Research Note

Use of Green Fluorescent Protein to Detect Expression of *nif* Genes of *Azoarcus* sp. BH72, a Grass-Associated Diazotroph, on Rice Roots

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A *gfp* (green fluorescent protein) cassette for transcriptional fusions has been developed to study gene expression in *Azoarcus* sp. BH72 in association with plant roots. The bacteria expressed nitrogenase genes (*nifHDK*) in the rhizosphere, on root tips, and in epidermal cells of rice seedlings. Green fluorescent protein fusions also visualized promoter activity of single cells in soil.

Additional keywords: nitrogen fixation.

A variety of nitrogen-fixing bacteria such as *Azospirillum* spp. (Tarrand et al. 1978), *Herbaspirillum* spp. (Baldani et al. 1996), *Acetobacter diazotrophicus* (Gillis et al. 1989), and *Azoarcus* spp. (Reinhold-Hurek et al. 1993), occur on or in roots of grasses, some even endophytically inside roots and stems (James et al. 1994; Hurek et al. 1994b). Most *Azoarcus* spp. were isolated from Kallar grass (*Leptochloa fusca* (L.) Kunth) (Reinhold-Hurek et al. 1993; Hurek and Reinhold-Hurek 1995), a pioneer plant grown on salt-affected, low-fertility soils in the Punjab of Pakistan (Sandhu and Malik 1975). *Azoarcus* sp. BH72 can colonize and invade roots of Kallar grass and rice seedlings in similar patterns in gnotobiotic culture, without causing symptoms of plant disease despite the endophytic growth (Hurek et al. 1994b). Recently, we have obtained evidence that an *Azoarcus* sp. occurs naturally in association with rice roots (Hurek et al. 1997). The colonization of rice roots is of special interest, since rice is an agriculturally important crop that may provide microaerobic niches for nitrogen fixation when grown under flooded conditions. Low O₂ concentrations are essential for N₂ fixation by the strictly respiratory *Azoarcus* spp. (Hurek et al. 1987). At extremely low O₂ concentrations (30 nM), *Azoarcus* sp. BH72 can develop into a hyperinduced state, which is characterized by augmented rates and efficiencies of N₂ fixation (Hurek et al. 1994a, 1995).

Positive effects of diazotrophs such as *Azospirillum* spp. on yield of gramineous plants have largely been attributed to bacterial phytohormones rather than to nitrogen fixation

(Okon and Labandera-Gonzalez 1994). However, nitrogen fixation may significantly contribute to plant nutrition in gramineous systems, as has been shown for several sugar cane cultivars in Brazil (Lima et al. 1987). Since gramineous plants do not form nitrogen-fixing symbiotic structures such as root nodules, it is crucial for an understanding of the mechanisms of interactions to know sites of bacterial colonization and expression of bacterial genes, especially structural genes of nitrogenase *nifHDK*.

Bacterial gene expression on or in plant roots can be visualized with transcriptional fusions of bacterial target genes to reporter genes. Recently, the jellyfish green fluorescent protein (GFP) has been introduced as a marker for gene expression (Chalfie et al. 1994). Advantages of this reporter system are that external substrates or co-factors are not required, and diffusible products of an enzyme reaction are not involved. Since gene expression can be monitored and reliably localized in vivo without damage of the tissue, GFP appears to be a promising reporter system to study plant-microbe interactions. It was applied under control of a constitutive promoter to visualize rhizobia (Gage et al. 1996). However, to our knowledge, the GFP has not been used for measurement of bacterial gene regulation in association with plants. In this study, we report the construction of a promoterless *gfp* gene cassette for transcriptional fusions and its suitability to study expression of nitrogenase genes of *Azoarcus* sp. BH72 in association with rice roots.

A *gfp* cassette for transcriptional fusions in *Azoarcus* sp. BH72 was constructed as follows. Translational stop signals in all three reading frames (bold, see below) were introduced by polymerase chain reaction (PCR) upstream of a consensus sequence of the putative ribosomal binding site of *Azoarcus* sp. BH72 *nif* genes (underlined), which was fused to the start codon of *gfp* (italics); the cassette was flanked by *EcoRI* restriction sites (double underlined). A *gfp* mutant was used that shows increased fluorescence, stability, and a red shift in the excitation wavelength due to an F64L, S65T double substitution (Cormack et al. 1996). It was amplified with PCR primers binding to the 5'-end (5'-GGAATTC**TGATTGATTGA**GGAGAGTTTCAACATGAGTAAAGGAGAAGAACTT-3') and the 3'-end (5'-GGAATTC**AATTGGAAGTCTGGACAT**TT-3') of *gfp*. The PCR fragment was cloned into the *EcoRI*

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restriction site of the plasmid pSK Bluescript (Stratagene, La Jolla, CA) under the control of the *lacZ* promoter in *Escherichia coli* DH5 α . For a transcriptional fusion with *nifH*, a 1.45-kb *EcoRI*-*HindIII* fragment of pBGVN3 (Hurek et al. 1995) carrying 800 bp of the *nifH* gene and 640 bp of upstream sequences, including a σ^{54} -type promoter sequence, was cloned into pUC18, resulting in pEN322. The *gfp* cassette was inserted into the *EcoRI* site of the *nifH* gene in pEN322, and this construct was integrated into the chromosome of *Azoarcus* sp. BH72 (Reinhold et al. 1986; Reinhold-Hurek et al. 1993) by homologous recombination after electroporation (Fig. 1A), resulting in the mutant strain *Azoarcus* sp. BHFN35.

Bacteria were grown in VM medium (Reinhold-Hurek et al. 1993) for electroporation, and in SM medium (Reinhold et al. 1985) as inoculum for plant experiments. Nitrogen-fixing cells were grown in liquid nitrogen-free SM medium in gas-tight, stoppered flasks under a reduced-oxygen atmosphere (Karg and Reinhold-Hurek 1996). Rice seeds (*Oryza sativa* var. nihonbare) were surface sterilized, grown in agar medium, and inoculated as previously described (Hurek et al. 1994b), except that no proline and only 5 mg of malate per liter were added to the plant growth medium. Alternatively, a nonsterile, dried saline-sodic soil from a Kallar grass field in Shakot, Pakistan, was saturated with sterile, bi-distilled water and inoculated with 10^9 bacteria per 5 ml of soil. To visualize fluorescence of roots or bacteria, samples were incubated under oxic conditions at 4°C for 1 to 2 h to allow the formation of the GFP chromophore (Cody et al. 1993), and then inspected with a Zeiss Axioplan 2 microscope with a band pass excitation filter BP450-490, a 510 dichromatic beam splitter, and a barrier emission filter LP520. Micrographs were recorded on a Kodak Ectachrome 64 film, or by a 3 Chip RGB Colour Camera (Hamamatsu Photonic Systems, Bridgewater, NJ). Digital confocal imaging was achieved with Improvision Openlab software (Improvision, Coventry, England). Quantification of fluorescence in suspended cells was carried out in a Fluorolite 1000 fluorometer (Dynatech, Denkendorf, Germany).

When wild-type GFP (Chalfie et al. 1994) or a GFP mutant with a single amino acid substitution (Heim et al. 1994) were expressed in *Azoarcus* sp. BH72, no significant fluorescence was observed (not shown). In contrast, a GFP mutant that carries a double amino acid substitution and shows increased fluorescence (Cormack et al. 1996) yielded sufficient light emission to visualize gene expression in single cells of *Azoarcus* sp. BH72 (Fig. 1C). The nonefficient variants have been used successfully in mycobacteria (Kremer et al. 1995), *Bacillus subtilis* (Webb et al. 1997), and *Rhizobium meliloti* (Gage et al. 1996); thus, the application of GFP in new bacterial species requires careful optimizing. Lack of sufficient fluorescence might be due to mRNA instability or to inefficient folding or chromophore formation of the protein. Multiple copies of *gfp* on plasmids might increase the yield of fluorescence; however, our strategy was to use a single gene copy integrated into the bacterial chromosome to allow long-term experiments without application of selective antibiotics. The fluorescence obtained by the transcriptional fusion of *gfp* to the *nifH* promoter correlated well with *nifH* gene expression. Bright fluorescence was observed only in exponentially grown cells that had high rates of nitrogen fixation (acetylene reduction) (Fig. 1B,C, 18 h) and thus high expression levels of *nif*

genes. Otherwise, fluorescence faded within a few seconds (Fig. 1C, 12 h, 38 h), or no fluorescence was detected in the stationary phase (Fig. 1C, 46 h) or under conditions of repression of *nif* genes (aerobic growth on ammonia; Fig. 1C, 0 h). The repression of the *nif-gfp* fusion was confirmed by quantification of fluorescence in bacterial suspensions. *Azoarcus* sp. BHFN35 grown on ammonia (0.1%) showed the background levels of fluorescence of the *gfp*-lacking wild-type strain, whereas nitrogen-fixing cells (Fig. 1B, 18 h) showed 1,167 units per OD₅₇₈ = 1. This repression by combined nitrogen under free-living conditions was also observed when bacteria were grown in association with plants in the presence of KNO₃ (0.1%).

It is difficult to assess cellular gene expression in soil with transcriptional fusions, because some soil microorganisms show background activity of most of the commonly used reporter proteins, such as β -glucuronidase (Jefferson et al. 1987). Moreover, detection levels are not sufficient to allow visualizing of single cells, e.g., for luciferase (Kloepper and

Fig. 1. Expression of a *nifH-gfp* fusion in nitrogen-fixing *Azoarcus* sp. strain BHFN35. **A**, Physical map of the integration into the chromosome of a pUC18-derived plasmid carrying a *nifH-gfp* fusion. The fusion and the uninterrupted *nifHDK* operon are under the control of the original σ^{54} -type promoter. E, *EcoRI* restriction site. **B**, Growth (open square, OD₅₇₈) and specific acetylene reduction rate (solid diamond) of an N₂-fixing culture under reduced-oxygen atmosphere (solid circle, *O₂* concentration in headspace). **C**, In vivo green fluorescent protein (GFP) fluorescence of cells sampled at different points of the growth curve when nitrogen fixation rates were absent (0 h), low (12 h), high (18 h) or ceased in stationary phase (38 h, 46 h); for comparison, epifluorescence micrographs were taken with identical exposure times.

Fig. 2. Expression of a *nifH-gfp* fusion of *Azorhizobium* sp. strain BHGN35 in soil (A–D) and in association with roots of rice seedlings (E–H) 5 or 10 days after inoculation, respectively. Light and corresponding epifluorescence micrographs of soil-bacterial aggregates (A, B), or single bacteria (C, D) in wet soil showing green fluorescent protein (GFP) fluorescence. Digital confocal images of (E), layers of bacterial cells attached to lateral roots (F), fluorescing bacteria attached to the elongation/differentiation zone of a root tip, and (G, H), fluorescing bacteria inside a layer of root cap cells that were sloughed off for microscopy. Digital confocal images are composed of one (E, H) or 60 (F) optical sections. Boxed regions at higher magnification show single fluorescing bacteria. Corresponding light micrographs (A, C, G) are with interference-contrast or phase-contrast illumination. Bars = 100 μ m or 5 μ m in box (E), 100 μ m or 10 μ m in box (F), or 10 μ m (H).

Beauchamp 1992). On the other hand, the GFP might be a promising alternative. We were able to detect *nifH-gfp* expression in single cells (Fig. 2D) and in soil-bacterial aggregates (Fig. 2B) in an unsterile, sandy-clay loam inoculated with *Azoarcus* sp. BHFN35, the blue-green GFP fluorescence being clearly distinct from yellow-green background fluorescence of soil particles. There was no background activity from soil-inhabiting microorganisms, blue-green fluorescing bacteria being undetectable in uninoculated controls (not shown). Therefore, this method could be useful to track bacteria, e.g., genetically engineered microorganisms, or to analyze gene expression and its spatial distribution in complex, heterogeneous soil systems. The potential of GFP to monitor bacteria in quartz sand (Burlage et al. 1996) or in other environments such as activated sludge (Eberl et al. 1997) or aquatic environments (Leff and Leff 1996) has been demonstrated.

nif gene expression of *Azoarcus* sp. BH72 was also visualized in association with roots of rice seedlings, indicating that the rice rhizosphere may provide suitable conditions for N_2 -fixing *Azoarcus* spp. even when only negligible amounts of carbon source (5 mg per liter) were added. The autofluorescence of roots themselves was yellowish and clearly distinct from blue-green GFP fluorescence (Fig. 2E,F). Strong fluorescence was found in bacterial aggregates adjacent to lateral roots, thick layers being visible when the agar matrix was still attached (Fig. 2E). *gfp*-expressing bacteria were also tightly adhered above root tips at the zone of elongation and differentiation (Fig. 2F). This correlates with the observation that this zone is the site of primary colonization and infection of Kallar grass and rice roots (Hurek et al. 1994b). Occasionally, root epidermis cells were filled with tightly packed, fluorescing bacteria (Fig. 2G,H), indicating that *nif* genes can be expressed even when bacteria are inside plant cells. These plant cells are most likely degenerated (Hurek et al. 1994b); however, plant cell content of combined nitrogen does not appear to repress bacterial *nif* genes. The *nifH* expression has also been observed for *Azospirillum brasilense* on the surface of wheat roots with *lacZ* (Arsène et al. 1994) and *gus* fusions (Vande Broek et al. 1993); expression was reported to be rare without addition of external carbon sources or reduced oxygen concentrations (Vande Broek et al. 1993). Transcriptional *gfp* fusions have two advantages: (i) since the signal is the fluorescence of the cellular protein itself and is thus not diffusible, in contrast to the primary product of X-Gal or X-Glu staining, the site of expression can be unequivocally localized; and (ii) only strong gene expression can be visualized in *Azoarcus* sp. BHGN35 (Fig. 1), assuring that the signal is significant and not due to an accumulation of cleaved substrate during a long incubation period, which may still be obtained at low gene expression levels.

Thus, the application of the transcriptional *gfp* fusions developed here may contribute to an understanding of functions and dynamics of plant-microbe interactions.

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LITERATURE CITED

- Arsène, F., Katupitiya, S., Kennedy, I. R., and Elmerich, C. 1994. Use of *lacZ* fusions to study the expression of *nif* genes of *Azospirillum brasilense* in association with plants. *Mol. Plant-Microbe Interact.* 7:748-757.
- Baldani, J. I., Pot, B., Kirchhof, G., Falsen, E., Baldani, V. L. D., Olivares, F. L., Hoste, B., Kersters, K., Hartmann, A., Gillis, M., and Döbereiner, J. 1996. Emended description of *Herbaspirillum*: Inclusion of [*Pseudomonas*] *rubrisubalbicans*, a mild plant pathogen, as *Herbaspirillum rubrisubalbicans* comb. nov., and classification of a group of clinical isolates (EF Group 1) as *Herbaspirillum* species 3. *Int. J. Syst. Bacteriol.* 46:802-810.
- Burlage, R. S., Yang, Z. K., and Mehlhorn, T. 1996. A transposon for green fluorescent protein transcriptional fusions: Application for bacterial transport experiments. *Gene* 173:53-58.
- Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W., and Prasher, D. C. 1994. Green fluorescent protein as a marker for gene expression. *Science* 263:802-805.
- Cody, C. W., Prasher, D. C., Westler, W. M., Prendergast, F. G., and Ward, W. W. 1993. Chemical structure of the hexapeptide chromophore of the *Aequorea* green-fluorescent protein. *Biochemistry* 32:1212-1218.
- Cormack, B. P., Valdivia, R. H., and Falkow, S. 1996. FACS-optimized mutants of the green fluorescent protein (GFP). *Gene* 173:33-38.
- Eberl, L., Schulze, R., Ammend, A., Geisenberger, O., Erhart, R., Sternberg, C., Molin, S., and Amann, R. 1997. Use of green fluorescent protein as a marker for ecological studies of activated sludge communities. *FEMS Microbiol. Lett.* 149:77-83.
- Gage, D. J., Bobo, T., and Long, S. R. 1996. Use of green fluorescent protein to visualize the early events of symbiosis between *Rhizobium meliloti* and alfalfa (*Medicago sativa*). *J. Bacteriol.* 178:7159-7166.
- Gillis, M., Kersters, K., Hoste, B., Janssens, D., Kroppenstedt, R. M., Stephan, M. P., Teixeira, K. R. S., Döbereiner, J., and De Ley, J. 1989. *Acetobacter diazotrophicus* sp. nov., a nitrogen-fixing acetic acid bacterium associated with sugar cane. *Int. J. Syst. Bacteriol.* 39: 361-364.
- Heim, R., Prasher, D. C., and Tsien, R. Y. 1994. Wavelength mutations and posttranslational autooxidation of green fluorescent protein. *Proc. Natl. Acad. Sci. USA* 91:12501-12504.
- Hurek, T., Egner, T., and Reinhold-Hurek, B. 1997. Divergence in nitrogenases of *Azoarcus* spp., *Proteobacteria* of the β -subclass. *J. Bacteriol.* 179:4172-4178.
- Hurek, T., Reinhold, B., Fendrik, I., and Niemann, E. G. 1987. Root-zone-specific oxygen tolerance of *Azospirillum* spp. and diazotrophic rods closely associated with Kallar grass. *Appl. Environ. Microbiol.* 53:163-169.
- Hurek, T., and Reinhold-Hurek, B. 1995. Identification of grass-associated and toluene-degrading diazotrophs, *Azoarcus* spp., by analyses of partial 16S ribosomal DNA sequences. *Appl. Environ. Microbiol.* 61:2257-2261.
- Hurek, T., Reinhold-Hurek, B., Turner, G. L., and Bergersen, F. J. 1994a. Augmented rates of respiration and efficient nitrogen fixation at nanomolar concentrations of dissolved O_2 in hyperinduced *Azoarcus* sp. strain BH72. *J. Bacteriol.* 176:4726-4733.
- Hurek, T., Reinhold-Hurek, B., Van Montagu, M., and Kellenberger, E. 1994b. Root colonization and systemic spreading of *Azoarcus* sp. strain BH72 in grasses. *J. Bacteriol.* 176:1913-1923.
- Hurek, T., Van Montagu, M., Kellenberger, E., and Reinhold-Hurek, B. 1995. Induction of complex intracytoplasmic membranes related to nitrogen fixation in *Azoarcus* sp. BH72. *Mol. Microbiol.* 18:225-236.
- James, E. K., Reis, V. M., Olivares, F. L., Baldani, J. I., and Döbereiner, J. 1994. Infection of sugar cane by the nitrogen-fixing bacterium *Acetobacter diazotrophicus*. *J. Exp. Bot.* 45:757-766.
- Jefferson, R. A., Kavanagh, T. A., and Bevan, M. W. 1987. GUS fusions: β -glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* 6:3901-3908.
- Karg, T., and Reinhold-Hurek, B. 1996. Global changes in protein composition of N_2 -fixing *Azoarcus* sp. strain BH72 upon diazotroph formation. *J. Bacteriol.* 178:5748-5754.
- Kloepper, J. W., and Beauchamp, C. J. 1992. A review of issues related to measuring colonization of plant roots by bacteria. *Can. J. Microbiol.* 38:1219-1232.
- Kremer, L., Baulard, A., Estaquier, J., Poulain-Godefroy, O., and Loch, C. 1995. Green fluorescent protein as a new expression marker in my-

- cobacteria. *Mol. Microbiol.* 17:913-922.
- Leff, L. G., and Leff, A. A. 1996. Use of green fluorescent protein to monitor survival of genetically engineered bacteria in aquatic environments. *Appl. Environ. Microbiol.* 62:3486-3488.
- Lima, E., Boddey, R. M., and Döbereiner, J. 1987. Quantification of biological nitrogen fixation associated with sugar cane using a ^{15}N -aided nitrogen balance. *Soil Biol. Biochem.* 19:165-170.
- Okon, Y., and Labandera-Gonzalez, C. A. 1994. Agronomic applications of *Azospirillum* - an evaluation of 20 years worldwide field inoculation. *Soil Biol. Biochem.* 26:1591-1601.
- Reinhold, B., Hurek, T., and Fendrik, I. 1985. Strain-specific chemotaxis of *Azospirillum* spp. *J. Bacteriol.* 162:190-195.
- Reinhold, B., Hurek, T., Niemann, E.-G., and Fendrik, I. 1986. Close association of *Azospirillum* and diazotrophic rods with different root zones of Kallar grass. *Appl. Environ. Microbiol.* 52:520-526.
- Reinhold-Hurek, B., Hurek, T., Gillis, M., Hoste, B., Vancanneyt, M., Kersters, K., and De Ley, J. 1993. *Azoarcus* gen. nov., nitrogen-fixing proteobacteria associated with roots of Kallar grass (*Leptochloa-fusca* (L.) Kunth) and description of two species *Azoarcus indigens* sp. nov. and *Azoarcus communis* sp. nov. *Int. J. Syst. Bacteriol.* 43:574-584.
- Sandhu, G. R., and Malik, K. A. 1975. Plant succession - A key to the utilization of saline soils. *Nucleus* 12:35-38.
- Tarrand, J. J., Krieg, N. R., and Döbereiner, J. 1978. A taxonomic study of the *Spirillum lipoferum* group, with description of a new genus, *Azospirillum* gen. nov. and two species, *Azospirillum lipoferum* (Beijerinck) comb. nov. and *Azospirillum brasilense* sp. nov. *Can. J. Microbiol.* 24:967-980.
- Vande Broek, A., Michiels, J., Van Gool, A., and Vanderleyden, J. 1993. Spatial-temporal colonization patterns of *Azospirillum brasilense* on the wheat root surface and expression of the bacterial *nifH* gene during association. *Mol. Plant-Microbe Interact.* 6:592-600.
- Webb, C. D., Teleman, A., Gordon, S., Straight, A., Belmont, A., Lin, D. C. H., Grossman, A. D., Wright, A., and Losick, R. 1997. Bipolar localization of the replication origin regions of chromosomes in vegetative and sporulating cells of *B. subtilis*. *Cell* 88:667-675.