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Pyocyanin, a Virulence Factor Produced by *Pseudomonas aeruginosa,* Alters Root Development Through Reactive Oxygen Species and Ethylene Signaling in *Arabidopsis*

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Pyocyanin acts as a virulence factor in *Pseudomonas aeruginosa*, a plant and animal pathogen. In this study, we evaluated the effect of pyocyanin on growth and development of *Arabidopsis* seedlings. Root inoculation with *P. aeruginosa* PAO1 strain inhibited primary root growth in wild-type (WT) *Arabidopsis* seedlings. In contrast, single *lasI*– and double *rhlI*–/*lasI*– mutants of *P. aeruginosa* defective in pyocyanin production showed decreased root growth inhibition concomitant with an increased phytostimulation. Treatment with pyocyanin modulates root system architecture, inhibiting primary root growth and promoting lateral root and root hair formation without affecting meristem viability or causing cell death. These effects correlated with altered proportions of hydrogen peroxide and superoxide in root tips and with an inhibition of cell division and elongation. Mutant analyses showed that pyocyanin modulation of root growth was likely independent of auxin, cytokinin, and abscisic acid but required ethylene signaling because the *Arabidopsis etr1-1*, *ein2-1*, and *ein3-1* ethylene-related mutants were less sensitive to pyocyanin-induced root stoppage and reactive oxygen species (ROS) distribution. Our findings suggest that pyocyanin is an important factor modulating the interplay between ROS production and root system architecture by an ethylene-dependent signaling.

The ecophysiology of plants cannot be understood without the microbial populations that proliferate outside and inside roots. Rhizobacterial species may impact root physiology through production of plant hormones such as auxin or cytokinins, by stimulating root growth, or by altering root system architecture. Moreover, many bacterial species provide protection against pathogens, tolerance to abiotic stress, and resistance to insect or herbivore attack, and even allelopathy may be due to root-associated microorganisms (Friesen et al. 2011; Ortiz-Castro et al. 2009). Bacteria communicate with plants through secreted signaling factors. These are small, diffusible molecules that are specifically released and then recognized by eukaryotic tissues. By producing different classes of signals, the bacteria can be recognized as pathogens or symbionts leading to very different host responses (Ortiz-Castro and López-Bucio 2013; Ortiz-Castro et al. 2011).

The *Pseudomonas* genus comprises ubiquitous gram-negative bacteria distributed in different environments and contains pathogenic species for plants (i.e., *Pseudomonas syringae* and *P. aeruginosa*). Other species have the ability to colonize the rhizosphere (i.e., *P. aeruginosa, P. fluorescens, P. putida, P. aureofaciens*, and *P. chloraphilis*), where they can act as plant-beneficial bacteria by antagonizing pathogens or through production of compounds that influence plant-disease resistance and growth (Venturi 2006). The ability of *P. fluorescens* CHA0 and *P. aeruginosa* 7NSK2 to induce resistance in grapevine against *Botrytis cinerea* was recently demonstrated. Both strains also triggered an oxidative burst and phytoalexin accumulation in grape cells and primed leaves for accelerated phytoalexin production upon challenge with *B. cinerea* (Verhagen et al. 2010). Redox-active pyocyanin (PCN) secreted by *P. aeruginosa* 7NSK2 triggers systemic resistance to *Magnaporthe grisea* but enhances *Rhizoctonia solani* susceptibility in rice (De Vleeschauwer et al. 2006). These results suggest that *Pseudomonas*-derived metabolites can induce reactive oxygen species (ROS), which act as a double-edged sword in the interaction of rice with the hemibiotroph *M. grisea* and the necrotroph *R. solani.*

Gram-negative bacteria produce and use *N*-acyl-*L*-homoserine lactones (AHL) for cell-to-cell communication through a regulatory mechanism known as quorum sensing (QS), which links perception of bacterial cell density to gene expression (Fuqua et al. 1994). QS coordinates many physiological processes such as symbiosis, production of virulence factors, resistance to oxidative stress, antibiotic resistance, motility, and biofilm formation (Miller and Bassler 2001). In *P. aeruginosa*, two main QS signals, *N*-(3-oxododecanoyl)-*L*-homoserine lactone (C12-AHL) and *N*-butyryl-*L*-homoserine lactone (C4-AHL), are synthesized by the AHL synthases encoded by the *lasI* and *rhlI* genes, respectively. At high bacterial density, the transcription factor LasR binds to C12-AHL; whereas RhlR, another transcriptional regulator, binds to C4-AHL to activate the transcription of virulence genes (Bosgelmez-Tinaz 2003; de Kievit and Iglewski 2000; Fuqua and Greenberg 2002; Rumbaugh et al. 2000).

Roots have developed the capacity to recognize bacterial QS signals and adjust growth and development in response to

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these metabolites (Mathesius et al. 2003; Ortiz-Castro et al. 2008; von Rad et al. 2008). Recently, genetic, chemical, and plant-growth data were presented showing that, in *P. aeruginosa*, the *lasI* QS system controls the production of three diketopiperazines (DKP)—namely, cyclo(L-Pro-L-Val), cyclo(L-Pro-L-Phe), and cyclo(L-Pro-L-Tyr)—that were involved in plant growth promotion by this bacterium. Analysis of all three bacterial DKP in *Arabidopsis thaliana* seedlings provided detailed information indicative of an auxin-like activity, based on their efficacy at modulating root architecture, activation of auxin-regulated gene expression, and response of auxin-signaling related mutants (Ortiz-Castro et al. 2011).

P. aeruginosa is most studied for its importance as a human and plant pathogen. Surprisingly, many studies have revealed extensive conservation in its virulence mechanisms to infect evolutionary divergent hosts. One of these conserved virulence factors is PCN. For example, PCN participates in the fast killing of *Caenorhabditis elegans*, *Drosophila melanogaster*, and *Mus musculus*, likely by producing ROS (Lau et al. 2003, 2004a and b; Mahajan-Miklos et al. 1999). PCN is synthesized from chorismate through a series of complex steps mediated by proteins encoded by two *phzABCDEF*G operons, and by the *phzH*, *phzM*, and *phzS* genes, which modify precursors into the tricyclic compound (Mavrodi et al. 2001; Rada and Leto 2013). PCN synthesis is regulated by QS, as several reports indicate that mutations in the *lasI-lasR* and *rhlI-rhlR* QS systems result in the loss of PCN production (de Kievit and Iglewski 2000; Rumbaugh et al. 2000; Schaber et al. 2004; Siehnel et al. 2010). Moreover, PCN itself functions as a QS signal, as indicated by the fact that i) it demonstrates cell density-dependent accumulation, ii) it is a small diffusible molecule that is recognized by adjacent cells, and iii) it triggers a specific transcriptional response (Dietrich et al. 2006), further complicating our understanding of its mechanisms of action.

Despite in vitro studies demonstrating that PCN interferes with multiple cellular functions in animals, its importance during bacteria–plant interactions is uncertain. This is partially caused by the difficulty in defining the contribution of PCN among the numerous virulence factors produced by *P. aeruginosa* during infection. Currently, the response of plant cells or whole organs to *P. aeruginosa*-produced PCN is unknown, and whether this compound causes cell damage or regulates fundamental cellular processes in plants remains to be clarified. To gain insight into how PCN might be functionally integrated into *P. aeruginosa* physiology during interaction with plants, the contribution of PCN to growth and development was assessed by comparing the *Arabidopsis* primary and lateral root responses to *P. aeruginosa* PAO1 and the QS-related mutants *rhlI*–, *lasI*–, and *rhlI–/lasI*– in direct interaction of the bacteria with the root system. We also provide detailed pharmacological evidence of PCN bioactivity on *Arabidopsis* seedlings and analyzed the growth of primary roots in response to PCN in wild-type (WT)-, auxin-, cytokinin-, ethylene-, and abscisic-acid-related *Arabidopsis* mutants. Our data conclusively indicate that PCN acts as a signaling molecule for root development likely affecting ROS production and ethylene signaling.

RESULTS

AHL-mediated QS plays a role in growth and development of *Arabidopsis* modulated by *P. aeruginosa.*

We first tested whether direct colonization of the *Arabidopsis* root with *P. aeruginosa* PAO1 and QS-related single *rhlI*– and *lasI*–, and double *rhlI–/lasI*– mutants could affect growth of seedlings. In several experiments and times of co-cultivation, *P. aeruginosa* PAO1 caused primary root growth inhibition, and the shoot system was unable to grow (Fig. 1B, G, and L). These effects were similar to those caused by the *P. aeruginosa rhlI*– mutant, defective on the AHL synthase that produces C4-AHL (Fig. 1C, H, and M). In contrast, co-cultivation with the *P. aeruginosa lasI*– single mutant defective on 3-oxo-C12-AHL synthesis or with the *rhlI–/lasI*– double mutant failed to cause inhibition of growth and dramatically increased root and shoot biomass production of seedlings at 3, 6, and 9 days of co-cultivation (Fig. 1D, I, and N; and E, J and O, respectively). These data indicate that C12-AHL–mediated QS controls the production of factors that repress primary root growth. Interestingly, an analysis of hydrogen peroxide (H_2O_2) in root tips of *Arabidopsis* seedlings co-cultivated for 9 days with WT *P. aeruginosa* and AHL-related mutants revealed a decrease in H_2O_2 in roots co-cultivated with WT and *rhlI* mutants and an increased accumulation of H_2O_2 in both *lasI*– and *rhlI–/lasI*– mutants (Fig. 2A to E). This highly contrasting response indicates that diffusible factors released by WT *P. aeruginosa* modulates the levels of H_2O_2 and perhaps other ROS likely involved in root system adjustment.

AHL-mediated QS regulates PCN production in *P. aeruginosa.*

P. aeruginosa releases PCN as a main virulence factor (De Vleesschauwer et al. 2006). To determine whether QS-related single *rhlI*– and *lasI*– and double *rhlI–/lasI*– mutants of *P. aeruginosa* could be defective on the production of PCN, we determined production of this metabolite in bacterial cell cultures by spectrophotometric analyses. It was found that PCN levels drastically decreased in single *rhlI*– and *lasI*– and double *rhlI–/lasI*– mutants when compared with *P. aeruginosa* PAO1 (Fig. 3). These data show that AHL-modulated QS plays an important role in PCN production.

PCN alters *A. thaliana* root system architecture.

To determine whether *Arabidopsis* plants could sense PCN and investigate how this compound affects plant morphogenesis, we evaluated *Arabidopsis* root developmental responses to pharmacological application of PCN. With this aim, *Arabidopsis* seedlings were germinated and grown on 0.2× Murashige-Skoog (MS) agar medium supplemented with PCN concentrations from 0.6 to 40 μM and primary root growth was measured 10 days after germination (d.a.g.). PCN treatments showed a dose-dependent inhibitory effect of primary root growth, with 10 μM PCN causing a 70% reduction in primary root length (Fig. 4A; Supplementary Fig. S1). In contrast, an induction of lateral root formation was evident from 0.6 to 2.5 μM PCN, while inhibitory effects were recorded at higher concentrations (Fig. 4B). A stimulatory effect of PCN in lateral root density (LRD) was also observed with a threefold increase from 2.5 to 20 μM concentration of this compound when compared with solvent-treated seedlings (Fig. 4C).

PCN alters root hair development.

Root hairs are root epidermal cells that participate in nutrient and water uptake and increase the exploratory potential of the root system. To analyze whether PCN could alter root hair development, we performed experiments in which *Arabidopsis* WT (Col-0) seedlings were germinated and grown on the surface of agar plates containing different concentrations of PCN from 0.6 to 40 μM. Root hair parameters were analyzed at 7 d.a.g. on primary roots of solvent-treated or PCN-treated seedlings. To investigate the effects of PCN on root hair density, we measured the trichoblast length and root hair length on seedlings subjected to different concentrations of this compound. Trichoblasts are the hair-forming root epidermal cells that form cell files along the root surface. We found a dose-

dependent decrease in trichoblast length in response to PCN treatment (Fig. 5A), which correlated with increased root hair number and root hair length (Fig. 5B and C). Root hair development was located closer to the primary root tip in plants grown in medium supplied with 40 μM PCN (Supplementary Fig. S2), clearly indicating the progression of cell differentiation toward the root meristem region. These results suggest that PCN can be perceived by roots and alter root system architecture and root hair development.

PCN alters cell division without affecting cell viability or integrity.

Previous reports demonstrated the toxicity of PCN in different organisms. However, the effects of PCN inhibiting primary root growth of *Arabidopsis* seedlings suggested that this compound could play an important role in cell division or elongation. To investigate the patters of cell division in response to PCN, we analyzed the expression of *CycB1:uidA*, which is expressed only in cells in the G2/M phase of the cell cycle and is a marker of mitotic activity (Colón-Carmona et al. 1999), and *pPRZ1:uidA*, which marks only active meristems (Sieberer et al. 2003). The inhibition of primary root growth under PCN concentrations of 5 μM or higher correlated with the reduction in the number of cells expressing *CycB1:uidA* in the primary root meristem and β-glucuronidase (GUS) expression of *pPRZ1:uidA* transgenic seedlings (Fig. 6A to H). We also analyzed the gene expression of the cell nuclei marker *AtHistH2B:YFP* (*yellow fluorescent protein*) by confocal laser-scanning microscopy in seedlings stained with propidium iodide (PI) to determine whether PCN could cause cell death or damage of root tissues. Visualization of *AtHistH2B:YFP* in the nuclei of cells 7 d.a.g. showed that PCN-treated roots were, indeed, viable. In these cells, PI was unable to penetrate, even at concentrations higher than 40 μM (Fig. 6I to L). Quantification of meristem length and number of cells expressing *CycB1:uidA* clearly documented the repressing effects of PCN on cell proliferation in primary roots (Fig. 6M and N). These results suggest that PCN regulates cell division without affecting cell integrity or meristem viability.

PCN did not activate auxin inducible gene expression in *Arabidopsis* roots.

Auxin is an important phytohormone involved in the modulation of several development processes in the root system. To

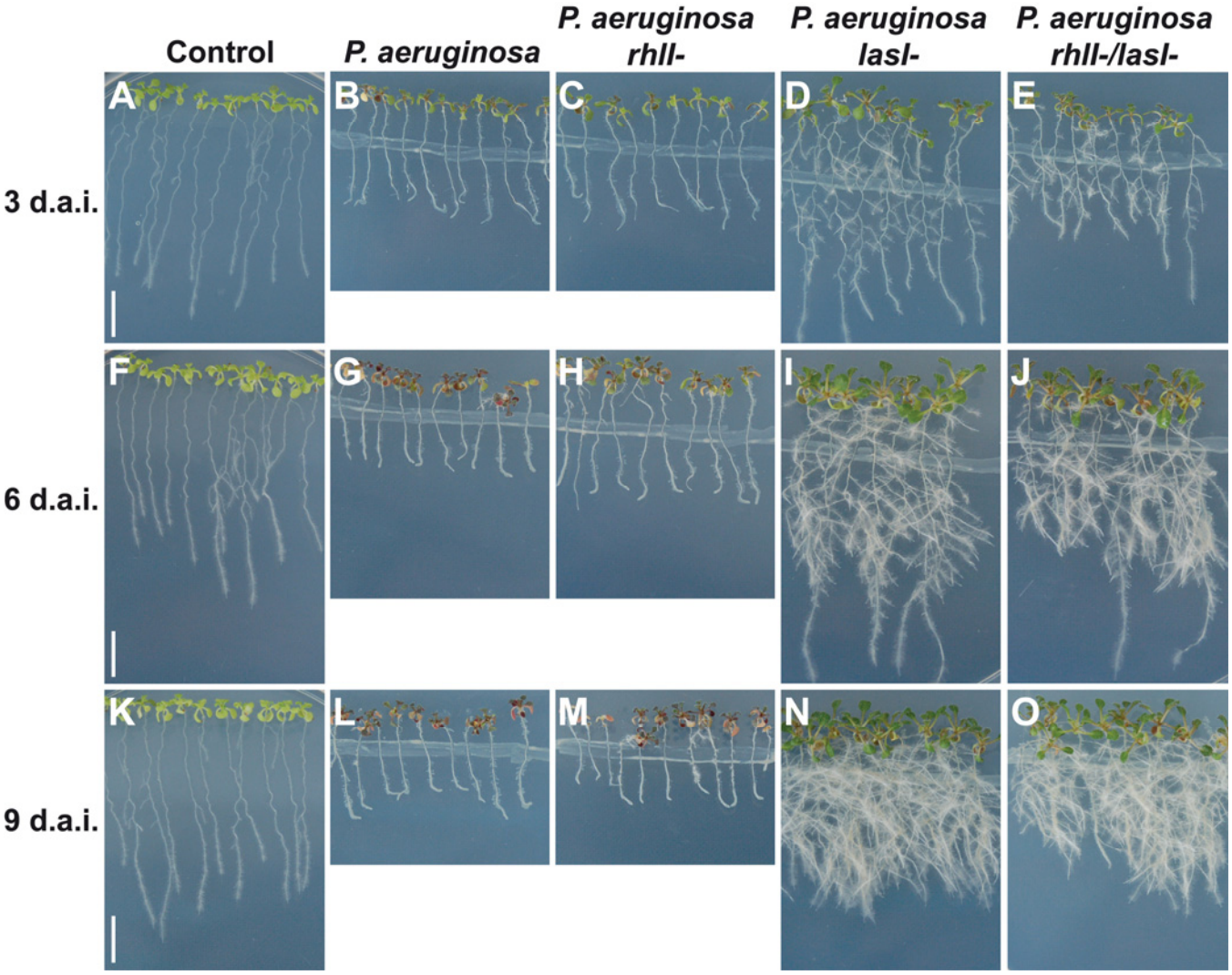

Fig. 1. Effect of co-cultivation with *Pseudomonas aeruginosa* wild-type (WT) and quorum-sensing (QS) mutant strains on plant growth. Six-day-old *Arabidopsis thaliana* seedlings were co-cultivated with WT *P. aeruginosa* or mutants defective on the AHL synthases LasI, RhlI, or RhlI/LasI at direct contact and grown for **A** to **E,** 3; **F** to **J,** 6; and **K** to **O,** 9 days. Representative photographs were taken for plates from each treatment. This experiment was repeated three times with similar results. Notice the damage and inhibitory effect on root system architecture caused by the *P. aeruginosa* WT and *rhlI*– mutant and the strong induction on root system architecture and greening plants effect of the *P. aeruginosa lasI*– and *rhlI–/lasI*– mutants. Scale bar = 1 cm.

test whether PCN may or not function via auxin-regulated processes, we analyzed expression of *DR5:uidA* (Ulmasov et al. 1997) and *BA3:uidA* (Oono et al. 1998) auxin-inducible markers in transgenic *Arabidopsis* seedlings treated with PCN. Histochemical staining of roots of transgenic *DR5:uidA* and *BA3:uidA* seedlings that were grown for 10 days on 0.2× MS medium supplemented with solvent, indole-3-acetic acid (IAA), or the indicated concentrations of PCN is shown in Figure 7. *DR5:uidA* expression in solvent-treated seedlings is located at the edges of the cotyledons and mainly at the root tip region (Fig. 7A and G). *DR5:uidA* seedlings grown in a concentration of 3 μM IAA showed GUS activity throughout the shoot and primary root (Fig. 7B and H), whereas *BA3:uidA* seedlings supplied with the same IAA concentration expressed GUS specifically at the root elongation region (Fig. 7N and T). When *DR5:uidA* and *BA3:uidA* seedlings were grown on PCN-supplied medium, the GUS expression remained similar in the shoot and primary root tip (Fig. 7C to F, I to L, O to R, and U to X). These results suggest that PCN did not induce auxin-responsive gene expression in *Arabidopsis* seedlings.

PCN inhibits primary root growth of auxin-, cytokinin-, and abscisic-acid-related *Arabidopsis* mutants.

Several mutants with alterations in root development have been identified using screens for resistance to growth inhibitory amounts of phytohormones. Because PCN did not activate

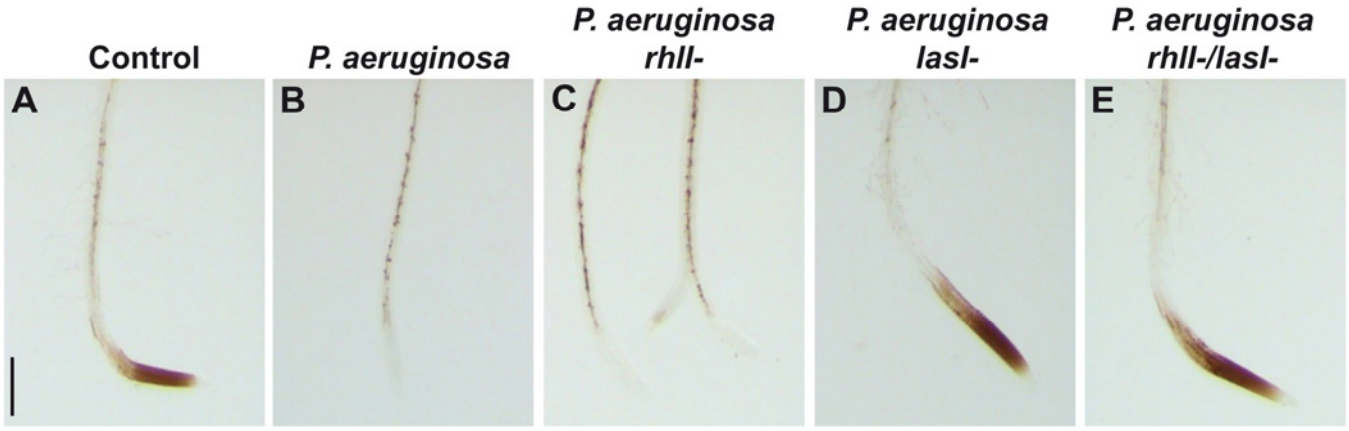

Fig. 2. Effect of co-cultivation of *Pseudomonas aeruginosa* wild-type (WT) and quorum-sensing (QS) mutant strains on H_2O_2 accumulation in the primary root meristem of *Arabidopsis thaliana* seedlings. Four-day-old *A. thaliana* seedlings were co-cultivated with WT *P. aeruginosa* or mutants defective on the AHL synthases RhlI, LasI, or RhlI/LasI at a distance of 5 cm from the primary root tip and grown for 8 days. Representative photographs of primary root of **A,** control seedlings or co-cultivated with **B,** WT *P. aeruginosa* and **C,** *rhlI*; **D,** *lasI*; or **E,** *rhlI/lasI* mutants. *Arabidopsis* seedlings were treated with a solution of 3,3′-diaminobenzidine (DAB). In the presence of H_2O_2, DAB polymerizes, forming a dark, red-brown coloration in plant tissues. This experiment was repeated three times with similar results. Scale bar = 500 μm.

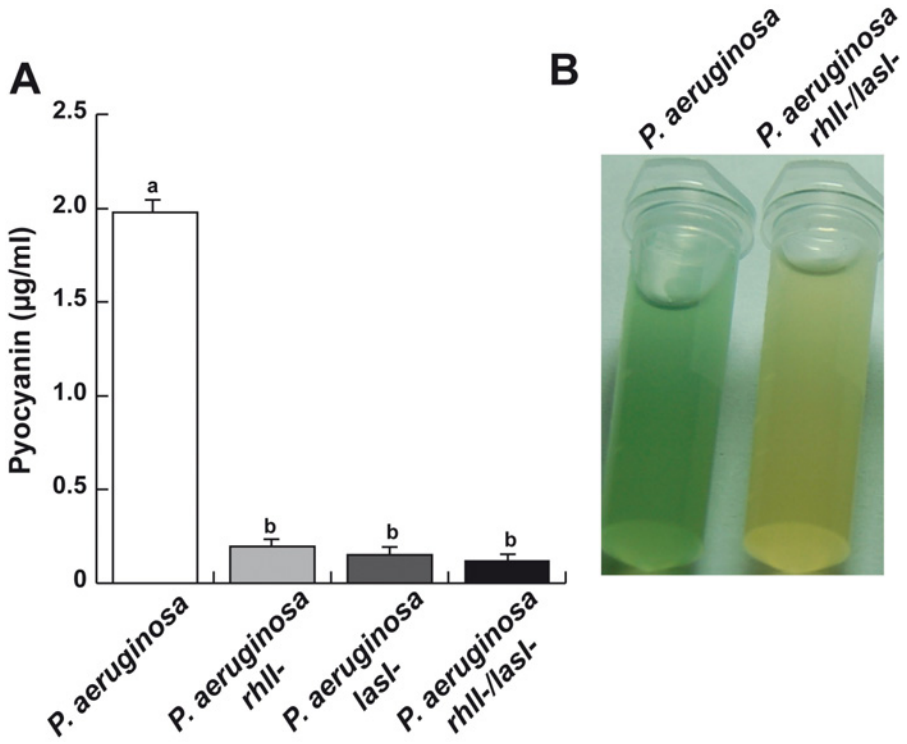

Fig. 3. Pyocyanin production in *Pseudomonas aeruginosa* wild-type (WT) and quorum-sensing (QS) mutant strains. **A,** Pyocyanin production: cells were grown in Luria-Bertani medium at 37°C for 48 h, the supernatant fractions were separated, and the amount of pyocyanin (μg ml^{-1}) in each fraction was determined by the chloroform:acid extraction procedure. Values represent the mean of three independent experiments ± standard deviation. **B,** Representative photograph of pyocyanin production as observed by the green color of culture grown in liquid medium.

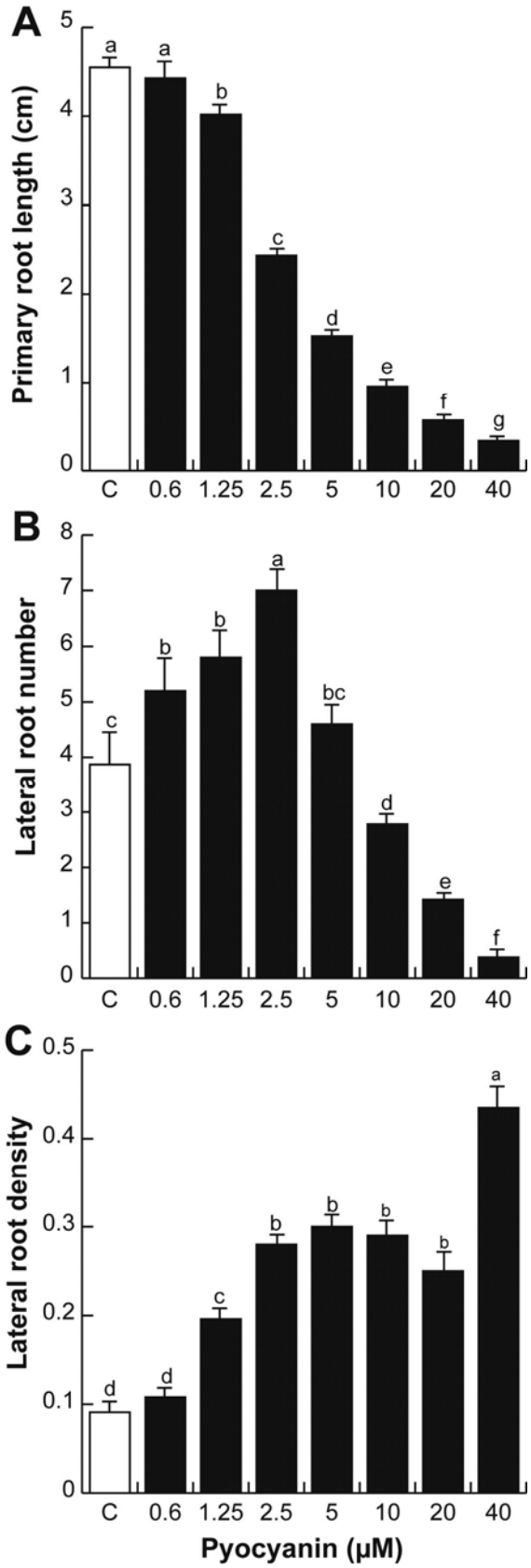

Fig. 4. Effect of pyocyanin on *Arabidopsis* root system architecture. *Arabidopsis* wild-type (WT) (Col-0) seedlings were germinated and grown for 10 days under increasing pyocyanin concentrations. **A,** Primary root length; **B,** lateral root number; **C,** lateral root density. Values shown represent the mean ± standard deviation (n = 30). Different letters represent means statistically different at the 0.05 level. The experiment was repeated three times with similar results.

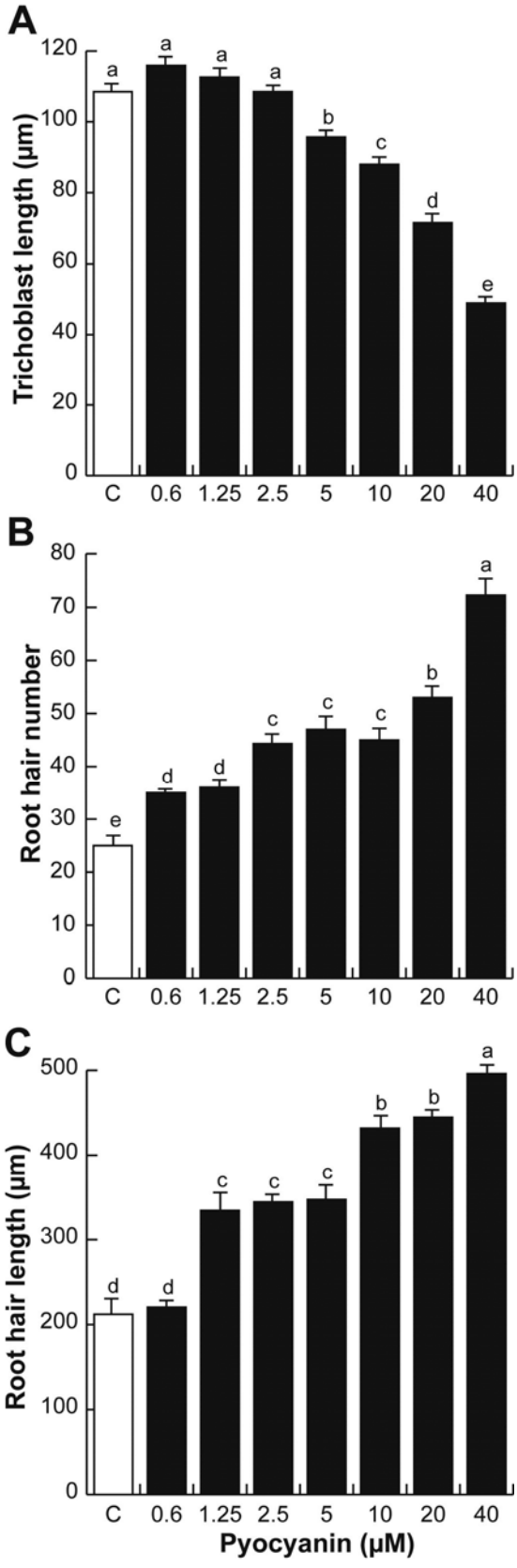

Fig. 5. Effects of pyocyanin on epidermal cell differentiation. **A,** Trichoblasts length; **B,** root hair number; **C,** root hair length. *Arabidopsis thaliana* seedlings were grown for 5 days on 0.2× Murashige-Skoog medium supplemented with the indicated concentrations of pyocyanin. Data points indicated mean ± standard deviation (n = 20). Results show mean of 10 epidermal cells located in the root hair forming zone of the primary root. This experiment was repeated twice with similar results. Different letters indicate statistical differences at $P < 0.05$.

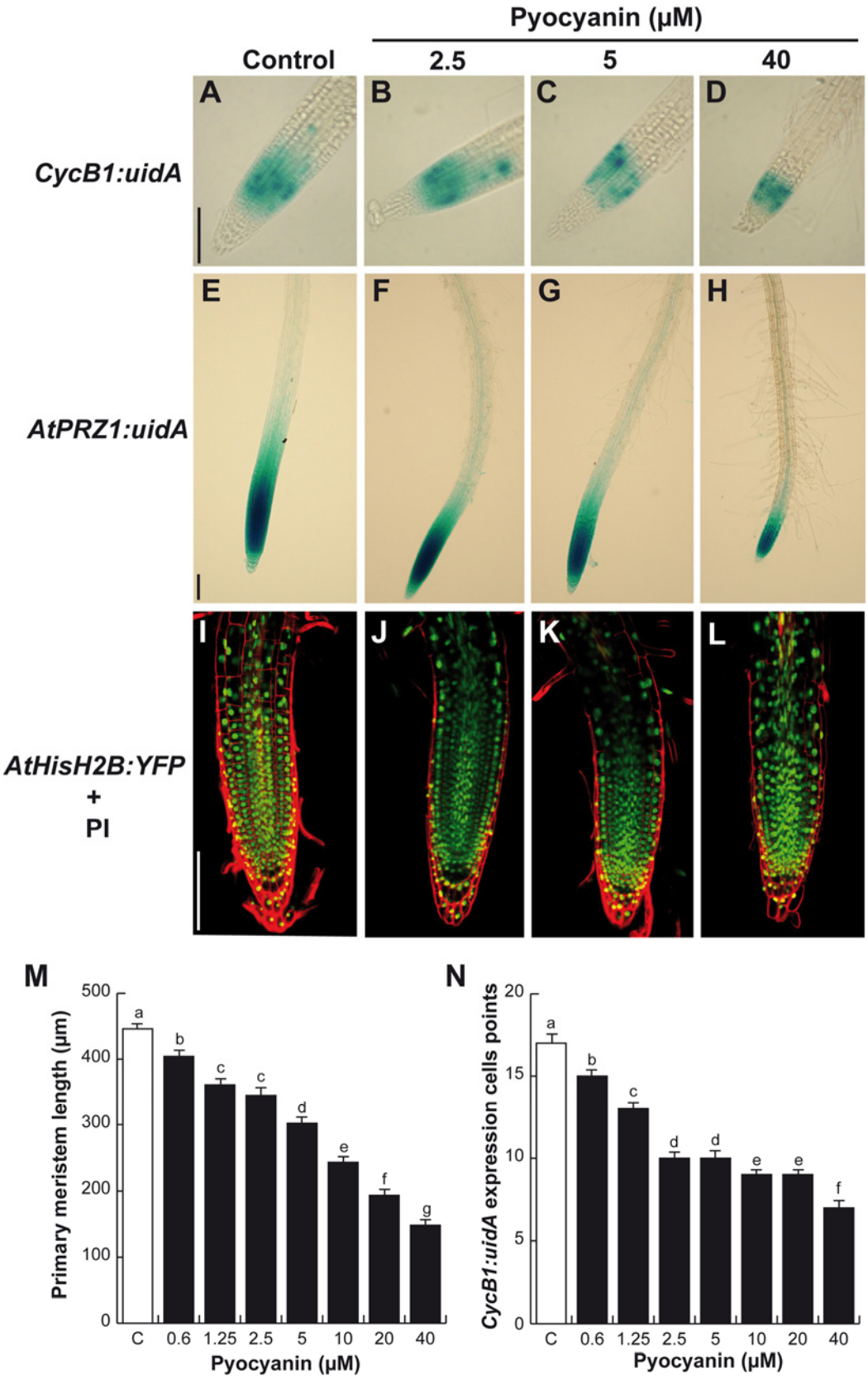

Fig. 6. Effect of pyocyanin on cell division and meristem viability. *Arabidopsis thaliana* seedlings expressing the *CycB1:uidA*, *AtPRZ1:uidA*, or *AtHistH2B:YFP* markers were grown for 5 days on 0.2× Murashige-Skoog medium supplemented with the indicated concentrations of pyocyanin. **A** to **H,** Plants were stained for β-glucoronidase activity and cleared to show gene expression. **I** to **L,** Transgenic *Arabidopsis* seedlings expressing the *AtHisH2B:YFP* marker were stained with propidium iodide to determine cell structure and viability. Photographs show representative individuals from at least 20 stained plants. The experiment was replicated twice with similar results. Scale bar = 100 μm. **M,** The *CycB1:uidA* expression domain in response to pyocyanin was measured and **N,** cells expressing this marker were counted. Data points represent the mean ± standard deviation (n = 20). The experiment was replicated two times with similar results. Different letters indicate statistical differences at $P < 0.05$.

auxin-inducible markers, we decided to confirm whether PCN operates or not in a genetically defined auxin pathway. With this aim, *Arabidopsis* WT (Col-0) seedlings and auxin-related mutants *tir1afb2afb3*, *arf7arf19*, *axr1-3*, and *aux1-7* were evaluated in primary root growth response assays to 10 µM PCN. PCN treatment caused a 70% inhibition in primary root growth in WT plants compared with solvent-treated seedlings (Supplementary Fig. S3). When *tir1afb2afb3, arf7arf19, axr1-3*, and *aux1-7* were grown in medium supplied with 10 µM PCN, the inhibition in primary root growth was similar to that observed in WT plants. The results of both auxin-responsive gene expression and the root response of auxin-related mutants to PCN suggest that auxin is not involved in plant perception of PCN.

In addition, we evaluated the involvement of cytokinin, abscisic acid, and ethylene signaling in response to PCN by evaluating the primary root growth of *Arabidopsis* double mutants defective on cytokinin receptors (*cre1-12ahk2-2* and *cre1-12ahk3-3*), abscisic acid signaling (*abi1* and *abi3*), and ethylene signaling (*ein2-1* and *ein3-1*). The primary root growth of auxin, cytokinin, and abscisic acid mutants was normally inhibited by PCN, indicating that these phytohormones are unlikely mediating the cellular effects of PCN. Interestingly, an analysis of ethylene response mutants (*ein2-1* and *ein3-1*) showed a small yet statistically significant resistance of primary root growth to inhibition by PCN, indicating that ethylene might be a signal that mediates the plant response to PCN.

A role of ethylene signaling in root response to PCN.

To further define the particular role of ethylene signaling in the *Arabidopsis* developmental responses to PCN, we investigated the sensitivity of primary root responses to several PCN concentrations of *Arabidopsis* WT seedlings and *etr1-1, ein2-1*, and *ein3-1* mutants. PCN was supplied to the growth medium in concentrations from 0.3 to 10 µM and the primary root growth of all four lines was measured. Interestingly, we found than *etr1-1* and *ein2-1* showed resistance to inhibition of primary root growth compared with WT seedlings, while *ein3-1* did not show clear resistance to PCN (Fig. 8A). To further determine the participation of ethylene signaling in the responses to PCN, we used $AgNO_3$, a well-known blocker of ethylene action. We found that, when *Arabidopsis* seedlings were grown on medium supplemented with 5 µM PCN and 5 µM $AgNO_3$, the inhibitor reduced the effect of PCN in both primary root growth and root hair development (Fig. 8B and C to J). This restoration of primary root growth in plants grown on medium supplemented with PCN and $AgNO_3$ correlated with normalization of root hair differentiation process caused by PCN (Fig. 8G to J). These results suggest that ethylene signaling plays a role in root architectural responses to PCN.

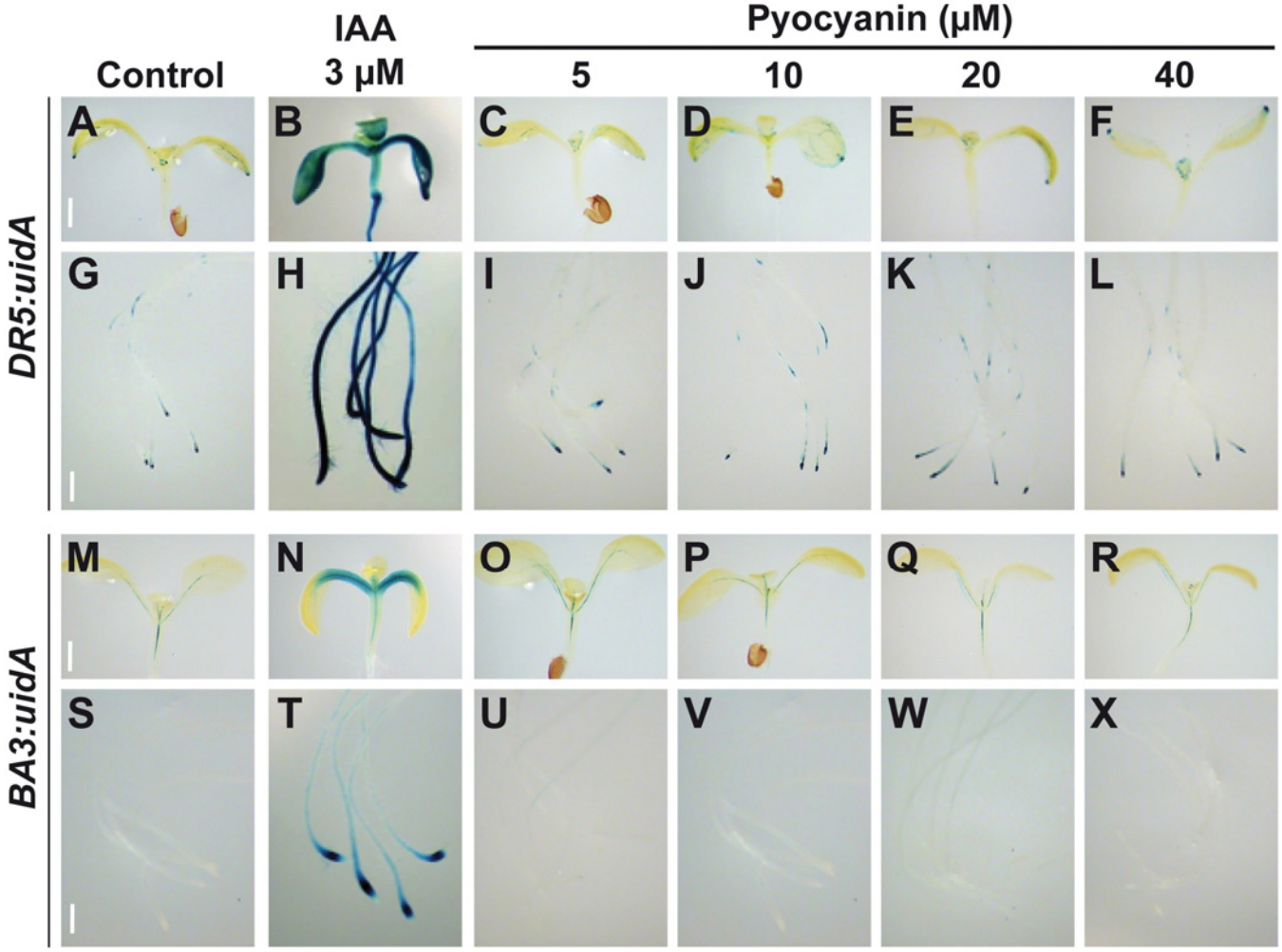

Fig. 7. Effect of pyocyanin on auxin-regulated gene expression. **A** to **L,** *DR5:uidA* and **M** to **X,** *BA3:uidA* gene expression in transgenic seedlings grown on 0.2× Murashige-Skoog (MS) agar medium for 6 days and then transferred into 24-well cell culture plates (10 seedlings per well) containing 2 ml of 0.2× MS liquid medium supplied with the indicated concentrations of indole-3-acetic acid (IAA) or pyocyanin and incubated for 10 h. Seedlings were stained for β-glucuronidase activity and cleared for microscopy analysis. Photographs show representative individuals from at least 30 stained plants (scale bars = 500 µm).

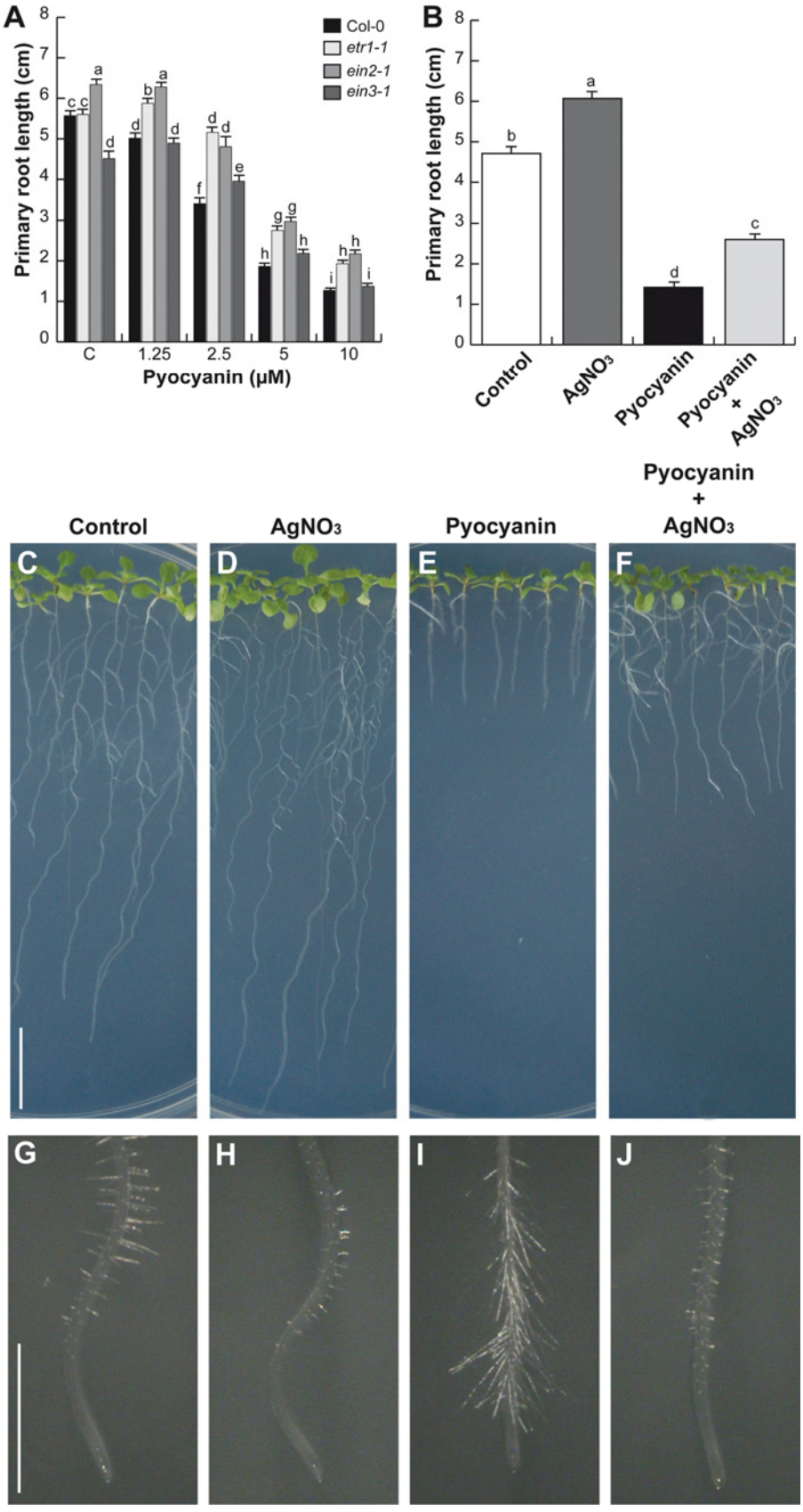

Fig. 8. Role of ethylene signaling in pyocyanin-induced primary root inhibition of *Arabidopsis* seedlings. **A,** *Arabidopsis thaliana* wild-type (WT) and *etr1-1, ein2-1*, and *ein3-1* ethylene mutant seedlings were grown for 12 days on 0.2× Murashige-Skoog (MS) medium supplemented with the indicated concentrations of pyocyanin. **B,** *Arabidopsis* WT (Col-0) seedlings were grown for 12 days on 0.2× MS agar medium supplemented with 5 μM pyocyanin and $AgNO_3$. Data points show the mean ± standard deviation ($n = 30$). Representative photographs of **C** to **F,** *Arabidopsis* root system architecture and **G** to **J,** root hair development under the different treatments are shown. Different letters indicate means that differ statistically at $P < 0.05$. The experiment was repeated three times with similar results.

PCN induces ROS production dependent of ethylene signaling.

An important toxicity mechanism by which PCN damages eukaryotic hosts is the production of ROS (Liu and Nizet 2009). To test whether the effects of PCN on primary root growth were accompanied by an induction of ROS, we analyzed ROS accumulation in primary root tips by confocal microscopy using fluorochromes to detect total ROS and superoxide (O^{2-}). To test the role of ethylene signaling in ROS induction by PCN, we grew *Arabidopsis* seedlings on 0.2× MS medium supplemented with or without 5 μM PCN, $AgNO_3$, or PCN plus $AgNO_3$ and, 7 d.a.g., the seedlings were incubated with 2′,7′-dichlorofluorescein diacetate (H2DCF-DA) or dihydroethidium (DHE) to detect total ROS and O^{2-} in the primary root tip by confocal microscopy. As expected, we found that total ROS and O^{2-} increased in plants treated with PCN (Fig. 9C and H). This increase in ROS was similar to that induced by paraquat, a generator of ROS commonly used to evaluate ROS production in different systems (Fig. 9E and J). Interestingly, when plants are supplied with the ethylene perception blocker $AgNO_3$, the levels of ROS and O^{2-} were reduced in control seedlings (Fig. 9B and G) or in seedlings supplied with PCN (Fig. 9D and I). Quantification of fluorescence confirms that PCN provokes an ROS accumulation which is dependent of ethylene signaling (Fig. 9K and L).

In another series of experiments, we analyzed the levels of H_2O_2 in ethylene-related mutants treated with different concentrations of PCN, whose levels clearly changed in *Arabidopsis* root tips (Fig. 10). We found that PCN reduces the levels of H_2O_2 on primary root tips in a dose-dependent way (Fig. 10A to F). However, in *etr1-1*, *ein2-1*, and *ein3-1* seedlings, the levels of H_2O_2 were sustained even at concentrations of 1.25 and 2.5 μM PCN that drastically affect root growth (Fig. 10G to X). This sustained production of H_2O_2 indicates that the PCN mechanism of signaling involves the ethylene pathway and that it is probably related to the resistance of primary root growth when the plants are grown on PCN.

DISCUSSION

Plant roots are colonized by an immense number of microbes, referred to as the root microbiome. Selected strains of beneficial soilborne bacteria can protect against abiotic stress and prime the plant immune system against a broad range of pathogens. *Pseudomonas* spp. rhizobacteria represent one of the most abundant genera of the root microbiome. Rhizobacteria can influence root architecture; most prominently, by enhancing lateral root formation and root hair development. This can be done by producing phytohormones or bacterial QS signals that are perceived at the root tip to adjust cell proliferation and growth.

Our previous work has shown that co-cultivation of *Arabidopsis* seedlings with *P. aeruginosa* inhibits primary root growth, which is determined by the rate of cell division in the meristematic zone and the extent of cell expansion in the elongation zone. This leads to an acceleration of lateral root growth as a result of increased rates of cell division in the pericycle (Ortiz-Castro et al. 2011). Interestingly, co-cultivation of *Arabidopsis* with the QS-related mutants *rhlI–*, *lasI–*, and *rhlI–/lasI–* caused a decreased inhibition of root growth and a concomitant phytostimulation (Fig. 1), which can be likely explained by either a decreased production of virulence factors or stimulation of root developmental processes as *P. aeruginosa lasI–* and *rhlI–/lasI–* mutants overproduce cyclodipeptides with auxin activity (Ortiz-Castro et al. 2011). Most likely, the beneficial effects of co-cultivation with *lasI–* and *rhlI–/lasI–* bacterial strains may be due to a combination of both processes.

The beneficial effect of *Pseudomonas* spp. to plants by means of regulating root architecture was recently confirmed by Zamioudis and associates (2013). By employing a germ-free experimental system, the authors showed the ability of selected *Pseudomonas* strains to promote plant growth and drive developmental plasticity in the roots of *Arabidopsis* by inhibiting primary root elongation and promoting lateral root and root hair formation. By studying cell-type-specific developmental markers and employing genetic and pharmacological approaches, it was demonstrated the crucial role of auxin signaling and transport in rhizobacteria-stimulated changes in the root system architecture of *Arabidopsis*. The authors further show that *Pseudomonas* spp.-elicited alterations in root morphology and that rhizobacteria-mediated systemic immunity are mediated by distinct signaling pathways.

Root growth depends on maintaining the proper balance between cell division and differentiation. In the primary root, cells originate from a stem cell center at the tip. Progeny of these stem cells rapidly divide in a transit-amplifying zone known as the meristem, after which they undergo massive increases in cell volume in the elongation zone. Once fully elongated, cells enter the maturation zone, in which they differentiate into various cell types.

One of the factors of virulence and survival of *P. aeruginosa* is the production of secondary metabolites (i.e., phenazines, which have antibiotic properties) including PCN (1-hydroxy-5-methylphenazine), a blue-green pigment with redox properties (Lau et al. 2004a; Liu and Nizet 2009). PCN synthesis is regulated by the *lasR* and *rhlR* QS systems (de Kievit and Iglewski 2000; Rumbaugh et al. 2000; Schaber et al. 2004; Siehnel et al. 2010) and, in agreement with these previous results, we found a decreased production of PCN in *lasI* and *rhlI/lasI P. aeruginosa* mutants (Fig. 3). Although all three QS-related *P. aeruginosa* mutants tested have a significant defect in PCN production, the *rhlI* mutant inhibited growth and affected root architecture similarly to the WT Pao1 strain. This result indicates that loss of PCN production is not the only factor by which *P. aeruginosa* inhibits root growth or decreases H_2O_2 level in the root tip. A second factor which affects root growth and is present in *rhlI* mutants is C12-AHL. Our previous work demonstrated that C12-AHL but not C4- or C6-AHL is very active in inhibiting primary root growth (Ortiz-Castro et al. 2008), thus explaining why *rhlI* mutants still repress primary root growth.

PCN can easily penetrate biological membranes and directly accept electrons from reducing agents such as NADPH and reduced glutathione, then transfer the electrons to oxygen to generate ROS such as H_2O_2 and O^{2-} at the expenses of host antioxidant systems such as glutathione and catalase (O'Malley et al. 2004). Several reports have documented that PCN is an important virulence factor of *P. aeruginosa*. Its induction through quorum signaling correlated with the biofilm growth stage of the bacterium. Although PCN has a wide range of toxic effects in animal cells, the proposed basis of its toxicity is production of O^{2-} anions and downstream ROS by oxidizing NADPH (O'Malley et al. 2004; Lau et al. 2004a; Liu and Nizet 2009). To the best of our knowledge, there is a lack of information about the levels of PCN released by *P. aeruginosa* when colonizing plant roots. However, some reports, mainly from animal systems, have shown that PCN accumulates in low micromolar levels in *P. aeruginosa* host cells. Wilson and associates (1998) showed that PCN levels varied between 78.5 and 128.5 μM in the sputum of patients with cystic fibrosis (CF). Hunter and associates (2012) analyzed the level of PCN of 47 CF patients, identifying concentrations up to 48 μM in patients with severe CF. In another study by Price-Whelan and associates (2007), the PCN concentration reached 100 μM in

the stationary phase. Dietrich and associates (2006) showed that PCN activates genes related to redox homeostasis, iron acquisition, and virulence by using DNA microarrays and quantitative reverse-transcriptase polymerase chain reaction. In this work, the authors reported that *P. aeruginosa* PAO1 and PA14 produce 10 and 55 µM PCN, respectively, during the stationary phase, and demonstrated that PCN can act as signaling molecule at these concentrations.

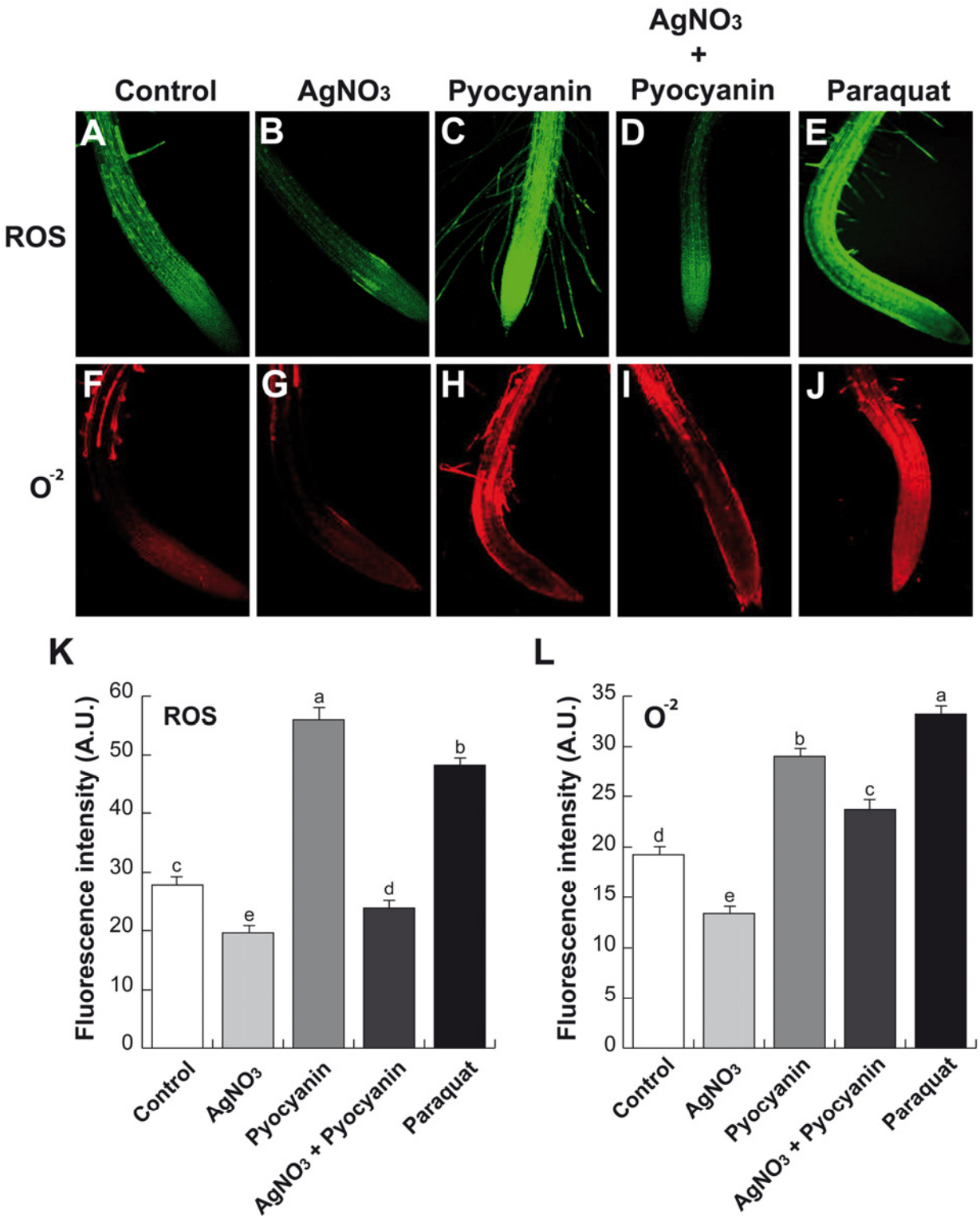

Fig. 9. Role of ethylene signaling in reactive oxygen species (ROS)-induced production by pyocyanin. Representative photographs of the detection of endogenous **A** to **E,** ROS and **F** to **J,** O^{2-} with 2′,7′-dichlorofluorescein diacetate (H2DCF-DA) and dihydroethidium (DHE), respectively, which were determined in primary roots of *Arabidopsis* seedlings grown for 7 days on 0.2× Murashige-Skoog agar medium supplemented with 5 µM pyocyanin and $AgNO_3$ or with 0.1 µM paraquat. H2DCF-DA and DHE fluorescence signals from primary root tips (n = 10) for **K,** ROS and **L,** O^{2-} were quantified using the ImageJ program. The graph is expressed in arbitrary units. Values in K and L represent the mean ± standard deviation (n = 30). Different letters are used to indicate means that differ statistically at $P < 0.05$. The experiment was repeated three times with similar results. Photographs are representative individuals of at least 10 seedlings analyzed. Scale bar = 100 µm.

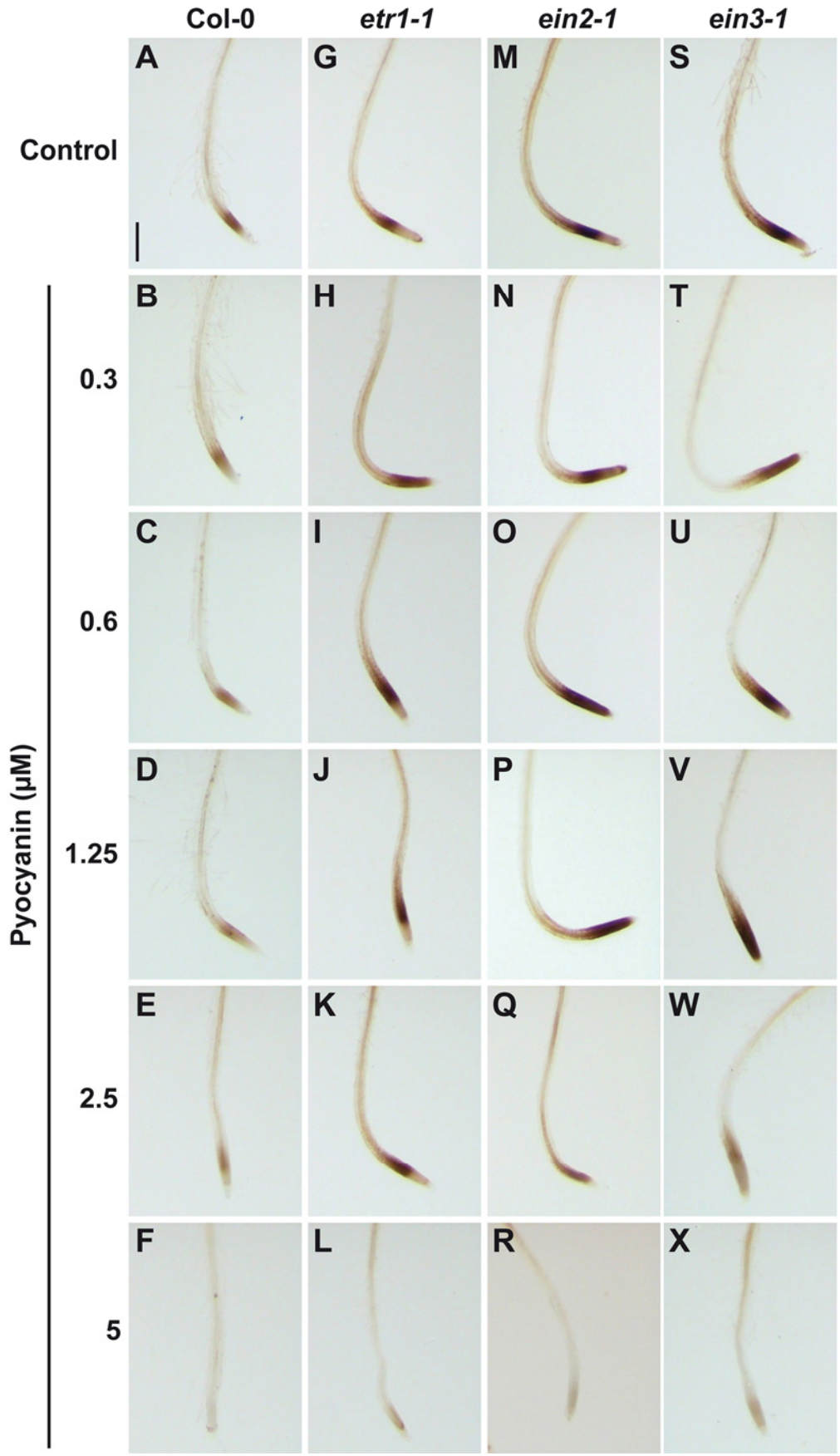

Fig. 10. Effect of pyocyanin on H_2O_2 accumulation in the primary root meristem of *Arabidopsis thaliana* wild-type (WT) (Col-0) and ethylene-related mutants *etr1*, *ein2*, and *ein3*. Histochemical detection of H_2O_2 with 3,3′-diaminobenzidine staining in **A** to **F,** *Arabidopsis thaliana* WT (Col-0) and **G** to **L,** *etr1-1*; **M** to **R,** *ein2-1*; and **S** to **X,** *ein3-1*. *Arabidopsis* seedlings were grown for 7 days on 0.2× Murashige-Skoog medium supplemented with the indicated concentrations of pyocyanin. Photographs show representative individuals from at least 30 stained plants. Scale bar = 500 µm.

Based on the finding that PCN is capable of killing fungi and is toxic to nematodes, we hypothesized that the eukaryotic cellular pathways that are affected by PCN could be evolutionarily conserved and, therefore, by using a plant model system, it would be possible to define whether PCN causes toxicity to cells or regulates fundamental cellular processes such as division, elongation, or differentiation at concentrations naturally present when colonizing host cells. Moreover, diverse bacterial species proliferate in the rhizosphere and release PCN and other phenazines with potential biocontrol activities (Bosgelmez-Tinaz 2003; Fuqua and Greenberg 2002). This would suggest that natural phenazines such as phenazine-1-carboxylic acid and PCN can accumulate in the plant rhizosphere in amounts sufficient not only for inter- and intraspecies signaling but also for the direct inhibition of competing organisms.

Despite numerous reports of PCN-mediated cellular injuries, the response of plant cells or whole organs to *P. aeruginosa*-produced PCN is unknown. A lack of information might have led to an underestimation or miss-estimation of the mechanisms by which *P. aeruginosa* causes cell damage or phytostimulation. Knowledge about the activity of PCN in plants and the cellular pathways that are affected may be of practical value in agriculture, and it was the objective of this research to clarify some aspects of PCN activity in *Arabidopsis* seedlings. We found that PCN can be directly perceived by roots to adjust growth and development and no toxicity symptoms were evident, indicating that the activity of PCN in plants might be rather different from that reported for animal cells. PCN was found to inhibit primary root growth and stimulate lateral root and root hair formation in a dose-dependent way in low micromolar concentrations (Figs. 4 and 5). In this regard, PCN activity is similar to the previously reported activities of other bacterial QS signals (namely, C12-AHL), which regulate root system architecture in a highly specific way, depending on the length of the acyl-side chain (Ortiz-Castro et al. 2008). These results indicate that bacteria may affect root development by producing not only AHL but also PCN and, possibly, other phenazines with signaling roles in plant cells.

Accumulating evidence indicates that ROS play an essential role in the basic mechanism of cell growth and in the establishment of cell shape. This fundamental role in cell growth is likely to be widespread in plant parts, as shown in the polarized tip growth of root hairs. These structures are long, thin extensions growing out perpendicularly from trichoblasts, one of the cell types of the root epidermis. The presence of root hairs greatly increases the surface area of the root available for the absorption of nutrients and water and for interaction with soil particles and bacteria. Because PCN decreases trichoblast cell length and increases root hair elongation (Fig. 5), it is tempting to speculate that plant perception of QS signals and PCN affect both the production and localization of ROS and then the growth mechanisms that determine the shape of trichoblast change. Our data indicating that PCN affects primary roots, root hairs, and lateral root development through production of ROS are consistent with available genetic and pharmacological evidences. For instance, the ROOT HAIR DEFECTIVE2 (RHD2)/AtrbohC protein defective on a respiratory burst oxidase homolog (RBOH) enzyme, which catalyzes the reduction of oxygen to generate the O^{2-} anion, is required for root hair elongation. The roots of plants homozygous for loss-of-function *rhd2* mutations have decreased levels of ROS and are 20% shorter than the WT, indicating that cell expansion is defective in these plants (Foreman et al. 2003; Renew et al. 2005). On the other hand, by using inhibitors such as diphenylene iodonium, it has been suggested that NOX-derived ROS control cell expansion in maize (*Zea mays*) roots (Liszkay et al. 2004). Recently, it was found that silencing PvRbohB in transgenic *Phaseolus vulgaris* roots had a negative impact on LRD. In this work, the downregulation of PvRbohB affected both the growth and ROS levels in young lateral roots. Interestingly, the PvRbohB promoter was induced during lateral root primordium initiation in the pericycle, and remained active throughout lateral root development. This study identifies RBOHs as potentially important players in lateral root development in *P. vulgaris.* The particular impact of such regulation of root hair and lateral root growth by bacterial molecules such as PCN in the interactions between plants and bacteria remains to be determined. The above-described information indicates that ROS-mediated configuration of the root system is not an *Arabidopsis*-specific response and, thus, PCN might be active in crops.

Although root treatment with PCN did not induce visible cell death in transgenic *Arabidopsis* seedlings expressing the *AtHisH2B:YFP* marker stained with PI, a marked reduction in root meristem length and expression of *CycB1:uidA* and *AtPRZ1:uidA* was observed (Fig. 6), indicating that PCN repress cell division. It could be proposed that the PCN-induced generation of ROS might lower proliferating cell activity, thus decreasing primary root growth. These results suggest that redox regulation plays an important role in maintaining root meristem activity. Moreover, this is supported by previous findings that differences in O^{2-} and H_2O_2 accumulation in the root tip significantly affect root growth and differentiation (Dunand et al. 2007; Tsukagoshi et al. 2010). Our data indicate that PCN modulates the balance between cell proliferation and differentiation by directly regulating the accumulation of ROS in the root tip.

Contradictory information exists regarding the role of plant hormones in regulation of ROS production. Transcriptional regulation of ROS controls transition from proliferation to differentiation in the *Arabidopsis* primary root. This pathway seems to function independently of auxin and cytokinin signaling (Tsukagoshi et al. 2010). In contrast, in *Lepidium sativum* (cress), five respiratory burst oxidase homologs (Lesarbohs) were sequenced and it was found that their expression patterns were similar to their *Arabidopsis* orthologs throughout the life cycle. Cress plants in which *LesarbohB* expression was knocked down showed a root phenotype associated with defective auxin-related genes (Müller et al. 2012). These transgenic plants further displayed altered expression of auxin marker genes, including those encoding the auxin-responsive proteins 14 and 5 (IAA14 and IAA5), and LATERAL ORGAN BOUNDARIES DOMAIN16, an auxin-responsive protein implicated in lateral root initiation. It was speculated that ROS produced by rbohs play a role in root development via auxin signaling. Our data are in agreement with those of Tsukagoshi and associates (2010) in that the effects of PCN on ROS induction are independent of auxin signaling, considering the following evidence. First, PCN did not activate the expression of the auxin-inducible reporter markers *DR5:uidA* and *BA3:uidA* (Fig. 7); second, the auxin-related *tir1afb2afb3*, *arf7 arf19*, *axr1-3,* and *aux1-7* mutants displayed similar primary root growth inhibition in response to PCN when compared with WT seedlings. In addition, the primary root growth of cytokinin- and abscisic acid-related mutants also were normally inhibited by PCN, indicating that the genes defective in these mutants are unlikely mediating the cellular effects of PCN in the primary root.

The ROS distribution at the primary root tip shows that localized O^{2-} accumulation in the meristematic zone is necessary for proliferation, whereas H_2O_2 accumulates in the elongation zone when cells arrest division and begin differentiation (Tsukagoshi et al. 2010). Because PCN is a redox-active compound and has been demonstrated before to be capable of generating ROS in animal systems, we investigated whether PCN

treatment activates the oxidative machinery of *Arabidopsis* roots. By means of a combination of fluorophores that specifically react with ROS and using confocal microscopy, we found that PCN supply to *Arabidopsis* seedlings grown in vitro leads to enhanced ROS and O^{2-} levels in primary root tips. PCN treatment increased O^{2-} accumulation in the root elongation zone (Fig. 9), while co-cultivation with *Pseudomonas aeruginosa* (Fig. 2) or PCN supply (Fig. 10) decreased H_2O_2 accumulation in the same region, which was coincident with the inhibitory effects of PCN on cell division and elongation. Thus, disrupting the spatial distribution of O^{2-} or H_2O_2 may compromise normal root growth. Staining for the presence of these ROS in the root showed a clear correlation between growth rate and the relative distribution of different ROS species in the meristematic and elongation zones. Interestingly, differences in the localization of O^{2-} and H_2O_2 in seedlings treated with PCN (Fig. 9) or co-cultivated with *P. aeruginosa* WT and QS-related mutants *lasI*, *rhlI*, and *rhlI/lasI* (Fig. 2) suggest that these ROS can function as intercellular signaling molecules and not only as toxicity factors, as reported in animal cells.

The PCN-elicited accumulation of ROS was partially blocked when supplied together with the ethylene blocker $AgNO_3$ (Fig. 9), and the H_2O_2 decrease was lower in the ethylene-related mutants *etr1-1*, *ein2-1*, and *ein3-1* than in WT seedlings. These data correlate with the greater resistance of ethylene-related mutants *etr1* and *ein2-1* to primary root growth inhibition caused by PCN (Fig. 8), further indicating that ethylene plays an important role in mediating the ROS response to PCN. To the best of our knowledge, the particular distribution of O^{2-} and H_2O_2 in primary root tips of ethylene-related mutants has not been previously investigated. However, while analyzing the flg22-triggered ROS production in *Arabidopsis* seedlings, Mersmann and associates (2010) identified ethylene signaling as a critical component of the oxidative burst in response to this bacterial elicitor because *etr1-1* and *ein2-1* mutants were strongly diminished in flg22-induced ROS accumulation. Ethylene has diverse functions in plant–microbe interactions (van Loon et al. 2006). It is important for defense against necrotrophic fungi (Chagué et al. 2006) but its contribution to bacterial resistance remains unclear. Our data demonstrated that, among the PCN responses tested, the ethylene-insensitive mutants were resistant to the PCN effect, decreasing H_2O_2 accumulation in the elongation zone of the primary root (Fig. 10). This suggests that ethylene plays a dual function in response to bacterially produced PCN: it may contribute to defense responses, possibly through regulation of ROS production, and, at the same time, in ROS-modulated root system architecture. Our work underscores the importance of PCN as a signaling molecule in plant–bacteria interactions as a modulator of cellular programs that determine the configuration of the root system. Understanding the contribution of QS in pathogenesis and symbiosis, particularly the role played by AHL in the production of virulence factors or compounds with a role in auxin (Ortiz-Castro et al. 2011) or ethylene signaling (this work), should contribute to the development of new strategies for protecting plants against pathogens or increase plant productivity.

MATERIALS AND METHODS

Plant material and growth conditions.

A. thaliana (Col-0); the transgenic lines *CycB1:uidA* (Colón-Carmona et al. 1999), *AtPRZ1:uidA* (Sieberer et al. 2003), *DR5:uidA* (Ulmasov et al. 1997), *BA3:uidA* (Oono et al. 1998), and histone *AtHisH2B:YFP* (Boisnard-Lorig et al. 2001); and mutant lines *etr1-1* (Hua and Meyerowitz 1998), *ein2-1* (Guzmán and Ecker 1990), *ein3-1* (Chao et al. 1997), *tir1afb2afb3* (Dharmasiri et al. 2005), *arf7arf19* (Okushima et al. 2007), *aux1-7* (Pickett et al. 1990), *axr1-3* (Lincoln et al. 1990), *abi1* (Ma et al. 2009), *abi3* (Koornneef et al. 1984; Nambara et al. 1992), and *cre1-12ahk2-2* and *cre1-12ahk3-3* (Higuchi et al. 2004; Mähönen et al. 2006) were used for all experiments. Seed were surface sterilized with 95% (vol/vol) ethanol for 5 min and 20% (vol/vol) bleach for 7 min. After five washes with sterile distilled water, seed were germinated and grown on agar plates containing 0.2× MS medium (Murashige and Skoog 1962). MS medium (Murashige and Skoog basal salts mixture; catalog M5524) was purchased from Sigma-Aldrich (St. Louis). The suggested formulation is salts at 4.3 g $liter^{-1}$ for a 1× concentration of medium; we used 0.9 g $liter^{-1}$, which we consider and refer to as 0.2× MS. This medium lacks amino acids and vitamins. PCN was purchased from Sigma-Aldrich. The compound was dissolved in dimethyl sulfoxide and used at the indicated concentrations. In control seedlings, we added the solvent in amounts equal to those present in the greatest concentration of compound tested. Phytagar (micropropagation grade) was purchased from Phytotechnology (Shawnee Mission, KS, US.A.). Plants were placed in a plant growth chamber (Percival Scientific AR-95L) with a photoperiod of 16 h of light, 8 h of darkness, light intensity of 100 μmol m^2 s^{-1}, and temperature of 22°C.

In vitro plant-bacteria co-cultivation assays.

Bacterial strains used in this work were *P. aeruginosa* PAO1 (WT) and *P. aeruginosa* single mutants *rhlI*– and *lasI*– and double mutant *rhlI–/lasI*– (Li et al. 2007). The bacterial strains were evaluated in vitro for their pathogenic or plant-growth-promotion ability, using the *Arabidopsis* Col-0 ecotype. Bacterial densities of 2.5×10^8 CFU were inoculated by streaking on agar plates containing 0.2× MS medium. Six-day-old germinated *Arabidopsis* seedlings (10 seedlings per plate) were transferred and located over the bacterial streak site and grown for a further 3-, 6-, and 9-day period. The plates were placed in the growth chamber (Percival Scientific AR-95L) in a completely randomized design. All experiments were replicated at least three times.

Analysis of plant growth and statistical analysis.

Growth of primary roots was registered using a rule. Lateral root number (LRN) was determined by counting the lateral roots present in the primary root from the tip to root/stem transition. LRD was determined by dividing the LRN by the primary root length and was expressed as LRD cm^{-1}. The length of the meristems was determined as the distance between the quiescent centers to the cell file where cells started to elongate. For all experiments, data were statistically analyzed in the SPSS 10 program (SPSS, Chicago). Univariate and multivariate analyses with a Tukey's post hoc test were used for testing differences in growth and root developmental responses in the WT and ethylene-related mutants. Different letters are used to indicate means that differ significantly ($P < 0.05$).

Microscopy.

The *A. thaliana* root system was analyzed with a stereoscopic microscope (Leica MZ6; Leica Microsystems, Wetzlar, Germany). Total lateral roots were counted at ×30 magnification. Primary root meristems were analyzed in semipermanent preparations of cleared roots using a composed microscope (Axiostar Zeiss Plus; Carl Zeiss, Göttingen, Germany) at ×100 or ×400 magnifications. Images were captured with a Sony Cybershot DSC-S75 digital camera (Sony Electonics Inc., Oradell, NJ, U.S.A.) adapted to the microscope and processed with the Zeiss Axio Vision 4AC software (Carl Zeiss).

Histochemical analysis.

Transgenic plants that express the *uidA* reporter gene (Jefferson et al. 1987) were stained in 0.1% 5-bromo-4-chlorium-3-indolyl, β-D-glucuronide in phosphate buffer (NaH_2PO_4 and Na_2HPO_4, 0.1 M, pH 7) with 2 mM potassium ferrocyanide and 2 mM potassium ferricyanide for 12 h at 37°C. Plants were cleared and fixed as previously described by Malamy and Benfey (1997). The processed roots were included in glass slips and sealed with commercial nail varnish. For each marker line and treatment, at least 10 transgenic plants were analyzed.

H_2O_2 production was detected by the endogenous peroxidase-dependent staining procedure using 3,3′-diaminobenzidine (DAB) uptake (Thordal-Christensen et al. 1997). Control or PCN-treated of *A. thaliana* WT and ethylene mutant seedlings were placed in a solution of DAB at 1 mg ml^{-1}, pH 3.8, and incubated in dark for 2 h. Subsequently, they were immersed in boiling 96% (vol/vol) ethanol for 10 min and then stored in 96% (vol/vol) ethanol. For each treatment, at least 15 treated seedlings were analyzed. A representative plant was chosen for each treatment. H_2O_2 production was visualized as a reddish-brown precipitated coloration and photographed using a stereoscopic microscope.

PI staining and YFP detection.

For confocal microscopy, solvent- or PCN-treated transgenic *Arabidopsis* seedlings expressing the histone *AtHisH2B:YFP* construct (Boisnard-Lorig et al. 2001) were mounted on microscope slides into a solution of PI. For fluorescent staining with PI, recently collected plants with intact root systems were transferred to a solution of PI at 10 mg/ml for 3 min. Seedlings were rinsed in water and mounted in 50% glycerol on microscope slides. The same sample was recorded separately at wavelengths specific to both PI fluorescence with an 568 nm excitation line and a emission window of 585to 610 nm, and YFP emission with a 505- to 550-nm band-pass emission filter (488-nm excitation line), after which the two images were merged to produce the final image. Primary root meristems were analyzed by imaging mounted samples with an inverted confocal microscope (Olympus FV1000).

PCN quantification.

PCN was extracted from the supernantant fraction of *P. aeruginosa* grown in Luria-Bertani medium at 37°C for 48 h. Supernatant (1 ml) was mixed with 1 ml of chloroform and the lower organic layer was separated. To this layer, 1 ml of 0.2 HCl was added and the PCN rich organic layer was separated to give a pink to deep-red solution. The absorbance of this solution was measured at 520 nm. Concentrations, expressed as micrograms of PCN produced per milliliter of culture supernantant, were determined according to Essar and associates (1990).

ROS and O^{2-} detection

General ROS and specific O^{2-} anion were monitored by incubating *Arabidopsis* seedlings with 10 μM fluorescent probes H2DCF-DA and DHE, respectively, in 10 mM Tris-HCl (pH 7.4) (Gomes et al. 2005). *Arabidopsis*-treated seedlings were incubated for 30 min in darkness and washed three times for 5 min with fresh buffer. Fluorescence signals from at least 10 treated and control seedlings were detected using a confocal microscope (Olympus FV1000). Fluorescence signals were quantified by counting pixel numbers in the green channel by employing ImageJ software.

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LITERATURE CITED

Boisnard-Lorig, C., Colon-Carmona, A., Bauch, M., Hodge, S., Doerner, P., Bancharel, E., Dumas, C., Haseloff, J., and Berger, F. 2001. Dynamic analyses of the expression of the HISTONE::YFP fusion protein in *Arabidopsis* show that syncytial endosperm is divided in mitotic domains. Plant Cell 13:495-509.

Bosgelmez-Tinaz, G. 2003. Quorum sensing in gram-negative bacteria. Turk. J. Biol. 27:85-93.

Chagué, V., Danit, L. V., Siewers, V., Schulze-Gronover, C., Tudzynski, P., Tudzynski, B., and Sharon, A. 2006. Ethylene sensing and gene activation in *Botrytis cinerea*: A missing link in ethylene regulation of fungus-plant interactions. Mol. Plant-Microbe Interact. 19:33-42.

Chao, Q., Rothenberg, M., Solano, R., Roman, G., Terzaghi, W., and Ecker, J. R. 1997. Activation of the ethylene gas response pathway in *Arabidopsis* by the nuclear protein ETHYLENE-INSENSITIVE3 and related proteins. Cell 27:1133-1144.

Colón-Carmona, A., You, R., Haimovitch-Gal, T., and Doerner, P. 1999. Spatio-temporal analysis of mitotic activity with a labile cyclin–GUS fusion protein. Plant J. 20:503-508.

De Kievit, T. R., and Iglewski, B.H. 2000. Bacterial quorum sensing in pathogenic relationships. Infect. Immun. 68:4839-4849.

De Vleesschauwer, D., Cornelis, P., and Höfte, M. 2006. Redox-active pyocyanin secreted by *Pseudomonas aeruginosa* 7NSK2 triggers systemic resistance to *Magnaporthe grisea* but enhances *Rhizoctonia solani* susceptibility in rice. Mol. Plant-Microbe Interact. 19:1406-1419.

Dharmasiri, N., Dharmasiri, S., and Estelle, M. 2005. The F-box protein TIR1 is an auxin receptor. Nature 435:441-445.

Dietrich, L. E. P., Price-Whelan, A., Petersen, A., Whiteley, M., and Newman, D. K. 2006. The phenazine pyocyanin is a terminal signaling factor in the quorum sensing network of *Pseudomonas aeruginosa*. Mol. Microbiol. 61:1308-1321.

Dunand, C., Crevecoeur, M., and Penel, C. 2007. Distribution of superoxide and hydrogen peroxide in *Arabidopsis* root and their influence on root development: Possible interaction with peroxidases. New Phytol. 174:332-341.

Essar, D. W., Eberly, L., Hadero, A., Crawford, I. P. 1990. Identification and characterization of genes for a second anthranilate synthase in *Pseudomonas aeruginosa*: Interchangeability of the two anthranilate synthases and evolutionary implications. J. Bacteriol. 172:884-900.

Foreman, J., Demidchick, V., and Bothwell, J. H. F. 2003. Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. Nature 422:442-446.

Friesen, M. L., Porter, S. S., Stark, S. C., von Wettberg, E. J., Sachs, J. L., and Martinez-Romero, E. 2011. Microbially mediated plant functional traits. Annu. Rev. Ecol. Evol. Syst. 42:23-46.

Fuqua, C., and Greenberg, E. P. 2002. Listening in on bacteria: Acyl-homoserine lactone signalling. Nat. Rev. Mol. Cell Biol. 3:685-695.

Fuqua, W. C., Winans, S. C., and Greenberg, E. P. 1994. Quorum-sensing in bacteria: The luxR-LuxI family of cell density-responsive transcriptional regulators. J. Bacteriol. 176:269-275.

Gomes, A., Fernandes, E., and Lima, J. L. F. C. 2005. Fluorescence probes used for detection of reactive oxygen species. J. Biochem. Biophys. Methods 65:45-80.

Guzmán, P., and Ecker, J. R. 1990. Exploiting the triple response of *Arabidopsis* to identify ethylene-related mutants. Plant Cell 2:513-523.

Higuchi, M., Pischke, M. S., Mahonen, A. P., Miyawaki, K., Hashimoto, Y., Seki, M., Kobayashi, M., Shinozaki, K., Kato, T., Tabata, S., Heleriutta, Y., Sussman, M. R., and Kakimoto, T. 2004. In planta functions of the *Arabidopsis* cytokinin receptor family. Proc. Natl. Acad. Sci. U.S.A. 101:8821-8826.

Hua, J., and Meyerowitz, E. 1998. Ethylene responses are negatively regulated by a receptor gene family in *Arabidopsis thaliana*. Cell 94:262-271.

Hunter, R. C., Klepa-Ceraj, V., Lorenzi, M .M. Grotzinger, H. Martin, T. R., and Newman, D. K. 2012. Phenazine content in the cystic fibrosis respiratory tract negatively correlates with lung function and microbial complexity. Am. J. Respir. Cell Mol. Biol. 47:738-745.

Jefferson, R. A., Kavanagh, T. A., and Bevan, M. W. 1987. GUS fusion: β-glucuronidase as a sensitive and versatile fusion marker in higher plants. EMBO (Eur. Mol. Biol. Organ.) J. 6:3901-3907.

Koornneef, M., Reuling, G., and Karssen, C. M. 1984. The isolation and

characterization of abscisic acid-insensitive mutants of *Arabidopsis thaliana*. Physiol. Plant. 61:377-383.
Lau, G. W., Goumnerov, B. C., Walendziewicz, C. L., Hewitson, J., Xiao, W., Mahajan-Miklos, S., Tompkins, R. G., Perkins, L. A., and Rahme, L. G. 2003. The *Drosophila melanogaster* toll pathway participates in resistance to infection by the gram-negative human pathogen *Pseudomonas aeruginosa*. Infect. Immun. 71:4059-4066.
Lau, G. W., Hassett, D. J., Ran, H., and Kung, F. 2004a. The role of pyocyanin in *Pseudomonas aeruginosa* infection. Trends Mol. Med. 10:599-606.
Lau, G. W., Ran, H., Kong, F., Hassett, D. J., and Mavrodi, D. 2004b. *Pseudomonas aeruginosa* pyocyanin is critical for lung infection in mice. Infect. Immun. 72:4275-4278.
Li, L. L., Malone, J. E., and Iglewski, B. H. 2007. Regulation of the *Pseudomonas aeruginosa* quorum-sensing regulator VqsR. J. Bacteriol. 189:4367-4374.
Lincoln, C., Britton, J. H., and Estelle, M. 1990. Growth and development of the *axr1* mutant of *Arabidopsis*. Plant Cell 2:1071-1080.
Liszkay, A., van der Zalm, E., and Schopfer, P. 2004. Production of reactive oxygen intermediates by maize roots and their role in wall loosening and elongation growth. Plant Physiol. 136:3114-3123.
Liu, G. Y., and Nizet, V. 2009. Color me bad: Microbial pigments as virulence factors. Trends Microbiol. 17:406-413.
Ma, Y., Szostkiewicz, I., Korte, A., Moes, D., Yang, Y., Christmann, A., and Grill, E. 2009. Regulators of PP2C phosphate activity function as abscisic acid sensors. Science 324:1064-1068.
Mahajan-Miklos, S., Tan M. W., Rahme, L. G., and Ausubel, F. M. 1999. Molecular mechanisms of bacterial virulence elucidated using a *Pseudomonas aeruginosa-Caenorhabditis elegans* pathogenesis model. Cell 96:47-56.
Mähönen, A. P., Bishopp, A., Higuchi, M., Nieminen, K. M., Kinoshita, K., Törmäkangas, K., Ikeda, Y., Oka, A., Kakimoto, T., and Helariutta, Y. 2006. Cytokinin signaling and its inhibitor AHP6 regulate cell fate during vascular development. Science 311:94-98.
Malamy, J. E., and Benfey, P. N. 1997. Organization and cell differentiation in lateral roots of *Arabidopsis thaliana*. Development 124:33-44.
Mathesius, U., Mulders, S., Gao, M., Teplitski, M., Caetano-Anollés, G., Rolfe, B. G., and Bauer, W. 2003. Extensive and specific responses of a eukaryote to bacterial quorum-sensing signals. Proc. Natl. Acad. Sci. U.S.A. 100:1444-1449.
Mavrodi, D. V., Bunsall, R. F., Delaney, S. M., Soule, M. J., Phillips, G., and Thomashow, L. S. 2001. Functional analysis of genes for biosynthesis of pyocyanin and phenazine-1-carboxamide from *Pseudomonas aeruginosa* PAO1. J. Bacteriol. 183:6454-6465.
Mersmann, S., Bourdais, G., Rietz, S., and Robatzek, S. 2010. Ethylene signaling regulates accumulation of the FLS2 receptor and is required for the oxidative burst contributing to plant immunity. Plant Physiol. 154:391-400.
Miller, M. B., and Bassler, B. L. 2001. Quorum sensing in bacteria. Annu. Rev. Microbiol. 55:165-199.
Müller, K., Linkies, A., Leubner-Metzger, G., and Kermode, A. R. 2012. Role of a respiratory burst oxidase of *Lepidium sativum* (cress) seedlings in root development and auxin signalling. J. Exp. Bot. 63:6325-6334.
Murashige, T., and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. 15:473-497.
Nambara, E., Naito, S., and McCourt, P. 1992. A mutant of *Arabidopsis* which is defective in seed development and storage protein accumulation is a new *abi3* allele. Plant J. 2:435-441.
Okushima, Y., Fukaki, H., Onoda, M., Theologis, A., and Tasaka, M. 2007. ARF7 and ARF19 regulate lateral root formation via direct activation of LBD/ASL genes in *Arabidopsis*. Plant Cell 19:118-130.
O'Malley, Y. Q., Reszka, K. J., Spitz, D. R., Denning, G. M., and Britigan, B. E. 2004. *Pseudomonas aeruginosa* pyocyanin directly oxides glutathione and decreases its levels in airway epithelial cells. Am. J. Physiol. Lung Cell Mol. Physiol. 287:94-103.
Oono, Y., Chen, Q. G., Overvoorde, P. J., Kohler, C., and Theologis, A. 1998. *age* mutants of *Arabidopsis* exhibit altered auxin-regulated gene expression. Plant Cell 10:1649-1662.
Ortiz-Castro, R., and López-Bucio, J. 2013. Small molecules involved in transkingdom communication between plants and rhizobacteria. Pages 295-307 in: Molecular Microbial Ecology of the Rhizosphere, Vol. I. F. J. De Bruijn, ed. Wiley-Blackwell, Hoboken, NJ, U.S.A.
Ortiz-Castro, R., Martínez-Trujillo, M., and López-Bucio, J. 2008. N-acyl-L-homoserine lactones: A class of bacterial quorum-sensing signals alter post-embryonic root development in *Arabidopsis thaliana*. Plant Cell Environ. 31:1497-1509.
Ortiz-Castro, R., Contreras-Cornejo, H. A., Macías-Rodriguez, L., and López-Bucio, J. 2009. The role of microbial signals in plant growth and development. Plant Signal. Behav. 4:1-12.
Ortiz-Castro, R., Díaz-Pérez, C., Martínez-Trujillo, M., del Río, R. E., Campos-García, J., and López-Bucio, J. 2011. Transkingdom signaling based on bacterial cyclodipeptides with auxin activity in plants. Proc. Acad. Natl. Sci. U.S.A. 108:7253-7258.
Pickett, F. B., Wilson, A. K., and Estelle, M. 1990. The *aux1* mutation of *Arabidopsis* confers both auxin and ethylene resistance. Plant Physiol. 94:1462-1466.
Price-Whelan, A., Dietrich L. E. P., and Newman, D. K. 2007. Pyocyanin alters redox homeostasis and carbon flux through central metabolic pathways in *Pseudomonas aeruginosa* PA14. J. Bacteriol. 189:6372-6381.
Rada, B., and Leto, T. 2013. Pyocyanin effects on respiratory epithelium: Relevance in *Pseudomonas aeruginosa* airway infections. Trends Microbiol. 21:73-81.
Renew, S., Heyno, E., Schopfer, P., and Liszkay, A. 2005. Sensitive detection and localization of hydroxyl radical production in cucumber roots and *Arabidopsis* seedlings by spin trapping electron paramagnetic resonance spectroscopy. Plant J. 44:342-347.
Rumbaugh, K. P., Griswold, J. A., Iglewski, B. H., and Hamood, A. N. 2000. The role of quorum sensing in the in vivo virulence of *Pseudomonas aeruginosa*. Microbes Infect. 2:1721-1731.
Schaber, J. A., Carty, N. L., McDonald, N. A., Graham, E. D., Cheluvappa, R., Griswold, J. A., and Hamood, A. N. 2004. Analysis of quorum sensing-deficient clinical isolates of *Pseudomonas aeruginosa*. J. Med. Microbiol. 53:841-853.
Sieberer, T., Hauser, M. T., Seifert, G. J., and Lusching, C. 2003. PROPORZ1, a putative *Arabidopsis* transcriptional adaptor protein, mediates auxin and cytokinin signals in the control of cell proliferation. Curr. Biol. 13:837-842.
Siehnel, R., Traxler, B., An, D. D., Parsek, M. R., Schaefer, A. L., and Singh, P. K. 2010. A unique regulator controls the activation threshold of quorum-regulated genes in *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. U.S.A. 107:7916-7921.
Thordal-Christensen, H., Zhang, Z., Wei, Y., and Collinge, D. B. 1997. Subcellular localization of H_2O_2 in plants. H_2O_2 accumulation in papillae and hypersensitive response during the barley-powdery mildew interaction. Plant J. 11:1187-1194.
Tsukagoshi, H., Busch, W., and Benfey, P. B. 2010. Transcriptional regulation of ROS controls transition from proliferation to differentiation in the root. Cell 143:606-615.
Ulmasov, T., Murfett, J., Hagen, G., and Guilfoyle, T. 1997. Aux/IAA proteins repress expression of reporter genes containing natural and highly active synthetic auxin response elements. Plant Cell 9:1963-1971.
van Loon, L. C., Geraats, B. P. J., and Linthorst, H. J. M. 2006. Ethylene as a modulator of disease resistance in plants. Trends Plant Sci. 11:184-191.
Venturi, V. 2006. Regulation of quorum sensing in *Pseudomonas*. FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Rev. 30:274-291.
Verhagen, B. W. M., Trotel, P., Couderchet, M., Höfte, M., and Azziz, A. 2010. *Pseudomonas* spp.-induced systemic resistance to *Botrytis cinerea* is associated with induction and priming of defence responses in grapevine. J. Exp. Bot. 61:249-260.
von Rad, U., Klein, I., Dobrev, P. I., Kottova, J., Zazimolova, E., Fekete, A., Hartmann, A., Schmitt-Kopplin, P., and Durner, J. 2008. Response of *Arabidopsis thaliana* to N-hexanoyl-DL-homoserine-lactone, a bacterial quorum sensing molecule produce in the rhizosphere. Planta 229:73-85.
Wilson, R., Sykes, D. A., Watson, D., Rutman, A., Taylor, G. W., and Cole, P. J. 1998. Measurement of *Pseudomonas aeruginosa* phenazine pigments in sputum and assessment of their contribution to sputum toxicity for respiratory epithelium. Infect. Immun. 56:2515-2517.
Zamioudis, C., Mastranesti, P., Dhonukshe, P., Blilou, I., and Pieterse, C. M. J. 2013. Unraveling root developmental programs initiated by beneficial *Pseudomonas* spp. bacteria. Plant Physiol. 162:304-318.