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ORIGINAL ARTICLE

Assessment of SCAR markers to design real-time PCR primers for rhizosphere quantification of *Azospirillum brasilense* phytostimulatory inoculants of maize

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Azospirillum, inoculant quantification, plant growth-promoting rhizobacteria, real-time PCR, root colonization, sequence characterized amplified region markers.

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Abstract

Aims: To assess the applicability of sequence characterized amplified region (SCAR) markers obtained from BOX, ERIC and RAPD fragments to design primers for real-time PCR quantification of the phytostimulatory maize inoculants *Azospirillum brasilense* UAP-154 and CFN-535 in the rhizosphere.
Methods and Results: Primers were designed based on strain-specific SCAR markers and were screened for successful amplification of target strain and absence of cross-reaction with other *Azospirillum* strains. The specificity of primers thus selected was verified under real-time PCR conditions using genomic DNA from strain collection and DNA from rhizosphere samples. The detection limit was 60 fg DNA with pure cultures and 4×10^3 (for UAP-154) and 4×10^4 CFU g^{-1} (for CFN-535) in the maize rhizosphere. Inoculant quantification was effective from 10^4 to 10^8 CFU g^{-1} soil.
Conclusion: BOX-based SCAR markers were useful to find primers for strain-specific real-time PCR quantification of each *A. brasilense* inoculant in the maize rhizosphere.
Significance and Impact of the Study: Effective root colonization is a prerequisite for successful *Azospirillum* phytostimulation, but cultivation-independent monitoring methods were lacking. The real-time PCR methods developed here will help understand the effect of environmental conditions on root colonization and phytostimulation by *A. brasilense* UAP-154 and CFN-535.

Introduction

Azospirillum strains are well known as plant growth-promoting rhizobacteria (PGPR). Phytostimulatory traits identified in these bacteria include free-living nitrogen fixation (James 2000), production of phystohormones (Dobbelaere *et al.* 2003) or nitric oxide (Creus *et al.* 2005) and deamination of the ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC; Prigent-Combaret *et al.* 2008). By stimulating root proliferation and elongation, *Azospirillum* PGPR may lead, in turn, to improved uptake of water and nutrients (Okon and Kapulnik 1986; Jacoud *et al.* 1999).

Plant growth-promoting effects of *Azospirillum* inoculants have been documented with different types of crops (often cereals), under different soil and climatic conditions, and they may lead to improved crop yield (Charyulu *et al.* 1985; Okon and Labandera-Gonzalez 1994; Dobbelaere *et al.* 2001; Pedraza *et al.* 2009). In certain countries, crop inoculation with *Azospirillum* phytostimulatory strains is receiving attention as a means to enable a reduction in nitrogen fertilizer usage without compromising crop yield, in a context of lower-input agriculture (Fuentes-Ramirez and Caballero-Mellado 2006; El Zemrany *et al.* 2006).

One of the main conditions necessary for effective phytostimulation by *Azospirillum* is rhizosphere survival

of the inoculant in sufficient numbers (Dobbelaere *et al.* 2002), even though the preliminary interaction between *Azospirillum* and seed is already important (Jacoud *et al.* 1999). This means that techniques are required for effective monitoring of inoculant cell number in the rhizosphere. However, very few tools are available to assess the fate of wild-type *Azospirillum* strains after inoculation. In the case of *Azospirillum lipoferum* CRT1, a PCR approach targeting the 16S-23S rDNA internal spacer region can be used for inoculant detection (Baudoin *et al.* 2009). However, many important PGPR strains of *Azospirillum* belong to the *A. brasilense* species (Lucy *et al.* 2004). Strain-specific antibodies and/or molecular probes are available for the identification of *A. brasilense* strains Wa3 (Aßmus *et al.* 1997), Sp7 and Sp245 (Rothballer *et al.* 2003) by fluorescent *in situ* hybridisation (FISH), but these tools do not allow strain-specific quantification.

Quantitative PCR approaches are promising to quantify individual strains in complex environments, and in the rhizosphere they have been implemented in the case of MPN-PCR (Rosado *et al.* 1996; Mirza *et al.* 2006) and competitive PCR (Johansen *et al.* 2002; Mauchline *et al.* 2002; Rezzonico *et al.* 2005; Gobbin *et al.* 2007). More recently, real-time PCR has become the method of choice for quantifying rhizosphere populations because it enables high specificity, sensitivity, and speed (Sørensen *et al.* 2009). This method can be based on the measurement of fluorescence generated by SYBR Green, a commercial dye that binds nonspecifically to double-stranded DNA after each PCR cycle. The cycle at which the fluorescence crosses the threshold line, known as C_T, is directly proportional to the amount of DNA present in the sample. In the rhizosphere, however, real-time PCR has only been used so far for the quantification of indigenous bacterial groups (Mavrodi *et al.* 2007) and its applicability to monitor bacterial inoculant strains remains to be assessed.

The objective of this study was to develop real-time PCR protocols for strain-specific quantification of two prominent phytostimulatory *A. brasilense* PGPR, i.e. strains UAP-154 and CFN-535, which have been used in hundreds of thousands hectares as inoculants for cereals (Dobbelaere *et al.* 2001; Fuentes-Ramirez and Caballero-Mellado 2006). To this end, sequence characterized amplified region (SCAR) markers (i.e. markers corresponding to PCR amplicons of known DNA sequence) that are strain specific were sought by random or rep-PCR genomic fingerprinting and used to develop primers, then selected primer pairs were screened for specificity. The primer pairs obtained were further assessed for SYBR Green-based real-time PCR quantification of the two *A. brasilense* strains in soil and the maize rhizosphere.

Material and Methods

Bacterial strains

All *Azospirillum* strains (Table 1) were routinely grown at 28°C with shaking in N-free NFb medium (Nelson and Knowles 1978) supplemented with 2·5% v/v LBm (i.e. Luria-Bertani medium containing only 5 g NaCl l^{-1}; Pothier *et al.* 2007). *Pseudomonas* strains were grown in LBm, *Agrobacterium* in Levure Peptone Glucose (LPG; Roy *et al.* 1982) and *Rhizobium* in Yeast Extract Mannitol (YEM; Vincent 1970). Colony counts of *Azospirillum* strains in media or gnotobiotic rhizosphere samples were performed after spreading dilutions on RC plates (Rodriguez Caceres 1982) and a 72-h incubation at 28°C.

DNA preparation

Three methods were used to obtain DNA. Genomic DNA from bacterial log cultures was extracted using DNA Tissue kit (Macherey & Nagel, Düren, Germany) according to manufacturer's instructions. Rhizosphere DNA in the experiment where sterile soil was used was extracted by thermal shock (Baudoin *et al.* 2009). The aliquots were heated for 10 min at 100°C and placed directly on ice for 5 min. DNA from the other rhizosphere samples and from bulk soil samples was extracted with the FastDNA® SPIN® kit (BIO 101 Inc., Carlsbad, CA, USA). To this end, 250–300 mg samples (described later.) were transferred in Lysing Matrix *E* tubes from the kit, and DNA was extracted and eluted in 50 μl of sterile ultra-pure water, according to the manufacturer's instructions. DNA concentrations were assessed by OD measurements at 260 nm NanoDrop (Nanodrop technologies, Wilmington, DE, USA).

BOX, ERIC and RAPD amplifications

Molecular profiles of strains UAP-154 and CFN-535, as well as *A. brasilense* strains Cd, L4, NC9, NC16, PH1, R5(15), Sp245, Sp7, Wb1, Wb3, WN1, WS1 and ZN1 (Table 1) were generated using BOX-A1R, ERIC and RAPD primers (Table 2), as described respectively by Fancelli *et al.* (1998); Rademaker *et al.* (1998) and Versalovic *et al.* (1998). For each type of PCR amplification, the profiles were compared using GelCompar II (Applied Maths, Sint-Martens-Latem, Belgium) and subjected to clustering analysis, based on the presence/absence of bands (Jaccard similarity coefficient) and the unweighted pair group method with arithmetic (UPGMA) mean, using Primer v6 software (PRIMER-E, Plymouth, UK). Strain-specific bands (based on electrophoretic mobility) were identified.

Table 1 Strains used in this study

Species and strains	Host plant	Origin	Reference*
Azospirillum brasilense			
UAP-154; CFN-535	Maize	Mexico	Dobbelaere *et al.* 2001
ZN1	Maize	Pakistan	Blaha *et al.* 2006
L4	Sorghum	France	Blaha *et al.* 2006
Sp245	Wheat	Brazil	Blaha *et al.* 2006
Wb1; Wb3; WS1; WN1	Wheat	Pakistan	Blaha *et al.* 2006
PH1	Rice	France	Blaha *et al.* 2006
R5(15)	Rice	Cuba	Blaha *et al.* 2006
Cd	*Cynodon dactilon*	USA	Blaha *et al.* 2006
Sp7	Digitaria	Brazil	Blaha *et al.* 2006
NC9	Soil	Mali	Blaha *et al.* 2006
NC16	Soil	Mali	Vial *et al.* 2006
Azospirillum lipoferum			
CRT1	Maize	France	Blaha *et al.* 2006
Br17	Maize	Brazil	Vial *et al.* 2006
B506; B510; B518	Rice	Japan	Blaha *et al.* 2006
RSWT1	Rice	Pakistan	Blaha *et al.* 2006
TVV3	Rice	Vietnam	Blaha *et al.* 2006
4B	Rice	France	Blaha *et al.* 2006
N4	Cotton	Pakistan	Blaha *et al.* 2006
Br10	Soil	Brazil	Vial *et al.* 2006
NC4	Soil	Mali	Vial *et al.* 2006
Pseudomonas fluorescens			
F113	Sugar beet	Ireland	Ramette *et al.* 2003
Pf-153	Tobacco	Switzerland	Gobbin *et al.* 2007
C10-186; S7-29	Tobacco	Switzerland	Ramette *et al.* 2003
Q37-87	Wheat	USA	Ramette *et al.* 2003
K94-41	Cucumber	Czech Republic	Wang *et al.* 2001
P97-1	Cucumber	Bhutan	Wang *et al.* 2001
Pseudomonas sp. CHA0	Tobacco	Switzerland	Ramette *et al.* 2003
Rhizobium etli CFN-42	Bean	Mexico	Romero *et al.* 1991
Agrobacterium tumefaciens C58	Prunus	USA	Blaha *et al.* 2006

*Reference from the current article, from which further information can be accessed on earlier work on these strains.

Conversion of BOX, ERIC and RAPD fragments into SCAR markers

Strong strain-specific bands from BOX, ERIC and RAPD analyses that were at least 200-bp in length were excised from 1% agarose gels and purified using the Nucleospin ExtractII kit (Macherey & Nagel), following the manufacturer's instructions. The purified DNA fragments were then ligated into pGEM®-T Easy vector (Promega, Madison, WI, USA), according to manufacturer's instructions. *Escherichia coli* JM109 (Promega) was transformed with the resulting recombinant plasmids, as specified by the supplier, and grown overnight at 37°C on Luria-Bertani agar (Sambrook *et al.* 1989) supplemented with ampicillin (100 μg ml^{-1}), 0·5 mmol l^{-1} IPTG and 80 μg X-Gal ml^{-1}. White colonies were selected for colony PCR in 50 μl of PCR mix for specific PCR amplification with universal primers M13f and M13r (Promega) to check the presence of plasmid insert. The clones selected for sequencing were grown overnight with shaking at 37°C in LB + ampicillin (100 μg ml^{-1}), and plasmids were purified using the Plasmid kit (Macherey & Nagel). Plasmid inserts were then sequenced in both directions by Cogenics (Meylan, France). Sequences are available in GenBank (accession nos. GQ500126 and GQ500127).

Primer design

DNA sequences comparable to those of the SCAR markers were sought on the web using BlastN at http://pbil.univ-lyon1.fr/BLAST/blast_nuc.php, with default parameters, as well as in preliminary genome sequences of *A. brasilense* Sp245. Putative strain-specific sequences, i.e. sequences without any match in sequence search were selected to design primer sets for *A. brasilense* UAP-154 and CFN-535. Primer design was performed using

Table 2 Primers used in the study

PCR type and primers	Sequence	Reference
BOX PCR		
BOX-A1R	CTACGGCAAGGCGACGCTGACG	Versalovic *et al.* 1998
ERIC PCR		
ERIC 1R	ATGTAAGCTCCTGGGGATTCAC	Rademaker *et al.* 1998
ERIC 2	AAGTAAGTGACTGGGGTGAGCG	Rademaker *et al.* 1998
RAPD analysis		
Primer 1253	GTTTCCGCCC	Fancelli *et al.* 1998
Real-time PCR CFN-535		
F12*	AAGCGATCCCGACCTTGAGGCA	This work
F24*	TGTCGATGCCGACAGGCTTGACCA	This work
Real-time PCR UAP-154		
U2*	TGACGGCCAACACCAACGACTC	This work
U7*	TGCCGTCGATGAACGACGCCATCTG	This work

*Primers designed based on a BOX SCAR marker.

FastPCR software (http://www.biocenter.helsinki.fi/bi/programs/fastpcr.htm) based on (i) an amplicon size inferior to 300-bp and (ii) primers 18- to 22-bp in length. The Oligo 6·65 software (Molecular Biology Insights, West Cascade, CO, USA) was then used to screen and select primer pairs under the following criteria : (i) high melting temperature of primers (Tm *c.* approximately 60°C, estimated using the nearest-neighbour thermodynamic method), (ii) low Tm difference between primers (ΔTm < 2°C) and (iii) lack of predicted hairpin loops, duplexes and primer-dimer formation.

Primer selection using strain collection

Primer selection was implemented based on the successful amplification of the target strain and the absence of cross-reaction with nontarget strains. Three pools of bacterial genomic DNA were used as negative controls, i.e. an *A. brasilense* pool (13 nontarget strains + UAP-154 or CFN-535 when testing respectively, CFN-535 or UAP-154 primers), an *A. lipoferum* pool (11 strains) and a pool of other common rhizosphere bacteria (including the α-Proteobacteria *Rhizobium etli* and *Agrobacterium tumefaciens*, as well as gamma-Proteobacteria belonging to *Pseudomonas* genus) (Table 1). A first step of primer selection was performed under qualitative PCR conditions, with about 30 ng of gDNA. A second step was performed under quantitative PCR conditions, using 30 pg of gDNA and primer concentrations ranging from 500 nmol l^{-1} to 1 μmol l^{-1}. To check primer specificity, the observation of melting curves (described later) was completed by agarose gel electrophoresis of real-time PCR products.

Real-time PCR conditions

Real-time PCR was performed in 20-μl PCR volumes containing 10 μl LightCycler FastStart DNA Master SYBR Green I (Roche Applied Science, Indianapolis, IN, USA), 0·75 μmol l^{-1} of each primer, 0·2 μl of T4 gene 32 protein (Roche Applied Science) and 2 μl of template DNA. White 96-well microplates and a LC-480 LightCycler were used (Roche Applied Science). The cycling programme included a 10-min incubation at 95°C followed by 50 cycles consisting of 95°C for 30 s, 70°C for 30 s and 72°C for 30 s. Amplification specificity was studied by melting curve analysis of the PCR products performed by ramping the temperature to 95°C for 10 s and back to 65°C for 15 s followed by incremental increases of 0·1°C s^{-1} up to 95°C. Melting curve calculation and determination of Tm values were performed using the polynomial algorithm function of LightCycler Software ver. 1 (Roche Applied Science).

Generation of standard curves for genomic DNA

Genomic DNA from *A. brasilense* UAP-154 or CFN-535 was used to prepare ten-fold dilution series from 3×10^6 to 3×10^1 fg DNA μl^{-1} (in triplicate). Sterile water (2 μl) was used as negative control. The cycle threshold C_T, i.e. the number of PCR cycles necessary to reach the threshold fluorescence level, was automatically determined for each sample by the LightCycler software v.1 (Roche Applied Science) based on the second derivative maximum method. A standard curve for each strain was generated by plotting the C_T number against the logarithm of bacterial DNA concentration for the three independent replicates, using LightCycler Software v.1 (Roche Applied Science). Amplification efficiency (E) was calculated from the slope of the standard curve using the formula $E = 10^{-1/\text{slope}} - 1$.

Generation of standard curves for real-time PCR quantification in maize rhizosphere

Two contrasted soils were used for standard curves. One was sampled from the loamy surface horizon of a

French luvisol from a maize field at La Côte St André near Bourgoin (clay 16·2%, silt 44%, sand 40%, organic matter 2·1%, pH 7·0; El Zemrany *et al.* 2006), and the other from the sandy-clay-loam topsoil of a Mexican vertisol from a field at Zacatepec near Cuernavaca, Morelos (clay 30·9%, silt 7·3%, sand 61·8%, organic matter 1·9%, pH 7·6). Lysing-Matrix E tubes (BIO 101 Inc.) containing 250 mg lyophilized bulk soil were inoculated with one of the two *A. brasilense* strains to reach 10^3, 10^4, 10^5, 10^6, 10^7, or 10^8 CFU added per tube (three replicates per inoculation level per strain). No bacteria were added to the negative control. Colony counts were performed for each cell suspension used to generate standard curves. After 1 h of incubation at 4°C, DNA extraction was performed using the FastDNA® SPIN® kit (BIO 101 Inc.) as described previously. Real-time PCR was performed as described previously. For each strain, a standard curve was generated for each replicate by plotting C_T number versus log CFU added per g of soil. Amplification efficiency was calculated as described earlier.

Real-time PCR quantification of *Azospirillum* inoculants in the rhizosphere

Seeds of maize (*Zea mays*) hybrid PR37Y15 (Pioneer Hi-Bred International, Johnston, IA, USA) were surface sterilized by stirring in sodium dichloroisocyanurate-containing Bayrochlor Mini solution (Bayrol, Dardilly, France) for 15 min. Seeds were then washed 4–6 times with sterile water and germinated on water agar (8 g l^{-1}) for 2 d in the dark at 28°C. For each of the two *A. brasilense* strains, cells from overnight NFb liquid cultures were collected by centrifugation at 4 000 ***g*** for 10 min, gently washed and resuspended in 10 mmol l^{-1} $MgSO_4$ solution to obtain 10^8 CFU per ml. Germinated maize seeds were inoculated by soaking for 1 h in one of the bacterial suspensions. Sterile water was used for the negative control. One maize seedling was placed in each pot, which contained 600 g sieved (at 5 mm) nonsterile soil from La Côte St André (4 pots per treatment) adjusted to 20% (w/w) water content. The pots were placed for 10 days in a greenhouse with 18 h of light (under 400 W lamps; 22°C and 45–50% relative humidity) and 6 h of dark (18°C and 60–65% relative humidity) and fitted with an automated irrigation system.

On sampling, each root system was dug up and shaken vigorously to discard soil loosely adhering to the roots. Roots and tightly adhering soil were then transferred to a 1-litre bottle containing 300 ml of sterile distilled water, and the bottles were shaken for 15 min. The soil fraction was recovered by centrifugation for 30 min at 5600 ***g*** and flash-freezed in liquid nitrogen. Samples were then lyophilized for 48 h in Falcon tubes and homogenized by crushing in the tubes using a spatula and 250 mg were used for DNA extraction, as described in earlier section.

Assessment of real-time PCR data in comparison with colony counts

A microcosm experiment was performed under axenic conditions to compare real-time PCR data with colony counts. Maize seeds (cv. PR37Y15) were disinfected, pregerminated and inoculated (or treated with water), as described, using a cell suspension containing 10^8 CFU per ml of strains CFN-535 or UAP-154 (giving respectively about 10^4 and 10^6 CFU per seed, as indicated by colony counts). Two seedlings (one of the two was used for *Azospirillum* monitoring) were then added per microcosm, which consisted of 300 g of autoclaved La Côte St André soil placed in 150-cm^3 glass bottles and adjusted to 20% (w/w) water content. Each inoculated treatment and the negative control was studied using 12 microcosms, which were placed following a randomized block design (with four blocks) in a growth chamber at 75% relative humidity, with 16 h of light (30 W lamps) at 26°C and 8 h of dark at 18°C. At 1, 2 and 3 d after inoculation, root systems were sampled (as described previously) and transferred each into a 15-ml Falcon tube containing 5 ml of 10 mmol l^{-1} $MgSO_4$ solution. After high-speed vortexing (5 min), 1 ml from each of the 36 samples (3 treatments × 4 replicates × 3 samplings) was characterized by real-time PCR and colony counts, as described previously.

Statistics

Greenhouse and growth chamber experiments followed a randomized block design. Colony counts were expressed as log CFU per root system or per g of dry root, and real-time quantification data were converted to log CFU equivalents per root system or per g of lyophilized soil. The relation between log CFU data and C_T values was assessed by regression analysis when assessing standard curves and correlation analysis for the comparison of real-time PCR data with colony counts. Statistical analyses were performed at $P < 0{\cdot}05$, using S PLUS software (TIBCO Software Inc., Palo Alto, CA, USA).

Results

Molecular comparison of *Azospirillum* strains and identification of SCAR markers

Molecular profiles of *A. brasilense* UAP-154 and CFN-535 generated 11 BOX PCR bands each, and respectively

8 and 7 ERIC PCR bands and 5 and 4 RAPD bands. The profiles readily distinguished between the two strains, regardless of the method (Fig. 1). Based on size comparison with the entire collection of 26 *Azospirillum* strains (for each profiling method), only 11 of the 24 bands obtained for strain UAP-154 were specific to that strain, whereas another 11 bands out of the 22 CFN-535 bands were specific to strain CFN-535. A total of 9 (UAP-154) and 6 bands (CFN-535) were sequenced, but many of the SCAR markers thus obtained were discarded after *in silico* analysis because of their homology with DNA sequences recovered from Genbank database and Sp245 genome. Finally, four strain-specific SCAR markers were identified for strain UAP-154 (two from BOX PCR and two from RAPD analysis) and for strain CFN-535 (two from BOX PCR and two from ERIC PCR).

Selection of SCAR-based primers

Screening of primer pairs derived from strain-specific SCAR markers based on Tm criteria and absence of PCR-impairing structure formation gave 28 primer sets for strain UAP-154 and 21 for strain CFN-535 (Table 3). However, most primer sets were then discarded because of cross-reaction with nontarget strain(s), under qualitative or quantitative PCR conditions. Only one strain-specific primer set for strain UAP-154 and another for strain CFN-535 remained (Table 2), both designed from a BOX SCAR marker (Table 3).

Real-time PCR quantification of *Azospirillum* strain in laboratory cultures

The usefulness of real-time PCR to quantify each of the two *A. brasilense* strains in laboratory cultures was

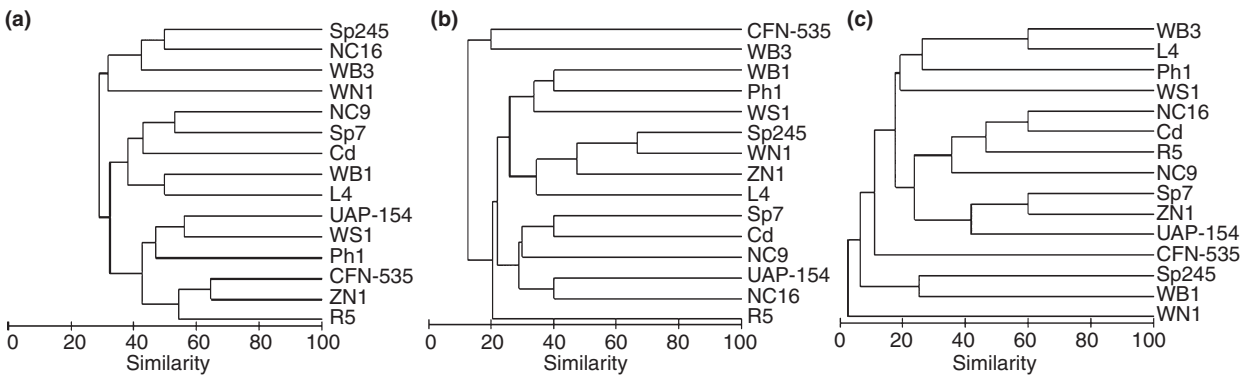

Figure 1 Genetic similarity of *A. brasilense* UAP-154 and CFN-535 and 13 other *A. brasilense* strains based on analysis of (a) BOX, (b) ERIC and (c) RAPD molecular profiles. The UPGMA clustering method was applied to a similarity matrix generated by GelCompar II software (Applied Maths) and calculated with the Jaccard coefficient.

Table 3 Strain-specific primers selection using three pools of genomic DNA

SCAR marker used (and length [bp])	Number primer sets*	Primer sets eliminated after qualitative PCR testing			Primer sets eliminated after quantitative PCR testing		
		Against other *A. brasilense* strains†	Against other bacteria‡	Number primers remaining	Against other *A. brasilense* strains	Against other bacteria	Number primers remaining
Strain UAP-154							
BOX 2 (500)	13	10	3	0	0	0	0
BOX 3 (900)	6	3	1	2	1	0	1
RAPD 1 (500)	5	5	0	0	0	0	0
RAPD 2 (800)	4	4	0	0	0	0	0
Strain CFN-535							
BOX 4 (400)	4	4	0	0	0	0	0
BOX 5 (500)	3	3	0	0	0	0	0
BOX 6 (800)	12	8	2	2	1	0	1
ERIC 3 (400)	5	3	2	0	0	0	0

*Primer sets validated in silico. Suitable primers were not found for a 250-bp ERIC-based SCAR marker for strain UAP-154.

†The A. brasilense pool is composed of the genomic DNA from 13 non-target strains + the other target strain.

‡Tests were performed separately for an *A. lipoferum* pool (11 strains) and a pool of non-*Azospirillum* rhizosphere bacteria (10 strains).

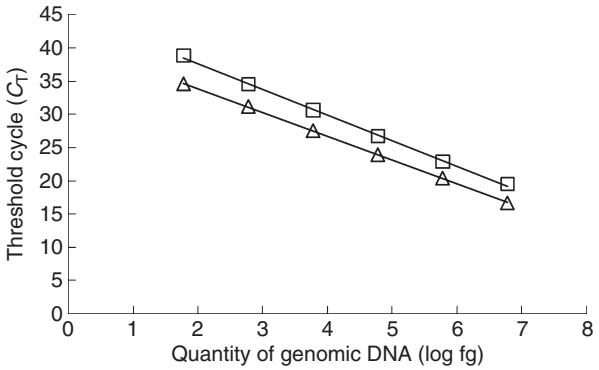

Figure 2 Real-time PCR standard curves for *A. brasilense* UAP-154 (□) and CFN-535 (Δ) *in vitro* generated by plotting C_T numbers against the quantity of genomic DNA added to the reaction mix. Means from three replicates are represented.

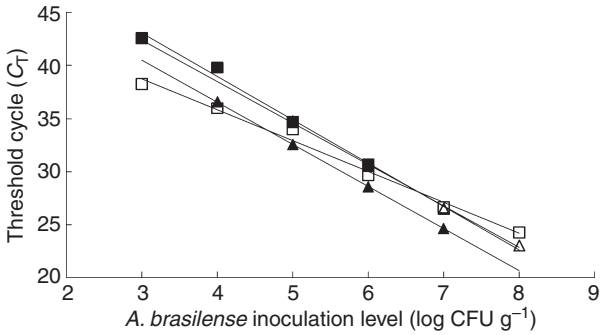

Figure 3 Real-time PCR standard curves for *A. brasilense* UAP-154 in nonsterile bulk soil from La Côte St André (■) and Zacatepec (□), and *A. brasilense* CFN-535 in nonsterile bulk soil from La Côte St André (▲) and Zacatepec (Δ). The standard curves were generated by plotting C_T numbers against the inoculation level of each *A. brasilense* strain. Means from three replicates are represented.

assessed based on standard curves established after adding various amounts of purified genomic DNA to PCR mix. The limit of detection was 60 fg DNA for strains UAP-154 and CFN-535, corresponding respectively to 20 and 7 CFU. For each strain, R^2 values higher than 0·99 were found after regression analysis between DNA amount and C_T from real-time PCR over a range of six orders of magnitude (Fig. 2). Amplification efficiencies were about 90% for the two strains (Table 4).

Real-time PCR quantification of *Azospirillum* strains in the maize rhizosphere

Quantification of *A. brasilense* strains in maize rhizosphere was based on standard curves obtained with DNA extracted from bulk soil inoculated with 10^3–10^8 CFU of either strain. With La Côte St André soil, R^2 values higher than 0·98 were found after regression analysis between inoculation levels (as log CFU g^{-1}) and C_T from real-time PCR analysis of soil, over a log CFU range of at least 4 orders of magnitude (Fig. 3). Amplification efficiencies were above 96% (Table 4). Similar results were obtained when using Zacatepec soil, except that amplification efficiency with strain CFN-535 was only 86% (Table 4). For both strains, the highest amplification efficiencies were recorded when total DNA extract had been diluted 100 (La Côte St André soil) and 200 times (Zacatepec soil).

For each strain, the standard curves were then used to estimate inoculant cell number in the rhizosphere of seed-inoculated maize plants, based on C_T value obtained by real-time PCR. In the maize rhizosphere, the detection limit was 4×10^3 (for UAP-154) and 4×10^4 CFU g^{-1} (for CFN-535), and the quantification limit was 4×10^3 CFU g^{-1} for both strains. When UAP-154 and CFN- 535 strains were monitored in the rhizosphere of La Côte St André soil at 10 d, they were found at respectively 1·4 to $4{\cdot}2 \times 10^6$ (mean $3{\cdot}0 \times 10^6$) and 7·7 to 15×10^5 (mean $1{\cdot}0 \times 10^6$) log CFU equivalents per root system, i.e. respectively 3·5 to $6{\cdot}0 \times 10^5$ (mean value $4{\cdot}9 \times 10^5$) and 1·3 to $2{\cdot}9 \times 10^5$ (mean $1{\cdot}9 \times 10^5$) log CFU equivalents per g of dry root.

Comparison of real-time PCR data with colony counts in axenic rhizosphere

The use of axenic rhizosphere microcosms enabled colony counts of the inoculants. *A. brasilense* UAP-154 and CFN-535, which were inoculated at respectively 10^6 and 10^4 CFU per seed, were recovered between 10^5

Table 4 Standard curve parameters and real-time PCR amplification efficiencies

Source of DNA	Strain	Detection limit	Quantification limit	Slope	Error	Amplification efficiency (%)
Bacterial culture	UAP-154	60 fg	60 fg	−3·571	0·0179	90·6
Bacterial culture	CFN-535	60 fg	60 fg	−3·580	0·0065	90·3
La Côte St André bulk soil	UAP-154	4×10^3 CFU g^{-1}	3×10^4 CFU g^{-1}	−3·365	0·0054	98·2
La Côte St André bulk soil	CFN-535	4×10^4 CFU g^{-1}	3×10^4 CFU g^{-1}	−3·415	0·0429	96·2
Zacatepec bulk soil	UAP-154	4×10^3 CFU g^{-1}	3×10^4 CFU g^{-1}	−3·283	0·0564	100
Zacatepec bulk soil	CFN-535	4×10^4 CFU g^{-1}	3×10^4 CFU g^{-1}	−3·709	0·0178	86·1

and 10^7 CFU per root system from days 1 to 3 after inoculation. When the same samples were studied by real-time PCR, a significant correlation was obtained between log CFU and C_T for strains UAP-154 ($r = 0{\cdot}86$, $P < 0{\cdot}001$) and CFN-535 ($r = 0{\cdot}84$, $P < 0{\cdot}001$) (Fig. 4).

Discussion

A. brasilense PGPR strains have been extensively studied for phytostimulation of cereal crops (Okon and Labandera-Gonzalez 1994; Dobbelaere *et al.* 2001) and to a lesser extent for biological control of phytoparasitic plants (Miché *et al.* 2000) and microbes (Bashan and de-Bashan 2002), soil weathering (Puente *et al.* 2006), waste recycling (de-Bashan and Bashan 2004) and improving drought tolerance in plants (Rodriguez-Salazar *et al.* 2009). The use of *A. brasilense* inoculants in Latin American countries has constantly increased in recent years, reaching in 2007 around 500 000 hectares of wheat and maize (Castro-Sowinski *et al.* 2007), but *Azospirillum* does not always survive well in soil or the rhizosphere (Bashan *et al.* 1995; Bashan 1999), which may affect inoculant performance (Dobbelaere *et al.* 2002). However, satisfactory methods for monitoring *A. brasilense* wild-type inoculants in the rhizosphere are lacking, which means cases of inoculation failure remain unexplained.

We describe here the development of a SYBR Green real-time PCR assay targeting SCAR markers for the maize inoculants *A. brasilense* UAP-154 and CFN-535. The use of SCAR markers is applicable to uncharacterized genomes, which was the case here. SCAR markers from BOX, ERIC and RAPD PCR amplicons were chosen because (i) BOX, ERIC and RAPD profiles can distinguish between *Azospirillum* strains including *A. brasilense* strains (Fani *et al.* 1993; Fancelli *et al.* 1998; Mirza *et al.* 2000; Vial *et al.* 2006; Baudoin *et al.* 2009), (ii) strain-specific RAPD markers have been used previously for the detection of an *A. lipoferum* strain in soil (Fancelli *et al.* 1998) and (iii) they proved useful to develop strain-specific primers for real-time PCR quantification of a biocontrol strain of the bacterial genus *Pantoea* (Nunes *et al.* 2008). Indeed, we found that BOX, ERIC and RAPD PCR discriminated effectively between *A. brasilense* strains, but the number of total and especially strain-specific bands was higher with BOX than with ERIC and RAPD PCR combined. However, it must be kept in mind that band yield of RAPD PCR might have been improved by changing primer sequence and/or concentration (Fani *et al.* 1993; Fancelli *et al.* 1998). Sequencing and BlastN analyses resulted in the identification of four strain-specific SCAR markers for each strain, and as half of them were obtained by BOX PCR it makes BOX PCR the method of choice to obtain SCAR markers for *A. brasilense* strains.

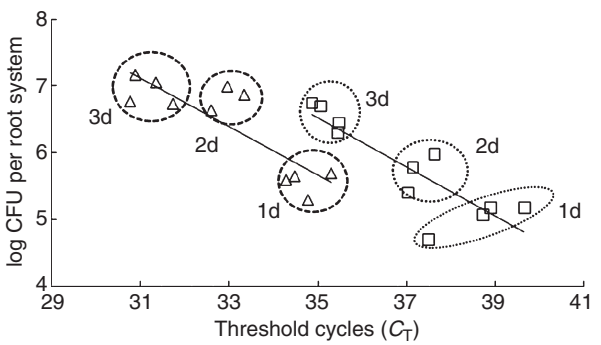

Figure 4 Relation between real-time PCR's C_T numbers and colony counts for *A. brasilense* strains UAP-154 (□) and CFN-535 (Δ) in axenic rhizosphere microcosms at 1, 2 and 3 days after inoculation. For visual clarity, data from a same sampling are gathered using dotted lines for each strain.

Many primer sets were derived from the SCAR markers, but most of them were later discarded because cross-reaction was found with nontarget strain(s), under qualitative or quantitative PCR conditions. It must be kept in mind that PCR was performed with rather high quantity of genomic DNA of the 34 rhizosphere strains used as negative controls (respectively 30 ng and 30 pg in qualitative and quantitative PCR). Lower DNA concentrations would probably have resulted in the selection of additional primers, but with a higher risk of cross-reaction with DNA from indigenous bacteria in subsequent rhizosphere experiments and the need to optimize real-time PCR conditions.

The two real-time PCR assays were validated based on (i) PCR efficiency higher than 85% *in vitro* and in soil, (ii) ecologically-relevant detection and quantification limits, (iii) significant correlation with colony counts under axenic rhizosphere conditions and (iv) the possibility to estimate population size of the inoculants (each found slightly above 10^5 log CFU equivalents per root system at 10 d) in the rhizosphere of maize grown in nonsterile La Côte St André soil. The detection limits of the two real-time PCR assays did not depend on the soil used, but differed according to the strain (4×10^3 and 4×10^4 CFU g^{-1} lyophilized soil for strains UAP-154 and CFN-535, respectively). The reason behind this difference is not known. As *Azospirillum* inoculation requires at least 10^5 CFU $plant^{-1}$ for effective phytostimulation (Kapulnik *et al.* 1985; Arsac *et al.* 1990; Okon and Itzigsohn 1995; Benizri *et al.* 2001), these detection limits were satisfactory. Had it not been the case, the detection limit could have been lowered by developing an alternative real-time PCR method using TaqMan probes.

One main advantage of the standard curve approach is the integration of some of the bias linked to DNA extraction, as standard curves relate cell numbers to C_T values obtained with DNA from inoculated nonsterile bulk soil. Indeed, the latter involves DNA extracted with the same procedure subsequently used in quantification experiments. This standard curve approach proved effective for the quantification of *Pseudomonas* groups in the wheat rhizosphere (Mavrodi *et al.* 2007) and here (as indicated by PCR efficiency results) to estimate the population densities of each *A. brasilense* strain.

In conclusion, SCAR markers were useful to design PCR primers for rhizosphere quantification of *A. brasilense* inoculants of maize and this approach is advocated for other *Azospirillum* strains used on cereal crops. The two real-time PCR assays will be used in future work to assess the influence of ecological conditions on root colonization and maize phytostimulation by *A. brasilense* UAP-154 and CFN-535.

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