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QUORUM SENSING AND 3-INDOLE ACETIC ACID DEGRADATION PLAY A ROLE IN COLONIZATION AND PLANT GROWTH PROMOTION OF *ARABIDOPSIS THALIANA* BY *BURKHOLDERIA PHYTOFIRMANS.*

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Nadie desafía a nadie por algo que uno no ha hecho, pensé. Pero luego pensé que la vida (o su espejismo) nos desafía constantemente por actos que nunca hemos realizado, en ocasiones por actos que ni siquiera se nos han pasado por la cabeza realizar…

Durante un segundo de lucidez tuve la certeza de que nos habíamos vuelto locos. Pero a ese segundo de lucidez se antepuso un supersegundo de superlucidez (si me permiten la expresión) en donde pensé que aquella escena era el resultado lógico de nuestras vidas absurdas. No era un castigo si no un pliegue que se abría de pronto para que nos viéramos en nuestra humanidad común. No era la constatación de nuestra ociosa culpabilidad sino la marca de nuestra milagrosa e inútil inocencia….

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Y sin más, dentro de mi escena interna

vueltas dan todas las vivencias

que por frustración agitaron mi cabeza y me situaron en salares infinitos,

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FIGURE INDEX

ABBREVIATIONS

3-OH-C8-HSL	3-hydroxyoctanoyl-L-Homoserine lactone
3-OXO-C14-HSL	3-oxo-tetradecanoyl-L-Homoserine lactone
ACC	1- aminocyclopropane-1-carboxylate
BCC	*Burkholderia cepacia* complex
C8-HSL	N-octanoyl-L-Homoserine lactone
FW	Fresh weight
GFP	Green fluorecence protein
GOI	Gene of interest
HK	Housekeeping
HSL	Homoserine lactone
IAA	Indole-3-acetic acid
ISR	Induction of systemic resistance
LB	Luria Bertani
LP.04	Four rosette leaves visible
LP.06	Six rosette leaves visible
MS	Murashige and Skoog
OD_{600}	Optic density to 600 nm
Bp	Base pair
PCR	Polymerase chain reaction

PGPR	Plant growth promoting bacterium
QS	Quorum sensing
RT	Reverse transcription
TLC	Thin-layer chromatography
X-gal	5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside

RESUMEN

La comunicación molecular en la rizósfera tiene un importante papel en la regulación de rasgos que caracterizan la asociación planta-bacteria. *Burkholderia phytofirmans* PsJN es una bacteria promotora del crecimiento de plantas (PGPR) bien caracterizada y capaz de establecer interacciones rizosféricas y endofíticas con diferentes especies de plantas. Para una efectiva promoción del crecimiento es necesario que las PGPR tengan una colonización competente del hospedero. Utilizando cepas mutantes de *B. phytofirmans* PsJN en los sistemas de quorum sensing, se obtuvo evidencia sobre la importancia de la comunicación célula a célula para una colonización eficiente de plantas de *Arabidopsis thaliana* y para el establecimiento de una interacción benéfica. Además, se observó que la degradación de la auxina ácido 3-indolacético (AIA) tiene un rol clave en la promoción del crecimiento de plantas y es necesaria para una colonización eficiente. La cepa silvestre de PsJN pero no la mutante *iacC*, incapaz de degradar AIA, puede re-establecer los efectos de promoción en las raíces de *A. thaliana* en presencia de AIA exógeno, indicando la importancia de esta característica en la promoción del crecimiento de la raíz primaria. Usando una línea transgénica de *A. thaliana* con la vía de señalización de auxinas suprimida (sobre-expresora del gen represor mir393) y analizando la expresión de los receptores de auxinas en plantas inoculadas, se evidenció que la señalización de auxinas en las plantas es necesaria para producir los efectos promotores de crecimiento producidos por esta PGPR. Los resultados que obtenidos también apuntan a que tanto la disminución de niveles de etileno (controlados por el gen *acdS*) como la señalización por auxinas interactúan en la planta, evidenciado por la respuesta de la planta a la cepa mutante en el gen señalado, que codifica para la enzima ácido 1-aminociclopropano-1-carboxílico desaminasa. Finalmente, se observó que el sistema

quorum sensing BphI.2/RsaL/BphR.2 es inducido por la molécula señal 3-OXO-C14-homoserina lactona y esta molécula también induce la expresión del regulador BphR.1, indicando una posible jerarquía entre estos dos sistemas de quorum sensing. Adicionalmente, el análisis de expresión en la cepa silvestre y las mutantes en la producción de homoserinas lactonas, mostró una regulación, mediante los sistemas quorum sensing, de la expresión en genes relacionados con la producción de exosolisacáridos y el gen *cheA,* involucrado en motilidad quimiotáctica.

ABSTRACT

Molecular communication in the rhizosphere plays an important role regulating traits involved in plant–bacteria association. *Burkholderia phytofirmans* PsJN is a well-known plant growth promoting bacterium (PGPR), which establishes rhizospheric and endophytic colonization in different plants. A competent colonization is essential for plant growth promoting effects produced by bacteria. Using appropriate mutant strains of *B. phytofirmans*, we obtained evidence for the importance of N-acyl homoserine lactone-mediated (quorum sensing) cell-to-cell communication in efficient colonization of *Arabidopsis thaliana* plants and the establishment of a beneficial interaction. We also observed that bacterial degradation of the auxin indole-3-acetic acid (IAA) plays a key role in plant-growth-promoting traits and is required for efficient rhizosphere colonization. Wild type *B. phytofirmans* but not the *iacC* mutant in IAA mineralization is able to restore promotion effects in roots of *A. thaliana* in the presence of exogenously added IAA, indicating the importance of this trait for promoting primary root length. Using a transgenic *A. thaliana* line with suppressed auxin signaling (miR393) and analyzing the expression of auxin receptors in wild-type inoculated plants, we provide evidence that auxin signaling in plants is necessary for the growth promotion effects produced by *B. phytofirmans*. The interplay between ethylene and auxin signaling was also confirmed by the response of the plant to a 1-aminocyclopropane-1-carboxylate deaminase bacterial mutant strain. Finally, we observed that BphI.2/RsaL/BphR.2 quorum sensing system is induced by 3-OXO-C14-homoserine lactones and this signal molecule is able to induce the BphR.1 regulator, indicating a possible hierarchy between both quorum-sensing systems. In addition, transcript analysis in wild type and mutant

strains of quorum sensing showed that exopolysaccharide production related genes and the *cheA* gene, involved in chemotactic motility, were regulated by these systems.

GENERAL INTRODUCTION

In order to improve their survival in the environment, microorganisms establish diverse biotic interactions. Some of the more complex chemical, physical, and biological interactions occur between plant roots and the microorganisms in the surrounding soil (Brencic and Winans, 2005). The rhizosphere represents a highly dynamic space for interactions between plant roots and pathogenic and beneficial soil microorganisms (Harsh et al. 2006, 2008; Bais et al. 2006). This is in part regulated by the chemicals excreted by the plant roots into the surrounding soil (Badri and Vivanco, 2009; Hartmann et al. 2009), including low molecular weight secondary metabolites and high molecular weight compounds, such as mucilage and proteins (Badri and Vivanco, 2009). Nutrient availability in the rhizosphere is higher than in the bulk soil and the presence of plant exudates creates a suitable environment for growth of microorganisms (Costa et al. 2006; 2007). In the rhizosphere, molecular communication between microorganisms and their plant hosts plays a fundamental role in pathogenesis, and in the establishment of beneficial interactions (Mark et al. 2005). Moreover, competition in the rhizosphere is related to the ability of microorganisms to use components such as organic acids and polymers as carbon sources (Lugtenberg et al. 1999; Matilla et al. 2007), as well as its ability to weaken or destroy competing organisms (Lugtenberg et al. 2001).

Plant growth promoting rhizobacteria (PGPR) produce beneficial effects on plant growth through several mechanisms such as nitrogen fixation (Hurek et al. 2002), improved nutrient uptake (Kraiser et al. 2011), phytohormone production (Idris et al. 2007), and induction of systemic resistance (ISR) (Bakker et al. 2007). A single

bacterium may possess more than one of these mechanisms (Ahmad et al. 2008). These bacteria are able to establish rhizosphere populations in host plants and even colonize inside of plants tissue, in the intercellular spaces named apoplast (Dong et al. 1994; Tejera et al. 2006). Recent studies show the importance of bacteria-to-bacteria communication in plant colonization by PGPR (Bais et al. 2004; Compant et al. 2005; Steindler et al. 2009). Bacteria can communicate by sensing and responding to small signaling molecules that make them responsive to neighboring bacteria (Whitehead et al. 2001). A variety of signaling molecules have been identified; among them, *N*-acyl-homoserine lactones (HSL) frequently synthesized by Gram-negative bacteria and oligopeptides synthesized by Gram-positive bacteria. These signaling molecules control gene expression through quorum sensing (QS) that operate as LuxIR-type or two component quorum sensing systems in Gram negative and positive bacteria, respectively (Whitehead et al. 2001; Miller and Bassler, 2001). Bacteria defective in QS signaling are less effective in host colonization (Bauer and Mathesius, 2004; Quiñones et al. 2005; Ortíz-Castro et al. 2009), and the role of QS regulating rice growth promotion by *Pseudomonas aeruginosa* PUPa3 has been also reported (Steindler et al. 2009).

In addition to QS molecules, other signal molecules may also play a role in plant-bacterial signaling. Among them, the auxin phytohormone indole-3-acetic acid (IAA) has been detected in culture supernatants of several rhizobacteria (Loper and Schroth 1986; Idris et al. 2007; Phi et al. 2008), suggesting that IAA may be a relevant signaling molecule in microorganisms (Bianco et al. 2006; Liu and Nester 2006; Spaepen et al. 2007; 2009; Yang et al. 2007; Van Puyvelde et al. 2011). For example, IAA triggers a broad gene-expression response in *Azospirillum brasilense* (Van Puyvelde et al. 2011),

and IAA synthesis is controlled by a positive feedback transcriptional mechanism (Vande Broek et al. 1999). IAA has been also reported as a signaling molecule in *Escherichia coli* (Bianco et al. 2006), *Agrobacterium tumefaciens* (Liu and Nester, 2006; Yuan et al. 2008), *Erwinia chrysanthemi* (Yang et al. 2007), and *Rhizobium etli* (Spaepen et al. 2009). IAA is the major naturally occurring auxin in plants. This molecule regulates many plant developmental processes including embryogenesis, root and stem elongation, phyllotaxy, apical dominance, photo and gravitropism, and lateral root initiation (Muday and DeLong, 2001). Genetic and biochemical analyses in Arabidopsis have led to the identification of a number of genes involved in auxin perception, signaling and transport (Calderón-Villalobos et al. 2010). The F-box proteins TIR1, AFB1, AFB2, and AFB3 are receptors for the plant hormone auxin (Calderón-Villalobos et al. 2010; Navarro et al. 2006). Additionally, TIR1 and AFB transcripts are targets of miR393, a conserved miRNA, that reduce auxin signaling in miR393-overexpressing lines of transgenic plants.

The production of IAA by PGPR has been involved in plant growth and root proliferation (Vande Broek et al. 2005; Idris et al. 2007). IAA is a quickly biodegradable in soils and in plant tissue preparations, which it has often attributed to natural bacterial contaminants (Leveau and Gerards 2008). Complete mineralization of and growth on IAA have been reported in various bacteria, for which plants represent natural sources of IAA. For that reason, mineralization of IAA or transformation into biologically inactive form, offers bacteria the potential for manipulation of IAA-related activities (Leveau and Gerards 2008). Notably, five different biosynthetic pathways and one pathway for IAA mineralization or transformation have been described in bacteria (Idris et al. 2007; Leveau and Gerards 2008).

Burkholderiales strains have received increasing attention during the past few years due to their biotechnological applications. Unfortunately, most of the studies have given emphasis in isolation, taxonomy, distribution and genetics of *Burkholderia cepacia* complex (BCC) species, related to the human opportunistic pathogen found in cystic fibrosis patients (Eberl, 2006; Vial et al. 2007). Recently, many members of a group with ability to colonize wide arrays of plant species have been found (Di Cello et al. 1997, Parke and Gurian-Sherman, 2001; Barret and Parker 2006). Moreover, specific traits affecting plant growth such as nitrogen fixation (both in nodules and in free living cells), phosphate solubilization and antagonism against pathogenic fungi have been described for *Burkholderiales* (Ait Barka et al. 2000, Elliot et al. 2007; Parke and Gurian-Sherman, 2001, Bontemps et al. 2010). A phylogenetic tree, based on 16S rRNA sequence analysis, shows the separation of the genus *Burkholderia* into two major clusters (Caballero-Mellado et al. 2007) (Fig.1). One of them, include pathogenic species such as *B. glumae*, *B. pseudomallei* and *B. mallei* and other 17 well characterized species referred to as BCC (Coenye and Vandamme, 2003). In BCC species, the QS is highly conserved, consisting of CepI, which synthesizes mainly *N*-octanoylhomoserine lactone (C8-HSL) and the CepR C8-HSL sensor-response regulator (Venturi et al. 2004; Eberl, 2006) and it is involved in the regulation of similar phenotypes in many different species of BCC members, including the regulation of virulence-associated factors (Kim et al. 2004; Solis et al. 2006; Devescovi et al. 2007; Suárez-Moreno et al. 2008). The second group, phylogenetically distant from the BCC, is formed by more than 25 novel environmental

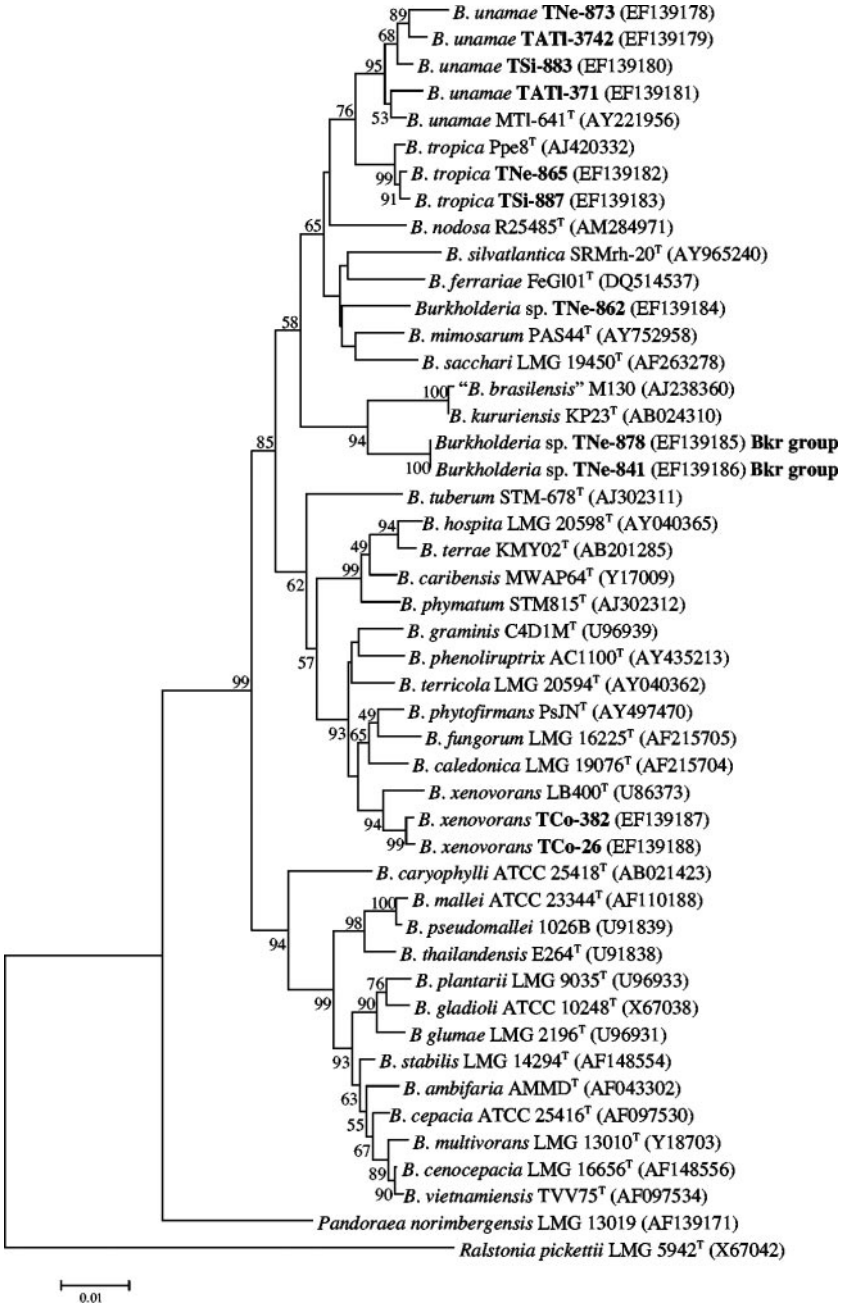

Figure 1. Phylogenetic tree based on 16S rRNA gene sequences showing the relatedness among the nitrogen-fixing *Burkholderia* species associated with tomato plants (strain designations in bold) and related *Burkholderia* species. These sequences were compared with previously published 16S rRNA gene sequences from *Burkholderia* species and related bacteria, such as *Ralstonia* and *Pandorea*. The multiple alignments of the sequences were performed with CLUSTAL W software. The tree topology was inferred by the neighbor-joining method, based on 1,310 DNA sites, and distance matrix analyses were performed according to Jukes and Cantor, using the program MEGA version 2.1. The bar represents one nucleotide substitution per 100 nucleotides. The nodal robustness of the tree was assessed using 1,000 bootstrap replicates. The NCBI GenBank accession number for each strain is shown in parentheses (Caballero-Mellado et al. 2007).

non-pathogenic species described later than the year 2000, which include mainly plant-associated, rhizospheric and/or endophytic bacteria (Caballero-Mellado et al. 2004; 2007; Goris et al. 2000; Reis et al. 2004; Perin et al. 2006; Suárez-Moreno et al. 2008). Besides of the differences based on 16s rRNA between both groups, the latter bacterial group have a conserved QS type LuxI/R, mediated by HSLs, named BraI/R originally described in *B. kururiensis,* which is related to the LasI/R and PpuI/R AHL QS systems of *P. aeruginosa* and *Pseudomonas putida*, respectively, and is present in almost 20 sequenced species of this novel beneficial *Burkholderia* group (Suárez-Moreno et al. 2008). Internal division of this cluster appear to reflect interactions with their respective hosts, as most species included in the BCC have shown pathogenic interactions with their hosts, while most species from the plant-associated clade are reported to be beneficial (Bontemps et al. 2010; Suárez-Moreno et al. 2012).

The PGPR *B. phytofirmans* PsJN promotes growth of horticultural crops such as tomato, potato, and grape (Genin and Boucher 2004; Spaepen et al. 2007; Theocharis et al. 2012) and belong to this new clade of beneficial *Burkholderia*. To date, only one plant-growth-promotion molecular mechanism has been proved experimentally in *B. phytofirmans* PsJN: the reduction of the plant ethylene hormone levels by 1-aminocyclopropane-1-carboxylate ACC deaminase (AcdS) (Compant et al. 2005; Vadassery et al. 2008). The phytohormone ethylene is a potent modulator of plant growth and development, involved in the plant developmental cycle, plant disease resistance, microbe–plant interactions and the response to abiotic stresses. Bacterial ACC deaminase, which cleaves ACC into ammonia and a-ketobutyrate, is important to plant growth promote because ameliorate plant stress by reduction of ethylene (Hardoim et al.

2008). *B. phytofirmans* PsJN mutants in the *acdS* gene, therefore lacking AcdS activity, are unable to promote the elongation of canola roots (Sun et al. 2009). Genome sequence analysis of *B. phytofirmans* PsJN shows the presence of two putative IAA synthesis pathways (the indole-3-acetamide and the tryptophan side chain oxidase pathways) and two putative QS systems associated with a HSL production, a BphI.1homoserine lactone synthase related with 3-OH-C8-HSL production and a BpI.2 synthase related with 3-OXO-C14-HSL, 3-OH-C14-HSL and 3-OH-C12-HSL production (Weilharter et al. 2011; Trognitz et al. 2009). In addition, it encodes one *iac* operon putatively involved in degradation of IAA, as reported for *Pseudomonas* spp. (Leveau and Gerards, 2008).

The preceding elements raise several questions concerning molecular signaling in plant – bacteria interactions. Among them, are QS systems of *B. phytofirmans* PsJN, involved in regulation of functions required for plant colonization? Has the IAA signaling molecule a role in plant growth promoting effects by *B. phytofirmans*? Are there any connections between QS and IAA signaling?

Based on such questions the hypotheses of this thesis were:

1. *The conserved QS system of the beneficial Burkholderia cluster, present in B. phytofirmans PsJN, regulates functions involved in rhizospheric and endophytic colonization.*

2. *B. phytofirmans PsJN induces the IAA signaling in A. thaliana to produce plant growth promoting effects.*

The overall objective of this thesis was determine the importance of molecular signaling present in *B. phytofirmans* PsJN for colonization and growth promotion of *A. thaliana* plants, comparing the effects of wild type *B. phytofirmans* PsJN and four mutants on this plant model.

The specific objectives of this thesis were:

1. Determine the importance of BphI.1/BphR.1 and BphI.2/RsaL/BphR.2 QS systems and IAA signaling molecule of *B. phytofirmans* PsJN to colonize and growth promote of *A. thaliana* plants.

    a. Analyze the ability of *bphI*.1 and *bphI*.2 single mutants in HSL synthases to colonize and growth promote *A. thaliana* plants.

    b. Analyze the ability of the *iacC* mutant, unable to degrade IAA, to colonize and promote growth of *A. thaliana* plants.

    c. Determine if IAA signaling in A. thaliana is requiring to plant growth promotion by *B. phytofirmans* PsJN.

2. Determine a possible regulation by BphI.1/BphR.1 and BphI.2/rsaL/BphR.2 QS systems of *B. phytofirmans* PsJN on genes with functions involved in colonization of *A. thaliana* plants, by real time qRT-PCR.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

B. phytofirmans PsJN was obtained from A. Sessitsch. Wild type PsJN and the four mutants were grown at 30°C on Dorn mineral salts medium (Dorn et al. 1974) containing 10 mM fructose, 5 mM 4-hydroxybenzoate or benzoate, or 2.5 mM IAA as the sole carbon and energy source and, if required, kanamycin (50 μg ml^{-1}) or spectinomycin (100 μg ml^{-1}) for 12 h. For growth tests of strains on 2.5 mM IAA, cells were grown for 36 h, biomass measured at OD_{600nm}, and three replicates were performed for each growth measurement.

Construction of *B. phytofirmans* PsJN null mutants.

Internal fragments of the *bphI*.1 gene (locus Bphyt_0126, HSL synthase of chromosome 1 QS system), *bphI*.2 gene (locus Bphyt_4275, HSL synthase of chromosome 2 QS system), *iacC* gene (locus Bphyt_2156, aromatic ring hydroxylating dioxygenase involved in catabolism of IAA), and *acdS* gene (locus Bphyt_5397, 1-aminocyclopropane-1-carboxylic acid deaminase) sequences were amplified by PCR, using the primer pairs in Table 1. The PCR products were cloned using the pCR2.1-TOPO system (Invitrogen, Carlsbad, CA, U.S.A.) to generate plasmids pCR2.1bpi.1, pCR2.1iacC, and pCR2.1acdS. The *bphI*.2 PCR product was cloned using the pCR8/GW/TOPO system (Invitrogen), to generate plasmid pCR8bpI.2. These plasmids

Table 1. Primer pairs used in this thesis.

Primers	Sequences
bpI.1-mutFw	GACGGAGGCCAGCAATATAA
bpI.1-mutRv	GTATGGGAGATGTCGCGATT
bpI.2-mutFw	GAACGTCACCAGTTCGTGAAT
bpI.2-mutRv	ATGGAGATCGACGGCTATGA
iacC-mutFw	GGTCA ACGTCTTGCA GAACC
iacC-mutRv	GTTTCGTCGTCGATCGATTT
acdS-mutFw	CGAATATCTGATCCCCGAAG
acdS-mutRv	AAGCCGATGTCGAAACCAT
gumD.1Fw	CTCCGCTTTTCGATGAGTTC
gumD.1Rv	ATGCTTTCTTCCGACAGTGG
gumD.2Fw	TGTACACGCCGTATTGAACG
gumD.2Rv	CGAGTGGTTGAAGAGGGAAA
gumD.3Fw	ACGATCTGGTGAACATCCGCTTCA
gumD.3Rv	ACAGGCGGTCGAAGATTTCCTTCT
gumD.4Fw	GAAGAGCACGGCAAGATCAC
gumD.4Rv	TGTAGCCCTTCACCAGATCC
flgBFw	CGACGACTACGGCAATCTG
flgBRv	GATTCGAAGTGCAGCGTGT
flhDFw	GGGGATTTCGGACCAGTTAG
flhDRv	AAACGGCACAACACCTGATT
fliFFw	CAAGGACTCGACCACCAACT
fliFRv	CGACCACACGCAGATAGTTC
flhBFw	CTGCCAATTCTCGCTTTCAC
flhBRv	GATTGAGGCGGTCGAATTT

cheAFw	GATCCGTTGACTCACCTCGT
cheAFw	TCGTCGCTCACTTCAATGAC
flp/fap.1Fw	CGTGACGGCTATTGAATATGG
flp/fap.1Rv	TGCTGCAAGACAGTGGAGAC
cpA.1Fw	CCTTTCGGTATTTCGCTGAC
cpA.1Rv	ACGGCGAAGACTTTGACATC
flp/fap.2Fw	GTGTGGCAGCGATCGAATAC
flp/fap.2Rv	TTGGTGGTCATGTTCTGCAC
cpA.2Fw	GTTGGTCGTGACAGGATTGA
cpA.2Rv	CCATCCACATCGTCATGC
bpI.1Fw	CCAGCAATATAAGCGGATCG
bpI.1Rv	GCTGGAACTGCACGATATGA
bpI.2Fw	GGCCTGGATCAGCATGTAGT
bpI.2Rv	GGCGCGAGTATTTCATGG
bpR.1Fw	CCAGGTCAGGCATACGATCT
bpR.1Rv	CTGCCTCATCAGCACACAGT
bpR.2Fw	TATTCAAATGGCGCTGACTG
bpR.2Rv	AATTCTGGCGGCATATCAAC
rsaLFw	ACTTCACCAAAACCGTGTCC
rsaLRv	TTATTGACCCGGGTTGAGC
16SFw	CGGGCTAGAGTATGGCAGAG
16Rv	CGTGCATGAGCGTCAGTATT
pBpI.2GAF	CGAGAAGCTTAGGCCTGAATCTCCTGAACGTGTTGGGGTA
pBpI.2GAR	TTGAGGCCCTTTCGTCTTCGGTCTGAACGCCGGTTTG
prsaLGAF	TTGAGGCCCTTTCGTCTTCCGTTGTCGAATTCCTGA
rsaLGAR	CGAGAAGCTTAGGCCTGAATAGTGCAGTGCTTGTCTCGAA
BpRg.2FW	ACTTCACCAAAACCGTGTCC
BpRg.2RV	AATGGCATCAAAACGGCTAC

were electroporated in *B. phytofirmans* PsJN to get one recombination event disruption of the target gene, and recombinants were selected on Luria-Bertani (LB) agar containing 50 μg/ml kanamycin to obtain strain PsJN *bphI*.1, strain PsJN *iacC*, and PsJN *acdS* null mutants and 100 μg/ml spectinomycin to obtain strain PsJN *bphI*.2 null mutant. Correct insertions in null mutant strains were confirmed by PCR and sequencing.

Construction of *lacZ* reporter fusions.

Putative promoter regions were fused to the *lacZ* gene from pKGWP0 (Marín et al. 2010). A PCR product comprising the 2–200 and 24–198 bp region upstream of the translational starts of the *bphI*.2 or *rsaL* genes, respectively, were obtained. Both fragments were amplified using primer pairs pbpI.2FW-bpI.2RV and prsaLFW-prsaLRV (Table 1) for *bpI*.2 and *rsaL* promoters, respectively, that contain 20 bp in terminal sequence that overlap to upstream *lacZ* gene in pKGWP0, which were joined into a covalently sealed molecule in a one-step isothermal reaction as described by Gibson et al. (2009), to get the two reporter vectors $P_{bphI.2}$-lacZ and P_{rsaL}-lacZ. The same assembly method was used to construct a derivate plasmid expressing *bphR*.2 gene. The *bphR*.2 gene was amplified using primer pairs bpRgFW-bpRgRV and the final fragment obtained had 20 bp in its terminal sequence that overlap to the downstream P_{BAD} promoter from pBS1 vector (Bronstein et al. 2005), to get the expression vector PBS1-BphR.2. The recombinant plasmids were electroporated in *B. phytofirmans* PsJN and selected in minimal medium supplemented with spectinomycin (100 mg ml^{-1}) and/or gentamycin (30

mg ml^{-1}). *B*-galactosidase assays were performed according to standard protocols (Miller, 1972) after 4 h of incubation, using 3-OXO-C14-HSL (1 μM and 2 μM) as inducer.

RNA extraction, cDNA synthesis, and qRT-PCR analysis on bacteria and plants.

For bacterial RNA extraction, cells of *B. phytofirmans* PsJN wild type and *bphI*.1, *bphI*.1 and *bphI*.12, single and double mutant strains were grown overnight in minimal medium with fructose to inoculate a fresh culture medium, let the cells grow until OD_{600nm} = 1.8, and then supplement this culture with 10 mM of fructose or plant root exudates and incubate for 1 h. Total RNA was obtained from 4 ml of each culture, using RNA protect bacteria reagent and RNeasy Mini Kit (QIAGEN, Chatsworth, CA, USA). The RNA was quantified using a GeneQuant 1300 Spectrophotometer (GE Healthcare, Piscataway, NJ, USA) and treated with TURBO DNase Kit (Ambion, Austin, TX, USA) to remove DNA contamination. The RT-PCR was performed using ImProm-II Reverse Transcription System (Promega Corporation, Madison, WI, USA) with 1 mg of RNA in 20 ml reactions.

For plant RNA extraction, plants of LP.04 stage (Boyes et al. 2001) (four rosette leaves visible, corresponding to 13 days after sowing) or LP.06 stage (Boyes et al. 2001) (six rosette leaves visible, corresponding to 19 days after sowing) were used. Ten plantlets for each treatment were collected in liquid nitrogen and ground with a pestle in an Eppendorf tube. Then, RNA was obtained using the Trizol method following the manufacturer's instructions (Invitrogen). For cDNA synthesis, 1 μg of total RNA treated with DNAse I (RQ1; Promega Corp., Madison, WI, U.S.A.) was reverse transcribed with

random hexamer primers using the Improm II reverse transcriptase (Promega Corp.), according to the manufacturer's instructions.

RT-PCR was performed using the Brilliant SYBR Green QPCR Master Reagent Kit (Agilent Technologies, Santa Clara, CA, U.S.A.) and the Eco Real-Time PCR detection system (Illumina, San Diego, CA, U.S.A.). The PCR mixture (15 µl) contained 2.0 µl of template cDNA (diluted 1:10) and 140 nM each primer. Amplification was performed under the following conditions: 95°C for 10 min; followed by 40 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s; followed by a melting cycle from 55 to 95°C. Relative gene expression calculations were conducted as described in the software manufacturer's instructions: an accurate ratio between the expression of the gene of interest (GOI) and the housekeeping (HK) gene was calculated according to the following expression 2–(ΔCtGOI – HK). Then, gene expression levels were normalized to the average value of the expressions in the control treatment. AtSAND (AT2G28390) was used as the HK gene in plants using previously described primer pairs (Czechowski et al. 2005) and 16S rRNA (Bphyt_R0071) was used in bacteria. For bacterial qRT-PCR analysis primer pairs for gumD.1 (Bphyt_4056), gumD.2 (Bphyt_6264), gumD.3 (Bphyt_0818), gumD.4 (Bphyt_1955), flgB (Bphyt_3794), flhD (Bphyt_3820), fliF (Bphyt_3770), flhB (Bphyt_3804), cheA (Bphyt_3815), flp/fap.1 (Bphyt_1650), cpA.1 (Bphyt_1651), flp/fap.2 (Bphyt_4612), cpA.2 (Bphyt_4613), bphI.1 (Bphyt_0126), bpI.2 (Bphyt_4275), bphR.1 (Bphyt_0125), bphR.2 (Bphyt_4273) and rsaL (Bphyt_4274) genes were used (Table 1). For plant qRT-PCR analysis primer pairs TIR1 (AT3G62980), AFB1 (AT4G03190), AFB2 (AT3G26810) and AFB3 (AT1G12820)

have been described elsewhere (Vidal et al. 2010). All experiments were performed in three biological and two technical replicates.

Green fluorescence protein labeling.

Strain PsJN and its mutants were tagged with the GFP marker gene using a mini-Tn5 system (Mathysse et al. 1996), which forms stable genomic insertions. Wild type PsJN and PsJN *bphI*.1 and PsJN *bphI*.2 mutants were conjugated with *E. coli* PRK2073, as helper, in a triparental mating, and *E. coli* S17, which contains the plasmid with mini-Tn5GFP construct carrying tetracycline resistance. Transconjugants carrying the GFP marker were selected on LB containing tetracycline at 10 μg/ml. GFP-labeled cells were examined by an Optical Fluorescence microscope in Model Nikon Eclipse 50i (Nikon, Tokyo) equipped with GFP HYQ and G-2E/C filters.

HSL extraction and visualization.

HSLs were purified from spent supernatant and separated by using a reversed-phase thin-layer chromatography (TLC) plates as previously described (Suárez-Moreno et al. 2008). For visualization, the TLC plates were overlaid with a thin layer of AB top agar seeded with *A. tumefaciens* NTL4(pZLR4) in the presence of 100 μg of X-Gal (5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside)/ml, as described previously (Suárez-Moreno et al. 2008). Dried HSLs extracts were resuspended in dichloromethane and 2 μL were spotted in each line.

Motility assays.

Swimming assays were performed in Dorn mineral salts medium (Dorn et al. 1974) and 0.3% agar plates containing 5 mM of fructose. The inoculation was performed by spotting 1 µl of a bacterial suspension having an $OD_{600\ nm}$ of 1.2. The swimming zones were measured after 24 h of incubation at 30°C, as described in Atkinson et al. (2006), for wild type species and their QS mutant derivatives.

Measurement of IAA synthesis.

Bacterial cells were grown with 5 mM fructose at OD_{600nm} =1, which corresponds to the logarithmic phase of growth. At this point, bacterial cells were incubated with 2.5 mM tryptophan (as inducer of IAA synthesis) for 3 h; then, IAA synthesis was measured on supernatants of cultures using Salkowski reagent as described previously (Glickmann and Dessaux 1995). Three replicates were performed for each IAA synthesis measurement.

Preparation of bacterial inoculants.

For plant colonization and growth effect experiments, *B. phytofirmans* PsJN and its four mutant derivative strains (PsJN *bphI*.1, PsJN *bphI*.2, PsJN *acdS*, and PsJN *iacC*) were routinely grown in Dorn mineral salts medium (Dorn et al. 1974) with 2 mM fructose in an orbital shaker (150 rpm) for 24 h at 30°C. Cell suspensions from each inoculum were then obtained and adjusted to approximately 10^8 CFU/ml, as determined

by plate counting. Then, each strain at 10^4 CFU/ml was homogeneously inoculated on 1% agar plates containing Murashige and Skoog (MS) basal salt mixture (Sigma-Aldrich, St. Louis).

Plant growth.

A. thaliana ecotype Col-0 and the *A. thaliana* transgenic line ox-miR393 (Navarro et al. 2006; Vidal et al. 2010) were used. Seeds were surface sterilized with 50% (vol/vol) commercial chlorine bleach for 7 min and washed three times in sterile distilled water. Then, seeds were kept at 4°C for 2 days in the absence of light to produce stratification. After that, seeds were sown in sterile plastic petri dishes with 1% agar plates containing MS basal salt mixture (Sigma-Aldrich) inoculated or not with bacteria. Eight seeds were sown in each plate and six plates were used for each treatment: control without bacteria, wild type, and *bphI*.1, *bphI*.2, *iacC*, and *acdS* mutant strains. To perform exogenous IAA degradation assays, seeds were germinated and grown for 2 weeks on plates with MS medium containing 0.001 or 1 µM IAA inoculated or not with either wild-type or *iacC* mutant strains. Plates were placed vertically, sealed with parafilm, and arranged in a completely randomized design. The plant growth chamber was run with a cycle of 12 h of light and 12 h of darkness and a temperature of 22 ± 2°C. Three biological replicates were carried out.

Plant root exudates.

Plant root exudates were obtained based in a procedure described by Hoegenka et al. (2003). Magenta culture vessels were adapted for sterile hydroponic growth using 250

μm polypropylene mesh as substrate for plant growth and a support stand constructed from two notched rectangles of polycarbonate. After 15 days, shoots were removed and root exudates and control eluate (liquid from control cultures) were collected. Root exudates were filter-sterilized (0.22-m filter, Millipore, Billerica) and stored at 20°C in the dark until use.

Determination of rhizospheric and endophytic bacterial colonization.

For rhizospheric colonization tests, 3-week-old plants were removed from inoculated MS media agar and washed in phosphate buffer solution, with vortex agitation. Extracted liquid material was serially diluted with Dorn mineral salts medium before plating on Dorn medium plates supplemented with benzoate as the sole carbon and energy source. The CFU/mg FW was determined after 48 h of incubation at 30°C. For endophytic colonization tests, 3-weeks-old plantlets inoculated with GFP-labeled PsJN strains were removed from the agar plates, surface sterilized with 70% ethanol for 1 min followed by 1% commercial chlorine bleach and a 0.01% Tween 20 solution for 1 min, then washed three times in sterile distilled water (adapted from Compant et al [2005]). Plating the distilled water from a final wash on R2A medium routinely controlled sterility on these plants. Then, the sterilized plant material was macerated in sterile mortars and the disrupted tissue was re-suspended in 1 ml of sterile 50 mM phosphate buffer to obtain an aqueous extract. CFU/mg FW was determined by serial dilutions of these extracts in R2A agar plates after 48 h of incubation at 30°C, and plates were examined under UV light using an Optical Epifluorescence Nikon Eclipse 50i microscope (Nikon, Tokyo).

Experiments were conducted with six plants analyzed for each treatment, in two biological replicates.

Microscopy analyses.

To determine rhizosphere colonization by GFP-marked strains, treated and untreated plant root surfaces were examined by optical epifluorescence and confocal microscopy. Epifluorescence images were taken using a Nikon Eclipse 50i microscope (Nikon) equipped with GFP HYQ and G- 2E/C filters, and photographs were taken with a DS Fi1 digital camera (Nikon). Confocal microscope images were obtained using Olympus FluoView 1000 confocal laser scanning (Olympus, Tokyo) equipped with high-performance sputtered filters.

Determination of plant growth parameters.

Twenty-five plantlets from each inoculated treatment as well as 25 non inoculated plantlets were analyzed. Root length and lateral roots number were measured directly in harvested plants, and FW was recorded as previously described (Compant et al. 2005; Nowak et al. 1995; Sessitsch et al. 2005). The chlorophyll contents were also determined following a published procedure (Porra et al. 1989). Chlorophyll was extracted from leaves of *A. thaliana* in N,N-9- dimethylformamide for 24 h at 4°C in the dark, and chlorophyll a and chlorophyll b concentrations were measured simultaneously by

spectrophotometry. For root hair number measurements, segments at a distance of 1 cm from the root tip of the primary root were analyzed using light microscopy.

Statistical analysis.

Data for plant growth parameters and population density (CFU/mg FW) were statistically analyzed using one-way analysis of variance, to compare multiple means. The chlorophyll contents and CFU/mg FW were subjected to logarithmic transformation before data analysis. When analysis of variance showed significant treatment effects, Tukey's honestly significant difference ($p < 0.5$) test was applied to make comparisons between treatments. Statistical analyses of plant gene expression were performed using the Mann-Whitney Test for nonparametrics data in the statistical software package STATISTICA (version 6.0; StatSoft Inc., Tulsa, OK, U.S.A.).

RESULTS

Effect of *B. phytofirmans* PsJN on growth of *A. thaliana*.

Based on its effects on other plant species (Frommel et al. 1991; Pillay and Nowak 1997; Ait Barka et al. 2000; Compant et al. 2005), it was supposed that *B. phytofirmans* PsJN would also promote growth of *A. thaliana*. Different growth parameters were evaluated in plants inoculated with strain PsJN, as well as in mock-inoculated controls. After 3 weeks of inoculation, the presence of strain PsJN increased FW (169%), primary root length (121%), root hair number (197%), and chlorophyll content (130%) of *A. thaliana* as compared with non-inoculated plants (Fig. 2A). The number of lateral roots, however, remained unchanged. AcdS has been reported as required for canola growth promotion by this bacterium (Sun et al. 2009); therefore, we tested the effect of a strain PsJN *acdS* mutant on *A. thaliana* growth. In contrast with the wild type strain, plants inoculated with this mutant did not increase primary root length, chlorophyll content, or the number of root hairs and only partially increased FW (126%) (Fig. 2A), indicating that AcdS activity and, therefore, ethylene levels, play a role in *A. thaliana* growth promotion produced by *B. phytofirmans* PsJN.

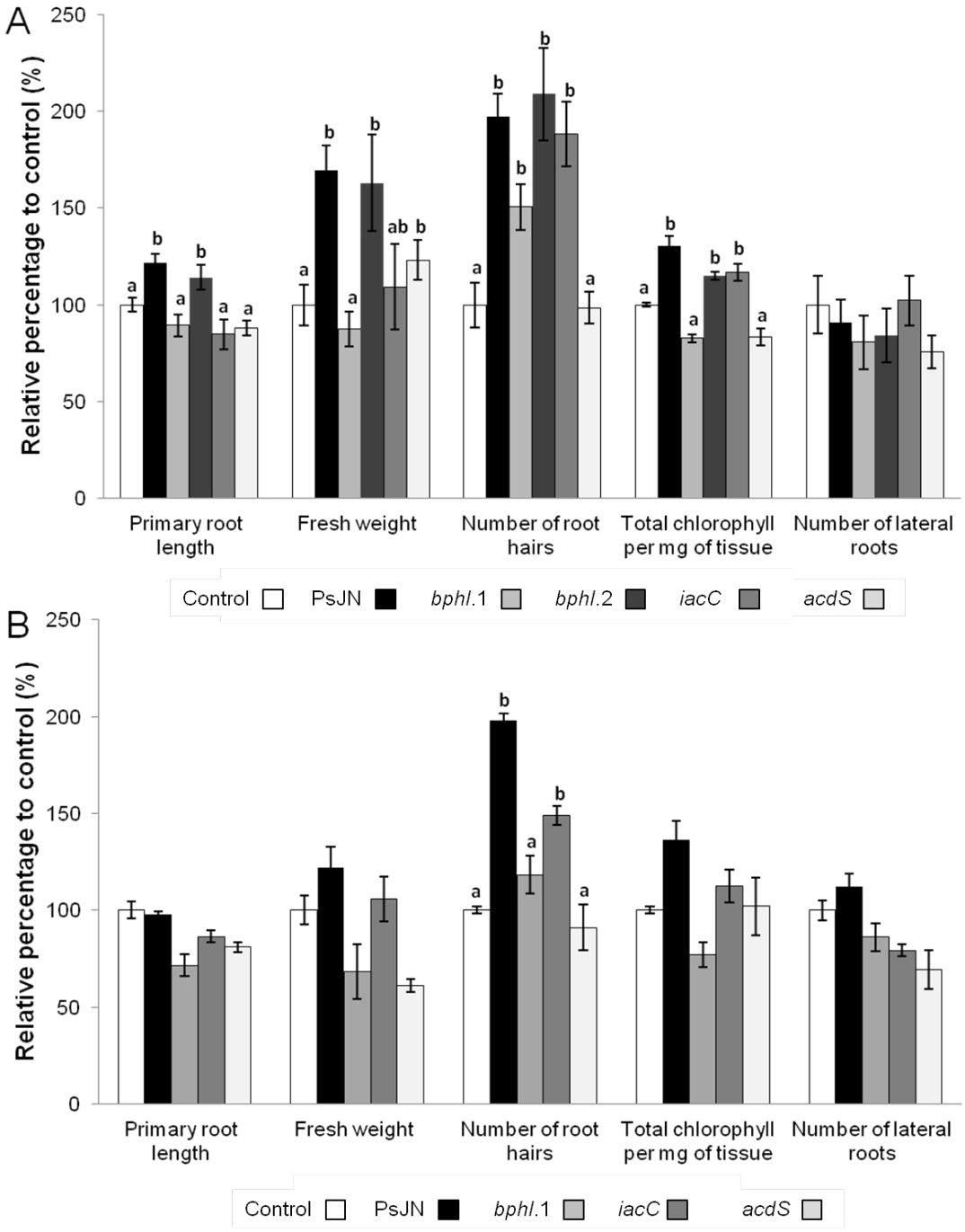

Figure 2. Plant growth and metabolic parameters in A, gnotobiotic *Arabidopsis thaliana* col-0 and B, *A. thaliana* miR393 overexpressor lines, culture systems inoculated with *Burkholderia phytofirmans* PsJN, its *bphI*.1, *bphI*.2, *iacC*, and *acdS* mutants, or non-inoculated (control). Growth parameters were measured 3 weeks after inoculation. Bars show mean percentage values with respect to control plants, and the error bars indicate standard deviations from average of three biological replicate experiments for each treatment. Different letters are used to indicate means that differ significantly (one-way analysis of variance Tukey's honestly significant difference tests, $p < 0.05$).

Effects of *B. phytofirmans* PsJN QS mutants on growth promotion and colonization of *A. thaliana*.

To test the role of tow QS systems of *B. phytofirmans* PsJN (Fig. 3) in the ability to promote plant growth, we used strain PsJN *bphI*.1 and *bphI*.2 HSL synthase mutants. In comparison with the wild type strain, *bphI*.1 and *bphI*.2 mutants produced significantly lower levels of 3-hydroxy-C8-HSL, and the *bphI.2* mutant did not produce C14-3-oxo-HSL (Fig. 4). In addition, the *bphI*.1 mutant displayed a decreased swimming motility (Fig. 5). These mutant strains grow on several carbon sources, such as fructose, 4-hydroxybenzoate, or benzoate, at the same levels as the wild-type strain (OD_{600nm} = 1.0, after 12 h of culture), indicating that their general metabolism is not affected. Concerning turnover of IAA (see next section), the *bphI*.1 and *bphI*.2 mutants synthesized IAA (19.6 and 19.9 μg/ml, respectively) to an amount similar to the wild-type strain (20.0 μg/ml), and the three strains grow with 2.5 mM IAA as the sole carbon and energy source at the same level: OD_{600nm} = 0.6, after 26 h of growth. After 3 weeks of inoculation, the *B. phytofirmans* PsJN *bphI*.1 mutant was unable to increase primary root length (89%), FW (87%), and chlorophyll content (82%) of *A. thaliana*, in contrast with the wild-type strain (Fig. 2A). Only the number of root hairs increased (150%) in plants inoculated with this mutant, although to a lower extent compared with the wild-type strain. In contrast, plants inoculated with the *bphI*.2 mutant increased primary root length, number of roots hairs, chlorophyll content, and number of lateral roots to an extent similar to that of plants inoculated with the wild type strain (Fig. 2A).

To determine whether differences in growth promotion of *A. thaliana* by *B. phytofirmans* PsJN and the *bphI*.1 mutant were due to different capabilities for

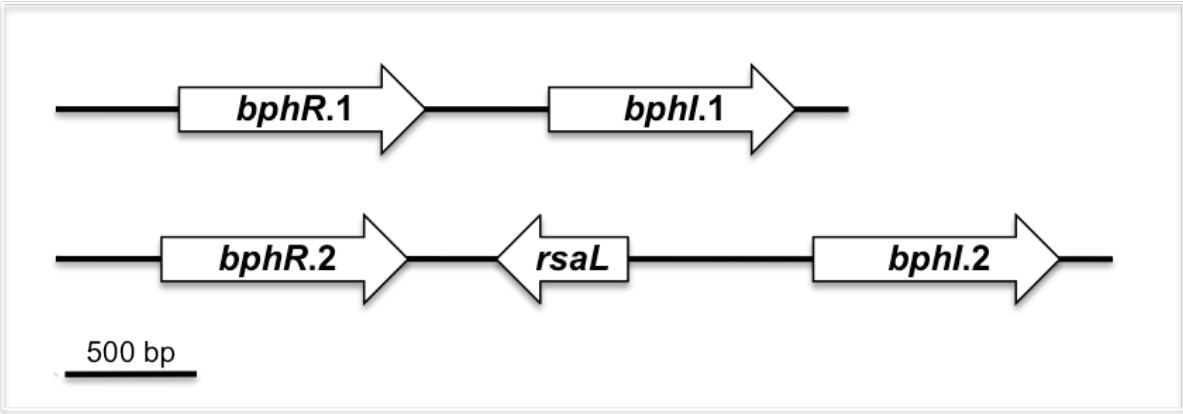

Figure 3. A map of DNA fragments from *B. phytofirmans* PsJN, containing bphI.1/bphR.1 and bphI.2/rsaL/bphR.2 QS systems genes from chromosome 1 and chromosome 2, respectively.

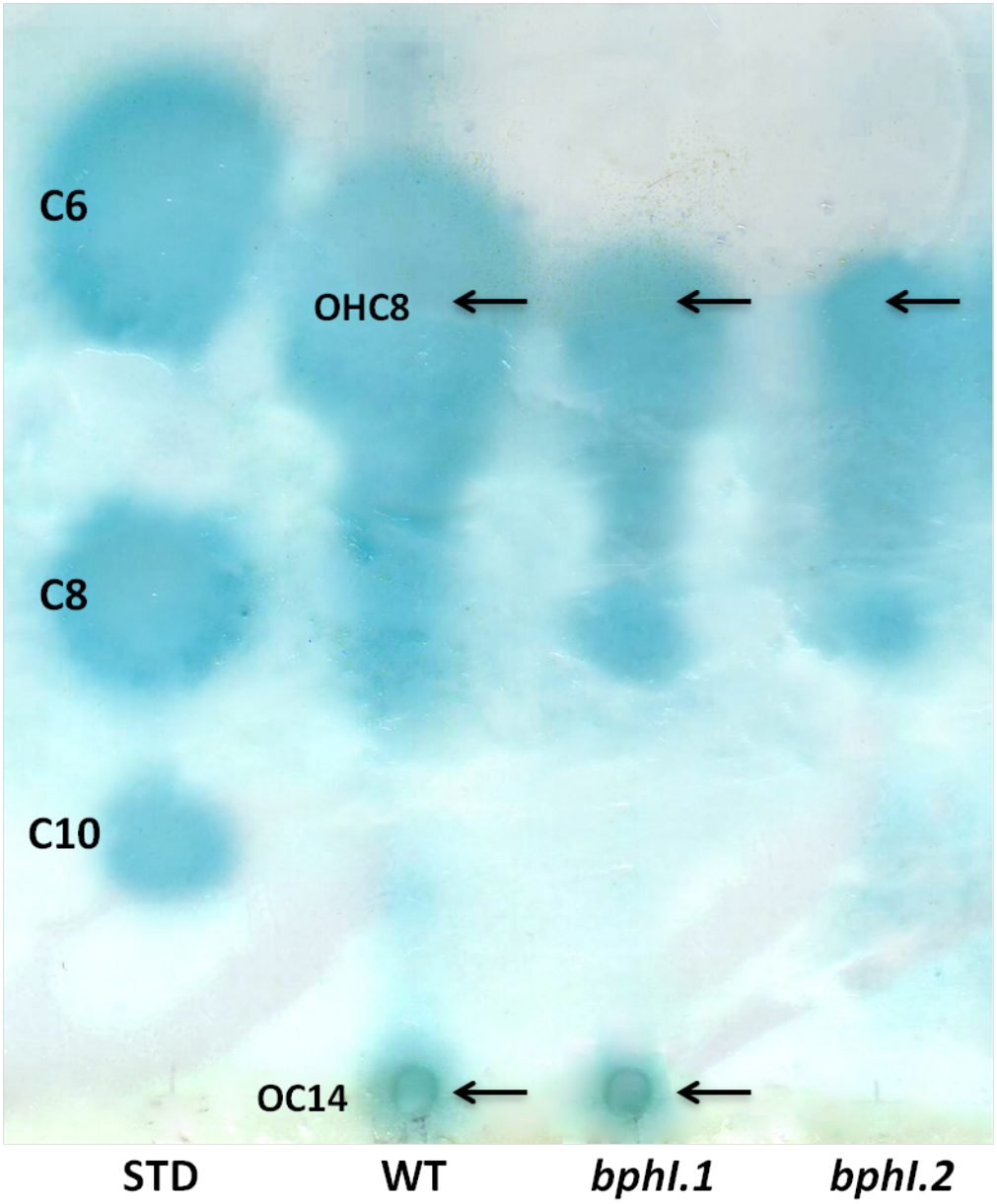

Figure 4. Thin layer chromatography analysis of N-acyl-homoserine lactones (HSL) produced by wild-type strain PsJN (WT), the PsJN *bphI*.1 mutant and the PsJN *bphI*.2 mutant. Arrows indicate OHC8 (3-hydroxy-C8-homoserine lactone) produced by WT, *bphI*.1 and *bphI*.2 mutants and OC14 (C14-3-oxo-homoserine lactone) produce by WT and *bphI*.2 mutant. Other spots could be associated with OH-C14-HSL and OH-C12-HSL production by this strain. STDs, synthetic standards of HSL used as reference, C6, hexanoyl homoserine lactone; C8, octanoyl-homoserine lactone; C10, decanoyl-homoserine lactone.

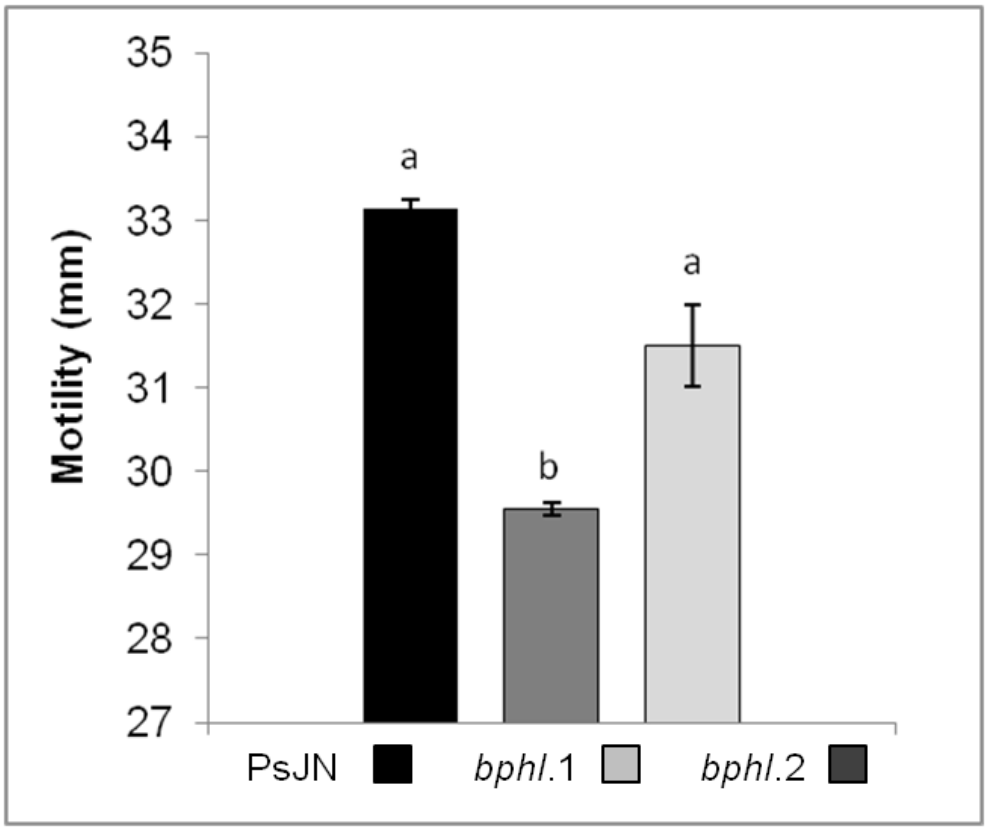

Figure 5. Swimming motility assay of wild type PsJN, and mutants *bphI*.1 and *bphI*.2. The assay was run on 0.3 % agar plates containing 5 mM fructose. Bacteria were point-inoculated and grown at 30°C for 24 h, as described in Atkinson et al. 2006. Values represent difference in radius between the middle and the outer ring. The bars show mean values, and the error bars indicate standard deviations from 8 plates analyzed. Different letters are used to indicate means that differ significantly (One way ANOVA Tukey's HSD tests; $p < 0.05$).

rhizospheric and endophytic colonization, bacterial colonization of this plant was evaluated in gnotobiotic cultures. Strain PsJN *bphI*.1 showed a reduced ability for rhizospheric colonization of *A. thaliana* (8 ± 0.1 log CFU/mg FW) compared with the wild type and the *bphI*.2 mutant (9.84 ± 0.01 and 9 ± 0.3 log CFU/mg FW, respectively).

Epifluorescence and confocal microscopy analysis of *A. thaliana* roots inoculated with the wild type PsJN or the *bphI*.1 mutant green fluorescent protein (GFP) tagged strains showed green fluorescent bacterial cells mainly attached to lateral root emergences and root tips and highly spread on hair roots; however, strong adherence to tissues was observed only with the wild-type strain (Fig. 6) and the *bphI*.2 mutant (data not shown), whereas the *bphI*.1 mutant cells are present but seem to move loosely on the root surface. Endophytic colonization of *A. thaliana* by both *bphI*.1 and *bphI*.2 mutants was statistically lower (5 ± 0.9 and 4.9 ± 0.2 log CFU/mg FW, respectively) than with the wild- type strain (7.9 ± 0.5 log CFU/mg FW). These results indicate that QS signaling is required for rhizospheric and endophytic colonization of strain PsJN in *A. thaliana* roots.

Effect of an IAA degradation mutant of *B. phytofirmans* on growth promotion and colonization of *A. thaliana*.

To study whether IAA degradation by *B. phytofirmans* PsJN has a role on plant growth promotion, the mutant *iacC*, completely unable to grow on IAA because it lacks a functional aromatic ring hydroxylating dioxygenase involved in the IAA degradation pathway (R. A. Donoso, unpublished results), was constructed and analyzed. This mutant

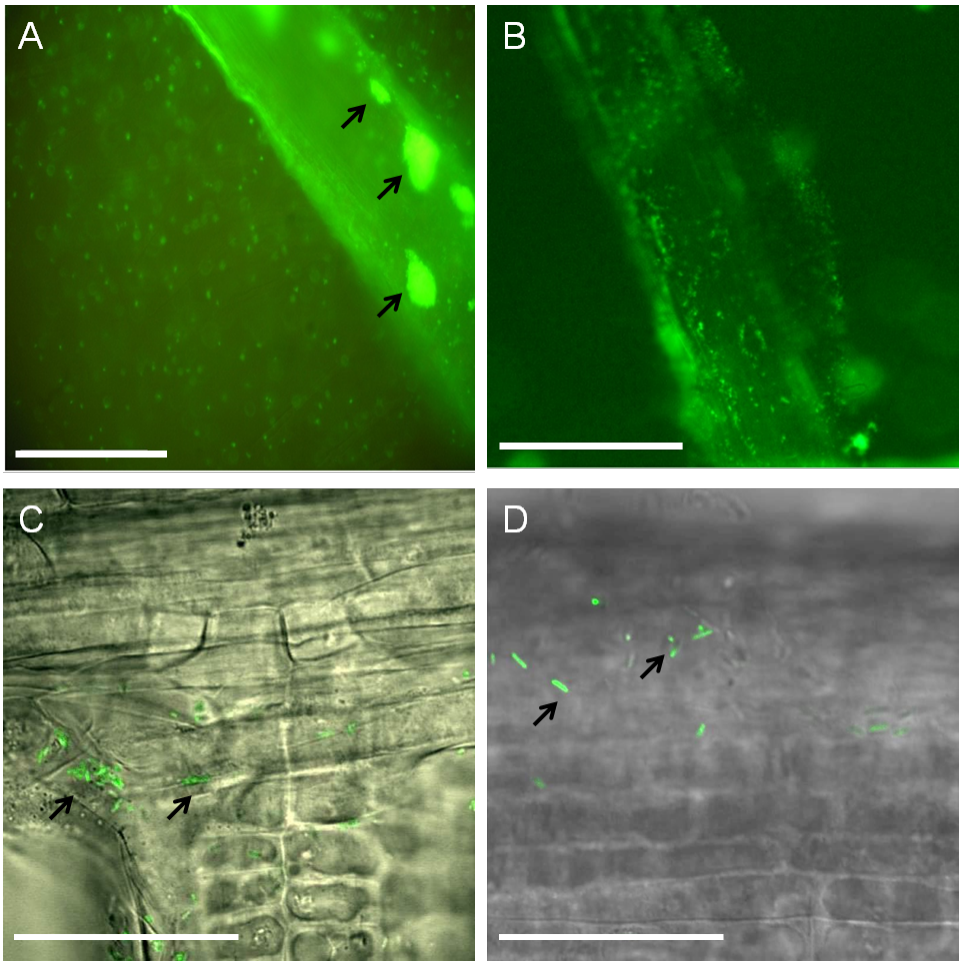

Figure 6. Representative examples of epifluorescence and confocal images of root colonization of *Arabidopsis thaliana* plants by wild type PsJN::GFP and *bphI*.1::GFP mutant. A, Epifluorescence microscopy of root surface colonization by strain PsJN; bacterial cells are observed as colonies attached to the surface of the root forming a film (arrows). B, Epifluorescence microscopy root surface colonization by *bphI*.1 mutant, bacterial cells are observed around the roots, on root hairs surface, but not being part of a film. C, Confocal microscopy of root colonization by strain PsJN, bacterial cells are observed as colonies attached to lateral root emergence and in an intercellular position, between the epidermal layer (arrows). D, Confocal microscopy of root colonization by the *bphI*.1 mutant; bacterial cells are observed over the root surface, but not attached to this surface (arrows). Scale bars: (A, B) 100 µm, (C, D) 10 µm.

grows on different carbon sources such as fructose, 4-hydroxybenzoate, or benzoate at the same yields and rates as the wild-type strain, and produces normal extracellular levels of IAA (18 μg/ml). After 3 weeks of inoculation of a gnotobiotic *A. thaliana* culture with the *iacC* mutant, no differences were observed in number of root hairs, chlorophyll content, and number of lateral roots with respect to plants inoculated with the wild-type PsJN (Fig. 2A). However, the growth promotion effects of the wild-type strain on FW and primary root length were not observed with the *iacC* mutant (Fig. 2A), indicating that degradation of IAA by *B. phytofirmans* PsJN is required for full plant growth promotion. Neither the rhizospheric (9.4 ± 0.1 versus 9.84 ± 0.01 log CFU/mg FW) nor the endophytic (6.8 ± 0.6 versus 7.9 ± 0.5 log CFU/mg FW) colonization levels in *A. thaliana* were affected in this mutant compared with the wild type PsJN. To further study the effect of IAA degradation on growth promotion by *B. phytofirmans*, the abilities of the wild-type strain and *iacC* mutant to abolish the effects of exogenously added IAA on root development were compared. Two concentrations of IAA were exogenously added when seeds were placed on plates. A sharp decrease in primary root lengths and leaf senescence was verified at 1 μM IAA (Fig. 2A and B). The inoculation with the wild type strain completely prevented the primary root length inhibition effect of such concentration of IAA, whereas the *iacC* mutant only partially reverted such inhibition (Fig. 7A and B).

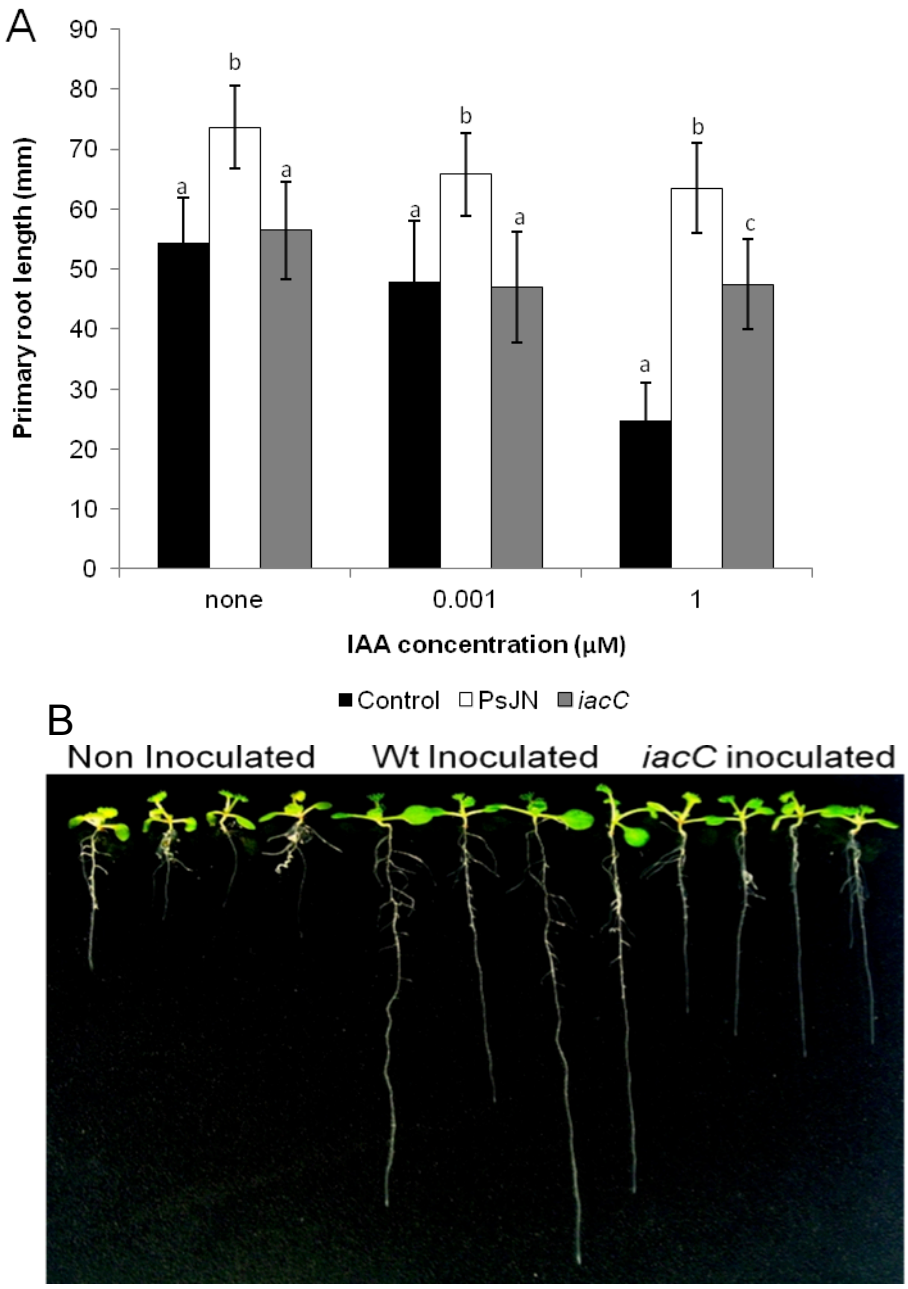

Fig. 7. A, Effect of exogenous addition of indole-3-acetic acid (IAA) on the elongation of primary roots in plants of *Arabidopsis thaliana* inoculated with *Burkholderia phytofirmans* PsJN or its *iacC* mutant. Different letters are used to indicate means that differ significantly in root lengths (one-way analysis of variance Tukey's honestly significant difference tests, $p < 0.05$). B, Photograph of 10-day-old seedlings grown exposed to 1 µM exogenous IAA.

Effect of *B. phytofirmans* PsJN and its mutants in growth promotion of a transgenic *A. thaliana* with reduced auxin signaling.

To determine whether IAA signaling in the plant is required for plant growth promotion by *B. phytofirmans* PsJN, a transgenic line of *A. thaliana* overexpressing miR393 (ox-miR393) was used. These plants have reduced expression of the auxin receptor genes *TIR1*, *AFB1*, *AFB2*, and *AFB3* and reduced auxin signaling (Navarro et al. 2006). Gnotobiotic cultures of ox-miR393 plants were inoculated with the wild type PsJN strain or the *iacC*, *bphI*.1, and *acdS* mutants. Three weeks after the inoculation, the wild-type strain and some of its mutants failed to produce an increase in primary root length, FW, or total chlorophyll content, as seen in wild type plants (Fig. 2B). Only the number of root hairs increased in the transgenic plant inoculated with the wild-type strain and the *iacC* mutant (Fig. 2B). *B. phytofirmans* colonized the rhizosphere of gnotobiotic cultures of ox-miR393 plants at normal levels (9.8 ± 0.1 log CFU/mg FW). However, no *B. phytofirmans* endophytes were found in ox-miR393 plants. The transcript levels of the auxin receptor genes *TIR1*, *AFB1*, *AFB2*, and *AFB3* were analyzed in wild-type plants inoculated with strain PsJN using quantitative real time polymerase chain reaction (qRT-PCR) (Fig. 8). Inoculation did not affect the expression of these genes in plants of four leaves (13 days after sowing). However, *AFB1* and *AFB3* genes were up regulated in PsJN inoculated plants at the stage of six leaves (19 days after sowing).

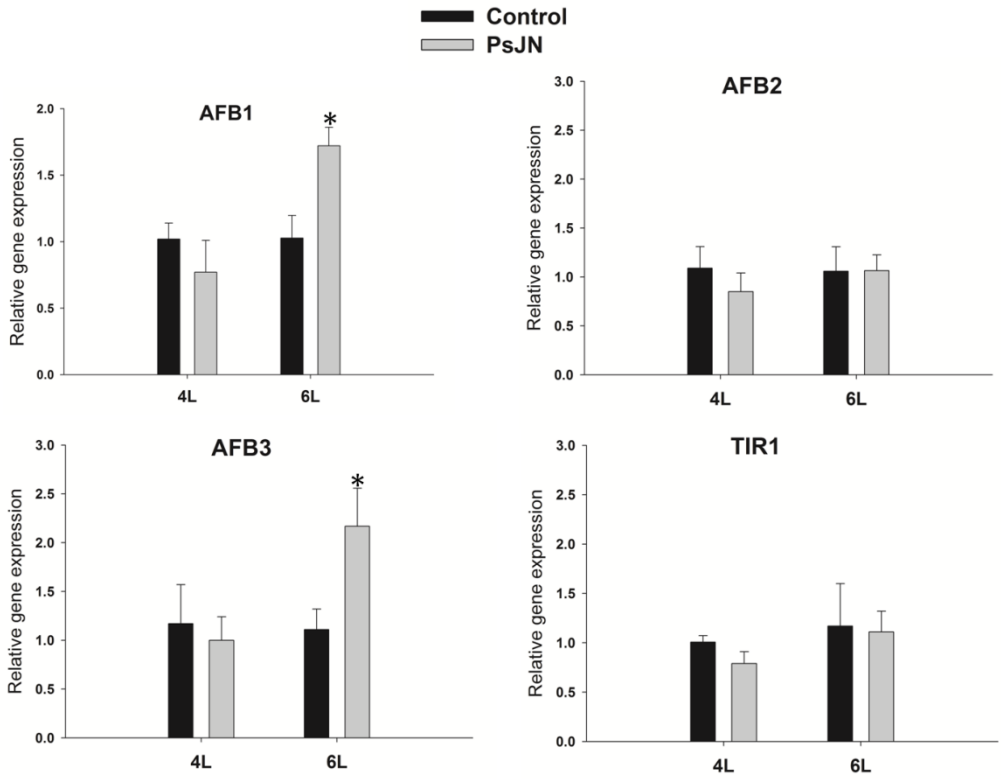

Fig. 8. Effect of *Burkholderia phytofirmans* PsJN inoculation in the gene expression of auxin receptors in wild-type plants. Quantitative real-time polymerase chain reaction determinations of relative levels of gene expression in complete plants at four rosette leaves (4L) or six rosette leaves (6L) stages. The housekeeping gene AtSAND was used to normalized transcript. Data are means ± standard errors of three biological replicates. Asterisk indicates statistical significance (*AFB1*: Mann-Whitney U test, Z = –1,963, P < 0.05; *AFB3*: Mann-Whitney U test, Z = –1,727, $p < 0.05$).

The role of the two QS systems in the regulation of selected genes.

In order to find if the two QS systems regulate the expression of motility and exopolysaccharide synthesis genes in the presence or absence of plant root exudates, the transcript levels of representative genes were analyzed in wild type PsJN strain and *bphI*.1, *bphI*.2 and *bphI*.12, single and double mutants, using qRT-PCR. We evaluated the expression levels of four undecaprenyl phosphate glucose phosphotransferase genes (*gumD*.1, *gumD*.2, *gumD*.3 and *gumD*.4) corresponding to the first genes of four operational clusters related with exopolysaccharide synthesis. *gumD*.1 and *gumD*.2 genes were up regulated in the *bphI*.1 mutant strain (Fig. 9A), but when cells were induced with plant root exudates genes *gumD*.1, *gumD*.2 and *gumD*.3 were down regulated in the *bphI*.1 mutant strain (Fig. 9B). Similar results were observed in expression levels of chemotactic motility *cheA* gene which was up regulated in the *bphI*.1 and *bphI*.12 single and double mutant strains in the absence of root exudates but down regulated in the *bphI*.1 mutant and up regulated in the *bphI*.2 and *bphI*.12 single and double mutants, in the presence of root exudates (Fig. 9C-D). No differences were observed in expression levels of flagellar motility related genes (*flgB, flhD, fliF, flhB*) in the evaluated conditions (Fig. 9C-D).

3-oxo-C14-HSL induces the expression of *bphI*.2, *rsaL* and *bphR*.1 genes of QS system.

In order to get some insight into the role of the BphI.1/R.1 and BphI.2/RsaL/R.2 regulons, we analyzed the transcript levels of genes from each QS system in wild type PsJN , *bphI*.1, *bphI*.2 and *bhpI*.12 mutant strains. We evaluated the expression of QS

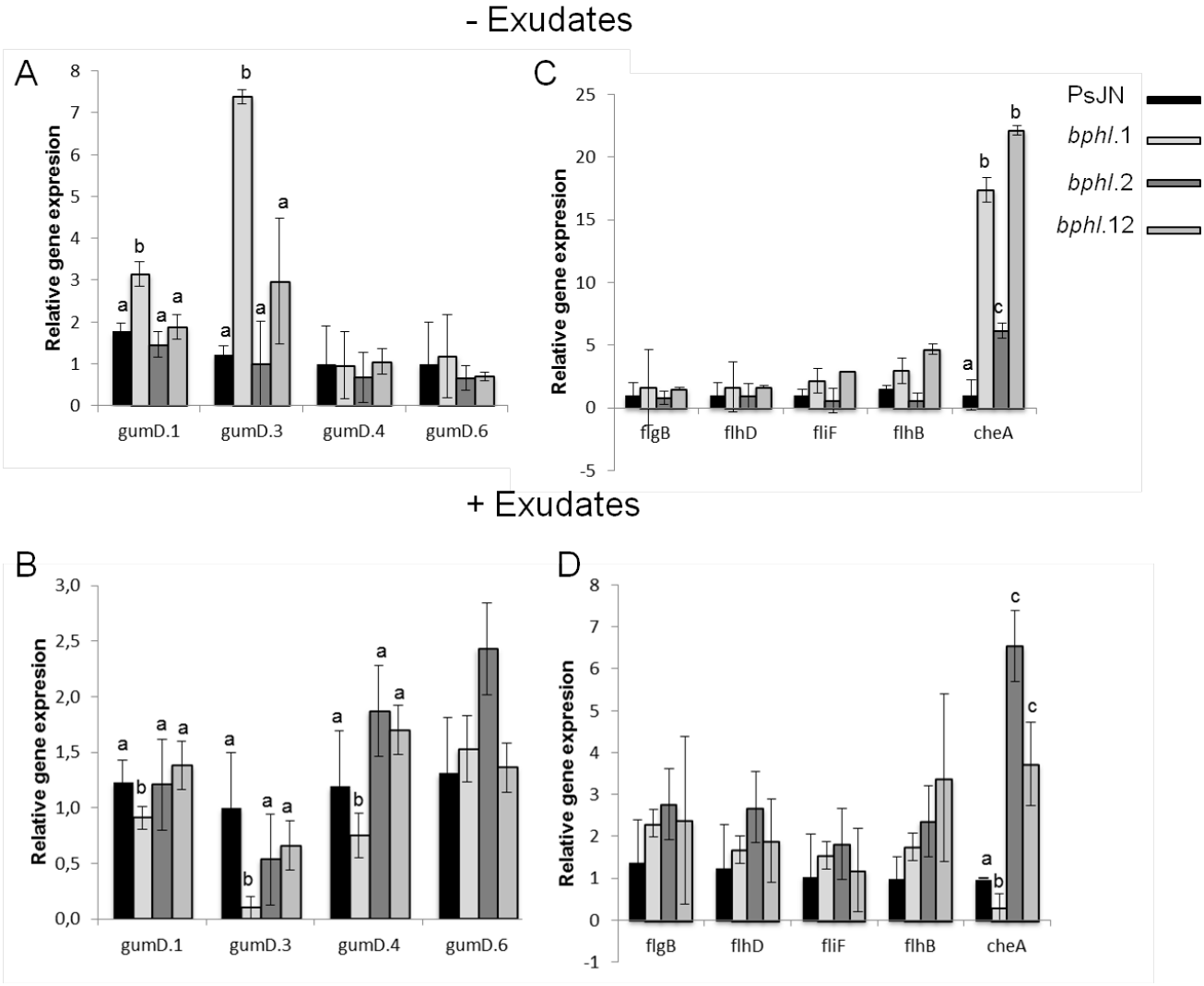

Figure 9. Role of two QS systems in regulation of selected genes. Real-time PCR analysis of exopolysaccharide and flagellar motility related genes in the presence or not of plant root exudates. *Burkholderia phytofirmans* PsJN wild type and *bphI*.1, *bphI*.2 and *bphI*.12 single and double mutant strains were grown in Dorn minimum medium with 10 mM of fructose (A,C) or plants root exudates (B,D) and incubated for 1 h and total RNA was isolated. Data are means ± standard errors of two biological replicates. Different letters are used to indicate means that differ significantly (one-way analysis of variance Tukey's honestly significant difference tests, $p < 0.05$).

genes of both systems in PsJN wild type, *bphI*.1, *bphI*.2 and *bphI*.12, single and double mutants, using qRT-PCR. We observed that *bphR*.1 gene was up regulated in the *bphI*.1 mutant strain but not in *bphI*.2 and *bphI*.12 single and double mutants, respectively (Fig. 10). In addition, *bphI*.2, *bphR*.2 and *rsaL* genes were down regulated in *bphI*.2 and *bphI*.12 single and double mutants (Fig. 10), indicating that 3-OXO-C14-HSL up-regulates the transcription of these genes.

The *Pseudomonas* homologue of the RsaL protein located between the *bphI*.2 and *bphR*.2 genes in *B. phytofirmans* PsJN, was reported to negatively regulate the transcription of the LasI HSL synthase in *P. aeruginosa and P. putida* (Rampioni et al. 2009). We were interested to determine whether RsaL from *B. phytofirmans* negatively regulates *bphI*.2. We overexpressed *bphR*.2 gene in wild type PsJN strain in the presence of 3-OXO-C14-HSL and its promoter activity was evaluated in $P_{bhpI.2}$ –*lacZ,* P_{rsaL} –*lacZ* or *rsaL*-$P_{bhpI.2}$ –*lacZ* transcriptional fusions. Similar to transcript levels results, we observed that the promoter activity of *bphI*.2 and *rsaL* genes was induced about 100 and 400 Miller units, respectively, in the presence of 1 μM 3-OXO-C14-HSL. When *rsaL* gene was present in the respective transcriptional fusion, *bphI*.2 gene promoter activity did not produce significant levels of *B*-galactosidase, even after exposure to 2 μM of 3-OXO-C14-HSL, indicating repression by RsaL regulator (Fig. 11).

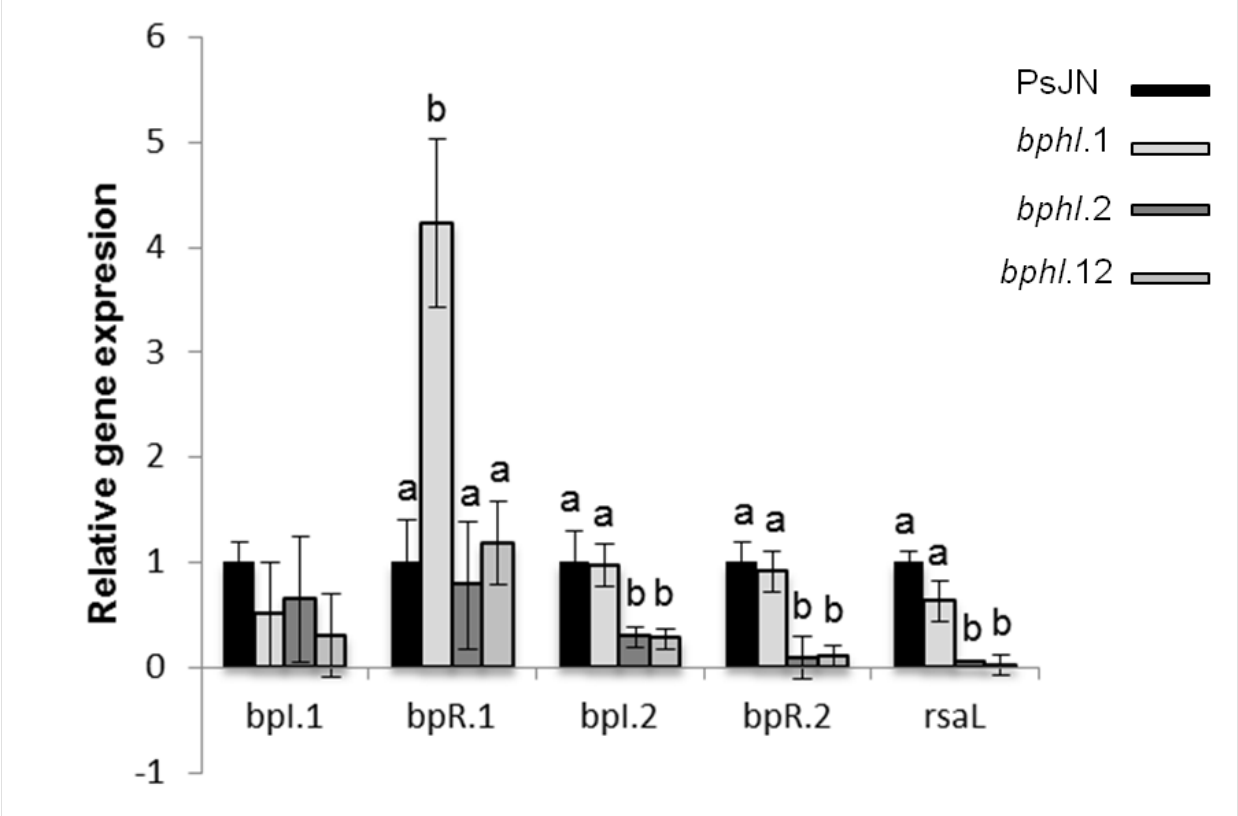

Figure 10. Transcriptional levels of quorum sensing (QS) genes in *Burkholderia phytofirmans* PsJN and *bphI*.1, *bphI*.2 and *bphI*.12 single and double mutants. Real-time PCR analysis of QS system genes from chromosome 1 and 2 was carried out in stationary phase of growth. *B. phytofirmans* PsJN wild type and *bphI*.1, *bphI*.2 and *bphI*.12 single and double mutants were grown in Dorn minimal medium with 10 mM of fructose at OD_{600nm}= 1.8 nm and total RNA was isolated. Data are means ± standard errors of two biological replicates. Different letters are used to indicate means that differ significantly (one-way analysis of variance Tukey's honestly significant difference tests, $P < 0.05$).

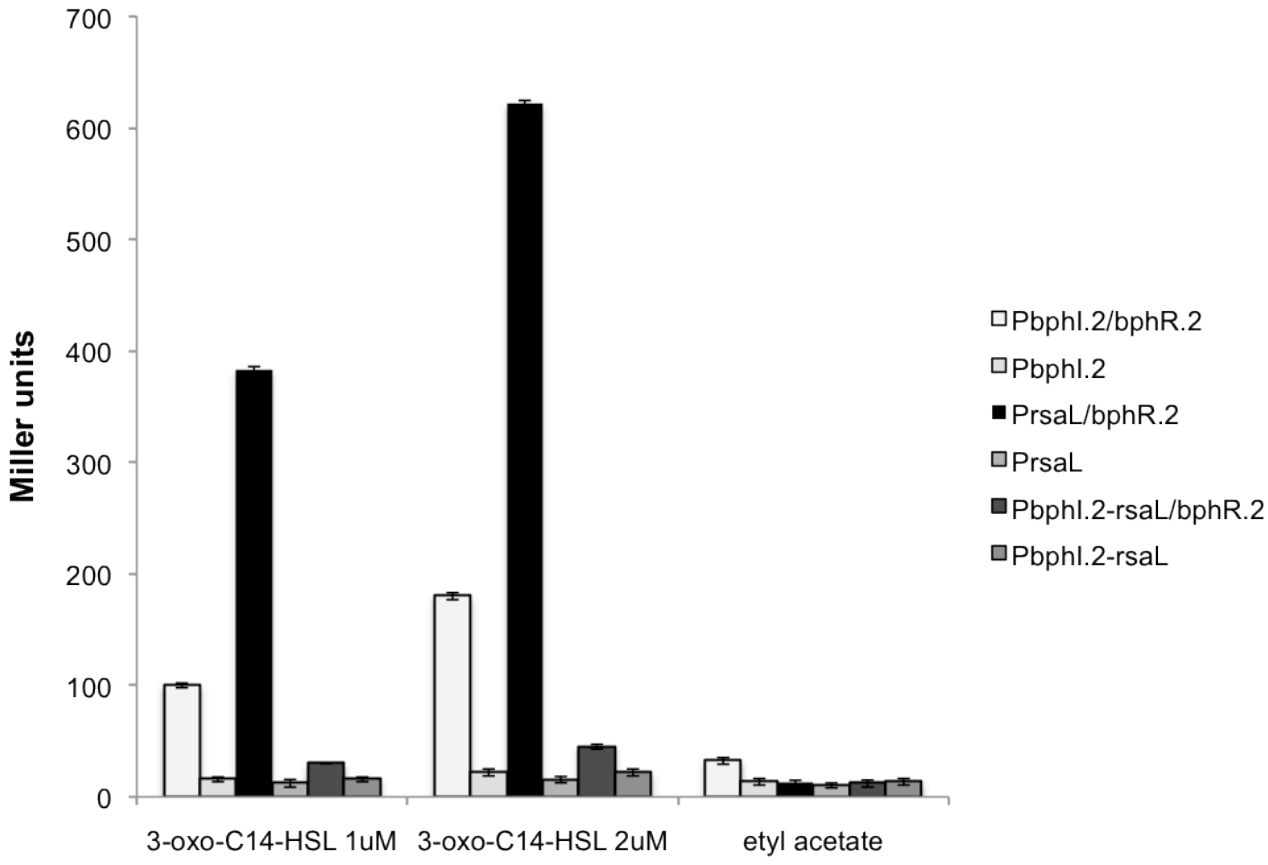

Figure 11. *bphI*.2 and *rsaL* gene promoter activities in wild type *Burkholderia phytofirmans* PsJN. The *B*-galactosidase activities were measured after 12 h of growth in the presence of 1 µM or 2 µM 3-oxo-C14-homoserine lactone (HSL) from transcriptional fusions $P_{bhpI.2}$–*lacZ,* P_{rsaL}–*lacZ* or *rsaL*-$P_{bhpI.2}$–*lacZ*, in presence or not of an overexpressed *bphR*.2 plasmid. Ethyl acetate was used as negative control of induction. All experiments were performed in triplicate, and mean values with standard deviations are indicated.

DISCUSSION

B. phytofirmans colonizes the rhizosphere and internal tissues of its plant hosts (Compant et al. 2005; Sessitsch et al. 2005). In this work, we showed that the plant model *A. thaliana* is also colonized by strain PsJN. We also found that AcdS activity and QS are required for plant growth promotion and rhizospheric and endophytic colonization in *A. thaliana*. We report, for the first time, the involvement of IAA in plant growth promotion by this well-known PGPR. An IAA degradation mutant is not effective in promoting plant growth and in preventing adverse effects of exogenously added IAA; the plant growth promotion effects are partially abolished in *A. thaliana* plants with reduced IAA signaling, and the expression of some auxin receptors is up-regulated in inoculated plants. In this context, it is worth mentioning that batch cultures of *B. phytofirmans* degrading IAA present two fold longer lag phases compared with other aromatic carbon sources (data not shown), and the synthesis of IAA by *B. phytofirmans* occurs before IAA degradation, suggesting that IAA turnover is carefully controlled in this bacterium (R. A. Donoso, unpublished results). Furthermore, qRT-PCR detection of *iacC* gene transcripts and *iacC* gene promoter transcriptional fusion analysis clearly demonstrated that IAA regulates its degradation and is able to significantly induce *iacC* gene expression (R. A. Donoso, unpublished results).

Although some hints of the mechanism or mechanisms used by *B. phytofirmans* to promote plants growth have been proposed (Ait Barka et al. 2000; Sessitsch et al. 2005), thus far only the involvement of AcdS in primary root length stimulation in canola had been described (Sun et al. 2009). In our model plant, the effect of PsJN inoculation on root elongation is certainly lesser than the elongation observed with other plant models

such as canola (Sun et al. 2009); however, we have shown it is reproducible and statistically significant, which allows us to differentiate the effects of the wild type strain and the *acdS* mutant strain. Interestingly, tests with the IAA degradation mutant (*iacC*) also showed no improvement of primary root lengths. It has been described that root growth can be stimulated or inhibited depending on the concentration of IAA produced by bacteria (López-Bucio et al. 2007; Persello-Cartieux et al. 2001). In plants inoculated with the *iacC* mutant, IAA levels in roots should be higher than in roots inoculated with the wild type PsJN strain, because this mutant cannot degrade IAA and, thus, the plant would be more susceptible to primary root shortening. Leveau and Lindow (2005) have described similar effects in radish plants inoculated with *P. putida* 1290, a strain that catabolizes IAA. This can be explained through the cross-talk between auxin and ethylene synthesis in plants, where auxin increases ACC synthase gene transcription, stimulating ethylene synthesis and, conversely, ethylene promotes expression of IAA biosynthetic genes increasing IAA levels at the root tip (Růzicka et al. 2007; Swarup et al. 2007; Strader et al. 2010). Therefore, high concentrations of IAA lead to high levels of ethylene, decreasing root cell length (Strader et al. 2010). Consistently, we found that strain PsJN abolishes the effect of exogenous IAA that decreases primary root length and leaf senescence and that *A. thaliana* plants inoculated with the *iacC* mutant showed only a partial reversion of the effect of exogenous IAA. Although we cannot discard the possibility that the *iacC* mutant partially transforms exogenously added IAA, this partial effect may be also explained through cross talk with the AcdS effect, which is still operative in the *iacC* mutant, that reduces levels of ethylene and that is visible in the stimulation root elongation and reduction of leaf senescence. It would be expected that

the *acdS* and *iacC* genes double mutant strain completely revert observed effects of exogenously added IAA with the *iacC* mutant strain. These observations suggest that both AcdS and IAA degradation activities are important in primary root growth promotion by *B. phytofirmans* PsJN (Table 2).

The use of an *A. thaliana* transgenic line overexpressing miR393 provided additional support for the involvement of plant IAA signaling in plant growth promotion by *B. phytofirmans* PsJN. The positive effects of bacterial inoculation almost completely disappeared in ox-mi393 plants. Although rhizospheric colonization still takes place in this transgenic line, endophytic colonization by *B. phytofirmans* was grossly impaired. Similar results were found with ox-miR393 plants inoculated with pathogen *P. syringae* DC 3000, where repression of auxin signaling restricted its growth inside plants (Navarro et al. 2006). The results obtained with this *A. thaliana* transgenic line which, among other effects in auxin signaling, has reduced expression of auxin receptor genes (Navarro et al. 2006), prompted us to test the effect of inoculation with strain PsJN on the expression of these genes, finding up-regulation of the expression of *AFB1* and *AFB3* genes in a developmental time controlled manner. Furthermore, our lab has analyzed recently global transcription changes in Arabidopsis plants inoculated with strain PsJN, finding that genes implicated in auxin pathway were significantly regulated (Poupin et al. 2013).

Bacterial IAA synthesis has been proposed as an important feature in pathogenic bacteria-plant interactions (Comai and Kosuge 1982; Navarro et al. 2006) as well as in phytostimulation processes (Patten and Glick, 2002a and b) and, consequently, should play a relevant role in plant growth promotion mechanisms in *B. phytofirmans* and other PGPR. The results reported here support that possibility; unfortunately, the construction

Table 2. Resume of growth promoting effects in *A. thaliana* plants inoculated by different *B. phytofirmans* PsJN strains.

Strain	**Characteristic**	**Positive Effect on *A. thaliana* Plants Growth.**
PsJN	Wild type strain	Primary root legth; fresh weight;number of root hairs; total chorophyll per mg of tissue
bphI.1	Homoserine lactone sinthase, of chromosome 1, null mutant strain	Number of root hairs
bphI.2	homoserine lactone sinthase, of chromosome 2, null mutant strain	Primary root legth; fresh weight;number of root hairs; total chorophyll per mg of tissue
iacC	IAA degrading null mutant strain	Number of root hairs; total chorophyll per mg of tissue
acdS	ACC desaminase null mutant strain	Fresh weight

of IAA synthesis strain PsJN mutants, which may allow testing the effects on plant growth promotion, is not simple because this strain possesses at least two putative IAA biosynthetic pathways: the indole-3-acetamide pathway and the tryptophan side chain oxidase pathway (Weilharter et al. 2011). In addition, we carried out homology searches, in the strain PsJN genome, of other IAA synthesis pathways and we found putative marker genes for the indole-3-pyruvate, indole-3-acetonitrile, and tryptamine pathway. We have generated single mutants for all these marker genes but these mutants still synthesize IAA (R. A. Donoso, unpublished results), indicating that strain PsJN carries multiple pathways to synthesize this compound. This is similar to the case of *Azospirillum brasilense*, in which at least three different pathways of IAA synthesis are functional (Prinsen et al. 1993).

Other molecular signaling processes may contribute to PGPR performance. Recently, it has been described that the PGPR *Azospirillum lipoferum* cell-to-cell communication QS system mediated by HSL is implicated in rhizosphere competence and adaptation to plant roots (Boyer et al. 2008). Furthermore, a QS system has been involved in the regulation of plant growth promoting traits of *P. aeruginosa* PUPa3 (Steindler et al. 2009). We reported here that a *bphI*.1 mutant in the QS system of *B. phytofirmans*, which produces lower HSL levels than the wild type strain, is unable to promote growth on *A. thaliana*. This is probably due to the lower rhizosphere colonization and null endophytic colonization ability of the mutant compared with the wild type strain. Consistently, low motility, and grossly impaired adherence to *A. thaliana* root surface were observed in the *bphI*.1 mutant compared with the wild-type strain. Analysis of transcript levels of genes involved in flagellar motility *flgB, flhD, fliF,*

flhB did not show differences between wild type and mutants, but the chemotaxis *cheA* gene was up-regulated in *bphI*.1 and *bphI*.12 single and double mutants. Several studies have demonstrated that motility is important in root colonization and non-motile mutants belong to the less competitive class of colonizing bacteria (Swart et al. 1994; Dekkers et al. 1998; Chin-A-Woeng et al. 2000; Lugtenberg et al. 2001), but when mutants are tested alone in gnotobiotic systems the ability to colonize the root is not significantly different from that observed in wild type strains (Lugtenberg et al. 2001). In contrast, *cheA* gene mutants tested in gnotobiotic systems are severely defective in colonization, indicating that motility is required for root colonization and chemotaxis is very important in the initial process (Kravchenko and Makarova, 1993; Dekkers et al. 2000; Lugtenberg et al. 2001).

A particular importance have also the ability of bacteria to form microcolonies on different parts of the roots, from the tip to the elongation zone, that can grow into large population sizes on roots to form mature biofilms (Rudrappa et al. 2008b). Diverse exopolysaccharides produced by different strains have been involved in biofilm structures (Mack et al. 1996; Miller and Bassler, 2001; Gotz, 2002; Waters and Bassler, 2005). We evaluated four *gumD* genes involved in exopolysaccharide production in *B. phytofirmans,* either as uronic acids (D-glucuronic acid, D-galacturonic and D-mannuronic acids) or ketal groups linked to pyruvate. Similar to what was observed with the chemotaxis *cheA* gen, we found that *gumD*.1 and *gumD*.3 were up regulated in the *bphI*.1 mutant. These results suggest two possibilities: i) that 3-OH-C8-HSL has a negative regulation on these genes or ii) that 3-OXO-C14-HSL exerts up-regulation on these genes. Because surface of plant roots is very dynamic and between 10% and 40% of photosynthates are secreted

as root exudates (Lugtenberg et al 1999; Rudrappa et al. 2008a; 2008b), providing a source of organic compounds important for roots colonization, we determined the transcript level of these genes in the presence of root exudates. We observed that transcript levels of chemotaxis *cheA* gene were down regulated in *bphI*.1 and *bphI*.12 mutant strains in the presence of plant root exudates (Fig. 8D). The same result was observed in transcript levels of *gumD*.1, *gumD*.3, and *gumD*.4 genes that were down regulated in the *bphI*.1 mutant strain (Fig. 8B). Transcriptomic analysis of *B. phytofirmans* in the presence of plant root exudates showed that genes involved in stress responses are up regulated (González B., unpublished results), suggesting that QS genes expression may be reduced in the stress conditions generated by the presence of plant root exudates. Moreover, compounds that are capable of inhibiting or interfering with QS signals have been identified. It is possible that plants may also synthesize and secrete compounds that mimic the activity of bacterial HSL signaling compounds. Several types of QS-inhibiting phytochemicals, such as polyphenols, are capable of affecting biofilm formation in some bacteria (Nazzarro et al. 2013). The AHL signal-mimic activities detected in pea (Pisum sativum) exudates might play important roles in stimulating HSL-regulated behaviors in certain bacterial strains while inhibiting these behaviors in others. This suggests that there is significant crosstalk between different bacterial species and plant roots within the rhizosphere, which is mediated through precise combinations of signal transduction and response regulation. Structures of most AHL signal-mimic compounds have not been elucidated, however, some basic structures of biomolecules that acts as a signals in the rhizosphere have structural similarities to AHL molecules (Zhuang et al. 2013), even the structure of IAA involved in plant signaling before

discussed in this study. All these molecules may perfectly being part of plant root exudates thus explaining impaired gene regulation by QS system observed here (Brencic and Winans, 2005; Boyer and Wisniewski-Dyé, 2009). Additional experiments on gene expression analysis in QS mutants at different times are required to track the transcript levels of *cheA* and *gumD* genes at the beginning of roots colonization.

Regulation by a QS system may influence key steps leading to biofilm formation required for plant growth promotion. This may explain low rhizosphere and endophytic colonization and lack of plant growth promoting traits observed with the PsJN *bphI*.1 mutant, and low endophytic levels of the *bphI*.2 mutant. The production of exopolysaccharide polymer has been reported in strain PsJN and in other plant associated *Burkholderia* spp., and it is believed to be involved in the plant-bacterium interaction (Ferreira et al. 2010). Interestingly, impaired exopolysaccharide production affected endophytic colonization by a QS mutant in *B. kururiensis* (Suárez-Moreno et al. 2010). Although the production of cell wall degrading endoglucanases by strain PsJN plays a role in grapevine endophytic colonization (Compant et al. 2005), both *bphI* mutants and the wild-type PsJN strain showed no differences in endoglucanase levels (data not shown), indicating that lower endophytic colonization found with *A. thaliana* is not due to the lack of this cell wall degrading activity.

Studies in roots of tomato plants growing in nonsterile soil provide strong evidence that AHL molecules are produced by the bacterial consortium naturally colonizing and strongly support the view that these molecules may act as signals for coordinating the functions of the different populations within the community (Steidle et al 2001; Gotschlich et al. 2001). It is probably that QS systems of *B. phytofirmans* PsJN,

are functional in rhizosphere, but anyway all effects observed in *A. thaliana* by *B. phytofirmans* PsJN, related with colonization and/or plant growth promoting traits mediated by QS system and IAA signaling, need to be demonstrated by using a soil based system.

We also studied how QS systems of *B. phytofirmans* PsJN are regulated. We establish that 3-OXO-C14-HSL induces *bphI*.2/*rsaL*/*bphR*.2 genes of QS system of chromosome 2 and RsaL represses the activity of *bphI*.2 promoter in the presence of 3-OXO-C14-HSL. The same HSL molecule is the QS signal in *B. kururiensis* M130, *B. unamae* MT641 and *B. xenovorans* LB400 (Suárez-Moreno et al. 2010). We also observed that 3-OXO-C14-HSL induces the expression of the *bphR*.1 gene regulator in the *bphI*.1 mutant strain, indicating a possible hierarchical regulation. The conserved QS systems in beneficial *Burkholderia* are related to the LasI/R and PpuI/R HSL QS systems of *P. aeruginosa* and *P. putida*, respectively (Suárez-Moreno et al. 2008). These QS systems are characterized as hierarchical, specifically in *P. aeruginosa*, where LasR-3-C12-HSL activates transcription of *rhlIR* and *rhlI* genes, belonging to the second QS system of *P. aeruginosa.* An RsaL repressor is present in the intergenic region between the *lasR* and *lasI* genes, which is directly activated by LasR-3OC12-HSL and inhibit QS by repressing *lasI* transcription (Schuster and Greenberg, 2006). RsaL repressor controls the expression of over 200 genes in *P. aeruginosa*, half of which are also QS-controlled (Rampioni et al., 2009). It is possible that RsaL repressor in *B. phytofirmans* PsJN has a similar function, but additional studies are necessary to confirm its role in PsJN strain.

Based on expression analysis in wild type and QS mutant strains, we propose a model for QS systems including target genes and regulation in *B. phytofirmans* PsJN (Fig. 12).

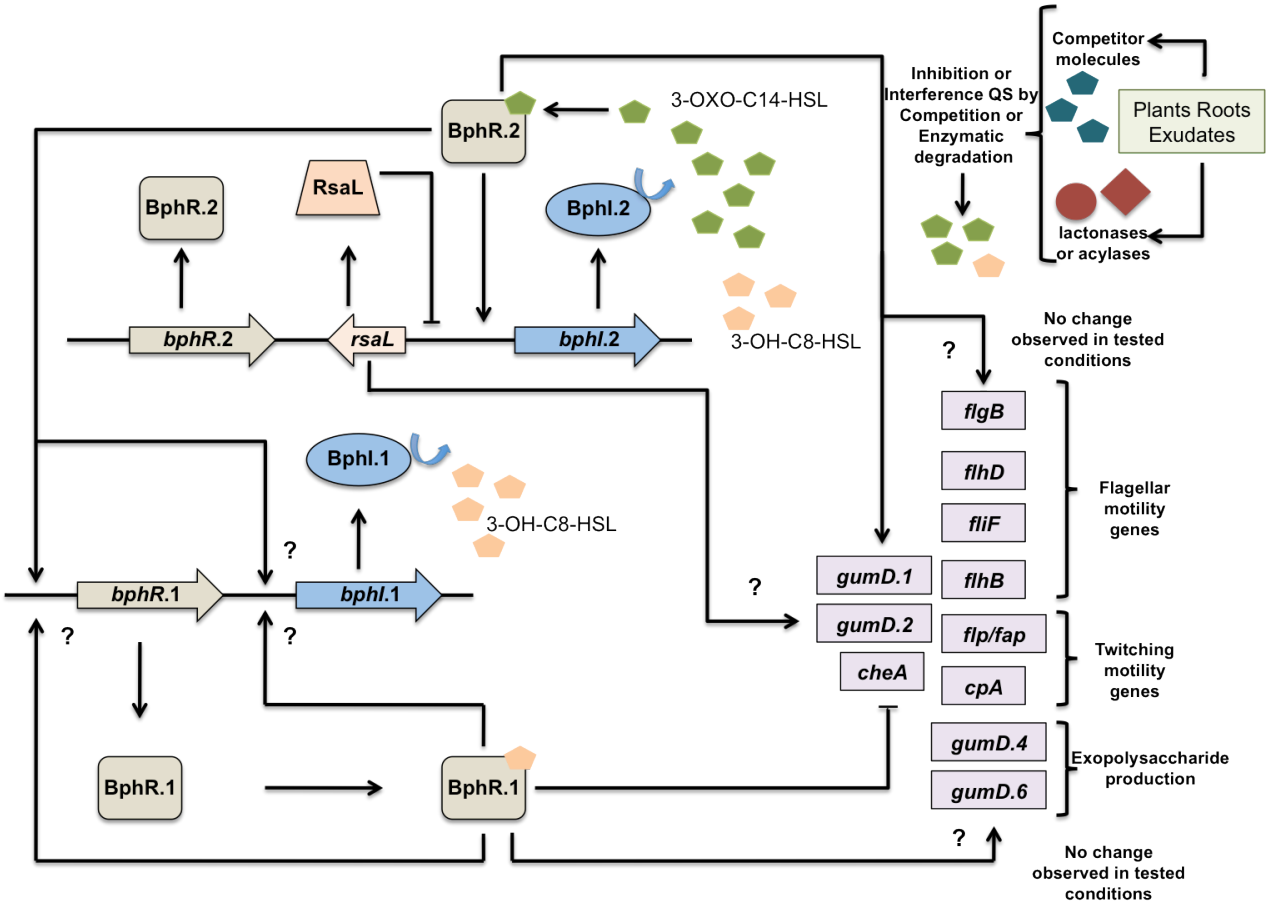

Figure 12. Schematic model of the two homoserine lactone QS systems of *Burkholderia phytofirmans* PsJN. The BphR.1 receptor responds to 3-OH-C8-HSL produced by the BphI.1 HSL synthase and BphR.2 responds to 3-OXO-C14-HSL produced by BphI.2 HSL synthase. BphR.2-3-OXO-C14-HSL activates expression of *bphI*.2, *rsaL*, *bphR*.2 and *bphR*.1 genes, whereas RsaL represses *bphI.2* transcription. BphR.2-3-OXO-C14-HSL seems to regulate positively chemotaxis *cheA* and exopolysaccharide production relates *gumD*.1 and *gumD*.3 genes. On the contrary, it is probable that BphI.1-3-OH-HSL regulates negatively these genes. However, it is currently uncertain whether the expression of flagellar and twitching motility and other exopolisaccharide production related genes are or not under BphI/R regulation, as has been demonstrated in *P. aeruginosa* (Juhas et al. 2005) and *P. putida* (Steidle et al. 2002). Similarly, it is also unclear whether RsaL can down-regulate these or another genes (Rampioni et al. 2009). Factors affecting directly HSL synthesis or accumulation within the cell, as possible compounds present in plant roots exudates, could inhibit or interfere in QS by competing or enzymatic degradation of these signal molecules (Brencic and Winans, 2005; Boyer and Wisniewski-Dyé, 2009).

CONCLUSIONS

** *B. phytofirmans* PsJN promotes growth of *A. thaliana*, increasing the root length, fresh weight and total chlorophyll content and colonizes the rizhosphere and internal tissues.

** The ACC deaminase (AcdS) activity and bacteria-bacteria communication, mediated by 3-hydroxy-C8-HSL, are required for *A. thaliana* growth promotion and rhizosphere and endophytic colonization by *B. phytofirmans* PsJN.

** The ability to degrade IAA by *B. phytofirmans* PsJN is important for effective *A. thaliana* growth promotion and in preventing adverse effects of exogenously added IAA.

** Auxin receptors are up regulated in *A. thaliana* inoculated with *B. phytofirmans* PsJN supporting the involvement of IAA in plant growth promotion by this bacterium.

** Chemotaxis and exopolysaccharide production related genes, are regulated by QS systems and could be relevant to colonize *A. thaliana* plants by *B. phytofirmans* PsJN.

** The characterization of the QS systems in *B. phytofirmans* PsJN, one of species from the new plant-associated *Burkholderia* cluster, demonstrate that BphI.1/R.1 and BphI.2/R.2 have different ways to regulate genes with functions related to plants colonization, as will be predicted because their differences in HSL signal molecules production and response.

** The 3-OXO-C14-HSL QS signal molecule up regulates transcriptional levels of *bphI*.2, *rsaL*, *bphR*.2 and *bphR*.1 genes, suggesting that a transcriptional regulatory hierarchy mediated by 3-OXO-C14-HSL is probably present between BphI.1/R.1 and BphI.2/RsaL/BphR.2 QS systems.

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