Investigation of the role of the endofungal bacterium *Rhizobium radiobacter* in the tripartite Sebacinalean symbiosis

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Table of contents

List of Abbreviations

ABA	Abscisic acid
BSA	Bovine Serum Albumin
BI-1	BAX inhibitor-1
cDNA	Complementary DNA
CLSM	Confocal laser scanning microscopy
DEPC	Diethylpyrocarbonate
DAPI	4', 6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleosidtrophosphate
dpi	days post inoculation
EDTA	Ethylenediaminetetraacetic acid
EF-Tu	Elongation factor TU receptor
ETI	Effector-triggered immunity
FISH	Fluorescence *in situ* hybridization
flg22	A 22-amino-acid-long peptie derived from flagellin
GFP	Green Fluorescent Protein
ISR	Induced systemic resistance
ITS	Internal transcribed spacer
JA	Jasmonic acid
LB	Lysogeny Broth
LL-Piri	Long-term laboratory cultured *P. indica*
MAMP	Microbe-associaed molecular pettern
MOPS	3-(N-morpholino) propanesulfonic acid
MQ H2O	Milli-Q water
mRNA	Messenger RNA
MS	Murashige Skoog
MTI	MAMP-triggered immunity

OD   Optical density

PAMP   Pathogen associated molecular patterns

PBS   Phosphate-buffered saline

PCR   Polymerase chain reacctoin

PI   Propidium Iodide

P. indica   *Piriformospora indica*

PRR   Pattern recognition receptor

PTI   PAMP-trigged immunity

q-PCR   Quantitative real-time PCR

RNA   Ribonucleic acid

Re-Piri-1   re-isolated *P. indica* subculture-1

Re-Piri-2   re-isolated *P. indica* subculture-2

RE-Piri   re-isolated *P. indica*

rpm   Rounds per minute

*Rr*F4   *Rhizobium radiobactor* F4

RT   Room Temperature

SA   Salicylic acid

SAR   Systemic aquired resistance

SDS   Sodium dodecyl sulfate

SEM   Scanning electron microscopy

SOC   Super Optimize Broth

SP-Piri   Single-protoplast-derived *P. indica*

T3SS   Type III secretion system

T4SS   Type IV secretion system

Tef   Translation elongation factor

TEM   Transmission electron microscopy

Ubi   Ubiquitin

VBNC   Viable but nonculturable

WT   Wild-type

X-Gluc   5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid

1 Introduction

1.1 Plant-microbe interactions

The interactions between plants and microbial organisms play a crucial role in the evolution and development of land plant (Chisholm *et al*. 2006). The microbes associated with a plant host can be categorized as mutualists, parasites and pathogens (Newton *et al.* 2010) (Fig. 1.1). The dynamics detection of plant-microbe interaction is revealing that many microbes change their relationships with plant as the switches of life cycle stages and the change of environmental conditions (Kogel *et al*. 2006; Huang *et al.* 2009).
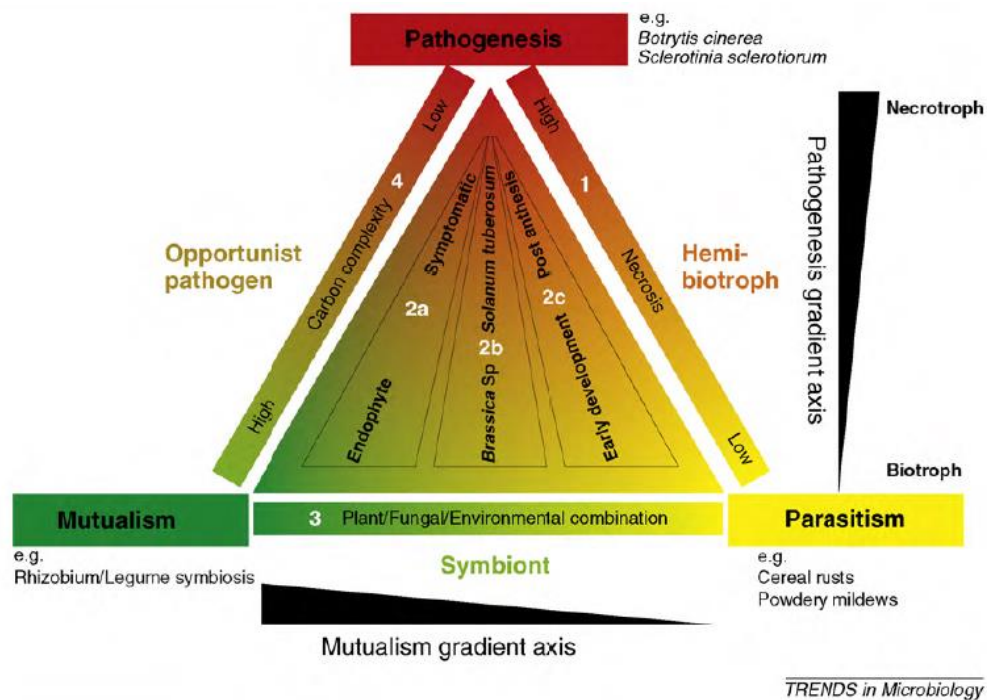
Figure 1.1 Trophic space of microbe with plant (taken from Newton *et al.* 2010). This triangle pattern represents the trophic relationships of plant-microbe interaction, in which there are three key trophic states: mutualism, parasitism and pathogenicity. The mutualism gradient axis is represented in the vertical axis from mutualism to parasitism for symbiotic relationships. The pathogenesis gradient axis is represented in the vertical axis from necrotroph to biotroph. Individual microbes can predominantly occupy specific trophic spaces in these ranges, but frequently change among different trophic states during different stages of their life cycles in response to environmental, host developmental or microbe-specific. Examples of trophic interactions in the corners of the triangle:

Botrytis cinerea Sclerotinia sclerotiorum (pathogenesis); Rhizobium, Legume symbiosis (Mutualism); Cereal rusts Powery mildews (Parasitism).

Microbes behave as pathogens on plant in different mechanisms with various symptoms and damages at certain stages of the life cycle and environmental condition. At one extreme, necrotrophic pathogens such as fungi *Botrytis cinerea* (grey mould), *Monilinia fructicola* and *Sclerotinia sclerotiorum* (stem rot) or the bacterium *Ercinia carotovora* penetrate plant, kill host cells and decompose the plant tissue to provide themselves with enough food (Jan *et al.* 2006; Rojo *et al.* 2003). Necrotrophs normally secrete lytic enzymes and toxins resulting in the death of tissue, subsequently decompose the tissue and use the nutrition released from the dead tissue. Biotrophic pathogens are relying on living plant tissue for their own propagation, they feed on host resources without casing necrotic lesions or other damage, such as powdery mildew (*Blumeria graminis f. sp. hordei*) and rust (*Puccinia triticina*) (Panstruga 2003). Hemi-biotrophic pathogens, such as barley ramularia leaf spot (*Ramularia collo-cygni*) and potato late-blight (*Phytophthora infestans*), are symptomless pathogens between necrotrophic and biotrophic pathogens. At the initially biotrophic infection phase, hemibiotrophy cause no or very less symptom while there are necrotic lesions formed by the haustoria in the later growth phase (Sowley *et al.* 2010).

In the symbiotic plant-microbe association, the plant host obtains advantages from the microbial colonization instead of suffering from any "pain". The benefits here are based on a well-balanced status between both partners (Kogel *et al*. 2006). In the mutualism relationship, promoted growth of the host normally induced by mineral nutrients support, abiotic stress tolerance and increased resistance against pathogens (Redman *et al.* 2002; Colditz *et al.* 2005; Arnold *et al.* 2003). The microbes in this relationship get nutrient supply without disturbing the development of the host. Mycorrhizal fungi and endophytes are typical examples who are involved as mutualistic microbes. Basidiomycete *Piriformospora indica* is the representative

mutualistic fungus in the microbe-plant root interaction, it provides various benefits to the host plants, such as barley, Arobidopsis, tomato and wheat (Waller F. *et al.* 2006; Deshmukh *et al.* 2006; Sharma *et al.* 2008; Qiang *et al.* 2012).

1.1.1 Plant innate immunity

In order to ensure plant development and proliferation, plants built a series of defense strategies, which is related to the innate immune system of animals (Ausubel 2005), to against most of the microbial attackers. During the evolution, there are two branches of innate immune system of plant developed to resistant against the majority of the microbes (Chisholm *et al.* 2006; Jones and Dangl 2006). Microbes enter the inner part of plant through the wounds on the stem, stomata on the leaves or other natural openings. After penetrating plant cell walls, the plasma is exposed in front of the microbes, where the microbes meet with the transmembrane pattern recognition receptors (PRRs), which recognize the pathogen- or microbial-associated molecular patterns (PAMPs or MAMPs), such as bacterial flagellin, EF-Tu (elongation factor Tu) and fungal chitin (Chisholm *et al.* 2006; Zipfel and Felix 2005; Zipfel *et al.* 2006). The perception of a microorganism on the plasma membrane triggers primary immune response pattern-triggered immunity (PTI). This first layer immune system halts the microbial colonization by inducing oxidative burst (ROS), mitogen-activated protein kinase signaling cascade (MAP3K) or callose deposition (Schwessinger and Zipfel 2008).

Once microbes acquired the capacity to restrain the primary immune defense, effector-triggered immunity (ETI) as the second layer immune system is motivated in plant and functioned in a more specialized mechanism (Dodds and Rathijen 2010; Pieterse *et al.* 2009). Microbial pathogens secret effector protein into the cytosol of plant host cell and interfere with PTI in the intracellular space.The effectors from diverse kingdoms can be recognized directly or indirectly by NB-LRR (nucleotide binding and leucine rich repeat) protein encoded by R-genes (Dangl and Jones 2001), thereby trigger the ETI, which results in the disease resistance and hypersensitive cell

death response (HR) in plant host (Fig. 1.2). PTI is weak, while ETI is stronger, faster and can be triggered again and again. A 'zigzag' model containing four phases illustrates the immune system in plant host (Jones and Dangl 2006).

Effectors' strategies and mechanisms were studied mainly in bacterial phytopathogens. Individual pathogenic bacteria strain can encode 20-30 effectors, and delivers into host cytoplasm using the type III secretion systems TTSS (Staskawicz *et al.* 2001; Chang *et al.* 2005; Cunnac *et al.* 2009). *P. syringae* AvrPto effector suppresses papillae formation on the cell wall, AvrE and HopPtoM effectors inhibit the callose deposition to against the defense based on cell wall (DebRoy *et al.* 2004; Hauck *et al.* 2003), AvrPtoB effector behaves as a mimic of ubiquitin ligases and transfers the ubiquitin to suppress ETI-associated PCD (programmed cell death) in tomato (Abramovitch *et al.* 2003; Janjusevic *et al.* 2005; Rosebrock *et al.* 2007). *Xanthomonas* effectors XopD, AvrXv4 and AvrBsT interfere with SUMO protein conjugation pathway in plant (Roden *et al.* 2004), AvrBs3 effector family alerts gene transcriptions in plant nucleus to downregulate host defense (Yang *et al.* 2000), TAL (transcription activator-like) effectors induce the expression of symptom development associated host genes (Boch *et al.* 2009). Compared with the data on effectors in bacteria, the function and mechanism of eukaryotic effectors are rarely known. The effectors from fungal- and oomycete-pathogen are secreted through the endomembrane system and delivered by unknown mechanism into plant cells (Kamoun 2007; Panstruga and Dodds 2009).
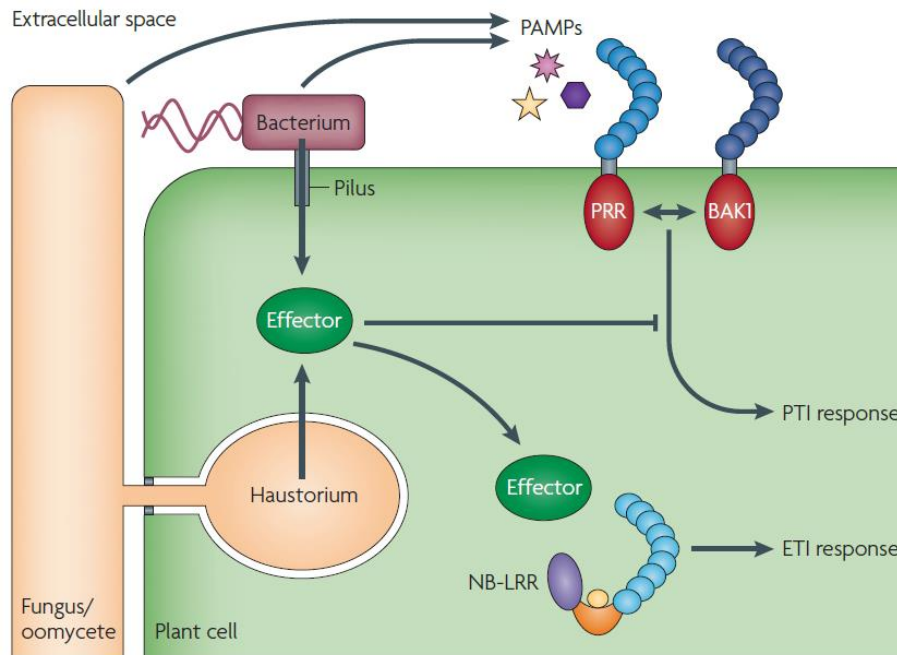
Fig. 1.2 Schematic representation of plant innate immunity (taken from Dodds and Rathjen 2010). Bacterial pathogens multiply in the extracellular space of plant cells, while fungal and oomycete pathogens mostly form hyphae in the extracellular space or feeding structure haustoria and invasive hyphae in the intercellular space. Molecules known as PAMPs were released from varied pathogens in the extracellular space, recognized by PRRs on the plant cell surface and triggered the PTI response. PRR normally contains one extracellular domain LRR (leucine-rich repeat) and one intercellular kinase domain, interacts with BAK1 (brassinosteroid insensitive 1-associated kinase 1) protein to elicit PTI pathway. Effectors from bacterial pathogens were delivered through a type-III secretion pilus into plant cells, whereas the effectors from fungal and oomycete pathogens were delivered by unknown mechanism through haustoria or other structures into the host cell. Most of these effector proteins are recognized by NB-LRR receptors and initiate ETI pathway, and suppress PTI on the other hand. NB-LRR consist a LRR domain, a NB domain and an amino-erminal toll or coiled-coil domain.

1.1.2   Plant systemic immunity

Once there is plant defense response triggered on the infection site, plant systemic defense response is normally activated in the distal parts. The activated systemic immunity can protect undamaged plant tissue from subsequent pathogen invasions. Systemic acquired resistance (SAR) provides long-lasting disease resistance in

5

healthy plant tissue against the secondary infection mainly with biotrophic pathogens (Durrant and Dong 2004). SAR needs the accumulation of salicylic acid (SA) and the expression of pathogenesis-related (PR) genes (Vernooij *et al.* 1994; Bostock 2005). The conserved *NON-EXPRESSOR OF PATHOGENESIS-RELATED1* (*NPR1*) gene is considered to be a positive regulator in SAR signaling pathway, and also functions at the downstream of SA (Fig. 1.3) (Pieterse *et al.* 2010; Fobert and Despres 2005; Dong 2004).

Induced systemic resistance (ISR) is associated with beneficial soil-borne microorganisms, for instance rhizobacterial strains and mycorrhizal fungi that promote the plant growth and increase plant resistance to bacterial and fungal pathogens (Pieterse *et al.* 1998; Van Loon *et al.* 1998; Pozo and Azcon-Aguilar 2007). Microbe-associated molecular patterns (MAMPs) from the beneficial microbes colonized on roots are recognized by the plant host, which results in effective systemic resistance on the shoot part of the host (Bakker *et al.* 2007; Van Wees *et al.* 2008). In contrast to the 'broad spectrum' of SAR, ISR is less and predominantly effective against necrotrophic pathogens and chaw insects. Jasmonic acid and ethylene (JA-ET) dependent signaling and the associated genes, such as *PDF1.2*, *MYC2* and *ERF1*, play essential role in the ISR pathway (Ton *et al.* 2002; Van Oosten *et al.* 2008).
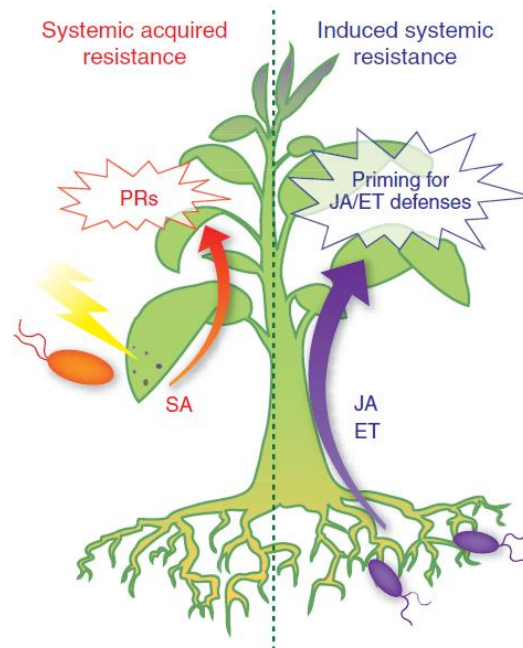
Fig. 1.3 A model of systemic immune responses in plant (taken from Pieterse *et al.* 2010). Systemic acquired resistance (SAR) is induced by a mobile signal from locally pathogen infected tissue, the signal is transported through plant vascular system to distal tissues and actives the defense responses in healthy systemic tissues in the plant host. Salicylic acid (SA) plays a crucial role in the SAR pathway, since it can regulate the expression of genes encoded pathogenesis-related proteins (PRs). Induced systemic resistance (ISR) is activated by the beneficial microorganisms colonized on plant roots. The mobile signals created from plant root travel to the above-ground plant parts, activate systemic immunity against pathogen attack in shoot part. JA-ET (jasmonic acid-ethylene) signaling pathways are essential in ISR. The expression of JA- and ET-associated genes is evident after pathogen attack in ISR-expressing plant.

1.2   Beneficial soil-borne microorganisms

Some soil-borne microorganisms can improve plant nutrition and assistant plant host to deal with the biotic and abiotic stresses. Such beneficial belowground microbes are known as plant growth-promoting rhizobacteria (PGPR) and plant growth-promoting fungi (PGPF) (Van Wees *et al.* 2008). The beneficial effects on plant not only results in a higher yield, but also have a high relevance in natural and agricultural ecosystems because of the reduced industrial fertilizer pollution in agricultural soils and water (Yang *et al.* 2009; Weyens *et al.* 2009).

1.2.1 Plant growth-promoting rhizobacteria (PGPR)

Plant growth-promoting rhizobacteria (PGPR) are a small portion of rhizosphere microbes (2-5%) that promote plant growth directly or protect plant from soil-borne diseases indirectly (Lugtenberg and Kamilova 2009). Rhizosphere, one layer of soil influenced by plant root, is a hot spot for microbes in the underground. Plant roots secrete metabolite via root exudate that can be utilized by the PGPR as nutrients. The root exudates released by plant include many organic compounds, such as amino acids, organic acids, sugars, vitamins, nucleotides, phenolics, putresccine and carbon (Uren 2007). PGPR promotes plant growth through increasing the supply of nutrients to the host plant. For instance, the biological N_2 fixer rhizobia is a well studied PGPR for the ability to fix N_2 in the legume family through nitrogenase activity. The increased nutrients in plant rhizosphere also involve the solubilized phosphates and facilitated absorption of iron (Nautiyal *et al.* 2000; Kim *et al.* 1998; Von Wiren *et al.* 2000). The positive effects on root growth and morphology change increase root surface area, which can help the plant host to take up more nutrients from the surrounding environment (Bashan and Dubrovsky 1996; Vessey and Buss 2002; Galleguillos *et al.* 2000; German *et al.* 2000; Holguin and Glick 2001). Plant growth promotion can be measured by the yield or the weight and length of shoots and roots. Endophytic bacteria represent a small subgroup of PGPR, they attach on the root surface and subsequently enter the root interior and colonize in the intercellular spaces (Hallmann and Berg 2007; Gray and Smith 2005; Hardoim *et al.* 2008; Rosenblueth and Martinez-romero 2006). The colonization of PGPR on root is changing as plant changes the chemical and physical composition at rhizosphere via exudations, water potential, soil pH and O_2 pressure (Griffiths *et al.* 1999; Tavaria and Zuberer 1998; Xu 2000). The penetration mostly rake place at the cracks on root, for instance the lateral root base, root hairs and root tips (Fig. 1.4). In addition, the cell-wall degrading enzyme (CWDE) secreted by bacteria plays crucial role in the invasion process (Compant *et al.* 2010; Lodewycks *et al.* 2002). The relationships between PGPR and plant hosts can be categorized into rhizospheric and endophytic relying on the

microbial localization (McCulley 2001). For instance, *Kluyvera ascorbate* colonizes on the upper two-thirds on the root surface of canola plant (Ma *et al.* 2001). Root junctions among epidermal cells and lateral root protrusion sides are the most popular colonization position for PGPR, which are also thought to be the entry point for PGPR (O'Callaghan *et al.* 2001; Spencer *et al.* 1994).
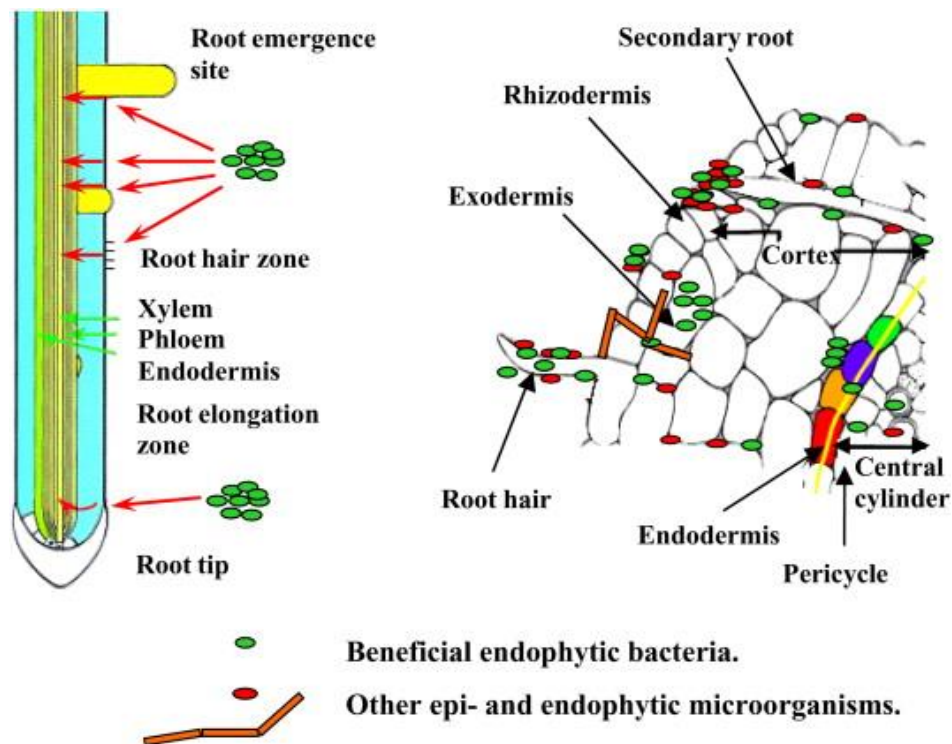
Fig. 1.4 Plant root colonization sites by endophytic bacteria (taken from Compant *et al.* 2010).
The use of PGPR as biological control is an environment-friendly approach against pathogenic microorganisms including fungi, bacteria, viruses, insects and nematodes. The mechanisms for rhizobacteria to exhibit biological control are varied. Antibiotics produced by gram-negative bacteria function via antagonism mechanism, such as the implication of lipopeptide biosurdactants from B. subtilis and pseudomonads in biological control (De Bruijn *et al.* 2007; Ongena *et al.* 2007). Signal interference mechanism is based on the degradation of AHL, which is required in the biofilm formation and the cell-wall-degrading enzymes synthesis (Lin *et al.* 2003; Shephard *et al.* 2008). Predation and parasitism based on enzymatic destruction of fungal cell wall are the main biocontrol mechanism in fungi, like *Trichoderma* species (Harman *et al.* 2004). ISR mechanism, which was discovered in the resistance to *Fusarium* wilt of carnation from rhizobacterium *Pseudomonas* sp. Stain WCS417, can be

triggered by variety of beneficial microbes (Van Peer *et al.* 1991; Van Wees *et al.* 2008; Van der Ent *et al.* 2009; Segarra *et al.* 2009). The successful biocontrol bacterium need also compete on nutrients and niches on the root with other organisms (Chin-A-Woeng *et al.* 2000).

1.2.2 *Rhizobium*-Legume symbiosis

Rhizobium spp. is Gram-negative bacteria that fix N_2 for the leguminous family, such as soybean, peanut, pea and alfalfa. *Rhizobium* spp. forms nodules on the roots, differentiates into nitrogen-fixing bacteriods, and converts stable nitrogen gas in the soil into biologically useful form ammonia, which can be used as nitrogen source and taken up by the plant host (Van Rhijin *et al.* 1995; Spaink *et al.* 1998;). The establishment of this symbiosis between these two partners is based on a complicated molecular dialogue, starting even when rhizobia getting closer to root hairs in the soil (Kijne *et al.* 1992). The association stimulates the deformation and curling of root hair, and the secretion of flavonoids from plant roots activates the expression of *nodD* genes in rhizobia (Long *et al.* 1989; Long *et al.* 1996). Bacteria are released from the infection threads, which are originated from root hair and controlled by the Nod factors, into the developing nodule cells coming from the reprogramming of the root cortical cells (Oldroyd *et al.* 2011). During the release of bacteria, an un-walled infection droplet is formed from the invagination of infection thread membrane (Brewin 2004; Rae *et al.* 1992). Bacteria are surrounded by the symbiosome membrane through the symbiotic interface - infection droplet (Parniske 2000; Roth and Stacey 1989). Thereafter bacteria differentiate into bacteroids with the expression of enzyme nitrogenase and are able to fix the nitrogen (Vasse *et al.* 1990). The symbiosome membrane makes the exchange of carbohydrates and fixed nitrogen happened between plant host and bacteria (Oldroyd *et al.* 2011). In addition to *Rhizobium*, *Azospirillum* is also a N_2-fixer for wheat, sorghum and maize (Okon *et al.* 1998).

1.2.3   Plant growth promoting mycorrhizal fungi

Some plant species form symbiotic alliance on the root system with certain mycorrhizal fungi, in this mutualistic association plant benefits with more accessible minerals from the soil while fungi take advantage of carbohydrates from plant root exudates (Bonfante and Anca 2009).

Arbuscular mycorrhizal fungi (AMF) form a symbiosis through improving the supplement of phosphate, nitrogen and water to plant host and obtaining carbohydrate from plant in return (Finlay 2008; Solaiman and Saito 1997; Bago *et al.* 2003). AMF as the most widespread symbiosis belongs to a monophyletic phylum Glomeromycota, and as one number of endomycorrhiza part of the fungal hyphae of AMF is inside of plant cells (Fitter 2005; Schübler *et al.* 2001; Hibbett *et al.* 2007). The symbiotic development between arbuscular mycorrhiza and plant host results in the formation of the tree-shaped structure arbuscules (Parniske 2008). The exchange of nutrients and signals among plant host and fungus partners is supposed to occur in these symbiotic interface arbuscules. The structure of arbuscule in plant cell comprises three parts, fungal plasma membrane, periarbuscular space (PAS) and plant-derived periarbuscular membrane (PAM). The hyphe branches of fungus are surrounded by a plant-derived PAM at the outside layer of the subcellular structure arbuscule. The apoplastic interface PAS is formed between the PAM and fungal plasma membrane (Harrison 2005). As the change of nutrients distribution in the soil over time, Arbuscules have a short lifetime and are normally degraded about 8.5 days (Alexander *et al.* 1989; Javot *et al.* 2007).

The development of AM fungal hyphae changes dramatically with the existence of plant-derived signals (Fig. 1.4). Strigolactones were discovered as endogenous plant hormones in various plants, such as pea, *Arabidopsis thaliana* and rice (Gomez-Roldan *et al.* 2008; Umehara *et al.* 2008). It was also discovered that strigolactones can stimulate the spore germination of AMF, induce the hyphae branching, alter the mitochondrial activity and the fungal physiology as well (Akiyama *et al.* 2005; Besserer *et al.* 2006). So strigolectones act as signals in the

communication systems between plant host and symbiotic fungi at the presymbiotic phase (Parniske 2005). Fungal signalling molecules Myc factors and plant receptors are getting more attention currently in the induction of symbiosis-specific fungus-plant responses. One example of Myc factor ENOD11- was illustrated to be able to induce the transcriptional activation of some symbiosis-related genes (Kosuta *et al.* 2003). LysM domain plays important role in the plant receptors, such as Nod factor and chitin receptors (Smit *et al.* 2007; Radutoiu 2007). Plant cells prepare the intracellular environment for the infection of biotrophic fungi, which is called prepenetration apparatus (PPA) (Genre *et al.* 2005; Siciliano *et al.* 2007). The subcellular structure PPA is formed a few hours after the hyphopodium formation, the formation of PPA is relying on the migration of nucleus in the plant cell, the movement of nucleus determine the fungal growth path in the cells (Genre *et al.* 2008).
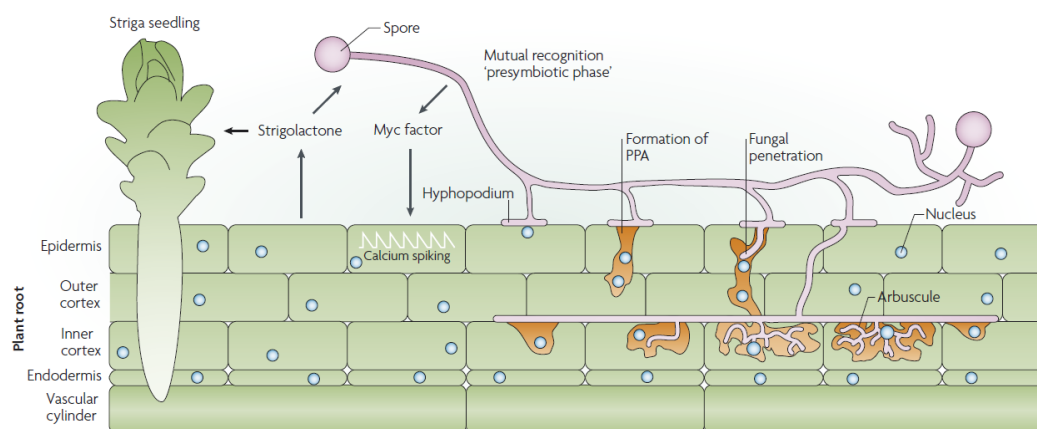
Fig. 1.4 The development of arbuscular mycorrhiza in plant root (taken from Parniske 2008). Strigolactones coming from root exude induce the hyphal branching, spore germination, physiological activity of fungal hyphae and spore, and seeds germination for some plants, for instance parasitic plant Striga. Mycorrhiza factors (Myc) produced by fungi have the ability to induce calcium spiking in the outer layer of plant root epidermal cells and the expression of some symbiosis-related genes in plant. AMF form hyphopodium that originated from mature hyphae instead of germination tubes. Subsequently, a prepenetration apparatus (PPA) is formed in plant cell. Thereafter, the fungal hypha goes through the hyphopodium into PPA in plant cell, and enters cortex layer under the guide of PPA.

There, fungi leave plant cell, branch laterally along root axis in apoplast. These hyphae induce the PPA-like structure in cortical cells, then branch and form arbuscules in these cells. Fresh spores are synthesized normally at the tip of individual hyphae outside of plant root.

Seven genes in plant are identified to be required in the AM symbiosis and rhizobia symbiosis (Kistner *et al.* 2005). These genes are involved in signal transduction network, which plays an important role in the intracellular accommodation for AMF symbiosis and rhizobia symbiosis in plant host cells. *SYMRK* gene encodes a receptor-like kinase that is portrayed as a key to open the symbiotic signalling pathway, this receptor-like kinase can perceive the extracellular signal coming from microbial symbiosis and transduce the signal through its intracellular kinase domain (Markmann *et al.* 2008; Gherbi *et al.* 2008). Proteins encoded by *CASTOR* gene and *POLLUX* gene have similar domain structures and identified sequence similarity, those proteins are seen as counter-ion channels, such as potassium-permeable cation channel, induce calcium spiking, but is not calcium release channel (Peiter *et al.* 2007; Oldroyd and Downie 2008; Imanizumi-Anraku *et al.* 2005). *NUP85* and *NUP133* genes encode nucleoporins proteins these are required in the temperature-dependent initiation of symbiosis and act in the upstream of the calcium spiking (Kanamori *et al.* 2006; Saito *et al.* 2007; Alber *et al.* 2007; Lusk *et al.* 2007). *CCaMK* gene encodes a calcium-calmodulin-dependent protein kinase, which can perceive the calcium induced by AMF in plant host cell (Mitra *et al.* 2004; Kistner *et al.* 2005). The protein encoded by *CYCLOPS* contains a functional signal from nuclear localization, induces the arbuscule development during the symbiosis, and was illustrated to interact with CCaMK in both planta and yeast (Lévy *et al.* 2004). The analysis of these genes in infected plant starts uncovering the signalling networks in symbiotic AMF.

1.3   *Rhizobium radiobacter* F4

1.3.1   The genus *Agrobacterium*

The genus *Agrobacterium* was originally established by Conn in 1942 (Approve Lists

1980) in tumor-inducing *Agrobacterium tumefaciens*, hairy root organism *Agrobacterium rhizogenes* and the nonphytopathogenic strains *Agrobacterium radiobacter*, later this genus was supplemented with biovar I, biovar II and biovar II (Sawada *et al.* 1993). The genus *Rhizobium* is well-known nitrogen-fixing bacteria that form nodule on legumes. *Agrobacterium tumefaciens* is one of the serious plant pathogens in the world with a broad plant host in dicotyledon. The crown gall disease is caused by *A. tumefaciens* and results in a severe agronomic loss. The genome of *A. tumefaciens* C58 is about 5.67 megabase, contains one circular chromosome, one linear chromosome, one tumor-inducing plasmid pTiC58, and another plasmid pAtC58 (Wood *et al.* 2001; Goodner *et al.* 2001; Slater *et al.* 2013). The gall tumor is caused by the discrete T-DNA gene on the tumor-inducing (Ti) plasmid. The T-DNA is transferred into plant cell and integrated randomly into the plant genome. The expression of T-DNA gene alters the synthesis of plant hormones leading the cell proliferation into tumor, and the synthesis of enzyme leading to the bacterial nutrient source opines (Zupan *et al.* 2000; Binns and Thomashow 1998). Tumor-inducing genes can be replaced by exogenous DNA fragment, the reconstructed Ti-plasmid is introduced back into *A. tumefaciens* and transfers the desired gene into plant host. Because of this characteristics, *A. tumefaciens* as an ideal tool for gene transfer plays essential role in transgenic plant research (Topping *et al.* 1995; Newell 2000). The similar transport, metabolic and regulatory systems between *A. tumefaciens* and nitrogen-fixing *Rhizobiaceae* indicate a close evolutionary relationship in the divergence of pathogenic and symbiotic lifestyles (Young *et al.* 2001; Oke and Long 1999).

1.3.2   Endofungal bacteria

Endofungal bacterial are symbionts of fungi residing within the fungal mycelium and spores, were original defined as bacterium-like objects (BLOs) in endomycorrhizal fungi in 1970 (Mosse 1970; Macdonald and Chandler 1981; Scannerini and Bonfante 1991; Bonfante and Anca 2009). The lifecycle of those endobacteria in the fungal host

has two hypothetical mechanisms vertical transmission and horizontal transmission. In the vertical transmission, endobacteria replicate inside the host mycelium and transferred to the next generation by the vegetative spores. During the horizontal transmission, the fungal host could release the endobacteria from the cell and be infected by other competitive bacteria (Lackner *et al.* 2009; Partida-Martinez *et al.* 2007). Normally the mutualistic symbionts tend to be the vertical transmission whereas the pathogenic symbionts prefer the horizontal transmission, but in some cases mutualisms choose the horizontal transmission as well under the help of a type III secretion system (Yamamura 1997; Dale *et al.* 2002).

Some of the arbuscular mycorrhizal fungi harbor endosymbiotic bacteria including Gram-positive and Gram-negative bacteria in the fungal cytosol (Parniske 2008; Naumann *et al.* 2010). For example, one Glomeromycotan fungus *Geosiphon pyriformis* forms a symbiosis in the bladders with *Nostoc* cyanobacteria that has the ability of photosynthesis and nitrogen fixation (Schluesser *et al.* 2002). The rod-shaped endobacterium *Candidatus* Glomeribacter gigasporarum was identified in the AM fungus *Gigaspora margarita*, and vertically transmitted through the vegetative spore generations (Bianciotto *et al.* 2003; Bianciotto *et al.* 2004). Several studies showed that the ectomycorrhizal fungi harbor intracellular bacteria, such as the existence of *Alphaproteobacteria* inside the mycelium of *Laccaria bicolor* S238N (Bertaux *et al.* 2003; Bertaux *et al*. 2005). The endofungal bacterium *Burkholderia rhizoxininca* was detected as mycotoxins producer in the rice pathogenic fungus *Rhizopus microspores* (Partida-Martinez and Hertweck 2005; Lackner *et al.* 2009; Moebius *et al.* 2014). Diverse endohyphal bacteria were detected to dwell in the hyphae of phylogenetically diverse foliar fungal endophytes (Hoffman and Arnold 2010). The endobacteria associated with zygomycete fungus *Mortierella alpine* produce the quorum-sensing molecule N-acylhomoserine lactones (AHL) (Kai *et al.* 2012a and 2012b). One of the oldest plant-associated fungi endogone host unique mollicutes-related endobacteria (Desirò *et al*. 2015). Endobacteria associated with the genera of *Sebacina* and *Piriformospora* belong to the Gram-negative bacteria

Rhizobium and *Acinetobacter*, as well as the Gram-positive bacteria *Rhodococcus* and *Paenibacillus* as detected by cultivation in independent analysis based on 16S rRNA gene sequence analysis (Scharma *et al.*, 2008). The most comprehensively studied example of a tripartite Sebacinalean symbiosis is that of *Piriformospora indica* because it was possible to isolate the endobacterium and to grow independent of the fungal host. This enobacterium was identified by 16S rRNA gene sequencing as the Alphaproteobacterium *Rhizobium radiobacter* (syn. *Agrobacterium radiobacter;* syn. *Agrobacterium tumefaciens*) strain F4 (*Rr*F4) (Sharma *et al.* 2008).

The successful isolation of endobacteria from fungal hosts is a big progress on the way to explore the endobacterial lifestyle and the evolution procedure. The full genome sequencing of the isolated bacteria gives a glance into the mutualistic relationships. Bioinformatic analysis suggested that the endobacteria probably help to keep the fitness of the fungus through the synthesis of some antibiotic- or toxin-resistance molecules and vitamin B12 (Ghignone *et al.* 2012). Many of the obligate endofungal bacteria showed a reduced genome size compared with the free living bacteria species in the same genera such as the endobacteria *Burkholderia rhizoxinica* (Lackner *et al.* 2011; Naito *et al.* 2015; Fujimura *et al.* 2014; Torres-Cortés *et al.* 2015). The fungus-bacterial interaction has preceded the development of fungus symbiosis with plant.

1.3.3   Endofungal *Rhizobium radiobacter* F4

Sharma and coworker reported an endofungal bacterium *Rhizobium radiobacter* F4, which was detected in the cytoplasm of the endophytic sebacinalean fungus *Piriformospora indica* and subsequently isolated from the fungus host and successfully pure cultured on agar plate (Sharma *et al.* 2008). This bacterium is a rod-shaped bacterium 1-1.5 µm in length, it is described as *Alphaproteobacterium Rhizobium radiobacter* (syn. *Agrobacterium radiobacter*; syn. *Agrobacterium tumefaciens*) strain F4 (*Rr*F4).. After the successful isolation of *Rr*F4 bacteria, Sharma did the biological activity assay on model crop plant barley. Intriguingly, the plant

infected by bacteria *Rr*F4 showed certain growth promotion and pathogen resistance against barley powdery mildew, which are quite similar to the biological activity induced by fungus *P. indica*.

These endobacteria were detected by Fluorescence *in situ* hybridization (FISH) with a 16S rRNA targeting universal bacterial probe and a respective *Rhizobium*-specific probe, amplified by PCR with universal 16S rRNA primers and specific *Rr*F4's ITS primers. All these results showed the existence of one specific endobacteria endobacterium, *Rhizobium radiobacter* in the fungus *P. indica*, however the very few hybridization dots in FISH indicate the very few number of endobacteria exciting in the fungus host. The genomic DNA ratio between the endofungal *R. radiobacter* and *P. indica* was 0.035: 100, which further confirmed the low number of endobacteria in *P. indica* (Sharma et al. 2008). The same case was found in the mycelium of ectomycorrhizal *Laccaria bicolor*, which was reported by Bertaux that there were 1-20 bacteria per fungal cell (Bertaux *et al.* 2003; Bertaux *et al.* 2005).

1.4   The mutualistic fungus *Piriformospora indica*

The root endophytic fungus *Piriformospora indica* belongs to the order Sebacinales in the phylum Basidomycota, and was originally isolated from two shrubs' rhizosphere in 1996 in Indian Thar Desert, north-western Rajasthan (Weiß *et al.* 2004; Verma *et al.* 1998; Varma *et al.* 1999). *P. indica* produces pear-shaped chlamydospores with 8-25 nuclei in each spore, establishes biotrophic relationship with its plant host through the mycorrhizal hyphae (Verma *et al.* 1998; Waller *et al.* 2005). Molecular phylogenetic analyses revealed that *P. indica* has a close relationship with the species in the heterogeneous *Sebacina vermifera* complex, which shows similar biological benefits to plant host (Weiß *et al.* 2004; Deshmukh *et al.* 2006; Sharma and Kogel 2009). As a mutualistic endophyte, *P. indica* is the most studied model fungus in Sebacinales. The discovery of endofungal bacteria harbored by *P. indica* constitutes an intricate tripartite relationship among plant, fungus and bacteria.

1.4.1   Biological beneficial activity mediated by *P. indica*

P. indica colonizes a broad spectrum of monocotyledonous and dicotyledonous plants without any preference, induces resistance to leaf and root pathogens, promotes biological biomass and yield, and increases tolerance to abiotic stress such as salt stress (Peškan-Berghöfer *et al.* 2004; Waller *et al.* 2005; Deshmukh *et al.* 2006; Schäfer and Kogel 2009; Varma *et al.* 2012; Oberwinkler *et al.* 2014; Ye *et al.* 2014). The mechanism of *P. indica* functioning as disease control agent was mediated through induced systemic resistance (ISR) and depended on the jasmonic acid signaling pathway (Stein *et al.* 2008; Jacobs *et al.* 2011). Pathogenic assessments were performed in dico- and monocotyledonous with variety of pathogens, such as crop plant barley infected by powdery mildew fungal pathogen, wheat infected with *Xanthomonas translucens* pv. *translucens* DSM 18974^{T} , model plant *Arabisopsis thaliana* against *Golovinomyces orontii* and tomato against *Fusarium oxysporum* (Serfling *et al.* 2007; Waller *et al.* 2005; Stein *et al.* 2008). The strong growth promotion mediated by *P. indica* was revealed to rely on phospholipid signaling pathway and improved nitrate supply in cereals and *Arabidopsis thaliana*. The synthesis of phosphatidic acid (PA) is involved in the induced growth, in which phospholipases D (PLDα1 and PLDδ) are required in the upstream of the synthesis and PDKs 1.1 and 1.2 are involved in the downstream, there after the MPKs 3, 6 and Ca^{+} are activated (Camehl *et al.* 2011). In addition, some signaling components like LRR proteins and MATH protein and phytohormones such as gibberellin, cytokinins and ethylene are revealed to contribute to the growth promotion (Oelmüller *et al.* 2005; Chandler *et al.* 2002; Shahollari *et al.* 2007; Camehl *et al.* 2010; Jacobs *et al.* 2011; Vadassery *et al.* 2008; Schäfer *et al.* 2009; Sun 2008; Yadav *et al.* 2010). Several independent studies illustrated that the salt stress tolerance induced by *P. indica* is associated with changed antioxidative capacities in plant host (Baltruschat *et al.* 2008; Waller *et al.* 2005; Kumar *et al.* 2009; Vadassery *et al.* 2009).

1.4.2   Colonization pattern of *P. indica*

Root penetration and colonization strategies of *P. indica* on broad plant hosts were

revealed by cell biological studies and cytological analysis. The effective colonization starts from the germination of chlamydospores, fungal hyphae forms extracellular network in one day on the root surface (Deshmukh *et al.* 2006; Schäfer *et al.* 2009). Subsequently, biotrophic colonization phase starts, hyphae enter into the epidermal layer and branch through the intercellular space, intercellular hyphae in signal rhizodermal and cortical cells were visible at three days. Cell death-dependent colonization phase following after the biotrophic colonization was occurred after three days, cell death was proved by the disintegration of cytoplasm and endomembrane, and the incomplete endoplasmic reticulum (ER) and nucleus in the cell (Jacobs *et al.* 2011; Qiang *et al.* 2012). Fungus penetrates and colonizes adjacent cells after the completely filling in single cells, and there are necks formed on hyphae at the traverse site through cell walls (Deshmukh *et al.* 2006). Gradual increase of *P. indica* proliferation was detected on the maturation zone, abundant mycelium colonization on root surface and net-like inter- and intra-cellular hyphae were observed in the rhizodermal and cortical layers at seven days. Sporulation was mostly occurred at 14 days, single spores developed at the hyphal tips and a stack of spores were formed in root hair cells starting from the basal parts of root hair. The continuous research on barley, Arabisopsis and tomato has revealed that the maturation zone is the hot spot for *P. indica* while the elongation and meristematic zones are normal free from colonization (Deshmukh *et al.* 2006; Schäfer *et al.* 2009; Jacobs *et al.* 2011; Zuccaro *et al.* 2011; Qiang *et al.* 2012).

Phytohormones are involved in the innate immune suppression that is prerequisite for the successful colonization and the board host rang of *P. indica*. Plant host recognizes the mutualist *P. indica* through its MAMPs, which trigger the MTI in plant root and restrict the colonization and penetration of *P. indica* through different interaction stages (Jacobs *et al.* 2011). *P. indica* recruits JA to achieve the suppression of MTI through the oxidative burst in the early stage, SA- and indole glucosinolate-mediated defense play the dominant role through manipulating gene expression in the later stages. The root response to mutualistic *P. indica* is similar to the perception system

and immune response in leaves with pathogen (Jacobs *et al.* 2011; Qiang *et al.* 2012).

1.4.3   Genome and transcriptome analyses of *P. indica*

Zuccaro and colleagues did the genome sequencing and transcriptome analyses of *P. indica*, which provide a chance to look deeper insight the fungal lifestyle on the genome level (Zuccaro *et al.* 2011). Genome sequencing data showed that this fungus possesses a 24.97 Mb genome, and as a heterokaryon fungus contains two genetically distinct nuclei (Zuccaro *et al.* 2009; Zuccaro *et al.* 2011). Through the comparison of corresponding domains and protein binding motifs, it was shown that *P. indica* owns complex intracellular signaling pathways that sense and receive the signals from external environment and plant host. Three carbohydrate binding motifs are dominantly found, LysM functions on fungal cell wall as chitin-binding protein, WSC was described as sensor in yeast for the cell wall integrity, and CBM1 acts on plant cell wall as fungal hydrolytic enzyme (De Jonge *et al.* 2010; Varna *et al.* 1997; Boraston *et al.* 2004; Gaulin *et al.* 2004). About 10% of genes induced in *P. indica* during the colonization on root encoded small secreted proteins SSPs, which may function as effectors involved in the suppression of plant immunity, penetration and growth in plant cells (Qiang *et al.* 2012; Rafiqi *et al.* 2013). One candidate effector (PIIN_08944) was suggested to play a role during the root colonization of *P. indica* through suppression iof SA-mediated immune responses in plant host (Akum *et al.* 2015).

1.4.4   Comparison of arbuscular mycorrhiza and *P. indica*

Arbuscular mycorrhiza (AM) in the phylum Glomeromycota form peri-fungal membrane surrounding the AMF hyphae during the infection with plant (Bonfante and Genre 2010). The similar structure, which was called plant-derived membrane and used to separate the hyphae from plant cytoplasm, was sometimes found with *P. indica* during the fungal interaction with plant host (Lahrmann *et al.* 2013; Lahrmann and Zurrcaro 2012; Jacob *et al.* 2011). It was reported that the successful invasion of AMF into plant root cells requires a set of plant common symbiosis genes (CSGs)

involving in the signal transduction pathway, such as synthesis of SYMRK, CCaMK and CYCLOPS (Singh *et al.* 2014; Antolin-Llovera *et al.* 2014; Singh and Parniske 2012). However, the colonization and growth promotion mediated by *P. indica* in plant are independent from the classical CSGs, since there were intracellular hyphae and spores of *P. indica* formed in CSG mutants of *L. japonicus* (Banhara *et al.* 2015). *A. thaliana* as a number in the Brassicaceae family does not have one specific set of CSGs which results in the failure to interact with AMF. These research illustrated different pathways are exploited by *P. indica* and AMF during the infection with plant host.

1.5   Tripartite bacteria-fungi-plant interactions

Microbes ubiquitously colonize on the terrestrial plants in a high abundance in the ecosystem, in consequence an intricate and finely tuned tripartite interaction is established among bacteria, fungi and plant partner (Fig. 1.5) (Bonfante and Anca 2009). Soil fungi colonize on roots and form a bridge between soil and plant through hyphae, which increase the root surface area and promote the nutrient flow in the system. This bridge connection is even established among different plants boosting the horizontal nutrient movement. In this bipartite relationship, there is quite often additional bacterium partner who may colonize on the surface of mycorrhizal roots/extraradical hyphae, or live as endofungal bacteria in fungal cytoplasm, which make the relationship complicated and change the bipartite relationship into tripartite interaction.

There is spatially and physiologically specificity about microbial biodiversity in the rhizosphere. Investigations showed that bacterial assemblage on roots are mainly determined by fungi and influenced by soil pH. However, fungus themselves are mostly influenced by plant community composition, such as the ectomycorrhizal fungi *Paxillus involutus* (Kisa *et al.* 2007; Roesti 2005; Singh *et al.* 2008; Vestergård *et al.* 2008). Streptomycetes have been identified as modulators of plant symbiosis, and facilitate the root colonization of pathogenic fungi through repressing plant responses (Schrey and Tarkka 2008). The fungus *Glomus intraradices* could grow and

sporulate independently of its plant host in the presence of bacterium *Paenibacillus validus* that might release raffinose, which indicate an interesting argument that bacteria may give out some compounds mimicking plant molecules (Bouwmeester *et al.* 2007; Hildebrandt *et al.* 2002; Hildebrandt *et al.* 2006). The effect of bacteria on plant-fungus microhabitat is multifaceted, ranging through the production of phytohormones to alleviating pathogens or other abiotic stress (Artursson *et al.* 2006; Bonfante and Anca 2009). Bacteria produce extracellular polymers assisting the attachment to ensure a physical contract with fungi, excrete organic acids as carbon source for associated fungi (Bianciotto *et al.* 2001; Frey-Klett *et al.* 2007). Bharadwaj et al. found that high number of spore-associated bacteria could inhibit the plant pathogen *Rhizoctonia solani* (Bharadwaj *et al.* 2008).

Fig. 1.5 Illustration of interactions established among plants, micorrhizal fungi and bacteria in the root rhizosphere (taken from Bonfante and Anca 2009). Ectomycorrhiza and arbuscular mycrorrhiza were illustrated on the left side and right side of plant root respectively. Endobacteria (white color) are presenting in spores and intraradical mycelium of AM fungi; Rhizosphere bacteria (red color) are beneficial or detrimental to other partners through the release of some diffusible factors; Other bacteria (green color) may play positive effects through physical contact with fungus-root surface. Arrows illustrate the release of some diffusible factors, for instance strigolactones, auxin-like molecules volatiles and some Myc factors, that are further perceived by reciprocal partners.

From loosely or tightly associated bacteria to endobacteria resided in the fungal cytosol, a more intriguing tripartite relationship is exemplified in the context of toxinogenic *Burhholderia-Rhizopus* symbiosis. *Rhizopus microspores* is a zygomycete fungus and causes rice seedling blight disease, which results in severe loss in agriculture for famers (Iwasaki *et al.* 1984; Iwasaki *et al.* 1986). The endosymbionts *Burkholderia rhizoxinica* living in *Rhizopus microspores* act as a producer of rhizoxin, which is further processed by the fungal host into phytotoxin rhizoxin. This phytotoxin rhizoxin blocks mitosis in the plant cells by binding to the β-tublin, kills the rice plant and causes rice seedling blight (Partida-Martinez and Hertweck 2005; Partida-Martinez *et al.* 2007a; Scherlach *et al.* 2006; Lackner *et al.* 2009). During this process, the endofungal bacteria help host fungi producing phytotoxin to kill rice plant, while the host fungi provides a protective shelter for its bacteria partner, then both of them benefit from the nutrients released from dead rice plant (Scherlach *et al.* 2012). Fungal self-resistance to the antimitotic toxin was correlated with the rhizoxin sensitivity to β-tublin sequences (Schmitt *et al.* 2008). *Burkholderia rhizoxinica* is vertically transmitted in the fungal vegetative reproduction, controls fungal sporulation since the cured fungi doesn't have spores anymore, has the capability to infect the fungus host again (Partida-Martinez *et al.* 2007b; Nadine *et al.* 2014). Genome sequencing of this culturable microbe gave detailed insight into the evolution and metabolic connection of this symbiont relationship (Lackner *et al.* 2011a and 2011b).

1.6 Objectives

The root endophyte *Piriformospora indica* establishes a biotrophic relationship with a broad spectrum plants, promotes biomass and crop yield, induces resistance to leaf and root pathogens, and increases tolerance to abiotic stress. The discovery of the endofungal bacterium *Rhizobium radiobacter* F4 living in *P. indica*'s cytoplasm suggested that P. indica forms an intricate tripartite-relationship with the plant host and the bacterium.

In my thesis, I addressed the question who is the partner responsible for growth promotion and biotic/abiotic resistance induced in the plant host, the endophyte *P. indica*, the endobacterium *Rr*, or both the fungus and the bacterium when functioning together? For this aim, I explored the colonization pattern of GUS/GFP-labeled *Rr*F4 on barley and Arabidopsis roots with fluorescence/light microscope, confocal laser scanning microscopy, scanning and transmission electron microscopy. In order to reveal what is happening to the bacteria during the colonization of *P. indica* with the plant, the number and release situation of the endobacteria was analyzed. To compare the biological activity mediated by *P. indica* with and without endobacteria, I developed several strategies to cure *P. indica* from its endobacterium.

2   Materials and Methods

2.1   Bacterial and fungal materials

2.1.1   *Piriformospora indica*

Piriformospora indica-DSM11827 was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany. This isolate came from one original sample collected in the Thar desert, India in 1997 (Varma *et al*. 1998). *P. indica* was propagated on modified *Aspergillus* complex medium (CM medium) at room temperature (Pham *et al*. 2004). Chlamydospores were harvested from the 3-4 weeks old culture on agar plate, and sub-cultivated in Erlenmeyer flask filled with liquid CM medium shaking 130 rpm at room temperature.

CM medium (1L)	
20×salt solution	50 mL
Glucose	20 g
Peptone	2 g
Yeast extract	1 g
Casamino-acid	1 g
1000×Microelements	1 mL
Agar-agar	15 g
A. dist. H_2O	950 mL

20×salt solution (1L)	
$NaNO_3$	120 g
KH_2PO_4	30.4 g
KCl	10.4 g
$MgSO_4 \cdot 7H_2O$	10.4 g

1000×Microelement (1L)	
$ZnSO_4 \cdot 7H_2O$	2.65 g
H_3BO_3	1.5 g
$MnCl_2 \cdot 4H_2O$	6.0 g
KI	0.75 g
$CuSO_4 \cdot 5H_2O$	0.13 g
$Na_2Mo_7O_4 \cdot 2H_2O$	0.0024 g

2.1.2 *Rhizobium radiobacter* **F4**

Rhizobium radiobacter F4 is a subculture of strain PABac-DSM, which was isolated from *P. indica* DSM 11827 by Sharma in 2008 (Sharma *et al.*, 2008). *Rr*F4 was cultured on solid YEP medium at room temperature for one and a half days or in liquid YEP medium in Erlenmeyer flask on shaker 130 rpm at room temperature. For long time preservation, *Rr*F4 was cultured in liquid YEP medium till $OD_{600}=0.6$, then supplemented with 20% (v/v) glycerol and stored at -80 ℃.

YEB medium (1L)	
Beaf extract	5 g
Yeast extract	1 g
Caseinhydrolysate	5 g
Sucrose	5 g
$MgSO_4 \cdot 7H_2O$	0.49 g
Agar-agar	15 g
Adjust the pH to 7.2	

2.1.3 **Generation of β-glucuronidase (GUS) - and GFP-*Rr*F4**

For histochemical localization of *Rr*F4 in roots, GUS (ß-glucuronidase) and GFP (green fluorescent protein) tagged *Rr*F4 were generated with the plasmid pLH6000 (DNA cloning service, Germany).

2.1.3.1 **Generation of electro-competent *Rr*F4 cells**

The electro-competent *Rr*F4 cells were made first. A single colony of *Rr*F4 was inoculated in 5 mL LB medium with 100 μg/mL gentamycin, and cultured overnight at 28℃ with vigorous shaking 220 rpm; Sub-cultured in 15 mL LB medium for 4-6 h until $OD_{600}=1.0-1.5$; Transfer the bacteria on ice for 10 min, and centrifuge 10 min by 4 500 rpm at 4 ℃; Afterwards re-suspend the pellet into sterile ice-cold 10% glycerol, centrifuge 10 min by 4500 rpm at 4℃ (two times); Finally re-suspend the cells in ice-cold 10% glycerol, aliquot into 50 μL and freeze in liquid nitrogen.

2.1.3.2 Electroporation

The GUS-containing plasmid was generated by introducing *GUS* gene into the plasmid pPCV812 under the control of a *mannopine synthase* (*mas*) promoter (Imani *et al*., 2002). 0.5 mL plasmid DNA (10-100 ng) was mixed with 50 μL competent cells, applied the pulse by Gene Pulser Xcell^TM (Bio-RAD, UAS). The cells were re-suspended in 1 mL SOC medium after 5 min on ice and incubated at 28 ℃ for 1 h, spread the cells on LB medium with 100 μg/mL gentamycin. The X-Gluc (Duchefa, Netherland) was used in this research as GUS substrate (Jefferson, 1987).

The GUS-containing plasmidFor GFP-*Rr*F4, a codon optimized *GFP* (Cormack *et al*., 1996) was cloned into a pGEM-T Easy vector (Promega, Germany), cut by EcoR1 restriction enzyme and inserted into plasmid Plh6000 under the constitutive control of the *E. coli ribosomal protein* promoter (Post *et al*., 1980), re-cut by restriction enzyme EcoR1 and inserted into pLH6000. GFP-*Rr*F4 was confirmed by fluorescence microscopy with GFP-specific filter sets.

LB medium (1 L)	
Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
Agar-agar	15 g
Adjust pH to 7.0, sterilize by autoclaving	

SOC medium (100 mL)	
Trytone	2 g
Yeast extract	0.5 g
5 M NaCl	0.2 mL
1 M KCl	0.25 mL
1 M MgCl2	1 mL
1 M Glucose	2 mL
Sterilize by autoclaving	

X-Gluc (50 mL)	
X-Gluc	50 mg
0.1 M Phosphate buffer (pH 7.0)	49 mL
5 mM Potassium ferricyanide (MW 329.2)	500 µL
5 mM Potassium ferrocyanide (MW 422.4)	500 µL
Triton x-100	50 µL

Sterilize with a 0.2 µm filter, store at -18℃ at dark.

0.1 M Phosphate buffer (418 mL)	
0.2 M KH_2PO_4 (2.7g/100ml)	87 mL
0.2 M K_2HPO_4 (6.96g/200ml)	122 mL
A. dist. H_2O	209 mL

Adjust pH 7.0, autoclave.

2.2   Plant materials and growth conditions

Barley (*Hordeum vulgara* L.) cultivar Golden Promise and *Arabidopsis thaliana* ecotype Colombia-0 (Col-0, N1092) were used in this research.

2.2.1   Germination of seeds

For aseptically grown barley seedlings, the seeds were firstly surface sterilized in 70% (v/v) ethanol for 5 min, followed by a subsequent exposure to NaClO (6% active chlorine) solution with two drops of Tween 20 for 1.5 h. Thereafter, seeds were rinsed in pH 3.0 distilled water once and normal distilled water three times in clean bench. After peeling off the glumes, seeds were placed on sterilized filter paper in aseptic jar and germinated three days in the culture room (24 ℃,16/8 h photoperiod).

For aseptically grown *Arabidopsis thaliana*, the seeds were sterilized in 70% ethanol for 1 min and NaClO (3% active chlorine) solution for 10 min, rinsed in distilled water as above and dried on sterilized filter paper in clean bench. The dried seeds were placed on solid 1/2 MS medium with sucrose at 4 ℃ for two days and then germinated in the culture room for one week.

2.2.2   Root inoculation

Three-week-old *P. indica* cultures were used in root inoculation experiment. The

chlamydospores were collected with autoclaved 0.002% tween-20 water by gently scratching the surface of *P. indica* culture on petri dishes with spatula. The spores were filtered with miracloth to remove the mycelium and centrifuged at 3500 rpm for 7 min to pellet the spores. Subsequently, the pellet was suspended in tween-20 water after washing three times. The spore concentration was detected with microscopy and adjusted to 100000 spores per mL. Three days old barley seedlings were dip-inoculated in *P. indica* spores solution for 2 h, while the tween-20 water was used as mock.

GUS/GFP-*Rr*F4 and wild type *Rr*F4 were cultured in YEB medium at 28 ℃ overnight, centrifuged and re-suspended in 10 mM $MgSO_4.7H_2O$ solution with OD_{600}=1.4. Subsequently, three days old barley seedlings or one week old *Arabidopsis thaliana* seedlings were dip-inoculated in the bacteria solution for 30 min, while 10 mM $MgSO_4.7H_2O$ solution was used as mock.

2.2.3   Seedling growth conditions

After root inoculation, barley seedlings were grown on 1/2 MS medium in culture room or in pot in a climate chamber. The pot was containing 3:1 mixture of expanded clay (Seramis®, Masterfoods) and Oil Dri® (Damolin). The growth condition in the climate chamber was 16 h photoperiod, 22/18 ℃ day/night and a photon flux density of 160 µmol $m^{-2} s^{-1}$. The seedlings in per pot were fertilized once every week with 30 mL Wuxal N solution (Schering, N/P/K: 12/4/6).

Arabidopsis thaliana seedlings were grown vertically on 1/2 MS medium in squared petri dishes (Greiner Bio-One). The controlled growth condition for *Arabidopsis thaliana* was 8/16 h light/dark, 22/18 ℃ and 60% rel. humidity. The roots were harvested at 0 day post-inoculation (dpi), 5, 7, 14 and 21dpi, used for q-RT-PCR or staining.

1/2 Murashige-Skoog (MS) medium (1L)	
MS	2.20 g
Sucrose	5 g
Adjust PH 5.8	
Gelrite	4 g

2.3 Basic molecular biological methods
2.3.1 Genomic DNA extraction

The genomic DNA from bacteria, fungi and plant were extracted with different approaches during this study.

The DNA of pure *Rr*F4 was extracted with the Freeze-thaw method: the overnight cultured *Rr*F4 was centrifuge at 8000 rpm for 5 min, the pellet was washed with 500 µL 1×PBS and stored at -20 ºC. For freeze-thaw DNA extraction, 500 µL distilled water was added to re-suspend the sample, then the sample was heated for 1 min at 110 ºC and frozen at -20 ºC (repeated for three times), finally the genomic DNA from *Rr*F4 was ready to use.

The biomass of fungal mycelium was collected from liquid culture or agar-plate, then immediately frozen in liquid nitrogen and ground into fine powder with mortar and pestle. The plant root was harvested from jar, pot or square petri dish, then frozen and ground into fine powder as above. 100-200 mg powder was used for DNA extraction by Plant DNeasy kit (QIAGEN, GmbH, Hilden, Germany), or 400-500 mg power used for DNA isolation with the NucleoSpin® Soil Kit (Macherey-Nagel, Germany) with lysis buffer SL1 according to manufactures' instructions. DNA was eluted in 30 µL Milli-Q water, the concentration was measured using NanoDrop ND-1000 (Peqlab Biotechnology GmbH, Erlangen, Germany). DNA samples with concentration 200 ng/µL were used for PCR or stored at -20 ºC.

2.3.2 Polymerase chain reaction (PCR)

The conventional amplification was performed in a PCR Thermo cycler (Biometra,

GmbH, Germany). Two types of DNA polymerase (GoTag®DNA Polymerase, Promega, Germany; DCS Pol, Germany) were used to amplify genes of interest from different DNA templates. The standard PCR mixture and thermal cycle program are given below. The elongation time normally depends on the size of the PCR product (1kb/min), while the annealing temperature is adjusted to the primers.

GoTag DNA Polymerase (25 µL)	PCR mixture
5×Buffer	2 µL
dNTPs 2 mM	0.5 µL
$MgCl_2$ 25 mM	1.5 µL
Fwd primer 10 µM	0.3-1.0 µL
Rev primer 10 µM	0.3-1.0 µL
DNA Polymerase 5 u/µL	0.25 µL
Template	1 µL
A. dist. H_2O	Up to 25 µL

DCS Pol PCR mixture		Thermal cycle program		
10× BD Buffer	2.5 µL	95 °C	3 min	
dNTPs 2 mM	2.5 µL	95 °C	30 sec	
$MgCl_2$ 25 mM	2.5 µL	X °C	30 sec	30-35 cycles
Fwd primer 10 µM	0.3-1.0 µL	72 °C	1 min/kb	
Rev primer 10 µM	0.3-1.0 µL	72 °C	10 min	
DCS-Polymerase	0.3 µL			
Template	1 µL			
A. dist. H_2O	Up to 25 µL			

2.3.3   Total RNA extraction

Plants root were harvested at different time points, quickly frozen in liquid nitrogen, ground into fine powder and stored at -80 ºC. Total RNA was extracted using TRIzol (Invitrogen, Karlsruhe, Germany). 1 mL TRIzol reagent was added into 100-150 mg tissue powder, vortexed for 15 sec and incubated 5 min at room temperature (RT). 200 µL chloroform was added, mixed well by vortexing and centrifuged at 15000 rpm and

4 ºC for 20 min after 3 min of incubation at RT. Subsequently, the supernatant was transferred to a 1.5 mL eppendorf tube containing 500 µL isopropanol and incubated for 10 min at RT. Then the sample was centrifuged at 15000 rpm and 4 ºC for 30 min for RNA precipitation. The pellet was washed with 1 mL 75% (v/v) ethanol (centrifugation 13 000 rpm at 4 ºC for 5 min) and dried in a clean bench for 10-15 min. The RNA was resuspendend in 30 µL DEPC by incubatation at 65 ºC for 5 min. The concentration of total RNA was measured using a NanoDrop ND-1000 (Peqlab Biotechnology GmbH, Erlangen, Germany) with wavelength of 260 nm and 280 nm. RNA extracts were stored at -80 ºC.

2.3.4 Reverse transcriptional polymerase chain reaction (RT-PCR)

The total RNA was transcribed into cDNA using the Quantitect® Reverse Transcription kit (QIAGEN, GmbH, Hilden, Germany). According to the protocol, 1000 ng RNA was mixed with 2 µL 7×gDNA Wipeout buffer with DEPC water to the volume of 17 µL, and incubated at 42 ºC for 2 min. Then 1 µL RT-primer mix, 4 µL 5×Quantiscript RT buffer and 1 µL Quantiscript reverse transscriptase were added to the final volume of 23 µL. Subsequently, the reaction was performed in a Thermo cycler (Biometra, GmbH, Germany) with the program below. The cDNA was diluted to 10 ng/µL with DEPC water, used for quantitative Real-Time PCR analysis immediately or stored at -80 ºC for later analysis.

Reverse transcription program

22 ºC 3 min

42 ºC 30 min

85 ºC 3 min

4 ºC 10 min

2.3.5 Quantitative Real-Time PCR (qPCR)

The quantitative real-time PCR was performed on a 7500 Fast Real-Time PCR System (Applied Biosystems, CA, USA) to quantify the relative/absolute amount of

*Rr*F4/*P. indica* to see the number of *Rr*F4 cells in *P. indica* or the relative expression of some transcripts. The standard curve approach is often used for the absolute quantification. It is prepared from samples with known template concentration, and the concentration of unknown target gene is calculated by the interpolation of its PCR signal into the standard curve. In this research the internal transcribed spacer (ITS) region was used to quantify the amount of *Rr*F4. The standard curve was generated through two steps of amplifications. First, the genome of *Rr*F4 was amplified with universal bacterial primer pair E786 (5'-GATTAGATACCCTGGTAG-3') and 23Sr (5'-GGGTTBCCCCATTCRG-3'), the 2182 bp PCR product containing partially 16S rRNA, the ITS regions and partially 23S rRNA was subsequently amplified with *Rr*F4 specific primer pair ITS-Rh-F (5'-TCAGCACATAACCACACCAATCGCG-3') and ITS-Rh-R (5'TGCTTTGTACGCTCGGTAAGAAGGG-3') and generated a 266 bp PCR product. The quantification of *P. indica* was relying on the *Tef* (transcriptional enhancer factor-1) gene. The standard curve for the quantification of *P. indica* was generated through the universal fungal primer pair tefO (5'-GGGTGCTCGACAAGCTCAA-3') and tefJ (5'-ACACATCCTGGAGTGGGAGA-3') with 800 bp PCR product and nest PCR with *P. indica* specific primers PiTef-For (5'-TGCGTCGCTGTCAACAAGATG-3') and PiTef-Rev (5'-ACCGTCTTGGGGGTTGTATCC-3') with 162 bp PCR product. the standard curves were generated by the dilution of nest PCR products with the serial number of target genes from 10^2 to 10^9. The amplification plot, melt curve and standard curve were shown below. All the samples were coming from three independent biological experiments and measured with three technological replicates. There are two phases in a qRT-PCR program. The first phase is standard PCR amplification and fluorescence determination, the second phase is the production of melting curve, which is an assessment of the dissociation of double-stranded DNA fragments during raising temperature and indicates the specific amplification of primers. The melting peak shows 50% of PCR products are denatured. The cycle threshold (Ct) value was recorded and the relative expression $2^{-\Delta Ct}$ value was

calculated with the method from Livak & Schmittgen (Livak and Schmittgen 2001).

PCR mixture (1x)

Green JumpStart Taq ReadyMix (SigmaAldrich)	10 µL
Primer fwd 10 µM	0.3-1.0 µL
Primer rev 10 µM	0.3-1.0 µL
Template	variable
A. dist. H_2O	Up to 20 µL

Thermal cycle program

95 °C	4 min	
95 °C	30 sec	
60 °C	30 sec	40 x
72 °C	30 sec	
83 °C (optional)	30 sec	
95 °C	15 sec	
68 °C	60 sec	Melting curve
95 °C	30 sec	
24 °C	15 sec	

2.3.6 Agarose gel electrophoresis

DNA and RNA samples were visualized and detected with agarose gel electrophoresis. 2 µL DNA samples or 20 ng PCR products were mixed with 10×DNA loading buffer and loaded on 0.8-2% (w/v) agarose gel containing about 1 mg/ml ethidium bromide. The gel was running in 1×TBE buffer at 70-150 V for 45-60 min. The 1 kb plus DNA ladder (Invitrogen, Darmstadt, Germany) was used on the agarose gel as standard marker. RNA samples were mixed with 2×RNA loading dye (Fermentas, St.

Leon-Roth, Germany), denatured at 95 °C for 5 min, and loaded on 1% agarose gel containing 1×MOPS buffer and 5% (v/v) formaldehyde. Subsequently, a UV-Transluminator (Fröber Larbortechnik, Lindau, Germany) was used to detect all the signals on the gel, a camera (digitStore, INTAS, Göttingen, Germany) was used to document the gels.

10 x TBE (Tris-Borate-EDTA)	10 x MOPS buffer pH 7.0
0.9 M Tris	200 mM MOPS
0.9 M Boric acid	50 mM sodiumacetate
0.025 M EDTA	10 mM EDTA
Adjust pH 8	adjust pH 7.0
A. dist. H_2O up to 1 L	A. dist. H_2O up to 1 L

2.3.7 Denaturing gradient gel electrophoresis (DGGE)

DGGE is a technique to separate DNA fragments with the same length but different G+C content and nucleotides according to the mobilities under the increasing gradient of formamide and urea based denaturing gel conditions. DGGE as community fingerprint pattern can be used to characterize microbial populations in the environment (Muyzer and Smalla 1998). The DNA used for DGGE analysis was extracted by NucleoSpin® Soil Kit (Macherey-Nagel, Germany) with adjusted protocol as described in 2.3.1. The bacterial 16S rRNA gene was used as target gene, amplified by primers 799f/1492r (799f: 5'-AACMGGATTAGATACCCKG-3'; 1492r: 5'-TACGGYTACCTTGTTACGACTT-3') (Chelius et al. 2001) and followed by a nest PCR with 42 bp GC clamp on the 5'end of the GC primer GC968f/1378r (GC968f:5'-AACGCAAGAACCTTAC-3';1378r:5'-CGGTGTGTACAAGGCCCGG GAACG-3') (Heuer et al. 1997). PCR products 25 µl mix with 5 µl loading dye were loaded on a pre-warmed polyacrylamide gradient gel, DGGE standard containing 16S rRNA genes from six bacteria strains was added on the gel (*Novosphingobium fuchskuhlense* FNE08-7[T], *Massilia timonae* CCUG 45783[T], *Novosphingobium acidiphilum* FSW06-204d[T], *Achromobacter denitrificans* DSM 30026[T], *Acidovorax*

delafieldii LMG 5943[T] and *Nocardia jiangxiensis* DSM 17684[T]).

Polyacrylamid gels containing formamide and urea (40-70%) were used for the DGGE analysis. The solution A, solution B and stacking gel were prepared from 0% and 80% stock solutions supplemented with TEMED and APS. Then solutions were poured in the front chamber through a pipe connected with a pump (Minipuls 2, Gilson. Inc). The casting was stopped before the gel reaching the comb. Stacking gel was pipetted on the top of the gel to generate slots on the DGGE gel. The comb was taken out after one h, the gel was stored over night at 4 ℃. The electrophoresis was performed in a tank containing 17 L pH 7.4 1×TAE buffer. The samples were loaded after the gradient gel was pre-warmed up to 60 ℃ under 100 V. The electrophoresis was performed with the parameters 100 V, 60 ℃ and 22 h. The bottom glass plate attached with gel was stained in ethidium bromide (30 µL ethidium bromide mixed with 500 mL pure water) for 20 min and washed in pure water for 10 min. The gel images were taken with imaging system software (Fluor-STM Multilmageer, BioRad) by exposure under UV 10-40 seconds.

Loading dye			50×TAE buffer (pH 7.4)	
Glycerol	7 mL		Tris base	242 g
Bromphenol blue	250 µL		Glacial acetic acid	57.1 mL
Xylene cyanol	250 µL		EDTA (0.5M, pH 8.0)	100 mL
DNase and RNase free water	2.5 mL		Pure water	Up to 1000 mL

Stock solution 80% (150 mL)			Stock solution 0% (150 mL)	
Urea	50.4 g		50 TAE	3 mL
Formamide	48 mL		40% Acrylamid/bisacrylamid	26.25 mL
50 TAE	3 mL			
40% Acrylamid/bisacrylamid	26.25 mL			

Stacking gel	
Stock solution 0%	9 mL
Stock solution 80%	0
TEMED	8 µL
APS (10%)	100 µL

Solution A (40%)			Solution B (70%)	
Stock solution 0%	12 mL		Stock solution 0%	3 mL
Stock solution 80%	12 mL		Stock solution 80%	21 mL
TEMED	17 µL		TEMED	17 µL
APS (10%)	86 µL		APS (10%)	86 µL

2.4 *In situ* detection of bacteria and fungi

2.4.1 Fluorescence *in situ* hybridization (FISH)

FISH is a widely used technique for the *in situ* analysis of microbial communities. The fluorescently labelled oligonucleotide probes are targeted to the ribosomal RNA (rRNA) and give out fluorescence signals under the fluorescence microscopy (Amann and Fuchs 2008). In this research FISH method was used to detect the endobacterium in the fungal materials. The applied FISH protocol used in this research was based on the description from Manz *et al.* (1996) with some modifications. The procedure was carried out as the flowing:

Fixation: All the samples were fixed to stabilize the cells and permeabilize the cell membranes. Overnight culture of bacteria or three weeks old fungal culture from agar plates were fixed in 50% ethanol (NaCl : ethanol 1 : 1) for 3-4 h at 4 °C or 4% ice-cold 1×PFA for 3-12 h at 4 °C. The solution was removed by centrifuge at 6000 rpm and 5 min at 4 °C. Thereafter, the samples were washed in 1×PBS for three times. Finally they were re-suspended in 1×PBS : 99.9% ethonal 1 : 1 and stored at -20 °C.

Lysozyme treatment: The fixed samples were pre-treated with lysozyme to enhance the permeabilization before hybridization step. Samples were pipetted and dried on the 6 recesses-microscope slide coated with gelatin (0.1% gelatin and 0.01% chromium potassium sulfate). 20 µL 1 mg/mL lysozyme was added to each samples on the slide and incubated in root temperature for 20 min. Remove the lysozyme from the slide carefully with 1 mL ice-cold 1×PBS, subsequently dip the slide into ice-cold 1×PBS for 15 sec and dry the slide at 46 °C.

10 µg/mL Proteinase K was used to treat the samples at 37 °C for 30 min, then inactive the proteinase K by incubating in 0.01 M HCl at room temperature for 10 min.

Wash the samples with ice-cold 1×PBS and dry the slide at 46 °C.

Dehydration: The dried samples were dehydrated in an increasing ethanol series 50%, 80% and 96% (v/v) three min for each.

Hybridization: 9 µL hybridization buffer containing corresponding concentration of formamide were mixed with 1 µL of each probe (50 ng/µL) and added into the each sample. The slide was put into a 50 mL tube with moistened tissue and incubated for 1.5 h at 46 °C in a dark hybridization oven.

Washing: The hybridization buffer on the slide was washed away with washing buffer, which was pre-warmed at 48 °C. The samples were incubated in the 50 mL washing buffer at 48 °C for 15 min. Afterwards the slide was washed by pure H_2O and dried at RT in the dark.

Microscopy: 10 µL DAPI was applied to each sample on the slide, incubate at RT for 3 min and remove the DAPI solution by rinsing with distilled water. The air-dried samples were mounted in AF1 anti-fading reagent (Citifluor Ltd., London, UK) and covered the samples with a cover slide, then observed with epi-fluorescence microscope (Leica DM 5000B, Germany). The excitation and emission wavelength were 488 nm and 530 nm for FITC labeled EUB-338, 358 nm and 461 nm for DAPI staining.

Hybridization buffer for FISH

Formamid %	5M NaCl µL	1M Tris-HCl µL	10%SDS µL	Formamid µL	A. dist. H_2O µL	Total volume mL
0%	360	40	2	0	1600	2
20%	360	40	2	400	1200	2
25%	360	40	2	500	1100	2
30%	360	40	2	600	1000	2
35%	360	40	2	700	900	2

Washing buffer for FISH

Formamid %	0.5M EDTA µL	1M Tris-HCl (pH 8.0) mL	10% SDS µL	5 M NaCl µL	A. dist. H$_2$O mL	Total volume mL
0	500	1	50	9000	39.45	50
5	500	1	50	6300	42.15	50
10	500	1	50	4500	43.95	50
15	500	1	50	3180	45.27	50
20	500	1	50	2250	46.2	50
25	500	1	50	1590	46.86	50
30	500	1	50	1120	47.33	50
35	500	1	50	800	47.65	50
40	500	1	50	560	47.89	50
45	500	1	50	400	48.05	50
50	500	1	50	280	48.17	50
55	500	1	50	200	48.25	50

Phylogenetic oligonucleotide Probes and % Formamide

Probe	Target	Specificity	% Formamide
EUB-338 I, II, III	16S rRNA	All bacteria	0-35
Rh-1247	16S rRNA	Rhizobium (Rhizobium, Agrobacterium, Ochrobactrum)	35
ALF-968	16S rRNA	Alphaproteobacteria (Sphingomonas, Agrobacterium, Rhodobacter)	20-35
BET-42a	16S rRNA	Betaproteobacteria (Nitrosomonas, Acidovorax, Sphaerotilus, Comamonas)	35
GAM-42a	16S rRNA	Gammaproteobacteria (Enterobacteria, Pseudomonas)	35
ALF-1b	16S rRNA	Alphaproteobacteria	20
HGC-69a	16S rRNA	Actinomaycetes (Streptomyces, Nocardia, Corhnebacteria)	25
EUK-516	18S rRNA	Eukaryotes	0
Univ1392		All organism (Bacteria, Archea, Eukaryotes)	0
CF-319a+b		Cytophaga-Flavobacterium-Flexibacter groups	35
PLA-46		Planktomycetes	25
PLA-886		Planktomycetes	25

10 X PBS	
NaH$_2$PO$_4$	12 g
Na$_2$HPO$_4$	14.2 g
NaCl	75.70 g
A. dist. H$_2$O	1000 mL
pH 7.2 - 7.4	

5M NaCl	
NaCl	292.0 g
A. dist. H$_2$O	1000 mL

0.5M EDTA			10 % SDS (sodium dodecyl sulphate)	
EDTA ($C_{10}H_{16}N_2O_8$)	146.10 g		SDS	10 g
A. dist.H_2O	900 mL		A. dist. H_2O	100 mL
Adjust pH 8.0 (NaCl)			Filter sterilization	
A. dist. H_2O	Up to 1000 mL			

1M Tris-HCl			Lysozyme solution 1mg/mL	
Tris ($NH_2C(CH_2OH)_3$)	121.14 g		Lysozyme	10 mg
A. dist.H_2O	900 mL		0.5 M EDTA (pH 8.0)	100 µL
Adjust pH 8.0 (HCl)			1 M Tris-HCl (pH 8.0)	100 µL
A. dist.H_2O	Up to 1000 mL		Pure water	800 µL

The oligonucleotide probes used in this research were EUB-338-mix (EUB-338, EUB-338-Ⅱ, an EUB-338-Ⅲ) (sequence 5' GCTGCCTCCCGTAGGAGT3') for bacteria (Daims et al. 1999), EUK-516 (sequence 5' ACCAGACTTGCCCTCC3') for fungus (Amann et al. 1990) and Rh-1247 (sequence 5' TCGCTGCCCACTGTG3') for *Rhizobium* (Ludwig et al. 1998). The probes were labeled with fluorescence FITC or Cy3.

2.4.2 Sybr green I (SG-1) staining

Sybr green I is a double stranded DNA staining dye, it stains DNA of fungi and bacteria. The *P. indica* material was fixed in 99.9% ethonal : 1×PBS (1 : 1) overnight at 4 ℃, then filtered onto black 0.22 µL GTBP brown Isopore^{TM} membrane filter using a vacuum pump. Subsequently, the filter was placed onto one glass slide, 5 µL Syber Green I moviol staining solution (Lunau *et al.* 2005) was dropped on the filter and followed by covering with cover slide. Thereafter, the slide was observed with epi-fluorescence microscopy.

2.4.3 GUS-staining

Sampled barley or *Arabidopsis thaliana* roots were washed in 70% ethanol for 1 min and distilled water for 1 min. Subsequently, the roots were treated with water sonication (Sonorex RK106, Berlin, Germany) for 30 sec and three times. Thereafter

X-Gluc was added and inoculated with the samples in 15 mL tube at 37 ℃ for overnight. X-Gluc was converted into a blue colored product by the β-glucuronidase expressed by the transformed tissue cells (GUS assay; Jefferson, 1987).

2.4.4 WGA-staining and Congo red staining

Root samples were washed in distilled water three times and then fixed in fixation solution for 24 h. The fixation solution contained chloroform: ethanol : trichloroacetic aci (20% : 80% : 0.15%). Subsequently, the materials were treated with 10% KOH solution for 30 sec and washed in 1×PBS buffer (pH 7.4) for three times (5 min for each time). Thereafter, the root materials were embedded in staining solution containing 10 μg/mL WGA Alexa Fluor® 488 (Molecular Probes, Karlsruhe, Germany) and 0.02% Silwet L-77 in 1×PBS buffer. Root material was stained for 10 min in the staining solution. During this time, the vacuum infiltration was applied to the root staining for three times (each time 25 mm Hg for 1 min). Finally, the roots were put on a glass slide for microscopy after washing in 1×PBS buffer. The florescence from WGA-AF 488 was detected with epi-fluorescence microscopy (Axioplan 2, Zeiss, Oberkochen, Germany). The emission and absorption wave length were 470/20 nm and 505-530 nm, respectively. The same method was used for Congo red (Merck, Darmstadt, Germany) staining, which was detected by microscopy with emission at 546/12 nm and absorption at 590 nm.

2.5 Microscopy analysis

Different kinds of microscope were used in this research for different samples and purposes. FISH samples were detected with epi-fluorescence microscope and confocal laser scanning microscopy (CLAM). GUS-*Rr*F4 bacteria and related root samples were observed with light microscope, transmission electron microscopy (TEM) and scanning electron microscopy (SEM). GFP-*Rr*F4 and related root samples were analyzed with epi-fluorescence microscope and CLSM.

Light microscopically analysis of cross section with GUS-stained barley roots GUS-stained root samples were fixed for cross section analysis (Grieb 1992). The

roots were fixed in ethanol : eisessig : formaldehyde 9 : 0.5 : 0.5 solution for 48 h, dehydrated in 70% (v/v) ethanol for 4 h, and H_2O : ethanol : tert-butanol 15 : 50 : 35 for 2 h. Afterwards, treated with ethanol : tert-butanol 25 : 75 for 2 h, sopropand : tert-butanol 25 : 25 for 2 h, and tert-butanol for 12 h. Thereafter, embedded in tert-butanol : paraffin 1 : 1 for 48 h at 60 ℃. Then the samples were cut by microtome (Reichert, Vienna, Austria) into 30 μm thick sections and mounted on the microscopical slide.

Partial function of paraffin here is making the sample cutting easier, but there will be negative impact from the paraffin when observe the sample under microscopy. So the de-parafinization step was followed to remove paraffin from the slide. The slides were imbedded in xylol solution twice with each time 5 min. Subsequently, the slides were dehydrated in graded ethanol series (96-, 70-, 50%; 5 min each step), and again in the ethanol series (50-, 70-, 96%, 5 min each step). Thereafter, the slides were imbedded in xylol solution for 15 min, covered the sample with a cover slide when the cross section samples were dry. Finally, the slides were investigated by a Leica DM IL microscope (Leica, Wetzlar, Germany).

2.5.1 Confocal laser scanning microscopy (CLSM)

The confocal laser scanning microscopy (CLSM) was used to analyze the colonization pattern of GFP-tagged *Rr*F4 on barley and *Arabidopsis* seedlings. The seedlings were carefully separated from the ½ MS medium and washed in water. Roots were imaged directly with CLSM for the intact image or cross sectioned in a cryo-microtome after fixation in 3% formaldehyde with 0.05 M cacodylat buffer, freezed protection with sucrose, embedding in Tissue-Tek and freezing in isopropanol. Sections (30 μm thick) were embedded in Mowiol or RotiMount FluorCare (Carl Roth, Germany). Specimens were placed on glass slides and covered with cover slides (no.: 1.5; thickness: 0.17 μm) and imaged using a Leica TCS SP5 VIS confocal laser scanning microscope and a 63 x 1.30 GLYC objective providing an increased working distance. The 488 nm excitation laser line and 496-550 nm

emission filter were used to detect the GFP fluorescence. Images were scanned at 400 Hz using 512x512 or 1024x1024 pixel resolutions. The pinhole was set to max 1 airy unit providing a min voxel depth of 504 nm. Z-stacks were recorded using a step size below the calculated thickness of the optical sections. Fluorescence and bright field images were processed using the LAS-AF package (Leica, Germany).

2.5.2 Transmission electron microscopy (TEM)

Selected root areas were subsequently dissected, embedded in gelatine (Fluka, Germany), post fixed in 1% osmium tetroxide, washed and incubated in 1% aqueous uranyl acetate (Polysciences) overnight at 4 °C. Specimens were dehydrated in an ethanol series 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, Germany) at 120kV under zero-loss conditions and images were recorded at slight under focus using a 1k x 1k slow-scan proscan ccd camera.

2.5.3 Scanning electron microscope (SEM)

The colonization pattern of *Rr*F4 on root surface was analyzed with SEM. Roots with branches were fixed in 1% osmium tetroxide, washed in buffer and dehydrated in an ethanol series. Finally they 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 3-5kV. Images were recorded using a secondary electron (SE)-detector with the voltage of the collector grid biased to + 300 V to improve the signal-to-noise ratio and reveal optimal topographical contrast.

2.6 Re-isolation of *P. indica*

Barley seedlings were inoculated with the spores of *P. indica* as described in 2.2, and cultured with ½ MS medium under axenic condition in the culture room. After two weeks of incubation, roots were harvested under sterile conditions (clean bench) and cut into small segments. The root fragments were washed with 70% (v/v) ethanol for 1 min, followed by 3% (x/v) NaClO for 1, 3, 5 and 10 min. The root fragments of

different treatments were put on CM agar plates or liquid CM flaskes and cultured at RT. *P. indica* formed colonies around the root segments after 5 days, then sub-cultured on new CM agar plates for further analysis such as the endobacteria quantification and detection.

2.7   Curing *P. indica* from endobacterium

In order to cure *P. indica* from the endobacterium, *P. indica* protoplasts were treated with antibiotics.

2.7.1   Fungal protoplast formation

The chlamydospores of *P. indica* were collected from three weeks old culture with 0.002% tween 20-water, cultured in 200 mL liquid CM medium shaking with 130 rpm at 28 ℃. After 7 days of incubation, the culture was filtered through miracloth and washed with 0.9% NaCl solution. Subsequently, the mycelium was crashed in liquid CM medium in blender with 7 sec at high level and 7 sec at low level. The crashed mycelium was cultured in liquid CM medium for 3 days before using for protoplast isolation.

0.2 g lysing enzymes from *Trichoerma harzianum* (Sigma-Aldrich, USA) was dissolved in 10 mL SMC buffer (1.33 M sorbitol, 50 mM $CaCl_2$, 20 mM MES buffer pH 5.8), sterilized with 0.2 μm filter and kept in 4 ℃ before using. Three days old *P. indica* culture was filtered through miracloth. In order to have mycelium, 50 mL 0.9% NaCl solution was used to wash away the spores or other contaminants from the miracloth. Thereafter the mycelium was dissolved in lysing enzyme solution and incubated in 37 ℃ hybridization oven for 1 h by slightly shaking. The protoplast formation was checked with microscope. To stop the enzymes activity, 10 mL ice cold STC buffer (1.33 M sorbitol, 50 mM $CaCl_2$, 10 mM TrisHCl; pH 7.5) was added to each 10 mL protoplasts sample through miracloth filter. Protoplasts were collected by centrifuge at 4 000 rpm 4℃ for 10 min, and gently suspended in 1 mL STC buffer in a 1.5 mL eppendorf tube by pipetting. Finally, the protoplasts were dissolved in 100

µL cold STC buffer after washing with STC buffer for two times (the best concentration is 10^7-10^9 protoplasts per mL).

2.7.2 Treatment with antibiotics

The fungal protoplasts were plated on CM medium plates containing 300 µg/mLspectinomycin and 300 µg/mL ciprofloxacin, while protoplasts plated on CM medium without antibiotics as control. This was termed as protoplast generation 1 (G1). The germination of single protoplast was clearly viewed on the plate after one week, ten colonies formed from ten protoplasts were picked up and transferred to ten fresh CM medium plates containing both spectinomycin and ciprofloxacin as above. After culturing for three weeks, fungal samples were taken from each plates and endobacteria were detected by real-time PCR with specific ITS primer. The chlamydospores were collected from the plates depending on the endobacteria detection result, protoplast formation was performed as before and cultured on CM medium with spectinomycin and ciprofloxacin, which was termed as protoplast G2. Ten colonies were picked up from each plate and transferred to fresh plates again. The endobacteria were detected in the same way. Protoplasts treatment with antibiotics went till G3 generation.

2.8 Infection of *P. indica* with GFP-/GUS-*Rr*F4

In order to see if *Rr*F4 can infect *P. indica* and go back to its fungal host again, the GFP- and GUS- tagged *Rr*F4 were used here.

2.8.1 Protoplasts formation from *P. indica* and *Rr*F4

GFP-/GUS-*Rr*F4 were cultured in 10 mL LB medium with 50 µg/mL spectionomycin at 37 °C, 190 rpm overnight. 250 µL overnight culture was transferred and cultured in 25 mL same medium until OD_{600}=0.7. The bacteria were pelleted by centrifugation at 5 000 rpm for 10 min, washed with 25 mL distilled water for three times, and re-suspended in 2.5 mL 1×SMMP buffer. Subsequently, lysozyme was added into the bacteria suspension with the concentration 10 mg/mL and incubated at 37 °C for 12-16 h without shaking. The bacteria protoplasts were pelleted by centrifugation 5

000 rpm for 6 min after observing with microscope, washed with 1×SMMP twice and dissolved into 2.5 mL 1×SMMP buffer by pipetting up and down to mix well. Small aliquots were generated with 300 µL volume, used it directly or stored at -80 °C (maximum 6 months). The protoplasts of *P. indica* were formed as above, while the protoplasts from *Rr*F4 were isolated as flowing.

2.8.2 Fusion

300 µL protoplasts of *P. indica* were mixed with 300 µL protoplast of bacteria in 2 mL fusogen. The mixture was vortexed gently for 30 sec followed by standing for 30 sec, then added 7 mL 1×SMMP immediately and centrifuged at 5 000 rpm for 20 min at RT. The pellet was dissolved into 1 mL 1×SMMP and incubated for 3-4 h at 30-37 °C. Finally, 200 µL mixtures were plated evenly on each LB plate and cultured at 30 °C.

1×SMMP (25 mL)	
2×SMM	13.8 mL
4×PAB	10 mL
5%BSA	1.25 mL

It can stay at 4 °C for 2 days.

2×SMM (200 mL)	
1.0 M Sucrose	68.4 g
0.02 M Tris	0.49 g
0.01 M MgCl$_2$	0.41 g
0.04 M Maleic acid	0.93 g

Adjust pH 6.8 with 10N NaOH, then autoclave for 12 min

4×PAB (Bacto Perrasam Broth) (500 mL)	
Nutrient Broth (Gibro)	8 g
Bactopepton (Difco)	7 g
Yeast Extract	6.2 g

Adjust pH 6.8, then autoclave

Fusogen (100 mL)	
PEG 6000	40 g
2×SMM (without autoclaving)	50 mL

Add water up to 100 mL, adjust pH 6.8, then autoclave for 12 min

2.8.3 Co-cultivation of GFP-*Rr*F4 and *P. indica*

GFP-*Rr*F4 suspension with $OD_{600}=0.1$ was added to three days old germinated *P. indica* spores solution in liquid CM medium. Thereafter the mycelium of *P. indica* was daily investigated by epi-fluorescence microscopy. The protoplasts of *P. indica* were inoculated with GFP-*Rr*F4 suspension with $OD_{600}=0.1$ in Eppendorf tube for 40 min, thereafter cultured on CM medium with 300 μg/mL spectinomycin. The material was analysed by microscopy after one week.

2.9 Biological activity of *P. indica* and *Rr*F4 assays

Barley Golden Promise were inoculated with *P. indica* or *Rr*F4, and cultured in climate chamber for three weeks as describes in 2.2.3. Plants cultured in pots containing 3:1 expanded clay (Seramis®, Masterfoods) and Oil Dri® (Damolin) were used for the growth promotion assay. The shoot and root weights were measured in each treatments. Plants cultured in pots containing soil were used for the pathogenic assay. The third leaves from three-week old plants were used for a detached leaf-segment test to see the plant systemic resistance. Barley powdery mildew *Blumeria graminis* f. sp. *hordei* was used as pathogen in the assessment. The leaf segments were inoculated with 15 conidia mm-2 spores of *B. graminis* f. sp. *hordei* for 10 min, and kept on water agar plates (1.5% agar) with 5% benzimidazole under darkness for 6 days. Then the pustules on the leaves segments were counted under microscope.

3   Results

3.1   Detection of the endobacterium *R. radiobacter* in *P. indica*

The endofungal bacterium associated with the fungus *P. indica* was detected in the fungal tissue by FISH using the fluorescently labeled universal bacterial 16S rRNA binding probe EUB-338. The FISH results showed the presence of few endobacteria in both mycelium (Fig. 3.1 A) and chlamydospores (Fig. 3.1 B) of *P. indica* which was cultured in liquid CM medium. Since the cell wall of fungi could be a barrier for the entrance of the FISH probe into the fungal cells to bind to the ribosomal RNA of endobacteria, crushed hyphae and protoplasts from *P. indica* were analyzed by FISH in parallel. The low abundance of endobacteria in *P. indica* was also illustrated with crushed *P. indica* material (Fig. 3.1 C). The nucleic acid staining dye 4',6-diamidino-2-phenylindole (DAPI) was used together with the FISH probe EUB-338-FITC (green fluorescent signal) to exclude unspecific fluorescence signals. While probe EUB-338-FITC stained only bacterial cells (Fig. 3.1 D), DAPI stained bacterial cells and nuclei of fungi and thereby confirmed the presence of endobacteria (Fig. 3.1 E) in the fungal tissue (Fig, 3.1 F) In this experiment, pure *Rr*F4 cells were used as positive control to control the efficiency of the probe binding (Fig. 3.1 G, H, I). Compared with the rod-shaped pure *Rr*F4, the endobacterial cells of the *Rhizobium* were smaller in size and had a coccoid-shape in *P. indica*. Probe EUB-338-Cy3 (red fluorescence signal) was used for confocal laser scanning microscopy to further confirm the existence of the endobacteria within fungal tissue (Supplement Fig. 7.1). Probe Rh-1247, which is specific for bacteria in the Rhizobium group, was also used to detect the endobacteria in *P. indica* (data not shown).
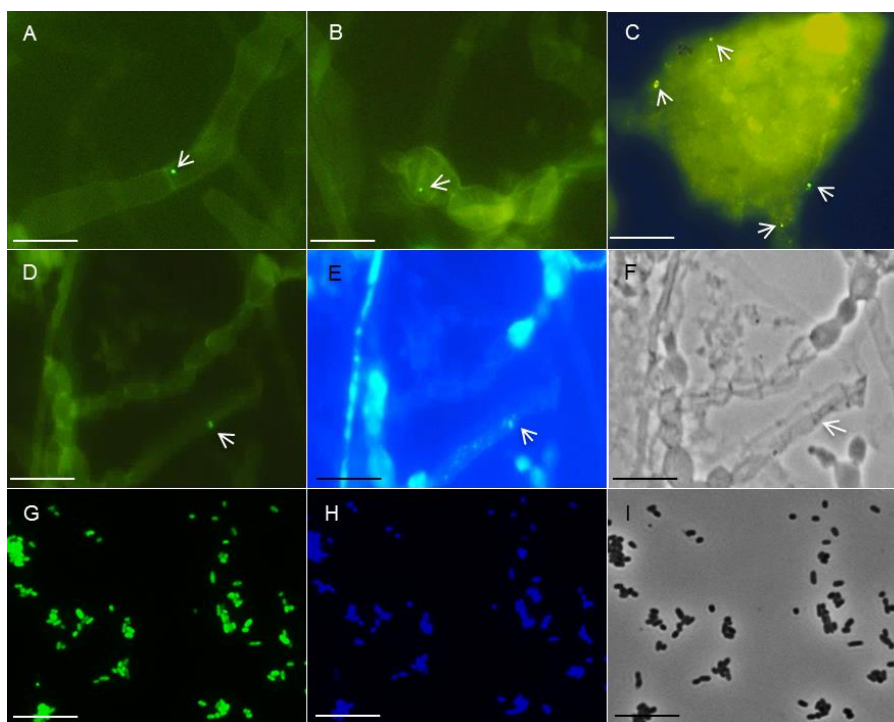
Fig. 3.1 FISH detection of endobacteria in *P. indica* lab cultures by fluorescence microscopy. *P. indica* was cultured in liquid CM medium for three weeks, fixed and analysed with FISH using a univseral bacterial probe (A, B). A: endobacterial cells detected in hyphae; B: endobacterial cells detected in chlamydospores; Three-week-old *P. indica* was crashed with blender and fixed for FISH (C). *P. indica* cultured in petri dish with solid CM medium was used for endobacteria detection, the same field showed in different channels (D, E, F); D: stained with FISH (green signal); E: DAPI staining (blue signal); F: light microscopical image. Pure *Rr*F4 was cultured overnight in modified YEB medium and fixed for FISH detection as positive control (G, H); G: pure *Rr*F4 stained by FISH; H: light microscopic image. The probe used in FISH detection was the bacteria probe EUB-338-FITC (green fluorescence). White arrows point to the detected endobacterium. All microscopic analyses were done at 1,000 fold magnification; all bars indicated 10 μm.

3.2 Growth of *Rr*F4 stain in pure culture

The endofungal bacterium was isolated from *P. indica* and named as strain *Rhizobium radiobacter* F4 (*Rr*F4, Glaeser *et al*. 2015). The size of *Rr*F4 cells was 1.2-2.0 μm in length and 0.7-0.9 μm in width. To get an indication of the growth stage of cultures applied for different kind of experiments, the growth behavior of *Rr*F4 in liquid

culture was evaluated in four independent cultures. As shown below, with the starting of OD_{600}=0.01, slow growth occurred at the beginning of the incubation experiment because RrF4 needed to adapt to the new growth condition. This lag phase lasted approximately 4 hours (lag phase). Subsequently, bacterial cell division became faster and got optimal between 4-17 hours (logarithmic phase). After 17 hours of incubation the cell density did not increase anymore, the producing of new bacterial cells and the dying of old bacterial cells were at a dynamic balance (stationary phase) (Fig. 3.2). RrF4 culture at the logarithmic phase was used for all the root inoculation experiments.
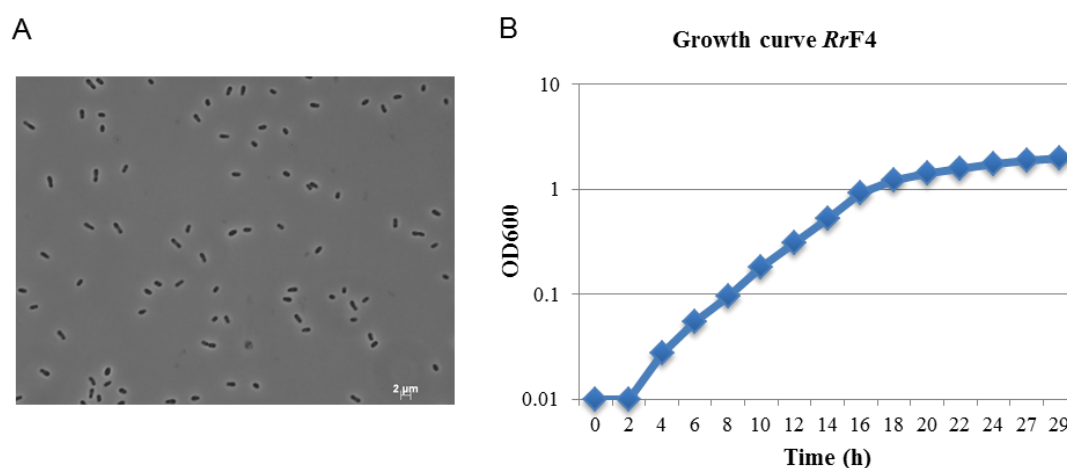
Fig. 3.2 Pure culture of RrF4 in liquid medium. RrF4 cells were visualized with microscopy, and the length and width of the bacterial cells were measured (A). Growth curve of pure RrF4 culture was measured by spectrophotometry (B). RrF4 was first cultured in YEB medium to OD_{600}=0.2. Then 20 mL of RrF4 culture were taken out from the original culture and sub-cultured in four independent flasks filled with 100 mL YEB medium at 130 rpm min^{-1} room temperature (starting OD_{600}=0.01). The sub-culture in these four independent flasks was measured every 2 hours till the stationary phase. The curve is based on mean values of four independently growing cultures. The standard deviation was always below 5% and is therefore not shown. Growth phases: lag phase (0-2 h), log phase or exponential phase (2-17 h), late exponential phase (around 17 h), stationary phase (18-29 h).

3.3 Multiplication of *Rr*F4 on the barley root surface

The aim of this experiment was to compare the root colonization pattern of free *Rr*F4 with the fungal host *P. indica*; the colonization of *Rr*F4 on barley roots was assessed microscopically with GUS- and GFP-tagged *Rr*F4 cells. Pure *Rr*F4 were labelled with GFP and GUS reporter genes, respectively. The green or blue bacteria cells were visualized by microscopy (Fig. 3.3 A, B). Surface sterilized barley seeds were germinated in autoclaved jars on sterile filter paper. After three days, germinated barley seedlings were dip-inoculated with *Rr*F4 cell suspension (OD$_{600}$=1.2) and incubated for 30 min under a clean bench. Inoculated seedlings were transferred into autoclaved jars supplied with ½ MS medium, and cultured in the culture room (24℃, 16h/8h photoperiod) until harvest.
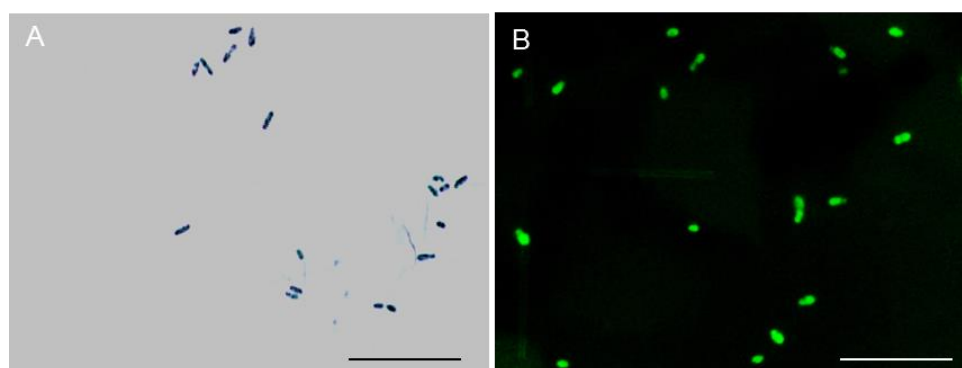

Fig. 3.3 GUS- and GFP-tagged *Rr*F4 cells. The pure culture of *Rr*F4 cells were labelled with reporter gene GUS (A) and GFP (B). A: Blue GUS-tagged *Rr*F4 cells were visualized by light microscopy after incubating with substrate X-Gluc. B: Green GFP-tagged *Rr*F4 cells were visualized using fluorescence microscopy. The bar indicates 10 μm (Reporter bacteria were produced with support of Dr. J. Imani, IPAZ).

3.3.1 Colonization of *Rr*F4 on primary barley roots

Root samples were harvested at 5, 7, 14 and 21 dpi, incubated overnight in the substrate X-Gluc and investigated by light or CLSM microscopy. The colonization of primary roots with *Rr*F4 cells increased from 5 to 21 dpi (Fig. 3.4 A, B, C and D). We observed the sample at each time point, with higher magnification at later time points

(Fig. 3.5 A, B, C and D). At 5 dpi, GUS-tagged *Rr*F4 cells colonized the maturation zone I of primary roots; blue colonization (GUS-*Rr*F4 cells) covered an area of approximately one centimeter in length at the root tip (Fig. 3.5 A). At 7 dpi, the colonized area increased in length, but the root cap was still virtually free from bacteria colonization (Fig. 3.5 B). At 14 dpi, bacteria had spread into the maturation zone II but only few cells were detected in the elongation- and meristematic zones, the root cap still remained virtually free of bacteria (Fig. 3.5 C). The same pattern of bacterial colonization was also observed at 21 dpi (Fig. 3.5 D).
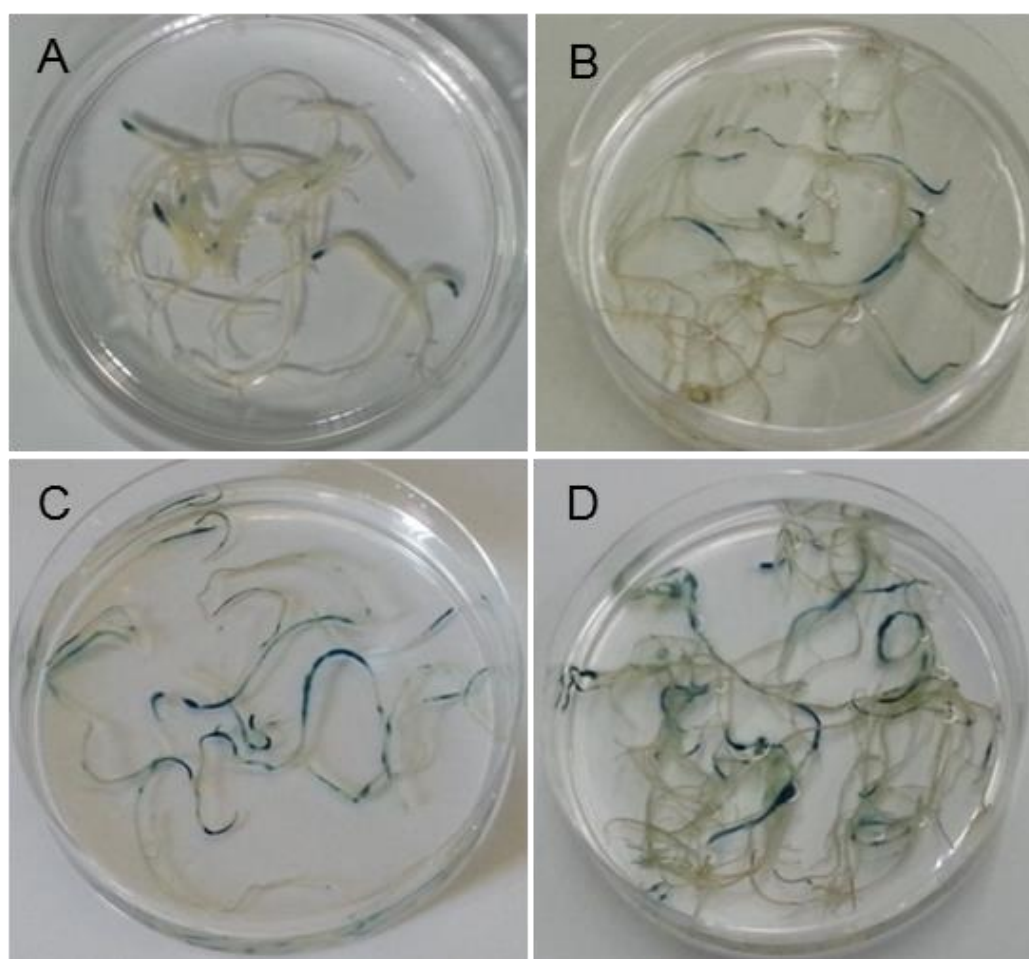
Fig. 3.4 Multiplication of GUS-tagged *Rr*F4 cells on barley roots. Root samples were harvested at four time points after dip-inoculation (dpi), 5 (A), 7 (B), 14 (C) and 21 (D) dpi. After washing in 70% (v/v) ethanol for one time and treating with sonication for three times, roots were embedded into the solution of the substrate X-Gluc, and incubated at 37℃ for overnight. The root area colonized by bacteria stained blue.
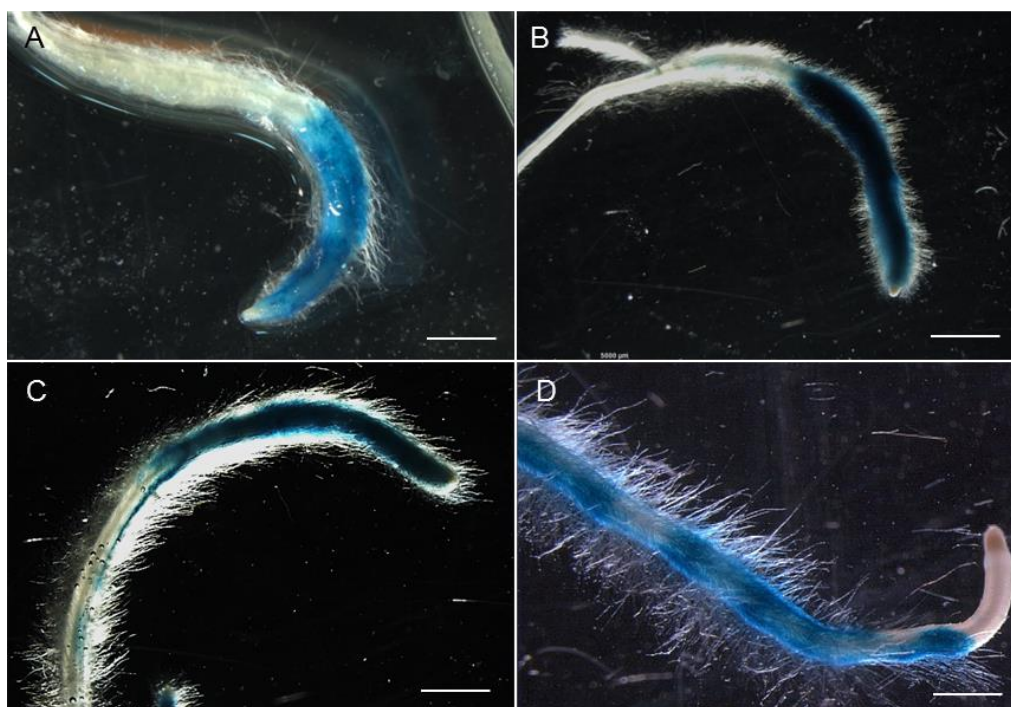
Fig. 3.5 Multiplication of GUS-tagged *Rr*F4 cells on barley roots shown at higher magnification. Single roots were taken from the stained roots as showing above in Fig. 3.3 at 5 dpi (A), 7 dpi (B), 14 dpi (C) and 21 dpi (D). All bars indicat 3,000 µm.

Microscopically observation of stained roots showed a distinct pattern of dark and bright blue staining in the root hair zone (Fig. 3.5 D). This dark-bright staining suggested higher *Rr*F4 proliferation at specific sites on the root surface. In addition, single root cells filled with dark blue *Rr*F4 (GUS-labelled) were observed on the root surface of the maturation zone I at 7 dpi and 14 dpi (Fig. 3.6 A and B). Transmission electron microscopy (TEM) confirmed that these cells were heavily colonized with bacterial cells (Fig. 3.6 C). The same phenomenon was also found with GFP-tagged *Rr*F4 by confocal laser scanning microscopy (CLSM) analysis (Fig. 3.6 D). TEM analysis and additionally tested DAPI staining (data not shown) did not show the presence of a nuclei in those cells which indicate that those plant cells were dead. Whether dead cells were invaded by bacteria or the plant cells died after invasion could not be clarified with the obtained data.
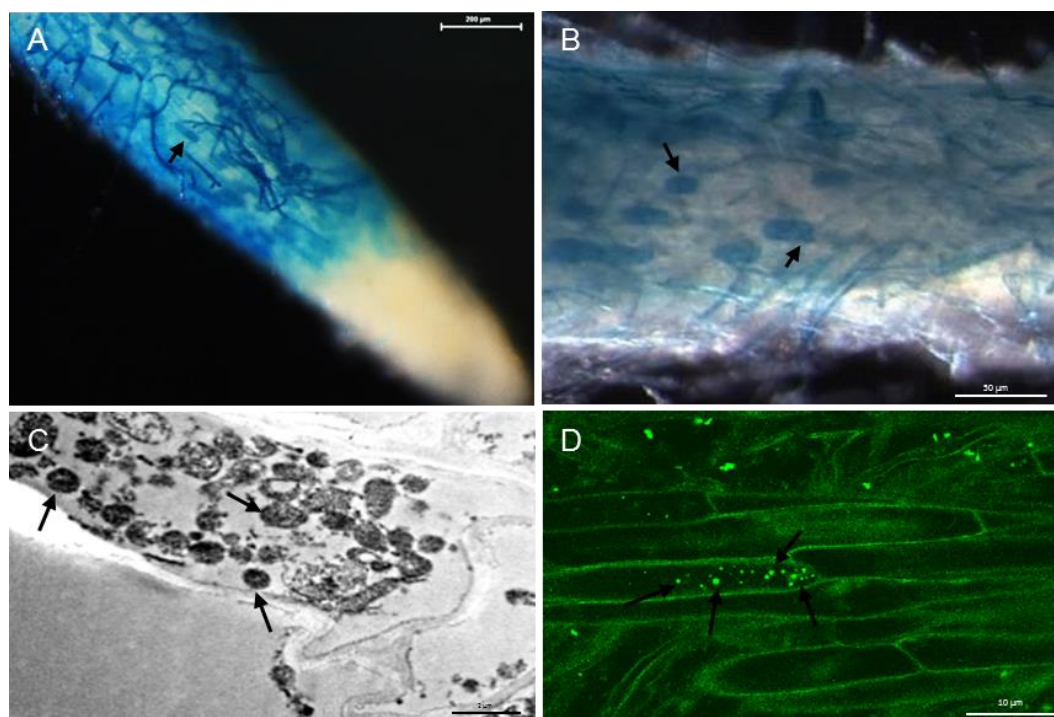
Fig. 3.6 Colonization of single root surface cells by *Rr*F4. Dark blue cells on the root surface were observed under the light microscope at 7 (A) and 14 dpi (B). Transmission electron microscopy (TEM) scanned single root cells filled with bacteria (C). Confocal laser scanning microscopy (CLSM) showed single root cells filled with GFP-tagged *Rr*F4 bacterial cells (D). Arrows show the presence of bacterial cells. (B and C were published in Glaeser *et al.* 2015)

3.3.2 Colonization at root junctions and lateral barley roots

Beside the colonization of primary barley roots, *Rr*F4 cells were also found to form conglomerates at root junctions and lateral roots at 14 and 21 dpi. Epifluorescence microscopy using a GFP-filter showed the colonization of GFP-tagged *Rr*F4 on the lateral root protrusion site at 14 dpi (Fig. 3.7 A) and proliferated at the base of fresh lateral roots at 21 dpi (Fig. 3.7 B), in a similar manner as primary roots. Root samples colonized by GUS-tagged *Rr*F4 cells were investigated by light microscopy, blue bacterial cells were constantly detected at root junctions as the growth of lateral root both at 14 and 21 dpi (Fig.3.7 C and D). At 21 dpi, the root hair zones of lateral roots were colonized with the same pattern as in primary roots (Fig. 3.8 A and B). The

colonization pattern on lateral roots was mutual verified with GUS-tagged *Rr*F4 and GFP-tagged *Rr*F4.
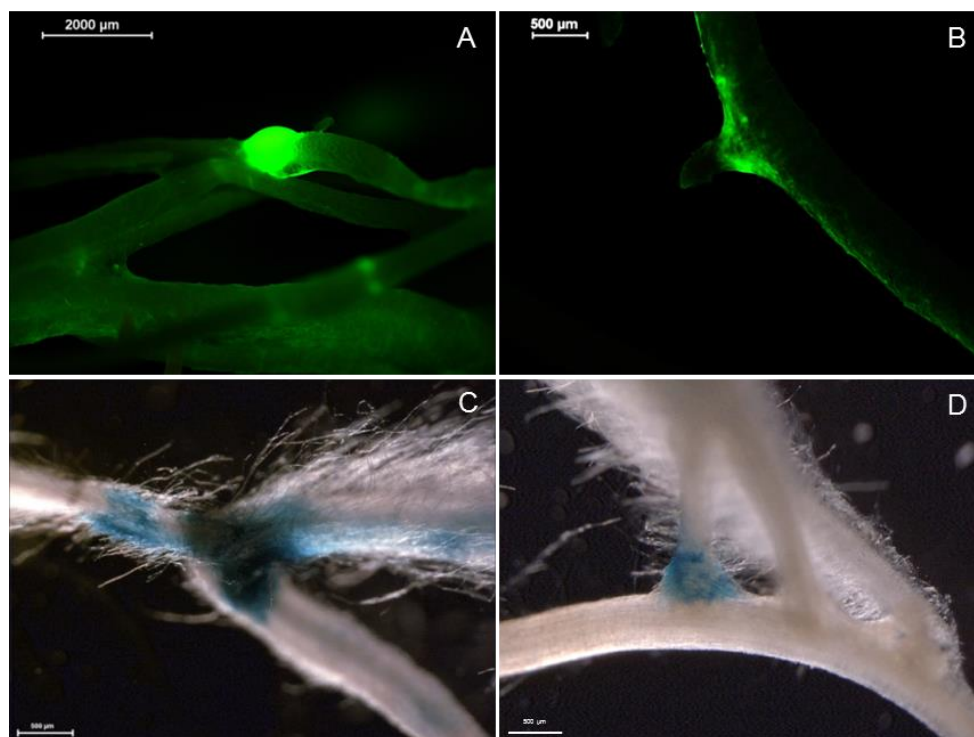

Fig. 3.7 Colonization of GUS- and GFP-tagged *Rr*F4 on barley root junctions. Barley seedlings were inoculated with GFP-tagged *Rr*F4 and cultured in sterilization condition. Harvested root samples were subsequently observed under the microscope with the GFP filter; green fluorescence was detected at the lateral root protrusion site at 14 dpi (A) and fresh lateral root base at 21 dpi (B). Barley roots treated with GUS-expressing *Rr*F4 were observed with the light microscope; blue bacterial colonization was detected at the root junctions at 14 (A) and 21 dpi (B).
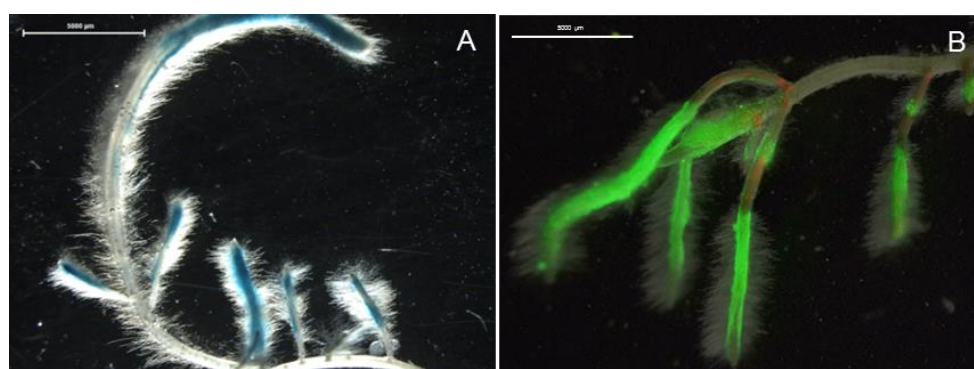

Fig. 3.8 Colonization pattern of GUS- and GFP-tagged *Rr*F4 on lateral roots. Barley root samples

were harvested at 21 dpi. GUS-expressing *Rr*F4 were detected on the root hair zone of lateral roots (A). Green fluorescence was at roots inoculated with GFP-expressing *Rr*F4 on the root hair zone of lateral roots (B). (B was published in Glaeser *et al.* 2015)

3.3.3 *Rr*F4 cell aggregates and single cells on root surface

The surface colonization of barley primary roots was further investigated by SEM and TEM. Consistent with the above findings of GFP and GUS-tagged, root-cap and elongation zones were much less colonized by *Rr*F4 (Fig. 3.9 A-D) compared to root hair zones, where *Rr*F4 formed dense surface attached biofilms (Fig. 3.9 E-G). Root cap colonizing *Rr*F4 cells formed cell aggregates located in cracks between root cap cells but did not fully cover the cell surface (Fig. 3.9 A and B). These cell aggregates and single surface attached cells were cross-linked by fiber-like structures (Fig. 3.9 C and D). Larger cell aggregates (micro-colonies), often developed at the sites of root hair protrusion rather than being attached to fully developed root hairs (Fig. 3.9 E and F). The dense surface attached biofilm in the maturation zone of primary roots, as shown in Fig. 3.9 E, was investigated in more detail by TEM. *Rr*F4 form an approximately 5 µm thick multilayer biofilm with cells embedded by a dense matrix of extracellular polymeric substances (Fig. 3.9 G).
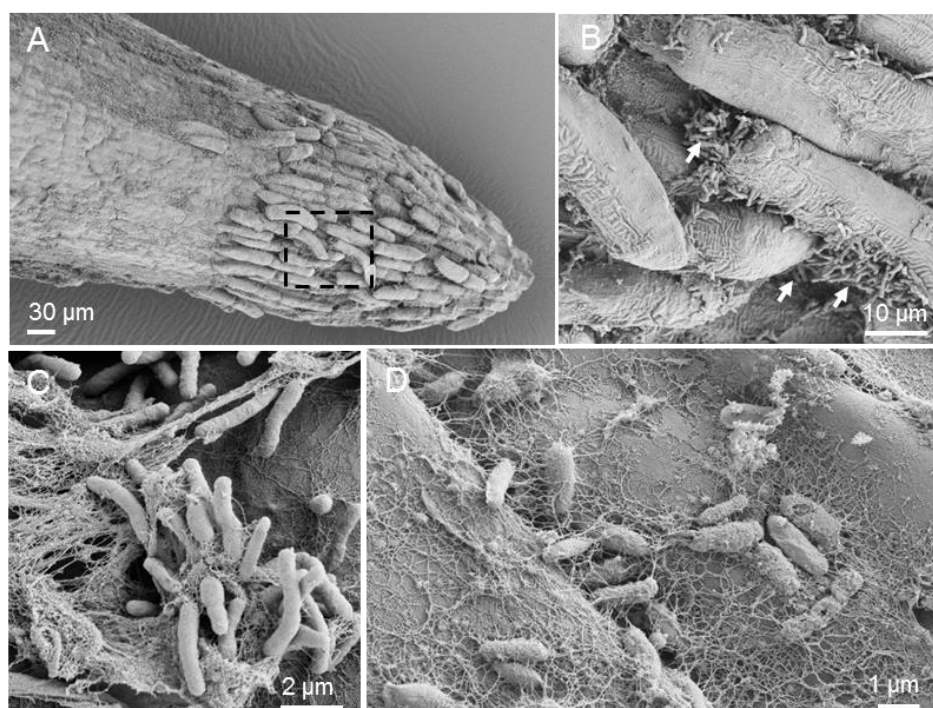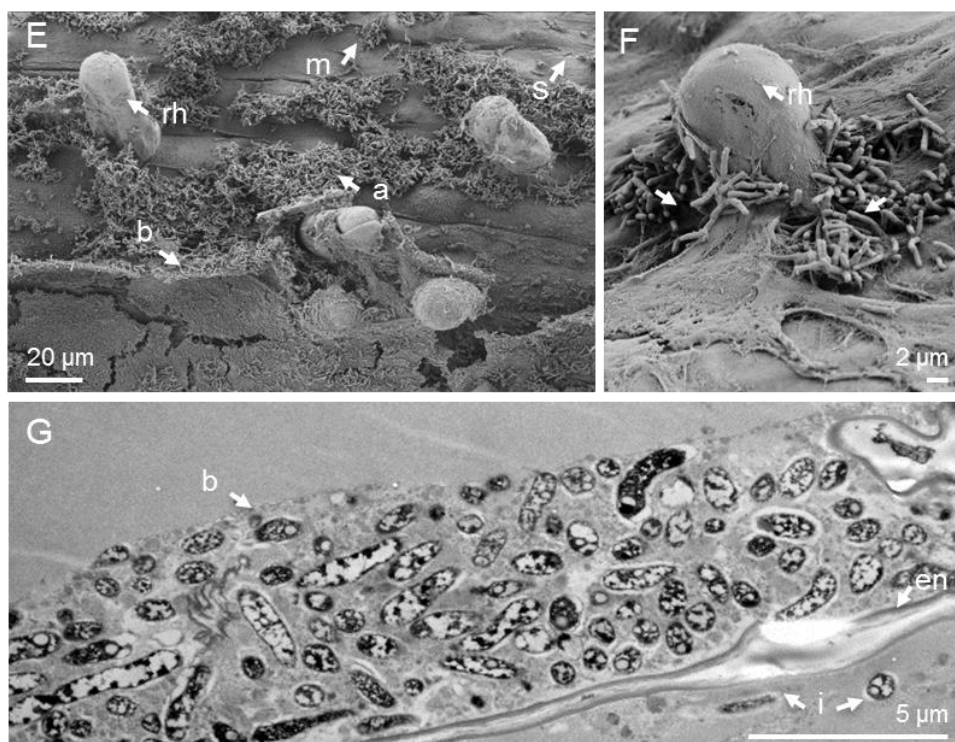
Fig. 3.9 Colonization of barley primary roots by *Rr*F4 analyzed by scanning and transmission electron microscopy. A, B: *Rr*F4 at the root tip (B is a zoom out of A showing *Rr*F4 cells at the root cap cells, square). C: *Rr*F4 cell aggregates at the root tip. D: Single *Rr*F4 cells attached to the root surface distal to the root 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 *Rr*F4 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) *Rr*F4 cell aggregates around the root hair protrusion site; assumed area of penetration into the root tissue (arrow). (G) Surface-attached dense biofilm of *Rr*F4 cells. Bacterial cells are embedded in an extrapolysaccharide (EPS)-like structure; biofilm at the root surface (b). (Glaeser *et al.* 2015)

3.4 Localization of *Rr*F4 in the inner barley root tissue

To explore the localization of *Rr*F4 in the inner root tissue, cross-sections of barley roots were analyzed with cytological method. Three-day-old barley seedlings were dip-inoculated with GUS- or GFP-tagged *Rr*F4 suspension (OD_{600}=1.2) for 30 min under aseptic condition, thereafter cultured in sterilized jars supplied with ½ MS medium. The root samples were harvested at 5, 7, 14 and 21 dpi and horizontal cross-sections of root areas showing blue stain were prepare for further microscopically analysis.

Cross-section of barley roots colonized by GUS-tagged *Rr*F4 was analyzed by microscopy (Fig. 3.10 A). At 5 dpi, blue GUS staining was showing on the root surface, around the root hairs and the rhizodermis (Fig. 3.10 B). At 7 dpi, the blue staining extended to the rhizodermis, exodermis and cortical tissues, which indicated the invasion of GUS-tagged *Rr*F4 cells into the root cortex (Fig. 3.10 C). Later (21 dpi) blue staining also was seen in the endodermis (Fig. 3.10 D). At higher magnification (1,000 fold), it was clearly shown that the endodermal cells were surrounded by blue bacteria (Fig. 3.10 E). To test whether the bacteria moved to upper plant parts the shoots of the barely seedlings were examined by microscopy after incubation with substrate X-Gluc. No blue stained bacterial cells were detected in the stem, leaf or leaf sheath (Supplement Fig. 7.2). Consistent with this, PCR amplification of *Rr*F4 specific ITS targets with DNA extracted from the shoot part of barley was also negative. Re-isolation of *Rr*F4 from leave tissue was also not successful.

The colonization pattern of *Rr*F4 in the inner barley root tissue was confirmed with GFP-tagged *Rr*F4 (Fig. 3.11). Green fluorescence *Rr*F4 cells were consistently detected on the root hair and in the rhizodermis, exodermis and cortex layers. At 21 dpi, *Rr*F4 cells were detected in cell junctions of endodermal cells and in the central cylinder. Inside the central cylinder, bacteria were also seen in the intercellular spaces while intracellular colonization was not detectable.
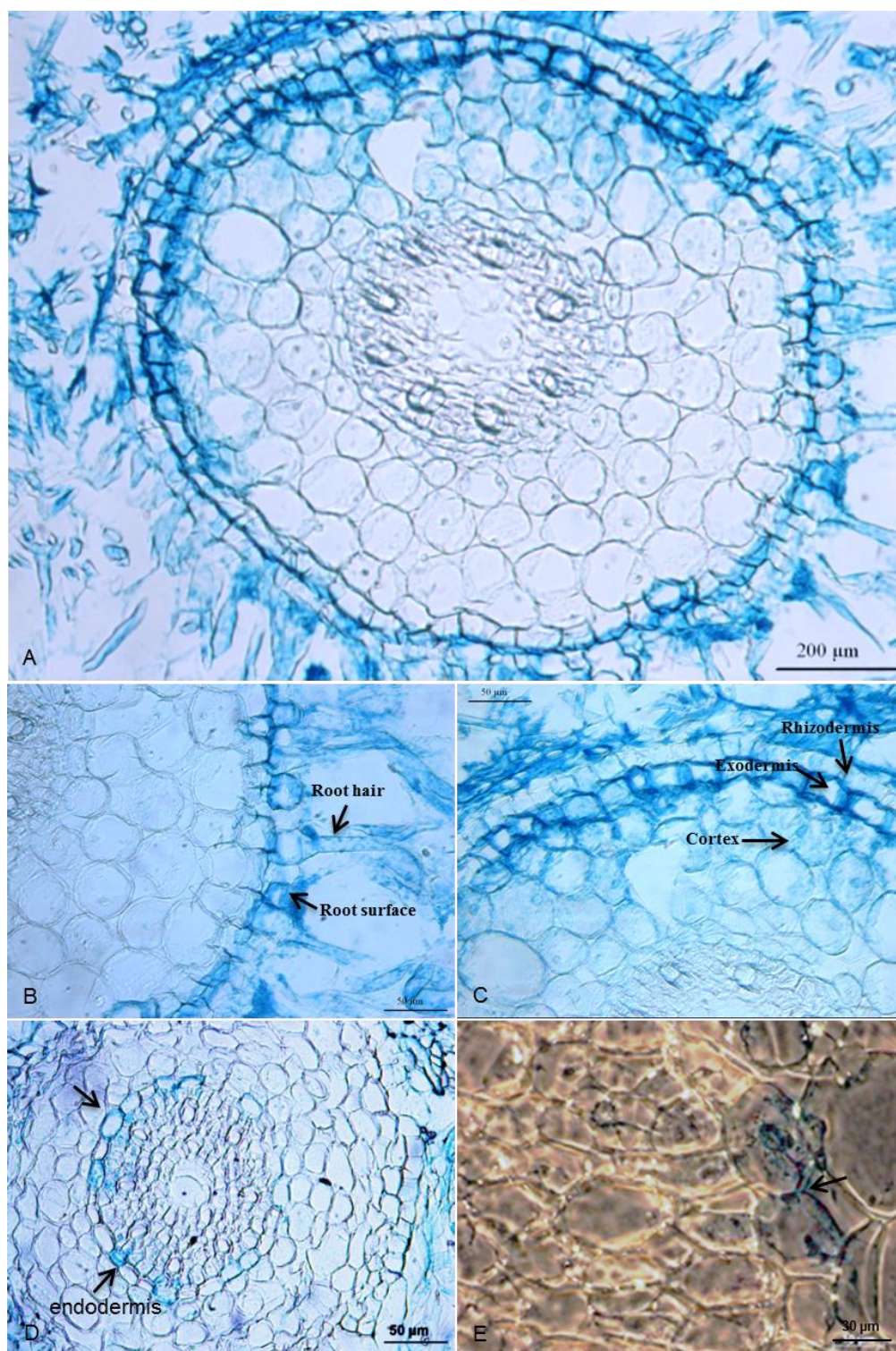
Fig. 3.10 Light microscopy of barley primary root cross-sections. The localization of *Rr*F4 in the root hair zone and inner root tissue was analyzed after inoculation with GUS-tagged *Rr*F4. A: overview of root cross-section at 5 dpi (root hairs were destroyed during the sonication and fixation steps in sample preparation). B: zoom out of Fig. A showing blue *Rr*F4 cells on the root surface and root hair. C: blue strained bacteria in the different layers of the root tissue at 7 dpi (rhizodermis, exodermis and

cortex). D, E: bacteria around the endodermal cells at 21 dpi. (A and D were published in Glaeser *et al.* 2015).
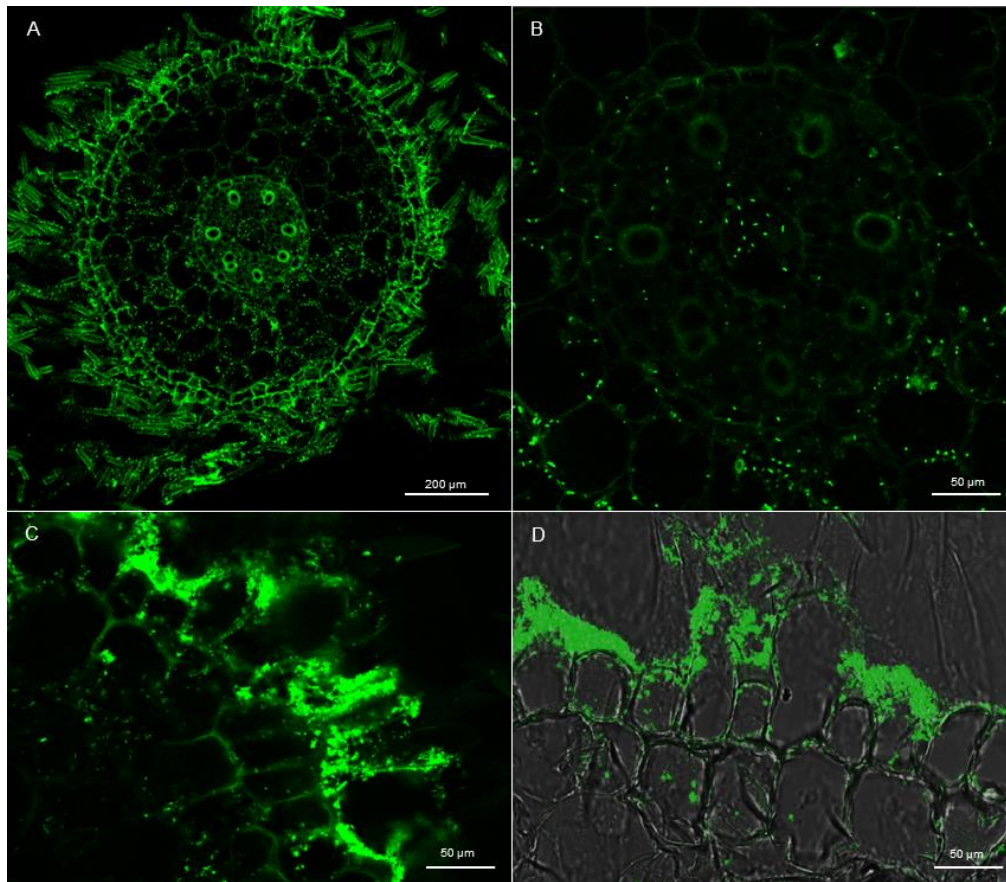

Fig. 3.11 Confocal laser scanning microscopy of barley primary root cross-sections. The localization of *Rr*F4 in barley roots was analyzed after inoculation with GFP-tagged *Rr*F4. A: overview of a root cross-section in the root hair zone at 21 dpi. B: a zoom out of central cylinder showing green fluorescence *Rr*F4 cells in the root endodermis and central cylinder. C, D: green bacteria on the rhizodermis, exodermis and cortex layers. (D was published in Glaeser *et al.* 2015)
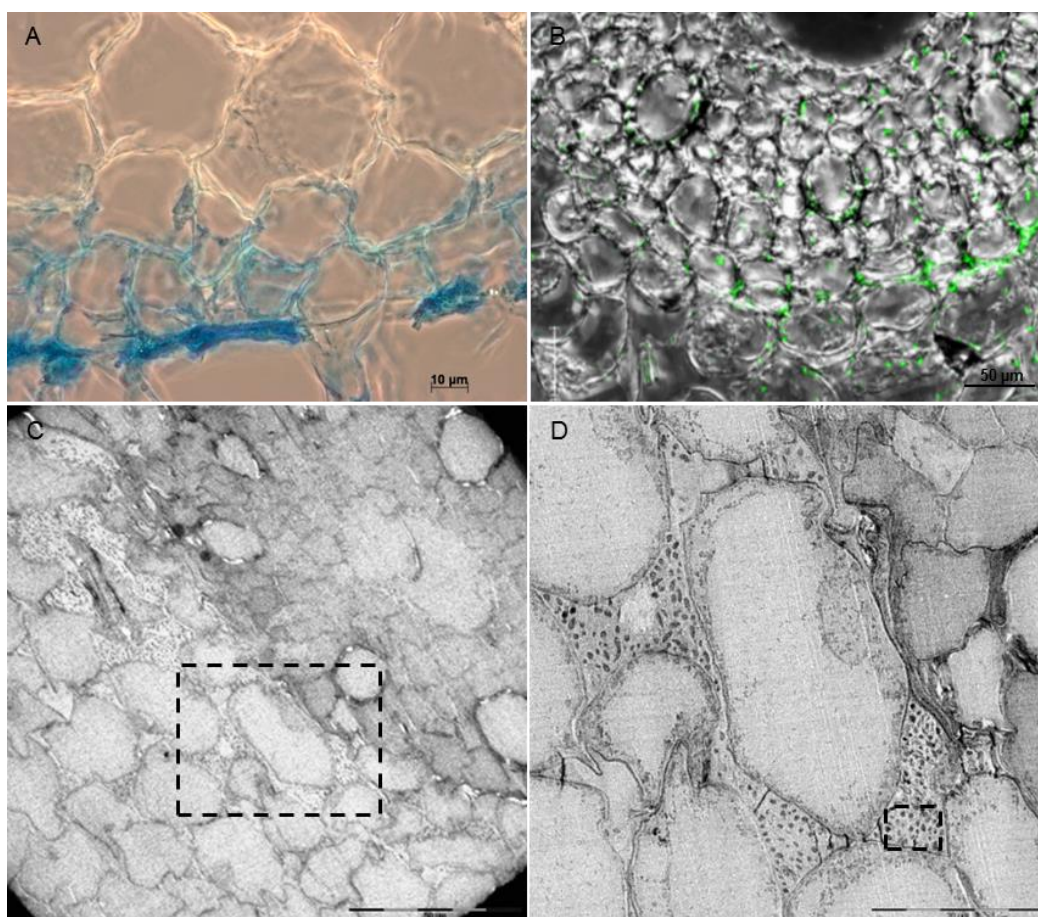
Fig. 3.12 Localization of *Rr*F4 in the cortex and endodermis of barley primary root cross sections. The localization of *Rr*F4 in the cortex of barley root was analyzed after inoculation with GUS and GFP-tagged *Rr*F4. A: GUS-tagged *Rr*F4 were localized in the intercellular space. B: GFP-tagged bacterial showed the same colonization pattern in the intercellular cells. C: bacteria surrounded the cortical cells in the intercellular space was observed. D: a zoom out of one single cortical cell and many small bacterial cells around, square box indicated bacteria cells. (B was published in Glaeser *et al.* 2015)

Analysis with GUS-expressing *Rr*F4 showed that bacteria colonized in the intercellular spaces among rhizodermal and exodermal cells (Fig. 3.12 A). Cross-section with GFP-tagged *Rr*F4 showed bacteria in the intercellular spaces of cortical cells, endodermal cells and the central cylinder (Fig. 3.12 B). More details are shown by TEM. A dense colonization of the intercellular spaces was shown in the cortex tissue and up to the endodermis (Fig. 3.12 C, D).

3.5 Colonization of Arabidopsis roots by *Rr*F4

The colonization pattern of Arabidopsis roots was assessed using GUS- and

GFP-tagged bacteria. GUS-expressing *Rr*F4 cells were colonized on the maturation zone of Arabidopsis roots while the root cap was free of bacteria. The colonization of GUS-expressing *Rr*F4 on Arabidopsis roots was increased from 5 to 14 dpi (Fig. 3.13 A, B and C). No blue staining was obtained on control seedlings without inoculation with GUS-expressing *Rr*F4 but incubated with substrate X-Gluc which excluded plant derived glucuronidase activity (Fig. 3.13 D, E and F). Confocal laser scanning microscopy of Arabidopsis roots inoculated with GFP-tagged *Rr*F4 showed single *Rr*F4 cells as well as dense, locally restricted aggregates at the surface of root hair zones at 7 dpi and 14 dpi, the surface of root hairs and, in higher abundance, at the base of root hair cells (Fig. 3.14 A, B). At a later time point (21 dpi) *Rr*F4 also colonized on the sites of secondary root emergence (Fig. 3.14 C, D). The central vascular systems (xylem) of primary and secondary roots were heavily colonized (Fig. 3.14 E). But still, *Rr*F4 cells were not detectable by q-PCR and FISH in the shoot parts of the Arabidopsis.

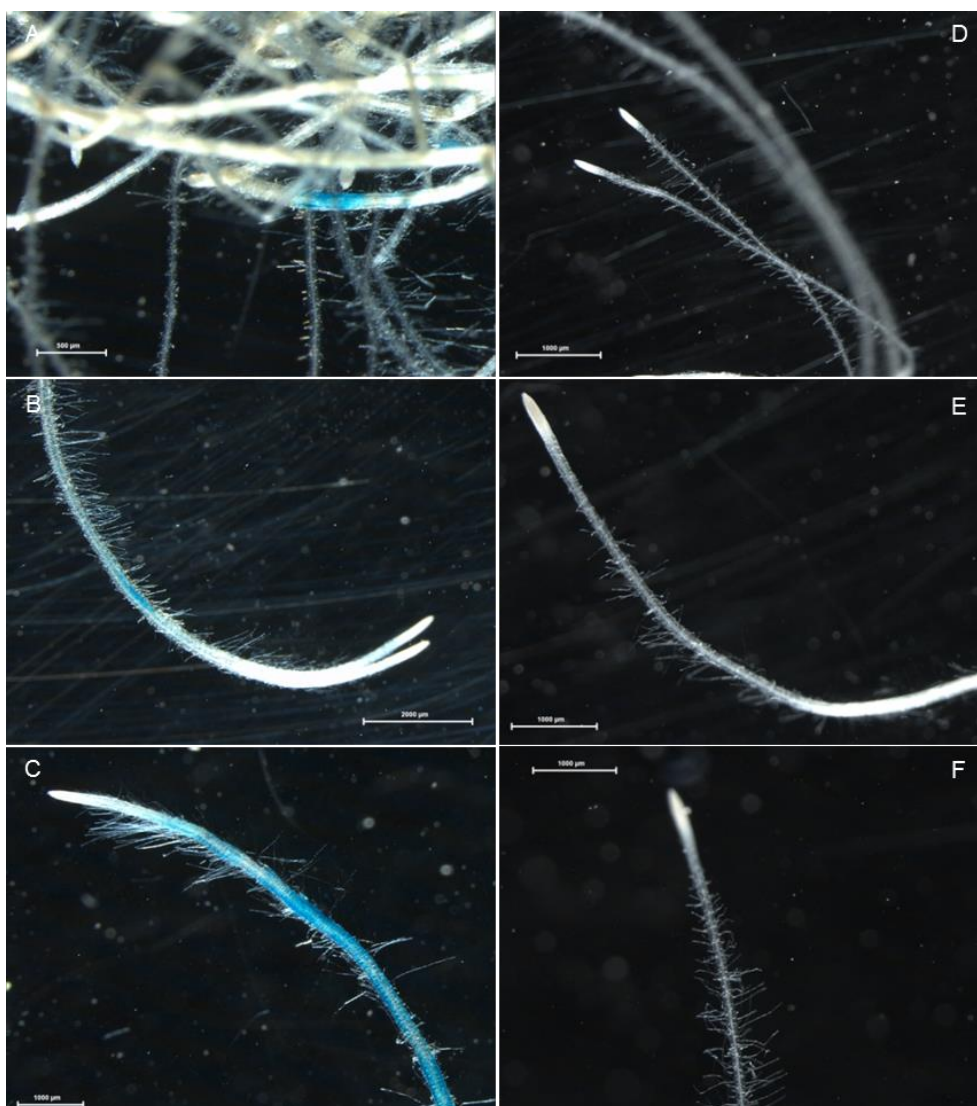**Fig. 3.13 The colonization pattern of GUS-tagged *Rr*F4 on *Arabidopsis thaliana*.** A, B, C: the root hair zone of Arabidopsis inoculated with GUS-tagged *Rr*F4 became blue after incubated with the substrate X-Gluc at 5, 7 and 14 dpi, while the Arabidopsis seedlings control (D, E, F) without GUS-tagged *Rr*F4 treatment, showed no blue coloration after incubation with X-Gluc. (A, B and C were published in Glaeser *et al.* 2015)
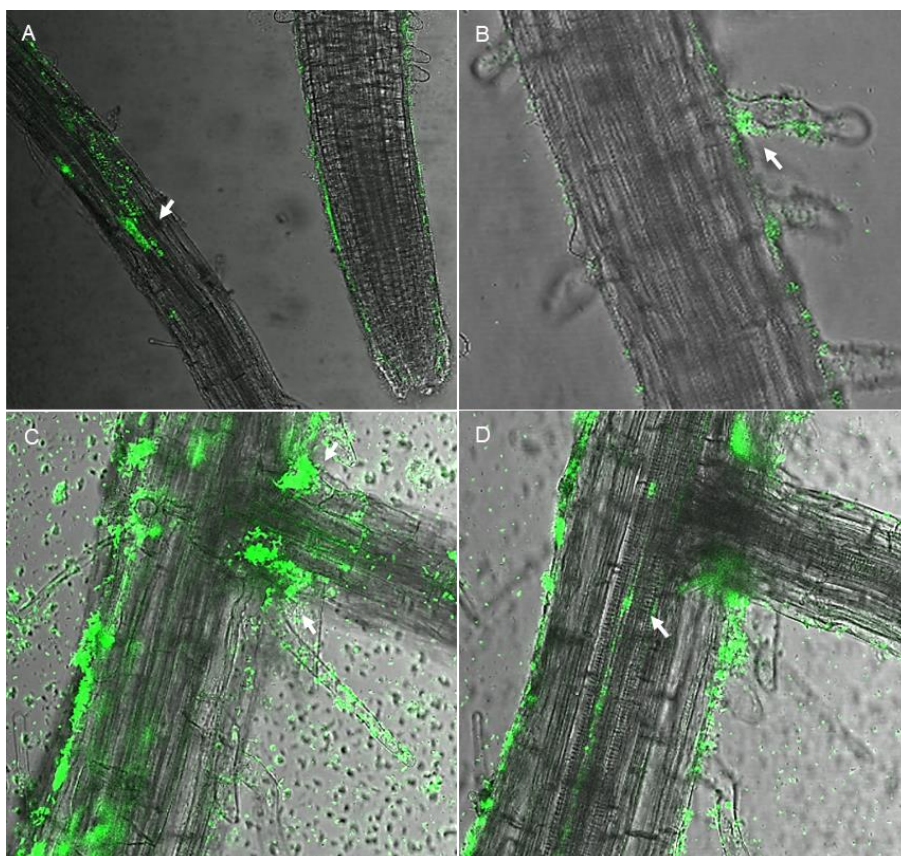
Fig. 3.14 Colonization of primary Arabidopsis roots by GFP-tagged *Rr*F4. Arabidopsis roots were analyzed at 7 dpi and 21 dpi. A: *Rr*F4 colonization of the root surface in the root hair zone with single attached bacteria, thin biofilms and dense locally restricted aggregates (arrows). B: colonization 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: Bacterial cells inside the plant's vascular system (arrow: xylem). C and D represent different layers of a CLSM screen on Arabidopsis roots. (Published in Glaeser *et al.* 2015)

3.6 The impact of cell death regulator BAX inhibitor-1on the colonization of *Rr*F4

P. indica initially colonizes living cells, which die during the colonization process. *P. indica* was shown to proliferate in dead host cells. Since endobacterium Rhizobium sp. has a strong relationship with *P. indica*, it was investigated whether also *Rr*F4 invades living cells and causes host cell death. First, the expression of the *HvBI-1* gene in wild type barley cultivar Golden Promise was analyzed. Quantitative PCR analysis showed

that the expression of *HvBI-1* was slightly increased in barley root during the plant development, while the expression was slightly but not significantly increased when seedlings inoculated with *Rr*F4 were compared with the non-inoculated plants (Fig. 3.15). Barley mutants, which overexpress the functional GFP-HvBI-1fusion protein, were used to determine the colonization density of *Rr*F4. The amount of *Rr*F4 was measured in independent GFP–HvBI-1 barley mutant lines by q-PCR. It was shown that the relative amount of *Rr*F4 in the transgenic line E14L1 slightly but not significantly increased at 7 dpi compared with the colonization on wild type barley (Fig. 3.16).
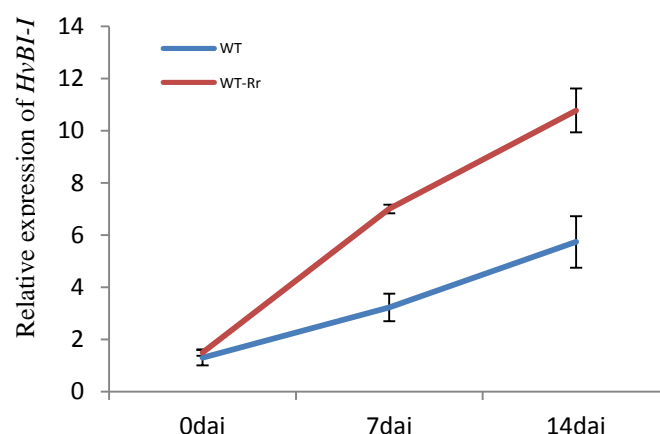

Fig. 3.15 Impact of *Rr*F4's colonization on the expression of *HvBI-1* in barley roots. The expression of *HvBI-1* was analyzed by quantitative PCR at 7 dpi and 14 dpi. The relative amount was increased in both treated and untreated barley samples from 0 to 14 dai. Mean values and Error bars of three independent biological replicates are shown.
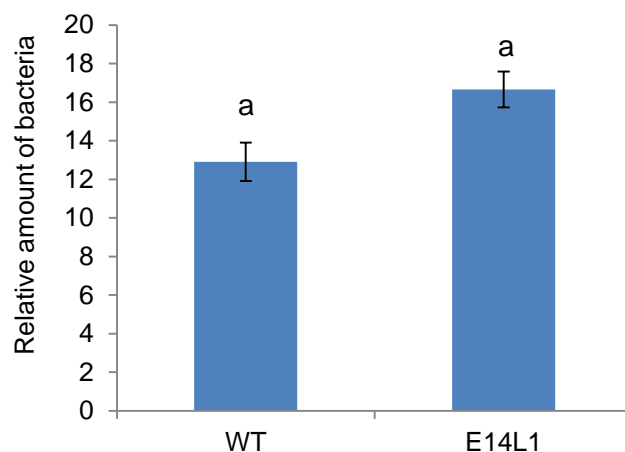
Fig. 3.16 Relative amount of *Rr*F4 in wt barley and the transgenic barley *HvBI-1* line. WT represents wild-type barley (Golden Promise). E14L1 represent an independent transgenic HvBI-1 Golden Promise line. The relative amount of *Rr*F4 was based on the ITS region in *Rr*F4 relative to the barley *ubiquitin* gene. Mean values and error bars of three independent biological replicates are given. Letters on the top of the bars indicate statistically differences tested by T-test (P<0.05). Same litters indicate no significant differences.

3.7 Quantification of endobacterium *R. radiobacter* in *P. indica*

3.7.1 Limited detection of bacteria *R. radiobacter*

An *Rr*F4 ITS-target standard curve was generated to quantify the amount of Rhizobium cells in *P. indica*. The standard curve consisted of eight concentrations of *Rr*F4 ITS targets 1×10^2 to 1×10^9 per µL. The amplification efficiency of the ITS Q-PCR, which determined by the standard curve, was 69% (y=-4.391x + 45.872, R^2=0.988). Melt curve of Q-PCR products indicated the problem of the detection limit for this primer system. Primer dimers were formed at low concentrations of target DNA (100 targets per PCR reaction) or in NTCs. Those had a lower melting point (Tm = 78ºC) than *Rr*F4's ITS target (Tm=88°C) in the Q-PCR (Fig. 3.17). Because the ITS amplification products did not melt at 83ºC, while the amplification products from primer dimers were totally molten at that temperature, this temperature 83ºC was ajusted to the Q-PCR cycles before fluorescence measurement to avoid the unspecific quantification of primer dimers. In the dilution with 100 targets, only primer dimers

could be detected as indicated by melt curve analysis (Tm = 78ºC). The detection limit by Q-PCR was 100 ITS targets per Q-PCR reaction. In order to detect the endobacterium by Q-PCR, approximately 500 mg of fungal tissue was necessary for DNA extraction.
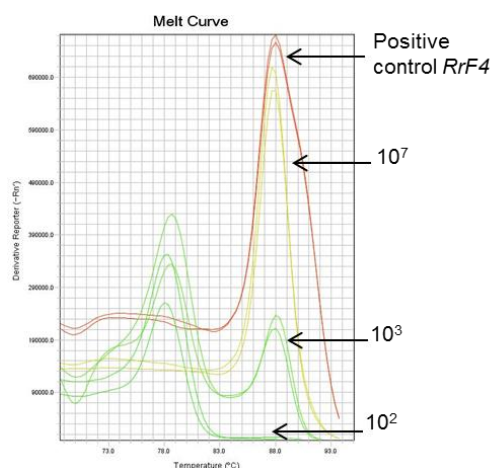

Fig. 3.17 Melt curve of PCR amplified ITS fragments and primer dimers in Q-PCR. Three different concentrations of ITS targets (10^7, 10^3 and 10^2 per PCR reaction) and pure RrF4 were used to see the primer efficiency. There was melting peak (T_m) with 10^7 and 10^3 targets at 83ºC as in pure RrF4. There was only melting peak from primer dimer with 10^2 targets at 78ºC instead of ITS melting peak at 83ºC.

3.7.2 Quantification of endobacteria *R. radiobacter* in *P. indica* colonized barley roots

The amount of endobacteria *R. radiobacter* was quantified with *P. indica* colonized barley roots and compared to axenic *P. indica* cultures. The relative amounts of *P. indica* and endobacteria *R. radiobacter* in barley roots were determined relative to the amount of the barley housekeeping gene *ubiquitin*. The colonization of *P. indica* was stronger for seedlings cultured in jars with ½ MS meidum than those cultured in pots filled with 2:1 mixture of expanded clay and Oil-Dei, since the relative amount of *P. indica* in jars growth condition was higher than in pot growth condition (Fig. 3.18 A). The relative amount of endobacteria was constantly detected in barley roots grown in jars at 7 and 14 dpi, while in pots it was only detectable at 7 dpi (Fig. 3.18 B). The

absolute amount of endobacteria *R. radiobacter* was calculated with the standard curve of *Rr*F4's ITS amplification products (Fig. 3.18 C), while the absolute amount of *P. indica* was calculated using a standard curve based on the amplification of the fungal *Tef* gene (Fig. 3.18 D). Based on the genome sequence, *Rr*F4 has likely three ribosomal rRNA operons and therefore three ITS regions on the bacterial genome. The number of targets determined by the Q-PCR quantification needs to be divided by three to get the number of endobacteria cells.
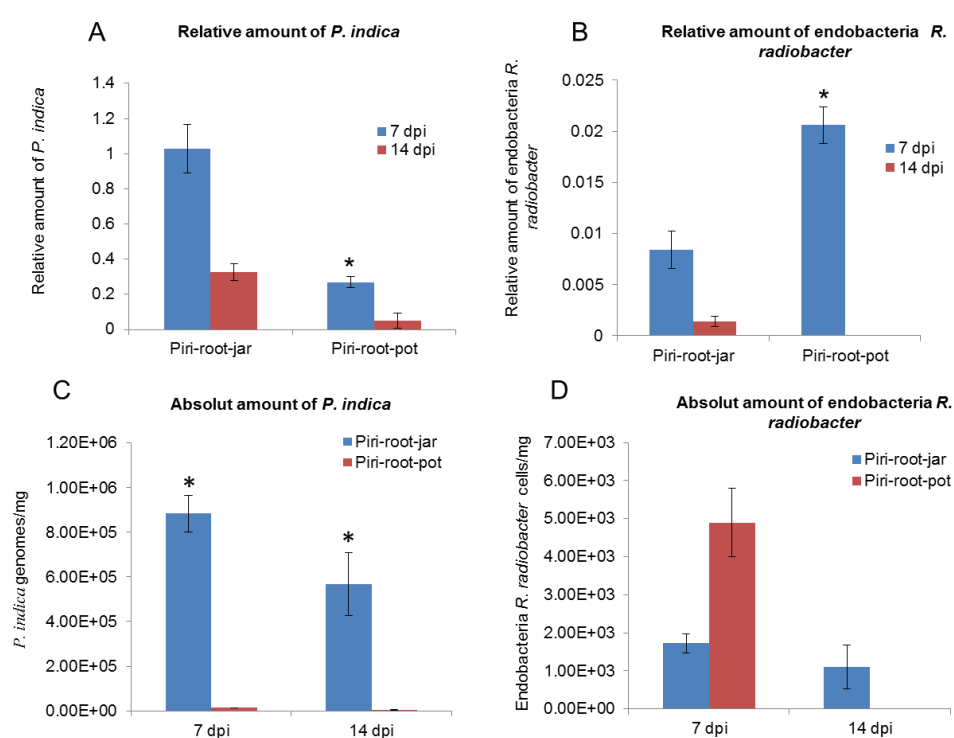
Fig. 3.18 Quantification of endofungal *R. radiobacter* and *P. indica* in barley roots under different growth conditions. Three-day-old barley seedlings were dip-inoculated with *P. indica* spores for 1.5 hour, subsequently cultured in sterilized jars with ½ MS medium or pots with Seramis and Oil-Dri (3:1). Roots were harvested at 7 and 14 dpi. A: The relative amount of *P. indica* was quantified with *Tef* and related to barley *ubiquitin*; B: The relative amount of endobacteria was quantified with *Rr*F4's ITS related to barley *ubiquitin*; C: The absolute amount of *P. indica* calculated with standard curve of *Tef* gene; D: The absolute amount of endobacteria calculated with standard curve of ITS region from *Rr*F4. Error bars indicate standard errors based on three independent biological replicates. Asterisks indicate statistical significant difference (Student's t-test *$P<0.05$; **$P<0.01$).

The experiment above showed plants growing in sterile condition with ½ medium is a better system for the measurement of endobacterium *Rhizobium* than plants growing in pots. The abundance of Rhizobium bacteria was detected and compared in *P. indica* in the presence and absence of the plant host. The relative amount of endobacteria in *P. indica* was calculated with ITS related to *Tef* gene from *P. indica*. Q-PCR results showed that the relative amount of endobacteria in *P. indica* colonized on barley roots was significantly higher than in axenic *P. indica* at all the time points, and the amount detected in root samples at 7 dpi was higher than at 14 dpi (Fig. 3.19).
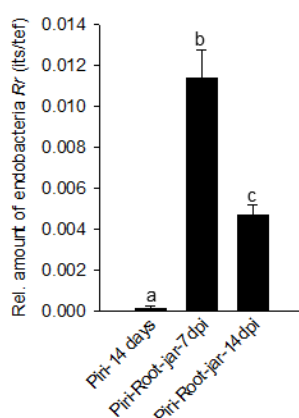
Fig. 3.19 Quantification of endobacterium *R. radiobacter* in the presence and absence of the plant host. Three-day-old barley seedlings were inoculated with spores of *P. indica*, and cultured in sterile jars. At the same time the spores were cultured in liquid CM medium in flask. Barley roots were harvested at 7 dpi and 14 dpi, while the *P. indica* from liquid culture was harvested after 14 days. The relative amount of endobacteria from each sample was quantified with Q-PCR. Mean values and error bars based on three independent biological replicates are given. Different letters on the top of the bars indicate statistically significant differences tested by one-way analysis of variance performed with the Tukey test (P<0.05).

3.7.3 Quantification of endobacteria *R. radiobacter* in *P. indica* supplied with root extracts

Root extracts obtained from grinded root tissue and small root pieces obtained from mechanical cutting were added into the liquid culture of *P. indica* spores as growth supplement, and harvested after one week for endobacteria *R. radiobacter* quantification, respectively. The relative amount of endobacteria increased in both

cases, but the increase in both *P. indica* cultures was not significant compared with the relative endobacteria amount in axenic *P. indica* culture (Fig. 3. 20).

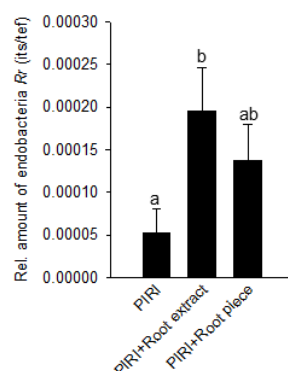**Fig. 3.20 The relative amount of endobacterium *R. radiobacter* in *P. indica* cultured with root extracts and small pieces of roots, respectively.** Sterilized barley seeds were germinated under sterile growth condition for five days, roots from these seedlings were harvested under the clean bench. Half of the root material was cut into small pieces, added into liquid culture of *P. indica* spores and cultured at 130 rpm/min room temperature. The other half of the root material was grinded with sterilized mortar and pestle in liquid CM medium, and filtered through 0.2 μm filter. The filter-sterilized root extraction was added into liquid culture of *P. indica* spores and cultured in the same way. After cultivation for 7 days the relative amount of endobacteria was quantified and calculated based on the genome ratio. Mean values and error bars based on three independent biological replicates are given. Different letters on the top of the bars would indicate statistically significant differences tested by one-way analysis of variance performed using the Tukey test (P<0.05).

3.8 Re-isolation of *P. indica*

Since the amount of endobacterium *Rhizobium* was increased when *P. indica* colonized on barley roots，fresh *P. indica* was re-isolated from barley roots, and the endobacteria were quantified in this freshly re-isolated *P. indica* (RE-Piri). Barley roots were surface sterilized and cultured with CM medium on agar plate or liquid CM medium. After one week of incubation, spores or mycelia of *P. indica* resident inside the roots broke the root cell wall, propagated and formed colony on the agar medium around the root (Fig. 3.21 A, B, C) or mycelium around the root tissue and root hair in liquid culture (Fig. 3.21 D and E). The re-isolated *P. indica* subculture-1 (Re-Piri-1) was further transferred to a fresh plate which represented as re-isolated *P.*

indica subculture-2 (Re-Piri-2) (Fig. 3.21 F). The biomass from Re-Piri-1 and Re-Piri-2 were taken from the plates and used for DNA extraction with Macherey-nagel kit. The amount of endobacteria was quantified and compared with long-term lab cultured *P. indica* (LL-Piri) by Q-PCR. Higher amount of endobacteria were detected in RE-Piri and decreased again after sub-cultivation on agar plates. As shown in Fig. 3.22, the relative amount of endobacteria was significantly increased in Re-Piri-1 compared to axenic LL-Piri culture. The amount of endobacteria was significantly higher in Re-Piri-1 than in Re-Piri-2.

Fig. 3.21 Re-isolation of *P. indica* from barley roots. Barley seedlings were inoculated with *P. indica* and cultured on ½ MS agar in sterile jars for two weeks. The inoculated roots were surface sterilized (washed with 70% ethonal for 1 min and 3% NaClO for 5 min), cut into small pieces and cultured on CM solid and liquid medium. After two weeks, *P. indica* colonies were formed around root pieces which was recorded as re-isolated-*P. indica*-1 (Re-Piri-1) (A, B, C, D and E). The Re-Piri-1 was sub-cultured on CM agar plate as re-isolated-*P. indica*-2 (Re-Piri-2) (F).
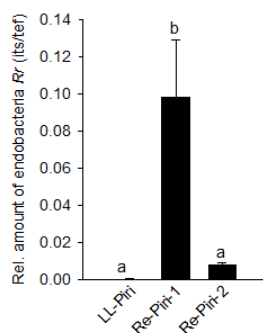

Fig. 3.22 Quantification of endobacterium *R. radiobacter* in RE-Piri (Re-Piri-1 and Re-Piri-2)

compared to LL-Piri. The relative amount of endofungal bacteria was quantified with ITS targets of *Rr*F4 related to *Tef* gene of *P. indica*. Three-week-old cultures were used for quantification. The relative amount of endobacteria was quantified in Re-Piri-1, Re-Piri-2 and compared with LL-Piri. Mean values and standard errors based on three independent biological replicates. Different letters on the top of the bars would indicate statistically significant differences tested by one-way analysis of variance performed using the Tukey test (P<0.05).

3.9 Invasion of free living *Rr*F4 into *P. indica* was not observed

Pure culture of GFP-tagged *Rr*F4 cells were co-cultured with *P. indica* to test if *Rr*F4 can invade the fungal host where it was originally isolated from. Spores of *P. indica* were cultured in liquid CM medium and germinated for three days. Subsequently different concentrations of GFP-tagged *Rr*F4 cells were added to the *P. indica* culture. The co-culture was observed under the fluorescence microscopy after cultivation for one day, two days and three days. Many GFP-tagged *Rr*F4 cells were sticking at the surface of hyphae especially at the tips (Fig. 3.23A) and on chlamydospores (Fig. 3.23B), but green fluorescent *Rr*F4 cells were not detected within hyphae or spores (Fig. 3.23). In order to introduce *Rr*F4 into the fungal host, protoplasts of *P. indica* were generated to merge with protoplast of GFP-tagged *Rr*F4, but it was still not successful to bring *Rr*F4 back to fungus host (data not showed).
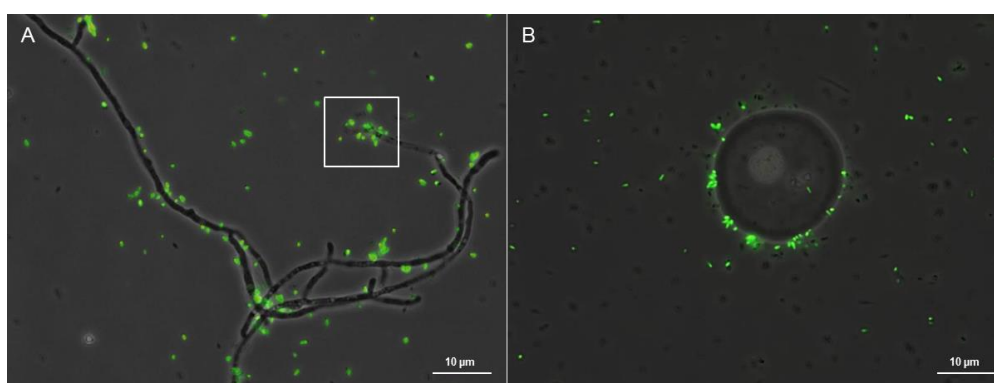
Fig. 3.23 GFP-tagged *Rr*F4 sticking around the hyphae (A) and chlamydospores (B) of *P. indica*. Chlamydospores were collected from three-week-old *P. indica* cultures on agar plates, and cultured for three-day in CM medium to germinate. Overnight cultured GFP-tagged *Rr*F4 bacteria were collected, re-suspended and added to the *P. indica* culture with final bacterial concentrations of OD_{600}=0.1, 0.01 and 0.001. The co-culture was observed under the fluorescence microscopy after one day, two days and

three days. Figure A and B showed the fluorescence microscopic analysis after one day co-culture. A: The GFP-tagged *Rr*F4 stacking around the hyphae. B: the GFP-tagged *Rr*F4 surrounding the spores. The square box showed GFP-tagged *Rr*F4 around the tip of hyphae.

3.10 Curing *P. indica* through antibiotics treatments and single protoplast cultivation

The biological activity and functional mechanism of *Rr*F4 were exploited because of the successful isolation of the endobacterium *Rhizobium radiobacter*. It has not been possible to cure *P. indica* so far, therefore further trials were performed here to further expose the connection among the endobacterium, the fungus *P. indica* and the plant host. Treatments with antibiotics are often used to kill endofungal bacteria and to obtain a cured fungus (Partida-Martinez and Hertweck 2005). We decided to carry out our experiment bythis method as well. In order to avoid that the fungal cell wall prohibits the entrance of antibiotic into the fungal cells, protoplasts of *P. indica* were used for the antibiotic treatments. CM medium containing 300 μg/mL spectinomycin and 300 μg/mL ciprofloxacin were testified to be very efficient on killing *Rr*F4 cells grown as pure culture. Therefore those concentrations were used in this experiment. Five-day-old single fungal colonies that were derived from single protoplasts were transferred to a new culture plate. No differences were obtained between antibiotic treated and untreated *P. indica* cultures at the beginning of the cultivation, but after three generations, the germination of *P. indica* was obviously delayed when the protoplasts cultured on the round petri dish with medium containing antibiotics (Fig. 3.24 A, B). Light microscopical imaging showed that the fungal colonies on medium with antibiotics were smaller than the colonies on medium without antibiotics. The single colony was picked up and cultured on new agar media, the diameter of the colony without antibiotic treatment was approximately 6 cm, while the diameter of the colony on antibiotic containing medium had a size of only 3 cm (Fig. 3.24 C, D). During the experiment, no apparent morphology change was obtained on the hyphae of *P. indica*, but the number of spores formed by *P. indica* with antibiotic treatment was significantly reduced (Fig. 3.24 E, F). With q-PCR analysis, endobacteria could

not be detected in four samples from the third generation of culture. Because of the detection limitation of the ITS q-PCR system of 100 ITS targets in the PCR reaction, further tests are needed to check for endobacteria in these single-protoplast-derived *P. indica* cultures (SP-Piri) (see chapter 3.11).

The single-protoplast-derived *P. indica* culture and control *P. indica* culture were cultivated in liquid CM medium for one week, respectively. The mycelia were taken from these liquid cultures, stained with DAPI and observed with fluorescence microscopy. Changed nuclei structures were observed among them. The nuclei in the hyphae of SP-Piri were in rod shape (Fig. 3.25 A), while the nuclei from control *P. indica* were in spherical shape (Fig. 3.25 B).
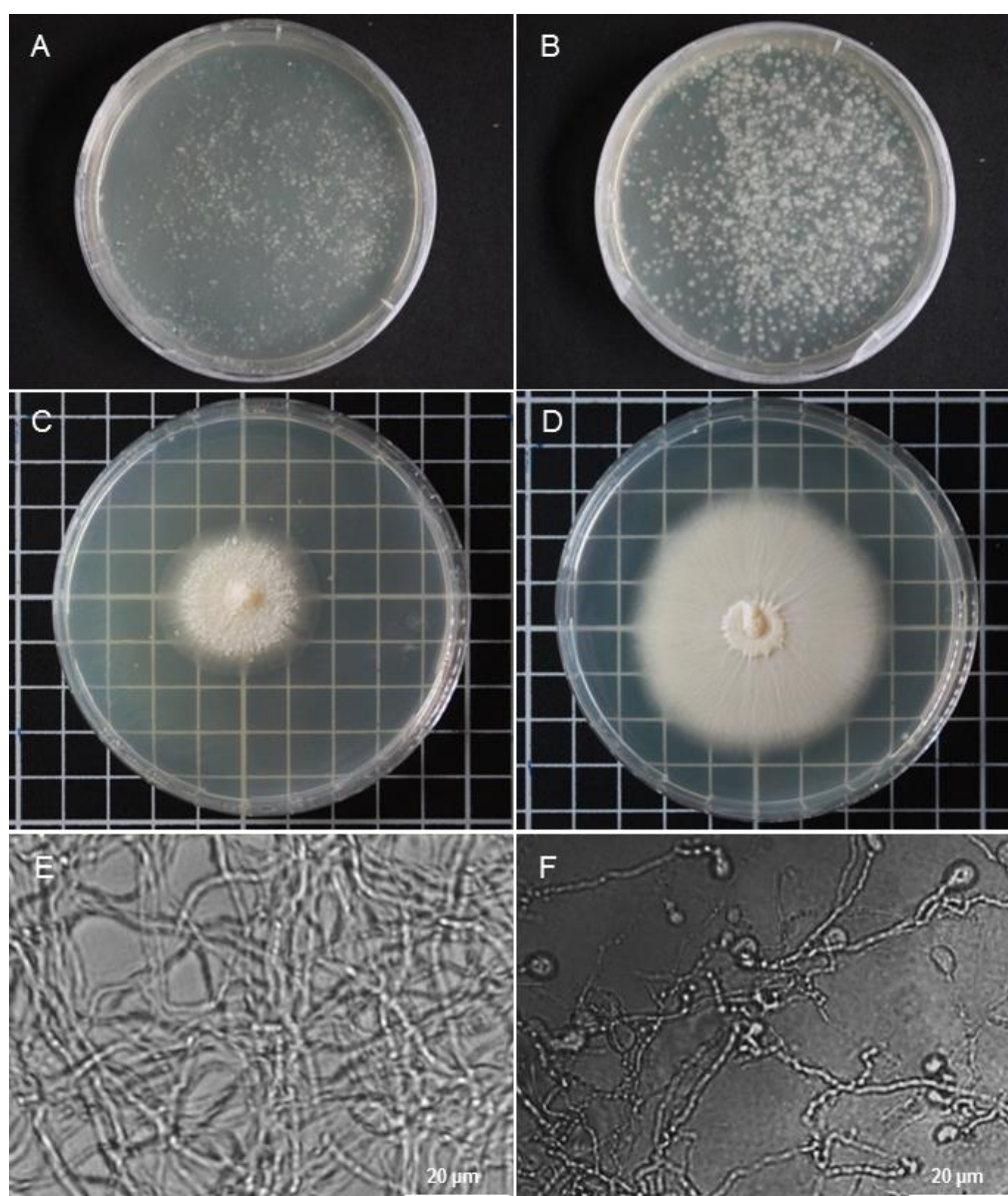
Fig. 3.24 morphological changes of *P. indica* cultures after antibiotics treatment and single protoplast cultivation. The protoplasts of *P. indica* were extracted and cultured on CM medium with spectinomycin and ciprofloxacin (A, C, E,) and without antibiotics (B, D, F). A: protoplasts germinated for 5 days on CM medium containing 300 μg/mL spectinomycin and 300 μg/mL ciprofloxacin. B: protoplasts germinated for 5 days on CM medium without antibiotics. C: single colony picked up from A and propagated on medium with antibiotics for 10 days. D: single colony picked up from B and propagated on medium without antibiotics for 10 days. E: the mycelia of *P. indica* growing on the medium with antibiotics were observed under microscope. F: the mycelia and spores of *P. indica* growing on the medium without antibiotics were observed under the microscope.
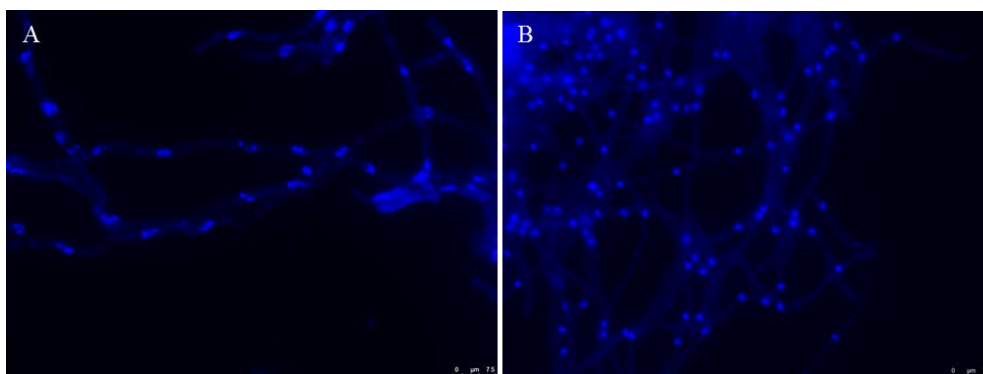
Fig. 3.25 Nuclei structure in the hyphae of single-protoplast-derived *P. indica* and control *P. indica*. Single-protoplast-derived *P. indica* culture and control *P. indica* culture were cultivated in liquid CM medium with 130 rpm/min for one week. The mycelia were stained with DAPI and observed under fluorescence microscopy. A: mycelium from single-protoplast-derived *P. indica* culture, the nuclei were stained with DAPI resulting in blue fluorescence under fluorescence microscopy; B: mycelium from control P. indica culture.

3.11 Biological activity conferred by SP-Piri, LL-Piri and RE-Piri

To address the question whether single-protoplast-derived *P. indica* and freshly re-isolated *P. indica* exhibit biological activities comparable with long term lab-cultured *P. indica*, barley seedlings inoculated with SP-Piri, LL-Piri and RE-Piri, respectively, were cultured in pots containing 2:1 mixture of expanded clay and Oil-Dri. Fresh weights from shoot and root of the seedlings were measured to test for growth promotion, while the systemic resistance was assessed with barley powdery mildew infection.

3.11.1 Root colonization patterns from SP-Piri, LL-Piri and RE-Piri

During the cultivation for biological activity assay, the roots of barley seedlings at 7 dpi were investigated microscopically with WGA-staining to visualize the colonization density and pattern of different *P. indica* cultures. The staining showed a high amount of pear-shaped chlamydospores and mycelium in the roots inoculated with RE-Piri (Fig. 3.26 A), while a much less colonization was obtained for roots infected by LL-Piri (Fig. 3.26 B). The lowest infection rate was determined for the roots inoculated with SP-Piri, the culture showed a very low colonization of the root

tissue by the SP-Piri and most of the structures obtained were mycelium (Fig. 3.26 C). Similar results were obtained in three independent biological replicates.
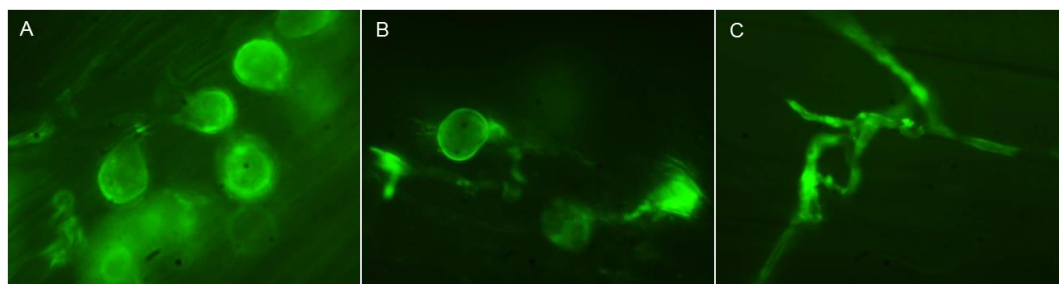
Fig. 3.26 WGA-staining of barley roots colonized by *P. indica* at 7 dpi. Three-day-old seedlings were dip-inoculated with mycelium of SP-Piri, LL-Piri, and RE-Piri, respectively, and cultured for 7 days in a growth chamber. Roots were harvested from one-week-old plants and stained with WGA for fluorescence microscopy analysis. A: pear-shaped chlamydospores and mycelium in the root colonized by RE-Piri; B: spores and mycelium in the root infected by LL-Piri; C: mycelium colonized on the barley root inoculated with SP-Piri.

3.11.2 Quantification of endobacteria *R. radiobacter* in root colonized by SP-Piri, LL-Piri and RE-Piri

Q-PCR analysis was used to determine the abundance of endobacteria in barley roots colonized by single-protoplast-derived *P. indica* culture (SP-Piri), re-isolated *P. indica* culture (RE-Piri) and long term lab-cultured *P. indica* culture (LL-Piri). Three-day-old seedlings were dip-inoculated with mycelium from SP-Piri, fresh RE-Piri and LL-Piri, respectively. Root samples were harvested at 7 dpi, and used to quantify the colonization density of *P. indica* and the amount of endobacteria present in the root colonized with different *P. indica* cultures. The *Tef* gene of *P. indica* was used for the quantification of the fungus, while the specific ITS primer system was used for the quantification of the endobacteria Rhizobium. The fresh RE-Piri showed a significant highest colonization density of the roots of barley seedlings grown on 1/2 MS in jar, no significant colonization difference were obtained between SP-Piri and LL-Piri (Fig. 3.27 A). The relative amount of endobacteria in roots colonized by RE-Piri was significantly higher compared with LL-Piri and SP-Piri colonized roots (Fig. 3.27 B).

There was still endobacteria detectable in roots colonized by SP-Piri, although the amount was significantly reduced compared with LL-Piri and RE-Piri (Fig. 3.27 B). The constantly detected endobacteria in SP-Piri indicates that single protoplast cultivation and antibiotics treatment did not curing *P. indica* from its endobacteria completely.
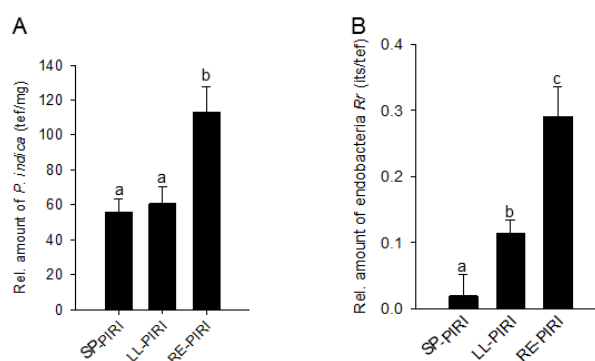
Fig. 3.27 Colonization of different *P. indica* cultures on barley roots and quantification of endobacterium *R. radiobacter* inside. Three-day-old barley seedlings were dip-inoculated with mycelium from SP-Piri, fresh RE-Piri and LL-Piri, respectively. The seedlings were cultured in a growth chamber and harvested after one week for quantification. A: the colonization of different *P. indica* cultures on barley roots. The amount of *P. indica* was quantified with q-PCR detection of the fungal *Tef* gene. B: genome ratio of endobacteria Rhizobium and *P. indica* (endobacteria/Piri) on barley roots. The amount of endobacteria was quantified with ITS of *Rr*F4 and uniformed with *Tef* gene of *P. indica*. SP-Piri: single-protoplast-derived *P. indica* cultue; LL-Piri: long term lab-cultured *P. indica*; RE-Piri: fresh *P. indica* re-isolated from barley roots. Mean values and standard errors of three independent biological replicates are given. Different letters on the top of the bars indicate statistically significant differences tested by one-way analysis of variance performed with the Tukey test (P<0.05).

3.11.3 Fresh weight measurement and pathogen resistance assessment

Three-week-old plants inoculated with different *P. indica* cultures were harvested for biomass measurement. Growth promotion was observed from barley plants inoculated with LL-Piri and RE-Piri compared to the control (non-inoculated plants) before harvest (Fig. 3.28 A). Compared with control, shoot fresh weight was increased in all the plants treated with *P. indica* cultures (Fig. 3.28 B). The shoot weight was

significantly increased in barely seedlings treated with RE-Piri (17.7%) and LL-Piri (15.9%) compared to the control seedlings which were not inoculated with any *P. indica* culture. Although slight growth promotion was obtained by the visual observation from SP-Piri inoculated plants, the 7.5% increase induced by SP-Piri was not significantly different from control plants. There was no significant difference among plants infected with SP-Piri, LL-Piri and RE-Piri. The average root weights showed increase in plants treated by SP-Piri, LL-Piri and RE-Piri compared to control plants, but the increase was not significant (Fig. 3.28 C).

To determine the systemic resistance among different *P. indica* cultures, the third leaves from three-week-old plants inoculated with *P. indica* cultures were used in detached-leaves pathogen assay. Conidia from powdery mildew pathogen were blwn to infect the detached leaf-segment, pustules were counted after 6-day infection. In contrast to control plants, the number of pustules formed on leaves was decreased in all the plants treated with *P. indica* cultures (Fig. 3.28 D). The pustules on leaves from plants inoculated with RE-Piri and LL-Piri were significantly reduced compare with control, nevertheless there was no significant reduction on plant infected with SP-Piri. The maximum systemic resistance against *Bgh* was induced in *Rr*F4 treated plants (26.9%) followed by infection from RE-Piri (14.5%) and SP-Piri (14.5%). The lowest systemic resistance was from the infection of LL-Piri (3.4%).
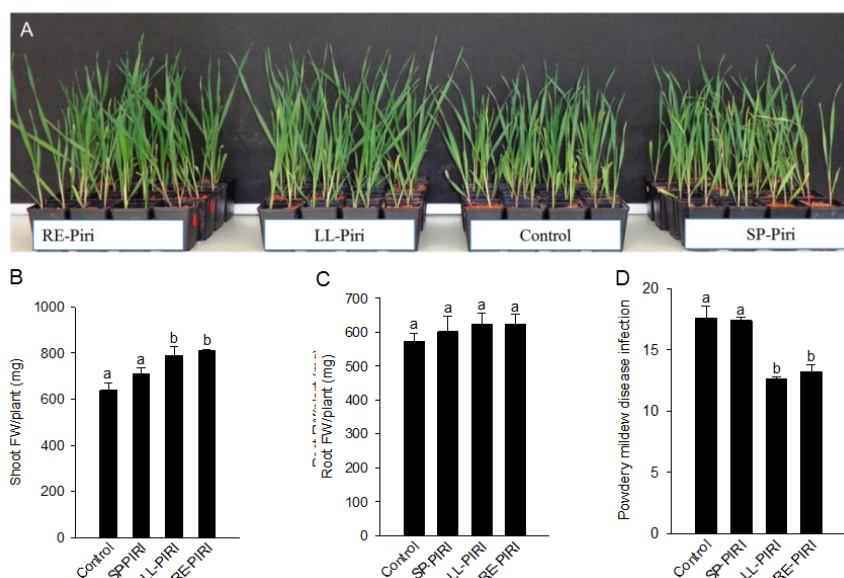
Fig. 3.28 Biological activity conferred by SP-Piri, LL-Piri, RE-Piri and *Rr*F4 on barley. Three-day-old barley seedlings were inoculated with mycelium of SP-Piri, LL-Piri and RE-Piri, respectively, and cultured in growth chamber. A: three-week old plants inoculated with RE-Piri, LL-Piri, SP-Piri and control; B: the fresh weight from shoot; C: the fresh weight from root; D: number of pustules on the detached-leaves. Bars indicate standard errors based on three independent biological replicates. Letters on the top of the bars indicate statistically significant differences tested by one-way analysis of variance performed with the Tukey test (P<0.05).

4   Discussion

*Rr*F4 treatment increased plant biomass and resistance to powdery mildew *Blumeria graminis* f. sp. *hordei* in barley, the pathogenic bacterium *Xanthomonas translucens* pv. *Translucens (Xtt)* in wheat and *Pseudomonas syringae* pv. *tomato* DC3000 (Pst) in Arabidopsis (Scharma *et al.* 2008; Glaeser *et al.* 2015), while its fungal host *P. indica* conferred promotion on biomass and yield, induction of resistance to leaf and root pathogens, and increase in the tolerance to salt stress (Peškan-Berghöfer *et al.* 2004; Waller *et al.* 2005; Sherameti *et al.* 2005; Schäfer and Kogel 2009; Jacobs *et al.* 2011; Varma *et al.* 2012; Oberwinkler *et al.* 2014; Ye *et al.* 2014). The beneficial effects induced by *Rr*F4 in plants suggest a crucial role of the bacterial partner from *P. indica* and raise the question whether *P. indica* induces pathogenic resistance and biomass formation on the broad spectrum plant host alone.

4.1   Colonization pattern of *Rr*F4 on the root surface

*Rr*F4 labeled with GUS- and GFP- reporter genes were used to visualize the colonization pattern of bacterium *Rr*F4 on barley and Arabidopsis. It was clearly showed in barley root that GUS-labeled *Rr*F4 colonized on the maturation zone I at the early inoculation period, spread to maturation zone II on the primary root later on, and proliferated at lateral roots with the same pattern as in primary root after longer inoculation period. During the whole growth period, root cap was virtually remained free of bacteria colonization while elongation zone and meristematic zone were seldom colonized (Glaeser *et al.* 2015). The resembled root colonization pattern was observed from *P. indica*. Fungal colonization and proliferation were gradually increased on the root maturation zone, while the elongation zone was colonized with occasional hyphal structures, and root cap was a litter bit heavier infested by hyphae than the elongation zone but much less than the colonization on maturation zone (Deshmukh *et al.* 2006; Schäfer *et al.* 2009; Jacobs *et al.* 2011). On the basis of *P. indica*'s colonization pattern on Arabidopsis, we drew a comparison draft about the colonization pattern of *Rr*F4 and its fungal host *P. indica* in the meristematic zone,

elongation zone and maturation zone on Arabidopsis roots, and it is published in Glaeser *et al.* 2015 (Fig. 4.1). These data indicate the free-living *Rr*F4 not only has similar biological function on plant host, but also has quite similar colonization pattern as its fungal host *P. indica* on the plant.
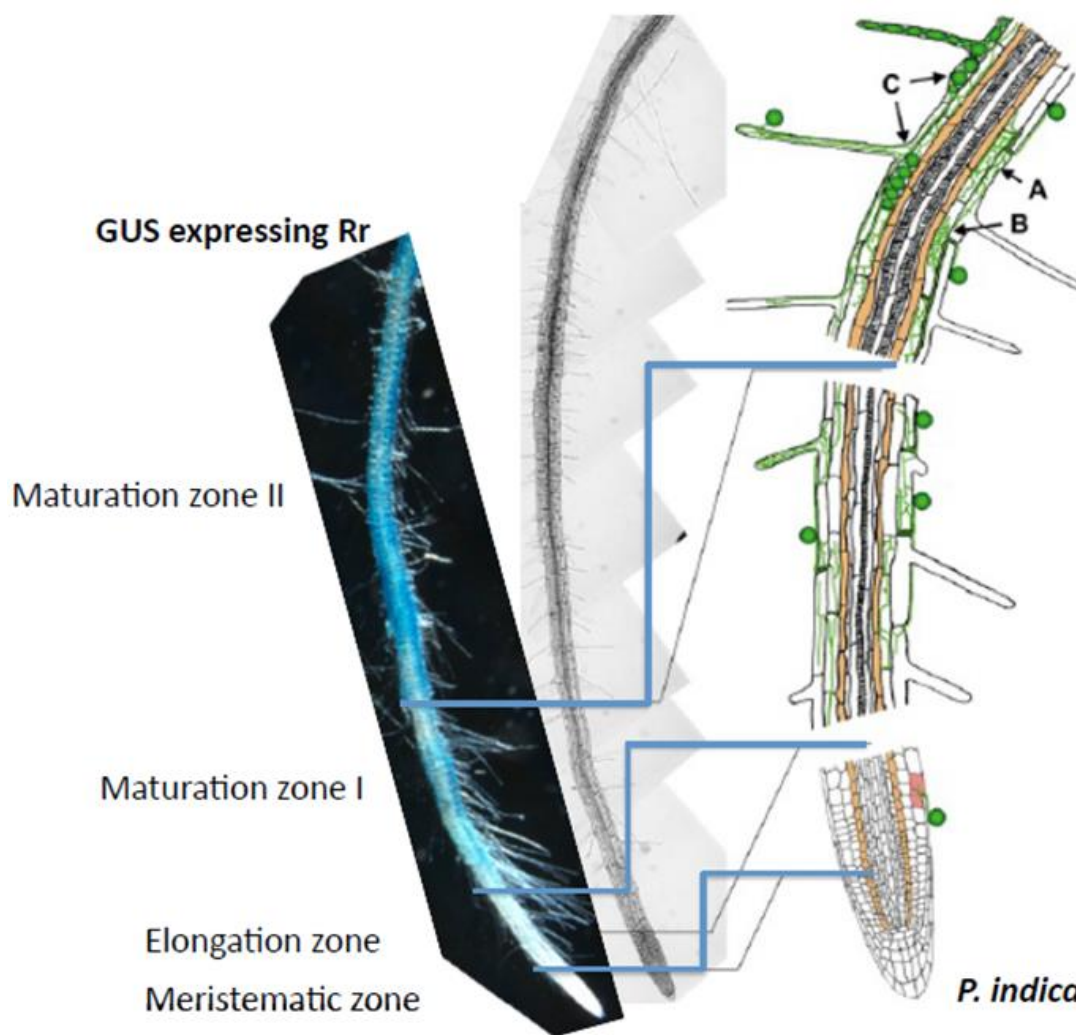
Fig. 4.1 Comparison of the colonization pattern of *Rr*F4 and its fungal host *P. indica* on Arabidopsis roots (taken from Glaeser *et al.* 2015). Root colonization of *P. indica* is mainly on the maturation zone II of Arabidopsis, while *Rr*F4 additionally colonized on the elongation zone, maturation zone I and meristematic zone as well.

Dense biofilms formed by *Rr*F4 on the root surface were detected by scanning and transmission electron microscopy, the multilayer biofilm were approximately 5 μm

thick with bacterial cells embedded in a dense matrix of extracellular polymeric substances. This matrix could be formed by extracellular polysaccharides, extracellular DNA or (glyco) proteins (Flemming and Wingender 2010). Several genes responsible for the synthesis of polysaccharide were found in the genome of *Rr*F4 (Glaeser *et al.* 2015). The polysaccharide can be succinoglycan, glucans and outer membrane lipopolysaccharides, they are well known to be involved in the formation of extracellular biofilm matrix of '*Agrobacterium tumefaciens*' strains (Goodner *et al.* 2001; Heindl *et al.* 2014; Matthysse 2014).

Fiber-like structures were detected to cross-link bacterial cells on root surface. It was previously reported that the production of cellulose is necessary for the efficient root surface attachment for *Agrobacterium tumefaciens* and leads to the loose aggregation of cells on root (Heindl *et al.* 2014; Matthysse 2014; Matthysse 1983). Based on these findings, we presumed the fiber-like structures could be cellulose. Through the analysis of *Rr*F4's genome sequencing, we found that two gene clusters, which are required for the cellulose production in *R. radiobacter* C58, *celABCG* and *celDE*, presented on *Rr*F4's linear chromosome (Glaeser *et al.* 2015). This molecular information got from genome analysis confirmed our presumption that fiber-like structures among *Rr*F4 cells are cellulose.

Many bacteria cells were preferentially proliferated at the lateral root protrusion and root hair protrusion, formed bacterial conglomerates at primary and lateral root junction as the growth of the lateral roots. Bacterial cell micro-colonies were also located in the cracks among plant cells on the root cap instead of fully cover the cell surface. The colonization of *Rr*F4 on plant doesn't induce more root hairs compared with the untreated control plants. Compant and colleagues reported that the cracks on plant root, such as lateral root bases, root hairs and root tips, are normally served as entry sides for bacterial penetration (Compant *et al.* 2005; Compant *et al.* 2010; Reinhold-Hurek and Hurek 1998). These evidences in combination with results shown here indicate that *Rr*F4 probably make use of the lateral root emergence, root hair protrusion and cracks on the root as the passive entrance into the inner root tissue.

4.2 Intercellular localization of *Rr*F4 in the root cortex and central cylinder

Cross-section of barley roots were used to analyze the bacterial colonization in the inner roots. GUS-tagged and GFP-tagged *Rr*F4 bacteria were visible in the root cortex (rhizodermis, exodermis and cortical tissues) after seven days inoculation. Light microscopy and TEM with the ultrathin root cross-section showed that most of the bacteria were detected among the rhizodermal cells and cortical cells in the intercellular spaces. After 21 days inoculation, GUS-*Rr*F4 cells were detected in the intercellular space between the endodermal cells and the central cylinder, while GFP-*Rr*F4 cells were also seen in the intercellular spaces of central cylinder. The finding of bacterial colonization in the endodermis is crucial, since the *Rr*F4's fungal host *P. indica* was not observed in the inner part of root beyond the endodermis to central cylinder layer (Deshmukh *et al.* 2006; Jacobs *et al.* 2011). We can not exclude that bacteria *Rr*F4 were able to pass the endodermis into central cylinder, it is also possible that *Rr*F4 cells invaded the vascular tissue via the non-maturated tissue of the elongation zone where differentiation has not been completed yet. However, bacteria cells were not found in the over ground part shoot tissue, since the blue or green bacterial (GUS-*Rr*F4 and GFP-*Rr*F4) were neither visible under microscopes nor culturable on medium from stem, leaf or leaf sheath, and the specific ITS target of *Rr*F4 could not be detected by qPCR in shoot and leaves. Those data suggested that, *Rr*F4 is different from other endophytic bacteria (Rothballer *et al.* 2008; Hardoim *et al.* 2008; Van der Ent *et al.* 2009), it does not spread systemically into the upper plant tissue.

*Rr*F4 cells were assessed to colonize in the interior of Arabidopsis roots. Bacterial cells were consistently seen on the surface of root hair zones, at the base of root hair and emergence site of secondary root from 7 dpi to 21 dpi. The central vascular system of primary and secondary Arabidopsis roots were heavily colonized compared with the barley roots.

4.3 Single root cell colonization strategies of *Rr*F4

Bax inhibitor-1 (BI-I) is a negative regulator of cell death that suppresses cell death through Bcl-2 in mammals, and is located in the membrane of endoplasmic reticulum

(Xu and Reed, 1998). There are no homologs of the cell death inducer BAX in plants, but BI-1 in barley and other plants were shown to suppress cell death induced by BAX (Hückelhoven, 2004; Eichmann *et al.* 2006; Watanabe and Lam 2009). Although the relative expression of *HvBI-1* was increased during barley root development, *Rr*F4-treated barley plants showed an additional increase in expression compared with non-treated plants from 0 dai to 14 dai, while *P. indica* colonized plants showed a reduced expression of *HvBI-1* compared with non-colonized plants (Deshmukh *et al.* 2006). Quantification of *Rr*F4 in transgenic HvBI-1 line showed no significant difference compared with the amount in wt barley, while the quantification of *P. indica* in those lines showed significant reduction (Deshmukh *et al.* 2006). These experimental data and the comparison with the data from *P. indica* indicates that the colonization of *Rr*F4 doesn't induce cell death in plant host.

Our previous study showed that *Rr*F4 multiplies at the maturation zone on the primary and secondary roots, enters the inner roots through the lateral root protrusions and root hair protrusion sides, propagates at the rhizodermal, cortical and endodermal layers and spreads into the central cylinder (Gleaser *et al.* 2015). During the experiment, we observed that there were single root cells heavily colonized by GUS-tagged *Rr*F4 on root hair zone. Confocal laser scanning microscopy analysis showed cells within several GFP-tagged *Rr*F4 on the root surface at early time pints, single cell was filled by GFP-tagged *Rr*F4 except the nucleus at the later time points. Root cross-section analysis illustrated *Rr*F4 colonized in the epidermis and went into the root cortex through the intercellular space. These data reveal *Rr*F4's cell colonization strategies that *Rr*F4 multiplies in the intercellular space firstly, afterwards some *Rr*F4 invade the barrier of cell wall and successfully infect the dead root cells in the maturation zone, after some time from that, *Rr*F4 proliferates quickly in the dead cells till the dead cell is full of bacteria except the nucleus. The dead cells here could be caused by programmed cell death (PCD), which was carried out in a self-regulated process in the plant host.

4.4 Endobacteria in stationary phase-VBNC

Some gram-negative bacteria have extraordinary ability to survive under nutrient-shortage or harsh environment. They enter into a stationary phase like state that involves morphological and physiological adaptations, when the environment is not sufficient enough to keep the steady growth (Llorens *et al.* 2010). In this stationary phase like state, bacterial cells become smaller as the result of reductive division and dwarfing, more resistant against different assaults since the formation of cell envelopes, and non-proliferative because of the starving (Nystrom 2004; Llorens *et al.* 2010). We adventurously assume that *Rr*F4 is at a stationary phase-like viable but nonculturable (VBNC) in fungal host *P. indica* in axenic culture, which could explain the very low numbers of endobacteria *Rr*F4 in the fungal hyphae and spores and the smaller spherical shape of endobacteria compared with the free-living axenic *Rr*F4 culture in liquid or solid medium. In this stationary phase, bacterial cells are more resistant to antibiotics, which could be one reason for the insufficient antibiotics treatment to cure endobacteria *Rr*F4 from its fungal host. Bacteria resuscitate from the stationary phase to growth phase when there is third partner plant supplying with enough nutrition and better propagation conditions, which could explain the increased amount of *Rr*F4 when *P. indica* colonized on plant root. A clinical strain of *Vibrio parahaemolyticus* Vp5 was explored to be recoveried from VBNC after upshift temperature from 4 ℃ to 20 ℃ and 37 ℃ (Coutard *et al.* 2007).

FtsZ is the primary cytoskeletal protein responsible for cell division in almost all the bacteria. In the bacterial cell, the ftsZ protein assembles into a protofilament that will further form into a cytokinetic Z ring at the center of the cell. This Z ring is tethering to the bacterial membrane through the binding of C-terminal peptide with FtsA protein, and supplying the bacterial cell division with cytoskeletal framework (Osawa *et al.* 2008; Peters *et al.* 2007; Pichoff and Lutkenhaus 2005). *FtsZ* as a marker gene for cell division, the expression of *FtsZ* in one endocellular bacterium *Candidatus* Glomeribacter gigasporarum was analyzed and showed to be at the highest value when the fungal host *Gigaspora margarita* colonized on plant (Anca et al. 2009). It will be interesting in the following work to measure the expression of *FtsZ* in

endobacteria Rhizobium during the tripartite interaction.

4.5 Secretion system

The co-culture of GFP-tagged *Rr*F4 and *P. indica* showed GFP-*Rr*F4 cells sticking on the hyphae and chlamydospores instead of entry into hyphae. Moebius and coleagues found that the type 2 secretion system (T2SS) of endosymbiotic Burkholderia is central for the active invasion into hyphe of the living fungus *Rhizopus* (Moebius *et al.* 2014). They said that during the invasion T2SS releases chitinase, chitosanase and chitin-binding proteins to soften the cell wall of fungus and allow the bacterial entry into fungal hyphae (Moebius *et al.* 2014). Based on this information, we checked the genome information of *Rr*F4. Not surprisingly, the whole gene cluster that is responsible for the encoding of T2SS components is missing in the genome of *Rr*F4 compared with the endosymbiont bacterium *Burkholderia*. Instead, *Rr*F4 has a Type IV secretion system, which mediates the transfer and conjugation of plasmid DNA fragment from bacteria in to the genome of plant, fungi and human cells. T4SS is a remarkable characteristic of *Agrobacterium tumefaciens*, which plays a crucial role in the transgenic technology and shows very close relationship to stain *Rr*F4 in phylogenetic tree (Fronzes *et al.* 2009; Sharma *et al.* 2008). The missing T2SS in *Rr*F4 could be the reason for the un-successful invasion of *Rr*F4 into fungal host *P. indica*.

Our co-culture experiment showed that *Rr*F4 sticking around the fungus especially the tip of hyphae and spores, which are the active part from *P. indica.* Those hyphae tips and germinating spores can be potential entry sides in the long term evolution between fungus *P. indica* and bacteria *Rr*F4. In natural environments there are many microbes in the soil, there are nutrition competition and limit, under a certain circumstance with a fine microbe balance, *P. indica* could be able to uptake *Rr*F4 from the soil in the long evolution from the active hyphae tips and germinating spores.

4.6 Removing endobacterium from *P. indica*

In general, there are two possibilities to explore the critical role of the endobacteria R.

radiobacter in the tripartite relationship. One way is the function analysis of pure bacteria *Rr*F4 on plant host without the influence from fungus host *P. indica*, which has been achieved as the successful insolation of endobacteria from fungus (Sharma *et al.* 2008; Glaeser *et al.* 2015). The other one is the functional exploration of cured *P. indica* on plant host without the impact coming from the endosymbiont. So it is an urgent target to remove the endobacteria from *P. indica* and obtain the cured *P. indica*. Partide-Martinez and Hertweck obtained cured fungus *Rhizopus microspores* from endosymbiont *Burkholderia rhizoxinica* through the antibiotics treatment (Partide-Martinez and Hertweck 2005). Alternatively, repeated passages through single-spore inoculation with plant host was used to dilute the initial *Candidatus* Glomeribacter gigasporarum (*Ca*Gg) bacterial population eventually leading to cured AM *Gigaspora margarita* spores (Lumini *et al*. 2007). In order to generate endobacteria-free fungus *P. indica*, we combined single-protoplast propagation and antibiotics treatment. In this experiment, single protoplast was used instead of single chlamydospore because of the consideration that fungal cell wall could protect endofungal bacteria being killed by applied antibiotics. *P. indica* cultures showed no endobacterial detection were further inoculated with barley seedlings, unfortunately, *Rr*F4 bacteria were again detected in the plant root samples. However, the relative amount of *Rr*F4 is significantly reduced compared with root samples colonized by wt *P. indica*. Sharma reported similar result that endobacteria were coming up after culture in medium without antibiotics (Sharma *et al.* 2008). From these data, we concluded that single-protoplast-derived *P. indica* cultures were partially cured fungus with reduced amount of endobacteria and therefore termed as partially cured *P. indica* (pcPIRI).

During the experiment, we observed that the germination of chlamydospores derived from single clones were delayed and single colonies were approximately 50% smaller in size and produced less spores compared to colonies grown without antibiotics. Although we cannot exclude the effect coming from antibiotics treatment, those morphological changes could be induced by the reduced number of endobacteria.

There are some research showed endobacteria mediated the fitness of its fungal host, for instance, the cured *Rhizopus microspores* showed no sporulation (Partida-Martinez *et al.* 2007; Partide-Martinez and Hertweck 2005). The cured fungus *Gigaspora margarita* showed limited changes in spore morphology, while the symbiosis with this endobacterium *Ca*Gg increases the environmental fitness and bioenergetics potential of the AM fungal host (Lumini *et al*. 2007; Salvioli *et al.* 2015).

4.7   Amount variation of endobacteria *R. radibacter* in *P. indica*

It is proofed by specific FISH detection, PCR amplification and successful bacteria isolation that the endobacterium *R. radiobacter* is presenting in *P. indica* in a very low amount, sometimes even under the detection of Q-PCR. This low amount and limited detection are the bottlenecks to analysis the function of endobacterium in *P. indica* and eventually in the tripartite relationship with plant. From our experiment, we found that the amount of the endobacterium significantly increased in plant roots treated with *P. indica* than the axenic *P. indica* culture from agar plate. Higher amount of endobacterial *R. raiobacter* were was also detected in grinded root extract and small root pieces. This new finding about significantly increased amount of *R. radiobacter* in the root sample colonized by *P. indica* shed new light on the further study.

Subsequently, fresh *P. indica* culture was re-isolated from the surface sterilized root. Single colonies growing out from barley root were sub-cultured on new agar plate. Quantification results showed that the relative amount of endobacterium in re-isolated *P. indica* (RE-Piri) were significantly higher than the endobacterium amount in long-term laboratory cultured *P. indica* (LL-Piri), while the relative bacterial amount in fresh *P. indica* was significantly higher than that in sub-cultured *P. indica*. The higher amount of endobacteria in fresh *P. indica* isolated from infected barley roots, and the significantly decrease in the subculture on axenic agar plate further indicate that plant host promotes the propagation of endobacterium.

N-acyl homoserine lactones (AHLs) are one kind of signaling molecules produced in many Gram-negative bacteria, and mediate the bacterial behavior through a density-dependent communication quorum sensing (QS) (Schenk and Schikora 2015;

Dong *et al.* 2011; Teplitski *et al.* 2000). The short chain AHLs induce growth promotion effect on a plant due to an impact to the phytohormone auxin, while long chain AHLs have positive impact on plant defense mechanisms (von Rad *et al.* 2008; Bai *et al.* 2012; Liu *et al.* 2012; Schikora *et al.* 2011; Schenk *et al.* 2012, 2014). AHL-induced resistance in plant is called AHL-priming (Schenk and Schikora 2015). Variety of oxo- and hydroxyle-C8- to C12-HSL compounds were detected in the concentrated *Rr*F4 extracts through Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) analysis (Dan Li 2010). QS signal molecules *p*-coumaroyl-HSL, methylated *p*-coumaroyl-HSL and ethylated p-coumaroyl-HSL were produced by *Rr*F4, when *p*-coumaric acid was supplied in the culture medium (Dan Li 2010). *P*-coumaric acid is a natural compound produced by plant on the rhizosphere as precursor compound for plant polymer lignin (Whetten and Sederoff 1995). They reached the possibility that *Rr*F4 requires *p*-coumaric acid secreted from plant to produce AHL signaling molecular *p*-coumaroyl-HSL (Dan Li 2010). My colleague generated *Rr*F4 mutant that is impaired in AHL production. The colonization of this mutant *Rr*F4 on plant roots is significantly reduced compared with wt *Rr*F4, and the growth promotion and disease resistance on plant host induced by the mutant were also low than wt *Rr*F4 (data not published yet). Based on this previous research, our results on the variable cell numbers of endobacteria with/without the plant host suggest that the proliferation of endobacterium in *P. indica* needs the chemical *p*-coumaric acid from the plant host to synthesis long chain *p*-coumaroyl-HSL, methylated *p*-coumaroyl-HSL and ethylated p-coumaroyl-HSL, which act as homoserine lactone quorum-sensing signals to increase plant resistance to pathogens in turn.

The increased amount of endobacteria *R. radiobacter* in plant root, and in the presence of root extractions, root pieces infected by *P. indica*, and the significant reduced amount in the sub-cultured *P. indica* isolated from the plant indicate that the endobacterium getsmore benefit from the tripartite relationship with both fungal and plant hosts than in the bipartite relation with only the fungus. Meanwhile, this result

also leads to more complicate questions on whether the endobacteria are released from the fungus during fungal colonization of plants, and whether the increased amount of endobacteria is inside the fungus or endobacteria released from fungus host into root tissue and propagated in a large number in roots.

4.8 Characteristics of SP-Piri and RE-Piri

Colonization and induced biological activity were compared among three kinds of *P. indica* cultures. Single-protoplast-derived *P. indica* (SP-Piri) has very low amount of endobacteria, long term laboratory *P. indica* (LL-Piri) has normal amount, while re-isolated *P. indica* (RE-Piri) has highest endobacterial amount compared with the other two cultures. The quantification with inoculated plants confirmed the difference among these three cultures in bacteria. The genome ratio *Rr*F4/Piri was significantly low in barley root colonized by SP-Piri than by LL-Piri, while the genome ratio from RE-Piri colonizing plants was significantly higher than from LL-Piri plants.

Compared with control plants, RE-Piri treated plants and LL-Piri treated plants had significantly increased shoot weighs, which is identical to the results in many publications that *P. indica* promoted the growth of plant host (Waller *et al.* 2005; Sharma *et al.* 2008; Qiang *et al.* 2012). But there was neither significant difference between SP-Piri plants and control nor SP-Piri plants and LL-Piri plants, the biological promotion induced by SP-Piri was between control plants and LL-Piri plants. These results indicate that with low amount of endobacterium, the fungus host, as SP-Piri, mediates reduced plant growth promotion compared with normal *P. indica*; with high amount of endobacterium, the fungus host, as RE-Piri, induces increased plant host promotion. Taken together, we reach the conclusion that endobacterium mediates growth promotion of plant host, contributes to the biological activity induced by fungus host *P. indica*.

The colonization of SP-Piri, LL-Piri and RE-Piri on barley seedlings were quantified by *Tef* gene with standard curve. The quantification results showed that RE-Piri had the best colonization on barley roots, while there was no significant difference between LL-Piri and SP-Piri. WGA-staining with treated barley roots showed strong

mycelium and spores colonization from RE-Piri inoculated seedlings, whereas less colonization in LL-Piri roots and few mycelium colonization in SP-Piri roots. Those results suggested that the amount of endobacterium has an influence on the fungus sporulation and the fungal colonization on plant host.

4.9   Conclusions

The aim of this study was to investigate the role of endobacterium *R. radiobacter* in the tripartite symbiosis with fungal and plant hosts. Plant growth promotion and pathogen resistance induced by the isolated enobacterium *R. radiobacter* stain F4 has been showed in previous research (Sharma et al. 2008). In the present work, the focus was on the colonization pattern of *Rr*F4 on the plant host, the variation of the endofungal *R. radiobacter* with and without plant host under different growth conditions, the morphology change of *P. indica* with decreased endobacteria, and the biological activity on plant mediated by *P. indica* with different amount of endobacterial *R. radiobacter* cells.

GUS- and GFP- tagged *Rr*F4 were used to explore the colonization pattern on barley and *Arabidopsis thaliana* roots. *Rr*F4 were detected on the root maturation zone I at early inoculation period, spread into maturation zone II and proliferate on the lateral roots with the same pattern later on, root caps were virtually free of bacteria while meristematic and elongation zones were rarely colonized by *Rr*F4 through the whole period of study. Bacterial cells were cross-linked by fiber-like structures and formed multilayer biofilms on the root surface.

*Rr*F4 not only colonized the root surface but also entered into the inner root. Bacterial cells were mostly detected on the root hairs, in the intercellular space in rhizodermis, exodermis, endodermis and central cylinder, but not in the upper parts like stem and leaf. Occasionally, some single root cells in root hair zone were heavily colonized by *Rr*F4 cells. Bacterial cell colonies were formed in the root hair and lateral root protrusions, root junctions between primary and lateral roots, and cracks among cells on the root cap. Nonetheless, the colonization of *Rr*F4 on plant roots did not induce more root hairs or lateral roots.

Quantification results showed that the low relative amount of endobacteria *R. radiobacter* in axenic *P. indica* was increased in plant root samples inoculated with *P. indca*. Subsequently, fresh *P. indica* was re-isolated from barley roots. Re-isolated *P. indica* harbored higher amount of endobacteria *R. radiobacter* than long-term laboratory cultured *P. indica*, and the relative amount was significantly reduced in subcultures. *Rr*F4 could not go back to the fungal host *P. indica* under laboratory condition.

Single-protoplast-derived *P. indica* cultures produced less chlamydospores, showed delayed germination of protoplast, slowly growth of the fungus, and reduced amount of endobacteria. There was no significant plant growth promotion induced by single-protoplast-derived *P. indica*, while there was significant promotion mediated by long-term laboratory cultured *P. indica* and re-isolated fresh *P. indica*. RI-Piri had the best colonization on barley roots, while there was no significant difference between LL-Piri and PC-Piri. RI-Piri had high activity on plant and produced lots of mycelium and spores on roots, whereas less colonization from LL-Piri and only very few mycelium colonization from PC-Piri colonized roots.

5   Summary/Zusammenfassung

5.1   **Summary**

Endofungal bacteria as symbionts residing in the fungal mycelium and spores were first describes as Bacteria-Like Organisms (BLOs) in 1970's (Mosse 1970). The bipartite relationship between fungi and terrestrial plants on the rhizosphere becomes complicated tripartite interaction as the discovery of endobacteria. In the last decade, research focussed on the endobacteria in arbuscular mycorrhizal fungi (Bonfante and Anca 2009; Naumann *et al*. 2010; Salvioli *et al.* 2015), in ectomycorrhizal basidiomycete fungus (Bertaux *et al.* 2005), and in rice pathogenic fungus *Rhizopus microsporus* (Partida-Martinez and Hartweck 2005; Moebius et al. 2014). We extended the former work to explore and establish a model tripartite symbiosis system among the Sebacinalean fungus *Piriformospora indica*, the Alphaproteobacterium *Rhizobium radiobacter* and a broad spectrum of plants.

The free-living bacterial strain *R. radiobacter* F4 (*Rr*F4) showed similar colonization pattern as its fungal host *P. indica* in plant roots. *Rr*F4 cells heavily colonizedthe root maturation zone, embedded in dense polysaccharides biofilms and were interwoven in cellulose fiber-like structures on the root surface. Thereafter, *Rr*F4 cells entered into inner tissue through lateral root emergence, root hair protrusion and cracks on the root, proliferated in the intercellular space in the rhizodermal and cortical layers into the central vascular system. Nevertheless, *Rr*F4 only invaded and colonized the dead root cells instead of inducing plant host cell death. The systemic resistance mediated by *Rr*F4 is based on the jasmonate-based ISR pathway, and defense gene expression resembles that observed with *P. indica* (Gleaser *et al.* 2015).

The increased amount of endobacteria *R. radiobacter* in plant roots inoculated with *P. indica*, and the significantly reduced amount of endobateria in the sub-cultured *P. indica* that was isolated from plant roots indicate endobacteria *R. radiobacter* obtain more benefit from the tripartite relationship with both fungal and plant hosts than in the bipartite relation with only the fungus. It is an open question whether the endobacteria *R. radiobacter* released from *P. indica* into root tissue during the fungal

colonization on plant.

It was not successful to cure *P. indica* from the endobacteria through antibiotics treatment and single protoplast cultivation. However, we obtained a *P. indica* culture with reduced amount of endobacteria *R. radiobacter*, and partially cured *P. indica* showed low beneficial activity on plants. We presume the endobacteria *R. radidobacter* inside *P. indica* are at a stationary phase, which is the reason for the low number, small size and resistance to antibiotics in axenic culture. The endobacteria *R. radiobacter* resuscitate from stationary phase to growth phase when there is third partner plant supplying with enough nutrition and better propagation conditions, which results in the increased amount of endobacteria *R. radiobacter* when *P. indica* colonized on plant root.

Taken together, this study underlines the crucial role of endofungal bacteria in the tripartite symbiosis. Further studies are needed to better understand the mechanism of *R. radiobacter* in the tripartite interaction, such as the regulation of mRNA on transcriptome level.

5.2 Zusammenfassung

Endofungale Bakterien wurden als Symbionten, die im pilzlichen Myzel residieren, zuerst in den 1970ern als Bakterien-ähnliche „Bacteria-Like Organisms" (BLOs) beschrieben (Mosse 1970). Mit der Entdeckung von Endobakterien ist aus der zweiteilige Symbiose von Pilzen und terrestrischen Pflanzen eine komplizierten dreiteiligen Symbiose geworden. Im letzten Jahrzehnt konzentrierte sich die Forschung auf Endobakterien in Arbuskulären Mykorrhizapilze (Bonfante and Anca 2009; Naumann *et al*. 2010; Salvioli *et al.* 2015), in ektomykorrhizalen Basidiomyceten (Bertaux *et al.* 2005) und in den *Reispflanzen* befallenden *Rhizopus microspores* (Partida-Martinez and Hartweck 2005; Moebius *et al.* 2014). Bei Sharma *et al.* (2008) wurde erstemals gezeigt, dass auch der endophytische Sebacinaleen Pilz *Piriformospora indica* Wirt eines Endobakteriums ist. Dieses wurde als *Rhizobium radiobacter* (syn. *Agrobacterium tumefaciens*) identifiziert und konnte in Reinkultur kultiviert werden (Stamm *R. radiobacter* F4, *Rr*F4). In der vorliegenden Studie wurde die bisherige Arbeit zu dieser dreiteiligen Symbiose zwischen *P. indica,* dem Alphaproteobakterium *Rhizobium radiobacter* und einem breitem Spektrum von Pflanzen näher untersucht.

Das freilebende Bakterium *Rr*F4 zeigte ein dem Wirtspilz ähnliches Kolonisierungsmuster von Pflanzenwurzeln. *Rr*F4 Zellen kolonisieren die Wurzeloberfläche der Reifungszone der Wurzel in erheblichem Maße, die Zellen sind mit faserartigen Zellulose-ähnlichen Strukturen auf der Wurzeloberfläche angehaftet und bilden Biofilme an der Wurzeloberfläche in denen die Zellen in eine dichte Polysaccharid Schicht eingebettete sind. Durch Ausbuchtungen der Wurzelhaarzellen und die Austrittsstellen der Seitenwurzeln scheinen *Rr*F4 Zellen ins innere Gewebe der Wurzel einzutreten; dort vermehren sie sich im Interzellularraum der rhizodermalen und kortikalen Wurzelschichten. Entgegen dem Wirtspilz dringen *Rr*F4 Zellen auch in die zentralen Leitungsbahn vor. Mikroskopisch wurden einzelne Wurzelhaarzellen an der Wurzeloberfläche nachgewiesen die gefüllt waren mit *Rr*F4 Zellen. Da keine Zelltod-Induktion durch *Rr*F4 in den Wurzeln nachgewiesen werden

konnte ist anzunehmen das *Rr*F4 nur in tote Wurzelzellen eindringt und sich dort vermehrt. Die durch *Rr*F4 vermittelte systemische Resistenz basiert auf den Jasmonsäure-abhängigen Signalweg der induzierten systemischen Resistenz. Die Expression von Abwehr-Genen bei der Pflanze entspricht größtenteils der beobachteten Expression bei *P. indica* (Glaeser *et al.* 2015).

Im Rahmen der Arbeit konnte gezeigt werden das die Anzahl an Bakterienzellen in der Tripartiden Symbiose im Vergleich zu einer Laborkultur von *P. indica* signifikant zunehmen. Wurde der Pilz von der Wurzel re-isoliert konnte gezeigt werden das die Konzentration an Bakterienzellen mit jeder weiteren Subkultivierung des Pilzes auf Agarplatten wieder abnahm.

Dies zeigt, dass die Abundanz der Endobakterien vielmerh abhängig ist von der dreiseitigen Symbiose mit *P. indica* und der Wirtpflanzen als von der zweiteiligen Symbiose mit *P. indica*. Es ist immer noch eine offene Frage, ob während der Kolonisierung durch den Pilz Endobakterien aus *P. indica* im Wurzelgewebe ausgesscheidet werden.

Während *Rr*F4 in Reinkultur wächst war es auch in dieser Arbeit nicht möglich, *P. indica,* durch Antibiotikabehandlung kombiniert mit der Kultivierung einzelner Protoplasten, Bakterien-frei zu bekommen. Es war jedoch möglich eine *P. indica* Kultur zu selektieren, die mit einer geringeren Anzahl von Endobakterien besiedelt ist. Diese *P. indica* Kultur zeigte eine reduzierte Aktivität hinsichtlich der Pflanzenwachstumsförderung. Diese Ergebnisse lassen vermuten das das Endobakterium in der dipartiden Symbiose in einen sogenannten „viable but not culturable" VBNC Status übertreten indem die Bakterienzelle ähnlich zu Zellen in der stationären Phase sind. Die Zellen haben eine reduzierte metabolische Aktivität, sind kleiner und unempfindlich gegen Antibiotika. Bei Kontakt mit der Wirtspflanze treten die Bakterienzellen vermutlich wieder in einen normalen aktivwachsende Zellform über, was bei einer Kolonisierung von Pflanzwurzeln durch *P. indica* zu einer erhöhten Anzahl von Endobakterien führt.

Zusammenfassend betont diese Studie die entscheidende Rolle der endofungalen

Bakterien in der dreiteiligen Symbiose von *P. indica,* dem Endobakterium und den entsprechenden Wirtspflanzen. Weitere Untersuchungen sind erforderlich, um ein tieferes Verständnis der Rolle von *Rr*F4 in der dreiteiligen Beziehung zu erhalten, wie zum Beispiel die Regulierung der Gen Expression auf Transkriptionsebene.

6   Reference

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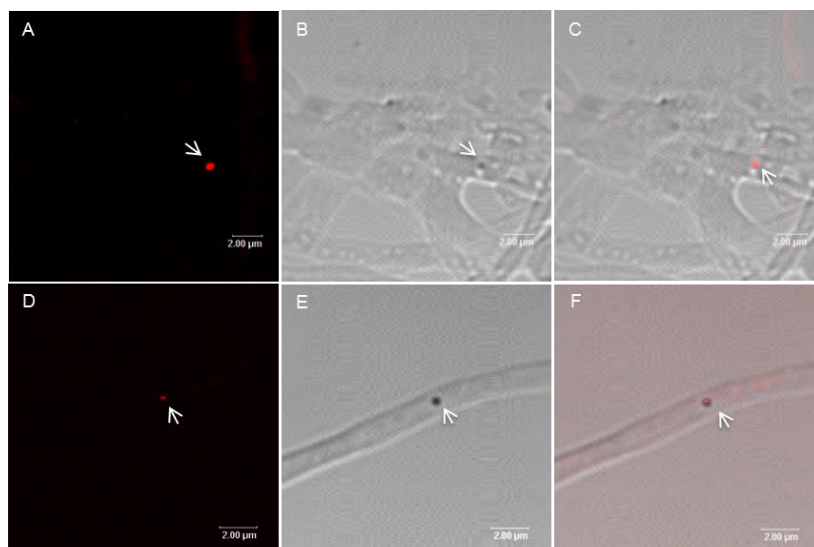
7 Supplement

Fig. 7.1. FISH analysis of endobacteria in *P. indica* by confocal laser scanning microscopy (CLSM). *P. indica* material was fixed and hybridized with probe EUB-338-Cy3. Endobacteria in fungal mycelium were detected under UV (A and D), and under light microscope (B and E). The fluorescence pictures overlaid with light pictures showed in figure C and F. CLSM was at 5000-fold magnification.
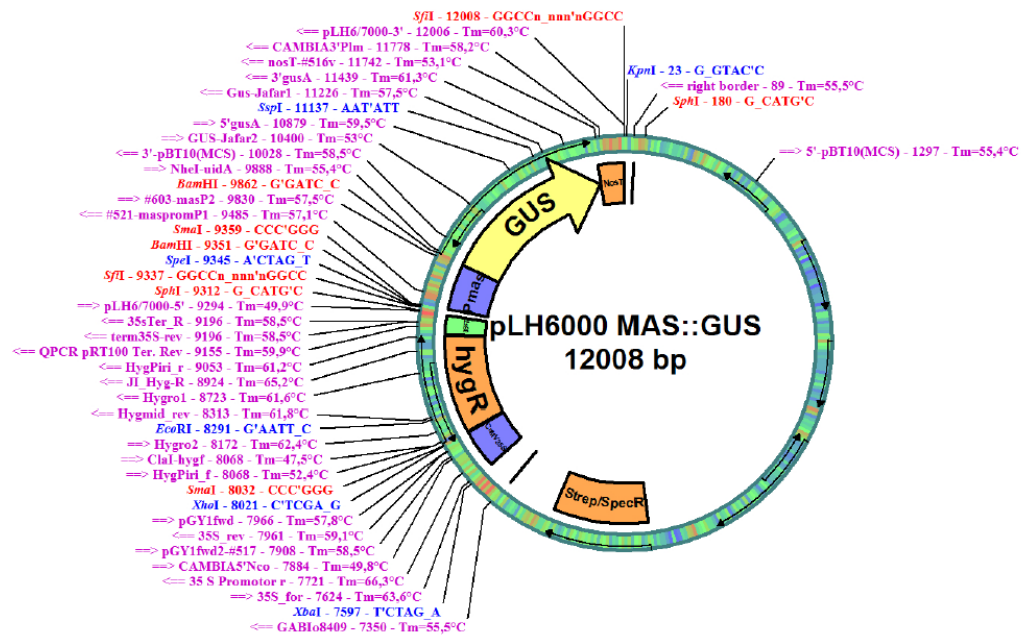
Fig. 7.2 The GUS-containing plasmid map for GUS-tagged *Rr*F4.

8 Erklärung

Ich erkläre:Ich habe die vorgelegte Dissertation selbständig und ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis" niedergelegt sind, eingehalten."

Gießen, Feb 2016

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