

## ORIGINAL ARTICLE

# Non-pathogenic *Rhizobium radiobacter* F4 deploys plant beneficial activity independent of its host *Piriformospora indica*

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The Alphaproteobacterium *Rhizobium radiobacter* F4 (*RrF4*) was originally characterized as an endofungal bacterium in the beneficial endophytic Sebacinalean fungus *Piriformospora indica*. Although attempts to cure *P. indica* from *RrF4* repeatedly failed, the bacterium can easily be grown in pure culture. Here, we report on *RrF4*'s genome and the beneficial impact the free-living bacterium has on plants. In contrast to other endofungal bacteria, the genome size of *RrF4* is not reduced. Instead, it shows a high degree of similarity to the plant pathogenic *R. radiobacter* (formerly: *Agrobacterium tumefaciens*) C58, except vibrant differences in both the tumor-inducing (pTi) and the accessor (pAt) plasmids, which can explain the loss of *RrF4*'s pathogenicity. Similar to its fungal host, *RrF4* colonizes plant roots without host preference and forms aggregates of attached cells and dense biofilms at the root surface of maturation zones. *RrF4*-colonized plants show increased biomass and enhanced resistance against bacterial leaf pathogens. Mutational analysis showed that, similar to *P. indica*, resistance mediated by *RrF4* was dependent on the plant's jasmonate-based induced systemic resistance (ISR) pathway. Consistent with this, *RrF4*- and *P. indica*-induced pattern of defense gene expression were similar. In clear contrast to *P. indica*, but similar to plant growth-promoting rhizobacteria, *RrF4* colonized not only the root outer cortex but also spread beyond the endodermis into the stele. On the basis of our findings, *RrF4* is an efficient plant growth-promoting bacterium.

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## Introduction

Although soil-borne beneficial microbes are prevalent, their phylogenetic, spatial and functional

diversity is widely unresolved (Berg *et al.*, 2014). Complex symbiotic interactions including bacteria, fungi and their host plants are especially little understood, and their global prevalence is widely unknown (Lackner *et al.*, 2009; Hoffman and Arnold 2010; Naumann *et al.*, 2010). To address this issue, we have been studying the tripartite Sebacinalean symbiosis, comprising fungi of the order *Sebaciales* (Basidiomycota), phylogenetically diverse endofungal bacteria and a broad range of monocotyledonous and dicotyledonous host plants (for review, see Qiang *et al.*, 2012a). A first analysis of the distribution on a world-wide scale claimed that Sebacinalean symbioses are prevalent in all continents (Weiss *et al.*, 2011; Riess *et al.*, 2014) potentially making them a vital part of global soil ecosystems. Although the molecular mechanisms by which the Sebacinalean symbiosis is established and by which the plant benefits from its fungal partners is largely understood, the role of the endofungal bacteria is still

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unclear (Peškan-Berghöfer *et al.*, 2004; Waller *et al.*, 2005; Deshmukh *et al.*, 2006; Camehl *et al.*, 2010; Zuccaro *et al.*, 2011; Qiang *et al.*, 2012b).

The root endophyte *Piriformospora indica* (syn. *Serendipita indica*) is a model fungus of the *Serendipitaceae* of the *Sebacinales* (Oberwinkler *et al.*, 2014). Since its discovery in the Indian Thar dessert in 1996 (Varma *et al.*, 1999), *P. indica* and related *Sebacina vermifera* strains were shown to promote biomass, yield and health of a broad spectrum of plants (Varma *et al.*, 2012; Ye *et al.*, 2014). Genetic and biochemical assessments of the resistance mechanism that is induced by *P. indica* in *Arabidopsis thaliana* (*Arabidopsis*) against a wide spectrum of leaf and root pathogens showed a requirement of jasmonate synthesis and signaling and thus an operable induced systemic resistance (ISR) pathway (Stein *et al.*, 2008; Jacobs *et al.*, 2011).

In 2008, Sharma and coworkers (Sharma *et al.*, 2008) reported for the first time that members of the *Serendipitaceae* regularly undergo complex symbioses involving plants and endofungal bacteria of different genera. Endofungal bacteria were previously detected in Glomeromycotan arbuscular mycorrhiza symbioses (Bonfante and Anca, 2009; Naumann *et al.*, 2010), in the ectomycorrhizal fungus *Laccaria bicolor* (Bertaux *et al.*, 2003, 2005), in the rice pathogenic fungus *Rhizopus microsporus* (Partida-Martinez and Hertweck, 2005), in hyphae of phylogenetically diverse foliar fungal endophytes (Hoffman and Arnold, 2010), in the soil isolate *Mortierella alpina* (Kai *et al.*, 2012) and the plant symbiotic Endogone *Mucoromycotina* fungi (Desirò *et al.*, 2015). Endofungal bacteria, associated with fungi of the genera *Piriformospora* and *Sebacina*, belong to two genera of Gram-negative (*Rhizobium* and *Acinetobacter*) and two genera of Gram-positive (*Paenibacillus* and *Rhodococcus*) bacteria. The most comprehensively studied example of a tripartite Sebacinalean symbiosis is the association of *P. indica* with the Alphaproteobacterium *Rhizobium radiobacter* (syn. *Agrobacterium radiobacter*; syn. *Agrobacterium tumefaciens*) strain F4 (*RrF4*). Fluorescence *in situ* hybridization using a *Rhizobium*-specific probe confirmed the stable endocellular association of small numbers of *RrF4* cells within *P. indica* chlamydospores and hyphae, and a ratio of 0.035 ng of bacterial DNA per 100 ng of *P. indica* DNA was determined by quantitative PCR (qPCR) analysis (Sharma *et al.*, 2008). This result coincides well with the low number of different bacteria (1–20 per fungal cell) that were detected in the ectomycorrhiza fungus *Laccaria bicolor* (Bertaux *et al.*, 2003; Bertaux *et al.*, 2005). Intriguingly, *RrF4* could be isolated from powdered fungal mycelia and propagated in axenic cultures, showing that the bacterium is not entirely dependent on its fungal host. However, various attempts to stably cure *P. indica* of its resident bacterial cells repeatedly failed, raising the possibility that *RrF4* is essential for *P. indica* survival. Consistent with an intricate

association, no other bacteria have been isolated from various laboratory cultures of *P. indica*.

In an attempt to further elucidate the plant growth-promoting function of the endofungal *RrF4*, its genome was sequenced and compared with closely related non-endofungal *R. radiobacter* and other related *Agrobacterium* strains. We also show that *RrF4*'s root colonization pattern and beneficial activity are widely reminiscent of those induced by its fungal host *P. indica*. Our data suggest that the ubiquitous Sebacinalean symbiosis is a novel source for beneficial bacteria potentially useful for agronomic applications.

## Materials and methods

### *RrF4* strain

Experiments were performed with *Rhizobium radiobacter* (syn. *Agrobacterium radiobacter*; syn. *Agrobacterium tumefaciens*) F4 (*RrF4*), a subculture of strain PABac-DSM isolated from *P. indica* DSM 11827 (Sharma *et al.*, 2008). Generation of *RrF4* strains expressing  $\beta$ -glucuronidase (GUS) and green fluorescent protein (GFP) is described in the Supplementary Materials and Methods.

### *RrF4* genome sequence analysis

Genomic DNA of strain *RrF4* was isolated and shotgun libraries were prepared for sequencing. Emulsion PCR, emulsion breaking of DNA-enriched beads and sequencing of the shotgun libraries were performed on a second-generation pyrosequencer (454 GS FLX Titanium, Roche, Mannheim, Germany) using Titanium reagents and Titanium procedures as recommended by the developer following protocols for shotgun sequencing.

Quality filtering of the pyrosequencing reads was performed using the automatic standard signal processing pipeline of the GS Run Processor (Roche) to remove failed and low-quality reads from raw data and to remove adaptor sequences.

The initial assembly of the data from 454 pyrosequencing was performed using the GS FLX Newbler software 2.0.01 (Roche) with a minimum overlap length of 40 bp and a minimum overlap identity of 90% followed by a comparative alignment to the genome of *R. radiobacter* (syn. *Agrobacterium tumefaciens*, *A. radiobacter*) C58, which finally resulted in four distinct contigs. The data were uploaded into GenDB (Meyer *et al.*, 2003) and subjected to an automatic annotation. Blast ring images were generated using BRIG (Alikhan *et al.*, 2011). Genome comparisons were performed in EDGAR (Blom *et al.*, 2009). This Whole Genome Shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession JZLL00000000. The version described in this paper is version JZLL01000000.

### Plant material and growth conditions

*Arabidopsis thaliana* ecotype Columbia-0 (Col-0, N1092) and the following mutants were obtained from the Nottingham Arabidopsis Stock Centre (NASC): *npr1-1* (Cao *et al.*, 1994), *npr1-3*, *jar1-1* (Staswick *et al.*, 1992), *ein2-1* (Guzman and Ecker, 1990), *jin1* (Berger *et al.*, 1996) and *NahG* (Gaffney *et al.*, 1993). Seeds were germinated on half strength MS medium supplemented with 0.5% (w/v) sucrose and solidified with 0.4% (w/v) gelling agent (Gelrite, Duchefa, BH Haarlem, The Netherlands) under short day conditions (8 h light at 22 °C/18 °C (day/night) and a photon flux density of 183  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) in 100 mm square Petri dishes. Alternatively, plants were grown in pots containing soil (Archut Fruhstorfer Erde, Type P, HAWITA Gruppe, Lauterbach, Germany) or a vermiculite ( $\varnothing$  0–3 mm, Deutsche Vermiculite Dämmstoffe GmbH, Sprockhövel, Germany)—sand mixture (3:1).

Seeds of wheat cv. Bobwhite and barley cv. Golden Promise (GP) were germinated on sterilized wet filter paper in sterile glass jars for 3 days. Plants were grown in a growth chamber at 22 °C/18 °C (day/night cycle), with 60% relative humidity, and a photoperiod of 16 h (240  $\mu\text{mol m}^{-2} \text{s}^{-1}$  photon flux density). Before germination, all seeds were surface-sterilized with ethanol 70% (v/v) for 3 min (wheat/barley) or 1 min (Arabidopsis) and 25% sodium hypochlorite for 90 min (wheat/barley) or 10 min (Arabidopsis) under continuous shaking, washed once with sterilized water (pH 3) and rinsed four times with sterile distilled water.

### Inoculation of root with *RrF4*

*RrF4* was grown overnight in modified LB broth (1% casamino hydrolysate, 0.5% yeast extract and 0.5% NaCl, pH 7.0 supplemented with 100  $\mu\text{g ml}^{-1}$  gentamycin) at 28 °C and 150 r.p.m. GUS- and GFP-expressing *RrF4* were cultured in the presence of 100  $\mu\text{g ml}^{-1}$  spectinomycin. Bacterial cells were collected by centrifugation (3202 g, 10 min), washed and resuspended in 10 mM  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  buffer. Roots of 3-day-old barley/wheat seedlings or 7-day-old Arabidopsis seedlings, respectively, were dip-inoculated for 30 min in *RrF4* suspensions ( $\text{OD}_{600} = 1.0\text{--}1.4$ ). Control seedlings were dipped into 10 mM  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ .

### Quantification of *RrF4* in roots

The relative abundance of *RrF4* in root tissues was quantified by qPCR targeting the internal transcribed spacer (ITS) between the 16S rRNA and 23S rRNA genes of the ribosomal RNA operon of *RrF4* (Sharma *et al.*, 2008; see Supplementary Table S1 and Supplementary Materials and Methods).

### Visualization of root colonization by *RrF4*

The colonization of plant roots was visualized using GUS- and GFP-expressing *RrF4* strains combined

with light-, epifluorescence- and confocal laser scanning microscopy. Root cross-sections also were analyzed by transmission electron microscopy (TEM) (see Supplementary Materials and Methods).

### Gene expression analysis in roots

Roots of 3-day-old barley seedlings were dip-inoculated with a solution of *RrF4* ( $\text{OD}_{600} = 1.4$ ) for 30 min and with a fungal spore solution (500 000-spore per ml) for 1.5 h. Seedlings were transferred to pots containing a 2:1 mixture of expanded clay (Seramis, Masterfoods, Verden, Germany) and Oil-Dri (Damolin, Mettmann, Germany) under long day condition. Fertilization was carried out weekly with 20 ml of a 0.1% WUXAL top N solution (N/P/K: 12/4/6; Aglukon, Düsseldorf, Germany) per pot containing one plant. Total RNA extraction, cDNA synthesis and qPCR analysis were performed according to Jacobs *et al.* (2011).

### Plant infection with *Pseudomonas*

*Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*; Preston, 2000) was obtained from Dr N. Schlaich, RWTH Aachen, Germany. *Pst* was grown on liquid King's B medium (King *et al.*, 1954) supplemented with 50  $\mu\text{g ml}^{-1}$  rifampicin and 50  $\mu\text{g ml}^{-1}$  kanamycin overnight at 28 °C. Bacterial cells were collected by centrifugation (3202 g; 10 min), washed and suspended in 10 mM  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  containing 0.02% Silwet L-77 to a concentration of  $10^8$  colony-forming units (CFU) per ml ( $\text{OD}_{600} = 0.2$ ).

For the analysis of induced resistance, roots of 2-week-old Arabidopsis plants were dip-inoculated with *RrF4* ( $\text{OD}_{600} = 1$ ) for 30 min and grown in soil. After 2 weeks, leaves were sprayed with *Pst* (0.5 ml bacterial solution on each plant). One day before challenge inoculation, plants were placed in transparent boxes with closed lids to increase the relative humidity for optimal infection. Gene expression values were normalized to the housekeeping gene *AtUBQ5-4* values using the  $2^{-\Delta\text{Ct}}$  method (Schmittgen and Livak, 2008). Primers used in this study are listed in Supplementary Table S1.

### Statistical analysis

Statistical analyses were performed in SigmaPlot 12 (Systat Software) using one-way analysis of variance or Student's *t*-tests after data were tested for normality distribution (Shapiro-Wilk test) and equal variance.

## Results and Discussion

### Comparative genome analysis and phylogenetic placement of *RrF4*

The genome of *RrF4* is organized in a circular (2.8 Mbp) and a linear chromosome (2.06 Mbp), a tumor-inducing plasmid pTiF4 (0.21 Mbp), and an



accessory plasmid pAtF4 (0.54 Mbp) in the same manner as *R. radiobacter* C58 (formerly: *A. tumefaciens* C58; *Agrobacterium* biovar I, genomovar G8) (Table 1; Goodner *et al.*, 2001). This unusual genome structure, containing a linear chromosome, has been observed only for *Agrobacterium* biovar I strains (Slater *et al.*, 2013). A phylogenetic tree calculated based on the core genome of *RrF4* and *Agrobacterium* biovar I, II and III reference strains (Supplementary Figure S1a) and average amino acid identity analyses (Supplementary Figure S1b) showed the assignment of *RrF4* to biovar I strains with very close relationship to C58 (amino acid identity=99.8%). The presence of all genomovar G8-specific gene cluster defined by Lassalle *et al.* (2011) indicated the assignment of *RrF4* genomovar G8 in the same manner as C58 though some difference in C58-specific gene cluster indicated genetic distinction of both strains (Supplementary Table S2).

Comparison of the gene content of the circular chromosome of *RrF4* and the four biovar I reference strains showed that *RrF4* shared 87.3% of its genes (2369 of 2713 genes) with all biovar I strains, but 94.5% with strain C58 (2563 of 2713 genes), among those 163 genes (6.4% of the total genes) were only

shared with C58 (Figure 1a). Moreover, 73.9% (1356 of 1834 genes) of the genes from the linear chromosome were shared with all biovar I strains but 98.2% (1702 of 1834 genes) with C58, among those 305 genes (17.9% of the total genes) were only shared with C58 (Figure 1b). Several of the genes encoded by the circular and linear chromosome of C58 and *RrF4* are relevant not only for pathogenicity but also for the plant-microbe interaction in general (Goodner *et al.*, 2001; Heindl *et al.*, 2014). Among those, we detected homologs that were reported to be involved in the interaction of plant growth-promoting bacteria with host plants (Supplementary Table S3). In contrast to other, obligate endofungal bacteria that live in symbiosis with their fungal hosts, *RrF4* does not have a reduced genome (Lackner *et al.*, 2011; Ghignone *et al.*, 2012; Fujimura *et al.*, 2014; Torres-Cortés *et al.*, 2015; Naito *et al.*, 2015), which may indicate a facultative symbiosis of *RrF4* with *P. indica*. The high similarity (in size and gene content) of the chromosomal genomes of *RrF4* and C58 indicated that *RrF4* did not lose any essential genes or partial genetic pathways. The circular and linear chromosomes of *RrF4* had 100 and 80 singleton open reading frames, respectively, not present in C58. Most of these open reading frames were of unknown function and may be candidates for future studies to elucidate a potential role for the endofungal growth of *RrF4* (Supplementary Table S1) and/or fitness of its fungal partner *P. indica*.

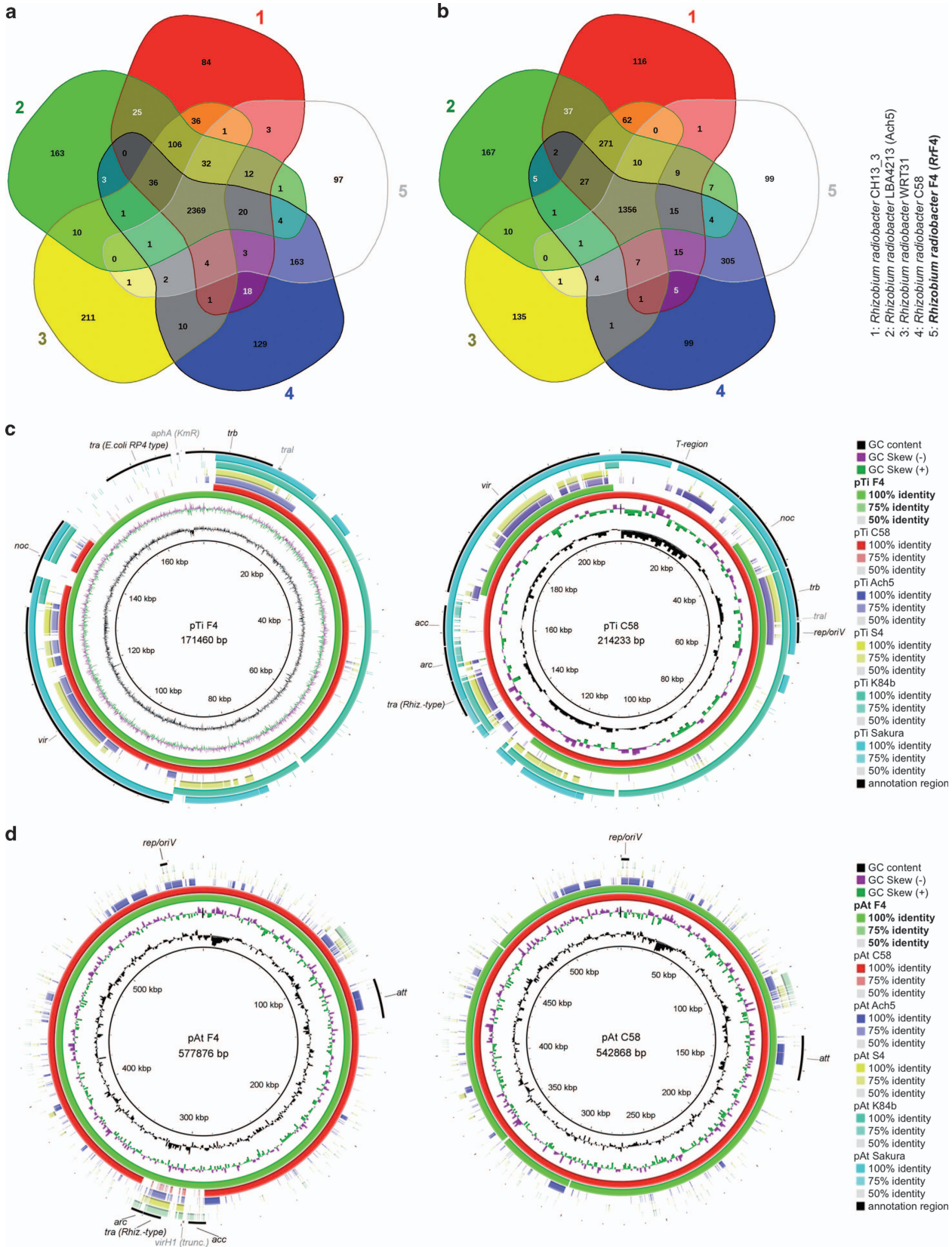
Whereas *RrF4* and C58 showed a high degree of similarity based on the circular and linear chromosomes, the plasmids were more diverse (Figures 1c and d). These differences partly stem from gene translocations in *RrF4* from the pTi to pAt plasmid, partly from unique sequences in both strains (Table 1). The *acc* operon, responsible for agrocinopine A+B uptake and catabolism, the *arc* operon for agrocinopine regulation of conjugation, a truncated version of the *virH1* gene and the *tra* operon described by Piper *et al.* (1999) were translocated in *RrF4* from plasmid pTi to pAt. The latter one also includes the quorum sensing regulator gene *traR* but not the AHL synthase *traI*, which remains upstream of the *trb* operon on pTi in both strains. Furthermore, *RrF4* harbors an additional set of *tra* genes on the pTiF4 plasmid which shows highest similarity to the conjugation genes on the RP4 plasmid of *Escherichia coli*. Most interestingly, in contrast to C58, *RrF4* lacks the complete transfer

**Table 1** Comparison of chromosome and plasmid sizes and GC contents of the genomes of *RrF4* and C58 and comparison of the location and presence of gene regions on the two plasmids of *RrF4* and C58

	Circ. chr.	Lin. chr.	pTi	pAt
<i>Size and G+C content of chromosomes and plasmids</i>				
<i>RrF4</i> size (Mbp)	2.80	2.06	0.17	0.58
C58 size (Mbp)	2.84	2.08	0.21	0.54
<i>RrF4</i> GC content (%)	59.41	59.32	58.63	57.42
C58 GC content (%)	59.38	59.28	56.67	57.33
<i>Feature</i>	<i>RrF4</i>		<i>C58</i>	
<i>Localization of gene regions on the pAt and pTi plasmids</i>				
T-region (incl. ipt)	missing		pTi	
<i>Acc</i>	pAt		pTi	
<i>arc</i> (incl. traR)	pAt		pTi	
<i>tra</i> ( <i>Rhiz.</i> -type)	pAt		pTi	
<i>tra</i> ( <i>E. coli</i> pRP4) type)	pTi		missing	
<i>virH1</i>	pAt (truncated by 200 bp)		pTi	

Abbreviations: Circ. chr., circular chromosome; Lin. chr., linear chromosome. Genome sequence of C58 was obtained from GenBank of NCBI (Genome Acc. number: AE007869.2-AE007872.2).

**Figure 1** Venn diagrams showing shared and unique genes of *RrF4* and biovar I reference strains. (a) Circular chromosome, (b) linear chromosome. (c, d) BRIG-comparison of different *Rhizobium* pTi and pAt plasmids. The name of the reference plasmid sequence is given in the center of the rings, the concentric circles show the similarities of the compared plasmids as explained in the legend on the right of every picture. *acc* = agrocinopine A+B uptake and catabolism operon; *arc* = agrocinopine regulation of conjugation operon; *noc* = nopaline catabolism region; T-region = transfer T-DNA region at plant infection; *tra* (*Rhizobium*-type) = genes for conjugation/DNA metabolism homolog to *Rhizobium* spp.; *tra* (*E. coli* RP4 type) = genes for conjugation/DNA metabolism homolog to *E. coli* RP4 plasmid; *trb* = operon for plasmid transfer (mating pair formation), incl. *traI*; *vir* = virulence gene cluster; *aphA* = Kanamycin resistance gene; *rep/oriV* = replication genes *repABC*; *att* = gene cluster required for attachment to plant cells. Acc. numbers of reference genomes are listed in the legend of Supplementary Figure S1.



DNA region and some adjacent genes belonging to the nopalinal catabolic (*noc*) region. It is hard to speculate which role the additional *E. coli*-like *tra* genes might have for the non-pathogenic *RrF4*. While the ‘*Rhizobium*-like’ *tra* genes are usually involved in the transfer of the T-region to the infected plant cells and are regulated by a *luxI/luxR* type quorum sensing system (Fuqua and Winans, 1994), the *E. coli tra* genes are required for bacterial conjugation (Frost *et al.*, 1994).

Although it shares nearly identical circular and linear chromosomes with the pathogenic *A. tumefaciens* C58, *RrF4* is non-pathogenic. As shown previously, and consistent with our finding, curing C58 from its pTi plasmid results in a non-pathogenic strain with albeit weak plant growth-stimulating activity (Walker *et al.*, 2013). Nevertheless, at present, we do not know all the factors needed by *RrF4* to associate with its fungal host *P. indica*. Lackner *et al.* (2011) denoted the intrahyphal *Burkholderia rhizoxinica* as a bacterium with a ‘genome in transition’ because it showed to some extent a reduced genome size compared with free-living *Burkholderia* species which was not as strongly reduced as found in several other endobacteria. Although *RrF4* did not show a reduced genome size, changes in the structure and gene content of both, the pTi and pAt plasmids may hint to an adaptation to a specific ecological niche. The gene content alone, however, will not be sufficient to explain the endofungal lifestyle of *RrF4*; further studies must elucidate the molecular communication in the tripartite association between bacterium, fungus and plant.

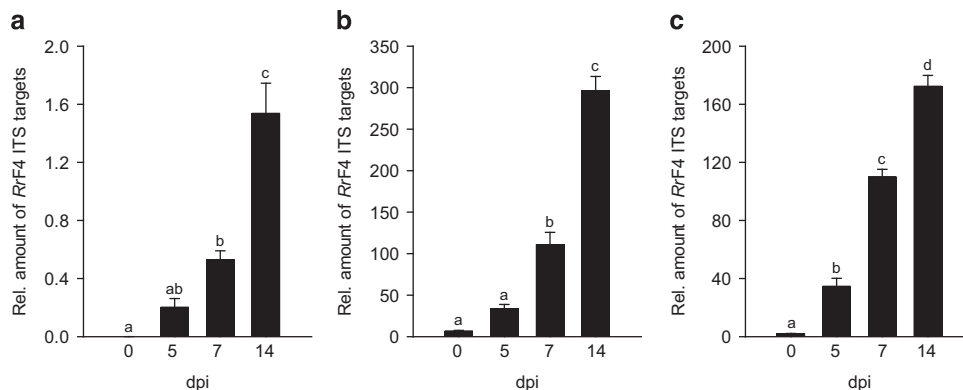
#### *RrF4* promotes plant growth

Treatment of barley roots with free-living *RrF4* increased plant biomass and powdery mildew resistance (Sharma *et al.*, 2008). To further expand this finding, we assessed the potential of *RrF4* to

mediate growth promotion in *Arabidopsis*. To this end, roots of 7-day-old seedlings were dip-inoculated with *RrF4* ( $OD_{600} = 1.0$ ) and subsequently transferred to the three different growth substrates soil-sand mixtures (3:1), vermiculite-sand mixtures (3:1) and half strength MS medium. Two to three weeks after inoculation, plants showed a significant increase in shoot and root fresh weight (FW), and strongly accelerated lateral root formation, regardless of the substrate in which they were grown (Supplementary Figures S2a–f). This finding adds another level of complexity to the yet controversial discussion as to whether *P. indica* induces biomass formation in *Brassicaceae* (Lahrman *et al.*, 2013). Our data doubtless show that the bacterial partner of *P. indica* is able to strongly induce growth promotion in *Arabidopsis*.

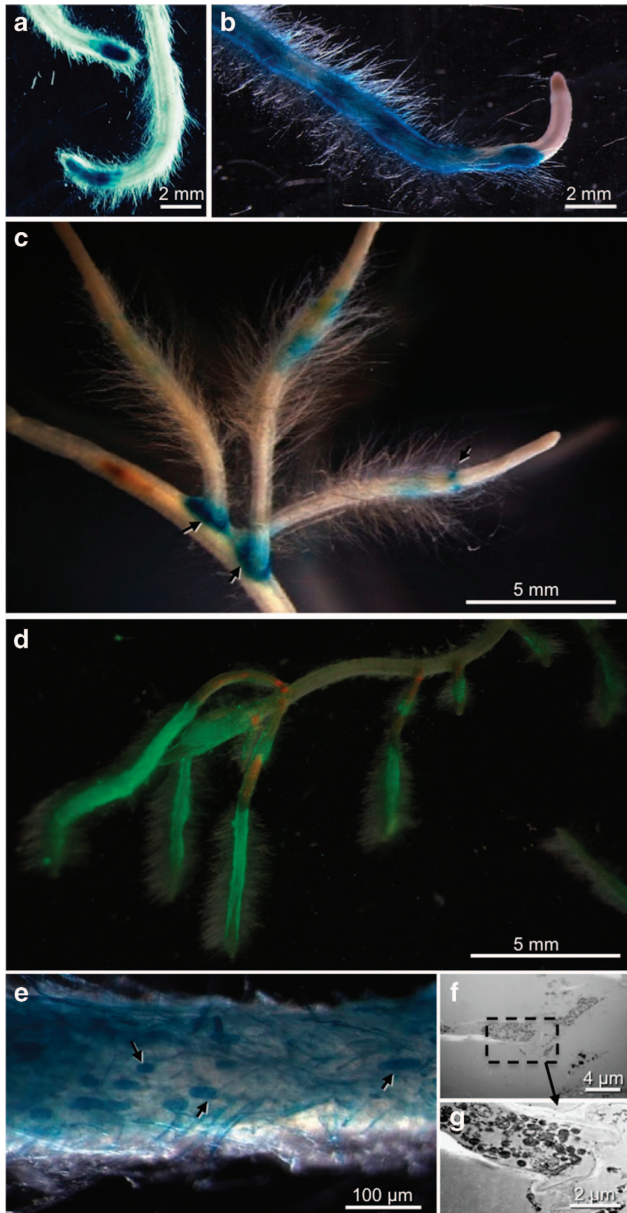
#### *RrF4* colonizes roots and proliferates independent of its fungal host

We asked whether free-living *RrF4* can colonize plant roots and multiply independently of its fungal host. Because axenically grown plants are most suitable for bacterial quantification and microscopy, *Arabidopsis* seedlings grown on half strength MS medium were used for this analysis. At various time points after dip-inoculation, root material was collected and *RrF4* was quantified by qPCR targeting the ITS of the ribosomal RNA operon. Before DNA extraction, roots were sequentially washed in 70% ethanol and distilled water and sonicated, to remove excess of bacteria from the surface. Upon this treatment (30 min after dip-inoculation = 0 days post inoculation (dpi)), the amount of *RrF4* cells that remained attached to the roots was below the detection limit. During 2 weeks, *RrF4* cells proliferated in the apical 4 cm of the roots as indicated by a significant increase in *RrF4* ITS targets relative to plant *ubiquitin* (Figure 2a). Consistent with an increase in the relative abundance, the absolute



**Figure 2** Proliferation of *RrF4* in roots of *Arabidopsis* (a), barley (b) and wheat (c). Roots of 7-day-old seedlings (*Arabidopsis*) or 3-day-old seedlings (barley, wheat) were dip-inoculated with *RrF4*. Genomic DNA was extracted at the indicated time point and *RrF4* ITS targets quantified by qPCR relative to *Arabidopsis* or barley *ubiquitin* or wheat *alpha tubulin*, respectively. Error bars indicate standard errors based on three independent biological replicates. Different letters on top of the bars indicate statistically different differences tested by one-way analysis of variance (all pairwise multiple comparison) performed with the Tukey test ( $P < 0.05$ ).





**Figure 3** Localization of GUS- and GFP-expressing *RrF4* in barley roots. GUS-expressing *RrF4* on a primary root at 5 (a) and 14 dpi (b), and on lateral protrusions and hair zones of secondary roots at 21 dpi (c). GFP-expressing *RrF4* on hair zones (30 dpi) visualized by epifluorescence microscopy (d). (e–g) Single (dead) rhizodermal cells colonized by GUS-tagged *RrF4* at 21 dpi. Light microscopy (e) and TEM (f, g) of GUS-tagged *RrF4*. Dark blue stained plant cells (arrows) are densely packed with bacteria. Dashed box: area heightened in (f); arrows: bacterial cells.

amounts of *RrF4* cells per gram root FW increased (Supplementary Table S4). At 14 dpi, bacterial cell numbers reached  $2.9 (\pm 0.5) \times 10^9$  cells per gram Arabidopsis root FW.

In parallel, the propagation of *RrF4* was assessed in roots of barley and wheat seedlings (Figures 2b and c). Higher ratio values for ITS targets vs root gene targets suggests a more intense bacterial colonization of these graminaceous plants. In

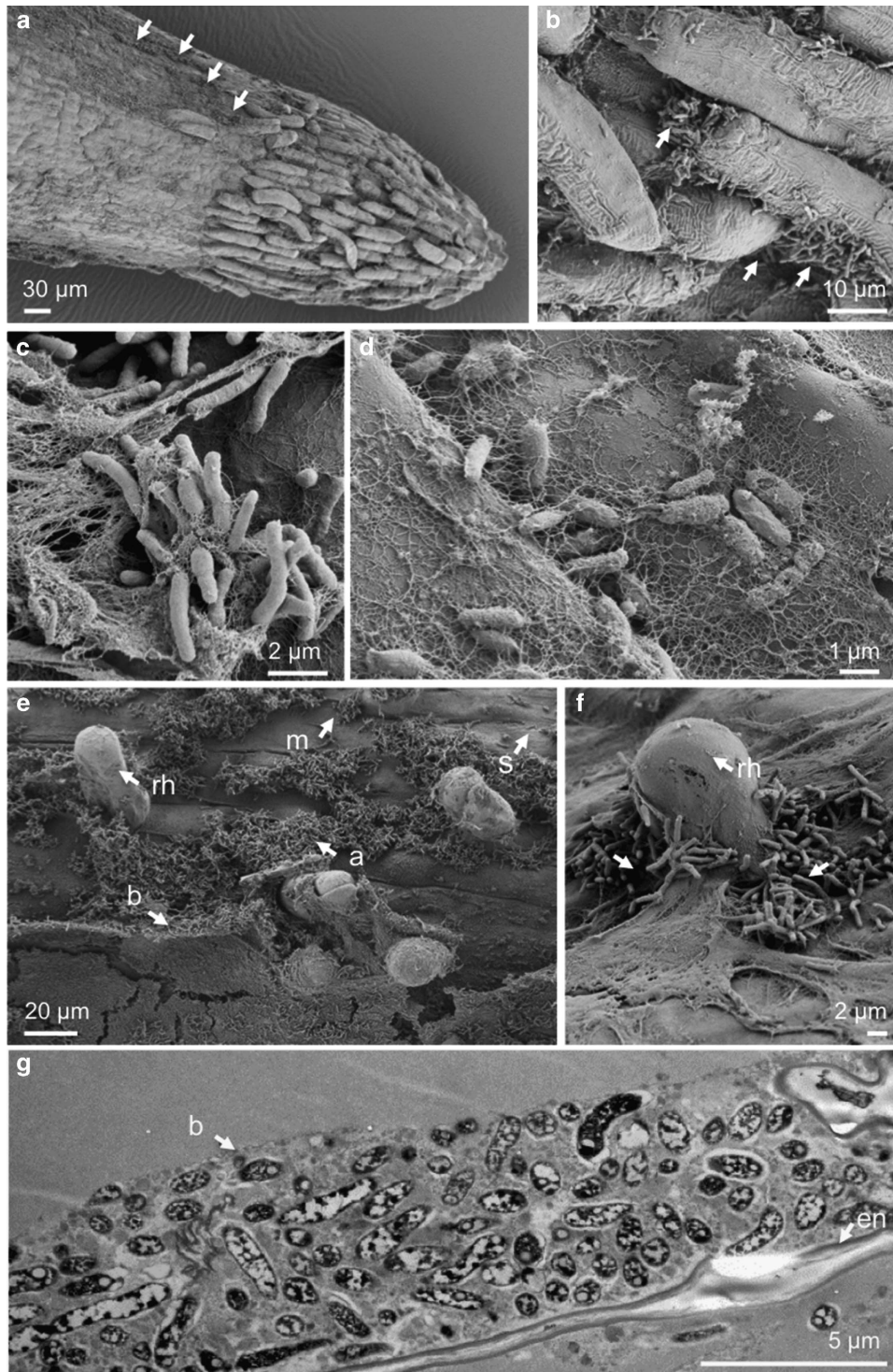
contrast to *Arabidopsis*, relatively high amounts of *RrF4* cells remained attached to the root surface after dip-inoculation (0 dpi; barley:  $2.1 (\pm 0.4) \times 10^8$ ; wheat:  $3.3 (\pm 2.0) \times 10^8$  cells per gram FW of roots (Supplementary Table S4)), which may indicate that *RrF4* bacteria interact more efficaciously with the surface of graminaceous plants. At 14 dpi, bacterial cell numbers reached  $8.1 (\pm 1.4) \times 10^9$  (barley) and  $6.0 (\pm 2.5) \times 10^9$  (wheat) per gram root FW, suggesting that *RrF4* interacts and multiplies in graminaceous plants with high efficiency.

#### *RrF4* multiplies at the root surface

Next, colonization of barley roots was assessed microscopically with GUS- and GFP-tagged bacteria (Figure 3). At 5 dpi, GUS-expressing *RrF4* cells were seen in the maturation zone I of primary roots in an area covering approximately 1 cm in length (Figure 3a). At later time points (14 dpi), bacteria had spread into the maturation zone II while the elongation and meristematic zones as well as the root cap remained virtually free of bacteria (Figure 3b). This colonization pattern conspicuously resembles the root colonization pattern of *P. indica* (Deshmukh et al., 2006). Microscopy also revealed a distinct pattern of dark and bright blue staining in the root hair zone suggesting specific sites of higher *RrF4* proliferation at the root surface (Figure 3b). At later time points, root hair zones of lateral roots also were colonized with the same pattern as in primary roots. Bacterial conglomerates were particularly present at lateral root protrusions (Figure 3c) that probably serve as entry sites. Inoculation of roots with GFP-tagged *RrF4* cells confirmed this pattern. Microscopic assessment at 30 dpi revealed strong bacterial colonization of primary and secondary roots (Figure 3d). Inoculation of wheat and Arabidopsis roots with *RrF4* virtually showed the same colonization pattern (Supplementary Figure S3) and accordingly resembled the pattern observed with *P. indica* (Supplementary Figure S4; also see Jacobs et al., 2011, Figure 1).

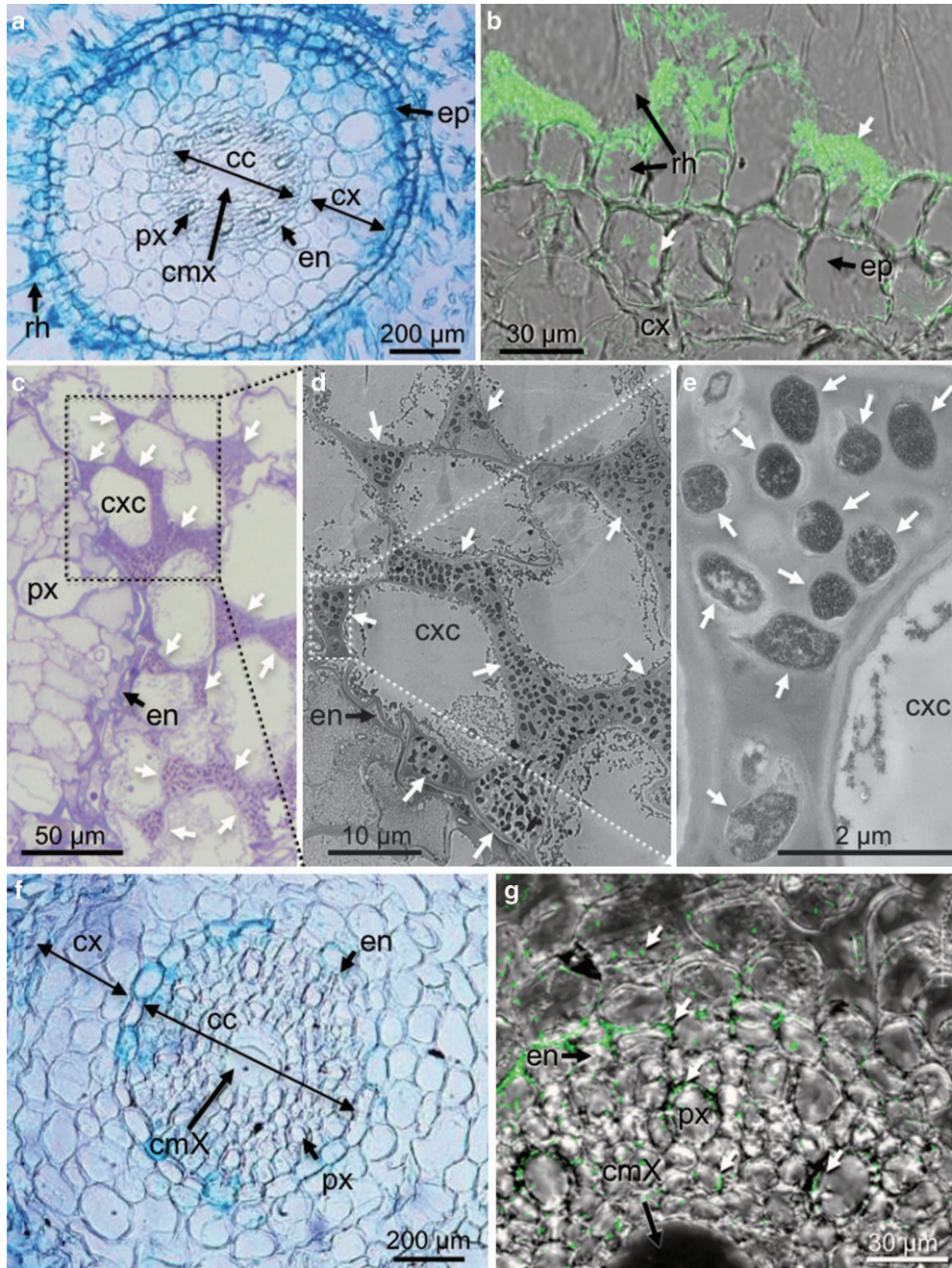
GUS staining showed single dark blue GUS-stained rhizodermal cells suggesting that these cells were heavily colonized by *RrF4* (Figure 3e), which was further confirmed by TEM (Figures 3f and g). The high number of bacterial cells as well as the fact that intact cytoplasm and plant cell organelles were neither detected by TEM nor by light microscopy after 4',6-diamidino-2-phenylindole staining (data not shown) indicate that the colonized plant cells were dead. Whether these plant cells were dead before bacterial colonization remains to be resolved.

The colonization of barley primary roots was further investigated by TEM and scanning electron microscopy. Consistent with the above findings, root-cap and elongation zones were much less colonized by *RrF4* (Figures 4a–d) compared with root hair zones, where *RrF4* formed dense surface-attached biofilms (Figures 4e–g). Root cap-colonizing



**Figure 4** Colonization of barley primary roots by *RrF4* analyzed by scanning (a–f) and transmission (g) electron microscopy. (a, b) *RrF4* at the root tip (b is a zoom out of a showing *RrF4* cells at cracks of the root cap cells, arrows). (c) *RrF4* cell aggregates at the root tip. (d) Single *RrF4* cells attached to the root surface distal to the tip area. Bacterial cells are cross-linked by fiber-like structures. (e) Different stages in biofilm formation at the rhizoplane of the root hair zone: single *RrF4* cells attached to the rhizoplane (s); micro-colonies formed through multiplication of single attached cells (m); larger cell aggregates (a), thick surface attached biofilm (b); root hairs (rh). (f) *RrF4* cell aggregates around the root hair protrusion site; assumed area of penetration into the root tissue (arrow). (g) Surface-attached dense biofilm of *RrF4* cells. Bacterial cells are embedded in an extracellular polysaccharide (EPS)-like structure forming a four to five cell layers thick biofilm at the root surface (b).





**Figure 5** Localization of *RrF4* in the root hair zone of barley primary roots. (a, b) Root cross-sections showing the colonization of the root surface and the cortical tissue 7 dpi with (a) GUS- and (b) GFP-tagged *RrF4*. (c–e) Light microscopic (c) and TEM (d, e) images of ultra-thin cross-sections of the primary root shown in (a) illustrating the intercellular localization of *RrF4* in the cortex; (c) overview; (d) *RrF4* cells in the intercellular space; (e) zoom to single intercellular rod-shaped *RrF4* cells. (f, g) Cross-sections showing colonization of the central cylinder at 21 dpi by GUS- (f) or GFP-tagged *RrF4* cells (g). (a, f) Light microscopic and (b, g) confocal laser scanning images. Epidermis (ep), root hair cells (rh), cortex (cx), cortex-cells (cxc), endodermis (en), central cylinder (cc), peripheral xylem vessels (pX) central metaxylem vessels (cmX). Right arrows highlight bacterial cells.

*RrF4* cells formed cell aggregates located in cracks between root cap cells but did not fully cover the cell surface (Figures 4a and b). These cell aggregates and single surface-attached cells were cross-linked by fiber-like structures (Figures 4c and d). It was previously described that cellulose production leads to a loose aggregation of *R. radiobacter* cells (Matthysse, 1983) and is required for

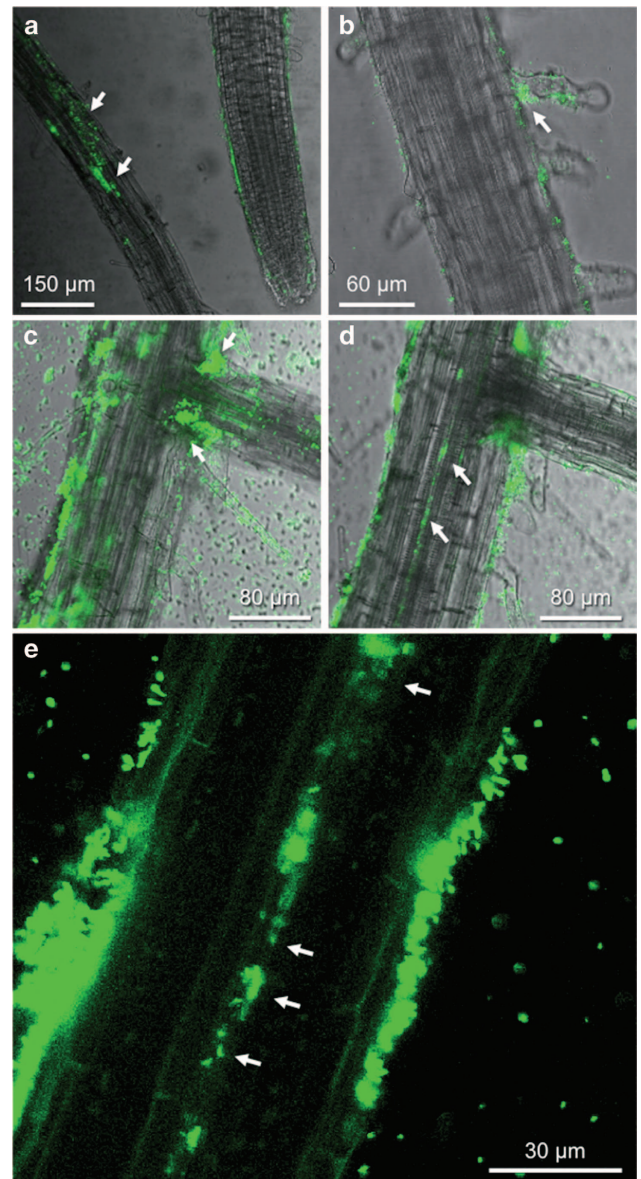
efficient root surface attachment of ‘*Agrobacterium tumefaciens*’ (reviewed by Heindl *et al.*, 2014; Matthysse, 2014). Because the two gene clusters required for cellulose production in *R. radiobacter* C58, *celABCG* and *celDE*, are present at the linear chromosome of *RrF4* (Supplementary Table 3), we presumed that the fiber-like structures can be cellulose.



Larger cell aggregates (micro-colonies) often developed at the sites of root hair protrusion rather than being attached to fully developed root hairs (Figures 4e and f). Root hair protrusion sides serve as bacterial entrance into the inner root tissue (Compant *et al.*, 2005). TEM showed a detailed picture of the approximately 5- $\mu$ m-thick multilayer biofilm with *RrF4* cells embedded by a dense matrix of extracellular polymeric substances (Figure 4g). This matrix may be formed by extracellular polysaccharides, extracellular DNA or (glyco)proteins (Flemming and Wingender, 2010). Like C58, the genome of *RrF4* contains several genes for polysaccharide synthesis such as cellulose, succinoglycan, glucans and outer membrane lipopolysaccharides (Goodner *et al.*, 2001; Supplementary Table S4), which are known to be involved in the formation of extracellular biofilm matrix of '*Agrobacterium tumefaciens*' strains (Heindl *et al.*, 2014; Matthyse, 2014).

*RrF4* colonizes the intercellular space of the root cortex and, unlike *P. indica*, proceeds into the central cylinder. Cross-sections of barley roots were analyzed for bacteria colonizing the inner root (Figure 5). At 7 dpi, GUS-tagged or GFP-tagged *RrF4* were visible in the root cortex (Figures 5a and b). Bacteria were often detected at cell junctions of rhizodermal and cortical cells. Light microscopy and TEM of ultrathin root cross-sections showed a dense colonization of the intercellular spaces in the cortex tissue up to the endodermis (Figures 5c–e). Root hair cells and cortex cells were not colonized, which implies an extracellular colonization pattern.

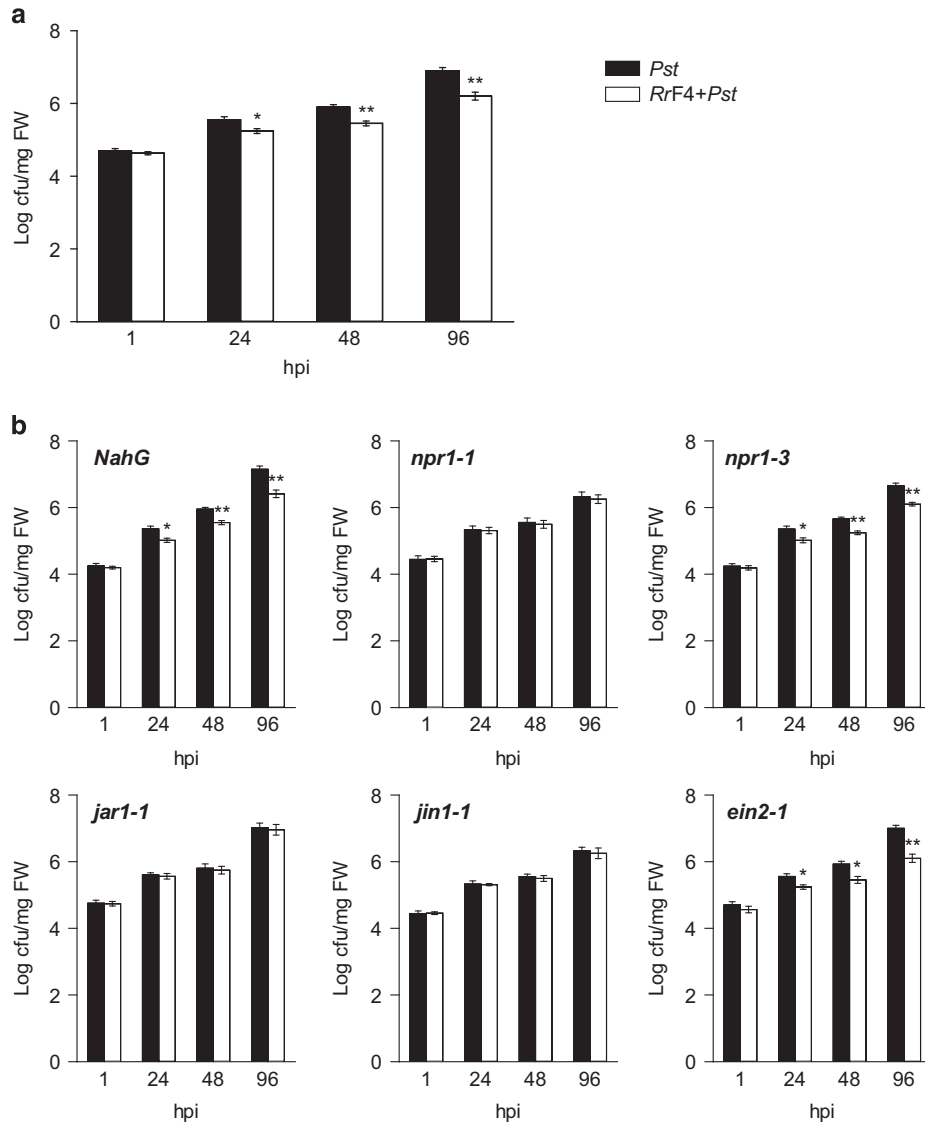
At later time points (21 dpi), *RrF4* cells were detected at cell junctions of endodermal cells and in the central cylinder. While we cannot exclude that *RrF4* is able to pass the endodermis, it is more likely that bacterial cells invaded the vascular tissue via the non-maturated tissue of the elongation zone where differentiation has not been completed yet. Inside the central cylinder, bacteria also were seen in the intercellular spaces, whereas intracellular colonization was not unambiguously detectable (Figures 5f and g). This finding is crucial insofar as colonization of the inner part of the root beyond the endodermis has not been observed with *RrF4*'s fungal host *P. indica* (Deshmukh *et al.*, 2006; Jacobs *et al.*, 2011). At 21 dpi, very few single GFP-tagged *RrF4* cells also were detected inside the stem but not in the leaves. Consistent with this, *RrF4*-specific ITS targets could not be amplified by qPCR from shoot and leaf extracts (at 14 and 21 dpi), and *RrF4* cells also could not be cultured from surface-sterilized leaf tissue. The later data suggest that, in contrast to other endophytic bacteria (Rothballer *et al.*, 2008; Hardoim *et al.*, 2008; Van der Ent *et al.*, 2009), *RrF4* cells do not spread systemically into the upper plant tissue.



**Figure 6** Colonization of Arabidopsis roots by GFP-tagged *RrF4*. Images were taken at 7 (a, b) and 21 (c–e) days after root dip-inoculation. (a) *RrF4* colonization of the root surface in the root hair zone with single attached bacteria, thin biofilms and dense locally restricted aggregates (arrows). (b) Colonization of the root hairs mainly at the cell bottom (arrows). (c) Localization of bacteria forming biofilms and aggregates at the root surface of the primary root and at the sites of lateral root protrusions (arrows). (d, e) Bacterial cells inside the plant's vascular system (arrow: xylem). (c–e) Different layers of a confocal laser scanning microscopy screen through a root, (c) surface, (d, e) central layer showing the central vascular system.

#### *RrF4* colonizes the interior of Arabidopsis roots

The colonization pattern in Arabidopsis roots was assessed using GFP-tagged bacteria. At 7 and 14 dpi, single *RrF4* cells as well as dense, locally restricted aggregates were seen at the surface of root hair zones, the surface of root hairs and, in higher abundance, at the base of root hair cells (Figures 6a–d). Compared with barley, biofilms were less dense (data not



**Figure 7** *RrF4*-mediated ISR to *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) in (a) *Arabidopsis* wild type and (b) mutants indicative of SA-, JA-, and ethylene based defense (b). Two-week-old roots were dip-inoculated with *RrF4*, and seedlings were grown in soil under short-day condition. Two weeks later, leaves were spray-inoculated with *Pst*, harvested at the indicated time points and assessed for *Pst* infection by determining colony-forming units (cfu) per milligram fresh leaf. Mutants *jar1-1*, *jin1-1* and *npr1-1*, which are compromised in JA-based ISR, are also compromised for *RrF4*-induced *Pst* resistance, while SA-defence-associated *NahG* and *npr1-3* as well as ethylene-associated *ein2-1* are not. Error bars indicate standard errors based on three independent biological replicates. Asterisks indicate statistical significant difference (Student's *t*-test \* $P < 0.05$ ; \*\* $P < 0.01$ ).

shown), and bacterial cells were again not detected within root hair cells. At a later time point (21 dpi), *RrF4* also colonized the sites of secondary root emergence. Cracks at secondary root emergence sites may be used by *RrF4* for passive entrance into the inner root tissue as was suggested for several endophytic bacteria (Reinhold-Hurek and Hurek, 1998; Compant *et al.*, 2005). In contrast to barley, the central vascular system (xylem) of primary (Figures 6d and e) and secondary roots (data not shown) were heavily colonized. However, like in barley, *RrF4* cells were hardly detectable in the green parts of the plant.

#### *Plant defense gene expression in response to RrF4*

We addressed the question whether *RrF4* activates genes that are indicative of the plant hormones salicylic acid (SA), jasmonate (JA) and gibberellin (GA), which are involved in plant defense (Spoel *et al.*, 2003). The barley genes *Pathogenesis-related 1b* (*PR1b*), *PR10*, *Jasmonate-induced protein 23* (*JIP23*), *1-Deoxy-D-xylulose-5-phosphate synthase* (*DXS*), *Ent-kaurene synthase 1* (*KS1*) and *Ent-kaurene synthase like 4* (*KSL4*) are responsive to *P. indica* (Deshmukh and Kogel 2007; Schäfer *et al.*, 2009; Pedrotti *et al.*, 2013) and were therefore used for our analysis. A direct qPCR-based comparison of



gene expression in barley roots in response to *RrF4* and *P. indica*, respectively, showed a similar activation pattern for all tested genes (Supplementary Figure 5). SA-induced (*PR1b*, *PR10*) and GA-induced (*DXS*, *KS1*, *KSL4*) genes were upregulated both by *RrF4* and *P. indica*, whereas the JA-induced gene *JIP23* was downregulated at 3 dpi as compared with controls. The only detectable difference in gene expression between *RrF4* and *P. indica* concerns *JIP23* at 7 dpi as the gene is induced by *RrF4* but not by *P. indica*.

#### *RrF4* mediates ISR to *Pseudomonas syringae*

*P. indica*-induced resistance in Arabidopsis against the powdery mildew fungus *Golovinomyces orontii* involved the activation of the ISR pathway (Stein et al., 2008). To assess whether *RrF4* induces ISR, Arabidopsis mutants indicative of SA signaling (SA-degrading SA hydroxylase (*NahG*), non-expressor of *PR 1-3* (*npr1-3*)), JA signaling (*jasmonate-responsive 1-1* (*jar1-1*), *jasmonate-insensitive 1* (*jin1*), *npr1-1*), and ethylene signaling (*ethylene insensitive 2-1* (*ein2-1*)) were assessed for *RrF4*-mediated resistance to *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*). Dip-inoculation of wild-type roots with *RrF4* reduced the number of *Pst* bacteria in leaves showing that *RrF4* induces systemic resistance to bacterial pathogens (Figure 7a). Similarly, and consistent with the report on *P. indica*, pretreated *NahG*, *npr1-3* and *ein2-1* mutants, like wild-type plants, displayed systemic resistance against *Pst* compared with non-pretreated plants (Figure 7b). In contrast, *jar1-1*, *jin1* and *npr1-1* plants were fully compromised for *RrF4*-mediated resistance (Figure 7b), showing that the jasmonate-based ISR pathway is also required for *RrF4*-mediated systemic resistance.

To further substantiate this finding, expression of JA-, SA- and ET-responsive genes was assessed in Arabidopsis leaves in response to *RrF4* (inducer) and *Pst* (challenger) inoculations. *PR1*, *Vegetative storage protein 2* (*VSP2*), *Ethylene receptor factor 1* (*ERF1*) and *Plant defensin 1.2* (*PDF1.2*) were determined at 24 and 48 h after inoculation by qPCR. JA marker genes *VSP2* and *PDF1.2* were induced while the SA marker gene *PR1* and ethylene marker gene *ERF1* were not induced as compared with plants that were challenged with *Pst* but not pretreated with *RrF4* (Supplementary Figure S6). Together, these data show that *RrF4*, like its host *P. indica*, induces disease resistance via the ISR pathway.

#### *RrF4* induces systemic resistance to *Xanthomonas translucens* pv. *translucens* in wheat

*RrF4*'s activity also was assessed in wheat against the pathogenic bacterium *Xanthomonas translucens* pv. *translucens* (*Xtt*), the causal agent of bacterial leaf streak of cereals. Roots of 3-day-old wheat seedlings were dip-inoculated in a suspension of *RrF4*, and subsequently transferred to soil. Three weeks later, leaves were spray-inoculated with a suspension of

*Xtt*, and assessed for infection symptoms at 5 and 7 dpi. Leaves from plants pretreated with *RrF4* showed reduced bacterial leaf streak symptoms (43% at 5 dpi; 34% at 7 dpi) compared with plants not pretreated with *RrF4* (Supplementary Figures S7a and b). Consistent with this, at 7 dpi, total chlorophyll content of leaves infected with *Xtt* was 20% lower when plants were not pretreated with *RrF4* (Supplementary Figure S7c).

## Conclusions

Our study shows that the non-pathogenic Alphaproteobacterium *R. radiobacter RrF4*, which is intricately associated with its fungal host *Piriformospora indica*, is genetically very similar to the well-studied plant pathogenic *R. radiobacter* biovar I strain C58 (genomovar G8). Highly similar chromosomal genetic content of *RrF4* and C58 indicate the high potential for *RrF4*'s interaction with host plants, whereas differences in the plasmid may explain its non-pathogenic nature. Further studies are needed to understand the basis of specific genomic features supporting *RrF4*'s endofungal lifestyle. The failure to cure *P. indica* from *RrF4* still hampers a conclusively prediction of the bacterium's role in the Sebacinalean symbiosis. Interestingly, however, induced resistance responses and defense gene expression in barley, wheat and Arabidopsis were hardly distinguishable when either induced by *RrF4* or *P. indica*. Thus, our data support the possibility that the beneficial biological activity previously assigned to *P. indica* may be at least partly allotted to the bacterium *RrF4*. Finally, *RrF4*'s biological activity was in several aspects comparable with other plant growth-promoting rhizobacteria indicating that endofungal bacteria of the Sebacinalean symbiosis are a valuable source of beneficial bacteria with agronomical potential.

## Conflict of Interest

The authors declare no conflict of interest.

## Acknowledgements

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