases by bacterial growth, resulting in an enhanced infection of roots by *Azoarcus* sp. strain BH72 (Hurek et al. 1994b). We found a similar effect for root-associated *nifH::gusA* expression: when we compared 400 mg versus 5 mg of malic acid (15 replicates in three independent experiments), we observed at days 11 to 13 after inoculation an increased histochemical GUS staining (70 to 95% versus 50 to 70% of plants stained), a significantly increased quantitative GUS activity per root system ($1,305 \pm 693$ versus 255 ± 99 GUS units per mg of protein), and an increased acetylene reduction activity (5.4 ± 13.8 versus 0.88 ± 1.31 nmol C_2H_4 per h per plant). This stimulating effect has also been observed for epiphytic, wheat-root-associated *nifH* expression of *Azospirillum brasilense* (Vande Broek et al. 1993). The GUS activity per root-associated bacterium was high in *Azoarcus* sp. strain BHGN3.1, whether high or low amounts of carbon had been added: based on the CFU of the same root systems used for GUS measurements, bacterial Miller units of $30,780 \pm 23,410$ versus $17,420 \pm 2,200$ were calculated, which are higher than maximum values obtained in pure cultures. This may be due to possible underestimations of bacterial numbers per root in CFU counts.

Root-associated *nifH::gusA* expression of *Azoarcus* sp. strain BHGN3.1 was subject to repression by combined nitrogen as in free-living cultures, as had also been observed for *Azospirillum brasilense* (Arsène et al. 1994). No histochemical GUS staining was observed upon addition of 10 mM KNO_3^- to the plant medium, and the root-associated GUS activity was lowered to almost background level (31 ± 10 units per mg of protein).

Histochemical staining for the activity of the GUS reporter gene revealed specific sites for nitrogenase expression of *Azoarcus* sp. strain BHGN3.1 not only on but also inside rice roots. Individual bacterial cells expressing nitrogenase genes were visible in the cortical layers of KOH-cleared plant roots (Fig. 3C). In order to verify internal colonization, *nifH* expression, and identity of bacteria, well-stained rice roots were resin-embedded into LR White acrylic resin, sectioned, and immunolabeled as described previously (Hurek et al. 1994b; Hurek and Villiger 1992). *Azoarcus* spp.-specific antibodies

(Hurek et al. 1994b) detected microcolonies in the aerenchyma, the gas-filled cortex region common to flooded grasses (Fig. 3D,E). In successive sections, the blue GUS stain was clearly visible in these microcolonies and in single, large bacteria (Fig. 3G). Immunogold-silver staining of successive sections with antibodies specific for the iron protein of nitrogenase (Hurek et al. 1994b) showed that these microcolonies of *Azoarcus* sp. strain BHGN3.1 not only expressed the *nifH* gene but also translated the iron protein (Fig. 3F). Colonization of the cortex by blue-stained bacteria was detected in all instances when well-stained (++ or +) roots were sectioned near sites of heavily colonized emergence points of lateral roots. However, the blue color of GUS staining was present not exclusively inside the bacterial cells, but also next to the plant cell walls (Fig. 3F,G), revealing a disadvantage of GUS as a reporter for a microaerobic environment. A dye monomer is released by cleavage of the substrate X-Gluc that will only precipitate as insoluble blue dimer in the presence of O_2 . Lack of immediate dimerization will therefore result in diffusion followed by precipitation at distant locations (Hurek et al. 1994b; Reinhold-Hurek and Hurek 1998a).

This problem may be overcome with the reporter protein GFP, which allows the detection of the reporter protein in vivo without damaging the plant tissue and without staining, thus avoiding artefacts of diffusion. The GFP-expressing strain BHFN35 carried the *gfp* gene fused to the *nifH* gene, similar to strain BHGN3.1 (Egener et al. 1998). Fluorescence microscopy with digital confocal imaging was carried out with a Zeiss Axioplan 2 microscope and digital confocal imaging software (Egener et al. 1998). Three-dimensional reconstructions of optical sections were obtained with NIH image software version 1.62b7 (NIMH, Bethesda, MD) (Reinhold-Hurek and Hurek 1998b). In well-infected root systems, in addition to external colonization sites as found in the GUS experiments, long stretches of typical blue-green fluorescence were visible (Fig. 3H), which were apparently localized inside the roots, looking more intense in a deeper focus plane approximately 30 μ m below the root surface (Fig. 3I). A three-dimensional reconstruction of optical sections taken at 2 μ m distance confirmed the presence of GFP fluorescence deep in the cortex (Fig. 3J). Light microscopy on semithin sections after resin-embedding of the same root piece (Fig. 3K) as well as transmission electron microscopy from a successive section (Fig. 3L) corroborated the presence of large bacterial microcolonies in the aerenchyma. Since strong GFP fluorescence correlates with high levels of gene expression in *Azoarcus* sp. strain BHFN35 (Egener et al. 1998), *nif* gene expression in the rice cortex appeared to be very active. This was also supported by the observation that incubation times for development of GUS staining had been very short (30 to 120 min) in well-colonized roots. Epiphytic *nif* gene expression on grass roots has been reported for *Azospirillum brasilense* (Arsène et al. 1994; Vande Broek et al. 1993) and *Azoarcus* sp. strain BHFN35 (Egener et al. 1998). However, to our knowledge, this is the first time that significant endophytic *nif* gene expression has been visualized in roots of gramineous plants.

The expression of bacterial *nif* genes in intercellular spaces in the cortex implies that apoplastic transport is responsible for nutrient flow, although the movement by diffusion in the plane of the cell wall is 10^3 to 10^4 times slower than the diffusion rate in water (Dong et al. 1994). However, apoplastic

transport is not necessarily a constraint for active N₂ fixation, since also in actively N₂-fixing root nodules of *Parasponia* spp., rhizobia are persistent in infections threads surrounded by plant cell wall material (Trinick and Hadobas 1988). In sugar cane stems, the apoplastic fluid contains sucrose in high concentrations (12.5%, pH 5.5) (Dong et al. 1994), conditions that are optimal for the sugar cane endophyte *Acetobacter diazotrophicus* (Gillis et al. 1989; Stephan et al. 1991). In roots of gramineous plants, the composition of apoplastic organic material in comparison to root exudates still needs to be elucidated.

The in situ expression of nitrogenase genes inside grass roots allows some insights into the environmental conditions of this microhabitat. The oxygen tensions inside the tissue of rice roots are apparently permissive for *nif* gene expression of *Azoarcus* sp. strain BH72, which tolerates up to 20 µM O₂ in pure culture. This correlates with estimates of O₂ concentrations in rice roots by others. The cortex of mature rice roots disrupts to form large aerenchymatic air spaces, which allow transfer of air into roots in an otherwise anoxic flooded soil. O₂ concentrations were estimated to be 30 µM (Armstrong 1978) inside rice roots in flooded conditions or 0 to 10 µM in two-dimensional root mats of rice (Gilbert and Frenzel 1998). Aggregation of respiring bacteria might reduce the O₂ concentration further, leading to optimal conditions for *nif* gene expression. Without addition of N to the plant medium, conditions were also permissive for endophytic *nifH* gene expression in root intercellular spaces, suggesting that combined nitrogen occurs only in low concentrations in the apoplast.

Evidence for endophytic *nifH* gene expression of *Azoarcus* spp. was previously obtained from noninoculated, soil-grown Kallar grass roots by in situ hybridization studies (Hurek et al. 1997). Nevertheless, attempts to isolate *Azoarcus* spp. in parallel experiments had failed (Reinhold-Hurek et al. 1993), suggesting that unculturable strains might have been responsible for the observed activity. Our present study demonstrates that this activity (i) can indeed be carried out by a culturable *Azoarcus* strain and (ii) can be detected in another host plant, rice. Yet, the success of infection of this alternative host is very variable in the laboratory systems we use. Genetic evidence that *Azoarcus* spp. may occur in natural association with rice (Hurek et al. 1997; Ueda et al. 1995) raises the possibility of isolating strains with better colonization capacities for this host. The demonstration that the interior of rice roots is a suitable microhabitat for highly active expression of *nif* genes is a first step toward an improvement of endophytic, putatively beneficial associations with rice for a more sustainable agriculture.

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