Quorum-Sensing Systems in the Plant Growth-Promoting Bacterium *Paraburkholderia phytofirmans* PsJN Exhibit Cross-Regulation and Are Involved in Biofilm Formation

Ana Zúñiga, Raúl A. Donoso, Daniela Ruiz, Gonzalo A. Ruz, and Bernardo González

Facultad de Ingeniería y Ciencias, Universidad Adolfo Ibáñez, Millennium Nucleus Center for Plant Systems and Synthetic Biology, and Center of Applied Ecology and Sustainability (CAPES), Santiago, Chile

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Quorum-sensing systems play important roles in host colonization and host establishment of *Burkholderiales* species. Beneficial *Paraburkholderia* species share a conserved quorum-sensing (QS) system, designated Brl/R, that controls different phenotypes. In this context, the plant growth-promoting bacterium *Paraburkholderia phytofirmans* PsJN possesses two different homoserine lactone QS systems BpI.1/R.1 and BpI.2/R.2 (Brl/R-like QS system). The BpI.1/R.1 QS system was previously reported to be important to colonize and produce beneficial effects in *Arabidopsis thaliana* plants. Here, we analyzed the temporal variations of the QS gene transcript levels in the wild-type strain colonizing plant roots. The gene expression patterns showed relevant differences in both QS systems compared with the wild-type strain in the unplanted control treatment. The gene expression data were used to reconstruct a regulatory network model of QS systems in *P. phytofirmans* PsJN, using a Boolean network model. Also, we examined the phenotypic traits and transcript levels of genes involved in QS systems, using *P. phytofirmans* mutants in homoserine lactone synthases genes. We observed that the BpI.1/R.1 QS system regulates biofilm formation production in strain PsJN and this phenotype was associated with the lower expression of a specific extracytoplasmic function sigma factor *ecf26.1* gene (implicated in biofilm formation) in the *bpl.1* mutant strain.

The nutrient-rich rhizosphere is essential for plant-microorganism associations, since plant exudates enrich the soil material in close contact with plant roots and, therefore, contain much larger microbial numbers and activities than the nonrooted bulk soil; thus, it is from the rhizosphere that microbes are recruited to play some beneficial or pathogenic functions in plants (Bakker et al. 2013). Plant growth-promoting bacteria (PGPB) are one of the best-studied groups of beneficial bacteria because of their increasing agronomic importance. PGPB are able to colonize root surfaces, forming tight rhizospheric interactions and, occasionally, establishing endophytic populations (Dekkers et al. 1998; Idris et al. 2007; Lery et al. 2011). PGPB induce beneficial effects on plant

growth through several mechanisms, such as nitrogen fixation, improvement of nutrient absorption, phytohormone production, and increasing tolerance to biotic and abiotic stresses (Bhattacharyya and Jha 2012; Compant et al. 2008; Idris et al. 2007; López-Bucio et al. 2007; Lugtenberg and Kamilova 2009; Kraiser et al. 2011; Vejan et al. 2016; Zúñiga et al. 2013). Remarkably, key characterized genes involved in plant growth-promoting effects and plant colonization are regulated by quorum-sensing (QS) systems (Pérez-Montañón et al. 2013; Quiñones et al. 2005; Steindler et al. 2009; Walker et al. 2004; Zúñiga et al. 2013). It has been reported that QS systems regulate several bacterial functions in a cell density-dependent manner, such as exopolysaccharide biosynthesis, motility, biofilm formation, and siderophore biosynthesis, among others (Chapalain et al. 2013; Fuqua and Greenberg 2002; Goo et al. 2015; Packiavathy et al. 2013; Pérez-Montañón et al. 2013), acting as a regulator of bacterial cooperation, thus rendering such bacteria social in nature (Darch et al. 2012; Diggle et al. 2007; Goo et al. 2015). In gram-negative bacteria, the *N*-acyl-homoserine lactone (AHL) signal molecules are frequently synthesized to communicate through QS (Whitehead et al. 2001) and plants are able to detect and respond to AHL signal (Hernández-Reyes et al. 2014; Ortiz-Castro et al. 2009; Teplitski et al. 2011). Studies of plant responses to AHLs demonstrated that these molecules affect plant functions such as transcriptional regulation, response to stress and hormones turnover (Hernández-Reyes et al. 2014; Mathesius et al. 2003). In addition, plants are able to take up AHLs in a process dependent on the length of their acyl chain, modifying the growth of roots and rosettes (Götz et al. 2007; Ortiz-Castro et al. 2008; von Rad et al. 2008).

In PGPB such as *Pseudomonas aeruginosa* PUPa3, QS mediated by AHL regulates rice growth promotion (Steindler et al. 2009), while some PGPBs defective in QS signaling are less effective in host colonization (Bauer and Mathesius 2004; Ortiz-Castro et al. 2009; Quiñones et al. 2005; Zúñiga et al. 2013). Because of differences in PGPB ecology and physiology, QS-dependent regulation is highly diverse between species and strains (Bertini et al. 2014; Goo et al. 2015; McAnulla et al. 2007; Ryu et al. 2013; Sánchez-Contreras et al. 2007). QS systems possess regulatory genetic networks whose study might take advantage of the use of system biology methods to model and understand the dynamic behavior of these complex genetic systems (Audretsch et al. 2013; Dallidis and Karafyllidis 2014).

The *Burkholderia* genus has been well-defined as a phenotypic, metabolic, and ecologically diverse bacteria, with its members inhabiting diverse niches (Coenye and Vandamme

Corresponding author: B. González; Telephone: +1 56-2-23311619; Fax: +1 56-2-23311906; E-mail: bernardo.gonzalez@uai.cl

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2003; Pérez-Pantoja et al. 2012; Suárez-Moreno et al. 2012). Despite the fact that several different members of the genus are not pathogens, the most comprehensive characterization of *Burkholderia* genus has been conducted on those strains that are opportunistic human pathogens (Seo et al. 2015; Valvano et al. 2005). A phylogenetic tree based on 16S rRNA gene sequences of over 80 *Burkholderia* species shows a noticeable division in two clades (Depoorter et al. 2016). One of these clades comprises more than 25 nonpathogenic species mainly characterized for their beneficial association with plants, most of them being described as PGPB (Caballero-Mellado et al. 2007; Suárez-Moreno et al. 2010, 2012). Recently, this group of *Burkholderia* species has been proposed as belonging to a separate genus, namely, *Paraburkholderia* (Sawana et al. 2014). Of the above-mentioned nonpathogenic cluster, 20 species share a conserved AHL-based QS system, designated BraI/RsaL/BraR_{kur} because it was first reported in *P. kururiensis* (Suárez-Moreno et al. 2008). The BraI/RsaL/BraR_{kur} QS system responds to 3-oxo-C14-AHL, and it has a regulatory protein named RsaL that represses the transcription of the AHL synthase encoded by the *bral* gene (Suárez-Moreno et al. 2008). The *Burkholderia cepacia* complex (BCC) pathogenic species possess a QS system named CepI/CepR, which produces *N*-octanoyl homoserine lactone (C8-AHL) and it is unrelated to the BraI/RsaL/BraR_{kur} QS system (Suárez-Moreno et al. 2008, 2010, 2012). To date, no evidence has been reported relating both QS systems in a hierarchical connection (Choudhary et al. 2013). In members of order Burkholderiales, these QS systems regulate important phenotypic traits, principally those involved in colonization and niche invasion, in both pathogenic and beneficial species (Choudhary et al. 2013; Gamage et al. 2011; Malott et al. 2005). Furthermore, to control the phenotypic characteristics already mentioned, QS systems are part of complex interactions with a network of regulators, among which a sigma factor has been involved in QS hierarchical control in some species of *Paraburkholderia* (Aguilar et al. 2003; Solis et al. 2006; Wongtrakoongate et al. 2012).

P. phytofirmans PsJN is a PGPB that establishes rhizospheric and endophytic colonization in different plants and produces positive effects in horticultural crops like tomato, potato, and grape (Compant et al. 2008; Fernandez et al. 2012; Theocharis et al. 2012). *P. phytofirmans* PsJN possesses a conserved BraI/RsaL/BraR QS system (named BpI.2/RsaL/BpR.2) and an additional QS system named BpI.1/BpR.1, present in only a few of the plant-beneficial environmental *Paraburkholderia* spp. (as in *P. xenovorans*, named XenI2/R2) (Coutinho et al. 2013). In a previous study, we reported that an AHL synthase BraI-like mutant of *P. phytofirmans* PsJN (designated *bpl*.2) was still able to promote growth of *Arabidopsis thaliana* (Zúñiga et al. 2013). Similar results in plant growth-promoting effects were obtained using two different cultivars of *Zea mays* inoculated with the PsJN *bpl*.2 mutant (Coutinho et al. 2013). We also reported that no growth promotion in *A. thaliana* plants was observed when they were inoculated with the strain PsJN AHL synthase *bpl*.1 mutant, which also exhibited significantly low root-colonization levels compared with the wild-type strain (Zúñiga et al. 2013). These findings indicate regulatory effects on unidentified genes and functions involved in colonization and plant-growth promotion by the BpI.1/R.1 QS system of *P. phytofirmans* PsJN. In this context, functions of the additional BpI.2/rsal/R.2 QS system remain unknown. Determination of the dynamic behavior and interplay of both *P. phytofirmans* PsJN QS systems may provide a way to control and improve beneficial associations with plants.

The purpose of this work was to describe the possible hierarchic structure of the QS genes networks and biofilm

production control in *P. phytofirmans* PsJN when this bacterium is exposed to *A. thaliana* plant roots. We reported a cross-regulation between both QS systems mediated principally by the BpR.1 and RsaL regulators and that these systems apparently control a sigma factor related to biofilm production. We also carried out a temporal study to understand and reconstruct the regulatory network of both QS systems using a Boolean network model, thus allowing us to propose a network regulation model for these two strain PsJN QS systems in the presence of *A. thaliana* plant roots.

RESULTS

QS systems gene expression in *P. phytofirmans* PsJN in the presence of *A. thaliana* plant roots.

To address the function of QS systems in the first steps of plant colonization and growth promotion, we evaluated the temporal expression profile of QS genes in the PsJN wild-type strain after its inoculation in *A. thaliana* roots, using a hydroponic system of growth (Supplementary Fig. S1). We observed important differences between unplanted (control) and planted conditions in transcript levels of the five QS genes tested (Fig. 1). *bpl*.1 and *bpR*.1 gene transcript levels presented significant differences at 24 and 48 h after inoculation (Fig. 1). Transcript levels of both genes seem to be equally regulated in the unplanted control treatment but are differently regulated after 1 h of the exposure to the plant (Fig. 1), while the *bpR*.1 gene appears to be repressed in planted conditions or induced in unplanted conditions. Clear differences were also observed in *bpl*.2 and *bpR*.2 gene transcript levels at specific evaluated times in the PsJN strain, changing the pattern of expression observed in bacteria exposed to plant roots versus control treatment. *bpl*.2 gene transcript levels appear to be down-regulated after 30 min postinoculation in medium with growing plants in comparison with the control treatment (Fig. 1), whereas those transcripts from *bpR*.2 presented a significant burst at 24 h postinoculation (Fig. 1). *rsal* transcript levels were similar in control and planted treatments (Fig. 1). In general, transcript levels of QS genes changed when bacteria were inoculated in medium with growing plants, indicating a specific response to plant presence, but their expression pattern is complex to predict. To gain more insight about interaction of QS systems in strain PsJN when exposed to the presence of *A. thaliana* roots, we used the expression pattern data of *bpl*.1, *bpR*.1, *bpl*.2, *bpR*.2, and *rsal* genes (transcript levels of genes from bacteria in the presence of plant roots) to reconstruct a QS network. Using the Boolean network as a mathematical tool, we predicted a graph (Fig. 2) on which nodes represent the genes, proteins, or signaling molecules and the edges are the interactions between the parts of the QS systems. We obtained a network in which the 3-OH-C8-AHL-BpR.1 complex would positively regulate *bpl*.1 and *bpR*.1 gene expression and repress *bpl*.2 and *rsal* gene expression (Fig. 2). In turn, 3-oxo-C14-AHL-BpR.2 complex positively regulates *bpl*.2 and *bpR*.1 gene expression and represses *bpl*.1 and their own expression (Fig. 2). The network also indicates that *rsal* represses *bpl*.2 gene expression and positively regulates *bpR*.2 (Fig. 2).

Transcript levels of QS systems regulatory genes in wild-type *P. phytofirmans* PsJN and QS mutants.

To test the cross-regulation in the QS network predicted by the model, we evaluated transcript levels of QS system genes in wild-type strain *P. phytofirmans* PsJN and of homoserine lactone synthases (producer of AHLs) in strain PsJN mutants. *P. phytofirmans* PsJN QS system BpI.1/R.1 produces 3-OH-C8-AHL and the BpI.2/RsaL/R.2 QS system generates predominantly 3-oxo-C14-AHL and 3-OH-C14-AHL (Choudhary

et al. 2013; Coutinho et al. 2013). We had previously observed that PsJN mutant strains in the AHL-producing genes *bplI* and *bplJ* generated significantly lower levels of 3-OH-C8-AHL, and the *bplI* mutant did not synthesize 3-oxo-C14-AHL (Zúñiga et al. 2013), suggesting that these QS systems are not mutually regulating the synthesis of these signal molecules and that, possibly, these QS systems are not cross-regulated, contrary to what the QS network model predicted. To address this point, we determined the transcripts levels of the *bpR*.1, *bpR*.2, and *rsaL* QS regulatory genes, using the quantitative reverse transcription-polymerase chain reaction (qRT-PCR) technique. We evaluated strains that had inactivated *bplI*, *bplJ*, or both *bplI* and *bplJ* genes and compared them with the wild-type strain at stationary phase of growth (minimal medium), when the concentrations of AHL molecules were expected to be at their highest. We observed a *bpR*.1 gene transcript levels three times lower in the *bplI* single and *bplI*-*bplJ* double mutants than transcript levels observed in the wild-type strain (Fig. 3A), indicating that the 3-OH-C8-AHL-BpR.1 complex probably autoregulates their own expression. This was also analyzed evaluating transcript levels of the *bpR*.1 gene in cells of the wild-type strain induced with 3-OH-C8-AHL and 3-oxo-C14-AHL molecules at exponential growth phase, when the concentrations of AHL molecules were expected to be at their lowest, for 1 h. We observed that *bpR*.1 gene transcript levels were up-regulated (25×) in the presence of 3-OH-C8-AHL, as compared with noninduced transcript levels (Fig. 3B), supporting the idea that 3-OH-C8-AHL-BpR.1 complex autoregulates their own expression. In contrast, *bpR*.2 and *rsaL* gene transcript

levels were up-regulated in the *bplI*-*bplJ* double mutant strain compared with the wild-type strain (Fig. 3A) but were down-regulated in the *bplI* and *bplJ* single mutants, 10 and 200×, respectively. No changes were observed in transcript levels of the *bpR*.2 gene when cells were induced with 3-OH-C8-AHL and 3-oxo-C14-AHL molecules (Fig. 3B), dismissing a direct regulation of this gene by either that 3-OH-C8-AHL-BpR.1 or 3-oxo-C14-AHL-BpR.2 complex or both. In contrast, the *rsaL* gene was down-regulated in the presence of 3-OH-C8-AHL and up-regulated in presence of 3-oxo-C14-AHL compared with those of noninduced strain PsJN cells, indicating a possible direct regulation of this gene by 3-OH-C8-AHL-BpR.1 and 3-oxo-C14-AHL-BpR.2 complexes. Additionally, we observed that the *bplI* gene was induced with 3-OH-C8-AHL and the *bplJ* gene was induced by 3-oxo-C14-AHL, suggesting a direct regulation by 3-OH-C8-AHL-BpR.1 and 3-oxo-C14-AHL-BpR.2 complexes, respectively.

We also tested the promoter activities of *bplI*, *bplJ*, and *rsaL* genes in *Escherichia coli*, induced with 3-OH-C8-AHL and 3-oxo-C14-AHL molecules, to determine a possible direct regulation by BpR.1 and BpR.2 QS regulators on these promoters. We observed that 3-oxo-C14-AHL increased 20-fold the promoter activity of *bplJ* and *rsaL* genes (Fig. 3C), confirming a direct regulation by the 3-oxo-C14-AHL-BpR.2 complex. Moreover, the presence of 3-OH-C8-AHL produced a 10-fold increase in the promoter activity of the *bplJ* gene, suggesting that BpR.2 is promiscuous and is able to respond to other AHL molecules. No activity was observed in the *rsaL* promoter in the presence of 3-OH-C8-AHL. No induction

Fig. 1. Quorum-sensing system gene expression of *Paraburkholderia phytofirmans* PsJN in the presence of *Arabidopsis thaliana* roots. Quantitative reverse transcription-polymerase chain reaction determinations of relative transcript levels of the genes *bplI*, *bplJ*, *bpR*.1, *bpR*.2, and *rsaL* in the wild-type strain at 0, 5, and 30 min, 1, 2, 24, and 48 h postinoculation. Data are means $\pm$ standard errors of three biological replicates. Solid and dashed lines indicate the pattern expression of *P. phytofirmans* PsJN QS genes in hydroponic systems in the absence or the presence of the plant, respectively. Asterisks are used to indicate means that differ significantly (one-way analysis of variance significant difference tests, $P < 0.05$).

activity was observed in the *bpl.1* promoter in the presence of 3-OH-C8-AHL or 3-oxo-C14-AHL molecules. These results indicate that the 3-OH-C8-AHL-BpR.1 complex regulator is not able to autoactivate *bpl.1* promoter. This was confirmed by evaluating the activation of the $P_{bpl.1}$ promoter, using the same construction assessed in *E. coli* but in the presence of AHL obtained from spent supernatants of PsJN wild-type strain, as inducer, in which $P_{bpl.1}$ promoter displayed low activities but significantly higher as compared with control treatment induced with ethyl acetate (Supplementary Fig. S2). Additionally, we confirmed the activation of the $P_{bpl.1}$ promoter in *P. phytofirmans* PsJN wild-type strain, in which its activation was 10-fold greater at the stationary phase of growth (optical density [OD] = 1.5) than in the exponential phase of growth (OD = 0.3) (Supplementary Fig. S3), indicating the need of an additional AHL or a regulator present in PsJN wild type to obtain a higher activation of the $P_{bpl.1}$ promoter.

P. phytofirmans PsJN QS system regulation in biofilm formation.

Biofilm formation has been related to effective rhizospheric colonization by PGPB (Meneses et al. 2011; Rinaudi and Giordano 2010). To determine a possible regulation of this trait by QS systems in the PsJN strain, we compared QS mutants with the wild-type strain. Remarkably, biofilm formation was significantly reduced in the *bpl.1* and *bpl.1-bpl.2* double mutants grown on 20 mM fructose as carbon source (Fig. 4A). It should be noted that both the *bpl.1* single mutant (Zúñiga et al. 2013) and the *bpl.1-bpl.2* double mutant (data not shown) presented very low levels of colonization and plant growth promotion. In contrast, no significant differences were observed in 10 mM of fructose in the *bpl.2* mutant compared with the wild-type strain (Fig. 4A). Biofilm formation was partially recovered in the *bpl.1* mutant strain when both 3-OH-C8-AHL and 3-oxo-C14-AHL were added to the growth medium (Supplementary Fig. S4). Previous data suggest that biofilm production in *P. phytofirmans* PsJN is influenced by an ECF sigma factor belonging to the ECF26 subfamily, according to Staroń et al. (2009) classification, encoded by the *ecf26.1* gene (*unpublished results*). The *ecf26.1* mutant

strain produced significantly less biofilm compared with the wild-type strain (Fig. 4B). In order to explore interactions between the QS system and sigma factor *ecf26.1*, its transcript levels were determined in the wild-type and QS mutants. We observed differences between *ecf26.1* gene transcript levels, with the *bpl.1* mutant transcript levels three times lower than the wild-type strain and those of the *bpl.2* mutant two times higher than the wild-type transcript levels (Fig. 5). It is quite possible that the corresponding transcript levels of the *bpl.1-bpl.2* double mutant, essentially the same of those of the wild-type strain, resulted from the balance of the contrasting effects in the single mutants (Fig. 5). Transcript levels of the *ecf26.1* gene reported here correlated with those of biofilm formation (Fig. 4B) and the decreased total AHL production in the *ecf26.1* mutant (data not shown), suggest a possible regulation of the *ecf26.1* gene by QS systems.

DISCUSSION

We reported reduced production of biofilm in the *bpl.1* single mutant strain compared with the wild-type strain. We also observed lower transcript levels of gene *ecf26.1* sigma factor in the *bpl.1* mutant strain compared with the wild-type strain. Additionally, we described, for the first time, a regulatory network that represents QS system interactions when *P. phytofirmans* PsJN was exposed to *A. thaliana* roots, indicating the importance to the global dynamic in QS gene expression and the genes they regulate. In this network, we observed that expression of both *bpl.1* and *bpR.1* genes are induced by 3-OH-C8-AHL, but the same AHL seems to repress *rsaL* gene expression, which is supported by induction data (Fig. 3B). On the other hand, the network indicates that 3-oxo-C14-AHL induces *rsaL*, *bpR.1*, and *bpl.2* gene expression (Fig. 3B) and evidence also supports the direct induction of *bpl.2* gene promoter by the 3-oxo-C14-AHL-*bpR.2* complex (Fig. 3C). Taken together, the presence of an additional regulator to activate the *bpl.1* gene promoter in the PsJN strain, the analysis of QS gene transcript levels in single and double mutants (Fig. 3A), and the expression pattern of QS genes in the wild type, indicate that QS systems seem to be a complex network that exhibit cross-regulation and an unclear dynamic in the presence of plant roots.

The importance of the bacterial ability to form microcolonies on different parts of the roots is known, from the tip to the elongation zone, hence, they can grow into large populations to form mature biofilms on plant roots (Rudrappa et al. 2007). In a previous study, we observed that the *bpl.1* mutant form less biofilm on roots affecting the final colonization of *A. thaliana* (Zúñiga et al. 2013). Here, we observed less biofilm formation by the *bpl.1* mutant compared with the wild-type strain (Fig. 4A). Interestingly, the *bpl.2* mutant strain does not present differences in biofilm formation compared with wild-type strain (Fig. 4A). Experiments in *Pseudomonas fluorescens* showed that biofilm formation in abiotic surfaces has a different structure than a biofilm formed in roots, which would indicate contrasting genetic programs that probably are involved in distinct environmental growth contexts (Barahona et al. 2011). Alternatively, sigma factors are, to some extent, responsible for this differential response, since they coordinate gene expression under different growth conditions or diverse environmental stimuli (Ho and Ellermeier 2012; Tettmann et al. 2014). Some evidence indicates that particular sigma factors are involved in biofilm formation in *Pseudomonas* strains (Ho and Ellermeier 2012; Masák et al. 2014; Tettmann et al. 2014). Here, we reported that transcript levels of the sigma factor *ecf26.1*, which is directly involved in biofilm formation, were lower in the *bpl.1* mutant strain compared with the PsJN wild-type strain,

Fig. 2. Model of regulatory network of quorum-sensing systems in *Paraburkholderia phytofirmans* PsJN. The model shows interactions of each node (*bpl.1*, *bpl.2*, *bpR.1*, *bpR.2*, and *rsaL*) with positive or negative regulations. The threshold vector (containing the threshold values for each node) is 0.32, -0.38, -0.86, 0.62, -0.79, respectively. The regulatory network was reconstructed under the threshold Boolean network model using a genetic algorithm, considering the synchronous updating scheme.

although no differences were observed in transcript levels of *bpl.1* gene in the *ecf26.1* gene mutant. In addition, we observed less AHL production in the *ecf26.1* mutant strain, as compared with the wild-type strain (data not shown), suggesting that QS control this ECF sigma factor or vice versa. Even more, we observed that the *ecf26.1* gene was slightly up-regulated in *bpl.2* compared with PsJN wild-type strain (Fig. 4B), which could be related to a tendency of this mutant to produce more biofilm in other carbon sources than plant root exudates. It has been shown that sigma factors and QS systems have cross-regulation to control gene expression of each other (Chin-A-Woeng et al. 2000, 2005; Schuster et al. 2003, 2004; Wongtrakongate et al. 2012). In fact, stationary phase RpoS sigma factor affects the expression of more than 40% of quorum-controlled genes identified in *Pseudomonas aeruginosa* (Schuster et al. 2003; 2004). Similar effects of RpoS have been observed in *Burkholderia pseudomallei*, in which hierarchical control of RpoS over QS-controlled genes was reported (Wongtrakongate et al. 2012). More experiments are required to determine if lower transcript levels of the *ecf26.1* gene observed in the *bpl.1* mutant were due to a direct cross-regulation between the sigma factor and the QS system mediated by 3-OH-C8-AHL-*bpl.1*R complex or because another intermediary regulator is operating.

It is known that functions of QS genes, the synthases and regulators, are subjected to various and sometimes different regulation processes (Duan and Surette 2007; Venturi 2006), implicating these genes can be independently regulated, as we observed in results on QS gene expression. Here, we confirmed that both *bpl.2* effector and *rsaL* repressor genes from the BraI/RsaL/BraR-like QS system were induced by 3-oxo-C14-AHL in *P. phytofirmans* PsJN (Fig. 3). The conserved QS systems in beneficial *Paraburkholderia* spp. are related to the LasI/R and PpuI/R AHL QS systems of *Pseudomonas aeruginosa* and *Pseudomonas putida*, respectively (Suárez-Moreno et al. 2008), in which similar gene-expression control patterns have been observed (Duan and Surette 2007). These QS systems are hierarchical and, in the case of *Pseudomonas aeruginosa* LasR-3-oxo-C12-AHL, activate the transcription of the *rhIR* and *rhII* genes, which belong to the second QS system of *Pseudomonas aeruginosa*. Also, it is known that the RsaL repressor controls the expression of over 200 genes in *Pseudomonas aeruginosa*, half of which are also QS-controlled (Rampioni et al. 2009). It is possible that the RsaL repressor in *P. phytofirmans* PsJN has a similar function; mutant strains and transcriptional studies are required to confirm its role in this strain. In the present study, we report that the BpI.1/BpR.1 QS system responds to 3-OH-C8-AHL, increasing transcript levels of the *bpl.1* and *bpR.1* genes, but an additional regulator for the 3-OH-C8-AHL-BpR.1 complex is necessary to a higher

activation of *bpl.1* promoter (Fig. 3). It is possible that a specific sigma factor could be required to better *bpl.1* promoter activation in the PsJN strain, and we propose that *ecf26.1* sigma factor could be a good candidate. We also showed that transcript levels of the *rsaL* gene were down-regulated in the presence of 3-OH-C8-AHL (Fig. 1B), suggesting a cross-regulation between BpI.1/BpR.1 and BpI.2/*rsaL*/BpR.2 QS systems in *P. phytofirmans* PsJN strain. No changes were observed in the *bpR.2* gene transcript levels in the presence of

Fig. 3. Transcriptional levels of quorum-sensing (QS) system genes in *Paraburkholderia phytofirmans* PsJN. **A**, Relative transcript levels of QS regulatory genes *bpl.1*, *bpR.2*, and *rsaL* in *P. phytofirmans* PsJN wild-type strain and in the *bpl.1*, *bpl.2*, and *bpl.1-bpl.2* single and double mutants at stationary phase of growth (optical density at 600 nm [OD₆₀₀] = 1.8). **B**, Relative transcript levels of QS system genes in *P. phytofirmans* PsJN wild-type strain induced with 2 μ M 3-OH-C8-AHL (acyl-homoserine lactones) and 3-oxo-C14-AHL, at exponential growth phase OD₆₀₀ = 0.7. Ethyl acetate (EA) solvent was used as a control in noninduced tests. Data are means $\pm$ standard errors of three biological replicates. Different letters are used to indicate means that differ significantly (one-way analysis of variance Tukey's honestly significant difference tests, $P < 0.05$). **C**, *bpl.1*, *bpl.2*, and *rsaL* gene promoter activities in *Escherichia coli*, in the presence of 2 μ M 3-OH-C8-AHL or 3-oxo-C14-AHL. EA was used as negative control of induction. Data are means $\pm$ standard errors of three biological replicates.

3-oxo-C14-AHL. There are several bacterial species that harbor in their genome multiple AHL-based QS systems that can be interconnected in an independent, synergistic or repressed regulatory network (Cha et al. 1998; Atkinson et al. 2008). Examples of this are found in the above-mentioned *Pseudomonas aeruginosa* (Lee and Zhang 2015; Schuster and Greenberg 2006; Schuster et al. 2003), in which the LasR regulator can form a multimer and bind *las*-box sequences only in the presence of 3-oxo-C12-AHL (Kiratisin et al. 2002) and RhlR dimerizes and binds DNA both in the presence and absence of C4-AHL (Medina et al. 2003; Ventre et al. 2003; Venturi 2006), creating a complex QS regulatory network, as we have observed in *P. phytofirmans* PsJN.

Using the temporal expression profile of QS systems of *P. phytofirmans* PsJN in the presence of plant roots, we were enabled to observe the changes in QS genes in response to the plant. The differences found in transcript levels corresponding to each QS system gene in planted and unplanted conditions

Fig. 4. Analysis of biofilm formation. **A**, Biofilm formation of *Paraburkholderia phytofirmans* PsJN wild-type strain and the *bpl.1*, *bpl.2*, and *bpl.1-bpl.2* single and double mutant strains on 20 mM fructose. **B**, Biofilm formation of *P. phytofirmans* PsJN wild-type strain and the *ecf26.1* mutant strain on 20 mM fructose. Biofilm formation was measured after 48 h of incubation. Bars are means $\pm$ standard errors of three biological replicates. Different letters are used to indicate means that differ significantly (one-way analysis of variance Tukey's honestly significant difference tests, $P < 0.05$).

Fig. 5. Relative transcript levels of the *ecf26.1* gene in *Paraburkholderia phytofirmans* PsJN wild-type strain and the *bpl.1*, *bpl.2* and *bpl.1-bpl.2* single and double mutants at stationary phase of growth (optical density at 600 nm [OD_{600}] = 1.8). Data are means $\pm$ standard errors of three biological replicates. Different letters are used to indicate means that differ significantly (one-way analysis of variance Tukey's honestly significant difference tests, $P < 0.05$).

indicate that autoinducer and response-regulator genes could be independently regulated, influenced by environmental context and playing different roles under diverse conditions. Similar results were observed in temporal expression profiles of *Pseudomonas aeruginosa* QS genes, in which the hierarchic structure of the networks is regulated in response to changes in the environment (Duan and Surette 2007).

It is known that cell-to-cell communication in bacteria implies a complex regulatory network that is very attractive to disciplines such as engineering, because mathematical equations can provide models of networks that allow the understanding of cell-to-cell communication at a systemic level (Dallidis and Karafyllidis 2014; Goryachev 2011). Here, we used a Boolean network to represent a regulatory model of QS systems. Even though this tool presents some limitations, it enabled us to integrate all the information about QS systems regulation and get a successful model of QS network. This network was obtained using transcript levels of QS system genes expressed on inoculated PsJN bacteria in plant roots, which gave us information about the regulon formed by genes involved. Moreover, this modeling approach could be a useful tool to apply in other Burkholderiales species that share similar QS systems, to take all the information about each species and produce global models of QS networks in this genus in different environmental contexts.

MATERIALS AND METHODS

Bacterial strains and plasmids.

P. phytofirmans PsJN was obtained from A. Sessitsch. Wild-type PsJN and the three mutant strains (*bpl.1*, *bpl.2*, *bpl.1-bpl.2*) were grown on Dorn mineral salts medium (Dorn et al. 1974) containing 10 mM fructose as the sole carbon and energy source and, if required, kanamycin (50 $\mu\text{g ml}^{-1}$), spectinomycin (100 $\mu\text{g ml}^{-1}$), or gentamicin (30 $\mu\text{g ml}^{-1}$), at 30°C for 12 h.

Construction of *P. phytofirmans* PsJN mutants.

Mutant strains *bpl.1* and *bpl.2* were constructed as described by Zúñiga et al. (2013). To obtain the *bpl.1-bpl.2* double mutant, we introduced a second plasmid in the *bpl.2* mutant, constructed using the *bpl.1* PCR product (employing *bpl.1*mut primer pairs, listed in Supplementary Table S1), to assemble it in a pGEM-Tgen⁺ vector modified with a gentamicin antibiotic resistance. This plasmid was electroporated in the *P. phytofirmans* PsJN *bpl.2* mutant to get one recombination event disruption of the target gene, and recombinants were selected on Luria-Bertani (LB) agar, containing spectinomycin (100 $\mu\text{g/ml}$) and gentamicin (30 $\mu\text{g/ml}$), to obtain the PsJN *bpl.1-bpl.2* double mutant. Correct insertions in mutant strains were confirmed by PCR and sequencing.

RNA extraction, cDNA synthesis, and qRT-PCR analyses.

Cells of *P. phytofirmans* PsJN wild type and *bpl.1*, *bpl.2*, and *bpl.1-bpl.2* single and double mutant strains were grown overnight in minimal medium with 10 mM fructose, to inoculate a fresh culture medium. For the stationary growth phase expression analysis, we grew the cells until optical density at 600 nm (OD_{600}) = 1.8. To carry out expression analysis in wild-type PsJN induced with AHLs, we grew cells until OD_{600} = 0.7 (exponential phase of growth), and then, we supplemented this culture with 2 μM 3-OH-C8-AHL or 3-oxo-C14-AHL or with solvent ethyl acetate as a control and, finally, incubated cultures for 1 h. To evaluate gene expression in the presence of plants, we inoculated roots of *A. thaliana* plants, with 14 days of growth on hydroponic system, with 1×10^4 cells of PsJN wild-type strain for 48 h, and then, we took culture samples after 0, 5,

and 30 min, 1, 2, 24, and 48 h. The same hydroponic growth system, was used to inoculate bacteria but without plants, as a control treatment.

To extract RNA, we took 4 ml of each culture, using RNA Protect bacteria reagent and RNeasy mini kit (Qiagen, Chatsworth, CA, U.S.A.). The RNA was quantified using a GeneQuant 1300 Spectrophotometer (GE Healthcare, Piscataway, NJ, U.S.A.) and was treated with TURBO DNase kit (Ambion, Austin, TX, U.S.A.) to remove DNA contamination. The cDNA synthesis was performed, using ImProm-II reverse transcription system (Promega Corp., Madison, WI, U.S.A.), with 1 µg of RNA in 20 µl of reaction.

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 expression $2^{-\Delta\text{CtGOI} - \text{HK}}$. Then, gene expression levels were normalized to the average value of the expressions in the control treatment. 16S rRNA (Bphyt_R0071) was used as a HK gene. qRT-PCR analysis primer pairs for *efc26.1* (Bphyt_1666), *bpl.1* (Bphyt_0126), *bpl.2* (Bphyt_4275), *bpR.1* (Bphyt_0127), *bpR.2* (Bphyt_4277), and *rsaL* (Bphyt_4276) genes were used.

Construction of *lacZ* reporter fusions.

Putative promoter regions were fused to the *lacZ* gene from pKGWP0 (Marín et al. 2010). PCR products comprising approximately 200 bp of the upstream region contiguous to translational starts of the *bpl.1*, *bpl.2*, and *rsaL* genes were obtained. The amplified DNA fragments were then cloned into the *XhoI-KpnI* restriction site of pKGWP0, forming P_{bpl.1}-*lacZ*, P_{bpl.2}-*rsaL-lacZ*, and P_{rsaL}-*lacZ* plasmids, were transferred into *E. coli*, and were selected in LB medium supplemented with 100 µg of spectinomycin per milliliter. *bpR.1* and *bpR.2* gene sequences were amplified using bpR.1.g and bpR.2.g primer pairs. Primers were designed containing an additional 20 bp at the terminal sequence that overlap with the downstream P_{BAD} promoter from the pBS1 vector (Bronstein et al. 2005), which were joined into a covalently sealed molecule in a one-step isothermal reaction, as described by Gibson et al. (2009). The recombinant plasmid PBS1-BpR.1 was electroporated in *E. coli* P_{bpl.1}-*lacZ* and PBS1-BpR.2 was electroporated in *E. coli* P_{bpl.2}-*rsaL-lacZ* and P_{rsaL}-*lacZ*. *E. coli* strains were selected in LB medium supplemented with spectinomycin (100 µg ml⁻¹) and gentamycin (30 µg ml⁻¹). β-Galactosidase assays were performed according to standard protocols (Miller 1972) after 4 h of incubation, using 3OH-C8-AHL and 3OXO-C14-AHL (2 µM) as inducer.

Biofilm formation.

Biofilm formation was evaluated using the microtiter plate biofilm assay as described by Merritt et al. (2005). We grew *bpl.1*, *bpl.2*, and *bpl.1-bpl.2* mutants and PsJN wild-type strain in 2 ml of Dorn mineral salts medium (Dorn et al. 1974) containing 10 mM fructose until stationary phase. Then, 1:100 diluted cultures in Dorn mineral medium were used to inoculate microtiter plate wells filled with Dorn mineral medium containing 20 mM fructose. After 48 h of incubation at 30°C, planktonic bacteria were removed and each well was washed

with distilled water. Biofilm mass was measured using 0.1% crystal violet ethanolic solution. The OD₅₇₀ was determined by spectrophotometry.

Growth of *A. thaliana*.

A. thaliana ecotype Col-0 was used. Seeds were surface sterilized with 50% (vol/vol) commercial chlorine bleach for 7 min and were 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, 200 seeds were sown in sterile Magenta culture vessels, 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. The plant growth chamber was run with a cycle of 16 h of light and 8 h of darkness and a temperature of 22 ± 2°C. After 14 days, plants were inoculated. Three biological replicates were carried out for each treatment.

Boolean network modeling.

Let x be a finite set of n variables (genes), $x = \{x_1, \dots, x_n\}$, with $x_i \in \{0, 1\}$ for $i = 1, \dots, n$. A Boolean network is a pair (G, F) , where $G = (V, E)$ is a finite directed graph with V being the set of n nodes and E the set of edges. F is a Boolean function, $F: \{0, 1\}^n \rightarrow \{0, 1\}^n$ composed of n local functions $f_i: \{0, 1\}^n \rightarrow \{0, 1\}$. In this work, we used the synchronous or parallel updating scheme, i.e., at each time step, all the nodes are updated at the same time. The dynamics of the network (how the values of the nodes change through time) eventually converges to attractors (steady states), which can be either a fixed point where, once the network reaches that state, it can never leave it or a limit cycle, where the network returns to a previous state with a certain periodicity. The set of states that can lead the network to a specific attractor is called the basin of attraction. For the reconstruction of Boolean networks from data, it has become more common to see the threshold Boolean network version, which considers weights for the edges and threshold values for each node, then, the update is carried using a Boolean Heaviside function that depends linearly on its inputs (Goles et al. 2013). More formally, in a threshold Boolean network, updates of each node are computed by

$$x_i(t+1) = H\left(\sum_{j=1}^n w_{ij}x_j(t) - \theta_i\right)$$

$$H(z) = \begin{cases} 1, & \text{if } z \geq 0 \\ 0, & \text{if } z < 0 \end{cases}$$

with w_{ij} the weight of the edge coming from node j into node i , and θ_i the activation threshold of node i . The weights and thresholds are the network's parameters.

Regulatory network reconstruction using a threshold Boolean network.

The expression levels of QS system genes were obtained through time from cells collected at the exponential phase of growing (OD₆₀₀ = 0.7). RNA samples were then taken after 5, 15, 30, 60, and 120 min, 24, and 48 h. The transcript levels of *bpl.1*, *bpl.2*, *bpR.1*, *bpR.2*, and *rsaL* genes were binarized using the k-means clustering algorithm with $k = 2$. Using these binary data, we inferred a threshold Boolean network, using an evolutionary computation approach (Ruz et al. 2015). In particular, a genetic algorithm was used to find the network's parameters. In the case of the weights, we restricted the search for values within the set $\{-1, 0, 1\}$, whereas, for the threshold values, we consider continuous values within the range $[0, 1]$.

Statistical analysis.

Data for biofilm formation and gene transcript levels were statistically analyzed, using one-way analysis of variance, to compare multiple means. When analysis of variance showed significant treatment effects, the Tukey's honestly significant difference ($P < 0.05$) test was applied to make comparisons between treatments.

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