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Cyclic Lipopeptides of *Bacillus amyloliquefaciens* subsp. *plantarum* Colonizing the Lettuce Rhizosphere Enhance Plant Defense Responses Toward the Bottom Rot Pathogen *Rhizoctonia solani*

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The commercially available inoculant *Bacillus amyloliquefaciens* FZB42 is able to considerably reduce lettuce bottom rot caused by *Rhizoctonia solani*. To understand the interaction between FZB42 and *R. solani* in the rhizosphere of lettuce, we used an axenic system with lettuce bacterized with FZB42 and inoculated with *R. solani*. Confocal laser scanning microscopy showed that FZB42 could delay the initial establishment of *R. solani* on the plants. To show which secondary metabolites of FZB42 are produced under these in-situ conditions, we developed an ultra-high performance liquid chromatography coupled to time of flight mass spectrometry–based method and identified surfactin, fengycin, and bacillomycin D in the lettuce rhizosphere. We hypothesized that lipopeptides and polyketides play a role in enhancing the plant defense responses in addition to the direct antagonistic effect toward *R. solani* and used a quantitative real-time polymerase chain reaction–based assay for marker genes involved in defense signaling pathways in lettuce. A significant higher expression of *PDF 1.2* observed in the bacterized plants in response to subsequent pathogen challenge showed that FZB42 could enhance the lettuce defense response toward the fungal pathogen. To identify if surfactin or other nonribosomally synthesized secondary metabolites could elicit the observed enhanced defense gene expression, we examined two mutants of FZB42 deficient in production of surfactin and the lipopetides and polyketides, by expression analysis and pot experiments. In the absence of surfactin and other nonribosomally synthesized secondary metabolites, there was no enhanced *PDF 1.2*–mediated response to the pathogen challenge. Pot experiment results showed that the mutants failed to reduce disease incidence in lettuce as compared with the FZB42 wild type, indicating, that surfactin as well as other nonribosomally synthesized secondary metabolites play a role in the actual disease suppression and on lettuce health. In conclusion, our study showed that nonribosomally synthesized secondary metabolites of FZB42 are actually produced in the lettuce rhizosphere and contribute to the disease suppression by mediating plant defense gene expression toward the pathogen *R. solani*.

The plant-associated *Bacillus amyloliquefaciens* subsp. *plantarum* (Borriss et al. 2011) has been widely studied and is commercially available as a bioinoculant (RhizoVital42; ABiTEP GmbH, Berlin). The type strain FZB42 has been sequenced and fully annotated (Chen et al. 2007). Several studies have shown its beneficial effects on plant growth and disease suppression (Chowdhury et al. 2013; Idris et al. 2004, 2007). The strain exhibits a high genetic capacity to nonribosomally synthesize secondary metabolites like antifungal cyclic lipopeptides, such as bacillomycin D, surfactin, fengycin, and antibacterial polyketide compounds (Chen et al. 2007, 2009; Koumoutsi et al. 2004).

The soil-borne pathogen *Rhizoctonia solani* AG1-IB is known to cause bottom rot on lettuce and occurrs wherever lettuce grows (Blancard et al. 2006). In general, *Rhizoctonia* diseases are difficult to control and effective strategies are often lacking. This has stimulated considerable research in developing environmentally friendly control methods including the use of beneficial bacteria and fungi, and several of these microbial inoculants have been reported as effective in suppression of *Rhizoctonia* diseases (Grosch et al. 2005, 2012). In a recent study, we could show that *B. amyloliquefaciens* FZB42 considerably reduced severity of bottom rot on pot- and field-grown lettuce (Chowdhury et al. 2013). The control effect shown by FZB42 could rely on the potential antifungal activity of the several bioactive secondary metabolites. However, until now, it is not demonstrated that the concentration of secondary metabolites secreted by FZB42 or other representatives of plant-associated bacilli in the plant rhizosphere is sufficient for inhibition of the pathogen by direct antibiotic action.

The response shown by a plant species to a particular pathogen is very specific and varies according to the lifestyle and the infection strategy of the pathogen concerned (Pieterse et al. 2009). The first line of plant defense responses toward phytopathogens are usually elicited from several specific compounds known as

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pathogen-associated molecular patterns (PAMPs) that are secreted or harbored by the pathogens (Jones and Dangl 2006). The exposure to the pathogen leads to a local immune response referred to as PAMP-triggered immunity, which may be followed in the distal noninfected parts as a systemic acquired resistance response (Shah and Zeier 2013; Spoel and Dong 2012). Beneficial bacteria used in biological control often show a similar mode of immune response, termed induced systemic resistance (ISR), in plants (De Vleesschauwer and Höfte 2009; Kloepper et al. 2004). ISR emerged as an important mode of action, whereby beneficial microorganisms in the rhizosphere prime the plant for enhanced defense against a range of pathogens (Pieterse et al. 2014; van Loon et al. 1998). The ISR induced by beneficial bacteria is characterized by a weak, transient, and localized defense response, but this priming leads to an enhanced expression of host defense-related genes upon subsequent attack by a pathogen (Conrath et al. 2002; Van Wees et al. 2008). Various *Bacillus* strains have been reported to elicit ISR in different plants, such as tomato, bell pepper, muskmelon, watermelon, sugar beet, tobacco, *Arabidopsis* sp., cucumber, loblolly pine, and tropical crops like cayenne pepper (Choudhary and Johri 2009; Kloepper et al. 2004). Several studies have shown that volatile organic compounds (Farag et al. 2013; Rudrappa et al. 2010; Ryu et al. 2003, 2004) and cyclic lipopeptides like surfactin and fengycin (Cawoy et al. 2014; Desoignies et al. 2013; Henry et al. 2011; Jourdan et al. 2009; Ongena et al. 2005, 2007) are important secondary metabolites produced by *Bacillus* spp. that are capable of eliciting immune responses in plants. There has also been speculation that, in some specific plant-pathogen systems, more than one mechanism could be acting synergistically to suppress diseases (García-Gutiérrez et al. 2013; Rudrappa et al. 2010).

This study was performed to understand the molecular and ecological interactions between FZB42 and *R. solani* in the rhizosphere of lettuce. A recently established, chemically defined axenic system with lettuce and a *gfp*$^{+}$ (green fluorescent protein)-tagged wild-type strain of FZB42 (Budiharjo et al. 2014) was used in this study. Using confocal laser scanning microscopy (CLSM), we could visualize the establishment of *R. solani* on the plants in the presence of FZB42 in the rhizosphere of lettuce. We examined the in-situ production of selected secondary metabolites by FZB42 in the lettuce rhizosphere, using ultra-high performance liquid chromatography coupled to time of flight mass spectrometry (UHPLC-qToF-MS). To examine the defense gene expression in lettuce, a quantitative real-time polymerase chain reaction (qRT-PCR)-based assay was developed for several putative genes, such as pathogenesis-related protein 1 (*PR1*), defensin (*PDF 1.2*), and lipoxygenase involved in salicylic acid (SA)-dependent or jasmonic acid and ethylene (JA/ET)-dependent signaling pathways. We were further interested to identify if surfactin or other nonribosomally synthesized secondary metabolites of FZB42 could be molecular determinants for the regulation of defense gene expression in lettuce plants. Therefore, we examined two mutants of FZB42, mutant CH1 deficient in production of surfactin and mutant CH5 deficient in the synthesis of nonribosomally synthesized lipopetides and polyketides (Chen et al. 2009). Finally, we conducted pot experiments with lettuce bacterized with FZB42 or one of the two mutants and cultivated in the presence of the pathogen *R. solani*, to assess the effect of surfactin and of lipopeptides and polyketides in the actual disease suppression and on lettuce health. Taken together, our results could show, for the first time, that lipopeptides are actually produced by FZB42 in the lettuce rhizosphere and surfactin and (possibly) other nonribosomally synthesized secondary metabolites play an important role in the actual suppression of bottom rot caused by *R. solani*. We could also demonstrate that FZB42 can mediate the plant defense response in addition to the direct antibiotic effect of secondary metabolites in the biocontrol of *R. solani*.

RESULTS

FZB42 is able to delay the initial establishment of the fungal pathogen on the plants.

To study the initial interaction between the pathogen *R. solani* and FZB42 in the rhizosphere of lettuce, we used a quartz sand based axenic system. Lettuce seedlings from surface-sterilized seed were treated with a *gfp*$^{+}$-tagged strain of FZB42 (FB01mut1) (Table 1) and were allowed to grow for 7 days in a growth chamber before the pathogen was inoculated, using *R. solani*–overgrown potato dextrose agar (PDA) pieces. We observed, by CLSM, a successful establishment of FZB42 in the rhizosphere of lettuce over a period of 7 days (data not shown). Plants without FZB42 treatment but with *R. solani* inoculation were used as control. Six plants per time point were sampled, with an interval of 6 h, starting at 0 h postinoculation (hpi) with *R. solani* and ending at 72 hpi. CLSM observations after *R. solani* inoculation showed no growth of fungal hyphae on the root surface until 18 hpi, in both control and the FZB42-treated plants (Fig. 1). Observations after 24 hpi showed that the fungus grows toward the roots and on the hypocotyl of the control plants. In contrast, the FZB42-inoculated plants did not show the presence of any fungal hyphae on both the roots and the hypocotyl (Fig. 1, 24 h). However, 30 h after pathogen inoculation, all FZB42-inoculated plants also showed the presence of fungal hyphae on the roots to the hypocotyl region (Fig. 1, 30 h).

‘Damping off’ symptoms, characterized by brown lesions on hypocotyls followed by seedling collapse, appeared by 48 hpi in control plants whereas by 72 hpi in the FZB42-inoculated plants. In the presence of *R. solani*, we could microscopically observe that the density of FZB42 gradually decreased with time (Fig. 1). The fungal pathogen could grow in a higher density on the control plant tissue within 24 hpi and disintegration of plant cell wall was observed, possibly due to the production of cell wall–degrading enzymes by the fungal pathogen (Wibberg et al. 2013). Therefore, we selected this time point for our subsequent experiments using this model system. Thus, the study of the initial steps of bacterial, fungal, and plant interaction in the quartz sand model axenic system showed that the presence of FZB42 could delay the initial

Table 1. Bacterial strains used in this study

Strain	Genotype	Description	Reference
FZB42	Wild type	Producer of lipopeptides and polyketides	BGSC 10A6
FB01mut1	FZB42; Δ *amyE*:: em^{r} *gfp*$^{+}$	Labeled by green fluorescent protein (GFP)	Fan et al. 2011
CH1	FZB42; Δ *srf*:Cm^{R}; Δ *amyE*:: em^{r} *gfp*$^{+}$	Defective in surfactin production, with GFP marker	Chen et al. 2009, this study
CH5	FZB 42; *ΔyczE*, Δ *sfp*::Cm^{R}; Δ *amy*E::em^{r} *gfp*$^{+}$	Defective in production of lipopeptides and polyketides, with GFP marker	Chen et al. 2009, this study
CH1-Rif	FZB 42; Δ *srf*:Cm^{R}; Δ *amyE*:: em^{r} *gfp*$^{+}$ *rif*$^{+}$	Spontaneous rifampicin resistant mutant of CH1	This study
CH5-Rif	FZB42; *ΔyczE*; Δ *sfp*::Cm^{R}; Δ *amy*E::em^{r} *gfp*$^{+}$ *rif*$^{+}$	Spontaneous rifampicin resistant mutant of CH5	This study

establishment of the fungus on the surface of plant roots and in the hypocotyl region for a few hours as compared with the non-bacterized control. This was clearly an indication that FZB42 exercises some mechanism of inhibition to delay the advancement of *R. solani* to the plants.

FZB42 produces detectable amounts of secondary metabolites in the presence of *Rhizoctonia solani*.

To investigate the production of three nonribosomally synthesized lipopeptides by FZB42 in comparison with its mutants, mixed culture experiments were performed. We established a UHPLC-qToF-MS method for the detection of secondary metabolites. These experiements were performed in flasks containing potato dextrose broth (PDB) medium in the presence of *R. solani* and the bacterium. The goal was to demonstrate that FZB42 and the mutants CH1 (*srf*) and CH5 ($yczE^{-}$, sfp^{-}) (Table 1) were able to produce the lipopeptides in the presence of *R. solani* and to evaluate if our UHPLC-qToF-MS method was able to detect them in the presence of fungal secondary metabolites. *R. solani* was allowed to grow in PDB for 20 h before the inoculation with FZB42 or its mutants. After 24 h of coinoculation, samples were measured as described below. Mass spectrometric analyses could detect the presence of the cyclic lipopeptides of FZB42, namely, surfactin, fengycin, and bacillomycin D in the culture media. Comparing mutants against FZB42, we observed a remarkable decrease of

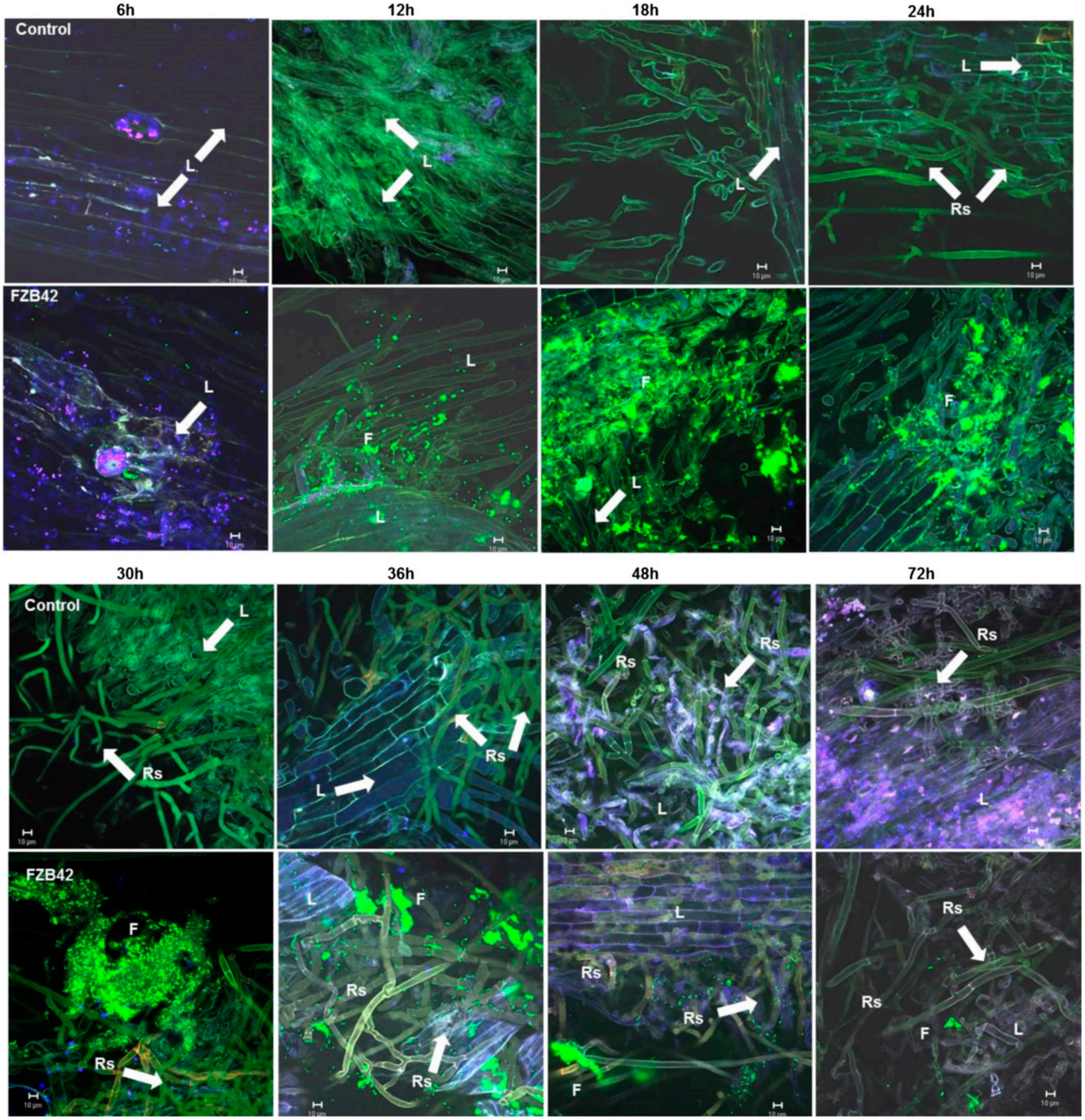

Fig. 1. Development of *Rhizoctonia solani* with *Bacillus amyloliquefaciens* FZB42 (lower panel) in the rhizosphere of lettuce. Confocal laser scanning microscopy pictures were taken at 6-h intervals up to 72 h postinoculation with *R. solani*. Green fluorescent protein–labeled derivatives of FZB42 (FB01mut1) were observed as fluorescing cells (F), using an excitation wavelength of 488 nm (argon laser). Emissions obtained using wavelengths of 543 and 633 nm (helium neon lasers) were used as control to visualize root structures (L) and hyphae of *R. solani* (Rs), as indicated. The disintegration of lettuce plant cell wall was observed as purple background due to autofluorescence.

bacillomycin D in CH1 (defective in surfactin production) without changes in fengycin concentration. As expected, the mutant CH5 did not show the production of any of the lipopeptides examined (Fig. 2). Thus, from this experiment, we could conclude that FZB42 is able to synthesize lipopeptides that could be detected in the presence of *R. solani.*

FZB42 produces secondary metabolites in the lettuce rhizosphere.

The actual production of lipopeptides by FZB42 in the lettuce rhizosphere has not been demonstrated to date. We performed UHPLC-qToF-MS analyses to follow the in situ production of targeted lipopeptides by FZB42 in the lettuce rhizosphere. Lettuce seedlings were bacterized with cell suspension of FZB42 and were cultivated in the axenic system for 7 days. One-week-old plants were inoculated with *R. solani* as described below. Bacterized plants without *R. solani* inoculation served as control. Three plants per treatment were sampled at time points 6, 12, 18, and 24 hpi (with *R. solani*). In all analyzed FZB42 bacterized rhizosphere samples (pathogen-inoculated and noninoculated ones), we could identify the three cyclic lipopeptides surfactin, fengycin, and bacillomycin D. The detected relative amounts were less in the non–pathogen inoculated compared with inoculated root extracts. The pathogen-infected samples bacterized with FZB42 showed decreased amount of surfactin within 12 hpi, followed by strong production of surfactin until 24 hpi (Fig. 3). A much lower intensity of fengycin production, although increasing, was observed over time. Monitored quantities of bacillomycin D decreased more slowly until 18 hpi but showed an enormous increase (approximately 250% in comparison with the 18 hpi time point) within the last 6 h. Thus, the results of mass spectrometric analyses showed the occurrence of the three investigated lipopeptides, with an increase in production of surfactin and bacillomycin D. Comparison of the relative amount of these metabolites and the parallel accomplished colonization studies furnishes

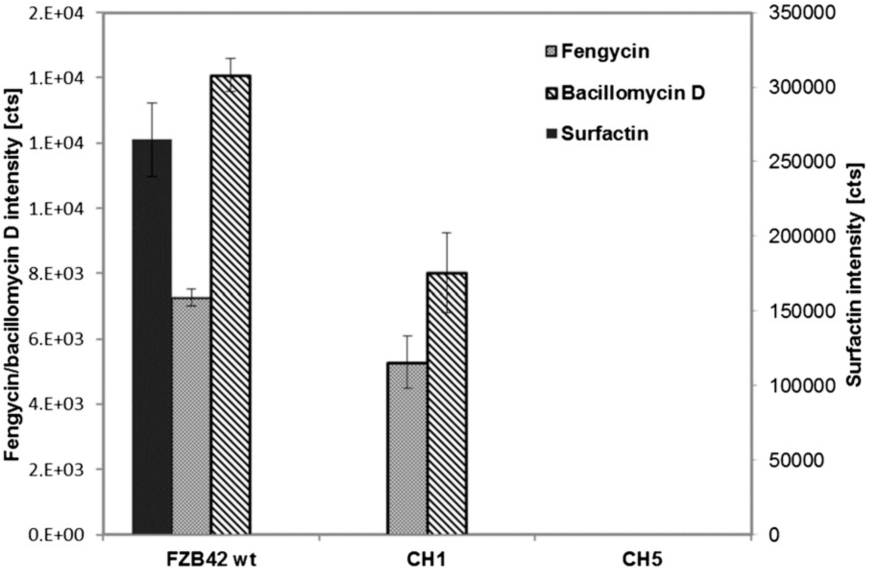

Fig. 2. Expression intensity of nonribosomally synthesized lipopeptides surfactin, fengycin, and bacillomycin D by *Bacillus amyloliquefaciens* FZB42 wild type (FZB42 wt) and its mutants, CH1 and CH5, in the presence of *Rhizoctonia solani*. Data are plotted as the average of three biological replicates ± standard deviation.

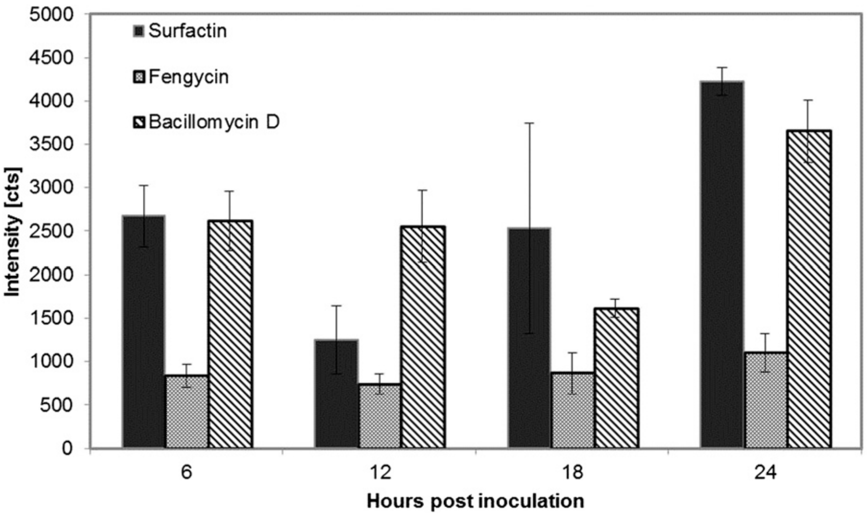

Fig. 3. Production of cyclic lipopeptides surfactin, fengycin, and bacillomycin D by FZB42 wild type in the rhizosphere of lettuce plants inoculated with *Rhizoctonia solani*. Data are plotted as the average of three biological replicates ± standard deviation. Principle component analysis of combined metabolite data shows clear separation of biological replicate clusters with increasing time postinoculation with *R. solani* (Supplementary Fig. S3).

proof of activation of the nonribosomal synthesis of secondary metabolites of FZB42 in the rhizosphere in the presence of *R. solani*. These lipopeptides may provide the necessary signal for inhibiting the pathogen by mediating the defense gene expression in the plants in addition to direct antibiosis of the pathogen.

FZB42 mediates the defense response in lettuce plants. To determine the signaling pathways mediated by FZB42, we analyzed the expression of several plant genes known to function in SA- or JA/ET-dependent immune signaling pathways (Table 2). Axenically grown lettuce seedlings were either bacterized with FZB42 or allowed to grow as control without FZB42 for 7 days, before introducing the fungus *R. solani* in the vicinity of the plants. Plants were excised 24 hpi and RNA extraction and qRT-PCR was performed. Expression of pathogenesis-related protein 1 (*PR1*), defensin (*PDF1.2*), and lipoxygenase (*LOX*) were monitored by qRT-PCR in plants in response to FZB42 treatment alone, to *R. solani* inoculation alone, and to *R. solani* inoculation in FZB42-bacterized (for 7 days) plants.

Plants bacterized with FZB42 alone displayed a higher level of *PR1* and *PDF 1.2* in comparison with the nonbacterized control (Fig. 4). However the expression of *LOX* was reduced in this case. In response to *R. solani*, the nonbacterized plants showed increased expression of *PDF 1.2* and *LOX*. The expression of *PR1* was suppressed by the fungus. This could indicate that lettuce plants respond to *R. solani* through the JA pathway. When challenged with *R. solani*, the plants bacterized with FZB42 showed significantly high expression (>12-fold) of *PDF 1.2* as compared with the control (Fig. 4). The expression of *LOX* was similar to that in the nonbacterized plants in response to *R. solani*. The expression of *PR1* was not significantly higher than in plants with FZB42 in the absence of *R. solani*, indicating that the expression is not further enhanced in the presence of the pathogen. The significantly higher expression of *PDF 1.2* observed in our experiments showed an induction of defense gene expression in the bacterized plants in response to a subsequent pathogen challenge through the expression of the JA/ET pathway. We also observed a higher expression of one selected transcription factor, *ERF* (data not shown), which could be involved in the JA/ET pathway.

Table 2. Genes with putative functions in salicylic acid (SA)-, jasmonic acid (JA)-, and ethylene (ET)-dependent defense signaling pathways in lettuce plants[a]

Gene	Putative function	Primers
PR1	SA inducible pathogenesis-related protein 1	5‘-GAGAAGGCCGATTATGATTA -3‘ 5‘-ATTATTGCATTGAACCCTTG -3‘
Def (PDF1.2)	JA/ET-inducible plant defensin factor (PDF1.2)	5′-GCCATCTTCTCTGCTTTTGAA-3′ 5′-ACACAAGACACTGCGACGAC-3′
LIPO	JA inducible lipoxygenase	5′-AAGAGCAGAAGCCACCCATA-3′ 5′-GTGGAAGGAACTGCGAGAAG-3′
ERF	ET response factor controlling ET-dependent expression of PDF 1.2	5′-CCGTTTGATTGTTCCGATTT-3′ 5′-TTCGGCTTCTTCACTGGATT-3′
GADPH	Glyceraldehyde-3-phosphate dehydrogenase	5′-AGGTAGCGATCAACGGATTC-3′ 5′-AGGTGGGATGCTTGTTTGAC-3′

[a] Primers were designed with Primer3Plus and were selected based on quantitative polymerase chain reaction primer recommendations.

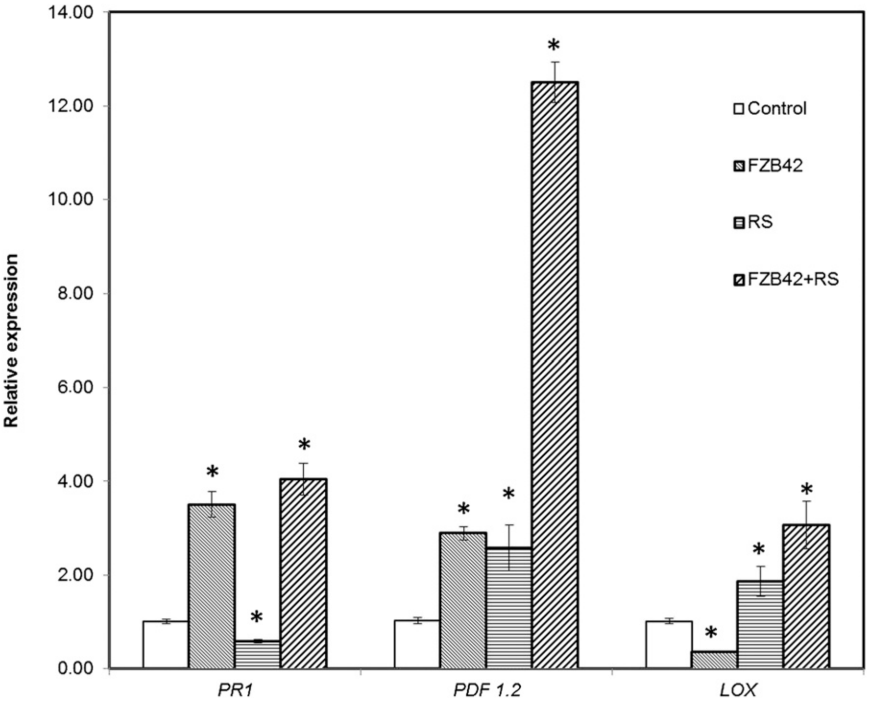

Fig. 4. Expression of pathogenesis related protein (*PR1*), plant defensing factor 1.2 (*PDF 1.2*), and lipoxigenase (*LOX*) genes involved in the salicylic acid- or jasmonic acid- and ethylene–dependent defense signaling pathways in lettuce. Data are expressed as the relative expression of the respective mRNAs normalized to the endogenous control *GADPH*. Control = noninoculated, FZB42 = bacterized with *Bacillus amyloliquefaciens* FZB42, RS = control plants at 24 h postinoculation (hpi) with *Rhizoctonia solani*, and FZB42+RS = FZB42 bacterized plants at 24 hpi with *R. solani*. Data are expressed as the average of six replicates ± standard deviation. *P* values were calculated using a *t* test; asterisks (*) indicate $P < 0.001$ and refer to the comparison between treated samples and noninoculated control.

Several nonribosomally synthesized secondary metabolites of FZB42 are involved in the regulation of defense responses in lettuce plants against *R. solani*.

The qRT-PCR data from above showed that the enhanced expression of *PDF 1.2* was an indicator for the regulation of plant defense response by FZB42. To examine the role of nonribosomally synthesized secondary metabolites in the regulation of defense responses against *R. solani* in lettuce, we bacterized axenically grown lettuce seedlings with the mutants CH5 (defective in *Sfp*-dependent nonribosomal synthesis of lipopeptides and polyketides) and CH1 (defective in surfactin production) and allowed them to grow for 7 days. The fungus *R. solani* was then inoculated in the vicinity of the plants, as described above. Plants were excised 24 hpi, and the expression of *PDF1.2* in the presence and absence of *R. solani* was examined. The mutant CH5 could elicit a higher expression of *PDF 1.2* (compared with FZB42) in lettuce plants in the absence of the fungal pathogen. However, when challenged with *R. solani*, the plants inoculated with either one of the mutants did not exhibit a significant expression of *PDF 1.2*, as compared with FZB42 (Fig. 5). Our results suggest that FZB42-mediated defense gene expression in lettuce involves surfactin and (possibly) other nonribosomally synthesized secondary metabolites.

Nonribosomally synthesized secondary metabolites produced by FZB42 are involved in disease suppression.

Having demonstrated in the axenic system that FZB42 can actually produce lipopeptides in the rhizosphere of lettuce and that the mutants for surfactin (CH1) and other secondary metabolites (CH5) do not show enhanced molecular defense responses, we wanted to examine if they actually play a role in the disease suppression. Two separate pot experiments were performed in a growth chamber to investigate the disease-suppressing effect of CH5 and CH1 compared with FZB42, using rifampicin-resistant mutants (Table 1) in the lettuce–*R. solani* pathosystem. Each treatment included four replicates, with six plants per replicate arranged in a randomized block design. Lettuce seedlings were drenched two times with spore suspension of FZB42 or its mutants and the pathogen were inoculated after planting of lettuce into the pots. The disease suppression effect of FZB42-Rif and the mutants were assessed based on the shoot dry mass (SDM) and the number of plants with disease symptoms (disease incidence [DI]) 4 weeks after planting in both experiments.

In both pot experiments, a significantly lower SDM was observed for nonbacterized lettuce plants in the presence of *R. solani* (Table 3). The negative effect of the pathogen on lettuce growth could be significantly reduced by the treatment of lettuce with FZB42. This was indicated by a comparable high SDM of plants treated with FZB42 in the presence of *R. solani* (FZB42-Rif+RS) and in the untreated control plants (Control) in both experiments. In contrast, a significantly lower SDM compared with the control was measured in the treatments with the mutants CH1 and CH5 in the presence of *R. solani* (CH1-Rif+RS, CH5-Rif+RS) (Table 3). Based on the data of DI (Table 3), we could observe that the number of plants with disease symptoms, as compared with the pathogen control, was significantly reduced only when the plants were treated with FZB42. The number of diseased plants was not significantly less than the pathogen control when the plants were treated with the mutants CH1 and CH5. Therefore, no significant disease control effects were shown by the mutants in both experiments. FZB42 as well as mutants CH1 and CH5 were able to colonize the rhizosphere of lettuce in a sufficient density during the entire growth period in both experiments (Supplementary Table S1). No significant decrease in colonization density was observed for the FZB42 or for the mutants, considering the total CFU counted within 4 weeks. A significantly lower number of spores, compared with vegetative cells, was revealed for both mutants and the FZB42 in the first experiment two weeks after planting and for CH5 also 4 weeks after planting. Thus, our results show that, although both the mutants CH1-Rif and CH5-Rif were able to successfully colonize the rhizosphere of lettuce, they did not show any significant effect on the control of *R. solani* infection and reduction of disease symptoms.

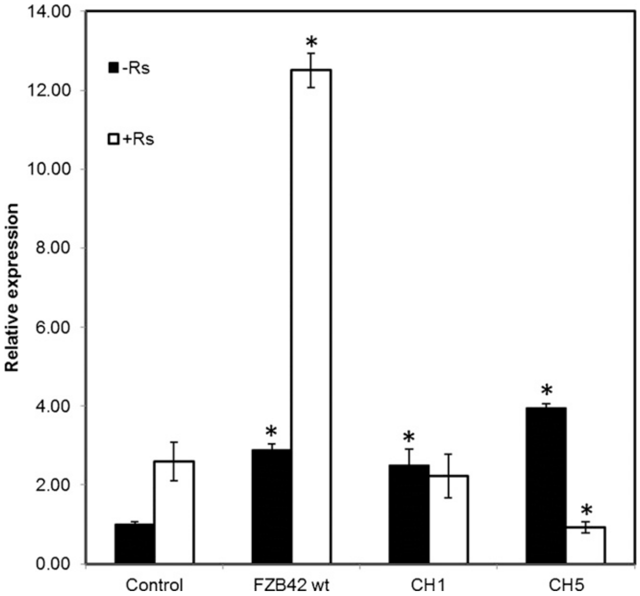

Fig. 5. Relative expression of *PDF 1.2* in lettuce plants. Control (nonbacterized) plants are compared with plants bacterized with *Bacillus amyloliquefaciens* FZB42 wild type (FZB42 wt) or with mutants CH1 (defective in surfactin production) and CH5 (defective in production of lipopeptide and polyketide). +RS = plants at 24 h postinoculation with *Rhizoctonia solani*, –RS = plants without *R. solani* inoculation. Data of the respective mRNAs were normalized to the endogenous control *GADPH*. Data are expressed as the average of six replicates ± standard deviation. Asterisks (*) indicate $P < 0.001$ and refer to the comparison between bacterized plants and controls under the same conditions.

DISCUSSION

Antibiosis of FZB42 and *R. solani* for root colonization.

In this study, we used a GFP-labeled derivative of FZB42 (FB01mut1) that showed a plant-specific behavior of colonizing plant roots (Dietel et al. 2013; Fan et al. 2011, 2012). Our results confirm the ability of FZB42 to establish succesfully in the rhizosphere of lettuce (Budiharjo et al. 2014). *R. solani* showed profuse growth and infection of, especially, the hypocotyl in

Table 3. Effect of *Bacillus amyloliquefaciens* FZB42-Rif and the mutants CH1-Rif and CH5-Rif on lettuce[a]

	Experiment 1		Experiment 2	
Treatment	SDM	DI (%)	SDM	DI (%)
Control	10.6 a	0	6.5 a	0
R. solani (+RS)	7.9 b	100 a	4.7 b	88 a
FZB42-Rif + RS	11.2 a	50 b	6.2 a	29 b
CH1-Rif + RS	8.5 b	65 a	5.4 b	57 a
CH5-Rif + RS	8.7 b	85 a	5.1 b	64 a

[a] Effect on shoot dry mass (SDM, shown in grams per plant) of lettuce (cv. Tizian) and disease incidence (DI) percent of plants with disease symptoms) caused by *Rhizoctonia solani* (+RS) under controlled conditions 4 weeks after planting, in two independent experiments. Different lower case letters indicate significant differences in SDM and DI, according to Dunnett's test procedure ($P = 0.05$).

control plants. The early steps of plant infection appear to be very similar independent of *R. solani* and host plant (Keijer 1996). The observed disintegration of the lettuce plant cell wall has also been described previously in *R. solani*–infected cauliflower (Pannecoucque and Höfte 2009). Host infection and colonization is associated with the production of cell wall–degrading enzymes, as underlined by genome analysis of the used *R. solani* isolate 7/3/14 (Wibberg et al. 2013). We could also observe that FZB42 cannot survive in the presence of the fungus, and its density decreases with time (Fig. 1). This could be due to one or both the production of antibacterial metabolites or competition for available root exudates by *R. solani*. Transcriptome analysis of the *R. solani* AG1-IB isolate supported its ability to produce antibiotics (Wibberg et al. 2014) that may act against FZB42 under specific axenic conditions more favorable for the pathogen, as evidenced by CLSM observations. In general, the microenvironment of the root in this axenic system is comparable to a battlefield, in that FZB42 and *R. solani* are in combat against each other, with the production of an array of metabolites to gain access to the plant.

The production of secondary metabolites by plant growth–promoting rhizobacteria provides them with an advantage over other microbes for root colonization in the highly competitive rhizosphere environment (Compant et al. 2010). An important aspect, which we also addressed in this work, is the proof that lipopeptides are actually synthesized by FZB42 in the lettuce rhizosphere. Only a few studies are available that indicate the detection of lipopeptides of bacilli in situ in the rhizosphere (Bais et al. 2004; Debois et al. 2014; Nihorimbere et al. 2012) and in the phyllosphere (Zeriouh et al. 2014). We developed a high throughput method using UHPLC-qToF-MS for fast detection of cyclic lipopeptides like surfactin, fengycin and bacillomycin D without time-consuming sample preparation or cost-intensive measurements. The method allows us to detect very low amounts of these secondary metabolites, such as those occurring in the rhizosphere after bacterization with FZB42. In a recent investigation, a matrix-assisted laser desorption-ionization mass spectrometry imaging method was used to examine the spatiotemporal changes in the secreted antibiome of *B. amyloliquefaciens* S499, a close relative of FZB42, on roots of *Arabidopsis thaliana*, tomato, and tobacco plants (Debois et al. 2014). Surfactin was identified as the major (over 90%) actively secreted lipopeptide compound. Very small amounts of iturins and fengycin were detected, mostly after 7 days of colonization, and no polyketides were found (Debois et al. 2014). Although this report seems to be in contrast to our results reporting detection of all three lipopeptides, on careful interpretation, it becomes clear that, in our axenic system, the first time point of measurement is 7 days after bacterization of the lettuce plants. Our earlier work shows that FZB42 can establish itself as biofilm on the lettuce roots in this period of time (Budiharjo et al. 2014). The lower amounts of fengycin detected in the rhizosphere of lettuce is in congruence with the report from Debois et al. (2014). Elaborated data showed an increasing production of surfactin and bacillomycin D by FZB42 within the first 24 h, in the presence of *R. solani*. The iturin bacillomycin D was identified as the most powerful antifungal metabolite produced by FZB42 (Chen et al. 2009). This increase in the production of bacillomycin D in the presence of *R. solani* implies some kind of recognition of the fungi by FZB42, which switches on its genetic machinery to produce this antifungal compound to attack the pathogen. This is in accordance with a recent investigation with different strains of *B. amyloliquefaciens*, including FZB42 showing enhanced production of iturins and fengycins in response to signals emitted by phytopathogens like *Fusarium oxysporum* and *Botrytis cinerea* (Cawoy et al. 2015) and another study demonstrating that iturins produced by *B. amyloliquefaciens* 41B1 could inhibit the germination of *Verticilium dahliae* microsclerotia in vitro and in the soil, with the result of a reduced disease severity of wilt on cotton under greenhouse conditions (Han et al. 2014).

Role of lipopeptides in mediating lettuce defense responses. Based on our hypothesis that FZB42 can mediate defense response in lettuce plants in addition to the direct antagonistic effect toward *R. solani*, we studied the expression of defense-related genes in lettuce plants. It has been observed that plants recognize the different molecular-associated molecular patterns (MAMPs) of beneficial microbes, similar to the recognition of pathogen-associated molecular patterns (PAMPs). This leads to an effective activation of the plant immune response (Van Wees et al. 2008; van der Ent et al. 2009; Pieterse et al. 2009). We could observe that FZB42 established itself in the rhizosphere of lettuce over a 7-day period and activated an expression of plant defense genes *PR1* and *PDF1.2*, even in plants not infected with the pathogen. The activated priming may result in enhanced response upon pathogen challenge, as observed. A similar effect was described on *Arabidopsis* inoculated with endophytic actinobacteria also capable of producing bioactive secondary metabolites (Conn et al. 2008). *Bacillus subtilis* UMAF6639 was also shown to moderately activate *LOX2* expression in melon plants before inoculation with pathogen (Zeriouh et al. 2014). It has been suggested that *Bacillus* spp. can actively block immune responses in *Arabidopsis* roots to establish a compatible interaction with their host (Niu et al. 2011). An early suppression of *Arabidopsis* defense genes in roots by *B. subtilis* FB17 to establish itself in the rhizosphere has also been reported (Lakshmanan et al. 2012). It has been speculated that JA signaling contributes partially to the suppression of root MAMP-triggered immunity. In our experimental setup, the plants were bacterized 7 days prior to the inoculation of *R. solani*, but the fungal hyphae were grown toward the roots and hypocotyl within 24 h (CLSM observations) and were in the vicinity of the bacterial cells. The transcriptome analysis of *R. solani* AG1-IB shows its ability to produce a number of compounds (Wibberg et al. 2014) that could be perceived by the plants triggering a PAMP-induced defense response, and we cannot specifically attribute a rhizobacteria ISR mechanism induced by FZB42, using our setup with the pathogen. However, we could clearly see that FZB42 mediates the plant defense response in distal tissues through an induction of expression of the JA/ET pathway, as indicated by rapid expression of *PDF 1.2* in the bacterized plants in response to pathogen challenge. This is in strong accordance with a recent report showing rapid and strong upregulation of basal defense genes in cotton plants treated with iturins from *Bacillus amyloliquefaciens* 41B-1 against *Verticillium* infection (Han et al. 2014). An earlier report also shows that *B. amyloliquefaciens* can induce systemic resistance in oilseed rape against *Botrytis cinerea* through JA-dependent *PDF 1.2* expression (Sarosh et al. 2009).

Several studies have shown that cyclic lipopeptides of bacilli can offer significant protection to plants against fungal pathogens by eliciting defense reactions (Jourdan et al. 2009; Ongena et al. 2007). Surfactins produced by the *Bacillus* sp. strain S499 have been shown to elicit systemic resistance in plants by the activation of the lipoxygenase-dependant oxylipin pathway (Ongena et al. 2007). The importance of surfactin produced by various *Bacillus* strains, including *B. amyloliquefaciens* FZB42, for the stimulation of plant defense has been recently examined (Cawoy et al. 2014). Using the leaf pathogen *Botrytis cinerea* to infect tomato plants, a considerable disease reduction was observed in *B. amyloliquefaciens* strains (S499, 98S and FZB42). This could be correlated to the relative amount of surfactin produced by the strains in a medium containing root exudates, and the levels of disease reduction were similar to those obtained by using 10 μM purified surfactin (Cawoy et al. 2014). To identify if secondary metabolites of FZB42 were indeed recognized as MAMPs by lettuce plants, we examined the mutant CH1, which is defective in the production of surfactin (*srf*), and the double mutant CH5 (Δ*sfp*, Δ*yczE*), deficient in the synthesis of all nonribosomally synthesized lipopeptides and polyketides of FZB42 (Chen et al. 2009). The results of failing expression of *PDF1.2* in plants inoculated with the mutants (Fig. 5) indicates that multiple nonribosomally synthesized secondary metabolites are probably involved in the mediation of defense response shown by the wild-type FZB42. The genes *sfp* (phosphopantetheinyl transferase [PPTase]) and *yczE* (integral membrane protein with unknown function) have been described to be essential for nonribosomal synthesis of lipopeptides and for synthesis of polyketides (Chen et al. 2009). A recent study showed that a *sfp* mutant of *B. amyloliquefaciens* SQR9M6 lost its antifungal activities against different tested fungi, including *R. solani* (Li et al. 2014). Interestingly, the mutant CH5 showed ability to thrive in the presence of *R. solani* (Supplementary Fig. S1). This might indicate being incapable of producing the antifungal secondary metabolites; the fungus does not detect this mutant as a threat and, in consequence, does not produce antibacterial compounds in its presence.

Biological control under complex soil conditions.

The conditions in field-grown lettuce are completely different from those in axenic model systems for studying the production of lipopeptides in the rhizosphere (Debois et al. 2014; Nihorimbere et al. 2012). Therefore, we conducted pot experiments that simulate actual field-like growth conditions for lettuce and disease development. The importance of nonribosomally synthesized metabolites, such as surfactin in actual disease suppression, was demonstrated by results of our pot experiments with the surfactin-negative mutant CH1. In an earlier dual confrontation assay against *R. solani* on PDA plates, CH1 showed an almost equal antagonistic activity compared with FZB42, as interpreted by the zone of inhibition (Supplementary Fig. S2). This result is explained by the fact that other antifungal compounds, like bacillomycin D, produced by this mutant CH1 were causing the inhibition of the fungal growth similar to FZB42. This was confirmed in another recent study with the mutant CH1 retaining similar inhibitory effect as compared with wild-type FZB42 on *Fusarium oxysporum* and *Botrytis cinerea*, indicating that iturin and fengycin but not surfactin are important antifungal compounds for direct antagonism of these two pathogens (Cawoy et al. 2015). However, using mutant CH1 in the pot studies, no efficient disease control effects were observed, although CH1 was able to successfully colonize the lettuce rhizosphere. This indicates that surfactin is important for disease suppression by mediating defense response of the plants and the production of other antifungal compounds alone is not sufficient to prevent the growth of the pathogen in the pots. In an earlier study, we have shown that FZB42 can positively influence the SDM of the plants in the presence of pathogen *R. solani* by reduction of the disease. In the absence of the pathogen, FZB42 did not show any growth-promoting effect on lettuce in the growth chamber (Chowdhury et al. 2013). The present study corroborates this observation. Therefore, our results indicate that, in addition to direct antibiosis due to the secondary metabolites of FZB42, an indirect mechanism of regulation of defense responses in lettuce plants through FZB42 is functional. The relevance of this induced resistance for this pathosystem is corroborated by the pathogenesis of *R. solani*, which infects the lower leaves of field-grown lettuce, while FZB42 is colonizing the roots.

Not much is known about the different signaling pathways involved in resistance mechanisms and their regulation in lettuce plants. Although we included some transcription factors for our expression study, it is rather speculative to draw conclusions from them due to lack of extensive studies with mutants targeting specific components in the SA, JA, and ET pathways. Therefore, the best estimation of the signaling pathways is based on the analogies to their counterparts in *Arabidopsis* and other plants. However, it should be taken into account that there might be important differences between the defense mechanisms in different plants (van Loon et al. 2006). The newly sequenced genome of lettuce (University of California Davis Genome Center) has opened up possibilities for investigating the expression of defense-related pathways in more detail. Further research in this direction would add valuable knowledge about the interactions of this commercially important plant with its beneficial microflora and pathogens.

In conclusion, our study involving mass spectrometric analyses, colonization studies, expression analysis, and pot experiments, indicates the importance of the production of lipopeptides by *B. amyloliquefaciens* FZB42 to control *R. solani* effectively. The stimulation of plant defense responses mediated by secondary metabolites of FZB42 seems to play an important role in the actual suppression of bottom rot in lettuce. The involvement of other factors, like volatiles (acetoin, and 2,3-butandiol), in induction of resistance against *R. solani* needs to be addressed in a separate study. Thus, the capacity to mediate plant defense responses is expected to work in concert with the direct antibiotic effects and their colonization efficiency. making FZB42 an effective plant growth-promoting and biocontrol agent.

MATERIALS AND METHODS

Bacillus strains and construction of mutants.

In this study, we used *Bacillus amyloliquefaciens* FZB42 wild type and mutants described in Table 1. All strains were grown in lysogeny broth (LB) medium (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) with respective antibiotics at 30°C. A spontaneous rifampicin-resistant colony was selected from an overnight culture in LB medium with rifampicin as described by Koumoutsi et al. (2004), resulting in the CH1-Rif mutant. The mutant CH5gfp was constructed by transformation of chromosomal DNA of CH5 into FB01mut1, according to (Chen et al. 2009). A spontaneous rifampicin-resistant mutant of CH5gfp was selected resulting in CH5gfp-Rif. The media and buffers used for DNA transformation of *Bacillus* cells were prepared as described by Kunst and Rapoport (1995). For pot experiments, spore suspensions prepared from FZB42 and its derivatives were used.

Preparation of *Rhizoctonia solani* inoculum.

The *Rhizoctonia solani* AG1-IB isolate 7/3/14 was obtained from the strain collection of the Leibniz Institute of Vegetable and Ornamental Crops, Großbeeren, Germany, was maintained on PDA (Merck, Darmstadt, Germany), and was used for inoculation of lettuce in the experiments. For the pot experiment, the inoculum was prepared as described by Schneider et al. (1997) on barley kernels.

Axenic system for the study of FZB42 and *R. solani* interaction in the lettuce rhizosphere.

Lactuca sativa cv. Tizian (Syngenta, Bad Salzuflen, Germany) seeds were surface-sterilized by treating with 1% Tween

80 for 2 min and 13% sodium hypochloride for 10 min, washing with sterile water (three times) in between. The sterilized seeds were placed on nutrient agar (Merck, Darmstadt, Germany) plates and were allowed to germinate in the dark for 2 days before being checked for bacterial or fungal contamination. Germinated lettuce seeds were soaked in a cell suspension of FB01mut1 adjusted to 10^7 to 10^8 CFU per milliliter with 0.3% NaCl for 1 h at room temperature. Bacterized seedlings were transferred to sterile plastic trays (Phytatray, Sigma-Aldrich, St. Louis) with sterilized quartz sand and 20 ml of Hoagland's solution (Sigma). The trays were placed in a growth chamber (Vötsch BioLine VB 1514) at 20 and 18°C with 12-h-light and 12-h-dark intervals. After 7 days of growth, lettuce plants at the four-leaf stage were inoculated with *R. solani* by placing PDA pieces (1 cm^2) with fungal hyphae 2 cm away from each plant and 1 cm deep in the quartz sand. Plants without FZB42 treatment but with *R. solani* inoculation were used as control. The plants were further cultivated in the growth chamber and six plants per time point were sampled at an interval of 6 h, starting at 0 hpi with *R. solani* and ending at 72 hpi. Plants were excised aseptically, were washed with sterile water to remove adhering quartz sand, and were cut in the hypocotyl region. Roots were mounted with antibleaching agent (Citifluor, Citifluor Ltd., London) on glass slides for microscopic observations.

CLSM.

CLSM was performed with a LSM 510 microscope (Carl Zeiss, Jena, Germany), using an excitation wavelength at 488 nm (argon laser) and collecting the emission band of 500 to 550 nm for GFP flourescence. Emissions obtained using wavelengths of 543 and 633 nm (helium neon lasers) were used as control to visualize root structure. Images were aquired and reconstructed using the Zeiss LSM Image browser.

Mixed-culture experiments to analyze secondary metabolites.

For in vitro mixed-culture experiments, flasks (three per treatment) containing 50 ml of 50% PDB medium were inoculated with one sclerotia of *R. solani* (produced on PDA plates) and were cultivated at 28°C and 90 rpm. After 20 h of fungal growth, the flasks were inoculated with 1 ml of each corresponding bacterial wild-type or mutant cell suspension (around 10^7 to 10^8 CFU/ml) and were further incubated for 24 h. Samples (10 ml) were taken from each flask 24 h after coinoculation with FZB42. Samples were centrifuged for 10 min at 5,000 rpm and 4°C, and pellets were separated from supernatants. In liquid nitrogen, quenched pellets were then extracted for 20 min with 80% methanol (CHROMASOLV LC-MS; Fluka, Sigma-Aldrich Laborchemikalien GmbH, Germany) in an ultrasonic bath, were evaporated using a SpeedVac system (Thermo Scientific, Waltham, MA, U.S.A.), and were solved in water for UHPLC-qToF-MS analyses.

Analyzing secondary metabolites in *Lactuca sativa* rhizosphere.

Germinated lettuce seeds were bacterized with FZB42 and were cultivated in the axenic system as described above for 7 days. Root colonization was confirmed by parallel experiments using CLSM. One-week-old plants were coinoculated with *R. solani* as described above. Plants bacterized with FZB42 but not inoculated with *R. solani* were used as control. Three plants per treatment were sampled at time points 6, 12, 18, and 24 hpi. Plants were cut at the hypocotyl region and the root system was cleaned carefully from adhering quartz sand. Complete roots were transferred in 2 ml of ice-cold solvent mixture of 80% methanol (CHROMASOLV LC-MS, Fluka) in water and was extracted about 20 min in tempered (0°C) ultrasonic bath. One-milliliter aliquots of supernatants were evaporated and resumed in water.

UHPLC-qToF-MS analyses of secondary metabolites.

Measurements were performed by an Acquity UPLC system (Waters Corporation, Milford, MA, U.S.A.) coupled to a time-of-flight mass spectrometer equipped with an Apollo II electrospray ionization source (maXis, Bruker Daltonik, Bremen, Germany). The reversed-phase seperation is adapted from Hu et al. (2008). Chromatographic separation was achieved on an Acquity BEH C18 column packed with 1.7-µm particles and dimensions of 1.0 × 50 mm (Waters Corporation). The temperatures of samples and column were set to 4°C and 40°C respectively. Each sample (5 µl) was injected through a partial loop. The mobile phases consisted of A (60% acetonitrile in water containing 10 mM ammonium formate) and B (90% i-propanol, 10% acetonitrile containing 10 mM ammonium formate and 0.1% formic acid) and were set to a flow rate of 0.2 ml/min. All chemicals were at least of HPLC grade and were supplied by Sigma. An elution gradient was adjusted for 4 min, holding the initial composition of 99% A and 1% B for 0.5 min, followed by a linear ramp up to 99% B within 1 min. By holding 99% B for 1.5 min, sufficient seperation of lipopeptides was achieved. Another linear gradient at 3 min set initial conditions and the pre-runtime of 2 min was used for column re-equilibration. The detection wavelength was set to 230 nm with a scan rate of 20 Hz, and mass spectra were acquired in positive ionization mode within a mass range of 100 to 2,000 *m/z*. Chromatograms and spectra were processed with DataAnalysis 4.0 (Bruker Daltonik).

Study of plant defense responses.

Lettuce seedlings were bacterized with cell suspension of FZB42 or mutants CH1 and CH5 and were cultivated in the axenic system, as described above. Control plants were drenched with 1 ml of sterile 0.3% NaCl instead of bacterial cell suspension. The trays were placed in a growth chamber (Vötsch BioLine VB 1514) at 20 and 18°C with 12-h-light and 12-h-dark intervals for 7 days to ensure the root colonization of FZB42. One-week-old plants were inoculated with *R. solani* as described above. Plants were incubated in the growth chamber for 24 h before RNA extraction.

Selection of target genes and primer design.

Target genes of lettuce showing similarity to a known or deduced function involved in the SA and JA/ET pathways were selected based on previously published expressed sequence tags (Klosterman et al. 2011) from the National Center for Biotechnology Information (NCBI) dEST database. Namely *PR1* (an SA-responsive marker gene), defensin (*PDF1.2*) (JA/ET response marker gene), lipoxygenase (*LOX*) (a JA-responsive marker gene) and an ethylene response factor (*ERF*) that could be expressed in the plant defense mechanism (Pieterse et al. 2009; van Loon et al. 2006) were used in this study. The complete sequences of the corresponding genes selected were obtained from the *Lactuca sativa* whole genome shotgun sequencing project at NCBI (BioProject database, PRJNA68025). BlastX (Altschul et al. 1997) searches were performed against the NCBI nr database to identify conserved regions in the selected genes. The candidate reference genes corresponding to ubiquitin (*Ubi*), protein phosphatase 2A (*PP2A*), glyceraldehyde-3-dehydrogenase (*GADPH*), and actin (*ACT*) were selected for normalization of gene expression in lettuce. The expression stability of these genes was analyzed using NormFinder software (Andersen et al. 2004). *GADPH* was identified as the most stable gene and was used as an endogenous control in qRT-PCR analysis.

The primer pairs used for the qRT-PCR analysis (Table 2) were designed according to the parameters established on the Primer3Plus program (Untergasser et al. 2007). The primer gene specificity was checked by PCR on cDNA and the amplicons were sequenced. The amplicon sequences were aligned against the *Lactuca sativa* whole-genome shotgun sequencing project, using BLAST at NCBI.

qRT-PCR.

qPCR was performed as described previously (Alquéres et al. 2013) with some modifications. Five plants were pooled, and total RNA was extracted from the aerial parts with TRizol (Life Technologies, Carlsbad, CA, U.S.A.), from frozen material, according to manufacturer's instructions and was followed by DNaseI (Promega Corp., Madison, WI, U.S.A.) treatment. RNA (1 µg) was used for cDNA synthesis, using the high-capacity cDNA reverse transcription kit with RNase inhibitor (Applied Biosystems, Foster City, CA, U.S.A.). Expression of selected genes were determined by RT-qPCR with Power SYBR green supermix (Applied Biosystems) using an iCycler thermal cycler (Applied Biosystems). cDNA dilutions (1 µl, 1:4) were used as PCR templates. Each PCR reaction contained 12.5 µl of 2× Power SYBR green supermix, 0.4 µM primers (Eurofins MVG Operon, Ebersberg, Germany), and 1 µl of template in a 25-µl reaction. PCR reactions were heated to 95°C for 3 min and, then, for 40 cycles with steps of 95°C for 30 s, 60°C for 30 s, and 60°C for 30 s. The generation of specific PCR products was confirmed by melting-curve analysis and gel electrophoresis. The $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) was employed for relative quantification. The quantity of interested genes was normalized to the quantity of the endogenous control gene for each condition. The experiments were done in biological triplicates (each with two technical replicates). Results were based on the average of triplicates and the standard deviation of the mean is shown.

Pot experiments for study of disease suppression effects of FZB42 mutants.

In two independent experiments, lettuce seeds (cv. Tizian) were cultivated as described by Chowdhury et al. (2013). Lettuce plants were planted at the two-leaf stage into pots (500 ml) filled with a mixture of quartz sand and Fruhsdorfer Einheitserde Typ P (Vechta, Germany; chemical analysis [mg/liter]: N = 120, P = 120, K = 170, Mg = 120, S = 100, KCl = 1, organic substance = 167, peat = 309; pH 5.9) at a 1:1 ratio (vol/vol) and cultivated at 22 and 15°C until harvest in a growth chamber (York, Mannheim, Germany) (16-h-day and 8-h-night cycle, 500 µmol m^{-2} s^{-1}, 60 and 80% relative humidity). For inoculation of lettuce with *R. solani*, two infested barley kernels were placed 1 cm deep and 2 cm apart from the seedlings 1 day after planting. The pots were watered lightly each day to maintain the substrate moisture and were fertilized weekly (0.2% Wuxal TOP N; Wilhelm Haug GmbH & Co. KG, Düsseldorf, Germany). Each treatment included four replicates with six plants per replicate arranged in a randomized block design. The disease suppression effect of FZB42-Rif and the mutants CH1-Rif and CH5-Rif were assessed based on the SDM and the number of plants with disease symptoms (DI) 4 weeks after planting in both experiments.

For bacterization of lettuce with FZB42-Rif and the mutants CH1-Rif and CH5-Rif, each plant was drenched with 10 ml of the corresponding spore suspension (10^7 spores/ml) at the two-leaf stage 1 week before pathogen inoculation and with a 20-ml spore suspension (10^7 spores/ml) at the three-leaf stage at planting.

The survival and root colonization efficiency of FZB42-Rif and the mutants CH1-Rif and CH5-Rif was evaluated 2 and 4 weeks after planting into the pots as described by Chowdhury et al. (2013).

Statistical analysis.

The data analysis was performed with the STATISTICA program (StatSoft Inc., Tulsa, Ok U.S.A.). The SDM, DI, and inoculant densities (CFU) were analyzed using analysis of variance with Dunnett's test procedure, with $P < 0.05$.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

National Center for Biotechnology Information BioProject database: www.ncbi.nlm.nih.gov/bioproject

NCBI dEST database: http://www.ncbi.nlm.nih.gov/dbEST