Effect of Clay Mineralogy on Iron Bioavailability and Rhizosphere Transcription of 2,4-Diacetylphloroglucinol Biosynthetic Genes in Biocontrol *Pseudomonas protegens*

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Pseudomonas strains producing 2,4-diacetylphloroglucinol (DAPG) can protect plants from soilborne phytopathogens and are considered the primary reason for suppressiveness of morainic Swiss soils to *Thielaviopsis basicola*-mediated black root-rot disease of tobacco, even though they also occur nearby in conducive sandstone soils. The underlying molecular mechanisms accounting for this discrepancy are not understood. In this study, we assessed the hypothesis that the presence of iron-rich vermiculite clay (dominant in suppressive soils) instead of illite (dominant in neighboring conducive soils) translates into higher levels of iron bioavailability and transcription of *Pseudomonas* DAPG synthetic genes in the tobacco rhizosphere. Rhizosphere monitoring of reporter gene systems *pvd-inaZ* and *phlA-gfp* in *Pseudomonas protegens* indicated that the level of iron bioavailability and the number of cells expressing *phl* genes (DAPG synthesis), respectively, were higher in vermiculitic than in illitic artificial soils. This was in accordance with the effect of iron on *phlA-gfp* expression in vitro and, indeed, iron addition to the illitic soil increased the number of cells expressing *phlA-gfp*. Similar findings were made in the presence of the pathogen *T. basicola*. Altogether, results substantiate the hypothesis that iron-releasing minerals may confer disease suppressiveness by modulating iron bioavailability in the rhizosphere and expression of biocontrol-relevant genes in antagonistic *P. protegens*.

Many plant-growth-promoting rhizobacteria have positive effects on plant health, which may take place via different modes of action; noticeably, induced systemic resistance (ISR) and direct phytopathogen inhibition (Haas and Défago 2005; Raaijmakers et al. 2009). Antimicrobial secondary metabolites often play a key role in these plant-protection effects, especially the *Pseudomonas* compound 2,4-diacetylphloroglucinol (DAPG), which can both induce systemic resistance (Iavicoli et al. 2003; Weller et al. 2012) and inhibit various root patho-

gens (Couillerot et al. 2009; Haas and Défago 2005). DAPG can also trigger gene expression of phytobeneficial functions in neighboring plant-protecting bacteria and fungi (Baehler et al. 2005; Combes-Meynet et al. 2011; Lutz et al. 2004).

Soilborne populations of DAPG-producing fluorescent *Pseudomonas* spp. are implicated in the natural suppressiveness of certain soils toward particular fungal phytopathogens (Kyselková and Moëgne-Loccoz 2012), such as *Gaeumannomyces graminis* var. *tritici* (take-all of wheat) (Weller 2007), *Fusarium oxysporum* f. sp. *pisi* (Fusarium wilt of pea) (Landa et al. 2002), and *Thielaviopsis basicola* (black root-rot of tobacco) (Frapolli et al. 2010). Soils specifically suppressive to black root rot of tobacco (and other crops) are mainly documented in the region of Morens (Switzerland). At Morens, disease suppressiveness is a microbial property of the soil (Stutz et al. 1986) involving DAPG-producing pseudomonads (Haas and Défago 2005), and inactivation of DAPG production ability in the Morens isolate *Pseudomonas protegens* CHA0 abolished its ability to suppress black root rot (Keel et al. 1992).

Morens soils are of particular interest from a molecular plant-microbe interaction standpoint because, in that region, conducive soils (formed from sandstone sediments) also occur in the vicinity of suppressive soils formed from glaciers' shallow morainic deposits overlying the sandstone (Stutz et al. 1985). Yet, both types of soil belong to the same brunisol category and are chemically similar, except for the predominance of iron-releasing vermiculite clay in suppressive soils versus illite clay in conducive soils (Stutz et al. 1989). This led to the hypothesis (Almario et al. 2013; Ramette et al. 2003) that iron released from vermiculite triggers transcription of *Pseudomonas* DAPG synthetic genes in suppressive soils to levels higher than those in conducive soils, where DAPG-producing *Pseudomonas* populations are also present at high levels (Almario et al. 2013; Frapolli et al. 2010) but where iron availability is supposedly lower (Keel et al. 1989; Voisard et al. 1989). Indeed, *P. protegens* CHA0 can retrieve iron from vermiculite (Müller 2009). Earlier work with artificial soils mimicking texture and clay mineralogy of Morens suppressive (vermiculitic) and conducive (illitic) soils did show that i) total and soluble iron contents (Keel et al. 1989) and ii) plant protection by *P. protegens* CHA0 (Keel et al. 1989) and other DAPG-producing pseudomonads (Ramette et al. 2006) were higher in vermiculite-rich than in illite-rich soils. However, whether or not iron bioavailability for pseudomonads and transcription of *Pseudomonas* DAPG synthetic genes in the rhizosphere were higher in the presence of vermiculite has not been assessed thus far.

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Under soil-less laboratory conditions, root expression of *Pseudomonas* DAPG synthetic genes (*phlACBDE* operon) is influenced by biotic factors such as plant species, plant cultivar, pathogen presence, and plant health (de Werra et al. 2008; Jamali et al. 2009; Jousset et al. 2011; Rochat et al. 2010). Abiotic factors such as Zn, Mo, and Fe concentrations can also modulate DAPG production in *Pseudomonas* spp. depending on culture medium conditions (Dowling et al. 1996; Duffy and Défago 1999). Recently, Lim and associates (2012) showed that iron had a strong impact on the transcriptome and proteome of the DAPG producer *P. protegens* Pf-5 and, in particular, iron-induced expression of DAPG synthetic genes in vitro.

The objective of this study was to assess the hypothesis that iron bioavailability for pseudomonads and transcription of *Pseudomonas* DAPG synthetic genes in the tobacco rhizosphere are higher in the presence of vermiculite than illite, which are the dominant clay types in soils suppressive and conducive, respectively, to *Thielaviopsis* black root rot. To this end, the artificial vermiculitic and illitic soils of Keel and associates (1989) were used, along with a mechanistic approach based on expression analysis of reporter gene systems *pvd-inaZ* (iron availability) and *phlA-gfp* (transcription of DAPG synthetic genes) on rhizosphere-stable plasmids (Rochat et al. 2010), which was complemented by real-time reverse-transcriptase polymerase chain reaction (RT-PCR) analysis of *phlA-gfp* transcription in DAPG-producing *P. protegens*.

RESULTS

Effect of bioavailable iron on cell density and *phlA-gfp* expression of *P. protegens* in vitro.

Increasing the in vitro iron concentration up to 5 μM with FeEDDHA, a chelated iron form bioavailable to *Pseudomonas* spp. (Keel et al. 1989), enhanced cell density (estimated by optical density at 600 nm [OD₆₀₀] values at 38 h) (Fig. 1A) of *P. protegens* CHA0(*attTn7::miniTn7-Gm-P_{tac}-mcherry*; pME7100), in which a plasmid-borne copy of the DAPG synthesis promoter *phlA* is fused to the promoterless reporter gene *green fluorescent protein (gfp)* (Rochat et al. 2010). This strain is hereafter referred to as CHA0-*mche*(pME7100), as in Rochat

and associates (2010). Additional increases of FeEDDHA concentration did not enhance cell density of the strain any further.

Bioavailable iron also modulated the number of GFP⁺ cells (i.e., cells accumulating GFP following expression of the *phlA-gfp* fusion) in vitro, with a maximum relative green fluorescence (relative fluorescence units [RFU]) level at 2 μM FeEDDHA (Fig. 1B). Lower and higher iron concentrations resulted in lower RFU levels, indicating a maximal activity of the *phlA* promoter at 2 μM FeEDDHA in vitro. Similar results were found at a later sampling (42 h) or when repeating the experiment (not shown).

Effect of clay mineralogy on iron bioavailability for *P. protegens* on roots in artificial soil.

Ice nucleation activity of the iron-biosensor strain *P. protegens* Pf-5 *pvd-inaZ*, in which the iron-regulated promoter *pvd* is fused to the promoterless ice nucleation reporter gene *inaZ* (Loper and Lindow 1994), was lower (−57%; $P = 0.008$) at 7 days in tobacco rhizosphere samples from vermiculitic than illitic artificial soil (Fig. 2). Similar findings were made at other samplings (3 and 21 days). This means that rhizosphere bioavailability of iron for *P. protegens* was comparatively higher in the vermiculitic soil and, indeed, it was estimated at 3.0 μM Fe in the vermiculitic soil versus only 0.8 μM in the illitic soil, based on data in Figure 2 and an in vitro standard curve (Supplementary Fig. S1). The ice nucleation activity of the positive control strain Pf-5 *iceC* (in which *iceC* is transcribed from its native iron-constitutive promoter) was comparable in both types of soil (Fig. 2).

Effect of clay mineralogy on cell density and *phlA-gfp* expression of *P. protegens* on roots in artificial soil.

Seven days after root inoculation of *P. protegens* CHA0-*mche*(pME7100) in the absence of *T. basicola*, the number of inoculant cells on tobacco roots was higher in the vermiculitic than the illitic soil based on microscopy analysis (+35% number of total cells field^{−1}) (Fig. 3A), with a similar trend (+59%) by real-time PCR analysis but not significant at $P < 0.05$ (Fig. 3D).

When expression of the *phlA-gfp* fusion was assessed at 7 days on tobacco roots in the absence of *T. basicola*, on one hand, the number of GFP⁺ inoculant cells (i.e., green cells)

Fig. 1. Effect of nine iron concentrations (from 0.1 to 1,000 μM FeEDDHA) on **A**, cell density (optical density at 600 nm [OD₆₀₀]) and **B**, green fluorescent protein fluorescence levels (relative fluorescence units [RFU]) of *Pseudomonas protegens* CHA0-*mche*(pME7100) at 38 h (early stationary phase) in OSGly liquid medium. Means and standard errors are represented ($n = 6$). For each type of measurement, statistical relations between iron concentrations are shown using letters a to e (analysis of variance and Fisher's least significant difference tests; $P < 0.05$). Similar results were obtained when measurements were taken at 42 h. The experiment was run on two other occasions, with measurements taken at 38 and 42 h, and similar results were obtained in all cases.

was higher (+63%) in the vermiculitic than the illitic soil (Fig. 3B). This resulted from a higher number of root-colonizing cells (discussed above) and a trend (+21%, but not significant at $P < 0.05$) for a higher proportion of GFP⁺ cells in the vermiculitic soil (Fig. 3C). On the other hand, real-time PCR analysis of *phlA-gfp* expression on tobacco roots gave comparable *gfp* mRNA (Fig. 3E) and relative *gfp* mRNA levels (i.e., *gfp* mRNA/cell ratio) (Fig. 3F) in both soils.

Effect of iron addition on cell density and *phlA-gfp* expression of *P. protegens* on roots in artificial illitic soil.

To determine whether iron bioavailability in the tobacco rhizosphere was limiting for *phlA-gfp* expression in illitic soil, the effect of iron supplementation (2.4 mM FeEDDHA) was investigated in this soil. As expected, this enhanced the level of bioavailable iron, as indicated by a lower ice nucleation activity ($P < 0.001$) of the iron biosensor strain *P. protegens* Pf-5 *pvd-inaZ* at 7 days (i.e., $-4.4 \pm 0.23 \log$ [ice nuclei cell⁻¹] versus only $-1.6 \pm 0.09 \log$ [ice nuclei cell⁻¹] in the nonamended illitic soil, corresponding to 8 and 0.8 μM bioavailable Fe, respectively, estimated using a standard curve). Iron supplementation resulted in enhanced root colonization of tobacco roots by *P. protegens* CHA0-*mche*(pME7100) at 7 days in illitic soil, based on higher total microscopy count of inoculant cells colonizing tobacco roots (+119%; $P < 0.001$) (Fig. 4A) and higher

Fig. 2. Effect of clay mineralogy on ice nucleation activity of the iron biosensor strain *Pseudomonas protegens* Pf-5 *pvd-inaZ* and the positive control strain Pf-5 *iceC* in the tobacco rhizosphere at 3, 7, and 21 days after transplanting of bacteria-inoculated seedlings in artificial vermiculitic (white bars) or illitic soil (gray bars). Ice nucleation activity was computed as the number of ice nuclei per cell (means $\pm$ standard errors; $n = 6$ plants). Ice nucleation activity of the positive control strain Pf-5 *iceC* was the same in both soils at each sampling, as expected. The experiment was done twice (each time with three plants per treatment per sampling) with similar results, and results were pooled. For each strain, statistical differences between soils within each date are shown using letters a and b (Student's t tests; $P < 0.05$).

real-time PCR number of inoculant cells (+78%, $P = 0.026$) (Fig. 4D).

The number of GFP⁺ cells of *P. protegens* CHA0-*mche*(pME7100) on tobacco roots (expressed per microscopy field) at 7 days was higher following iron supplementation (+190%; $P < 0.001$) (Fig. 4B). This resulted from a higher number of root-colonizing cells (discussed above) as well as a higher proportion (+25%; $P = 0.003$) (Fig. 4C) of GFP⁺ cells (i.e., proportion of green cells). Real-time PCR analysis of *phlA-gfp* expression on tobacco roots showed that iron supplementation had no effect on *gfp* mRNA levels (Fig. 4E) but lowered relative *gfp* mRNA levels (−62%; $P = 0.006$) (Fig. 4F).

Effect of clay mineralogy on cell density and *phlA-gfp* expression of *P. protegens* on roots in artificial soil in the presence of *T. basicola*.

When system complexity was increased by introducing the phytopathogenic partner, it was important to verify that the genetic modifications done to *P. protegens* CHA0 had not abolished its ability to control black root rot. In the absence of bacterial inoculation, comparable black-root rot severity (i.e., percentage of root surface covered by *T. basicola* chlamydospores; approximately 23%) was observed at 27 days in the vermiculitic and illitic artificial soils (Supplementary Table S1). Inoculation of *P. protegens* CHA0-*mche*(pME7100) resulted in lower black root-rot severity in the vermiculitic soil (8% severity). There was a similar trend in the illitic soil but it was not significant at $P < 0.05$ and disease level remained as high as 17%.

In OSGly minimal medium (Baehler et al. 2005), which contains 20 μM FeEDDHA, *T. basicola* had no effect on cell density and *phlA-gfp* expression of *P. protegens* CHA0-*mche* (pME7100) (data not shown). This was observed regardless of *T. basicola* inoculation level in the medium (10^2 to 10^4 endoconidia ml⁻¹).

In both artificial soils, *T. basicola* inoculation affected cell density of *P. protegens* CHA0-*mche*(pME7100), because total microscopy cell counts were 25% lower (Fig. 3A) and real-time PCR counts at least 35% lower (Fig. 3D). With *T. basicola* inoculation, the number of GFP⁺ cells of *P. protegens* CHA0-*mche*(pME7100) were more than 30% lower than in the absence of the pathogen in both soils (Fig. 3B) but without any significant effect on the proportion of GFP⁺ cells (Fig. 3C). Real-time PCR results showed comparable levels of *gfp* mRNA in *T. basicola*-inoculated and noninoculated treatments (Fig. 3E), as well as a relative *gfp* mRNA level higher upon *T. basicola* inoculation but only in the illitic soil (Fig. 3F).

In the presence of *T. basicola*, cell density of *P. protegens* CHA0-*mche*(pME7100) on roots was higher in the vermiculitic than the illitic soil based on microscopy analysis (+33% number of total cells field⁻¹) (Fig. 3A); however, this was not the case with real-time PCR data (Fig. 3D). In the presence of *T. basicola*, the number of GFP⁺ cells on tobacco roots (expressed per microscopy field) was higher in the vermiculitic than the illitic soil (Fig. 3B), due to a higher cell density (discussed above) and a trend (not significant at $P < 0.05$) for a higher proportion of GFP⁺ cells (Fig. 3C). *gfp* mRNA levels (Fig. 3E), as well as relative *gfp* mRNA levels (Fig. 3F), did not differ significantly between the two *T. basicola*-inoculated artificial soils.

Spatial patterns of *P. protegens* colonization and *phlA-gfp* expression on roots.

Confocal laser-scanning microscopy (CLSM) analysis showed that *P. protegens* CHA0-*mche*(pME7100) colonized the three root zones (i.e., base, middle, and root tips), including

both the root surface and root hairs (the latter found mostly in the root tip zone). In the illitic soil, iron addition resulted in higher numbers of inoculant cells at the root surface (+143%) and on root hairs (+66%), higher numbers of GFP⁺ cells at the root surface (+224%) and on root hairs (+193%), and a higher proportion of GFP⁺ cells on root hairs (+60%) (Supplementary Table S2). At the root surface, samples from the vermiculitic soil displayed higher numbers of inoculant cells (+62%) and higher numbers of GFP⁺ cells (+86%) but a comparable proportion of GFP⁺ cells compared with the illitic soil. There was no difference between the two soils when considering root hairs. In the presence of *T. basicola*, however, the numbers of inoculant cells (+46%) and of GFP⁺ inoculant cells (+151%), as well as the proportion of GFP⁺ cells (+59%), were higher in the vermiculitic than the illitic soil for root hairs. These effects were not significant at the root surface.

Because root systems can be heterogeneous, cell distribution was also considered in more details. In all experiments, *P.*

protegens CHA0-*mche*(pME7100) was found as a mixture of individual cells, cell clumps (comprising 2 to 10 cells), and microcolonies (>10 cells) (Supplementary Fig. S2A). The resulting cell distribution pattern differed between root surface and root hairs (χ^2 test of independence; $P = 10^{-17}$), because the proportion of individual cells was larger (20 versus 12%) and that of microcolony cells was smaller (44 versus 62%) on root hairs than at the root surface. In contrast, cell distribution patterns did not change significantly when comparing soil treatments (i.e., effect of clay mineral, iron addition, or *T. basicola* inoculation) or root zones (i.e., base, middle, or root tips). Similar findings were made when considering GFP⁺ cells of *P. protegens* CHA0-*mche*(pME7100) (not shown).

DISCUSSION

The aim of this study was to assess the hypothesis that the bioavailability of iron and the expression of DAPG synthetic

Fig. 3. Effect of clay mineralogy and inoculation with *Thielaviopsis basicola* ETH D127 (10^3 endoconidia cm^{-3}) on cell density and *phlA-gfp* expression of *Pseudomonas protegens* CHA0-*mche*(pME7100) in the tobacco rhizosphere at 7 days after transplanting of bacteria-inoculated seedlings in artificial vermiculitic (white bars) or illitic soil (gray bars). *T. basicola* inoculation is indicated by hatched lines. **A**, **B**, and **C**, Confocal laser-scanning microscopy analysis of tobacco roots. Two experiments were conducted (giving similar results), each consisting of three plants per treatment and 10 confocal fields (225 μm by 225 μm by 1 μm^3 per field) per plant. Pooled results from the two experiments are presented ($n = 60$ fields). For each field, the proportion of cells accumulating green fluorescent protein [GFP] following expression of the *phlA-gfp* fusion (GFP⁺) was calculated by dividing the number of green cells by the total number of fluorescent cells. **D**, **E**, and **F**, Real-time polymerase chain reaction analysis of tobacco roots. One of the experiments above was used, with five plants per treatment ($n = 5$). For each plant, the relative *gfp* mRNA level was calculated by dividing the number of *gfp* mRNA copies by the number of cells. In each of the six panels, means and standard errors are shown. Statistical differences between the four treatment combinations (illite versus vermiculite $\times$ *T. basicola*-inoculated or not) are shown using letters a to c (two-factor analysis of variance and Fisher's least significant difference tests; $P < 0.05$; the interaction between the two factors was not significant).

genes for *P. protegens* in the tobacco rhizosphere are higher in the presence of vermiculite than illite clay, thereby substantiating the hypothesis that (suppressive) vermiculitic soils in Morens should provide more favorable conditions for iron acquisition and *phl* transcription by *P. protegens* compared with (conductive) illitic soils. Indeed, biosensor analyses indicated that both iron bioavailability for *P. protegens* and PhlA protein accumulation (number of GFP⁺ cells) were higher in the vermiculitic than the illitic soil. Soil parent material determines many soil characteristics, including the type of clay mineral prevailing in the soil, yet the consequences for the functioning of rhizosphere microorganisms and plant–microbe interactions, which were important in the current case, are rarely studied (Almario et al. 2013; Ulrich and Becker 2006).

To determine the importance of iron bioavailability for *phlA-gfp* expression in *P. protegens*, iron addition was implemented in vitro, and it showed that the amount of bioavailable iron in

vermiculitic soil (estimated at 3 μM Fe) promoted *P. protegens* CHA0 growth in vitro and was close to the iron concentration inducing maximal *phlA-gfp* expression (i.e., 2 μM) (Fig. 1B). Indeed, similar positive effects on both features (especially at the root surface rather than on root hairs) were observed when the illitic soil was amended with iron at 2.4 mM (thereby increasing iron bioavailability 10 times), a concentration raising plant-protecting activity of *P. protegens* CHA0 to levels found in vermiculitic soil (Keel et al. 1989; Voisard et al. 1989). These findings are compatible with the observation that iron deprivation affects *phl* transcription in *P. protegens* in vitro (Lim et al. 2012), and the hypothesis that higher iron availability in vermiculitic soils is required for disease suppression (Ramette et al. 2003).

The fact that *P. protegens* cells as well as GFP⁺ cells at the root surface were much less abundant in the illitic soil than in high-iron soils (i.e., vermiculitic soil and iron-amended illitic soil) probably relates to the fact that iron deprivation affects several traits of importance for rhizosphere competence, besides *phl* transcription (Lim et al. 2012). Iron deprivation also limits cell motility (Lim et al. 2012) but this type of effect was not apparent when considering spatial patterns of *P. protegens* root colonization and GFP accumulation. Localized antimicrobial production by rhizobacteria at pathogen entry points is expected to underpin pathogen biocontrol (Fukui et al. 1994) but, here, *phl* transcription took place throughout the root system. This may be relevant for inhibition of *T. basicola*, because the latter does not display preferential sites of root penetration (e.g., it can infect root hairs as well as the root surface) (Hood and Shew 1997). It has been suggested that *P. protegens* CHA0 could also act by inducing ISR pathways in tobacco (Almario et al. 2013; Troxler et al. 1997), because *P. protegens* CHA0 delays but does not alter *T. basicola* infection patterns, and physical interaction between both microorganisms is not necessary for black root rot suppression (Troxler et al. 1997). Indeed, DAPG triggers ISR pathways in *Arabidopsis* (Weller et al. 2012). DAPG-triggered ISR requires a functional EIR1 gene (Iavicoli et al. 2003), which suggests that root tips, where this gene is expressed (Müller et al. 1998), might be a prime location to trigger ISR. However, cell distribution pattern of *P. protegens* on tobacco root tips did not differ in vermiculitic and illitic soils.

The numbers of cells and of GFP⁺ cells (i.e., expressing *phlA-gfp*) of root-colonizing *P. protegens* CHA0 reached lower values when *T. basicola* was added (Fig. 3AB). Pathogen presence often increases cell density and expression of *phl* genes in *P. protegens* in other pathosystems, such as *Rhizoctonia solani*–bean (Jamali et al. 2009), *Pythium ultimum*–maize, or *P. ultimum*–cucumber (Notz et al. 2001). Phytopathogens may also counteract antagonistic fluorescent pseudomonads using different mechanisms (Duffy et al. 2003), ranging from survival inhibition by *P. ultimum* (Fedi et al. 1997) to fusaric-acid-mediated repression of DAPG synthesis by *F. oxysporum* (Duffy and Défago 1997). Here, the negative effects of *T. basicola* on *Pseudomonas protegens* did not take place in vitro (i.e., in the absence of roots). Using a split-root system, Jousset and associates (2011) showed that infection of barley roots by *Pythium ultimum* promoted expression of *phl* genes in root-colonizing *Pseudomonas protegens* CHA0, through a systemic effect on the plant. It might be that the effect of *T. basicola* on tobacco was also systemic, and further work will be needed to address this issue.

In this work, microscopy analysis of GFP⁺ cells, which reflects GFP accumulation (GFP protein exhibiting a half-life of days) (Andersen et al. 1998) at the root microscale, was complemented by quantification of *gfp* mRNA, which reflects recent *phlA-gfp* transcriptional activity (mRNA exhibiting half-lives of

Fig. 4. Effect of iron addition (2.4 mM FeEDDHA) on cell density and *phlA-gfp* expression of *Pseudomonas protegens* CHA0-mche(pME7100) in the tobacco rhizosphere at 7 days after transplanting of bacteria-inoculated seedlings in artificial illitic soil. **A, B, and C,** Confocal laser-scanning microscopy analysis of tobacco roots. Two experiments were conducted (giving similar results), each consisting of three plants per treatment and 10 confocal fields (225 μm by 225 μm by 1 μm^3 per field) per plant. Pooled results from the two experiments are presented ($n = 60$ fields). For each field, the proportion of cells accumulating green fluorescent protein [GFP] following expression of the *phlA-gfp* fusion (GFP⁺) was calculated by dividing the number of green cells by the total number of fluorescent cells. **D, E, and F,** Real-time polymerase chain reaction analysis of tobacco roots. One of the experiments above was used, with five plants per treatment ($n = 5$). For each plant, the relative *gfp* mRNA level was calculated by dividing the number of *gfp* mRNA copies by the number of cells. In each of the six panels, means and standard errors are shown. Statistical differences between iron-amended (black bars) and nonamended soil (gray bars) are shown using letters a and b (Student's *t* tests; $P < 0.05$).

minutes) (Taniguchi et al. 2010) at the scale of the whole root system. Even though both measurements may not correlate, combining both provides additional insights (Taniguchi et al. 2010). Here, *gfp* mRNA levels were comparable in all treatments, whereas green fluorescence levels varied. This could entail i) *phlA-gfp* transcription levels that were not constant in space (i.e., with a number of transcripts that varied from one cell to the next) or in time or ii) the effect of post-transcriptional mechanisms (Taniguchi et al. 2010). Indeed, DAPG synthesis is subjected to Gac-mediated post-transcriptional upregulation (Haas and Défago 2005) and DAPG production was abolished in a *gacA* mutant despite *phl* transcription (Hassan et al. 2010). How differences in *phl* transcription translate in terms of DAPG synthesis under rhizosphere conditions remains to be quantified.

In the current work, differences in cell density of *P. protegens* CHA0 from one soil treatment to the next were of moderate magnitude (1.3- to 2.2-fold), as found when comparing root colonization of different cereal species (Rochat et al. 2010), and were lower than when comparing different plant species (de Werra et al. 2008; Notz et al. 2001). Similarly, the percentage of GFP⁺ *P. protegens* cells varied little between soil treatments (from 36 to 50%), as found between cereal species (Rochat et al. 2010). The percentage of GFP⁺ *P. protegens* cells on tobacco roots was rather low in comparison with cereals, where it may reach 86 to 97% (Rochat et al. 2010). This is in accordance with the lower ability of dicotyledonous plants (such as tobacco) to promote expression of *phl* genes compared with monocots (Notz et al. 2001).

DAPG-producing pseudomonads correspond to at least seven *Pseudomonas* spp. from two main lineages (i.e., *P. protegens* and the '*P. fluorescens*' complex) (Frapolli et al. 2007, 2012), which display differences in rhizosphere ecology (Sharifi-Tehrani et al. 1998; Wang et al. 2001). *P. protegens* is a well-established model for analysis of *Pseudomonas* secondary metabolism (Kidarsa et al. 2011) and plant-protection mechanisms in Morens suppressive soils (Haas and Défago 2005) but species from the '*P. fluorescens*' complex are also present in the tobacco rhizosphere in Morens soils (Frapolli et al. 2008, 2010), and ecological approaches at the scale of all DAPG-producing pseudomonads will be necessary to assess whether the current findings apply to all members of the functional group in Morens field soils. We carried out a preliminary assessment under non-sterile soil conditions but root colonization by *P. protegens* CHA0-*mche*(pME7100) was insufficient (approximately 5.5 log per gram of root) for the microscopy and real-time RT-PCR methodology we used, with often no more than a few GFP⁺ cells per microscopy field and real-time PCR of *gfp* mRNA below detection limit, respectively. Additional method development will be necessary to achieve real-time PCR assessment of *phl* transcripts in natural populations of DAPG-producing pseudomonads combined with chemical analysis of DAPG production. In addition, biocontrol is often multifactorial (Haas and Défago 2005) and DAPG synthesis is not the only iron-regulated antagonistic function in *P. protegens* (Lim et al. 2012), which means that a broader assessment of biocontrol functions in root-colonizing *Pseudomonas* populations, and perhaps also in other plant-beneficial microbes (Kyselková et al. 2009), will be required to better understand black root rot suppressiveness of iron-rich vermiculitic Morens soil.

In conclusion, iron bioavailability for DAPG-producing *P. protegens* colonizing tobacco roots was higher in the presence of vermiculite than illite clay, and it translated to higher cell density and GFP accumulation (pointing to enhanced *PhlA* levels) in the rhizosphere in vermiculitic compared with illitic soil. These results substantiate the hypothesis that higher iron availability in vermiculitic (suppressive) than illitic (conductive) Morens soils can promote transcription of DAPG syn-

thetic genes and black root rot control by pseudomonads in the tobacco rhizosphere.

MATERIALS AND METHODS

Microbial cultures and tobacco growth.

P. protegens CHA0-*mche*(pME7100) is a *attTn7::miniTn7*-Gm-P_{tac}-*mcherry* derivative of *P. protegens* CHA0 that contains plasmid pME7100, in which a copy of the DAPG synthesis promoter *phlA* is fused to the promoterless reporter gene *gfp* (Rochat et al. 2010). The strain was cultured at 27°C on King's B agar (King et al. 1954) supplemented with gentamycin (8 µg ml⁻¹) and tetracycline (for plasmid selection; 125 µg ml⁻¹). For *phlA-gfp* expression experiments, the strain was grown in 20 ml of Luria-Bertani (LB) (Duchefa Biochemie BV, Haarlem, The Netherlands) broth supplemented with the same antibiotics at 27°C and 120 rpm. At 8 h, 1 ml was used to inoculate 100 ml of antibiotic-free LB broth and cells were grown at 27°C and 180 rpm. At 4 h, log-phase cells were harvested and washed twice with ultrapure sterile water, using 3,500 × g and 23°C for 10 min. The pellet was suspended in ultrapure sterile water and the OD₆₀₀ was adjusted to 0.03 or 0.05 (i.e., 1.0 × 10⁷ and 1.6 × 10⁷ cells ml⁻¹, respectively). The absence of fluorescence before inoculation was checked under a Zeiss Axioskope epifluorescence microscope (Carl Zeiss S.A.S., Oberkochen, Germany).

The iron-biosensor strain *P. protegens* Pf-5 *pvd-inaZ* (rifampicin and kanamycin resistant [Rif^r Km^r]) is *P. protegens* Pf-5 carrying a plasmid in which the iron-regulated promoter *pvd* is fused to the promoterless ice nucleation reporter gene *inaZ* (Loper and Lindow 1994). The positive control *P. protegens* Pf-5 *iceC* (Rif^r Km^r) is identical to strain Pf-5 *pvd-inaZ* except that *iceC* is transcribed from its native iron-constitutive promoter (Loper and Lindow 1994). The two *P. protegens* strains were cultured at 27°C on King's B agar supplemented with rifampicin (100 µg ml⁻¹) and kanamycin (50 µg ml⁻¹). For gene expression experiments, strains were grown in 100 ml of SM medium (Loper and Lindow 1987) supplemented with 10⁻¹ M FeCl₃ at 27°C and 180 rpm. At 24 h, stationary-phase cells were harvested and washed, the pellet was resuspended, and the OD₆₀₀ adjusted to 0.015 or 0.10 (i.e., 1.0 × 10⁷ and 6.7 × 10⁷ cells ml⁻¹, respectively), as indicated above.

T. basicola Ferraris strain ETH D127 (Berk. and Br.) was grown for 4 weeks in the dark on malt agar (Ramette et al. 2003). For inoculation, an endoconidial suspension adjusted to 10⁶ endoconidia ml⁻¹ was prepared as described by Ramette and associates (2003).

Tobacco (*Nicotiana glutinosa* L.) was grown in a growth chamber at 22°C (day, 16 h) and 18°C (night, 8 h) at 70% relative humidity. Seed were surface disinfected in 70% ethanol for 1 min and 10% H₂O₂ for 10 min, then rinsed with ultrapure sterile water. They were then germinated on 1.6% water agar for 1 week before transferring onto 0.6% Knop's agar (Keel et al. 1989). At 4 weeks, four-leaf plants were removed and roots were washed aseptically in ultrapure sterile water before inoculation and transplanting into the soil systems.

Soil systems.

Artificial soil systems were prepared as described by Keel and associates (1989). Briefly, quartz sand of different grain sizes (10% 1.5 to 2.0 mm, 9.3% 0.8 to 1.2 mm, 9.3% 0.5 to 0.75 mm, 28.6% 0.10 to 0.50 mm, and 42.8% 0.08 to 0.20 mm) was mixed with quartz powder (55% <40 µm) and clay (vermiculite or illite), in a 70:20:10 weight ratio. The artificial soils were autoclaved, moistened with ultrapure sterile water (10% [vol/wt] for vermiculite and 5% [vol/wt] for illite), and 20 g was used to fill 42-cm³ containers. When indicated, soil

was amended with 2.4 mM FeEDDHA (Fe^{3+} at $9.9 \mu\text{g g}^{-1}$ dry soil) (Keel et al. 1989) or inoculated with *T. basicola* at 10^3 endoconidia cm^{-3} . After transplanting, 1.1 ml of Knop's plant nutrient solution was added per container, water content was adjusted to 70% soil water-holding capacity (i.e., 20% [wt/wt] for vermiculitic soil and 15% [wt/wt] for illitic soil) with ultrapure sterile water, and the containers were sealed with porous sterile adhesive tape (Micropore Surgical Tape; 3M, Borken, Germany). No further watering was necessary except for the longer (27 days) biocontrol experiment, where soil water content was maintained by watering the pots every week.

Biocontrol activity of *P. protegens* CHA0-mche(pME7100) in artificial soils.

To compare the biocontrol activity of *P. protegens* CHA0-mche(pME7100) in the vermiculitic and the illitic artificial soils, 10 plants per soil (each plant in a separate container) were either inoculated with *T. basicola*, *P. protegens* CHA0-mche(pME7100), or both or were not inoculated, as indicated below. At 27 days, black root rot severity (i.e., percentage of root surface covered by *T. basicola* chlamydospores) was scored using a height-class disease scale (Stutz et al. 1986).

Monitoring of *phlA-gfp* in vitro in response to iron or *T. basicola*.

The experiment was carried out as described by Baehler and associates (2005). In all, 3.3×10^5 cells of *P. protegens* CHA0-mche(pME7100) were inoculated in 10 ml of OSGly medium (Baehler et al. 2005) containing i) 0.1 to 1,000 μM FeEDDHA or ii) 20 μM FeEDDHA and *T. basicola* at 10^2 to 10^4 endoconidia ml^{-1} . For each treatment, 200 μl were placed (in six replicates) in a 96-well microtitration plate with flat transparent bottom (Greiner Bio-one, Frickenhausen, Germany) and the plate was incubated for 42 h at 30°C with orbital shaking (500 rpm). Green fluorescence (excitation 480 nm, emission 520 nm), red fluorescence (excitation 587 nm, emission 661 nm), and absorbance (at 600 nm) were measured every 2 h in a Xenius microplate reader spectrofluorometer (SAFAS, Monaco). For each individual measurement, fluorescence value was divided by the corresponding OD_{600} value and the resulting specific fluorescence level expressed as RFU. The experiment was done twice with similar results.

CLSM monitoring of *phlA-gfp* in the tobacco rhizosphere.

Tobacco root systems were inoculated by dipping for 1 h in a cell suspension ($\text{OD}_{600} = 0.03$; approximately 10^7 cells ml^{-1}) of *P. protegens* CHA0-mche(pME7100), and seedlings were transplanted into artificial soil, as described above. At 7 days, plants were unearthed and roots were washed in ultrapure sterile water, mounted in Aqua-Poly/Mount (Polysciences, Eppelheim, Germany), and examined immediately by CLSM using a 510 Meta microscope (Carl Zeiss S.A.S.) equipped with an argon-krypton laser, as well as detectors and filter sets for green (i.e., 488 nm for excitation and 505 to 550 nm for detection) and red (i.e., 543 nm for excitation and >585 nm for detection) fluorescence. After acquisition of the transmitted image (in bright-field mode at $\times 400$ magnification), the three single-color images were overlaid as a single image using LSM software (release 4.2; Carl Zeiss S.A.S.). Three root systems were analyzed per treatment, 10 images per root system were taken, and the whole experiment was done twice.

RNA/DNA extraction and real-time PCR quantification of bacterial cells and *gfp* mRNA.

For real-time PCR and RT-PCR, the roots of five plants inoculated with *P. protegens* CHA0-mche(pME7100) were collected in each treatment, washed in ultrapure sterile water, and

immediately frozen in liquid nitrogen. Noninoculated plants were used as negative controls. After grinding the roots in liquid nitrogen using a pestle, nucleic acids were extracted using the protocol of Griffiths and associates (2000), as modified by Almario and associates (2013), and were suspended in 100 μl of RNase-free water.

Cells of *P. protegens* CHA0 were quantified according to von Felten and associates (2010). Real-time PCR was done directly from 10- μl samples of nucleic acids (diluted five times), using 0.5 μM primers CHA0_1_for (5'-CGACACATGACCAACATCGTTTCGA-3') and CHA0_1_rev (5'-GGCTACAAGGT CATTACAAAATCCAGTGAT-3'), which target a strain-specific sequence-characterized amplified region marker (von Felten et al. 2010). A LightCycler 480 (Roche Applied Science, Meylan, France) was used according to the manufacturer's instructions. An external standard curve generated from genomic DNA of *P. protegens* CHA0 (obtained using the NucleoSpin Tissue kit; Macherey-Nagel, Hoerd, France) was used for estimation of cell number in uncharacterized DNA samples. Amplification specificity was checked by melting curve analysis and gel electrophoresis of the amplification product. DNA samples were analyzed in triplicate and the mean cycle threshold (Ct) value was reported in the external standard curve to infer cell number in the sample.

For *gfp* mRNA quantification, a 40- μl sample of nucleic acids was treated with DNase I (Invitrogen, Cergy Pontoise, France) according to the manufacturer's instructions, and RNA was purified and eluted in 100 μl of RNase-free water using the RNeasy kit (Qiagen, Courtaboeuf, France). Because one DNase I treatment was insufficient to eliminate all DNA contamination, a second treatment was performed on the purified RNA sample. Finally, RNA was precipitated for 30 min at room temperature using 1 volume of isopropanol, pelleted by centrifuging the tubes for 15 min at $16,000 \times g$ and 4°C , and resuspended in 60 μl of RNase-free water. Reverse transcription for *gfp* cDNA synthesis was performed using 15 μl of RNA primer RTgfpR (5'-CTCTTTTCGTTGGGATCTTTTCG-3') (Baehler et al. 2005) at 0.1 μM and the Omniscript RT kit (Qiagen), following the manufacturer's instructions. *gfp* cDNA was subsequently purified using the MinElute purification kit (Qiagen) and used in real-time PCR. A negative control (i.e., the same but without reverse transcriptase) was also studied for each sample. Real-time PCR quantification of *gfp* cDNA was conducted following the protocol of Baehler and associates (2005) using *gfp* primers RTgfpF (5'-CCTGTCCTTTTACCAGACAACCA-3') and RTgfpR at 0.5 μM in a LightCycler 480 (Roche Applied Science), according to the manufacturer's instructions. An external *gfp* standard curve generated from plasmid pME7100 (obtained using the NucleoSpin Plasmid kit; Macherey-Nagel) was used for estimation of *gfp* copy number in uncharacterized cDNA samples. Amplification specificity was checked by melting curve analysis and gel electrophoresis of the amplification product. cDNA samples were analyzed in triplicate and the mean Ct value was reported in the external standard curve to infer *gfp* cDNA copy number in the sample.

Assessment of iron bioavailability in vitro and in the tobacco rhizosphere.

The response of the iron-biosensor *P. protegens* Pf-5 *pvd-inaZ* to increasing iron concentrations in vitro was assessed as described by Loper and Henkels (1997). The strain (6.6×10^6 cells) was inoculated into 20 ml of SM medium containing 0.1 to 1,000 μM FeEDDHA. Three 5-ml aliquots per concentration were incubated for 48 h at 20°C and 120 rpm before measuring ice nucleation activity. To this end, the number of ice nuclei was determined by the droplet freezing assay (Loper and Lindow 1997), after serially diluting samples and placing

40 droplets of 10 µl from each dilution on a paraffin-coated aluminum foil floating on a -5°C ethanol bath and recording the number of frozen droplets after 2 min. The number of ice nuclei was computed as $\ln[1/(1 - \text{fraction of frozen droplets})]/[\text{droplet volume} \times \text{dilution}]$, as proposed by Vali (1971), and was divided by the number of cells (estimated via OD₆₀₀ measurement). A standard curve was generated by plotting the ice nucleation activity (ice nuclei cell⁻¹) against the FeEDDHA concentration.

To assess iron bioavailability in the tobacco rhizosphere, root systems were inoculated as indicated above, by dipping for 1 min in a cell suspension (OD₆₀₀ 0.015, approximately 10⁷ cells ml⁻¹) of the iron biosensor strain Pf-5 *pvd-inaZ* or the positive control strain Pf-5 *iceC*. The tobacco seedlings were then transplanted in the artificial soils. Seven days later, plants were unearthed. Each root system and adhering soil was placed in 1 ml of 10 mM potassium phosphate buffer (pH 7.0) and sonicated for 5 min. Samples were serially diluted, cell numbers were determined 2 days after plating onto King's B medium supplemented with rifampicin and kanamycin, and ice nucleation activity was determined (as indicated above). Ice nucleation activity of the positive control strain Pf-5 *iceC* was constant in all treatments. Three plants were studied per soil-strain combination. The experiment was done twice.

Image and statistical analyses.

The cell counting option of BioImage_L software v.2.1 (Chávez de Paz 2009) with a noise correction set to default was used to quantify each type of fluorescent cells per CLSM image. The proportion of cells that expressed the *phlA-gfp* fusion and produced GFP (i.e., proportion of GFP⁺ cells) was calculated as the number of green cells divided by the total number of fluorescent cells in the image.

The root colonization pattern of bacteria was studied by recording for each CLSM image i) the corresponding root zone (i.e., the root base [first third], the middle part [second third], or the root tip [third third]), ii) the location of bacterial cells (root surface or root hair), and iii) the size of each bacterial colony detected, using the particle size analysis option in ImageJ software.

Statistical analyses were performed without data transformation (for disease severity) or after square root (for proportions), rank (for bacterial colony size), or log₁₀ (for all other variables) transformation. Means were compared by Student's *t* tests, or by analysis of variance followed with Fisher's least significant difference tests when more than two treatments were studied. Size distribution patterns of bacterial cells (i.e., the proportion of individual cells, cell clumps, and microcolonies) were compared using a χ^2 test of independence. Analyses were carried out at *P* < 0.05, using the R environment.

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