Promoter-trap identification of wheat seed extract-induced genes in the plant-growth-promoting rhizobacterium *Azospirillum brasilense* Sp245

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*Azospirillum* strains have been used as plant-growth-promoting rhizobacteria (PGPR) of cereal crops, but their adaptation to the root remains poorly understood. Here, we used a global approach based on differential fluorescence induction (DFI) promoter trapping to identify genes of the wheat isolate *Azospirillum brasilense* Sp245 that are induced in the presence of spring wheat seed extracts. Fluorescence-based flow cytometry sorting of Sp245 cells was validated using *P* _lacZ_, *P* _sbpA_ and *P* _nifH_ promoters and *egfp_. A random promoter library was constructed by cloning 1–3 kb Sp245 fragments upstream of a promoterless version of *egfp* in the promoter-trap plasmid pOT1e (genome coverage estimated at threefold). Exposure to spring wheat seed extracts obtained using a methanol solution led to the detection of 300 induced DFI clones, and upregulation by seed extracts was confirmed *in vitro* for 46 clones. Sequencing of 21 clones enabled identification of seven promoter regions. Five of them displayed upregulation once inoculated onto spring wheat seedlings. Their downstream sequence was similar to (i) a predicted transcriptional regulator, (ii) a serine/threonine protein kinase, (iii) two conserved hypothetical proteins, or (iv) the copper-containing dissimilatory nitrite reductase NirK. Two of them were also upregulated when inoculated on winter wheat and pea but not on maize, whereas the three others (including *P* _nirK_) were upregulated on the three hosts. The amounts of nitrate and/or nitrite present in spring wheat seed extracts were sufficient for *P* _nirK_ upregulation. Overall, DFI promoter trapping was useful to reveal *Azospirillum* genes involved in the interaction with the plant.

**INTRODUCTION**

Several *Azospirillum* strains have been used as plant-growth-promoting rhizobacteria (PGPR) (Dobbelaere et al., 2001; El Zemrany et al., 2006; Jacoud et al., 1999), which may lead to improved crop yield (Dobbelaere et al., 2001; Okon & Labanera-Gonzalez, 1994). Plant-beneficial properties widely documented in *Azospirillum* strains include associative nitrogen fixation, production of phytohormones such as indole-3-acetic acid (IAA), production of nitric oxide (NO), and deamination of the ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC) (Blaha et al., 2006; Creus et al., 2005; Lambrecht et al., 2000). The last three traits may lead to enhanced root system branching and root elongation, which in turn will favour the uptake of soil water and minerals (Glick et al., 1998; Jacoud et al., 1999; Okon & Kapulnik, 1986; Steenhoudt & Vanderleyden, 2000). The establishment of the associative symbiosis between *Azospirillum* PGPR and the plant starts at a very early stage, i.e. during bacterial colonization of germinating seeds (Jacoud et al., 1998). The effects of the bacterial partner on the plant have received considerable research attention. In contrast, however, the effects of the plant on *Azospirillum* have been less studied, and the adaptation of the latter to the root is still poorly understood.

**Abbreviations**: ACC, 1-aminocyclopropane-1-carboxylate; CLSM, confocal laser scanning microscope; DAR-4M AM, diaminorhodamine-4M acetoxymethyl ester; DFI, differential fluorescence induction; MCS, multiple cloning site; PGPR, plant-growth-promoting rhizobacteria; PTL, promoter-trap library.

The GenBank/EMBL/DDBJ accession numbers for the sequence data obtained in this work are given in Table 3 and Supplementary Table S1. A supplementary table of spring wheat seed extract-inducible cryptic fusions identified by DFI in *A. brasilense* Sp245 is available with the online version of this paper.
As for many other rhizobacteria, *Azospirillum* colonizes the plant at the expense of seed and root exudates. This involves chemotaxis and migration toward the plant, as well as microbial proliferation and microcolony/biofilm formation at the surface of seeds and roots (Reinhold et al., 1985; Vande Broek et al., 1998). *Azospirillum* can use as growth substrates a wide range of organic compounds present in the exudates of seeds and/or roots, such as malate, pyruvate and fructose (Alexandre et al., 2000; Fan et al., 2001; Vancura & Hanzlikova, 1972). Thus, although it is likely that adaptation to the plant involves a large set of bacterial genes, only a few of them have been identified so far in the case of *Azospirillum*. Therefore, it is relevant to broaden our understanding of gene expression changes undergone by *Azospirillum* during the establishment of the associative symbiosis with the plant.

Different approaches can be followed to identify bacterial genes induced under particular environmental conditions. This may involve the comparison of protein profiles (Guerreiro et al., 1997), as done for *Azospirillum brasilense* Sp7 and Sp245 (Van Bastelaere et al., 1993), but identification of the corresponding genes is not always straightforward. RNA approaches are possible, based on the use of gene expression arrays, differential display using arbitrarily primed PCR or cDNA-AFLP, or subtractive hybridization techniques (Bhagwat & Keister, 1992; Mark et al., 2005; McClelland et al., 1995; Valverde et al., 2006). The induction of gene transcription can also be studied after cloning short DNA fragments containing promoter sequences (i.e. promoter traps) upstream of reporter or selectable genes; this includes *in vivo* expression technology (IVET; Rainey, 1999), recombinase-based *in vivo* expression technology (RIVET; Zhang et al., 2006) and differential fluorescence induction (DFI; Allaway et al., 2001; Zhang & Cheng, 2006).

The objective of this work was to identify genes of the wheat isolate *A. brasilense* Sp245 that are induced in the presence of wheat seed extracts. The focus was on extracts from seeds rather than roots because (i) the preparation of seed extracts is fast and reproducible, (ii) seed and root exudates are largely similar (Vancura & Hanzlikova, 1972), and (iii) effective phytostimulation relies on early interaction between *Azospirillum* and germinating seeds (Jacoud et al., 1998). The genome of *A. brasilense* Sp245 is in the process of being sequenced, but the whole genome sequence was not available at the start of the project, and thus we selected a global approach based on DFI promoter trapping to identify seed extract-induced genes in this bacterium.

**METHODS**

**Bacterial strains and media.** Bacterial strains and plasmids are listed in Table 1. *A. brasilense* Sp245-Rif is a root-proficient, spontaneous rifampicin-resistant mutant of the wild-type strain Sp245. *A. brasilense* was grown at 28°C in modified Luria–Bertani (LB) medium (Sambrook et al., 1989), i.e. LBm (with only 5 g NaCl l⁻¹), or in AB medium after replacing glucose with malic acid (5 g l⁻¹; Sigma-Aldrich) as sole carbon source (i.e. ABmal) (Chilton et al., 1974). *Escherichia coli* was grown in LB medium at 37°C. Liquid cultures were incubated on a rotary shaker at 180 r.p.m. (50 r.p.m. for microtiter plates). One-third of the volume of the tubes was filled to ensure fully aerobic conditions. To implement oxygen-limited conditions (microaerobiosis), tubes were entirely filled with medium. Antibiotics (Euromedex) were added at the following final concentrations (µg ml⁻¹), unless otherwise specified: ampicillin (Ap), 100; gentamicin (Gm), 25; kanamycin (Km), 40; rifampicin (Rif), 50.

**Plasmid transfer and PCR conditions.** Bacterial conjugations were performed by bi- or triparental mating (Vial et al., 2004), and in the latter pRK2013 was used as a helper (Figurski & Helinski, 1979). Transconjugants were selected on LBm containing Ap and Gm (and Rif when Sp245-Rif was involved) after incubation at 28°C.

**Construction of an egfp promoter-trap library.** egfp was selected to construct a promoter-trap library of *A. brasilense* Sp245, by cloning short DNA fragments from Sp245 upstream of a promoterless egfp in the promoter-trap vector pOT1e (Table 1). EGF is an enhanced GFP variant designed for flow cytometry studies (Cormack et al., 1996). Plasmid pOT1e derives from pBBR1 (Allaway et al., 2001), a replicon stably maintained (at medium copy number) in several bacterial species including *A. brasilense* (Ramos et al., 2002).

Total genomic DNA of *A. brasilense* Sp245 was obtained as described by Sambrook et al. (1989) and was partially digested with BspI431 (Fermentas). Fragments 1–3 kb in length were purified by linear sucrose gradient (5–30%). Four successive fractions were recovered and ligated independently using T4 DNA ligase (Fermentas) to form a single library.

Each of the four ligation mixtures was introduced into *E. coli* DH10B by electroperoration using a Gene Pulser apparatus (Bio-Rad), according to the manufacturer's instructions. The cells were then plated on LB agar containing 10 µg Gm ml⁻¹. After overnight incubation, a total of 96,000 colonies were obtained. For each ligation mixture, the resulting colonies were scraped off from the plates and pooled, giving libraries PTL-Ec (for Promoter-Trap Library in *E. coli*) 1 to 4 (Table 1). Preliminary insert analysis was carried out on five colonies picked up at random from each library. This was done by PCR, using primers F2603 and F2604 (Table 2), as well as by restriction with AvaI (Fermentas).

Each of the four PTL-Ec libraries was transferred en masse by triparental mating in *A. brasilense* Sp245-Rif, as described above. A total of about 8.5 × 10⁷ individual Gm-resistant colonies were obtained. These colonies were pooled in LBm medium, giving libraries PTL-Ab (for Promoter-Trap Library in *A. brasilense* 1 to 4 (Table 1). Part of PTI-Ab1, PTL-Ab2, PTL-Ab3 and PTL-Ab4 were pooled, resulting in PTL-AbP. Bacteria in the libraries were propagated as little as possible to favour equal representation of clones.

**Construction of egfp transcriptional fusions with sbpA or nifH promoters.** Positive DFI controls were constructed using two characterized promoters from *A. brasilense* Sp245, i.e. the plant-
inducible promoter PsbA (Van Bastelaere et al., 1993) and the nitrate-repressed promoter PnifH (de Zamaroczy et al., 1989). The PsbA-egfp and PnifH-egfp transcription fusions were constructed by PCR amplification of PsbA (229 bp ampiclon, of which 180 bp were upstream of the sbpA ATG start codon) and PnifH (248 bp amiplicon, of which 205 bp were upstream of the nifH AGG start codon), respectively, and inserting them upstream of promoterless egfp gene in the BamHI site of the promoter-trap plasmid pOT1e. The PCR primers used to this end introduced BgII (for F2438) and BamHI (for F2440) restriction sites in the case of PsbA-egfp, and BgII restriction sites (for each of F2442 and F2443) in the case of PnifH-egfp.

After QIAquick PCR purification (Qiagen), performed according to the manufacturer’s instructions, the PCR products were cloned into pGEM-T easy (Promega). Plasmid DNA from positive clones was restricted with II-digested promoter-trap plasmid pOT1e and transferred to the manufacturer's instructions, and restricted with BgII and BamHI (for PspA-egfp) and BgII (for PnifH-egfp) (Fermentas). DNA fragments containing PspA-egfp or PnifH-egfp were then subcloned in BamHI-digested promoter-trap plasmid pOT1e and transferred into chemically competent cells of E. coli S17.1 λ pir (Sambrook et al., 1989). Preliminary insert analysis was carried out by PCR on some colonies, using primers F2603 and F2604 (Table 2). Afterwards, the correct orientation and sequence of the two egfp transcriptional fusions were confirmed by sequencing with primers F2603 and F2604, before introduction into A. brasilense Sp245-Rif by bireaparental mating (as described above).

**Collection of seed extracts.** Seeds of winter wheat (Triticum aestivum 'Soissons'), spring wheat (Triticum aestivum L. 'Fiorina'), maize (Zea mays 'LG24.50') and pea (Pisum sativum 'Wisconsin Perfection') were obtained respectively from Delley Seed and Plants Ltd (Delley, Switzerland), Florimond-Desprez (Cappelle en Péve, France), Limagrain (St Quentin Fallavier, France) and the John Innes Centre (Norwich, UK). Seeds (10 g) with no cracks or other visible deformities were added to 100 ml methanol and sonicated for 10 min using an S-line apparatus (Bioblock). The extract solution was passed through Whatman paper no. 3M, evaporated under vacuum at 37 °C to a volume of 5 ml, vacuum dried, weighed, resuspended in methanol to reach 50 mg dry matter ml⁻¹ and stored at −20 °C. Unless otherwise specified, this stock solution was diluted 50-fold in ABmal medium and filtered through a 0.22 μm pore-size filter before use. In most cases, the stock solutions of seed extract were prepared on two occasions. Unless otherwise indicated, seed extracts refer hereafter to spring wheat seed extracts, which were the ones used to find induced promoters. Amounts of nitrate and nitrite in spring wheat seed extracts were quantified (in duplicate) by ionic chromatography {Service Central d’Analyse du CNRS, Solaise, France).
**Flow cytometry parameters.** Flow cytometry was used to measure fluorescence levels and/or to select and sort fluorescent or non-fluorescent *A. brasilense* cells (Figs 1 and 2). Flow cytometry was performed with a FACSCalibur apparatus (Becton Dickinson Biosciences) fitted with a 15 mW argon laser emitting at 488 nm. FACSD流 solution (Becton Dickinson Biosciences) was used as sheath fluid for analysis and sterile (0.22 μm-filtered) phosphate-buffered saline (PBS) for cell sorting.

Bacterial suspensions were diluted in sterile PBS to reach 10⁶ cells ml⁻¹, based on OD₆₀₀ measurements. Bacteria were detected by forward scatter, side scatter, and fluorescence with logarithmic amplifiers. Prior to cell sorting and/or analysis, parameters were set using *A. brasilense* Sp245-Rif(pOT1e) as non-fluorescent control. One *A. brasilense* clone obtained in this work and expressing *egfp* constitutively was used as positive fluorescence control. At times, the machine was calibrated and scaled using a Bacteria Counting kit (Molecular Probes). Cell suspensions were diluted in order not to exceed 1000 or 1500 events s⁻¹ for cell sorting and for cell analysis, respectively.

For each sample, the fluorescence level was recorded for each of 50,000 events, unless otherwise specified. Data were plotted as histograms using CellQuest version 3.3 (Becton Dickinson Biosciences) and/or WinMDI version 2.8 (http://facs.scripps.edu/).

For cell sorting of the PTL-AbP library, the 'single-cell' mode of the FACSCalibur apparatus was used, and sorted cells were collected directly into 50 ml conical tubes. The resulting cell suspensions were passed through a 0.22 μm pore-size filter.

**DFI and flow cytometry analysis of the *egfp* promoter-trap library.** DFI-based flow cytometry cell sorting was used to screen the *egfp* promoter-trap library PTL-AbP for seed extract-inducible promoters of *A. brasilense* Sp245 (Fig. 2a). Ap, Rif and Gm were added to all growth media used afterwards. An aliquot (25 μl) from library PTL-AbP was used to inoculate 5 ml ABmal medium. After an 8 h incubation (exponential phase), 2.5 ml was transferred to 2.5 ml ABmal medium supplemented with spring wheat seed extracts (final extract concentration 0.5 g dry matter 1⁻¹). Cells were incubated for 20 h (during which cells went from exponential phase to stationary phase) and sorted, retaining fluorescent ones (Fig. 2b). A total of 10⁶ cells were assessed, which ensured full coverage of the PTL-AbP library based on the formula of Daugherty *et al.* (2000). The 11 % most fluorescent cells among the 10⁷ cells assessed (i.e. about 250,000 cells) were gathered on a filter, which was subsequently transferred to 20 ml ABmal medium supplemented with 0.2 ml methanol. After a 68 h incubation, the cells were sorted again, retaining non-fluorescent cells (about 5 %) in order to discard constitutive promoters (Fig. 2b).

The filter used to concentrate the cells was transferred to ABmal medium. After 54 h incubation, the cells were again induced with seed extracts for 20 h and then sorted, retaining the most fluorescent cells (about 18 %) (Fig. 2b). The sorted cells were plated on LBm agar within 1 h after sorting and incubated for 3 days at 28 °C.

Individual colonies were recovered and were first grown in a microtitre plate containing ABmal for 20 h in order to measure their relative fluorescence intensity (Xenius spectrophotofluorimeter microplate reader; Safas). Individual clones showing no relative fluorescence intensity [i.e. a fluorescence lower than that of the control strain Sp245-Rif(pOT1e)] were selected. Induction of those clones was confirmed by incubating them in ABmal medium containing either seed extracts or methanol (as a control) and measuring fluorescence after 20 h.

The *in vitro* induction of clones was assessed by flow cytometry analysis of *egfp* expression, as follows. Each clone was grown for 15–24 h in ABmal medium and 0.5 ml was mixed with 0.5 ml fresh ABmal medium with or without an inducing condition (described below) and grown for (depending on the experiment) another 5–20 h. The inducing conditions tested were: (i) seed extracts (0.5 g dry matter 1⁻¹), (ii) 5 mM D-galactose or L-arabinose (Sigma-Aldrich) as carbon source, and (iii) 5 mM sodium nitrate or sodium nitrite (Sigma-Aldrich) as nitrogen source. In the case of the *PnifH-egfp* fusion, the cells from 0.5 ml cultures were pelleted and resuspended in 1 ml ABmal medium without ammonium.

**Genetic characterization of DFI clones.** For independent confirmation of induction in response to seed extracts, plasmids were reintroduced into *A. brasilense* strains Sp245-Rif and Sp245 by biparental mating. To this end, plasmids isolated from *A. brasilense* DFI clones were first transferred into chemically competent cells of *E. coli* S17.1 Δpir. Plasmids were used as PCR template with primers

### Table 2. Primers (all designed in this work) and PCR conditions

<table>
<thead>
<tr>
<th>Target gene and primer</th>
<th>Primer sequence (5'→3')</th>
<th>Nucleotide position</th>
<th>Annealing temp. (°C)</th>
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<td>MCS*</td>
<td>CCGTTTACAACGATAAAAGC</td>
<td>4095</td>
<td>55</td>
</tr>
<tr>
<td>F2603</td>
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<td></td>
</tr>
<tr>
<td>nifH†</td>
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<td>55</td>
</tr>
<tr>
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</tr>
<tr>
<td>sbpA‡</td>
<td>GCCGCAAGCAAGATCTCAAGTG</td>
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<td>50</td>
</tr>
<tr>
<td>F2438</td>
<td>CGATACCGGAGGGGATCCCGG</td>
<td>1273</td>
<td></td>
</tr>
</tbody>
</table>

*Positions are relative to GenBank accession no. AJ131020.†F2442 and F2443 were derived from the *nifH* sequence of *A. brasilense* Sp245 (GenBank accession no. X51500). BglII sites introduced into primers are underlined.‡F2438 and F2440 were derived from the *sbpA* sequence of *A. brasilense* Sp245 (GenBank accession no. U40823). BglII and BamHI sites, respectively, introduced into primers are underlined.
F2603 and F2604, which anneal to loci flanking the multiple cloning site (MCS) of pOT1e. After QIAquick PCR purification (Qiagen), the DFI clones were sequenced with primer F2604 plus, in the case of a large PCR product, primer F2603 (Table 2).

Sequencing was performed by Genome Express (Meylan, France) and sequences were analysed with BLASTN and BLASTX algorithms (http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul et al., 1997). Identification of ORFs was done with FramePlot 2.3.2 (http://watson.nih.go.jp/jun/cgi-bin/frameplot.pl) (Ishikawa & Hotta, 1999). BPROM was used for prediction of promoters (http://www.softberry.com). SignalP 3.0 was used to predict the presence and location of signal peptide cleavage site in deduced amino acids sequences (http://www.cbs.dtu.dk/services/SignalP) (Bendtsen et al., 2004). Preliminary data of the A. brasilense genome sequencing project (available at http://genomics.ornl.gov/research/azo) were used to predict ORFs localized downstream of the promoters selected by the DFI promoter-trap technique. Accession numbers for sequence data obtained in the current work are reported in Table 3 and Supplementary Table S1 (available with the online version of this paper).

**Root colonization experiments.** Seeds (10 g) were surface-sterilized as described by Dobbelaere et al. (1999) with an additional final soaking step in sterile-filtered 2 % (w/v) sodium thiosulfate (Miché & Balandreau, 2001). Afterwards, seeds were rinsed three times with sterile water, left in sterile water for another 30 min and germinated on water agar (15 g l\(^{-1}\)) for 3 days in the dark at 28 °C. A. brasilense cells from overnight ABmal cultures were collected by centrifugation, gently washed and resuspended in 10 mM MgSO\(_4\). A suspension of 10\(^7\) c.f.u. was made by serial dilutions in sterile 10 mM MgSO\(_4\) (based on OD\(_{600}\)), and 100 µl of this cell suspension was inoculated per seedling.

In some of the experiments, 12 cm square Petri dishes containing water agar and four seedlings were used. Inert material (pieces of PVDF membrane filters; Millipore) placed in dishes containing water agar supplemented with a carbon source (5 g malic acid l\(^{-1}\)) was used as a plant-less control. The dishes were kept flat for 1 day, then inclined at 45° for 7–13 days, at 28 °C in the dark. Two plants were studied per treatment.

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**Fig. 1.** Flow cytometer fluorescence detection of A. brasilense Sp245-Rif cells harbouring promoter-trap plasmid pOT1e or derivatives after overnight growth in ABmal. Representative results for one of three replicates are shown. (a) Front SCatter (FSC)×Side SCatter (SSC) density plot of 50 000 events, which indicates the frequency distribution of Sp245-Rif cells in the culture sample according to respectively their size (FSC) and granularity (SSC). The bacterial population can be gated in a unique region designated R1. (b) Relative intensity of green fluorescence (FL1-H) emitted by Sp245-Rif(pOT1e) events gated in R1, and manual definition of M1 threshold for autofluorescence (which was of low level) of Sp245-Rif(pOT1e) cells. (c) FL1-H of the events gated in region R1 for PlacZ-egfp construct Sp245-Rif(pMP2444), showing a fluorescence shift in comparison with the control Sp245-Rif(pOT1e). (d) FL1-H in the control (grey area) or after exposure of the PsbpA-egfp construct Sp245-Rif(pR2.59) to PsbpA inducers β-galactose (solid line) or l-arabinose (dashed line). (e) FL1-H in the control (grey area) or after incubation of the PnifH-egfp construct Sp245-Rif(pR2.57) in ammonium-free ABmal (solid line). (f) FL1-H of sample consisting of 97 % non-fluorescent Sp245-Rif(pOT1e) cells and 3 % fluorescent Sp245-Rif(pMP2444) cells, in which egfp is controlled by PlacZ.
In the other experiments, a hydroponic system consisting of cotton-plugged, 180 x 15 mm Pankhurst glass tubes containing 8 ml nitrogen-free Marvin–Prevel–Charpentier–Lavigne (MPCL) medium (Jofré et al., 1998) and one seedling was used. The tubes were placed for 7 days in a growth chamber at 20 °C with 16 h light (150 μE m⁻² s⁻¹) and 8 h dark. Three plants were studied per treatment.

**Quantitative and qualitative analyses of fluorescence.**

Quantitative analysis of fluorescence in bacterial cultures was done with a Xenius spectrofluorimeter microplate reader (Safas) (excitation at 488 nm; emission at 510 nm; wavelength slit 10 nm). OD₆₀₀ and fluorescence intensity (I) were measured with growth medium used as blank and A. brasilense Sp245-Rif(pOT1e) as negative control. The relative fluorescence intensity (Iᵣ) was calculated based on the following formula (Tang et al., 1999):

\[
Iᵣ = \frac{I}{OD₆₀₀} - \frac{I_{Ab}}{OD_{Ab}}
\]

where I₆₀₀ and OD₆₀₀ are obtained for strain Sp245-Rif(pOT1e).

Detection of NO by the specific fluorescent probe diaminorhodamine-4M acetoxymethyl ester (DAR-4M AM; Calbiochem) was carried out in A. brasilense cells, as described by Creus et al. (2005). Briefly, cells grown in ABmal with spring wheat seed extracts were resuspended in 20 mM HEPES/NaOH pH 7.8, incubated for 2 h in the presence of 15 μM DAR-4M AM, washed, and examined by epifluorescence microscopy as described below.

Microscopy observations of cultures were made using an Axioskop 40 epifluorescence microscope (Carl Zeiss) with standard phase-contrast lenses. Fluorescent cells were observed in bacterial cultures was done with a Xenius spectrofluorimeter microplate reader (Safas) (excitation at 488 nm; emission at 510 nm; wavelength slit 10 nm). OD₆₀₀ and fluorescence intensity (I) were measured with growth medium used as blank and A. brasilense Sp245-Rif(pOT1e) as negative control. The relative fluorescence intensity (Iᵣ) was calculated based on the following formula (Tang et al., 1999):

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**Fig. 2.** Identification and isolation of DFI clones of *A. brasilense* Sp245-Rif responding to spring wheat seed extracts. (a) Scheme describing the selection of clones. Fluorescence-activated sorting of cells carrying the egfp promoter-trap library was carried out in three rounds. In the first round, the bacteria were grown in the presence of seed extracts (obtained using a methanol solution) and only fluorescent cells were retained. In the second round, the bacteria selected were grown without seed extracts and non-fluorescent cells were retained. In the third round, the bacteria were grown again in the presence of seed extracts to confirm the seed extract-inducible status of the promoters. After the third round, the cells were plated to obtain colonies used in subsequent experiments. (b) Flow cytometry analysis of the three sorting rounds of the PTL-AbP library. The amount of fluorescent cells was 11% at the end of the first round and 18% at the end of the third round, whereas 5% of the cells were non-fluorescent at the end of the second round.
were estimated by LSM calculation. Each treatment was studied using two 1 cm root pieces taken from two seedlings.

**RESULTS AND DISCUSSION**

**Validation of fluorescence-based flow cytometry cell sorting for DFI promoter analysis in A. brasilense Sp245**

Flow cytometry analysis of *A. brasilense* Sp245-Rif(pOT1e) (50 000 events) obtained in ABmal medium enabled us to manually define a live gate designated R1 (independently of fluorescence properties) (Fig. 1a), which provided the background level of relative intensity of green fluorescence (FL1-H) (Fig. 1b). On this basis, the ability of flow cytometry sorting to select fluorescent *A. brasilense* cells was then assessed using PlacZ, PsbpA, and PnifH constructs. The latter were constructed using egfp because none of the other reporter genes tested [gfpmt3, gfpUV and the unstable variant gfp(LVA)] gave any fluorescence. EGFp is stable, which does not allow observation of fast changes in gene expression but permits the integration and amplification of weak promoter responses, and this can be useful if genes are expressed only very briefly during the early stages of the associative symbiosis. First, the constitutively fluorescent PlacZ-egfp construct *A. brasilense* Sp245-Rif(pMP2444) resulted in a shift in the relative intensity of green fluorescence (Fig. 1c), in comparison with the control Sp245-Rif(pOT1e). Second, a significant up-shift in fluorescence was found (i) with the addition of PsbpA inducers such as D-galactose or L-arabinose to cultures of *A. brasilense* Sp245-Rif containing the PsbpA-egfp plasmid construct pR2.59 (Fig. 1d), and (ii) after transfer to ammonium-free ABmal broth of Sp245-Rif containing the PnifH-egfp plasmid construct pR2.57 (Fig. 1e). These observations were confirmed by epifluorescence microscopy.

The DFI approach requires that a minority of fluorescent cells can be distinguished from prevalent non-fluorescent cells, and here flow cytometry sorting of 3% constitutively fluorescent Sp245-Rif(pMP2444) cells from 97% non-fluorescent Sp245-Rif(pOT1e) cells was efficient (Fig. 1f). Only fluorescent colonies were obtained after plating onto LBm plates but colony number was lower than expected, which was probably due to the reduced viability of sorted cells (Ferrari et al., 2004). Taken together, these results indicate that DFI combined with flow cytometry cell sorting can be applied to *A. brasilense* Sp245.

**Representativeness of the A. brasilense Sp245 promoter-trap library**

The construction of the promoter-trap library involved pOT1e cloning of small DNA fragments of strain Sp245 upstream of a promoterless egfp, and the library was transferred into *E. coli* DH10B. The PTL-Ec library thus obtained consisted of about 96 000 transformants. Analysis of 20 randomly chosen transformants indicated that all contained an insert, with an average insert size estimated at 1.8 kb. According to the formula of Clarke & Carbon

<table>
<thead>
<tr>
<th>Clone</th>
<th>Insert size (bp)</th>
<th>GenBank accession no.</th>
<th>Organism, accession no. (in parentheses), sequence type, and levels of significance (E-value), identity and similarity for the most similar protein sequence</th>
<th>Upregulation in planta (on roots)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>DFI-8</td>
<td>1307</td>
<td>EF577485</td>
<td>Acidiphilium cryptum JF-5 (ZP_01145463) flagellar hook protein (FlgE), 2e⁻⁴⁶, 31 %, 47 %</td>
<td>++</td>
</tr>
<tr>
<td>DFI-26‡</td>
<td>678</td>
<td>EF577487</td>
<td>'Magnetospirillum magnetici' AMB-1 (YP_421636) predicted transcriptional regulator, 1e⁻²², 43 %, 56 %</td>
<td>++</td>
</tr>
<tr>
<td>DFI-31</td>
<td>755</td>
<td>EF577488</td>
<td>Bradyrhizobium sp. BTA1 (ZP_0060683) conserved hypothetical protein, 9e⁻³⁵, 56 %, 49 %</td>
<td>++</td>
</tr>
<tr>
<td>DFI-32</td>
<td>955</td>
<td>EF577489</td>
<td>Hyphomicrobium denitrificans A3151 (BAC0912) nitrite reductase (NirK), 1e⁻²⁰², 59 %, 73 %</td>
<td>++</td>
</tr>
<tr>
<td>DFI-51</td>
<td>252</td>
<td>EF577492</td>
<td>Synechococcus elongatus PCC 7942 (AAM81181) conserved hypothetical protein, 1e⁻¹⁰, 46 %, 56 %</td>
<td>++</td>
</tr>
<tr>
<td>DFI-102</td>
<td>1150</td>
<td>EF577494</td>
<td>Synechococcus sp. WH 7805 (ZP_01125336) VCBS protein, 0, 35 %, 46 %</td>
<td>−</td>
</tr>
<tr>
<td>DFI-164</td>
<td>324</td>
<td>EF577502</td>
<td>Magnetospirillum magnetotacticum MS-1 (ZP_00055357) serine/threonine protein kinase, 1e⁻¹², 36 %, 56 %</td>
<td>++</td>
</tr>
</tbody>
</table>

* Most similar protein in GenBank.
† Upregulation did not take place (no fluorescence; −), or was weak (+) or strong (++)
‡ Clone DFI-26 was isolated three times.
(1976), a 99% probability of having a given DNA sequence represented in a library of 2 kb fragments in the case of strain Sp245 (genome size about 7.1 Mbp) (Martin-Didonet et al., 2000) would require 16,346 clones with a correct orientation. Considering the two possible orientations of a cloned insert (upstream of egfp), this means that the library (96,000) represented nearly a threefold coverage of the Sp245 genome.

When the PTL-Ec library was transferred into A. brasilense Sp245-Rif, about 8.5 × 10⁶ transformants were obtained (library PTL-AbP). According to Poisson’s law (P = e⁻¹, where P = probability of missing a single recombinant, N = number of Sp245-Rif recombinants and n = number of DH10B recombinants), the PTL-AbP library has a probability P = 1.4 × 10⁻⁴ to miss a given clone. Therefore, based on these criteria, the representativeness of the A. brasilense Sp245 promoter-trap library was significant.

DFI screening and identification of promoters upregulated by wheat seed extracts

The PTL-AbP library was screened for promoters upregulated by wheat seed extracts by cell sorting with flow cytometry, as outlined in Fig. 2(a). Among the 300 induced clones thus identified, 279 single-colony isolates from the final sort were assessed for lack of fluorescence in microtiter plates in the absence of wheat seed extracts. Only 46 of the clones were confirmed as being upregulated by seed extracts. This discrepancy could result from (i) false-positive fluorescent clones recovered by flow cytometry (Ferrari et al., 2004), and/or (ii) differences in growth culture conditions between the two screenings.

Due to loss of viability in glycerol stocks, only 21 of these candidate clones were completely recovered by PCR using primers F2603/F2604 (Table 2) and sequenced. Comparison with preliminary data of the A. brasilense Sp245 genome sequencing project and GenBank indicated that the inserts of 12 of the 21 clones were located within putative ORFs and/or in the opposite direction of predicted genes (Supplementary Table S1). These cryptic fusions are attributed to incomplete genome annotations (Silby et al., 2004), and they are often found in promoter-trap studies (reviewed by Rediers et al., 2005). Considering the high number of antisense transcripts identified in this and comparable studies, they are unlikely to be artefacts. Rather, they are thought to downregulate gene expression (Rediers et al., 2005; Silby & Levy, 2004).

In contrast, the nine other clones included sequences (one of them found three times) of putative promoters. Therefore, all further analyses were performed with only seven of these clones. Epifluorescence microscopic observations indicated that all these clones were also induced when exposed in vitro to seed extracts obtained by soaking spring wheat seeds in water instead of methanol (not shown).

Sequence analysis of seed extract-induced fusions

The seven correctly oriented, seed extract-induced fusions obtained included sequences of putative promoters, and, in most cases, the start of downstream ORFs (Table 3, Fig. 3). Based on data of the A. brasilense genome sequencing project, ORFs of identical orientation and located less than 100 bp downstream of these ORFs were detected in the case of DFI-51 and DFI-164, suggesting possible operon structures. A first group of DFI clones (DFI-8 and DFI-102) corresponds to promoters of genes that might be involved in cell motility and adhesion. The DNA insert present upstream of egfp in clone DFI-8 corresponds to a partial ORF of 888 bp. The entire deduced amino acid sequence displays significant homology with sequences of FlgE flagellar hook proteins (COG1749; best match for Acidiphilium cryptum JF-5). The flgE gene encodes a short, highly curved tubular structure that connects the flagellar motor to the filament acting as a helical propeller. In clone DFI-102, the insert contains a partial ORF of 790 bp, whose deduced amino acid sequence displays significant
Fig. 4. Expression of seed extract-induced DFI clones DFI-102, DFI-31, DFI-51 and DFI-32 of A. brasilense Sp245-Rif in the root hair zone of spring wheat, maize and pea roots at 7 days after inoculation of seedlings, based on CLSM image analysis. Root surfaces and root hairs are shown in (c), (d), (f), (j), (k) and (l), whereas only root hairs are shown in (a), (b) and (e), and root surfaces in (g) and (h). Bacterial cells expressing EGFP are green, whereas white/grey and blue backgrounds correspond to the root image formed by the transmitted light and the reflected light, respectively. (m) Sagittal section of biofilm structure formed by the PnifH-egfp construct Sp245-Rif(pR2.57) on pea roots. Clones DFI-8, DFI-26 and DFI-164 displayed the same fluorescent patterns as DFI-102, DFI-31 and DFI-51, respectively. rs, root surface; rh, root hair; bc, clumps of non-fluorescent bacterial cells. In (l), the boundary of the root surface is indicated by a white dashed line.

homology with several VCBS proteins (best match for Synechococcus sp. WH 7805). These proteins contain a domain of about 100 residues that is repeated several times in large proteins found in numerous species of Vibrio, Colwellia, Bradyrhizobium and Shewanella (hence the name VCBS) and in smaller copy numbers in proteins from several other bacteria. A role in adhesion has been suggested for many members of this protein family but remains to be demonstrated.

A second group (clones DFI-26 and DFI-164) corresponds to promoters of genes involved in gene regulation pathways. The DNA insert present upstream of egfp in clone DFI-26 (the exact same insert was found in two other clones) corresponds to the promoter region of an ORF whose deduced amino acid sequence displays significant homology with predicted transcriptional regulators (COG1396; best match for ‘Magnetospirillum magnetotacticum’ AMB1). This predicted transcriptional regulator contains a type 3 helix–turn–helix and may belong to the xenobiotic response element family. In clone DFI-164, the insert contains a partial ORF of 38 bp. The entire deduced amino acid sequence of this ORF displays significant homology with serine/threonine kinases (COG0515; best match for Magnetospirillum magnetotacticum MS-1). The ORF downstream that may be part of the same operon encodes a hypothetical protein (best match for M. magnetotacticum MS-1). Serine/threonine kinases are involved in protein phosphorylation and regulate numerous processes in prokaryotes. Promoter-trapping in vivo induction of such a gene has already been reported for pathogens, where it contributes to virulence (Kilić et al., 1999; Wang et al., 1998), but apparently never for PGPR. However, the role of this gene in the associative symbiosis is unknown, all the more as the substrate(s) of the kinase remain(s) to be identified.

A third group (clones DFI-31 and DFI-51) corresponds to promoters of genes encoding conserved hypothetical proteins. The deduced amino acid sequence of the DFI-31 insert (partial ORF of 296 bp) displays significant homology with conserved hypothetical proteins containing a ferritin-like diiron-carboxylate domain (best match for Bradyrhizobium sp. BTAi1). Similar ORFs are recovered in several annotated genomes of rhizobia and they are located immediately upstream of the nosRZDFLYL operon, which is implicated in the reduction of nitrous oxide to dinitrogen. However, the physiological role of the proteins encoded by these genes has not been identified yet. In clone DFI-51, the insert possesses a partial ORF of 64 bp. The entire deduced amino acid sequence of this ORF shows significant homology with conserved hypothetical proteins of the COG2929 category (best match for Synechococcus elongatus PCC 7942). These hypothetical proteins are mostly found in phages, and upregulation of phage-derived genes has been observed during promoter-trap studies of eukaryote-associated bacteria (Camilli & Mekalanos, 1995; Dozois et al., 2003). The ORF downstream codes for a predicted transcriptional regulator belonging to the xenobiotic response element family (best match for Rhodospirillum rubrum ATCC 11170).

Finally, DFI-32 corresponds to a gene possibly involved in denitrification. The DNA insert located upstream of egfp in clone DFI-32 corresponds to a partial ORF of 341 bp, which displays significant homology with sequences of dissimilatory copper-containing nitrite reductases NirK (best match for Hyphomicrobium denitrificans A3151; 59 % identity and 73 % similarity). This is the first evidence for the presence of nirK in A. brasilense. NirK is implicated in the conversion of nitrite to nitric oxide. Denitrification has been studied in the rhizosphere (Ghiglione et al., 2002), but expression patterns of denitrification genes (such as nirK) have been neglected.

Overall, it appears that the seven promoters could correspond to broad functions already encountered in promoter-trap studies of bacteria–host interactions, i.e. (i) cell motility and adhesion, (ii) gene regulation and (iii) nitrogen metabolism (Ridders et al., 2005). However, the genes themselves differ from the ones previously identified.

**In planta expression of seed extract-induced fusions**

When constructs were assessed on roots, large numbers of fluorescent cells were observed, pointing to significant plasmid stability. On all three plant species studied, fluorescent derivatives of A. brasilense Sp245-Rif were found as single cells as well as numerous sparse clumps of cells, all over plant roots and especially in the root hair zone (Fig. 4), a colonization pattern already documented in Azospirillum spp. (Zhu et al., 2002). These cell clumps (biofilm structures) were about 26 μm high and up to 50 μm wide [illustrated for the PnifH-egfp construct Sp245-Rif(pR2.57) on pea; Fig. 4m]. The cells were larger and...
Plant-induced genes in *Azospirillum*

**Wheat**

(a)  rh  

(b)  rh  

(c)  rh  

**Maize**

(d)  rh  

(e)  rh  

(f)  rh  

**Pea**

(g)  rh  

(h)  rh  

(i)  rh  

(j)  rh  

(k)  rh  

(l)  rh  

(m)  26 µm  

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displayed a more coccoid shape (i.e. cyst-like cells) on roots than in liquid culture.

The seven promoters upregulated in vitro by wheat seed extracts were assessed on spring wheat 'Fiorina', another wheat cultivar (winter wheat 'Soissons'), another monocot (maize) and a dicot (pea). None of these DFI clones were fluorescent when their cells were colonizing the surface of PVDF membrane filters (i.e. in the plant-less control), or when present as planktonic cells. When inoculated singly onto spring or winter wheat seedlings, all seven DFI clones except DFI-8 and DFI-102 (Table 3) were fluorescent on roots at 7 days (illustrated for spring wheat in Fig. 4a, d, g, j), meaning that the corresponding promoters were upregulated in the presence of a wheat plant. In the case of spring wheat, observations were also made at 14 days, and similar induction results were found (data not shown).

That DFI-8 and DFI-102 inserts were induced in the presence of wheat seed extracts but not on wheat roots was unexpected. Perhaps the corresponding inducer(s) are lacking in root extracts, or certain compounds present in root exudates or at the root surface prevent induction of the two genes, and consequently they are only expressed at the earlier stages of plant development (i.e. on germinating seeds). This result might also be due to other differences in physico-chemical conditions between the two experimental set-ups.

On maize roots (at 7 days), clones DFI-51, DFI-164 and DFI-32 were fluorescent (Fig. 4h, k), as on wheat. However, the last one displayed low fluorescence levels, as a higher detector amplification gain was needed. The two non-fluorescent clones on wheat roots (DFI-8 and DFI-102) were fluorescent on maize roots (Fig. 4b), whereas two fluorescent clones on wheat roots (DFI-26 and DFI-31) were not fluorescent on maize roots (Fig. 4e). As for wheat, all seven clones except DFI-8 and DFI-102 were fluorescent on pea roots at 7 days (Fig. 4c, f, i, l). On pea, clones DFI-26, DFI-164 (not shown), DFI-32 (Fig. 4l), DFI-51 (Fig. 4i) were highly fluorescent, while DFI-31 (Fig. 4f) showed a lower fluorescence level based on comparison of detector amplification gains.

Therefore, all seven clones were fluorescent in planta, but host specificity of the induction depended on the DFI fusion, as three clones were induced on all four plants, whereas two clones were induced only on maize and the last two only on wheat and pea. These observations suggest that certain but not all plant stimuli are plant species-specific. The PGPR literature reports both host-specific and broad adaptations to the plant (Landa et al., 2002; Rezzonico et al., 2007), including in the case of Azospirillum spp. (Dobbelaere et al., 2002; Mirza et al., 2006). Here, importantly, differences were found between plant species but not when comparing different wheat cultivars. Unexpectedly, wheat was more similar to pea than to maize (another monocot) when considering gene induction results on roots, although pea was the sole dicot studied. Perhaps the particularity of the results for maize is related to its C4 metabolism (Fedi et al., 1992; Reinhold et al., 1985), but this hypothesis will require further assessment using a wider range of plants.

**In vitro characterization of seed extract-induced PnirK-egfp fusion**

Further analysis of the nirK clone DFI-32 (nitrite reductase) evidenced consensus, σ70-relevant −10 and −35 promoter sequences located close to the start codon. Also found was +25 TCCTGACGGTCGTCAAGT +46, which is a wyTTGAC-(N4)-GTCAArw recognition motif for the fumarate-nitrate reduction (FNR) transcriptional activator also present in other nirK promoters (Zumft, 2002). As for many periplasmic metalloenzymes, this nirK product has a signal peptide at its N-terminus (P>0.999), and probably a cleavage site between residues 26 and 27 (P=0.571).

The in vitro induction of nirK in response to spring wheat seed extracts was confirmed when the plasmid (pR2.54) containing the PnirK-egfp fusion was reintroduced into...
strain Sp245-Rif or introduced into strain Sp245 (data not shown). The fusion was also upregulated in the presence of other seed extracts, with a 7.5-fold induction for pea and a 9-fold induction for winter wheat (compared with an 11-fold induction for spring wheat).

As nitrate can induce nirK expression in rhizosphere denitrifiers (Baek & Shapleigh, 2005; Velasco et al., 2001), we investigated whether seed extract induction of nirK could be due to the presence of nitrate and/or of the NirK substrate nitrite: both are present in the rhizosphere (Binnerup & Sørensen, 1992). Whereas the expression level of the PnirK-egfp fusion was very low in ABmal medium lacking nitrate or nitrite, the addition of 5 mM sodium nitrate or sodium nitrite rendered late-exponential-phase cells fluorescent, increasing the expression of the fusion 21-fold and 14-fold, respectively (Fig. 5a). In the conditions tested during the DFI screening, the amounts of nitrate and nitrite provided by seed extracts were estimated as 0.34 and 0.09 mM, respectively. Expression of the fusion was increased 1.7-, 2.8-, 6.9- and 4.6-fold by adding 0.34 mM sodium nitrate (Fig. 5b, ii), 0.09 mM sodium nitrite (Fig. 5b, iii), 0.34 mM sodium nitrate and 0.09 mM sodium nitrite (Fig. 5b, iv), or 0.5 g dry matter l⁻¹ of wheat seed extracts (Fig. 5b, v), respectively. These results indicate that the amounts of nitrate and/or nitrite present in wheat seed extracts may, alone, account for the observed induction of DFI-32 by these extracts.

The occurrence of an FNR recognition motif points to oxygen-dependent regulation, and indeed oxygen availability can influence nirK expression in other denitrifiers. In A. brasilense Sp245, the expression level of PnirK-egfp was low under fully aerobic conditions, whereas cells displayed a 5.6-fold increase of fluorescence under microaerobic conditions. Microaerobic conditions, which are favourable for denitrification (Baek & Shapleigh, 2005), prevailed in the experimental set-ups used for in vitro DFI procedures and validations (data not shown). This is relevant for the ecology of A. brasilense in planta, as oxygen-limited conditions can occur in the rhizosphere (Højberg et al., 1999).

Fig. 6. Microscopy detection of NO with the NO-specific fluorescent probe DAR-4M AM in the PnirK-egfp fusion A. brasilense Sp245-Rif(pR2.54) after growth in ABmal containing spring wheat seed extracts. (a) Bright-field observation. (b) Epifluorescence observation using a 450–490 nm band-pass excitation filter and a 520 nm long-pass emission filter. This panel displays green fluorescence in EGFP-expressing cells. (c) Epifluorescence observation using a 530–545 nm band-pass excitation filter and a 565 nm long-pass emission filter. This panel depicts (in orange) the detection of NO in cells with DAR-4M AM. (d) Overlay of (b) and (c). EGFP-expressing cells producing NO are yellow (three are indicated with arrows) whereas those not producing NO are green (two are indicated with dashed arrows).
A. brasilense Sp245 denitrifies on wheat roots (Neuer et al., 1985), producing NO (Creus et al., 2005), and here evidence for NO production by strain Sp245-Rif under the current experimental conditions (i.e. in ABM1 supplemented with wheat seed extracts) was obtained at the single-cell level using the NO-specific fluorescent probe DAR-4M AM. Only some of the cells displayed red fluorescence, regardless of whether Sp245-Rif (not shown) or the PnirK-egfp fusion Sp245-Rif(pR2.54) was used (Fig. 6a). Overall, Sp245-Rif(pR2.54) consisted of a majority of green cells and three minor subpopulations corresponding to (i) non-fluorescent cells, (ii) red cells and (iii) cells displaying both red and green fluorescence.

Interestingly, the NirK product (NO) is an important signal molecule for plants (Delledonne, 2005) and NO produced during root colonization by Sp245 on tomato roots can stimulate lateral root formation (Creus et al., 2005). Therefore, the current results suggest that plant-induced expression of nirK may be relevant not only for denitrification but also for plant growth promotion. The fact that this interaction was functional on all four plants tested raises the possibility that A. brasilense Sp245 could act as a broad-host-range PGPR, and indeed this strain has been successfully used to promote growth of wheat, maize, sorghum and tomato (Bashan et al., 2004).

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REFERENCES


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