Root-Secreted Malic Acid Recruits Beneficial Soil Bacteria[1][C][W][OA]

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Beneficial soil bacteria confer immunity against a wide range of foliar diseases by activating plant defenses, thereby reducing a plant's susceptibility to pathogen attack. Although bacterial signals have been identified that activate these plant defenses, plant metabolites that elicit rhizobacterial responses have not been demonstrated. Here, we provide biochemical evidence that the tricarboxylic acid cycle intermediate L-malic acid (MA) secreted from roots of Arabidopsis (*Arabidopsis thaliana*) selectively signals and recruits the beneficial rhizobacterium *Bacillus subtilis* FB17 in a dose-dependent manner. Root secretions of L-MA are induced by the foliar pathogen *Pseudomonas syringae* pv *tomato* (Pst DC3000) and elevated levels of L-MA promote binding and biofilm formation of FB17 on Arabidopsis roots. The demonstration that roots selectively secrete L-MA and effectively signal beneficial rhizobacteria establishes a regulatory role of root metabolites in recruitment of beneficial microbes, as well as underscores the breadth and sophistication of plant-microbial interactions.

Many bacterial and fungal pathogens are not restricted to infecting aerial or root tissues exclusively. As such, communication between aboveground and belowground components can confer a survival advantage and potentially limit or prevent diseases. Current literature suggests that the metabolic levels of low-M_r compounds play a significant role in triggering plant innate defense responses (Klessig et al., 2000; Hückelhoven, 2007). A corresponding induced defense response initiating intraplant signaling between leaves and roots has been implicated in herbivory (van Tol et al., 2001; Rasmann et al., 2005). Indeed, plant basal levels of shoot defenses were significantly altered following not only root herbivory, but also artificial damage and application of plant defense hormones (van Dam et al., 2003; Soler et al., 2005; Rasmann and Turlings, 2007). Others have shown that shoot herbivory attack resulted in an increase in root concentrations of nicotine and protease inhibitors in *Nicotiana attenuata* and glucosinolates in *Brassica campestris* (Baldwin et al., 1994; Ludwig-Müller et al., 1997; van Dam et al., 2003; Soler et al., 2007). The importance of chemical signals as distress indicators has greatly benefited from pioneering work on volatile signaling (De Moraes et al., 1998, 2001; Kessler and Baldwin, 2001). Under herbivory attack, plants relay compounds such as hormones, exogenous volatile organic compounds, and non-horm1 sary metabolites as long-distance root-to-shoot signals (Erb et al., 2008). Wounding of plant tissues by insect feeding triggers the release of well-characterized volatile signals that attract natural enemies of insect herbivores (De Moraes et al., 2001; Kessler and Baldwin, 2001). Additionally, leaves of the donor plants also perceive intraplant volatile signals to adjust their defensive phenotype (Heil and Silva-Bueno, 2007). Apart from these studies on volatile distress signaling, there are very few studies (van Tol et al., 2001; Rasmann et al., 2005; Hiltpold and Turlings, 2008) on how plants respond by secreting chemicals in response to herbivore attack to recruit beneficial entomopathogenic nematodes to the rhizosphere. While considerable data exist on the occurrence of aboveground/belowground communication in the case of plant herbivory, evidence of similar phenomena in plant-pathogenic bacteria interactions is lacking.

Plants use an array of metabolites to defend themselves against harmful organisms and to attract others that are beneficial. For example, it has been widely documented in the case of root-rhizobial and root-mycorrhizal interactions that roots secrete secondary metabolites that act as messengers to attract *Rhizobium* and arbuscular mycorrhizal fungi (Kent Peters and Long, 1988; Besserer et al., 2006). Despite progress

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toward understanding the symbiotic-plant microbe interactions, little headway has been made in identifying the genetic and biochemical changes responsible for the attraction of nonsymbiotic rhizospheric microbes to plants. Plant associations with plant growth-promoting rhizobacteria (PGPR) provide protection from soil-borne plant pathogens by antagonistic mechanisms (Bais et al., 2004; Cavaglieri et al., 2005). Such bacteria, by colonizing the root surface, can also trigger induced systemic resistance (ISR) in aerial portions of the plant (Ryu et al., 2004). ISR by rhizobacteria was demonstrated using both Gram-negative and Gram-positive bacteria (Baker et al., 1985; Maurhofer et al., 1994; Zhou and Paulitz, 1994; Liu et al., 1995; Leeman et al., 1996; Benhamou and Belanger, 1998; Pieterse et al., 1998; Kloepper et al., 2004). *Bacillus subtilis* not only has been reported to trigger ISR (Kloepper et al., 2004; Ryu et al., 2004), but also has been implicated in promoting plant growth and protection from fungal infection (Dal-Soo et al., 1997; Emmert and Handelsman, 1999; Bacon et al., 2001; Estevez de Jensen et al., 2002; Ryu et al., 2003). Bacterial signals have been identified that activate plant defense responses through the ethylene pathway, independent of the salicylic acid (SA) pathway or the jasmonic acid pathway (Ryu et al., 2004). However, no plant metabolites involved in a positive feedback mechanism have been identified.

Although evidence exists for intraplant communication, to date there have been no reports demonstrating whether plants exude specific chemical signals through their roots to attract beneficial bacteria in the rhizosphere. Furthermore, it is unknown whether shoot infection by pathogenic bacteria induces recruitment of beneficial rhizobacteria to the root surface. Links between interorganism signaling under distress conditions, especially between aboveground and belowground tissues, are poorly understood. Such signaling, although potentially complex due to the involvement of significant physical distances, may be an important and effective strategy in plant defense that has thus far been overlooked. In an effort to address this deficiency, we used *Pseudomonas syringae* pv *tomato* (Pst DC3000)-Arabidopsis (*Arabidopsis thaliana*) and *B. subtilis* strain FB17 model systems. Here, we report that Pst DC3000-infected Arabidopsis foliage relay chemical signals below ground through root secretions. The root-secreted chemical specifically attracts and enhances FB17 root binding and biofilm formation on infected seedlings. We also show that the chemoattraction and biofilm promotion activity of a secreted component is an enantiomeric-dependent response.

RESULTS

Rhizobacteria Colonization of Roots Stimulated by Leaf Pathogen

To probe how plant pathogen attack may influence the recruitment of beneficial rhizosphere bacteria, root symbiont colonization was measured in the presence and absence of a foliar pathogen. Twenty-day-old Arabidopsis plants were rhizoinoculated with FB17. Subsequently, the plants were infected with Pst DC3000 by pressure infiltration into the leaves. By 5 d postinoculation, leaves infected by Pst DC3000 stimulated biofilm formation of the beneficial rhizobacteria FB17 both qualitatively as determined by confocal microscopy (Fig. 1A) and quantitatively by colony-forming units (CFU; Fig. 1B). In fact, within 5 d of Pst DC3000 leaf inoculation, a 4-fold increase in FB17 colonization was observed in the roots compared

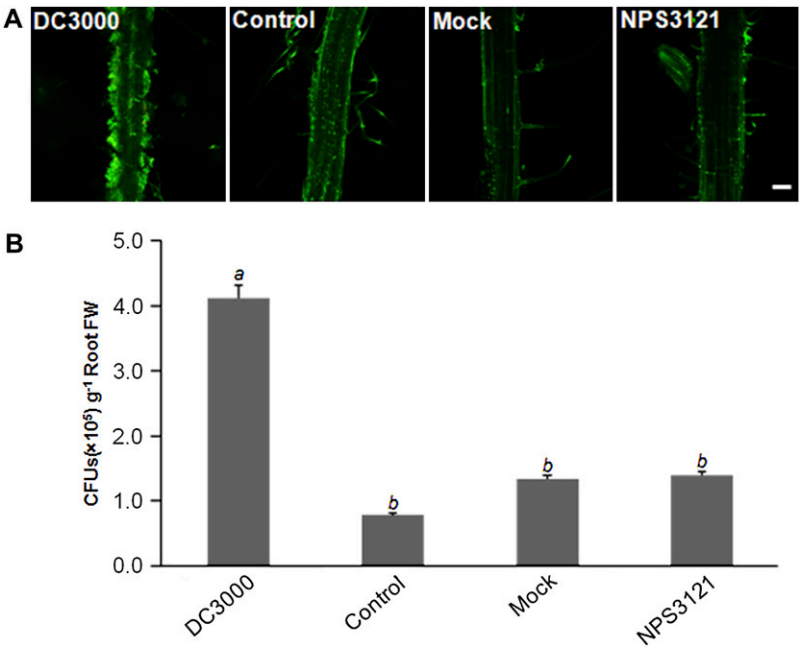

Figure 1. *B. subtilis* strain FB17 root colonization and biofilm formation with pathogen and nonpathogen leaf treatments. The data showed a higher colonization and biofilm formation 5 d posttreatment in response to aerial infection with Pst DC3000 than in untreated (control), water-injected (mock), or nonpathogenic NPS3121-inoculated leaves in representative root colonization experiments by confocal microscopy (A) and CFU quantification (B). The strong green fluorescence along the sides of the roots indicates the FB17 biofilm visualized by staining with SYTO13. Different letters indicate significant difference between the treatments ($P < 0.05$; ANOVA test; scale = 100 μm). [See online article for color version of this figure.]

with mock or nonpathogenic *P. syringae* pv *phaseolicola* (NPS3121) treatments [$F_{(3,20)} = 114.5$; $P < 0.05$]. This observation that aerial infection with Pst DC3000 caused change in root symbiont colonization implicated root exudate involvement in the beneficial microbe recruitment.

Leaf Infection Induces Malic Acid Root Secretions

To examine whether leaf infection can trigger changes in the composition of metabolites from root secretions, the root secretions from ecotype Columbia (Col-0) plants subjected to different aerial bacterial infection treatments, such as Pst DC3000, untreated (control), water injected (mock), or treated with nonpathogenic strain NPS3121, were collected. The root secretions were chemically analyzed by HPLC. Profiles of the concentrated root exudates from Pst DC3000-infected plants revealed a peak that exhibited a significant increase under the Pst DC3000 infection regime. The peak was further characterized by liquid chromatography-mass spectrometry analysis and retention time overlap and determined to be malic acid (MA; Fig. 2A); MA quantification from root exudates of differently treated plants indicated a 7-fold increase [$F_{(3,20)} = 212.1$; $P < 0.05$] in MA accumulation under

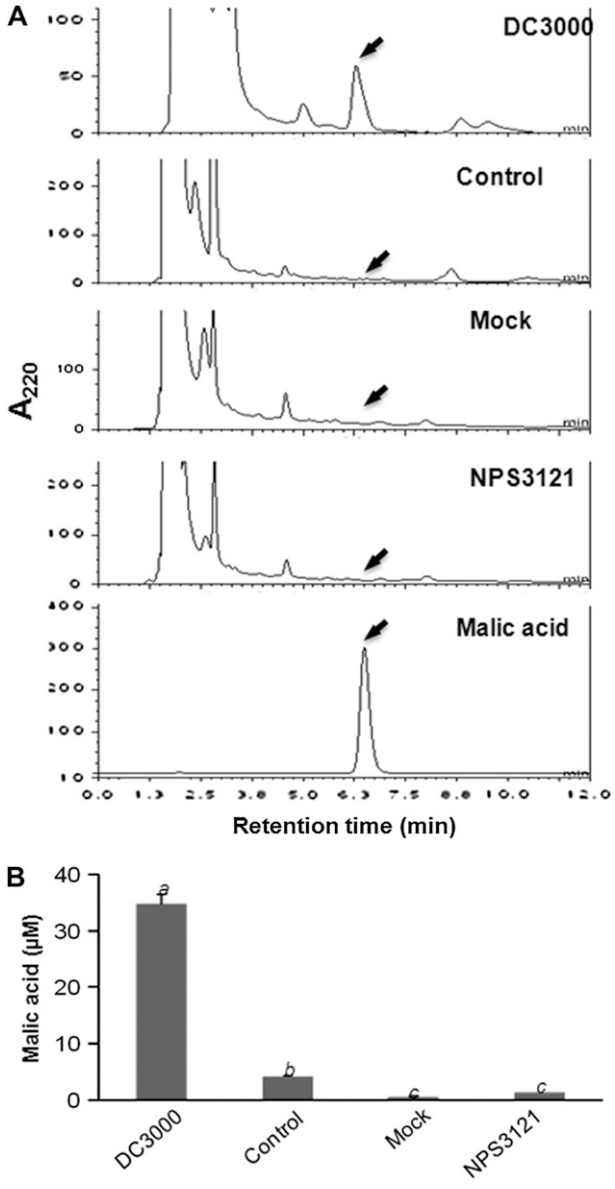

Figure 2. Root exudate composition with pathogen and nonpathogen leaf treatments. Greater MA was detected with aerial infection with Pst DC3000 than in untreated (control), water-injected (mock), or nonpathogenic NPS3121-inoculated leaves as observed by HPLC analysis with representative HPLC profiles (arrow indicates MA elution position; A) and MA peak-area quantification (B). Different letters indicate significant difference between the treatments ($P < 0.05$; ANOVA test).

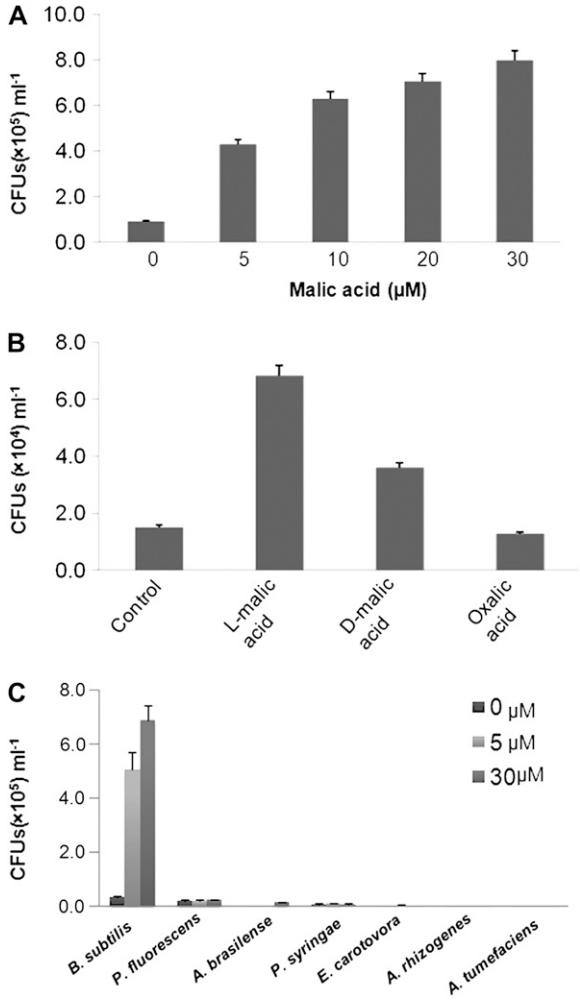

Figure 3. MA-specific chemotactic motility of *B. subtilis* strain FB17 measured by following capillary chemotactic assay. L-MA showed a dose-dependent chemotactic attraction of FB17 (A); structure-dependent chemotactic attraction of FB17 (B); and bacterial species-specific attraction to MA (C). Bacterial strains used in the chemotactic assay included *B. subtilis* strain FB17, *A. tumefaciens* strain LBA4404, *E. carotovora* strain AH2, *A. rhizogenes* strain Arqua-1, *A. brasilense* strain Cd, *P. fluorescens* (*Pf01*), and *P. syringae* (Pst DC3000). Data are the average of six replicates from two experiments conducted separately.

Pst DC3000 leaf infection compared to control, mock, and NPS3121 treatments (Fig. 2B). This pattern of MA accumulation in root secretions raised the question whether the recruitment of higher populations of FB17 to Pst DC3000-infected plants was caused by the MA root secretions or simply a secondary effect of the interaction itself.

FB17 Exhibits Positive MA Chemotaxis

To evaluate MA's ability to selectively recruit FB17, microbial motility at varying MA doses was measured by capillary chemotaxis assay (Gordillo et al., 2007). FB17 exhibits positive chemotactic behavior in a concentration-dependent manner within the dose range of 5 to 30 μM (Fig. 3A). The concentration range of 5 to 30 μM falls within the biological titers of MA secreted in planta through the root exudates (Fig. 2B). The non-natural D-MA, as well as the 4-carbon oxalic acid assayed at the high L-MA response level of 30 μM, exhibited significantly [$F_{(4,25)} = 117.17$; $P < 0.05$] lower efficacies compared to the natural L-MA form, albeit still significantly higher than the water control (Fig. 3B) with D-MA. To establish whether L-MA specifically chemoattracts FB17, other beneficial bacteria, such as *Pseudomonas fluorescens* strain *Pf01* and *Azospirillum brasilense* strain Cd, and pathogenic bacteria, such as Pst DC3000, *Erwinia carotovora* strain AH2, *Agrobacterium rhizogenes* strain Arqua-1, and *Agrobacterium tumefaciens* strain LBA4404, were assayed for MA. Our results revealed that none of the bacteria shows any significant motility toward L-MA [$F_{(2,25)} = 119.12$; $P < 0.05$] compared to FB17 (Fig. 3C), establishing the specific chemotactic role of L-MA in attracting PGPR strain FB17.

MA Transporter Mutant Fails to Recruit *B. subtilis* onto the Root Surface

Arabidopsis T-DNA knockout mutant *Atalmt1* for MA transporter *AtALMT1* deficient in root MA secretion (Hoekenga et al., 2006) was assayed to confirm the role of MA secretions in recruiting FB17. The amount of malate secreted from *Atalmt1* was highly reduced [$F_{(3,20)} = 152.62$; $P < 0.05$] either with or without Pst DC3000 aerial infection (Fig. 4A). Moreover, FB17 failed to colonize the root surface of *Atalmt1* under both infected and noninfected conditions as shown by both microscopic root binding and CFU data [$F_{(2,25)} = 100.23$; $P < 0.05$; Fig. 4, B and C]. To establish the role of *AtALMT1* conclusively, we exploited a previously reported approach (Hoekenga et al., 2006) and generated F_1 plants (genetically complemented for *AtALMT1*) from the cross *Atalmt1* × Landsberg *erecta*

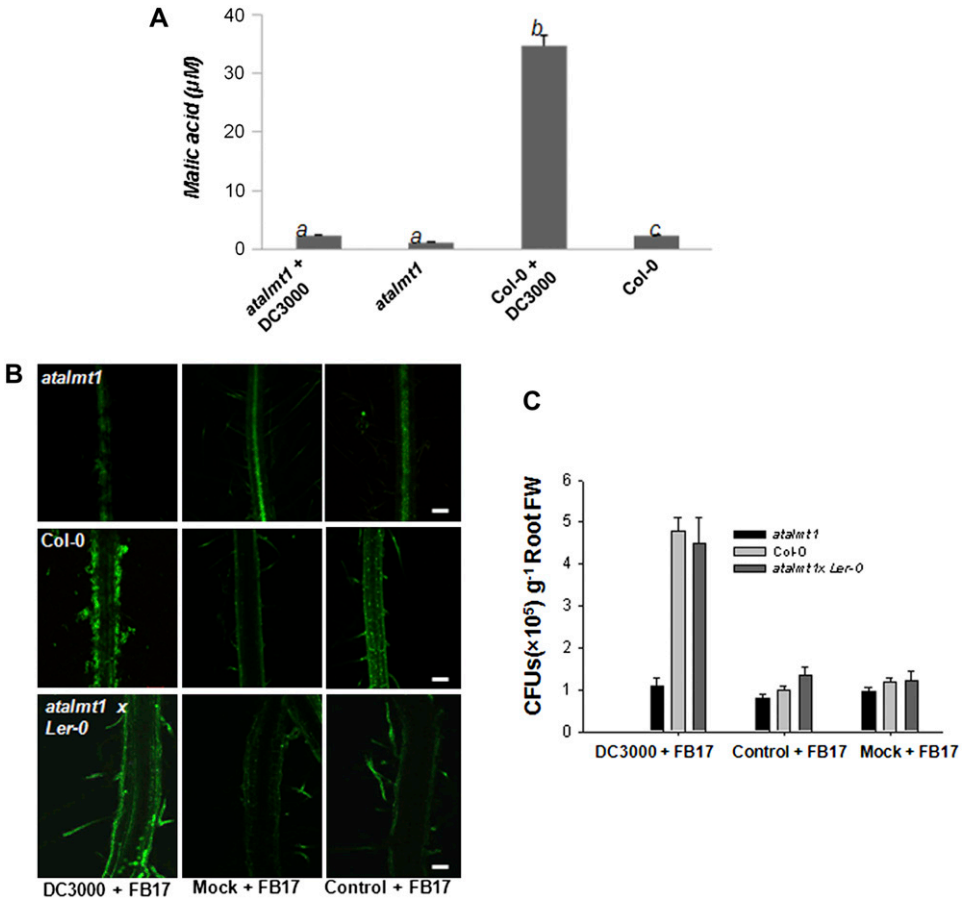

Figure 4. MA transporter mutant (*Atalmt1*) was ineffective in *B. subtilis* strain FB17 root recruitment. A, Reduced MA secretion in *Atalmt1* with or without Pst DC3000 infection (different letters indicate significant difference between the treatments ($P \leq 0.05$; ANOVA test) causes reduced FB17 binding and root colonization, shown with representative confocal images stained with SYTO13 (B). Top two rows = 100 μm; bottom row = 50 μm. C, Quantification of FB17 root binding by CFUs ($n = 6$). [See online article for color version of this figure.]

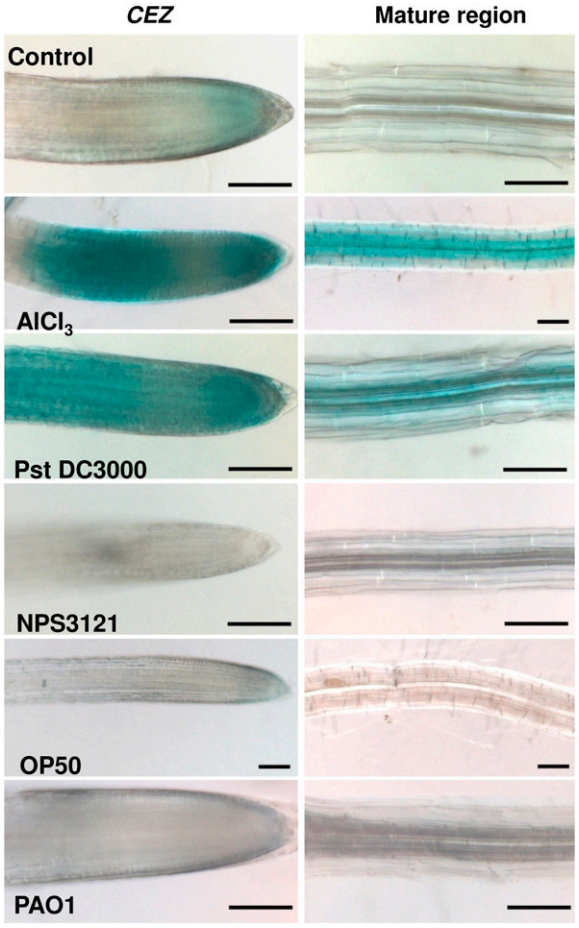

Figure 5. Effect of Pst DC3000 leaf infection on the expression of root *AtALMT1*. Twenty-day-old Arabidopsis line carrying *AtALMT1* promoter::*GUS* fusion construct grown on peat pellets was leaf infected with Pst DC3000 and other controls, such as NPS3121, *E. coli* OP50, and *P. aeruginosa* PAO1; 12-h-postinfection roots were stained for GUS. The figure shows a significantly higher GUS induction in response Pst DC3000 (on par with the positive control $AlCl_3$ [4 μM] root treatment) when compared to untreated controls and other bacterial strains such as NPS3121, OP50, and PAO1 (scale = 100 μm).

(L*er*-0). The F_1 generated lines were subsequently used for FB17 colonization studies under a Pst DC3000 infection regime. As expected, FB17 was able to colonize the F_1 line of *Atalmt1* × L*er*-0 in a similar fashion to wild-type Col-0 roots especially when leaf infected with Pst DC3000 (Fig. 4, B and C). In our hands, the *Atalmt1* × L*er*-0 F_1 line also restored malate release under Pst DC3000 infection (data not shown) as reported previously for aluminum (Al) treatment (Hoekenga et al., 2006).

Leaf Infection Induces Root *AtALMT1* Expression

To check whether aerial leaf infection with Pst DC3000 transcriptionally regulates *AtALMT1* expression, we employed an Arabidopsis transgenic line carrying an *AtALMT1* promoter::*GUS* fusion construct (Kobayashi et al., 2007). The 20-d-old *AtALMT1* promoter::*GUS* Arabidopsis lines were leaf infiltrated with Pst DC3000, NPS3121, *Pseudomonas aeruginosa* PAO1, *Escherichia coli* OP50, and also roots treated with 4 μM $AlCl_3$ as a positive control. The data showed significantly higher *AtALMT1*::*GUS* expression in the treatments with Pst DC3000 leaf infection similar to the $AlCl_3$ positive control in both root central elongation zone and mature region (Fig. 5). However, leaf infiltration with other bacteria, such as PAO1, OP50, and nonpathogenic NPS3121, showed no induction. These data indicated that the *AtALMT1* expression may be specific to leaf pathogenic interactions.

Plant Infected Root Exudates and L-MA Induce *B. subtilis* Biofilm Operons

Microscopic analysis demonstrated increased binding and biofilm formation of FB17 on the root surface. To examine whether biofilm formation was transcriptionally regulated by root secretions, we tested a key operon *yqxM* required for *B. subtilis* biofilm formation. We utilized *B. subtilis* strain Marburg carrying the

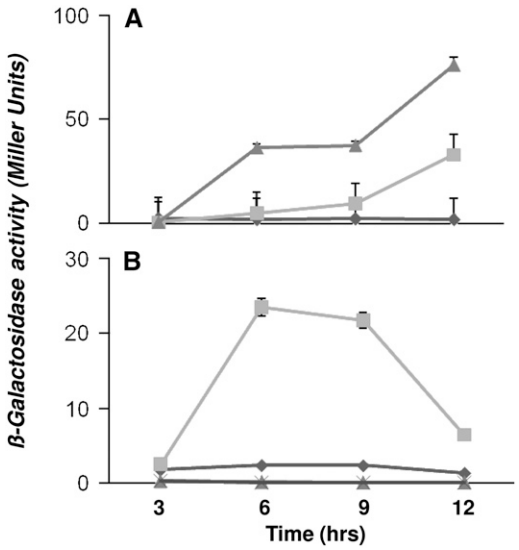

Figure 6. A, Effect of Arabidopsis root exudates on the transcription of the *yqxM* operon in *B. subtilis* strain Marburg carrying the *yqxM-lacZ* fusion (NRS1531). Strain NRS1531 was grown in biofilm medium under biofilm formation conditions at 37°C with root exudates (5%) from Pst DC3000-infected plants (triangles), uninfected (squares) plants, and control (diamonds) without root exudates, and β-galactosidase activity was measured at regular intervals and plotted as a function of time. These experiments were repeated on at least three independent occasions and a representative plot is shown. B, Effect of different MA isomers and oxalic acid on the transcription of the *yqxM* operon in *B. subtilis*. Strain NRS1531 was grown in biofilm medium under biofilm formation conditions at 37°C with 5 μM MA isomers, L-MA (squares) and D-MA (×), oxalic acid (triangles), control (diamonds) without MA isomers, or oxalic acid, and β-galactosidase activity was measured at regular intervals and plotted as a function of time. These experiments were repeated on at least three independent occasions and a representative plot is shown.

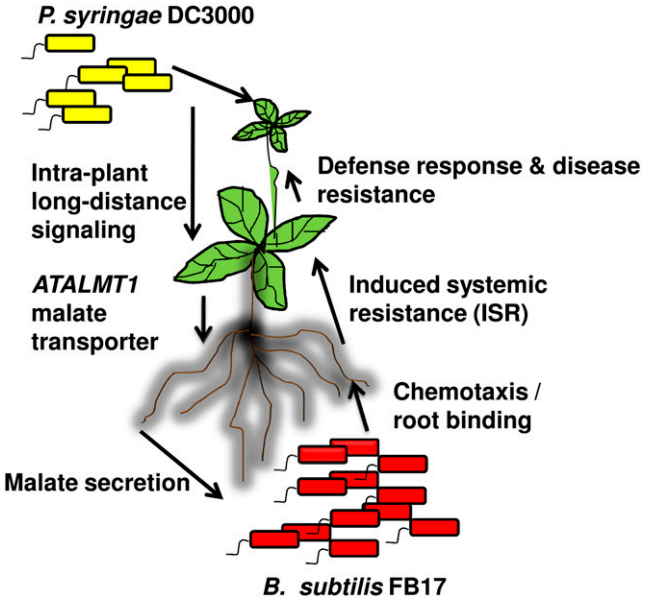

Figure 7. A schematic depicting the long-distance intraplant signaling to recruit rhizobacterium *B. subtilis* strain FB17 through secretion of MA postaerial infection by Pst DC3000. [See online article for color version of this figure.]

yqxM-lacZ fusion (NRS1531) to study the transcription. Biofilm operon regulation in the *lacZ* operon fusion line was monitored for β-galactosidase activity. The treatment with root exudates from Pst DC3000-infected plants resulted in higher induction of the *yqxM* operon between 6 and 12 h posttreatment when compared to untreated controls (Fig. 6A). However, the decline in the expression after 9 h may be a feedback response.

In addition to root exudates from aerially infected plants causing induction of the FB17 biofilm operon *yqxM*, MA alone also elevated expression of the biofilm operon *yqxM* (Fig. 6B). However, with the L-MA treatment, activity level was lower and the kinetics response was abbreviated compared to root exudates. Another isomer (D-MA) and the five-carbon unit oxalic acid did not stimulate β-galactosidase activity (Fig. 6B).

FB17 Root Colonization Triggers ISR and Protects Arabidopsis from Pst DC3000 Infection

To test whether plants associated with FB17 extend protection from disease, we inoculated the FB17 root-colonized Arabidopsis plants with the pathogen Pst DC3000. Consistent with our data on FB17 root colonization following Pst DC3000 leaf infection, the root colonization of FB17 resulted in protection of plants from Pst DC3000 infection. The Pst DC3000-infected FB17-colonized plants revealed reduced disease incidence, symptom development (chlorosis), and pathogen multiplication. The results were highly significant ($P < 0.05$; t test) compared to the control plants not colonized with FB17 and treated with Pst DC3000 (Supplemental Fig. S1, A and B).

To further test whether this protection offered by FB17 was due to the induction of ISR, we checked for known systemic resistance markers such as *PR1* gene expression and free SA levels in the leaves of FB17 root-colonized plants. We utilized Arabidopsis lines carrying *PR1*::*GUS* fusions to study the *PR1* expression. Plant roots colonized with FB17 showed higher *PR1*::*GUS* expression in the leaves on par with leaf SA-treated positive controls compared to uninoculated control plants. Other controls, where plants were root inoculated with OP50 and *Pf01*, failed to induce *PR1*::*GUS* expression in the leaves (Supplemental Fig. S2). Similarly, when free SA levels were analyzed (Scott et al., 2004), the Col-0 plant roots colonized with FB17 showed increased free SA titers compared to plants without FB17 colonization (data not shown).

P. fluorescens (*Pf01*) Fails to Bind to the Root Surface of Aerially Infected Arabidopsis Col-0

To test the specificity of the Arabidopsis FB17 interaction, *Pf01* root-inoculated plants were monitored with and without aerial infection. Consistent with chemotaxis assay data, infected plants failed to recruit *Pf01* to the root surface as observed previously for FB17. The Pst DC3000-infected Col-0 plants showed poor binding of *Pf01*, indistinguishable from the untreated and *Pf01*-only treatments (Supplemental Fig. S3A). Further, the *Pf01* root inoculation failed to protect plants from Pst DC3000 infection (Supplemental Fig. S3, B and C). This result suggested that plants specifically engaged with FB17 under foliar bacterial infection.

DISCUSSION

Certain beneficial rhizobacteria activate plant defenses and thereby mitigate the impact of foliar diseases (Ryu et al., 2004); however, plant recruitment of such soil microbes has yet to be established. Here, we showed that inducible defense responses triggered by plant pathogenesis included the induction of root secretions that effectively recruited beneficial rhizobacteria. Specifically, the major inducible root-secreted metabolite MA selectively activated FB17 chemotactic mobility in a dose-dependent manner (Fig. 7). Correspondingly, reduction in MA root secretions with the MA transporter mutant *Atalmt1* compromised beneficial bacteria root recruitment. This phenotype was restored when the *AtALMT1* mutation was genetically complemented. Furthermore, the leaf infection of *AtALMT1*::*GUS* fusion line with Pst DC3000 was able to induce root *AtALMT1* expression and it has been reported that *AtAlMT1* expression was also induced in response to Al (Hoekenga et al., 2006). However, in this study, we showed *AtALMT1* induction in response

to Pst DC3000 leaf infection occurred even in the absence of Al. The induction of *AtALMT1* and the efflux of MA in response to Pst DC3000 infection independent of Al revealed the multifunctional role of *AtALMT1*. It will be interesting to further elucidate the key pathogenic determinants, which are specifically involved in triggering *AtALMT1* induction. These results establish MA as the first member of a novel class of plant signals operative in rhizosphere attraction of beneficial microbes.

The enantiomeric specificity of naturally produced L-MA in triggering chemotactic mobility implicates receptor-mediated activation of bacterial directed chemotaxis. In addition to stereoselective chemical signals, *B. subtilis* motility is mediated by the pH and electrical potential of the cell exterior. Bacterial flagellar movement is driven by the motor protein complex Mot (Blair and Berg, 1990). The membrane-embedded MotAB complexes and their homologs constitute ion channels and couple flagellar rotation by proton motive or sodium motive forces across the cytoplasmic membrane (Manson et al., 1977; Blair and Berg, 1990; Berry and Armitage, 1999; McCarter, 2001). Interestingly, MotPS-dependent motility is greater in the presence of MA than in the presence of Glc (Ito et al., 2004).

Bacteria also form sessile communities called biofilms that are morphologically and physiologically differentiated from free-living bacteria (Bais et al., 2004; Rudrappa et al., 2007). The process of biofilm formation starts at the late exponential phase of bacterial growth. In addition to bacterial cell-to-cell signaling involvement in the development of such structures, FB17 colonizes and forms continuous biofilms on the roots of Arabidopsis plants aerially infected with Pst DC3000 unlike the nonhost strain NPS3121, which does not infect or produce disease symptoms with Arabidopsis (van Wees and Glazebrook, 2003). This establishes the specificity of biofilm formation with specific pathogen-infection regimes. Plant-generated MA was also specifically induced with Pst DC3000 and positively induced the biofilm operon *yqxM*. The operon *yqxM* is a member of a three-gene operon *yqxM-sipW-tasA*, where *tasA* encodes for a protein that is a major component of the biofilm matrix and *yqxM* is important for the proper localization of TasA to the matrix (Branda et al., 2006). In plants, increased accumulation of L-MA can be triggered by elevated biosynthesis of one or more of the tricarboxylic acid MA precursors, fumarate, oxaloacetate, or pyruvate, by fumarase, malate dehydrogenase, and malic enzyme, or by reduced MA turnover to pyruvate or oxaloacetate by malic enzyme and malate dehydrogenase, respectively (Casati et al., 1999). Alternatively, upregulation of the malate transporter (*AtALMT1*) augments root MA secretions (Kobayashi et al., 2007). Future lines of study will investigate the involvement of MA secretion on induction of bacterial volatile signaling in *B. subtilis*, which has been reported to be involved in the induction of ISR in *B. subtilis*-colonized plants (Ryu et al., 2004). Concomitantly, others have reported that a few organic acids, especially oxaloacetate, trigger the operon for *Bacillus* acetoin production *alsSD* (Schilling et al., 2007).

To establish the specificity of exuded malate toward FB17, other bacteria, including the PGPR *Pf01*, were assayed for chemotaxis. We speculated that the poor colonization patterns of *Pf01* under aerial pathogen infection regimes dictates that infected plants exhibit positive feedback specifically for FB17. In fact, aerially infected plants failed to show increased *Pf01* binding to the root surface. Our data showed restriction of pathogen multiplication through the induction of ISR by triggering the expression of the *PR1* gene in an SA-mediated mechanism following FB17 root colonization. Several studies had previously reported the induction of ISR by root-colonized PGPR (Ryu et al., 2004) or targeted inhibition of pathogen genes in a standard biocontrol setup (Duffy et al., 2004). Most of these studies were linked to production of a pathogen antagonist by the biocontrol strain. Thus far, the best example of chemical-based intraplant signaling was established recently, where the volatile compounds emitted by one plant part elicited defense responses in other parts of the same plant (Gershenzon, 2007; Heil and Silva-Bueno, 2007). There is no evidence that shows that plants are capable of selectively populating its rhizosphere with beneficial microbes to induce a typical ISR response. Our results showed evidence of the importance of interorganism signaling, although their ecological relevance needs to be examined. Understanding the two-way signaling event in terms of a bacterial metabolite component that malate elicits underscores the evolutionary development of acquired responses in plants to recruit beneficial microbes in its rhizosphere. Subsequent studies should reveal new gene-for-gene interactions that exist among plants and microbes.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) wild-type Columbia (Col-0) seeds were procured from Lehle Seeds. Arabidopsis T-DNA knockout mutant line *Atalmt1* was obtained from the Arabidopsis Biological Resource Center (ABRC).The plants were selfed for four generations to achieve homozygosity and the homozygous plants were used in this study. Arabidopsis transgenic lines carrying the *AtALMT1* promoter::*GUS* fusion construct were obtained from Dr. Hiroyuki Koyama. The *AtALMT1* genetic complemented F_1 line was generated by crossing *Atalmt1* × L*er*-0 by following a published protocol (Hoekenga et al., 2006). Crossing was performed between *Atalmt1* (Col-0 background) and Arabidopsis L*er*-0 ecotype (as a pollen donor). In the F_1 generation, the *AtALMT1* allele from L*er*-0 restored the *AtALMT1* phenotype in terms of Al-activated malate release (Hoekenga et al., 2006). Seeds were cultured on Murashige and Skoog (1962) solid medium with 3% Suc and allowed to germinate for 3 to 4 d until the roots and shoot emerged by incubating at 23°C ± 2°C under a 16-h-light/8-h-dark photoperiod. The plates were illuminated with cool-white fluorescent light with an intensity of 24 μmol m^{-2} s^{-1}.

Bacterial Strains

The PGPR *Bacillus subtilis* strain FB17 (obtained from Dr. Ray Fall, University of Colorado, Boulder, CO) was maintained on Luria-Bertani plates and *Pseudo-*

monas fluorescens Pf01 (obtained from Dr. George O'Toole, Department of Microbiology and Immunology, Dartmouth Medical School, Hanover, NH). The strain NRS1531 with biofilm operon-lacZ transcription fusions, *thrC*::*yqxM-lacZ* in wild-type Marburg background (obtained from Dr. Nicola R. Stanley-Wall, Division of Applied and Environmental Biology, School of Life Sciences, University of Dundee, Dundee, Scotland), was maintained on Luria-Bertani plates supplemented with 0.5 μg mL^{-1} erythromycin. *Pseudomonas syringae* strains Pst DC3000 and NPS3121 and *Escherichia coli* strain OP50 (obtained from Dr. Jorge M. Vivanco, Colorado State University, Fort Collins, CO) were maintained on Luria-Bertani plates with and without 50 μg mL^{-1} rifampicin, respectively. *Pseudomonas aeruginosa* strain PAO1 (obtained from Dr. Frederick M. Ausubel, Massachusetts General Hospital, Boston) was maintained on Luria-Bertani plates with 20 μg mL^{-1} rifampicin. *Agrobacterium tumefaciens* strain LBA4404 and *Erwinia carotovora* strain AH2 (obtained from Dr. Thomas Evans, Department of Plant and Soil Sciences, University of Delaware, Newark, DE) were maintained on Luria-Bertani plates. *Agrobacterium rhizogenes* strain Arqua-1 (obtained from Dr. Janine M. Sherrier, Department of Plant and Soil Sciences, University of Delaware, Newark, DE) and *Azospirillum brasilense* strain Cd (obtained from Dr. Yaacov Okon, Department of Plant Pathology and Microbiology, Hebrew University, Israel) were maintained on TY-agar plates. For growth of *B. subtilis* under in vitro biofilm formation conditions, cells were grown at 37°C in a Luria-Bertani-based biofilm medium supplemented with 15 mM $(NH_4)_2SO_4$, 80 mM K_2HPO_4 $3H_2O$, 44 mM KH_2PO_4, 3.4 mM sodium citrate, 1 mM $MgSO_4$, and 0.1% Glc.

Effect of *P. syringae* Infection on *B. subtilis* Root Binding and Biofilm Formation on the Roots of Arabidopsis

Arabidopsis Col-0 plants grown on sterile peat pellets in a growth chamber set for a photoperiod of 16 h light/8 h dark at 23°C ± 2°C and illuminated with cool fluorescent light with an intensity of 24 μmol m^{-2} s^{-1} for a 20-d period. The plants were root inoculated with FB17 (0.5 OD_{600}) by drenching with 10 mL of the culture in water. All the fully expanded leaves were pressure infiltrated with 100 μL of 0.02 OD_{600} culture of Pst DC3000. The cultures in sterile water were prepared by centrifuging the overnight cultures and washing the pellet to get rid of Luria-Bertani medium and resuspending the pellet in sterile water to obtain appropriate density. Different treatments included control root inoculated with FB17, mock (leaf infiltrated with sterile water + root inoculated with FB17), Pst DC3000 + FB17 (leaf infiltration of FB17 root-inoculated plants), and NPS3121 + FB17 (leaf infiltration of FB17 root-inoculated plants). The plants were transferred to sterile magenta boxes and incubated in the growth chamber for an additional 4 d. The experiment was terminated after 4 d and the cocultivated roots were collected and fixed in 4% paraformaldehyde to image for FB17 binding and biofilm formation. Similar experiments were carried out using the malate transporter mutant *Atalmt1*. Each treatment had six replicates and the experiment was repeated on two independent occasions.

Effect of *P. syringae* Infection on *P. fluorescens* (*Pf01*) Binding and Biofilm Formation on the Roots of Arabidopsis

All the conditions for this experiment were similar as explained above, except that the plants were root inoculated with *P. fluorescens Pf01* (0.5 OD_{600}) by drenching with 10 mL of the culture in water. All the observations that were recorded for the earlier experiment were recorded here also.

Collection and Analysis of Root Exudates from Aerially Infected Col-0 Plants

Individual Col-0 plants were grown on a sterile sponge pellet suspended into sterile 1% Murashige and Skoog liquid medium for 20 d. The fully expanded leaves of the 20-d-old plants were inoculated aseptically with 100 μL of 0.02 OD_{600} culture of *P. syringae* Pst DC3000 (prepared as above). The spent liquid medium from the infected and uninfected plants containing root exudates was collected after 5 d and lyophilized. For HPLC analysis, 100 mg of the lyophilized powder of the root exudates were dissolved in 500 μL of 25 mM potassium phosphate buffer (pH 2.5) and filtered; 30 μL of the filtrate were injected for HPLC analysis. The separation was performed with an isocratic mobile phase of 25 mM potassium phosphate buffer (pH 2.5) on an Altech organic acid column (Prevail, organic acid, 5 μm; 150 mm × 4.6 mm). The peaks obtained were compared with an array of standard organic acid peaks run under the same conditions. The major peak was identified by comparing the retention time with that of the matching standard. Root exudates collected similarly from the Arabidopsis malate transporter mutant *Atalmt1* were also processed and analyzed using the same conditions. Each treatment had 12 replicates and the experiment was repeated on at least two independent occasions.

Chemotaxis Assay

The capillary assay was performed as per the published description (Gordillo et al., 2007) with slight modifications. Briefly, the assay setup consisted of a 200-μL pipette tip as a chamber for holding 100 μL of bacterial suspension (0.1 OD_{600} prepared from overnight grown culture) in Luria-Bertani medium. A 4-cm 25-gauge needle (Becton-Dickinson) was used as the chemotaxis capillary and was attached to a 1-mL tuberculin syringe (Becton-Dickinson) containing 200 μL of the compound (5 μM of different MA isomers such as L-MA, D-MA, and also oxalic acid separately in Luria-Bertani liquid medium). After 3 h of incubation at room temperature, the needle syringe was removed from the bacterial suspension and the content diluted and plated in Luria-Bertani medium. Accumulation of bacteria in the capillaries was calculated as the average from the CFUs obtained in triplicate plates and the results were expressed as the mean of at least three separate capillary assays for each determination. The different bacterial strains assayed for chemotaxis included FB17, *Pf01*, Cd, Pst DC3000, AH2, Arqua-1, and LBA4404.

Effect of Pst DC3000 Leaf Infection on Root *AtALMT1*::*GUS* Expression

Transgenic Arabidopsis (Col-0) plants carrying *AtALMT1* promoter::*GUS* fusion construct for the experiments were grown on peat pellets in a growth chamber set for a photoperiod of 16 h light/8 h dark at 23°C ± 2°C and illuminated with cool fluorescent light with an intensity of 24 μmol m^{-2} s^{-1} for a 20-d period. The 20-d-old plants were leaf infiltrated with Pst DC3000, NPS3121, OP50, and PAO1 and a positive control of $AlCl_3$ root treatment was also included in addition to an untreated control. After 12 h of treatment, the roots were stained and imaged for the expression of *AtALMT*::*GUS* according to published protocol (Kobayashi et al., 2007).

Effect of *B. subtilis* Root Inoculation on the *P. syringae* (Pst DC3000) Infection

Arabidopsis Col-0 plants were grown on peat pellets in a growth chamber set for a photoperiod of 16 h light/8 h dark at 23°C ± 2°C and illuminated with cool fluorescent light with an intensity of 24 μmol m^{-2} s^{-1} for a 20-d period. The plants were root inoculated with *B. subtilis* FB17 (0.5 OD_{600}) by drenching with 10 mL of the culture in water. Fully expanded leaves were pressure infiltrated with 100 μL of 0.02 OD_{600} culture of Pst DC3000 the next day. Different treatments included control (without Pst DC3000 infiltration), control root inoculated with FB17, mock (leaf infiltrated with sterile water + FB17 root inoculated), Pst DC3000 (only leaf infiltration), and Pst DC3000 + FB17 (leaf infiltration of FB17 root-inoculated plants). The plants were transferred to magenta boxes and incubated in the growth chamber for an additional 4 d. The experiment was terminated after 4 d and the observations such as disease symptom development in terms of chlorosis, number of CFUs of Pst DC3000/g fresh weight of the leaf were recorded by extracting a known fresh biomass of the leaf and plating on Luria-Bertani plates containing 50 mg mL^{-1} rifampicin.

Effect of *B. subtilis* FB17 Root Inoculation on Whole-Plant *PR1*::*GUS* Expression

Transgenic Arabidopsis (Col-0) plants carrying *PR1*::*GUS* fusion construct were grown on peat pellets for a 20-d period. The 20-d-old plants were root inoculated with FB17 by drenching the pellet with 10 mL (0.5 OD_{600}) culture suspended in sterile distilled water. In addition, a separate set of *PR1*::*GUS* plants was also treated with other bacteria such as *Pf01* and OP50 by drenching the pellet with 10 mL (0.5 OD_{600}) of culture suspended in sterile distilled water. Positive controls, such as SA (0.1 mM) treatment on leaves and root were also performed by spraying a known concentration of SA on the leaves and roots of *PR1*::*GUS* plants. Four days after inoculation, the plants were stained for GUS assay using a GUS staining kit (obtained from Sigma-Aldrich) according to manufacturer's instructions. The control and treated plants were imaged for the expression of *PR1*::*GUS* in the leaves as per the

published protocol (Shapiro and Zhang, 2001). A representative image of at least 12 leaves was presented for each treatment. Each treatment had six replicates and the experiment was repeated on two independent occasions.

Microscopy

To view adherent FB17 cells and biofilm on the root surface by confocal scanning laser microscopy, the roots were stained at a dilution of 1:1,000 with SYTO13 (Invitrogen). Single median optical sections were captured with a Zeiss 10× Plan-Apochromat (numerical aperture 0.45) objective lens using a Zeiss LSM 510 NLO on an Axiovert 200M. The 488-nm laser line of the argon laser and a 505-nm long-pass filter were used for excitation and emission, respectively. All data were acquired 24 h postinoculation and posttreatment with *B. subtilis* FB17 (5-μL culture of 0.02 OD_{600}) of 10-d-old plants grown in 4 mL of liquid Murashige and Skoog medium with 1% Suc. Each experiment was repeated twice with three replicates each and a representative image of at least 20 roots imaged for each treatment was presented.

β-Galactosidase Assay

To study the effect of root exudates collected from Pst DC3000-infected Col-0 plants and MA on the transcription of the *yqxM* promoter under biofilm formation condition, *B. subtilis* strain Marburg carrying the *yqxM-lacZ* fusion (NRS1531) was utilized. The β-galactosidase units produced per minute were estimated as per the published protocol (Rudrappa et al., 2007). Each treatment had six replicates and the experiment was repeated on at least two independent occasions and a representative plot is shown.

Statistical Analysis

All data were averaged from two separate experiments unless mentioned otherwise and further analyzed for variance followed by a Student's *t* test and ANOVA with the Benjamini-Hochberg correction (Benjamini and Hochberg, 1995) for multiple testing when necessary using statistical package JMP 7.0 and Microsoft Excel XP. The data means were considered significantly different at the probability of $P \leq 0.05$.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Effect of *B. subtilis* FB17 root colonization on Pst DC3000 leaf infection.

Supplemental Figure S2. Effect of *B. subtilis* FB17 root colonization on *PR1*::*GUS* expression.

Supplemental Figure S3. Effect of *P. fluorescens* (*Pf01*) root colonization on Pst DC3000 leaf infection.

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