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Review

Piriformospora indica—a mutualistic basidiomycete with an exceptionally large plant host range

XIAOYU QIANG[1], MICHAEL WEISS[2], KARL-HEINZ KOGEL[1,*,†] AND PATRICK SCHÄFER[1,*]

[1]*Research Centre for Biosystems, Land Use, and Nutrition, Institute of Phytopathology and Applied Zoology, Justus Liebig University, D-35392 Gießen, Germany*
[2]*Universität Tübingen, Institut für Evolution und Ökologie, Organismische Botanik, Auf der Morgenstelle 1, D-72076 Tübingen, Germany*

SUMMARY

Piriformospora indica is a basidiomycete of the order Sebacinales, representing a model for the study of mutualistic symbiosis and, beyond that, the plant immune system. The fungus colonizes the roots of a wide range of vascular plants, increasing their growth, seed yield and adaptation to abiotic and biotic stresses. The fungal colonization of roots begins with a biotrophic growth phase, in which living cells are colonized, and continues with a cell death-dependent phase, in which root cells are actively killed by the fungus. The complexity of sebacinalean symbiosis is further enhanced by the presence of endocellular bacteria which may represent significant determinants for a successful outcome of the symbioses. Molecular ecological analyses have revealed an exceptional relevance of sebacinoid fungi in natural ecosystems worldwide. This natural competence could be rooted in their phenotypic adaptability, which, for instance, allows *P. indica* to grow readily on various synthetic media and to colonize distinct hosts. In molecular and genetic studies, *P. indica*'s mutualistic colonization strategy has been partly unravelled, showing that the jasmonate pathway is exploited for immune suppression and successful development in roots. Research on *P. indica* supports efforts to make the bioprotective potential of the fungus accessible for agricultural plant production. The decoding of *P. indica*'s genome has revealed its potential for application as bioagent and for targeted improvement of crop plants in biotechnology-based approaches.

INTRODUCTION

Piriformospora indica was isolated in association with a spore of *Glomus mosseae* from the rhizosphere of two shrubs in the Indian Thar Desert, north-western Rajasthan, in the 1990s (Verma *et al*., 1998). Soon after, the fungus was characterized as a mutualistic endophyte because of its ability to colonize plant roots without causing any visible disease symptoms in roots or shoots. Instead, it confers strong growth promotion and induces disease resistance to microbial pathogens (Peškan-Berghöfer *et al*., 2004; Schäfer and Kogel, 2009; Varma *et al*., 1999; Waller *et al*., 2005). Molecular phylogenetic analyses have revealed that *P. indica* belongs to the basidiomycetous order Sebacinales (Weiß *et al*., 2004). Within this order, *P. indica* shows close relationships to species of the heterogeneous *Sebacina vermifera* complex and to multinucleate *Rhizoctonia* species, all of which convey similar beneficial effects to their host plants (Deshmukh *et al*., 2006; Sharma and Kogel, 2009). The natural host of *P. indica* has not been determined, but closely related species have recently been detected as natural root endophytes of plants from several continents (Weiß *et al*., 2011), whereas *S. vermifera* species have been sampled from diverse terrestrial orchids (Warcup, 1988; Weiß *et al*., 2004). The agricultural potential of *P. indica* is deduced from the wide range of mutualistic symbioses it establishes with an apparently unlimited number of plants. Recent efforts at our institute have resulted in the successful sequencing and annotation of the *P. indica* genome (Zuccaro *et al*., 2011). *Piriformospora indica* thus represents a genetically accessible model to study the molecular basis of processes associated with fungal accommodation and the establishment of root symbioses. The identification of an intricate association of *P. indica* with the bacterium *Rhizobium radiobacter* has added another level of complexity to the organization of the symbiosis (Sharma *et al*., 2008). In sum, these analyses build a base for the application of *P. indica* and related mutualists in the field and for the performance of targeted improvements of agriculturally relevant crop traits. This review provides an extensive update on various aspects of plant root–*P. indica* symbioses, with an emphasis on the determinants associated with plant benefits, the role of the endobacteria herein and the plant processes influencing root colonization. In addition, we present the current knowledge on the systematics of the mutualistic order Sebacinales, the global distribution of its members and their importance for diverse ecosystems.

SYSTEMATIC POSITION, TAXONOMY AND MORPHOLOGY OF THE SEBACINALES

Molecular phylogenetic analyses (Weiß *et al*., 2004) have revealed that *P. indica* is a member of the basidiomycetous order

*Correspondence: Email: karl-heinz.kogel@agrar.uni-giessen.de; patrick.schaefer@agrar.uni-giessen.de
†Present address: Boyce Thompson Institute for Plant Research, Ithaca, NY 14850, USA.

Sebacinales (Basidiomycota: Agaricomycetes; Hibbett *et al*., 2007). Until the advent of molecular methods for fungal determination, only a few morphospecies were known from this fungal group. Since then, more and more sebacinoid fungi have been detected as mycobionts of plant roots in molecular ecological studies, and it has become apparent that Sebacinales harbours an enormous biodiversity (Weiß *et al*., 2011).

Basidiomes of Sebacinales have a more or less gelatinous habit. Micromorphologically, they are characterized by longitudinally septate basidia, a lack of hyphal clamp connections and of sterile hymenial elements (hyphidia) and, often, by the presence of thickened cell walls in substrate hyphae (Weiß *et al*., 2004). Verified teleomorphic genera in the Sebacinales include *Sebacina* and *Serendipita* (morphologically defined by resupinate basidiomes or the absence of macroscopically visible basidiomes), *Tremellodendron* (with coralloid basidiomes), *Efibulobasidium* (pustulate basidiomes), *Craterocolla* (pulvinate basidiomes) and *Tremelloscypha* (infundibuliform basidiomes). However, molecular phylogenetic studies indicate that these morphologically defined genera represent artificial groups (Weiß *et al*., 2004), and more research integrating molecular data and morphology is needed to establish phylogenetically sound specific and generic concepts in the Sebacinales. Mitosporic specimens in the Sebacinales have been assigned to the anamorphic genera *Chaetospermum* (Rungjindamai *et al*., 2008), *Ditangium* (the mitosporic stage of *Craterocolla*) and to the so far monotypic *Piriformospora*.

Although the exact phylogenetic position of the Sebacinales within the Agaricomycetes is still unclear, it has been shown that they can be divided into two major clades, which have been informally designated as group A and group B (Fig. 1; Weiß *et al*., 2004). Macroscopically visible basidiomes have so far only been observed in Sebacinales group A. The only teleomorph in group B, characterized by the formation of single clusters of basidia and the production of elongated, vermiform basidiospores, has been described as *Sebacina* (= *Serendipita*) *vermifera* (Oberwinkler, 1964), and has since been observed repeatedly in fungal cultures isolated from several terrestrial Australian orchids (Warcup, 1988). Molecular studies suggest that *S. vermifera* is, in fact, a broad complex of cryptic species (Weiß *et al*., 2004), and it is very possible that all teleomorphic stages of members of group B are similar to *S. vermifera*.

The anamorphic *P. indica*, originally isolated from a spore of *Glomus mosseae* (Verma *et al*., 1998), also belongs to group B (Fig. 1; Weiß *et al*., 2004). Interestingly, *P. indica* is phylogenetically closely related to strain DAR 29830 (Fig. 1; Weiß *et al*., 2011), a multinucleate *Rhizoctonia*, which was originally isolated from a spore of *Glomus fasciculatum* (Williams, 1985). Although a teleomorphic stage of DAR 29830 has not been observed so far, another strain, identical to DAR 29830 in culture characteristics and hyphal modifications, has been successfully transformed into a teleomorphic stage reported as 'similar to, but distinct from, *Sebacina vermifera*' (Milligan and Williams, 1987). It is therefore very possible that *P. indica* possesses an as yet undetected teleomorphic stage.

SEBACINALES—ECOLOGY AND DISTRIBUTION

Ecologically, Sebacinales is characterized by its associations with plant roots. No other known fungal group shows a broader spectrum of mycorrhizal types. Sebacinalean fungi are involved in ectomycorrhizae (e.g. Glen *et al*., 2002; Tedersoo *et al*., 2003; Urban *et al*., 2003), orchid mycorrhizae (e.g. Huynh *et al*., 2009; Selosse *et al*., 2002), arbutoid mycorrhizae (Selosse *et al*., 2007), ericoid mycorrhizae (Allen *et al*., 2003; Selosse *et al*., 2007), cavendishioid mycorrhizae (a specific mycorrhizal association of hemiepiphytic Ericaceae in the Andean mountain rain forest; Kottke *et al*., 2008; Setaro *et al*., 2006) and also in the jungermannioid mycothallus, a fungal association of leafy liverworts (Kottke *et al*., 2003).

In addition to the unique spectrum of mycorrhizal types in which sebacinalean fungi are involved, they also frequently occur as symptomless root endophytes of land plants (Weiß *et al*., 2011). Interestingly, the closest relatives of *P. indica*, known so far from molecular studies, were all detected as root endophytes from field-collected plants (Fig. 1; Weiß *et al*., 2011). The numerous *in vitro* experiments performed to study the endophytic associations of *P. indica* with various host plants during recent years may thus model a type of symbiosis which is an important component of terrestrial ecosystems.

Mycorrhizal associations are not evenly distributed over Sebacinales groups A and B (Weiß *et al*., 2011). Ectomycorrhizal associations have only been reported from group A, whereas ericoid and cavendishioid associations have only been observed in group B. Endophytic Sebacinales have been detected in group A as well as in group B. As endophytic associations are prevalent in more basal groups, it has been hypothesized that this mode of interaction with plant roots is the basal type in the Sebacinales, from which the various mycorrhizal types have evolved (Weiß *et al*., 2011). However, this hypothesis needs to be tested in further studies.

From the available data, it is clear that sebacinalean fungi occur on all continents, including Antarctica (Bridge and Newsham, 2009). However, data are still too sparse to derive detailed hypotheses regarding the phylogeography of the Sebacinales (Weiß *et al*., 2011).

PLANT HOSTS AND THEIR BENEFITS FROM COLONIZATION BY *P. INDICA*

Among the beneficial effects transferred by the fungus to a large range of mono- and dicotyledonous host plants, growth promotion, seed yield increase and enhanced resistance/tolerance to

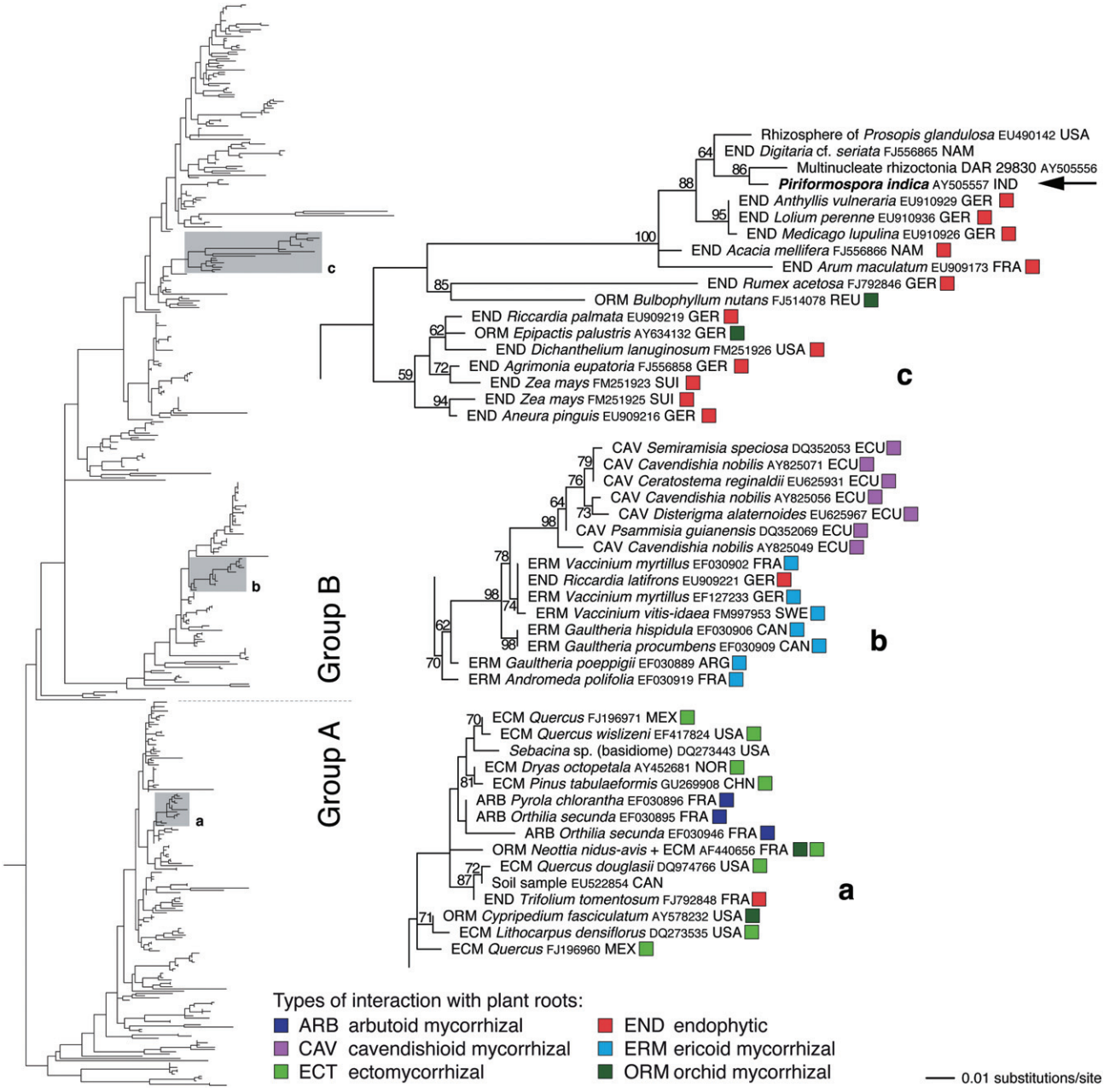

Fig. 1 An overview of the phylogenetic relationships in the Sebacinales, based on a representative sampling of partial nuclear-encoded ribosomal large subunit sequences. Most of the available sequences are from mycobionts detected in plant roots in the field. The alignment was calculated using MAFFT (Katoh and Toh, 2008); a phylogenetic hypothesis was constructed using maximum likelihood analysis as implemented in RAxML (Stamatakis, 2006). Detailed subtrees illustrate the phylogenetic relationships of the three clades highlighted in the full tree (a from Sebacinales group A; b, c from group B); subtree sequences are identified by their GenBank accession numbers. The placement of *Piriformospora indica* in clade c is indicated by the arrow. Numbers on the branches are bootstrap support values obtained from 1000 replicates (only values ≥50% are shown); branch lengths are scaled in terms of the number of expected substitutions per nucleotide. Coloured boxes indicate the type of symbiosis. Country codes used: ARG, Argentina; CAN, Canada; CHN, China; ECU, Ecuador; FRA, France; GER, Germany; GBR, Great Britain; IND, India; ITA, Italy; MEX, Mexico; NAM, Namibia; NOR, Norway; REU, Reunion; SUI, Switzerland; USA, United States of America. For more details of Sebacinales phylogeny, see Weiß *et al*. (2011).

Fig. 2 *Piriformospora indica* and its beneficial effects on colonized plants. (A, B) *Piriformospora indica* grows and forms chlamydospores on solid and liquid artificial media. (C) Tomato cv. Hildaris is protected by *P. indica* against *Fusarium oxysporum*. Note the growth-promoting effect in plants colonized by *P. indica* in comparison with control plants. (D, E) Systemic protection of leaves against *Blumeria graminis* f.sp. *hordei* (*Bgh*) after barley root colonization by *P. indica*. (F, G) Protection against *Golovinomyces orontii* (*Go*) in *Arabidopsis*.

biotic and abiotic stresses are agronomically most relevant (Blechert *et al*., 1999; Fakhro *et al*., 2010; Peškan-Berghöfer *et al*., 2004; Varma *et al*., 1999; Verma *et al*., 1998; Waller *et al*., 2005). The exploitation of *P. indica* may be facilitated in future by its ability to grow on axenic artificial solid or liquid media (Fig. 2A,B). *Piriformospora indica*-colonized plants acquire resistance against a variety of leaf and root pathogens. During the past 8 years of research, we and others have gathered plenty of data on *P. indica*'s potential for disease control, including the devastating root diseases caused by various *Fusarium* species (Fig. 2C, Table 1). However, *P. indica*'s plant protective potential might be affected by environmental factors. For instance, the replication of Pepino mosaic virus was higher in *P. indica*-colonized tomato under low-light conditions. Enhanced viral replication was transient and vanished over time. By contrast, high-light regimes reduced viral titres in *P. indica*-colonized tomato (Fakhro *et al*., 2010). Most convincingly, it has been demonstrated in barley, wheat and *Arabidopsis* that the fungus induces systemic resistance against biotrophic leaf pathogenic powdery mildew fungi (Fig. 2D–G) (Serfling *et al*., 2007; Stein *et al*., 2008; Waller *et al*., 2005). Based on an analysis of respective *Arabidopsis* mutants, the systemic resistance response is dependent on an operative jasmonic acid (JA) pathway, arguing for the involvement of an induced systemic resistance (ISR)-type response (Stein *et al*., 2008). A direct physiological connection of root encounters and systemic effects was shown by a noninvasive electrophysiological approach (Felle *et al*., 2009). As soon as 5–8 min after chlamydospore application to barley roots, a transient acidification of the leaf apoplast could be detected. In the long term, leaves were primed for stronger resistance against the leaf pathogen *Blumeria graminis* f.sp. *hordei*. None of these responses was observed after root treatment with active chitin fragments.

Piriformospora indica-mediated effects are apparently associated with altered antioxidative capacities in colonized plants, as conveyed by several independent studies. For instance, barley roots colonized by *P. indica* were more tolerant against salt stress and displayed an increase in glutathione and ascorbate contents, as well as dehydroascorbate reductase activity (Waller *et al*., 2005). Consistent with this, the colonization of salt-sensitive cultivar Ingrid resulted in salinity tolerance and an increase in its antioxidative capacity comparable with that of the salinity-tolerant cultivar California Mariout (Baltruschat *et al*., 2008). On colonization by *P. indica*, an adjusted antioxidative potential in maize roots correlated with resistance against *Fusarium verticillioides* (Kumar *et al*., 2009). Moreover, ascorbate levels were enhanced and two genes [*monodehydroascorbate reductase 2* (*MDAR2*) and *dehydroascorbate reductase 5* (*DHAR5*)] involved in the reduction of the antioxidant ascorbate were induced in *Arabidopsis* roots and shoots during early interaction stages with *P. indica* (Vadassery *et al*., 2009a). As mutants lacking either MDAR2 or DHAR5 were hypersusceptible to *P. indica*, enhancement in antioxidants might also control root colonization. These studies also hint at the possibility that an imbalanced colonization might convert the mutualistic into a detrimental interaction.

The physiological nature of the seed yield increase and growth promotion has been the subject of a couple of studies. In addition to very strong growth promotion activity in cereals and some dicot plants (Varma *et al*., 1999; Waller *et al*., 2005), the Oelmüller lab also showed *P. indica*-mediated growth promotion in *Arabidopsis*. A leucine-rich repeat protein (LRR protein) and MATH domain-containing protein are required for *P. indica*-induced growth promotion (Oelmüller *et al*., 2005; Shahollari *et al*., 2007). Both proteins have a predicted plasma membrane localization, and therefore are thought to be far upstream in signalling associated with the mutualistic outcome. Further studies have revealed the importance of phospholipid signalling for growth promotion (Camehl *et al*., 2011). Based on genetic and physiological studies, two phospholipases D (PLDα1 and PLDδ) are recruited by *P. indica* and are involved in the generation of phosphatidic acid (PA).

Table 1 Plant pathogens and respective diseases control as a result of root colonization by *Piriformospora indica*.

Pathogen	Disease/symptom	Host	Reference
Blumeria graminis f.sp. *graminis*	Powdery mildew	Barley	Waller *et al*. (2005)
Blumeria graminis f.sp. *tritici*	Powdery mildew	Wheat	Serfling *et al*. (2007)
Golovinomyces orontii	Powdery mildew	*Arabidopsis*	Stein *et al*. (2008)
Cochliobolus sativus	Root rot	Barley	Waller *et al*. (2005)
Fusarium graminearum	Fusarium root rot	Barley	Deshmukh and Kogel (2007)
Fusarium culmorum	Fusarium root rot	Barley, wheat	Serfling *et al*. (2007); Waller *et al*. (2005)
Fusarium verticillioides	Maize root disease	Maize	Kumar *et al*. (2009)
Fusarium oxysporum	Fusarium wilt	Tomato	H. Baltruschat, K.-H. Kogel, unpublished data
Rhizoctonia solani	Rhizoctonia root rot	Barley	H. Baltruschat, K.-H. Kogel, unpublished data
Thielaviopsis basicola	Black root rot	Tomato	H. Baltruschat, K.-H. Kogel, unpublished data
Pepino mosaic virus	Yellow leaf mosaic	Tomato	Fakhro *et al*. (2010)
Pseudocercosporella herpotrichoides	Eyespot	Wheat	Serfling *et al*. (2007)
Verticillium dahliae	Verticillium wilt	Tomato	Fakhro *et al*. (2010)

Downstream of PA, 3′-phosphoinositide-dependent protein kinases (PDKs) 1.1 and 1.2 are required to activate the kinases OXI1 (oxidative signal-inducible 1) and AGC2-2 (cAMP-DEPENDENT PROTEIN KINASE A/PROTEIN KINASE G/PROTEIN KINASE C 2-2). Eventually, the mitogen-activated protein kinases (MPKs) 3 and 6 are activated. In addition, a cell wall extract (CWE) of *P. indica* also induced, although to a lesser extent, growth promotion in *Arabidopsis*. The CWE increased cytosolic Ca^{2+} currents and activated MPK6, which was required for CWE-induced growth promotion (Vadassery *et al*., 2009b). PLDα1 is known to be activated by Ca^{2+}. Therefore, Ca^{2+} influx after *P. indica* recognition by root cells might represent one of the first triggers involved in the activation of the growth promotion pathway (Camehl *et al*., 2011). In addition, hormones, such as cytokinins and ethylene, have been suggested to be involved in *P. indica*-mediated growth promotion (Camehl *et al*., 2010; Vadassery *et al*., 2008). Gibberellin (GA) signalling is apparently recruited by *P. indica* for correct root colonization (Jacobs *et al*., 2011; Schäfer *et al*., 2009) and might contribute to growth promotion. Plant mutants with constitutive GA signalling or plants treated with GA show enhanced plant growth (Chandler *et al*., 2002; Sun, 2008). It will be interesting to see whether the described signalling components (e.g. LRR proteins, MATH domain-containing protein) are connected to hormone signalling and the plant's antioxidative status to mediate the beneficial effects.

In arbuscular mycorrhizal symbioses, a bilateral exchange of nutrients occurs: plants are supplied with phosphate and nitrogen, and fungi receive carbohydrates (Smith and Smith, 2011). Interestingly, phosphate supply is apparently not directly associated with growth promotion. Even in cases in which the mycorrhizal interaction results in plant growth depression, fungi transfer significant amounts of phosphate to their hosts. The physiological reason for growth promotion is therefore still unclear (Smith and Smith, 2011). However, arbuscular mycorrhizae predominantly take up phosphate as orthophosphate (Pi) and nitrogen as NH_4^+ or NO_3^- from the soil by their extraradical mycelium. Pi is mostly transported as polyphosphate within hyphae, whereas arginine is the predominant transport form of nitrogen. The nutrients are transported to the intraradical hyphae which are directly connected to root cortical cells. Here, nutrients are converted and root cells are supplied with Pi and NH_4^+. Although several phosphate and ammonium transporters of plants have been identified, and these are mostly inducible by mycorrhizae, the mechanism of nutrient uptake by roots remains to be resolved (Smith and Smith, 2011). Several studies have demonstrated the significance of an improved nutrient supply, made available by *P. indica*, for growth promotion. Blechert *et al*. (1999) observed that the colonization of protocorms and roots of autotrophic *Dactylorhiza* spp. (Orchidaceae) by *P. indica* was associated with the formation of hyphal coils (pelotons). In orchid mycorrhizae, these pelotons are surrounded by perifungal membranes and interfacial matrices separating them from the host cytoplasm. These plant–fungus interfaces specifically operate in nutrient exchange (Peterson and Massicotte, 2004). It is unknown whether *P. indica* establishes similar interfaces for nutrient exchange in colonized root cells. Recent studies have monitored an improved phosphate supply to maize shoots by *P. indica* (Yadav *et al*., 2010). Consistent with this, knock-down of a high-affinity phosphate transporter (PiPT) in *P. indica* resulted in reduced phosphate transfer to maize shoots and correlated with the inability of the fungus to promote plant growth. *Piriformospora indica*-induced growth promotion might be further dependent on an improved nitrate supply. The colonization of tobacco and *Arabidopsis* roots by *P. indica* was associated with elevated nitrate reductase activity and nitrate uptake in roots (Sherameti *et al*., 2005). As reported for arbuscular mycorrhizal symbioses (Bonfante and Genre, 2010), *P. indica* might also receive carbohydrates from its hosts. Our studies have revealed lower hexose and starch contents in *P. indica*-colonized roots (Schäfer *et al*., 2009). Accordingly, Sherameti *et al*. (2005)

observed the induction of *SEX1*, which encodes a glucan water dikinase and is involved in starch degradation.

INTRICATE ASSOCIATION WITH ENDOBACTERIA

Recent work has shown that members of Sebacinales regularly undergo complex tripartite symbioses involving plants and bacteria of different genera (Sharma *et al*., 2008), which is reminiscent of the associations previously detected in glomeromycotan arbuscular mycorrhizal symbioses (Bonfante and Anca, 2009; Frey-Klett *et al*., 2007). The endobacteria that have so far been identified in the Sebacinales belong to two genera (*Rhizobium* and *Acinetobacter*) of Gram-negative as well as two genera (*Paenibacillus* and *Rhodococcus*) of Gram-positive bacteria; they are associated with three phylogenetically and morphologically distinct members of the *Sebacina vermifera* species complex. The fact that neither of the fungal species could be cured so far from their bacterial occupier indicates an intricate association. Consistent with this, a critical role of these bacteria in sebacinoid symbiosis cannot be excluded. The most comprehensively studied example of a tripartite sebacinoid symbiosis is the association of *P. indica* with the α-Proteobacterium *Rhizobium radiobacter* (syn. *Agrobacterium tumefaciens*) PABac-DSM (Sharma *et al*., 2008). Fluorescence *in situ* hybridization (FISH) using a *Rhizobium*-specific probe confirmed the endocellular association of PABac-DSM with fungal spores and hyphae. The bacterium is present in low numbers (2–20 per fungal cell), consistent with quantitative reverse transcription-polymerase chain reaction (qRT-PCR) data revealing a ratio of 0.02–0.035 ng of bacterial DNA per 100 ng of *P. indica* DNA. By PCR analysis, the *virD2* gene was identified, indicating that the Ti plasmid is present in PABac-DSM. However, the *isopentenyltransferase* (*IPT*) gene, which is associated with cytokinin biosynthesis, could not be detected, which may explain the nonpathogenic nature of this bacterial strain.

In contrast to bacteria associated with members of the *Sebacina vermifera* species complex, *R. radiobacter* PABac-DSM could be isolated from *P. indica* and propagated in axenic cultures, demonstrating that the bacterium is not entirely dependent on its fungal host (Sharma *et al*., 2008). Interestingly, antibiotics, used to kill or stop bacterial growth in pure bacterial cultures with high efficacy, were virtually ineffective in removing the endocellular bacteria from the fungus. The culture of *P. indica* for 2 months in the presence of ciprofloxacin (200 mg/mL) and/or spectinomycin (300 mg/mL) did not eliminate PABac-DSM from the fungus, although these antibiotics were found to be highly efficient in inhibiting the growth of PABac-DSM *in vitro*. This finding most obviously raises the question as to the significance of this bacterium for the sebacinoid symbiosis. In this respect, it was also noted that higher concentrations of bacteria were harmful, if not lethal, to the fungus, raising the possibility that the fungus needs to control the concentration of endobacteria to avoid pathogenic facets.

In order to test the possibility that *R. radiobacter* PABac-DSM contributes to the beneficial effects typically observed in the sebacinoid symbiosis, the bacterium was applied directly to plants. Intriguingly, PABac-DSM induced beneficial responses in barley similar to those reported for *P. indica*, among them higher biomass and ISR to powdery mildew (Sharma *et al*., 2008). Although the number of bacteria needed to induce these beneficial activities was much higher than detected by qRT-PCR in *P. indica*-colonized roots, these findings are widely consistent with a report on the potential of *R. radiobacter* for the improvement of plant performance in integrated production systems (Humphry *et al*., 2007). A clue to the contribution of PABac-DSM to the sebacinoid symbiosis comes from the recent analysis of its metabolomic composition. A research team at the Helmholtz Zentrum München (D. Li *et al*., unpublished data) found that the bacterium produces a spectrum of different *N*-acyl homoserine lactones with acyl chains of C8, C10 and C12 and hydroxy- or oxo-substitutions at the C3 position. These compounds are well-known autoinducers with quorum sensing activity in bacterial populations. Intriguingly, when tested on plants, the same compounds exhibited a broad spectrum of physiological responses (von Rad *et al*., 2008; Schuhegger *et al*., 2006), and also induced ISR in barley, raising the possibility that at least parts of the beneficial activities brought about by *P. indica* stem from these bacterial molecules (Schikora *et al*., 2011). Together, the discovery of bacterial associations in the Sebacinales further adds to increasing evidence that interactions of fungi with bacteria are more widespread than expected and that their dynamics may be crucial in ecosystems.

GENOME ORGANIZATION OF *P. INDICA*

Piriformospora indica possesses a 25-Mb genome, which was sequenced in parallel to cDNAs obtained from different developmental stages (Zuccaro *et al*., 2009, 2011). A plethora of information comes from the elucidation of the genome sequence, which cannot be discussed in detail here. Key findings of the genomic analysis relate to the life style of the fungus and corroborate its potential to follow sequentially a biotrophic and a saprotrophic nutrition allocation during the colonization of plant roots. A combination of single nucleotide polymorphism (SNP) analysis and comparative confocal laser scanning microscopy (CLSM) revealed the presence of two haploid genomes indicative of a dikaryotic mycelium. Some 11 768 putative protein encoding genes were identified, including multigene families with large expansion proposed to function in the regulation of cellular responses to stress and nutrient allocation. In contrast with ectomycorrhiza, cell wall-degrading enzymes are strongly expanded, and a comparison of the saprotrophic and the symbiotic transcriptome, when the fungus is maintained on artificial media or in association

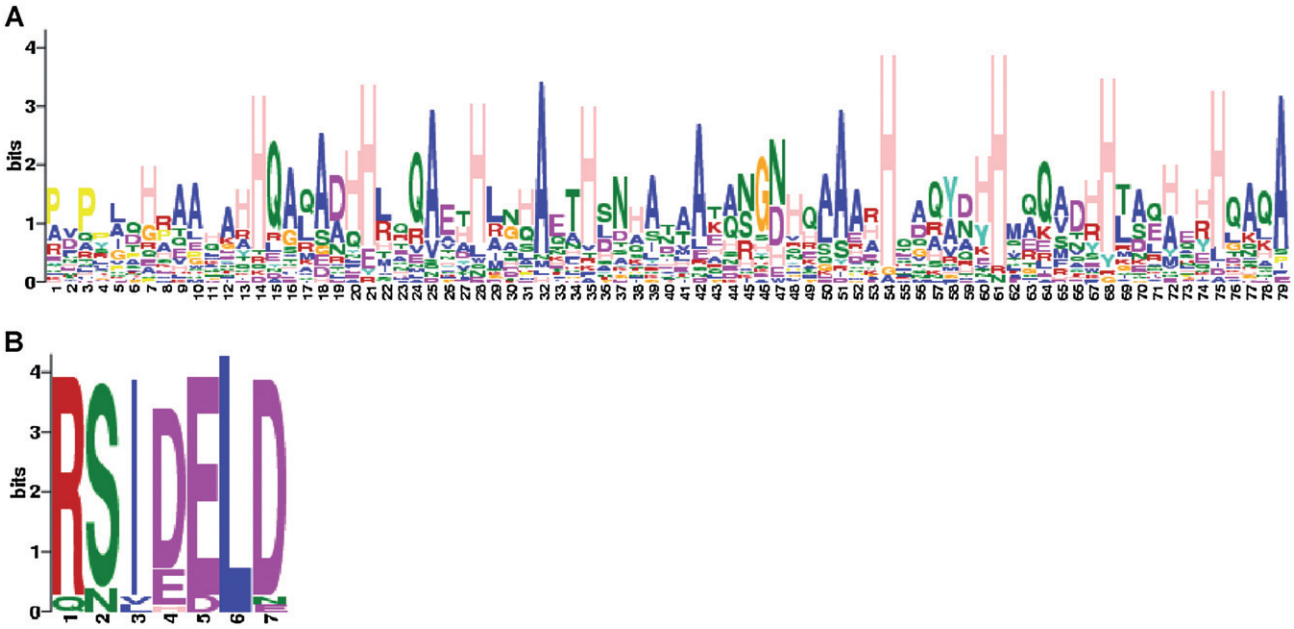

Fig. 3 Conserved motifs of some putative *Piriformospora indica* effectors. Potential effectors were analysed for conserved motifs with the *de novo* motif-finding algorithm MEME (Bailey *et al*., 2009). In several effectors, a central motif with a conserved and regular distribution of histidine and alanine (A) and the seven-amino-acid RISDEL motif (B) were found.

with roots, respectively, revealed a tightly regulated, symbiosis-associated gene activity.

Piriformospora indica-specific domain expansion was observed for carbohydrate-binding proteins. Some of these proteins resemble lectins and may function to mask ligands. These include domains that are possibly involved in carbohydrate binding, such as the chitin-binding domain LysM. Altogether, some 120 *P. indica* genes contain at least one carbohydrate-binding motif, and most possess a signal peptide and no transmembrane domains. In fungi, chitin-binding domains are mainly found associated with hydrolytic enzymes acting on the fungal cell wall. Most interestingly, many of the lectin-like *P. indica* proteins are plant responsive. What role this *P. indica* expansion of lectin-like proteins plays in the symbiosis remains unclear.

About 10% of the genes induced during the colonization of barley roots encode small secreted proteins, which may function as effectors. Most of these proteins are *P. indica* specific and poorly related to each other. Using a yeast signal sequence trap (YSST) method, a range of putative secreted *P. indica* peptides were isolated (Klute, 2011). In addition to some LysM peptides, probably the most interesting candidate detected as a 'hypothetical open reading frame (orf') showed similarities to at least 30 other small secreted proteins with a length of 110–130 amino acids (Zuccaro *et al*., 2011). The amino acid sequences of these proteins have a conserved and regular distribution of histidine and alanine, and a highly conserved pattern of seven amino acids 'RISDELD' at the C terminus of 26 proteins, and are therefore designated as DELD proteins (Fig. 3A,B). Most of the DELD proteins are induced during symbiosis. The RISDELD motif and similar motifs are rarely present in other fungal proteins. Tridimensional structure prediction showed that all DELD proteins most probably form two helices interrupted by a central conserved glycine. Together, recent work on the *P. indica* genome suggests that DELD is a new gene family with a conserved domain of unknown function secreted during root colonization.

ROOT COLONIZATION STRATEGIES OF *P. INDICA*

The broad host range of *P. indica*, which encompasses vascular plants and even mosses, indicates that the mutualist has evolved highly effective colonization strategies. Recent cell biological studies in *Arabidopsis* and barley have revealed that the fungus preferentially colonizes the maturation zone of roots and is hardly observable at the root meristematic and elongation zones (Deshmukh *et al*., 2006; Jacobs *et al*., 2011) (Fig. 4A). At a cellular level, the fungus colonizes living root cells by direct penetration (Figs 4B and 5A) (Jacobs *et al*., 2011). This biotrophic colonization is accompanied by a broad-spectrum suppression of root innate immunity. On the basis of molecular and genetic analyses, plant roots, like leaves, are equipped with an effective innate immune system, and immune suppression by *P. indica* is a prerequisite for successful root colonization (Jacobs *et al*., 2011). As immune suppression occurs in *Arabidopsis* and barley roots (Jacobs *et al*., 2011; Schäfer *et al*., 2009), this might further explain the large host range of the fungus. The underlying mechanisms

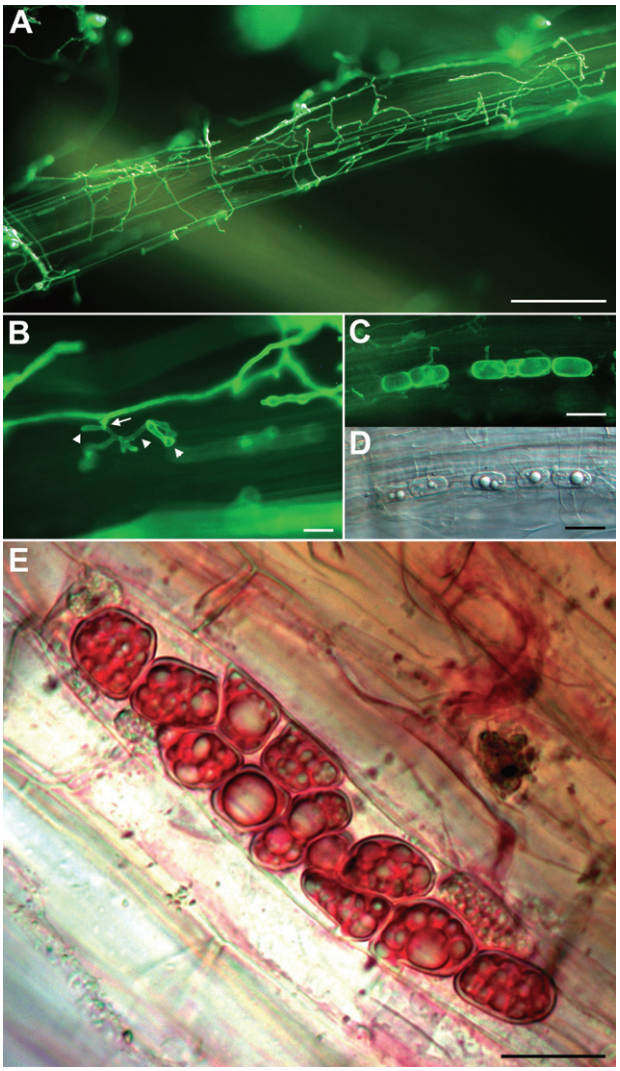

Fig. 4 Colonization of *Arabidopsis* root by *Piriformospora indica*. (A) Large areas of the root's maturation zone are colonized by *P. indica* at 7 days after inoculation (dai). (B) Root colonization is achieved by direct cell penetration (arrow) and by the subsequent establishment of intracellular hyphae (arrowheads). (C, D) Root colonization is associated with the development of intracellular spores in colonized cells. (C) Fluorescence image of intracellular spores. (D) Bright field image of (C). (E) Chlamydospores of *P. indica* in tomato root cells. Fungal structures were stained with WGA-AF488 (Invitrogen, Darmstadt, Germany) and visualized by fluorescence microscopy (excitation/emission: 470–520/505–530 nm) using an Axioplan 2 Microscope (Zeiss, Jena, Germany) (A–C), or stained with fuchsin–lactic acid and visualized by bright field microscopy (E). Bars: 60 μm (A); 10 μm (B–E).

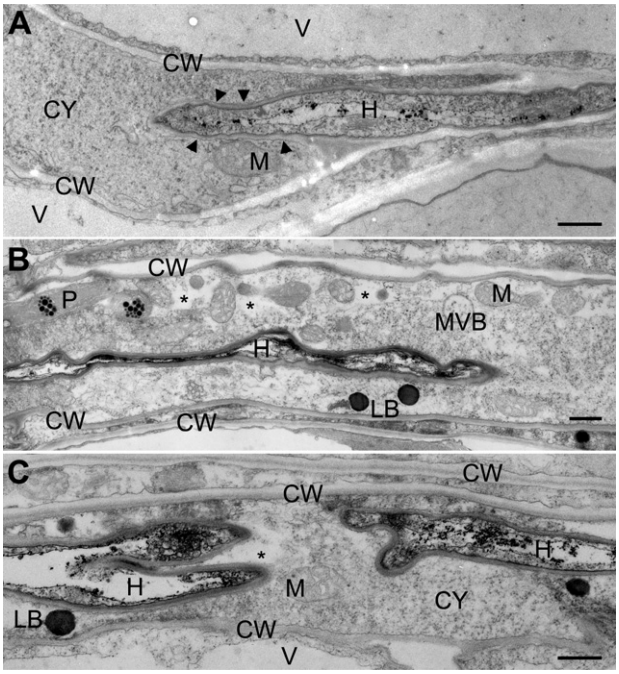

Fig. 5 Ultrastructural alterations in *Piriformospora indica*-colonized *Arabidopsis* cells. (A) Early biotrophic colonization stage at which colonized root cells are alive as indicated by the integrity of the cytosol and mitochondria. The vacuoles in noncolonized neighbouring cells show an intact tonoplast. The intracellular hypha is surrounded by the plant plasma membrane (arrows). (B, C) As intracellular colonization proceeds, colonized cells undergo cell death indicated by lysis of the cytosol (asterisks), whereas organelles do not show morphological alterations. CW, root cell wall; CY, cytosol; H, hypha; LB, lipid bodies; M, mitochondrium; MVB, multivesicular bodies; P, plastids; V, vacuole. Bars, 2 μm.

are unknown, but phytohormones are largely involved. Barley and *Arabidopsis* mutants impaired in gibberellic acid (GA) and JA metabolism, respectively, show elevated root immune responses, together with reduced root colonization (Jacobs *et al*., 2011; Schäfer *et al*., 2009). Further studies have revealed that *P. indica* depends on JA to suppress early immune responses (e.g. root oxidative burst) as well as salicylic acid (SA)- and glucosinolate-related defence pathways (Jacobs *et al*., 2011). Consistent with this, *Arabidopsis* mutants impaired in SA and glucosinolate-associated defence are more susceptible to *P. indica* (Jacobs *et al*., 2011; Sherameti *et al*., 2008). Excitingly, the genome of *P. indica* is now available, and thus allows the identification of effector molecules that target immune signalling components.

Compelling evidence suggests that *P. indica*'s early biotrophic interaction with its plant hosts is followed by a cell death-dependent colonization phase (Fig. 5B,C). The first evidence was provided with barley, when ectopic overexpression of the negative cell death regulator BAX INHIBITOR-1 (BI-1) resulted in reduced colonization of roots (Deshmukh *et al*., 2006). BI-1 is known to be an integrator of endoplasmic reticulum (ER) stress, thereby supporting cell integrity and viability under unfavourable conditions. In addition to its involvement in cell death, the ER is essential for correct processing of immunity-related proteins with an extracellular (Wang *et al*., 2005) or plasma membrane-associated destination, including the pattern recognition receptor EFR (Nekrasov *et al*., 2009; Saijo *et al*., 2009). EFR activates basal immunity after recognition of the bacterial elongation factor TU and effectively

halts bacterial invasion of plants (Zipfel *et al*., 2006). Recent cell biological analyses of *Arabidopsis* roots defined ultrastructural alterations during cell death-associated colonization by *P. indica* (X. Qiang *et al*., unpublished data). Based on these studies, *P. indica* induces ER stress in colonized roots but, at the same time, suppresses the adaptive ER stress response pathway (unfolded protein response, UPR). Similar to the situation in mammals, the inability of colonized cells to relieve ER stress via the UPR results in the activation of a pro-apoptotic signalling cascade. Consequently, vacuolar collapse was identified to be downstream of ER stress and to represent a key element of the *P. indica*-induced cell death pathway. This vacuolar collapse is essential for both cell death execution and root colonization, and is mediated by vacuolar processing enzymes (VPEs). Respective *Arabidopsis* mutants lacking VPEs were incapable of undergoing cell death-associated vacuolar collapse and exhibited reduced fungal colonization. It is assumed that the fungus uses these dead cells for intracellular sporulation (Fig. 4C–E). Taken together, mutualistic *P. indica* gains its large host range by a biphasic colonization strategy, which comprises biotrophic accommodation by effective host immune suppression, followed by an ER stress-induced caspase-dependent vacuolar cell death. The exact execution of both colonization phases is a prerequisite for a successful symbiosis with *Arabidopsis*. In terms of root immunity, the disturbance of ER integrity by *P. indica* might impair the secretion of immunity-associated proteins (e.g. PR1, PRRs). In addition to the suppression of early immune signalling, this disturbance of immune execution might further disarm the root, thereby facilitating root colonization. Notably, we observed the initiation of ER disturbance early during biotrophic colonization (3 days after inoculation).

PERSPECTIVE

Concerning its beneficial nature, *P. indica* and its relatives in the Sebacinales have considerable potential as biopesticides and plant strengtheners. Particular efforts should therefore be made to define the molecular and biochemical bases of sebacinalean symbioses and their physiological effects on crop plants, as that may be a prerequisite for the introduction of sebacinalean fungi into crop production. Equally important is the definition of the root colonization strategies of *P. indica*. In addition to determining the relevance of colonization for the transfer of beneficial traits, *P. indica* has been extremely valuable in understanding the orchestration of root innate immunity. In particular, the *Arabidopsis–P. indica* system allows a comparison between root and leaf innate immunity. Further, we may gain an insight into how roots discriminate between pathogens and mutualists. These aspects are of outstanding importance for future breeding strategies. Considering its immune suppressive capacities, *P. indica* might also represent a unique tool to uncover the weak points of plant innate immunity. The recently accomplished annotation of the *P. indica* genome will undoubtedly support all research efforts related to *P. indica*, but also to other mutualistic plant-colonizing fungi. Moreover, we have just established a stable barley root transformation system that allows the fast and robust analysis of *P. indica* protein functions in all aspects of root development, as well as abiotic stress tolerance and biotic interactions in crop plants (Imani *et al*., 2011).

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