The *Pseudomonas* Secondary Metabolite 2,4-Diacetylphloroglucinol Is a Signal Inducing Rhizoplane Expression of *Azospirillum* Genes Involved in Plant-Growth Promotion

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During evolution, plants have become associated with guilds of plant-growth-promoting rhizobacteria (PGPR), which raises the possibility that individual PGPR populations may have developed mechanisms to cointeract with one another on plant roots. We hypothesize that this has resulted in signaling phenomena between different types of PGPR colonizing the same roots. Here, the objective was to determine whether the *Pseudomonas* secondary metabolite 2,4-diacetylphloroglucinol (DAPG) can act as a signal on *Azospirillum* PGPR and enhance the phytostimulation effects of the latter. On roots, the DAPG-producing *Pseudomonas fluorescens* F113 strain but not its *phl*-negative mutant enhanced the phytostimulatory effect of *Azospirillum brasilense* Sp245-Rif on wheat. Accordingly, DAPG enhanced Sp245-Rif traits involved in root colonization (cell motility, biofilm formation, and poly- β -hydroxybutyrate production) and phytostimulation (auxin production). A differential fluorescence induction promoter-trapping approach based on flow cytometry was then used to identify Sp245-Rif genes upregulated by DAPG. DAPG enhanced expression of a wide range of Sp245-Rif genes, including genes involved in phytostimulation. Four of them (i.e., *ppdC*, *flgE*, *nirK*, and *nifX-nifB*) tended to be upregulated on roots in the presence of *P. fluorescens* F113 compared with its *phl*-negative mutant. Our results indicate that DAPG can act as a signal by which some beneficial pseudomonads may stimulate plant-beneficial activities of *Azospirillum* PGPR.

The rhizosphere is a very active microbial habitat driven by the organic substrates released by the plant as root exudates (Bais et al. 2006). Trophic conditions are important in determining the assembly and functioning of the rhizosphere microbial community (Björnlund et al. 2006). However, the ecology of rhizosphere microorganisms is also influenced by the synthesis of inhibitory plant and microbial compounds involved in

parasitism, antagonism, or competition (Bouwmeester et al. 2007; Couillerot et al. 2009), as well as molecular signals (i.e., low molecular weight compounds that are emitted by an organism and recognized by another at nanomolar to micromolar concentration, and that elicit a response in the latter) (Bais et al. 2006; Hirsch et al. 2003). These signals are important for the establishment of mutualistic and associative interactions between microorganisms and plant roots (Cooper et al. 2007; Pothier et al. 2007).

Mutualistic plant–bacteria interactions have been extensively studied in the case of the nitrogen-fixing symbiosis with legumes or actinorhizal plants and, at least in the former case, the signaling phenomena are well documented (Gibson et al. 2008; Spaink 2000). In contrast, the signaling involved in associative symbiosis between plant-growth-promoting rhizobacteria (PGPR) and roots are poorly understood. Most signaling phenomena studied in the case of multitrophic interactions involving PGPR concern the impact of i) root-secreted compounds on the selection of root-associated bacteria (Ramey et al. 2004) and the control of their plant-beneficial activities (Pothier et al. 2007; Rudrappa et al. 2008; Teplitski et al. 2000); ii) bacterial secondary metabolites, including phytohormones, on root growth and plant defense (Lambrecht et al. 2000; Pieterse et al. 2003); and iii) quorum-sensing pheromones in the regulation of microbe–microbe social relationships (Boyer et al. 2008; Daniels et al. 2006). Because PGPR might have coevolved with plants, it could be that other types of signaling phenomena are also involved when considering the social relations between different types of PGPR colonizing the same roots (Wei and Zhang 2006). However, this possibility has been neglected so far.

Among molecular signals released in the rhizosphere, the *Pseudomonas* secondary metabolite 2,4-diacetylphloroglucinol (DAPG) is one of the most intriguing compounds synthesized by PGPR. This phenolic was originally studied for its antimicrobial properties, and it was found to be antifungal, antibacterial, antiviral, antihelminthic, and phytotoxic when used at a high concentration (Haas and Keel 2003; Weller 2007). However, at lower concentrations, DAPG acts as a signal molecule influencing gene expression of biocontrol traits in plant-protecting pseudomonads and fungi (Baehler et al. 2005; Brodhagen et al. 2004; Lutz et al. 2004). DAPG can also be a signal molecule for plants, inducing systemic resistance (Iavicoli et al. 2003; Bakker et al. 2007), stimulating root exudation (Phillips et al. 2004), and enhancing branching of the root system via the auxin-dependent plant signaling pathway (Brazelton et al. 2008). Whether it can also have a signaling effect on

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non-*Pseudomonas* PGPR is not known and was the focus of the current work.

DAPG-producing pseudomonads play an important role in disease-suppressive soils, where plants are protected from soil-borne diseases by naturally occurring microbial populations (Frapolli et al. 2008; Weller et al. 2007). In microarray comparisons of indigenous populations in soils suppressive or conducive to tobacco black root rot or wheat take-all, it came as a surprise that species of *Azospirillum*, a key genus of phyto-stimulatory PGPR, colonized roots jointly with DAPG-producing pseudomonads in suppressive soils (Kyselková et al. 2009; Sanguin et al. 2009). *Azospirillum* PGPR display various plant-beneficial traits; i.e., production of nitric oxide (Creus et al. 2005) and auxin phytohormones (Costacurta and Vanderleyden 1995), ACC deaminase activity (Prigent-Combaret et al. 2008), associative nitrogen fixation (Bally and Elmerich 2007), and control of parasitic plants (Miché et al. 2000) and bacteria (Bashan and de-Bashan 2002). Their phyto-stimulatory effects have been shown under greenhouse experiments and field conditions (Dobbelaere et al. 2001; El Zemrany et al. 2006). The possibility that biocontrol *Pseudomonas* PGPR could interact with *Azospirillum* PGPR has been shown before (Naiman et al. 2009; Vázquez et al. 2000) but the mechanisms involved remain to be identified.

In this work, our goal was to determine whether DAPG can act as a signal molecule on *Azospirillum* spp. To this end, the impact of the DAPG production ability of fluorescent *Pseudomonas* spp. on the phyto-stimulatory effect of *Azospirillum brasilense* Sp245-Rif on wheat was analyzed. In addition, the impact of DAPG on traits involved in Sp245-Rif root colonization and plant-growth promotion was evaluated in vitro. Finally, strain Sp245-Rif was exposed to DAPG at concentrations found in the rhizosphere, and a differential fluorescence induction (DFI) approach (Allaway et al. 2001) based on fluorescence-activated cell sorting (FACS) was then used to identify Sp245-Rif genes upregulated in the presence of DAPG.

RESULTS

Effect of *Pseudomonas fluorescens* F113 and its *phl*-negative mutant F113G22 on plant growth promotion by *A. brasilense* Sp245-Rif.

Under gnotobiotic conditions, root development of spring wheat inoculated with *A. brasilense* Sp245-Rif alone was significantly better at 7 days than that of noninoculated plants based on total root length, average root diameter, root surface area, or total root volume (Table 1). Coinoculation of *A. brasilense* Sp245-Rif with DAPG-producing strain *P. fluorescens* F113 led to plant root development that, depending on the measurement, was comparable (for total root length and root

surface area) or enhanced (for average root diameter, total root volume, and number of roots) in comparison with that obtained with strain Sp245-Rif alone. When *phl*-negative mutant *P. fluorescens* F113G22 was used instead of *P. fluorescens* F113 in coinoculation with *A. brasilense* Sp245-Rif, a trend (not significant at $P < 0.05$ however) for a decrease of plant root development was observed, leading to results comparable with those recorded when using strain Sp245-Rif alone. This trend was not linked to differences in *Azospirillum* root colonization levels, because *A. brasilense* Sp245-Rif was recovered at 6.2 ± 0.8 (inoculated singly), 6.7 ± 0.4 (in the presence of *P. fluorescens* F113), and 6.5 ± 0.6 log CFU plant⁻¹ (in the presence of *P. fluorescens* F113G22) from wheat roots at 7 days (Table 1).

Effect of synthetic DAPG on selected Sp245-Rif phenotypes.

As an effort to understand the enhanced plant growth-promoting effect of Sp245-Rif/F113 coinoculation, the impact of DAPG on several Sp245-Rif traits implicated in root colonization and plant growth promotion was analyzed in vitro. None of the DAPG concentrations tested up to 500 μ M resulted in *Azospirillum* growth inhibition, whereas growth was affected at 1,000 μ M DAPG (Supplementary Fig. S1). DAPG had no effect on *Azospirillum* swarming (data not shown), whereas swimming of strain Sp245-Rif was significantly higher at intermediate DAPG concentrations of 0.01 and 1 μ M but lower again at 0.1 μ M DAPG (Fig. 1). This increase only at two intermediate DAPG concentrations was confirmed on two other occasions (data not shown). Biofilm formation on polystyrene reached a maximum for an intermediate DAPG concentration of 0.1 μ M (Fig. 2). Cell accumulation of poly- β -hydroxybutyrate (PHB) storage material increased linearly with DAPG concentration (Fig. 3), which was confirmed by epifluorescence microscopic observations.

DFI identification of *A. brasilense* promoters upregulated by DAPG.

The signaling effect of DAPG on the *A. brasilense* Sp245-Rif transcriptome was thereafter analyzed using a promoter-trapping approach. When the promoter-trap library PTL-AbP of strain Sp245-Rif (Pothier et al. 2007) was screened for promoters upregulated by DAPG, flow cytometry cell sorting gave 1,920 DFI clones at 0.01 μ M DAPG and 960 at 10 μ M DAPG. DAPG induction of the most fluorescent DFI clones was confirmed in microtiter plates (at the corresponding DAPG concentration) in two independent experiments. Among them, 158 (at 0.01 μ M DAPG) and 60 (at 10 μ M DAPG) DFI clones displayed an induction factor higher than 1.5 in both microtiter plate experiments. Sequence comparison of 96 DFI

Table 1. Effects of single or double inoculation with *Azospirillum brasilense* Sp245-Rif, 2,4-diacetylphloroglucinol (DAPG)-producing *Pseudomonas fluorescens* F113, or its *phl*-negative mutant F113G22 on root system development of wheat at 7 days (mean $\pm$ standard error, $n = 5$)^a

Root system parameters	Control ^b	Single inoculation			<i>Pseudomonas</i> co-inoculated with Sp245-Rif	
		Sp245-Rif	F113	F113G22	F113	F113G22
Total root length (cm plant ⁻¹)	15.4 $\pm$ 2.6 b	28.1 $\pm$ 2.4 a	18.9 $\pm$ 3.3 b	24.3 $\pm$ 3.2 ab	30.6 $\pm$ 2.7 a	27.9 $\pm$ 1.8 a
Root surface area (mm ² plant ⁻¹)	347 $\pm$ 66 c	738 $\pm$ 72 ab	439 $\pm$ 82 c	519 $\pm$ 75 bc	932 $\pm$ 76 a	774 $\pm$ 62 a
Average root diameter (mm)	0.67 $\pm$ 0.04 c	0.83 $\pm$ 0.02 b	0.74 $\pm$ 0.07 bc	0.66 $\pm$ 0.04 c	0.97 $\pm$ 0.03 a	0.88 $\pm$ 0.04 ab
Total root volume (mm ³ plant ⁻¹)	60 $\pm$ 10 d	160 $\pm$ 20 bc	90 $\pm$ 20 cd	90 $\pm$ 10 cd	230 $\pm$ 20 a	170 $\pm$ 20 ab
Number of roots per plant	24 $\pm$ 5 b	52 $\pm$ 9 b	36 $\pm$ 8 b	49 $\pm$ 8 b	101 $\pm$ 16 a	51 $\pm$ 7 b
Colonization (log CFU plant ⁻¹) ^c		6.2 $\pm$ 0.8 b	7.7 $\pm$ 0.5 a	7.4 $\pm$ 0.3 a	6.7 $\pm$ 0.4 b/7.6 $\pm$ 0.6 a	6.5 $\pm$ 0.6 b/7.3 $\pm$ 0.8 a

^a For each data row, statistical differences between treatments are indicated with lowercase letters (analysis of variance and Fisher's least significant difference tests; $P < 0.05$). Plant parameters were compared between the six treatments (i.e., the noninoculated control, the three single inoculations, and the two double inoculations). For the colonization data, the seven populations evaluated on the roots following single- and double-strain inoculations were compared with each other.

^b Noninoculated control.

^c Colonization by inoculants.

clones (i.e., 64 of 158 DFI clones at 0.01 μM DAPG and 32 of 60 DFI clones at 10 μM DAPG) with the *A. brasilense* Sp245 whole-genome sequence and GenBank database indicated that 37 of 64 inserts (at 0.01 μM DAPG) and 15 of 32 inserts (at 10 μM DAPG) encompassed putative promoters, whereas the 44 others were within putative open reading frames (ORF) or in the opposite direction of predicted genes.

Sequence analysis of DAPG-induced promoters.

Based on *A. brasilense* Sp245 genome data and BlastP analyses, the 52 DFI clones corresponding to putative promoters were grouped in seven functional classes according to function prediction of the corresponding ORF located downstream. The first class (cell transport and nutrient scavenging) included three DFI clones (Table 2). Their deduced protein sequences are homologous to i) a secretion protein of the HlyD membrane fusion protein type (clone IID3) and ii) two ABC transporters, LivG (IIIB2) and an iron permease (IIB4).

The second class (central intracellular metabolism) included 16 DFI clones, a majority displaying deduced protein sequences involved in energy production and electron transfer, such as i) cytochrome c551 peroxidase (clones IB1 and IIG4) and cytochrome c peroxidase (IIG6), ii) aconitate hydratase (IIIC6 and IIIF2) implicated in the Krebs and glyoxylate cycles, iii) cytochrome c oxidase (IIID1) and polyphosphate kinase (PPK) (IIA4) relevant for oxidative phosphorylation, and iv) carbon-monoxide dehydrogenase (IF10) (Table 2). For the other clones, the deduced protein sequence is homologous to i) the EutB subunit of the cobalamin-dependent ethanolamine ammonia-lyase (clone IID7), ii) an α -D-galactoside galactohydrolase (IIE6), iii) an enoyl-CoA hydratase/isomerase involved in butanoate and PHB metabolism (IIIC1), iv) a malic enzyme catalyzing the anaplerotic reaction supplying pyruvate from malate in the Krebs cycle (IIIA8), v) a UvrD helicase II (IE7), and vi) the translation elongation factor EF-G (IID8, IIA8 and IIF3).

Fig. 1. Effect of synthetic 2,4-diacetylphloroglucinol (DAPG) (0.001 to 100 μM) on cell motility of *Azospirillum brasilense* Sp245-Rif on soft agar. Diameters (mm) of spreading through the medium at 3 days are shown as means $\pm$ standard deviation ($n = 6$). Letters indicate significant differences among treatments ($P < 0.05$).

Fig. 2. Effect of synthetic 2,4-diacetylphloroglucinol (DAPG) (0.001 to 10 μM) on biofilm formation of *Azospirillum brasilense* Sp245-Rif on polystyrene. Cell adhesion data (at 6 days) are expressed as means $\pm$ standard deviation ($n = 8$). Letters indicate significant differences among treatments ($P < 0.05$).

The third class (post-translational modification and proteasome) included seven DFI clones (Table 2). Their deduced protein sequences are homologous to i) a post-translational methionine aminopeptidase (clone IIIH7); ii) an M20/M25/M40 peptidase (IIF5) and a serralsin (IIH4) involved in proteolysis; iii) a periplasmic chaperone corresponding to an outer membrane lipoprotein (IIIC8) and the chaperone DjlA (IB12 and IA9); and iv) a stress-induced, FtsH protease-controlling band7-stomatin-like protein (IH8).

The fourth class (biotic interactions) included three DFI clones (Table 2). The deduced protein sequence of one of them (clone IB8) is homologous to the nitrogen fixation-related proteins NifX-NifB, which are responsible for synthesis of the iron-molybdenum dinitrogenase cofactor (Rubio and Ludden 2008). Another corresponds to Sp245's own phenylpyruvate decarboxylase-encoding gene, *ppdC* (clone IIIC3), which is involved in synthesis of phytohormone 3-indole acetic acid (IAA) (Dobbelaere et al. 1999). The deduced protein sequence of the third DFI clone (clone IIA3) is homologous to Sp245's own flagellar hook protein FlgE. The same region was previously identified as upregulated by wheat seed exudates (Pothier et al. 2007). Another *A. brasilense* Sp245-Rif wheat seed exudate-induced gene (i.e., the nitrite reductase-encoding *nirK*) was observed as upregulated in the presence of DAPG (0.01 μ M) but this clone was not obtained here by flow cytometry.

The fifth class (transcription regulation) contained five DFI clones (Table 2). They encode putative transcriptional regulators of the XRE (clone IIIE2), AlgH (IF11), AsnC (IIB3), or GntR family (IIC8), or a putative anti- σ -factor antagonist with a sulfate transporter and anti- σ domain (IB4).

The sixth class (stimulus perception and signaling) includes two DFI clones (Table 2). They correspond to a putative CstS1-like hybrid sensor kinase/response regulator (clone IIIE5) and a putative adenylate/guanylate cyclase involved in cyclic nucleotide biosynthesis (IIH5).

The seventh class gathers DFI clones whose predicted amino acid sequences did not give any hit in GenBank search (clones IE12, IC12, IB5, ID8, IIIG1, IH7, and IIF6) or displayed homol-

ogy with proteins of unknown function (IE1, IE3, IIID6, IIB7, IIIG6, IIH7, IIG5, IIC4, and IIC6). The deduced protein sequences for four of the latter are homologous to conserved hypothetical proteins with PDZ (i.e., protein-protein interactions), and FIST or CBS domains (i.e., ligand-protein interactions).

Quantification of in vitro DAPG induction of the *A. brasilense* Sp245-Rif *ppdC* gene expression and indolic compound production.

The effect of DAPG concentration on expression of IAA gene *ppdC* was investigated in more detail, this time in the presence of IAA precursor tryptophan. First, fluorescence measurements indicated that expression of the *ppdC* clone IIIC3 significantly increased with DAPG concentrations between 0.001 and 10 μ M, reaching a maximum at 1 μ M DAPG (Fig. 4A). Second, a Salkowski colorimetric assay showed that production of IAA and related indole compounds was significantly higher at 0.001 to 1 μ M DAPG in comparison with the control (Fig. 4B).

In planta expression of four plant-interaction-relevant DFI clones in the presence of *P. fluorescens* F113 or *phl*-negative mutant F113G22.

When assessed in planta, four DFI clones of particular relevance for interaction with the plant (i.e., corresponding to *ppdC*, *flgE*, *nirK*, and *nifX-nifB*) were found both as single cells and large cell clumps at 7 days after inoculation, based on transmitted light observations (data not shown). They were over the whole root system of spring wheat but colonization was higher in the root hair zone.

When inoculated singly, all four DFI clones were fluorescent on roots at 7 days, meaning that the corresponding promoter was transcribed, even though no DAPG-producing bacterium was present (Supplementary Fig. S2). More specifically, *ppdC* and *flgE* were expressed in the root hair zone, *nifX-nifB* in the root hair zone and on border cells at the root tip, and *nirK* along the entire root system except the apex.

Fig. 3. Effect of synthetic 2,4-diacetylphloroglucinol (DAPG) (0.001 to 100 μ M) on poly- β -hydroxybutyrate accumulation in *Azospirillum brasilense* Sp245-Rif. Data are expressed as means $\pm$ standard deviation ($n = 4$). The concentration of 0 μ M DAPG was arbitrarily put at 10^{-6} μ M DAPG. The linear regression equation and its R^2 value are shown ($P = 0.0037$). Standard errors were 1.9 (estimated slope) and 4.9 (intercept).

Qualitative distribution of fluorescent cells on roots at 7 days was not modified when plants had been coinoculated with a pseudomonad 2 days after *Azospirillum* inoculation; however, the total root surface area with fluorescent cells of the *ppdC-egfp* and *flgE-egfp* clone was reduced in the presence of *P. fluorescens* F113G22 (Fig. 5). Lower fluorescence levels of DFI clones at 7 days were observed in the presence of *phl*-negative mutant F113G22 compared with the control (1.8-fold reduction with *ppdC* to 5.3-fold reduction with *nirK*) but the trend is not significant in the case of the *ppdC* clone (Fig. 5). In the presence of the DAPG-producing wild-type strain F113, however, fluorescence levels of the four DFI clones were i) like those in the control but ii) higher than those in the presence of the mutant F113G22 (Fig. 5).

DISCUSSION

This work dealt with the possibility that DAPG production by root-colonizing biocontrol pseudomonads could interfere positively with phytostimulation by *Azospirillum* PGPR. Indeed, coinoculation of wheat with the DAPG-producing strain *P. fluorescens* F113 and the PGPR *A. brasilense* Sp245-Rif

resulted in enhanced root diameter, total root volume, and root number in comparison with single Sp245-Rif inoculation (Table 1). For root number, this positive effect did not occur when the *phl*-negative mutant F113G22 was coinoculated instead of F113, suggesting that DAPG production was involved. One possibility is that DAPG could act directly on the plant. First, DAPG may influence root system architecture, as shown on tomato (Brazelton et al. 2008), but here, neither *P. fluorescens* F113 strain nor its *phl*-negative mutant had any effect when inoculated singly. Second, DAPG may also enhance root exudation (Phillips et al. 2004), which might favor *Azospirillum* spp. survival or functioning. However, the total number of Sp245-Rif cells colonizing wheat roots was not significantly higher in the presence of F113 or F113G22 (Table 1). Third, perhaps the plant physiological responses induced by DAPG (Iavicoli et al. 2003) facilitate plant receptivity to Sp245-Rif's phytohormonal effects; however, this hypothesis was outside the scope of this study.

The other possibility is that DAPG could act as signal on *Azospirillum* spp. themselves, inducing the expression of plant-beneficial genes, and this was the focus of the current work. To this end, the impact of DAPG on Sp245-Rif transcriptome was

Fig. 4. Effect of synthetic 2,4-diacetylphloroglucinol (DAPG) (0.001 to 10 μM) on *ppdC* gene expression and 3-indole acetic acid (IAA) production in differential fluorescence induction clone IIC3 of *Azospirillum brasilense* Sp245-Rif after 6 days in ABmal medium supplemented with tryptophan at 100 μg ml⁻¹. **A**, Relative enhanced green protein fluorescence of the *ppdC-egfp* fusion was quantified using a spectrofluorimeter. **B**, Equivalent IAA concentration (for IAA and related indole compounds) was obtained using a colorimetric Salkowski method. Data are expressed as means ± standard deviation ($n = 4$), and letters indicate significant differences among treatments ($P < 0.05$).

analyzed by promoter trapping using flow cytometry. This was done at 10 μ M DAPG, which corresponds to i) average DAPG concentrations reported in the rhizosphere (Bonsall et al. 1997) and ii) concentrations that improve lateral root production in tomato (Brazelton et al. 2008), as well as at a thousand-fold lower concentration (0.01 μ M DAPG), which might be relevant to colonization patterns of *Azospirillum* and *Pseudomonas* spp. on wheat roots (as both bacteria may colonize spatially distant sites). Indeed, the majority of Sp245-Rif promoters upregulated by DAPG were obtained with 0.01 μ M DAPG. This is consistent with the concept that DAPG can act on Sp245-Rif as a molecu-

lar signal, eliciting a broad cell response at very low concentration. Indeed, several DFI clones obtained at 0.01 μ M DAPG corresponded to genes involved in signal transduction pathways, such as those encoding the Cst1 response regulator (IIIIE5); five transcriptional regulators (IIIE2, IF11, IIB3, IIC8, and IB4), including an anti- σ factor (IB4); and four unknown proteins potentially involved in protein-protein interactions (IE1, IE3, IIID6, and IIC4). DAPG also induces a putative adenylate/guanylate cyclase (IIIH5) required for the biosynthesis of cyclic nucleotides, which are cell second messengers that control a wide range of cellular processes (Pesavento and Hengge 2009).

Table 2. Potential 2,4-diacetylphloroglucinol (DAPG)-induced promoters identified by differential fluorescence induction (DFI) in *Azospirillum brasilense* Sp245-Rif

Clone ^y	Accession no.	Gene, function	Induction level ^x		Comparison with closest database sequence ^z
			0.01 μ M	10 μ M	
Class I: cell transport and nutrient scavenging					
IIB4	GU291813	ABC transporter	■		<i>Microcoleus chthonoplastes</i> PCC 7420 (YP002616034), ABC transporter, permease protein (potentially involved in iron III uptake), 0e0, 65%, 78%
IID3	GU291817	Secretion protein	■		<i>Parvibaculum lavamentivorans</i> DS-1 (YP001412779), HlyD-family secretion protein (COG0845, coiled-coil domain), 5e-116, 65%, 81%
IIIB2	GU291827	<i>livG</i>		■■■	<i>Desulfovibrio vulgaris</i> subsp. <i>vulgaris</i> Hildenborough (YP009936), branched-chain amino acid ABC transporter, ATP-binding protein, 3e-66, 50%, 68%
Class II: central metabolism					
IID8**	GU291818	<i>fusA</i>	■		<i>Rhodospirillum centenum</i> SW (YP002298082), translation elongation factor, EF-G (GTPases, COG0480), 0e-0, 62%, 78%
IIA8*	GU291812	<i>fusA</i>	■		<i>R. centenum</i> SW (YP002298082), translation elongation factor, EF-G (GTPases, COG0480), 0e-0, 62%, 78%
IIIF3**	GU291820	<i>fusA</i>	■		<i>R. centenum</i> SW (YP002298082), translation elongation factor, EF-G (GTPases, COG0480), 0e-0, 62%, 78%
IIIE6	GU291819	<i>melA</i>	■		<i>Rhodopsseudomonas palustris</i> CGA009 (NP945731), α -D-galactoside galactohydrolase, melibiase (EC 3.2.1.22), 9e-177, 70%, 81%
IID7	GU291848	<i>eutB</i>	■		<i>Bradyrhizobium</i> sp. BTAi1(YP001239723), ethanolamine ammonia-lyase heavy chain EutB, 0e0, 78%, 86%
IIG4**	GU291849	<i>ccp</i>	■		<i>Magnetospirillum gryphiswaldense</i> MSR-1 (CAM76415), cytochrome c551 peroxidase Ccp, 1e-156, 77%, 88%
IB1**	GU291804	<i>ccp</i>	■■■		<i>M. gryphiswaldense</i> MSR-1 (CAM76415), cytochrome c551 peroxidase Ccp, 1e-156, 77%, 88%
IIA4	GU291811	<i>ppk</i>	■		<i>Rhodospirillum centenum</i> SW (YP002297760), polyphosphate kinase PPK (EC2.7.4.1), 0e-0, 77%, 89%
IIG6	GU291824	<i>mauG</i>	■		<i>Pseudovibrio</i> sp. JE062 (ZP05083047), Di-haem cytochrome c peroxidase (COG1858), family Ccp, 3e-125, 61%, 73%
IF10	GU291807	<i>coxL</i>	■■		α -Proteobacterium BAL199 (ZP02189787), carbon-monoxide dehydrogenase CoxL (acceptor, EC1.2.99.2), 0e0, 56%, 70%
IE7	GU291844	<i>uvrD</i>	■■■		<i>M. gryphiswaldense</i> MSR-1 (CAM76107), DNA helicase II (COG0210), 0e-0, 64%, 76%
IIIC6*	GU291829	<i>acnA</i>		■	<i>R. centenum</i> SW (YP002299535), aconitate hydratase AcnA (EC4.2.1.3), 0e0, 76%, 85%
IIIF2*	GU291832	<i>acnA</i>		■■	<i>R. centenum</i> SW (YP002299535), aconitate hydratase AcnA (EC4.2.1.3), 0e0, 76%, 85%
IIIA8	GU291845	<i>maeB</i>		■	<i>R. centenum</i> SW (YP002296658), NAD-dependent malic enzyme (EC 1.1.1.40), 0e0, 64%, 79%
IIID1	GU291830	<i>coxB</i>		■■■	<i>R. centenum</i> SW (YP002299791), cytochrome c oxidase subunit II CoxB (EC1.9.3.1), 2e-86, 65%, 78%
IIIC1	GU291828	Butanoate metabolism		■■■	<i>Ochrobactrum intermedium</i> LMG 3301 (ZP04682997), enoyl-CoA hydratase/isomerase, crotonase (EC4.2.1.55), 2e-112, 78%, 88%
Class III: post-translational modifications and proteasome					
IIIF5	GU291821	Proteolysis	■		<i>Aurantimonas</i> sp. SI85-9A1 (ZP01228661), peptidase M20/M25/M40 family (EC 3.4.13.20), 1e-138, 57%, 70%
IIH4	GU291825	Serralysin	■		<i>Azospirillum brasilense</i> (AAS83088), putative protease-like protein, serralysin family (EC 3.4.24.40), 1e-68, 36%, 48%
IA9*	GU291803	Chaperonin	■■		<i>R. centenum</i> SW (YP002296874), putative DnaJ-like protein Dj1A, 3e-66, 57%, 72%
IB12*	GU291805	Chaperonin	■■		<i>R. centenum</i> SW (YP002296874), putative DnaJ-like protein Dj1A, 3e-66, 57%, 72%
IIIH7	GU291838	<i>map</i>		■	<i>M. magnetotacticum</i> MS-1 (ZP00055468), methionine aminopeptidase, peptidase M Map (EC 3.4.11.18, COG0024), 1e-117, 75%, 85%

(continued on next page)

^x Induction levels at DAPG trapping concentration of DFI clones assessed in microtiter plates at the corresponding promoter-trapping DAPG concentrations are indicated by black squares ■■■ = level higher than 3; ■■ = level between 3 and 1.5; and ■ = level between 1.5 and 1.3.

^y Asterisks: * = identical clone isolated more than one time and ** = clones with a distinct insert for the same gene.

^z Organism (accession number), function, levels of significance = E-value, identity, and similarity for the most similar protein sequence.

Of the 96 DFI clones sequenced, 44 were within putative ORF or in the opposite direction of predicted genes, as found in all promoter trap studies (Rediers et al. 2005), and were discarded. These cryptic fusions are attributed to incomplete genome annotations (Silby et al. 2004). Considering the high number of antisense transcripts identified in this and comparable studies, they are unlikely to be artifacts. Rather, they are thought to downregulate gene expression (Rediers et al. 2005; Silby and Levy 2004). Although this approach surveys only a fraction of the upregulated genes (illustrated here with *nirK*), results clearly indicated that the 52 correctly oriented promoters upregulated by DAPG corresponded to very different cell

functions (Table 2), meaning that the DAPG signal may have a broad impact on *Azospirillum* ecology, as follows.

First, several genes identified are involved in stress response, which may be important to prepare *Azospirillum* cells located in the immediate vicinity of *Pseudomonas* DAPG producers for subsequent build-up of DAPG concentration, the compound being inhibitory at high concentration (Cronin et al. 1997; Keel et al. 1992), including for Sp245-Rif. Among those genes, *eutB* (clone IID7) is involved in the recycling of membrane components (Stojiljkovic et al. 1995), which might be important to counteract negative effects of concentrated DAPG on *Azospirillum* plasma membrane and cell viability (our

Table 2. (continued from preceding page)

Clone ^y	Accession no.	Gene, function	Induction level ^x		Comparison with closest database sequence ^z
			0.01 μ M	10 μ M	
IIC8	GU291833	<i>lola</i>		■	<i>R. centenum</i> SW (YP002298972), outer membrane lipoprotein carrier protein <i>Lola</i> , 2e-59, 61%, 81%
IH8	GU291846	Stomatin		■	<i>Xanthomonas campestris</i> pv. <i>campestris</i> B100 (YP001902680), stomatin-like membrane protein (band 7 protein family, <i>qmcA</i> homolog, COG0330), 2e-119, 65%, 81%
Class IV: biotic-interactions					
IIA3	GU291810	<i>flgE</i>	■		<i>A. brasilense</i> Sp245 (ABV72264), flagellar hook protein <i>FlgE</i> , 6e-167, 100%, 100%
IB8	GU291806	<i>nifX-nifB</i>	■ ■		<i>Rhodospseudomonas palustris</i> BisA53 (YP780140), nitrogen fixation-related protein (IPR003731), 7e-25, 44%, 72%
IIIC3	GU291854	<i>ppdC</i>		■ ■	<i>A. brasilense</i> Sp245 (P51852), phenylpyruvate decarboxylase (EC 4.1.1.74), 0e0, 99%, 99%
Class V: transcriptional regulation					
IIB3	GU291851	AsnC family	■		<i>Sinorhizobium medicae</i> WSM419 (YP001327273), AsnC family putative transcriptional regulator, 1e-55, 65%, 83%
IIC8	GU291816	GntR family	■		<i>M. gryphiswaldense</i> MSR-1 (CAM77897), GntR family transcriptional regulator, 1e-49, 55%, 68%
IF11	GU291808	AlgH family	■ ■		<i>Rhodospirillum centenum</i> SW (YP002299806), AlgH family putative transcriptional regulator (COG1678), 2e-76, 74%, 85%
IB4	GU291839	Regulator	■ ■ ■		<i>Rhodospseudomonas palustris</i> BisA53 (YP780132), anti- σ -factor antagonist, STAS domain (COG1366), 7e-14, 41%, 61%
IIIE2	GU291834	XRE family		■ ■	<i>Rhodospirillum rubrum</i> ATCC 11170 (YP427129), XRE family transcriptional regulator with cupin sensor domain, 1e-67, 69%, 81%
Class VI: stimulus perception and signaling					
IIIE5	GU291835	Response regulator		■	<i>R. centenum</i> SW (AAP22932), hybrid sensor kinase/response regulator CstS1 (COG0784, REC superfamily), 5e-11, 35%, 54%
IIIH5	GU291837	Cyclic nucleotide biosynthesis		■	<i>Bradyrhizobium</i> sp. ORS278 (YP001206407), putative adenylate/guanylate cyclase (EC4.6.1.1, COG2114), 1e-98, 46%, 62%
Class VII: others					
IIF6	GU291822	Function unknown	■		Protein of unknown function, no significant similarity
IIC6	GU291847	Function unknown	■		<i>Vibrio splendidus</i> 12B01 (ZP00990141), exported protein of unknown function (COG0303), 1e-06, 37%, 58%
IIG5	GU291823	Function unknown	■		<i>R. rubrum</i> ATCC 11170 (YP425862), hypothetical protein Rru_A0771, 2e-118, 27%, 42%
IIH7	GU291826	Siderophore biosynthesis	■		<i>R. centenum</i> SW (YP002299259) conserved protein of unknown function (COG4321, predicted arylsulfate sulfotransferase-related proteins), 4e-16, 45%, 62%
IIB7	GU291814	Putative nuclease	■		<i>R. centenum</i> SW (YP002298514), conserved protein of unknown function with a DUF820 domain (COG4636, PF05685, predicted nucleases), 1e-30, 44%, 58%
IIC4	GU291815	Signal transduction system	■		<i>M. magneticum</i> AMB-1 (YP420955), CBS domain-containing protein (PF0057), 2e-24, 45%, 65%
IE12	GU291852	Function unknown	■ ■		Protein of unknown function, no significant similarity
IC12	GU291853	Function unknown	■ ■		Protein of unknown function, no significant similarity
ID8	GU291841	Function unknown	■ ■		Protein of unknown function, no significant similarity
IH7	GU291809	Function unknown	■ ■		Protein of unknown function, no significant similarity
IB5	GU291840	Function unknown	■ ■ ■		Protein of unknown function, no significant similarity
IE1*	GU291842	Signal transduction system	■ ■ ■		<i>Ruegeria pomeroyi</i> DSS-3 (YP166747), uncharacterized conserved protein GfdT (FIST and FIST_C superfamilies), 2e-89, 48%, 63%
IE3*	GU291843	Signal transduction system	■ ■ ■		<i>R. pomeroyi</i> DSS-3 (YP166747), uncharacterized conserved protein GfdT (FIST and FIST_C superfamilies), 2e-89, 48%, 63%
IIIG1	GU291850	Function unknown		■	Protein of unknown function, no significant similarity
IIID6	GU291831	Putative signaling protein		■	<i>Labrenzia alexandrii</i> DFL-11 (YP002613697), conserved hypothetical protein with a PDZ domain (IPR001478)
IIIG6	GU291836	Siderophore biosynthesis		■	α -Proteobacterium BAL199 (ZP02186779), hypothetical protein (COG4321, predicted arylsulfate sulfotransferase-related proteins), 8e-11, 50%, 73%

Fig. 5. Semiquantitative analysis of in planta fluorescence levels of *Azospirillum brasilense* Sp245-Rif differential fluorescence induction (DFI) clones expressing the **A**, *nirK-egfp*; **B**, *flgE-egfp*; **C**, *nifX-nifB-egfp*; and **D**, *ppdC-egfp* fusions on wheat after single inoculation (C), and coinoculations in the presence of *Pseudomonas fluorescens* F113 or its *phl*-negative derivative F113G22. For each DFI clone, confocal images were acquired using the same detector amplification gain to allow semiquantitative comparison of fluorescence between treatments. The *bioImage_L* software 1.0 was used to quantify the threshold level of green fluorescence intensity in the analyzed images (white bars) and the percentage of the area covered by green fluorescent cells (black bars). For each treatment, means were calculated from one or two images per root system, and at least three independent root systems were considered. Data are expressed as the means $\pm$ standard deviation ($n = 3$ to 6). Letters indicate significant differences among treatments ($P < 0.05$, Kruskal-Wallis paired comparison). For DFI clones expressing the *nirK-egfp* and *nifX-nifB-egfp* fusions, green fluorescence cover percents were not significantly different between treatments.

transmission electronic microscopy data, *unpublished*). DAPG is known to cause plasma membrane disorganization also in fungi (de Souza et al. 2003). Whether DAPG can damage other cell components in strain Sp245-Rif is not known but DAPG upregulation of genes encoding proteases (IIIH7, IIF5, and IIH4), a predicted nuclease (IIB7), and a UvrD helicase (IE7) means it could be the case. Alternatively, DAPG upregulation of these genes is perhaps useful to counteract stress conditions expected in the rhizosphere (Miché and Balandreau 2001). With regard to this hypothesis, DAPG induces DFI clone IIIC1 (Table 2), which encodes an enoyl-CoA hydratase/isomerase potentially involved in PHB biosynthesis (Fiedler et al. 2002). PHB accumulation, which increased linearly with DAPG concentrations (Fig. 3), favors *A. brasilense* survival under stress conditions (Kadouri et al. 2003; Kamnev et al. 2008). PHB production was probably fueled by consumption of the culture medium carbon source (malate), because malate utilization genes *maeB* and *acnA* were induced by DAPG. Accordingly, these three genes were induced at the higher DAPG concentration tested. It is worth noting that genes homologous to hydrolase-encoding *phlG*, so far the only bacterial gene known to be involved in DAPG resistance (in *Pseudomonas* strain CHA0) (Bottiglieri and Keel 2006), are not present in the Sp245-Rif genome but another unknown resistance mechanism might be involved in this strain.

Second, one DFI clone (IIA3) was the flagellar hook protein gene *flgE* and exemplifies genes involved in *Azospirillum* spp. establishment in the rhizosphere. DAPG upregulation of this gene was confirmed at DAPG concentrations of both 0.01 and 10 μM (data not shown) and correlated with enhanced motility (Fig. 1). In *Azospirillum* spp., the polar flagellum is required for motility toward the root (Croes et al. 1993) and mediates the initial steps of cell adsorption to the root surface and of biofilm formation (Turnbull et al. 2001; Zamudio and Bastarrachea 1994). Accordingly, the ability of Sp245-Rif to form biofilm increased with DAPG concentrations (Fig. 2). Consistent with this, *ppk* (clone IIA4), which also contributes to cell motility and biofilm formation (Brown and Kornberg 2008; Daniels et al. 2004), was upregulated by DAPG.

Third, DAPG induced Sp245-Rif genes potentially involved in sensing rhizosphere constituents. *livG* (strongly upregulated by 10 μM DAPG) codes an ABC transporter for branched-chain amino acids, whose supply by legumes regulates the symbiosis with *Rhizobium* spp. (Prell et al. 2009). Moreover, several DAPG-induced genes (i.e., *ccp*, *coxB*, *coxL*, and *mauG*) are involved in electron transfer and might play a key role in energy taxis response, which promotes root colonization by *A. brasilense* (Alexandre et al. 2004; Greer-Phillips et al. 2004).

Fourth, DAPG induced genes involved in plant growth promotion, such as *ppdC* (IIIC3; IAA production), *nirK* (DFI-32; nitric oxide production), and *nifX-nifB* (IB8; nitrogen fixation). DAPG upregulation of *ppdC* was confirmed by further in vitro analysis of *ppdC* gene expression and production of IAA in the presence of increasing concentrations of DAPG (Fig. 4). DAPG can also stimulate lateral root production by interacting with the plant auxin pathway in tomato (Brazelton et al. 2008), which means that DAPG-mediated phytostimulation might involve both a direct effect on the plant and an indirect effect via *Azospirillum* IAA production. The NirK denitrification product nitric oxide is another signal molecule stimulating lateral root formation (Creus et al. 2005; Molina-Favero et al. 2008), whose effects on the plant are expected to add up to those of IAA. DAPG also enhanced the expression of genes *nifX-nifB* involved in nitrogen fixation, which is interesting because nitrogen fixation has often been portrayed as a plant-beneficial trait of less importance in *Azospirillum* spp. (Barbieri et al. 1986; Dobbelaere et al. 2003).

On roots, the enhanced expression of Sp245-Rif genes important for phytostimulation in the presence of F113 compared with its *phl*-negative mutant was confirmed. Confocal analysis showed that *nirK*, *ppdC*, *flgE*, and *nifX-nifB* were highly induced in the presence of the DAPG-producing *Pseudomonas* strain. The enhanced green protein fluorescence on confocal images from DFI clones inoculated with F113 was not due to an increase in the number and size of *Azospirillum* cells but, clearly, to the induction of the expression of these *Azospirillum* genes by the DAPG-producing *Pseudomonas* strain. These genes were already expressed when *Azospirillum* spp. were alone on wheat roots (Fig. 5), raising the possibility that plant signals may act in synergy with DAPG to activate the expression of Sp245-Rif genes important for phytostimulation. In the presence of the *phl*-negative mutant, the expression of the four genes was strongly decreased. Inactivation of DAPG production in F113 may have reduced the plant-beneficial activity of *Azospirillum* spp. without altering their culturability, such as by enhancing the production of the *Pseudomonas* secondary metabolite hydrogen cyanide. Thus, DAPG may alleviate the downregulation of *Azospirillum* genes observed with the mutant strain. The possibility cannot be excluded that the distinct effects of F113 and F133G22 on *Azospirillum* gene transcription might involve indirect effects of *Pseudomonas* strains through modifications of plant host physiology. The comparison of the influence of the *Pseudomonas* DAPG producer versus its *phl*-negative mutant on *Azospirillum*-mediated effects on plant growth suggests that DAPG enhances *Azospirillum* plant-growth promoting activities. From these observations, it is tempting to speculate that DAPG has become a signal for beneficial plant-microbe interactions during co-evolution of plants with its guild of PGPR partners.

MATERIALS AND METHODS

Bacterial strains and media.

Bacterial strains and plasmids are listed in Table 3. *A. brasilense* was grown at 28°C in Luria-Bertani medium (Sambrook et al. 1989) containing NaCl at only 5 g liter⁻¹ (i.e., LBm), AB medium (Chilton et al. 1974) containing malic acid at 5 g liter⁻¹ as carbon source (i.e., ABmal), or Nfb (Nelson and Knowles 1978) supplemented with 1/40 (vol/vol) LBm (i.e., Nfb*). *P. fluorescens* strains were grown at 28°C in sucrose asparagine iron medium (i.e., SA-Fe) (Cronin et al. 1997). Liquid cultures were incubated on a rotary shaker at 50 rpm (for microtiter plates) or 180 rpm. Only one-third of the tubes were filled to ensure fully aerobic conditions. Antibiotics (Euromedex, Souffelweyersheim, France) were added at the following final concentrations: ampicillin (Ap), 100 $\mu\text{g ml}^{-1}$; chloramphenicol (Cm), 30 $\mu\text{g ml}^{-1}$; gentamicin (Gm), 25 $\mu\text{g ml}^{-1}$; and rifampicin (Rif), 50 $\mu\text{g ml}^{-1}$.

Effect of synthetic DAPG on adhesion and motility of *A. brasilense* Sp245-Rif.

The effect of synthetic DAPG (Toronto Research Chemicals Inc., North York, Canada) on adhesion of *A. brasilense* Sp245-Rif to polystyrene was investigated in 24-well microtiter plates (Nalgene NUNC, Roskilde, Denmark). DAPG was dissolved in methanol (from 0.0001 to 1 mM DAPG) and 15 μl was added to 1.5 ml of ABmal medium in each well. Methanol (1% vol/vol) was used as DAPG-negative control. Sp245-Rif was inoculated at 1.5×10^6 cells ml⁻¹ by adding aliquots from a 48-h Nfb* culture. The effect of DAPG concentrations (from 0.001 to 10 μM DAPG) was analyzed twice using independent Sp245-Rif cell cultures and four replicates per treatment ($n = 8$). The microtiter

plates were incubated 6 days at 28°C, and biofilm formation was measured as described by Dorel and associates (1999). Briefly, the liquid medium (and the planktonic bacteria it contained) was carefully removed using a pipette. Biofilms developed on polystyrene surfaces were gently washed off using 500 µl of ABm medium, and biofilm bacteria were suspended in 1.5 ml of cold ABm medium by pipetting up and down. In both samples (planktonic bacteria and biofilm bacteria), cell numbers were estimated by measuring the optical density at 600 nm (OD₆₀₀). Adhesion was computed as the ratio between biofilm bacteria and total bacteria.

The effect of synthetic DAPG on motility of *A. brasilense* Sp245-Rif was assessed on soft agar LBm plates containing 0.2% (swimming ability) or 0.7% (swarming ability) noble agar (Difco Laboratories, Detroit) and 0.001 to 100 µM DAPG. Methanol (1% vol/vol) was used as a DAPG-negative control. Strain Sp245-Rif was inoculated at 1×10^7 cells by adding 5 µl of overnight LBm culture on top of the plates. The plates were incubated for 3 days face up at 28°C; then, diameters of motility halos were measured. The analysis was done twice using independent Sp245-Rif cell cultures, with three replicates per treatment ($n = 6$).

Effect of synthetic DAPG on PHB accumulation by *A. brasilense* Sp245-Rif.

The impact of 1% (vol/vol) methanol (DAPG-negative control) and DAPG (0.001 to 100 µM) on PHB accumulation inside intact cells of *A. brasilense* Sp245-Rif was evaluated by staining cells with Nile Red, as described by Degelau and associates (1995). For all staining assays, Nile Red (1 mg ml⁻¹) was prepared in acetone. Planktonic Sp245-Rif cells (obtained as described above) were diluted in phosphate buffer saline (PBS) to reach 10^6 cells ml⁻¹, based on OD₆₀₀ measurements. An aliquot of 0.5 ml of Sp245-Rif bacterial suspension was mixed with 0.5 ml of cold absolute ethanol and 10 µl of Nile Red solution. The mixture was incubated for 30 to 40 min at room temperature. Because fluorescence signals are proportional to cell PHB content (Degelau et al. 1995), red fluorescence level was quantified by flow cytometry, using a FACS-Canto II (Becton Dickinson Biosciences, Pont de Claix, France)

fitted with a HeNe laser emitting at 633 nm and with an allophycocyanin filter for emission. FACSDiva and WinMDI software were used for acquisition and parameter analysis, respectively.

Effect of synthetic DAPG on IAA production ability of *A. brasilense* Sp245-Rif.

To assess the effect of synthetic DAPG on IAA production ability of *A. brasilense* Sp245-Rif(pOT1e-*ppdC-egfp*) (i.e., DFI clone IIC3), the strain was grown in 1.5 ml of ABmal (in 24-well microtiter plates) supplemented with tryptophan at 100 µg ml⁻¹ and either 1% (vol/vol) methanol (DAPG-negative control) or DAPG (0.001 to 10 µM). The plates were incubated for 6 days at 28°C and cell density was estimated based on OD₆₀₀ measurements. IAA production ability was investigated at two complementary levels. First, the enhanced green protein fluorescence of the *ppdC-egfp* fusion was measured in 150 µl of each culture, using a Xenius spectrofluorimeter microtiter plate reader (Safas, Monaco, France) (excitation at 488 nm, emission at 510 nm, wavelength slit of 10 nm). Second, 100 µl of each culture was removed and centrifuged, and the supernatant was assayed for the presence of IAA and related indole compounds using the PC Salkowski colorimetric method as described by Glickmann and Dessaux (1995).

Promoter trapping of *A. brasilense* Sp245-Rif genes induced by synthetic DAPG.

DFI and flow cytometry cell sorting were used to screen the *egfp* promoter-trap library PTL-AbP (Pothier et al. 2007) and identify DAPG-inducible promoters in *A. brasilense* Sp245-Rif. The library, with 96,000 clones, represents nearly a three-fold coverage of the Sp245 genome, and the representativeness of the *A. brasilense* Sp245-Rif promoter-trap library was significant (Pothier et al. 2007).

Ap, Rif, and Gm were added to all growth media used afterward. An aliquot (25 µl) from the PTL-AbP library was used to inoculate 5 ml of ABmal medium. After an 8-h incubation (i.e., until exponential phase), 250 µl were transferred to 25 ml of ABmal medium supplemented with 0.01 or 10 µM DAPG. Cells were incubated for 40 h (i.e., until stationary phase) and

Table 3. Bacterial strains, plasmids, and libraries used in this study

Strains, plasmids and library	Description ^z	Reference or source
Strains		
<i>Azospirillum brasilense</i> Sp245-Rif	Root-proficient, spontaneous rifampicin-resistant mutant of Sp245	Pothier et al. 2007
Sp245-Rif(pR2.54)	DFI clone DFI-32 (i.e., Sp245-Rif strain containing <i>PnirK-egfp</i> transcription fusion in pOT1e; Gm ^r)	Pothier et al. 2007
Sp245-Rif(pOT1e- <i>soxR-egfp</i>)	Positive fluorescent control strain for the establishment of flow cytometry parameters	Pothier et al. 2007
Sp245-Rif(pOT1e- <i>flgE-egfp</i>)	DFI clone IIA3 (i.e., Sp245-Rif strain containing <i>PflgE-egfp</i> transcription fusion in pOT1e; Gm ^r)	This study
Sp245-Rif(pOT1e- <i>nifX-egfp</i>)	DFI clone IB8 (i.e., Sp245-Rif strain containing <i>PnifX-egfp</i> transcription fusion in pOT1e; Gm ^r)	This study
Sp245-Rif(pOT1e- <i>ppdC-egfp</i>)	DFI clone IIC3 (i.e., Sp245-Rif strain containing <i>PppdC-egfp</i> transcription fusion in pOT1e; Gm ^r)	This study
<i>Pseudomonas fluorescens</i> F113	Strain isolated from sugar beet	Shanahan et al. 1992
<i>P. fluorescens</i> F113G22	F113 derivative, with a Tn5::lacZY insertion in <i>phl</i> operon, Km ^r	Shanahan et al. 1992
<i>Escherichia coli</i> DH5α	Host for cloning; F ⁻ φ80lacZΔM15 Δ(lacZYA-argF)U169 <i>deoR recA1 endA1 hsdR17(r_k⁻/m_k⁺) phoA supE44 thi-1 gyrA96 relA1 λ⁻</i>	Invitrogen
<i>E. coli</i> S17.1 λ <i>pir</i>	Host for conjugal plasmid transfer; <i>recA endA thi pro hsdR</i> Δ ⁺ ΩRP4-2-Tc::Mu-Km::Tn7; Sm ^r Tp ^r	de Lorenzo and Timmis 1994
Plasmids		
pOT1e	Promoter probe vector based on pBBR1MCS-5 replicon. Contains promoterless <i>egfp</i> and MCS between two transcriptional terminators; Gm ^r	Allaway et al. 2001
Promoter library		
PTL-AbP	Promoter trap library in <i>A. brasilense</i> Sp245-Rif containing 1- to 3-kb fragments upstream <i>egfp</i> in pOT1e	Pothier et al. 2007

^z Gm^r, Km^r, Sm^r, and Tp^r = resistant to gentamicin, kanamycin, streptomycin, and trimethoprim, respectively.

sorted, retaining fluorescent cells. This was done with an FACS Vantage SE (Becton Dickinson Biosciences) fitted with a 15-mW argon laser emitting at 488 nm, using the single-cell mode of the apparatus. Sterile (0.22- μ m filtered) PBS was used as sheath fluid for analysis and for cell sorting. In total, 10^7 cells were assessed, which ensured full coverage of the PTL-AbP library based on the formula of Daugherty and associates (2000). Prior to cell sorting or analysis, flow cytometry parameters were set using *A. brasilense* Sp245-Rif(pOT1e) as the nonfluorescent control and one *A. brasilense* clone, Sp245-Rif(pOT1e-*soxR-egfp*), expressing *egfp* constitutively as the positive control (Pothier et al. 2007). This resulted in the selection of the 11.7 and 2.31% most fluorescent cells among the 10^7 cells assessed for the 0.01 and 10 μ M DAPG conditions, respectively, each of which was gathered into a 5-ml BD Falcon tube (Dickinson Biosciences) and transferred to 20 ml of ABmal medium. After 40 h of incubation, the cells were sorted again, retaining nonfluorescent cells (approximately 58 and 4.5% for 0.01 and 10 μ M DAPG experiments, respectively), in order to discard constitutive promoters.

For final selection, the sorted cells were transferred to ABmal medium, grown for 40 h, induced again with DAPG at 0.01 or 10 μ M during 40 h, and sorted. The most fluorescent cells (4.34 and 15.0% for 0.01 and 10 μ M DAPG, respectively) were subsequently distributed into wells (in 96-well plates) containing LBm agar and incubated for 2 days at 28°C (filling 20 and 10 microtiter plates for the 0.01 and 10 μ M DAPG conditions, respectively).

Confirmation of DAPG induction of the clones was sought by incubating each clone in ABmal medium containing 1% methanol or DAPG (0.01 or 10 μ M depending on DFI condition) for 48 h and measuring fluorescence using a spectrofluorimeter microtiter plate reader as described above. Fluorescence intensity of sorted clones (i.e., I_{abs}) and Sp245-Rif(pOT1e) control (i.e., I_{abc}), as well as OD₆₀₀ of sorted clones (i.e., OD_{600s}) and Sp245-Rif(pOT1e) control (i.e., OD_{600c}) were measured, using growth medium as blank. Relative fluorescence intensity (Ir) was calculated based on the formula: $Ir = I_{abs}/OD_{600s} - I_{abc}/OD_{600c}$. Individual clones showing at least a 1.3-fold increase in relative fluorescence intensity compared with the methanol condition were selected.

Sequence analysis of Sp245-Rif DFI clones' promoters.

To characterize DAPG-upregulated Sp245-Rif DFI clones selected by DFI promoter trapping, their inserts were amplified with PCR primers F2603 and F2604 (Pothier et al. 2007). PCR amplifications were performed according to the *Taq* polymerase manufacturer (Invitrogen, Cergy Pontoise, France). In each reaction tube, 100 ng of template DNA was used in 50 μ l. The amplification cycle consisted of an initial 5 min at 95°C; 35 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s (or 90 s for large amplicons) at 72°C; followed by a final 7-min extension at 72°C. For large inserts (> 2kb), the plasmids were isolated from the DFI clones using the NucleoSpin Plasmid kit (Macherey-Nagel, Hoerd, France) and were sequenced with primer F2604. Sequencing was performed by Cogenics (Meylan, France) and sequences were analyzed with BLASTN and BLASTX algorithms (Altschul et al. 1997). ORF identification was done with FramePlot 2.3.2 (Ishikawa and Hotta 1999). Preliminary data of the *A. brasilense* Sp245 genome sequencing project were used for ORF prediction. Sequences obtained in the current work are deposited under GU291803 to GU291854.

In vitro DAPG induction of Sp245-Rif DFI clones.

The in vitro induction of four Sp245-Rif DFI clones was assessed by spectrofluorimetry analysis of *egfp* expression, as

follows. Each clone was grown for 24 h in ABmal Gm Rif medium. Culture aliquots (in volumes containing 1.5×10^7 cells) were transferred to 1.5 ml of fresh ABmal Gm Rif medium containing 1% methanol (control), 0.01 or 10 μ M DAPG, and grown for 48 h at 28°C. Strain Sp245-Rif(pOT1e) was used as nonfluorescent control. Quantitative analysis of fluorescence in bacterial cultures (100 μ l) was done as described above.

In planta induction experiments.

Seed of spring wheat (*Triticum aestivum* cv. Fiorina, 10 g), obtained from Florimond-Desprez (Cappelle en Pévèle, France), were surface-sterilized as described by Pothier and associates (2007), and germinated on water agar (8 g liter⁻¹) for 3 days in the dark at 28°C. The inoculants were obtained by overnight growth in liquid SA-Fe for *P. fluorescens* F113 and its *phl*-negative mutant F113G22 or ABmal Gm for *A. brasilense* strains. The cells were collected by centrifugation (6,000 rpm), gently washed, and resuspended in 0.8% NaCl solution, and the suspensions adjusted (based on OD₆₀₀) to 1×10^7 (for *Pseudomonas* spp.) or 2×10^8 CFU ml⁻¹ (for *Azospirillum* spp.). Inoculation of each *A. brasilense* Sp245-Rif DFI clone, alone or 2 days later together with F113 or F113G22, was done by adding 50 μ l of the corresponding cell suspension per seedling. The same volume of 0.8% NaCl solution was added for single inoculations or in the noninoculated control. Square plates (120 by 120 by 17 mm (Greiner Bio One, Frickenhausen, Germany) containing water agar (8 g liter⁻¹) and four seedlings were used. The plates were placed vertically for 7 days in a growth chamber at 20°C with 16 h of light (150 μ E m⁻² s⁻¹) and 8 h of darkness. Four plants (i.e., two plants from two independent experiments) were studied per treatment.

For confocal laser scanning microscopy, root pieces 1 to 2 cm in length were mounted in Aqua-Poly/Mount (Polysciences, Eppelheim, Germany) and examined immediately. Semiquantitative comparisons of fluorescence intensities of DFI clones on roots were made using a 510 Meta microscope (Carl Zeiss S.A.S., Oberkochen, Germany) equipped with an argon-krypton laser, detectors, and filter sets for green fluorescence (i.e., 488 nm for excitation and 510 to 531 nm for detection). After acquisition of the transmitted image (in bright-field mode), the two single-color images were overlaid as a single image using LSM software (release 3.5; Carl Zeiss S.A.S.). At least two independent root systems were analyzed per treatment and five images per root system, and the whole experiment was done twice. The same detector amplification gains were used for semiquantitative comparison of fluorescence levels. The *bio-Image_L* software 1.0 was used to quantify the threshold level of green fluorescence intensity in the images analyzed and the surface area covered by green fluorescent cells (Chávez de Paz 2009).

Plant-growth promotion assay.

To assess growth promotion of spring wheat cv. Fiorina by *A. brasilense* Sp245-Rif in coinoculation with *P. fluorescens* F113 or F113G22, strain Sp245-Rif was grown overnight in 50 ml of ABmal medium. Cells were centrifuged and resuspended in 0.8% NaCl solution to obtain 10^9 cells ml⁻¹. Then, 5×10^8 Sp245-Rif cells were mixed with 50 ml of water agar (0.8%), which was introduced into 120-by-120-by-17-mm square plates. Noninoculated plants were used as control. Four disinfect seeds (as described above) were then laid onto the plates, which were incubated vertically in a growth chamber at 20°C with 16 h of light (150 μ E m⁻² s⁻¹) and 8 h of darkness. Then, 2 days later (to avoid any growth inhibition effect of strain Sp245-Rif), 50 μ l containing 10^6 cells of *P. fluorescens*

F113 or F113G22 or 50 µl of 0.8% NaCl were added on seeds. At 7 days, root system architecture was examined by image analysis (WinRHIZO 2002c; Régent Instrument Inc., Québec City, Canada) to quantify total root length, root surface area, average root diameter, total root volume, and root numbers, using at least 20 plants (five plates containing 4 plants) per treatment.

Statistical analysis.

For PHB contents, the area under the curve of emitted fluorescence (660 nm) was taken and log transformed, and its relation with DAPG concentration was assessed by linear regression (linear model, $P < 0.05$) using S-plus software (TIBCO Software Inc., Palo Alto, CA, U.S.A.). Plant and bacterial data were processed by analysis of variance followed, when appropriate, with Fisher's least significant difference tests or by Kruskal-Wallis paired comparison. Stats were done at $P < 0.05$, using S-plus software 6.1.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

National Center for Biotechnology Information BLAST database:
www.ncbi.nlm.nih.gov/BLAST
 National Institutes of Health BLASTN and BLASTX database:
<http://www.ncbi.nlm.nih.gov/BLAST>
 National Institutes of Health Watson database: watson.nih.gov
 Oak Ridge National Laboratory *Azospirillum brasilense* Sp245 analysis
 data: genome.ornl.gov/microbial/abra/23apr08