Microbe-Associated Molecular Patterns (MAMPs)-triggered root responses mediate beneficial rhizobacterial recruitment in *Arabidopsis*.

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Our recent study demonstrated that foliar infection by *Pseudomonas syringae* pv. tomato (hereafter *PstDC3000*) induced malic acid (MA) transporter (*ALMT1*) expression leading to increased MA titers in the rhizosphere. MA secretion in the rhizosphere increased beneficial rhizobacteria *Bacillus subtilis* FB17 (hereafter FB17) titers causing an induced systemic resistance (ISR) response in plants against *PstDC3000*. Having shown that a live pathogen could induce an intra-plant signal from shoot-to-root to recruit FB17 belowground, we hypothesized that pathogen-derived microbe-associated molecular patterns (MAMPs) may relay a similar response specific to FB17 recruitment. The involvement of MAMPs in triggering plant innate immune response is well studied in the plant’s response against foliar pathogens. In contrast, MAMPs-elicited plant responses on the roots and the below-ground microbial community are not well understood. It is known that pathogen-derived MAMPs suppress the root immune responses, which may facilitate pathogenicity. Plants subjected to known MAMPs such as a flagellar peptide, flagellin (flg22), and a pathogen-derived phytotoxin, coronatine (COR), induced a shoot-to-root signal regulating *ALMT1* for recruitment of FB17. Micrografts using either a COR insensitive mutant (*coi1*) or flagellin insensitive mutant (*fls2*) as the scion and *ALMT1pro:GUS* as the rootstock revealed that both COR and flg22 are required for a graft transmissible signal to recruit FB17 belowground. The data suggest that MAMPs-induced signaling to regulate *ALMT1* is salicylic acid (SA) and JAR1/JIN1/MYC2 independent. Interestingly, a cell culture filtrate of FB17 suppressed flg22-induced MAMPs-activated root defense responses, which are similar to suppression of COR-mediated MAMPs-activated root defense, revealing a diffusible bacterial component that may regulate plant immune responses. Further analysis showed that the biofilm formation in *B. subtilis* negates suppression of MAMPs-activated defense responses in roots. Moreover, *B. subtilis* suppression of MAMPs-activated root defense does require JAR1/JIN1/MYC2. The ability of FB17 to block the MAMPs-elicited signaling pathways related to antibiosis reflects a strategy adapted by FB17 for efficient root colonization. These experiments demonstrate a remarkable strategy adapted by beneficial rhizobacteria to suppress a host defense response which may facilitate rhizobacterial colonization and host-mutualistic association.
Plant roots are the first organs that come in contact with diverse belowground microflora. Rhizospheric microbes, which utilize plant root exudates for growth and multiplication (Lugtenberg et al., 2001; Bais et al., 2006; Rudrappa et al., 2008a), are attracted to the rhizosphere, and may have either beneficial or deleterious effects on the plant. Classical examples are beneficial mycorrhizal fungi that provide the host with an enhanced root surface for absorbing water and mineral nutrients, notably phosphate (Harrison, 2005), and Rhizobium that fix atmospheric nitrogen into ammonium that can be used by the plant for amino acid biosynthesis (Spaink, 2000). Several other types of beneficial soil-borne microbes, such as plant growth-promoting rhizobacteria (PGPR) and fungi, can stimulate plant growth by suppressing plant diseases (van Loon et al., 1998) or insect herbivory (van Oosten et al., 2008). The biological control activity is exerted either directly through antagonism of soil-borne pathogens or indirectly by eliciting a plant-mediated resistance response (van Loon et al., 1998; Pozo and Azcón-Aguilar, 2007).

The resistance responses mediated by PGPRs are either through systemic acquired resistance (SAR) or induced systemic resistance (ISR), both of which function systemically throughout the plant (Conrath et al., 2002). PGPRs activate ISR (van der Ent et al., 2009) while SAR is triggered by necrotizing pathogens (Conrath et al., 2002). SAR is controlled by the salicylic acid (SA)-dependent signaling pathway, and its onset involves local and systemic increases in endogenously synthesized SA, leading to activation of the regulatory protein NPR1 and the subsequent NPR1-dependent expression of genes encoding pathogenesis-related (PR) proteins, including PR1, PR2, and PR5 (van Loon and van Strien, 1999). Non-pathogenic PGPRs regulate ISR by jasmonic acid (JA)- and ethylene (ET)-dependent signaling pathways and are associated with the downstream regulation of plant defensin 1.2 (PDF1.2) (van Oosten et al., 2008). Pieterse and associates (1998) reported that ISR triggered by Pseudomonas fluorescens WCS417r signals resistance responses through JA- and ET-dependent pathways. Rhizobacteria-mediated ISR has been demonstrated in a variety of plants including bean, carnation, cucumber, radish, tobacco, tomato, and the model plant Arabidopsis thaliana (van Loon et al., 1998). Beneficial rhizobacteria trigger ISR by priming the plant for potentiated activation of various cellular defense responses, which are subsequently induced upon pathogen attack (Conrath et al., 2006). The potentiated responses include oxidative burst (Iriti et al., 2003), cell wall reinforcements (Benhamou et al., 1996), accumulation of defense-related materials and enzymes (Chen et al., 2000), secondary metabolite production (Ongenaa et al., 2000), and impediment of infection processes of pathogens such as inhibition of sporangia and zoospore germination (Yan et al., 2002). Lipopolysaccharides (LPS), siderophores, or SA from rhizobacteria also are indispensable for successful disease protection (De Meyer...
et al., 1999; See review by Ramamoorthy et al., 2001). In connection with ISR and PGPR, Niu and coworkers (2011), reported that *Bacillus cereus* AR156 induces ISR in *Arabidopsis* by simultaneously activating SA- and JA/ET-dependent signaling pathways.

Despite progress toward understanding the microbe-mediated plant responses in plant-microbe interactions, little headway has been made in identifying the genetic and biochemical changes responsible for the attraction of beneficial symbiotic rhizospheric microbes. Under herbivory, wounding of plant tissues by insect feeding triggers the release of volatile signals that attract natural enemies of insect (Kessler and Baldwin, 2001). Upon insect infestation, plants release compounds such as hormones, exogenous volatile organic compounds, and secondary metabolites as long-distance root-to-shoot signals (reviewed by Erb et al., 2009). Whitefly infestation of pepper plants elicits SA-dependent signaling in leaves while roots showed increased colonization of Gram positive bacterial populations (Yang et al., 2011), suggesting a signaling event between aboveground and belowground plant parts. 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. Some of the recent reports indicate that the hormonal activation of ISR by ET/JA or SAR by SA showed no significant effect on the density and structure of the rhizosphere bacterial community (Doornbos et al., 2011). On the contrary, a recent report suggests that ATP binding cassette (ABC) transporters involved in root secretions may structure the rhizospheric microbiome (Badri et al., 2008). Carbon enrichment of the rhizosphere especially carboxylate excretion and acidification at the root surface might have a strong general impact on structuring rhizospheric microbial communities (Marschner et al., 2002). Tricarboxylic acids such as malic acid (MA) and citrate are suitable carbon sources for many microorganisms (López-Bucio et al., 2000; reviewed by Pineda et al., 2010). A recent study from our group showed that inoculation of *Arabidopsis* leaves with the foliar pathogen *Pseudomonas syringae* pv. *tomato* (*Pst*DC3000) induced MA excretion in roots (Rudrappa et al., 2008a). The study revealed that *Pst*DC3000 infected shoots relay chemical signal(s) underground through root MA secretion, resulting in specific chemotaxis to recruit rhizobacteria *Bacillus subtilis* strain FB17 (FB17). The authors further demonstrated that infection of *Arabidopsis* leaves up-regulated root *ALMT1*. These findings were further validated by Chen and coworkers (2012), wherein exudates of tomato roots strongly stimulated *B. subtilis* biofilm formation *ex planta* and that an abundant small molecule in the exudates, L-MA, was able to stimulate biofilm formation at high concentrations in a manner that was dependent on the KinD CACHE domain.

When pathogenic bacteria infect, plants recognize molecules common to many classes of microbes called microbe-associated molecular patterns (MAMPs), such as bacterial flagellin (flg22) (Felix et al., 1999) and bacterial elongation factor *Tu* (Kunze et al., 2004). Other MAMPs include chitin, a
major component of the fungal cell wall (Wan et al., 2008), lipopolysaccharides (Zeidler et al., 2004), and peptidoglycans (PGNs; Gust et al., 2007). MAMPs in leaves including flg22 induce overlapping genes (Zipfel et al., 2004). MAMPs in leaves trigger an oxidative burst, ET, and nitric oxide leading to activation of defense response genes (reviewed by Zhang and Zhou 2010). First, purified flagellin from *Pseudomonas putida* WCS358, as well as LPS from *P. fluorescens* WCS417r and *P. putida* WCS358, were shown to trigger ISR against *P. syringae* in *Arabidopsis* (Meziane et al., 2005). Further studies showed that flg22-induced gene expression and regulation is SA-independent and SA-dependent in early and late phases after flg22 administration (Vlot et al., 2009). Second, *Rhizobium Nod* factors, which are structurally related to chitin, are recognized by LysM receptor kinases in legume roots (Limpens et al., 2003). From the abovementioned studies, it is clear that MAMPs-based interactions modulate plant innate immunity to create a ‘primed’ state. But, many pathogens have evolved strategies to counteract the plant immune response, including, the involvement of direct injection of virulence effectors through the type III secretion system (Block et al., 2008). Many *P. syringae* pathovars secrete coronatine (COR), a low molecular weight phytotoxin that functions in leaves as a mimic of JA-Ile (Kunkel and Brooks, 2002). By activating the JA pathway, COR triggers a mutually antagonistic interaction between the SA and JA signaling pathways and suppresses SA signaling, a key component in basal resistance against *P. syringae*. In addition, COR suppresses the flg22-elicited activation of the *Arabidopsis* gene *NHO1*, which is important for resistance against *Pseudomonas* infection (Li et al., 2005). Finally, COR suppresses MAMPs-induced stomatal closure, believed to block epiphyte pathogens such as *P. syringae* from entering the interior of leaves through these natural openings (Melotto et al., 2006). In contrast with leaves, relatively little is known about MAMPs-mediated responses in roots. Recently, the presence of a sensitive MAMPs-triggered immune system was described in *Arabidopsis* roots (Millet et al., 2010). In the same study, it was shown that MAMPs elicit callose deposition on roots and exudation of the antimicrobial compound camalexin. A microarray analysis of the initial phase of colonization by *B. subtilis*, showed a suppression of root defense-related gene expression (Niu et al., 2011).

The root-colonizing PGPR, FB17, represents a useful model to study principles of plant root-microbe interactions in terms of mutualism and root colonization. As a mutualist, FB17 confers beneficial traits such as increased abiotic stress tolerance and disease resistance to plants (Zhang et al., 2008; 2010; Rudrappa et al., 2008a; 2010; reviewed by Choudhary and Johri, 2009). Herein, we show that foliar applied MAMPs and COR elicited FB17 colonization in *Arabidopsis* roots through activation of root *ALMT1* expression and those FLS2- and COI1-dependent graft-transmissible long-distance signals recruited FB17 belowground. In addition, we show that MAMPs activation of *ALMT1* was independent of SA and transcription factor JIN1/MYC2 in JA pathways. Our results reveal that the suppression of MAMPs-activated defense response by *B. subtilis* was independent of biofilm formation and dependent
on JIN1/MYC2 in the JA signaling pathway. The demonstration that FB17 selectively down-regulated root-specific defense genes and MAMPs-triggered innate plant responses establishes a strategy adapted by FB17 to effectively colonize host roots and avoid antagonism.
Results

Foliar MAMPs exposure elicits root colonization by FB17 in Arabidopsis

To determine if a foliar spray of MAMPs could influence the recruitment of beneficial rhizosphere bacteria, root-specific colonization was measured in the presence and absence of different MAMPs. Twenty-day-old Arabidopsis wild type Col-0 (WT) and ALMT1 knock out (almt1) plants were rhizo-inoculated with the beneficial rhizobacteria, FB17, and then subsequently foliar sprayed with different MAMPs and phytoxin COR. Post 3 d of treatments, leaves sprayed with MAMPs/COR or infected with PstDC3000 stimulated FB17 colonization both qualitatively (confocal microscopy) (Fig. 1A) and quantitatively (colony-forming units CFUs) (Fig. 1B). Remarkably, within 3 d of COR (5 µM) or flg22 (1 µM) treatment, there was a ~10-fold increase in FB17 colonization compared to the mock treatment (Fig. 1B). To validate the specificity in FB17 root colonization post flg22 or COR treatment, three additional MAMPs, lipopolysaccharides (LPS; 500 µg mL⁻¹), chitin (500 µg mL⁻¹), peptidoglycan (PGN; 500 µg mL⁻¹), were also tested for FB17 colonization in Arabidopsis roots. The response to these three MAMPs were much weaker and more variable than flg22, COR, or PstDC3000-elicited responses (Fig. 1A & B). These data, that aerial MAMPs treatments specifically flg22 and phytoxin COR could induce FB17 root colonization, suggest that plants sense and trigger an intra-plant response to recruit beneficial rhizobacterial belowground.

Aerial MAMPs trigger root ALMT1 expression

Our laboratory previously reported that PstDC3000 aerial infection enhanced ALMT1 mediated-MA secretion in the rhizosphere leading to increased FB17 root colonization (Rudrappa et al., 2008a). Concomitantly, the data above also showed that foliar spray with flg22 or COR led to induced FB17 binding which may be mediated through root ALMT1 expression. To test this further, we employed almt1 which is known to be deficient in root MA secretion (Hoekenga et al., 2006). FB17 showed significantly lower extent of colonization on the root surface of almt1 under flg22, COR, or mock (water control) or positive control PstDC3000 infected conditions as shown by both microscopic root binding (Fig. 1A) and CFU data (Fig. 1B). To substantiate that ALMT1 expression is activated by flg22 or COR, we employed Arabidopsis transgenic line carrying an ALMT1pro::GUS fusion construct. The in vitro grown ALMT1pro::GUS and WT were foliar sprayed with flg22 (1 µM), COR (5 µM), LPS (500 µg mL⁻¹), chitin (500 µg mL⁻¹), PGN (500 µg mL⁻¹), water or PstDC3000 (OD₆₀₀=0.1; positive control). ALMT1pro::GUS expression was higher in the root in the flg22 or COR treatments (Fig. 1C). Similarly, semi quantitative RT-PCR (sqRT-PCR) analyses of ALMT1 expression shows, increased levels of ALMT1 in the COR and
flg22 (~6 and 5.5 fold respectively) treated plants compared with the mock treatments (Fig. 1D).

However, the other bacterial-derived MAMPs such as chitin, LPS, and PGN did not show any induction of ALMT1 expression (Supplementary Fig. 1A & B), suggesting that COR or flg22 may be involved in FB17 root-symbiotic colonization response.

In a previous study (Millet et al., 2010), it was shown that bacterial MAMPs flg22, chitin, and PGN were recognized in roots and induced genes involved in the plant immune response. Therefore, to further confirm this finding of a flg22 specific induction of ALMT1 expression, chitin, LPS, PGN, or flg22 were applied to leaves of Arabidopsis transgenic lines carrying an CYP71A12pro:GUS, MYB51 pro:GUS and WRKY11 pro:GUS fusion construct and expression of MAMPs responsive defense marker genes CYP71A12, MYB51 and WRKY11 in leaves were quantified by employing GUS staining (Supplementary Fig. 2A) and expression of MAMPs responsive defense marker genes were monitored by sqRT-PCR in WT plants (Supplementary Fig. 2A & B). Out of the three genes, MYB51 and WRKY11 showed MAMPs responsive induced defense expression in leaves post MAMPs treatment (Supplementary Fig. 2A & B). The data clearly showed that the concentration of other MAMPs (chitin, LPS, and PGN) used in our study was adequate to induce MAMPs-responsive defense genes locally (Supplementary Fig. 2A & B). These experiments confirm that all MAMPs and COR induce defense markers in leaves but just COR and flg22 are able to induce root ALMT1.

MAMPs insensitive mutants, coi1 and fsl2, negate induction of ALMT1 expression and FB17 recruitment

Having shown that a foliar spray of either COR or flg22 triggers FB17 root binding in Arabidopsis, we next evaluated the involvement of FLS2 and COI1 in recruiting FB17 belowground. It has been reported that FLS2 represents a functional flg22 receptor (FLS2) (Gomez-Gomez et al., 2001) and COR blocks SA-signaling and stomatal closure through COI1, an E3 ubiquitin ligase involved in JA signaling and a key component of the defense response against necrotrophic pathogens and insect herbivores (Xie et al., 1998). We compared the levels of ALMT1 expression in the roots of coi1 (COR insensitive) and fsl2 (flagellin receptor mutant) sprayed with COR or flg22. There was a significant reduction ($P \leq 0.05$) of ALMT1 in coi1 sprayed with COR and in fsl2 sprayed with flg22 compared with the positive control PstDC3000 (Supplementary Fig. 3A). Moreover, flg22 sprayed on coi1 and COR sprayed on fsl2 showed increased root ALMT1 accumulation, although, the expression was less compared to PstDC3000-infected plants. To test if a foliar spray of COR or flg22 modulates the FB17 root colonization patterns, root inoculation of FB17 was conducted on WT, coi1, and fsl2 background and quantified (Supplementary Fig. 3B). In the fsl2 background, the overall root FB17 colonization was lower in the flg22 treated and PstDC3000 treated compared to WT controls. Similarly, COR treated or PstDC3000 treated coi1 plants had lower root FB17 colonization compared with WT plants, indicating
that FLS2 and COI1 mediated signaling pathways are required for ALMT1 expression (Supplementary Fig. 3A &B).

Both flg22 and COR trigger a FLS2 and COI1 dependent graft-transmissible long-distance signal to recruit FB17 belowground

To confirm that both COR- and flg22-induced ALMT1 involves an intraplant shoot-to-root ALMT1 signal, insensitive mutants coi1 and fls2 scions were micrografted onto ALMT1 pro:GUS rootstocks. Post maturation of the micrografts, the leaves were sprayed with flg22, COR, or PstDC3000; after 24 h of treatment, whole plants were stained for GUS expression (Fig. 2A & B) and ALMT1 expression in rootstocks quantified by sqRT-PCR (Fig. 2C & D). Untreated grafts were used as controls.

ALMT1 pro:GUS expression in the roots of fls2-scion micrografts stained deeper blue after being sprayed by COR and PstDC3000 compared to grafts sprayed with flg22 (Fig. 2A). ALMT1 pro:GUS expression in the roots of coi1 -scion micrografts were deeper blue after being sprayed with either flg22 or PstDC3000 compared to grafts sprayed with COR (Fig. 2B). In contrast, wild type scions grafted over ALMT1 pro:GUS rootstocks showed an ALMT1 expression with flg22, COR or PstDC3000 treatments (Supplementary Fig. 4). However, in both micrograft sets, PstDC3000-treated plants displayed the deepest blue staining suggesting regulation of ALMT1 through FLS2 and COI1. As described above, the ALMT1 pro:GUS stocks with fls2 scion grafts sprayed with COR or PstDC3000 showed increased root ALMT1 expression compared to grafts sprayed with flg22 (Fig. 2C). Similarly, ALMT1 pro:GUS stocks with coi1 scion grafts sprayed with flg22 or PstDC3000 showed increased root ALMT1 expression compared to grafts sprayed with COR based on sqRT-PCR analysis (Fig. 2D). These results indicate that in the flagellin receptor mutant, fls2, the induction of root-ALMT1 expression is prevented when sprayed with flg22 but still occurs when sprayed with COR or treated with PstDC3000.

Having shown through the chimeric graft experiments that an intraplant mobile signal may be involved to link aerial MAMPs recognition and root ALMT1 components, we next checked the implications of this intra-plant long distance signaling in root FB17 colonization. In accordance, with the increased ALMT1 expression, the fls2/ALMT1 pro:GUS grafts sprayed with COR or PstDC3000 revealed higher FB17 root colonization compared to those sprayed with flg22 (Supplementary Fig. 5A). Interestingly, the same trend was obtained, wherein, the coi1/ALMT1 pro:GUS grafts sprayed with flg22 or PstDC3000 showed higher FB17 binding than those sprayed with COR (Supplementary Fig. 5B). These data suggest there is functional regulation of aboveground MAMPs and belowground ALMT1 cross-talk with regard to beneficial microbe recruitment belowground.

flg22 and COR activation of ALMT1 expression is SA/JAR1/JIN1/MYC2 independent
More than a decade of research has shown that SA acts as a major player in systemically transmitting defense signals in many plant systems. Many genes that show induced expression in response to MAMPs are also induced by SA. Tsuda and coworkers (2008), showed an intimate interaction between MAMPs-triggered responses and SA-mediated signaling mechanisms revealing SA accumulation post flg22 treatment. Herein, we examined the dependence of flg22-triggered ALMT1 expression on SA. To test this, ALMT1 expression in roots was quantified in two SA impaired lines, ics1-1 and npr1-1 that were foliar treated with flg22 (Fig. 3A). Surprisingly, both in ics1-1 and npr1-1 observed normal flg22-elicited root ALMT1 expression comparable to root ALMT1 expression in WT treated with flg22 (Fig. 3A). For further confirmation of non-involvement of SA in mediating flg22-triggered ALMT1 expression, the seedlings were treated with exogenous SA and ALMT1 expression was monitored in ALMT1pro:GUS and quantified by sqRT-PCR. In both experiments, seedlings with exogenous SA did not activate ALMT1 expression (Fig. 3B). In addition, our data clearly showed that SA induced PR1 compared to ALMT1 expression (Fig. 3B), suggesting that the concentration of SA used in our study was adequate for gene expression. Together, these results show that the systemic induction of root ALMT1 expression by flg22 is independent of SA signaling.

It is generally understood that COR and methyl jasmonate (MeJA) are similar in both structure and function (Uppalapati et al., 2005). COR might activate JA signaling downstream of JAR1 (involved in JA-Ile synthesis) and by recruiting transcription factors other than JIN1/MYC2 (Dombrecht et al., 2007). To test the involvement of JA-signaling pathway in COR-triggered root ALMT1 expression, mutants (jar1-1, jasmonic acid resistant 1 and jin1-9, jasmonate insensitive 1) impaired in JA-signaling pathways were tested. The jar1-1 and jin1-9 were foliar treated with COR and root ALMT1 expression was measured (Fig. 3C). Surprisingly, COR was involved in induction of root ALMT1 expression in both jar1-1 and jin1-9, confirming the non-involvement of JAR1/JIN1/MYC2 in JA-signaling pathway in COR-triggered root ALMT1 expression. Subsequently, a structural analogue of COR, MeJA, was applied exogenously to the WT seedlings and root ALMT1 expression was monitored. Interestingly, exogenous treatment of MeJA did not induce ALMT1 expression in roots (Fig. 3D). In contrast, supplementation of MeJA to WT plants induced PDF1.2, a marker gene used routinely for characterization of the jasmonate-dependent defense responses (Fig. 3D). This data suggests that although COR mimics MeJA structurally, it may induce a differential intraplant signaling response to trigger ALMT1 expression that may not involve JAR1/JIN1/MYC2 in JA signaling.

Aerial exposure of MAMPs induce root defense responses similar to live pathogen

A common root defense mechanism adapted by various plant species is to biosynthesize and exude a plethora of antimicrobial compounds into the rhizosphere (Badri and Vivanco, 2009). Our data
above clearly showed that post-aerial treatment with MAMPs, an intraplant signal regulates ALMT1 to recruit FB17 belowground. Millet and coworkers (2010) and Denoux et al. (2008) showed that flg22 treatment upregulated the expression of three defense related genes, CYP71A12, MYB51, and WRKY11. CYP71A12 encodes a cytochrome P450 that is very similar to CYP71A13, which catalyzes the conversion of indole-3-acetaldoxime to indole-3-acetonitrile during camalexin biosynthesis (Nafisi et al., 2007). MYB51 is a transcription factor essential for the regulation of indole-glucosinolate biosynthesis (Gigolashvili et al., 2007). The transcription factor WRKY11 is a negative regulator of basal resistance in Arabidopsis (Journot-Catalino et al., 2006). Millet and coworkers (2010) also showed that MAMPs treatment in roots upregulated CYP71A12, MYB51, and WRKY11 defense genes. To analyze the mutualistic association of FB17 and Arabidopsis roots, we speculated that post-MAMPs treatment, FB17 may modulate root defense genes for efficient colonization.

To test the hypothesis that FB17 may intervene with MAMPs-triggered plant innate immunity, we evaluated the promoter:GUS transgenic lines of CYP71A12, MYB51, and WRKY11 post treatment with flg22, COR, PstDC3000, or mock. All three GUS reporter genes (CYP71A12, MYB51 and WRKY11) were activated in the roots post foliar flg22 or PstDC3000 treatment only (Supplementary Fig. 6A). To validate the reporter expression data of root defense response post foliar flg22 or COR treatment, we examined the induction of CYP71A12, MYB51, and WRKY11 gene expression using semi-quantitative RT-PCR in WT roots (Supplementary Fig. 6B). The sqRT-PCR data validated the histochemical GUS expression that both PstDC3000 and flg22 but not COR upregulated root defense responses in plants. These data also suggest that upon aerial infection, plants may elevate the overall innate defense response to mitigate the pathogenesis. The question is how do plants differentiate between pathogens and non-pathogens in terms of modulating defense response and how do beneficial microorganisms intervene with the root defense response to be host-associated?

FB17 suppresses MAMPs-triggered root defense response

The work above showed that MAMPs trigger a root-defense response similar to a foliar pathogen; next, we evaluated how FB17 bypasses the elevated root defense. To test the possible mechanism of FB17 colonization on the root under a primed defense scenario, we examined the promoter:GUS transgenic lines of CYP71A12, MYB51, and WRKY11 subjected to aerial treatment with flg22 and/or root inoculation with FB17. Foliar treatment with flg22 highly induced CYP71A12, MYB51, and WRKY11 compared to the FB17 alone or the mock. Interestingly, FB17 strongly suppressed the flg22-elicited activation of the CYP71A12, MYB51, and WRKY11 (Fig. 4A). This is validated by measuring the relative expression of CYP71A12, WRKY11, and MYB51 by sqRT-PCR following flg22 treatment and/or FB17 inoculation (Fig. 4B). To test which component of the FB17 is necessary or sufficient for the suppression
of innate immunity, the cell free lysate (CFL) and heat killed (HK) bacteria were tested for their ability to suppress the MAMPs-triggered responses in the roots (Supplementary Fig. 7). The HK fraction failed to suppress CYP71A12pro:GUS or MYB51pro:GUS reporter response in roots elicited by flg22 (Supplementary Fig. 7). In contrast, CFL treatment actively suppressed the flg22-mediated root defense responses (Supplementary Fig. 7). These results suggest that live bacteria or a diffusible bacterial component may suppress the flg22-mediated root defense responses. The results also indicated that beneficial microorganisms may actively block the innate defense responses in the roots in order to establish a compatible interaction with the host.

Bacillus subtilis suppression of MAMPs-triggered root defense response is independent of biofilm formation

The work above showed that a B. subtilis-derived component actively suppressed the flg22-mediated defense response in Arabidopsis roots. It is known that the biofilm formation and colonization ability of Bacillus and other endospore forming Gram-positive bacteria is dependent on extracellular matrix and TasA (Branda et al., 2006). We hypothesized that TasA protein may play a role in the suppression of root defense genes in Arabidopsis. To test this, WT and the promoter:GUS transgenic lines of CYP71A12, MYB51, and WRKY11 were subjected to foliar treatment with flg22 and/or root inoculated with parental B. subtilis strain 3610 (a parental strain for tasA mutant) or tasA::mls mutant. A quantitative real time PCR (qRT-PCR) was also performed using root tissues to estimate the expression levels of CYP71A12, MYB51, and WRKY11. Like FB17, reporter lines treated with wild type 3610 (3610) showed active suppression of flg22-mediated CYP71A12, MYB51, and WRKY11 (Fig. 4C). Interestingly, a CFL from 3610 also suppressed the flg22-mediated CYP71A12 and MYB51 defense responses. Contrastingly, a HK component of 3610 was impaired in suppressing the flg22-mediated CYP71A12 and MYB51 root defense responses (Fig. 4C). In marked contrast, the tasA::mls was impaired in suppressing the flg22-mediated CYP71A12 and MYB51 defense responses in roots. As expected and previously reported (See Millet et al., 2010), COR suppressed callose deposition in Arabidopsis roots (Fig. 4D). To study further MAMPs signaling activation and its response on callose deposition, we tested plants root treated with FB17/tasA::mls and flg22. FB17 suppressed the flg22-elicited callose deposition in Arabidopsis roots (Fig. 4D). To further test whether the 3610 and tasA::mls strains differ in their ability to form biofilms with FB17, we used the methods of Hamon and Lazazzera (2001) to measure adherence of the bacterium to the wells of a microtiter plate. As shown in Figure 5A, the tasA::mls formed significantly less biofilm compared with its parental strain, 3610 and FB17. To determine if tasA had a colonization ability on biotic surfaces, we measured colonization on roots. Our results showed that tasA::mls is impaired in root colonization compared to FB17 and 3610 (Fig. 5B & C).
To distinguish whether biofilm formation *per se*, was critical for suppressing the MAMPs-activated defense responses in host plants, we tested mutants in *B. subtilis* defective in the production of the extracellular polysaccharide (EPS) matrix. Two mutants of FB17 defective in the *epsG* (*DFB79*) and *epsO* (*DFB82*) genes predicted to be required for EPS synthesis, failed to form biofilms compared with the parental strain FB17 (Supplementary Fig. 8A). To test for suppression of MAMPs-triggered defense responses, the biofilm deficient mutants *DFB79*, *DFB82*, and *tasA::mls* were presented to flg22 treated *CYP71A12* <em>pro:GUS</em>, *MYB51* <em>pro:GUS</em>, and *WRKY11* <em>pro:GUS</em> plant cell lines. Unlike the *tasA* mutant, the EPS-defective *epsG* (*DFB79*) and *epsO* (*DFB82*) mutants actively suppressed the flg22−triggered *CYP71A12*, *MYB51*, and *WRKY11* defense responses comparably to FB17 or PY79 treated plants (Supplementary Fig. 8B). These results suggest that biofilm formation in *B. subtilis* is not critical for suppression of MAMPs-triggered immunity (MTI).

*B. subtilis* mediated suppression of flg22-triggered root defense response is JAR1/JIN1/MYC2 dependent

It is widely accepted that the flg22-triggered defense response in roots is suppressed by pathogen secreted phytotoxin COR or beneficial microbes primarily dependent on the JA pathway (Millet et al., 2010; Jacobs et al., 2011). To evaluate if *B. subtilis*-mediated suppression of flg22 is JA dependent, we used three-week-old seedlings of WT, *jar1-1*, and *jin1-9*. Seedlings were sprayed with flg22 or mock treated and root inoculated with FB17. Subsequently, expression of root defense genes (*CYP71A12*, *MYB51*, and *WRKY11*) and root FB17 colonization was quantified (Fig. 5D-E). Interestingly, in JA-signaling mutants (*jar1-1* and *jin1-9*), flg22-mediated *CYP71A12*, *MYB51*, and *WRKY11* expression remained unchanged compared to the WT plants (Fig. 5D). To substantiate whether reduced FB17 colonization in *jar1-1* and *jin1-9* roots was associated with elevated immunity, we checked total CFU in JA mutants. We found reduced FB17 colonization in flg22-treated *jar1-1* and *jin1-9* compared to WT roots (Fig. 5E), suggesting that inability of FB17 to suppress MTI may result in reduced FB17 root titers. These data suggest that flg22-activated root defense in *Arabidopsis* is independent of JIN1/MYC2 in JA-signaling but FB17-mediated suppression of flg22-activated root defense is JAR1/JIN1/MYC2 dependent. These findings are consistent with the general convention that the plant growth-promoting rhizobacterial colonization in host plant roots may require the suppression of MAMPs signaling to protect the beneficial bacteria against MAMPs-elicited defense in the early phase of root colonization (Zamioudis and Pieterse, 2012).

**Regulation of root defense response and ALMT1 expression by MAMPs**

Since foliar MAMPs treatment elicited root defense genes and also induced *ALMT1* expression, we tested the epistatic relationship between *ALMT1* and root defense genes. For this, we tested the
ALMT1 expression analyses on insertion mutants cyp71A12, myb51, and wrky11 in the absence and presence of foliar MAMPs. Briefly, twenty-day-old seedlings of insertion mutants, cyp71A12, myb51, and wrky11, were treated with PstDC3000, COR, or flg22. Post 24 h of treatment, ALMT1 root expression was evaluated using sqRT-PCR. As per our previous report, WT plants treated with PstDC3000 showed enhanced root ALMT1 expression (Rudrappa et al., 2008a; Fig. 6A). The WT plants treated with COR or flg22 also showed increased root ALMT1 expression (Fig. 6A). Interestingly, the mutant lines cyp71A12, myb51, and wrky11 also revealed similar trends in ALMT1 expression when treated with PstDC3000, MAMPs or COR as compared to WT plants (Fig. 6A). Inversely, we also evaluated the MAMPs-triggered root defense response in almt1 plants subjected to aerial PstDC3000 and MAMPs treatments. The data showed similar expression levels of root defense response genes as shown for WT plants (Fig. 6A). These data confirm that MAMPs activation of root defense response genes functions in parallel and independent of ALMT1 responses. We next evaluated the functional response of ALMT1 expression by visualizing the FB17 colonization in cyp71A12, myb51, and wrky11 mutants. The leaves of WT, cyp71A12, myb51, and wrky11 plants were inoculated with PstDC3000 or MAMPs and subsequently root drenched with FB17. Post 72 h, both WT and mutant plants treated with PstDC3000 or MAMPs showed enhanced FB17 colonization (Supplementary Fig. 9).

Our previous results and those of others (Rudrappa et al., 2008a; Hoekenga et al., 2006) have shown the specificity of root ALMT1 activation to PstDC3000 and aluminum (Al<sup>3+</sup>) treatments. To further verify that ALMT1 expression is independent of MAMPs-triggered root-defense responses, we used the promoter:GUS transgenic lines of CYP71A12, MYB51, and WRKY11 against MA and AlCl<sub>3</sub>. It has been shown that MAMPs and AlCl<sub>3</sub> trigger ALMT1 expression. We wondered that if MAMPs could trigger ALMT1 expression leading to increased MA titers helping FB17 association, how would an increase in MA affect root defense responses? Interestingly, a direct treatment of MA (100 nM) and AlCl<sub>3</sub> (4 µM) to promoter:GUS transgenic plants of CYP71A12, MYB51, and WRKY11 showed unaltered expression of all the three root-specific defense genes (Supplementary Fig. 10), suggesting the non-interference of the MAMPs-triggered metabolite (MA) with the root defense genes. These data suggest that xenobiotic agents, which are able to induce ALMT1 expression in roots, do not disturb immunity in roots. Moreover, ALMT1 does not interfere with the root immune responses.

Defense-suppressive function of COR is abolished upon root colonization by FB17

Having shown that FB17 abolishes the flg22-mediated root defense, we next wanted to see how co-treatment with MAMPs and FB17 modulates PstDC3000 pathogenesis in Arabidopsis. To initiate this experiment, three-week-old WT plants were foliar pretreated with COR, flg22, and post 24 h PstDC3000 was coinoculated in the absence or presence of root inoculated FB17. Post 72 h of treatment, the growth
of PstDC3000 was monitored by CFUs (Rudrappa et al., 2008a; Millet et al., 2010). Previous experiments showed that application of flg22-treated plants triggered resistance against PstDC3000 (Zipfel et al., 2004). In contrast, treatment of plants with COR resulted in down regulation of flg22-triggered immune response in Arabidopsis roots (Millet et al., 2010). In accordance with the published data, our results showed flg22-induced resistance against PstDC3000 and that resistance was more pronounced during co-treatment with flg22 and FB17 inoculation (Fig. 7). In parallel, plants treated with COR showed enhanced susceptibility to PstDC3000 (Fig. 7). In addition, plants co-treated with COR and FB17 showed resistance to PstDC3000 (Fig. 7). In light of the fact that both COR and FB17 suppress the flg22-induced root defense responses (Supplementary Fig. 12) with FB17 abolishing COR-mediated susceptibility, FB17 may act through an unknown pathway to up-regulate disease resistance against PstDC3000.
Recently, several reports have established the role of beneficial PGPRs in improving plant health by increasing tolerance to pathogens, insect pests, abiotic stress, including drought and salinity (reviewed by Choudhary and Johri, 2009; Zhang et al., 2008; 2010). Among these PGPR strains, FB17 rhizoinoculated onto the roots of *Arabidopsis* plants reduced disease severity thus inhibiting the proliferation of foliar pathogen *Pst*DC3000 through induction of JA/ET-mediated ISR and SA-mediated SAR (Rudrappa et al., 2010). The magnitude of colonization on the root by beneficial microbes is limited by several factors that include root surface bio-chemistry and composition of root exudates (Rudrappa et al., 2008b; Doornbos et al., 2011; Chen et al., 2012). It has been speculated that components of root exudates may play a critical role in establishing a beneficial microbiome in the rhizosphere (Bais et al., 2006; Lugtenberg et al., 1999). The composition of root exudates depends on plant species and cultivar, development stage and stress factors (Uren 2000). There is little knowledge in the field as to how plant roots respond to biotic stress, i.e. foliar pathogen attack. It is known that plants, upon foliar attack, modulate their primary and secondary metabolic pathways to augment pathogen defense. This is shocking given plant roots respond to various belowground biotic and abiotic stresses by synthesizing complex toxins and also play an active part in environmental sensing. In this report, we demonstrated that MAMPs elicit potential root responses to trigger beneficial rhizobacterial recruitment. We show that foliar MAMPs treatments induce a graft transmissible intraplant shoot-to-root signal to elicit a malate transporter leading to increase in FB17 colonization. We also found that FB17 suppresses MAMPs-triggered innate immune responses in roots (Fig. 8). The ability of plant roots to activate a malate driven beneficial microbe recruitment by recognizing specific MAMPs may play a critical role in efficient colonization by rhizobacteria, and in turn, may limit access of pathogenic microbes to the roots.

**Foliar MAMPs trigger root FB17 colonization**

In plants, increased accumulation of MA is triggered by elevated biosynthesis of one or more MA precursors such as fumerate, oxaloacetate, or pyruvate (Casati et al., 1999). Alternatively, upregulation of *ALMT1* elicits root MA secretions (Kobayashi et al., 2007). It has been argued that most of the Gram-positive and Gram-negative microbes prefer simple sugars as a carbon source compared to complex carboxylic acids. A recent work shows that *B. subtilis* prefers MA as a carbon source and inflicts metabolite repression to take up other carbon sources for an efficient MA uptake (Kleijn et al., 2010 & reviewed by Fernie and Martinoia, 2009). This recent data validates the specificity and preference of *B. subtilis* for MA. Our previous studies showed that aerial plant pathogenesis involved the induction of MA
in root secretions that effectively recruited beneficial rhizobacteria (Rudrappa et al., 2008a; 2010). We also showed that the pathogenic phyllosphere pathogen, \textit{Pst} DC3000, induced root \textit{ALMT1} expression (Rudrappa et al., 2008a). The present study reveals that pathogen-derived MAMPs especially flg22 and phytotoxin COR are sufficient to mimic the live pathogen response of induced root \textit{ALMT1} expression in \textit{Arabidopsis} (Fig. 1). Unlike COR and flg22, other tested MAMPs, chitin, PGN, and LPS, failed to elicit \textit{ALMT1} expression; thus raising a hypothesis that plants have evolved root responses to specific MAMPs depending on the nature of the invading pathogen. It is known that several species of \textit{Bacillus} biosynthesize PGNs (Daniel and Errington, 2003). The specificity of MAMPs to elicit \textit{ALMT1} expression, thereby enhancing FB17 colonization, suggests that plants recognize pathogenic and non-pathogenic MAMPs differently. Surprisingly, FB17 failed to elicit \textit{ALMT1} expression in roots (Supplementary Fig. 11). The fact that aerial treatment with flg22 or COR induced root \textit{ALMT1} expression suggests the involvement of various downstream signaling components of FLS2 and COI1 pathways to target \textit{ALMT1}.

Foliar MAMPs induce root \textit{ALMT1} signal

Genetic approaches can be useful for dissection of signaling pathways that might shed light on the recruitment of beneficial microbes in the rhizosphere. It is known that there exists a root-to-shoot signaling pathway mediated through \textit{BYPASS1} that regulates leaf venation patterning (van Norman et al., 2004). The roots of \textit{BYPASS1} over-produce a mobile component that functions non-cell-autonomously to arrest shoot growth. Several plant hormones are known to travel long distances, from the root to the shoot, and to affect shoot processes. An excellent example is the control of shoot branching, which is regulated by the root-derived signaling molecule recently identified as strigolactone (Gomez-Roldan et al., 2008). Similarly, cytokinins have been implicated as long-distance signals that communicate the nitrogen status from the root to the shoot (Takei et al., 2001; see also Kudo et al., 2010). The signal that communicates drought conditions from the root to the shoot is widely believed to be mediated through abscisic acid (ABA) (Bahrun et al., 2002). While there are some examples of root-to-shoot signaling regulating developmental phenotype and hormone translocation, there is no information that dictates the existence of a shoot-to-root, intraplant signal relaying a plant’s response with beneficial microbes. Our results show for the first time the connection between foliar spray of MAMPs and root \textit{ALMT1} components to regulate a positive feedback response in plants. The fact that FB17 root treatment failed to increase root \textit{ALMT1} expression, suggests a pathogen specific induction of malate efflux for rhizobacterial recruitment (Supplementary Fig. 11). This also strengthens our model (Fig. 8) and provides a molecular validation that foliar treatment launched an intraplant signal connecting shoot-to-root, to attract beneficial microbes belowground. Using \textit{fls2} or \textit{coi1} scions and \textit{ALMT1}$_{pro}$:\textit{GUS} rootstocks showed
the activation of ALMT1, suggesting the existence of a mobile signal (Fig. 2 and Supplementary Fig. 5A, B).

SA/JAR1/JIN1/MYC2 independent regulation of flg22 or COR induced ALMT1 expression

Different bacterial MAMPs including flg22 induce overlapping genes (Zipfel et al., 2004) and further studies showed that flg22-induced gene expression and regulation is SA-independent and SA-dependent in early and late phases after flg22 administration (Vlot et al., 2009). This intriguing observation suggests that the perception of MAMPs by plants leads to the induction of early responsive genes through an SA-independent signaling mechanism resulting in SA accumulation (Tsuda et al., 2008). When SA has accumulated to a sufficiently high level, expression of some of the early responsive genes is kept high by an SA-dependent signaling mechanism (Tsuda et al., 2008). This suggests that plants exploit SA-mediated signaling to maintain MAMPs-triggered defense expression (Tsuda et al., 2008). However, our observations showed that MAMPs-triggered root ALMT1 expression was independent of the SA signaling pathway (Fig. 3). Our studies clearly indicate that MAMPs triggered ALMT1 expression involves the FLS2 pathway. It is surprising as flg22 has been shown to involve SA, JA, and ET pathways in parallel for induction of resistance against foliar pathogens (Zipfel et al., 2004).

There is a possibility that downstream components of FLS2 may hold a key in MAMPs-triggered regulation of ALMT1. In parallel, it is known that PstDC3000 utilizes COR, a structural mimic of the signaling molecule JA-Ile, to suppress MAMPs-activated defense response in Arabidopsis roots (Millet et al., 2010). Our data show that regulation of root ALMT1 by COR is dependent on ubiquitin ligase COI1, a key regulator of JA signaling. This data and the fact that COR modulates the genes involved in the pathways to JA (Uppalapthi et al., 2005), prompted us to evaluate the importance of JA in COR-COI1-mediated regulation of ALMT1. In contrast, MeJA failed to induce root ALMT1 expression and COR regulation of ALMT1 was found independent of JA signaling. This is surprising as COR and JA are considered structural analogues and are speculated to induce similar plant responses and have impact on multiple phytohormone pathways (Uppalapati et al., 2005). Although both COR and MeJA are involved in various physiological responses, we do not understand to what extent COR mimics MeJA. There are striking structural differences in COR and MeJA and this may evade the regulation of ALMT1 by JA.

B. subtilis suppresses MTI in roots to confer beneficial symbiotic interactions

In beneficial symbiotic interactions, especially legume-rhizobia and mycorrhizal fungi, rhizobium and mycorrhizae have evolved mechanisms to efficiently suppress the host immune systems to establish successful infections (Reviewed by Zamioudis and Pieterse, 2012). Similarly, the beneficial symbiotic microbes such as PGPR, that often grow endophytically inside roots or on root surfaces, may also
minimize stimulation of host’s immune system. It was recently reported that the beneficial bacterium, *P. fluorescens* WCS417r, suppresses the MAMPs-triggered response in *Arabidopsis* roots that is responsible for deposition of callose in the root elongation zone elicited by the flagellar peptide flg22 (Millet et al., 2010). In the present study, applying FB17 to *Arabidopsis* roots resulted in the early suppression of defense genes that may facilitate FB17 colonization on *Arabidopsis* roots. This is surprising as the general convention dictates that beneficial rhizobacteria are known to elicit root-defense responses (Verhagen et al., 2004). However, it is possible that the suppression of MAMPs signaling is necessary for successful root colonization by PGPRs. We speculate that the early phases of root colonization by PGPRs require the suppression of MAMPs signaling to protect the bacteria against MAMPs-elicited antimicrobial exudates. Interestingly, FB17 showed resistance against MAMPs-elicited *Arabidopsis* root exudates (data not shown), explaining the efficiency of FB17 to colonize *Arabidopsis* roots. There is a tempting possibility that FB17 may down regulate the root-defense response in the initial phases of root-microbe interaction for the establishment of the biofilm community. It is also known that FB17 specifically induces the expression of *PR1* and *PDF1.2* in *Arabidopsis* leaves but not in the roots (Rudrappa et al., 2010). Similarly, *B. subtilis* strain AR156, when applied to *Arabidopsis* roots resulted in the expression of four defense related genes (*PR1, PR2, PR5*, and *PDF1.2*) in the leaves but not in the roots (Niu et al., 2011).

In addition, the observation that FB17 suppresses MTI in the roots is at odds with the prevailing view that MAMPs are the molecular determinants responsible for ISR (Millet et al., 2010). To our knowledge, FB17 does not produce COR or compounds with related structures. Therefore, it is likely that FB17 suppresses the MAMPs-triggered response in roots via a different mechanism. The fact that the heat-killed FB17 treatment negated the suppression of MAMPs-triggered root response supports the view that a small diffusible proteinaceous molecule may mediate this suppression. On a similar line, the inability of the TasA mutant (*tasA::mls*) to suppress flg22-mediated innate response indicates the importance of amyloid fibers in down-regulating plant-defense response. Functional amyloids that participate in normal biological processes in various organisms include harpins in *Xanthomonas campestris* and *P. syringae* (Oh et al., 2007), and fimbriae described in *Mycobacterium tuberculosis* (Alteri et al., 2007). Of all the amyloid fibers, type-III dependent harpins in plant pathogenic bacteria are known to interact with plants to cause hypersensitive response-type of programmed cell death (Oh et al., 2007). Interestingly, a transcriptomic analysis of *Arabidopsis* plants treated with harpins revealed genes related to cell wall biogenesis, cellular communication and signaling (Livaja et al., 2008). In addition, harpin-treated *Arabidopsis* plants also showed down regulation of important plant defense genes such as WRKY transcription factors and oxidative burst-associated genes like NADPH oxidases (Livaja et al., 2008). *B. subtilis* has recently been shown to produce amyloid fibers which were important to regulate the structural integrity in *B. subtilis* biofilms (Romero et al., 2010). To our knowledge, other than harpins, no
other amyloid fibers have been reported to interact with plants. It is surprising that given the abundance of amyloid fibers in biofilms of both Gram-positive and Gram-negative bacteria, how little we know about the role of amyloid fibers in plant-microbe interactions. Interestingly, TasA protein was described as having antibiotic activity (Stöver and Driks, 1999).

Why would *Bacillus* species produce amyloid fibers in its association with plants? The widespread existence of amyloid producing rhizobacteria suggests that the production of antibiotic rich amyloid fibers could be a strategy to outcompete other microbes in the rhizosphere. It could also be speculated that amyloid fiber producing *Bacillus* species could colonize better on plant roots. Interestingly, our data showed that TasA plays a critical role in regulating colonization on plant roots. The importance of root colonization on *B. subtilis*-mediated MTI suppression was immediately ruled out as *B. subtilis* mutants defective in another structural component of biofilm formation, specifically biosynthesis of the EPS matrix, actively suppressed MTI in roots. Thus, MTI suppression is co-regulated with biofilms but biofilm formation *per se* is not required for MTI. Since association of *Bacillus* species induces defense response in plants against pathogens, it could be speculated that *B. subtilis*-derived extracellular components may not elicit defense responses in the root, but may trigger a long distance signal from the roots to leaves mediating ISR in the leaves by simultaneously activating the SA- and JA/ET-signaling pathways in an NPR-1 dependent manner (Rudrappa et al., 2010; Niu et al., 2011). Conversely, *B. subtilis*-derived extracellular components may not play any role in ISR mediated signaling against foliar pathogens and only involve host suppression for efficient colonization. On a similar line, it would be interesting to check the importance of known *Bacillus* biofilm mutants on induction of ISR response in host plants.

**FB17 down regulates flg22-mediated innate defense responses in Arabidopsis roots**

Our data showed that flg22 up-regulated root *ALMT1* expression leading to increased colonization by FB17. In parallel, the data presented here also reveal that FB17 abolishes the flg22-mediated root-defense response which may explain FB17 efficiency to bind to roots. Our data falls in line with the published data showing that plants pretreated with flg22 were more resistant to *PstDC3000* (Zipfel et al., 2004). It was also shown that flg22-mediated resistance requires the activation of SA, JA, and ethylene pathways in parallel and that knocking out a single pathway alone does not abolish the induction of resistance (Zipfel et al., 2004). The increased protection against *PstDC3000* under mixed treatment of FB17 and flg22 may be explained by the added ability of both MAMPs and FB17 to modulate multiple defense pathways (Zipfel et al., 2004; Rudrappa et al. 2008a; 2010).

In parallel, COR, a polykeytide and a phytotoxin produced by various species of *P. syringae*, down regulates innate defense responses in plants through ubiquitin ligase COI1, a key regulator of JA
(Melotto et al., 2006; Tsuda et al., 2008; Millet et al., 2010). Various lines of studies have shown that COR intervenes with the SA/JA antagonistic pathway to suppress a defense response in plants leading to susceptibility against *PstDC3000* (Brooks et al., 2005; Melotto et al., 2006). In contrast, a recent study showed that COR-induced suppression of plant defense is independent of SA-JA antagonism (Brooks et al., 2005; Katsir et al., 2008; Millet et al., 2010). Our data and that of Millet et al. (2010) showed that COR suppresses the root-defense response in *Arabidopsis*. It was shown that COR abolishes the flg22-mediated root defense response in *Arabidopsis* that is dependent on COI1 and JIN1/MYC2 (Millet et al., 2010). At the molecular level, JA-SA antagonism is known from studies in *coi1* and *jin1-9*, where both mutants displayed increased SA signaling post bacterial challenge (Kloek et al. 2001). Similarly, JA signaling is recruited by beneficial mycorrhizal fungi *Piriformospora indica* to suppress the early root MTI responses (Jacobs et al., 2011). Since both *jin1-9* and *jar1-1* mutants exhibited an enhanced MAMPs-induced immune response in response to FB17 colonization (Fig. 5), JA signaling contributes at least partially to the suppression of root MTI. This was further validated by decreased FB17 colonization under MAMPs treatment in JA mutants, suggesting that JAR1/JIN1/MYC2-mediated immune activation may restrict FB17 root colonization.

At this juncture, it is hard to explain how and why FB17 suppresses root innate immune response. On the contrary, from the published work, it is known that FB17 mediates resistance against *PstDC3000* through JA/ET-mediated ISR and SA-mediated SAR by triggering *PDF1.2* and *PR1* expression (Rudrappa et al., 2010). It would be interesting to determine how FB17, in spite of abolishing MAMPs-triggered root responses, protects plants against aerial pathogens. We speculate that the FB17 may adapt a temporal suppression of MAMPs-triggered root response in order to facilitate root colonization.
**Materials and Methods**

**Plant Material and Growth Conditions:** *Arabidopsis* WT seeds were procured from Lehle Seeds (Round Rock, TX, USA). *Arabidopsis* insertion lines *coi1* (SALK_45434C), *fls2* (SALK_093905C), *jar1*-1 (SALK_030821C), *jin1*-9 (SALK_061267C), *npr1*-1 (CS3726), *almt1* (SALK_005672C), *myb51*-1 (SM_3_16332), *cyp71A12* (GABI_127H03-1) and *wrky11* (SALK_141511C) were obtained from the *Arabidopsis* Biological Resource Center (ABRC). *Arabidopsis* transgenic lines *CYP71A12pro:*GUS, *MYB51pro:*GUS and *WRKY11pro:*GUS were obtained from Dr. Frederick M. Ausubel, Harvard Medical School, Boston, MA 02114. Fifty to hundred seeds were sown in 90 mm Petri plates containing two layers of filter paper (Whatman 70 mm Ø, Cat No 1001-070) and wetted with 4 mL of sterile Nano water and allowed to germinate for 3 to 4 d until the root 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 110 μmol·m⁻²·s⁻¹. Five uniform seedlings were transferred on to sterile wire-mesh in Magenta® boxes containing sterile liquid medium [0.5X Murashige and Skoog (1962) (MS) basal media, 2.5 mM 2-(N-morpholino) ethane sulphonic acid (MES), pH 5.8] for two weeks. Each box with 5 seedlings was considered as one biological replicate.

**Treatment of seedlings with MAMPs:** MAMPs were used at the following concentrations unless otherwise specified: For foliar spray experiments, 5 μM COR, 1 μM flg22, 500 μg mL⁻¹ chitin, 500 μg mL⁻¹ PGN, 500 μg mL⁻¹ LPS were used. Plants grown on wire-mesh were carefully removed and sprayed with COR or other MAMPs and transferred to a new sterile Magenta® box containing basal media as described above. After 24 h, experiments were terminated and roots were collected and used for RNA isolation or GUS staining. A 10 mg mL⁻¹ chitin solution was prepared by autoclaving 250 mg of chitin (Sigma-Aldrich) suspended in 25 mL of water for 30 min. The solution was then centrifuged and the supernatant collected.

**Bacterial strains and infections:** *Pst*DC3000 infection assays: *Pseudomonas syringae* pv. tomato (*Pst*DC3000) (obtained from Dr. Jorge M. Vivanco, Colorado State University, Fort Collins, CO) was maintained on Luria-Bertani (LB) plates with 50 mg L⁻¹ rifampicin. A single colony from a freshly streaked plate with antibiotic selection was used to grow overnight cultures from which approximately OD₆₀₀ = 0.02-0.5 inoculum was prepared and used in all the experiments. For routine plant-based studies, cells were grown in LB medium at 37°C, 220 rpm. Cells collected by centrifugation were washed twice with sterile 10 mM MgCl₂ and were re-suspended in 10 mM MgCl₂ to a final density of OD₆₀₀=0.1 for foliar dip of *Arabidopsis*. Fully expanded leaves of
Arabidopsis, plants grown on sterile peat pellets, were dipped in 25 mL of OD\textsubscript{600}=0.1 culture of PstDC3000 with 0.0125% Silwet L-77 for 5 min.

B. subtilis root colonization: Bacillus subtilis strain FB17 (obtained from Dr. Ray Fall, University of Colorado, Boulder, CO) was maintained on LB plates, wild strain 3610, \textit{tasA::mls} (erythromycin and lincomycin resistant) (obtained from Dr. Roberto Kolter, Harvard Medical School, Boston MA) and PY79, DFB79 (\textit{epsG::TnYLB-1}, Kanamycin resistant), DFB82 (\textit{epsO::TnYLB-1}, Kanamycin resistant) (obtained from Dr. Daniel B Kearns, Indiana University, Bloomington, IN), were maintained on LB plates with specific antibiotic selections. All strains were streaked from a freezer stock onto low salt LB plates (Tryptone 10 gL\textsuperscript{-1}, yeast extract 5 gL\textsuperscript{-1}, NaCl 5 gL\textsuperscript{-1}) with appropriate antibiotics. An LB liquid culture of 10 mL was started with bacteria from the plates that had been stored at -4\textdegree C for less than two weeks. After 12 h at 30\textdegree C, a subculture using 1:100 dilution was incubated further at 30\textdegree C, when the OD\textsubscript{600} reached 0.8-1.0, bacterial cells were spun down and re-suspended in sterile 10 mM MgCl\textsubscript{2} to obtain appropriate density of OD\textsubscript{600}=0.5. Treatments included \textit{Arabidopsis} roots inoculated with FB17, PstDC3000 + FB17, foliar sprayed MAMPs and COR with root inoculated FB17. The plants were incubated in the growth chamber for an additional 3 d when the experiment was terminated. Bacterial populations were monitored by serial dilution assays.

Treatment of seedlings with bacterial strains for GUS assays: PstDC3000, FB17, 3610, \textit{tasA::mls}, PY79, DFB79 and DFB82 were cultured on LB medium with appropriate antibiotics. For infection of seedlings grown in Magenta\textregistered boxes, bacteria were grown overnight in LB supplemented with an appropriate antibiotic at 30\textdegree C, bacteria were centrifuged, washed with water, and re-suspended in 0.5X MS medium to a final OD\textsubscript{600} of 0.002. Three-week-old seedlings were treated by adding 1ml of bacterial suspension and incubated for 24 h. For experiments using cell-free lysate (CFL) and heat-killed (HK) bacteria, FB17, 3610, \textit{tasA::mls} were grown overnight and diluted with water to OD\textsubscript{600} of 2. For CFL preparation, bacteria were filtered using 0.22 μm syringe filters and, for HK, the bacteria were maintained at 65\textdegree C for 6h. Three-week-old seedlings were treated by adding 1:1 of HK or CFL with 0.5X MS liquid medium with 2.5 mM MES into each Magenta\textregistered box and flg22. After 24 h of incubation, the experiments were terminated and seedlings were checked for GUS activity.

Root exudation collection and micro-titer assay: Individual WT and transgenic plants were grown steriley on wire-mesh in liquid basal medium. Fully expanded leaves of three-week-old plants were sprayed with different MAMPs or with PstDC3000 (prepared as above) aseptically. The spent liquid medium from the infected and uninfected plants containing root exudates were collected after 2 d and
lyophilized. The lyophilized samples were diluted with sterile water to get 2X concentration. FB17 and
PstDC3000 were suspended in the exudate to OD$_{600}=0.001$ and distributed to 96-well plates and
incubated 30°C for 24 h. Bacterial populations were monitored by serial dilution assays.

**In vitro assay for solid surface biofilm formation:** In vitro biofilm formation of FB17, 3610, tasA::mls, PY79, DFB79, and DFB82 was monitored based on the methods of O'Toole and Kolter (1998) with slight
modification. The biofilm growth medium based on Hamon and Lazazerra (2001) was LB medium plus
0.015 mM ammonium sulfate, 10 mM potassium phosphate (pH 7), 3.4 mM sodium citrate, 1 mM
MgSO$_4$, and 0.1% (w/v) Glc. The inoculum was obtained by growing the cells in biofilm growth medium
and shaking to mid-exponential growth and then diluting the cells to OD$_{600}$ of 0.02 in fresh biofilm
growth medium. Samples of 700 µL of the diluted cells were aliquoted to each of 12 sterile round bottom
culture tubes with caps (12 × 75 mm; from VWR Scientific), and the cultures grown at 37°C without
agitation for 48 h. Cells that had adhered to the tubes were treated with 0.1% crystal violet (CV) for 10–
15 min at 25°C without agitation, the tubes were drained of liquid via pipet, gently rinsed several times
with water, and allowed to dry at 25°C overnight and photographed. The CV that had stained the cells
was solubilized in 1 mL of 80% (v/v) ethanol and 20% (v/v) acetone. Biofilm formation was quantified
by measuring the OD$_{630}$ for each well using an Opsys MR-Dynex plate reader (Chantilly, VA, USA). The
assay was performed on three separate occasions.

**RNA isolation, reverse transcription-polymerase chain reaction (RT-PCR):** Total RNA was isolated
from roots 24 h post treatment. RNA was extracted using PureLink RNA isolation buffer according to the
manufacturer’s instruction manual (Invitrogen, CA, USA). Possible contaminant genomic DNA in RNA
extract was removed using Turbo DNAfree™ kit (Ambion). The gene-specific primers for the genes
ALMT1 (Forward: 5'-GGCCGACCGTGCTATACGAG-3', Reverse: 5'-GAGTTGAATTACTTACTGAAG-3'),
CYP71A12 (Forward: 5'-GATTATCACCTCGGTTCCT-3', Reverse: 5'-CCACTAATACTTCCCAGATTA-3'),
MYB51 (Forward: 5'-ACGGACAAAAACCGATCAAG-3'; Reverse: 5'-AAGCCGAGGCAAACACTAAA-3'),
PRI (Forward: 5'-TTCTTCTTTAGCCCAAACTCGAGGTAATACGCAG-3'), PDF1.2 (Forward: 5'-GGTGGAAAGCACAGTTTGTGGAAGAATTAG-3', Reverse: 5'-AATACACACGATTAGCACC-3'), WRKY11 (Forward: 5'-ACGGACAAAAACCGATCAAG-3'; Reverse: 5'-AAGCCGAGGCAAACACTAAA-3') and UBQ1 (Forward:
TCGTAAGTACAATCAGTGATGACAGATG, Reverse: CACTGAAACAGAAAAACCAACACCCT), were
synthesized (Invitrogen). First-strand complementary DNAs were synthesized from 500 ng of total RNA
in 20 µL final volume using M-MuLV reverse transcriptase and oligo-dT (18-mer) primer (Fermentas
PCR amplifications were performed using PCR mixture (15 μL) that contained 1 μL of RT reaction product as template, 1x PCR buffer, 200 μM dNTPs mix (Fermentas GmbH), 1 U of Taq DNA polymerase (Promega), and 0.1 μM of each primer depending on the gene. PCR was performed at initial denaturation at 94°C for 4 min, 22 or 26 cycles (30 sec at 94°C; 30 sec at 60°C; 30 sec at 94°C), and final elongation (8 min at 72°C) using a thermal cycler (Bio-rad). The PCR products were separated on 1.4% agarose gel, stained with ethidium bromide (0.001%), and documented in a gel documentation system and the bands were quantified using E.A.S.Y. WIN 32. Each band was normalized against the intensity obtained with the same cDNA using the UBQ1 primers. For qRT-PCR, 1 µg of total RNA was reverse transcribed using ProtoScript® First Strand cDNA Synthesis Kit from New England Biolabs. qRT-PCR was performed using a Mastercycler ep realplex machine (Eppendorf) and 5 PRIME-RealMaster Mix SYBR ROX (5-PRIME). The program used for qRT-PCR was as follows: 3 min at 95°C, 45 cycles of 15 s at 95°C/30 s at 53°C, followed by a melt curve from 70 to 95°C. Expression values were normalized to that of the UBQ1.

**Micrografting:** Seeds were sterilized for 5 min in 3% bleach (v/v) [5.25% (w/v) sodium hypochlorite] followed by 1 min in 70% ethanol (v/v), rinsed four times with sterile Nano water, and re-suspended in 1 mL sterile Nano water. Twenty to thirty seeds were sown in 90 mm Petri plates containing six layers of filter paper (Whatman 70 mm Ø, Cat No 1001-070) overlaid with a nitrocellulose membrane (Whatmann OPTITRAN BA-S 85, 0.45µm, Ø82mm, CONV.-No D102536) and wetted with 4 mL of sterile Nano water. Plates were oriented vertically under 24 h of 140 μmol.m⁻².s⁻¹ at 22°C (Arabidopsis chamber AR36L2, Percival Scientific Inc). Seedlings 3-5 d post-sowing were butt grafted (Turnbull et al., 2002). Grafts healed in plates oriented vertically under 10 h of 50 μmol.m⁻².s⁻¹ at 27°C (Arabidopsis chamber AR22L, Percival Scientific Inc.) for 2-4 d after which grafted seedlings were placed in 90 mm Petri plates containing 1/2 MS and 1% agar (w/v) and returned to the 24 h, 140 μmol.m⁻².s⁻¹, 22°C incubator. Adventitious root growth from scions was monitored and removed periodically. The rootstocks used were ALMT1pro:GUS and WT and the scions used were ALMT1pro:GUS, WT, fls2, and coi1. GUS and ALMT1 expression in grafts and FB17 growth in the roots were measured as described herein.

**ALMT1pro:GUS expression assays in grafts:** Micrografted plants were grown steriley on wire-mesh in 6-well microtitre plates and leaves were sprayed with different MAMPs. After 24 h post treatment, plants were stained for GUS (Sigma-Aldrich) according to manufacturer’s instructions. The plants were vacuum-infiltrated for 5min and then incubated at 37°C for 1h. Tissues were cleared in 95% ethanol and photographed using a Nikon D90 camera.
Callose staining: Roots were harvested post 1 day of inoculation with COR or flg22 or flg22+FB17 or Flg22 + tasA::mls or mock (water control), and samples were fixed in 3:1 ethanol:acetic acid solution for several hours. The fixative was changed several times to ensure both thorough fixing and clearing of the tissues, which was essential for good callose detection in the roots. Tissues were rinsed with water several times and rehydrated in 70% ethanol for 2 h, 50% ethanol for an additional 2h, and water overnight. Decolorization was done by adding 10% NaOH and placed at 37°C for 1 to 2 h. Tissue was rinsed several times with water prior to incubation for 30 min in darkness in 0.01% (w/v) aniline blue dissolved in 150 mM K2HPO4 (pH 9.5). Callose deposits were detected using META spectral detector on the LSM5 DUO microscope (excitation, 365 nm; emission, 420 nm).

Microscopy: In order to view adherent FB17 cells on the root surface by laser scanning confocal microscopy, the roots were stained with SYTO®13 (Invitrogen, Molecular Probes, Eugene, OR). Experiments were performed 24 h post-inoculation and post-treatment with FB17 to three-week-old plants grown in Magenta® boxes. Images were captured with a 25X C-Apochromat objective (numerical aperture 1.2) on a Zeiss LSM 510 NLO attached to an Axiovert 200M with Zeiss AIM software (Rel. 3.2). Images were acquired with the 488 nm line excitation of an Argon laser using a 505 nm long pass emission filter. GUS stained plants were viewed with a Zeiss M²Bio Fluorescence Stereomicroscope with the 1.6X lens and digitally acquired with a Zeiss Axio Cam color camera using AxioVision software.

Data analysis: All data presented are the mean values of at least six replicates unless mentioned otherwise, and the data have been presented as mean ± SE. The data were analyzed by one-way analysis of variance (ANOVA) using Microsoft Excel 2010® (Microsoft Corporation, Washington), and post-hoc mean separations were performed by Duncan’s Multiple Range Test (DMRT) at \( p \leq 0.05 \) using software SPSS version 12.0.
ACKNOWLEDGEMENTS: Authors thank Drs. Frederick M. Ausubel and Roberto Kolter for Arabidopsis transgenic lines \textit{CYP71A12}_{pro:GUS}; \textit{MYB51}_{pro:GUS}, \textit{WRKY11}_{pro:GUS} reporter lines and \textit{tasA::mls} mutant. Authors thank both the anonymous reviewers for insightful comments. H.P.B. acknowledges the support from University of Delaware Research Foundation (UDRF), NSF Award IOS-0814477 and DE EPSCoR program. Y-S W. acknowledges support from the National Center for Research Resources (5P30RR031160-03) and the National Institute of General Medical Sciences (8 P30 GM103519-03).
Legend to Figures:

Figure 1: Foliar MAMPs elicit FB17 colonization on Arabidopsis roots:

(A) Confocal images showing differential root FB17 colonization. Three-week-old in vitro seedlings of WT or almt1 plants were foliar sprayed with flg22 (1 µM), COR (5 µM), chitin (500 µg mL⁻¹), LPS (500 µg mL⁻¹), PGN (500 µg mL⁻¹), Pst DC3000 (OD₆₀₀=0.1) or an equal volume of water as the control and rhizo-inoculated with FB17 (OD₆₀₀=0.001) for 24 h. The green fluorescence in the panels shows FB17. Scale bars: 50 µm.

(B) FB17 growth quantification on the roots by colony forming units (CFUs). Three-week-old pellet-grown WT or almt1 plants were foliar sprayed with flg22 (1 µM), COR (5 µM), chitin (500 µg mL⁻¹), LPS (500 µg mL⁻¹), PGN (500 µg mL⁻¹), Pst DC3000 (OD₆₀₀=0.1) or an equal volume of water as control and rhizo-inoculated with FB17 (OD₆₀₀=0.5) of 4 ml/pellet and incubated for 72 h. Data represents the mean ± SE. * p≤0.05, two tailed ‘t’ test, (SE values are from 24 independent measurements from two experiments).

(C) flg22 and COR foliar treatment elicits ALMT1 expression in the root central elongation, meristematic and maturation regions. Three-week-old in vitro grown transgenic seedlings carrying ALMT1pro:GUS promoter were foliar sprayed with flg22 (1 µM), COR (5 µM) or Pst DC3000 (OD₆₀₀=0.1), or an equal volume of water as experimental control. GUS staining of ALMT1pro:GUS seedlings was performed 24 h post-treatment. Scale bars; 50 µm, common to all panels.

(D) Measurement of ALMT1 expression in the roots of plants foliar sprayed with MAMPs. Total RNA was isolated and semi-quantitative RT-PCR (sqRT-PCR) performed. Data represents mean ± SE. Lower case letters indicate the statistical significance among different treatments according to DMRT at p≤0.05 (SE values are three technical replicates of one experiment, repeated twice with similar results).

Figure 2: MAMPs trigger a FLS2 and COI1 dependent graft-transmissible to trigger root ALMT1 expression:

(A and B) Two weeks after graft maturation, micrografts were transferred to liquid medium for 4 d and foliar sprayed with flg22 (1 µM), COR (5 µM) or Pst DC3000 (OD₆₀₀=0.1). Plants were incubated for 24 h and subjected to GUS staining. The images are a representative sample of six plants. Scale bars; 4 mm, common to all panels. Graft notation is fls2/ALMT1pro:GUS, where fls2 is scion and ALMT1pro:GUS is rootstock and for coi1/ALMT1pro:GUS, where coi1 is scion and ALMT1pro:GUS is rootstock.

(C and D) Measurement of root ALMT1 expression in the micrografts fls2/ALMT1pro:GUS and coi1/ALMT1pro:GUS foliar sprayed with flg22 (1 µM), COR (5 µM) or Pst DC3000 (OD₆₀₀=0.1). Total RNA was isolated after 24 h of incubation and sqRT-PCR performed. Data represents the mean ± SE. The
lower case letters represent statistical difference at $p \leq 0.05$ according to DMRT ($\text{SE}$ values are three technical replicates of one experiment).

Figure 3: flg22 induced ALMT1 expression is SA/JAR1/JIN1/MYC2 independent:

(A) Measurement of root ALMT1 expression by sqRT-PCR in WT and icsl-1 and nprl-1. Three-week-old in vitro grown WT, icsl-1 and nprl-1 were foliar sprayed with flg22 (1 µM), COR (5 µM) or PstDC3000 (OD$_{600}=0.1$), an equal volume of water as control and incubated for 24 h. The total root RNA was isolated and was subsequently analyzed for relative expression level of ALMT1.

(B) Measurement of ALMT1 and PRI expression by sqRT-PCR in WT treated with flg22 (1 µM) or SA (100 µM). RNA isolation was performed 24 h post treatment. Insert: ALMT1 expression in ALMT1$_{pro}$::GUS plants treated with flg22 (1 µM) or SA (100 µM) was analyzed 24 h post treatment. Scale bar=50µm.

(C) Measurement of root ALMT1 expression by sqRT-PCR in WT and mutant’s jar1-1 and jin1-9. Three-week-old in vitro grown WT, jar1-1 and jin1-9 were foliar sprayed with 1 µM flg22, 5 µM COR or PstDC3000 (OD$_{600}=0.1$), an equal volume of water as control and incubated for 24 h. The total root RNA was isolated and was subsequently analyzed for relative expression level of ALMT1.

(D) Measurement of ALMT1 and PDF1.2 expression by sqRT-PCR in WT plants treated with COR (5 µM) and MeJA (100 µM). Insert: ALMT1 expression in ALMT1$_{pro}$::GUS treated with COR and MeJA. GUS staining and RNA isolation was performed 24 h post treatment. Scale bar=50µm. For all the panels, data presented as mean ± SE. The lower case letters represent statistical difference at $p \leq 0.05$ according to DMRT ($\text{SE}$ values are three technical replicates of one experiment, repeated twice with similar results).

Figure 4: Rhizobacteria FB17 suppresses MAMPs elicited defense responses in the root.

(A) Transgenic seedlings carrying a CYP71A12$_{pro}$::GUS; MYB51$_{pro}$::GUS or WRKY11$_{pro}$::GUS reporter construct in WT background were grown in vitro and simultaneously treated with flg22 (1 µM) or with equal volume of water as control and FB17 (OD$_{600}=0.001$) and incubated for 24 h before GUS staining. Scale bars; 4 mm, common to all panels.

(B) Three-week-old in vitro-grown Arabidopsis WT plants simultaneously treated with flg22 (1 µM) or with an equal volume of water as control and FB17 (OD$_{600}=0.001$) and incubated for 24 h, total RNA from roots was isolated and semi-quantitative RT-PCR was performed. Data represents the mean ± SE. * $p\leq 0.05$, ** $p\leq 0.001$; two tailed ‘t’ test, n=3.

(C) Three-week-old in vitro-grown Arabidopsis WT plants simultaneously treated with flg22 (1 µM) + 3610 or tasA::mls (OD$_{600}=0.001$), flg22 (1 µM) + culture free lysate (CFL of 3610 or tasA::mls) and flg22 (1 µM) + heat killed (3610 or tasA::mls) and incubated for 24 h before RNA isolation. Real time
PCR was performed and expression of *CYP71A12*, *MYB51*, and *WRKY11* was quantified. Data presented as mean ± SE. The lower case letters represent statistical difference at \( p \leq 0.05 \) according to DMRT (SE values are three technical replicates of one experiment, repeated twice with similar results).

**Figure 5:** *tasA::mls* failed to form root colonization and FB17 mediated suppression of flg22-elicited root defense response is JAR1/JIN1/MYC2 dependent.

**A** Surface attachment and biofilm formation by FB17, 3610 wild-type and *tasA::mls* strains. OD\(_{630}\) of solubilized crystal violet (CV) from solid surface assays over time for tested *B. subtilis* strains; Data presented as mean ± SE. The lower case letters represent statistical difference at \( p \leq 0.05 \) according to DMRT (SE values are three technical replicates of one experiment, repeated twice with similar results).

**B** Confocal images showing differential root colonization by FB17, 3610 wild-type and *tasA::mls* mutant strains. Three-week-old *in vitro* seedlings of WT were foliar sprayed with *PstDC3000* (OD\(_{600}\)=0.1) or an equal volume of water as the control and rhizo-inoculated with FB17 or 3610 wild-type and *tasA::mls* (OD\(_{600}\)=0.001) for 24 h. The green fluorescence in the panels shows biofilm. Scale bars: 50 \( \mu m \).

**C** Evaluation of rhizobacterial growth on the *Arabidopsis* roots by colony forming units (CFUs). Three-week-old pellet- grown WT plants were foliar sprayed with *PstDC3000* (OD\(_{600}\)=0.1) or an equal volume of water as control and rhizo-inoculated with FB17 or 3610 wild-type and *tasA::mls* (OD\(_{600}\)=0.5) of 4mL/pellet and incubated for 72 h. Data represents the mean ± SE. * \( p \leq 0.05 \), ** \( p \leq 0.001 \); two tailed ‘t’ test, \( n=3 \).

**D** Measurement of root *CYP71A12*, *MYB51*, and *WRKY11* expression by semi-quantitative RT-PCR in WT and mutant’s *jar1-1* and *jin1-9*. Three-week-old *in vitro* grown WT, *jar1* and *jin1-9* plants were treated with 1 \( \mu M \) flg22, or flg22+FB7 or an equal volume of water as control and incubated for 24 h. The total root RNA was isolated and was subsequently analyzed for relative expression level. Data presented as mean ± SE. *ns* represents non-significant within the compared means, statistical difference at \( p \leq 0.05 \) according to DMRT.

**E** FB17 growth quantification on the roots by colony forming units (CFUs). Plants (WT, *jin1-9* and *jar1-1*) were foliar sprayed with flg22 (1 \( \mu M \)) or an equal volume of water as control and rhizo-inoculated with FB17 (OD\(_{600}\)=0.5) of 4mL/pellet and incubated for 72 h. Data represent the mean ± SE. The lower case letters represent statistical difference at \( p \leq 0.05 \) according to DMRT (SE values are three technical replicates of one experiment, repeated twice with similar results).
**Figure 6:** Independent regulation of root defense response and ALMT1 expression by MAMPs.

(A) Measurement of ALMT1 expression in cyp71A12, myb5, wrky11 and WT plants. Three-week-old Arabidopsis mutants cyp71A12, myb5, wrky11 and WT were treated with flg22 (1 µM), COR (5 µM), PstDC3000 (OD600=0.1) or equal volume of water as control and total RNA was collected from roots after 24 h of incubation. Semi-quantitative RT-PCR was performed and relative expression of ALMT1 was quantified. Data represent the mean ± SE. The lower case letters represent statistical difference at $p \leq 0.05$ according to DMRT (SE values are three technical replicates of one experiment, repeated twice with similar results).

(B) Measurement of CYP71A12, MYB5, and WRKY11 expression in almt1 plants. Three-week-old Arabidopsis mutants almt1 were treated with flg22 (1 µM), COR (5 µM), PstDC3000 (OD600=0.1), or equal volume of water as control and total RNA was collected from roots after 24 h of incubation. Semi-quantitative RT-PCR was performed and relative expression of CYP71A12, MYB5 and WRKY11 was quantified. Data represent the mean ± SE. * $p \leq 0.05$, ** $p \leq 0.001$; two tailed ‘t’ test (SE values are three technical replicates of one experiment, repeated twice with similar results).

**Figure 7:** Defense-suppressive function of COR is abolished upon root colonization by FB17.

Three-week-old Arabidopsis WT plants were foliar sprayed with flg22 (1 µM), COR (5 µM) or equal volume of water as control. After 24 h of incubation, plants were infected with PstDC3000 by leaf-dip. After 72 h of infection, PstDC3000 growth was measured as CFUs. Data represent the mean ± SE. The lower case letters represent statistical difference at $p \leq 0.05$ according to DMRT (SE values are three technical replicates of one experiment, repeated twice with similar results).

**Figure 8:** A schematic depicting the long distance intra-plant signaling involving foliar MAMPs treatment and root ALMT1 to recruit rhizobacteria Bacillus subtilis FB17. The schematic shows that MAMPs-triggered signal recruits FB17 through ALMT1 belowground along with elicitation of root defense with foliar MAMPs treatments. In contrast, FB17 suppresses MAMPs-elicited root-defense response. The dashed lines show the pathogen and MAMP-induced responses. The schematic shows that FB17 in the initial phase of root colonization suppresses root-defense genes possibly for biofilm formation.
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Figure 1: Foliar MAMPs elicit FB17 colonization on Arabidopsis roots:  (A) Confocal images showing differential root FB17 colonization. Three-week-old in vitro seedlings of WT or almt1 plants were foliar sprayed with flg22 (1 µM), COR (5 µM), chitin (500 µg mL⁻¹), LPS (500 µg mL⁻¹), PGN (500 µg mL⁻¹), PstDC3000 (OD₆₀₀=0.1) or an equal volume of water as the control and rhizo-inoculated with FB17 (OD₆₀₀=0.001) for 24 h. The green fluorescence in the panels shows FB17. Scale bars: 50 µm. (B) FB17 growth quantification on the roots by colony forming units (CFUs). Three-week-old pellet-grown WT or almt1 plants were foliar sprayed with flg22 (1 µM), COR (5 µM), chitin (500 µg mL⁻¹), LPS (500 µg mL⁻¹), PGN (500 µg mL⁻¹), PstDC3000 (OD₆₀₀=0.1) or an equal volume of water as control and rhizo-inoculated with FB17 (OD₆₀₀=0.5) of 4mL/pellet and incubated for 72 h. Data represents the mean ± SE. * p≤0.05, two tailed 't' test, (SE values are from 24 independent measurements from two experiments). (C) flg22 and COR foliar treatment elicits ALMT1 expression in the root central elongation, meristematic and maturation regions. Three-week-old in vitro grown transgenic seedlings carrying ALMT1 pro:GUS promoter were foliar sprayed with flg22 (1 µM), COR (5 µM) or PstDC3000 (OD₆₀₀=0.1), or an equal volume of water as experimental control. GUS staining of ALMT1 pro:GUS seedlings were performed 24 h post-treatment. Scale bars; 50µm, common to all panels. (D) Measurement of ALMT1 expression in the roots of plants foliar sprayed with MAMPs. Total RNA was isolated and semi-quantitative RT-PCR (sqRT-PCR) performed. Data represents mean ± SE. Lower case letters indicate the statistical significance among different treatments according to DMRT at p≤0.05, (SE values are three technical replicates of one experiment, repeated twice with similar results).
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(C and D) Measurement of root ALMT1 expression in the micrografts fls2/ALMT1$_{pro}$:GUS and coi1/ALMT1$_{pro}$:GUS foliar sprayed with flg22 (1 µM), COR (5 µM) or PstDC3000 (OD$_{600}$=0.1). Total RNA was isolated after 24 h of incubation and sqRT-PCR performed. Data represents the mean ± SE. The lower case letters represent statistical difference at p≤0.05 according to DMRT (± values are three technical replicates of one experiment).
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(B) Measurement of *ALMT1* and *PR1* expression by sqRT-PCR in WT treated with flg22 (1 µM) or SA (100 µM). RNA isolation was performed 24 h post treatment. Insert: *ALMT1* expression in *ALMT1$_{pro}$:GUS* plants treated with flg22 (1 µM) or SA (100 µM) was analyzed 24 h post treatment. Scale bar=50µm.

(C) Measurement of root *ALMT1* expression by sqRT-PCR in WT and mutants *jar1-1* and *jin1-9*. Three-week-old *in vitro* grown WT, *jar1-1* and *jin1-9* were foliar sprayed with 1 µM flg22, 5 µM COR or *PstDC3000 (OD$_{600}$=0.1), an equal volume of water as control and incubated for 24 h. The total root RNA was isolated and was subsequently analyzed for relative expression level of *ALMT1*.

(D) Measurement of *ALMT1* and *PDF1.2* expression by sqRT-PCR in WT plants treated with COR (5 µM) and MeJA (100 µM). Insert: *ALMT1* expression in *ALMT1$_{pro}$:GUS* treated with COR and MeJA. GUS staining and RNA isolation was performed 24 h post treatment. Scale bar=50µm. For all the panels, data presented as mean ± SE. The lower case letters represent statistical difference at *p*≤0.05 according to DMRT (*n* is three technical replicates of one experiment, repeated twice with similar results).
Figure 4: Rhizobacteria FB17 suppresses MAMPs elicited defense responses in the root. (contd.)
Figure 4: Rhizobacteria FB17 suppresses MAMPs elicited defense responses in the root.

(A) Transgenic seedlings carrying a CYP71A12pro:GUS; MYB51pro:GUS or WRKY11pro:GUS reporter construct in WT background were grown in vitro and simultaneously treated with flg22 (1 µM) or with equal volume of water as control and FB17 (OD₆₀₀=0.001) and incubated for 24 h before GUS staining. Scale bars; 4 mm, common to all panels.

(B) Three-week-old in vitro-grown Arabidopsis WT plants simultaneously treated with flg22 (1 µM) or with an equal volume of water as control and FB17 (OD₆₀₀=0.001) and incubated for 24 h, total RNA from roots was isolated and semi-quantitative RT-PCR was performed. Data represents the mean ± SE. * p≤0.05, ** p≤0.001; two tailed ‘t’ test, n=3.

(C) Three-week-old in vitro-grown Arabidopsis WT plants simultaneously treated with flg22 (1 µM) + 3610 or tasA::mls (OD₆₀₀=0.001), flg22 (1 µM) + culture free lysate (CFL of 3610 or tasA::mls) and flg22 (1 µM) + heat killed (3610 or tasA::mls) and incubated for 24 h before RNA isolation. Real time PCR was performed and expression of CYP71A12, MYB51 and WRKY11 was quantified. Data presented as mean ± SE. The lower case letters represent statistical difference at p≤0.05 according to DMRT (SE values are three technical replicates of one experiment, repeated twice with similar results).

(D) Flg22-elicited deposition of callose in Arabidopsis roots. WT seedlings were co-treated with flg22 (1 µM), or with COR (5 µM), and root treated with FB17 or tasA:mls and incubated for 24 h. The red punctate spots on roots depict callose deposition. Scale bar: 50 µm for all the panels.
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(B) Measurement of CYP71A12, MYB5, and WRKY11 expression in almt1 plants. Three-week-old Arabidopsis mutants almt1 were treated with flg22 (1 µM), COR (5 µM), PstDC3000 (OD600=0.1), or equal volume of water as control and total RNA was collected from roots after 24 h of incubation. Semi-quantitative RT-PCR was performed and relative expression of CYP71A12, MYB5 and WRKY11 was quantified. Data represents the mean ± SE. * p≤0.05, ** p≤0.001; two tailed 't' test. (SE values are three technical replicates of one experiment, repeated twice with similar results).
Figure 7: Defense-suppressive function of COR is abolished upon root colonization by FB17.

Three-week-old Arabidopsis WT plants were foliar sprayed with flg22 (1 µM), COR (5 µM) or equal volume of water as control. After 24 h of incubation, plants were infected with PstDC3000 by leaf-dip. After 72 h of infection, PstDC3000 growth was measured as CFUs. Data represents the mean ± SE. The lower case letters represent statistical difference at ps0.05 according to DMRT (se values are three technical replicates of one experiment, repeated twice with similar results).
1. Initial root attachment by PGPR *B. subtilis*.
2. Pathogen induced ALMT1 expression.
3. Malic acid secretion and up regulation of *epsA* and *yqxM* biofilm operons in *B. subtilis*.
4. *B. subtilis* suppresses root MAMP-triggered immunity (MTI) through an unknown mechanism which is independent of biofilm formation.
5. Flg22 triggers up-regulation of CYP71A12 and MYB51 independent of ALMT1.
6. *B. subtilis* induces ISR via SA, NPR1 and ETH.

**Figure 8:** A schematic depicting the long distance intra-plant signaling involving foliar MAMPs treatment and root ALMT1 to recruit rhizobacteria *Bacillus subtilis* FB17. The schematic shows that MAMPs-triggered signal recruits FB17 through ALMT1 belowground along with elicitation of root defense with foliar MAMPs treatments. In contrast, FB17 suppresses MAMPs-elicited root-defense response. The dashed lines show the pathogen and MAMP-induced responses. The schematic shows that FB17 in the initial phase of root colonization suppresses root-defense genes possibly for biofilm formation.