RESEARCH ARTICLE

Genome wide profiling of *Azospirillum lipoferum* 4B gene expression during interaction with rice roots

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Abstract

Azospirillum-plant cooperation has been mainly studied from an agronomic point of view leading to a wide description of mechanisms implicated in plant growth-promoting effects. However, little is known about genetic determinants implicated in bacterial adaptation to the host plant during the transition from free-living to root-associated lifestyles. This study aims at characterizing global gene expression of *Azospirillum lipoferum* 4B following a 7-day-old interaction with two cultivars of *Oryza sativa* L. *japonica* (cv. Cigalon from which it was originally isolated, and cv. Nipponbare). The analysis was done on a whole genome expression array with RNA samples obtained from planktonic cells, sessile cells, and root-adhering cells. Root-associated *Azospirillum* cells grow in an active sessile-like state and gene expression is tightly adjusted to the host plant. Adaptation to rice seems to involve genes related to reactive oxygen species (ROS) detoxification and multidrug efflux, as well as complex regulatory networks. As revealed by the induction of genes encoding transposases, interaction with root may drive bacterial genome rearrangements. Several genes related to ABC transporters and ROS detoxification display cultivar-specific expression profiles, suggesting host specific adaptation and raising the question of *A. lipoferum* 4B/rice cv. Cigalon co-adaptation.

Introduction

Rhizosphere constitutes an important microhabitat characterized by sustainable interactions between plants and rhizobacteria, which are essential for plant-growth and health. Plants exude up to 11% of fixed carbon via their roots, supporting rhizosphere microbial populations (Bais *et al.*, 2006; Jones *et al.*, 2009). In return, rhizobacteria provide nutrients and improve plant growth via specific mechanisms such as nitrogen fixation and phytohormone secretion (Richardson *et al.*, 2009). These beneficial interactions involve rhizobial symbionts (mutualism) or plant growth-promoting rhizobacteria (PGPR, cooperation). Whereas mutualistic associations require partner recognition and a specific molecular crosstalk between plants and invading bacteria (Oldroyd *et al.*, 2011), mechanisms involved in the cooperation between PGPR and plants have been overlooked (Drogue *et al.*, 2012).

Among PGPR, members of the genus *Azospirillum* are known to colonize roots of important cereals and other grasses, and constitute one of the dominant population in rice rhizosphere (Steenhoudt & Vanderleyden, 2000; Lu *et al.*, 2006). Several *Azospirillum* strains exert phytostimulatory effects on plant growth and crop yields, and therefore constitute a promising alternative to reduce chemical inputs in the context of sustainable agriculture (Bashan *et al.*, 2004). This plant growth-promoting effect was originally attributed to *Azospirillum* ability to fix atmospheric nitrogen, but the contribution of *Azospirillum* biological nitrogen fixation in plant growth promotion is still debated (Bashan & de-Bashan, 2010). It is well admitted that *Azospirillum* PGPR effect is mainly due to the production of several phytohormones allowing an increase in the number of lateral roots and root hairs, which results in higher nutrient and water uptake by the plant (Somers *et al.*, 2004). Nevertheless, production of nitric oxide (NO) was also evidenced as strongly involved in the *Azospirillum*-induced root branching (Molina-Favero *et al.*, 2008). Next to increasing the number of lateral roots and root hairs, *Azospirillum* also enhances root exudation

(Heulin *et al.*, 1987) and modifies the chemical structure of root cell wall (El Zemrany *et al.*, 2007). More recently, the composition of plant secondary metabolites was shown to vary according to *Azospirillum* strain/plant cultivar combinations, reviving the question of host specificity in phytostimulating rhizobacteria (Walker *et al.*, 2011; Drogue *et al.*, 2012; Chamam *et al.*, 2013). So far, global analyses of *Azospirillum* were performed only by means of bacterial cultivation in presence of root exudates (Van Bastelaere *et al.*, 1999; Pothier *et al.*, 2007) or auxin indole-3-acetic acid (Van Puyvelde *et al.*, 2011), but up till now, no global response of bacterial cells directly grown in contact with the plant has been realized.

When inoculated on two rice cultivars *Oryza sativa* L. *japonica* group (cv. Cigalon and cv. Nipponbare), *Azospirillum lipoferum* 4B displays a similar rhizoplane colonization pattern on both cultivars, but promotes plant growth and modifies secondary metabolic profiles more dramatically on its original cultivar Cigalon (Chamam *et al.*, 2013). In order to identify bacterial genes regulated during *Azospirillum*-rice cooperation and distinguish genes potentially involved in cultivar-specific interaction, a global gene expression analysis of *A. lipoferum* 4B cells associated with rice roots of the two aforementioned cultivars was performed. Global gene expression was monitored on a whole genome expression array based on the genome sequence of *A. lipoferum* 4B (Wisniewski-Dyé *et al.*, 2011), with RNA samples obtained from root-adhering bacteria. This study provides an overview of *Azospirillum* gene expression during the cooperation with rice roots.

Materials and methods

Bacterial strain and growth conditions

The plant growth promoting bacteria *A. lipoferum* 4B (Thomas-Bauzon *et al.*, 1982) was grown overnight (180 r.p.m.) at 28 °C in nitrogen-free basal broth supplemented with 2.5% of low salt Luria-Bertani, that is, Nfbm, as described by Vial *et al.* (2006). Bacterial cells were harvested in late-exponential phase, that is, at OD_{580} around 1.2.

Seed sterilization, germination conditions, plant inoculation, and plant growth conditions

Two rice (*O. sativa* L.) cultivars belonging to the Japonica group, cv. Cigalon (C. Louvel, Centre Français du Riz, Arles, France) and cv. Nipponbare (J.B. Morel, BGPI, Montpellier, France) were used. Rice seeds were surface sterilized by washing for 40 min in a sodium hypochlorite solution containing 1 g of Na_2CO_3, 30 g of NaCl, and 1.5 g of NaOH per liter of distilled water (Hurek *et al.*, 1994). Seeds were then rinsed five times for 3 min in demineralized sterile water, and chlorine traces were removed by washing three times for 7 min in sterile-filtered 2% (w/v) sodium thiosulfate, and by rinsing five times for 3 min in demineralized sterile water (Miché *et al.*, 2003). Surface sterilized seeds were germinated on sterile plant agar (8 g L^{-1}; Sigma Chemical Co, Saint Louis, MO) for 2 days in the dark at 28 °C. A 10 mL aliquot of bacterial cells in late-exponential phase was transferred in a 50 mL BD Falcon™ tube (BD, Franklin Lakes, NJ) for further RNA plantktonic cell extraction (see below); the rest of the culture was centrifuged, resuspended at a concentration of 2.10^9 cells mL^{-1}, mixed with 50 mL of plant agar (8 g L^{-1}; to a final concentration of 2.10^7 cells mL^{-1}) and introduced into 120 × 120 × 17 mm square plates as previously described (Chamam *et al.*, 2013). For both rice cultivars, five disinfected germinated seeds were laid onto the plates and 30 plates were realized. All the plates were incubated vertically, for 7 days in a growth chamber (MLR350; SANYO, UK) with a photoperiod of 16 h at 28 °C (light 150 μE m^{-2} s^{-1}), and 8 h at 22 °C in the dark. Two inoculations were performed independently.

Growth of bacteria on artificial root surfaces

In order to mimic root surface, four cellulose acetate filters (Sartorius Stedim Biotech GmbH, Goettingen, Germany) were used as artificial root surfaces and placed onto 120 × 120 × 17 mm square plates (Greiner Bio-One Ltd, Stonehouse, UK) containing 50 mL of Nfbm plant agar (8 g L^{-1}). Three hundred microliters of bacterial cell suspensions used for plant inoculation was inoculated on top of each filter. All the plates containing the inoculated artificial-root devices were incubated in the same conditions as for plant growth experiments. Two inoculations were performed independently resulting in two independent samples per conditions.

Bacterial cells isolation from planktonic, sessile, and plant conditions

Four different conditions (two independent samples per condition) were used for the transcriptome analysis: a planktonic condition (liquid shaken culture), a sessile condition corresponding to the artificial-root device and two plant conditions corresponding to inoculation of rice plantlets. For the planktonic condition, 20 mL of RNA-protect Bacteria Reagent (Qiagen, Courtaboeuf, France) was added to 10 mL of bacterial cells in late-exponential phase and the mixture was centrifuged during 20 min, at 15 °C, 13 000 ***g***. The supernatant was discarded and the pellet was immediately frozen using liquid nitrogen and

stored at −80 °C. For the sessile and the plant conditions, 2 × 4 filters and 2 × 35 plant root systems were respectively pooled in two 50 mL BD Falcon™ tubes containing 8 mL of TE buffer and 16 mL of RNAprotect Bacteria Reagent (Qiagen). Bacterial cells were recovered by vortexing vigorously, four times for 1 min. For each condition, the content of the two 50 mL BD Falcon™ tubes (i.e. bacteria recovered from eight filters or 70 plant root systems for each cultivar) was pooled into a new tube and centrifuged during 20 min, at 15 °C, 13 000 ***g***. Supernatants were discarded and the pellets were immediately frozen using liquid nitrogen and stored at −80 °C.

RNA isolation, amplification, and cDNA synthesis

For each condition (two independent samples per condition), the bacterial cell pellet (10^9 cells for planktonic and sessile conditions, 10^8 cells for plant conditions) was resuspended in 960 μL of suspension buffer (Prigent-Combaret *et al.*, 2012) and transferred in 1.5-mL tubes containing 400 mg of glass beads (Sigma). Cell lysis was realized by shaking for 1 min with the TissueLyser II equipment (Qiagen), cooling for 2 min at 4 °C and shaking again for 1 min. After a centrifugation of 5 min, at 4 °C, 15 500 ***g***, the aqueous phase containing ribonucleic acids was recovered, and 1 mL of TRIzol® Reagent (Invitrogen, Carlsbad, CA) was added. After incubation of 5 min at room temperature, 100 μL of phenol/chloroform/isoamyl alcohol (25 : 24 : 1) were added, the samples were homogenized, incubated during 5 min at room temperature and centrifuged for 10 min, at 4 °C, 15 500 ***g***. A second phenol/chloroform/isoamyl alcohol extraction (200 μL) was done and ribonucleic acids were precipitated overnight at −20 °C in a solution containing two volumes of 100% ethanol, 0.1 volume of 7.5 M ammonium acetate, and 0.01 volume of 5 g L^{-1} glycogen. Samples were centrifuged during 15 min, at 4 °C, 15 500 ***g***, and the pellets were rinsed twice with 70% ethanol before resuspension. About 10 μg of RNA was obtained for planktonic and sessile conditions, and 1 μg for plant conditions. PCR (16S rRNA gene) on all samples confirmed that the DNase I (Invitrogen) treatment had removed all remaining DNA. RNA integrity was assessed using Agilent RNA 6000 Pico Kit (Agilent Technologies, Waldbronn, Germany) and the Agilent 2100 Bioanalyzer (Agilent Technologies) device.

In order to increase mRNA representation in RNA samples, 1 μg of total RNA was digested with mRNA ONLY™ Procaryotic mRNA isolation kit (Epicentre Biotechnologies, Madison, WI) according to the manufacturer's protocol. The RNA samples were then amplified using MessageAmp™II-Bacteria Kit (Ambion Inc, Austin, TX), with an amplification step of 6 h in order to obtain enough RNA for cDNA synthesis and to minimize amplification associated bias, according to Spiess *et al.* (2003). Amounts of amplified RNA ranged from 10 to 50 μg.

The microarray cDNA was synthesized with the SuperScript® Double-Stranded cDNA Synthesis Kit (Invitrogen) following the provided protocol. After optimization, the use of a mix (1 : 1) of random primers (Promega Corporation, Madison, WI) and Oligo-dT(15) primers (Promega) was chosen to maximize the length and the quantity of cDNA fragments obtained.

Microarray design, hybridization and data analysis

An *A. lipoferum* 4B whole genome expression array (4 × 72 K) was designed by Roche Nimblegen, Inc. (Madison, WI), based on the genome sequence (Wisniewski-Dyé *et al.*, 2011), as follows: five probes (length, 60 nucleotides) per gene, covering 6154 genes (127 genes with no probes, 44 transcripts with < 5 probes) and two replicates of probes per 72 K (technical replicates). Each cDNA sample was labeled (Cy3) and hybridized by Roche Nimblegen according to their standard protocol. The eight cDNA samples (four conditions, two independent replicates) were randomly distributed on microarrays.

Data preprocessing and analysis were performed using ARRAYSTAR 4 software (DNASTAR, Inc., Madison, WI) and the web available Analysis of NimbleGen Arrays Interface (ANAIS; Simon & Biot, 2010). The robust multi-array average method associated with quantile normalization was applied at probe values (Bolstad *et al.*, 2003; Irizarry *et al.*, 2003). Probe values were summarized to gene values using median polish procedure. Analysis of variance with a false discovery rate (FDR) adjustment method was applied to determine the FDR adjusted *P*-value (P_{adj}; Benjamini & Hochberg, 1995) and genes differentially expressed were selected using a P_{adj} threshold of 0.05 and Log_2 fold change ($|Log_2(FC)|$) cutoff of 1. In a first analysis, the planktonic condition was used as reference to determine genes regulated in the sessile condition (Supporting Information, Table S2). In a second analysis, both the planktonic and the sessile conditions were used to evidence genes differentially expressed during the interaction with rice roots (Tables S3 and S4).

The data have been submitted in the National Center for Biotechnology Information Gene Expression Omnibus (GEO accession number IGSE42450).

RT-qPCR

Gene expression levels were validated by performing reverse transcription quantitative real-time PCR (RT-qPCR) on a group of nine representative genes using LightCycler® 480 SYBR Green I Master kit (Roche Diagnostics GmbH, Mannheim, Germany) on a LightCycler® 480 Real-Time PCR System (Roche; Table S5). Two genes showing an invariant expression were used as reference genes: *uppS* (AZOLI_1074) and *nadE* (AZOLI_p30432). Single strand cDNA was synthesized using the Omniscript® Reverse Transcription kit (Qiagen). An amount of 500 ng of total RNA used in the array experiment was incubated for 5 min at 65 °C with 2 μL of random primers (Promega) in a final volume of 14.75 μL. A volume of 5.25 μL of a solution containing 2 μL of 10× Buffer, 2 μL of 5 mM dNTPs, 1.25 μL of 10 units μL^{-1} RNase inhibitor, and 1 μL of 4 units μL^{-1} Omniscript Reverse Transcriptase, was added to each sample before incubating for 1 h at 37 °C. From these reactions, 2 μL of cDNA were used as template for RT-qPCR reaction. DNA contamination was checked with reactions that lacked reverse transcriptase as negative controls. Specific primers were designed using the Primer3Plus interface (Untergasser *et al.*, 2007) with the following criteria: product size ranges 150–250, primer size comprised between 20 and 21 bases, optimal primer T_m 60 °C (Table S1). Real-time PCR conditions were: a denaturation stage of 10 min at 95 °C; an amplification stage of 50 cycles of 30 s at 94 °C, 15 s at 65 °C and 15 s at 72 °C; and a melting curve stage of 5 s at 95 °C and 1 min at 65 °C increased to 97 °C with a ramp rate of 0.11 °C s^{-1}. All reactions were performed in three technical replicates and carried out in LightCycler 480 Multiwell plate 96 (Roche) with adhesive sealings foils (Roche) in a final volume of 20 μL containing 4 μL of nuclease free water, 2 μL of each primer (5 μM), 10 μL of master mix, and 2 μL of the template. Primer efficiencies were determined by standard curves with serial dilution of DNA (5 $\log_{10}$ concentrations). The planktonic control was used as the calibrator condition and relative gene expression was calculated using the Pfaffl method (Pfaffl, 2001).

Expression ratios obtained by RT-qPCR were plotted vs. the respective microarray values. Prior to performing correlation analyses, the data were tested for normality using the Shapiro–Wilk test. Because the data are not normally distributed, correlation analyses were done with Spearman's Rho test (Morey *et al.*, 2006). Further RT-qPCR validations were done prior to RNA amplification to ensure that no bias effects were introduced.

Results and discussion

Global gene expression analysis of *A. lipoferum* 4B cells

To identify *Azospirillum* genetic determinants regulated during the cooperation with rice and evaluate the impact of rice genotype on bacterial transcriptome, liquid cultures of *A. lipoferum* 4B (planktonic condition) were inoculated on roots of two rice cultivars (cooperative conditions), as well as on artificial-root devices (sessile condition). Global gene expression analysis was realized using microarray with RNA samples obtained from root-adhering bacteria recovered 7 days after inoculation, a stage at which enhanced rice root growth is detectable (Chamam *et al.*, 2013). Rice inoculation was performed with bacterial cells grown to late-exponential phase as such *Azospirillum* cells were shown to accumulate poly-β-hydroxybutyrate (carbon storage compounds) and exopolysaccharides (cell aggregation), essential factors that improve stress resistance, survival and root attachment (Kadouri *et al.*, 2003; Bahat-Samet *et al.*, 2004). Recovery of a sufficient quantity of mRNA from root-adhering cells revealed to be a challenging task that prompted us to pool several root systems and to perform RNA amplification (see Materials and methods).

Hierarchical clustering analysis of microarray data reveals that cooperative conditions display the most dissimilar gene expression patterns compared to planktonic

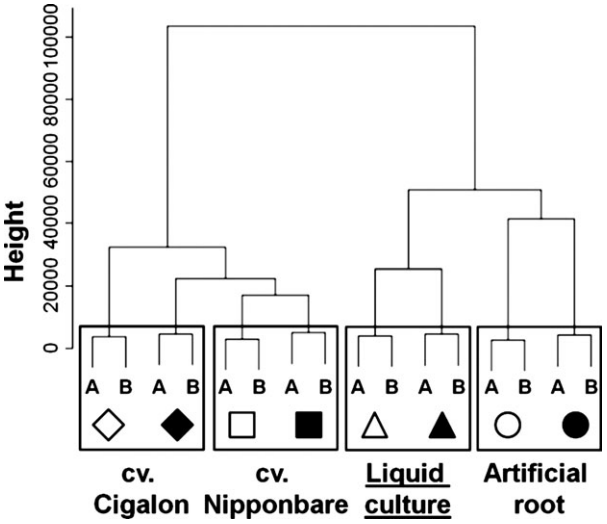

Fig. 1. Hierarchical clustering analysis of *Azospirillum lipoferum* 4B gene expression data. Triangles indicate gene expression data for the planktonic condition, circles for the sessile condition, squares for cv. Cigalon root-associated condition and diamonds for cv. Nipponbare root-associated condition. Black and white symbols indicate the two independent biological replicates. A and B letters indicate the two probe replicates per slide (technical replicates).

and sessile conditions (Fig. 1). In a first analysis, genes differentially expressed in *Azospirillum* cells recovered from artificial-root devices were determined using the planktonic condition as a reference (Table S2). In a second analysis, both planktonic (P) and sessile (S) conditions were used as references to identify differentially expressed genes during the cooperation with each rice cultivar, resulting in two sets of differentially expressed genes for cv. Cigalon (4B_CigS and 4B_CigP) and two sets of differentially expressed genes for cv. Nipponbare (4B_NipS and 4B_NipP; Tables S3 and S4). About 40% of up-regulated genes and 50% of down-regulated genes encode proteins of unknown function.

To validate microarray data, reverse transcription quantitative polymerase chain reaction (RT-qPCR) was performed on nine genes. Expression ratios obtained by RT-qPCR were plotted vs. the respective microarray values showing that the RT-qPCR is in agreement with microarray data (Fig. 2, Table S5).

Genes regulated in sessile cells compared with planktonic cells

A total of 148 genes are differentially expressed (34 up-regulated and 114 down-regulated) in cells recovered from artificial-root devices compared with planktonic cells (Table S2). Functional classification reveals that genetic determinants involved in (1) transport and binding proteins, (2) regulatory functions and signal transduction, (3) energy metabolism, (4) cellular processes, and (5) cell envelope are mostly repressed (Fig. 3). As expected, genes directly involved in chemotaxis and swimming motility of *Azospirillum* (*fliI1*, *flgB1*, *flgF1*, *fliQ3* *cheY1*, *cheY5*) are down-regulated in cells recovered from artificial-root devices. In addition, genes related or potentially related to membrane biosynthesis, *lpxC*, *lolD*, *mepA*, the putative glycosyl transferase AZOLI_2268 and the putative septum formation initiator AZOLI_1328 are repressed. On the contrary, differentially expressed genes related to protein synthesis (four genes), transcription (three genes) and central intermediary metabolism (two genes) are exclusively induced (Fig. 3). Two of the six *rpoH* copies harbored by *A. lipoferum* 4B genome (*rpoH4* and *rpoH6*) and four genes encoding ribosomal proteins (*rpsA*, *rpsN*, *rpsK*, and *rplM*) are up-regulated.

In agreement with microscopic observations evidencing that *Azospirillum* adhering to artificial-root devices form several layers of aggregated cells (data not shown), that is,

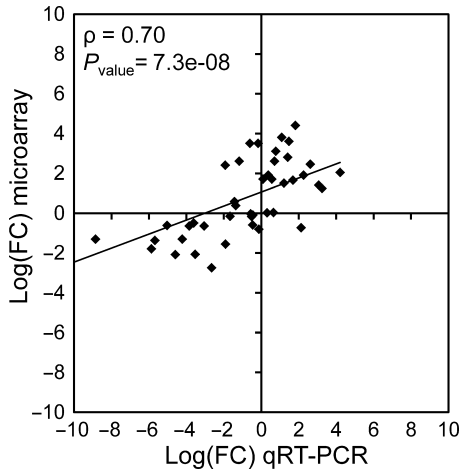

Fig. 2. Correlation of microarray and RT-qPCR results. Expression ratios of nine representative genes were determined using RT-qPCR, for 4B_CigP, 4B_CigS, 4B_NipP, 4B_NipS, and the sessile condition. Each microarray value (3 per gene) and RT-qPCR value (3 per gene) were $\log_2$ transformed and plotted against each other for comparison (Table S5). Correlation analyses were done with Spearman's Rho test.

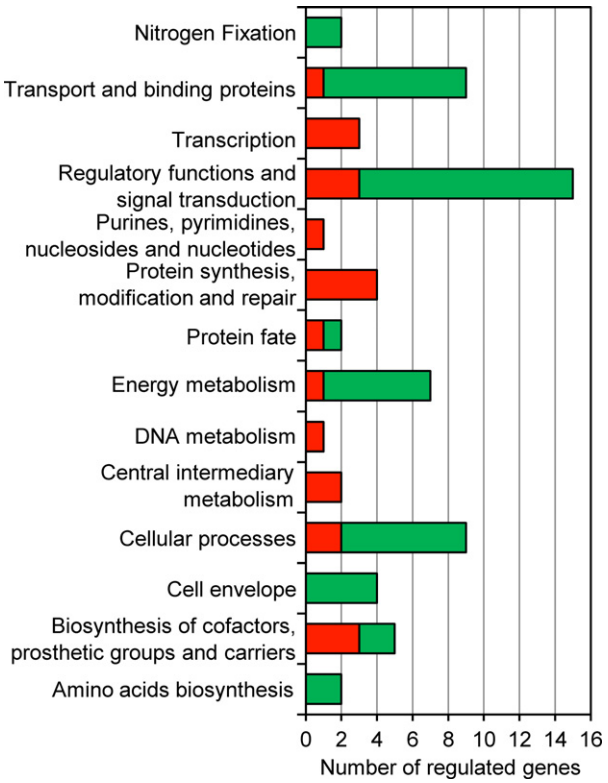

Fig. 3. Functional categories of genes regulated on artificial-root device. *Azospirillum lipoferum* 4B cells recovered from artificial roots were compared to planktonic cells (reference condition). The number of genes that are up-regulated (red) or down-regulated (green) are shown per functional category. The functional classification (Bioprocess) was done according to the AzospirilluScope database (https://www.genoscope.cns.fr/agc/microscope/home/index.php). Only 66 of the 148 genes are shown here, the remaining 82 genes encoding proteins of unknown function.

a spatial organization similar to the one observed in biofilms, expression profiles suggest that these cells grow in a sessile state. Indeed, flagellar motility was shown to be necessary for biofilm development of *Pseudomonas aeruginosa* but these structures were no longer required for maintenance of a mature biofilm (Whiteley *et al.*, 2001). In addition, several *rps* and *rpl* genes encoding ribosomal components were previously reported to be regulated in *Escherichia coli* and *P. aeruginosa* biofilms (Schembri *et al.*, 2003; Dötsch *et al.*, 2012).

In our study, 99 of the 148 genes differentially expressed on the artificial-root devices display the same regulation in both Cigalon- and Nipponbare-associated cells compared to planktonic cells, that is, 4B_CigP and 4B_NipP (Table S2); functional annotation could assign a role for only 45 of those 99 genes (Table 1). Genes involved in chemotaxis (*cheY1* and *cheY5*), motility (*flgF1* and *fliI1*), and membrane biosynthesis (*lolD*, *mepA* and *lpxC*) are repressed in both artificial-root device and rice-associated cells (4B_CigP and 4B_NipP). On the contrary, several genes related to stress response (*nhaA1*, *cspA2*, *msrA*) and two of the four genes encoding ribosomal proteins discussed above are induced in the three conditions. Interestingly, the gene encoding the transcriptional regulator FlcA, previously shown to control flocculation and wheat root surface colonization in *Azospirillum brasilense* Sp7 is also induced in these conditions (Pereg-Gerk *et al.*, 1998). All these results evidence similarities between the state of *A. lipoferum* cells grown on artificial-roots (sessile state) and the state of cells recovered from rice roots. As previously described for *Pseudomonas putida* recovered from maize rhizosphere, *A. lipoferum* root-associated cells grow in a sessile-resembling state (Matilla *et al.*, 2007). These results are in agreement with the rhizoplane colonization pattern repeatedly observed for *A. lipoferum* 4B (Chamam *et al.*, 2013).

Adaptation to the host plant

As described above, both planktonic and sessile conditions were used as references to determine genes regulated ($P_{adj} < 0.05$ and $|Log_2(FC)| > 1$) during the cooperation with each rice cultivar, resulting in four sets of genes: 4B_CigP and 4B_CigS for cv. Cigalon; 4B_NipP and 4B_NipS for cv. Nipponbare (Tables S3 and S4). For each cultivar, expression profiles were compared to unveil genes that are up-regulated or down-regulated whatever the reference (Fig. 4). As for cv. Cigalon, 76 up-regulated genes and 42 down-regulated genes were identified (including respectively 36 and 22 genes encoding proteins of unknown function). The association with cv. Nipponbare appears to induce a wider range of gene expression changes with 369 up-regulated genes and 66 down-regulated genes (including respectively 137 and 30 genes coding proteins of unknown function). While most of the genes related to (1) transport and binding proteins, (2) transcription, and (3) protein fate are induced on both cultivars, genes involved in regulatory functions and energy metabolism are mostly repressed for cv. Cigalon and mostly induced for cv. Nipponbare (Fig. S1). These results highlight a cultivar-dependent transcriptome response of *A. lipoferum* 4B established on rice roots, in the tested conditions.

Besides cultivar-specific responses, comparison of genes regulated by cv. Cigalon and cv. Nipponbare evidences that 75 genes are up-regulated and 26 genes are down-regulated regardless of the rice cultivar (Figs 4 and 5). The involvement of plant-mediated stresses in the established cooperation is reflected by the induction of genes involved in reactive oxygen species (ROS) detoxification like *ohr* (organic hydroperoxide resistance protein), *hybF* (maturation of hydrogenases 1 and 2), and AZOLI_p 50438 (putative oxidoreductase), as well as genes potentially involved in cell damage repair (Fig. 5). Among the latter, *msrA* and *msrB* genes encode a ubiquitous peptide methionine sulfoxide reductase known to be implicated in oxidized protein repair mechanisms (Ezraty *et al.*, 2005). The induction of genes encoding MDR efflux pumps of the RND family (*acrA2*), the phage shock protein operon (*pspABC*) and genes implicated in heat shock response (*hspD2*, *groES1*) indicates that *A. lipoferum* 4B faces and adapts to diverse stress conditions. In particular, the *psp* operon was shown to be significantly induced in *E. coli* cells associated to lettuce roots (Hou *et al.*, 2012).

These results suggest that ROS detoxification and multidrug efflux are important features of *A. lipoferum* 4B cooperative cells and not only during the very early stages of root colonization. This is consistent with the fact that ROS are continuously produced in plant and that root exudates contain a large number of compounds that mediate positive and negative plant–bacteria interactions (Bais *et al.*, 2006; Pauly *et al.*, 2006). Indeed, ROS were suggested to play a role in signaling processes during bacteria–plant symbioses (Pauly *et al.*, 2006), and a RND-type efflux system was reported to play a host-specific role in *Bradyrhizobium*-legume symbiosis (Lindemann *et al.*, 2010). Whereas the role of MDR pumps in the establishment of bacteria–plant interactions has been mainly investigated in phytopathogens, some pieces of evidence tend to demonstrate their implication in plant beneficial bacteria (Matilla *et al.*, 2007; Ramachandran *et al.*, 2011). Plant-exuded metabolites were also shown to regulate a wide range of *Azospirillum* genes, and some of them might induce both stress responses and signaling pathways (Pothier *et al.*, 2007; Van Puyvelde *et al.*, 2011).

Table 1. Sessile-regulated genes that display a similar regulation in Cigalon- and Nipponbare-associated *Azospirillum* cells*

			$Log_2(FC)^{\dagger}$		
Gene_ID	Gene name	Product	Sessile	4B_CigP	4B_NipP
Up-regulated genes					
AZOLI_0157	*msrA*	Peptide methionine sulfoxide reductase	1.21	3.80	4.28
AZOLI_0309		Acylphosphatase	3.05	1.63	1.46
AZOLI_0474	*rpsN*	30S ribosomal protein S14	1.44	1.71	2.64
AZOLI_0552		Monothiol glutaredoxin	3.17	4.08	4.89
AZOLI_1271	*rpoH4*	RNA polymerase sigma factor (32)	1.74	2.57	2.93
AZOLI_1336		Putative oligoketide cyclase/dehydratase	1.63	2.41	3.12
AZOLI_2205	*nuoB*	NADH-quinone oxidoreductase	2.21	1.28	3.45
AZOLI_p10093	*rplM*	50S ribosomal subunit protein L13	2.11	2.35	3.31
AZOLI_p19698	*rpoH6*	RNA polymerase sigma factor (32)	1.49	2.85	3.21
AZOLI_p30180	*nhaA1*	Sodium-proton antiporter	2.60	4.85	5.27
AZOLI_p40437	*cspA2*	Cold shock protein, DNA binding	3.13	2.45	2.63
AZOLI_p40461		Putative response regulator, LuxR/FixJ family	4.35	2.59	3.62
Down-regulated genes					
AZOLI_0086		Two-component response regulator	−1.06	−1.22	−1.10
AZOLI_0508		Putative acyl dehydratase	−4.70	−4.45	−2.51
AZOLI_0546		Fe-S cluster assembly protein	−4.10	−4.30	−4.51
AZOLI_0674	*nifE*	Nitrogenase Mo-cofactor synthesis	−2.79	−3.07	−3.27
AZOLI_1161		Acireductone dioxygenase ARD	−1.66	−2.48	−2.67
AZOLI_1232		Putative NADH-ubiquinone oxidoreductase	−3.25	−3.70	−2.90
AZOLI_1328		Putative septum formation initiator	−3.07	−3.09	−3.10
AZOLI_1738	*flgF1*	Flagellar basal-body rod protein FlgF	−1.13	−1.38	−1.10
AZOLI_1748		Putative transcriptional regulator, XRE family	−1.37	−1.28	−1.11
AZOLI_1890		Putative 2Fe-2S ferredoxin	−1.68	−3.37	−3.18
AZOLI_1914	*cheY5*	Chemotaxis response regulator CheY	−2.70	−2.32	−1.84
AZOLI_2140	*lpxC*	UDP-3-*O*-acyl GlcNAc deacetylase	−4.64	−3.86	−3.41
AZOLI_2177	*lolD*	Lipoprotein ABC transporter	−1.09	−1.54	−1.18
AZOLI_2268		Putative glycosyl transferase, family 2	−1.94	−2.55	−1.79
AZOLI_2311	*nifB*	Nitrogenase FeMo-cofactor synthesis	−5.11	−6.27	−5.48
AZOLI_2333		Putative transcriptional regulator (CheY-like)	−1.18	−4.09	−3.28
AZOLI_2396	*mepA*	Murein endopeptidase	−2.05	−2.21	−1.89
AZOLI_2425	*cheY1*	Chemotaxis response regulator CheY	−1.46	−1.30	−1.54
AZOLI_2578		Putative diguanylate phosphodiesterase	−3.18	−2.78	−2.67
AZOLI_2756		Putative transcriptional regulator, MarR family	−2.72	−2.96	−2.87
AZOLI_2795		Cytochrome *C* peroxidase	−2.08	−1.94	−2.17
AZOLI_2821		Putative transcriptional regulator (CheY-like)	−1.28	−1.51	−1.38
AZOLI_p20340		Putative chemotaxis regulator CheY	−3.26	−3.50	−3.48
AZOLI_p20355	*poxB*	Pyruvate oxidase	−1.08	−1.05	−1.38
AZOLI_p20550		Putative permease of the major facilitator superfamily	−1.42	−1.26	−1.51
AZOLI_p30036		Putative transcriptional regulator, LysR family	−2.11	−1.89	−2.05
AZOLI_p40131		Putative H+/gluconate symporter	−4.39	−2.81	−1.93
AZOLI_p40279		Transcriptional regulator, MarR family	−1.82	−2.71	−2.60
AZOLI_p40342		Putative Hydrogenase expression/formation protein hupK	−1.29	−2.28	−2.55
AZOLI_p40435	*fliI1*	Flagellum-specific ATP synthase	−3.76	−2.85	−2.93
AZOLI_p40473		Amino acid ABC transporter	−1.78	−2.11	−1.87
AZOLI_p40532		Protein-tyrosine-phosphatase	−1.66	−2.78	−2.54
AZOLI_p60016		Putative Fe-S oxidoreductase	−2.28	−3.09	−2.13

*Only genes with assigned functions are listed (i.e. 45 of 99 genes). The remaining genes (five up-regulated and 49 down-regulated) encode proteins of unknown function or conserved proteins of unknown function and are displayed in Table S2.

†Relative to planktonic condition, only genes differentially expressed in the three conditions (Sessile, 4B_CigP and 4B_NipP) are shown (Table S2).

Interestingly, adaptation to the host plant may also induce *A. lipoferum* 4B genome rearrangements (Fig. 5). Indeed, two copies of genes encoding transposases of insertion sequences are up-regulated (AZOLI_0073, AZOLI_0093) regardless of rice cultivar, indicating a potential enhanced transposition activity in root-associated cells. Because genome rearrangements mediated by insertion sequences may lead to gene inactivation or

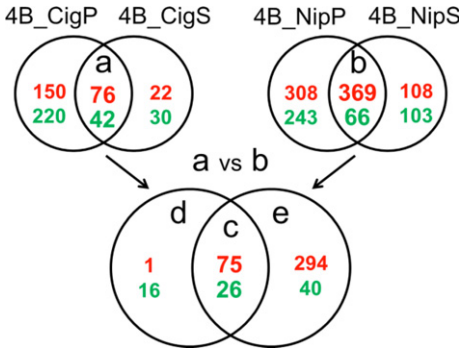

Fig. 4. Venn diagramm of genes differentially expressed in root-associated cells ($|Log_2(FC)| \geq 1$ and $P_{adj} < 0.05$). Two reference conditions (planktonic and sessile) were used to evidence genes up-regulated (red) and down-regulated (green) in *Azospirillum lipoferum* 4B associated with rice roots ($|Log_2(FC)| \geq 1$ and $P_{adj} < 0.05$). Genes regulated regardless of the reference condition were evidenced for cv. Cigalon (a) and cv. Nipponbare (b) by comparing the respective list of genes; 4B_CigP compared with 4B_CigS (Table S3) and 4B_NipP compared with 4B_NipS (Table S4). Then these two sets were compared to distinguish *A. lipoferum* 4B genes regulated regardless of the rice cultivar (c) from those specifically regulated with cv. Cigalon (d) or cv. Nipponbare (e).

neighboring-gene regulation, transposition is generally maintained at low levels in bacterial cells (Mahillon & Chandler, 1998). However, induction of transposases and genes involved in DNA rearrangements were reported for *P. putida* in maize rhizosphere (Matilla *et al.*, 2007). *In vitro*, several *Azospirillum* strains, including *A. lipoferum* 4B, display large-scale genomic rearrangements associated to phase variation; these events might occur in the rhizosphere as a nonswimming strain displaying all the features of the 4B variant has been isolated simultaneously and at the same frequency than strain 4B (Bally *et al.*, 1983; Vial *et al.*, 2006). Recent genomic analyses of *Azospirillum* revealed that most of the genes encoding critical functions for the association with plants were horizontally acquired (Wisniewski-Dyé *et al.*, 2011, 2012). Thus, understanding whether DNA rearrangements are induced by a general stress response or a particular plant signal may unravel mechanisms leading to *Azospirillum* genome evolution.

A high number of genes implicated in regulatory functions and signal transduction are regulated in cells interacting with rice roots of both cultivars (Figs 5 and S1). A total of 12 genes encoding transcriptional regulators that belong to various families (AraC, ArsR, GntR, OmpR, and TetR) are differentially expressed (nine up-regulated and two down-regulated), suggesting that different signals are perceived by *Azospirillum* in the root micro-environment. In particular, two genes encoding TetR regulators (AZOLI_1032, AZOLI_2103) are induced, so that members of the TetR family, known to control genes involved in multidrug resistance, catabolic pathways, osmotic stress resistance and pathogenicity, could play a key role in plant–bacterial signaling in the rhizosphere (Ramos *et al.*, 2005; Matilla *et al.*, 2007). Transcriptional regulators of the GntR family may also control key determinants of *Azospirillum*-plant cooperation, as previously suggested by the regulation of AZOBR_50003 in response to the presence of auxin (indole-3-acetic acid) in *A. brasilense* Sp245 liquid cultures (Van Puyvelde *et al.*, 2011). The induction of the *pchR* transcriptional regulator of pyochelin biosynthesis is of particular interest as pyochelin is a siderophore shown to be implicated in the induction of systemic resistance against fungus pathogen in tomato and rice (Audenaert *et al.*, 2002; De Vleesschauwer *et al.*, 2006). However, the *pch* operon is not differentially expressed in the tested conditions so that the role of *pchR* in *A. lipoferum* 4B-rice interaction should be further investigated.

Transcriptional regulation also involves the induction of RNA polymerase sigma factors. *Azospirillum* strains (and more particularly *A. lipoferum* 4B) harbor a remarkably high number of *rpoH* paralogues. While most of the *Alphaproteobacteria* harbor two copies of genes encoding RpoH sigma factors, this gene is present in five copies in the strains *A. brasilense* Sp7 and *A. brasilense* Sp245, whereas six copies are found in the *A. lipoferum* 4B genome (Wisniewski-Dyé *et al.*, 2011; Kumar *et al.*, 2012). Interestingly, two copies (*rpoH4* and *rpoH6*) are similarly induced in sessile cells associated to artificial and rice roots (see above) and one copy is induced (*rpoH1*) only in rice-associated cells, suggesting that *rpoH* alleles are finely regulated during the adaptation of *A. lipoferum* 4B to rice roots. Sigma factors are known to play a key role in bacteria–plant beneficial interactions and particularly in the expression of beneficial properties of bacterial symbionts. Indeed, *Rhizobium* and *Sinorhizobium* mutated in the *rpoH1* gene were affected in nitrogen fixation and nodule formation (Mitsui *et al.*, 2004; Martínez-Salazar *et al.*, 2009). Moreover, *rpoH* was suggested to regulate auxin production in *A. brasilense* (Spaepen *et al.*, 2007).

Surprisingly, genes involved in nitrogen fixation are not induced in root-associated cells, suggesting that no significant biological nitrogen fixation occurs in the tested conditions. However, the implication of nitrogen fixation in plant growth improvements mediated by *Azospirillum* is still debated and growth promotion is supposed to result from combination of unrelated mechanisms (Bashan & de-Bashan, 2010). Indeed, *A. lipoferum* 4B genome harbors other genetic determinants potentially involved in plant-beneficial functions such as *acdS* encoding a protein involved in 1-aminocyclopropane-1-carboxylate deamination and *nirK* encoding a protein involved in NO

Log(FC) 4B_CigS	Log(FC) 4B_NipS	Log(FC) 4B_CigP	Log(FC) 4B_NipP	Label	Gene	Product
4.3	4.4	5.6	5.7	AZOLI_0242	*pspB*	DNA-binding transcriptional regulator of *psp* operon
3.8	4.4	3.1	3.6	AZOLI_1541		sensor histidine kinase
3.3	3.7	3.3	3.9	AZOLI_0243	*pspC*	DNA-binding transcriptional activator of *psp* operon
3.1	4.6	3.2	4.7	AZOLI_p40056		putative N-acetyltransferase
4.2	4.7	4.1	4.6	AZOLI_0241	*pspA*	transcriptional regulator of *psp* operon
4.0	4.3	4.0	4.3	AZOLI_p10013		putative non-ribosomal peptide synthetase
3.7	4.6	3.9	4.8	AZOLI_p20171	*acrA2*	multidrug efflux transporter, AcrA component
3.5	4.7	4.1	5.2	AZOLI_p40055	*rpoH1*	RNA polymerase sigma factor (sigma32)
4.1	3.7	5.0	4.6	AZOLI_p30017	*hybF*	protein involved with the maturation of hydrogenases 1 and 2
2.6	3.2	3.8	4.3	AZOLI_0157	*msrA*	peptide methionine sulfoxide reductase
2.5	3.1	4.0	4.5	AZOLI_2686	*groES1*	small subunit of chaperonin GroESL
2.2	2.6	4.9	5.3	AZOLI_p30180	*nhaA1*	sodium-proton antiporter
2.5	3.0	3.2	3.7	AZOLI_0828		acyl-CoA thioesterase (Tol-Pal associated)
2.3	3.0	3.0	3.7	AZOLI_p50224	*msrB*	peptide methionine sulfoxide reductase msrB
2.6	3.8	2.2	3.4	AZOLI_p30494		putative glutathione S-transferase with thioredoxin-like domain
2.0	3.5	3.0	4.6	AZOLI_p30260		putative FeS cluster assembly protein
5.0	5.3	2.2	2.5	AZOLI_p30202		putative Formate/nitrite transporter
2.3	1.9	1.5	1.0	AZOLI_0073		transposase of ISAli3, IS630 family. ORFB
2.0	1.5	1.6	1.1	AZOLI_0093		transposase of ISAli3, IS630 family. ORFB
1.4	1.3	1.3	1.2	AZOLI_1893	*zur*	zInc uptake transcriptional regulator
1.7	3.5	1.7	3.5	AZOLI_2103		putative transcriptional regulator, TetR family
2.8	2.6	2.6	2.4	AZOLI_p20158	*pchR*	regulatory protein Pchr (AraC family)
1.8	2.0	2.9	3.1	AZOLI_2439	*hspD2*	small heat shock protein; HSP20-like chaperone
1.9	2.3	2.2	2.6	AZOLI_0918	*ohr*	organic hydroperoxide resistance protein, OsmC superfamily
1.7	2.0	2.1	2.4	AZOLI_2324	*tatE*	Sec-independent protein translocase
2.0	2.7	2.1	2.8	AZOLI_1257		putative glucosyl transferase
2.0	2.7	2.0	2.7	AZOLI_3088		putative GCN5-Acetyltransferase
2.0	2.9	1.7	2.6	AZOLI_3017		putative oxygen-independent coproporphyrinogen III oxidase
1.6	2.5	2.3	3.1	AZOLI_2492	*def1*	formylmethionine deformylase
1.8	2.7	2.1	3.0	AZOLI_p50438		putative flavin dependant oxidoreductase
1.9	2.6	1.3	1.9	AZOLI_1032		putative transcriptional regulator, TetR family
1.3	2.4	1.3	2.4	AZOLI_0005		two-component response transcriptional regulator (OmpR family)
1.2	2.2	1.0	2.0	AZOLI_2533	*acnA*	aconitase
1.0	1.8	1.5	2.3	AZOLI_p20515	*kdpB*	potassium translocating ATPase, subunit B
1.4	2.0	1.7	2.3	AZOLI_2345		putative peptidase, PmbA-like
1.6	2.1	1.8	2.3	AZOLI_p50336	*cyoA*	cytochrome o ubiquinol oxidase subunit II
1.4	1.9	1.5	1.9	AZOLI_p40490		transcriptional regulator, GntR Family
1.2	1.4	1.7	1.9	AZOLI_p50211		putative permease of the drug/metabolite transporter superfamily
-3.0	-2.9	-3.2	-3.0	AZOLI_0173	*cmk*	cytidylate kinase
-1.7	-1.6	-3.4	-3.2	AZOLI_1890		putative 2Fe-2S ferredoxin
-2.4	-1.7	-3.6	-2.9	AZOLI_1719		putative lipoprotein RlpA-like
-2.4	-2.1	-3.3	-2.9	AZOLI_p10636		putative sugar nucleotidyltransferase
-3.1	-2.2	-4.1	-3.3	AZOLI_2333		putative transcriptional regulator (CheY-like receiver domain)
-2.0	-1.5	-2.8	-2.3	AZOLI_2147	*ppx*	exopolyphosphatase
-2.1	-2.2	-1.8	-1.8	AZOLI_0785	*dctP*	TRAP-type C4-dicarboxylate transporter
-3.7	-2.4	-2.7	-1.4	AZOLI_p10807	*cycA*	cytochrome c
-4.0	-3.6	-4.8	-4.4	AZOLI_0214		transcriptional regulator, ArsR family
-4.7	-4.4	-3.9	-3.6	AZOLI_p10813		response regulator receiver (CheY-like protein)
-4.0	-4.1	-3.8	-4.0	AZOLI_p1tRNA3		Pro tRNA
-4.0	-4.1	-4.0	-4.2	AZOLI_p1tRNA4		Pro tRNA
-3.5	-4.1	-3.8	-4.4	AZOLI_p20319		putative universal stress protein

Fig. 5. *Azospirillum lipoferum* 4B genes regulated regardless of the rice cultivar. Subset of *A. lipoferum* 4B genes with known function that are differentially expressed regardless of the reference condition and the rice cultivar ($|\text{Log}_2(\text{FC})| \geq 1$ and $P_{adj} < 0.05$). Genes are classified according to hierarchical clustering analysis (Euclidean distance method). Red represents up-regulations and green represents down-regulations.

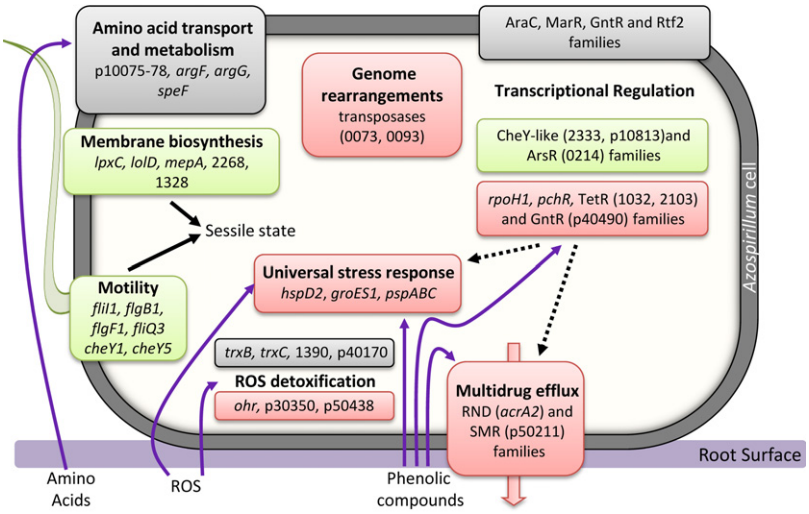

Fig. 6. Overview of gene expression during *Azospirillum* adaptation to rice roots. Up-regulated functions are highlighted in red and down-regulated functions in green. Functions displaying cultivar-specific responses are highlighted in gray. Black dotted arrows symbolize potential regulatory link between the functions. Purple arrows symbolize potential link between plant-exuded compounds and regulated functions. Nameless genes are identified according to their label without the prefix AZOLI_.

production, but they do not appear to be up-regulated in the tested conditions (Pothier *et al.*, 2008; Prigent-Combaret *et al.*, 2008; Wisniewski-Dyé *et al.*, 2011). However, spatial variations previously reported for *Azospirillum* gene expression along the root (Vande Broek *et al.*, 1993; Combes-Meynet *et al.*, 2011) may lead to an underestimation of the regulation of these key determinants.

Evidence for host-specific adaptation in *Azospillum*-rice cooperation

Whereas *A. lipoferum* 4B genes described above are regulated regardless of the rice cultivar, other key determinants appear to be differentially expressed in a cultivar-dependent manner regardless of the reference condition, as illustrated in Fig. 4. Indeed, 17 genes are differentially expressed (one up-regulated and 16 down-regulated) only during the interaction with cv. Cigalon and 334 genes (294 up-regulated and 40 down-regulated) only with cv. Nipponbare (Tables S3 and S4). A vast majority of the up-regulated genes are related to regulatory functions and signal transduction, transport and binding proteins, energy metabolism and central intermediary metabolism. Three putative sensor histidine kinases (AZOLI_1900, AZOLI_p20362, and AZOLI_p30320) and two putative diguanylate cyclases (AZOLI_0003 and AZOLI_p10650) are up-regulated only with cv. Nipponbare. Moreover, 16 transcriptional regulators belonging to AraC, MarR, Rtf2, GntR families display Nipponbare-specific expression profiles (14 up-regulated and two down-regulated). These results highlight the potential impact of plant genotype variability on regulatory networks established by *A. lipoferum* 4B root-associated cells.

Cultivar-specific differences are also observed for 15 components of ABC transporters and at least three components of multidrug efflux system. In particular, an operon potentially involved in amino acids transport (AZOLI_p10075-78) seems preferentially regulated with cv. Nipponbare (Table S4). In addition, *argF*, *argG,* and *speF*, three genes involved in arginine and proline metabolism, are induced only with cv. Nipponbare. These inductions are consistent with the presence of arginine and proline in root exudates of 7-day-old rice plants but to our knowledge, differences in amino acids concentrations have never been investigated at the cultivar level (Bacilio-Jiménez *et al.*, 2003). Tight adjustments and plant-specific response of bacterial transportome were previously reported in adaptation of *Rhizobium leguminosarum* to host and nonhost legume or nonlegume rhizospheres (Ramachandran *et al.*, 2011). In addition, the impact of plant cultivar on bacterial transcriptomic response was evidenced for *P. aeruginosa* grown in liquid culture with sugarbeet exudates (Mark *et al.*, 2005).

The composition and structure of PGPR communities are conditioned by plant genotypes and several lines of evidence are in favor of a genotype-specific adaptation of cooperative phytostimulating rhizobacteria (Hartmann *et al.*, 2008; Bouffaud *et al.*, 2012; Drogue *et al.*, 2012). Recently, plant secondary metabolite profiling evidenced specific interaction between *A. lipoferum* 4B and its original host cultivar (cv. Cigalon; Chamam *et al.*, 2013). In this context, the fact that gene expression changes are of lower importance in *A. lipoferum* 4B cells associated with cv. Cigalon suggests that evolutionary processes could have led to a more specialized interaction (Fig. 4). Moreover, several genes encoding thioredoxin (*trxB*, *trxC*, AZOLI_p40170) or superoxide dismutase (AZOLI_1390) are specifically induced with cv. Nipponbare, suggesting that *A. lipoferum* 4B faces a more important oxidative stress when associated with this cultivar than with the one the strain was originally isolated (cv. Cigalon). The

hypothesis of co-adaptation of both partners can be supported by specific changes in root exudation, and root metabolites profiles induced in a strain dependent manner. Indeed, strains *A. lipoferum* 4B and *A. brasilense* A95 (isolated from rice, France) caused an increased rice exudation whereas *A. brasilense* R07 (isolated from rice, Senegal) and *A. lipoferum* B7C (isolated from maize) did not stimulate rice exudation, compared with sterile control (Heulin *et al.*, 1987). Moreover, inoculation of *Azospirillum* on maize and rice induces modifications of secondary metabolite profile in both roots and shoots depending on *Azospirillum* strain/cultivar combinations, which suggests specific adaptation of the whole plant partner (Walker *et al.*, 2011; Chamam *et al.*, 2013). Finally, the fact that rice ethylene responses to beneficial diazotrophic bacteria, including *A. brasilense*, appears to be controlled by both plant and bacterial genotypes (Vargas *et al.*, 2012) opens interesting issues that may be investigated by rice transcriptome profiling.

Conclusion

This study represents the first report of cultivar-specific response for a PGPR recovered from roots. Similarities observed between sessile and root-adhering cells evidence the sessile-like state of *Azospirillum* cells associated to rice roots. However, adaptation to rice involves a wide range of gene up-regulations indicating that *Azospirillum* may face and adapt to various stress conditions (Fig. 6). Some of these stresses could be mediated by ROS, toxic compounds involved in defense response of the host plant, suggesting that the plant immune system could play a role in the establishment of *Azospirillum*-rice cooperation. Our results highlight the tight adjustment of regulatory networks as well as the potential induction of genome rearrangements in root-associated cells 7 days after inoculation. Several genes related to ABC transporter and ROS detoxification display cultivar-specific expression profiles, suggesting host-specific adaptation and raising the question of *A. lipoferum* 4B/rice cv. Cigalon co-adaptation.

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Authors' contribution

B.D. and H.S. contributed equally to this study.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Number of *Azospirillum lipoferum* 4B regulated genes grouped by functional categories.
Table S1. Specific primers for qPCR.
Table S2. Genes regulated in *Azospirillum* sessile cells compared to planktonic cells ($|\text{Log}_2(\text{FC})| \geq 1$ and $P_{adj} < 0.05$).[a]
Table S3. Genes differentially expressed in 4B_CigP and 4B_CigS ($|\text{Log}_2(\text{FC})| \geq 1$ and $P_{adj} < 0.05$).[a]
Table S4. Genes significantly regulated in 4B_NipP and 4B_NipS ($|\text{Log}_2(\text{FC})| \geq 1$ and $P_{adj} < 0.05$).[a]
Table S5. RT-qPCR validation of microarray data.