Plants Respond to Pathogen Infection by Enhancing the Antifungal Gene Expression of Root-Associated Bacteria

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Plant health and fitness widely depend on interactions with soil microorganisms. Some bacteria such as pseudomonads can inhibit pathogens by producing antibiotics, and controlling these bacteria could help improve plant fitness. In the present study, we tested whether plants induce changes in the antifungal activity of root-associated bacteria as a response to root pathogens. We grew barley plants in a split-root system with one side of the root system challenged by the pathogen *Pythium ultimum* and the other side inoculated with the biocontrol strain *Pseudomonas fluorescens* CHA0. We used reporter genes to follow the expression of ribosomal RNA indicative of the metabolic state and of the gene *phlA*, required for production of 2,4-diacetylphloroglucinol, a key component of antifungal activity. Infection increased the expression of the antifungal gene *phlA*. No contact with the pathogen was required, indicating that barley influenced gene expression by the bacteria in a systemic way. This effect relied on increased exudation of diffusible molecules increasing *phlA* expression, suggesting that communication with rhizosphere bacteria is part of the pathogen response of plants. Tripartite interactions among plants, pathogens, and bacteria appear as a novel determinant of plant response to root pathogens.

Plant roots interact with a multitude of beneficial and antagonistic microorganisms which significantly affect plant fitness (van der Heijden et al. 2008). Plants allocate a considerable fraction of their assimilates to the root system and exude large amounts of mono- and polysaccharides, organic acids, phenolic compounds, amino acids, and proteins (Bais et al. 2006). These exudates function as resources selecting and fueling a specific bacterial community (Mazzola 2002). In return, symbiotic rhizosphere microorganisms provide services to the plant, including protection against fungal pathogens by producing antibiotics and eliciting plant defense (Compant et al. 2005).

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Plants adjust the quantity and composition of exudates in response to root pathogens and symbionts, bacterial products, and nutrient availability (Paterson et al. 2006; Phillips et al. 2004); and exudate composition modifies the gene expression pattern of root-associated bacteria (Mark et al. 2005). This suggests that plants actively interact with these bacteria (Badri and Vivanco 2009), and we hypothesized that plants can stimulate the production of antifungal compounds of rhizosphere bacteria in response to the presence of pathogens. We investigated this tripartite interaction between plants, pathogens, and antagonistic bacteria in a split-root system with barley. One side of the root system was infected with the oomycete *Pythium ultimum*, a pathogen causing damping-off disease (Martin and Loper 1999). The other side was inoculated with the biocontrol bacterium *Pseudomonas fluorescens* CHA0, an efficient rhizosphere colonizer which protects crop plants against pathogenic fungi by producing antifungal compounds (Haas and Keel 2003; Voisard et al. 1994). Preventing direct contact between biocontrol bacteria and fungal pathogen, the split-root system allowed investigating pathogen-induced but plant-mediated effects on bacterial gene expression. We used reporter fusions to follow in situ the expression pattern of the ribosomal RNA operon *rrnBP1*, indicating the bacterial metabolic state (Ramos et al. 2000), and the antifungal gene *phlA*, involved in the synthesis of 2,4-diacetylphloroglucinol (DAPG) (Bangera and Thomashow 1999; Schnider-Keel et al. 2000). DAPG is a major biocontrol toxin of *P. fluorescens* CHA0 and strongly inhibits *Pythium* spp. growth (de Souza et al. 2003). In a separate experiment, we screened root exudates of noninfected and infected barley plants and explored potential signal molecules involved in the communication between plants and biocontrol pseudomonads. Using high-performance liquid chromatography (HPLC), we analyzed the changes in exudation patterns after infection and confirmed the potential of the candidate signal molecules to elicit in vitro the expression of antifungal genes by *P. fluorescens*.

RESULTS

Effect of *Pythium* and *Pseudomonas* spp. on plant growth.

Plant fresh weight at harvest was 112.9 ± 6.2 mg (mean $\pm$ standard error). Infection by *Pythium ultimum* reduced plant biomass by 30% in noninoculated plants ($F_{1,14} = 26.6$, $P = 0.001$) but had no effect on plants inoculated with *P. fluorescens*.

($F_{1,14} = 0.1$, $P = 0.748$). Infection affected neither the shoot-to-root ratio of barley plants ($F_{1,14} = 1.26$, $P = 0.280$) nor the biomass of roots of the two root halves ($F_{1,14} = 0.30$, $P = 0.590$), suggesting that pathogen infection did not result in preferential allocation of plant resources toward the uninfected root half.

Plant impact on bacterial metabolism.

On average, roots were colonized by bacteria at $5.28 \pm 1.1 \times 10^7 \text{ g}^{-1}$ of root. The colonization was not significantly affected by infection with *Pythium ultimum* ($F_{1,25} < 0.1$, $P = 0.980$), and bacterial basal activity (rRNA expression) was not influenced by infection ($F_{1,25} < 0.1$, $P = 0.93$). *Pythium* infection, however, modified the gene expression pattern of *P. fluorescens*. In control plants, the expression of *phlA* in *P. fluorescens* was negatively correlated with the expression of the ribosomal operon *rrnBP1* ($r = -0.75$, $P = 0.001$), suggesting a competition between bacterial investment in growth versus investment in the production of antibiotics. In contrast, gene expression in bacteria colonizing infected plants was reversed ($F_{1,25} = 20.1$, $P < 0.001$ for treatment–rRNA *rrnBP1* interaction) (Fig. 1). In infected plants, the expression of the two bacterial genes was positively correlated ($r = 0.62$, $P = 0.016$), indicating changes in bacterial metabolism following pathogen infection of the distant root half.

The relative expression of *phlA* increased parallel to root biomass in noninfected plants ($F_{1,25} = 12.3$, $P = 0.004$) (Fig. 2), suggesting that larger plants supported a higher antifungal activity by rhizosphere bacteria. However, the expression of *phlA* was 18% higher in infected plants ($F_{1,25} = 9.4$, $P = 0.004$), and the ratio between relative *phlA* expression and root biomass of infected plants increased 10-fold compared with control plants ($F_{1,12} = 8.4$, $P = 0.013$ for root–treatment interaction) (Fig. 2).

Changes in root exudation after *Pythium* infection.

Vanillic, fumaric, and *p*-coumaric acids were detected in extracts of root exudates. The infection by *Pythium ultimum* modified the exudation of these three compounds ($F_{3,18} = 4.1$, $P = 0.029$ for infection–root side interaction). Exudation of vanillic, *p*-coumaric, and fumaric acids on the local (infected) side was not affected by *Pythium ultimum* (Fig. 3), whereas their exudation on the systemic side increased by factors of 5.1, 4.0, and

Fig. 1. Relationship between the expression of the rRNA *rrnBP1* and *phlA* genes in *Pseudomonas fluorescens* CHA0 on roots of uninfected plants (closed symbols) and plants infected by *Pythium ultimum* (open symbols) in a split-root experiment where one side of the root system was inoculated with *Pythium ultimum* (or left uninoculated) and the other side with *P. fluorescens*. Dashed lines show the respective regression lines for the two treatments.

2.9, respectively. Moreover, in infected plants, the exudation increased with the size of the root system ($F_{3,18} = 3.34$, $P = 0.037$ for infection–root side–root weight interaction).

No inhibitory effects of the tested compounds on the growth of *Pythium ultimum* could be detected even at elevated concentrations (1 mM), suggesting that they do not act as antibiotic against this pathogen.

In vitro effects of root exudates on *phlA* expression.

All tested compounds increased the expression of *phlA*; the effect depended on the concentration of the compounds and could be well modeled with Michaelis-Menten kinetics ($P < 0.001$ for all tested compounds) (Fig. 4). However, the induction of *phlA-gfp* expression differed between the compounds. Half maximal induction was achieved at 2.5, 5.4, and 37.2 μM for vanillic, fumaric, and *p*-coumaric acids, respectively, showing that *phlA* gene expression is most sensitive to vanillic acid.

DISCUSSION

We present a new tripartite interaction among plants, root pathogens, and biocontrol rhizosphere bacteria. As indicated by changes in *phlA* expression, plants responded to pathogen infection by stimulating the activity of antifungal genes of associated rhizobacteria. Because expression levels of *phlA* are directly correlated with DAPG production (Baehler et al. 2005), the observed induction of *phlA* gene expression suggests an increased antifungal activity of the bacteria. Remarkably, no contact with root pathogens was required for this induction, suggesting that signaling from the pathogen to the bacteria was mediated via systemic signaling of the plant. In bacteria colonizing uninfected plants, the expression of *phlA* and *rrnBP1* was negatively correlated, suggesting a trade-off between the investment of resources into primary metabolism and into the production of antibiotics. These observations

Fig. 2. Variation in the expression of the *phlA* gene of *Pseudomonas fluorescens* CHA0 with root biomass on roots of uninfected plants (closed symbols) and plants infected by *Pythium ultimum* (open symbols) in a split-root experiment where one side of the root system was inoculated with *Pythium ultimum* (or left uninoculated) and the other side with *P. fluorescens*. Dashed lines show the respective regression lines for the two treatments. *phlA* expression was normalized using the rRNA expression level as standard. Dashed lines show linear regressions for each treatment.

corroborate with the assumption that rhizosphere bacteria are carbon limited (Normander et al. 1999). On the roots of infected plants, however, this relationship was inverted; the expression of the *phlA* gene was positively correlated with that of *rrnBP1*. This suggests that bacteria were not only more active but also invested these resources into the production of antifungal compounds. This is in agreement with the observations of Phillips and associates (2004) that plants increase root exudation in response to antifungal metabolites such as DAPG, which results in a feedback loop improving biocontrol of fungal pathogens by rhizobacteria. Infected roots and roots inoculated with *P. fluorescens* CHA0 were physically separated; therefore, the results demonstrate that the switch

Fig. 3. Induction of the exudation of vanillic, p-coumaric, and fumaric acid by barley plants grown after infection by *Pythium ultimum* in split-root systems. Exudation was measured in infected (local side) and uninfected roots halves (systemic side) in healthy (control) and infested plants (infected). Exudation is expressed in micromoles per gram of root dry weight. Asterisks indicate differences between selected treatments as indicated by Tukey's honestly significant difference test (N.S., nonsignificant; * and ** indicate $P < 0.05$ and 0.01 , respectively).

in bacterial gene expression was induced via plant signaling. These results contrast previous observations that infection of plant leaves by pathogens (*Botrytis cinerea*) results in down-regulation of the expression of antifungal genes in *P. fluorescens* in the rhizosphere (de Werra et al. 2008). Pathogen attack can induce changes in exudation (Bezemer and van Dam 2005), and plant response may differ for aboveground and below-ground pathogens.

Root biomass also played an important role in the strength of the interaction between rhizosphere bacteria and the fungal plant pathogen. The expression of the *phlA* gene was positively correlated with root biomass, and pathogen infection strengthened this relationship. In barley, exudation is negatively correlated with root size (Liljeroth et al. 1990), which is likely to affect the interaction between plants and rhizosphere bacteria.

Fig. 4. Effect of vanillic, p-coumaric, and fumaric acid on the expression of *phlA* in *Pseudomonas fluorescens* CHA0 grown in minimal ammonium-glucose medium without antibiotics amended with glucose at 4 g liter^{-1} as sole carbon source (OS-Glc). Gene activity was measured as green fluorescence (relative fluorescence) in strain CHA0 carrying the *phlA*-gfp[AAV] reporter fusion. Fluorescence was measured after 6 h and normalized to the control treatment (methanol). The regression curve is based on Michaelis-Menten fitting.

Plant root exudates function as belowground signaling molecules shaping rhizosphere microbial populations (Badri et al. 2009), and exudation is a dynamic process adjusted according to the needs of the plant (Paterson et al. 2006). Both beneficial rhizosphere bacteria and pathogens affect root exudation (Kamilova et al. 2006; Phillips et al. 2004). Root infection results in marked changes in the exudation of phenolic and organic acids (Lanoue et al. 2010; Wurst et al. 2009), and our results show that these changes may form the base of a tripartite interaction among plants, fungal pathogens, and rhizosphere bacteria. Indeed, vanillic and fumaric acid, signal compounds which changed markedly in concentration after pathogen infection, induced the expression of *phlA* even at very low concentration. This suggests that small changes in exudation may result in increased expression of antifungal genes by *P. fluorescens*.

Rhizosphere bacteria and fungal root pathogens are linked via plant carbon resources exuded by roots (Notz et al. 2002; Paterson et al. 2006). Interestingly, neither the expression of *rrnBP1* nor the colonization of roots by *P. fluorescens* was influenced by the infection of roots with *Pythium ultimum*, suggesting that, in addition to resource-based linkages, the interaction between plant-associated microorganisms is modulated by plant signals affecting bacterial secondary metabolism.

Remarkably, local exudation of infected roots was little influenced by the infection with *Pythium ultimum*, whereas exudation of vanillic, *p*-coumaric, and fumaric acids of noninfected roots at the other compartment of the split-root system increased up to fourfold. This is surprising considering the usual increase in secondary metabolite exudation in the presence of pathogens (Bais et al. 2002; Lanoue et al. 2010). This may have been due to degradation of the studied secondary compounds by the pathogen or by reduced resource allocation by the plant to infected roots (Henkes et al. 2008). Changes in root exudation after pathogen infection mainly have been assessed at the scale of the whole root system, overlooking the spatial heterogeneity of exudation. Thus, the split-root system used in this study may help to clarify questions regarding local or systemic exudation of plant roots upon infection.

The induced exudates had no effect on the mycelium growth of *Pythium ultimum*. This is surprising, because vanillic and *p*-coumaric acid have been reported to inhibit fungal growth (Kim et al. 2006; Lanoue et al. 2010) and our results indicate that, in addition to toxicity, organic and phenolic acids form part of the pathogen response of plants by acting as signal molecules for symbiotic rhizobacteria. Beneficial antibiotics such as DAPG also may have phytotoxic side effects (Brazelton et al. 2008), and plants may only benefit from fostering their production in the presence of pathogens. Thus, root exudates not only participate in the control of belowground pathogens (Bais et al. 2005; Lugtenberg and Kamilova 2009) but also are involved in the interaction between plant roots and beneficial rhizobacteria.

The exudation of signal molecules by part of the roots not exposed to the fungal pathogen suggests that plant–bacteria signaling not only results from local interaction with the patho-

gen (e.g., due to nutrient leakage) (Notz et al. 2001; Lanoue et al. 2010) but also is triggered systemically by pathogen infection. Plants can sense and respond to signal compounds produced by beneficial rhizosphere bacteria (Lugtenberg and Kamilova 2009), and bidirectional communication between plants and bacteria may occur. Our results indicate that this signaling allows plants to rapidly respond to fungal pathogens via systemic responses. Further analyses in gnotobiotic systems are needed to test the direction of this signaling as well as the signal cascades involved. These experiments, however, are challenging because they have to overcome the difficulties of distinguishing between plant and bacterial metabolites, and plant signal compounds transformed by rhizobacteria.

Overall, this study proposes plant interaction with beneficial rhizosphere bacteria as a new component of pathogen response. Efficient communication between plants and root-associated bacteria may significantly increase plant fitness under pathogen pressure, and understanding the plant traits linked to this interaction may help to establish new disease-resistant cultivars.

MATERIALS AND METHODS

Microorganisms, plasmids, and culture conditions.

Bacteria and plasmids used in this study are listed in Table 1. The strains were kept on nutrient agar plates with the corresponding antibiotics. Prior to inoculation, bacteria from a single colony were grown in liquid Luria Bertani (Sambrook and Russell 2001) at 25°C for 12 h with agitation (200 rpm). Bacterial cells from early exponential phase cultures (optical density at 600 nm [OD₆₀₀] = 0.3) were harvested by centrifugation (4,600 rpm, 4°C, 10 min), washed in 0.9% NaCl, and resuspended in Neff's modified amoeba saline (AS) (Page 1988). The metabolic state of the bacteria was estimated with a reporter miniTn7-*gfp* chromosomal insert placed under the control of the growth-rate-dependent promoter P_{rrnBP1} (Lambertsen et al. 2004). To follow the expression of the antifungal gene *phlA* in the rhizosphere, a *mcherry*-based reporter fusion placed under the control of the *phlA* promoter on plasmid pME9012 was used (Table 1). A reporter fusion based on an unstable green fluorescent protein (GFP) variant carried by plasmid pME7102 (Baehler et al. 2005) was used for monitoring *phlA* expression in vitro. These two reporter fusions have been shown to reflect the actual *phlA* expression level by the bacteria (Baehler et al. 2005), and the expression of the *phl* operon is itself correlated with the rate of DAPG production by *P. fluorescens* CHA0 (Notz et al. 2001). Standard methods were used for plasmid extraction and transformation of bacteria (Sambrook and Russell 2001) and for Tn7-based transposition (Schnider-Keel et al. 2000).

Pythium ultimum was kept routinely on 20% V8 agar (V8 at 200 ml liter⁻¹, CaCO₃ at 10 g liter⁻¹, and agar at 20 g liter⁻¹). An actively growing mycelium inoculum was prepared on autoclaved millet seed as described elsewhere (Notz et al. 2001). Plates were incubated at room temperature in the dark for 7 days and regularly mixed to ensure homogenous distribution.

Table 1. Strains and plasmids used in this study

Strain or plasmid	Genotype and phenotype ^a	Reference
<i>Pseudomonas fluorescens</i>		
CHA0	Wild type, biocontrol strain	Voisard et al. 1994
CHA0-P _{rrnBP1} - <i>gfp</i>	CHA0::attTn7-P _{rrnBP1} - <i>gfp</i> -a; Gm ^r	This study
Plasmids		
Psm1973	miniTn7(Gm)P _{rrnBP1} - <i>gfp</i> -a; Gm ^r	Lambertsen et al. 2004
pME7102	<i>phlA</i> - <i>gfp</i> [AAV] transcriptional fusion; reporter for DAPG biosynthetic gene expression; Tc ^r	Baehler et al. 2005
pME9012	<i>phlA</i> - <i>mcherry</i> transcriptional fusion; reporter for DAPG biosynthetic gene expression; Km ^r	Rochat et al. 2010

^a Gm^r, Km^r, and Tc^r indicate gentamicin, kanamycin, and tetracycline resistance, respectively. DAPG = 2,4-diacetylphloroglucinol.

Plants.

Seed of barley (*Hordeum vulgare* cv. Barke) were dehusked by immersion in 50% H₂SO₄ with agitation for 90 min and washed twice in 0.5 M NaHCO₃ solution to neutralize the acid. Seed were sterilized by soaking in 70% ethanol for 2 min and 2% AgNO₃ for 30 min with agitation. Silver residues were removed by five successive washing cycles in 0.9% NaCl and distilled water of 10 min each. The seed were germinated individually on water agar (8 g liter⁻¹). After 6 days, the plantlets were checked for contamination, transferred to the split-root microcosm (discussed below), and inoculated according to the treatments.

Microcosm construction and inoculation.

The split-root microcosms used for reporter fusion assays were constructed from 250-by-160-by-15-mm polycarbonate plates (Supplementary Fig. S1). One plant seedling was placed in each microcosm and the roots were equally distributed into the two split-root chambers. Prior to establishment of the seedlings, the microcosms were autoclaved and each chamber half (100 by 150 by 5 mm) was filled with 15 ml of sterile 1/5 Long Ashton nutrient solution (Hewitt 1966) in 2% agar. Sterile seedlings were added aseptically after cooling of the microcosms and inoculated as described below. The microcosms were covered with a 6-mm-thick polycarbonate lid fixed tightly with paper clamps. The opening of the microcosms around the shoot base was sealed with autoclaved Terostat VII sealing mass (Henkel, Düsseldorf, Germany). Microcosms for HPLC analysis of root exudates were built of two 12-ml glass tubes with polyethylene lid (Neolab, Heidelberg, Germany). The tubes were washed twice in ethyl acetate, autoclaved, glued together, and filled with 10 ml of HPLC H₂O (Roth, Karlsruhe, Germany).

Four treatments were set up: noninoculated, *Pythium ultimum*, *P. fluorescens*, and *Pythium ultimum* + *P. fluorescens*. To avoid direct contact between the organisms, *Pythium ultimum* and *P. fluorescens* were always inoculated onto different halves of the split-root system (referred to as “local”, in contact with the pathogen, and “systemic”, the response side without pathogen). The local side of the split-root system received 500 mg of the *Pythium* inoculum (discussed above) or 500 mg of uninfected sterile millet seed as control. For reporter fusion assays, the systemic side was inoculated with 1 ml of a 1:1 mixture of the two reporter strains of *P. fluorescens* CHA0 (CHA0-P_{trmB}-gfp and CHA0/pME9012, OD₆₀₀ = 0.1); in the control treatment 1 ml of AS was added.

In the microcosms used for HPLC measurement, the local side of the root system was infected with chopped *Pythium* mycelium. The systemic side was left sterile. The opening was sealed with autoclaved silicon grease. Plants were grown with a 16-h light phase (22°C, 500 μmol s⁻¹ m⁻²) and 8 h of darkness (18°C) at 60% relative humidity for 6 days. In all, 14 and 6 replicates were set up per treatment for the reporter assay and the HPLC measurement, respectively.

Measurement of bacterial activity.

Six days after inoculation, the microcosms were destructively sampled. Roots inoculated with *P. fluorescens* were placed in 10 ml of cold phosphate-buffered saline buffer containing 0.8% formaldehyde, and root-associated bacteria were extracted by vigorously shaking the roots for 20 min. Samples were kept at 4°C until analysis. In situ expression of the *phlA-mcherry* and P_{trmBPI}-gfp reporter fusions was analyzed with a FACScalibur flow cytometer (Becton and Dickinson, San Jose, CA, U.S.A.) equipped with a 15-mW air-cooled argon ion laser excitation light source (488 nm). GFP and mCherry fluorescence emissions were measured at 530 and 661 nm, respec-

tively, and amplified with a logarithmic gain. Forward scatter (FSC) signals were collected using a photodiode with an amplification factor of 10, a threshold of 253, and a log gain. Side scatter signals were measured using a photomultiplier tube set at 350 V, a threshold of 72, and a log gain.

Data were collected using CellQuest software (Becton and Dickinson) and analyzed with WinMDI 2.8 (The Scripps Research Institute FACS Core Facility webpage). Gating was done on the base of the FSC signal. Fluorescence signals were gated using the complementary single fluorescent strains as blank in order to eliminate the background fluorescence.

Bacteria colonizing the roots were counted with an inverted fluorescence microscope after staining with 4',6-diamidino-2-phenylindole as described elsewhere (Normander et al. 1999).

Extraction of root exudates.

Samples of the media (5 ml) from each of the treatments were filtered through a 0.22-μm filter (Millipore, Bedford, MA, U.S.A.), acidified to pH 2 with 1 N HCl, and extracted in 5 ml of ethyl acetate on an orbital shaker at 300 rpm (Bühler, Hechingen, Germany) at room temperature for 1 h. The organic phase was evaporated under nitrogen and the residue taken up in 70 μl of methanol for HPLC analyses.

HPLC–diode array detection analysis.

The HPLC apparatus consisted of a Waters system equipped with a gradient pump (Waters 600 controller; Milford, MA, U.S.A.), a cooled autosampler (Waters 717 plus), and a UV–visible photodiode array detector set (Waters 996) to acquire data from 200 to 400 nm. Empower 2 software from Waters was used for instrument control, data acquisition, and data processing. Analyses were performed on a 3-μm column (250 by 4 mm, Multospher 120 RP18HP; CS-Service, Langerwehe, Germany) at room temperature (21°C). The mobile phase consisted of aqueous phosphoric acid (0.1% wt/vol; eluent A) and acetonitrile (eluent B) pumped at 0.5 ml min⁻¹ into the HPLC system (Lanoue et al. 2010). The gradient started at 5% B and increased linearly to 72% in 60 min, followed by washing and reconditioning of the column. Wavelength detection was set at 219 nm for vanillic acid (retention time [RT] = 30.11 min), 310 nm for *p* coumaric acid (RT = 35.4 min), and 210 nm for fumaric acid (RT = 9.79 min). Wavelength detection was set at 219 nm for vanillic acid, 310 nm for *p*-coumaric acid, and 210 nm for fumaric acid. Compounds in extracts were identified according to their UV spectra and retention time by comparison with authentic standards.

Chemicals.

Standard compounds vanillic acid, *p*-coumaric acid, and fumaric acid were purchased from Sigma-Aldrich (St. Louis). Acetonitrile (LiChrosolv), methanol (LiChrosolv), and ethyl acetate (LiChrosolv) were obtained from Merck (Darmstadt, Germany). Ultrapure water was obtained from a Millipore Milli-Q water purification system.

Effects of pure compounds on *phlA* expression.

In order to assess the effect of single exudated chemicals as signal compound affecting antifungal gene expression, *P. fluorescens* CHA0 carrying pME7102 (Table 1) was grown in a minimal ammonium-glucose medium without antibiotics amended with glucose at 4 g liter⁻¹ as sole carbon source (OS-Glc) (Schnider-Keel et al. 2000) in 96-well microtiter plates (Greiner Bio-one, Frickenhausen, Germany). The test compounds of *p*-coumaric, vanillic, and fumaric acid were dissolved in methanol and added at a final concentration of 50, 25, 12.5, 6.25, 3.63, and 1.82 μM (four replicates each). Bacterial cultures (200 μl per well) were incubated at 25°C with agitation

(500 rpm). Green fluorescence (excitation at 485 nm and emission at 520 nm) and OD₆₀₀ were recorded at regular intervals with an M200 microplate reader (Tecan, Crailsheim, Germany) during 6 h. Relative fluorescence (RFU) was expressed as the ratio between total green fluorescence value and OD₆₀₀. The autofluorescence of *P. fluorescens* CHA0 was used for background correction. Methanol without test compounds was used as control. As indicated by preliminary experiments, vanillic, p-coumaric, and fumaric acids are not used as a carbon source by *P. fluorescens* CHA0 because the strain was unable to grow on minimal medium supplemented with 1 mM of any of these compounds (data not shown). RFU after 6 h was normalized by calculating the percentage change relative to the control level. The stimulatory activity of the tested compounds as a function of their concentration was assessed with the single substrate Michaelis-Menten kinetics as described elsewhere (Kay et al. 2005) to retrieve the molecule concentration required for inducing half the maximal change in *phlA* expression.

Statistical analysis.

Geometrical means for FL1 (GFP) and FL3 (mCherry) signals were normalized prior to analysis. Gene expression of the bacteria was analyzed using a general linear model with a full factorial design. The model investigated the effect of infection by *Pythium ultimum* and of root size on the normalized *phlA* value and on the relationship between the expression level of rRNA and *phlA*. The effect of infection on the ratio between primary and secondary metabolism was determined by analyzing the expression of *phlA* as a function of the infection status and the expression of rRNA *rrnBPI*. The effect of *Pythium* infection on vanillic, p-coumaric, and fumaric acid root exudation was analyzed by two-factor analysis of variance (ANOVA) with the factors infection (*Pythium* or Control) and root side (local or systemic). Effects of single compounds on the expression of *phlA* in vitro were tested at five time points with a nested ANOVA. All statistical analyses were performed in STATISTICA 7.1 (Statsoft Inc., Tulsa, OK, U.S.A.). All experiments were repeated with similar results.

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The Scripps Research Institute FACS Core Facility software webpage:
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