***Paraburkholderia phytofirmans* PsJN Protects *Arabidopsis thaliana* Against a Virulent Strain of *Pseudomonas syringae* Through the Activation of Induced Resistance**

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Paraburkholderia phytofirmans PsJN is a plant growth-promoting rhizobacterium (PGPR) that stimulates plant growth and improves tolerance to abiotic stresses. This study analyzed whether strain PsJN can reduce plant disease severity and proliferation of the virulent strain *Pseudomonas syringae* pv. *tomato* DC3000, in *Arabidopsis* plants, through the activation of induced resistance. *Arabidopsis* plants previously exposed to strain PsJN showed a reduction in disease severity and pathogen proliferation in leaves compared with noninoculated, infected plants. The plant defense-related genes *WRKY54*, *PR1*, *ERF1*, and *PDF1.2* demonstrated increased and more rapid expression in strain PsJN-treated plants compared with noninoculated, infected plants. Transcriptional analyses and functional analysis using signaling mutant plants suggested that resistance to infection by DC3000 in plants treated with strain PsJN involves salicylic acid-, jasmonate-, and ethylene-signaling pathways to activate defense genes. Additionally, activation occurs through a specific PGPR-host recognition, being a necessary metabolically active state of the bacterium to trigger the resistance in *Arabidopsis*, with a strain PsJN-associated molecular pattern only partially involved in the resistance response. This study provides the first report on the mechanism used by the PGPR *P. phytofirmans* PsJN to protect *A. thaliana* against a widespread virulent pathogenic bacterium.

Plants serve as ecological niches for diverse communities of microbes that work in concert with the plant to survive in a wide range of stressful environmental conditions (Coleman-Derr and Tringe 2014). Because roots deposit up to 40% of the plant's photosynthetically fixed carbon, microorganisms are predominantly hosted in the rhizosphere, representing this small zone around the roots, one of the most energy-rich habitats on earth (Bais et al. 2006). The rhizosphere is an important ecological niche in which several and complex plant-microbe interactions occur, which can be beneficial, neutral, or harmful to plants

(Bais et al. 2006). Among the beneficial interactions are those involving plant growth-promoting rhizobacteria (PGPR) that colonize the rhizosphere of many plant species generating positive effects, such as increased plant growth, reduced susceptibility to diseases, and improved tolerance to abiotic stresses (Kloepper et al. 2004; Lugtenberg and Kamilova 2009; Pinedo et al. 2015; Poupin et al. 2013; van Loon 2007; Yang et al. 2009).

Diverse pathogens and pests can attack plants in complex natural environments. In agriculture, this leads to tremendous annual crop losses, representing a total value of over €450 billion worldwide (Vos et al. 2013). As such, elucidating the mechanisms of plant defense has become increasingly important. Two main levels of defense have been described (Jones and Dangl 2006). The first level, which involves recognition of potentially pathogenic microorganisms, is ensured by pattern recognition receptors that detect pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs) (Schwessinger and Zipfel 2008). This recognition leads to a basal resistance called PAMP- or MAMP-triggered immunity (PTI or MTI), conferring low-level resistance to virulent pathogens (Jones and Dangl 2006). The second level is represented by a stronger and more specific resistance mechanism, the effector-triggered immunity (ETI), which involves a direct or indirect interaction between the product of a host resistance gene and its cognate pathogen-encoded effector protein (Jones and Dangl 2006; Xin and He 2013). Among ETI manifestations is the hypersensitive response, in which necrotic lesions develop at one or more sites of pathogen entry (Senthil-Kumar and Mysore 2013; Vlot et al. 2009). As a consequence of the ETI response, systemic acquired resistance (SAR) is activated. This is a long-lasting and broad-based resistance to infection by a wide variety of pathogens (Fu and Dong 2013; Ryals et al. 1996; Senthil-Kumar and Mysore 2013; Vlot et al. 2009). Reactive oxygen species and salicylic acid (SA) are essential for a successful PTI, ETI, and SAR responses (Herrera-Vásquez et al. 2015). Not only the hormone SA is important for the regulation of the plant immune responses but, also, jasmonic acid (JA) and ET hormones. JA and ET signaling can act synergistically to activate defense genes, mainly against necrotrophic pathogens (through the ET response factor [ERF] branch of the JA pathway) and against insect herbivory (through the MYC branch) (Pieterse et al. 2009). A fine-tuning crosstalk between SA and JA hormones plays a central role in the modulation of the plant immune signaling network (Caarls et al. 2015). Relative concentrations of each hormone, timing, and sequence

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of initiation of the SA and JA signaling are important to determine whether the interaction between both pathways will be antagonistic, neutral, or synergistic (Caarls et al. 2015; Koornneef et al. 2008; Liu et al. 2016; Mur et al. 2006; Pieterse et al. 2012).

Biological control using PGPR has been recognized as an attractive tool for plant disease management in modern agriculture and is considered as an alternative or a supplemental way to reduce the use of agrochemicals (Babalola 2010; Beneduzi et al. 2012; Bhattacharyya and Jha 2012; Compant et al. 2005a). PGPR have several mechanisms to fight pathogens, such as antagonism, production of antibiotics, signal interference (e.g., degradation of quorum-sensing *N*-acyl homoserine lactones), competition for nutrients and niches, competition for ferric ions by siderophore production, and the activation of a plant defense system known as induced systemic resistance (ISR), in which plants are primed against pathogen attack (Haas and Défago 2005; Lugtenberg and Kamilova 2009; Weller et al. 2012). For many biological control agents, ISR has been recognized as the mechanism that, at least partly, explains the reduction in disease severity and incidence. ISR is a state of enhanced defensive capacity developed by a plant following colonization by PGPR or beneficial fungi (PGPF) (Naznin et al. 2014; Van der Ent et al. 2009; van Loon 2007; van Loon et al. 1998). In plants primed by ISR, defense responses are not activated directly but are accelerated upon pathogen or insect attack, resulting in enhanced resistance to the attacker (Van Wees et al. 2008). Fitness costs associated with induced defense arise from allocation and ecological costs. Therefore, the defense arsenal in primed plants remains dormant until pathogen infection, such that priming does not confer major fitness costs under pathogen-free conditions (van Hulten et al. 2006). The limited costs of the primed state are often compensated by the benefits in environments with pathogen or herbivore pressure (Vos et al. 2013).

A long and growing list of PGPR, principally *Bacillus*, *Pseudomonas*, and *Serratia* species, can elicit induced resistance and protect against a broad spectrum of pathogens in different plant species, including several crops, the model plant *Arabidopsis*, and even woody species (Choudhary and Johri 2009; De Vleeschauwer and Höfte 2009; Pineda et al. 2010). Remarkably, plant-protective responses by bacteria belonging to genus *Burkholderia* are less-studied at the molecular level, although this genus encompasses some of the more diverse and environmentally adaptable plant-associated bacteria able to establish a wide range of relationships with their hosts, including free-living in the rhizosphere as well as epiphytic, endophytic, obligate endosymbionts, and phytopathogens (Coenye and Vandamme 2003; Compant et al. 2008). In this context, *Paraburkholderia phytofirmans* PsJN (formerly *Burkholderia* [Sawana et al. 2014]) is a PGPR able to produce positive effects in horticultural crops such as tomato, potato, and grape (Ait Barka et al. 2006; Compant et al. 2005b; Kurepin et al. 2015; Miotto-Vilanova et al. 2016; Nowak et al. 1998; Sessitsch et al. 2005) but also in *Arabidopsis* (Pinedo et al. 2015; Poupin et al. 2013, 2016) and switchgrass (Wang et al. 2015). This bacterium harbors genetic characteristics that would allow efficient use of plant-related molecules as carbon and energy source, as well as a successful rhizospheric and endophytic colonization (Donoso et al. 2017; Mitter et al. 2013; Zúñiga et al. 2013). It has been also reported that this bacterium stimulates growth of inoculated plants and induces physiological changes enhancing plant adaptation to environmental stresses such as cold, salinity, and drought (Ait Barka et al. 2006; Fernández et al. 2012; Ledger et al. 2016; Naveed et al. 2014; Pinedo et al. 2015; Su et al. 2015; Theoharis et al. 2012). Nevertheless, little is known regarding the ability of *P. phytofirmans* PsJN to protect plants from pathogens. Only a couple of studies have reported biocontrol effects of strain PsJN

in a whole plant in which its inoculation was shown to enhance resistance to the necrotrophic pathogen *Botrytis cinerea* in grapevine plants (Ait Barka et al. 2002; Miotto-Vilanova et al. 2016).

This study reports, for the first time, that *P. phytofirmans* PsJN acts as biocontrol agent of *A. thaliana* plants, reducing disease susceptibility against the virulent strain *Pseudomonas syringae* pv. *tomato* DC3000. The protection conferred by strain PsJN proceeds through the activation of induced resistance in the plant, activating SA-, JA- and ET-dependent signaling pathways. Furthermore, using strain PsJN derivatives or inactivated forms, it was determined that the recognition and, therefore, the protection provided by this PGPR strain in *Arabidopsis* is very specific and requires the bacterium to be metabolically active.

RESULTS

P. phytofirmans PsJN protects *A. thaliana* plants against virulent *Pseudomonas syringae* DC3000.

In order to determine if strain PsJN protects its host against a phytopathogenic bacterium, infection assays in plants cocultivated with or without strain PsJN were performed with a virulent strain of *Pseudomonas syringae* pv. *tomato* DC3000 (Whalen et al. 1991; Xin and He 2013). To discriminate the effect of metabolically active bacteria from that of inactive or dead bacteria, a treatment with heat-killed bacterium (HK-PsJN) was included. The results indicated that plants cocultivated with strain PsJN were resistant to the disease triggered by DC3000 during 28 days postinfection (dpi) (Fig. 1). The onset of symptoms, a gray-brown lesion and chlorosis, became evident from the third week, in control plants (21 dpi) (Fig. 1A). Additionally, disease severity determinations in infected plants yielded significant differences from day 21 onward among plants cocultivated with strain PsJN and plants cocultivated with HK-PsJN or noninoculated control plants (Fig. 1B).

DC3000 growth in plant leaves was monitored to determine whether the lack of symptom development correlated with restriction of pathogen multiplication. Results showed that plants cocultivated with strain PsJN controlled the pathogen population at its initial levels in all measured times (Fig. 1C). In contrast, in noninoculated plants and in plants cocultivated with HK-PsJN, the population of DC3000 was two orders of magnitude higher than in plants inoculated with metabolically active strain PsJN (Fig. 1C).

To determine if the protective response triggered by strain PsJN on *Arabidopsis* plants in early stages of its life cycle is sufficient to maintain the protection against the disease along the entire life cycle of the plant, a long-term assay was carried out. Results indicated that, after 56 dpi, 11-week-old plants cocultivated with strain PsJN exhibited a healthy phenotype (Fig. 2), with senescent leaves due its age (77 days after sowing [DAS]) (Pinedo et al. 2015; Poupin et al. 2013). In contrast, plants noninoculated with strain PsJN were completely chlorotic and dead (Fig. 2). Infected *A. thaliana* plants cocultivated with strain PsJN harbored a DC3000 population of 1×10^4 CFU per milligram of fresh weight (FW), the same order of magnitude of the CFU initially inoculated, even after 56 dpi. In the case of nonprotected, control plants, CFU of strain DC3000 could not be quantified, since the required normalization with plant FW was not possible due to extensive weakening of the tissues.

Specific interaction between *A. thaliana* plants and *P. phytofirmans* PsJN is required for a protective effect against virulent *Pseudomonas syringae* DC3000.

Most studies on biocontrol have interrogated only PGPR of interest and do not include non-PGPR strains as a control for specificity of the defense response. To study if the protective state triggered by strain PsJN on *Arabidopsis* was specific, tests

Fig. 1. Plant symptomatology, disease severity, and proliferation of virulent *Pseudomonas syringae* pv. *tomato* DC3000 in infected *Arabidopsis thaliana* plants cocultivated or not with *Paraburkholderia phytofirmans* PsJN. **A**, Representative photographs of *Arabidopsis* rosettes exposed to different treatments: plants cocultivated with *P. phytofirmans* PsJN, cocultivated with heat-killed bacteria (HK-PsJN), and noninoculated plants infected with a virulent strain of *Pseudomonas syringae* pv. *tomato* DC3000. Results were recorded at 0, 7, 14, 21, and 28 days after infection (dpi). **B**, Disease severity in strain PsJN-inoculated, HK-PsJN-inoculated, and noninoculated *Arabidopsis* plants, after DC3000 infection. Disease severity was measured 7, 14, 21, and 28 dpi and the number of leaves per plant showing disease symptoms, where 0% means no leaves present symptoms and 100% means that all leaves present symptoms. Each bar represents the average of at least eight individual plants and its standard error. Different letters on the bars indicate statistically significant differences among treatments (one-way analysis of variance [ANOVA], $P < 0.05$; Tukey's test, $P < 0.05$). **C**, Proliferation of virulent DC3000 (disease progression) in rosettes of *Arabidopsis* plants cocultivated or not with strain PsJN and HK-PsJN. Each point represents the log-transformed mean of eight individual plants (CFU per gram of leaf fresh weight) and its standard error. Asterisks indicate statistically significant differences between control and the other treatments in each time (one-way ANOVA, $P < 0.05$; Tukey's test, $P < 0.05$). Results are representative of four independent experiments.

with two additional gram-negative bacteria belonging to phylum Proteobacteria were performed, using *Escherichia coli* K12 as a representative of a bacterium noninteracting with plants and *Cupriavidus pinatubonensis* JMP134, a non-PGPR belonging to the same family (*Burkholderiaceae*) of *P. phytofirmans* PsJN, previously reported to colonize *Arabidopsis* plants (Ledger et al. 2012). Results indicated that in the first two weeks after infection, plants cocultivated with strains K12 and JMP134 presented a transiently smaller population of DC3000 than noninoculated plants, whereas plants cocultivated with strain PsJN exhibited a pathogen population significantly lower than the non-PGPR treatments (Fig. 3A). At 21 dpi, the proliferation of DC3000 was similar for noninoculated plants and plants inoculated with strains K12 and JMP134 and were three orders of magnitude higher than in plants inoculated with strain PsJN (Fig. 3A). The symptomatology observed 21 days after infection with these treatments (Fig. 3B) was in agreement with the effective control of DC3000 levels in the plant tissues.

Metabolically active *P. phytofirmans* PsJN cells allowed a lower accumulation of reactive oxygen species in *A. thaliana* plants and were required for pathogen protection.

To support the protective role of metabolically active strain PsJN, an additional assay measuring the phytopathogen population and symptomatology of disease in *Arabidopsis* plants using killed bacteria but conserving their cell integrity (K-PsJN) was performed. The K-PsJN treatment was also designed to determine if the protective response could be triggered by a MAMP of *P. phytofirmans* PsJN. Results showed that, in the first week after infection, plants cocultivated with K-PsJN exhibited a lower level of DC3000 proliferation than noninoculated plants, suggesting that MTI could be activated in these plants (Fig. 4A). However, from the second week after infection, plants cocultivated with K-PsJN presented the same level of DC3000 proliferation as noninoculated plants, which was significantly higher than the DC3000 proliferation of PsJN-treated plants (Fig. 4A). Moreover, the symptomatology of the disease 21 days after the infection event was consistent with the quantitative measurement of the population (Fig. 4B). As disease development induces oxidative stress, a histochemical assay to detect the reactive oxygen species superoxide anion (O_2^-) in *Arabidopsis* plants was carried out 21 days after DC3000

infection. The results indicated that plants cocultivated with metabolically active strain PsJN accumulated less O_2^- (visualized as a blue precipitate) than control and K-PsJN cocultivated plants (Fig. 4C).

***P. phytofirmans* PsJN induces transcriptional changes in SA and JA/ET responsive-defense genes in *A. thaliana* plants infected with virulent *Pseudomonas syringae* DC3000.**

To better understand the defense mechanisms involved in *Arabidopsis* protection mediated by *P. phytofirmans* PsJN against *Pseudomonas syringae* DC3000, the transcriptional profiles of well-characterized defense-related genes of *A. thaliana* were studied. The expression pattern of *PATHOGENESIS-RELATED GENE 1* (*PR1*; AT2G14610), a key marker of the SA signaling pathway, and *PLANT DEFENSIN 1.2* (*PDF1.2*; AT5G44420), a key marker of the JA/ET signaling pathways, were measured by analyzing mRNA accumulation in whole noninoculated plants (control) and in plants cocultivated with strain PsJN before and after DC3000 infection (0 and 1, 6, and 24 h, respectively). *PR1* belongs to a group of genes that code for proteins with antimicrobial activity (van Loon et al. 2006) and is the best-characterized and most common marker for the SA signaling pathway (Vlot et al. 2009). A molecular cascade relying on salicylate-dependent signals to *PR1* via NPR1, WRKY, and TGA transcription factors has been well established (Eulgem 2005; Vlot et al. 2009). Results indicated that *PR1* gene was significantly up-regulated in plants cocultivated with *P. phytofirmans* PsJN 1, 6, and 24 h after DC3000 infection (Fig. 5). The expression patterns of two other important genes of the SA signaling pathway, *ISOCORISMATE SYNTHASE 1* (*ICS1*; AT1G74710) and the transcription factor WRKY54 (AT2G40750), were analyzed as well. *ICS1* was up-regulated in *Arabidopsis* plants cocultivated with strain PsJN before infection (time 0 h) and *WRKY54* was down-regulated at the same time. Nevertheless, *WRKY54* was up-regulated 1 and 24 h after infection in strain PsJN-treated plants (Supplementary Fig. S1A).

Because crosstalk between the defense hormones SA and JA plays a central role in the modulation of the plant immune signaling network (Pieterse et al. 2009), the study of the *PDF1.2* expression pattern was also addressed. *PDF1.2* encodes a plant defensin with antimicrobial properties and is activated by the transcription factor ERF1 (Penninckx et al. 1996), which is an important node of convergence of JA and ET signaling pathways (Lorenzo et al. 2003; Pieterse et al. 2009). Results of quantitative reverse transcription-polymerase chain reaction (qRT-PCR) indicated that *PDF1.2* was significantly up-regulated in plants cocultivated with *P. phytofirmans* PsJN before infection and 6 h after infection (Fig. 5). With the JA/ET signaling pathways, the expression levels of four related genes were analyzed, *LIP-OXYGENASE 2* (*LOX2*; AT3G45140), *VEGETATIVE STORAGE PROTEIN 2* (*VSP2*; AT5G24770), the transcription factors MYC2 (AT1G32640), and *ERF1* (AT3G23240). As expected by the previous result for *PDF1.2*, the expression level of *ERF1* was significantly higher in strain PsJN-treated plants 1 and 6 h after DC3000 infection, whereas *LOX2* was up-regulated by strain PsJN and, after the infection event, its expression was down-regulated in strain PsJN-treated plants. Additionally, MYC2 was up-regulated 6 h after infection and down-regulated 24 h post-infection (hpi) in strain PsJN-treated plants. On the other hand, no significant changes were detected in *VSP2* expression between control and strain PsJN-treated plants.

***P. phytofirmans* PsJN induces SA production in *A. thaliana* plants.**

Induction by strain PsJN of genes encoding for the ICS1 enzyme, the SA-marker gene *PR1*, and the SA-inducible gene

Fig. 2. Long-term protection of *Arabidopsis thaliana* plants cocultivated with *Paraburkholderia phytofirmans* PsJN. Two representative photographs of PsJN-treated and nontreated plants infected with the virulent strain of *Pseudomonas syringae* DC3000. Photos were taken 77 days after sowing and 56 days after infection. At the end of the plant life cycle, strain PsJN-treated plants show suppressed disease symptoms and, conversely, non-inoculated plants present complete chlorotic and dead tissues.

WRKY54 suggested that SA should be produced in response to this PGPR. In order to confirm this hypothesis, the amounts of free SA and glycosylated SA (SAG) were measured in plants cocultivated with *P. phytofirmans* PsJN. As shown in Figure 6, significantly higher levels of free SA and SAG were detected in plants inoculated with strain PsJN, representing 1.57 μg SA per gram of FW, in comparison with control plants, which presented 0.35 μg SA per gram of FW. The same pattern was observed 24 h after DC3000 infection, where PsJN-treated

plants presented significantly more free SA and SAG than control plants (Fig. 6). After 48 h of the infection, there were no significant differences in the amount of SA between plants treated or not with *P. phytofirmans* PsJN (Fig. 6).

***P. phytofirmans* PsJN does not induce protection in mutant plants impaired in SA and JA/ET signaling pathways.**

To reinforce the findings obtained at the molecular level with the transcriptional analyses, in which the involvement of SA

Fig. 3. Response to *Pseudomonas syringae* pv. *tomato* DC3000 infection of *Arabidopsis thaliana* plants cocultivated with plant growth promoting rhizobacteria (PGPR) or non-PGPR strains. **A**, Proliferation of virulent DC3000 in rosettes of *Arabidopsis* plants cocultivated with or without *Paraburkholderia phytofirmans* PsJN and plants cocultivated with the non-PGPR *Escherichia coli* K12 and *Cupriavidus pinatubonensis* JMP134. Measurements were done at 7, 14, and 21 days postinfection (dpi). Each bar represents the log-transformed mean of six individual plants (CFU per gram of leaf fresh weight) and its standard error. Different letters on the bars indicate statistically significant differences among treatments (one-way analysis of variance, $P < 0.05$; Tukey's test, $P < 0.05$). Results are representative of two independent experiments. **B**, Symptomatology of infected *A. thaliana* plants over three weeks. Representative photographs of *A. thaliana* rosettes exposed to different bacterial treatments (plants cocultivated with strains PsJN, JMP134, and K12 and noninoculated plants). Results were recorded at 0, 7, 14, and 21 days postinfection (dpi).

and JA/ET signaling pathways seems to be important for the protection conferred by *P. phytofirmans* PsJN, infection assays with several plants mutant in SA, JA, and ET pathways were performed. The results of single mutant plants infected with DC3000 showed that strain PsJN still protects *sid2-2* plants (*SA induction deficient 2*), a mutant deficient in SA biosynthesis (Wildermuth et al. 2001) (Fig. 7A). Similarly, *coi1-1* (*coronatine insensitive 1*), a JA-insensitive mutant (Xie et al. 1998), and *ein2-1* (*ET insensitive 2*), an ET-insensitive mutant (Alonso et al. 1999), could resist disease as well as the wild-type plants

when they were inoculated with strain PsJN (Fig. 7A). These results suggest that more than one hormone signaling pathway is involved in the induced resistance triggered by *P. phytofirmans* PsJN in *Arabidopsis* plants. Therefore, an approach using double, triple, and quadruple mutants was applied (Tsuda et al. 2009). The set of double mutants comprised the three possible combinations of SA, JA, and ET pathways. So, *dde2-2/ein2-1*, *ein2-1/sid2-2*, and *dde2-2/sid2-2* double mutants preserve the SA, JA, or ET signaling pathways, respectively. The triple and quadruple mutants, *dde2-2/ein2-1/sid2-2*, and

Fig. 4. Response to *Pseudomonas syringae* DC3000 infection of *Arabidopsis thaliana* plants cocultivated with metabolically active or inactive *Paraburkholderia phytofirmans* PsJN. **A**, Proliferation of virulent DC3000 (disease progression) 21 days after infection in rosettes of *Arabidopsis* plants cocultivated with or without strain PsJN and mercuric chloride-treated bacteria (K-PsJN). Each bar represents the log-transformed mean of six individual plants (CFU per gram of leaf fresh weight) and its standard error. Different letters on the bars indicate statistically significant differences among treatments (One-way analysis of variance, $P < 0.05$; Tukey's test, $P < 0.05$). Results are representative of two independent experiments. **B**, Symptomatology of *A. thaliana* plants after 21 days of infection. Representative photographs of *A. thaliana* rosettes exposed to different treatments: plants cocultivated with strain PsJN, K-PsJN, and noninoculated and infected with the virulent DC3000. **C**, Histochemical detection of superoxide anion in *A. thaliana* after 21 days of infection. Representative photographs of *Arabidopsis* plants cocultivated or not with strain PsJN or with K-PsJN, after DC3000 infection.

dde2-2/ein2-1/pad4-1/sid2-2 are defective in SA, JA, and ET signaling pathways. DDE2 (DELAYED DEHISCENCE 2) is essential for JA biosynthesis (Park et al. 2002), whereas PAD4 (PHYTOALEXIN DEFICIENT 4) is important for SA accumulation in response to some SA-inducing stimuli and has an SA-independent immune function (Glazebrook et al. 2003; Jirage et al. 1999). Three weeks after challenge with DC3000, only mutants impaired in the three hormonal signaling pathways exhibited no significant differences in DC3000 population between control and strain PsJN-treated plants (Fig. 7B). Altogether, these results confirmed that *P. phytofirmans* PsJN protects *A. thaliana* through the activation of defenses dependent of the SA- and JA/ET signaling pathways.

A nonmotile mutant strain of *P. phytofirmans* PsJN has reduced ability to protect *A. thaliana* plants against virulent *Pseudomonas syringae* DC3000.

It has been reported that a synthetic 22-amino acid flagellin peptide derivative from *P. phytofirmans* PsJN is differentially recognized by the flagellin sensing system (FLS2-flg22) of grapevine and *Arabidopsis* (Trdá et al. 2014). In order to elucidate the role of flagellin bacterial elicitor of the defense response triggered by strain PsJN in *Arabidopsis* plants, a *P. phytofirmans* strain defective in the *fliA* gene was generated (PsJN Δ fliA). This gene encodes an alternative sigma factor (σ 28) necessary for the transcription of flagellin and other proteins required in late flagellar biogenesis in different bacteria, including *Burkholderia* species (Anderson et al. 2010;

Fig. 5. Expression patterns of *Arabidopsis thaliana* defense-related genes in response to *Paraburkholderia phytofirmans* PsJN, alone (0 h) or in the presence of *Pseudomonas syringae* DC3000 (1, 6, and 24 h). Quantitative reverse transcription-polymerase chain reaction determinations of relative expression of *PR1* (PATHOGENESIS-RELATED GENE 1) and *PDF1.2* (PLANT DEFENSIN 1.2) genes in complete plants, 13 days after sowing, cocultivated or not with strain PsJN. Total RNA was extracted before DC3000 infection (0 h) and 1, 6, and 24 h after infection. Data are means $\pm$ standard error of three to five biological replicates per treatment, each considering tissue from four plants and two technical replicates. Normalization was performed with the housekeeping SAND family gene (AT2G28390). Asterisks indicate statistically significant differences among treatments in a particular time (one-way analysis of variance, $P < 0.05$; Student's *t* test, one asterisk [*] $P < 0.05$; two asterisks [**] $P < 0.01$; three asterisks [***] $P < 0.001$).

Fig. 6. Salicylic acid (SA) levels in *Arabidopsis thaliana* plants cocultivated with or without *Paraburkholderia phytofirmans* PsJN. Free SA (gray bars) and glycosylated SA (SAG) (white bars) levels in complete plants 13 days after sowing (four rosette leaves, LP.04 stage) were quantified in *Arabidopsis* plants inoculated with or without strain PsJN (0 h) and also 24 and 48 h postinfection with DC3000. Data are means $\pm$ standard deviation of three biological replicates, where each biological replicate represents a pool of 0.5 g of whole plants. Asterisks indicate statistically significant differences among treatments (two-way analysis of variance, $P < 0.05$; Tukey's test, two asterisks [**] indicate $P < 0.01$; three asterisks [***] $P < 0.001$; ns, no significant differences).

Chilcott and Hughes 2000; Kim et al. 2007). The *P. phytofirmans fliA* mutant was rendered nonmotile (Fig. 8A), with a similar phenotype to that reported for *Burkholderia glumae* (Kim et al. 2007). Additionally, the mutant strain PsJN Δ fliA produced less and more fragile biofilm than the wild-type strain (Fig. 8B), which is consistent with studies in strains of *Burkholderia cenocepacia* and *Burkholderia pseudomallei* that found flagellum-mediated motility is important for biofilm formation (Saldías et al. 2008; Tunpiboonsak et al. 2010). Results of the infection assay in plants cocultivated with the mutant strain PsJN Δ fliA, the wild-type strain PsJN, and noninoculated plants showed that strain PsJN Δ fliA had reduced ability to protect *Arabidopsis* plants compared with the

wild-type strain, as measured by DC3000 population (Fig. 8C) and disease symptomatology (data not shown). However, the mutant strain still provided a degree of protection compared with non-inoculated plants (Fig. 8C).

The levels of endophytic and epiphytic colonization of this mutant strain were similar to the wild-type strain (data not shown).

DISCUSSION

This report demonstrates, for the first time, that *P. phytofirmans* PsJN protects, throughout the entire life cycle, *A. thaliana* plants against the common phytopathogen *Pseudomonas syringae* pv.

Fig. 7. Response to *Pseudomonas syringae* DC3000 infection of *Arabidopsis* hormonal mutant plants cocultivated with or without *Paraburkholderia phytofirmans* PsJN. Proliferation of virulent DC3000 21 days after infection in rosettes of *Arabidopsis* wild-type plants and **A**, single mutant plants (*sid2-2*, *coi1-1*, *ein2-1*), or **B**, double, triple, and quadruple mutant plants (*dde2-2/ein2-1*, *dde2-2/sid2-2*, *dde2-2/ein2-1/sid2-2*, and *dde2-2/ein2-1/pad4-1/sid2-2*), cocultivated with or without strain PsJN. Each bar represents the log-transformed mean of six individual plants (CFU per gram of leaf fresh weight) and its standard error. Asterisks indicate statistically significant differences among treatments. For data in A, unpaired *t* test, two asterisks (**) indicate $P < 0.01$; three asterisks (***), $P < 0.001$. For data in B, Mann-Whitney test, one asterisk (*) indicates $P < 0.05$; two asterisks (**), $P < 0.01$; ns, no significant differences. Results are representative of two independent experiments.

tomato DC3000. This phenomenon is not explained by production of antimicrobials by strain PsJN thus affecting growth of this phytopathogen (data not shown). This protection is due to the activation of induced resistance in the plant, which strongly activates the expression of key defense-related genes belonging to the SA-, JA- and ET-signaling pathways. Protection conferred by *P. phytofirmans* PsJN to *Arabidopsis* plants to the pathogenic bacterium *Pseudomonas syringae* requires metabolically active cells, as plants cocultivated with heat-killed bacteria (HK-PsJN) or inactivated bacteria with conserved cell integrity (K-PsJN) exhibit disease symptoms caused by *Pseudomonas syringae* pv. *tomato* infection and higher population levels of the pathogenic bacterium

in comparison with strain PsJN-treated plants. Metabolically active PsJN cells are also required to confer salt tolerance to *A. thaliana* (Ledger et al. 2016; Pinedo et al. 2015). The specificity of *Arabidopsis* protection to *Pseudomonas syringae* DC3000 infection by *P. phytofirmans* PsJN is supported by the inability of two other bacterial strains (*E. coli* K12 and *C. pinatubonensis* JMP134) to prevent disease symptoms and to control phytopathogen population levels, indicating that plant protection over time is not provoked by shared MAMPs. MAMPs or 'general elicitors,' such as flagellin, lipopolysaccharides, and elongation factor Tu from gram-negative bacteria (Mishina and Zeier 2007), are recognized by the plant innate immune system through the action of

Fig. 8. Motility, biofilm production, and *Pseudomonas syringae* DC3000 biocontrol in *Arabidopsis thaliana* plants cocultivated with the *Paraburkholderia phytofirmans* mutant strain PsJNΔfliA. **A**, Representative photograph of swimming motility assay 5 days after placing the initial inocula onto the plate. Swimming motility was tested using a minimal medium containing 10 mM fructose and 0.25% agar. *P. phytofirmans* PsJN (wild type) was able to swim (growth zone present around initial inoculum). In contrast, the mutant strain PsJNΔfliA did not show a growth zone around its initial inoculum. The assay was done in triplicate. **B**, Biofilm production assay of strain PsJN and its *fliA* mutant derivative growing 72 h in microplates containing 50 mM mannitol. Biofilms were exposed to 0.1% (wt/vol) of crystal violet dye after washing with abundant water. Dry biofilms were exposed to absolute ethanol and, after 30 min solutions were measured through absorbance at 600 nm by spectrophotometry. Biofilm formation was normalized by the growth of the respective strain. Each bar represents the mean of five independent experiments and its standard error. Asterisks indicate statistically significant differences among treatments (Mann Whitney test, $P < 0.01$). **C**, *Pseudomonas syringae* DC3000 population levels in *Arabidopsis* plants alone or cocultivated with wild-type strain *P. phytofirmans* or its *fliA* mutant derivative. Measurements were taken 21 days after infection. Each bar represents the log-transformed mean of six individual plants (CFU per gram of leaf fresh weight) and its standard error. Different letters on the bars indicate statistically significant differences among treatments (one-way analysis of variance, $P < 0.05$; Tukey's test, $P < 0.05$).

pattern recognition receptors located at the plasma membrane and result in MTI (Pieterse et al. 2009; Thilmony et al. 2006; Trdá et al. 2014). Successful recognition of these MAMPs results in a mild but effective activation of the plant immune responses in systemic tissues, H₂O₂ production, pathogenesis-related (PR) gene induction, and callose deposition (Mishina and Zeier 2007; Van Wees et al. 2008). However, virulent pathogens have acquired effectors that suppress MTI and the disease unavoidably proceeds, resulting in effector-triggered susceptibility (Pieterse et al. 2009; Thilmony et al. 2006). Results reported here are in concordance with this observation, as plants cocultivated with *E. coli* or *C. pinatubonensis* only contained the disease (*Pseudomonas syringae* pv. *tomato* proliferation) at the beginning of infection (14 dpi) (Fig. 3), suggesting that MTI was triggered, leading to the activation of the *PR1* gene (Supplementary Fig. S2). Accordingly, *P. phytofirmans* PsJN must elicit a stronger defense mechanism than MTI (i.e., induced resistance) in *Arabidopsis* plants to limit the disease development over the time.

The activation of defense-related genes can be due to one or more of the SA, JA, or ET signaling pathways, which, in turn, are highly interconnected (Memelink 2009; Pieterse et al. 2009; 2012; Van der Does et al. 2013). Nevertheless, the particular hormone-signaling pathway activated by the colonization of an ISR-inducing PGPR in order to cope with a pathogen attack is specific to the plant species, the pathogenic bacterium, and the PGPR strain combination (De Vleeschauwer and Höfte 2009). For instance, the ISR-inducing PGPR *P. aeruginosa* 7NSK2 triggers ISR in *Arabidopsis* plants in a SA-independent manner (Ran et al. 2005), but the same PGPR in tobacco plants triggers ISR through the SA signaling pathway (De Meyer et al. 1999). On the other hand, *Bacillus subtilis* FB17 activates ISR in *Arabidopsis* through the SA and ET pathway (Rudrappa et al. 2010). Remarkably, *B. cereus* AR156 can trigger ISR via simultaneous activation of the SA- and JA/ET-signaling pathways, resulting in an enhanced level of induced protection in *Arabidopsis* due to the activation of *PR1*, *PR2*, *PR5*, and *PDF1.2* defense genes (Niu et al. 2011). Thus, several genes representative of SA and JA/ET pathways were selected to study induction of resistance in *Arabidopsis* by strain PsJN. In the case of the *ICS1* gene, which encodes the enzyme isochorismate synthase, which is required to synthesize SA in plants (Strawn et al. 2007; Wildermuth et al. 2001), its expression level was higher in strain PsJN-treated plants compared with control plants prior to DC3000 infection. This is in agreement with the fact that strain PsJN stimulated the production of SA in *Arabidopsis* plants before and 24 h after the challenge with the pathogenic bacterium (Fig. 6). Accordingly, it has been previously reported that, in cell cultures of *Vitis vinifera*, free SA (265 ng per gram of FW) was detected in response to *P. phytofirmans* PsJN inoculation (Bordiec et al. 2011). As expected, the expression level of the *PR1* gene was significantly higher in plants cocultivated with *P. phytofirmans* PsJN in all measured times after *Pseudomonas syringae* pv. *tomato* infection (1, 6, and 24 h) (Fig. 5). *PR1* is a late SA-responsive gene that has its peak of expression 24 h after SA treatment or after an incompatible interaction with an avirulent phytopathogen (Asai et al. 2014; Blanco et al. 2009; Uquillas et al. 2004). However, *PR1* expression in *Arabidopsis* is even later (72 and 120 hpi) with a virulent phytopathogen challenge (Asai et al. 2014). Notably, *Arabidopsis* plants treated with strain PsJN and infected with virulent DC3000 showed increased expression of the defense gene *PR1* significantly earlier. Additionally, expression of a large set of *WRKY* genes is induced by SA, including *WRKY54* and *WRKY70*, the latter of which can regulate the expression of SA-responsive genes, such as *PR1* (Van der Does et al. 2013). In a previous transcriptomic study by our group (Poupin et al. 2013), *P. phytofirmans* PsJN

was shown to upregulate the expression of *WRKY70* in *Arabidopsis* plants. Notably, results reported in the present study indicated that, in plants cocultivated with strain PsJN, the expression of *WRKY54* was down-regulated before *Pseudomonas syringae* pv. *tomato* infection; however, after the infection event, its expression was up-regulated in the same plants. Remarkably, single *sid2-2* mutant plants (deficient in SA biosynthesis) infected with DC3000 still were protected by *P. phytofirmans* (Fig. 7A). In addition, strain PsJN deficient in flagella (*PsJNΔfliA*) that did not show increased *PR1* gene expression provided a degree of protection against this pathogen attack (Fig. 8C), suggesting that the SA signaling pathway is only partially involved in the protection of *Arabidopsis* by *P. phytofirmans* PsJN.

Because a fine-tuning crosstalk between the defense hormones SA and JA plays a central role in the modulation of the plant immune signaling network (Mur et al. 2006), a selected group of key genes belonging to the JA/ET signaling pathway was analyzed. In *Arabidopsis*, the JA-responsive genes *PDF1.2* and *VSP2* are highly sensitive to suppression by SA in an NPR1-dependent manner, when ET signaling is not stimulated (León-Reyes et al. 2009; Pieterse et al. 2012). Otherwise, when ET signaling is stimulated, the NPR1 dependency of SA-JA crosstalk is bypassed (León-Reyes et al. 2009). The final outcome of the SA-JA signal dynamic during the complex interactions between plants and their attackers can be shaped by the ET present in the signal signature produced upon attack (Pieterse et al. 2012). For instance, in *Arabidopsis*, ET potentiates SA-responsive *PR1* expression (De Vos et al. 2007; Niu et al. 2011; Pieterse et al. 2012). Results reported here indicate that *LOX2*, an enzyme involved in JA biosynthesis induced by wounds or mechanical damage (Chauvin et al. 2013), was up-regulated in strain PsJN-treated plants before DC3000 infection, which is in agreement with that reported previously by Poupin et al. (2013). However, after the infection event, *LOX2* expression was down-regulated in plants cocultivated with strain PsJN over all measured times. This result is in accordance with previous reports that demonstrate that endogenously accumulated SA has an inhibitory effect on *LOX2* gene expression and JA formation (Spoel et al. 2003). Additionally, *PDF1.2* gene is synergistically induced by a combination of JA and ET (Memelink 2009). As expected, the expression level of *PDF1.2* was significantly higher due to the inoculation of strain PsJN, which is according to previous reports (Poupin et al. 2013) and was also up-regulated 6 h after DC3000 infection in strain PsJN-treated plants (Fig. 5).

Many reports describe an antagonistic interaction between SA and JA pathways, but synergistic interactions have been reported as well (Liu et al. 2016; Mur et al. 2006). The relative concentrations of SA and JA in plants are important in determining gene expression levels. For instance, a transient synergistic enhancement in the expression of genes associated with both JA (*PDF1.2*) or SA (*PR1*) signaling pathways when both hormones SA and JA are applied at low concentrations was observed in tobacco plants (Mur et al. 2006). Even more, a recent report has shown that an early induction of JA-responsive genes and de novo JA synthesis following SA accumulation is activated through the SA receptors NPR3 and NPR4 instead of the JA receptor COI1 (Liu et al. 2016). It is quite possible that a synergistic interaction between SA and JA would be taking place in *Arabidopsis* plants inoculated with *P. phytofirmans* PsJN, because *ICS1* and *LOX2* were activated in plants cocultivated with strain PsJN, and, after *Pseudomonas syringae* pv. *tomato* infection, *PR1* and *PDF1.2* were highly induced in the same plants.

Since *P. phytofirmans* PsJN induced the expression of *ICS1*, *PDF1.2*, and *LOX2* genes (Fig. 5) and stimulated the production

of SA (Fig. 6), *A. thaliana* plants inoculated with this PGPR strain were better prepared, at least at the molecular and metabolic level, to face the disease caused by the virulent strain of *Pseudomonas syringae* DC3000. Furthermore, early expression of the defense gene *PR1*, 1 hpi instead of its normal timing (72 hpi) (Asai et al. 2014), suggests that *Arabidopsis* plants cocultivated with strain PsJN were in a priming state before infection. Therefore, these plants could respond faster and stronger upon pathogen attack, resulting in enhanced resistance. Additionally, the activation of *WRKY54*, *MYC2*, and *ERF1* defense genes in strain PsJN-treated plants after *Pseudomonas syringae* pv. *tomato* challenge likely contributed to reduced disease severity and to prevent pathogen proliferation in the leaves.

Moreover, the participation of the SA, JA, and ET hormones in the induced resistance triggered by *P. phytofirmans* PsJN in *Arabidopsis* plants was confirmed with several mutant lines impaired in these hormonal signaling pathways, in which, only in the triple and quadruple mutants *dde2-2/ein2-1/sid2-2* and *dde2-2/ein2-1/pad4-1/sid2-2*, strain PsJN could not protect plants against the pathogenic bacterium (Fig. 7). Concerning results using double mutant lines, strain PsJN can protect its host if at least one hormonal signaling pathway remains intact (Fig. 7B). Therefore, it is quite clear that the PGPR *P. phytofirmans* PsJN induced resistance in *A. thaliana* by activating, either, SA-, JA- or ET-dependent signaling pathways. Mediation of induced resistance by these three signaling pathways has also been reported for the PGPR *B. cereus* AR156 in *A. thaliana* (Niu et al. 2011); however, PGPR-mediated induced resistance by the three most important hormonal pathways for plant defense has not been widely reported so far. It is very interesting that *P. phytofirmans* PsJN can activate the SA and JA/ET signaling pathways on plants that it colonizes, because plants will be better prepared to face an attack by either biotrophic or necrotrophic pathogens. In fact, a recently published study (Miotto-Vilanova et al. 2016) showed that strain PsJN protects grapevine plants from a necrotrophic pathogen (*B. cinerea*) by the activation of *PR1* and JA-related genes and, also, by an accumulation of starch and callose deposition in leaves.

After demonstrating that strain PsJN is able to trigger induced resistance in *Arabidopsis* plants, we sought to determine which effector molecules from strain PsJN elicit the defense response in the plant. To tackle this challenge, infection assays with killed but cellularly intact strain PsJN (K-PsJN, MAMPs remained intact) were carried out. Seven days after the infection, K-PsJN-treated plants presented fewer CFU of the pathogen than control plants (Fig. 4A); however, this difference disappeared on 14 and 21 dpi. These results can be explained by the activation of MTI; nevertheless, this innate immune response is not sufficient to contain the proliferation of the virulent strain DC3000 in K-PsJN-treated plants, because this pathogenic bacterium represses three genes associated with basal defense response in *Arabidopsis*, *FLS2*, *FRK1*, and *WRKY22* (Thilmony et al. 2006). This is reflected in the DC3000 population at 14 and 21 days after infection, when plants treated with K-PsJN do not have significant differences with respect to noninoculated plants (Fig. 4A). Therefore, strain PsJN MAMPs such as flagella or lipopolysaccharides, removed from their functional context and considered solely for their molecular composition, are not sufficient to fully induce resistance in plants. Additionally, the mutant lacking flagella (PsJN Δ fla) still displays a significantly high level of control of the pathogen population size in comparison with noninoculated plants (Fig. 8), reinforcing the idea that the flagellum (or the ability to move) could be only one component of a set of elicitors (or functions) able to induce resistance in *Arabidopsis*.

The notion that metabolically active *P. phytofirmans* PsJN triggers induced resistance in *Arabidopsis* plants is also supported by a study done with a synthetic flg22 peptide derived from *P. phytofirmans* PsJN in *A. thaliana* and *V. vinifera* (Trdá et al. 2014). The authors demonstrated that the flagellin receptor of *V. vinifera* (VvFLS2) differentially recognizes flg22 from strain PsJN from pathogenic bacteria, suggesting that flagellin from the beneficial PGPR *P. phytofirmans* has evolved to evade the grapevine immune recognition system. Accordingly, there is no growth inhibition, nor inhibition of H₂O₂ production, in grapevine plants. The opposite effects were observed in *Arabidopsis* plants, in which flg22 of *P. phytofirmans* induced H₂O₂ production and growth inhibition in a similar fashion to effects triggered by the pathogen-derived flg22 peptides from *Pseudomonas aeruginosa* or *Xanthomonas campestris* (Trdá et al. 2014). This antecedent reinforces the idea that *P. phytofirmans* PsJN needs to be metabolically active to modulate plant regulatory mechanisms, including defense mechanisms. This is aligned with the fact that colonization of roots by ISR-eliciting PGPR requires local suppression of MAMP-triggered immunity to protect the PGPR against MAMP-triggered production of antimicrobial compounds such as phytoalexins (Millet et al. 2010; Pieterse et al. 2014). Downregulation of root immune responses has been previously described for the PGPR genus *Trichoderma* and for ISR-inducing PGPR such as *B. subtilis* FB17 and *Pseudomonas fluorescens* WCS417 (Pieterse et al. 2014). Specifically, *Pseudomonas fluorescens* WCS417 suppresses the activation of defense genes in *Arabidopsis* roots that are triggered by flg22 (Millet et al. 2010). Based in the results shown by Poupin et al. (2013) and Trdá et al. (2014), it can be suggested that *P. phytofirmans* PsJN exerts a similar suppression as *Pseudomonas fluorescens* WCS417 in *Arabidopsis* roots.

To the best of our knowledge, the present study is the first report showing that the PGPR *P. phytofirmans* PsJN protects *A. thaliana* against a virulent pathogenic bacterium (*Pseudomonas syringae* DC3000) and, also, established that the underlying mechanism of the plant protection is the activation of induced resistance. Moreover, strain PsJN can trigger induced resistance in *Arabidopsis* plants either by the SA-, JA-, or ET-signaling pathways; therefore, plants should be better prepared to face an attack either by biotrophic or by necrotrophic pathogens.

MATERIALS AND METHODS

Plant growth conditions and bacterial inoculation treatments.

P. phytofirmans PsJN was routinely grown in minimal saline medium (Dorn et al. 1974), containing 10 mM fructose, in an orbital shaker (120 rpm) at 30°C. Cell suspensions were subsequently collected and adjusted to 1×10^8 CFU per milliliter, as determined by plate counting. *A. thaliana* wild-type plants were Col-0 ecotype, as were as the background of all mutant lines used in this study. *Arabidopsis sid2-2* (Wildermuth et al. 2001) and *coi1-1* mutants (Xie et al. 1998) were previously described. Seeds of *ein2-1* (CS3071) (Alonso et al. 1999), *dde2-2/ein2-1* (CS65997), *dde2-2/sid2-2* (CS65999), *dde2-2/ein2-1/sid2-2* (CS66004), and *dde2-2/ein2-1/pad4-1/sid2-2* (CS66007) (Tsuda et al. 2009) were obtained from the Arabidopsis Biological Resource Center. Seeds were surface-sterilized with 50% sodium hypochlorite (100% commercial laundry bleach) containing 0.1% Tween 20, were rinsed four times with sterile water, and were kept at 4°C for 4 days, to promote uniform germination. To prepare the inoculated pots (Phytatray 1552; Sigma-Aldrich, St. Louis) for gnotobiotic assays, the initial inoculum of strain PsJN (1×10^8 CFU/ml) was homogeneously diluted in half strength Murashige and

Skoog medium (50% MS) 0.8% agar (Murashige and Skoog 1962) just before gelling, to reach a final inoculum of 1×10^4 CFU/ml of medium. This inoculum size was previously reported by Poupin et al. (2013) as the optimum to provoke positive effects in *A. thaliana* plants. Then, sterilized and synchronized seeds were sown, eight per pot, in pots with 50% MS medium inoculated with or without the strain. Pots were placed in a growth chamber at 22°C with a photoperiod of 16 h light and 8 h dark.

To assess the effect of heat inactivated bacteria (HK-PsJN, mortality corroborated by plate counting), bacterial cells were heated at 95°C for 20 min and were then used as inoculum at a dilution of 1×10^4 CFU/ml, as described above. Additionally, to evaluate the effect of metabolically inactive but structurally intact (cell membrane and their MAMPs) cells of strain PsJN, an inoculum of strain PsJN was treated with mercuric chloride (HgCl_2). The procedure was as follows: 45 ml of an overnight strain PsJN culture was centrifuged three times at $10,000 \times g$ for 5 min at room temperature to wash the minimal medium culture salts. The pellet was then resuspended in 20 ml of 50 mM phosphate buffer, and the cells were exposed to 20 mM HgCl_2 for 1 min. In order to completely remove the HgCl_2 and thus prevent damage in plants, the culture was centrifuged three times at 8,000 rpm for 5 min at room temperature and was resuspended every time in 40 ml of 50 mM phosphate buffer. The final resuspension was carried out with 10 ml of 50 mM phosphate buffer, to adjust cell concentration to an optical density at 600 nm (OD_{600}) of 0.6 (same concentration as the living cells culture). Plate counting corroborated mortality, and cell integrity was verified by optical microscope visualization after staining with safranin dye.

To assess the specificity of strain PsJN in triggering induced resistance in *A. thaliana*, two additional treatments were performed. One group of plants was sown, in pots containing 50% MS, 0.8% agar, and *Escherichia coli* K12 at 1×10^4 CFU/ml, as a bacterium not associated with plants, and another group was sown in the same conditions but with *C. pinatubonensis* JMP134 at 1×10^4 CFU/ml, as an environmental bacterium neutral for plants (Ledger et al. 2012).

Infection of plants and proliferation assays.

A virulent strain of the plant pathogen *Pseudomonas syringae* pv. *tomato* DC3000 was grown on King's B medium in an orbital shaker (120 rpm) at 28°C, supplemented with rifampicin (50 µg/ml) and kanamycin (50 µg/ml) as selection antibiotics. Three-week-old plants (21 DAS) from each treatment were infected on rosettes by spraying 100 µl per pot with an inoculum of DC3000 (5×10^4 CFU/ml) in 10 mM MgCl_2 or only 10 mM MgCl_2 , for mock treatments, based on a previously described procedure (Innerebner et al. 2011). Mock treatments never showed any effects on plants. Pots were returned to the growth chamber at 22°C with the same photoperiod as above.

In order to measure disease progression, above-ground plant parts (rosettes) from eight independent plants per treatment were taken at 0, 7, 14, 21, and 28 dpi. The FW of each plant was recorded. Subsequently, to determine DC3000 cell numbers contained in the infected plant tissues, collected rosettes were placed individually into 2-ml tubes containing 1.3 ml of 100 mM phosphate buffer (pH 7) supplemented with 0.2% (vol/vol) of the surfactant silwet L-77 (Lehle Seeds, Round Rock, TX, U.S.A.) (Innerebner et al. 2011). Samples were placed into an orbital shaker (120 rpm) at 28°C for 1.5 h. After washing, tenfold serial dilutions were plated onto King's B agar plates supplemented with rifampicin (50 µg/ml) and kanamycin (50 µg/ml). Plates were incubated at 28°C for 48 h and the number of antibiotic-resistant CFU was determined. CFU were normalized by the FW of plants (CFU per milligram of FW) and the

values were log10 transformed. Plants severely damaged by disease could not be handled to obtain enough biomass material for accurate FW determinations. Statistical analyses were done with R software version 3.2.1 (R Development Core Team 2011). One-way analysis of variance (ANOVA) and a Tukey's test were carried out for each timepoint independently.

Symptomatology and plant disease severity index.

Disease symptoms of infected *A. thaliana* plants were recorded by photographing rosette areas every week since the day of infection with DC3000 until 28 dpi. The disease severity index was measured based on the number of leaves per plant showing disease symptoms (necrotic or water-soaked spots surrounded by chlorosis), where 0% means that no leaf presents symptoms and 100% means that all leaves present symptoms (protocol adapted from Hossain et al. [2007]). At least eight individual plants per treatment were used for this measurement.

Histochemical detection of O_2^- .

At 21 dpi, *Arabidopsis* plants were incubated in phosphate-buffered saline solution (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , 2 mM KH_2PO_4) containing 0.06 mM nitroblue tetrazolium, to detect the superoxide radical (O_2^-), after which the plant individuals were cleared in 80% ethanol at 60°C to remove chlorophyll (protocol adapted from Carvalho et al. [2008]). Images were taken with a digital camera coupled to a stereo microscope (Leica S6D, Wetzlar, Germany).

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

Sterilized and synchronized *A. thaliana* seeds were sown in square petri dishes with 50% MS medium 0.8% agar, inoculated or not with *P. phytofirmans* PsJN. Plates were placed vertically in a growth chamber at 22°C with a photoperiod of 16 h light and 8 h dark. At 13 DAS, *Arabidopsis* plants with four visible leaves (LP.04 stage [Boyes et al. 2001]) were challenged with DC3000 or left as controls (mock treatment) under the same procedure described above. Immediately after infection, four randomly selected mock-treated plants were sampled and stored in 1.5-ml Eppendorf tubes containing RNAlater (Ambion, Austin, TX, U.S.A.), according to the manufacturer's instructions. Five pools with four plants each were collected per treatment (noninoculated plants, mock and strain PsJN-inoculated plants, mock). In the qRT-PCR results, these treatments correspond to 0 h. At 1, 6, and 24 h after DC3000 infection, four randomly selected *Pseudomonas syringae* pv. *tomato*-infected plants were sampled and stored in 1.5-ml Eppendorf tubes containing RNAlater (Ambion), according to the manufacturer's instructions. Five pools with four plants each were collected per treatment (noninoculated plants, *Pseudomonas syringae* pv. *tomato* and strain PsJN-inoculated plants, *Pseudomonas syringae* pv. *tomato*). In the qRT-PCR results, these treatments correspond to 1, 6, and 24 h. For RNA extraction, plants were ground with a pestle in the Eppendorf tube without the RNAlater solution. Then, total RNA of each sample was extracted, using the Trizol reagent (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer's recommendations.

For cDNA synthesis, 1 µg of total RNA treated with DNase I (RQ1, Promega, Madison, WI, U.S.A.) was reverse transcribed with random hexamer primers, using the Improm II reverse transcription (Promega), following the manufacturer's instructions. Real time 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.), as described by Poupin et al. (2013). The PCR mixture (15 µl) contained 2.0 µl of template cDNA (diluted 1:10) and 140 nM of each primer. Amplification was performed under the following conditions:

95°C for 10 min, followed by 40 cycles of 94°C, 30 s; 58 to 62°C, 30 s; and 72°C, 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: $2^{-(\Delta CtGOI-HK)}$ (Dauelsberg et al. 2011). Then, gene expression levels were normalized to the average value of the treatment with less gene expression. Data presented here were normalized, using *AtSAND* (AT2G28390) amplification as the housekeeping gene (Czechowski et al. 2005). Primer pairs used in this study were designed with Primer 3 software (Koressaar and Remm 2007; Untergasser et al. 2012) and were confirmed with Primer-BLAST (National Center for Biotechnology Information). Sequences of all primers and their references (if applicable) are listed in Supplementary Table S1. In all cases, the reaction specificities were tested with melt gradient dissociation curves and 2% agarose electrophoresis gels of each PCR product. All experiments were performed with five biological and two technical replicates. One-way ANOVA and a Student's *t* test were carried out for each timepoint independently.

Construction and phenotypic evaluation of a nonmotile mutant strain of *P. phytofirmans*.

The 5' and 3' ends of the *fliA* gene sequence from *P. phytofirmans* PsJN, the gentamicin 3'-acetyltransferase gene sequence of pBS1 plasmid (Bronstein et al. 2005), the aminoglycoside 3'-phosphotransferase gene sequence, and the pUC replication origin of plasmid pCR2.1-TOPO (Invitrogen) were amplified by PCR, using primer pairs FliA1Fw-FliA1Rv, FliA2Fw-FliA2Rv, GmFw-GmRv, KmFw-KmRv, and OriFw-OriRv (Supplementary Table S2), respectively, and were ligated in a single-reaction method for assembling multiple overlapping DNA molecules (Gibson et al. 2009), to generate suicidal plasmid pMut-*fliA*. Then, this plasmid was electroporated in *P. phytofirmans* PsJN to obtain a two-recombination event, generating a replacement of the functional *fliA* gene by aminoglycoside 3'-phosphotransferase gene, which confers kanamycin resistance (Supplementary Fig. S3A). Accordingly, the *P. phytofirmans* *fliA* mutant was selected on Luria Bertani agar containing 50 µg of kanamycin per milliliter. Correct insertions in the mutant strain were confirmed by sensitivity to gentamycin, PCR, and sequencing. Also a growth curve in 5 mM glucose of PsJNΔ*fliA* and the wild-type strain was carried out (Supplementary Fig. S4). To evaluate swimming motility the wild-type strain (PsJN) and its *fliA* mutant derivative (PsJNΔ*fliA*) were grown to stationary phase and 1 µl of initial inoculum was placed onto plates containing minimal medium, 10 mM fructose, and 0.25% of agar. Five days after incubation at 30°C, colony phenotypes were photographed. The assay was done in triplicate. In order to assess the biofilm production of strain PsJN and PsJNΔ*fliA*, they were grown 72 h in microplates containing 50 mM mannitol. Biofilms were incubated with 0.1% (wt/vol) of crystal violet dye for 30 min, after washing with abundant water. Dry biofilms were exposed to absolute ethanol and, after 30 min, solutions were measured through absorbance at 600 nm by spectrophotometry. Biofilm formation was normalized by the growth of each strain (OD₆₀₀). Five independent experiments were performed.

SA extraction and quantitation.

Three pools of 0.5 g of *A. thaliana* plants (13 DAS) cocultivated with or without *P. phytofirmans* PsJN in the same conditions as plants used for the qRT-PCR analysis were harvested before DC3000 infection (0 hpi) and also 24 and 48 hpi. Free and total SA (the sum of free and glycosylated SA) were extracted and were quantified as described by Verberne et al.

(2002). The SA extracts were measured by high-pressure liquid chromatography (Waters Alliance, Milford, MA), coupled to a fluorescence detector and using an Atlantis T3 column (silica-based column, 250 × 4.6 mm, reversed-phase C18).

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