RESEARCH ARTICLE

Endophytic root colonization of gramineous plants by *Herbaspirillum frisingense*

Michael Rothballer[1], Barbara Eckert[1], Michael Schmid[1], Agnes Fekete[2], Michael Schloter[3], Angelika Lehner[4], Stephan Pollmann[5] & Anton Hartmann[1]

[1]Department Microbe–Plant Interactions, Helmholtz Center Munich, German Research Center for Environmental Health (GmbH), Munich, Germany; [2]Institute of Ecological Chemistry, Helmholtz Center Munich, German Research Center for Environmental Health (GmbH), Munich, Germany; [3]Institute of Soil Ecology, Department Terrestrial Ecogenetic, Helmholz Center Munich, German Research Center for Environmental Health (GmbH), Munich, Germany; [4]Vetsuisse Faculty, Institute for Food Safety and Hygiene, University of Zurich, Zurich, Switzerland; and [5]Department of Plant Physiology, Ruhr-University Bochum, Bochum, Germany

Correspondence: Anton Hartmann, Department Microbe–Plant Interactions, Helmholtz Center Munich, German Research Center for Environmental Health (GmbH), Ingolstädter Landstr. 1, D-85764 Neuherberg/Munich, Germany. Tel.: +49 0 89 3187 4109; fax: +49 0 89 3187 3376; e-mail: anton.hartmann@helmholtz-muenchen.de

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Abstract

Herbaspirillum frisingense is a diazotrophic betaproteobacterium isolated from C4-energy plants, for example *Miscanthus sinensis*. To demonstrate endophytic colonization unequivocally, immunological labeling techniques using monospecific polyclonal antibodies against two *H. frisingense* strains and green fluorescent protein (GFP)-fluorescence tagging were applied. The polyclonal antibodies enabled specific *in situ* identification and very detailed localization of *H. frisingense* isolates Mb11 and GSF30^T within roots of *Miscanthus* × *giganteus* seedlings. Three days after inoculation, cells were found inside root cortex cells and after 7 days they were colonizing the vascular tissue in the central cylinder. GFP-tagged *H. frisingense* strains could be detected and localized in uncut root material by confocal laser scanning microscopy and were found as endophytes in cortex cells, intercellular spaces and the central cylinder of barley roots. Concerning the production of potential plant effector molecules, *H. frisingense* strain GSF30^T tested positive for the production of indole-3-acetic acid, while Mb11 was shown to produce *N*-acylhomoserine lactones, and both strains were able to utilize 1-aminocyclopropane-1-carboxylate (ACC), providing an indication of the activity of an ACC-deaminase. These results clearly present *H. frisingense* as a true plant endophyte and, although initial greenhouse experiments did not lead to clear plant growth stimulation, demonstrate the potential of this species for beneficial effects on the growth of crop plants.

Introduction

Within the *betaproteobacteria*, the genus *Herbaspirillum* comprises several mostly diazotrophic species, some of which exhibit the potential of endophytic and systemic colonization of a number of plants (Schmid *et al.*, 2005). Root-colonizing *Herbaspirillum seropedicae* could be detected on root surfaces and as endophytes in intercellular spaces, as well as within intact root cells (Olivares *et al.*, 1997). *Herbaspirillum rubrisubalbicans* was described as a diazotrophic endophyte with slight pathogenicity in some sugar cane varieties (Baldani *et al.*, 1996; Olivares *et al.*, 1997). Plants of the *Gramineae* family are colonized by *H. seropedicae* and *H. rubrisubalbicans*, but other plant species can also serve as hosts (Baldani *et al.*, 1996). Döbereiner *et al.* (1993) postulated that the endophytically colonizing species *H. seropedicae* supplies the plant with bacterially fixed nitrogen, which was experimentally proven by Elbeltagy *et al.* (2001) in rice (*Oryza officinalis*) after inoculation with *Herbaspirillum* sp. strain B501. In recent years, many new *Herbaspirillum* species have been characterized, including three isolates from plant origin: *Herbaspirillum frisingense* from the C4-fiber plants *Miscanthus* spp. and *Pennisetum purpureum* (Kirchhof *et al.*, 2001), *Herbaspirillum lusitanum* from root nodules of *Phaseolus vulgaris* (Valverde *et al.*, 2003) and *Herbaspirillum hiltneri* from surface-sterilized wheat roots (Rothballer *et al.*, 2006). Within the *H. frisingense* isolates, one group was retrieved

from washed roots (Mb group) while another one originated from washed leaves and stems (GSF group) of *Miscanthus* spp. grown in Freising, Germany (Kirchhof *et al.*, 2001). In this study, two *H. frisingense* strains representing these two groups were examined further for their colonization behaviour on *Miscanthus* × *giganteus*, a close relative of the plant they were originally isolated from, and additionally on barley plants for comparison to demonstrate a more general potential for endophytic colonization. The perennial grass genus *Miscanthus* was particularly interesting in this context, as its rapid growth and low nitrogen demand suggested a stimulatory effect of nitrogen-fixing, root-colonizing bacteria. *Miscanthus sinensis* belongs to the *Gramineae* family and reaches up to a height of 3–4 m in only one growth season in Germany. In particular, the variety *Miscanthus* × *giganteus*, a naturally occurring, sterile hybrid of *M. sinensis* and *Miscanthus sacchariflorus* (Greef & Deuter, 1993), has been tested for its industrial utilization, as the chemical structure of the fibers is similar to those of wood. Therefore, it could be used for manufacturing paper, pulp or cardboard, and it might serve as raw material for chemical products, or in pelletized form as an energy source. In Germany, yields of 20–30 ton ha^{-1} $year^{-1}$ (Lewandowski & Kicherer, 1997) account for the suitability of this grass as a renewable resource for energy production with an even CO_2 balance. However, the cultivation of energy crops like *Miscanthus* is only sustainable if costly agricultural procedures, agrochemicals and fertilization can be minimized. Because *Miscanthus* represses to a large extent the emergence of weeds due to its dense growth and because no severe diseases are known to date (Walsch & McCarthy, 1998), only the question of fertilization needs to be addressed. In several experiments, it has been shown that the use of a mineral nitrogen fertilizer had no significant effect on biomass (Schwarz *et al.*, 1994; Christian *et al.*, 1997a, b; Himken *et al.*, 1997). Christian *et al.* (1997a, b) examined nitrogen distribution and balance after the application of a fertilizer labeled with ^{15}N. Only 19% of the total nitrogen was found to be derived from the introduced fertilizer. Alternative nitrogen sources such as biological nitrogen fixation might therefore play an important role. Consequently, Kirchhof *et al.* (1997) investigated the occurrence of diazotrophic bacteria in *Miscanthus*. In low numbers, cells of *Azospirillum lipoferum* and a species unknown at that time, later described as *H. frisingense* (Kirchhof *et al.*, 2001), could be detected. However, it has not yet been demonstrated to what extent these bacteria are able to colonize plants endophytically. Endophytic colonization is considered to be a very important trait in plant growth-promoting rhizobacteria (PGPR), because endophytes have a much more intimate and stable interaction with their plant hosts. Furthermore, no information is available yet on possible mechanisms of plant growth promotion via the production of effector or signaling molecules by *H. frisingense*. Indole 3-acetic acid (IAA) production by rhizosphere bacteria is known to stimulate plant growth via several effects (Dobbelaere *et al.*, 2001), and the production of this phytohormone was also documented for *H. seropedicae* (Bastián *et al.*, 1998). Another candidate mechanism for plant growth stimulation is the presence of enzymatic activity to hydrolyze 1-aminocyclopropane-1-carboxylate (ACC), the precursor of the plant hormone ethylene in the rhizosphere (Glick *et al.*, 1998). Most recently, bacterial autoinducer signaling molecules of the *N*-acylhomoserine lactone (AHL) type were also found to exert positive effects on diverse plants (Mathesius *et al.*, 2003; Schuhegger *et al.*, 2006).

In this communication, we report on inoculations of barley and *Miscanthus* plants with *H. frisingense* strains $GSF30^T$ and Mb11, the detailed localization of the colonizing bacteria on and in the roots and the occurrence of diverse plant growth-stimulatory traits in these bacteria.

Materials and methods

Bacterial strains and culture conditions

Herbaspirillum frisingense strain $GSF30^T$ (DSM 13128) was isolated from washed, crushed leaf material, strain Mb11 (DSM 13130) from washed, crushed roots of *M. sacchariflorus* on a nitrogen-free, semi-solid medium (Kirchhof *et al.*, 2001). All *Herbaspirillum* isolates were grown at 30 °C on an NB complex medium; for the transposon-tagged strains, 50 μg of kanamycin was added. *Escherichia coli* strain HB101 with plasmid pRK600 (Figurski & Helinski, 1979) was grown on Luria–Bertani (LB) medium (Bertani, 1951) containing 10 μg mL^{-1} chloramphenicol. *Escherichia coli* strain MV1190 harboring plasmid pJBA28 (Andersen *et al.*, 1998) was cultivated on LB medium supplemented with 50 μg mL^{-1} kanamycin.

Inoculation method, growth conditions and harvesting of *Miscanthus* and barley cultivars

Plants of *Miscanthus* × *giganteus* and *M. sinensis* 'Goliath' were obtained by meristem and subsequent callus cultures from shoot tips (LBP: Landesanstalt für Bodenkultur und Pflanzenbau, Freising, Germany). Inoculation of these sterile tissue culture plants was performed after they had been grown for 2–3 weeks on MS-medium (Murashige & Skoog, 1962) to develop fine roots.

Seeds of barley (*Hordeum vulgare*) variety 'Barke' were obtained from the Saatzucht Josef Breun GdbR (Herzogenaurach, Germany). To eliminate contaminating microorganisms, seeds for the monoxenic system were treated with 1% (v/v) Tween 20 (Sigma, Steinheim, Germany) in sterile deionized water for 2 min, surface sterilized with 70% ethanol (5 min) and washed three times with sterile deionized water. Subsequently, seeds were incubated in sodium hypochlorite

solution (6–14% Cl active; Riedel-de Haën, Seelze, Germany) for 20 min and washed five times with sterile deionized water. After incubating the seeds for 3–4 days at 30 °C in the dark on NB plates, only those seeds that showed no visible contamination were picked for incubation.

Inoculation of *Miscanthus* and barley plants was performed when roots were between 1 and 2 cm in length using the inoculation method described by Schloter & Hartmann (1998) with slight modifications: overnight cultures of the respective *H. frisingense* strain were washed twice with phosphate-buffered saline (1 × PBS, 10 mM NaH_2PO_4, 10 mM Na_2HPO_4, 130 mM NaCl, pH 7.2) and diluted to a concentration of 10^8 cells mL^{-1} 1 × PBS. Seeds or calli were incubated in this bacterial suspension for 1 h before planting.

Monoxenic cultivation was performed in sealed glass tubes (3 cm width, 50 cm length) filled with 100 g of autoclaved quartz sand and 10 mL of sterile modified Hoagland's solution (Terry, 1980; Rothballer *et al.*, 2003). Plants were grown under greenhouse conditions at a temperature of 15–25 °C during the day and 10–15 °C during the night. After 3 days to 8 weeks, roots were harvested by taking the whole plant out of the glass tube with quartz sand, and rinsing off the adhering material with sterile 1 × PBS.

Tagging of *H. frisingense* with a *gfp* containing transposon

The plasmid pJBA28 (Andersen *et al.*, 1998), harboring a mini-Tn5 transposon with a kanamycin resistance cassette, and a constitutively expressed *gfp* (modified form mut3) gene (Cormack *et al.*, 1996) under control of a $P_{A1/04/03}$ promoter were transferred to *H. frisingense* strains $GSF30^T$ and Mb11 via conjugative transfer by triparental mating (Kristensen *et al.*, 1995). *Herbaspirillum frisingense* transconjugants were selected by plating on MMAB agar (Vanstockem *et al.*, 1987) supplemented with 50 μg mL^{-1} kanamycin and 6 μg mL^{-1} nalidixic acid.

FISH analysis

For FISH, 1 mL of a pure overnight culture was fixed with 4% paraformaldehyde for at least 1 h at 4 °C (Amann *et al.*, 1990). Hybridization with fluorochrome (Fluorescein, Cy3)-labeled oligonucleotide probes followed the protocols described by Manz *et al.* (1992) and Amann *et al.* (1992).

Detection of bacteria using confocal laser scanning microscopy (CLSM)

For the visualization of the *gfp*-tagged *H. frisingense* cells, freshly harvested whole roots or hand-cut root slices were used and placed on a glass slide. For preparing the longitudinal slices the roots were placed on a glass slide and cut into halves with a razor blade. Whole roots or slices were embedded in Citifluor (Citifluor Ltd., Canterbury, UK), and green fluorescent protein (GFP)-derived fluorescence was detected using a CLSM (LSM510 Meta: Zeiss, Oberkochen, Germany). GFP was excited at 488 nm by an argon ion laser. Two helium neon lasers provided excitation wavelengths of 543 and 633 nm, resulting in unspecific autofluorescence of the root material, which enables visualization of the root structure. The three signals were combined and depicted as a red green blue (RGB) image. Additionally, specific GFP fluorescence was verified by scanning the characteristic wavelength spectrum of a signal in question with the implemented function of the LSM510 Meta system. An optical sectioning of the root was achieved by moving the focus position deeper into the sample in 1 μm steps, producing *z*-stacks of individual pictures from the same *xy* area with a penetration depth of up to 60 μm. The resulting set of pictures was displayed as an orthogonal view with the help of the LSM510 software package provided by Zeiss.

Production and purification of polyclonal antisera

Overnight cultures (10 mL) of *H. frisingense* Mb11 and $GSF30^T$ were centrifuged at 6000 ***g*** for 10 min, washed twice with sterile 1 × PBS and diluted to a concentration of about 10^9 bacteria mL^{-1}. The suspension was transferred into a Petri dish and the bacteria were inactivated by ultraviolet radiation for 90 min. Two milliliters were taken up with a sterile syringe and mixed with 200 μL of Freund's Adjuvant complete (Sigma) for enhancing immune response. This was injected subcutaneously into a female, 6-month-old New Zealand rabbit. These immunizations were repeated weekly for 6 weeks with equal concentrations of inactivated bacterial suspensions containing 300 μL of Freund's Adjuvant incomplete (Sigma). Control blood samples were taken from the rabbit before first immunization, as well as 3 and 5 weeks later, and analyzed for antibody production in white polystyrene micro titer plates by standard sandwich chemoluminescence ELISA (detection reagent RPN 190; Amersham-Pharmacia Biotec, Freiburg, Germany) according to the manufacturer's recommendations. One week after the last immunization, the anesthetized animal was bled by a heart puncture. After the collected 50–100 mL of blood coagulated at 4 °C overnight, the serum was removed and centrifuged (1200 ***g***, 10 min). The resulting cell-free antiserum was stored at − 20 °C until processed further.

Purification of the polyclonal antisera was performed by adsorption to an Econo-Pac® protein A cartridge (BioRad, Munich, Germany) following the manufacturer's protocol. For a first screening of the protein A purified sera, we used cells from an overnight culture, which were treated with 50%, 70% and 90% ethanol, followed by a fixation step using 4% paraformaldehyde for 1 h at 4 °C. A penetration of antibodies into the cell interior after this procedure has been shown before (Metz *et al.*, 2003). After the determination of

cross-reactions the respective strains, with which an unspecific binding had been detected, were used for a second purification step. For this, 500 mL of an overnight culture was harvested, washed and resuspended in 5 mL 1 × PBS. To this suspension, 200 μL of purified antiserum was added and incubated at room temperature for 2 h with slow stirring. Afterwards, bacteria were separated by centrifugation and the supernatant was supplemented with sodium azide to inhibit bacterial or fungal growth. The purified and tested antisera were stored at 4 °C.

Optimal concentrations of the purified antisera were identified by performing ELISA with various dilutions of the purified antisera but constant numbers of target bacteria, as well as a distantly related bacterium (*E. coli*) as a negative control. The dilution, which resulted in the most intense signal compared with the negative control, was considered as optimal and used for all further experiments.

Immunogold labeling and detection

Plant material was cut into 1-cm pieces, washed thoroughly with 1 × PBS and fixed with 10 mL of 1 × PBS containing 3% (w/v) paraformaldehyde and 0.1% (w/v) glutaraldehyde at 4 °C overnight. Fixation was stopped by washing with 50 mM NH_4Cl in 1 × PBS. Samples were dehydrated with rising ethanol concentrations (70%, 90% and 100%, 10 min each) and thereafter embedded in a series of Unicryl dilutions (ethanol: Unicryl 2:1, 1:2, and pure Unicryl, 1 h each). The plant pieces were then transferred to gelatin capsules and infiltrated with pure Unicryl overnight. Finally, the samples were kept at 55 °C for 2 days to solidify.

For transmission electron microscopy (TEM), ultra-thin sections (60–80 nm) were prepared with an ultramicrotome (MT-7, RMC; Tucson, Arizona) and placed on a TEM grid. Subsequently, the immunogold labeling was carried out following the protocol of James *et al.* (1994), using an Antirabbit AuroProbe EM (Amersham-Pharmacia Biotec) with 15-nm gold particles. Contrast staining was performed with uranyl acetate (30 min) and lead citrate (4 min). As a control, the procedure was carried out without the purified polyclonal antiserum and with preimmune serum (control serum before first immunization of the rabbit) instead of the antiserum. The prepared samples were examined with a Zeiss TEM 10CR.

For light microscopy (Axioplan2; Zeiss), semi-thin sections (0.5–0.7 μm) were made, heat-fixed on glass slides and labeled with 5-nm gold particles according to the immunogold labeling procedure of James *et al.* (1994), as described above. This was followed by a silver enhancement according to the protocol of the IntenSE BL Silver Enhancement kit (Laboratory Janssen Life Science Products, Olen, Belgium). Slices were then contrasted with 0.1% (w/v) toluidine blue solution containing 0.1% (w/v) borax for 3 min, dried and embedded in Entellan® (Merck, Darmstadt, Germany).

Detection of AHL, IAA and ACC utilization

AHL production by *H. frisingense* was analyzed by a combination of ultra-performance liquid chromatography (UPLC) and ultra-high-resolution MS (FT-ICR-MS) as described elsewhere (Li *et al.*, 2006; Fekete *et al.*, 2007).

IAA measurements were performed based on a GC-MS method described by Müller & Weiler (2000). Briefly, for sample preparation cultures were grown in triplicate in M9 minimal medium supplemented with 0.4% of glucose and with 500 μg mL^{-1} of tryptophan, as well as without tryptophan. The bacteria were harvested by centrifugation and 100 μL of the supernatant was transferred to a fresh tube. The samples were supplemented with $[^2H]_2$-IAA (Cambridge Isotopes) as an internal standard at a concentration of 10 nmol mL^{-1}. Then, 12.5 μL of concentrated HCl and subsequently 250 μL of ethyl acetate were added and samples were vortexed for at least 10 s. Afterwards, samples were centrifuged for 1 min (18 000 ***g***) for phase separation. The upper organic phase was transferred to a fresh tube and dried in a speedvac. After an HPLC purification step the dried residue was dissolved in etheral CH_2N_2 to convert IAA to its methyl ester. After 10 s, the ether and excess CH_2N_2 were removed in a gentle stream of nitrogen. The residue was dissolved in 50 μL $CHCl_3$ and analyzed by GC-MS.

ACC utilization was detected according to Brown & Dilworth (1975) with cultures grown overnight, diluted by a factor of 10^7 and spread on mineral medium agar plates containing NH_4Cl or ACC or no nitrogen source. The plates were then incubated at 30 °C for 10 days. Growth on plates with ACC as the sole nitrogen source, but negligible growth without any nitrogen source (fixation of atmospheric nitrogen can only take place under microaerobic conditions) indicated the presence of ACC deaminase in the bacteria tested.

Results

Localization of GFP-labeled cells by CLSM

After conjugative transfer of the plasmid pJBA28 carrying the mini-Tn5-transposon with the constitutively expressed *gfp* and the kanamycin resistance genes, GFP-tagged mutants were selected. One tagged colony each of strain $GSF30^T$ and Mb11 was selected, singled out and tested for contaminations by FISH, using the species-specific oligonucleotide probe Hfris446 (Kirchhof *et al.*, 2001). The growth behavior of the tagged strains was tested and did not differ from the wild type in any respect. A GFP-labeled *Azospirillum brasilense* Sp7 strain (Rothballer *et al.*, 2003) served as a control for nonendophytic colonization of roots, and noninoculated control plants were also grown for comparison.

Inoculated sterilized barley seedlings were grown for 2–3 weeks in a monoxenic quartz sand system under greenhouse conditions. Plants were then harvested and single, intact

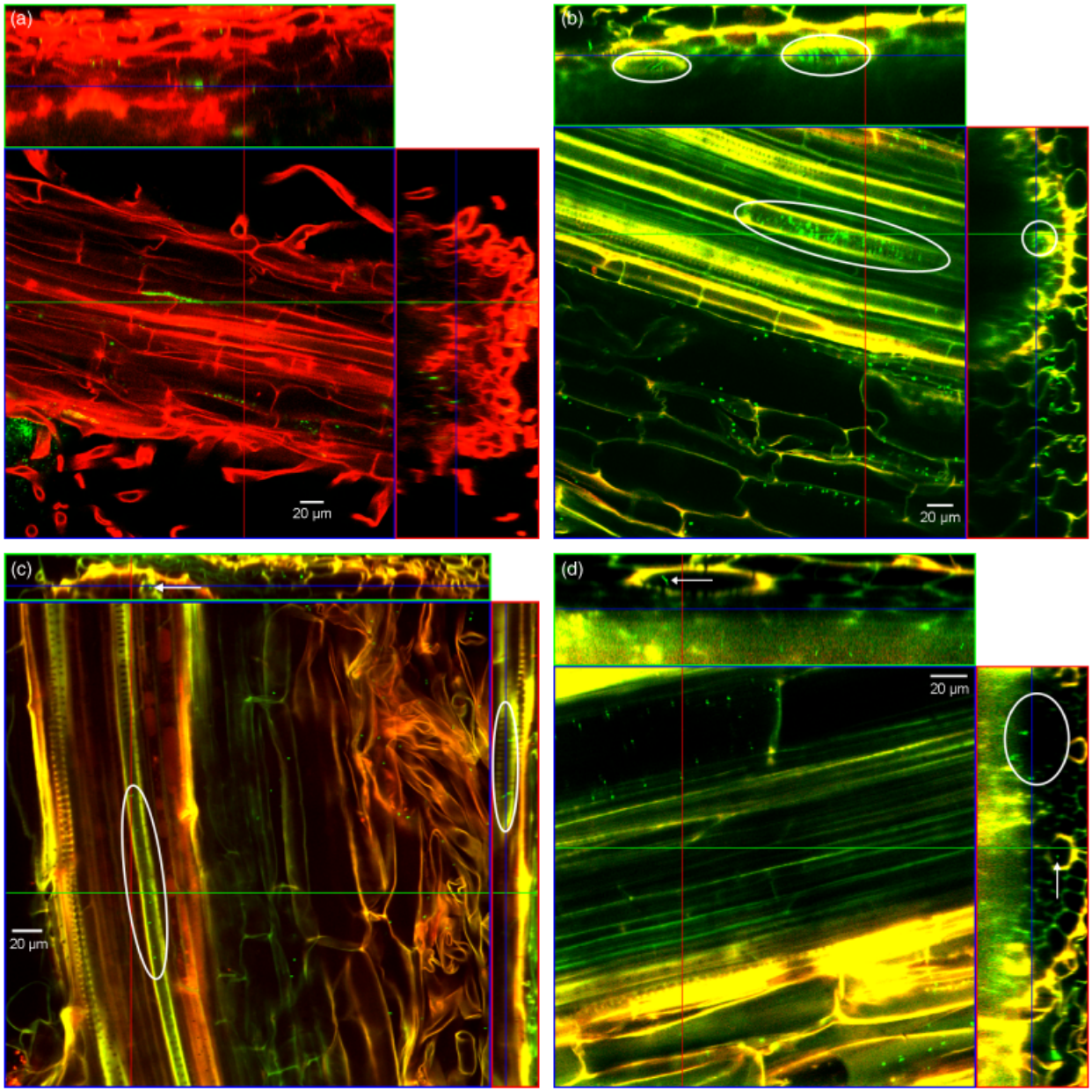

Fig. 1. Orthogonal optical sections of a three-dimensional confocal image created from a *z*-stack of *xy*-scans. The top view, framed in blue, shows one picture from the centre of this *z*-stack. The red and green lines represent vertical or horizontal optical cuts through the stack, which result in the side view images framed in red and green, respectively. In these side views the blue line marks the position, where the top view image is located within the *z*-stack. Depicted is an optical section through intact barley roots (*Hordeum vulgare*) of the variety Barke after 3 weeks of growth in the monoxenic system and *Herbaspirillum frisingense* (with chromosomal *gfp*mut3 label) as inoculum. Images were all taken from the root hair zone. (a) Colonization of intercellular spaces: Strain GSF30^T is detected at several positions between cortex cells of an uncut side root. The right side view window shows a diagonal optical sectioning of almost the complete, intact side root. (b) Xylem vessels of the central cylinder are completely colonized by strain GSF30^T (indicated by white circles), as well as several cortex cells. (c) A xylem vessel of an uncut central cylinder of a barley root colonized by strain Mb11 (indicated by white circles and arrows). (d) Endophytic colonization of a large xylem vessel (indicated by white circle in the side view window) and a small one (white arrows).

roots or roots that were cut once longitudinally were examined by a CLSM. When cut samples had to be used for detecting the colonization of the central cylinder, only slices with an intact endodermis were analyzed.

GFP-labeled cells could be found in great numbers on the root surface, and in all parts of the inner tissue, i.e. the cortex and the central cylinder (Fig. 1b). Frequently, cells of *H. frisingense* were localized in high numbers in intercellular

spaces of the outer cortex but also within intact root cells (about 100 cells per *z*-stack representing $320 \times 320 \times 60\,\mu m^3$; see Fig. 1a and c). In most cases, a massive colonization of the xylem vessels within the central cylinder was recognized, especially in older parts of the root (Fig. 1d). The pronounced ability of *H. frisingense* to colonize roots endophytically was clearly demonstrated by these microscopic studies. There was no detectable difference between the colonization behavior of strain GSF30^T and Mb11.

Endophytic colonization study by immunogold labeling

Two polyclonal antisera were produced specific for the *H. frisingense* strains Mb11 and GSF30^T. After purification with a protein A column, the antisera AntiMb11 and AntiGSF30 were tested for cross-reactivity with the following strains: *H. frisingense* (Mb group, $n = 10$), *H. frisingense* (GSF group, $n = 7$), *H. frisingense* (from *Pennisetum*, $n = 2$), *H. rubrisubalbicans* ($n = 7$), *H. seropedicae* ($n = 2$), *Herbaspirillum* spp. (clinical isolates, $n = 3$), *A. lipoferum*, *Azospirillum irakenase*, *Azospirillum amazonense*, *A. brasilense*, *Ochrobactrum anthropi*, *Sinorhizobium meliloti*, *Pseudomonas alcaligenes*, *Pseudomonas fluorescens*, *E. coli* JM105, *Arthrobacter citreus*, *Agrobacterium tumefaciens*, *Xanthomonas campestris*, *Burkholderia cepacia*, *Janthinobacterium lividum* and *Alcaligenes faecalis* (all type strains, if not indicated otherwise). A cross-reactivity level of $< 5\%$ of the maximum signal obtained with the strain used for immunization was considered as negative. The purified polyclonal antisera cross-reacted with other *H. frisingense* strains, which were also derived from *Miscanthus*, but not with *H. frisingense* isolates from *Pennisetum* (data not shown). This cross-reaction could not be reduced by incubating the respective purified antiserum with the cross-reacting strain, as this resulted in loss of sensitivity of the purified antiserum. To ensure specific differentiation of the target strains, we cross-incubated both purified sera with the cell extracts of the respective nontarget strain (serum against Mb11 with strain GSF30^T and serum against GSF30^T with strain Mb11). The only other cross-reaction was detected with *P. alcaligenes* (type strain, DSM 50342), which was therefore also incubated with both purified antisera in order to increase specificity. After this step, the cross-reaction with *P. alcaligenes* was below the detection level for both AntiMb11 and AntiGSF30. For optimal results the purified antisera had to be diluted 100-fold.

Miscanthus × *giganteus* plants from axenic tissue cultures were inoculated with *H. frisingense* strains Mb11 and GSF30^T. *Azospirillum brasilense* Sp7 served as a negative control for endophytic colonization and noninoculated control plants were cultivated as well. Inoculated plants were grown for 3 days to 8 weeks under greenhouse conditions in monoxenic systems. Light microscopic examination of immunostained samples after silver enhancement revealed *H. frisingense* cells on the rhizoplane, in outer cortex cells (Fig. 2) already after 3 days, in endodermis cells (Fig. 3) and in xylem vessels after 7 days (Figs 3 and 4). There was no visible difference in the colonization behavior between the strains Mb11 and GSF30^T. *Herbaspirillum frisingense* was also found in leave tissue, indicating an efficient systemic spreading of these bacteria (not shown).

An immunogold labeling, followed by an electron microscopic detection of the colonized root samples identified the bacteria as *H. frisingense* Mb11 or GSF30^T and allowed the documentation of several xylem vessels almost completely filled with bacteria (Fig. 4b). A comparison with noncolonized xylem vessels (Fig. 4a) showed xylem walls to be intact and not degraded in the presence of the bacteria. In the control plants with *A. brasilense* Sp7, only rhizoplane colonization was observable (not shown). When uninoculated control plants were incubated with both ploYclonal sera, no unspecific cross-reaction was observed with plant material.

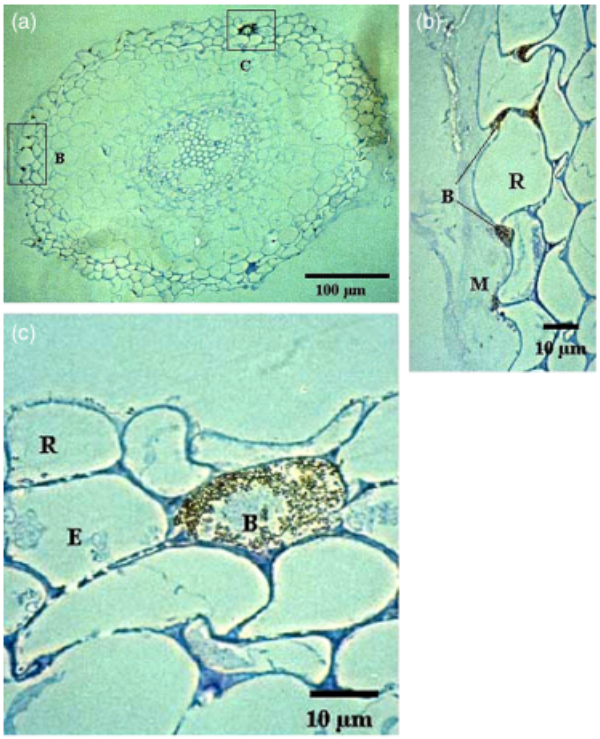

Fig. 2. (a) Light microscopic image of a root cross-section from a *Miscanthus* × *giganteus* plant inoculated with *Herbaspirillum frisingense* Mb11, 3 days after inoculation. Immunogold labeling with AntiMb11 and subsequent enhancement with silver staining. (b) (Detail of a) Bacterial cells (B) of *H. frisingense* Mb11 densely colonized the rhizoplane beneath the mucigel layer (M), penetrated the root surface and entered the intercellular spaces of the rhizodermis cells (R). (c) (Detail of a) An exodermis cell (E) is densely colonized by *H. frisingense* Mb11.

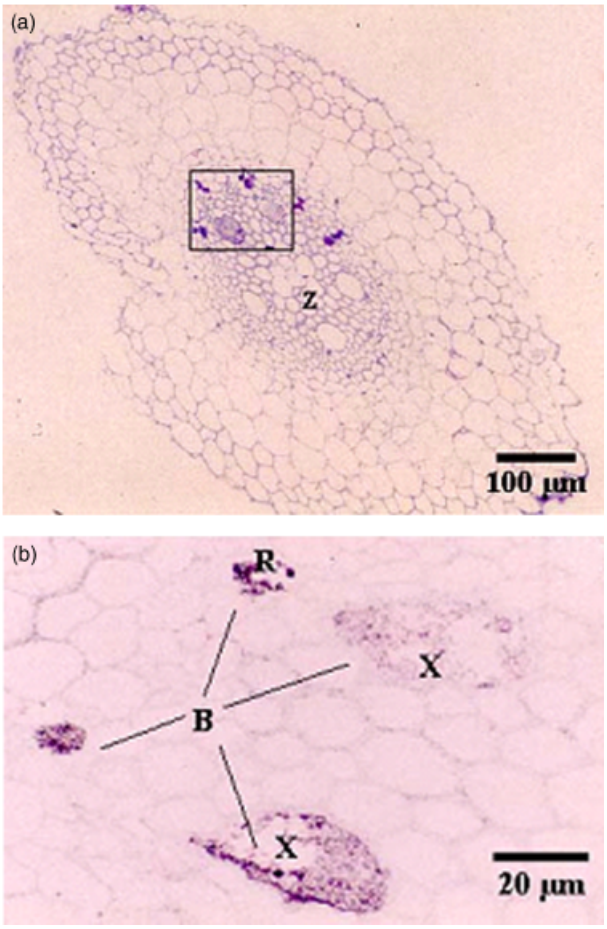

Fig. 3. Light microscopic images of a root cross-section from a *Herbaspirillum frisingense* Mb11 inoculated *Miscanthus* × *giganteus* plant 1 week after inoculation. (a) Stained with toluidine blue. Some xylem vessels in the central cylinder (Z) are colonized. (b) (same object as in a) Immunogold labeling with AntiMb11 and subsequent enhancement with silver staining. Bacteria (B), colonize xylem vessels (X) and some endodermis cells (R).

IAA production of *H. frisingense*

The IAA production was measured using the GC-MS method according to Müller & Weiler (2000). It was ensured that cell densities were comparable by determining the OD of the bacterial cultures at $OD_{436\,nm}$ before centrifuging the cells to obtain the culture supernatant for the GC-MS analysis. After a 36-h growth period in M9 mineral medium supplemented with glucose and tryptophan, 13.9 nmol mL^{-1} free IAA could be detected for *H. frisingense* strain GSF30^T cultures, whereas for strain Mb11 the concentration was 3.0 nmol mL^{-1}, which was only slightly above the value for the *E. coli*-negative control (not shown). The measured amount for the *A. brasilense* Sp7-positive control was 24.4 nmol mL^{-1}. Without adding tryptophan no IAA production could be detected.

AHL production by *H. frisingense*

The *H. frisingense* strains Mb11 and GSF30^T were analyzed for their AHL production using a combination of UPLC- and FT-ICR-MS analyses. After cultivation overnight in rich medium (NB) only for strain Mb11 could the production of minor amounts of OH-C14, C14 and C14 : 1 homoserine lactones be detected with the FT-ICR-MS method but not with UPLC, which is less sensitive, especially due to matrix effects of the growth medium. For other *Herbaspirillum* species, like *Herbaspirillum huttiense*, *Herbaspirillum chlorophenolicum*, *H. hiltneri* and *H. lusitanum* (all type strains), as well as for *H. frisingense* strain GSF30^T, no AHLs were detected with both methods.

ACC utilization of *H. frisingense*

ACC utilization was determined by growing the *H. frisingense* strains Mb11 and GSF30^T on mineral medium plates containing ACC as the only nitrogen source. An ACC-degrading strain, *Burkholderia phytofirmans* (type strain, DSM 17436) (Sessitsch *et al.*, 2005), was used as a positive

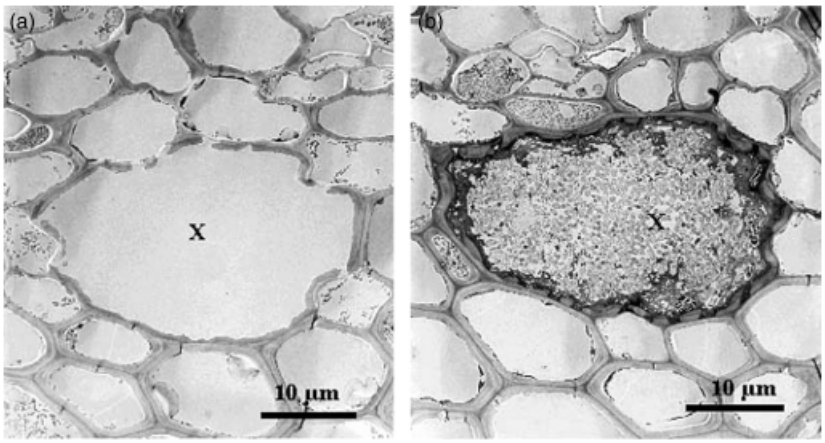

Fig. 4. TEM micrograph of a xylem vessel (X) of a *Miscanthus* × *giganteus* plant 1 week after inoculation with *H erbaspirillum frisingense* Mb11. Immunogold labeling with AntiMb11 and subsequent enhancement with silver staining. (a) Not colonized xylem vessel with intact cellular wall. (b) Completely colonized xylem vessel with obviously undegraded cell wall.

control. The results clearly demonstrated the ability of both *H. frisingense* strains to utilize ACC as a substrate, as they grew equally well on NH_4Cl-containing plates and on ACC-containing ones, whereas they showed almost no growth on the plates without a nitrogen source. They exhibited the same growth abilities as the positive control *B. phytofirmans*. When *H. hiltneri* $N3^T$ was tested for comparison, it did not grow on the ACC-containing and nitrogen-free medium, but grew normally on the NH_4Cl-supplemented plates. The ability of *H. frisingense* Mb11 and $GSF30^T$ to utilize ACC as the sole nitrogen source, while *H. hiltneri* $N3^T$ could not, possibly accounts for the activity of an ACC deaminase in the *H. frisingense* strains.

Discussion

Herbaspirillum seropedicae and H. rubrisubalbicans (Olivares *et al.*, 1997) can colonize the interior of cereals preferentially. To prove endophytic colonization unambiguously, it is not sufficient to demonstrate that a rhizosphere bacterium or phylogenetic marker genes can be isolated or retrieved from surface-sterilized plant tissue (roots or leaves). Primarily, an *in situ* detection with specific labeling techniques and highly resolving microscopic methods is required (Hurek *et al.*, 1994; James & Olivares, 1998; Hartmann *et al.*, 2000). For this reason, we applied two different methods, namely immunogold and GFP labeling, to identify and localize root-colonizing bacteria.

Both *H. frisingense* strains were labeled with the constitutively GFP expressing transposon from plasmid pJBA28. With this method, it was possible to visualize the target cells on the rhizoplane and up to 60 μm within the root cortex, where they were colonizing intercellular spaces as well as apparently intact root cells. The advantage of this method is the possibility to localize bacterial cells in root material that was not cut or in any way chemically treated. On the other hand, in order to detect cells in the central cylinder it proved essential to remove the cortex cells, as the rhizodermis in particular absorbs a lot of the laser light needed for excitation of fluorescent signals in deeper parts of the root tissue and itself exhibits a bright fluorescence. Only those slices were selected where cortex cells had been removed without harming the endodermis surrounding the central cylinder. With this, a transfer of cells from the outer to the inner parts of the roots during the cutting process could be ruled out. Therefore, we were able to demonstrate undoubtedly the massive colonization of barley root tissues within the central cylinder and especially xylem vessels.

Accordingly, in *Miscanthus*, an endophytic colonization of the xylem by the *H. frisingense* strains could be demonstrated with the immunogold labeling method and an electron microscope after only 7 days; colonization of cortex cells was observed after only 3 days (Figs 2–4). This kind of rapid colonization is not unusual for endophytes, for example James & Olivares (1998) described the sporadic colonization of sugar cane xylem vessels 2 days after inoculation with *H. seropedicae*. Both *H. rubrisubalbicans* and *H. seropedicae* were frequently found in intercellular spaces, as well as parenchymal and xylem cells of the central cylinder after 4 days (Olivares *et al.*, 1997). As soon as the xylem vessels are entered, the bacterial colonizers can be passively transported throughout the whole plant, which facilitates a fast systemic spreading. Evidence for such a systemic colonization by *H. frisingense* was found during this study in *Miscanthus* leaves after only 1 week. Thus, it can be stated that *H. frisingense* seems to be a very potent endophytic colonizer unlike for example *A. brasilense* Sp245 (Rothballer *et al.*, 2003).

There was no evidence for a pathogenic or other detrimental effect (e.g. through clogging of xylem vessels) of *H. frisingense*, as it was described for *H. rubrisubalbicans*, which causes light symptoms of mottled stripe disease in sugar cane (Baldani *et al.*, 1996). Instead, preliminary studies for the effect of the two *H. frisingense* strains on growth of both barley and *Miscanthus* gave first indications for a potential to promote plant growth, especially when both strains were applied together. However, more extensive studies with different plant host/inocula combinations have to be conducted to make final conclusions in this direction. No signs of cell wall degradation were noticeable, as it was documented for example for *Azoarcus* sp. (Reinhold-Hurek *et al.*, 1993, 2006). As both *H. frisingense* strains were found as endophytes in the plant interior the only pathway to enter the plant would be through root cracks at the emerging point of side roots, root injuries or the root tip. Especially because *H. frisingense* was found in the central cylinder, this suggested that endophytic colonization could be initiated at the emerging points of side roots. The central cylinder of roots is histologically isolated from the cortex by the endodermis, which protects the plant from transversal intrusion of bacteria. But when side roots are formed, this natural barrier breaks up and thereby allows bacteria to penetrate into the central cylinder. The infection pathway through cracks in the exo- and endodermis of the root caused by emerging side roots has already been demonstrated for *H. seropedicae* (Olivares *et al.*, 1995) and other rhizosphere bacteria like *Azospirillum* sp. (Patriquin *et al.*, 1983), *Gluconacetobacter diazotrophicus* (James *et al.*, 1994) and *Azoarcus* sp. (Reinhold & Hurek, 1988).

The considerable IAA production of *H. frisingense* $GSF30^T$ observed is in accordance with earlier observations for *H. seropedicae* (Bastián *et al.*, 1998). Comparable with the plant growth-promoting bacteria belonging to the genus *Azospirillum* (Dobbelaere *et al.*, 2001), IAA production can stimulate root growth and modify root physiology, leading to improved plant growth. The detected AHL compounds

might be another hint pointing in that direction, as other rhizosphere bacteria have been found to produce this kind of signaling substances as well, for example some strains of *A. lipoferum* (Vial *et al*., 2006) or *Pseudomonas putida* IsoF (Steidle *et al*., 2001). With the help of AHLs, the bacteria could gain information about distribution and density of neighboring cells as well as the surrounding diffusion space (Hense *et al*., 2007), or even cause specific systemic responses in the plant as in the case of *Serratia liquefaciens* MG11 and *P. putida* IsoF in Microtom^{R} tomato plants (Schuhegger *et al*., 2006). The influence of bacterial AHLs on eukaryotes has also been demonstrated in the case of *Medicago truncatula*, where AHLs cause the upregulation of auxin-responsive genes among other changes in protein expression (Mathesius *et al*., 2003). The putative ACC-deaminase activity in both *H. frisingense* strains could provide an additional possibility for these bacteria to reduce plant growth inhibition. An increase of ethylene levels in the root system under unfavorable conditions could lead to pronounced plant growth stimulation (Glick *et al*., 1998).

In conclusion, *H. frisingense* strains GSF30^{T} and Mb11 were found to be very effective endophytic colonizers of *Miscanthus* and barley. *Herbaspirillum frisingense* shows considerable potential as a beneficial plant endophyte and its application should be further considered for agricultural practice.

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