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An Engineered Device for Indoleacetic Acid Production under Quorum Sensing Signals Enables *Cupriavidus pinatubonensis* JMP134 To Stimulate Plant Growth

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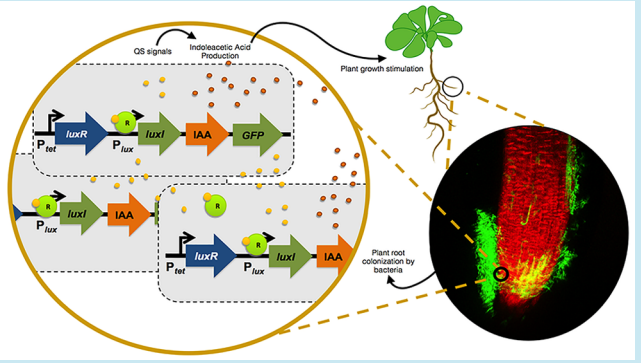
Supporting Information

ABSTRACT: The environmental effects of chemical fertilizers and pesticides have encouraged the quest for new strategies to increase crop productivity with minimal impacts on the natural medium. Plant growth promoting rhizobacteria (PGPR) can contribute to this endeavor by improving fitness through better nutrition acquisition and stress tolerance. Using the neutral (non PGPR) rhizobacterium *Cupriavidus pinatubonensis* JMP134 as the host, we engineered a regulatory forward loop that triggered the synthesis of the phytohormone indole-3-acetic acid (IAA) in a manner dependent on quorum sensing (QS) signals. Implementation of the device in JMP134 yielded synthesis of IAA in an autoregulated manner, improving the growth of the roots of inoculated *Arabidopsis thaliana*. These results not only demonstrated the value of the designed genetic module, but also validated *C. pinatubonensis* JMP134 as a suitable vehicle for agricultural applications, as it is amenable to genetic manipulations.



KEYWORDS: *Arabidopsis thaliana, Cupriavidus pinatubonensis, quorum sensing, synthetic beneficial interaction*

Microorganisms are one of the most strategic resources for overcoming the problems produced by excessive application of chemical fertilizers and pesticides in agriculture.[1] In particular, plant-growth-promoting rhizobacteria (PGPR), a well characterized group of biological fertilizers, increase the acquisition of nutrients, modify plant hormone levels, and protect the host from pathogen agents.[2−4] However, to properly generate beneficial effects on plants, a better understanding of the particular features of beneficial bacteria and their interactions with host plants and with other microorganisms is required. For example, the expression of genes involved in plant growth promotion mechanisms by PGPRs changes according to biotic and abiotic factors of the rhizosphere.[5] Yet, it is estimated that up to 90% of PGPRs inoculated in legume crops yield almost no effect, an observation explained by low viability of these bacteria in the soil.[6] For this reason, field inoculation with different PGPR strains in plants of agronomic interest often shows discrepancies with *in vitro* results, evidenced by a decrease in root colonization, as well as inconsistent effects on plant growth, calling into question the effectiveness of PGPR applications in agriculture.[5,7] The use of PGPR members with different plant growth promotion strategies, a PGPR consortium, is a common approach to generate synergistic effects in inoculated plants.[5,8] However, interactions between inoculated bacterial strains may yield unexpected effects due to competition or crosstalk, affecting plant colonization, bacterial gene expression, and consequently, beneficial effects. Among the important bacterial traits for plant colonization are motility and quorum sensing (QS), which confer competitive advantages and promote survival in a particular niche.[2,9] In this context, QS has an important role in host colonization because it regulates genes involved in cooperative traits.[10,11] Using the QS system,

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bacteria are able to sense their population via self-inducing molecules, which activate a response only when population density, and therefore the signal molecule in the extracellular environment, is high.[10] QS regulates numerous key processes relevant to biotechnological applications, including biofilm formation, catabolic gene expression, biosurfactant production, and exopolysaccharide synthesis.[12,13] The homoserine lactone (HSL) QS system has been used to engineer novel behaviors in bacteria with predictive gene expression response to HSL signal concentration.[14−16] QS has been also used to synchronize gene expression in a population, thereby mitigating intrinsic population variability and improving the yields in engineered strains.[16] The QS-based strategy is particularly interesting in PGPRs because their activity at plant roots is a consequence of all active cells expressing a particular gene involved in plant growth promotion. As such, if key traits are regulated by a QS system, their contribution would be observed only when the population reaches a significant, specific density, that is, when bacteria are able to colonize the plant roots.

In this study, we aimed to engineer the neutral (non-PGPR) rhizobacteria *Cupriavidus pinatubonensis* JMP134 to produce a synthetic plant growth promoting bacterium capable of coordinating the expression of genes involved in a particular beneficial effect on plant through a HSL-QS system. *C. pinatubonensis* JMP134 has distinct advantages in the rhizosphere because it can metabolize some components of plant root exudates, allowing the bacteria to colonize root surfaces.[17] We introduced a positive feedback QS circuit in *C. pinatubonensis* JMP134 to induce gene expression in a cell-density dependent manner. The population harboring the plasmid with this construct activates gene expression at a level similar to induction with HSL, as measured by GFP fluorescence. The introduced construct enables regulation of the expression of the IAA synthesis genes in strain JMP134, which is coordinated by an increase in the bacterial population density at the root level. Our study reports characterization of a synthetic QS system in *C. pinatubonensis* JMP134 and points to a promising model for biotechnological applications, as the strain can readily undergo genetic modifications and transformation and is intrinsically receptive to gene expression control by QS.

RESULTS AND DISCUSSION

C. pinatubonensis JMP134 as a Host for Synthetic QS Networks and Beneficial Plant−Bacteria Interactions. *C. pinatubonensis* JMP134 belongs to the *Burkholderiales* order and is well-characterized due to its catabolic features, especially those related to the degradation of aromatic compounds.[18] Although several HSL based quorum-sensing systems have been identified in *Burkholderiales* strains, none of them have been detected in strain JMP134.[18,19] This feature makes this strain a good host in which to construct and apply different genetic circuits controlled by synthetic HSL-QS systems. We chose the well-characterized LuxI/LuxR-type QS system because of its simplicity, standardization, and frequent application in synthetic biology.[15,16,20,21] Analysis of the strain's genome revealed no ORFs with significant identities to putative *luxI* and *luxR* genes of the *Vibrio fischeri* QS system. We also confirmed the lack of a functional HSL QS system in strain JMP134, as no HSL production was detected by thin-layer chromatography in supernatants from stationary growth phase (Supplementary Figure S1). This strain is also particularly interesting to test for improvement of the plant-bacteria interaction because it colonizes plant rhizosphere and degrades plant root exudates components.[17,22] Besides its ability to grow and colonize plant roots without any adverse effect for host, this bacterium does not possess putative genes involved in the promotion of plant growth, and its interactions with plants are considered to be neutral.[17] For these reasons, we chose the strain JMP134 to engineer a synthetic beneficial plant-bacteria interaction.

Characterization of a Lux-like QS System in _C. pinatubonensis_ JMP134 To Create an Autoinducible Regulation of Gene Expression. A genetic circuit with a positive feedback for cell density-dependent control of gene expression was constructed with the QS system from marine bacterium *V. fischeri*.[10,23,24] *luxI* and *luxR* genes and P_{lux} promoter, identified as key elements of the QS system in this bacterium, have been used previously to construct artificial cell-to-cell communication systems.[14,24−27] We constructed a genetic circuit able to produce and respond to *N*-(3-oxohexanoyl)-L-homoserine lactone, 3-oxo-C6-HSL, increasing the transcription of the target gene, GFP (Figure 1A). The strain harboring this circuit was called P_{lux}-*luxI*-GFP (Figure 1A), and was able to synthesize 3-oxo-C6-HSL (Supplementary Figure S1). We also constructed a control strain harboring a genetic circuit with inducible expression of GFP by 3-oxo-C6-HSL; this strain was called P_{lux}-GFP. To characterize the behavior of these circuits, we measured GFP expression in each strain using a fluorescence plate reader and a flow cytometer. We calculated the output of the circuits in strain JMP134 using the relative measurements of each circuit normalized by the fluorescence of wild type strain JMP134 with the pSEVA-GFP plasmid but without the promoter (Figure 1B). The dynamic response of the P_{lux}-*luxI*-GFP strain suggested a cell density-dependent curve of GFP production after 10 h of growth which corresponds to an optical density at 600 nm ($OD6_{00\ nm}$) of 0.23, indicating a positive-feedback loop in the synthesis of 3-oxo-C6-HSL (Figure 1B,C). This response was similar to the fluorescence curve observed in the strain P_{lux}-GFP in the presence of 1 μM of 3-oxo-C6-HSL between 9 and 17 h of growth. A delay was observed in the growth curve of strain P_{lux}-GFP induced with 3-oxo-C6-HSL compared with the non-induced strain (Figure 1C). The growth curve also showed an earlier stationary phase attained by the strain P_{lux}-*luxI*-GFP compared to the strain P_{lux}-GFP, induced or not with 3-oxo-C6-HSL. This phenomenon might reflect the metabolic burden of the device expression in the growth of the strain JMP134.[28] We did not observe any GFP production from the control strain in the absence of 3-oxo-C6-HSL (Figure 1B). Likewise, the strain JMP134 harboring a control circuit named P_{lux}-*rhlI*-GFP with a constitutive expression of *luxR*, and an inducible expression of *rhlI* (for *N*-butyryl-L-homoserine lactone synthase and C4-HSL synthesis) and GFP genes by 3-oxo-C6-HSL, was unable to produce GFP without the induction of exogenous 3-oxo-C6-HSL (Supplementary Figure S2), which corroborated the device synthesis of 3-oxo-C6-HSL and its orthogonality in the strain JMP134.

In general, the signaling mediated by HSL and QS activation in the population is attributed principally to the number of cells and the volume available for growth. Both factors can vary independently and affect the final cell density at which the positive feedback loop is activated.[12] On the basis of this criterion we analyzed the device induction in the strains P_{lux}-GFP and P_{lux}-*luxI*-GFP, grown in a flask after its synchronization, induced or not with 1 μM of 3-oxo-C6-HSL, by flow

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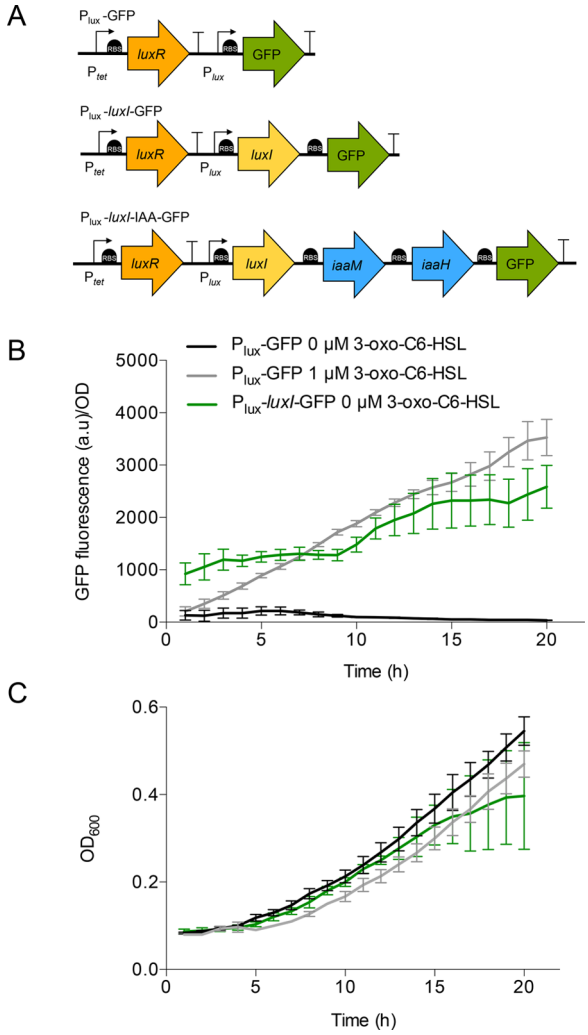

Figure 1. Engineering a device for positive feedback gene induction using a quorum sensing (QS) system in *Cupriavidus pinatubonensis* JMP134. (A) Genetic scheme of QS operon constructions harbored by different strain JMP134 derivatives; P_{lux}-GFP, P_{lux}-*luxI*-GFP, and P_{lux}-*luxI*-IAA-GFP. Parts of the device design: P (promoter), RBS (ribosome binding site), arrow (gene), T (terminator), for details sequences see Table 1. GFP fluorescence levels over time (B) and growth ($OD_{600\ nm}$) (C), in response to 0 or 1 μM of 3-oxo-C6 homoserine lactone (HSL) in strain P_{lux}-GFP compared to the autoinduction curve from strain P_{lux}-*luxI*-GFP without HSL. Each data point was normalized by $OD_{600\ nm}$ in panel B. Each fluorescence level is accompanied by an additional bar representing the standard deviation of the mean for that particular data set.

cytometry. The analysis showed a clear difference in the signal of GFP produced by the strain P_{lux}-*luxI*-GFP, after reaching an $OD_{600\ nm}$ of 0.3 (Figure 2A). Those strains induced with 1 μM 3-oxo-C6-HSL produced 20 and 60 times more GFP signal at $OD_{600\ nm}$ 0.1 than the noninduced strains P_{lux}-*luxI*-GFP and P_{lux}-GFP, respectively (Figure 2A). The device expression in the strain P_{lux}-*luxI*-GFP was slightly leaky, producing differences in the signal of GFP in the population. The positive feedback loops from diverse bacteria have this behavior by nature, which is desirable depending of the environment condition.[29−31] Here, the strain P_{lux}-*luxI*-GFP showed a complete synchronization in GFP signal, after reaching an $OD_{600\ nm}$ 0.4 (Figure 2A). It means that in these growth conditions, the population of the strain P_{lux}-*luxI*-GFP was able to produce a sufficient amount of autoinducer to synchronize the entire population to produce GFP signal at the same level. We also confirmed this phenomenon observing the levels of expression of homoserine lactone synthase protein LuxI by SDS-PAGE, which was detectable only after $OD_{600\ nm}$ 0.6 (Supplementary Figure S4). The regulation of the QS system in environments include a variety of factors that affect principally the autoinducer molecule and the signaling process.[32] In general it is assumed that a synchronous expression of genes is due to a density-dependent regulatory process in the population.[33] But now, the spatial distribution of cells is also considered, giving importance to the diffusion sensing.[34] Therefore, the genesis, diffusion, interception, as well as degradation produced by HSL-degradation enzymes (quorum quenching (QQ) enzymes) are involved in the complex regulatory network which influences the synthesis and accumulation of autoinducer signals within bacterial cells.[12,32,35,36] Here we obtained a simplified autoinducer device able to synchronize the gene expression of GFP in the strain JMP134 by a positive feedback loop after an $OD_{600\ nm}$ 0.4, corresponding to a middle exponential phase of growth. This type of autoinduction can be useful in complex environments, as the rhizosphere, in which the spatial distribution of bacteria is not homogeneous and small clusters of cells could be synchronized readily although the temporal changes in the diffusion rate and nutrient availability as it has been described by Hense et al., 2007.[34]

QS-Dependent IAA Biosynthesis in *C. pinatubonensis* JMP134 Creates a Synthetic Beneficial Interaction with *A. thaliana*. Plant beneficial bacteria promote plant growth through a variety of mechanisms.[22,37−40] One such mechanism is the synthesis of auxins, in particular the indole-3-acetic acid (IAA), which is involved in root proliferation.[41−46] In bacteria, IAA is synthesized from tryptophan, which is found at different concentrations in root exudates depending on the plant genotype.[5,47] Because we were interested in building synthetic beneficial plant-bacteria interaction with an autoinducible expression of beneficial target genes, we constructed an IAA QS-dependent biosynthesis device in strain JMP134. We used 2-tryptophan monooxygenase (*iaaM* gene) and indole-3-acetamide hydrolase (*iaaH* gene) enzymes from the L-tryptophan-dependent indole-3-acetamide biosynthesis pathway from *Pseudomonas savastanoi*.[48] We assembled both genes downstream from the P_{lux}-*luxI* sequence in the plasmid pSEVA-P_{lux}-*luxI*, creating the new plasmid P_{lux}-*luxI*-IAA-GFP (Figure 1A). The flow cytometry analysis of the strain harboring the plasmid P_{lux}-*luxI*-IAA-GFP, showed a feedback loop activation after the strain reached an $OD_{600\ nm}$ of 0.5 of growth, but only after an $OD_{600\ nm}$ of 1.0 was the GFP signal in the population completely synchronized (Figure 2B, Supplementary Figure S3). However, the GFP signal in this strain was less than those observed in the strain P_{lux}-*luxI*-GFP at $OD_{600\ nm}$ of 0.5 (Figure 2, Supplementary Figure S3 and Table 2), even in the presence of 1 μM of 3-oxo-C6-HSL, due probably to the metabolic burden produced by the expression of device genes in the growth strain. The levels of expression for LuxI, IaaM, and IaaH proteins were confirmed by a SDS-PAGE in the strain P_{lux}-*luxI*-IAA-GFP and presented a slight overexpression in the extract of protein from cultures at an $OD_{600\ nm}$ of 1.0 (Supplementary Figure S4). Similar to the strain P_{lux}-*luxI*-GFP, the strain P_{lux}-*luxI*-IAA-GFP presented an early stationary phase of growth after the device expression, compared to the curve from wild type and P_{lux}-GFP strains (Supplementary Figure S5) indicating

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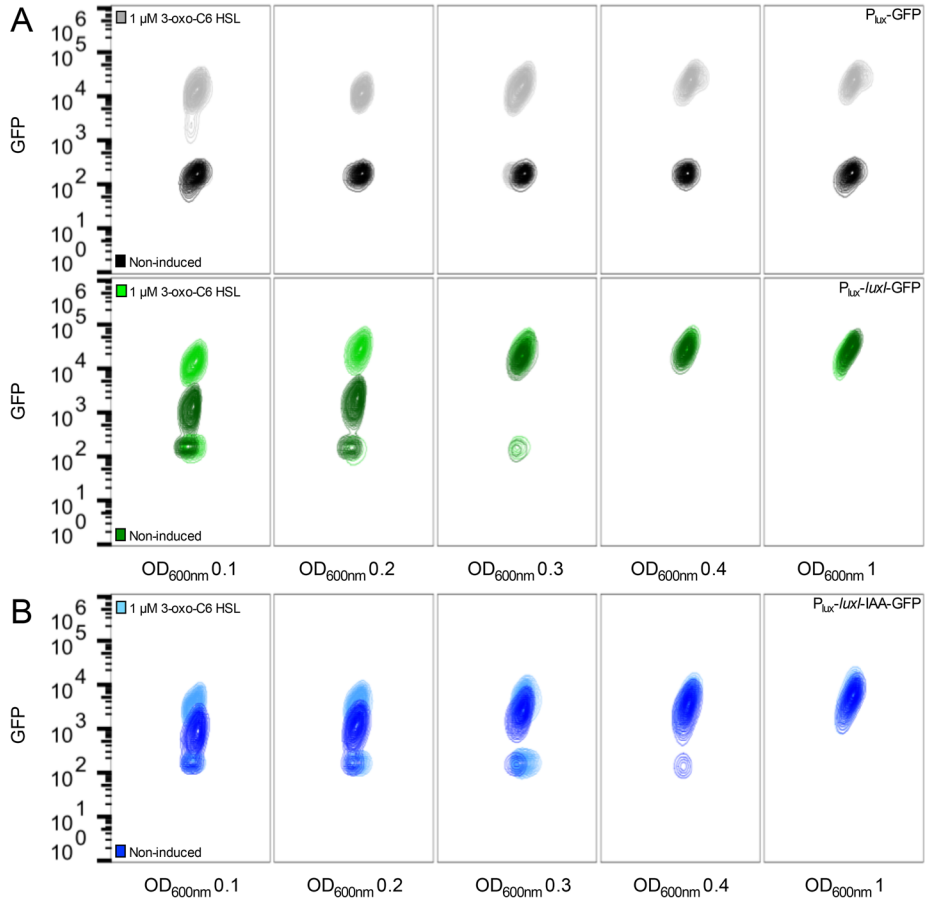

Figure 2. Device response to 3-oxo-C6-HSL induction. Flow cytometry contours showing GFP fluorescence plotted as a function of cell $OD_{600\ nm}$ of strains: P_{lux}-GFP (A, upper panel), P_{lux}-*luxI*-GFP (A, bottom panel), and P_{lux}-*luxI*-IAA-GFP (B), induced with 1 μM 3-oxo-C6-HSL or not. The contour intervals are at 2% of population. Supplementary Table T2 shows the number of events for each plot. The width of each contour represents forward scatter.

as well, the metabolic burden of the device expression in the growth of this strain.[28] Nevertheless, the synthesis of 330 μM of IAA was confirmed by high-performance liquid chromatography UV detection (HPLC-UV) 1 h after the addition of 1 mM of tryptophan as a precursor, in the strain P_{lux}-*luxI*-IAA-GFP, only after reaching an $OD_{600\ nm}$ of 0.6 of growth. No IAA synthesis was detected at an $OD_{600\ nm}$ of 0.2 in the presence of tryptophan, confirming the autoinduction of the device only at higher $OD_{600\ nm}$ (Supplementary Figure S6).

To test if the synthesis of IAA in strain JMP134 enables it to promote plant growth, we inoculated *Arabidopsis thaliana* plants with the strain P_{lux}-*luxI*-IAA-GFP. After 14 days of growth we evaluated parameters related to auxin effects in plants. Significant effects were observed in lateral root number, primary root length, fresh weight, and rosette area of inoculated plants (Figure 3A, Supplementary Figure S7 and Supplementary Table T3). As reported previously,[17] the wild type and P_{lux}-*luxI*-GFP strains were not able to modify growth of *A. thaliana* plants in any of the parameters measured above (Figure 3A, Supplementary Figure S7). It is know that small-chain (C6 and C8) HSLs do not produce any effect on root architecture of *A. thaliana* plants, as we confirmed in plants inoculated with P_{lux}-*luxI*-GFP strain.[49] A large proportion of microorganisms isolated from the rhizosphere possesses the ability to synthesize and release auxins as secondary metabolites.[50] The positive effect of bacterial IAA production on plants has been well documented in *Bradyrhizobium* and *Azospirillum*.[51,52] IAA enhances root development, facilitating nutrient uptake[3,53] as we observed in plants inoculated with strain P_{lux}-*luxI*-IAA-GFP (Figure 3A, Supplementary Figure S7). At the plant root level, IAA softens the cell wall rigidity, thereby increasing the amount of plant root exudates and providing additional nutrients to support bacterial growth.[3,54] Several aromatic compounds are present in plant root exudates which can be metabolized by aromatic ring cleavage pathways that converge in the formation of α-ketoadipate, an intermediate that can be incorporated into the central metabolism.[17,55] The use of this pathway by *C. pinatubonensis* JMP134 is important for growth at plant root surfaces and subsequent plant rhizosphere colonization.[17] To address the root colonization of P_{lux}-*luxI*-IAA-GFP and the *in vivo* expression of *luxI*-IAA-GFP synthetic operon, we evaluated GFP expression in the roots of a transgenic line of *A. thaliana*. We created an *in vivo* red fluorescent marker for plants that enables an experimental design using GFP-based markers in bacteria. This reporter labels plant membrane through a membrane-tagged protein LTI6b At3g05890[56,57] fused to the red fluorescent protein mKate2.[58] We observed a homogeneous pattern of colonization at the surface of roots by strain P_{lux}-*luxI*-IAA-GFP, principally at the root tip and surrounding hair roots (Figure 3D). The same pattern was observed in roots inoculated with the P_{lux}-*luxI*-GFP strain (Figure 3C). We used the wild type strain JMP134 without any fluorescent reporter system as a control of roots colonization. The number of cells at the plant roots was similar in wild type strain JMP134

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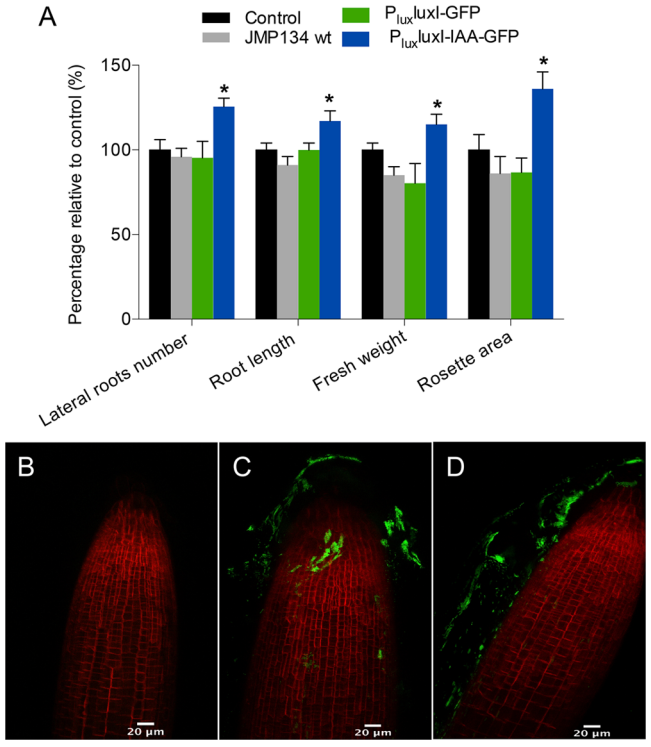

Figure 3. Beneficial effects in *Arabidopsis thaliana* plants inoculated by *Cupriavidus pinatubonensis* JMP134 harboring the engineered device for IAA production under quorum sensing signals. (A) Plant growth in gnotobiotic *A. thaliana* culture systems inoculated with wild type strain JMP134, P_{lux}-*luxI*-GFP, or P_{lux}-*luxI*-IAA-GFP strains. Growth parameters were measured 3 weeks after inoculation. Bars show mean percentage values with respect to control plants, and the error bars indicate standard deviations from an average of three biological replicate experiments for each treatment. The asterisk indicates statistically significant differences between treatments (one-way analysis of variance Tukey's honestly significant difference tests, $P < 0.05$). (B–D) Representative examples of confocal images of root colonization of *A. thaliana* *35S:mKate-LTI6b-NosT* plants by strains: wild type JMP134, without GFP reporter (B); P_{lux}-*luxI*-GFP (C); and P_{lux}-*luxI*-IAA-GFP (D). It is possible to observe the GFP produced by strains with activated QS system harboring their respective plasmids.

(5.65 ± 0.7 $\log_{10}$ colony forming units (CFU)/mg fresh weight (FW)) and strains P_{lux}-*luxI*-IAA-GFP (4.51 ± 0.9 $\log_{10}$ CFU/mg FW) and P_{lux}-*luxI*-GFP (5.93 ± 1.2 $\log_{10}$ CFU/mg FW), 14 days postinoculation. As PGPR displays more than one mechanism to promote plant growth, it is difficult to determine the real impact of bacterial IAA biosynthesis on plant growth.[5] However, our results demonstrate that IAA synthesis alone is able to promote growth in *A. thaliana* through an engineered synthetic beneficial plant-bacteria interaction. In conclusion we have demonstrated the value of the autoiducible device to synthesize IAA in plants, but also validated *C. pinatubonensis* JMP134 as a suitable vehicle for agricultural or soil remediation applications, as it is amenable to genetic manipulations.

METHODS

Strains and Media. *C. pinatubonensis* JMP134 was obtained from Deutsche Sammlung von Mikroorganismen and Zellkulturen GmbH, Braunschweig (DSMZ), Germany. Strain JMP134 wild type and its derivatives were grown at 30 °C in mineral salt medium Dorn,[59] supplemented with 10 mM benzoate or fructose and the addition of gentamicin (Gm; 30 $\mu g\ mL^{-1}$), when required. *Escherichia coli* Mach (Invitrogen, Carlsbad, CA, USA) cells were grown at 37 °C in Luria–Bertani (LB) medium and used as hosts for cloning procedures. Growth biomass tests were measured at an optical density at 600 nm (OD_{600}) and three replicates were performed for each growth measurement.

Plasmid Construction. Plasmids used in this study are listed in Table 1. We used the composite device BBa_F2620 for constitutive expression of *luxR*,[60] assembled in a Standard European Vector Architecture (pSEVA) vector with a RBS and a promoter-less green fluorescent protein (GFP) cargo, pSEVA637.[61,62] pSEVA plasmids have an optimal architecture for construction of complex prokaryotic phenotypes and are commonly used to engineer environmental Gram negative bacteria.[62−65] All plasmids were assembled in pSEVA637,[61,62] using the Gibson assembly method.[66] Each DNA fragment was obtained by PCR using oligonucleotides, ordered from Sigma-Aldrich Co. (St. Louis, MO, USA), and listed in Supplementary Table 1. To obtain the pSEVA-P_{lux}-GFP plasmid, we amplified the entire DNA fragment containing the P_{tet} promoter, *luxR* gene, and P_{lux} promoter from BBa_F2620 standard part from BioBrick Repository (primer numbers 1–2). These primers contain a 20-bp terminal sequence homologous to the terminus of the pSEVA637 fragment (primer numbers 3–4) to be linked by Gibson assembly. To obtain the pSEVA-P_{lux}-*luxI*-GFP plasmid, we assembled the amplified *luxI* gene from BBa_K516011 standard part from BioBrick Repository (primer numbers 5–6) and DNA fragment from pSEVA-P_{lux}-GFP using primers 3–7. Finally, to obtain the plasmid pSEVA-P_{lux}-*luxI*-IAA-GFP we assembled the amplified gene sequences *iaaM* and *iaaH* from BBa_K515100 standard part from BioBrick Repository (primer numbers 8–9) and DNA fragment from pSEVA-P_{lux}-*luxI*-GFP using primers 3–10. All plasmid

Table 1. Bacterial Strains and Plasmids Used in This Study[a]

strain or plasmid	relevant phenotype and/or genotype	ref or source
Strains		
C. pinatubonensis JMP134	benzoate$^+$, fructose$^+$, IAA$^-$, HSL$^-$	DSMZ (Lykidis et al., 2010)
E. coli Mach	ΔrecA1398 endA1 tonA 80ΔlacM15 ΔlacX74 hsdR(rK mK)	Invitrogen, Carlsbad, CA, USA
Plasmids		
pSEVA637	Gm^R; *oriV*(PBR1); GFP	Martinez-García et al.,
pSEVA-P_{lux}-GFP	Gm^R ; P_{lux}-GFP; *oriV*(PBR1)	this study
pSEVA-P_{lux}–*luxI*-GFP	Gm^R ; P_{lux}-*luxI*-GFP; *oriV*(PBR1)	this study
pSEVA-P_{lux}–*luxI-IAA*-GFP	Gm^R ; P_{lux}-*luxI*- IaaH-IaaM-GFP; *oriV*(PBR1); IAA$^+$	this study
pSB1C3-BBa_K515100	*Cm*^{R}; P_{veg}-IaaH-IaaM from *P. savastanoi*	BioBrick Repository Spring 2014 Distribution
pSB1C3-BBa_K516011	*Cm*^{R}; RBS-*luxI* (Synthesizes 3-oxo-C6-HSL)	BioBrick Repository Spring 2014 Distribution
pSB3K3-BBa_F2620	Km^R; P_{tet}-*luxR*-P_{lux}; *oriV*(P15A)	Canton et al., 2008

[a]Abbreviations used in this table are as follows: Gm, gentamicin; Cm, chloramphenicol; Km, kanamycin; GFP gene encoding a green fluorescent protein; IaaH, gene encoding indole-3-acetamide hydrolase; IaaM, gene encoding 2-tryptophan monooxygenase; *luxI*, gene encoding homoserine lactone synthase; IAA$^+$, ability to produce indole-3-acetic acid.

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constructs were confirmed by Sanger DNA sequencing (Macrogen, Korea). Plasmids were electroporated in *C. pinatubonensis* JMP134 and selected in LB medium with antibiotic.

Plate Reader Fluorescence and Flow Cytometry Analysis. For plate reader experiments, 2 mL of culture of Dorn minimal medium supplemented with 10 mM fructose and 30 μg mL^{-1} gentamicin (for strain harboring different versions of pSEVA plasmids) were inoculated with single colonies of strains from a freshly streaked plate of Dorn minimal medium with 10 mM benzoate. Cultures were grown for 15 h at 30 °C with shaking. Then, the cultures were washed once in fresh medium and diluted until $OD_{600\ nm}$ 0.05 in 5 mL of fresh medium and grown to an $OD_{600\ nm}$ 0.1 under the same conditions. Subsequently, 200 μL aliquots of each of the cultures were transferred into a flat-bottomed 96 well plate (Cellstar Uclear bottom, Greiner Bio-One, Solingen, Germany) and 2 μL of 100 μM of 3-oxo-hexanoyl−homoserine lactone (3-oxo-C6HSL) (Sigma-Aldrich, St. Louis) were added to each well. Six replicate wells were measured for each treatment. Three wells were each filled with 200 μL of medium to measure the absorbance background. The plate was incubated in a synergyMX well-plate reader (Biotek, Bad 21 Friedrichshall, Germany) at 30 °C and assayed with an automatically repeating protocol of absorbance measurements (600 nm absorbance filter) and fluorescence measurements (488 nm excitation filter, 525 nm emission filter) for 20 h, with 1 h between repeated measurements. For flow cytometry analysis, single colonies of strains from a freshly streaked plate of Dorn minimal medium with 10 mM benzoate were grown overnight at 30 °C with shaking in Dorn minimal medium with 10 mM fructose. Cells were washed twice with fresh medium and diluted in 20 mL of fresh medium with fructose until $OD_{600\ nm}$ 0.01 and incubated until the cultures reached $OD_{600\ nm}$ 0.1. At this point, cells were divided into two samples: one was induced by the addition of 1 μM 3-oxo-C6HSL, and the other was kept as a noninduced. Cultures were incubated as described above, and an aliquot of each sample was withdrawn every hour after induction. A MACSQuantTM VYB cytometer (Miltenyi Biotec, Bergisch Gladbach, Germany) was used for GFP analysis. The flow cytometry analysis was carried out on at least 20 000 cells, and the data was processed using FlowJo v.10.0.8 software (FlowJo LLC, Ashland, OR, USA).

SDS-PAGE. To determine the expression of LuxI, IaaM, and IaaH proteins, we run a SDS-PAGE from culture of strains P_{lux}-*luxI*-GFP and P_{lux}-*luxI*-IAA-GFP at an $OD_{600\ nm}$ of 0.2, 0.6, and 1. The cultures were centrifuged at 13 000*g* for 10 min to collect the cells. The pellets were weighed, and 10 mg was used for cell extract preparation in 50 μL 1× SDS sample buffer and heated at 95 °C for 10 min. After centrifugation for 10 min at 13 000*g*, 5 μL of the denatured sample was fractionated by SDS-PAGE in Novex wedgewell 4−12% Tris-Glycine gel (Invitrogen).

Measurement of IAA Synthesis. To determine the IAA synthesis we grew the strain P_{lux}-*luxI*-IAA-GFP in 50 mL of minimal medium with 10 mM of fructose as carbon source, until an $OD_{600\ nm}$ of 0.2 and 0.6 and then we exposed the cells to 1 mM of tryptophan for 3 h. Cells were centrifuged at 5.000*g*. The supernatant containing IAA was filtered (filter unit of 0.22 nm pore diameter), and its pH was adjusted to 2.5 using hydrochloric acid. The IAA was extracted from the aqueous medium with 1 volume of ethyl acetate (two times). The pooled organic layer was dried over anhydrous Na_2SO_4 and filtered, and the solvent was rotary evaporated. The samples were resuspended in ethyl acetate at 10× and diluted in buffer 40% methanol−60% H_2O containing 0.1% (v/v) phosphoric acid. The presence of IAA was determined by high-performance liquid chromatography−UV detection (HPLC-UV) from 20 μL of the sample, injected into Agilent Technologies 1260 Infinity II LC System (Agilent Technologies, Les Ulis, France) equipped with a Kromasil 100-3.5 C18 4.6 × 150 mm column. Methanol−H_2O mixtures containing 0.1% (v/v) phosphoric acid were used as the solvent, at a flow rate of 0.75 mL/min. The column effluent was monitored at 210 nm. Retention time for IAA methanol−H_2O (40:60) solvent IAA was 14.4 min. A calibration curve was achieved by injection of 20 μL of IAA at 0.1 to 100 μM.

Plants Construction, Inoculation, and Growth Parameters. *A. thaliana* ecotype Col-0 and the *A. thaliana* transgenic line *35S:mKate-LTI6b-NosT* were used. 35S:mKate-LTI6b-NosT was created by assembling 35S promoter (PCR-amplified from,[67] mKate2 (PCR-amplified as in ref 68) and LTI6b gene (PCR-amplified from 35S::EGFP-LTI6b;[57]) into pGreenII backbone by Gibson Assembly.[66] This vector was then used to transform *A. thaliana* using the floral dip method.[69] Seeds were surface sterilized with 50% (v/v) commercial chlorine bleach for 7 min and washed three times in sterile distilled water. Then they were kept at 4 °C for 2 days in the absence of light to produce stratification. After that, seeds were sown in sterile plastic Petri dishes with 1% agar plates containing MS basal salt mixture (Sigma-Aldrich, St. Louis) inoculated or not inoculated with bacteria. Strains JMP134 wt, P_{lux}-*lux*-GFP, and P_{lux}-*lux*-IAA-GFP were adjusted to approximately 10^4 CFU/mL, as determined by plate counting. Each strain was homogeneously inoculated on 1% agar plates containing Murashige and Skoog (MS) basal salt mixture (Sigma-Aldrich, St. Louis). Eight seeds were sown in each plate and six plates were used for each treatment: control without bacteria, JMP134 wild type, P_{lux}-*lux*-GFP, or P_{lux}-*lux*-IAA-GFP strains. Plates were placed vertically, sealed with parafilm, and arranged in a completely randomized design. The plant growth chamber was run with a cycle of 12 h of light and 12 h of darkness and a temperature of 22 ± 2 °C. Twenty-five plantlets from each inoculated or not inoculated treatment were analyzed. Rosette growth was registered photographically using Adobe Photoshop Cs3 software (Adobe Systems Incorporated, San Jose, CA, USA). Root lengths and lateral root numbers were measured directly in harvested plants, and fresh weight was recorded as previously described.[70] Rhizospheric colonization tests were performed using 2-week-old inoculated plants washed in phosphate buffer solution with vortex agitation. Extracted liquid material was serially diluted with Dorn mineral salts medium and grown in Dorn medium plates supplemented with 5 mM benzoate. The CFU/mg FW was determined after 48 h of incubation at 30 °C. Three biological replicates were carried out.

Confocal Microscopy Analysis. To determine rhizosphere colonization by GFP-marked strains, treated and untreated plant root surfaces were examined by confocal microscopy. Confocal microscope images were obtained using Leica TCS LSI confocal laser scanning (Wetzlar, Allemagne), using PLANAPO 5*x*/0.5 LWD objective.

Statistical Analysis. Data for plant growth parameters were statistically analyzed using one-way analysis of variance. When analysis of variance showed significant treatment effects, Tukey's honestly significant difference ($P < 0.05$) test was

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applied to make comparisons between treatments. Statistical analyses were performed using the statistical software package STATISTICA (version 6.0; StatSoft Inc., Tulsa, OK, U.S.A.).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.8b00002.

Further details of supplemental figures and tables (PDF)

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A.Z., F.d.l.F., F.F., and B.G. designed the experiments. A.Z., F.d.l.F., and F.F. performed the experiments. C.L. performed HPLC experiments. B.G., V.D., J.B., F.F., A.Z., and F.d.l.F., aided in interpreting the results. B.G., V.D., and J.B. provided material source. A.Z. wrote the manuscript. All authors provided critical feedback and helped shape the research and manuscript. All authors approved the manuscript.

Notes

The authors declare no competing financial interest.

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