Molecular and cytological investigations of the fungal endophyte *Piriformospora indica* and its interactions with the crop plant barley

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I Introduction

1.1 Root Symbiosis

Some of the most complex chemical, physical and biological interactions experienced by terrestrial plants are those that occur between the roots and their surrounding soil environment (rhizosphere). Rhizosphere interactions include root-root, root-insect, and root-microbe associations. Plant roots exude an enormous range of potentially valuable small molecular weight compounds into the rhizosphere. Many microbes grow and interact in the rhizosphere by utilizing nutrients directly or indirectly originating from plants. Some microorganisms can even colonize plant roots endophytically (endon gr. = within, phyton = plant) and exert beneficial or harmful effects on plant growth and development. Positive effects on plants may come from providing essential nutrients as a result of their colonization of the rhizosphere (*Azotobacter*, *Azospirillum*, phosphate solublizing bacteria and cyanobacteria) or by a direct symbiotic association with the root (*Rhizobium*, Mycorrhizae fungi and *Frankia*). They may also regulate physiological processes in ecosystems by decomposing organic matter, fixing atmospheric nitrogen, secreting growth promoting substances, increasing the availability of mineral nutrients and protecting against plant pathogens (Bais et al., 2006). At molecular level best characterized symbiotic systems are rhizobium and mycorrhizae which share and represent one of the first symbiosis signalling pathways.

1.1.1 Rhizobia-legumes symbiosis

Rhizobia form symbiotic associations with leguminous plants by fixing atmospheric nitrogen in root nodules. These interactions are very host specific as *Sinorhizobium meliloti* nodulates *Medicago, Melilotus,* and *Trigonella* genera, whereas *Rhizobium leguminosarum bv. viciae* nodultes *Pisum, Vicia,* Lens and *Lathyrus* genera.

A lipochitosaccharide-based signal molecule that is secreted by *Rhizobium*, named Nod factor (NF), induces root nodule formation in legumes. Genetic analysis in the legume species *Lotus japonicus* and *Medicago truncatula* have led to the identification of many components of the NF signalling cascade (Geurts et al., 2005). At least three of these genes do not function exclusively in the *Rhizobium* symbiosis but are also essential for the formation of mycorrhiza. LysM receptor kinases (LysMRKs) are good candidates to bind NFs, which contain a N-N-acetylglucosamine backbone, but the direct binding of NFs remains to be demonstrated (Madsen et al., 2003). Several other components that are essential for most of the early steps in NF signalling have been identified, and these are activated directly downstream of the NF receptors. In *M. trunculata*, these genes are named DOES NOT MAKE INFECTIONS1

(DMI1), DMI2 and DMI3, and NODULATION SIGNALING PATHWAY1 (NSP1) and NSP2 (Catoira et al., 2000; Oldroyd and Long, 2003). MtDMI1 has similarities to ligand-gated cation channels, whereas MtDMI2 is a receptor kinase (Endre et al., 2002; Ane et al., 2004) and MtDMI3 encodes a calcium and calmodulin-dependent protein kinase (CCaMK) (Levy et al., 2004; Mitra et al., 2004). NSP1/2Genes that are orthologous to MtDMI have been identified in pea and *L. japonicus* (Endre et al., 2002; Stracke et al., 2002; Levy et al., 2004; Mitra et al., 2004; Imaizumi-Anraku et al., 2005).

NFs are perceived by LysM receptor kinases (LysM-RKs). These activate at least two downstream signaling pathways, one depending on the DMI proteins and a DMI-independent pathway for which no specific genes have been identified yet. The signal is transduced from LysM-RKs to DMI1 and DMI2, which are upstream to calcium spiking. Ca spiking will be followed by DMI3 NSP1/NSP2 that lead to the activation of a first subset of symbiosis related genes. Subsequently, a second cluster of genes is activated, which is dependent on HAIR CURLING (MtHCL) in *M. trunculata* and NODULE INCEPTION (NIN) in *L. japonicus* and pea (Catoira et al., 2001; Borisov et al., 2003). The DMI pathway is also essential for mycorrhizal-based signaling triggered by a hypothetical Myc receptor (Geurts et al., 2005) (Fig A)

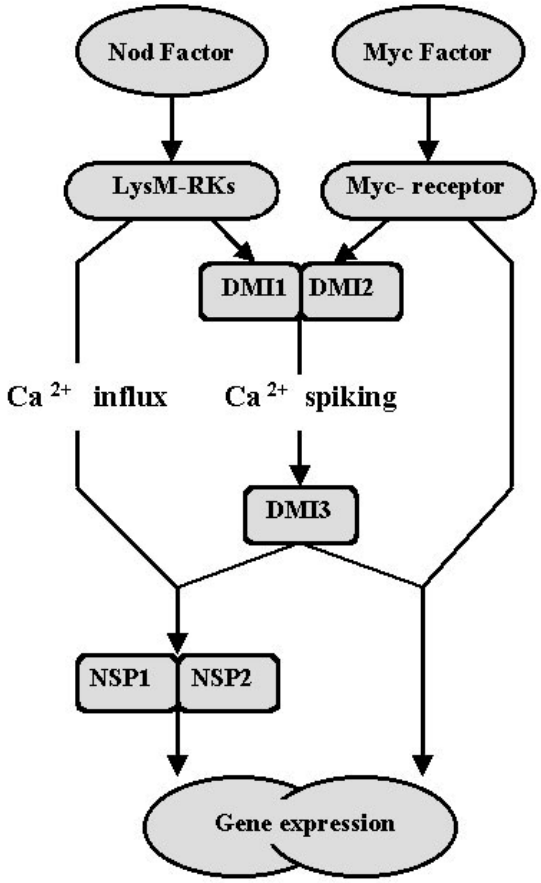

Fig A Early symbiosis signaling
Nod factor is perceived by LysM receptor kinases. These activate DMI-independent and/or DMI-dependent pathway. External Ca2+ influx and perinuclear Ca2+ spiking, are the main differences between them. The DMI pathway is also essential for mycorrhizal-based signaling triggered by a hypothetical Myc receptor. (Modified from Geurts et al., 2005)

1.1.2 Mycorrhizal symbiosis

Ecto-mycorrhizae and arbuscular mycorrhizae are the two classical types of mycorrhizal associations. Among these, the most widespread is the arbuscular mycorrhizal (AM) fungi found in vascular flowering plants (Harrison, 2005). The arbuscular mycorrhiza is an

endosymbiotic fungus, which inhabits root cortical cells and obtains carbon provided by the plant while it transfers mineral nutrients from the soil to cortical cells. The AM fungi are obligate biotrophs and depend entirely on the plant as carbon source for reproduction. The inability of AM fungi to grow in the absence of plant roots (e.g. under axenic culture conditions) has impeded the studies of these organisms. AM fungi usually outlast in the absence from host roots as resting spores in the soil. All AM fungi are members of the Glomeromycota, which is currently subdivided into four orders (Schußler et al., 2001). So far, approximately 150 species of AM fungi have been described (Kramadibrata et al., 2000), which are thought to be asexual. A recent study found that *Glomus intraradices*, has a haploid genome of 15 Mb (Hijiri and Sanders, 2004). The host range of AM comprehends legume species of which *Medicago, Melilotus, Trigonella*, *Pisum, Vicia,* Lens and *Lathyrus* genera are the most prominently studied while *Medicago truncatula* is becoming a model plant to study these symbiotic interactions.

1.1.2.1 Biotrophic interfaces for the exchange of nutrients

Upon spore germination the hyphal germ tubes of AMF grow through the soil in order to find a host plant. Once a host root has been recognized, the fungus forms a penetration organ on the root surface so called appressorium to enter the root. There are two morphological types of AMF: the Paris-type and the Arum-type. In the Arum-type of associations the fungus grows mostly intercellularly through the outer cortex, although occasionally a hypha directly traverses a cell, forming an intracellular coil. Once inside the inner cortex, the fungus forms dichotomously branched hyphae, called arbuscules, within the cortical cells. Arbuscules are terminally differentiated structures, which develop from side branches of the long intercellular hyphae. These elaborated organs form inside the plant cell but they remain separated from the plant cell cytoplasm by an extension of the plant plasma membrane that surrounds the fungus and follows the contours of the hyphal branches (Bonfante-Fasolo, 1984). Plant cell wall biosynthesis continues from this extended membrane while the narrow space in between the membrane and the fungal cell wall is filled with a extracellular matrix whose composition is reminiscent of plant primary cell walls (Balestrini et al., 1996). Phosphate is delivered to the plant across the arbuscule–cortical cell interface, and recently, plant phosphate transporters involved in this process were identified (Harrison et al., 2002; Paszkowski et al., 2002; Rausch and Bucher, 2002). Although there is no direct proof, it is anticipated that carbon is taken up by arbuscules. The arbuscule–cortical cell interface shares some structural and functional similarities to the symbiotic interface of the rhizobium-legume symbiosis, and the

haustorial-plant interface formed by biotrophic fungal pathogens (Smith and Smith, 1989; Harrison, 1999; Parniske, 2000). The AM symbiosis is a highly compatible association, and under phosphate-limiting conditions, intraradical development of the fungus can occur in more than 80% of the root length. In addition to the intraradical growth phase, the fungus also maintains an extraradical mycelium that can extend several centimeters from the root. The fungal hyphae within the root are connected to the extraradical mycelium and form a single continuum. The extraradical hyphae acquire phosphate, initiate the colonization of other roots and in most species, are also the site of sporulation.

The scrutiny of the signaling pathways underlying the establishment of AM symbioses is the focus of past and present research projects. Although signal molecules used by AMF to initiate the symbiosis are still unknown, recent studies give strong evidence for their existence. Lately, the cloning of three signaling proteins in legumes was a landmark step in the understanding of the signaling events exploited by AMF and *Rhizobia* to establish the symbiotic associations (Endre et al., 2002; Stracke et al., 2002; Ane et al., 2004; Levy et al., 2004; Mitra et al., 2004). In many plant-microbe symbioses, detection or attraction of the partner occurs prior to direct contact. In some instances a molecular dialog initiates events that are essential for the progression of the physical interaction. In the absence of host signals, micorrhizal spores germinate and grow for some time before they retract the cytoplasm from newly formed hyphae. In the presence of host signals, germinating hyphae branch and proliferate in order to reach the host. Akiyama et al. (2005) recently discovered a signaling molecule and hyphal branching factor secreted by plants that was defined as strigolactone. It is predicted that AMF also produce signals analogous to Nod factors that are required for initial symbiotic events (Albrecht et al., 1995; Catoira et al., 2000). So far, direct evidence for a "Myc factor" signal is lacking. The plant secreted "Branching factor" and mycorrhizal "Myc factor" would be one of the first signaling molecules involved in mycorrhizal signal transduction pathways.

All mycorrhizal mutants reported in legumes so far were identified from small populations of nodulation mutants and consequently represent genes required for both symbioses (Duc et al., 1989; Sagan et al., 1995; Wegel et al., 1998; Marsh and Schultze, 2001). For *Rhizobium*-legume interactions, the input signal has been identified. It has been shown that the outcome of symbiosis depends on the Nod factor receptors NFR1/LYK3 and NFR5 (Limpens et al., 2003; Madsen et al., 2003; Radutoiu et al., 2003). For the AM symbiosis, the input and, consequently, the beginning of "symbiotic pathways" are not yet clear. There might be additional receptors required, or alternatively, symbiosis is initiated with the SYMRK/DMI2

receptor kinase. The identities of the DMI1 and DMI3 proteins suggest that ion fluxes and calcium signaling are of central importance for the AM symbiosis. Whether AMF induce calcium spiking remains to be determined since the CCaMK (DMI3) is predicted to have the potential to respond to more than one calcium event and to distinguish subtly different calcium signatures (Levy et al., 2004; Mitra et al., 2004). The signaling steps downstream of DMI3 are currently unknown, although there is a set of genes, which are commonly regulated by both type of symbiosis (Fig A).

1.1.3 "Fungal endophytes" and root symbioses

Previously, only mycorrhizal fungi were considered mutualistic symbionts of plant roots. Per definition, fungi that colonize plants without causing visible disease symptoms at any specific moment (Petrini, 1991; Wilson, 1995; Stone et al., 2000) are called fungal endophytes. In all ecosystems, many plant parts are colonized by fungal endophytes. Recently, it has been recognized that many endophytic fungi can participate in mutualistic symbioses with host roots (Brundrett, 2002; Sieber, 2002). For instance, non-mycorrhizal microbes such as *Phialocephala fortinii*, *Cryptosporiopsis* spp. (Rommert et al., 2002; Schulz et al., 2002), dark septate endophyte (DSE) (Sieber, 2002), *Piriformospora indica* (Verma et al., 1998), *Fusarium* spp. and *Cladorrhinum foecundissimum* (Gasoni and Stegman De Gurfinkel, 1997; Kuldau and Yates, 2000; Sieber, 2002) *Chaetomium* spp. (Vilich et al., 1998) have been shown to improve the growth of their hosts after root colonisation. Within these symbioses, fungi most probably benefit by obtaining a reliable nutritional source while hosts may acquire multiple advantages beside an improved growth. Various hosts inoculated with root endophytes displayed an increased tolerance to abiotic stresses and induced resistance. The ascomycetous genera *Epichloë* and *Balansia*, and their anamorphs *Neotyphodium* and *Ephelis* are one of the best studied grass endophytic associations. They grow systemically, rarely epicuticularly, and intercellularly within all above-ground plant organs. These grass fungal endophytes are generaly transmitted through the seeds and they provide herbivore resistance to their host plant (Bacon et al., 2000).

Endophytic colonisations are associated with various plant organs. Depending on the invader and the interaction, endophytic colonization may be limited to roots (e.g. DSE or *P. indica*), confined to the leaves or needles (e.g. *Lophodermium* spp. or *Rhabdocline parkeri*), observed intercellularly in both roots and shoots (e.g. *Fusarium moniliforme*), or adapted to growth within the bark (e.g. *Melanconium apiocarpum*) (Stone, 1986; Fisher and Petrini, 1990; Bacon and Hinton, 1996; Verma et al., 1998; Deckert et al., 2001). Like pathogenic fungi,

mutualists developed several strategies to enter host plants, for example, generation of infection structures like appressoria and haustoria (e.g. *Discula umbrinella* (Stone et al., 1994)), direct host cell walls penetration (e.g. *Rhabdocline parkeri* (Stone, 1987)), or host infestation through stomata and substomatal chambers (e.g. *Phaeosphaeria juncicola* (Cabral et al., 1993)). In almost all system, detailed cytological analysis of root colonization is missing but endophytic growth within the roots is often shown to be extensive. Root colonisation can also be both inter- and intracellular. Morphologically and physiologically, endophytic root colonisations have the variability. Endophytic infection can be local or extensive and it may show either latency or virulence (Bacon and Yates, 2005; Schulz and Boyle, 2005). Thus this plasticity of endophytic interactions can be found at every level. Equally, endophytes mirror the different possible evolutionary life strategies (Brundrett, 2002), such as DSE occasionally penetrated the vascular bundles in asymptomatic interactions (Barrow, 2003), that turns to be frequently associated with pathogenicity (Schulz and Boyle, 2005). Therefore, it is not astonishing that endophytes can display variable life history strategies of symbioses, ranging from facultative saprobic to parasitic to exploitive mutualistic. Certain endophytes can even grow saprophytically on dead or senescing tissues following an endophytic growth phase indicating an assemblage of different evolutionary life models (Stone, 1987). The nature of endophytic colonization of plants does not only depend on its adaptation to a particular host or organ but also on innate but variable virulence patterns encountering host defence responses and environmental conditions (Schulz and Boyle, 2005).

1.1.4 *Piriformospora indica*

Ajit Verma and his collaborators firstly described *Piriformospora indica* in 1998 as a cultivable, micorrhiza-like fungus. The fungus was originally found in soil samples from the rhizosphere of the woody shrubs *Prosopsis juliflora* and *Zizyphus nummularia* growing in the western part of Rajasthan, which is a typical desert region of the Indian subcontinent (Thar). It was named according to its characteristic pear-shaped chlamydospores (Verma et al., 1998). Depending on the ultra structure of hyphae (presence of dolipore septa) and 18s rDNA sequence, *P. indica* was grouped in the class Hymenomycetes (Basidiomycota) (Verma et al., 1998). Serological classification showed close antigenic properties with mycorrhizal fungi (Varma et al., 2001). (Weiss et al., 2004) has further classified the fungus depending on alignment of nuclear rDNA sequence for the 5´ terminal domain of the ribosomal large subunit (nucLSU) into the newly defined order Sebacinales. In contrast to mycorrhizal fungi, this fungus can be cultured axenically on various synthetic simple and complex media at 25-

35ºC (Varma et al., 1999). Morphologically, *P. indica* hyphae are white and almost hyaline. They are thin walled irregularly septated and 0.7 to 3.5 µm in diameter. Septate hyphae often show anstmosis. Each hyphal segment is multinucleate with variable numbers of nuclei. Hyphal tips differentiate into chlamydospore of 16-25 µm length and 10-17 µm in width, which emerge individually or in clusters. Each spore contains 8-25 nuclei. So far, neither clamp connections nor sexual structures could be observed (Varma et al., 2001). When colonizing roots, *P. indica* tremendously improves the growth and overall biomass production of diverse hosts, including legumes (Varma et al., 1999; Varma et al., 2001; Singh et al., 2003). *P. indica* acts as a specific orchidaceous mycorrhizal fungus in *Dactylorhiza* spp.. The interaction of *P. indica* with protocorms has shown typical pelotons in a living host cell similar to orchid mycorrhiza. In addition, a pronounced growth promotional effect was seen with terrestrial orchids. In higher plants, the fungus was shown to form inter and intracellular hyphae in the root cortex, often differentiating into dense hyphal coils and chlamydospores (Blechert et al., 1999; Singh and Varma, 2000). In 2005, Waller et al. reported the potential of *P. indica* to induce resistance to fungal diseases, tolerance to salt stress and grain yield elevation in the monocotyledonous plant barley. The beneficial effects on the plant defense status is detected in roots against *Cochliobolus sativus* and *Fusarium culmorum* as well as to the leaf pathogen *Blumeria graminis* f. sp. *hordei*, demonstrating a systemic induction of resistance by a root-endophytic fungus. The systemically altered ‘‘defense readiness’’ was found to be associated with an elevated antioxidative capacity due to an activation of the glutathione–ascorbate cycle. The fungus also protects plantlets raised in tissue culture by overcoming the ‘transient transplant shock’ on transfer to field resulting in an almost 100% higher survival rate (Sahay and Varma, 1999).

AMF are the major model system to study mutualistic plant-fungus symbioses. However, the mechanisms leading to the establishment of symbioses and the resultant modifications on plant metabolism are far from being completely understood (Limpens and Bisseling, 2003; Breuninger and Requena, 2004; Marx, 2004; Parniske, 2004). Besides the complexity of the interaction between the plant and fungal partners, that is rooted by the limited availability of molecular tools. *Arabidopsis thaliana*, a common model to study plant development at the molecular and genetic level, is not among the hosts of mycorrhizal fungi. Furthermore, AMF are obligate biotrophs and cannot be cultured without hosts, which complicates a genetical manipulation (Newman and Reddell, 1987). In this respect, *P. indica* provides a promising model organism for the investigations of beneficial plant–microbe interaction. The endophyte is hosted by *Arabidopsis thaliana*, which is reflected by growth promotion (Peskan-

Berghofera et al., 2004; Shahollari et al., 2005) and resistance induction against the Arabidopsis powdery mildew *Golovinomyces orontii* (IPAZ, Giessen: unpublished data). In contrast to AMF, *P. indica* can be easily cultivated in axenic culture (Pham et al., 2004a) and potentiates its accessibility for stable transformation (see section 1.3). Using the *Arabidopsis thaliana* - *P. indica* system, Oelmueller and co-workers has described its involvement in plant protein modifications at the endoplasmic reticulum and plasma membrane (Peskan-Berghofera et al., 2004). They were also able to show a transient up regulation of *Arabidopsis* receptor kinase (Shahollari et al., 2005), nitrate reductase and glucan water dikinase (Sherameti et al., 2005) upon stimulation with *P. indica* before any visible colonization in *Arabidopsis* roots.

Thus *P. indica* has great potential in agriculture, forestry, horticulture and viticulture (Singh et al., 2003; Waller et al., 2005). Better understanding of *P. indica* symbiosis would open up numerous opportunities for the optimization of plant productivity in both managed and natural ecosystem, while minimizing risk of environmental damage. The properties of the fungus, *P. indica*, have been patented (Varma and Franken 1997, European patent office, Muenchen, Germany. Patent No 97121440.8-2105, Nov 1998).

1.2 Plant protection in the rhizosphere

Loss of carbon from plant roots promotes growth of many microorganisms and contributes to the development of the rhizosphere microflora. The rhizosphere contains beneficial and non-beneficial microorganisms of saprophytic, parasitic, mutualistic or symbiotic life style. Beneficial microorganisms interact with host plants as well as with other microorganisms of the rhizosphere in an antagonistic or mutualistic way. They can suppress the growth of pathogens and promote the growth of other beneficial microbe. There are many mechanisms involved in plant disease protection originating from the rhizosphere, such as improvement of plant nutrient status, changes in root morphology, the modification of microbial flora of rhizosphere and induced resistance or systemic resistance of plants (Bais et al., 2006). Biocontrol agents in the rhizosphere comprehend fungi and bacteria. Understanding the interactions in the rhizosphere can provide a biological control towards fungal diseases on seeds and roots.

1.2.1 Bacteria-fungal pathogen interactions

A range of different bacterial genera especially *Pseudomonas* species have been studied for the protection of plant fungal diseases. Many metabolites produced by these bacteria

(ammonia, butyrolactones, 2,4-diacetylphloroglucinol, HCN, kanosamine, oligomycin A, oomycin A, phenazine-1-carboxylic acid, pyoluterin) show anti-fungal properties (Milner et al., 1996; Keel and Défago, 1997; Nakayama et al., 1999; Thrane et al., 1999). Bacteria produce a range of iron chelating compounds (siderophores), which are thought to restrict the iron supply to pathogenic fungi, thereby restricting their growth (O'Sullivan and O'Gara, 1992; Loper and Henkels, 1999).

Apart form direct antifungal effects, bacteria can mediate a phenomenon called Induced Resistance, which is defined as the process of active resistance dependent on host plant physical or chemical barriers, activated by biotic or abiotic agents (Kloepper et al., 1992). Bacterially induced resistance is termed as induced systemic resistance (ISR). Most work in this area is focused on rhizophere colonized by *Bacillus* and *Pseudomonas* species where as all other forms are called as systemic acquired resistance (SAR). Major differences are that PR proteins such as PR1, β-1,3-glucanases are not universally associated with bacterially induced resistance (Hoffland et al., 1995; Pieterse et al., 1996) and salicylic acid (a known inducer of SAR) is not always involved in ISR (Pieterse et al., 1996).

1.2.2 Fungus-fungal pathogen interaction

The preferential use of fungi as biocontrol agents over bacteria comes from its ability to spread all over the rhizosphere due to the progression of hyphal growth. There are varieties of fungal species that have been studied for biocontrol properties in different rhizospheres. *Tricoderma* spp. (Whipps and Lumsden, 2001), non-pathogenic *Fusarium* and binucleate *Rhizocotonia* are the preferred biocontrol fungi being studied (Whipps, 2001). Competition for occupying niches or nutrients, production of antibiotics, induced resistance and mycoparasitism are the major biocontrol mechanisms (Howell, 1991; Postma and Rattink, 1991; Howell et al., 1993; Eparvier and Alabouvette, 1994; Herr, 1995, Morgan et al., 2005, Bais et al., 2006). Many of the previously described biocontrol fungi have recently been shown to work partially by induced resistance mechanisms. Non-pathogenic *Fusarium* (Hervás et al., 1995), *Pythium oligandrum*, non-pathogenic binucleate *Rhizoctonia*, *Penicillium oxalicum* (Whipps, 2001) as well as mycorrhizal fungi *Glomus intraradices* combined with non-VAM *Tagetes patula* (St. Arnaud et al., 1996) lead to induced resistance.

Mycoparasitic biocontrol fungi parasitize on hyphae, spores, *Sclerotia*, and other fungal structures by penetrating and degrading cell walls. Extracellular enzymes such as ß-1,3-glucanases, chitinases, cellulases, and proteases have been cloned from fungal microbes and especially from *Trichoderma* species (Archambault et al., 1998; Deane et al., 1998; Vázquez-

Garcidueñas et al., 1998). Transgenic tobacco and potato plants expressing these enzymes showed a high level of resistance against a broad spectrum of diseases (Lorito, 1998). Apart from mycoparasitism, many saprotrophic and endophytic fungi can provide plant growth promotion in the absence of any major pathogen (Inbar and Chet, 1994). Endophytic colonization of seed surface or roots, and promotion of growth are desirable regarding biocontrol activity (Kleifeld and Chet, 1992). Although transient plant growth inhibition following the application of some biocontrol agents to seeds or roots are well known (Bailey and Lumsden, 1998; Wulff et al., 1998). Consequently, a proven biocontrol agent for a given soil-borne plant pathogen may not always be capable of colonizing the rhizosphere or providing plant growth promotion. Despite many studies representing the disease protection in the rhizosphere by mutualistic endophytes, there is limited knowledge present on mechanisms by which mutualistic endophytes provide disease resistance. Interestingly, the non-mycorrhizal endophytic fungus *P. indica* (Verma et al., 1998), which was shown to promote growth of a range of plant species (Varma et al., 1999), additionally, protects crop plants from salt stress and fungal diseases like *Fusarium* root rot (Waller et al., 2005).

Fungi of the genus *Fusarium* are causal agents of severe plant diseases such as *Fusarium* head blight and crown root rot. After inoculation of barley roots with macroconidia, the necrotrophic fungus rapidly invades the root thus eliciting cell death of large areas of root tissue. Later, plant growth and biomass is severely diminished as portions of the plants' vascular system are not functional anymore. Recently, it has been shown that *P. indica* infested plants are more resistant to *Fusarium culmorum* as demonstrated by a 6-fold lower reduction of plant biomass compared with plants not inoculated with *P. indica*, but the possible mechanisms of disease protection are not known (Waller et al., 2005). These reports may reflect the usefulness of *P. indica*-barley-*Fusarium* experimental system to elucidate the mechanisms of disease protection in rhizosphere.

1.2.3 Method to quantify fungal infestation

Microscopic evaluation and quantification of disease resistance provided by endophytic fungi to other plant pathogens is exceptionally difficult, especially in those cases where both endophyte and pathogen occupy the same plant tissue. Scoring of disease symptoms or the use of molecular tools are the only reliable methods for this measurements. For *Fusarium* sp., the far easiest way to evaluate disease resistance is to score the visual symptoms. For pathogens that can kill complete plants, one can score survival rates to evaluate disease resistance. In the case of tissue necrotizing pathogens that do not kill their hosts, the degree of necrosis over

time can be used to monitor resistance. Although these techniques are relatively quick and easy to perform, there are certain disadvantages associated with these techniques. First, they are limited to those pathogen host interactions that result in the emergence of macroscopically visible disease symptoms. Secondly, these techniques only provide a measure for the extent of disease symptoms but not for the extent of pathogen colonization (Bent et al., 1992; Thomma et al., 1999). Therefore, it is of utmost importance to assess pathogen growth rather than disease symptoms.

The amount and composition of *P. indica* within roots can only be assessed by molecular methods. Here, similar methods of quantification used for AMF research can be introduced. There are two main methods available to quantify the colonization of roots by AM fungi. One is based on the quantification of fungal metabolites as sterols or chitin (Frey et al., 1994). While the second relies on the staining of roots followed by microscopic evaluation, although it is time consuming and labour intensive (McConigle et al., 1990). Recently, the quantitative polymerase chain reaction (PCR) became a popular and powerful tool for the identification and quantification of different fungi (Zeze et al., 1996; Redeker et al., 2000). By using real-time PCR, it possible to accurately quantify nucleic acids, which is very quick and compatible for statistical analysis. In 2004, the first report of real-time PCR application for the quantification of the AMF G*lomus intraradices* in colonized roots was published (Alkan et al., 2004). To study the disease protective impact provided by the endophytic fungus *P. indica* in barley roots, it is necessary to accurately quantify pathogen and endophyte development in co-infested plants. The use of real time PCR with microbe-specific primers would meet these requirements.

1.2.4 Defence gene expression during symbiosis

Plants have developed a range of sophisticated defence mechanisms. They commonly react to pathogens with an integrated set of responses including reinforcement of cell walls by deposition of lignin-like polymers and structural proteins, formation of low molecular weight antimicrobial agents (e.g. phytoalexins) and accumulation of pathogenesis related (PR) proteins (Bowles, 1990). The massive root colonization by AMF has prompted several studies on the regulation of plant defence-gene expression in response to mycorrhizal fungi. In some cases, an induction of defence responses was detected at early stages of root colonization followed by suppression at later stages of the symbiosis (Spanu et al., 1989; Lambais and Mehdy, 1993; Volpin et al., 1995). In other cases, there was no major change in the expression of plant defence-related genes in response to mycorrhizal root colonization

(Franken et al., 1994; Blee and Anderson, 1996). The overall impression from these studies was that plant defence-related genes are only weakly or transiently expressed in response to infection by symbiotic fungi (Bonfante and Perotto, 1995). However, very few studies have compared defense responses to pathogens and symbionts in the same plant (Wyss et al., 1991) (Gianinazzi-Pearson et al., 1992). Many studies have reported that AM symbiosis can reduce root disease caused by several soil-borne pathogens and also make plants more tolerant to abiotic stresses like salt and drought. Similarly, (Waller et al., 2005) have shown that *P. indica* activates plant defence responses systemically and locally when challenged with other pathogens. However, mechanisms underlying these protective effects are still not well understood.

1.3 Genetic transformation of filamentous fungi

An effective way to study the fungus-plant interactions is either to disrupt or overexpress plant genes in order to determine their influence within the association. Likewise, fungal genes can be silenced or overexpressed to judge their impact. Moreover, reporter proteins like Green Fluorescent Protein (GFP) or Red Fluorescent Protein DsRed can be transgenically expressed in filamentous fungi. These vital fluorescent markers in filamentous fungi allow the histological study of the fungal infection with higher resolutions in planta. Similarly, (Jansen et al., 2005) has used a constitutively GFP-expressing *Fusarium* wild-type strain to monitor the various stages of fungal infection and to demonstrate the development of *Fusarium* head blight on barley.

In filamentous fungi, the introduction of DNA by transformation permits a targeted gene disruption by replacing the wild-type allele on the genome with a mutant allele carrying the T-DNA insertion. The advantage of insertional mutagenesis, over the chemical or radiation mutagenesis, is that the mutated gene is tagged by transforming DNA and can subsequently be cloned using the sequence information provided by the T-DNA. A typical procedure for transforming filamentous fungi would be similar to the transformation of *Neurospora crassa* (Case et al., 1979), which involves the preparation of fungal protoplasts, delivery of the transforming DNA therein and selection of the generated transformants. Protoplast generation can be circumvented in two other methods of transformation: (1) biolistic and (2) *Agrobacterium tumefaciens* mediated gene transfer. In these types of transformations, intact fungal cells are either bombarded with gold particles coated with respective DNA or coincubated with *Agrobacterium tumefaciens* carrying transforming DNA on a binary vector. To identify the transformants, it is imperative that the gene conferring a selection advantage

(e.g. antibiotic) to the transformed cells is included within the transforming vector. Once the selection marker is integrated in to recipient genome, the transformants have the ability to grow in the presence of a specific antibiotic that is toxic to the untransformed wild-type strain. Nowadays, several dominant selectable markers are commonly used in fungal transformation procedures (Fincham, 1989). Both procedures have been widely used to transform filamentous fungi, but many fungi are recalcitrant to transformation and produce homo- or heterokaryon. Consequently, each fungal species needs its own customization and standardization of transformation procedure. But once standardized, the procedure can be applied to produce libraries of insertion mutants.

AMF are the major model system to study mutualistic plant fungal interaction. To date there is limited information available on AMF genes involved in mutualistic interactions (Harrison, 2005). The main obstacle is obligate biotrophic life style of AMF (Newman and Reddell, 1987). In opposite and as already metioned, *P. indica* can be easily cultivated in axenic culture (Pham et al., 2004b), which simplifies the provision of fungal material needed for fungal transformation. Successful isolation and regeneration of protoplast of axenically cultivable *P. indica* has been reported by Varma and coworkers (Varma et al., 2001). It is a task to standardize the successful genetic transformation and ectopic expression of marker genes in the saprophytic phase of *P. indica* to thoroughly study fungal genetics, which would help to understand the genome organization of this asexual endophyte. Thus transformation of *P. indica* may open up a new avenue of studying fungal genes in mutualistic interaction and utilizing the fungus in modern cropping systems.

1.4 Objectives

The endophyte *Piriformospora indica* is a model organism for species of the order Sebacinales, which harbors fungi that are involved in endophytic mutualistic symbioses with plants. The axenically cultivable *P. indica* increases biomass and grain yield in various plant species. In barley, it induces systemic resistance to the fungal leaf pathogen *Blumeria graminis* f. sp. *hordei*. Moreover, *P. indica* protects barley from abiotic stress. As other mutualistic endophytes, *P. indica* colonizes roots in an asymptomatic manner. Information on colonization patterns of these endophytes is very limited. This is partly due to the lack of histological studies that adequately describe the qualitative and quantitative distribution of endophytes within host roots. So far, the genetic demands of *P. indica* on host plants in order to achieve compatibility are unknown. Moreover, how the fungus penetrates plant roots, how roots are eventually colonized, or whether the mutualistic fungus has a facultative biotrophic

or necrotrophic lifestyle are the issues that have not been investigated. The aim of the present work was to analyse the fungal development and host reactions in the mutualistic symbiosis of the root endophyte *P. indica* and barley. Using histo-cytological approaches, it was the aim to examine the life style and colonization pattern of *P. indica* in barley. By introducing molecular procedures, a second focus was to define genetic requirements for barley root colonization by *P. indica* and to analyse the plant innate immunity response.

Fungi of the genus *Fusarium* are causal agents of severe plant diseases such as *Fusarium* head blight and crown root rot. Recently, it has been shown that *P. indica* infested plants are more resistant to *Fusarium culmorum* root rot. The second part of this work aims at analyzing whether *P. indica* also protects barley roots from root rot caused by *Fusarium graminearum*. To gain more insights into the mechanism of *P. indica* mediated root rot control, disease progression in roots infested and non-infested with *P. indica* were followed by means of a Q-PCR-based detection method. Furthermore, the plant response in *P. indica*-infested and non-infested barley roots after *F. graminearum* infection were determined by recording expression of transcription levels of pathogenesis-related (PR) genes.

There are still considerable limitations on genetic information of AM fungal genes involved in the diverse plant-mycorrhiza associations due to the obligate biotrophic life style of AMF and their inaccessibility for genetic transformation. In contrast to AMF, *P. indica* can be easily cultivated in axenic culture. The transformation of *P. indica* may open new avenues for studying those fungal genes important for the mutualistic interaction and for understanding the genome organization of this asexual endophyte. In previous studies, successful isolation and regeneration of protoplasts of axenically cultivable *P. indica* has been established. However, reported transformation method for *P. indica* has resulted in unstable transformants. For this purpose, it was attempted to standardize the biolistic gun mediated genetic transformation and ectopic expression of marker genes in the saprophytic phase of *P. indica*.

The root endophytic fungus *Piriformospora indica* requires host cell death for proliferation during mutualistic symbiosis with barley

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Fungi of the recently defined order Sebacinales (Basidiomycota) are involved in a wide spectrum of mutualistic symbioses (including mycorrhizae) with various plants, thereby exhibiting a unique potential for biocontrol strategies. The axenically cultivable root endophyte *Piriformospora indica* is a model organism of this fungal order. It is able to increase biomass and grain yield of crop plants. In barley, the endophyte induces local and systemic resistance to fungal diseases and to abiotic stress. To elucidate the lifestyle of *P. indica*, we analyzed its symbiotic interaction and endophytic development in barley roots. We found that fungal colonization increases with root tissue maturation. The root tip meristem showed no colonization, and the elongation zone showed mainly intercellular colonization. In contrast, the differentiation zone was heavily infested by inter- and intracellular hyphae and intracellular chlamydospores. The majority of hyphae were present in dead rhizodermal and cortical cells that became completely filled with chlamydospores. In some cases, hyphae penetrated cells and built a meshwork around plasmolyzed protoplasts, suggesting that the fungus either actively kills cells or senses cells undergoing endogenous programmed cell death. Seven days after inoculation, expression of barley *BAX inhibitor-1* (*HvBI-1*), a gene capable of inhibiting plant cell death, was attenuated. Consistently, fungal proliferation was strongly inhibited in transgenic barley overexpressing GFP-tagged HvBI-1, which shows that *P. indica* requires host cell death for proliferation in differentiated barley roots. We suggest that the endophyte interferes with the host cell death program to form a mutualistic interaction with plants.

biodiversity | mycorrhiza | rhizosphere | Sebacinales | systemic resistance

Most plants studied in natural ecosystems are infested by fungi that cause no disease symptoms. These endophytic fungi are distinguished from pathogens that lead to disease and reduce the fitness of their host plants (1). In many cases, endophytes form mutualistic interactions with their host, the relationship therefore being beneficial for both partners. Mutualism frequently leads to enhanced growth of the host. The beneficial effects for the plant can be a result of an improved nutrient supply by the endophyte as known for arbuscular mycorrhizal symbiosis, the most intensely studied mutualistic plant–fungus interaction (2). In addition to providing mineral nutrients, endophytes also can improve plant resistance to pathogens as demonstrated for arbuscular mycorrhiza fungi (AMF) in roots (3) and for a highly diverse spectrum of ascomycete endophytes in leaves (4, 5).

Mutualism requires a sophisticated balance between the defense responses of the plant and the nutrient demand of the endophyte. Hence, a mutualistic interaction does not imply absence of plant defense. Defense-related gene expression has been well studied during host colonization by obligate biotrophic AMF. Induction of defense genes was most prominent at early time points during penetration (6) but could also be detected during arbuscule development (7). On the other hand, there is clear evidence for impeded defense reactions during the establishment of mycorrhization. It is therefore a rather fine-tuned balance that keeps a mutualistic interaction in a steady state without disadvantages for both partners (8).

In the present work we aimed at studying fungal development and host reactions in the mutualistic symbiosis of the fungal root endophyte *Piriformospora indica* and barley (9, 10). The basidiomycete is a model organism for species of the recently described order Sebacinales, fungi that are involved in a uniquely wide spectrum of mutualistic symbioses (mycorrhizae) with plants (11). The axenically cultivable *P. indica* increases biomass and grain yield of crop plants. In barley, the endophyte induces root resistance against *Fusarium culmorum*, one of the fungal species causing head blight, and systemic resistance to barley powdery mildew *Blumeria graminis* f.sp. *hordei* via an unknown mechanism probably independent of salicylate or jasmonate accumulation. Moreover, *P. indica* protects barley from abiotic stress, such as high salt concentrations (10).

P. indica was originally discovered in the Indian Thar desert in northwest Rajasthan. *In vitro* experiments have shown a broad host spectrum of the fungus (12), including members of the Brassicaceae, like *Arabidopsis*, which are not colonized by AMF. As in barley, *P. indica* enhances seed yield, reduces the time for seed ripening, and increases tolerance to abiotic stress in *Arabidopsis* (13). How the fungus penetrates plant roots, how roots are eventually colonized, or whether the mutualistic fungus has a facultative biotrophic or a necrotrophic lifestyle are issues that have not yet been studied. In *Arabidopsis*, mycelium covers the surface of the roots. Hyphae penetrate root hairs and rhizodermis cells and eventually form chlamydospores in these cells (13). Our previous observations in barley revealed that the fungus, in contrast to obligate biotrophic AMF, colonizes dead root cells, suggesting a previously uncharacterized type of mutualism. Here

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Abbreviations: AMF, arbuscular mycorrhiza fungi; Ct, cycle threshold; dai, days after inoculation; nucLSU, nuclear gene coding for the large ribosomal subunit; PCD, programmed cell death; WGA-AF 488, wheat germ agglutinin-Alexa Fluor 488; WGA-TMR, wheat germ agglutinin-tetramethylrhodamine.

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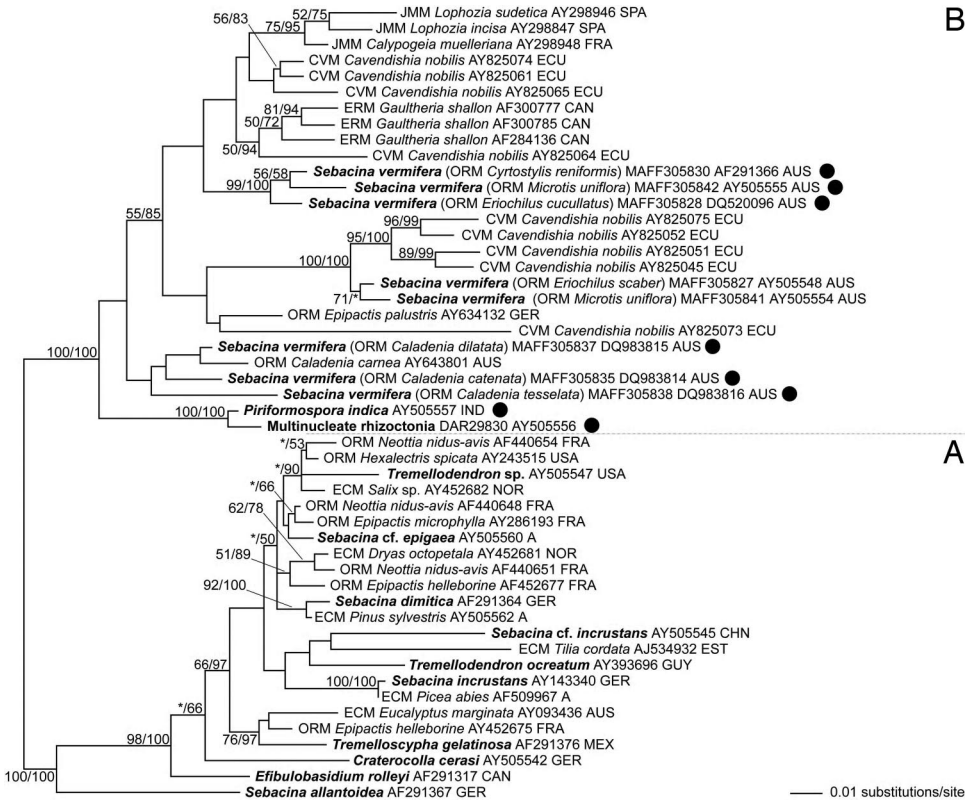

Fig. 1. Phylogenetic placement of the strains tested in this study within the Sebacinales, estimated by maximum likelihood from an alignment of nuclear rDNA coding for the 5′ terminal domain of the ribosomal large subunit. Branch support is given by nonparametric maximum likelihood bootstrap (first numbers) and by posterior probabilities estimated by Bayesian Markov chain Monte Carlo (second numbers). Support values of <50% are omitted or indicated by an asterisk. The tree was rooted according to the results of ref. 11, and subgroups discussed in ref. 11 are denoted with ''A'' and ''B.'' Sequences of the strains used in this study are indicated by black circles. Sequences from morphologically determined specimens or cultures are printed in bold. Sebacinalean sequences obtained from mycorrhizal plant roots are assigned to mycorrhizal types by the following acronyms: CVM, cavendishioid mycorrhiza (14); ECM, ectomycorrhiza; ERM, ericoid mycorrhiza; JMM, jungermannioid mycorrhiza; and ORM, orchid mycorrhiza. Proveniences are given as follows: A, Austria; AUS, Australia; CAN, Canada; CHN, People's Republic of China; ECU, Ecuador; EST, Estonia; FRA, France; GER, Germany; GUY, Guyana; IND, India; MEX, Mexico; NOR, Norway; and SPA, Spain.

we provide cytological and molecular evidence that *P. indica* proliferates in dead host cells and that colonization gradually increases with tissue maturation. The expression level of the cell death regulator *BAX inhibitor-1* (*HvBI-1*) appears critical for *P. indica* development in barley, suggesting that the recently discovered endophyte interferes with the host cell death machinery.

Results

***P. indica* Belongs to the Recently Defined Order Sebacinales.** Based on the nuclear genes coding for the large ribosomal subunit (nucLSU), available strains of the *Sebacina vermifera* species complex (Sebacinales group B) are closely related to *P. indica* (Fig. 1). We addressed the question whether strains of the *S. vermifera* complex exhibit comparable biological activities as *P. indica*. To this end, barley seedlings were inoculated with *P. indica* or different isolates of *S. vermifera* and shoot length and biomass were determined (Table 1). Despite obvious variation, we found consistent biological activities in the same order of magnitude as with *P. indica*. To determine the potential for systemic induction of resistance, barley third leaves from endophyte-colonized and noncolonized, 21-day-old plants were inoculated with the conidia of *B. graminis* f.sp. *hordei*, and powdery mildew pustules were counted after 7 days. We found consistent resistance-inducing activity of all strains of the *S. vermifera* complex, although there was considerable variation of the fungal activity of the different isolates (Table 1). These data support the view that the order Sebacinales is a source of endophytes with a feasible agronomical impact.

Endophytic Development in Barley Roots. To track endophytic development in barley, root penetration and colonization were analyzed by fluorescence microscopy. In general, we observed a gradual increase of fungal colonization and proliferation associated with root maturation (Fig. 2*a*). Colonization initiates from chlamydospores, which, upon germination, finally form a hyphal network on and inside the root. Hyphae enter the subepidermal layer through intercellular spaces where they branch and continue to grow (Fig. 2 *b–e*). In young differentiated root tissue, the fungus then often colonizes and completely fills up single cells (Fig. 2 *f* and *g*) before adjacent cells are colonized, whereas an unrestricted net-like intra- and intercellular colonization pattern is observed in mature parts. Intracellulary growing hyphae show necks at sites where the fungus traverses a cell wall (Fig. 2*h*). Occasionally, subepidermal hyphae penetrate the space between the cell wall and plasma membrane of rhizodermal or cortical cells. After branching, these hyphae enwrapped protoplasts, which showed cytoplasmic shrinkage (Fig. 3*a*). At later colonization stages, fungal hyphae excessively occupied rhizodermal and cortical cells. In some cases, transverse cell walls of adjoining cortical cells were absent, with the protoplasts covered by a dense meshwork of fungal hyphae. Eventually arrays of single spores developed from intracellular hyphal tips (Fig. 3 *b* and *c*). The fungus also penetrated basal parts of root hair cells, in which branching hyphae form large numbers of chlamydospores starting from the base of the root hair until a stack of spores fills the root hair (data not shown). In addition to this intracellular spore

Table 1. Effect of different Sebacinales species on barley biomass and systemic resistance to powdery mildew

Species/isolate	Increase in shoot length, %	Increase in shoot fresh weight, %	Reduction in leaf infection by *B. graminis*, %
P. indica	13.66**	26.45**	70.85**
S. v./MAFF305830	23.25**	48.24**	79.45**
S. v./MAFF305842	16.87**	15.48*	56.36*
Multinucleate *Rhizoctonia*/DAR29830	7.56**	10.76*	56.27*
S. v./MAFF305828	14.97**	28.72**	10.89
S. v./MAFF305837	16.34**	32.01**	58.19**
S. v./MAFF305835	7.80*	9.82	50.74*
S. v./MAFF305838	7.72**	6.41	44.89*

Species/isolates are shown with their culture collection numbers. Isolates of *Sebacina vermifera* (*S. v.*) were obtained from the National Institute of Agrobiological Sciences (Tsukuba, Japan); the isolate DAR29830 was kindly provided by Karl-Heinz Rexer (University of Marburg, Marburg, Germany). Values are means of three independent experiments, each consisting of 60 endophyte-inoculated and mock-inoculated plants, respectively. Powdery mildew infection was calculated from the number of fungal colonies developing on third leaf segments 7 dai with *B. graminis* f.sp. *hordei,* race A6 (15). Asterisks denote statistically significant differences between the respective values of endophyte-colonized and noncolonized plants (*, $P < 0.05$, Student's *t* test; **, $P < 0.01$, Student's *t* test).

formation, chlamydospores also were generated in the mycelial mats at the root surface.

***P. indica* Proliferates in Dead Cells.** We addressed the question of whether cortical and rhizodermal cells heavily occupied by fungal hyphae and chlamydospores were alive. In a cell viability assay with the fluorescent marker fluorescein diacetate, colonized cells did not show enhanced green fluorescence, suggesting that they were dead. In addition, these cells did not show any visible cytoplasmic streaming. Staining of colonized root hairs with an Alexa Fluor-488-labeled anti-actin antibody failed to show any host cytoskeleton, whereas noncolonized root hairs showed intact actin filaments (data not shown). To confirm that fungal colonization associates with dead cells, we double-stained root segments with DAPI for intact plant nuclei and wheat germ agglutinin-Alexa Fluor 488 (WGA-AF 488) for fungal chitin. We found a close spatial association of strong fungal colonization (Fig. 3 *d* and *f*) and DAPI-negative cells (Fig. 3 *e* and *g*), further suggesting that massive development of *P. indica* takes place in dead host cells.

Microscopic analyses demonstrated a fungal colonization pattern that strongly associated with the developmental stage of the host tissue (Fig. 2*a*). To substantiate this finding, we determined the amount of *P. indica* in different root zones by quantitative PCR using *P. indica* genomic DNA as a template for the quantification of the *P. indica* translation elongation factor gene *Tef* relative to the plant ubiquitin gene. Ten days after inoculation, the roots were cut into 0.5-cm-long apical segments of the root tip with the root cap and a basipetal segment including the differentiation zone. Consistent with the cytological data, we found a 5-fold higher relative amount of *P. indica* in the differentiation zone as compared with the apical root segment (2.53 ± 0.23 compared with 0.52 ± 0.12).

Analysis of fungal growth in the apical elongation zone revealed fungal development in intercellular spaces and formation of subepidermal intercellular hyphal mats. In contrast to its development in the differentiation zone, neither host cell wall degradation nor heavy fungal sporulation could be observed in this tissue, supporting the notion that there is a correlation between root tissue and fungal development. Juvenile tissue, which is considered to display less developmental cell death, is thus less occupied by *P. indica*. To support this observation, we tested for genomic DNA fragmentation by probing gel blots of high-molecular-weight DNA isolated from different root segments with radioactively labeled DNA probes. Genomic DNA fragmentation results from programmed cell death (PCD). As expected, the proportion of low-molecular-weight DNA fragments resulting from DNA fragmentation was lower in root tips than in mature parts of the root. *P. indica* did not change the amount of DNA fragmentation in root tips, whereas a small increase of 5–9% low-molecular-weight DNA was detected in the mature zone 10 days after inoculation with *P. indica*. To visualize DNA fragmentation in the root tissue, we used *in situ* DNA nick-end labeling and observed DNA fragmentation in nuclei of protoplasts enwrapped by *P. indica* (Fig. 3*h*). However, this was a rare event perhaps indicating a transient status before nuclei completely dissolved in invaded cells. Taken together, these results indicate that invasive growth of *P. indica* mainly occupies dead and dying cells in barley roots. Consistently, the fungus infested only dead cells of the root cap at the root tip zone, whereas the central meristematic tissue was always free of fungal hyphae (Fig. 3*i*). In adjacent cortical tissue, the fungus was present in the intercellular spaces of cells differentiating into cortical and epidermal tissue apparently without affecting differentiation. Accordingly, lateral root development from cambial cells that differentiate in root tip meristems was not compromised in roots infested by *P. indica*.

We measured the ratio of fungus to plant DNA (fungus/plant DNA ratio, FPDR) over time to check whether *P. indica* overgrows barley roots at late interaction stages. We observed an early moderate increase of the FPDR (1.8-fold) followed by a decrease and a final steady state (data not shown). This pattern reflects the symbiotic interaction in which the fungus develops moderately, subsequently induces plant growth (reflected in a decrease of FPDR), and finally reaches a steady-state level of fungal structures in the plant root. This growth pattern indicates a final balance of root growth and fungal proliferation.

Balancing of Host Cell Death and Impact of the Cell Death Regulator BAX Inhibitor-1. Because the cytological analysis of root colonization suggested that *P. indica* proliferates in dead host cells, we addressed the question of whether root invasion by *P. indica* interferes with the host's cell death machinery. Therefore, we kinetically analyzed expression of barley *HvBI-1*. BI-1 is one of the few conserved cell death suppressor proteins that apparently controls PCD in all eukaryotes and is considered a regulator of endoplasmic reticulum-linked Ca^{2+} signaling. In plants, *BI-1* is often activated in response to biotic or abiotic stresses (15–17).

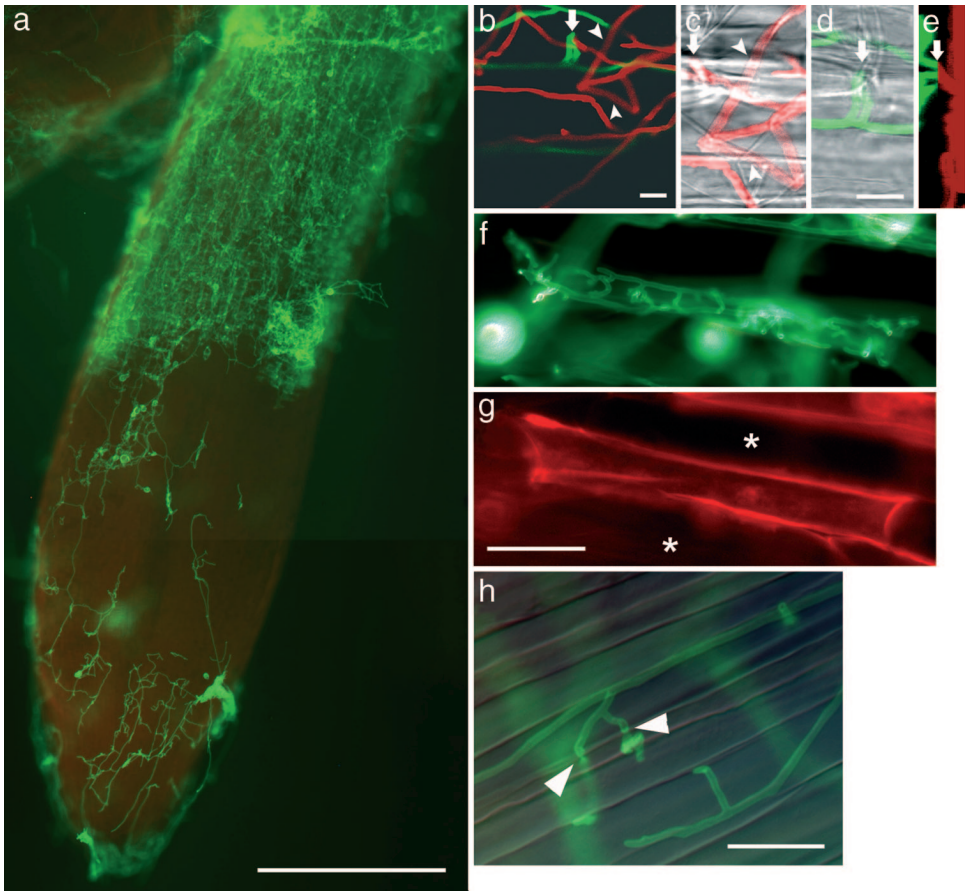

Fig. 2. Infestation pattern of *P. indica* in barley roots. (*a*) By 8 dai, hyphae excessively occupy rhizodermal and cortical cells of the differentiation zone. The elongation zone is less colonized, with occasional intercellular subepidermal hyphal structures. The root cap is heavily infested with hyphae. (*b–e*) After penetration (arrows) fungal hyphae colonize the subepidermal layer. (*b*) To better visualize the position of hyphae in the *z* axis, a confocal laser scanning image consisting of 30 frames of adjacent focal planes (*z* axis) was displayed as a maximum projection with the fluorescent signal of the wheat germ agglutinin-stained fungal hyphae displayed in red for the upper (abaxial) 15 frames and in green for the lower (adaxial, subepidermal) 15 frames. (*c* and *d*) For visualization of plant cell walls, two close-up bright-field images of two different focal planes are superimposed with the respective frames of the fluorescence images. Intercellular hyphae start branching and proliferate within the subepidermal space. (*c*) Subepidermal hyphae crossing cell walls (arrowheads) without exhibiting morphological changes (e.g., neck formation, as in *h*) revealing their periclinal localization. (*d*) The upper focal plane is characterized by hyphae penetrating the anticlinal space of adjacent rhizodermis cells. (*e*) Projection of the fluorescent signals of *c* and *d* in the *y* axis (vertical) and *z* axis (horizontal). Absence of fluorescent signals between adaxial (green) and abaxial hyphae (red) indicates a layer of rhizodermal cells free from hyphae. The penetration site is indicated by an arrow. (*f*) Colonization of a single cell within young differentiated tissue. After penetration, the cell is completely filled with intracellular hyphae before the colonization of adjacent cells. (*g*) The cell wall of the colonized cell is strongly stained with Congo red because of better dye accessibility compared with noncolonized neighbor cells (asterisks). Penetrated cells did not show autofluorescence. (*h*) Intracellular mycelium in mature root tissue. Overlay of bright-field image and fluorescence image. Intracellular hyphae form necks (arrowheads) at sites of cell wall crossing. Fungal structures are visualized by WGA-AF 488. [Scale bars: *a*, 300 μm; *b* and *d*, 10 μm (*c* and *d* are of the same scale); *f–h*, 30 μm.]

Quantitative PCR analysis of *HvBI-1* expression showed it slowly increasing during root development throughout the course of the experiment (Fig. 4*a*). In contrast, when roots were colonized by *P. indica*, *HvBI-1* expression was significantly reduced as compared with noncolonized roots from 7 days after inoculation (dai) onwards (Fig. 4*a*). These data support the idea that *P. indica* interacts with the host cell death machinery for successful development but does not cause plant stress.

To gain evidence for a role of host PCD and requirement of *HvBI-1* down-regulation for fungal success, we overexpressed a functional GFP–HvBI-1 fusion protein in barley under control of the constitutive cauliflower mosaic virus 35S promoter and analyzed fungal development. GFP–HvBI-1 expression was confirmed by PCR and by observation of the fluorescence of GFP–HvBI-1 at the nuclear envelope and in the endoplasmic reticulum in all transgenic plants used for further analysis (Fig. 5, which is published as supporting information on the PNAS web site). Root development in all independent GFP–HvBI-1 barley lines tested was macroscopically indistinguishable from wild type. We microscopically observed development of *P. indica* in GFP–HvBI-1 barley. Fungal epiphytic growth and sporulation were not strongly affected by GFP–HvBI-1. In contrast, invasive inter- and intracellular fungal growth was significantly reduced in GFP–HvBI-1 roots at 20 dai. To quantify the impact of GFP–HvBI-1 on fungal proliferation, the amount of *P. indica* was measured by quantitative PCR. At 20 dai, the relative amount of *P. indica* DNA in transgenic plants was only 20–50%, compared with wild-type plants depending on the transgenic line tested (Fig. 4*b*).

Discussion

P. indica and barley form a mutualistic symbiosis in which the endophyte colonizes the plant root, proliferates by inter- and intracellular growth and produces chlamydospores in dead root tissue. After establishment of the symbiosis the fungus confers improved growth, disease resistance and abiotic stress tolerance

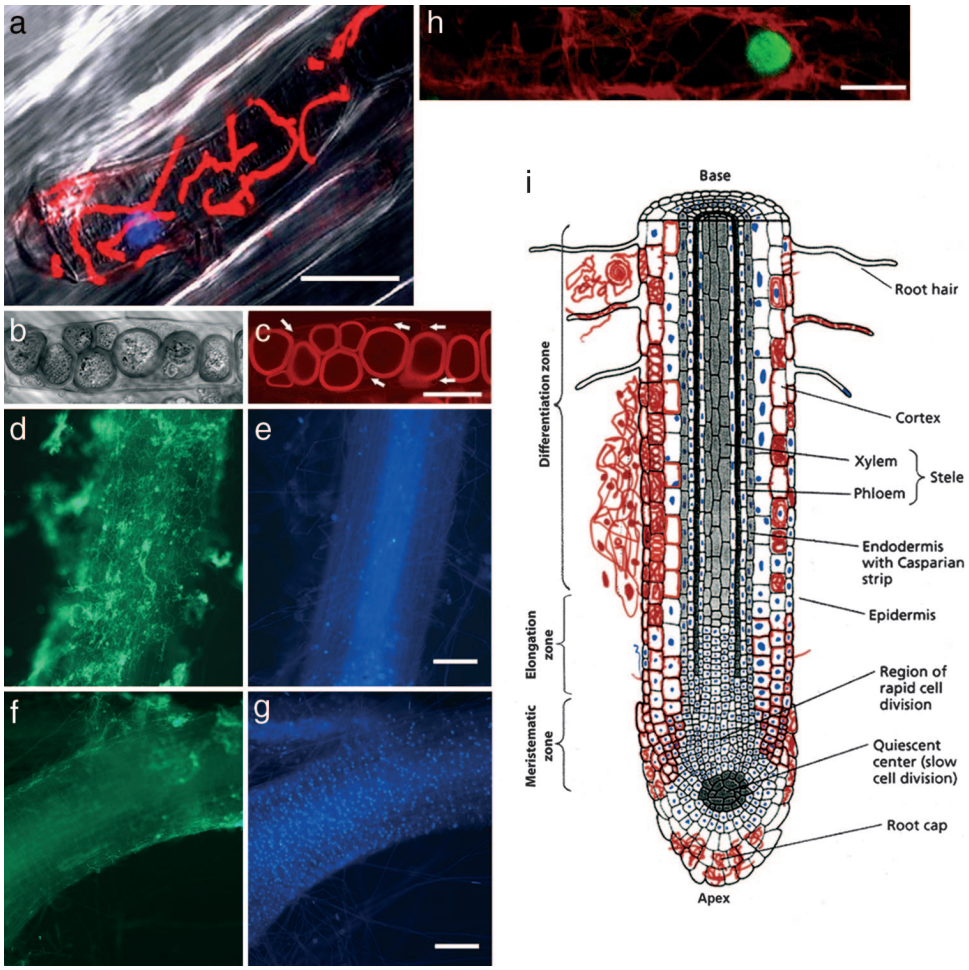

Fig. 3. Association of fungal structures with living and dead cells of the host tissue. (*a*) Fungal hyphae swathe a plant protoplast, which undergoes cytoplasmic shrinkage. Hyphae and nucleus stained with WGA-TMR and DAPI, respectively, are superimposed with the bright-field image. (*b*) Bright-field interference contrast image of chlamydospores in a root cortex cell. (*c*) Fluorescence image of the same cell stained with fuchsin-lactic acid. Arrows indicate hyphae on which the chlamydospores are formed. (*d–g*) Root colonization spatially associated with the absence of intact plant nuclei. Root segments (60 hours after inoculation) double-stained for intact plant nuclei (DAPI; *e* and *g*) and fungal hyphae (WGA-AF 488; *d* and *f*). (*d* and *e*) A root segment heavily colonized by fungal hyphae (*d*) contains only a few DAPI-stained nuclei (*e*). (*f* and *g*) A root segment with minor fungal colonization (*f*) contains a high number of DAPI-stained nuclei (*g*). (*h*) Hyphae swathing a cortical cell protoplast with a TUNEL-positive (green) nucleus. (*i*) Schematic drawing of a *P. indica*-infested root showing the different tissues and the associated colonization pattern, with hyphae depicted in red and DAPI-positive plant nuclei depicted in blue. (Scale bars: *a*, 30 μm; *c*, 10 μm; *d–g*, 300 μm; and *h*, 20 μm.) [Modified from ref. 37 (Copyright 1998, Sinauer, Sunderland, MA).]

to the host plant. Based on the nucLSU sequences our data show that strains of the *S. vermifera* species complex (Sebacinales group B) are closely related to *P. indica* (Fig. 1). These strains yield comparable biological activities in terms of biomass increase and systemic resistance to the biotrophic powdery mildew fungus (Table 1). Hence, the order Sebacinales, of which *P. indica* is considered a model organism, is a source of endophytes with a prospective agronomical impact.

To gain a better understanding of the cellular events leading to the establishment of the mutualistic symbiosis, we microscopically analyzed the interaction of the fungus with the root during the first days of development. After germination of chlamydospores, fungal hyphae grow closely aligned to the topography of rhizodermal cells before penetration of the root at the anticlinal interface of adjacent rhizodermal cell walls (Fig. 2 *b–e*). At such sites, hyphal branching initiates the formation of subepidermal intercellular networks. Intercellular growth is followed by the penetration of rhizodermal cells, which preferentially occurs in differentiated tissue. In young differentiated tissue, single penetrated cells are completely filled with fungal hyphae (Fig. 2 *f* and *g*). Such cells may provide resources for further invasive fungal growth. Mature root tissue is occupied by a network of intracellular hyphae, whose cell to cell "movement" is indicated by hyphal constrictions ("necks"; see Fig. 2*h*). In either case, fungal colonization proceeds by intra- and intercellular infestation of surrounding tissue and gradually increases with tissue maturation. Further proliferation of fungal hyphae finally leads to the development of extra- and intraradical "mats" of hyphae. At this stage, we visualized a clear spatial association of dead root tissue with strong mycelial growth. Dead tissue is characterized by the absence of intact plant nuclei, which were detectable in adjacent, less infected tissue (Fig. 3 *d–g*). This close association of host cell death with massive fungal growth suggests that the fungus contributes to host cell death. Although *P. indica* can induce cell death in poplar under specific conditions on artificial medium (18), fungal culture filtrate did not show any phytotoxic activity on barley (data not shown). At particular interaction sites, we obtained cytological evidence that the fungus can attack and enwrap living (DAPI-positive) protoplasts (Fig. 3*a*). Because *P. indica* can grow between and penetrate into living cells, we suggest that close association of the fungus with living tissue contributes to host reprogramming and, finally, cell death. The

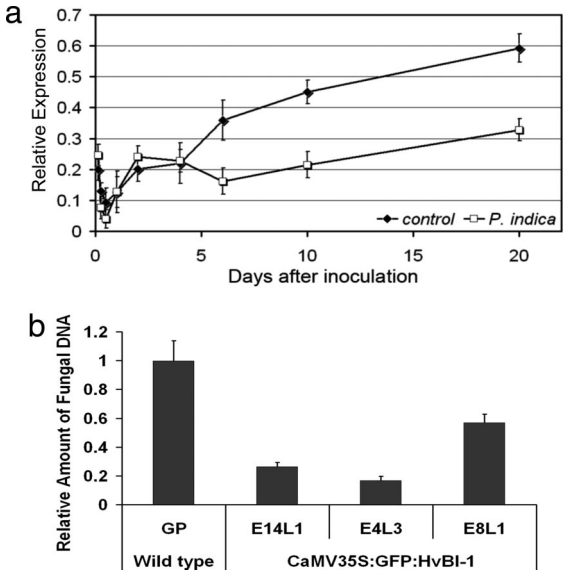

Fig. 4. Influence of HvBI-1 on the development of *P. indica* in barley roots. (*a*) Quantitative PCR analysis of *HvBI-1* expression. As compared with noncolonized roots, expression of the gene is significantly lower from 7 dai onward up to 20 dai. Error bars represent standard deviations. (*b*) The relative amount of *P. indica* DNA in transgenic GFP-HvBI-1 roots was determined at 20 dai. Error bars represent standard deviations. GP represents wild-type plants (Golden Promise). E14L1, E4L3, and E8L1 represent independent transgenic GFP-HvBI-1 GP lines, with five plants tested per line. All three lines were significantly different from the wild type ($P < 0.005$, Student's *t* test). Similar results were obtained in three experiments with plants of an independent transgenic GFP-HvBI-1 line.

inter- and intracellular growth pattern indicated that the fungus is also able to digest plant cell walls and we observed the elimination of transverse cell walls of adjoining cortex cells colonized by the fungus and/or filled with spores (data not shown). In summary, these observations indicate the fungus' capacity to attack and enter host tissue and to proliferate and sporulate in dead cells, most notably in the root differentiation zone (Figs. 2*a* and 3*i*). To obtain molecular evidence for a requirement of host cell death regulation, we analyzed the role of the cell death regulator HvBI-1. Although levels of *HvBI-1* mRNA slowly increased during barley root development, *P. indica*-colonized roots showed a significant reduction of *HvBI-1* mRNA levels compared with noncolonized roots from 7 dai onward (Fig. 4*a*). These data suggest a lowered threshold for PCD in endophyte-colonized roots and support the idea that *P. indica* influences intrinsic plant PCD. In barley leaves, *HvBI-1* is strongly expressed in incompatible interactions with the obligate biotrophic leaf pathogen *B. graminis* (15) and may have a role in restricting resistance-associated hypersensitive cell death reactions. The fact that *P. indica* attenuates expression of *HvBI-1* therefore indicates that PCD observed in the interaction with *P. indica* is different from hypersensitive cell death in pathogen defense. It remains to be shown what kind of PCD might be controlled by *P. indica*.

Previous work showed that HvBI-1 has a central role in the outcome of host–pathogen interactions (16, 19, 20). To functionally confirm the role of host PCD and a requirement of *HvBI-1* down-regulation for fungal proliferation, we constitutively overexpressed a GFP–HvBI-1 fusion protein in barley. All transgenic lines showed enhanced resistance to cell death induced by transient expression of mouse BAX in epidermal leaf cells (R. Eichmann, unpublished results). Comparison of the transgenic plants with the respective wild type showed a significant reduction of invasive growth of *P. indica* in GFP–HvBI-1 barley at 20 dai, when fungal proliferation is in a steady state. In contrast, transient overexpression of *HvBI-1* in barley leaf epidermis supported early biotrophic invasion of *B. graminis* into resistant barley (15, 21). Additionally, all GFP–HvBI-1 lines that restricted proliferation of *P. indica* showed enhanced susceptibility to a virulent isolate of *B. graminis*. This effect relied on a lower ability of the plant to stop the fungus by hypersensitive cell death, which is involved in basal barley disease resistance (V. Babaeizad, R. Eichmann, and J.I., unpublished results). Hence, the expression level of HvBI-1 might inhibit or support fungal proliferation depending on the microbial lifestyle. Quantification of *P. indica* confirmed that fungal growth was significantly restricted in GFP–HvBI-1 barley (Fig. 4*b*). Taken together, we provide genetic evidence that *P. indica* requires host cell death for successful proliferation. We suggest that the mutualistic symbiosis between *P. indica* and barley involves a sophisticated regulation of the plant's cell death machinery. The close spatial association of root cell death with massive infestation by *P. indica* might reflect the fungus' success to manipulate host cell PCD. Thereby *P. indica* might take advantage of naturally occurring root cell death in mature parts of the root. However, the main part of the root further develops and is not necrotized when colonized by the fungus.

Conclusion and Perspectives

The mutualistic symbiosis of crop plants and Sebacinales has a great potential for sustainable agriculture. In contrast to AMF, *P. indica* and other members from the same order mediate resistance to root pathogens and systemic resistance to biotrophic leaf pathogens. From an agronomical point of view, it is most promising that *P. indica* can enhance crop yield in cereals (10). Exploitation of endophytic fungi like *P. indica* may, however, not only complement crop production strategies, which presently rely on a high input of fungicides, but additionally may be an eminent source of molecular traits affecting both disease resistance and grain yield in cereals. For future utilization, it is important to gain additional information on effective application strategies (e.g., spore formulation), growth conditions, and the influence of environmental factors. The prospected huge biodiversity in the Sebacinales (11) and the physiological variation between the Sebacinales strains yield the perspective that for a given crop plant an optimal sebacinalean mutualist might become available. This latter notion is supported by the results of our molecular phylogenetic analysis (Fig. 1), which shows that the type of the interaction between Sebacinales and their plant hosts is probably influenced to a greater extent by the plant than by the fungus. Strains of the *S. vermifera* species complex that interacted with barley similar to *P. indica* were originally isolated from Australian orchids (11). In orchid mycorrhizae, however, the fungus invades vital cortical root cells of the host to form intracellular hyphal coils. The strains tested in the present study also are closely related to members of the Sebacinales that form cavendishioid mycorrhizas (14) with certain hemiepiphytic ericads (Fig. 1). In this mycorrhizal association, the fungal partner also predominantly invades vital cortical cells. It is evident that the mutualistic symbiosis between plants and fungi of the Sebacinales is a treasure chest to discover mechanisms to protect plants from biotic and abiotic stresses. Although evidence has been provided that the plant's antioxidant system plays a pivotal role in the *P. indica*-mediated stress tolerance (10), the precise mechanism and underlying signaling pathways remain to be elucidated. In this respect, *P. indica* has another important advantage: In contrast to AMF, *P. indica* colonizes *Arabidopsis*, and our recent results provide evidence that the fungus induces systemic resistance in this model plant similar to the resistance provided to the powdery mildew fungus in barley (our unpublished data). The power of the *Arabidopsis* signal transduction mutants available and reverse genetics will soon accelerate

disclosure of the molecular basis of the symbiosis and its beneficial effects on the host. Despite this perspective, differences in signaling pathways relevant for agronomically important traits exist between *Arabidopsis* and cereals, justifying strong emphasis on future cereal research.

Materials and Methods

Plant and Fungal Material and Plant Inoculation. Barley (*Hordeum vulgare* L.) cultivar Golden Promise was obtained from Jörn Pons-Kühnemann (University of Giessen, Giessen, Germany). *P. indica* isolate *WP2* was propagated as described (10). *S. vermifera* isolates (culture collection numbers; see Table 1) were propagated in MYP medium (aqueous solution of 7 g/liter malt extract, 1 g/liter peptone and 0.5 g/liter yeast extract).

For inoculation, barley kernels were sterilized with 6% sodium hypochloride, rinsed in water, and germinated for 2 days. Subsequently, seedling roots were immersed in an aqueous solution of 0.05% Tween-20 containing 5×10^5 ml^{-1} *P. indica* chlamydospores or homogenized mycelial solution (1 g/ml) of *S. vermifera*, respectively. Inoculated seedlings were grown in a 2:1 mixture of expanded clay (Seramis; Masterfoods, Verden, Germany) and Oil Dri (Damolin, Mettmann, Germany) (10).

Molecular Phylogenetic Analysis. We used nuclear DNA sequences coding for the 5′ terminal domain of the ribosomal large subunit to estimate the phylogenetic position of the Sebacinales strains used in the present study. An alignment covering a representative sampling of nucLSU sequences available for this fungal group was constructed with MAFFT 5.850 (22). The alignment was analyzed by using heuristic maximum likelihood as implemented in PHYML 2.4.4 (23), with a general time-reversible model of nucleotide substitution and additionally assuming a percentage of invariant sites and Γ-distributed substitution rates at the remaining sites (GTR+I+G; the Γ distribution approximated with four discrete rate categories), starting from a BIONJ tree (24). All model parameters were estimated by using maximum likelihood. Branch support was inferred from 1,000 replicates of nonparametric maximum-likelihood bootstrapping (25), with model parameters estimated individually for each bootstrap replicate. Additionally we performed a Bayesian Markov chain Monte Carlo analysis with MrBayes 3.1 (26). We ran two independent Markov chain Monte Carlo analyses, each involving four incrementally heated chains over two million generations, using the GTR+I+G model of nucleotide substitution and starting from random trees. Trees were sampled every 100 generations, resulting in an overall sampling of 20,000 trees per run, from which the first 5,000 trees of each run were discarded (burn in). The remaining 15,000 trees sampled in each run were pooled and used to compute a majority rule consensus tree to get estimates for the posterior probabilities. Stationarity of the process was controlled by using the Tracer program (27).

Generation of Transgenic Barley Plants. For constitutive overexpression and for tagging expression, we cloned a cDNA fusion of GFP and *HvBI-1* by digestion of pGY1-CaMV35S::GFP–HvBI-1 (15, 21) into appropriate sites of the binary vector pLH6000 (DNA Cloning Service, Hamburg Germany), which was then introduced into *Agrobacterium tumefaciens* strain AGL1 (28) to transform barley cultivar Golden Promise as described (29, 30). PCR analysis was used to confirm integration of the transfer DNA. The GFP reporter was visualized with either a standard fluorescence microscope or a confocal laser scanning microscope as described below.

Root Fixation, Staining and Microscopy, and DAPI Staining. Root segments were fixed as described in ref. 31, with noted exceptions. Fixed root segments were transferred to an enzyme solution containing 10 mg/ml driselase and chitinase, 16 mg/ml β-D-glucanase (InterSpex Products, San Mateo, CA) and 1 mg/ml BSA (Sigma, St. Louis, MO) dissolved in 25 mM phosphate buffer (PB) (4.0 g of NaCl/0.1 g of KCl/0.7 g of Na_2HPO4 $2H_2O$/0.1 g of KH_2PO4 in 500 ml water, pH 6.8) at room temperature for 15 min. After rinsing in PB, roots were further treated with 0.5% Triton X-100 in PB for 10 min. After additional rinsing in PB, plant nuclei were stained with 1 μg/ml DAPI for 30 min. During incubation, segments were vacuum-infiltrated three times for 1 min at 25 mmHg (1 mmHg = 133 Pa) and then rinsed with PB. Additionally, root material was stained with WGA-AF 488 as described below. All segments were analyzed with an Axioplan 2 microscope (excitation 365 nm and emission 420–540 nm; Zeiss, Jena, Germany).

A TUNEL assay was performed using an *in situ* cell death detection kit (Fluorescein; Roche Applied Science, Penzberg, Germany) according to the instruction manual. Root segments were fixed as described above. In addition, root segments were dehydrated and dewaxed by passage for 15 min through series of increasing concentrations of ethanol in water (from 10% to 100% in 10% increments) and back from 100% to 0% in 10% increments). Subsequently, segments were incubated in 50 μl of TUNEL reaction mixture. Grade 1 DNase I-treated roots were used as positive controls. Solutions were vacuum-infiltrated as described above and incubated for 60 min at 37°C in humidified atmosphere in the dark. Subsequently, segments were washed and transferred to 1× PB (pH 7.4) for destaining. Destained segments were counterstained with wheat germ agglutinin-tetramethylrhodamine (WGA-TMR) as described below. TUNEL-positive nuclei were excited at 488 nm and detected at 505–540 nm. Fluorescein diacetate vitality staining and actin staining of barley root was performed according to refs. 32 and 33, respectively.

Staining of *P. indica* in Root Tissue. Hyphae in root segments were either stained by 0.01% acid fuchsin-lactic acid (10) or with the chitin-specific dyes WGA-AF 488 and WGA-TMR (Molecular Probes, Karlsruhe, Germany). Depending on the studies, root material was either fixed for some experiments, dehydrated as described above, or transferred to trichloroacetic acid fixation solution [0.15% (wt/vol) trichloroacetic acid in 4:1 (vol/vol) ethanol/chloroform]. Subsequently, segments were incubated at room temperature for 10 min in 1× PBS (pH 7.4) containing each respective dye at 10 μg/ml. During incubation, segments were vacuum-infiltrated three times for 1 min at 25 mmHg. After rinsing with 1× PBS (pH 7.4), segments were mounted on glass slides. In cases that Congo red (Merck, Darmstadt, Germany) was used for counterstaining, it was added to WGA-AF 488 staining solution at a final concentration of 10 μg/ml. Confocal fluorescence images were recorded on a multichannel TCS SP2 confocal microscope (Leica, Bensheim, Germany). WGA-AF 488 was excited with a 488-nm laser line and detected at 505–540 nm. WGA-TMR was excited with a 543-nm laser line and detected at 560–630 nm. All segments that were analyzed with an Axioplan 2 microscope were either excited at 470/20 nm and detected at 505–530 nm for WGA-AF 488 or excited at 546/12 nm and detected at 590 nm for Congo red.

Genomic DNA Isolation, Real-Time PCR, and Transcript Analysis. The degree of root colonization was determined by using the $2^{-\Delta Ct}$ method (34). Cycle threshold (Ct) values were generated by subtracting the raw Ct values of the *P. indica* internal transcribed spacer or *Tef* gene (35) from the raw Ct values of plant-specific ubiquitin.

Roots were harvested, frozen, and ground in liquid nitrogen, and genomic DNA was isolated from ≈100 mg of root powder with the Plant DNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For quantitative PCR, 5–10 ng of total DNA was used. Amplifications were performed in 20

μl of SYBR green JumpStart *Taq* ReadyMix (Sigma–Aldrich, Munich, Germany) with 350 nM oligonucleotides, using an Mx3000P thermal cycler (Stratagene, La Jolla, CA). After an initial activation step at 95°C for 7 min, 40 cycles (94°C for 30 s, 60°C for 30 s, 72°C for 30 s, and 82°C for 15 s) were performed, and a single fluorescent reading was obtained after the 82°C step of each cycle. A melting curve was determined at the end of cycling to ensure amplification of only a single PCR product. Ct values were determined with the Mx3000P V2 software supplied with the instrument.

For quantitative two-step RT-PCR, 2 μg of total RNA were reverse-transcribed to first-strand cDNA with the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Aliquots of 20 ng of first-strand cDNA were subsequently used as a template for quantitative PCR with gene-specific primers. The plant-specific ubiquitin gene served as a control for constitutive gene expression in roots. Ubiquitin expression was consistent after inoculation with *P. indica* when compared with the amount of 18S ribosomal RNA. Specific PCR conditions were as described above, and comparative expression levels ($2^{-\Delta Ct}$) were calculated according to ref. 36. Expression levels are relative to the level of ubiquitin expression, which was constant in all RNA samples used and was set to 1. Values are the means of four samples of one biological experiment (infected roots) assayed by quantitative PCR in triplicate. The oligonucleotides used were as follows: ubiquitin (accession no. M60175), 5′-CAGTAGTGGCGGTCGAAGTG-3′ and 5′-ACCCTCGCCGACTACAACAT-3′; *P. indica* Tef (accession no. AJ249911) 5′-ACCGTCTTGGGGTTGTATCC-3′ and 5′-TCGTCGCTGTCAACAAGATG-3′; Bax inhibitor-1 (accession no. AJ290421) 5′-GTCCCACCTCAAGCTCGTTT-3′ and 5′-ACCCTGTCACGAGGATGCTT-3′; and *P. indica* ITS (accession no. AF 019636) 5′-CAACACATGTGCACGTCGAT-3′ and 5′-CCAATGTGCATTCAGAACGA-3′

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Supporting Figure

File in this Data Supplement:

Supporting Figure 5

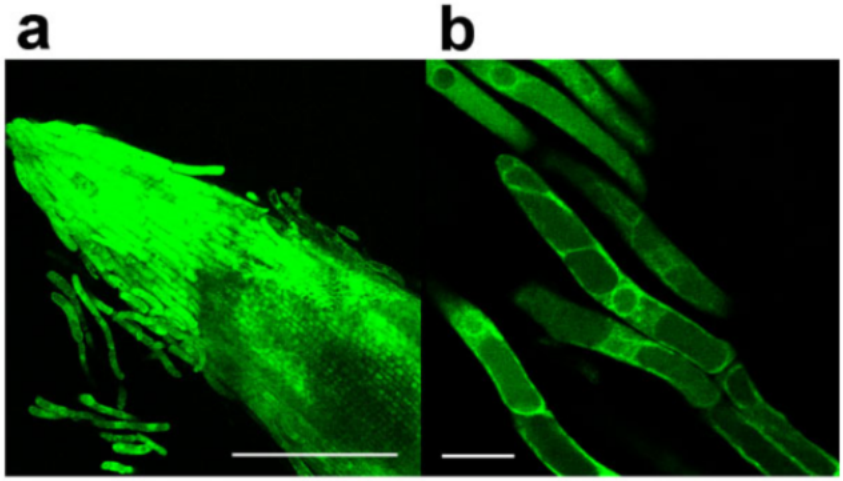

Fig. 5. Expression of the GFP-BAX inhibitor-1 (HvBI-1) fusion protein in roots of transgenic barley lines used in our study. (*a*) Confocal laser scanning microscope image of a root tip expressing GFP-HvBI-1 under the constitutive cauliflower mosaic virus 35S promotor. (*b*) Typical pattern of GFP-HvBI-1 fluorescence at the nuclear envelope and in the endoplasmic reticulum of detached barley root cells. (Scale bars: *b*, 300 μm and *c*, 20 μm.)

Piriformospora indica protects barley from root rot caused by *Fusarium graminearium*

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Abstract

The beneficial endophytic fungus *Piriformospora indica* colonizes barley (*Hordeum vulgare* L.) roots, which eventually results in higher plant biomass as well as protection against diseases and abiotic stress. Infection of the roots with pathogenic necrotrophic fungi of the genus *Fusarium*, in contrast, leads to necrotized roots and severe reduction of root and shoot biomass. We analyzed the interaction of *P. indica* with *F. graminearum* in barley roots and *in vitro*. Upon infestation with *P. indica*, roots were protected from *Fusarium* infections as evidenced by reduced root rot symptoms. Consistently, *Fusarium* quantification using quantitative polymerase chain reaction (Q-PCR) revealed a connection between reduced root rot symptoms and the relative amount of fungal DNA in the roots. *In vitro* analysis of the interaction of *P. indica* and *F. graminearum* under axenic culture conditions did not reveal reciprocal growth inhibition suggesting that direct antibiotic effects from *P. indica* are not responsible for reduced *Fusarium* development in barley roots. Expression of pathogenesis-related genes, which strongly increased in response to *F. graminearum* infections, was diminished in the presence of *P. indica*. This finding indicates that PR proteins do not play a crucial role in the *P. indica*-mediated resistance response to *Fusarium*.

Bio-control | pathogen | PR-genes | Q-PCR quantification | root endophyte

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Introduction

Leakage of nutrients into the rhizosphere provides a microenvironment for growth of many microbes in association with the plant root. These microbes follow the saprophytic, biotrophic or necrotrophic life styles. Many of these rhizosphere microorganisms colonize roots endophytically and provide beneficial effects on plant growth and health (WHIPPS, 2001; KOGEL et al., 2006). A prominent example are mycorrhiza fungi(HARRISON, 2005), which colonize the roots of the majority of terrestrial plant species. These fungi play an important role in enhancing plant growth, vigour, and nutrition. They also provide increased reproductive potential, improved root performance and stimulated defence against biotic and abiotic stresses (NEWSHAM et al., 1995; CORDIER et al., 1996; SLEZACK et al., 2000; AUGÉ, 2001; HARRISON, 2005). The recently discovered root endophytic fungus *Piriformospora indica* (VERMA et al., 1998), colonizes roots of a large number of plant species including cereals and brassicaceae (VARMA et al., 1999; PESKAN-BERGHOFER et al., 2004). The fungus belongs to the order Sebacinales (WEISS et al., 2004) that comprises several species showing the same beneficial properties on host plants. Endophytic growth of *P. indica* leads to enhanced growth and yield of the host. Because this fungus, in contrast to arbuscular mycorrhiza (AM) is axenically cultivable, it may serve as a model system to investigate mutualistic plant-fungal interactions. In barley, *P. indica* protects its host from salt stress and fungal diseases, besides its impact on biomass. The underlying mechanism of the fungus' beneficial activity is not yet understood but activation of the plant antioxidant system has been observed and thus implicated in the improvement of abiotic and biotic stress tolerance (WALLER et al., 2005).

Fungi of the genus *Fusarium* are causal agents of severe plant diseases such as *Fusarium* head blight and crown root rot (MCMULLEN et al., 1997; JANSEN et al., 2005). After inoculation of barley roots with macroconidia, the necrotrophic fungus rapidly invades the root thus eliciting cell death of large areas of root tissue. Later, plant growth and biomass is severely diminished as portions of the plants' vascular system are not functional anymore. Recently, it has been shown that *P. indica*-infested plants are more resistant to *Fusarium culmorum* as demonstrated by a 6-fold lower reduction of plant biomass compared with plants not inoculated with *P. indica* (WALLER et al., 2005). In the current study, we show that *P. indica* also protects barley roots from root rot caused by *Fusarium graminearum*. To gain more insights into the mechanism of *P. indica*-mediated root rot control, we followed disease progression in roots infested and non-infested with *P. indica* by means of a Q-PCR-based detection method. Moreover, we determined the plant response by recording expression of

transcription levels of pathogenesis-related (PR) genes in *P. indica*-infested and non-infested barley roots after *F. graminearum* infection.

Materials and Methods

Plant material and fungal inoculation

Barley (*Hordeum vulgare* L.) cultivar Maresi was grown in a 2:1 mixture of expanded clay (Seramis®, Masterfoods) and Oil Dri® (Damolin) in a growth chamber at 22°C/18°C day/night cycle, 60% relative humidity and a photoperiod of 16 h (240 µmol m^{-2} s^{-1} photon flux density), and fertilized weekly with 20 ml of a 0.1% Wuxal top N solution (Schering, N/P/K: 12/4/6). For inoculation, barley kernels were surface sterilized with 6% sodium hypochloride, rinsed in water, and germinated for 2 days. Subsequently, seedling roots were immersed in an aqueous solution of 0.05% Tween 20 containing $5x10^5$ ml^{-1} *P. indica* chlamydospores (VERMA et al., 1998). Root samples were checked microscopically for *P. indica* infestation. *P. indica* was propagated on defined modified *Aspergillus* medium (PHAM et al., 2004) and *F. graminearum* on synthetic nutrient agar (SNA) medium (NIRENBERG, 1981). Macroconidia of *Fusarium graminearum* strain 8/1 expressing the green fluorescent protein (GFP) (JANSEN et al., 2005) were suspended in 0.02% Tween 20 at a concentration of 50,000 ml^{-1} and used for inoculation of twelve-day-old *P. indica*-colonized plants and non-colonized 'control' plants by dipping in spore solution.. Mock inoculations were performed with water containing 0.02% Tween 20. Finally, roots were rinsed with water and harvested into liquid nitrogen at 0, 1, 3, 7 and 14 days after inoculation (dai) with *F. graminearum*.

Test for fungal antagonism

To test for direct antibiotic effects, an agar disc of 0.5 cm diameter completely covered by *F. graminearum* mycelium was placed in the centre of an agar plate and four *P. indica* agar discs were placed around at equal distance. In other sets, *P. indica* was surrounded by *F. graminearum* (see Fig. 2). Fungal cultures were propagated on SNA (NIRENBERG, 1981) plates at 18°C and mycelium growth was recorded after every five days.

Staining and microscopy

F. graminearum expressing GFP was visualized by confocal fluorescence microscopy using a multichannel TCS SP2 confocal system (Leica Microsystems, Bensheim, Germany). GFP was excited with a 488 nm laser line and detected at 505–530 nm. Fungal autofluorescence was excited with a 488 nm laser line and detected at 550–700 nm. *P. indica* roots were stained with 0.01% acid fuchsin-lactic acid (KORMANIK and MCGRAW, 1982). Fungal structures

in roots were visualized by mounting in 50% glycerol and examined with a Zeiss Axioplan 2 imaging microscope.

Genomic DNA isolation and real-time quantitative PCR

Roots were taken at specified intervals, frozen, crushed in liquid nitrogen and sampled in a 2-ml polypropylene tube. 400 µl AP1 buffer (Plant DNeasy kit; Qiagen, Hilden, Germany) and 4 µl of RNase (supplied with the Plant DNeasy kit) were added to each tube and incubated for 15 min at 65°C. The following isolation was performed according to the manufacturer's instructions (Plant DNeasy kit, Qiagen). Five to 10 ng of total DNA was used in each Q-PCR reaction. Amplifications were performed in 20 µl SYBR Green JumpStart^{TM} Taq ReadyMix^{TM} (Sigma) with 0.7 µl of 10 µM µl^{-1} oligonucleotide primers (see below). Amplifications were carried out with a Stratagene-Mx3000P (Stratagene Research). After an initial 7 min activation step at 95°C, 40 cycles (94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, 82°C for 15 s) were performed, and a single fluorescent reading was obtained after each cycle immediately following the 82°C step. A melting curve was determined at the end of cycling to ensure that only a single PCR product has been amplified. Cycle threshold (Ct) values were determined with the Mx3000P V2.00 (Stratagene Research) software. The primers were tested for equal amplification efficiencies of the target and reference by determining ΔCt variations with serial dilutions of 20 ng to 10 pg of *P. indica* and barley genomic DNA as well as *P. indica* and/or *F. graminearum* and barley genomic DNA (LIVAK and SCHMITTGEN, 2001). In separate reactions, the primers were also tested for template specificity by sequencing amplified products of the respective templates. Fungal templates used for plant specific primers and plant templates used for fungal specific primers gave no amplification product.

Quantification of *P. indica* and *F. graminearum* in barley roots

The ratio of fungal DNA to plant DNA was used to monitor fungal infection in barley roots. The *P. indica* specific *tef* gene and the *F. graminearum* specific *GFP* gene were used for determination of fungal DNA from respective Ct values. The barley-specific *Ubiquitin* gene was used for determination of plant DNA from its raw Ct values. For determination of *P. indica* progression, Ct curves were generated by using the $2^{-\Delta Ct}$ method where ΔCt is the difference between raw Ct values of the fungus-specific gene and the raw Ct values of the plant specific gene. The levels of *P. indica tef* or *Fusarium GFP* are relative to the level of ubiquitin, which was set at 1 (LIVAK and SCHMITTGEN, 2001).

PR gene expression analysis by quantitative RT-PCR

Table 1 Oligonucleotide primers used for PCR analysis.

Name	Sequence 5' to 3'
Hordeum vulgare L. *Ubiquitin* (Accession No M60175)	CAGTAGTGGCGGTCGAAGTG
	ACCCTCGCCGACTACAACAT
P. indica Translation Elongation Factor EF-1α (*tef*) (Accession No AJ249911)	ACCGTCTTGGGGGTTGTATCC
	TCGTCGCTGTCAACAAGATG
Green Fluorescent Protein (GFP) (JANSEN, et al., 2005)	AGAACGGCATCAAGGTGAAC
	ACTGGGTGCTCAGGTAGTGG
Hordeum vulgare L. *Pathogenesis-related 1b* (Accession No Z21494)	GGACTACGACTACGGCTCCA
	GGCTCGTAGTTGCAGGTGAT
Hordeum vulgare L. *Pathogenesis-related 5* (Accession No AY839293)	TAGAGCTTGCAGCAATGTCGACC
	CCTGAGCCCAGCTCGAAG
Hordeum vulgare L. *β-1,3-glucanase* (Accession No GI167056)	CACATCAAGGTGACCACGTC
	GGGTAGATGTTGGCCATGAG

Total RNA was extracted from crushed material using the RNAeasy plant mini kit, according to instruction manual (Qiagen GmbH, Hilden Germany). For quantitative two-step reverse-transcription polymerase chain reaction (Q-RT-PCR), 2 µg of total RNA from the above experiment were reverse transcribed to first strand cDNA by using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories GmbH, München, Germany) according to the manufacturers' instructions. The final reaction was diluted 5 times and aliquots of 20 ng of the first strand cDNA were subsequently used as template for Q-PCR with gene specific primers. The *Ubiquitin* on coding gene was used as a control for constitutive gene expression. Q-RT-PCR was carried out as described above and comparative expression levels ($2^{-\Delta Ct}$) were calculated according to (WULF et al., 2003). Expression levels are relative to the level of *Ubiquitin* expression, which was similar in all RNA samples used and set at 1.

Results

***Fusarium* root rot symptoms are delayed in *P. indica*-colonized barley roots**

For the assessment of the effect mediated by *P. indica* on *F. graminearum* development, 14-day-old plants were used that had been inoculated with *P. indica* chlamydospores at 2 days after germination. Control plants (CC) devoid of *P. indica* and *F. graminearum* remained healthy during the course of the experiment (Fig. 1A). Plants colonized by *P. indica* (CP) were more vital than control plants. Plants infected with *F. graminearum* (CF) showed severe symptoms of root and crown rot after 5 days of infection. In contrast, at the same time, plants colonized by *P. indica* and *F. graminearum* (PF) were free of visible symptoms and showed mild symptoms only at later times.

Microscopic analysis revealed extensive growth of *F. graminearum* on inoculated barley roots, with the mycelium completely covering the roots at 5 dai. At this time, crown tissue was necrotized, and occasionally intercellular growth of fungal hyphae was observed. Penetrated host cells showed plasmolysis or strong autofluorescence of the cell wall, indicating that they underwent cell death. In addition, newly formed macroconidia were present on the surface of

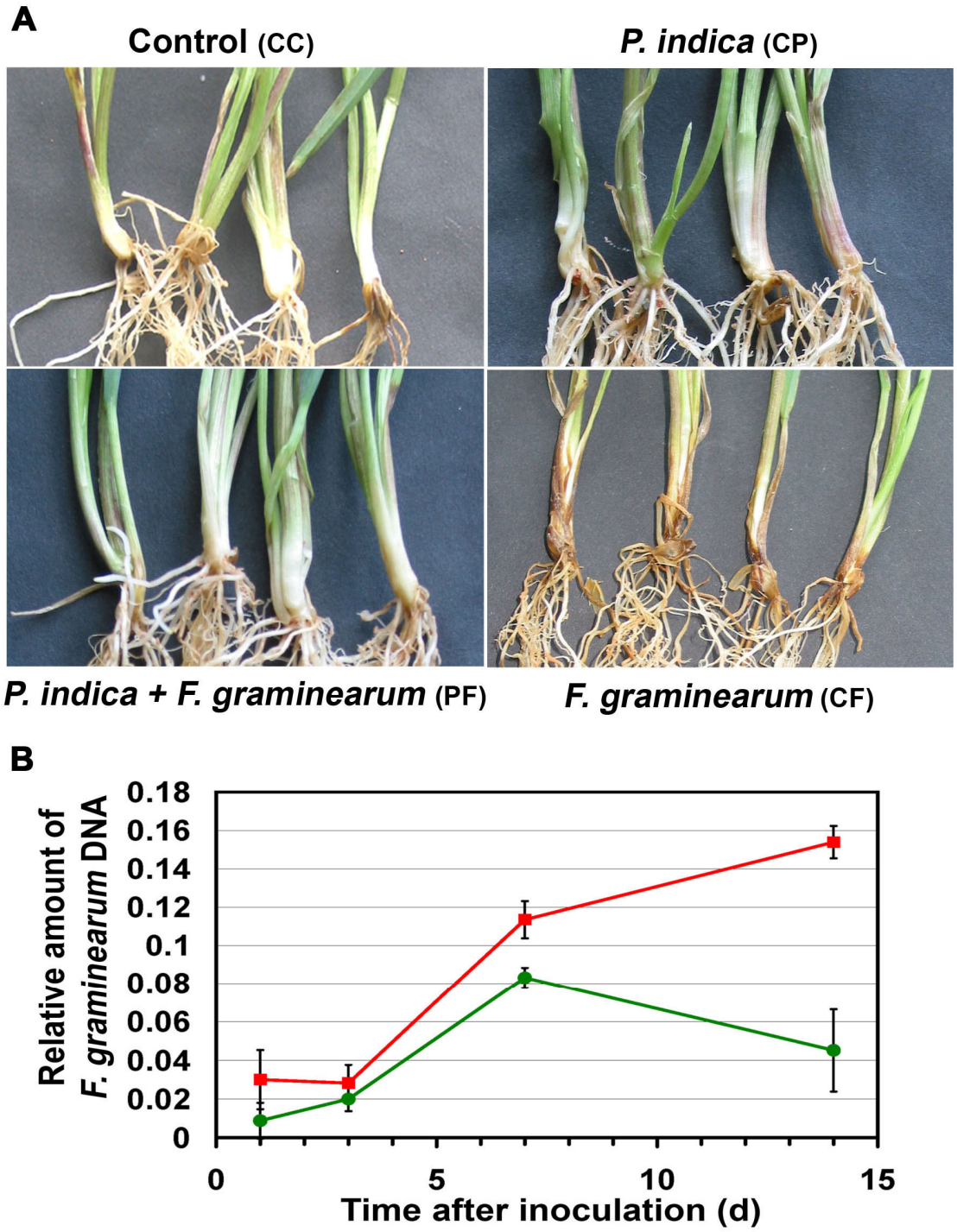

Fig. 1 Development and quantification of *Fusarium graminearum* in barley roots
A; Development of *F. graminearum* in barley roots
Crown root rot symptoms caused by *F. graminearum* in barley 5 days after inoculation with macroconidia. Control plants (CC); *P. indica*-infested plants (CP); *F. graminearum*-inoculated plants pre-inoculated with *P. indica* (PF); *F. graminearum* infected plants (CF).
B; Quantification of *F. graminearum* in barley roots
Relative amounts of the fungus were determined as the amount of fungal DNA relative to plant DNA; Squares: (CF) *F. graminearum*-inoculated plants; Circles: (PF) *F. graminearum*-inoculated plants pre-inoculated with *P. indica*. Each point on the curve represents (mean +/-SE) six individual pots with three plants per pot, (infected roots), (n=6) assayed by Q-PCR in triplicate. Y-axis represents relative amounts of *F. graminearum* DNA. Similar results were obtained in independent experiment.

the root and in crown tissue. In PF plants, *F. graminearum* showed a similar development, but both, the presence of the fungus and plant cell collapse could be observed less frequently as compared with CF plants, with no visible fungal hyphae in the crown tissue at 5 dai (data not shown).

Quantification of fungal DNA in barley roots by real-time quantitative PCR

We established an assay to quantify fungal biomass based on the relative quantification of fungal and plant DNA in barley roots by means of real-time quantitative PCR. Our regression curve of serial dilutions of the respective templates showed linearity of amplification over the dynamic range, for primers of the *P. indica tef* gene (BÜTEHORN et al., 2000), transgenic *GFP* for *F. graminearum* strain 8/1 and plant *Ubiquitin*. The results indicate assay specificity and sensitivity over a wide range of template concentrations (not shown).

F. graminearum quantification by Q-PCR revealed a connection between reduced root rot symptoms and the amount of fungal DNA. Pathogen development in CF plants was rapid, rising five fold over the 14 dai time point (Fig. 1B) and associated with extensive symptom development (Fig. 1A). At the end of the time course (14 dai), *F. graminearum* DNA became predominant in the CF samples, which could be ascribed both to fungal propagation and massive plant cell collapse. In contrast, development of *F. graminearum* in PF plants was gradual and associated with mild symptoms. At 14 dai, these plants had a less than 2-fold increase of *F. graminearum* DNA as compared with day 1 (Fig. 1B). We also determined development of *P. indica* in barley roots by using Q-PCR. Relative amounts of *P. indica* DNA showed an early moderate increase, followed by a decrease and eventually a steady state with minor changes during the course of the experiment (data not shown).

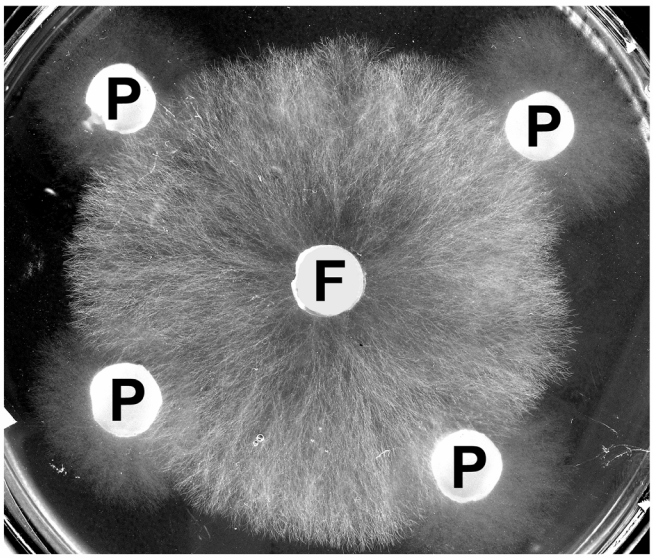

Fig. 2 Direct fungal antagonism
Agar plate co-cultivated with *Fusarium graminearum* (F); surrounded by *Piriformospora indica* (P). Fungal cultures were grown for five days.

***In vitro*-interaction of *P. indica* and *F. graminearum* in axenic cultures**

We analysed for direct antibiotic effects of *P. indica* on *F. graminearum* under axenic culture conditions. In one set of agar plates, we plated one fungal disc of *P. indica* surrounded by four discs of *F. graminearum* and in an other set we plated *F. graminearum* surrounded by *P.*

indica. After incubation for 5 days, neither *F. graminearum* nor *P. indica* growth was affected by the presence of the other fungus, and there was no zone of inhibition at the contact point of two fungal colonies (Fig. 2). Over time, *Fusarium* covered the entire plate. An independent experiment with four days pre-grown *P. indica* showed similar results, indicating that *P. indica* does not show any direct antagonistic activity against *F. graminearum*.

Influence of *P. indica* infestation on PR gene expression in roots challenged with *F. graminearum*

We tested if *P. indica* induces plant defence genes. A transient increase of *PR1b* transcript level in roots was observed at 1, 2 and 4 dai (Fig. 3). From 6 dai onwards, very low levels of *PR1b* were detected (Fig. 3). Similar results were obtained for *PR5* suggesting that the endophyte suppresses the primary accumulation of PR proteins during root colonization (data not shown).

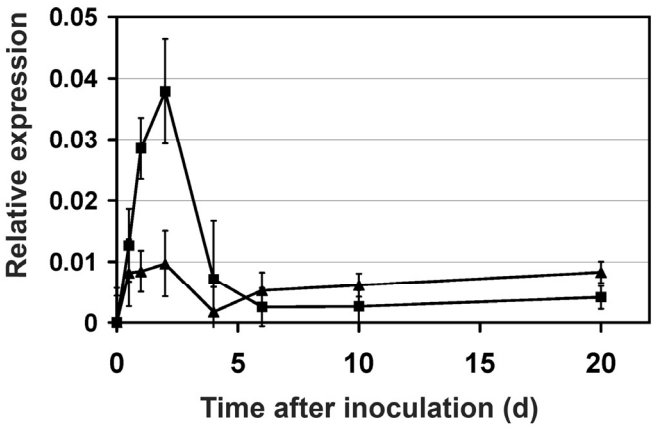

Fig. 3 Expression of the *PR1b* gene in response to *Piriformospora indica*
Levels of PR1b transcript were determined relative to the expression of the *Ubiquitin* transcript using Q-RT-PCR. Each point on the curve (mean +/-SE) represents six individual pots, with three plants per pot from a biological experiment, assayed by Q-PCR in triplicate. Similar results were obtained in an independent experiment. Y-axis represents relative amounts *PR1b* expression; X-axis represents days (d) after *P. indica* inoculation. *P. indica* inoculated (squares) and non inoculated barley roots (triangles).

Inoculation of roots with *F. graminearum* led to a strong induction of all tested PR genes (Fig. 4). *PR1b* transcript levels started to increase from 7 dai onwards up to a 50-fold increase at 14 days after *F. graminearum* inoculation. *PR5* transcript levels increased 2-fold from 0 to 3 dai and 8-fold until 14 dai. Induction of *ß-1,3-glucanase* (*PR2*) was fast and transient, with a maximum of a 100-fold increase at 3 dai and lowered to the level of about 10-fold at 7 to 14 dai. Roots challenged 12 days after *P. indica* pre-inoculation with *F. graminearum* showed a similar timing of PR gene induction as the roots inoculated with *F. graminearum* alone though the magnitude of *Fusarium*-induced gene expression in *P. indica* pre-inoculated plants was generally much weaker. For *PR1b*, a 9-fold and 11-fold induction at 7 and 14 dai, respectively, was detected, as compared with 24- and 50-fold induction in roots inoculated with *F. graminearum* alone. Induction of *PR5* was reduced by about 20% and 30% at day 7 and 14 dai, respectively. Induction of *ß-1,3-glucanase* was reduced by about 60% at 3 dai when transcript levels were highest.

Discussion

Our results demonstrate that *Piriformospora indica* and *Fusarium graminearum* do not exhibit direct antagonistic effects on each other *in vitro*, although *P. indica* provides protection against *F. graminearum* root rot in barley. We developed a real-time PCR-based assay to analyze *F. graminearum* development in barley roots. This method, together with the expression analysis of pathogenesis-related genes in the *F. graminearum*-infected root enabled us to characterize possible mechanisms responsible for the observed protection.

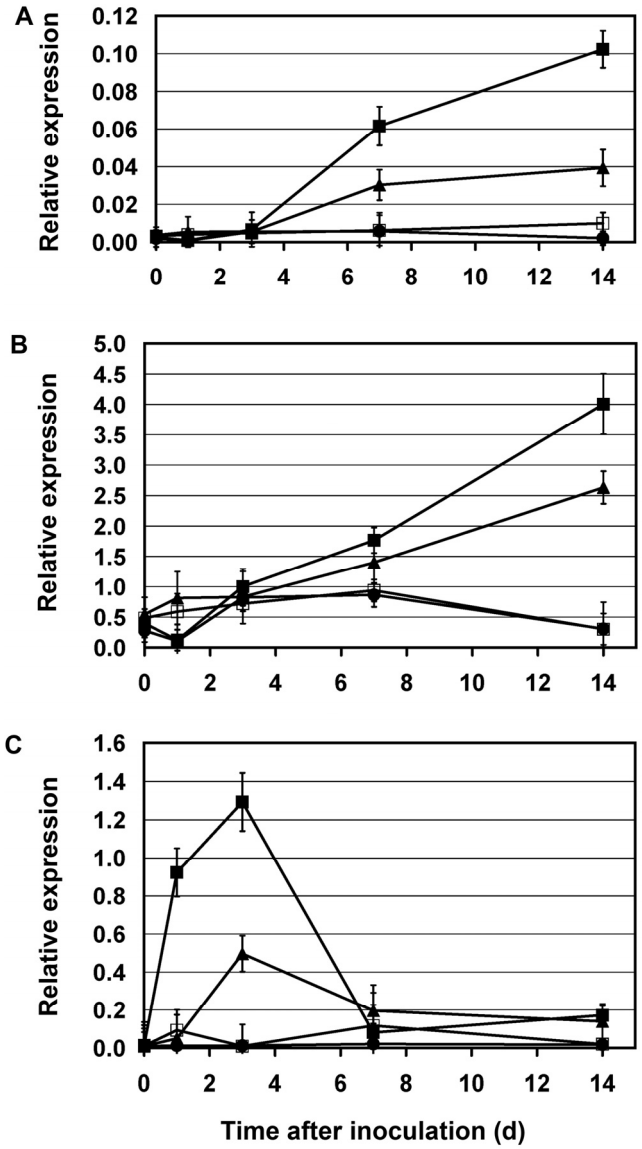

Fig. 4 Expression of PR genes after inoculation of barley roots with *Fusarium graminearum*
Levels of specific transcripts were determined relative to the expression of the barley *Ubiquitin* transcript using Q-RT-PCR. Each point on the curve (mean +/-SE) represents six individual pots, with three plants per pot from a biological experiment, assayed by Q-PCR in triplicate. Similar results were obtained in an independent experiment. The Y-axis represents relative amounts of PR gene expression. (A) *PR1b*; (B) *PR5*; (C) *β-1,3 glucanase* (*PR2*) Empty squares, (CC): Control plants; Filled circles, (CP): *P. indica* pre-inoculated plants; Filled squares, (CF): *F. graminearum*-inoculated plants; Filled triangles, (PF): *F. graminearum*-inoculated plants pre-inoculated with *P. indica*. The X-axis represents days (d) after *F. graminearum* inoculation.

Plant protection by *P. indica* has been shown for *Fusarium culmorum* (WALLER et al., 2005). In our current work, we have established that *P. indica* can also protect barley from root rot caused by *Fusarium graminearum*. Root rot was clearly visible in control plants (CF) by 5 days after *F. graminearum* infection, whereas *P. indica* pre-inoculated plants (PF) were almost indistinguishable from non-infected plants (CC&CP; Fig. 1A).

To quantify the amount of *F. graminearum* in barley roots, we determined the relative abundance of plant and fungal DNA in root tissues by performing real-time Q-PCR reactions targeted at specific fungal and plant genomic DNA sequences. Controls showed that the primer pairs used allow both sensitive and reliable DNA quantification over a wide dynamic range. As a

result, fungal abundance could be reliably quantified from a very early stage of infection until the final stage of fungal development.

For analyzing possible mechanisms of the observed protective effect of *P. indica*, we used plants with an established *P. indica* symbiotic interaction (12 days after inoculation) and inoculated these plants, in parallel with non-infested control plants, with *F. graminearum*. The *F. graminearum* DNA to plant DNA ratio increased 5-fold from 1 to 14 dai. This 5-fold increase is in contrast to the only 2.1-fold increase in roots pre-inoculated with *P. indica* and correlates well with weaker root rot symptoms in the latter interaction.

The ratio of *F. graminearum* to plant DNA in a root tissue reflects both fungal abundance and presence of intact plant cells. Therefore, this ratio is positively influenced by fungal growth as well as a higher degree of necrotized root tissue (lacking intact plant genomic DNA), giving an integrated and comprehensive view of the interaction. Our observed 5-fold increase in fungus to plant DNA ratio correlates to the microscopically observed extensive invasive growth of *F. graminearum* hyphae in inoculated roots. Penetrated host cells showed plasmolysis indicating cell death, which results ultimately in root rot symptoms. Q-PCR quantification can be used to assess plant resistance toward the fungus, which is an advantage compared with methods scoring disease symptoms. The latter methods are restricted to pathogen-host-interactions displaying macroscopically visible disease symptoms and do not provide information on the extent of pathogen colonization and proliferation. Several studies have shown that development of disease symptoms not always correlates with actual pathogen colonization (BENT et al., 1992; HOFFMAN et al., 1999; THOMMA et al., 1999).

To investigate protection of *P. indica*-infested roots against *F. graminearum*, we analyzed the expression of plant PR genes. We tested if the mutualistic fungus *P. indica* alone modulates plant defence genes. From day 6 after *P. indica* inoculation onwards, very low levels of *PR1b* were detected (Fig. 3). These results indicate that the endophyte suppresses the accumulation of defensive PR proteins. A transient induction of *PR1b* transcript levels was observed at 1, 2 and 4 dai. The intensity of this transient increase of a defence gene at an early stage of infection may vary depending on the density of inoculum and frequency of infection sites.

We have compared defence responses to a pathogen and a symbiont in the same plant genotype. Defence genes are indicators of induced resistance such as systemic acquired resistance. *P. indica*-infested plants (at 12 dai, see Fig. 3) did not show enhanced expression of three PR genes in the root, as compared with control plants. Infection with *F. graminearum* transiently induced expression of *ß-1,3-glucanase* from one day onwards while *PR1b* and *PR5*

expression remained increased until 14 dai. This induction was reduced by 30-60% in *F. graminearum* infected plants pre-inoculated with *P. indica* (Fig. 4).

Reduced *Fusarium*-induced PR gene expression in roots pre-inoculated with *P. indica* (Fig. 4) could be a result of lower amounts of the pathogenic fungus in these roots. It could therefore indicate the higher degree of resistance to *F. graminearum* in these plants. Alternatively, an active suppression of defence responses might be responsible for the diminished PR gene expression. Suppression of the defence response has been reported for interactions between plants and pathogenic fungi (BARZ et al., 1989; SHAUL et al., 2000) and was also observed for the AM – *Rhizoctonia solani* interaction (BONFANTE and PEROTTO, 1995; GUENOUNE et al., 2001). Whether such a suppressor is produced or induced by *P. indica* in the barley root remains to be shown.

Acknowledgements

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Genetic Transformation of the Plant-Growth-Promoting Root Endophyte *Piriformospora indica*

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Abstract:

Piriformospora indica is a recently discovered symbiotic basidiomycete inducing growth and pathogen resistance in infested plants. The fungus lives endophytically in root rhizodermis and cortex of a broad spectrum of host plants. Although *P. indica* is emerging as a model fungus for the study of mutualistic plant - fungus interactions, genetic transformation has not been accomplished yet. Here, we report transformation of the fungus by biolistic gene transfer and analysis of transgene expression in its saprophytic phase. The genes for hygromycin (*Hyg-B*) resistance and red fluorescent protein DsRed, both under the control of the constitutive *Aspergillus nidulans* glyceraldehydes 3-phosphate (gpd) promoter, were employed as markers. Patches of fluorescent hyphae and chlamydospores were observed upon fluorescence microscopic examination of hygromycin-resistant mycelia. The procedure described here lays the ground for the use of yet unavailable molecular genetic tools in analyzing a plant root endophytic fungus.

Piriformospora indica | Fungal transformation | biolistic gene transfer | *Hygromycin B* | *DsRed* | Symbiosis | mutualistic

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Introduction

Mutualistic symbiotic fungi play an indispensable role in upgrading growth, vigour, nutritional and hydratic status of plants (HARRISON, 2005). They also provide increased reproductive potential, improved root performance and stimulated defence against biotic and abiotic stresses (GIANINAZZI-PEARSON et al., 1996). In 1997, *Piriformospora indica* has been discovered in the Indian Thar-Desert (VERMA et al., 1998) and shown to exert many of the mutualistic capabilities of mycorrhiza fungi in a broad range of host plants (VARMA et al., 2001). Based on the nuclear genes coding for the large ribosomal subunit (nucLSU), the fungus' closest relatives are *Sebacina vermifera* species complex (Sebacinales group B) which form orchid mycorrhiza subsumed under the Sebacinales (WEISS et al., 2004; DESHMUKH et al., 2006). *P. indica* colonizes the roots of various plant species where it strongly promotes plant growth. Hosts include the important cereal crops rice, wheat and barley as well as many Dicotyledoneae including *Arabidopsis* (VARMA et al., 1999; PESKAN-BERGHOFERA et al., 2004). Upon root infestation with *P. indica*, barley is more tolerant to mild salt stress and more resistant to fungal diseases (WALLER et al., 2005). Thus, the fungus promises to serve as a model system to study the interaction of a symbiotic root endophyte with monocotyledonous plants. Transformation of axenically cultivatable *P. indica* may open up a new avenue of studying and utilizing the fungus in modern cropping systems. In contrast to arbuscular mycorrhiza fungi (AMF), the most extensively studied symbiotic fungi, *P. indica* can be easily cultivated in axenic culture where it asexually forms chlamydospores containing 8 to 25 nuclei (VERMA et al., 1998). Several methods, such as electroporation and polyethylene glycol (PEG)-mediated transformation of isolated protoplasts have been successfully employed for gene transfer into filamentous fungi, which were difficult to transform (for a review, see (CASAS-FLORES et al., 2004)). However, utilization of the PEG method to transform *P. indica* was not promising, because of the extremely low cell wall regeneration ability of protoplasts (our unpublished data). As a first step towards the genetic manipulation, we have developed a biolistic-mediated transformation system (HARRIER and MILLAM, 2001) by using DsRed as a marker of subcellular protein localization, a tracer of cell lineage and as label to follow development and colonization pattern of the fungus within the host plant. Here, we report the first molecular evidence for the successful genetic transformation and ectopic expression of marker genes in the saprophytic phase of the fungus.

Materials and methods

Plasmids, Growth conditions and Medium

For co-transformation of *P. indica* mycelium we used two plasmids: pAN7-1 (GenBank accession no. Z32698), bearing an *E. coli Hyg B* resistance gene flanked upstream by *Aspergillus nidulans* glyceraldehyde 3-phosphate dehydrogenase (gpd) promoter and downstream by the *A. nidulans* trpC transcription termination signal; and pPgpdA-DsRed containing the gene for DsRed-fluorescent reporter protein, an improved variant of DsRed from *Discosoma* sp. placed under the control of the constitutive *A. nidulans* glyceraldehyde 3-phosphate promoter (PgpdA) and the *A. nidulans* trpC transcriptional terminator (TtrpC) (MIKKELSEN et al., 2003).

The modified *Aspergillus* CM minimal medium containing ticcacilin 50 mg L^{-1} (PESKAN-BERGHOFERA et al., 2004) was used for cultivation of *P. indica* (strain WP3D). Circular mycelial disks (0.5 cm in diameter) were placed in the centre of petri dishes. Colonies were grown for 20 days on the CM medium until it reached 9 cm in diameter. Spores were isolated in sterile water containing 0.05% tween®-20 using a spreader and miracloth (Merck biosystems, UK). Two million chlamydospores were suspended in melted CM containing 0.75% agar. The spore suspension was placed in the centre of a petri plate containing 15 ml of CM medium. Spores were left for germination overnight at 24^{o}C in the dark. These plates were used for biolistic transformation.

Transformation

Biolistic PDS-1000/He Particle Delivery System (Bio-Rad, CA, USA) was used for the transformation. Gold particles (0.6 µM in diameter) coated with plasmid DNA were prepared according to the instrument's instruction manual (Bio-Rad). Overnight grown plates were placed in the Biolistic PDS-1000/He Particle Delivery System. The distance between the spores on the petri dish and gold particles on the macrocarrier was 5.0 to 14.5 cm. Bombardment was performed under a helium pressure of 650 to1300 psi (pounds per square inch). Bombardment using only gold particles without plasmid DNA was performed as a control experiment.

Selection and stabilization of co-transformants

After bombardment plates were incubated at 24^{o}C for up to 4 days. At the fifth day, plates were covered with 15 ml CM medium containing 100 µg ml^{-1} hygromycin and incubated further. Plates were periodically examined for colonies growing to the surface (putatively

hygromycin resistant). Hygromycin resistant and fluorescent colonies were transferred to fresh selective medium, containing 50 μg ml^{-1} hygromycin and incubated for one month. Co-transformants were sub-cultured further in medium containing 10 μg ml^{-1} hygromycin, and were finally transferred 37 days after the bombardment onto non-selective medium.

Confocal laser scanning microscopy

Colonies were periodically examined for DsRed fluorescence. Confocal fluorescence images were recorded on a multichannel TCS SP2 confocal system (Leica Microsystems, Bensheim, Germany). DsRed was excited with a 543 nm laser line and detected at 580–650 nm. Fungal auto-fluorescence was excited with a 488 nm laser line and detected at 550–700 nm followed by spectral unmixing using the Leica Confocal Software 2.5.1347a.

PCR and RT-PCR analysis

To verify whether the mosaic grown on non-selective media contained transformants, genomic DNA-PCR and RT-PCR were performed. Genomic DNA of wild type (wt) *P. indica* and transformants were prepared by using the DNeasy Plant mini Kit (QIAGEN GmbH, Germany) according to manufacturer's protocol. Isolated genomic DNAs were checked using standard agarose electrophoresis, and usually diluted 10-fold (10 ng) for use in PCR reactions. Two oligonucleotide primers of each of the coding regions of *DsRed, Hyg-B* and *translation elongation factor (Tef)* (GenBank accession no.AJ249911) were synthesized (Table 1). Genomic DNA isolated from a non-transformed *P. indica* strain was used as a control. PCR reactions were carried out using biotherm polymerase (Gene Craft GmbH Germany). Amplified DNAs were analyzed by EtBr staining after electrophoresis on 0.8% agarose gel electrophoresis.

Table 1 Primers used in PCR analysis

Name	Sequence 5'to 3'
DsRed	CCAAGCTGAAGGTGACCAAG
	TAGATGAAGGAGCCGTCCTG
Hygromycin	CGTGCTTTCAGCTTCGATGTAGG
	AAGATGTTGGCGACCTCGTATTG
Translation elongation factor	TCGTCGCTGTCAACAAGATG
	ACCGTCTTGGGGTTGTATCC
Ubiquitin	CAGTAGTGGCGGTCGAAGTG
	ACCCTCGCCGACTACAACAT

Similarly, RNA was extracted from same samples by using the RNeasy Plant mini Kit (QIAGEN GmbH, Germany) according to the instruction manual. Two μg of total RNA were reverse transcribed to first strand cDNA by using iScriptTM cDNA synthesis kit (bio-rad.com) according to the instruction manual. The final reaction was diluted 5 times and aliquots of 20 ng first strand cDNA were subsequently used as template for PCR with specific primers given above. The coding region for *P. indica Tef* was used as control for cDNA

quality. RNA without reverse transcription was used as a DNA contamination control. For primer sequences see Table 1.

Plant inoculation

Barley (*Hordeum vulgare* L.) cultivar Maresi (Lochow-Petkus GmbH Germany) was grown in a 2:1 mixture of expanded clay (Seramis®, Masterfoods) and Oil Dri® (Damolin) in a growth chamber at 22°C/18°C day/night cycle, 60 % relative humidity and a photoperiod of 16 h (240 µmol m^{-2} s^{-1} photon flux density), and fertilized weekly with 20 ml of a 0.1% Wuxal top N solution (Schering, N/P/K: 12/4/6). After kernels had been sterilized with 6% sodium hypochloride and germinated for 2 days, plantlets were inoculated with 0.5 x 10^6 ml^{-1} transformed chlamydospores before sowing. Transformed chlamydospores were isolated from plates in sterile water containing 0.05% tween-20, roots were dipped in spore solution for 2 hours and grown in expanded clay at above mentioned conditions. Roots were harvested into liquid nitrogen. Successful infection and presence of *P. indica* was measured by real-time PCR and microscopy.

For determination of amount of *P. indica* in barley roots qPCR-Ct curves were generated by subtracting the raw Ct values of the *P. indica* specific *tef* gene from the raw Ct values of plant specific *ubiquitin* gene for barley. The amount of *P indica* in barley roots is estimated by using $2^{-\Delta Ct}$ method where ΔCt is difference between raw Ct values of *tef* and *ubiquitin* gene. The levels of *P. indica tef* are relative to level of *ubiquitin*, which was set at 1 (LIVAK and SCHMITTGEN, 2001). The amount of *P. indica* DNA relative to plant DNA is used a mesure of colonization.

Results and discussion

Transformation

We performed micro-projectile-mediated biolistic co-transfer of equal amounts of plasmids pAN7-1 and pPgpd-DsRed into axenically grown mycelium of *Piriformospora indica* cultures. The plasmids contain the hph (*Hyg-B*) gene and the *DsRed-express* gene, respectively. In order to optimize transformation frequency, we varied target distance and pressure for micro-projectile transfer from 5.0 to 14.5 cm and 650 to 1300 psi, respectively. In our hands, optimal conditions for transformation

Table 2 Effects of target distance and helium pressure on the number of putative transformants.

Helium pressure (psi)	Target distance (cm)			
	5.0	8.0	11.0	14.5
650	0	0	0	0
1100	20	0	10	0
1300	50	30	0	0

were 0.6 µM gold micro-projectiles, 100 µg ml^{-1} plasmid DNA, 5.0 cm target distance, 1300 psi rupture disc, and 27 in. Hg chamber vacuum pressure. A distance of 5.0 cm and a pressure of 1300 psi obtained the maximum number of transformants (Table 2). These conditions accord well with those for *A. nidulans* transformation (FUNGARO et al., 1995) but differ from those used to transform the basidiomycete *Uromyces appendiculatus* (LI et al., 1993) were a higher pressure of 1550 psi and shorter distance of 2.5 cm were found to be more suitable.

Selection and stabilization of co-transformants

To find the optimal conditions for selection, *P. indica* was tested for axenic growth in the presence of various HygB concentrations. Mycelial growth was strongly reduced (by about 80%) at 10 µg ml^{-1} HygB and was completely inhibited at concentrations above 25 µg ml^{-1} HygB (data not shown). Therefore, we used 50 µg ml^{-1} HygB for the initial selection of HygB-resistant colonies in all subsequent transformation experiments. Four days after micro-projectile transfer, fungal colonies were covered with 15 ml CM medium containing 100 µg ml^{-1} HygB, giving a final concentration of 50 µg ml^{-1} HygB. Colonies growing towards the surface of the medium (putatively *hygromycin* resistant) were periodically examined for fluorescence. On agar plates with wild type fungus, no colony formation was observed under this condition. Emerging colonies that showed fluorescence were picked and transferred to fresh selective medium, containing 50 µg ml^{-1} HygB and incubated further for one month. Nearly 94% of these putative transformed colonies showed a reduced growth that was comparable to that of the wild type fungus. Several from the larger colonies were selected for microscopic and molecular analysis. This selective growth of colonies are interpreted as a transformation-dependent phenotype. The Hyg-B resistant colonies growing on selection medium ceased propagation upon prolonged cultivation (3–4 weeks). However these colonies were able to grow for short in several generations without forming chlamydospores when transferred to fresh selective medium (Fig. 1 A&B). This can be explained by the fact that a fraction of transformed nuclei of the multinucleated cells, albeit sufficient for mycelium survival, was not able to support the proliferation of antibiotic-resistant mycelia. Similar observations have been made with *A. nidulans* and other fungi (BARCELLOS et al., 1998) and references therein).

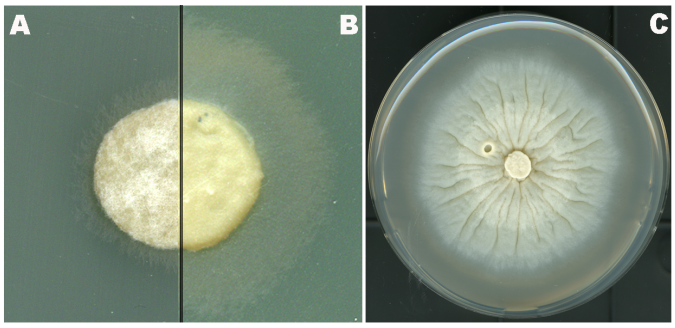

Fig. 1 Selection and stabilization of co-transformants
A; wild type *P. indica* colony on medium containing 50 µg ml^{-1} HygB. B; a putative transgenic *P. indica* colony on medium containing 50 µg ml^{-1} HygB; C; mosaic of transgenic and non transgenic *P. indica* on medium without HygB.

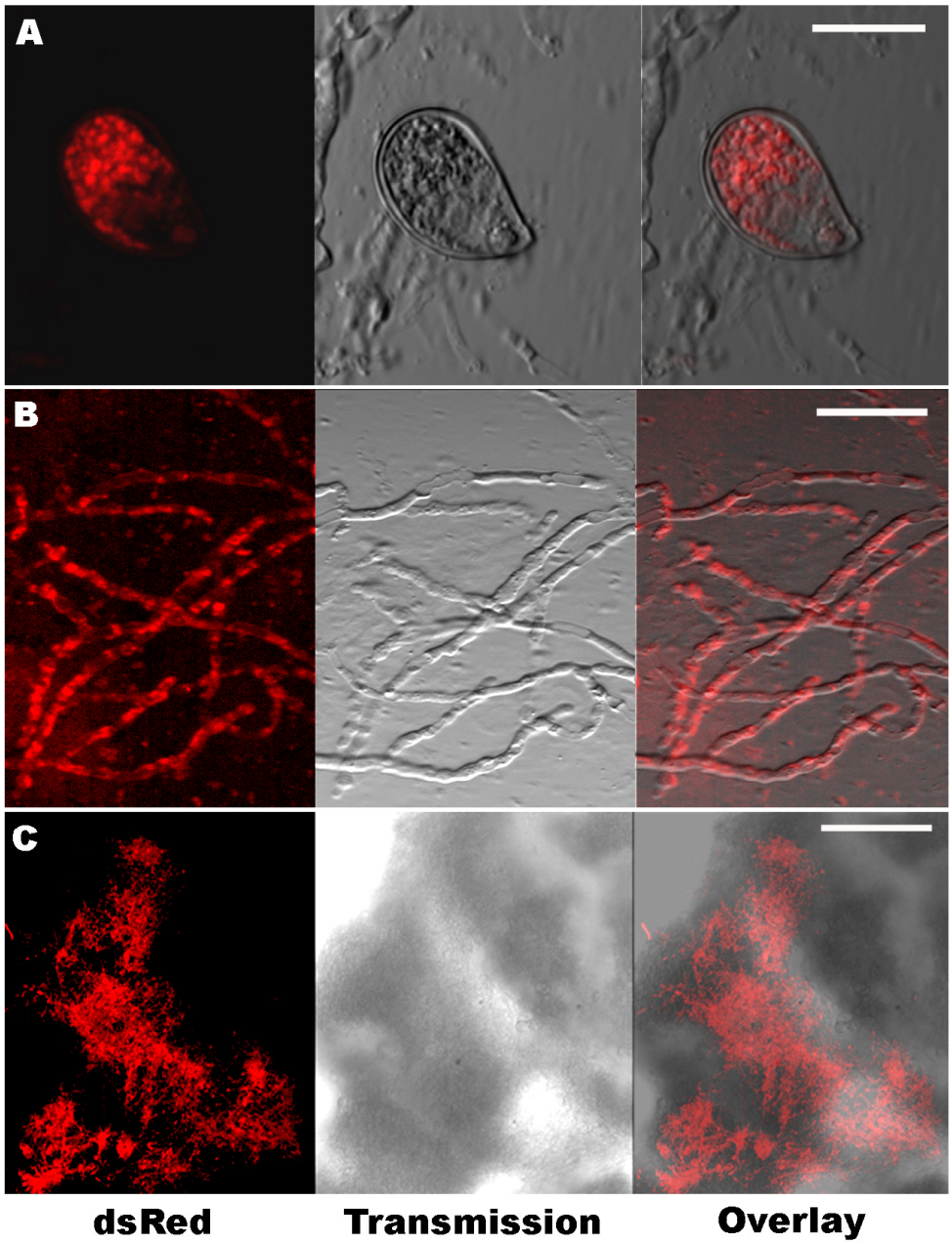

Fig. 2 Confocal microscopic visualization of DsRed expression in *P. indica* transformants
DsRed fluorescing chlamydospore of *P. indica* at 2 days after transformation, Bar = 16 µM; B, DsRed fluorescing hyphae of *P. indica* at 4 days after transformation, Bar = 16 µM; C, DsRed fluorescing colony (chlamydospores and hyphae) of *P. indica* at 30 days after transformation, Bar = 80 µM.

P. indica is made up by a meshwork of hyphae, tubular arrays of multinuclear incomplete septae that grow by tip extension with intervened unevenly multinucleate chlamydospores. Due to the lack of mononucleate cells (for instance, macroconidia as in *Neurospora crassa*), gene transfer to *P. indica* will give rise to a heterokaryon of transformed and untransformed nuclei, thus possibly making it more difficult to screen for transgene-associated phenotypes, such as the dominant selectable marker Hyg-B. To overcome this difficulty, we successively removed hygromycin selection pressure. On non-selective plates the fungus formed a mosaic of transgenic and non-transgenic hyphae as indicated by fluorescing and non-fluorescing areas of the colonies (Fig. 1 C).

DsRed expression and visualization

To estimate the extent of transformation, colonies on selective medium were periodically examined for dsRed fluorescence of peripheral hyphae from mycelia grown in the presence of Hyg-B. Figure 2 shows dsRed fluorescing hyphae and chlamydospores one month after bombardment. A diffuse background fluorescence was observed in parallel grown, mock-transformed mycelia (not shown). This contrasts with the strong and localized fluorescence observed in DsRed-expressing, transformed mycelia and chlamydospores (Fig. 2 A&B). Since fluorescing hyphae and chlamydospores are embedded in the nontransgenic mycelial mat (Fig 2 C), their relative abundance was difficult to quantify simply by visual inspection. The weak fluorescence observed at early times became progressively stronger upon prolonged co-cultivation. However, if one considers the high stability of DsRed and its variants (LORANG et al., 2001), this effect most likely reflects an enhanced intracellular accumulation of this protein rather than an increase in the percentage of transformed nuclei. DsRed fluorescence was mainly localized in the central part of the hyphae (see the image in the day 4 panel of Fig. 2 B.). To better visualize fluorescence distribution, transformed hyphae were subsequently analyzed with a confocal microscope. As shown in Fig. 2, DsRed fluorescence was found to be uniformly distributed within the internal part of the hyphal apex, with the exclusion of vacuoles.

PCR analysis of *P. indica* transformants

Presence of transgenic *Hyg-B* and *DsRed* in genomic DNA and their expression at transcript level was successfully confirmed by PCR analysis. Figure 3 shows in vertical pane 1; wild type *P. indica*, 2&3; transformants, 4; plasmid control. PCR on genomic DNA shows amplicon for *Hyg-B* and *DsRed*, respectively in horizontal pane A&B. Transcripts of same

genes are amplified on C-DNA (pane C: *Hyg-B* and pane D: *DsRed*). The quality of C-DNA was confirmed by *P indica* specific *tef* gene amplification (pane E). RNA used for reverse transcription was analysed by *tef* PCR for DNA contamination. (pane F). No detectable amplification product was obtained with specific oligonucleotide primers from reaction mixtures containing DNA from mock-treated *P. indica* mycelium (lane 1-A-D). Instead, an amplicon of the expected size identical to the product of the same reaction carried out on the plasmid control (lane 4 A-D) was obtained transformed *P. indica* mycelium (lane 2 and 3). The identity of the above PCR product as a *DsRed* and *hygromycin*-derived amplicon was confirmed by sequence analysis (data not shown). It is widely recognized that transformation efficiency decreases remarkably under non-selecting conditions (KAYA et al., 1990). In our PCR analysis we also found many PCR-negative colonies (one appeared in Fig. 4 for *Hyg-B*), especially when DNA from the mosaic stage level was used. In the case of individual *P indica* spores, however, this becomes more complex as the spores are multinucleate and contain many nuclei and only a small fraction of the nuclei present in the spore will be transformed. Expression of the resistance genes would be required to confer resistance to the whole spore and the resulting hyphae. One of the major disadvantages of the transformation of these fungal spores is their multinucleate status, and this will always lead to the formation of heterokaryons (HARRIER and MILLAM, 2001). It is not known how many nuclei have to be transformed before the functional expression of a reporter can be detected.

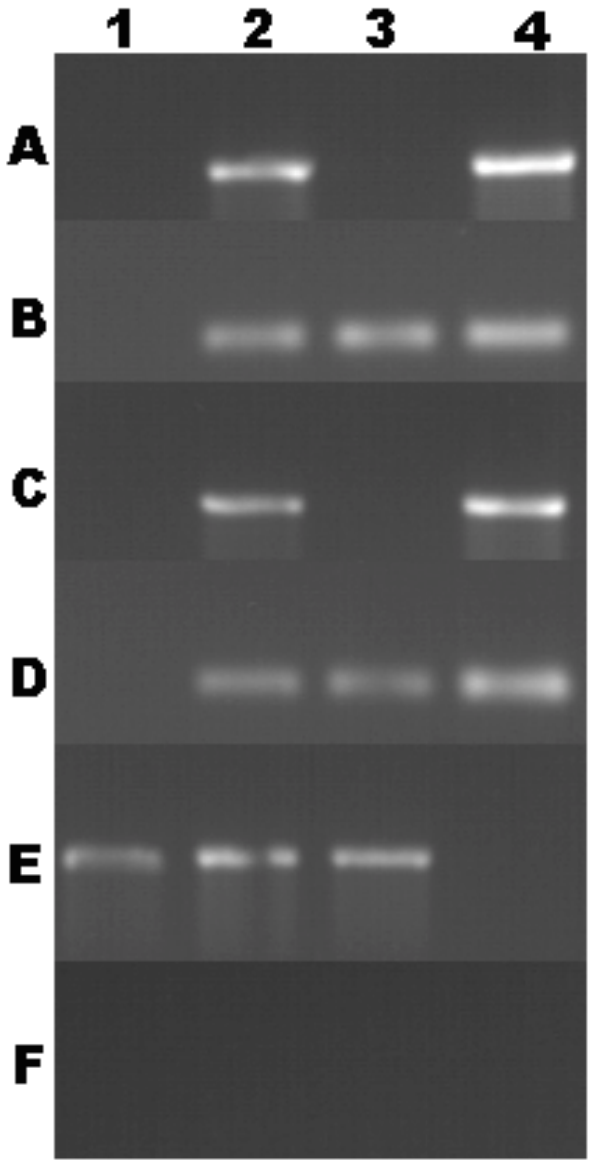

Fig. 3 PCR analysis of *P. indica* transformants
1; wild type *P. indica*, 2&3; transformants, 4; plasmid control; A&B, PCR on genomic DNA, Hyg-B and DsRed, respectively; C&D, PCR on cDNA, Hyg-B and DsRed, respectively, E; PCR on C- DNA, Tef, F; Tef PCR on RNA for DNA contamination control.

Furthermore, transformed nuclei will become diluted over subsequent generations. This was observed with *Gigaspora rosea*; with progressive cultivation, it became more difficult to detect the activity of the GUS gene that was used as a marker. Eventually, in the third generation of spores, it was impossible to detect GUS expression (HARRIER and MILLAM, 2001).

Transgenic *P. indica* displayed similar colonization of barley roots as the wild type strain

The capacity of transgenic fungi to colonize plant roots was investigated by inoculating roots of 4-day-old barley seedlings both with wild type and transgenic fungus. The amount of *P. indica* DNA relative to plant DNA is used as a measure of colonization. Colonized roots with wild type strains displayed a ratio for plant to fungus DNA of 2.8 while the respective value for the transformed strain was 2.7. We concluded that transgenic fungus colonizes barley roots with the same efficiency as the wild type strains. Consistently, microscopic analysis of root preparations showed no differences in colonization patterns. Moreover, the transformed fungus maintained its growth promoting activity.

Conclusions

Our work presents the first report on molecular modification of *P. indica* through biolistic transformation. We describe the transcriptionally active nature of the constitutive *A. nidulans* glyceraldehyde 3-phosphate promoter, and document the suitability of DsRed as a ‘‘reporter’’ gene in *P. indica*. The transformants could not be enriched through hygromycin selection, probably because of the inability to purify transformed homokaryons. The hygromycin-resistant, DsRed-expressing cells are thus likely produced by occurrence of low-frequency integration events, which in higher eukaryotes commonly account for 1% (or less) of the total transformants. Therefore, the enrichment and purification of transformed hyphal colonies from the mass of untransformed hyphae remains to be optimized. We have noticed that many of the transformed hyphae have produced spores, which may be also heterozygous, so that a possible way of isolating the transgenic portion of the mosaic could rely upon the enrichment of homozygous spores or part of the mycelium. However, this is not an easy task in *P. indica* because anastomosis has not been studied in detail and extreme fragility of the hyphae, which easily lose their entire cytoplasm when wounded. Unfortunately, for *P. indica* sexual spores or mononucleate cells have not been described. The procedures described here lay the ground for the use of previously unavailable molecular genetic tools such as RNA-mediated interference or antisense silencing of selected target genes or the overexpression of

homologous or heterologous genes for the study of the ecologically and agronomically important fungus *P. indica*.

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III Discussion

3.1 *Piriformospora indica* interferes with the host cell death program to form a mutualistic interaction with barley

Plants are often colonized by fungi that do not cause any disease symptoms. Many of these endophytic fungi exert beneficial effects on plant growth and development. Positive effects may come from providing essential nutrients as a result of their colonization (Harrison, 2005). The basidiomycete *Piriformospora indica* forms mutualistic symbioses with a broad spectrum of land plants. Its presence causes beneficial activities such as an increase in vegetative biomass and grain yield, local and systemic disease resistance, and tolerance to abiotic stresses (Waller et al., 2005). Based on nuclear genes coding for the large ribosomal subunit (nucLSU), the fungus' closest relatives within the order Sebacinales are *Sebacina vermifera* species (Sebacinales group B), which are classified as orchid mycorrhizae (Manuscript 1; Fig. 1 and (Weiss et al., 2004)). The understanding of the endophytic life strategy of *P. indica*, as starting point to elucidate the host benefits mediated by the fungus, is hampered by the lack of detailed cyto-histological studies, which could describe the qualitative and quantitative distribution of *P. indica* within the host cell. To gain a better understanding of the cellular events leading to the establishment of the mutualistic symbiosis, penetration and colonization of the fungus were analyzed by epifluorescence microscopy.

3.1.1 Endophytic development in barley roots

Fungal infestation of barley roots initiates from chlamydospores, which upon germination form hyphae. The newly formed hyphae creep on the root surface and some of the branches of the hyphae penetrated into the intercellular spaces. In maturation zone of roots some hyphae penetrate and pass through rhizodermal cells to subepidermal spaces by developing infection pegs. The melanized appressoria associated with classical foliar pathogen infection (Chumley and Valent, 1990) were not observed for *P. indica* hyphae on the surface of barley roots. Following penetration, intra- and intercellular growth of the fungus was visible in rhizodermal and cortical cell layers, similar to *Magnaporthe grisea* root infection (Sesma and Osbourn, 2004) or *Fusarium graminearum* epicarp infection (Jansen et al., 2005). The intracellular hyphae were thick with constrictions in places where they crossed the plant cell wall (Manuscript 1, Fig. 2h). In young differentiated tissue, single penetrated cells were completely filled with fungal hyphae (Manuscript 1, Fig. 2f and g). Such cells may provide resources for further invasive fungal growth. In mature parts of roots an unrestricted net-like intra- and intercellular colonization pattern was observed. Occasionally, subepidermal hyphae

penetrate the space between the cell wall and plasma membrane of rhizodermal or cortical cells. After branching, these hyphae enwrapped protoplasts, which showed cytoplasmic shrinkage (Manuscript 1, Fig. 3a). At later colonization stages, fungal hyphae excessively occupied rhizodermal and cortical cells. In some cases, transverse cell walls of adjoining cortical cells were absent while protoplasts were covered by a dense meshwork of fungal hyphae. Eventually, arrays of single spores developed from intracellular hyphal tips (Manuscript 1, Fig. 3b and c). The fungus also penetrated basal parts of root hair cells, in which branching hyphae formed large numbers of chlamydospores starting from the base of the root hair cell until a stack of spores fills the entire cell. In addition to this intracellular sporulation, chlamydospores were also generated in the mycelial mats developed at the root surface. In the root's elongation zone, the fungus usually infested intercellular spaces and formed a hyphal mat below the rhizodermis. No sporulation was observed in this tissue. Moreover, hyphae were always absent from intercellular spaces of meristematic cells even though hyphae were found in the intercellular spaces of cells differentiating into cortical and epidermal tissue. Intensive branched hyphae were especially observed in the areas of lateral root junctions. Obviously, the fungus branched and spread through ruptures produced in the cortex by emerging lateral roots. Since the endodermis of young radicle apparently acts as a barrier against penetration, the lateral root development was not affected by *P. indica*. Furthermore, newly developing adventitious roots were always free of *P. indica*. This suggests that the plant has evolved strategies to exclude the fungus from tissues, which is critical for root development. However, infections of stele and meristem can occur via wounds at lateral and adventitious root junctions suggesting that root health and the rate of suberization may serve as a key to the observed tissue specificity of *P. indica*. Above observations represent the phenotypic plasticity of *P. indica* in which infection patterns are altered by age and physiological status of root tissue. Similar plasticity was observed with other asymptomatic root endophytes like Dark Septate Endophyte(DSE), *Fusarium* sp. (Schulz and Boyle, 2005), which makes difficult to draw a definite picture on the symbiotic root interaction.

3.1.2 *P. indica* proliferates in dead cells

A clear spatial association of dead root tissue with strong mycelial growth was apparent as characterized by the absence of intact plant nuclei, which were detectable in adjacent, non-infected tissue (Manuscript 1, Fig. 3d–g). This close association of host cell death with massive fungal growth suggests that the fungus contributes to host cell death. Although *P.*

indica can induce cell death in poplar under specific conditions on artificial medium (Kaldorf et al., 2005) at particular interaction sites, cytological evidence was obtained that the fungus can attack and enwrap living (DAPI-positive) protoplasts (Manuscript 1, Fig. 3a). Since *P. indica* can grow intercellularly and penetrate into living cells, the close association of the fungus with living tissue might contribute to host reprogramming and finally, cell death. The inter- and intracellular growth pattern indicated that the fungus is also able to digest plant cell walls. Additionally, the elimination of transverse cell walls of adjoining cortex cells colonized by the fungus and/or filled with spores was observed.

Regarding the current study, it is difficult to categorize this mutualistic interaction as biotrophic, necrotrophic or hemibiotrophic. The infection of barley root tissue by *P. indica* displays great variance that is obviously related to the tissue age. Mature parts of roots show more invasive and intracellular growth while younger root parts show mainly intercellular growth. However, the clear and extraordinary association of strong mycelial growth with dead root tissue was mainly found in mature root parts. This compartmentalization of invasive and intercellular growth and living and dead tissue may demonstrate the ability of *P. indica* to grow as a hemibiotropic fungus. Therein, *P. indica* intercellularly infects living tissue as a biotroph but after latency period can cause host tissue to die. After cell death, the fungus might switch to a saprophytic life strategy and starts to sporulate. In other cases, *P. indica* might come as a saprotroph or necrotroph or biotroph depending on host and fungus genetics and/or environmental factors. Consistently while interaction with orchids, *P. indica* has been shown to be associated with living host cells (Varma et al., 2001).

For nutrient absorption, biotrophic fungi have usually developed sophisticated feeding structures, for example haustoria of powdery mildew fungi (e.g. *Blumeria graminis* f. Sp. *hordei*). AMF forms dichotomously branched hyphae, so called arbuscules, within cortical cells. Arbuscules are terminally differentiated organs, which are developed from side branches of long intercellular hyphae. These elaborated structures form inside the plant cell but remain separated from the plant cell cytoplasm by an extension of the plant plasma membrane that surrounds the fungus and follows the contours of the hyphal branches. This arbuscular interface is essential for the symbiotic delivery of phosphate to the plant (Harrison, 2005). *P. indica* remains associated with barley roots over the extended time period. However, the cytological analyses have not shown any obvious arbuscule-like morphologically specialized hyphal structures in cortical cells colonized by *P. indica.* Instead, many intercellular hyphae were found. In most strains of *Fusarium verticilliodes* and other endophytic fungi, the connection of such hyphal cell walls with plant cell walls may serve as

place for nutrient exchange (Honegger, 1986). Nutrient exchange between *P. indica* and its plant host remained to be shown.

3.1.3 Genetic determinants of cell death and *P. indica* proliferation in barley root

It is self-evident that the host cell death stops the development of biotrophic pathogens, whereas it is not unequivocally proven that cell death is supporting fungi committing a necrotrophic life style. Therefore, manipulation of plant cell death regulation could be a tool for understanding the role of host cell death in endophytic life strategies. Cell death is associated with plant development but also linked to pathogen defense in plants and animals. Execution of apoptosis as one type of programmed cell death in animals is irreversibly triggered by cytochrome c release from mitochondria via pores. This process is regulated for example by members of the Bcl-2 protein family such as pore-forming BAX or Bcl-2. This type of programmed cell death can be prevented by expression of BAX Inhibitor-1 (BI-1), a transmembrane domain containing protein that protects cells from the effects of BAX by an unknown mechanism. BAX, Bcl-2, and other related proteins are not present in plants. However, mammalian antagonists of BAX (Xu and Reed, 1998) like BI-1 and functional plant homologues of BI-1 were identified recently in Arabidopsis, rice and barley (Hückelhoven et al., 2001; Kawai-Yamada et al., 2001; Hückelhoven et al., 2003; Kawai-Yamada et al., 2004; Kawai-Yamada et al., 2006). BI-1 that can interact with Bcl-2 but not with BAX is localized at the endoplasmic reticulum and the nuclear envelope (Xu and Reed, 1998; Kawai-Yamada et al., 2001). Previous work showed that HvBI-1 has a central role in the outcome of host–pathogen interactions (Hückelhoven, 2004; Watanabe and Lam, 2006). In barley leaves, HvBI-1 is strongly expressed in incompatible interactions with the obligate biotrophic leaf pathogen *Blumeria graminis* sp. *hordei* (*Bgh*) (Hückelhoven et al., 2003) expression of BI-1 is rapidly up-regulated in plants during wounding or pathogen challenge (Hückelhoven, 2004) suggesting a role in response to abiotic and biotic stress related PCD. To obtain molecular evidence for a requirement of host cell death regulation, the role of the cell death regulator HvBI-1 was analyzed in the barley-*P. indica* association. Although levels of *HvBI-1* mRNA slowly increased during barley root development, *P. indica*-colonized roots showed a significant reduction of *HvBI-1* mRNA levels compared with non-colonized roots from 7 dai onward (Manuscript 1, Fig. 4a). The fact that *P. indica* attenuates the expression of *HvBI-1* suggests a lowered threshold for PCD initiation in endophyte-colonized roots and support the idea that *P. indica* influences intrinsic plant PCD. However, it remains to be seen how the

host constrains the risk of unhampered cell death execution after sacrificing some of its cells in order to limit fungal spreading.

To functionally confirm the role of host PCD, barley lines constitutively overexpressing a GFP–HvBI-1 fusion protein were examined. All transgenic lines showed enhanced cell survival after transient expression of mouse BAX in epidermal leaf cells (R. Eichmann, unpublished results). Comparison of the transgenic plants with the respective wild type showed a significant reduction of invasive growth of *P. indica* in barley overexpressing GFP–HvBI-1 at 20 dai, when fungal proliferation has reached a steady state level. In contrast, transient overexpression of HvBI-1 in the barley leaf epidermis supported early invasion of biotrophic *B. graminis* f. sp. *hordei* into resistant barley (Hückelhoven et al., 2003; Eichmann et al., 2004). Additionally, all GFP–HvBI-1 lines that restricted proliferation of *P. indica* showed enhanced susceptibility to a virulent isolate of *B. Graminis* f. sp. *hordei*. This effect relied on a lower ability of the plant to stop the fungus by hypersensitive cell death, which is involved in basal and R-gene mediated disease resistance in barley (V. Babaeizad, R. Eichmann, and J.I., unpublished results). Hence, the expression level of HvBI-1 might inhibit or support fungal proliferation depending on the microbial lifestyle. Quantification of *P. indica* by Q-PCR confirmed that fungal growth was significantly restricted in GFP–HvBI-1 barley (Manuscript 1, Fig. 4b) at 20 days after infection. Detailed cytological analysis and quantification of *P. indica* colonization of GFP–HvBI-1 barley at early infection stages has remained to be investigated. These studies may answer critical questions on microbial lifestyle at early stage of colonization.

Several studies in other pathosystem revealed that leaf cell death - and defense control can be linked. For instance, lesion mimic mutants such as *Arabidopsis lsd1* exhibit both spontaneous cell death and broad-spectrum resistance (Dietrich et al., 1994). Barley lines carrying recessive mutant *mlo* alleles at the *Mlo* locus, similar to *lsd1*, show spontaneous leaf cell death and non-race specific resistance to *Bgh* (Jørgensen, 1992; Schulze-Lefert and Panstruga, 2003). Thus, it is postulated that the barley MLO protein is a negative control element of cell death and of defense responses. Cell-survival mechanisms mediated by MLO probably negates plant defenses against *Bgh*, thereby allowing infection by the biotrophic fungus. Mlo mediated spontenous cell death is remained to be demonstrated in barley roots. However, *mlo* genotypes are highly susceptible to the hemibiotrophic pathogen *Magnaporthe grisea* and to necrosis-inducing culture filtrate from *Bipolaris sorokiniana* (Jarosch et al., 1999; Kumar et al., 2001). Therefore, the determination of *P. indica* proliferation in *mlo* mutant could give insights into mechanisms and the type of PCD, which are required for fungal propagation.

The effect of *mlo* mutation on proliferation of *P. indica* in the background of Pallas (*Mlo*) and Ingrid (*Mlo*) was determined by using the respective mutants P22 (*mlo*) and I22 (*mlo*). Two biological experiments of Pallas and one of Ingrid were analyzed at 20 days after *P. indica* inoculation. Microscopic analyses did not show any remarkable difference in fungal colonization. However, quantification of *P. indica* based on Q-PCR showed enhanced proliferation of *P. indica* in P22 barley roots in comparison to Pallas (0.1560±0.01 Vs 0.200±0.008), whereas I22 and Ingrid failed to show any difference in *P. indica* proliferation. This is consistent with the notion that spontaneous cell death phenotype of *mlo-5* mutation is stronger in the genetic background Pallas than in Ingrid (Peterhänsel et al., 1997). These results further indicate the role of cell death for *P. indica* proliferation in barley roots. The phenotypic plasticity of variable growth and colonization patterns of endophytes in host plants depend on host defense response, physiological status, environmental conditions and host genetic backround (Schulz and Boyle, 2005). The variable effects of the *mlo* mutation on *P. indica* proliferation in Pallas and Ingrid may represent a similar plasticity of the endophyte with its host genetic background. There in spontenous host cell death does not reflect in pronounced fungal proliferation may be due to genetic background and other physiological factors of Ingrid. Other studies with asymptomatic endophytes argue in favor of this hypothesis (Schulz and Boyle, 2005). Thus, various genetic or physiological conditions could drastically alter the behavior of *P. indica*. Similarly, *P. indica* has been shown to exert necrotrophic lifestyle on populus *Esch5* mutant plants cultivated on specific medium (Kaldorf et al., 2005), while on the other hand the interaction of *P. indica* with protocorms has shown typical pelotons in a living host cell similar to orchid mycorrhiza (Varma et al., 2001).

BI-1 overexpressing and knock-down plants do not exhibit a particular phenotype under common growth conditions (Ihara-Ohori et al., 2006; Watanabe and Lam, 2006). This suggests that BI-1 is not essential for plant development and growth. In contrast, mutations of *Mlo* lead to impulsive cell death in leaves in addition to dramatic increases in the spontaneous formation of cell wall appositions and early senescence, even in plants grown under sterile conditions. This indicates a role of *Mlo* in controlling plant defense and development related PCD (Jørgensen, 1992; Wolter et al., 1993). Overexpression of HvBI-1 in barley leads to dramatic reduction in *P. indica* proliferation whereas mutations of *Mlo* leads to marginal or no changes. These observations indicate that PCD observed in the interaction with *P. indica* is different from the Mlo mediated cell death in pathogen defense and development and underlines the fact that BI-1 possesses evolutionarily conserved functions that allow it to provide tolerance against specific cell death. It remains to be shown what kind of PCD might

be regulated by *P. indica*. Taken together, current genetic evidence demonstrates that *P. indica* requires host cell death for successful proliferation, which involves a sophisticated regulation of the plant's cell death machinery. The co-localization of root cell death with massive infestation by *P. indica* might reflect the fungus' success to manipulate host cell PCD. Thereby, *P. indica* might take advantage of root cell death programs in mature parts of the root.

3.2. Bioprotection provided by *Piriformospora indica* against barley root rot caused by *Fusarium graminearum*

3.2.1 *Fusarium* root rot symptoms are delayed in *P. indica*-colonized barley roots

There are varieties of fungal species including fungal endophytes, which demonstrate plant disease control properties in different rhizospheres (Whipps and Lumsden, 2001). As demonstrated by the present work, *P. indica* provides protection against *F. graminearum* root rot in barley. Root and crown rot symptoms were strikingly visible after *F. graminearum* inoculation of barley roots (CF), in contrast plants inoculated with *P. indica* (PF), in which *P. indica* mutualism was established before *Fusarium* inoculation showed no or mild symptoms of *Fusarium* root rot. Control plants (CC) devoid of *P. indica* and *F. graminearum* remained healthy during the course of the experiment. All plants colonized by *P. indica* (CP) were more vital than control plants.

There are four major mechanisms of biological plant disease protection 1) competition for occupying niches or nutrients, 2) production of antibiotics, 3) induced resistance and 4) mycoparasitism (Howell, 1991; Postma and Rattink, 1991; Howell et al., 1993) (Eparvier and Alabouvette, 1994; Herr, 1995). *P. indica* or *F. graminearum* do not exhibit direct antagonistic effects on each other, when confronted under axenic culture conditions. These results demonstrated that production of antimicrobials or mycoparasitism mechanisms are not involved in the bioprotective action provided by *P. indica*.

To date, there is no literature available describing infection pattern of *Fusarium* sp. to roots of barley plants. But many researches have studied early and late infection stages of root using GFP-expressing transgenic isolates of *Fusarium* in maize and tomato (Oren et al., 2003; Bolwerk et al., 2005). The main sites of plant penetration were the lateral roots and root hairs, where the fungus directly penetrates epidermal cells. (Murillo et al., 1999) found that fungal growth at this stage was mainly intercellular. These findings demonstrate the common life history strategy of *Fusarium* in plant roots, in which the pathogens first attach to the lateral

roots and root hairs and then penetrate directly through the epidermis of these tissues. After penetration, the fungus propagates in the intercellular spaces (Yates et al., 1997; Murillo et al., 1999; Lagopodi et al., 2002), where it continues to grow along the junctions of the plant epidermal cells covering large areas of the roots. Necroses start to appear several days after this event and are associated with a dense hyphal network that covered the necrotic tissues (Lagopodi et al., 2002). This implies two separated stages of fungal life in plant roots. While at initial stages the infection remains symptomless despite intercellular colonization, the second phase is associated with massive growth and severe necroses (Oren et al., 2003). In the more virulent pathogen *F. oxysporum*, the whole process proceeds much faster, and the transition from the initial symptomless phase to the necrotrophic phase occurs within a few days. Accordingly, the less aggressive pathogen *F. verticillioides* develops slower, leaving the plant more time to respond and restrict fungal growth (Bacon and Yates, 2005). On the other hand, in the present studies (manuscript 1) it was shown, that *P. indica* colonization increases with root tissue maturation and that the elongation zone showed mainly intercellular colonization. In contrast, the differentiation zone was heavily infested by inter- and intracellular hyphae and intracellular chlamydospores. The majority of hyphae were observed to be present at intercellular spaces and in dead rhizodermal and cortical cells that became finally completely filled with chlamydospores. The colonization strategy of *P. indica* in barley roots follows an asymptomatic mutualistic pattern throughout the plant life. Comparison of these two life history strategies denote that the first stage of *Fusarium* infection shares similar niches by occupying intercellular spaces. Therefore it is tempting to speculate that the competition for niches is one of the mechanisms of bioprotection provided by *P. indica*.

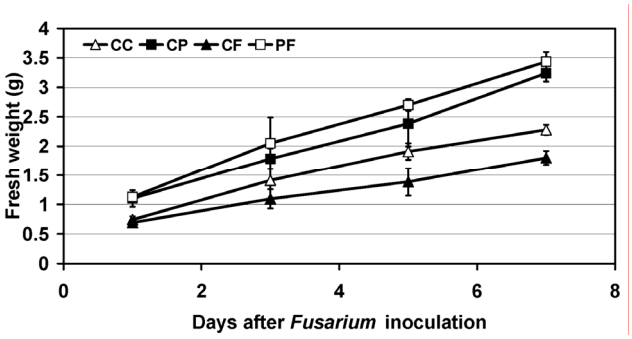

Fig I: Impact of *P. indica* on fresh weight and *Fusarium* root rot tolerance
Fresh weight of control (CC), colonized by *P. indica* (CP), colonized by *F. graminearum* (CF) and colonized by *P. indica* and *F. graminearum* (PF) plants.

The studies of Lagopodi et al. (2002) and Oren et al. (2003) on maize roots and by using GFP-expressing transgenic *Fusarium* demonstrated that systemic spread of *Fusarium* from root to mesocotyl tissue occurs during asymptomatic early stages of infection, resulting in crown rot during necrotic stage. In the present work, a similar infection pattern was observed in *Fusarium* inoculated control plants (CF). In contrast, *P. indica* pre-inoculated and *Fusarium* inoculated (PF) plants do not show symptoms of crown rot hinting towards

inhibition of systemic spread of *Fusarium* during early stage of *Fusarium* infection. Asymptomatic early spread of *Fusarium* represents a delicate balance between the fungus and the plant. Under conditions that favor symptomless infection, fungal growth may be restricted to specific tissues, where *Fusarium* penetrates only specific cells and in which it reproduces without damaging the surrounding cells (Oren et al., 2003). Under conditions that favor pathogenic development, more mycelium develops and the fungus switches to a more aggressive phase that probably involves secretion of hydrolytic enzymes and toxins (Nelson et al., 1993; Desjardins et al., 1995; Jardine and Leslie, 1999; Ruiz-Roldan et al., 1999; Daroda et al., 2001; Garcia-Maceira et al., 2001; Seo et al., 2001). The magnitude and temporal appearance of symptoms may vary from symptomless during the entire life cycle of the plant to severe rotting of the entire plant, depending on various physiological and environmental conditions. In many cases, diseased and asymptomatic plants occur in the same field planted with a genetically uniform host, similar environmental conditions and water availability (Dodd, 1980; Magan and Lacey, 1984; Drepper and Renfro, 1990; Magan et al., 1997; Nagy et al., 1997). Consistently, enhanced growth of *Fusarium*-infected plants has been reported previously and was attributed to the endophytic nature of the fungus (Leslie et al., 1990) (Yates et al., 1997). Amir and his group (Oren et al., 2003) reported that the same strain may cause growth enhancement and growth retardation, depending on the level of plant colonization, which is influenced by the amount of fungal inoculums present in the soil and at seeds. In experiments with *P. indica* and *Fusarium*, the biomass (fresh weight) of *Fusarium* inoculated and control plants were measured. Interestingly, no reduction in growth promotion was found in plants, which were colonized by both *P indica* and *F. graminearium* (PF), in comparison to other treatments (CC, CF). Growth promotional effects in PF-treated plants were similar to that of CP-treated plants (Fig I). These results might indicate that *F. graminearium* transition from endophytic to necrotic life style is delayed or disturbed in plants, which are pre-inoculated with *P. indica*.

3.2.2 Quantification of fungal DNA in barley roots by real-time quantitative PCR

In this study, a real-time PCR-based assay was developed to follow fungal progression on barley plants inoculated with the fungi *P. indica* and or *F. graminearium*. It is based on the relative quantification of plant and fungal DNA in infected tissues by performing two real-time PCR reactions targeted at fungal and plant sequences on inoculated samples. Control samples showed that the primer pairs used allow reliable DNA quantification over a very wide dynamic range, providing an excellent sensitivity to this technique. The current assay

allows to follow fungal development in plant root tissue during the whole time-course of infection. If lower inoculum levels were used, the assay sensitivity can still be easily improved by targeting repeated genomic sequences (e.g. ITS (Atkins et al., 2003)(Cullen et al., 2001) rather than the single copy gene *Tef* or *GFP*. The wide dynamic range of this Q-PCR-based method permits to score the outcome of the resistance interactions provided by *P indica* to barley roots against *F. graminearium*. In conclusion, this assay fulfils all requirements to discriminate slightly different levels of plant resistance in a statistically significant way.

F. graminearum quantification by Q-PCR revealed a correlation between reduced root rot symptoms and the amount of fungal DNA. Pathogen development in CF plants was rapid, as indicated by a five fold increase at 14 dai (Manuscript 2, Fig. 1B), and associated with extensive symptom development (Manuscript 2, Fig. 1A). At the end of the time course (14 dai), *F. graminearum* DNA became predominant in the CF samples, which could be ascribed both to fungal propagation and massive plant cell collapse. In contrast, development of *F. graminearum* in PF plants was gradually elevated and associated with mild symptoms. At 14 dai, these plants had a less than 2-fold increase of *F. graminearum* DNA as compared with day 1 (Manuscript 2, Fig. 1B). The ratio of *F. graminearum* to plant DNA in co-inoculated root tissue reflects both fungal abundance and presence of intact plant cells. Therefore, this ratio is positively influenced by fungal growth as well as a higher degree of necrotized root tissue (lacking intact plant genomic DNA), giving an integrated and comprehensive view of the interaction. The observed 5-fold increase of fungal to plant DNA correlates to the microscopically observed extensive invasive growth of *F. graminearum* hyphae in inoculated roots. Penetrated host cells showed plasmolysis indicating cell death, which results ultimately in root rot symptoms. Therefore, Q-PCR quantification can be used to assess plant resistance toward the fungus, which is advantageous compared with methods scoring disease symptoms. The latter methods bear certain disadvantages, for example, they are restricted to pathogen-host interactions displaying macroscopically visible disease symptoms and do not provide information on the extent of pathogen colonization and proliferation. Moreover, several studies have shown that development of disease symptoms do not always correlate with the degree of pathogen colonization (Bent et al., 1992; Hoffman et al., 1999; Thomma et al., 1999). In our case, normalization with plant DNA abundance leads to a simultaneous assessment of fungal growth and the respective plant response. Fungal biomass and fungal DNA/plant DNA ratio vary proportionally if plant DNA degradation or induction is negligible. However, this ratio is representative for the behavior of the two partners and is very accurate

to get an integrated view of the outcome of the interaction, and to assess plant resistance or susceptibility toward the fungus.

3.2.3 Influence of *P. indica* infestation on *PR* gene expression in barley roots challenged with *F. graminearum*

Many studies focused on the PR gene expression in plants associated with symbiotic fungi. In general, plant defense-related genes are only weakly or transiently expressed in response to infection by symbiotic fungi (for reviews, see (Bonfante and Perotto, 1995)). So far, defense gene expression in plants after *P. indica* inoculation was not analyzed. Therefore, it was tested if the mutualistic fungus *P. indica* by itself modulates plant defense genes. Very low levels of *PR1b* were detected, starting at 6 days after *P. indica* inoculation (Manuscript 2, Fig. 3). These results indicate that the endophyte actively suppresses the accumulation of PR1b and probably of other defensive proteins. A transient induction of *PR1b* transcript levels was observed at 1, 2 and 4 dai. Intensity of this transient increase of a defense gene at an early stage of infection may vary depending on the density of inoculum and frequency of infection sites. However, even the observed transient induction of PR1b is far lower compared to transcript levels observed during pathogenic interactions.

The activation of defense responses, such as deposition of lignins (Yates et al., 1997) and accumulation of PR proteins (Murillo et al., 1999), by *Fusarium* has been reported (Oren et al., 2003). My results show that, during symbiotic fungal colonization of barley roots, the plant-defense response is suppressed by the *P. indica* and in some amount can even overcome its elicitation by *F. graminearium* (Manuscript2, Fig. 4). Findings from previous studies suggested that AM fungi do not induce a strong plant defense response; however, the magnitudes of the molecular changes associated with AM colonization and the response elicited by pathogenic fungi in the same experimental system were not reported. To understand the protective action of *P. indica* in roots against *F. graminearum*, the expression of plant PR genes was analyzed. Therefore, the defense responses in roots to a pathogen and a symbiont were compared in the same plant genotype. *P. indica*-infested plants (at 12 dai, see Fig. 3) did not show enhanced expression of three PR genes in the root, as compared with control plants. In opposite, the infection of roots with *F. graminearum* induced the expression of *ß-1,3-glucanase* immediately while *PR1b* and *PR5* expression was increased at 7 dai. This induction was reduced by 30-60% in *F. graminearum*-infected plants pre-inoculated with *P. indica* (Manuscript 2, Fig. 4).

Reduced *Fusarium*-induced PR gene expression in roots pre-inoculated with *P. indica* (Manuscript 2, Fig. 4) could be a result of lower amounts of the pathogenic fungus in these

roots. This could indicate the higher degree of resistance to *F. graminearum* in these plants. Alternatively, an active suppression of defense responses might be responsible for the diminished PR gene expression. Suppression of the defense response has been reported for interactions between plants and pathogenic fungi (Barz et al., 1989; Shaul et al., 2000) and was also observed for the AM–*Rhizoctonia solani* interaction (Bonfante and Perotto, 1995). The nature of this suppression and whether such a suppressor is produced or induced by *P. indica* in the barley root remains to be shown.

3.3 Genetic Transformation of *Piriformospora indica*

3.3.1 Transformation of *P. indica*

Obligate biotrophic AMF are recalcitrant to culture axenically. Moreover, a genetic transformation would always require the presence of their host. Hence, any genetic approaches to decipher fungal genes involved in symbiosis are lacking. In contrast, *P. indica* has the advantage to be easily cultivated in axenic culture. The transformation of *P. indica* may open up a new avenue of studying the fungal genes pivotal for the mutualistic interaction and help to understand the genome organization of this asexual endophyte. One of the topics of present work was to genetically modify *P. indica* via biolistic transformation. For this reason the transcriptionally active nature of the constitutive *A. nidulans* glyceraldehyde 3-phosphate promoter was chosen in combination with *DsRed* to generate a reporter gene construct to stably transform *P. indica*.

P. indica develops a meshwork of hyphae consisting of multinuclear incompletely septated cells that grow by tip extension with intervened unevenly multinucleate terminal chlamydospores. While isolating chlamydospores by mechanical shearing of the mycelial mat bacteria were released into the spore suspension. Isolation and cultivation of these bacteria were performed to obtain a pure bacterial culture, which was further investigated by collaborator regarding colony character, biochemical properties and sequencing the ITS (Fig II). The association and function of bacteria within fungi has been observed in several cases (Partida-Martinez and Hertweck, 2005). Many studies have shown the occurrence of bacteria in association with mycorrhizae, e.g. in vital fungal cells (Bonfante-Fasolo and Scannerini, 1977; Nurmiaho-Lassila et al.,

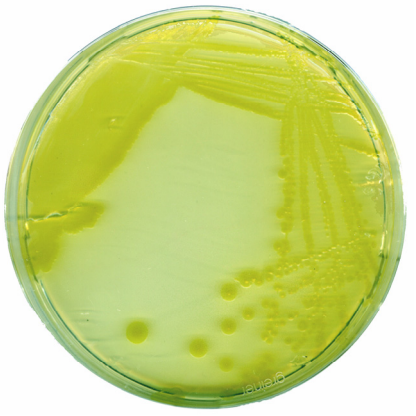

Fig II: Culture of bacteria isolated from *P. indica* spore solution, on luria-bertani agar.

1997)or in senescing mycorrhizal cells (Buscot, 1994; Mogge et al., 2000). The ultra-structural location and function of the bacteria associated with *P. indica* remained to be determined and will be one of the major objectives for the future.

In order to minimize potential artifacts during the transformation procedure due to the presence of bacteria, *P. indica* was grown in media containing broad-spectrum antibacterial antibiotics (Fig III). Success of this antibacterial antibiotic treatment to gain bacteria free fungus is remained to be shown which needs extensive biochemical cytological and molecular studies. Chlamydospores used in all subsequent transformation experiments were originating from *P. indica* cultures maintained in the presence of these antibacterial antibiotics. The biolistic gene gun-mediated transformation conditions were optimized to obtain a higher frequency of putative transformants. After standardization of different parameters the transformation set-up was finally came up similar to those adjustments used for *A. nidulans* transformation (Vainstein et al., 1994; Fungaro et al., 1995). During microprojectile bombardment, lower pressure and longer target distance gave relatively less transformants than high pressure and short target distance (Manuscript 3, Table1). It is considered that the difference between these conditions is strongly dependent on the type of target cells (e.g. conidia, spores, and mycelia) used for transformation. Given that *P. indica* chlaymydospores represent rigid structures with approximately 1.5 µm thick cell walls, (Pham et al., 2004a) a higher pressure is required to deliver DNA into spore cytoplasm. The constructs used for transformation comprehended plasmid pAN7-1 carrying the gene encoding *Hygromycin B* (*HygB*) and plasmid pPgpd-DsRed containing *DsRed*, both under the control of *A. nidulans* gpd promoter and trpC terminator. The gpd promoter was shown to be active in many fungal species including basidiomycetes (Punt et al., 1987). For the proper identification of transformed cells, it is imperative to determine the fungal ability to grow in the presence of a specific antibiotic. To find the optimal conditions for selection, *P. indica* was tested for its ability to axenically grow in the presence of various HygB concentrations. It was found that mycelial growth was strongly reduced (~80%) at 10 µg ml^{-1} HygB and was fully inhibited at concentrations above 25 µg ml^{-1} HygB (Fig III). Therefore, plasmid pAN7-1 was used for transformation and 50 µg

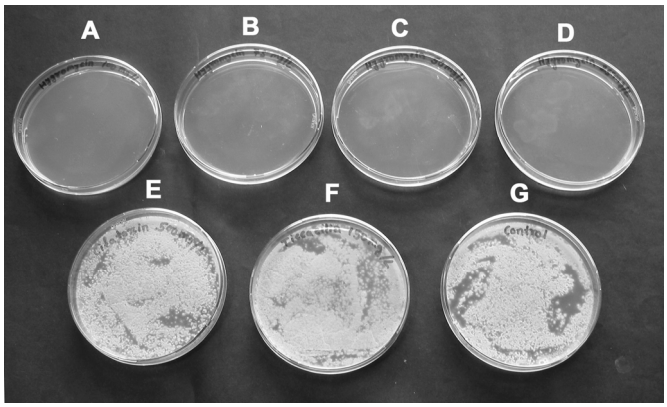

Fig III: Culture of *P. indica* on modified *Aspergillus* medium (G), in the presence of various HygB concentrations (A to D), in the presence of antibacterial antibiotics (E and F).

ml^{-1} HygB were adopted for the initial selection of HygB-resistant colonies in all subsequent transformation experiments.

3.3.2 Selection and stabilization of co-transformants

After transformation, putative HygB-resistant colonies were grown for one month on fresh selective medium, containing 50 µg ml^{-1} HygB. Nearly 94% of these putative transformed colonies showed reduced initial growth as compared to other transformed colonies. No substantial propagation of these Hyg-B- resistant colonies was observed upon prolonged cultivation (3–4 weeks). However these colonies displayed a short growth phase for several generations on fresh selective medium without forming chlamydospores (Manuscript 3, Fig. 1 A&B). This might be explainable by the introduction of the gene into a nuclear fraction of the multinucleated cells, which, although sufficient for mycelium survival, was not able to support the proliferation of antibiotic-resistant mycelia. Similar observations were made while transforming other multinucleate filamentous fungi including basidiomycetes (Benedetto et al., 2005). Due to the lack of mononucleate cells (for instance, macroconidia as in *Neurospora crassa*), gene transfer into the *P. indica* genome will result in a heterokaryon of transformed and untransformed nuclei, thus undoubtly limiting the success to screen for transgene-associated phenotypes, such as the dominant selectable marker HygB. To overcome this difficulty, hygromycin selection pressure was successively removed. On non-selective plates the fungus formed a mosaic of transgenic and non-transgenic hyphae as indicated by fluorescing and non-fluorescing areas of the colonies (Manuscript 3, Fig. 1). Epifluorescence microscopic analyses of putative transformants growing on HygB selective plates showed strong and localized fluorescence. DsRed fluorescence was found to be uniformly distributed within the internal part of the hyphal apex, with the exclusion of vacuoles. The weak fluorescence observed at early times became progressively stronger upon prolonged co-cultivation. This effect most likely reflects an enhanced intracellular accumulation of DsRed considering its high stability (Lorang et al., 2001). The transformants could not be enriched through hygromycin selection, probably because of the inability to purify transformed homokaryons. The hygromycin-resistant, DsRed-expressing cells are thus likely produced by occurrence of low-frequency integration events, which in higher eukaryotes commonly account for 1% (or less) of the total transformants. Therefore, the enrichment and purification of transformed hyphal colonies from the mass of untransformed hyphae remains to be optimized.

The confirmation of transformation came from PCR analysis of the unstable transformants by using transgene-specific oligonucleotides. The presence of the transgene was confirmed at the genomic DNA level and the transcript level by PCR and RT-PCR respectively. As a result of the PCR analysis it became obvious that many PCR-negative colonies were especially found in cases where DNA from the mosaic stage level was used as template. It is widely recognized that transformation efficiency decreases remarkably under non-selecting conditions (Kaya et al., 1990). However, it becomes even more complex in the case of individual *P indica* spores since they are multinucleate and only a small fraction of the nuclei present in the spore will be transformed. One of the major obstacles for transformation of these fungal spores is their multinucleate status, which will always lead to the formation of heterokaryons (Harrier and Millam, 2001). It is not known how many nuclei need to be transformed before the expression of reporter gene product can be detected. Furthermore, transformed nuclei will become diluted over subsequent generations as reported for *Gigaspora rosea*. Here, it became progressively more difficult to detect GUS gene activity in filial generations while it was impossible to detect any expression in spores of the third generation (Harrier and Millam, 2001).

As mentioned above, *P. indica* is associated with bacteria. Transformation of bacteria while transforming fungal spores is unavoidable possibility. However, it is not expected that the unstable transformants generated in this study are a result of bacterial transformation. For instance, the RT-PCR analysis provides evidence for the transcription of a gene, which is under control of eukaryotic promoter. Additionally, fluorescence microscopic analysis of dsRed expressing transformants show homogeneous dsRED protein accumulation in fungal cytoplasm, which is rather unexpected if genes are expressed in bacteria. Taken together, the transformants could most probably not be enriched through hygromycin selection, due to the inability to purify transformed homokaryons.

Enrichment of transgenic portions of the mosaic hyphal network would be a possible way to isolate homozygous transformants. However, this is not an easy task in *P. indica* since anastomosis has not been studied in detail so far, and the hyphae are extremely fragile and can easily lose their entire cytoplasm when wounded. Unfortunately, *P. indica* has not been shown to generate sexual spores or mononucleate cells. The procedures described in combination with previously unavailable molecular genetic tools — such as RNA-mediated interference or antisense silencing of selected target genes or the overexpression of homologous or heterologous genes, might support further strategies for genetically modifying the ecologically and agronomically important fungus *P. indica*.

IV Summary

Plant roots are potential hosts of a plethora of beneficial microorganisms including mycorrhizal fungi, rhizobial bacteria, and endophytic fungi of the newly defined order Sebacinales (Basidiomycota). The recently discovered root endophyte *Piriformospora indica* represents a model organism of this fungal order. The axenically cultivable *P. indica* colonizes roots of a large number of monocotyledonous and dicotyledonous plant species including cereals and Brassicaceae. The symbiosis is characterized by increased biomass and grain yield of interacting plants. In barley, the endophyte induces local and systemic resistance to fungal diseases and to abiotic stress. To further elucidate the lifestyle of *P. indica*, fungal development and host reactions were analyzed during the mutualistic symbiosis with barley roots. It was revealed that like other mutualistic endophytes, *P. indica* colonizes roots in an asymptomatic manner. The fungal colonization increases with root tissue maturation. The root tip meristem showed no colonization and the elongation zone showed mainly intercellular colonization. The differentiation zone was heavily infested by inter- and intracellular hyphae and intracellular chlamydospores. The majority of hyphae were present in intercellular spaces, dead rhizodermal and cortical cells that became completely filled with chlamydospores at later interaction stages. In some cases, hyphae penetrated cells and built a meshwork around plasmolyzed protoplasts, suggesting that the fungus either actively kills cells or senses cells undergoing endogenous programmed cell death. However, the expression of the barley *BAX inhibitor-1* (*HvBI-1*) gene, an inhibitor of plant cell death, was suppressed during colonization. Consistently, fungal proliferation was strongly inhibited in transgenic barley lines overexpressing GFP-tagged HvBI-1. The cytological observations and responses of the host genetic factor to fungal infestation showed that *P. indica* requires host cell death for proliferation in differentiated barley roots and the endophyte interferes with the host cell death program to form a mutualistic interaction with plants.

Infection of plant roots with pathogenic necrotrophic fungi of the genus *Fusarium* leads to necrotized roots and severe reduction of root and shoot biomass. Recently, it has been shown that *P. indica* infested plants are more resistant to *Fusarium culmorum.* In the current study, the interaction of *P. indica* with *Fusarium graminearum* in barley roots was analyzed. Upon infestation with *P. indica*, roots were protected from *Fusarium* infections as evidenced by reduced root rot symptoms. Consistently, *Fusarium* quantification using quantitative polymerase chain reaction (Q-PCR) revealed a correlation between reduced root rot symptoms and the relative amount of fungal DNA in the roots. Expression of pathogenesis related (PR) genes, which strongly increased in response to *F. graminearum* infections, was

diminished in the presence of *P. indica*. This finding indicates that PR proteins do not play a crucial role in the *P. indica*-mediated resistance response to *Fusarium*.

While *P. indica* is emerging as a model fungus to study mutualistic plant-fungus interactions, genetic transformation has not been accomplished yet. Transformation of the fungus by biolistic gene transfer and analysis of transgene expression in its saprophytic phase was performed. The transcriptionally active nature of the constitutive *Aspergillus nidulans glyceraldehyde 3-phosphate* (gpd) promoter and suitability of red fluorescent protein DsRed as a reporter gene in *P. indica* was established. The genes for hygromycin (Hyg-B) resistance and DsRed were employed as markers. Patches of fluorescent hyphae and chlamydospores were observed upon fluorescence microscopic examination of hygromycin-resistant mycelia. The procedure described here lays the ground for the use of yet unavailable molecular genetic tools in analyzing a plant root endophytic fungus.

IV Zusammenfassung

Pflanzenwurzeln sind potenzielle Wirte einer Vielzahl von Mikroorganismen einschließlich Mykorrhizapilzen, Rhizobien sowie endophytischen Pilzen der kürzlich bestimmten Ordnung Sebacinales (Basidiomycota). Der jüngst entdeckte Wurzelendophyt *Piriformospora indica* ist ein Modellorganismus dieser Pilzordnung. Der axenisch kultivierbare *P. indica* kolonisiert Wurzeln einer großen Anzahl ein – und zweikeimblättriger Pflanzenarten, einschließlich Getreidepflanzen und Brassicaceen. Die Symbiose ist durch eine erhöhte Biomasse und Ertragszuwächse in wirtspflanzen charakterisiert. In Gerste induziert der Endophyt lokale und systemische Resistenz gegen pilzliche Krankheiten und Toleranz gegenüber abiotischem Stress. Zur Aufklärung des Lebensstils von *P. indica* wurden Pilzentwicklung und Wirtsreaktionen in der mutualistischen Symbiose mit Gerstenwurzeln untersucht. Es wurde gezeigt, dass *P. indica* genau wie andere mutualistische Endophyten, Wurzeln in asymptomatischer Weise durch Erhaltung der Antagonismus Balance kolonisiert. Die pilzliche Kolonisierung steigt mit dem Alter des Wurzelgewebes. Das Gewebe der Wurzelspitze zeigte keine Besiedlung und die Elongationszone wies hauptsächlich interzelluläre Kolonisierung auf. Im Gegensatz dazu war die Differenzierungszone stark mit inter- und intrazellulären Hyphen und intrazellulären Chlamydosporen besiedelt. Die Mehrzahl der Hyphen wuchs interzellulär in toten rhizodermalen und kortikalen Zellen, die zu späteren Interaktionsstadien vollständig mit Chlamydosporen gefüllt waren. In einigen Fällen penetrierten die Hyphen Zellen und bildeten ein Netzwerk um plasmolysierte Protoplasten, was zu der Vermutung führt, dass der Pilz entweder Zellen aktiv tötet oder Zellen wahrnimmt,

die gerade endogenen programmierten Zelltod unterlaufen. Allerdings wurde eine reduzierte Expression des Gerstengens *BAX inhibitor-1 (HvBI-1)*, eines negativen Zelltodregulators, während der Wurzelkolonisierung gemessen. Damit übereinstimmend war die Verbreitung des Pilzes stark in transgenen Gerstenpflanzen inhibiert, die GFP-markiertes HvBI-1 überexprimierten. Die zytologischen Beobachtungen und der genetische Hinweis zeigen, dass der Zelltod in differenzierten Gerstenwurzelgewebe der Vermehrung von *P. indica* dient und dass der Endophyt in das Zelltodprogramm des Wirtes eingreift, um eine mutualistische Interaktion mit Pflanzen zu etablieren.

Infektion von Pflanzenwurzeln mit pathogenen nekrotrophen Pilzen der Gattung *Fusarium* führt zu nekrotisierten Wurzeln und schwerwiegender Reduktion von Biomasse in Wurzel und Spross. Kürzlich wurde gezeigt, dass mit *P. indica* infizierte Pflanzen eine höhere Resistenz gegenüber *Fusarium culmorum* aufweisen. In der vorliegenden Arbeit wurde die Interaktion von *P. indica* mit *Fusarium graminearum* in Gerstewurzeln analysiert. Nach Befall mit *P. indica* waren Wurzeln gegen Infektionen mit *Fusarium* geschützt, was sich durch reduzierte Symptome der Wurzelfäule zeigte. Damit einhergehend konnte durch Quantifizierung mittels quantitativer Polymerase Kettenreaktion (Q-PCR) eine Korrelation zwischen reduzierten Symptomen der Wurzelfäule und der relativen Menge an Pilz-DNA in Wurzeln hergestellt werden. Die nach *F. graminearum* Infektion stark erhöhte Expression von Pathogenese bezogenen (PR) Genen war bei Anwesenheit von *P. indica* reduziert. Dieses Ergebnis weist darauf hin, dass PR-Proteine keine entscheidende Rolle bei der *P. indica* vermittelten Resistenzantwort auf *Fusarium* spielen.

P. indica entwickelt sich mehr und mehr zum Pilzmodell für das Studium einer mutualistischen Pflanze-Pilz Interaktion, genetische Transformation konnte bisher jedoch noch nicht erreicht werden. Transformation des Pilzes mittels biolistischem Gentransfer und Analyse der Expression des Transgens in seiner saprophytischen Phase wurde im Rahmen dieser Arbeit durchgeführt. Der transkriptionell konstitutiv aktive *Aspergillus nidulans glyceraldehyde 3-phosphate* (gdp) Promotor und die Eignung von rot fluoreszierendem Protein DsRed als Reportergen wurde in *P. indica* etabliert. Die Gene für Hygromycin (Hyg-B) Resistenz und DsRed wurden als Marker verwendet. Bei Untersuchung von Hygromycin resistenten Mycelien im Fluoreszensmikroskop wurden Bereiche von fluoreszierenden Hyphen und Chlamydosporen beobachtet. Die hier beschriebene Prozedur legt die Basis für bisher nicht verfügbare molekulare genetische Werkzeuge zur Untersuchung endophytischer Pilze in Pflanzenwurzeln.

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List of abbreviation

AM Arbuscular mycorrhizal
Bgh *Blumeria graminis hordei*
BI-1 BAX Inhibitor-1
Ca^{2+} Calcium
CC Control plants
CCaMK Calmodulin-dependent protein kinase
CF Plants colonized by *F. graminearum*
CP Plants colonized by *P. indica*
DMI1 DOESN'T MAKE INFECTIONS1 mutants
DSE Dark septate endophyte
DsRed *Discosoma ssp.* Red
EtBr Ethidium bromide
GFP Green fluorescent protein
HCL HAIR CURLING mutant
HvBI-1 Hordeum vulgare L Bax inhibitor-1
HygB Hygromycin B
ISR Induced systemic resistance
ITS Inter-transcribed sequence
LysMRKs LysM receptor kinases
Mlo Mildew locus O
NF Nod factor
NIN NODULE INCEPTION mutant
NSP1 NODULATION SIGNALING PATHWAY1 mutant
nucLSU Nuclear large ribosomal RNA subunit
PCD Programmed cell death
PF Plants colonized by *P. indica* and *F. graminearum*
PR Pathogenesis related
Q-PCR Quantitative polymerase chain reaction
SAR Systemic acquired resistance
TM Transmembrane

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