The N-acyl homoserine-lactone depleted *Rhizobium radiobacter* mutant RrF4NM13 shows reduced growth-promoting and resistance-inducing activities in mono- and dicotyledonous plants

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**Abstract**

The Alphaproteobacterium *Rhizobium radiobacter* (syn. *Agrobacterium tumefaciens*, “A. fa brum”) can live in close association with the sebacoid fungus *Serendipita* (syn. *Piriformospora indica*) that forms a mutualistic Sebacinean symbiosis with a wide range of host plants. The endobacterial strain *R. radiobacter* F4 (*Rr*F4), which was originally isolated from the fungus, has plant growth promotion and resistance-inducing activities resembling the beneficial activities known from the endobacteria-containing *S. indica*. The mechanism by which free endobacterial cells influence growth and disease resistance of colonized host plants is not fully understood. Here, we show that *Rr*F4 produces a spectrum of quorum sensing-mediating N-acyl-homoserine lactones (AHLs) with acyl chains of C8, C10, and C12 as well as hydroxyl- or oxo-substitutions at the C3 position. In addition, and in line with previous findings showing that AHLs increase plant biomass and induce systemic resistance, the AHL-depleted lactonase-overexpressing transconjugant *Rr*F4NM13 was partially compromised in promoting growth and inducing resistance against bacterial pathogens in both *Arabidopsis thaliana* and wheat (*Triticum aestivum*). Scanning and transmission electron microscopy proved that *Rr*F4NM13, in contrast to *Rr*F4, does not form cellulose-like fiber scaffolds for efficient root surface attachment. Moreover, *Rr*F4NM13 does not penetrate into the intercellular space of the cortical tissue, which in contrast is strongly colonized by *Rr*F4. We discuss the possibility that AHLs contribute to the outcome of the Sebacinean symbiosis.

**Keywords** Quorum sensing · Homoserine lactones · Pseudomonas · Rhizobium · Piriformospora

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Introduction

Endofungal bacteria inhabit fungal cells and form beneficial relationships (symbioses) with their hosts (Bertaux et al. 2003, 2005; Bonfante and Anca 2009; Lackner et al. 2009). Members of the fungal Basidiomycota order Sebacinales regularly undergo complex tripartite symbioses involving plants and endofungal bacteria of different genera (Sharma et al. 2008; Glaeser et al. 2016; Guo et al. 2017; del Barrio-Duque et al. 2020). The most comprehensively studied example of a tripartite Sebacinales symbiosis is the association of the beneficial endophyte Serendipita indica with the Gram-negative Alphaproteobacterium Rhizobium radiobacter. The representative strain R. radiobacter F4 (RrF4) was originally isolated from S. indica (strain P. indica DSM 11827) and could be propagated in axenic cultures, demonstrating that the bacterium is not entirely dependent on its fungal host (Sharma et al. 2008; Glaeser et al. 2016).

In the present work, we followed the idea that the bacterial quorum sensing (QS) system of R. radiobacter is a critical factor in the interaction of the free-living cells of the endobacterium when interacting with plant roots. In Gram-negative bacteria, QS is mainly mediated by N-acyl-homoserine lactones (AHLs), which regulate a variety of bacterial activities such as motility, bioluminescence, biofilm formation, expression of virulence factors, antibiotic and exopolysaccharide (EPS) production and microbial community assembly during the anaerobic granulation process (Waters and Bassler 2005; Williams 2007; Davis et al. 2010; Hartmann and Schikora 2012; Ma et al. 2018). Several reports have described important activities of AHLs in regulating beneficial rhizobacteria-plant host communications (Schuhgeger et al. 2006; Pang et al. 2009; Mhlongo et al. 2018; Tang et al. 2020). Consistent with this, mutations disrupting AHL production or signaling affect the ability of most endobacteria to infect tissues/cells and to exert beneficial activity in their eukaryotic hosts (Parsek and Greenberg 2000; Gonzalez and Marketon 2003; Waters and Bassler 2005; Kai et al. 2012; Alabid et al. 2019).

AHLs differ in length and composition of their acyl side chains. Molecules with chain lengths of 4–18 carbon atoms frequently contain a carbonyl (oxo) or hydroxyl (OH) substitution on the third carbon atom (Williams 2007; Thomanek et al. 2013). Endofungal bacteria also produce AHLs as first shown for endobacteria of the zygomycete fungus Mortierella alpina A-178 (Kai et al. 2012), where they may play a role in the establishment and/or maintenance of the symbiotic interaction with the zygomycete. AHLs are also required for colonization of Arabidopsis thaliana roots and plant growth promotion by the plant growth-promoting rhizobacterium (PGPR) Burkholderia phytofirmans PsJN (Zuniga et al. 2013).

Plants are able to detect and react to bacterial QS molecules, which thereby influence the plants’ physiology (Mathesius et al. 2003; Schuhgeger et al. 2006; Ortiz-Castro et al. 2008; von Rad et al. 2008; Schikora et al. 2011; Han et al. 2016; Rankl et al. 2016; Schikora et al. 2016). Perception of AHLs by still unknown plant receptors leads to various responses, including altered gene regulation, induced defenses, modification of the cytoskeleton and hormonal imbalance (Bauer and Mathesius 2004). Both N-hexanoyl-l-homoserine lactone (C6-HSL) and N-butanoyl-l-homoserine lactone (C4-HSL) promote root growth in Arabidopsis, whereas AHLs with longer carbon chain lengths have an opposite effect as they reduce root growth (von Rad et al. 2008). Interestingly, longer-chained AHLs (C10- to C14-HSL), but not the shorter ones, induce resistance toward biotrophic and hemibiotrophic pathogens in plants such as barley and Arabidopsis (Schikora et al. 2011; Schenk et al. 2012).

Overall, the earlier reports discussed above suggest that plant responses to AHLs depend on AHL structure and concentration and the recipient plant species. Supporting this notion, AHL transport inside a plant also rests on the difference in the structural properties of the molecule. For example, both C6-HSL and C8-HSL translocated in barley from roots to shoots, while C10-HSL did not (Schikora et al. 2011). Likewise, C6-HSL, but not the C10-HSL or oxo-C14-HSL, was translocated from roots to shoots in Arabidopsis (Götz et al. 2007; Gonzalez and Marketon 2003).

Here, we show that the AHL-depleted transconjugant R. radiobacter RrF4NM13 is partly compromised in root colonization and does not induce the full beneficial activities in plant host, suggesting that QS plays a role for establishing a beneficial symbiosis during plant—R. radiobacter interactions.

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana ecotype Columbia (Col-0, N1092) was obtained from the Nottingham Arabidopsis Stock Centre (NASC). Seeds were surface-sterilized in 70% ethanol for 1 min, in 25% sodium hypochlorite for 10 min followed by washing with autoclaved water (pH 3) and four times with sterile distilled water. Seeds were dried on sterile filter paper and germinated in round petri dishes on half strength Murashige and Skoog (MS) medium (Murashige and Skoog 1962), containing glycine, myo-inositol, nicotinic acid, pyridoxine HCl and thiamine HCl supplemented with 0.5% sucrose and solidified with 0.4% gelrite (Duchefa biochemistry Haarlem, the Netherlands). Seeds were placed on sterile wet filter papers at 4 °C for 2 days to achieve synchronized...
germination. Subsequently, seedlings were grown under short-day conditions (8 h light at 22 °C/18 °C (day/night), with a photon flux density of 183 μmol m⁻² s⁻¹, and 60% relative humidity) in 100 mm square petri dishes (Greiner Bio-One GmbH, Frickenhausen, Germany).

Seeds of wheat cultivar (cv.) Bobwhite were surface-sterilized in 70% ethanol for 3 min and sodium hypochlorite for 1.5 h with continuous stirring. Seeds were germinated on wet filter paper in sterile glass jars for 3 days as described above. Dip-inoculated seedlings were transferred to sterile 1.7 L jars (four plants per jar), containing a solid half strength MS medium supplemented with 0.2% arabinose and tellurite, and kept in a climate chamber at 24 °C, 16 h light, 60% relative humidity and a photon flux density of 160 μmol m⁻² s⁻¹. For growth promotion assays, wheat plants were grown in pots containing a soil-sand (3:1) mixture (Archut Fruhstorfer Erde, Type P, Lauterbach, Germany). Roots were carefully cleared from adhering soil and sand particles, subsequently washed in sterile water to remove attached bacteria, separated from the shoot and dried on sterilized filter paper.

Generation of the lactonase-producing RrF4 transconjugant RrF4NM13

To generate RrF4 strains that produce less AHL, the broad-host-range expression vector pMLBAD, carrying the lactonase gene aiiA, which is inducible with 0.2% arabinose (Wopperer et al. 2006), was transferred into RrF4, using conjugation via triparental mating (Stotzky et al. 1990). E. coli HB101 pRK600 (Figurski and Helinski 1979) was used as helper strain for transferring plasmid DNA from donor to the recipient strain.

Preparation of R. radiobacter inoculum and root inoculations

Rhizobium radiobacter F4 (RrF4) was originally isolated from S. indica (strain’s old name: P. indica DSM 11827; Sharma et al. 2008), which is available from the IPAZ collection of microorganisms, Institute for Phytopathology, Justus Liebig University Gießen, Germany. RrF4 was grown overnight in modified LB medium (1% casamino hydrolysate, 0.5% yeast extract, 0.5% NaCl, pH 7.0, supplemented with 100 μg/mL gentamycin) at 28 °C and 150 rpm. The RrF4NM13 transconjugant was grown on nutrient broth medium (1% peptone, 0.3% bacto beef extract, 0.5% NaCl and 0.1% glucose), including 0.2% arabinose for the induction of the lactonase gene, and 200×potassium tellurite solution (5 mL/L) for resistance selection. Bacterial cells were collected centrifugation (3220×g; Eppendorf 5810R, Germany), washed, and resuspended in 10 mM MgSO₄·7H₂O. For inoculations, roots of three-day-old wheat and seven-day-old Arabidopsis seedlings were dipped for 30 min into bacterial suspensions (OD₆₀₀ = 1.0 for Arabidopsis, OD₆₀₀ = 1.4 for wheat). Control seedlings were dip-inoculated in 10 mM MgSO₄·7H₂O.

Root inoculation with S. indica

Serendipita indica (syn. P. indica; Weiß et al. 2016) originates from a sample collected in the Indian Thar desert in 1997 (Verma et al. 1998). The strain (old name P. indica DSM 11827) is available from the IPAZ collection of microorganisms. The fungus was propagated on modified complete medium (CM, 20 g/L glucose, 2 g/L peptone, 1 g/L yeast extract, 1 g/L casamino acids, 1 mL/L 1000×micromounters, and 50 ml/L 20×salt solution) at room temperature (RT) (Pham et al. 2004). Chlamydospores were collected from three-week-old S. indica cultures, washed and suspended in 0.002% tween-20 with 500,000 chlamydospores per mL. Three-day-old wheat seedlings were dip-inoculated in the chlamydomspore solution for 1.5 h. Seedlings dipped into tween-20 were used as control. Inoculated wheat seedlings were transferred to sterile jars containing a solid half strength MS medium supplemented with 0.2% arabinose and kept in a climate chamber as described above. Alternatively, they were grown in pots containing 3:1 mixture of expanded clay (Seramis, Mars GmbH, Germany) and Oil Dri (Damon, Germany), fertilized every week with an aqueous solution of Wuxal Super 8/8/6 (1:1000 v:v; Haug, Düsseldorf, Germany) in a climate chamber under a 16 h photoperiod and 22/18 °C day/night (60% rel. humidity, and a photon flux density of 240 μmol m⁻² s⁻²).

Preparation of Pseudomonas syringae and Xanthomonas translucens inoculation

Pseudomonas syringae pv. tomato DC3000 (Pst), which carried a resistance gene for rifampicin and the plasmid pVSP61 with resistance to kanamycin, was obtained from Dr. Nikolaus L. Schaich, RWTH Aachen, Germany. Pst was grown in liquid King’s B medium (2% bacto proteose peptone, 0.15% K₂HPO₄, 0.15% MgSO₄·7H₂O, 0.8% glycerol, 1.5% agar; King et al. 1954) supplemented with antibiotics (50 μg/mL rifampicin and 50 μg/mL kanamycin) for selection and grown overnight at 28 °C. Bacterial cells were collected by centrifugation, washed and resuspended in 10 mM MgSO₄·7H₂O, containing 0.02% Silwet L-77 (Momentive, Germany) to a final concentration of OD₆₀₀ = 0.2. Arabidopsis plants were sprayed each with 100 μL of this solution. Leaves were harvested at the indicated time points and
assessed for *Pst* infection by determining colony-forming units (cfu) per mg of fresh leaf.

*Xanthomonas translucens* pv. *translucens* DSM 18974† (Xtr) (Jones et al. 1917; Vauterin et al. 1995) was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ), Braunschweig, Germany. It was grown overnight in a liquid nutrient broth (0.5% peptone, 0.3% meat extract, pH 7.0) at 28 °C. Bacterial cells were collected by centrifugation, washed and resuspended in sterile 10 mM MgSO₄·7H₂O with 0.02% Silwet L-77 adjusted to a final concentration of OD₆₀₀ = 0.25. For wheat infection, leaves of three-week-old plants were sprayed with the disease symptoms.

High humidity for optimal infection until appearance of the symptoms were placed in transparent boxes with closed lids to maintain high humidity for optimal infection until appearance of the disease symptoms. 

### AHL detection and lactonase assay

For the qualitative detection of AHLs, transgenic bacteria were used as biosensors. Bacteria were grown under gentle shaking overnight in the dark at 28 °C till OD₆₀₀≈0.8 (LB medium with 100 µg/ml gentamycin; Duchefa biochemie). The bacterial culture was centrifuged and dichloromethane (CH₂Cl₂) was added to the supernatant with a ratio 1:10 for extraction of AHLs. The lower part was taken and then mixed with sodium sulfate (Na₂SO₄) for absorption of rest water overnight at 4 °C. Formed crystal particles were separated by sterile filter paper and dried by means of vacuum evaporation. The GFP expressing biosensor *Escherichia coli* MT102 carrying the plasmid pJBA89 (construct pUC18Not-luxR-Pluxl-RBSII-gfp (ASV)T0-T1) was used for detection of C₆ to C₁₄ AHLs (Andersen et al. 2001). *E. coli* was grown on LB medium with 100 µg/L ampicillin overnight at 21 °C. In the next day, different amount from AHLs extract (1, 5, 10, 25 and 50 µL) of cleared acetone extract were dropped on the bacteria lawn. Fluorescence was observed 2 h after incubation using an epifluorescence microscope (Axioplan 2, Zeiss, Oberkochem, Germany) with extinction wavelength 480/40 nm; emission filter 510 nm. For lactonase test assay, *Agrobacterium tumefaciens A* 136 was grown in LB medium overnight on a shaker at 28 °C (OD₆₀₀ = 0.9). This biosensor was used according to Han et al. (2016). Activity was determined by the appearance of the blue color on bacterial streaks.

### Extraction of AHLs from colonized wheat roots

Roots from three-day-old seedlings were dip-inoculated with either *RrF4*, *RrF4NM13*, or *S. indica*, and then grown in sterile glass jars containing a half strength MS medium (Duchefa) (supplemented with 0.2% arabinose). Roots were harvested at 7 dpi, immediately frozen in liquid nitrogen, collected in falcon tubes (Greiner, Germany), and stored at −80 °C. For AHL extraction, frozen roots were ground in liquid nitrogen. AHLs were first extraction in 50:50 vol% acetonitrile/chloroform; after solvent evaporation, they were resumed in acetonitrile and finally split by ultracentrifugation in pellets and supernatants before drying. The pellet was dissolved in acetone and vortexed.

### Determination of AHL species by UHPLC-qToF-MS

Dried pellets and supernatants of previously extracted roots or bacterial cells were resumed in 3 mL and 1 mL acidified ethyl acetate, respectively, and vortexed for 30 min. The samples were once more evaporated and dissolved in a mixture of ethyl acetate and acetonitrile (50:50 vol%, V₉₉, pellets = 500 µL; V₉₉, supernatants = 1 mL) for analyses (Mashburn and Whiteley 2005). AHL detection was performed by ultrahigh performance liquid chromatography time-of-flight mass spectrometry (UHPLC-qToF-MS, Bruker Daltonik, Bremen, Germany) as already described elsewhere (Buddrus-Schiemann et al. 2014). Briefly, 5 µL sample was separated through a BEH C₁₈ reversed-phase column (dimensions 1.0 mm × 150 mm, 1.7 µm particle size; Waters GmbH, Eschborn, Germany) at 40 °C and with a flow rate of 0.1 mL/min. The mobile phase consists of A: 10% acetonitrile in water (incl. 0.1% formic acid) and B: 100% acetonitrile. A linear gradient starting from 5% B and increasing to 90% B was applied, and UV absorption was recorded at 196.5 nm in addition to the mass spectrometric detection operated in positive electrospray ionization mode. Samples were reconciled with 18 external AHL standards of varying carbon chain lengths (C₄-HSL to C₁₄-HSL) and with or without 3-hydroxyl or 3-oxo-substitution, respectively. All chemicals and standards were purchased from Sigma-Aldrich (Taufkirchen, Germany).

### Quantification of *RrF4* and *RrF4NM13* in wheat roots by qRT-PCR

Bacteria-colonized roots of wheat cv. Bobwhite, grown in sterile glass jars containing a half strength MS medium supplemented with 0.2% arabinose, were harvested at 0, 5, 7 and 14 dpi. The upper four cm of roots were collected. In order to remove excess bacteria from the surface, roots were washed in 70% ethanol for 1 min, followed by distilled water for 1 min, and 3× sonication for 30 s in water using a Sonorex RK106 (Berlin, Germany). Afterward, roots were ground in liquid nitrogen and sampled in a 2 mL polypropylene tube. Genomic DNA was extracted from 100 to 200 mg root powder using the Plant DNeasy plant mini kit (Qiagen, Hilden,
et al. 2012). The relative abundance of RrF4 and RrF4NM13 in root tissue was quantified by quantitative PCR (qPCR) targeting the ribosomal RNA operon ITS-1 region of RrF4 (Sharma et al. 2008). qPCR was performed using 20 ng DNA as template, 150 nM of RrF4-specific internal transcribed spacer (ITS) primer (ITS-Rh-F/ITS-Rh-R; Sharma et al. 2008) and 350 nM plant gene-specific primer (alpha tubulin) (primers listed in Tab. S1). Each sample was pipetted in triplicate. Amplification procedures were performed as described (Glaeser et al. 2016). All q-PCR data acquired were based on three technical replicates and three biological experiments.

Evaluation of bacterial speck disease caused by Pst

For quantification of Pst, Arabidopsis leaves were harvested at time points 0, 24, 48, and 96 h post-inoculation (hpi). Leaves were weighed, rinsed with 70% ethanol for 1 min, sterile water for 1 min, and finally ground in 10 mM MgSO₄ × 7H₂O. Serial of dilutions were made from the homogenized plant materials and 10 µL from each serial dilution were dropped onto King’s B medium (Carl Roth, Germany) supplemented with 50 µg/mL rifampicin and 50 µg/mL kanamycin and incubated for 2 days at 28 °C. The colony-forming units (cfu) of rifampicin and kanamycin resistance Pst were counted.

Assessment of Xtt infections on wheat pants

Disease symptoms were scored as translucent stripes. For determination of disease severity on wheat leaves, a scale ranging from 0 to 9 based on diseased leaf area (0 = no disease symptoms, 1 = 10% leaf area, etc.) was used (Kandel et al. 2012).

RNA extraction, cDNA synthesis and analysis of expression of salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) responsive genes in wheat roots

Quantitative reverse transcriptase qRT-PCR was used for all gene expression analyses. Total RNA was extracted with 1 mL TRIzol® Reagent (Invitrogen, Karlsruhe, Germany) according to manufacturers’ protocol. cDNA synthesis was performed using a Qiagen Quantitect Reverse Transcription Kit. One µg RNA from each sample was used. The cycle number was set to 42. Forty nanograms of cDNA were used as a template for qRT-PCR. The 2⁻ΔΔCt method (Schmittgen and Livak 2008) was used to determine different gene expressions. All expression values were normalized to values of the housekeeping gene alpha tubulin. The primers used in this study are listed in Tab. S1. In order to check the efficiency of reverse transcription, PCR for the Alpha tubulin was performed. All expression values were normalized to the expression of Alpha tubulin (3 dpi values).

Fixation, staining and microscopy of colonized roots, electron microscopy

For electron microscopy, root branches were aldehyde fixed following established protocols, washed in buffer and dehydrated in an ethanol series. For surface analysis using scanning electron microscopy (SEM), roots were critical point dried, mounted on SEM holders and gold sputtered. Samples were observed in a FEG scanning electron microscope (DSM982 and MERLIN, ZEISS Germany) at 3–5 kV. Images were recorded using a secondary electron (SE)-detector with the voltage of the collector grid biased to +300 V in order to improve the signal-to-noise ratio and to reveal optimal topographical contrast. For transmission electron microscopy (TEM), selected root areas were dissected after fixation, embedded in gelatin (Fluka, Germany), post fixed in 1% osmium tetroxide, washed and incubated in 1% aqueous uranyl acetate (Polysciences, Hirschberg, Germany) overnight at 4 °C. Specimens were dehydrated in an ethanol series (30, 50, 70, 80, 90, 96 and 100% v/v, 20 min each) and embedded in LR White (Agar Scientific, UK). From the blocks cured by heat, ultrathin sections were cut and finally contrasted in uranyl acetate and lead citrate. Ultrathin sections were inspected in the TEM (EM912a/b—Zeiss) at 120 kV under zero-loss conditions and images were recorded at slight 50 under focus using a 2 k × 2 k slow-scan proscan ccd camera (Zeiss).

Results

R. radiobacter RrF4 produces AHLs

The endofungal lifestyle of the S. indica-associated RrF4 raises the question whether it has a functioning QS system. A previous report showed that the genome of RrF4 contains components of two AHL-based QS systems (Glaeser et al. 2016): The tra operon described by Piper et al. (1999) is present in the accessory plasmid pAtF4. This plasmid also contains the QS regulator gene traR, while the AHL synthase tral maps on the tumor-inducing plasmid pTiF4 upstream of the trb operon. Moreover, RrF4 harbors an additional set of tra genes on the pTiF4 plasmid showing highest similarity to the conjugation genes on the RP4 plasmid of E. coli.

Encouraged by these previous findings, we addressed the question whether RrF4 produces QS AHL-type metabolites. Chemical analysis using UHPLC-qToF-MS of bacterial extracts showed that RrF4 produces a spectrum of AHLs with acyl chains of C8, C10 and C12 as well as hydroxyl- or...
oxo-substitutions at the C3 position (Table 1). Sterile filtered extracts of RrF4 cultures (OD$_{600}$ ≈ 0.8) added to the axenically growing AHL reporter strain E. coli MT102 (Andersen 2001) induced a dose-dependent GFP signal, comparable to the treatment with an AHL used as positive control, while the AHL solvent acetone had no effect (Fig. S1).

Since we could not detect C14-HSL in axenic RrF4 cultures, though it is a highly active AHL with strong activity on priming and induced resistance (Schikora et al. 2011, 2016; Schuhegger et al. 2006), we assessed the possibility that C14-HSL is produced by RrF4 only during root colonization. In fact, we detected 3-oxo-C14-HSL in RrF4-colonized wheat roots at 7 dpi (Tab. S2).

Next, we wanted to know whether R. radiobacter also produces C14-HSL in the tripartite Sebacinalean symbiosis when it is intricately associated with its fungal host S. indica. To this end, we inoculated wheat roots with fungal chlamydospores. One week later, roots were harvested and analyzed for AHL by UHPLC-qToF-MS. We found that indeed roots inoculated with S. indica also contained higher amounts of 3-oxo-C14-HSL (Tab. S2).

### Table 1

| AHL | Neutral mass [M] | Measured mass [M + Na]$^+$ |
|-----|------------------|-----------------------------|
| N-(3-oxooctanoyl)-l-homoserine lactone | 3-oxo-C8-HSL | 241.1314 | 264.1206 |
| N-(3-hydroxyoctanoyl)-l-homoserine lactone | 3-OH-C8-HSL | 243.1471 | 266.1363 |
| N-(3-oxodecanoyl)-l-homoserine lactone | 3-oxo-C10-HSL | 269.1627 | 292.1519 |
| N-(3-hydroxydecanoyl)-l-homoserine lactone | 3-OH-C10-HSL | 271.1784 | 294.1676 |
| N-dodecanoyl-l-homoserine lactone | C12-HSL | 283.2147 | 306.2042 |
| N-(3-oxododecanoyl)-l-homoserine lactone | 3-oxo-C12-HSL | 297.1941 | 320.1832 |
| N-(3-hydroxydodecanoyl)-l-homoserine lactone | 3-OH-C12-HSL | 299.2097 | 322.1991 |

The AHL-degrading strain R. radiobacter RrF4NM13 is compromised in wheat colonization

To further substantiate our hypothesis that AHLs contribute to the beneficial activity of RrF4 during its interaction with a plant host, we generated the RrF4 mutant strain RrF4NM13 that ectopically expresses the AHL-degrading lactonase gene aiiA driven by an E. coli PBAD promoter, which is inducible with 0.2% arabinose (Wopperer et al. 2006). In contrast to RrF4, the AHL-depleted transconjugant RrF4NM13 did not stain positively with the AHL biosensor bacterium A. tumefaciens A136, indicating that AHLs were degraded by lactonase (Fig. 1). As seen from table S2, wheat roots inoculated with RrF4NM13 also contain less 3-oxo-C14-HSL than roots colonized with either RrF4 or S. indica.

Next, we quantified the bacterial cells in colonized wheat roots. qRT-PCR analysis showed that colonization of wheat cv. Bobwhite roots by RrF4NM13 was significantly (Student’s t test) reduced by 52% (5 dpi), 63% (7 dpi) and 56% (14 dpi) compared to colonization with RrF4 (Fig. 2a), while the empty vector control strain RrF4-ev showed the same colonization than RrF4.

**RrF4NM13 shows an aberrant colonization pattern on wheat roots**

In order to analyze the bacterial colonization pattern in more detail, roots of three-day-old wheat seedlings were inoculated with RrF4 and RrF4NM13 in jars on half MS medium under sterile conditions. SEM analysis of the colonized roots from the 7 dpi timepoint showed that RrF4 cells attached to the root surface with a dense cellulose fiber-like structure (CFS; Fig. 2b), while RrF4NM13...
formed elongated cells, which did not produce CFSs on the root surface (Fig. 2c). TEM analysis of root cross sections corroborated previous finding that \( RrF4 \) propagates in the intercellular space of the cortical root tissue of the root elongation zone (Fig. 2d; see Glaeser et al. 2016). In contrast, \( RrF4NM13 \) did not enter the inner root tissue (Fig. 2e), which could be a result of the altered surface attachment and/or the reduced number of surface colonizers. Light microscopic inspection of liquid cultures furthermore showed that \( RrF4 \) cells were motile, especially in the early exponential growth phase, while \( RrF4NM13 \) cells were impaired in motility (data not shown).

**RrF4NM13 shows reduced plant growth promotion activity**

To assess the effect of bacterial QS on plant growth promotion, roots of three-day-old wheat seedlings were dip-inoculated for 30 min with bacterial suspensions (\( OD_{600} = 1.4 \)) and transferred to soil-sand (3:1). As anticipated from earlier reports (e.g., Glaeser et al. 2016), \( RrF4 \) increased the shoot fresh weight (FW, by 29% at 21 dpi as compared with non-inoculated plants). In contrast, growth promotion activity of \( RrF4NM13 \) was significantly lower (increase in 7% shoot FW at 21 dpi; Fig. S2; ANOVA analysis, \( p < 0.05 \)).
Therefore, we assessed the expression of the JA biosynthesis gene *Allene oxide cyclase 1 (AOC1)*, which is involved in the production of 12-oxo-phytodienoic acid (OPDA), a precursor of jasmonic acid, and the *Plant defensin 1.2 (PDF1.2)* in wheat roots inoculated with bacteria. As anticipated, the expression of both genes as determined by qRT-PCR analysis was significantly (Tukey test; \( p < 0.05 \)) elevated at 3 and/or 7 dpi in response to *RrF4*, but not to *RrF4NM13* (Fig. S3a, b).

Similarly, the ethylene (ET) marker gene *ethylene receptor factor 1* (ERF1) was more strongly induced, confirming that *RrF4* induces a JA/ET response and also indicating that reduced AHL production affects this reaction (Fig. S3c). However, since *RrF4NM13* is impaired in root colonization, it remains unclear at this stage whether the lack of induction of the SA, JA and ET marker genes in the roots is a direct consequence of the reduced amount of AHL or alternatively a consequence of the reduced colonization of the roots by the bacteria.

**RrF4NM13 shows less beneficial activity on Arabidopsis**

We extended our analysis to the Arabidopsis host. Shoot and root fresh weights of *A. thaliana* Col-0 plants were determined 2 weeks after dip-inoculation of roots of seven-day-old seedlings in bacterial inducer suspensions (OD\(_{600} = 1.0\)) and culturing in square petri dishes on half MS medium, containing 0.2% arabinose. *RrF4NM13* slightly increased root fresh weight by 17% over buffer-treated controls (10 mM MgSO\(_4\times 7\)H\(_2\)O), compared to a strong increase in weight of 56% induced by *RrF4* and the positive (empty vector, ev) control *RrF4-ev* (Fig. 4a). Similarly, shoot fresh weight increased slightly, but significantly, (Tukey test; \( p < 0.05 \)) by 12% (*RrF4NM13*) and strongly by 38% (*RrF4; RrF4-ev*), respectively, compared with buffer control (Fig. 4b). Consistent with this, lateral root formation strongly increased in plants pretreated with *RrF4* and *RrF4-ev* at 14 dpi as compared to plants pretreated with *RrF4NM13* and buffer control plants (Fig. 4c).

To further assess the effect of *RrF4NM13* on systemic resistance against a pathogenic bacterium, roots of three-week-old Arabidopsis seedlings were treated in square petri dishes (half MS medium with 0.4% gelrite, including vitamins and 0.2% arabinose) with 100 µL *X. translucens* pv. *syringae* DC3000 (Pst; OD\(_{600} = 0.2\)). Three days later, leaves were spray-inoculated with the virulent bacterial pathogen *P. syringae* pv. *tomato* to determine the effect of *RrF4* on disease symptom development. *RrF4* and *RrF4NM13* suspensions were mock-inoculated with the virulent bacterial pathogen *P. syringae* pv. *tomato* to determine the effect of *RrF4* on disease symptom development. *RrF4* and *RrF4NM13* suspensions were mock-inoculated (Fig. 5), showing that *RrF4NM13*’s ability to induce resistance to *Pst* is weaker.

**Discussion**

We show here that quorum sensing by AHLs contributes as a factor to the beneficial activity of *RrF4*, which was originally isolated from the endophytic sebacinoïd fungus *S. indica*.
Significant for this notion was that AHL-depleted transconjugant RrF4NM13 is partially impaired in root colonization, induces lower systemic resistance in a monocotyledonous (wheat) and a dicotyledonous host plant (Arabidopsis) and shows reduced growth-promoting activity in these hosts. The data presented here substantiate the earlier finding that RrF4 promotes growth of host plants such as barley and Arabidopsis (Sharma et al. 2008; Glaeser et al. 2016). Wheat growth promotion was clearly detectable through an increase in the shoot and root fresh weights in soil-sand mixtures. Sharma et al. (2008) also showed a substantial production of indole-3-acetic acid (IAA), when RrF4 was grown in the presence of tryptophan. Other factors that may affect plant growth induced by RrF4 are siderophores and lipase. In fact, Li (2011) revealed in her dissertation that RrF4 produces siderophores and lipase. Consistent with this report, B. cepacia showed stimulation of growth of maize in iron-poor conditions by siderophore production (Bevivino et al. 1998). Furthermore, P. fluorescens CRPF9 induced a 17-fold increase in siderophore production, which led to an increase in rhizosphere colonization and promotion of growth in mung bean (Katiyar and Goel 2004). Finally, Park et al. (2009) reported that extracellular lipase activity is a property related to the plant growth-promoting effect of P. fluorescens RAF15.
RrF4 is closely related to *A. tumefaciens* (syn. *A. fabrum*) C58, a plant pathogenic strain of the same species. The genomes of both bacterial strains contain genetic components producing and perceiving AHL (White and Winans 2007; Haudecoeur and Faure 2010; Glaeser et al. 2016), suggesting that QS plays a role for both pathogenic and beneficial strains of the plant colonizing bacterium. Interestingly, RrF4 produces a spectrum of quorum sensing molecules of the N-acyl-homoserine lactones with acyl chains of C8, C10 and C12 as well as hydroxyl- or oxo-substitutions at the C3 position. Different studies claimed an effect of AHLs on the interaction between rhizobacteria and their host plants. Of note and consistent with our finding, a study conducted by Kai et al. (2012) showed that the production of AHLs by endofungal bacteria depends on the host fungus, in this case *Mortierella alpina* A-178.

Different AHLs affect root growth and disease resistance against biotrophic and hemibiotrophic pathogens in various plants, including cereals (von Rad et al. 2008; Schikora et al. 2011; Schenk et al. 2012). Consistent with these previous reports, we provide evidence that AHLs produced by RrF4 contribute both to growth-promoting activity and its ability to induce systemic resistance. Similarly, Han et al. (2016) showed that AHL production by *Acidovorax radiicus* N35 have a positive effect on root colonization, while an AHL defective mutant, just as RrF4NM13, showed less root colonization. The positive effect of AHLs on roots colonization could be explained by their effects on biofilm formation. QS in *Burkholderia phytofirmans* ps JN has an important role in biofilm formation and successful colonization of roots (Zuniga et al. 2013). Moreover, application of AHLs could promote the biofilm formation by *Acidovorax* sp. Strain MR-S7 (Kusada et al. 2014). RrF4NM13’s inability to produce cellulose-like fibers suggests a functional role of AHL production for the induction of biofilm formation at the surface of wheat roots. Consistent with this, cellulose fibers are known to be important for the attachment of *A. tumefaciens* cells to root surfaces (Matthysse 1983, 1987) and also for RrF4 (Glaeser et al. 2016).

Mathesius et al. (2003) reported that more than 150 proteins accumulated in response to C12- to C16-HSLs in roots of *Medicago truncatula*. Moreover, C10-HSL and C12-HSL induced secondary root and root hair formation, respectively (Ortiz-Castro et al. 2008; Von Rad et al. 2008; Schikora et al. 2011). Ortiz-Castro et al. (2008) demonstrated that C10-HSL modifies the root system architecture by inducing the expression of genes related to cell division and affects the plant enzymatic machinery to metabolize AHLs molecules. A recent study on C6-, C8- and C10-HSLs conducted on barley revealed that these AHLs influence the activity of specific detoxification enzymes, namely glutathione S-transferase and dehydroascorbate reductase in barley (Götz-Rösch et al. 2015).

We also show here that AHLs produced by RrF4 are involved in systemic resistance to pathogenic leaf bacteria *X. translucens* and *P. syringae* as revealed by attenuated resistance-inducing activity exerted by RrF4NM13 on wheat and Arabidopsis. In accordance with these findings, C6-HSL-producing *Serratia liquefaciens* MG1 can induce systemic resistance in tomato against the fungal leaf pathogen *Alternaria alternata* (Schuhhegger et al. 2006), and this effect is attenuated in the AHL-negative mutant *S. liquefaciens* MG44. Furthermore, the resistance induced by *Ensifer meliloti* (*Sinorhizobium meliloti*) against *P. syringae* in Arabidopsis plants was dependent on AHL accumulation (Zarkani et al. 2013). *E. meliloti*, which produces the long chain oxo-C14-HSL, also enhances defense responses against the fungal pathogen *Blumeria graminis* f. sp. *hordei* and against the oomycete *Phytophthora infestans* in barley and tomato plants, respectively (Hernández-Reyes et al. 2014). Interestingly, some plants produce QS substances that mimic N-acyl-homoserine lactones and thus interfere with the communication of pathogenic bacteria, a phenomenon called quorum quenching (Gao et al. 2003; Bauer and Mathesius 2004; Teplitski et al. 2010, Pérez-Montaño et al. 2013; Corral-Lugo et al. 2016). On the other hand, plant exudates can have a positive effect on the synthesis of AHLs (Schafer et al. 2008). The plant-associated bacterium *Rhodopseudomonas palustris* requires the plant-secreted precursor *p*-coumarate for the production of its signaling compound *N*-(*p*-coumaroyl)-*t*-homoserine lactone. In the same line, flavonoids released by legumes increase the expression of AHL synthesis genes in *Rhizobium* (Perez-Montano et al. 2011). Consistent with this, Li (2011) reported that the production of *p*-coumaroyl-HSL in RrF4 requires plant-secreted *p*-coumaric acid as a source, suggesting a strong relationship between RrF4 and its host plant.

**Conclusion**

We have shown that RrF4 requires AHLs to enter and maintain a mutualistic symbiosis with a monocotyledonous (wheat) and dicotyledonous (Arabidopsis) host plant. Evidence for an important role of AHLs in the natural *Sebacinae* tripartite symbiosis of *R. radiobacter* with *S. indica* and a host plant arises from observations that plant roots colonized with *S. indica* (which always contains *R. radiobacter* as an endobacterium) also accumulate AHLs. Since it has not been possible to cure *S. indica* from the endobacterium to date (Sharma et al. 2008; Glaeser et al. 2016; Guo et al. 2017), the fascinating question remains whether the fungus necessarily needs the endobacteria and the synthesis of AHLs to establish and/or maintain a successful symbiosis.
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Author’s contribution KH, JI, SG, AH and IA designed experiments and analyzed data; MH conducted TEM and SEM; MR, DL, PSK and JU designed, conducted and analyzed experiments on AHL detection, KH, IA, SG and AH wrote the manuscript.

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Data availability All data generated or analyzed during this study are included in this published article [and its supplementary information files].

Compliance with ethical standards

Conflict of interest The research described in the manuscript was not funded by private partners or industry. Ibrahim Alabid declares that he has no conflict of interest. Martin Hardt declares that he has no conflict of interest. Jafargholi Imani declares that he has no conflict of interest. Anton Hartmann declares that he has no conflict of interest. Michael Rothballer declares that he has no conflict of interest. Dan Li declares that he has no conflict of interest. Stefanie Glaeser declares that she has no conflict of interest. Karl-Heinz Kogel declares that he has no conflict of interest.

Ethics approval and consent to participate Approved by all authors.

Consent for publication All authors declare consent of publication.

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