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Brief report

Comparative *in situ* analysis of *ipdC–gfp*mut3 promoter fusions of *Azospirillum brasilense* strains Sp7 and Sp245

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Summary

Inoculation of wheat roots with *Azospirillum brasilense* results in an increase of plant growth and yield, which is proposed to be mainly due to the bacterial production of indole-3-acetic acid in the rhizosphere. Field inoculation experiments had revealed more consistent plant growth stimulation using *A. brasilense* strain Sp245 as compared with the strain Sp7. Therefore, the *in situ* expression of the key gene *ipdC* (indole-3-pyruvate decarboxylase) was examined in these two strains. Within the *ipdC* promoter of strain Sp245 a region of 150 bases was identified, which was missing in strain Sp7. Thus, three different translational *ipdC* promoter fusions with *gfp*mut3 were constructed on plasmid level: the first contained the part of the Sp245 promoter region homologous to strain Sp7, the second was bearing the complete promoter region of Sp245 including the specific insertion and the third comprised the Sp7 promoter region. By comparing the fluorescence levels of these constructs after growth on mineral medium with and without inducing amino acids, it could be demonstrated that *ipdC* expression in *A. brasilense* Sp245 was subject to a stricter control compared with strain Sp7. Microscopic detection of these reporter strains colonizing the rhizoplane documented for the first time an *in situ* expression of *ipdC*.

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The plant growth-promoting rhizobacterium (PGPR) *Azospirillum brasilense* has been known for a long time to exert beneficial influence on inoculated plants, such as maize and wheat (Dobbelaere *et al*., 2001). This stimulatory effect heavily depends on environmental factors, but also on the proper strain for inoculation (Fages, 1994; Dobbelaere *et al*., 2001). In most cases root-colonizing *A. brasilense* cells are embedded in the mucigel layer of the rhizoplane (Murthy and Ladha, 1987) with cell densities depending on the host plant and the colonizing bacterial strain (O'Hara *et al*., 1983). Furthermore, *A. brasilense* cells were shown to colonize not only injured root cells, but also the interior of intact roots (Umalia-Garcia *et al*., 1980).

Compared with other PGPR the ability of this bacterial species to fix atmospheric nitrogen seems to be of minor importance for plant growth stimulation (Patriquin *et al*., 1983). Instead, morphological changes of the colonized roots induced by an *A. brasilense* inoculum result in an increase in root surface, which improves water and mineral uptake (Okon and Kapulnik, 1986). The reason for this change in root morphology is suggested to be found in the production of the auxin indole-3-acetic acid (IAA) by the colonizing Azospirilla (Hartmann *et al*., 1983a), which leads to an increased production of root hairs (Hadas and Okon, 1987) and side roots (Barbieri *et al*., 1986). As early as 1979, Tien and colleagues reported evidences for the production of auxin-, cytokinin- and gibberellin-like substances by *A. brasilense* (Tien *et al*. 1979). But only the auxin IAA is produced in considerable amounts by cultures in the late logarithmic phase (Hartmann *et al*., 1983a). In order to prove the stimulating effect of IAA for plant growth, it was attempted to produce *A. brasilense* mutants no longer able to synthesize this auxin. After Tn*5* transposon mutagenesis *Azospirillum* mutants with strongly inhibited IAA production could be selected, which failed to exert a stimulatory effect on num-

ber of root hairs and length of side roots of inoculated plants (Barbieri *et al*., 1986). However, it has not been possible so far to construct completely IAA-negative mutants. A strain with a knockout mutation in one of the key genes in IAA synthesis still produced detectable amounts of IAA (Hartmann and Zimmer, 1994). This led to the conclusion that there are either several copies of the essential genes for IAA synthesis present in *A. brasilense*, or at least two synthetic pathways. Subsequent studies have substantiated that in fact three potential biochemical pathways exist for production of IAA. There are the indole-3-acetamid and the indole-3-pyruvate pathway, which both use tryptophan as a precursor, but there is also a tryptophan-independent synthetic pathway using by-products of the tryptophan biosynthesis (Prinsen *et al*., 1993). Within the indole-3-pyruvate pathway the enzyme indole-3-pyruvate-decarboxylase (IpdC) plays a major role in the conversion of indole-3-pyruvate to IAA. This reaction is influenced by the amino acids tyrosine and phenylalanine, which exhibit an upregulating effect on expression of the *ipdC* gene similar to the end-product IAA (Vande Broek *et al*., 1999).

The production of IAA is stimulated to a large extent by the addition of tryptophan (Hartmann *et al*., 1983a). In this context it was suggested that the excretion of IAA might serve as a way to reduce toxic concentrations of tryptophan (Bar and Okon, 1992). However, the presence of a tryptophan-independent IAA synthetic pathway seems to point towards a more prominent role of this metabolite in the interaction with plants. On the one hand, excretion of IAA by root-colonizing *A. brasilense* cells leads to an increased root branching and root surface area resulting in an elevated level of water and nutrient uptake by the plant (Okon and Kapulnik, 1986). On the other hand, this enlarged root surface is advantageous for the bacteria as well, as it provides more surface space for colonization (Dosselaere, 2000).

As IAA production plays such an important role in this plant–microbe relationship, a characterization of *Azospirillum* strains concerning quantity and regulation of IAA synthesis might be of major relevance for the application of these strains as inoculum in field trials. Therefore, it was the aim of this study to investigate expression of the *ipdC* gene in two *A. brasilense* strains, which are frequently used for plant growth promotion studies (Burdman *et al*., 2000; Pereg Gerk *et al*., 2000; Dobbelaere *et al*., 2001). It is known from field inoculation trials in Brazil (Baldani *et al*., 1987) that the strain *A. brasilense* Sp245 isolated from surface-sterilized wheat roots performed better in field experiments compared with the strain Sp7, an isolate from the rhizosphere soil of *Digitaria* (Tarrand *et al*., 1978). In-depth studies revealed a different colonization behaviour of wheat roots: in contrast to strain Sp7, *A. brasilense* strain Sp245 was able to penetrate the rhizodermis and establish in the root cortex (Schloter and Hartmann, 1998; Rothballer *et al*., 2003). The demonstration of this endophytic colonization was facilitated by generating constitutively *gfp* expressing transconjugands of *A. brasilense* Sp7 and Sp245 with the *gfp* variant mut3 (Ramos *et al*., 2002; Rothballer *et al*., 2003). To our knowledge no reporter constructs using a *gfp* marker gene have been created in this bacterium yet. In *A. brasilense* Sp245 an upregulating effect of tyrosine, phenylalanine and IAA on an *ipdC–gusA* fusion could be demonstrated by Dosselaere (2000), but the *gusA* reporter gene is not well suited for *in situ* studies on cellular level. Thus, we performed a comparative analysis of the promoter regions of the *ipdC* gene of *A. brasilense* strains Sp7 and Sp245 by constructing *gfp* promoter fusions. As expression of the *ipdC* gene of *A. brasilense* has not been shown in the rhizosphere so far, we also focused on the *in situ* expression of these *gfp* promoter fusions in *A. brasilense* cells colonizing the wheat root surface.

According to published sequence data for the *ipdC* gene of *A. brasilense* Sp7 (NCBI Accession No. X99587; Zimmer *et al*., 1998), the primers ipdC-P-ApaI and ipdC-P-NcoI were created to amplify the *ipdC* promoter region (for binding sites see Fig. 1). Using these modified primers, it was possible to attach an *Apa*I restriction site to the 5′-end and a *Nco*I site at the 3′-end of the amplificate. Genomic DNA from *A. brasilense* strain Sp7 as polymerase chain reaction (PCR) template resulted in a 170 bp fragment named i7P, while PCR with template DNA from strain Sp245 produced a fragment, which was 150 bp larger. Therefore, the primer pair ipdC2-EcoRI (5′-TACGAATTCGGCATCATTCGAAAGTCG-3′) and ipdC3-SacI (5′-TACGAGCTCACCTTGGCCTCCAGCC-3′) was used to amplify the whole *ipdC* promoter region and part of the *ipdC* gene (amplificate ipdC3, NCBI Accession No. AY628754). Sequence comparison revealed that the Sp7 *ipdC* promoter region was almost identical to a 140 bp segment directly upstream of the Sp245 *ipdC* start codon. Further upstream a 150 bp insertion followed in the *ipdC* promoter sequence of strain Sp245, which was not present in strain Sp7. Within this region two complementary sequences were found, which also exhibited a partially palindromic sequence motif (see Fig. 1). These characteristical motives point towards a possible relevance of this region in transcriptional regulation of the *ipdC* gene, as most known prokaryotic transcription factors bind to palindromic or pseudopalindromic DNA regions (Huffman and Brennan, 2002). Upstream of this region towards the beginning of the *gltX* promoter region a 30 bp segment could be found, which was again similar in both strains. An alignment of the homologous sequences of the *ipdC* promoter region of strain Sp7 and Sp245 is shown in Fig. 1.

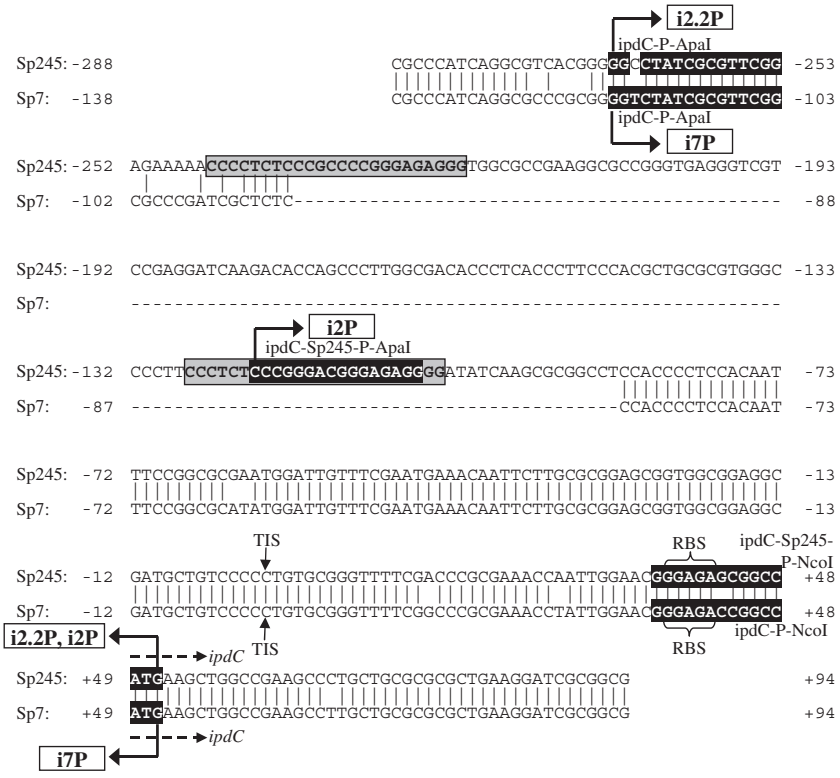

Fig. 1. Sequence alignment of the *ipdC* promoter regions of strain Sp245 and Sp7. Primer binding sites are marked in black, and the arrows indicate the amplified fragments used for constructing the promoter fusions. Between position –88 and –237 referring to the *ipdC* transcription initiation site (TIS) a 150 bp insertion is to be found in strain Sp245, which is missing in strain Sp7. Two complementary regions within this insertion are marked in grey. The TIS was determined by 5′RACE with the help of the 5′/3′RACE Kit (second generation, Roche, Penzberg, Germany) according to the protocol provided by the manufacturer. For strain Sp245 the same TIS was found by Vande Broek and colleagues (2005) with the primer extension method. Sequence data of strain Sp7 were taken from Zimmer and colleagues (1998), NCBI Accession No. X99587. Sequence data of strain Sp245 is from this study, NCBI Accession No. AY628754. RBS, ribosomal binding site.

To further investigate the functional implication of this insertion for the regulation of *ipdC* expression, two different Sp245 promoter fusions were designed. According to the obtained sequence data, one primer pair, ipdC-Sp245-P-ApaI and ipdC-Sp245-P-NcoI, was chosen in order to amplify exclusively the Sp245 promoter segment which is almost identical to the promoter sequence of strain Sp7. The resulting amplificate was 170 bp in size and termed i2P. A second primer pair, ipdC-P-ApaI and ipdC-Sp245-P-NcoI, was used to amplify a 320 bp fragment, which was named i2.2P and enclosed the whole *ipdC* promoter region of Sp245 including the specific insertion (for primer binding sites and amplified fragments, see Fig. 1). With these amplificates (i7P, i2P, i2.2.P) three translational promoter fusions with *gfp*mut3 were constructed and integrated in a pBBR1MCS-2 vector (Kovach *et al*., 1995), which was known to be stable in *A. brasilense* without antibiotic pressure (Rothballer *et al*., 2003).

The three constructs were transferred to *A. brasilense* strains Sp7 and Sp245 by conjugation via triparental mating (Kristensen *et al*., 1995, modified). Transconjugants were selected with MMAB (Vanstockem *et al*., 1987) agar plates containing nalidixic acid and kanamycin. Resistant strains were picked, analysed with FISH using *A. brasilense*-specific probe Abras 1420 (for details see Fig. 3) and named depending on the transferred plasmid as Sp7 i7P, Sp245 i2P or Sp245 i2.2P. The plasmids were also delivered to the respective other *A. brasilense* strain, giving rise to the transconjugants Sp245 i7P, Sp7 i2P and Sp7 i2.2P (see Table 1). Finally, *A. brasilense* strains Sp7 and Sp245 harbouring a constitutively expressed *gfp*mut3 gene (Rothballer *et al*., 2003) were also included as a control in the expression analysis to evaluate influencing factors of amino acid addition, which were not due to induction or repression of the *ipdC* promoter. The application of the instable variant *gfp*(ASV) did not produce fluorescently labelled cells in any case. Obviously the amounts of green fluorescent protein (GFP) accumulating in the cells was not sufficient for visualization with the CLSM, which was probably due to the instability of this GFP variant in combination with rather low expression levels of GFP in *A. brasilense*. Therefore, *gfp*mut3 had to be used. Employing this stable GFP variant, experimental timing had to be optimized, because of the continuous accumulation of GFP in the cell without considerable degradation. If cells that are grown without inducing amino acids were incubated for too long, the continuous increase of GFP would finally reach almost the same level as in cultures grown in the presence of amino acids and the inducing effect of the amino acids would be obscured. In order to demonstrate the maximum difference in expression activity between cultures grown on different media, incubation at 37°C was not to exceed 18 h. Afterwards cultures were stored for another 30 h at 4°C to enable the maturation of the GFP, which had been expressed during growth phase.

Table 1. Bacterial strains used in this study.

Bacterial strain	Description	Reference
Escherichia coli		
HB101	*recA thi pro leu hsd*$^-$ *M*$^+$ Sm^R	Kessler *et al.* (1992)
TOP10	F′ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*Z ΔM15 Δ*lac*X74 *rec*A1 *deo*R *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G	Invitrogen, Carlsbad, USA
Azospirillum brasilense		
Sp7	Nal^R, type strain; isolate from *Digitaria* rhizosphere	Tarrand *et al.* (1978)
Sp7 *gfp*mut3	Km^R, Nal^R, containing pBAH7	Rothballer *et al.* (2003)
Sp7 i7P	Km^R, Nal^R, containing pBBR-i7Pg(mut3)T	This study
Sp7 i2P	Km^R, Nal^R, containing pBBR-i2Pg(mut3)T	This study
Sp7 i2.2P	Km^R, Nal^R, containing pBBR-i2.2Pg(mut3)T	This study
Sp245	NalR wild-type isolate from surface-sterilized wheat roots	Baldani *et al.* (1987)
Sp245 *gfp*mut3	Km^R, Nal^R, containing pBAH7	Rothballer *et al.* (2003)
Sp245 i7P	Km^R, Nal^R, containing pBBR-i7Pg(mut3)T	This study
Sp245 i2P	Km^R, Nal^R, containing pBBR-i2Pg(mut3)T	This study
Sp245 i2.2P	Km^R, Nal^R, containing pBBR-i2.2Pg(mut3)T	This study

For strain Sp7 i7P (containing *gfp* fusion with whole *ipdC* promoter region of strain Sp7) an almost threefold increase of fluorescence intensity could be detected when grown on MMAB medium containing phenylalanine compared with growth on MMAB medium without amino acids. Also growth on MMAB medium with tyrosine or tryptophan resulted in a significant upregulation of *gfp* expression compared with cultivation on MMAB without amino acids. The highest *gfp* fluorescence was detectable in strains grown on NB medium (Fig. 2). In contrast, no media-dependent difference in fluorescence intensity was detected with transconjugant Sp7 i2.2P (containing *gfp* fusion with whole *ipdC* promoter region of strain Sp245) (Fig. 2). Fluorescence levels were considerably higher than in Sp7 i7P reaching or even surpassing the level of the constitutively *gfp* expressing transconjugant Sp7 pBAH7 (data not shown). Strain Sp7 pBAH7 also showed no significant upregulation induced by the media. Finally, strain Sp7 i2P (containing *gfp* fusion with truncated promoter region of strain Sp245) did not show any detectable fluorescence (not shown).

Cultivation of strain Sp245 i2.2P (containing *gfp* fusion with whole *ipdC* promoter region of strain Sp245) resulted in much clearer changes in fluorescence intensity compared with Sp7. On MMAB medium without amino acids the fluorescence was reduced to only 7% of the intensity attained in cultures grown on MMAB medium with tyrosine. As a whole the measured fluorescence intensities of strain Sp7 i7P were about 30% higher than of strain Sp245 i2.2P (Fig. 2). GFP fluorescence in strain Sp245 i2P (containing *gfp* fusion with truncated promoter region of strain Sp245) did not reach detectable levels. Strain Sp245 i7P (containing *gfp* fusion with whole *ipdC* promoter region of strain Sp7) exhibited a similar upregulating effect as strain Sp245 i2.2P but only about 50% of the fluorescence intensity. At last, transconjugant Sp245 pBAH7 (with constitutively expressed *gfp*) showed no dependence of fluorescence intensity on growth medium (data not shown).

The analysis of strain Sp245 i2.2P (containing whole *ipdC* promoter region of strain Sp245) produced results consistent with previous findings: Dosselaere (2000) reported expression studies of an *ipdC–gusA* promoter fusion (Vande Broek *et al.*, 1999), which also contained the whole promoter region of Sp245. They examined changes in expression level due to growth on MMAB medium supplemented with the amino acids phenylalanine or tyrosine. In this case strongest upregulation was recognized with tyrosine in the growth medium, whereas almost no GusA activity was detectable in cultures grown on MMAB medium without amino acids. The fact that the truncated Sp245 promoter fusion i2P did not enable any measurable *gfp* expression proved the importance of the entire Sp245-specific insertion for full promoter activity in strain Sp245. This was confirmed by studies with mutants in the *ipdC* promoter region (Vande Broek *et al.*, 2005). The reduction of fluorescence intensity by a factor of two in strain Sp245 i7P (containing whole *ipdC* promoter region of strain Sp7) supports this conclusion. Accordingly, the Sp245-specific insertion is in fact essential for a maximum upregulation of *ipdC* promoter activity, but there seem to be additional regulating factors involved.

In spite of the high similarity of the two *ipdC* promoter regions i2P and i7P, results for the expression analysis of strain Sp7 harbouring the respective *gfp* fusion of promoter region i7P were profoundly different. An increase in GFP fluorescence representing an upregulation of *ipdC* expression due to the presence of amino acids in the growth medium was detectable, but over all fluorescence intensity was clearly higher than in strain Sp245 i2.2P (containing whole *ipdC* promoter region of Sp245). In contrast to strain Sp245 i2.2P the upregulation response

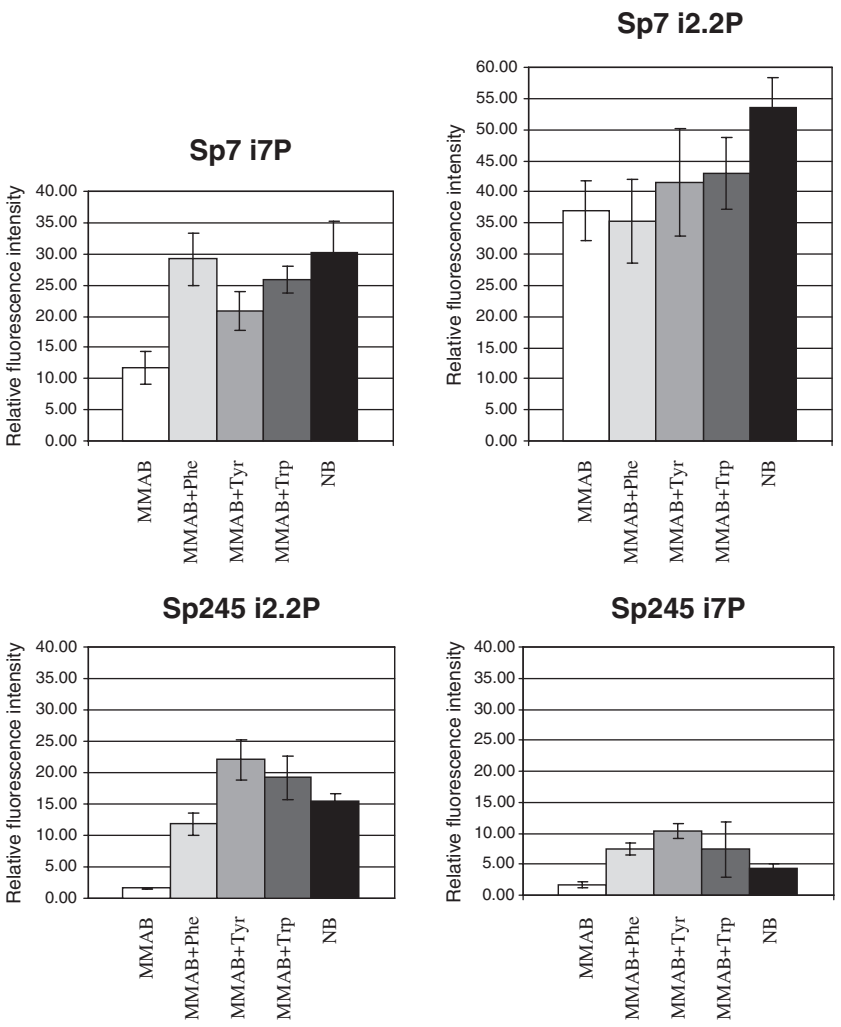

Fig. 2. Relative fluorescence intensity of transconjugands Sp7 i7P (construct with whole *ipdC* promoter region of strain Sp7), Sp7 i2.2P (construct with whole *ipdC* promoter region of strain Sp245), Sp245 i2.2P (construct with whole *ipdC* promoter region of strain Sp245) and Sp245 i7P (construct with whole *ipdC* promoter region of strain Sp7) after cultivation on different media. All strains were plated on NB and MMAB agar plates. Additionally, MMAB agar plates were used, which contained either phenylalanine, tyrosine or tryptophan (0.5 mM each). After incubation cultures were resuspended with PBS, diluted to an equal concentration and the fluorescence intensity was recorded with a CLSM. Six images of about 4000 cells were taken and a histogram enabled the calculation of fluorescence intensity per picture. From these data the mean value and twofold standard deviation (error bars) were calculated. Incubation of strains Sp7 i2P and Sp245 i2P (construct with truncated *ipdC* promoter region of strain Sp245) on agar plates containing amino acids did not result in any detectable fluorescence. In order to check whether the amino acids in the MMAB medium were consumed completely by the bacteria during incubation and would therefore not induce the promoter constructs, a water extraction of agar plates was performed at the end of the incubation period. The extract was analysed by capillary electrophoresis coupled to an UV-VIS detector (Beckmann P/ACE 5510), and compared with an extract from agar plates, which had not been inoculated with bacteria. With this method it could be demonstrated that after 18 h of incubation at least 80% of the amino acids were still present in the growth medium.

to phenylalanine was almost 50% higher than of tyrosine. Additionally the decrease in fluorescence intensity on medium without amino acids was not as pronounced (Fig. 2). This leads to the conclusion that expression of the *ipdC* gene in strain Sp245 is subject to a stricter control than in strain Sp7, which is also confirmed by the total absence of regulation in strain Sp7 i2.2P (containing whole *ipdC* promoter region of Sp245). The different stringency in the regulation of IAA could be decisive for the selection of an *Azospirillum* strain as an inoculum for growth stimulation of agricultural crops. Hartmann and colleagues (1983a) isolated mutants of *A. brasilense* strain Sp Cd (phylogenetically closely related to strain Sp7), which produced high amounts of IAA. When maize plants were inoculated with these mutants, an inhibitory effect on root and shoot growth was observed especially at elevated levels of nitrogen in the rhizosphere, which supports increased IAA production (Hartmann *et al.*, 1983b). Furthermore it has been demonstrated in several studies that an *Azospirillum* inoculum above a concentration of 10^8 cells per millilitre could also lead to a reduced root development (Okon and Kapulnik, 1986; Fallik *et al.*, 1988). Both results suggest an inhibitory effect of auxin on growth of inoculated plants, if a certain optimal concentration is surpassed. Considering this, strain Sp245, especially as colonizer of wheat root rhizodermal cells, seems to be better suited for an inoculation of agricultural crops, as the stricter regulation of IAA synthesis avoids an overproduction of auxin.

The transconjugants Sp7 i7P, Sp245 i2.2P and Sp245 i2P were also applied as reporter strains for the detection of *in situ* activation of the *ipdC* promoter on the rhizoplane of wheat roots (Brazilian summer wheat *Triticum aestivum*, PF839197) grown in a monoxenic quarz sand system. Again, as a control the constitutively *gfp* expressing strains Sp7 pBAH7 and Sp245 pBAH7 were inoculated as well. Two days after inoculation plant roots were harvested and GFP fluorescence was visualized with a CLSM. Using strain Sp7 i7P as inoculum there was no detectable difference concerning spatial distribution or flu-

orescence intensity compared with strain Sp7 pBAH7 (Fig. 3A). Intensely fluorescing cells of strain Sp7 i7P were distributed all over the wheat root surface and on root hairs. Fluorescing cells of strain Sp245 i2.2P were also clearly visible along the whole root (Fig. 3D), demonstrating *in situ* expression of the *ipdC* promoter fusion in Sp245, however, with clearly reduced intensity. In addition, the fluorescence intensity of both strain Sp7 and Sp245 did apparently not depend on the localization of a cell at any specific part of the rhizoplane. In contrast, GFP fluorescence of strain Sp245 i2P was not detectable. However, a colonization of the wheat roots by this strain could be confirmed performing FISH with an *A. brasilense*-specific probe (Fig. 3B and C).

The *in situ gfp* expression of the three *ipdC* reporter strains Sp7 i7P, Sp245 i2.2P and Sp245 i2P detected on wheat roots (Fig. 3) confirmed the results of the *in vitro* culture expression analyses. The activation of the GFP

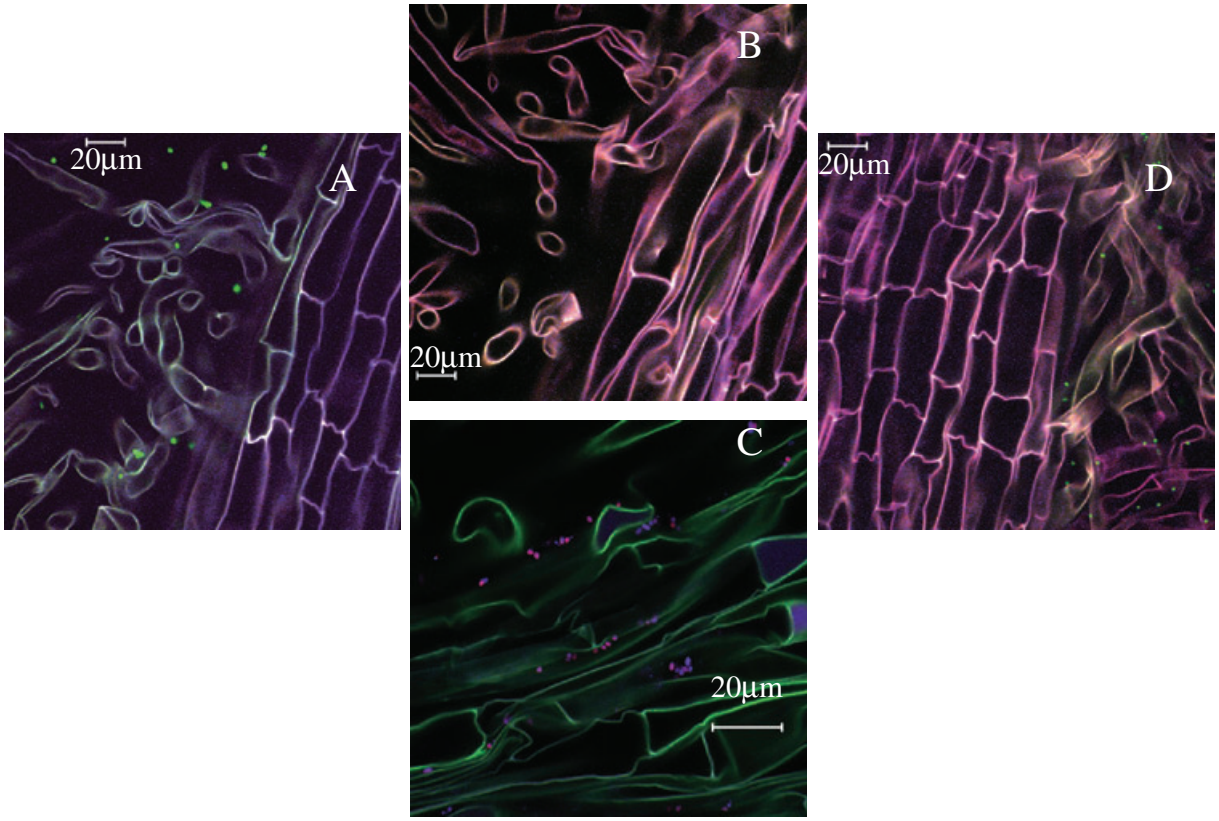

Fig. 3. Green fluorescent protein (GFP) expression by the various *A. brasilense* transconjugants on wheat roots and root hairs taken from a monoxenic system (CLSM images). Hybridization of roots with fluorochrome (Cy3, Cy5) labelled oligonucleotide probes was carried out in an Eppendorf tube with 160 µl of hybridization buffer and 20 µl of a 30 µg ml^{-1} working solution of each probe, but otherwise followed the protocols for pure culture hybridization described by Manz and colleagues (1992) and Amann and colleagues (1992). Two rRNA-targeted oligonucleotide probes were used, which were synthesized and fluorescently labelled by ThermoHybaid (Ulm, Germany). EUB-338 (Amann *et al.*, 1990), EUB-338-II and III (Daims *et al.*, 1999) were used as equimolar mixture and are specific for almost all members of the domain *Bacteria* at varying formamide concentrations. The 16S rRNA targeted probe Abras1420 (5′-CCACCTTCGGGTAAAGCCA-3′) (Stoffels *et al.*, 2001) in combination with a competitor (5′-CACCTTCGGGTAAAACCA-3′) enables specific identification of the species *A. brasilense* at a formamide concentration of 45% in the hybridization buffer. Fluorescing cells were detected by a confocal laser scanning microscope (LSM510 Meta, Zeiss, Oberkochen, Germany). An argon-ion-laser was used for excitation at 488 nm and a helium-neon-laser provided wavelengths of 543 nm and 633 nm for the excitation of Cy3 and Cy5 respectively. The Cy5 fluorescent dye emits in the far-red spectrum of light but a blue colour is assigned for illustration, whereas Cy3 is shown in its fluorescence colour red. After hybridization three single-colour images were produced with the CLSM and thereafter combined to one RGB (red green blue) picture. Due to the binding of both fluorescently labelled probes (EUB338 mix with Cy3 and Abras1420 with Cy5), *A. brasilense* cells were specifically identified by their magenta staining. For the visualization of the *gfp* expressing *A. brasilense* cells on wheat roots parts of freshly harvested roots were washed with 1× PBS and placed on a glass slide. They were embedded in Citifluor (Citifluor, Canterburry, UK) and GFP-derived fluorescence was detected using the CLSM. The same set of three lasers was used as described above. Green fluorescent protein was excited at 488 nm by an argon-ion-laser. A simultaneous detection of FISH and GFP signal was also attempted for all constructs, but due to the treatment with denaturing chemicals during the FISH protocol the GFP fluorescence was reduced dramatically, so that it was impossible to separate specific GFP fluorescence of cells from the background fluorescence.
A. Numerous intensely fluorescing cells of strain Sp7 i7Pg(mut3)T.
B. No GFP fluorescing cells of strain Sp245 i2P are visible in front of the autofluorescing background of the root.
C. Detection of strain Sp245 i2P (magenta) on wheat roots by FISH with the probes Abras-1420 (Cy5) and EUB-338-Mix (Cy3).
D. Clearly visible but compared with strain Sp7 i7P somewhat weaker GFP fluorescing cells of strain Sp245 i2.2Pg(mut3)T.

reporter constructs in the rhizosphere of wheat proves the inducing effect of root exudates on *ipdC* expression, as it was shown in this study that almost no *ipdC* promoter activity was recognizable without inducing amino acids. In this way the plant controls the synthesis of IAA by *A. brasilense* on the rhizoplane and endorhizosphere. Most interestingly, the expression level of the Sp245 *ipdC* reporter construct i2.2P was lower in the rhizosphere as compared with Sp7 *ipdC* reporter construct i7P. This probably reflects a more stringent regulation of *ipdC* expression in Sp245 when colonizing roots. Brandl and colleagues (2001) constructed an *ipdC–gfp* promoter fusion in *Erwinia herbicola* and demonstrated an *ipdC* promoter activation in cells colonizing leaves compared with cultures grown on minimal medium. Exactly as in *A. brasilense* IAA production increased, when *E. herbicola* cells were localized on the plant surface. Similar *in situ* reporter studies were performed by Egener and colleagues (1998) to visualize the activity of nitrogenase gene expression by *Azoarcus* sp. BH72 in association with rice roots. Using *gfp* promoter fusions high levels of *nifHDK* promoter activity could be recognized in different parts of the root. These findings, as well as the results presented in this study, are examples for the successful application of the GFP to elucidate *in situ* expression of putative key genes in plant–microbe interactions and to get further insights in the mechanisms of plant growth promotion.

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