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1 **Root Secreted Malic Acid Recruits Beneficial Soil Bacteria**

2 **Thimmaraju Rudrappa[1,2], Kirk J. Czymmek[2,3], Paul W. Paré[4] and Harsh P. Bais[1,2†]**

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4 [1]Department of Plant and Soil Sciences, University of Delaware, Newark, DE 19716; [2]Delaware
5 Biotechnology Institute, 15 Innovation Way, Newark, DE 19711; [3]Department of Biological
6 Sciences, University of Delaware, Newark, DE 19716; [4]Departments of Chemistry/Biochemistry
7 and Biology, Texas Tech University, Lubbock, TX 79409.

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33 [†]For correspondence: Email: hbais@udel.edu; Fax: 302-831-3447

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Abstract

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

Keywords: biofilm/*Arabidopsis*/*Bacillus*/root surface/malic acid/chemotaxis

INTRODUCTION

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 small molecular weight compounds play a significant role in triggering plant innate defense responses (Klessig et al., 2000; Hückelhoven, 2007). A corresponding induced defense response initiating intra-plant 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 not only following root herbivory, but also artificial damage and application of plant defense hormones (Rasmann and Turlings, 2007; Soler et al., 2005; van Dam et al., 2003). 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; van Dam et al., 2003; Ludwig-Müller et al., 1997; 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-hormone secondary 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 intra-plant 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 exists 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 which act as messengers to attract *Rhizobium* and arbuscular mycorrhizal fungi (Kent Peters and Long, 1988; Besserer et al., 2006). Despite progress towards understanding the symbiotic-plant microbe interactions, little headway has been made in identifying the genetic and biochemical changes responsible for the attraction of non-symbiotic 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 positive bacteria (Baker et al., 1985; Maurhofer et al., 1994; Zhou and Paulitz, 1994; Liu et al., 1995; Leeman et al., 1996; Pieterse et al., 1998; Benhamou and Belanger, 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 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 which activate plant defence responses through ethylene pathway, independent of salicylic acid or jasmonic acid pathway (Ryu et al., 2004). However, no plant metabolites involved in a positive feedback mechanism have been identified.

Although evidence exists for intra-plant 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 if shoot infection by pathogenic bacteria induces recruitment of beneficial rhizobacteria to the root surface. Links between inter-organism signaling under distress conditions, especially between above 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 thaliana* and *Bacillus subtilis* strain FB17 model system. Here we report that Pst DC3000 infected Arabidopsis foliage relay chemical signal(s) belowground 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 chemo-attraction and biofilm promotion activity of 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 *A. thaliana* plants were rhizo-inoculated with FB17. Subsequently, the plants were infected with Pst DC3000 by pressure infiltration into the leaves. By 5 days post-inoculation, leaves infected by Pst DC3000 stimulated biofilm formation of the beneficial rhizobacteria FB17 both qualitatively as determined by confocal microscopy (Figure 1A) and quantitatively by colony forming units (CFU) (Figure 1B). In fact within five days of Pst DC3000 leaf inoculation, a four-fold increase in FB17 colonization was observed in the roots compared with mock or non-pathogenic *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 (MA) Root Secretions

To examine whether leaf infection can trigger changes in the composition of metabolites from root secretions, the root secretions from Col-0 plants subjected to different aerial bacterial infection treatments such as Pst DC3000, untreated (control), water injected (mock), or treated with non-pathogenic strain NPS3121 were collected. The root secretions were chemically analyzed by high performance liquid chromatography (HPLC). Profiles of the concentrated root exudates from Pst DC3000 infected plants revealed a peak that exhibited a significant increase under Pst DC3000 infection regime. The peak was further characterized by mass spectral analysis (LC-MS) and retention time overlap and determined to be MA (Figure 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 Pst DC3000 leaf infection compared to control, mock and NPS3121 treatments (Figure 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 5-30 µM (Figure 3A). The concentration range of 5-30 µM falls within the biological titers of MA secreted

1 *in planta* through the root exudates (Fig. 2B). The non-natural D-MA as well as the 4-carbon
2 oxalic acid assayed at the high L-MA response level of 30 μM exhibited significantly
3 ($F_{(4,25)}$=117.17, *P<0.05*) lower efficacies compared to the natural L-MA form, albeit still
4 significantly higher than the water control (Figure 3B) with D-MA. To establish whether L-MA
5 specifically chemoattracts FB17, other beneficial bacteria such as *Pseudomonas fluorescens*
6 strain *Pf01, Azospirillum brasilense* strain Cd, and pathogenic bacteria such as Pst DC3000,
7 *Erwinia carotovora* strain AH2, *Agrobacterium rhizogenes* strain Arqua-1 and *Agrobacterium*
8 *tumefaciens* strain LBA4404 were assayed for MA. Our results revealed that none of bacteria
9 show any significant motility towards L-MA ($F_{(2,25)}$=119.12, *P<0.05*) compared to FB17 (Figure
10 3C) establishing the specific chemotactic role of L-MA in attracting PGPR strain FB17.
11
12 **MA Transporter Mutant Fails to Recruit *B. subtilis* onto the Root Surface**
13 An *A. thaliana* T-DNA knockout mutant *Atalmt1* for MA transporter *AtALMT1* deficient
14 in root MA secretion (Hoekenga et al., 2006) was assayed to confirm the role of MA secretions in
15 recruiting FB17. The amount of malate secreted from *Atalmt1* was highly reduced ($F_{(3,20)}$=152.62,
16 *P<0.05*) either with or without Pst DC3000 aerial infection (Figure 4A). Moreover, FB17 failed
17 to colonize the root surface of *Atalmt1* under both infected and non-infected conditions as shown
18 by both microscopic root binding and colony forming units (CFU) data ($F_{(2,25)}$=100.23, *P<0.05*)
19 (Figure 4B-C). To establish the role of *AtALMT1* conclusively we exploited a previously reported
20 approach (Hoekenga et al., 2006) and generated F1 plants (genetically complemented for
21 *AtALMT1*) from the cross *Atalmt1*×*Ler-0*. The F1 generated lines were subsequently used for
22 FB17 colonization studies under a Pst DC3000 infection regime. As expected FB17 was able to
23 colonize the F1 line of *Atalmt1*×*Ler-0* in a similar fashion to wild type Col-0 roots especially
24 when leaf infected with Pst DC3000 (Figure 4 B-C). In our hands, the *Atalmt1*×*Ler-0* F1 line also
25 restored malate release under Pst DC3000 infection (data not shown) as reported previously for
26 Al treatment (Hoekenga et al., 2006).
27
28 **Leaf Infection Induces Root *AtALMT1* Expression**
29 To check whether, aerial leaf infection with Pst DC3000 transcriptionaly regulates
30 *AtALMT1* expression, we employed an *A. thaliana* transgenic lines carrying an AtALMT1
31 promoter::GUS fusion construct (Kobayashi et al., 2007). The 20 day old *AtALMT1*
32 promoter::GUS Arabidopsis lines were leaf infiltrated with Pst DC3000, NPS3121, *Pseudomonas*
33 *aeruginosa* PAO1, *Escherichia coli* OP50 and also root treated with 4μM $AlCl_3$ as a positive
34 control. The data showed a significantly higher *AtALMT1*::GUS expression in the treatments with

1 Pst DC3000 leaf infection similar to the $AlCl_3$ positive control in both root central elongation
2 zone (CEZ) and mature region (Figure 5). However, leaf infiltration with other bacteria such as
3 PAO1, OP50 and non pathogenic NPS3121 showed no induction. This data indicated that the
4 *AtALMT1* expression may be specific to leaf pathogenic interactions.
5
6 **Plant Infected Root Exudates and L-MA Induce *B. subtilis* Biofilm Operons**
7 Microscopic analysis demonstrated increased binding and biofilm formation of FB17 on
8 the root surface. To examine if biofilm formation was transcriptionaly regulated by root
9 secretions we tested a key operon *yqxM* required for *B. subtilis* biofilm formation. We utilized *B.*
10 *subtilis* strain Marburg carrying the *yqxM-lacZ* fusion (NRS1531) to study the transcription.
11 Biofilm operon regulation in the *Lacz* operon fusion line was monitored for β- galactosidase
12 activity. The treatment with root exudates from Pst DC3000 infected plants resulted in higher
13 induction of the *yqxM* operon between 6 and 12 hours post-treatment when compared to untreated
14 controls (Figure 6A). However, the decline in the expression after 9 hours may be a feedback
15 response.
16 In addition to root exudates from aerially infected plants causing induction of the FB17
17 biofilm operon *yqxM*, MA alone also elevated expression of the biofilm operon *yqxM* (Figure
18 6B). However, with the L-MA treatment, activity level was lower and the kinetics response was
19 abbreviated compared to root exudates. Other isomer (D-MA) as well as the five-carbon unit
20 oxalic acid did not stimulate β-galactosidase activity (Figure 6B).
21
22 **FB17 Root Colonization Triggers ISR and Protects *A. thaliana* from Pst DC3000 Infection**
23 To test whether plants associated with FB17 extends protection from disease; we
24 inoculated the FB17 root colonized Arabidopsis plants with the pathogen Pst DC3000. Consistent
25 with our data on FB17 root colonization following Pst DC3000 leaf infection, the root
26 colonization of FB17 resulted in protection of plants from Pst DC3000 infection. The Pst DC3000
27 infected FB17 colonized plants revealed reduced disease incidence, symptom development
28 (chlorosis) and pathogen multiplication. The results were highly significant ($P<0.05$; t-test)
29 compared to the control plants not colonized with FB17 and treated with Pst DC3000
30 (Supplementary Figure 1A-B).
31 To further test whether this protection offered by FB17 was due to the induction of ISR
32 we checked for known systemic resistance markers such as PR1 gene expression and free SA
33 levels in the leaves of FB17 root colonized plants. We utilized Arabidopsis lines carrying
34 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 un-inoculated control plants. Other controls, where plants were root inoculated with OP50 and *Pf01*, failed to induce PR1::GUS expression in the leaves (Supplementary Figure 2). 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**

In order 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 (Supplementary Figure 3A). Further, the *Pf01* root inoculation failed to protect plants from Pst DC3000 infection (Supplementary Figure 3B-C). This result suggested that plants specifically engaged with FB17 under foliar bacterial infection.

1 **DISCUSSION**

2 Certain beneficial rhizobacteria activate plant defenses and thereby mitigate the impact of
3 foliar diseases (Ryu et al., 2004); however, plant recruitment of such soil microbes has yet to be
4 established. Here we showed that inducible defense responses triggered by plant pathogenesis
5 included the induction of root secretions that effectively recruited beneficial rhizobacteria.
6 Specifically, the major inducible root secreted metabolite MA selectively activated FB17
7 chemotactic mobility in a dose-dependent manner (Figure 7). Correspondingly, reduction in MA
8 root secretions with the MA transporter mutant *Atalmt1* compromised beneficial-bacteria root
9 recruitment. This phenotype was restored when the *AtALMT1* mutation was genetically
10 complemented. Furthermore, the leaf infection of *AtALMT1*::GUS fusion line with Pst DC3000
11 was able to induce root *AtALMT1* expression and it has been reported that *AtAlMT1* expression
12 was also induced in response to Al (Hoekenga et al., 2006). However, in the present study we
13 showed *AtALMT1* induction in response to Pst DC3000 leaf infection occurred even in the
14 absence of Al. The induction of *AtALMT1* and the efflux of MA in response to Pst DC3000
15 infection independent of Al revealed the multi-functional role of *AtALMT1*. It will be interesting
16 to further elucidate the key pathogenic determinants which are specifically involved in triggering
17 *AtALMT1* induction. These results establish MA as the first member of a novel class of plant
18 signals operative in rhizosphere attraction of beneficial microbes.

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

28 Bacteria also form sessile communities called biofilms that are morphologically and
29 physiologically differentiated from free-living bacteria (Bais et al., 2004; Rudrappa et al., 2007).
30 The process of biofilm formation starts at late exponential phase of bacterial growth. In addition
31 to bacterial cell-to-cell signalling involvement in the development of such structures, FB17
32 colonizes and forms continuous biofilms on the roots of Arabidopsis plants aerially infected with
33 Pst DC3000 unlike the non-host strain NPS3121 which does not infect or produce disease
34 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 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 via reduced MA turnover to pyruvate or oxaloacetate by malic enzyme and malate dehydrogenase, respectively (Casati et al., 1999). Alternatively, up regulation 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 towards 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 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 a chemical based intra-plant signaling was established recently, where the volatile compounds emitted by one plant part elicited defense responses in other parts of the same plant (Heil and Silva-Bueno, 2007; Gershenzon, 2007). There is no evidence which 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 inter-organism signaling although their ecological relevance needs to be examined. Understanding the two-way signaling event in terms of 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.

1 **MATERIALS AND METHODS**

2 **Plant Material and Growth Conditions**

3 *Arabidopsis thaliana* wild type cultivar Columbia (Col-0) seeds were procured from
4 Lehle Seeds (Round Rock, TX, and USA). Arabidopsis T-DNA knockout mutant line *Atalmt1*
5 was obtained from Arabidopsis Biological Resource Center (ABRC), Ohio State University,
6 Columbus, OH USA. The plants were selfed for four generations to achieve homozygosity and
7 the homozygous plants were used in the present study. Arabidopsis transgenic line carrying
8 *AtALMT1* promoter::GUS fusion construct was obtained from Dr. Hiroyuki Koyama, Laboratory
9 of Plant Cell Technology, Faculty of Applied Biological Sciences, Gifu University, 1-1
10 Yanagido, Gifu 501-1193, Japan. The *AtALMT1* genetic complemented F1 line was generated by
11 crossing *Atalmt1* × Ler-0 by following a published protocol (Hoekenga et al., 2006). The crossing
12 performed between *Atalmt1* (Col-0 background) and *A. thaliana* Ler-0 ecotype (as a pollen
13 donor). In the F1 generation the *AtALMT1* allele from Ler-0 restored the *AtALMT1* phenotype in
14 terms of aluminum (Al) activated malate release (Hoekenga et al., 2006). Seeds were cultured on
15 Murashige and Skoog's (Murashige and Skoog, 1962) (MS) solid medium with 3% sucrose and
16 allowed to germinate for 3-4 days until the roots and shoot emerged by incubating at 23 ± 2 °C
17 under 16 hr light and 8 hr dark photoperiod. The plates were illuminated with cool white
18 fluorescent light with an intensity of 24 µmol m^{-2} s^{-1}.

19

20

21 **Bacterial Strains**

22 The PGPR *B. subtilis* strain FB17 (obtained from Dr. Ray Fall, University of Colorado,
23 Boulder), was maintained on LB plates and *P. fluorescens Pf01* (obtained from Dr. George
24 O'Toole, Department of Microbiology and Immunology Dartmouth Medical School, Hanover,
25 NH). The strain NRS1531 with biofilm operon-lacZ transcription fusions, *thrC::yqxM-lacZ* in
26 wild type Marburg background (obtained from Dr. Nicola R. Stanley-Wall, Division of Applied
27 and Environmental Biology, School of Life Sciences, University of Dundee, Dundee, Scotland)
28 was maintained on LB plates supplemented with 0.5 µg ml^{-1} erythromycin. *P. syringae* strains Pst
29 DC3000, NPS3121 and *E. coli* strain OP50 (obtained from Dr. Jorge M. Vivanco, Colorado State
30 University, Fort Collins, CO) were maintained on LB plates with and without 50 µg ml^{-1}
31 rifampicin respectively. *P. aeruginosa* strain PAO1 (obtained from Dr. Frederick M. Ausubel,
32 Massachusetts General Hospital, Boston, MA) was maintained on LB plates with 20 µg ml^{-1}
33 rifampicin. *A. tumefaciens* strain LBA4404 and *E. caratovora* strain AH2 (obtained from Dr.

1 Thomas Evans, Department of Plant and Soil Sciences, University of Delaware, Newark, DE)
2 were maintained on LB plates. *A. rhizogenes* strain Arqua-1 (obtained from Dr. Janine M.
3 Sherrier, Department of Plant and Soil Sciences, University of Delaware, Newark, DE) and *A.*
4 *brasilense* strain Cd (obtained from Dr. Yaacov Okon, Department of Plant pathology and
5 Microbiology, Hebrew University, Israel) were maintained on TY-agar plates. For growth of *B.*
6 *subtilis* under *in vitro* biofilm formation conditions, cells were grown at 37 °C in a LB-based
7 biofilm medium supplemented with 15 mM $(NH_4)_2SO_4$, 80 mM $K_2HPO_4 \bullet 3H_2O$, 44 mM KH_2PO_4,
8 3.4 mM sodium citrate, 1 mM $MgSO_4$, and 0.1% glucose.
9

10 **Effect of *P. syringae* Infection on *B. subtilis* Root Binding and Biofilm Formation on the**
11 **Roots of *A. thaliana***

12 *A. thaliana* Col-0 plants grown on sterile peat pellets in a growth chamber set for a
13 photoperiod of 16 hr light and 8 hr dark at 23 ± 2 °C and illuminated with cool fluorescent light
14 with an intensity of 24 µmol m^{-2} s^{-1} for a twenty day period. The plants were root inoculated with
15 FB17 (0.5 OD_{600}) by drenching with 10 ml of the culture in water. All the fully expanded leaves
16 were pressure infiltrated with 100 µl of 0.02 OD_{600} culture of Pst DC3000. The cultures in sterile
17 water were prepared by centrifuging the overnight cultures and washing the pellet to get rid of LB
18 medium and re-suspending the pellet in sterile water to obtain appropriate density. Different
19 treatments included control root inoculated with FB17, mock (leaf infiltrated with sterile water +
20 root inoculated with FB17), Pst DC3000 + FB17 (leaf infiltration of FB17 root inoculated plants),
21 NPS3121 + FB17 (leaf infiltration of FB17 root inoculated plants). The plants were transferred
22 to sterile magenta boxes and incubated in the growth chamber for an additional four days. The
23 experiment was terminated after four days and the co-cultivated roots were collected and fixed in
24 4% para-formaldehyde to image for FB17 binding and biofilm formation. Similar experiments
25 were carried out using the malate transporter mutant *Atalmt1*. Each treatment had six replicates
26 and the experiment was repeated on two independent occasions.
27

28 **Effect of *P. syringae* Infection on *P. fluorescens* (*Pf01*) Binding and Biofilm Formation on**
29 **the Roots of *A. thaliana***

30 All the conditions for this experiment were similar as explained above except that the
31 plants were root inoculated with *P. fluorescens Pf01* (0.5 OD_{600}) by drenching with 10 ml of the
32 culture in water. All the observations that were recorded for the earlier experiment were recorded
33 here also.
34

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

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

17

18 **Chemotaxis Assay**

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

31

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

33 Transgenic Arabidopsis (Col-0) plants carrying *AtALMT1 promoter::GUS* fusion
34 construct for the experiments were grown on peat pellets in a growth chamber set for a

1 photoperiod of 16 hr light and 8 hr dark at 23 ± 2 °C and illuminated with cool fluorescent light
2 with an intensity of 24 µmol m^{-2} s^{-1} for a twenty day period. The twenty day old plants were leaf
3 infiltrated with Pst DC3000, NPS3121, OP50 and PAO1 and a positive control of $AlCl_3$ root
4 treatment was also included in addition to an untreated control. After 12hrs of treatment, the roots
5 were stained and imaged for the expression of *AtALMT*::GUS according to published protocol
6 (Kobayashi et al., 2007).
7
8 **Effect of *B. subtilis* Root Inoculation on the *P. syringae* (Pst DC3000) Infection**
9 *A. thaliana* Col-0 plants grown on peat pellets in a growth chamber set for a photoperiod
10 of 16 hr light and 8 hr dark at 23 ± 2 °C and illuminated with cool fluorescent light with an
11 intensity of 24 µmol m^{-2} s^{-1} for a twenty day period. The plants were root inoculated with *B.*
12 *subtilis* FB17 (0.5 OD_{600}) by drenching with 10 ml of the culture in water. Fully expanded leaves
13 were pressure infiltrated with 100 µl of 0.02 OD_{600} culture of Pst DC3000 the next day. Different
14 treatments included control (without Pst DC3000 infiltration), control root inoculated with FB17,
15 mock (leaf infiltrated with sterile water + FB17 root inoculated), Pst DC3000 (only leaf
16 infiltration), Pst DC3000 + FB17 (leaf infiltration of FB17 root inoculated plants). The plants
17 were transferred to magenta boxes and incubated in the growth chamber for an additional four
18 days. The experiment was terminated after four days and the observations such as disease
19 symptom development in terms of chlorosis, number of colony forming units of Pst DC3000 per
20 gram fresh weight of the leaf were recorded by extracting a known fresh biomass of the leaf and
21 plating on LB plates containing 50 mg ml^{-1} rifampicin.
22
23 **Effect of *B. subtilis* FB17 root Inoculation on Whole Plant *PR1::GUS* Expression**
24
25 Transgenic Arabidopsis (Col-0) plants carrying PR1::GUS fusion construct were grown
26 on peat pellets for a twenty day period. The twenty day old plants were root inoculated with FB17
27 by drenching the pellet with 10 ml (0.5 OD_{600}) culture suspended in sterile distilled water. In
28 addition a separate set of PR1::GUS plants were also treated with other bacteria such as *Pf01* and
29 OP50 by drenching the pellet with 10 ml (0.5 OD_{600}) culture suspended in sterile distilled water.
30 Positive controls such as SA (0.1mM) treatment on leaves and root were also performed by
31 spraying a known concentration of SA on the leaves and roots of PR1::GUS plants. Four days
32 after inoculation, the plants were stained for GUS assay using a GUS staining kit (obtained from
33 Sigma-Aldrich, USA) according to manufacturer's instructions. The control and treated plants
34 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 imaged was presented for each treatment. Each treatment had six replicates and the experiment was repeated on two independent occasions.

Microscopy

In order to view adherent FB17 cells and biofilm on the root surface by confocal scanning laser microscopy (CSLM), the roots were stained at a dilution of 1:1000 with SYTO®13 (Invitrogen, Molecular Probes, Eugene, OR). Single median optical sections were captured with a Zeiss 10X Plan-Apochromat (numerical aperture 0.45) objective lens using a Zeiss LSM 510 NLO on an Axiovert 200M. The 488nm laser line of the Argon laser and a 505nm long pass filter were used for excitation and emission, respectively. All data were acquired 24 hours post-inoculation and post-treatment with *B. subtilis* FB17 (5 µL culture of 0.02 OD_{600}) of 10 day old plants grown in 4 mL liquid MS medium with 1% sucrose. Each experiment was repeated twice with three replicates each and a representative image of at least twenty roots imaged for each treatment was presented.

ß-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 ß-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 the 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 a statistical package JMP®7.0. and Microsoft Excel XP. The data means were considered significantly different at the probability of $P \leq 0.05$.

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4

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1 **Figure Legends**
2
3 **Figure 1.** *Bacillus subtilis* strain FB17 (FB17) root colonization and biofilm formation with
4 pathogen and non-pathogen leaf treatments. The data showed a higher colonization and biofilm
5 formation 5 days post-treatment in response to aerial infection *Pseudomonas syringae* pv. tomato
6 DC3000 (Pst DC3000) than untreated (control), water injected (mock), or non-pathogenic
7 *Pseudomonas syringae* pv. *phaselicola* (NPS3121) inoculated leaves in representative root
8 colonization experiments by confocal microscopy (**A**) and colony forming unit (CFU)
9 quantification (**B**). The strong green fluorescence along the sides of the roots indicates the FB17
10 biofilm visualized by staining with SYTO13. Different letters indicate significant difference
11 between the treatments ($P<0.05$, ANOVA test), (Scale = 100 µm).
12
13 **Figure 2.** Root exudate composition with pathogen and non-pathogen leaf treatments**.** Greater
14 malic acid (MA) was detected with aerial infection *Pseudomonas syringae* pv. tomato DC3000
15 (Pst DC3000) than untreated (control), water injected (mock), or non-pathogenic *Pseudomonas*
16 *syringae* pv. *phaselicola* (NPS3121) inoculated leaves as observed by HPLC analysis with
17 representative HPLC profiles (arrow indicates MA elution position) (**A**) and MA peak-area
18 quantification (**B**). Different letters indicate significant difference between the treatments
19 ($P<0.05$, ANOVA test).
20
21 **Figure 3.** Malic acid (MA) specific chemotactic motility of *Bacillus subtilis* strain FB17 (FB17)
22 measured by following capillary chemotactic assay. L-MA showed a dose dependent
23 chemotactic attraction of FB17 (**A**); structure dependent chemotactic attraction of FB17 (**B**) and
24 bacterial species-specific attraction to MA (**C**). Bacterial strains used in the chemotactic assay
25 included *B. subtilis* strain FB17, *A. tumefaciens* strain LBA4404, *Erwinia caratovora* strain AH2,
26 *Agrobacterium rhizogenes* strain Arqua-1, *Azospirillum brasilense* strain Cd, *Pseudomonas*
27 *fluorescens* (*Pf01*) and *Pseudomonas syringae* (Pst DC3000). Data are the average of six
28 replicates from two experiments conducted separately.
29
30 **Figure 4.** Malic acid (MA) transporter mutant (*atalmt1*) was ineffective in *Bacillus subtilis* strain
31 FB17 (FB17) root recruitment**.** (**A**) Reduced MA secretion in *atalmt1* with or without
32 *Pseudomonas syringae* pv. tomato DC3000 (Pst DC3000) infection (different letters indicate
33 significant difference between the treatments ($p \leq 0.05$, ANOVA test) causes (**B**) reduced FB17
34 binding and root colonization shown with representative confocal images stained with SYTO 13

1 (Panel B, Scale: top two rows = 100 µm; bottom row = 50 µm). (**C**) Quantification of FB17 root
2 binding by colony forming units (CFUs) (n=6).
3
4 **Figure 5.** Effect of *Pseudomonas syringae* pv. tomato DC3000 (Pst DC3000) leaf infection on
5 the expression of root *AtALMT1*. Twenty-day old *A. thaliana* line carrying *AtALMT1*
6 promoter*::GUS* fusion construct grown on peat pellets was leaf infected with Pst DC3000 and
7 other controls such as *Pseudomonas syringae* pv. *phaselicola* (NPS3121), *E. coli* OP50, *P.*
8 *aeruginosa* PAO1, post 12hr infection roots were stained for β-glucuronidase. The figure shows a
9 significantly higher GUS induction in response Pst DC3000 (on par with the positive control
10 $AlCl_3$ [4µM] root treatment) when compared to untreated controls and other bacterial strains such
11 as NPS3121, OP50 and PAO1 (Scale = 100 µm).
12
13 **Figure 6.** (**A**) Effect of *A. thaliana* root exudates on the transcription of the *yqxM* operon in *B.*
14 *subtilis* strain Marburg carrying the *yqxM-lacZ* fusion (NRS1531). Strain NRS1531 was grown in
15 biofilm medium under biofilm formation conditions at 37°C with root exudates (5%) from Pst
16 DC3000 infected (-▲-), uninfected (-□-) plants and control (-◆-) without root exudates and ß-
17 galactosidase activity was measured at regular intervals and plotted as a function of time. These
18 experiments were repeated on at least 3 independent occasions and a representative plot is shown.
19 (**B**) Effect of different MA isomers and oxalic acid on the transcription of *yqxM* operon in *B.*
20 *subtilis.* Strain NRS1531 was grown in biofilm medium under biofilm formation conditions at
21 37°C with 5 µM MA isomers, L-MA (-□-), D-MA (-×-) and oxalic acid (-▲-), control (-◆-)
22 without MA isomers or oxalic acid and ß-galactosidase activity was measured at regular intervals
23 and plotted as a function of time. These experiments were repeated on at least 3 independent
24 occasions and a representative plot is shown.
25
26 **Figure 7.** A schematic depicting the long distance intra-plant signaling to recruit rhizobacteria
27 *Bacillus subtilis* strain FB17 (FB17) through secretion of malic acid (MA) post aerial infection by
28 *Pseudomonas syringae* pv. tomato DC3000 (Pst DC3000).
29
30

Figure 1

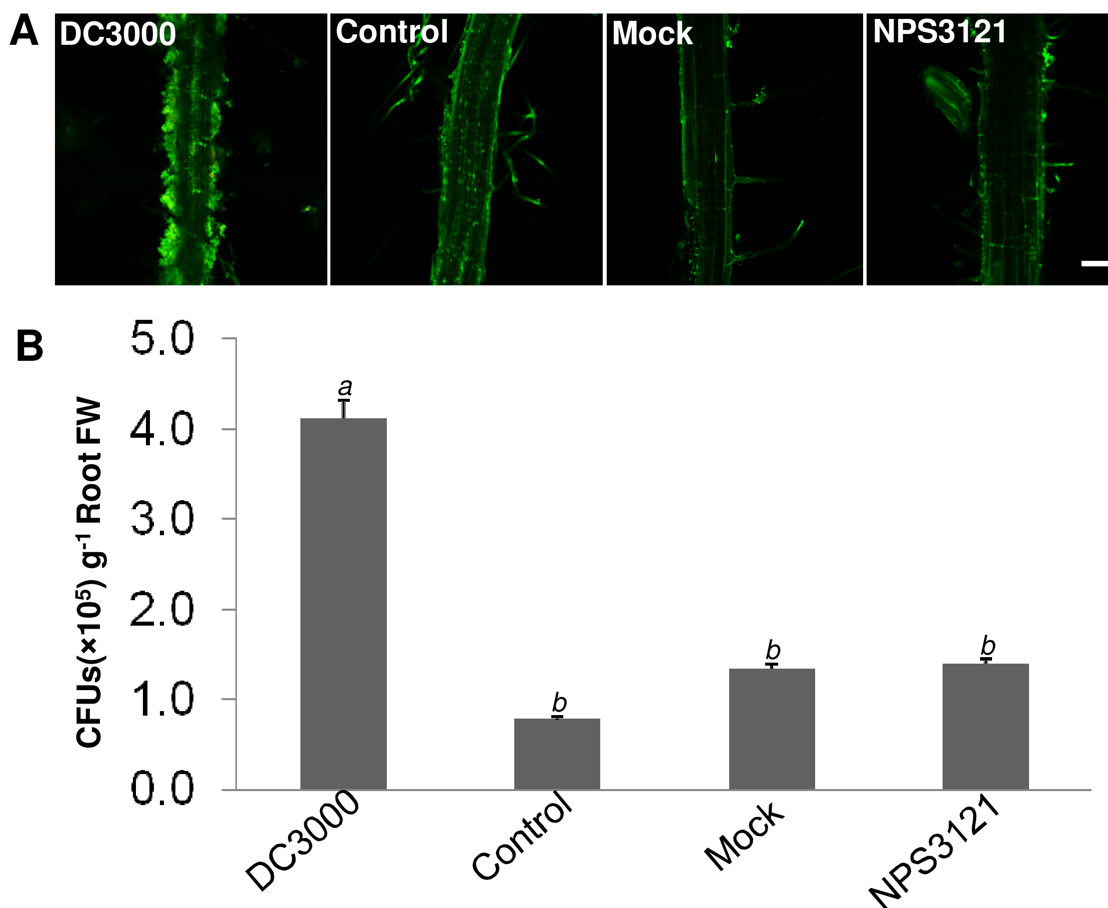

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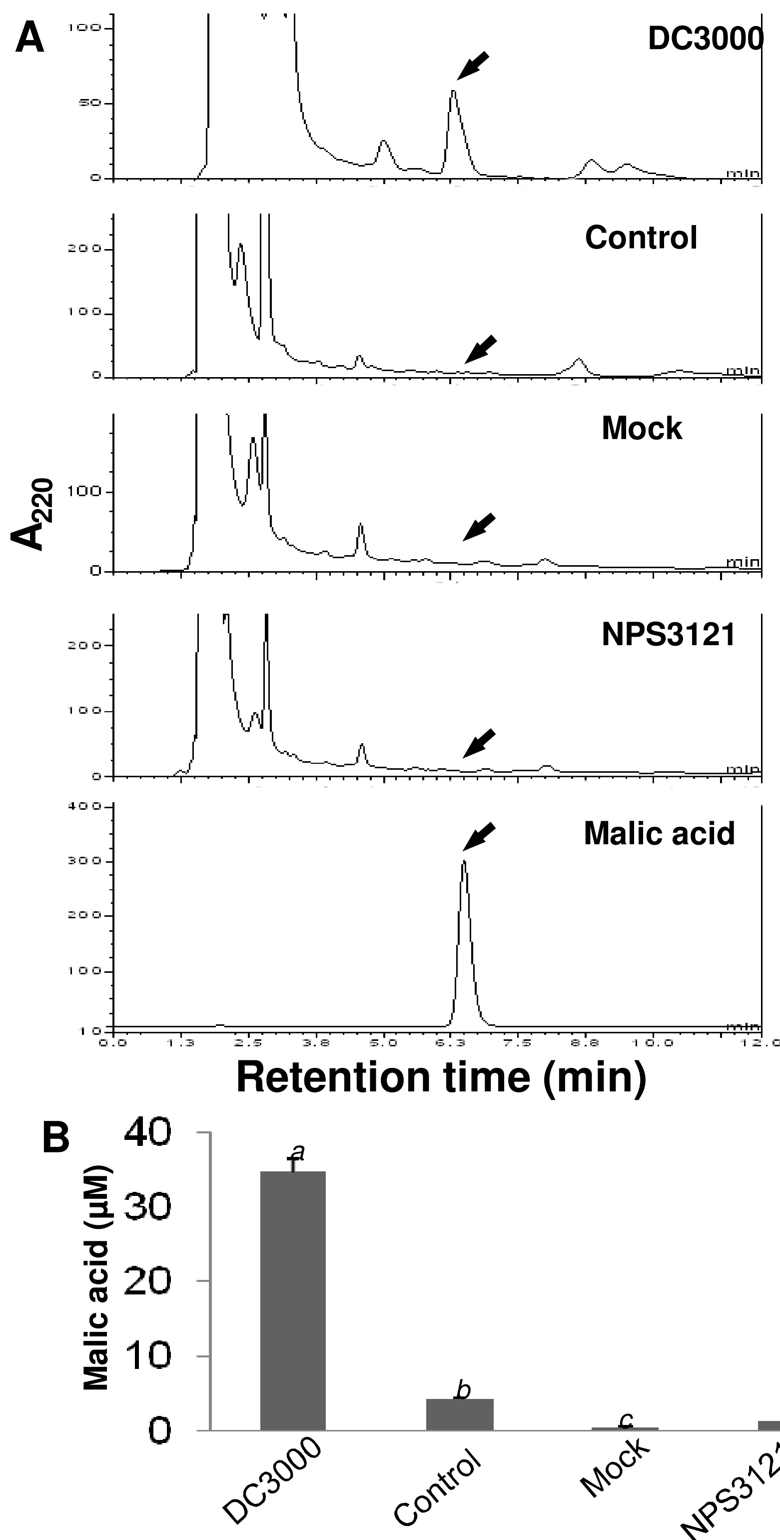
Figure 2



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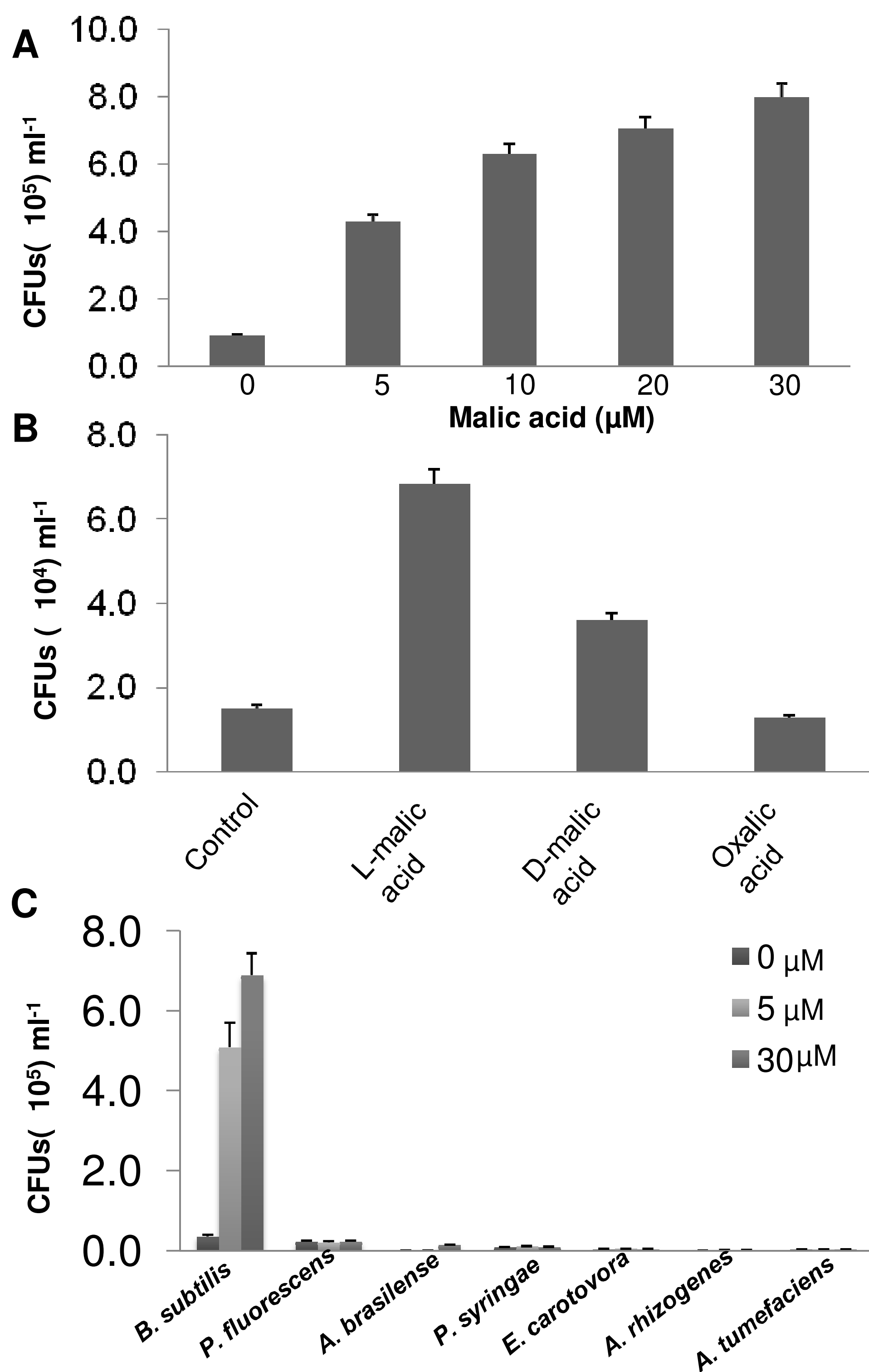

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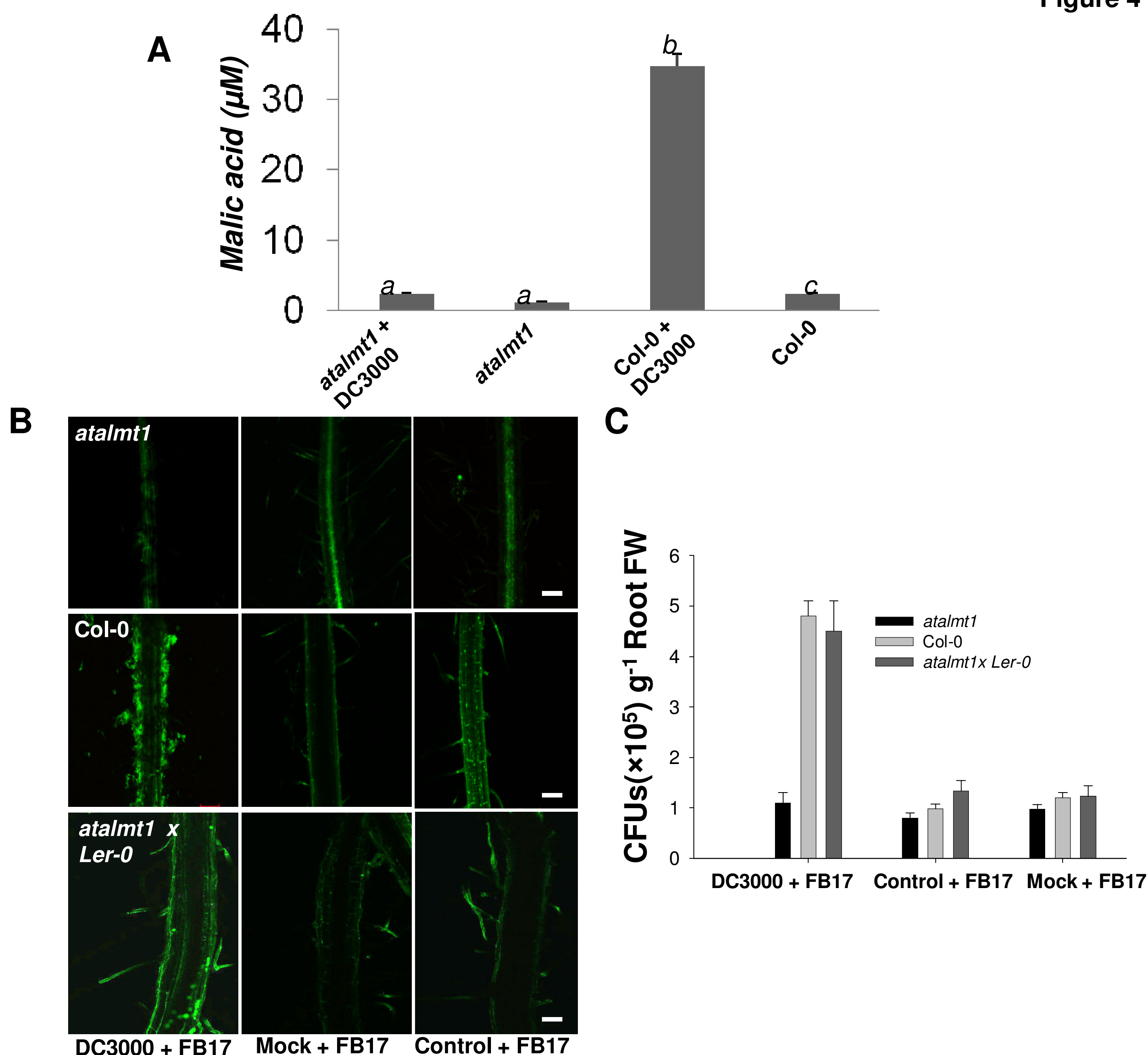
Figure 4



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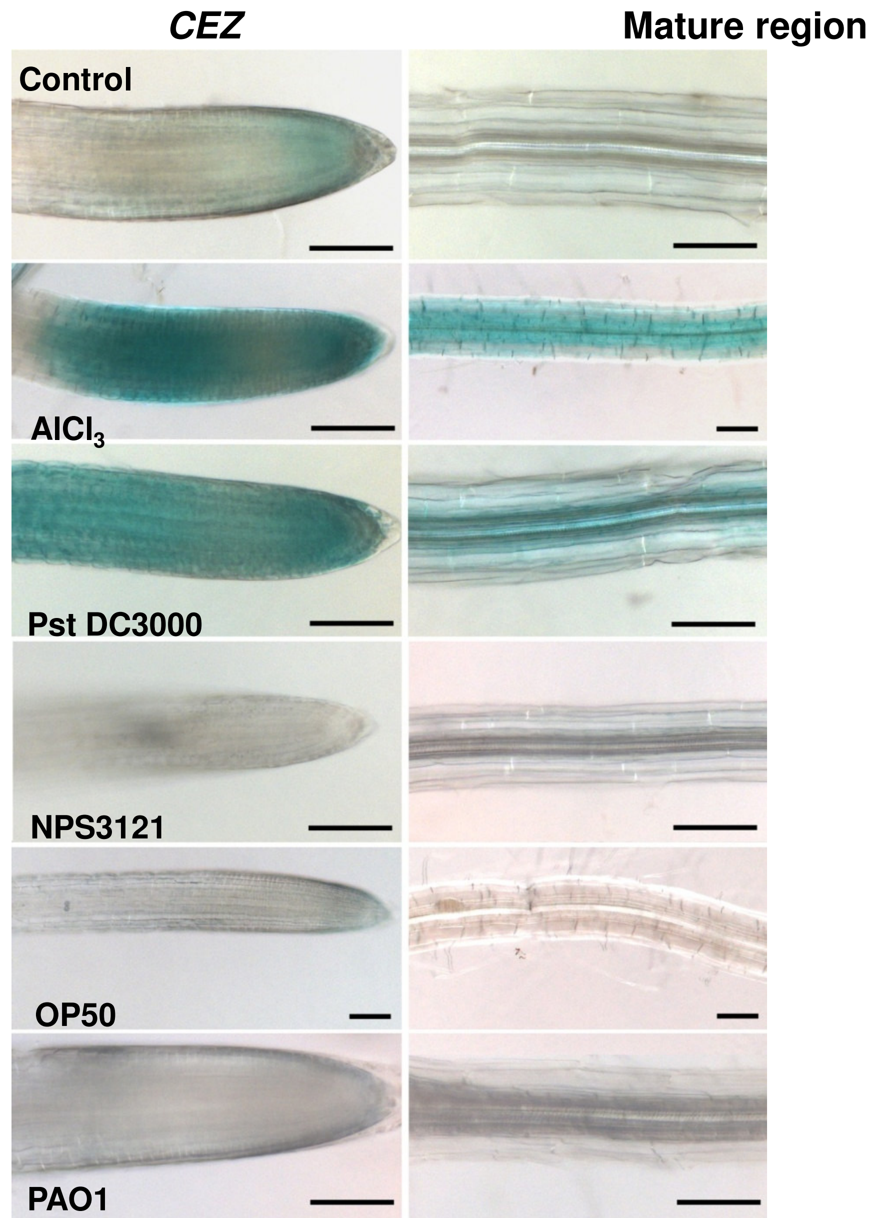

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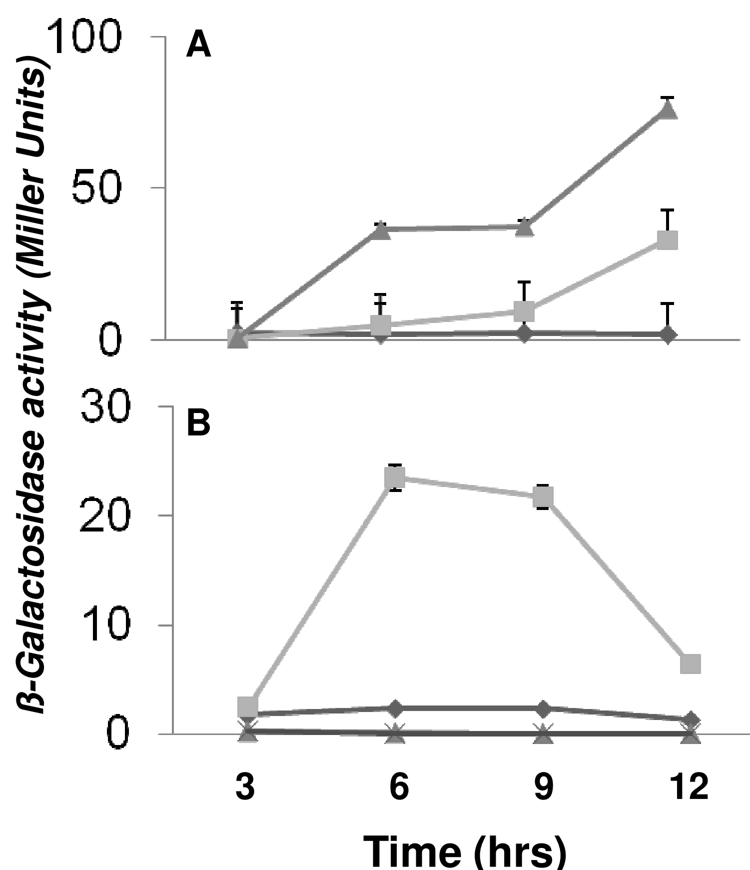

Figure 6. (**A**) Effect of *A. thaliana* root exudates on the transcription of the *yqxM* operon in *B. subtilis* strain Marburg carrying the *yqxM-lacZ* fusion (NRS1531). Strain NRS1531 was grown in biofilm medium under biofilm formation conditions at 37°C with root exudates (5%) from DC3000 infected (-▲-), uninfected (-■-) plants and control (-◆-) without root exudates and ß-galactosidase activity was measured at regular intervals and plotted as a function of time. These experiments were repeated on at least 3 independent occasions and a representative plot is shown. (**B**) Effect of different MA isomers and oxalic acid on the transcription of *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 (-■-), D-MA (-×-) and oxalic acid (-▲-), control (-◆-) without MA isomers or oxalic acid and ß-galactosidase activity was measured at regular intervals and plotted as a function of time. These experiments were repeated on at least 3 independent occasions and a representative plot is shown.

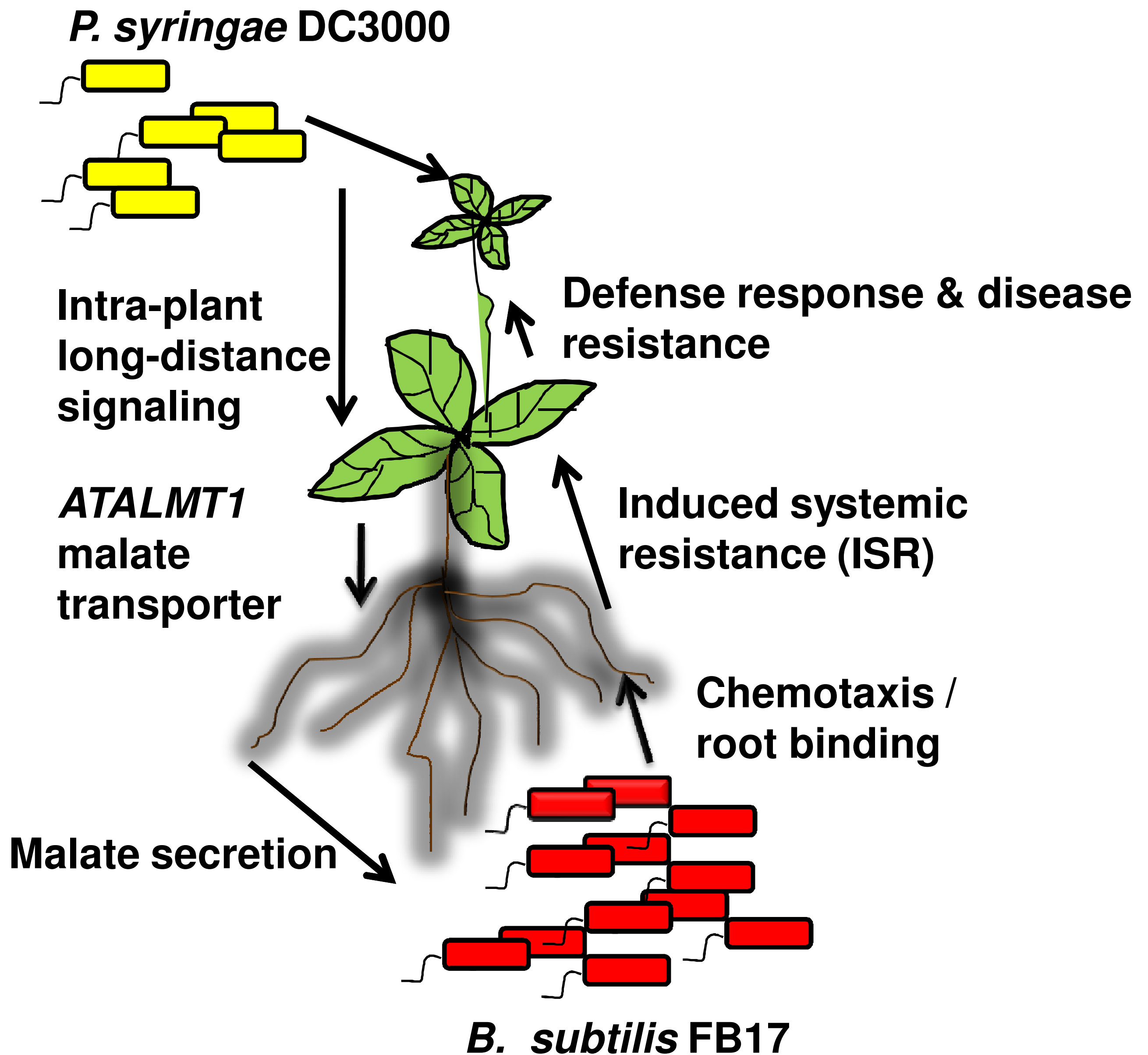

Figure 7. A schematic depicting the long distance intra-plant signaling to recruit rhizobacteria *Bacillus subtilis* strain FB17 (FB17) through secretion of malic acid (MA) post aerial infection by *Pseudomonas syringae* pv. tomato DC3000 (Pst DC3000).