Differential Contribution of Plant-Beneficial Functions from *Pseudomonas kilonensis* F113 to Root System Architecture Alterations in *Arabidopsis thaliana* and *Zea mays*

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Fluorescent pseudomonads are playing key roles in plant-bacteria symbiotic interactions due to the multiple plant-beneficial functions (PBFs) they are harboring. The relative contributions of PBFs to plant-stimulatory effects of the well-known plant growth-promoting rhizobacteria *Pseudomonas kilonensis* F113 (formerly *P. fluorescens* F113) were investigated using a genetic approach. To this end, several deletion mutants were constructed, simple mutants $\Delta phlD$ (impaired in the biosynthesis of 2,4-diacetylphloroglucinol [DAPG]), $\Delta acdS$ (deficient in 1-aminocyclopropane-1-carboxylate deaminase activity), Δgcd (glucose dehydrogenase deficient, impaired in phosphate solubilization), and $\Delta nirS$ (nitrite reductase deficient), and a quadruple mutant (deficient in the four PBFs mentioned above). Every PBF activity was quantified in the wild-type strain and the five deletion mutants. This approach revealed few functional interactions between PBFs in vitro. In particular, biosynthesis of glucose dehydrogenase severely reduced the production of DAPG. Contrariwise, the DAPG production impacted positively, but to a lesser extent, phosphate solubilization. Inoculation of the F113 wild-type strain on *Arabidopsis thaliana* Col-0 and maize seedlings modified the root architecture of both plants. Mutant strain inoculations revealed that the relative contribution of each PBF differed according to the measured plant traits and that F113 plant-stimulatory effects did not correspond to the sum of each PBF relative contribution. Indeed, two PBF genes ($\Delta acdS$ and $\Delta nirS$) had a significant impact on root-system architecture from both model plants, in vitro and in vivo conditions. The current work underscored that few F113 PBFs seem to interact between each other in the free-living bacterial cells, whereas they control in concert *Arabidopsis thaliana* and maize growth and development.

Plant growth-promoting rhizobacteria (PGPR) are root-colonizing bacteria able to provide growth and health benefits to plants through various mechanisms involving the expression of plant-beneficial functions (PBFs) in PGPR (Bruto et al. 2014; Lemanceau et al. 2017; Richardson et al. 2009; Vacheron et al. 2013). For example, PGPR can modulate plant hormonal signalization by producing phytohormones such as auxins, cytokinins, gibberellins, and nitric oxide (NO) (Bashan and de-Bashan 2010) or decreasing the emission of ethylene (Glick et al. 1998; Honma and Shimomura 1978; Vacheron et al. 2016a). PGPR may also help plant nutrition by solubilizing inorganic compounds, such as phosphate, or other mineral elements (Adesemoye and Kloepper 2009; Vessey 2003). Finally, some PGPR, in particular fluorescent pseudomonads, are producing antimicrobial compounds such as 2,4-diacetylphloroglucinol (DAPG), which protects plants against plant-pathogen microorganisms (Almario et al. 2014; Couillerot et al. 2009; Lantaigne et al. 2012) and activates plant defenses (Iavicoli et al. 2003). Some of these antimicrobial compounds can also act as a communication signal between bacteria (Combes-Meynet et al. 2011).

The availability of an ever-increasing number of sequenced and annotated PGPR genomes is allowing us to perform some comparative genomic analyses (Flury et al. 2016; Loper et al. 2012). The results of these analyses show the existence of combinations of PBF genes that are specific to some PGPR taxa (Bruto et al. 2014; Vacheron et al. 2016b). For example, in strains or species belonging to the *Pseudomonas fluorescens* group, the genes required for the synthesis of DAPG are present simultaneously with the ones required for cyanid hydrogen (HCN) synthesis (Ramette et al. 2003). In *Pseudomonas protegens*, the production of pyoluteorin is co-occurring with the production of DAPG (Ramette et al. 2011). The presence of co-occurring PBF genes in genomes suggests that these functions might be part of common bacterial regulatory or metabolic networks. Alternatively, Bashan and de-Bashan (2010) hypothesized that each PBF present in one PGPR genome would improve plant growth either simultaneously or successively. In this additive hypothesis, the overall improvement of plant growth would result from the sum of effects of each co-occurring PBF. Although the relative contribution of several antimicrobial compounds to biocontrol performance has been well studied (Calderón et al. 2013; Iavicoli et al. 2003; Romanowski et al. 2011; Voisard et al. 1989), the contribution of co-occurring PBFs in plant growth promotion is poorly documented.

Among fluorescent pseudomonads, the *Pseudomonas kilonensis* F113 strain (formerly *Pseudomonas fluorescens* [Almario et al.

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2017]) is a PGPR strain isolated from sugar-beet rhizosphere (Shanahan et al. 1992). That strain is well-known for its bio-control activity against a broad range of plant-pathogen micro-organisms (Cronin et al. 1997a and b; Dunne et al. 1998). Less known, but all the more important, F113 also promotes plant growth and development, particularly on maize (Vacheron et al. 2016a; Walker et al. 2012), probably because of its rhizosphere competence or, in other words, its ability to efficiently colonize plant roots and to compete with other rhizosphere-competent bacteria (Lugtenberg and Dekkers 1999). The analysis of the F113 genome sequence reveals the presence of multiple PBFs (Redondo-Nieto et al. 2013). We can identify the *phl* genes, which enable the production of DAPG (*phlD* encodes a polyketide synthase that is a key determinant of the DAPG biosynthesis) and, also, *gcd* encoding a glucose dehydrogenase involved in phosphate solubilization (Miller et al. 2010), *acdS* encoding the 1-aminocyclopropane-1-carboxylate (ACC) deaminase enzyme (Prigent-Combaret et al. 2008; Vacheron et al. 2016a), and *nirS* encoding the nitrite reductase involved in NO production (Choi et al. 2006). Specific associations of co-occurring PBFs were frequently observed among *Proteobacteria* (Bruto et al. 2014) and may reflect the existence of functional interactions occurring between PBFs. In F113, the interaction between the four PBFs (i.e., DAPG production, ACC deamination, phosphate solubilization, and NO production) may happen at a regulatory or metabolic level and could result, when combined all together, in enhanced plant growth. Following the additive hypothesis, we predict that the four co-occurring PBFs exert additive beneficial effects on the development of plant root-system architecture (RSA) and on the overall growth of plants by *P. kilonensis* F113. To test this hypothesis, we used a genetic approach and constructed five mutant strains: four deleted for each PBF and one with deletion of all four PBFs. These five mutants were used i) to analyze the functional relationships between F113 plant-beneficial activities and ii) to determine if they are all equally contributing to the plant beneficial effects of F113 on *Arabidopsis thaliana* in in vitro conditions and on maize (*Zea mays* L.) in a soil-pot experiment.

RESULTS

In vitro, most *P. kilonensis* F113 PBFs operate independently of each other.

The existence of functional interactions between the four co-occurring PBFs of *P. kilonensis* F113 was first tested in vitro and in absence of plants. In the wild-type F113 strain (WT) and in each various mutant strains (i.e., $\Delta acdS$, $\Delta phlD$, $\Delta nirS$, Δgcd and the quadruple mutant strain), we measured the activity of ACC deamination, phosphate solubilization, nitrite reduction and DAPG synthesis. As expected, the deamination of ACC into α -ketobutyrate was impaired in the $\Delta acdS$ and quadruple mutants (Fig. 1A). *AcdS* activity was weakly induced in Δgcd and $\Delta nirS$ compared with F113 (1.7-fold and 1.3-fold, respectively) and not significantly affected in the $\Delta phlD$ mutant (Fig. 1A). Moreover, the insoluble phosphate was no longer solubilized in the Δgcd and quadruple mutants (Fig. 1B). Furthermore, the glucose dehydrogenase *Gcd* activity was weakly but significantly decreased (1.7-fold) in the $\Delta phlD$ mutant but remained identical to the one of F113 in $\Delta nirS$ and $\Delta acdS$ (Fig. 1B). The $\Delta nirS$ and the quadruple mutants were unable to reduce nitrite (and consequently to produce NO) (Fig. 1C). The *NirS* activity in the $\Delta acdS$ or Δgcd or $\Delta phlD$ strains was identical to that of the F113 strain (Fig. 1C). As expected, there was no longer DAPG synthesis in the $\Delta phlD$ and quadruple mutants (Fig. 1D). Interestingly, there was a strong accumulation of DAPG in the Δgcd (82 times more than F113). By contrast the $\Delta acdS$ and $\Delta nirS$ strains produced as much DAPG as did F113 (Fig. 1D). Thus, the main functional interaction that occurs in vitro between all four different PBFs is between the cellular pathways containing the glucose dehydrogenase *Gcd* and the polyketide synthase *PhlD*, in which both are involved in the control of DAPG biosynthesis in *P. kilonensis* F113.

To further study the interaction between DAPG synthesis and the glucose dehydrogenase, the DAPG production and phosphate solubilization were compared between all double and triple deletion mutants (Supplementary Fig. S1). On one hand, no phosphate solubilization was observed in all *gcd* double

Fig. 1. Effect of the deletion of F113 plant beneficial functions (PBFs) on each PBF activity of the strain. **A**, 1-aminocyclopropane-1-carboxylate deaminase activity. **B**, Phosphate solubilization assay. **C**, N_2O quantification. **D**, 2,4-diacetylphloroglucinol (DAPG) quantification. ND = nondetected. Statistical differences between strains for each tested PBF are indicated with letters (analysis of variance and Tukey's honestly significant difference test, $P < 0.05$). Absence of letters indicates that differences were not significant.

and triple mutants. It is also significantly reduced in the $\Delta phlD\Delta acdS$ (1.5-fold compared with F113), $\Delta phlD\Delta nirS$ (1.3-fold compared with F113), and $\Delta phlD\Delta acdS\Delta nirS$ (1.7-fold compared with F113) mutants but in similar way than in the single $\Delta phlD$ mutant (1.7-fold compared with F113). Thus, apart from the small positive impact of DAPG production on the solubilization of phosphate, there is no other function that interacts with the latter. On the other hand, DAPG production is impaired in all $phlD$ double and triple mutants but is unaffected in a $\Delta acdS\Delta nirS$ mutant. Compared with the increase observed in a single gcd mutant (82 times higher than F113), DAPG production is much more increased in a $\Delta acdS\Delta gcd$ mutant (226 times higher than F113) but reduced in the $\Delta nirS\Delta gcd$ (30 times higher than F113) and $\Delta acdS\Delta gcd\Delta nirS$ mutants (26 times higher than F113). These results show that the cellular network involving the glucose dehydrogenase Gcd and the polyketide synthase PhlD is influenced negatively by $acdS$ and positively by $nirS$.

Deletion of PBF genes does not affect the fitness of *P. kilonensis* F113 in vitro but impairs its rhizosphere soil competitiveness.

When a PGPR does not enhance plant growth, it can be due to either the absence of a particular PBF in that strain, the poor expression of the PBF gene in the rhizosphere, or to a reduced rhizosphere competence. To assess if any of the tested PBFs can affect rhizosphere competence, we measured the growth of the WT strain and of every single and quadruple mutant strain in vitro and in vivo. In liquid culture, bacterial growth can be limited by the time required to divide (characterized by the growth rate) and the size of the cell population in the stationary phase (characterized by the final optical density [OD] reached in the liquid culture).

The growth rate of the quadruple mutant is higher than that of the WT or any given single mutant strain (Fig. 2; Supplementary Table S1). The growth rates of all simple mutants are similar to that of the WT (Fig. 2). In terms of final OD reached in stationary phase, the quadruple and the $\Delta phlD$ mutants reached the highest OD in the stationary growth phase compared with any of the single mutants and with the WT strain (Fig. 2). Since neither the $\Delta phlD$ nor the quadruple mutant synthesize DAPG in vitro (Fig. 1D), these results suggest that the production of DAPG might become toxic to F113 cells, at the stationary phase, in the tested conditions. To test that

hypothesis, the growth of F113, the $\Delta phlD$ mutant, and its complemented mutant was evaluated in the presence or absence of DAPG (Supplementary Fig. S2). First, the complemented $\Delta phlD$ mutant had a growth kinetic curve similar to that of F113. Second, the addition of DAPG reduced significantly the OD in the stationary growth stage of F113 (starting from 0.1 μg of DAPG per microliter). Third, the growth of $\Delta phlD$ was slowed down in the exponential phase and was reduced in the stationary growth phase by the addition of DAPG at 0.5 and 1 μg μL^{-1} . Fourth, the addition of DAPG (at 0.5 and 1 μg μL^{-1}) slowed down the exponential growth stage of the complemented $\Delta phlD$ mutant, but the OD reached in the stationary growth stage was quite similar to that of the complemented $\Delta phlD$ mutant and of F113. Thus, when DAPG concentration reaches 0.5 μg μL^{-1} , it negatively affects the growth of F113. Although the gcd mutant produced much more DAPG than the WT (and more than approximately 0.51 μg μL^{-1}), its growth is not affected. In any case, none of the simple and quadruple mutants have major deleterious effects on F113 cell growth allowing us to analyze their role during the interaction of F113 with plant.

To monitor the contribution of PBF genes in the fitness of F113 in maize rhizosphere, F113 and mutants were inoculated on maize seeds grown in natural soil, in the presence of the indigenous microbial communities. The same inoculation level of 5×10^6 bacteria per seed was used for each strain. F113 cell population was stable up to 8 days postinoculation (dpi) but decreased at 20 dpi (Fig. 3). At 8 dpi, all mutant strains were found to be less abundant than F113 in the rhizosphere, the $\Delta phlD$ and quadruple mutants being the least numerous (Fig. 3). At 20 dpi, there was no significant difference in rhizosphere colonization between F113 and the $\Delta phlD$ and Δgcd mutants, whereas $\Delta acdS$, $\Delta nirS$, and the quadruple mutants were less abundant than F113 (Fig. 3). Thus, all PBFs contribute to F113 rhizosphere competence but to various extents. In particular, $phlD$ is relevant for root colonization for up to 8 days and $acdS$ and $nirS$ genes are important for ensuring effective maize colonization over 20 days.

The *acdS*, *nirS*, and *gcd* genes affect maize growth.

F113 and PBF mutants were inoculated on maize seeds grown in pots filled with soil. Twenty days after, shoot and root architecture traits were measured, and the profiles obtained for each strain were compared using hierarchical clustering analyses

Fig. 2. Growth curve of *Pseudomonas kilonensis* F113 and its plant beneficial function mutants in King's B broth at 28°C. The data represent the means of five replicate cultures (standard errors were not presented but were lower than 10%). The experiment was done three times independently.

(HCA). Compared with the noninoculated condition, F113 significantly enhanced root growth (with a 50% increase of total root length and 46% increase of total root surface) (Supplementary Fig. S2) and shoot growth (with an enhancement of the total leaf surface of 44%) (Supplementary Table S3; Supplementary Fig. S3).

When comparing the inoculation effects of mutants to that of F113 on maize by HCA, two clusters were formed, F113, $\Delta phlD$, and Δgcd were grouped in one and $\Delta acdS$, $\Delta nirS$, and the quadruple mutant in the other (Fig. 4). In the first cluster, all the plant parameters appear evenly affected during inoculation, giving rise to a shape like a circle similar to the one obtained with F113. It suggests that there are either no differences or minor differences between F113 and the $\Delta phlD$ and Δgcd mutants. Consistent with that, there are no significant differences between F113 and $\Delta phlD$ for all plant parameters assessed, while the inoculation with Δgcd reduced significantly maize root length and surface with, respectively, 26 and 20% decrease compared with F113.

In the second cluster, the plant parameters appear to be unevenly affected, giving rise to an unsymmetrical shape. The three strains belonging to that cluster (i.e., $\Delta acdS$, $\Delta nirS$, and the quadruple mutant) have a similar impact on maize development and growth. Contrasting with the strains of the first cluster, the inoculation of $\Delta acdS$, $\Delta nirS$, and the quadruple mutant impacted the RSA significantly compared with that of F113, by reducing significantly the root length (–64, –59, and –71%, respectively), the root surface (–50, –50, and –57%, respectively), the number of root tips (respectively –62, –45, and –71%) and the root fresh weight (respectively, –31, –38, and –42%). Thus, the RSA modifications triggered by F113 on maize were lost when $\Delta acdS$, $\Delta nirS$ or the quadruple mutants were inoculated.

In conclusion, F113 PBF genes and, in particular, *acdS* and *nirS* appear to alter mostly maize root parameters but not shoot parameters.

F113 PBFs differently affect *A. thaliana* root hair elongation and primary root growth in gnotobiotic agar conditions.

To further investigate F113 PBF impact on roots, we turned to in vitro experiments, as they allow a careful monitoring of F113 effects on root development or root hairs. Because maize is a large plant, with a root system that is likely to be too large to be evaluated in vitro, we turned instead to *Arabidopsis*, as its root development shares some similarities with that of maize (Atkinson et al. 2014; Hochholdinger and Zimmermann 2008).

We first verified the effect of F113 inoculation and that of the various mutant strains on *Arabidopsis* primary root growth. F113 inoculation inhibited significantly (twofold) the primary root length of *A. thaliana* (Fig. 5A). When *A. thaliana* Col-0 was inoculated with $\Delta phlD$, a smaller reduction of the primary root growth (1.5-fold) compared with F113 was observed. However, the difference with F113 was not significant, implying that DAPG production only weakly contributed to the primary root length inhibition triggered by the WT strain (Fig. 5A). Contrarily, primary root length inhibition was lost when $\Delta acdS$, Δgcd , $\Delta nirS$, or the quadruple mutants were inoculated (Fig. 5A). Therefore, *acdS*, *gcd*, and *nirS* independently contribute to *Arabidopsis* primary root growth inhibition driven by F113 (Fig. 5A). Secondly, we investigated the effect of F113 and of the various mutant strains on the plant root-hair length. F113 inoculation led to significantly longer root hairs (Fig. 5B). Inoculation with the quadruple mutant generated the same plant phenotype as the noninoculated condition, suggesting that at least one of the deleted PBFs was responsible of enhanced root hair length (Fig. 5B). However, compared with F113 WT-inoculated plants, the average root-hair lengths were significantly reduced by 20% in $\Delta phlD$ -, 14% in $\Delta acdS$ -, 21% in Δgcd -inoculated plants, and up 41% in $\Delta nirS$ -inoculated plants (Fig. 5B). Therefore, none of F113 PBF functions is solely controlling root-hair elongation. Because the activation of the plant ethylene signaling pathway results in root-growth inhibition

Fig. 3. Comparison of maize root colonization levels between *Pseudomonas kilonensis* F113 and its plant beneficial function mutants. The gray bar corresponds to root colonization at the moment of the seed inoculation (day 0), black at 8 days postinoculation (dpi), and white at 20 dpi. Statistical differences across the time are represented with upper case letters A to C. Statistical differences between strains at a given time are represented with lower case letters a to c. The dotted line corresponds to the quantitative polymerase chain reaction detection limit.

and root-hair elongation (Pitts et al. 1998; Swarup et al. 2007) and is also an indicator of the activation of a defense-related mechanism (Broekgaarden et al. 2015), we looked at the effects of F113 and of the various mutant strains on plant ethylene emission by photoacoustic infrared spectrophotometry (Cristescu et al. 2013). Plant ethylene emissions increased significantly when F113 (1.5 fold) and $\Delta acdS$ (twofold) were inoculated compared with the noninoculated plant. The plant ethylene emission produced by $\Delta phlD$, Δgcd , $\Delta nirS$, and the quadruple mutant did not significantly differ from that of the noninoculated condition (Fig. 6). In conclusion, all F113 PBFs are contributing to the production of ethylene by *Arabidopsis*; but in a distinct manner, $acdS$ is reducing *Arabidopsis* ethylene content whereas $phlD$, gcd , and $nirS$ are increasing it.

DISCUSSION

In this work, we show that interactions between PBFs might be different when bacteria are growing in vitro or in contact with a plant, while functional overlapping between PBFs may occur by affecting the same plant pathway. It highlights that inactivation of one PBF gene can affect the activity of other ones and that all PBFs harbored by PGPR must be taken into account when analyzing the plant response effects of PGPR inoculation.

Functional interactions between co-occurring PBFs in *P. kilonensis* F113.

In vitro, the main functional interaction occurring in F113 cells between the four studied PBFs is between phosphate solubilization and biosynthesis of DAPG. We showed that the glucose dehydrogenase activity (Gcd) is repressing DAPG

biosynthesis in F113 (Fig. 1D), confirming observations formerly made in *Pseudomonas protegens* CHA0 (de Werra et al. 2009). In cells containing a functional Gcd, glucose-6P is transformed into gluconic acid, which is exported out of the cells, leading to medium acidification and phosphate solubilization (Stella and Halimi 2015). When gcd is deleted, a higher quantity of glucose-6P could be provided to the glycolysis, resulting, on the one hand, in increased ATP production by the cell and, on the other hand, in a higher amount of pyruvate that could then be incorporated to the tricarboxylic acid cycle to produce a higher amount of acetyl CoA, the precursor of DAPG (Bangera and Thomashow 1999; Clifford et al. 2016) (Supplementary Fig. S4). Interestingly, a metabolic interaction involving Gcd with another antimicrobial compound (i.e., pyoluteorin) has been evidenced in *P. protegens* CHA0 (de Werra et al. 2009).

DAPG was shown to act as an intracellular messenger controlling transcription of different PBF genes, such as pyoluteorin production in *P. protegens* CHA0 and Pf-5 (Clifford et al. 2016; de Werra et al. 2011; Kidarsa et al. 2011; Schnider-Keel et al. 2000), or as an intercellular messenger inducing PBFs in other PGPR, as in auxin production (Combes-Meynet et al. 2011). Therefore, we hypothesize that the Δgcd mutant strain could also show some specific modification at the transcriptional level of the expression of other PBF-encoding genes, resulting in potential functional compensation.

Distinct roles for F113 PBFs during colonization.

The functional importance of F113 PBF functions and its competitiveness against the native soil populations of rhizobacteria was tested on maize plants growing in pots filled with nonsterile soil, a setup close to natural conditions. For this experiment, we selected maize as a model because it is frequently grown in fields where the soil we used had been

Fig. 4. Comparison of the effects of *Pseudomonas kilonensis* F113 plant beneficial function mutants on maize growth by hierarchical clustering. For each plant parameter, ratios between data obtained with mutants and with F113 were calculated and expressed as percentages. Root- and shoot-measured parameters are colored, respectively, in brown and green on the star plot. LS#1, surface of leaf number 1 (same nomenclature for leaf 2 and 3); LSTot, total leaf surface; Chl, chlorophyll content in leaf number 3; FSM, fresh shoot mass; DSM, dry shoot mass; L, total root length; S, total root surface; D, root diameter; V, root volume; T, root tips; FRM, fresh root mass; DRM, dry root mass; R/S represents the ratio between the dry root biomass and the dry shoot biomass. Raw data were processed through hierarchical clustering analysis, using the Euclidean model and unweighted pair group method with arithmetic means as agglomeration method.

collected, suggesting that this soil is enriched in a maize-specific rhizosphere microbiota that is ideal to compare the rhizosphere competitiveness of a mutant vs. a wild-type strain. In addition, maize plantlets have a large root system, allowing an easy quantification of the colonization process.

Root secretion of organic substances is essential for the rhizosphere microbiota to thrive (Haichar et al. 2016). Since the root exudates are composed of organic molecules, the origin of which can be traced back to photosynthetic activity of the plant, it is important that the two timepoints selected to sample the maize root rhizosphere will not differ in the physiological state of the plant. We sampled maize roots 8 and 20 days after sowing. In each case, we found the maize seedlings are already performing photosynthesis and may release root exudate whose composition is not drastically dissimilar. Consistent with this hypothesis, the composition in secondary metabolites present in root exudates from five distinct maize inbred lines is less different between 8 and 10 days than it is between 3 and 5 days postgermination (V. Walker and C. Prigent-Combaret *unpublished results*).

At 8 dpi, there are less $\Delta phlD$ mutant bacteria in the maize root rhizosphere than F113 while, at 20 dpi, the $\Delta phlD$ mutant strain is equally abundant as the WT (Fig. 3). This suggests that DAPG is important at the beginning of the colonization

process. DAPG is a well-known antimicrobial compound secreted by F113 (Cronin et al. 1997a and b). Its secretion could limit the presence of competitor rhizobacteria or fungi in vivo, hence favoring an exclusive interaction between F113 and the host roots. Curiously, the Δgcd mutant strain is equally abundant as the WT strain at both timepoints (Fig. 3). Yet, the Δgcd mutant strain produces three times more DAPG in vitro than the WT strain (Fig. 1). Enhanced DAPG production may trigger some compensation mechanisms in the Δgcd mutant strain, allowing it to colonize maize roots as efficiently as the WT.

At 20 dpi, the colonization of the $\Delta nirS$, $\Delta acdS$, and the quadruple mutant is less efficient than that of F113, Δgcd , and $\Delta phlD$ (Fig. 3). $acdS^+$ strains are catabolizing ACC exuded by plant roots in α -ketobutyric acid (carbon source) and NH_4^+ (nitrogen source) (Glick et al. 1998). $acdS$ deletion can reduce the ability of F113 to fulfill its nutrient requirements in the rhizosphere, and thus, its fitness and root colonization ability. $NirS$ is allowing bacteria of the *Pseudomonas* genus, a strictly respiring genus, to respire nitrite in the microaerophilic or anaerobic conditions that are frequently encountered in the rhizosphere (Højberg et al. 1999). Thus, the loss of nitrogen-respiring capacity may explain the lower root colonization ability of the $\Delta nirS$ mutant that was previously observed (Ghiglione et al. 2002) as well as in our condition (Fig. 3). Since both $NirS$ and $acdS$ are

Fig. 5. Effects of the inoculation of *Pseudomonas kilonensis* F113 and its plant beneficial function mutants on the length of **A**, *Arabidopsis* primary roots and **B**, root hairs. NI = noninoculated condition. Conditions harboring the same letter were not significantly different (analysis of variance and Tukey's honestly significant difference test, $P < 0.05$).

important for the colonization at 20 dpi, one could have expected that in the quadruple mutant strain, the effects of the two mutations would add up, resulting in a strain with very poor ability to colonize at 20 dpi. Surprisingly, this is not the case (Fig. 3). This suggests the existence of potential epistatic interactions between the two genes *nirS* and *acdS* or with one or both *gcd* and *phlD* that our in vitro analysis failed to unravel (Fig. 1). It also might be that plant exudates or microbe-microbe interactions in the rhizosphere result in activation of other genes that compensate for the lack of *nirS* and *acdS*.

The maize colonization process by F113 has a significant impact on plant root and shoot growth.

To test if these different colonization patterns could affect maize growth in vivo, we measured 15 different physiological parameters (eight for shoots and seven for roots). To get a broad view of the effect of F113 on maize plants, we have used a hierarchical clustering analysis (HCA). Instead of focusing on each parameter, HCA considers parameter patterns that can be compared between the mutant and the WT strains (Fig. 4). The inoculation of maize with the $\Delta phlD$ or Δgcd strains resulted in a pattern similar to that of F113 (Fig. 4). Since *phlD* appears to play a role for maize root colonization at 8 dpi (Fig. 3), it suggests that F113 colonization of maize roots after 8 dpi is more important for the effects on maize growth than up to 8 dpi. Consistent with that interpretation, the inoculation of maize roots with the $\Delta nirS$ or $\Delta acdS$ or the quadruple mutant strain (all these genes are important for colonization at 20 dpi [Fig. 3]) results in a pattern different from that of the WT (Fig. 4).

Contrasting with F113, inoculation with $\Delta acdS$ or $\Delta nirS$ or the quadruple mutant resulted in a decrease in total root length, total root surface, and number of root tips and, at the same time, an increase in root diameter (Fig. 4). This indicates that *acdS* and *nirS*, two genes that are important for the fitness of F113, are also important for root growth promotion. How they could be beneficial to root maize growth remains unclear. For example, it could be a direct interference with the plant ethylene pathway for *AcdS* or with the plant NO pathways for *NirS*. At the level of the shoot, maize plants inoculated with $\Delta acdS$ or $\Delta nirS$ or the quadruple mutant show a weak decrease in leaf surfaces as well as in chlorophyll content, compared with F113 (Fig. 4). While the leaf surface of maize plants inoculated with

$\Delta acdS$, $\Delta nirS$, or the quadruple mutant are slightly higher than in the noninoculated plants, the chlorophyll content is lower. As chlorophyll fluorescence is reduced in stressed plants (Bolhr-Nordenkamp and quist 1993), this result could suggest that maize plants inoculated with $\Delta acdS$ or $\Delta nirS$ or the quadruple mutant are stressed, whereas F113 may alleviate plant stress. Taken together, the PBF functions that contribute to the long-term fitness of F113 are also the ones that have the strongest effects on the growth of maize plants.

Relative contribution of PBFs on *A. thaliana* root architecture.

To further study the contribution of the F113 various PBFs on root development, we performed in vitro experiments with plants. We have used *Arabidopsis* as a model plant because it is small, grows fast, has a relatively simple root development program (tap root), and its root hairs are very easily observable. In addition, maize and *Arabidopsis* root development programs share some similarities (Atkinson et al. 2014; Hochholdinger and Zimmermann 2008). Finally, F113, which was initially isolated from sugar beet roots, is known to colonize efficiently both monocotyledons such as wheat (Couillerot et al. 2011; De La Fuente et al. 2006) and maize (Couillerot et al. 2013; Von Felten et al. 2010) but, also, dicotyledonous crops such as alfalfa (Snchez-Contreras et al. 2002; Villacieros et al. 2003) or tomato (de Weert et al. 2002).

In vitro F113 inoculation resulted in *Arabidopsis* primary root growth inhibition and strong promotion of root hair elongation (Fig 5A and B). By contrast, the quadruple mutant showed no root architecture difference compared with the noninoculated *Arabidopsis*, suggesting that at least one F113 PBF or a combination of PBFs may trigger root architecture modifications (Fig. 5). Several plant hormones, such as auxin (controlling cell division and elongation) (Spaepen and Vanderleyden 2011) or ethylene (controlling root cell elongation) (Jung and McCouch 2013; Street et al. 2015; Swarup et al. 2007), control primary root elongation. However, F113 does not produce auxinic compounds (J. Vacheron, D. Muller, and C. Prigent-Combaret unpublished results) but, instead, produces DAPG (Fig. 1). One possibility is that DAPG may have an effect on the plant root system. Consistent with this, when tomato seedlings are grown in vitro and in the presence of 10 μ M DAPG, their

Fig. 6. Plant ethylene emission in response to the inoculation of *Arabidopsis* plantlets with *Pseudomonas kilonensis* F113 and its plant beneficial function mutants. Plant ethylene emission was calculated related to the plant ethylene emission for the noninoculated condition (NI). Conditions harboring the same letter were not significantly different (analysis of variance and Tukey's honestly significant difference test, $P < 0.05$).

primary root growth is inhibited (Brazelton et al. 2008). However, the $\Delta phlD$ mutant (which does not produce DAPG) remains able to inhibit *Arabidopsis* primary root elongation, and the Δgcd mutant (which overproduces DAPG) does not show further reduced primary root length compared with F113 (Fig. 5). In addition, both the $\Delta phlD$ and Δgcd mutants remain able to stimulate root hair length. These results imply that, aside from DAPG, other F113 PBFs are involved in the inhibition of primary root elongation and stimulation of root hair length in *A. thaliana*. Interestingly, the loss-of-function mutation in *nirS* significantly affects the inhibition of primary root growth (Fig. 5A) and root hair elongation (Fig. 5B). This may suggest that NO, produced by F113 NirS activity, as evidenced by using the specific fluorescent probe diaminorhodamine-4M acetoxymethyl ester (J. Vacheron, D. Muller, and C. Prigent-Combaret *unpublished results*), would act as an inhibitor of primary root growth and activator of root hair elongation. Consistent with this, NO is described as a positive regulator of root hair development (Lombardo and Lamattina 2012; Lombardo et al. 2006) and a negative regulator of primary root growth (Fernández-Marcos et al. 2011).

In the absence of PGPR, plant ethylene is known to inhibit primary root length and to activate root hair elongation (Cao et al. 1999; Pitts et al. 1998). F113 moderately increased plant ethylene emission, whereas all mutants but $\Delta acdS$ shared similar plant-ethylene emission as in noninoculated conditions (Fig. 6). Individually, each of the F113 PBFs contributes positively to ethylene production by the plant during the associative symbiosis with *A. thaliana* (Fig. 6). Contrasting with the other mutants, the $\Delta acdS$ strain colonization results in higher ethylene emission by the plant (Fig. 6). Hence, the AcdS activity negatively contributes to ethylene production. In our study, this mutant strain is the only simple mutant unable to degrade in vitro the ethylene precursor ACC (Fig. 1A). Assuming that the mutant strain behaves similarly in vitro and in vivo, the lack of AcdS activity results in higher ethylene emission by the plant (Fig. 6). However, this increase is not observed with the inoculation of the quadruple mutant, showing that the other PBFs activate plant ethylene production. Thus, it confirms that F113 colonization of the root system results in modulation of ethylene emission by the plant and that AcdS is not the only F113 PBF contributing to this effect. However, it remains to be seen if this emission is greater in the root, in the shoot, or both plant organs. It also confirms that AcdS activity is actively decreasing plant ethylene emission during the associative symbiosis. This result is consistent with a long-standing hypothesis on the function of AcdS during the interaction of AcdS⁺ bacteria with plants (Glick et al. 1998). Yet, this increased emission of ethylene in the presence of the $\Delta acdS$ mutant strain does not result in a shorter primary and longer root hairs compared with the effect of the WT strain (Fig. 5A and B). This absence of stronger root responses contrasts with previous observations suggesting that an absence of AcdS activity resulted in longer root hairs (Galland et al. 2012). But *Phyllobacterium brassicacearum* STM196, *Rhizobium leguminosarum* 128C53K, and *Pseudomonas* sp. strain UW4, the strains used in that previous work, may not have the same combination of PBFs as that of F113. Therefore, one should not rule out the possibility that this absence of stronger root responses with the $\Delta acdS$ strain is specific to F113 and its combination of PBFs. Another possibility that could explain the absence of stronger root responses, despite a significant increase in ethylene emission, is that F113 $\Delta acdS$ -inoculated plants are becoming insensitive to the effect of high ethylene on root cells.

Taken together, F113 may trigger both ethylene- and NO-dependent signaling pathways on *Arabidopsis*. Contrasting with the observations made on soil pot-grown maize, all F113s

PBFs tested in vitro on *Arabidopsis* contribute to modification of the root architecture.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions.

The bacterial strains and plasmids used in this study are listed in Supplementary Table S4. *P. kilonensis* F113 WT and its mutants were incubated at 28°C in King's B broth media (Simon and Ridge 1974) supplemented, when required, with gentamycin (15 µg ml⁻¹) or kanamycin (Km) (50 µg ml⁻¹). *Escherichia coli* was incubated at 37°C in Luria Bertani broth (Bertani 1951) supplemented with gentamycin (15 µg ml⁻¹) or ampicillin (75 µg ml⁻¹) and Km (50 µg ml⁻¹), when necessary. To assess the effects of DAPG on the growth of F113 and its mutants, DAPG (Santa Cruz Biotechnology) was added in King's B broth medium at three different concentrations (0.1, 0.5, and 1 mg ml⁻¹).

Growth kinetic assays.

Bacterial growth was monitored for 24 h at 28°C by measuring OD in a Bioscreen C analyzer (Labsystems) measuring the OD₆₀₀ every 15 min. Growth kinetic assays were performed three times independently with five technical repetitions.

Construction of deletion mutants and complementation of the *phlD* deletion mutant.

To construct F113 PBF deletion mutants, about 700-pb fragments were amplified from regions upstream and downstream from the PBF genes of interest, using specific primers (Supplementary Table S5), and fragments were subcloned into pGEM-T Easy (Promega). The upstream and downstream DNA fragments were digested with AatII/MunI and SacII/SacI for $\Delta phlD$ and Δgcd , AatII/PvuII and SacII/SacI for $\Delta acdS$, PvuII/MunI and SacII/SacI for $\Delta nirS$ and were then cloned, one after another, into pCM184, on both sides of the Km resistance-encoding gene (Marx and Lidstrom 2002). All restriction enzymes were supplied by ThermoFisher Scientific. All plasmid amplifications were done using *E. coli* JM109 chemically competent cells (Promega). For validation of the correct insertion of upstream and downstream DNA fragments into pCM184, sequencing, using primers S1F/S1R and S2F/S2R, was performed. pCM184 harboring upstream and downstream regions of the plant-beneficial genes of interest were transformed into F113 by electroporation (Højberg et al. 1999). Inactivation mutants resulting from a double recombination event of upstream and downstream regions were selected on King's B Km and were screened for plant-beneficial gene deletion by polymerase chain reaction (PCR) with specific primers. Then, the plasmid CM157 containing the *cre-lox* recombinase gene was introduced into F113 Km-resistant mutants in order to remove the antibiotic marker. To check the loss of the Km cassette, mutant strains were screened for their inability to grow on King's B Km. PCR using external primers was also used to validate the cassette loss. For complementation of the *phlD* deletion mutant, the coding region of the *phlD* gene of F113 was amplified by PCR, using primers phlDCompF1 and phlDCompR1, and was subcloned into pGEM-T Easy. The *phlD* gene was extracted from pGEM-T Easy using *ApaI/SacI* digestion and was inserted into the multiple cloning site of vector pBBR1MCS-5, under control of the lac promoter, resulting in pBBR1-MCS5:*phlD*. This plasmid was introduced into the $\Delta phlD$ mutant by electroporation (Højberg et al. 1999).

DAPG quantification by high-pressure liquid chromatography (HPLC).

DAPG production was measured by HPLC, according to the method developed by Bonsall et al. (1997). Briefly, samples

were analyzed with an Agilent 1200 series HPLC (Agilent Technologies) equipped with a degasser (G132A), a quaternary pump module (G1311A), an automatic sampler (G1329A), and a diode array detector (DAD G1315B). Separation of compounds was performed at room temperature with a C18 reverse-phase column. For each sample, 20 μ l was injected and eluted at 1 ml min⁻¹, using a step-by-step gradient increasing the acetonitrile (ACN) proportion in water; the gradient started at 40% of ACN for 4 min then, rose from 40 to 64% in 7.5 min, then came to 75% at 16.5 min, and ended at 100% at 18.5 min (100% maintained for 3 min before decreasing to 40% for 5 min). Chromatograms were recorded at 270 nm (maximum of absorbance of DAPG). The chemstation Agilent software was used for integration of chromatograms, and quantitation of DAPG was done, according to a reference curve, with the chemical standard (Sigma-Aldrich). This experiment was done independently three times.

ACC deaminase activity assay.

The ACC deaminase activity of strains was performed as described by Blaha et al. (2006). The method is based on the quantification of α -ketobutyrate produced by the deamination of ACC into α -ketobutyrate by the enzyme ACC deaminase. Total protein content in the assay was measured using the BCA protein assay kit (Pierce). ACC deaminase activity was assessed three times for each strain. The data are expressed in nanomoles of ketobutyrate per hour per milligram of protein. This experiment was done independently three times.

Phosphate-solubilizing assay.

The phosphate-solubilizing ability of strains was evaluated by measuring tricalcium phosphate solubilization halos on National Botanical Research Institute's Phosphate (NBRIP) agar medium. Strains were cultured for 1 day at 28°C in King's B broth and, then, were deposited four times (5 μ l each) on NBRIP agar medium. Solubilization halos were measured after 6 days of incubation at 28°C, using ImageJ software. This experiment was done three times independently.

Denitrification activity measurement.

The denitrification activity was estimated through the potential accumulation of N₂O in the culture media, which is one step of the denitrification process. The accumulation of N₂O was measured according to Bardon et al. (2016). Briefly, 20 ml of King's B media supplemented with 20 mM of KNO₃ were placed in a plasma flask sealed by a rubber stopper. An anaerobic condition was obtained by removing air and replacing it with a He/C₂H₂ mixture (90:10, vol/vol) in order to inhibit the reduction of N₂O to N₂ and to allow N₂O accumulation. Each strain was inoculated at a final OD₆₀₀ of 0.1. N₂O emissions were measured every 2 h for 24 h, using gas chromatography coupled to a microkatharometer detector (μ GC-R3000; SRA Instruments). The assay was performed twice with two technical replicates. N₂O emission was expressed as micrograms of N-N₂O per hour per milliliter of media. In addition, the ability of F113 to produce NO intracellularly was evaluated, using the specific fluorescent probe diaminorhodamine-4M acetoxymethyl ester (Calbiochem), as previously described by Pothier et al. (2007).

Bacterial inoculum preparation.

P. kilonensis F113 WT and related deletion mutants Δ *phlD*, Δ *acdS*, Δ *gcd*, Δ *nirS*, and the quadruple mutant (Δ *phlD* Δ *acdS* Δ *gcd* Δ *nirS*) were incubated overnight at 28°C in King's B broth medium. Cell cultures were centrifuged at 5,000 $\times$ g. The pellet was washed with sterilized water and was adjusted to 10⁷ bacteria per milliliter.

Bacterial inoculation effects on *Arabidopsis* RSA.

The seeds of *Arabidopsis thaliana* ecotype Columbia 0 (Col-0) were prepared according to Mantelin et al. (2006). After 7 days, plantlets were transferred to 50 ml of new plant agar medium containing F113 or mutant strains at 10⁷ cells per milliliter in square Petri dishes (120 $\times$ 120 mm). Inoculated plantlets were transferred to a phytotron set to the following parameters: 21°C day and 17°C night temperatures, 13-h light periods, 9,500 lux. Four *Arabidopsis* plantlets were disposed per Petri dish and six plates were prepared per condition. The seven conditions tested were *A. thaliana* inoculated with F113, with the four simple mutants, with the quadruple mutant, and the nonbacterized control. This experiment was made three times independently. After 7 days, three plants per Petri dish were scanned, in order to analyze the primary root elongation and root hair length, using ImageJ software (i.e., 18 analyzed plants per condition). The relative contribution of each tested PBF in the modulation of the *Arabidopsis* root architecture was calculated as follows.

$$\text{F113} - \text{control} = \alpha$$

$$\frac{[(\text{F113} - \text{mutant})/\alpha] \times 100}{\text{contribution to F113 effect}(\%)}$$

Arabidopsis ethylene emission.

Measurements of in vivo ethylene emission by *Arabidopsis* plantlets were performed using infrared laser spectroscopy coupled to photo-acoustic detection (Cristescu et al. 2013) (ETD-300, SensorSense). Practically, we carefully poured 600 μ l of sterile plant culture medium (as described above), inoculated or not with the various F113 strains at a final density of 2 $\times$ 10⁷ cells per milliliter, in a 2-ml sterile Eppendorf tube. Once solidified, we carefully introduced two wild-type 7-day-old *Arabidopsis* plantlets and closed the lid. Lids were punched to allow for regular breathing of the plants. This system was then transferred to a phytotron (MLR-352-PE, Panasonic) set to the following parameters: 21°C day and 17°C night temperatures, a 13-h light period, 9,500 lux. No measure was made during the first 48 h, to allow plant acclimation and to recover from stress associated to the transfer. Prior ethylene emission measurement, 200 μ l of plant liquid medium was added in each tube to keep the head space humid and, therefore, protecting the plant from the dry air coming from the compressed air bottle. Then, the lids of the tubes were removed and replaced by a rubber plug. Needles were pushed through the plug to allow the headspace to be flushed to the ethylene detector by compressed air. Every 90 min, the ethylene content in the headspace was measured while the tubes remained in the phytotron. That measurement was performed during two to three consecutive days, to collect enough data on ethylene content during the day. At the end of the experiment, plant fresh weight was measured to allow data normalization. Raw results were exported into a spreadsheet, in which they were normalized and processed to obtain at least 10 different measures of ethylene content in the presence of light in each condition.

Maize inoculation experiments in nonsterile soil.

The semilate maize hybrid PR37Y15 (Pioneer Semences) was used in this study. Maize seeds were surface-disinfected. Briefly, seeds were soaked in sodium hypochlorite (3.4%) for 1 h, with agitation. Five washes were performed with sterile water. Four maize seeds were sown in nonsterile soil taken from La Côte Saint-André near Lyon, France (Vacheron et al. 2016a) and were placed in 2-dm³ pots filled with 2.0 kg of sieved soil (5 mm) with a water content of 20% (vol/vol). Moisture was

maintained at 20% during the experiment. Seeds (four per pot) were inoculated with 100 µl of bacterial inoculum solution, corresponding to 10⁶ bacteria. Ten pots per condition were used. Inoculum impact on plant growth was assessed at 20 dpi by characterizing maize RSA, using WinRhizo (Reagent Instruments). Leaf surface was measured with the WinFolia image analysis software (Reagent Instruments). Chlorophyll content was measured with a chlorophyll content meter (Hansatech Instruments model CL-01). Root and shoot fresh and dry biomasses were weighed. Ten plants were analyzed per condition.

Monitoring the bacterial maize root colonization.

The measurement of root colonization was assessed at 0, 8, and 20 dpi, taking 10 seeds for each condition at each sampling time. Inoculated seeds or plants were harvested and disposed in a 50-ml Falcon tube and were then soaked in liquid nitrogen. Seeds and root systems were lyophilized and, then, were scratched to extract DNA from the seed surface and root-adhering soil. DNA extractions from 500 mg of rhizosphere soil or seed teguments were performed using FastDNA SPIN Kit for Soil (MP Biomedicals). DNA was also extracted from 1 ml of inoculum solution, to ascertain the cell concentration of the inoculants. The seed and root bacterial colonization was assessed by real-time PCR as described by Von Felten et al. (2010). Real-time PCR was performed using the LightCycler FastStart DNA Master SYBR Green I kit and a LC-480 LightCycler (Roche Applied Science). Five nanograms of extracted DNA were used to assess the abundance of seed and root colonization by the F113 parental and mutant strains, using F113_1_for and F113_1_rev primers with a detection limit of 10⁴ cells per seed and 10⁴ cells per gram of rhizosphere (Von Felten et al. 2010). Real-time PCR quantification data were converted to gene copy number per gram of lyophilized root, as described by Couillerot et al. (2013).

Statistical analysis.

Regarding assays on each plant-beneficial function, data were log-transformed, to respect normal distribution, and variances homogeneity and a one-way analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) tests were performed to detect significant differences between strains. *Arabidopsis* root hair length data were compared, using Kruskal-Wallis tests followed by posthoc nonparametric multiple comparisons. *Arabidopsis* primary root length data were square root-transformed in order to respect variance homogeneity and normality and a one-way ANOVA coupled with Tukey's HSD tests was performed. Ethylene emission data were expressed related to noninoculated condition and were compared, using ANOVA coupled with Tukey's HSD tests. The comparison of root colonization between strains at the same sampling time was analyzed by ANOVA and Tukey's HSD tests. The comparison of root colonization across the time for each strain was analyzed by ANOVA and Fisher's least significant difference tests. The effects of strain inoculation on maize growth parameters were compared, using ANOVA and Tukey's HSD tests and were represented using star plot representation coupled to hierarchical clustering analysis performed on the raw data (distance model: Euclidean; agglomeration method used: unweighted pair group method with arithmetic means). All data were presented as mean ± standard error. All analyses were performed at *P* < 0.05, using R studio software (R Studio Team 2015).

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