Host-related metabolic cues affect colonization strategies of a root endophyte

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The mechanisms underpinning broad compatibility in root symbiosis are largely unexplored. The generalist root endophyte *Piriformospora indica* establishes long-lasting interactions with morphologically and biochemically different hosts, stimulating their growth, alleviating salt stress, and inducing local and systemic resistance to pathogens. Cytological studies and global investigations of fungal transcriptional responses to colonization of barley and *Arabidopsis* at different symbiotic stages identified host-dependent colonization strategies and host-specifically induced effector candidates. Here, we show that in *Arabidopsis*, *P. indica* establishes and maintains biotrophic nutrition within living epidermal cells, whereas in barley the symbiont undergoes a nutritional switch to saprotrophy that is associated with the production of secondary thinner hyphae in dead cortex cells. Consistent with a diversified trophic behavior and with the occurrence of nitrogen deficiency at the onset of saprotrophy in barley, fungal genes encoding hydrolytic enzymes and nutrient transporters were highly induced in this host but not in *Arabidopsis*. Silencing of the high-affinity ammonium transporter *PiAMT1* gene, whose transcripts are accumulating during nitrogen starvation and in barley, resulted in enhanced colonization of this host, whereas it had no effect on the colonization of *Arabidopsis*. Increased levels of free amino acids and reduced enzymatic activity for the cell-death marker VPE (vacuolar-processing enzyme) in colonized barley roots coincided with an extended biotrophic lifestyle of *P. indica* upon silencing of *PiAMT1*. This suggests that PiAmt1 functions as a nitrogen sensor mediating the signal that triggers the *in planta* activation of the saprotrophic program. Thus, host-related metabolic cues affect the expression of *P. indica*'s alternative lifestyles.

root cortical cell death | RCD | broad-host range | biotrophy | mutualism

Upon plant colonization, fungi adopt different strategies to gain access to host nutrients. Whereas necrotrophs kill plant cells with subsequent saprotrophic nutrition, other fungi maintain biotrophic relationships with their hosts either transiently (hemibiotrophs) or as lifelong interactions. The degree of specialization to a particular host and the host's metabolic status may greatly influence plant colonization (1–4). Broad-host range root endophytes undergo long-term interactions with a large variety of plants, thereby playing a significant role in natural and managed ecosystems and in the evolution of land plants. To establish and maintain a compatible interaction with different hosts, these endophytes must respond and adapt to host-specific signals. Alternative lifestyles and colonization strategies in different host species thus may be a consequence of this adaptation to highly variable host environments. In this study, we addressed the question of whether endophytes adopt different strategies during colonization of distinct hosts or whether their success resides in a general colonization strategy. An interesting system to explore this issue is the mutualistic root endophyte *Piriformospora indica* (Basidiomycota, Sebacinales), with its large number of plant hosts. Among others, this generalist can establish a mutualistic interaction with roots of the agriculturally important monocot barley (*Hordeum vulgare*) and the dicot model

plant *Arabidopsis thaliana* (5, 6), two biochemically and morphologically distinct plants. Cytological studies in both hosts have shown that *P. indica* has a biphasic colonization strategy (7–10). Initial root cell invasions are biotrophic where colonized host cells maintain membrane integrity and invasive hyphae of *P. indica* remain enveloped by the host plasma membrane and thus are not accessible to cell wall stains such as wheat germ agglutinin–Alexa Fluor 488 (WGA-AF488) conjugate (7, 8, 11). Later, *P. indica* is found more often in dead or dying host cells (9, 10), especially in the root cortex of barley. Colonization at later stages was shown to be reduced by overexpression of the negative cell death regulator BAX inhibitor 1 in barley and to be mediated by an endoplasmic reticulum stress-triggered caspase-dependent cell death in *Arabidopsis* (9, 10). Questions arise as to what extent hemibiotrophy in a root endophyte reflects a general colonization strategy of the symbiont to benefit from different plants and how the symbiotic lifestyle is influenced by the hosts. Our study reveals that broad compatibility in a root endophyte is associated with phenotypic plasticity of the symbiont and with the expression of alternative lifestyle strategies in a host-dependent way.

Results

Expression Patterns of *P. indica* Genes, Including Those Encoding Small Secreted Proteins, Support a Diversified Colonization Strategy for Barley and *Arabidopsis*. We hypothesized that successful root colonization of different hosts would require host-related colonization strategies, and we studied these differences by a global characterization of fungal transcriptional responses to barley and *Arabidopsis* at different developmental stages. A customized Agilent microarray was designed to monitor *P. indica*'s gene expression during root colonization of plants grown on sugar-free minimal medium (PNM) at 3 (early biotrophic phase) and 14 (late saprotrophic phase) days post inoculation (dpi). Fungal material grown on PNM was used as a control. From the 11,463 *P. indica* genes represented on the microarray chip, about 2,400 genes were differentially regulated at the early biotrophic phase in barley compared with the control. At this time point, 3,162 fungal genes were differentially regulated in colonized *Arabidopsis* compared with the control. At the late colonization stage, far more genes (4,482 genes) were differentially regulated in

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colonized barley than in colonized *Arabidopsis* roots (1,948 genes; Dataset S1). In total, about 70% (2,023) of the *in planta*-induced genes (2,861 genes) encoding intracellular proteins and 60% (277 of 463 induced genes) of those encoding putative secreted proteins had host-specific expression profiles (*SI Appendix*, Fig. S1). In particular, 123 of the 216 induced genes encoding small secreted proteins (SSPs; <300 amino acids), also known as putative effectors (12), were either *Arabidopsis* or barley responsive (Fig. 1*A*), suggesting that colonization of different hosts may require exploitation of distinct effectors that can interact with elements characteristic to each host. SSPs recently were shown to facilitate colonization by manipulating host defense and reprogramming plant metabolism during symbiosis (13, 14). Many of these proteins are cysteine rich (15) or possess distinctive features, such as a regular pattern of histidine and alanine residues found in all members of the *P. indica*-specific DELD family (7, 8). Eighteen of the 29 genes encoding *P. indica* DELD proteins were plant responsive and largely induced in barley but to a lesser extent in *Arabidopsis* (Fig. 1*B* and Dataset S2). Additionally, a small set of 21 SSPs were identified that are expressed in both hosts at comparable symbiotic stages (Fig. 1*A*). These SSPs may represent general determinants that target conserved recognition and signaling pathways in roots. Congruent with the broad definition of effectors (12), at the early symbiotic stage, 16 and 14 of *P. indica*'s top 20 up-regulated SSPs in *Arabidopsis* and in barley, respectively, encoded proteins with no known functional domains. A different situation was found at 14 dpi, when 50% of *P. indica*'s SSPs induced during colonization of barley, but not of *Arabidopsis*, encoded putative hydrolases (mainly glycoside hydrolase families GH10, GH11, and GH61; Dataset S3). Taken together, these data are consistent with a diversified colonization strategy for barley and for *Arabidopsis*, especially at 14 dpi, and prompted us to further characterize the two interactions at this stage.
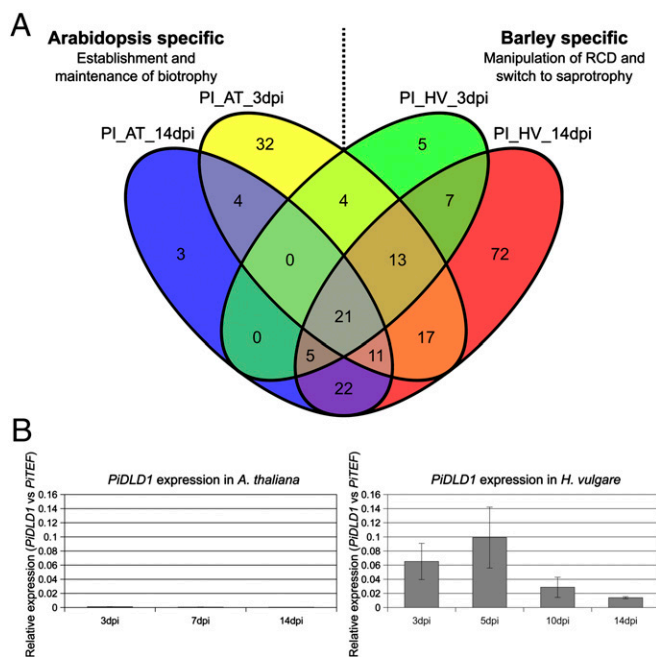

Fig. 1. Host-dependent expression profiles of *in planta*-induced *P. indica* genes encoding SSPs (<300 amino acids). (*A*) Number of SSPs significantly induced at 3 and 14 dpi during *Arabidopsis* (PI_AT) and barley (PI_HV) colonization, calculated vs. PNM control. (*B*) RT-qPCR analysis of *PiDLD1* (PIIN_05872), a member of the *P. indica*-specific putative effector family DELD, during colonization of *Arabidopsis* (*Left*) and barley (*Right*) at different time points from three independent experiments.

P. indica Undergoes Major Trophic, Phenotypic, and Transcriptional Rearrangements During Colonization of Barley, Whereas It Maintains a Predominant Biotrophic Nutrition in *Arabidopsis*.

Transcriptional data, together with cytological analyses, show that in barley, in response to a progressively increasing natural root cortical cell death (RCD) in older/basal root zones (16), *P. indica* undergoes a distinct nutritional shift from biotrophic to saprotrophic nutrition associated with the production of secondary thinner hyphae and with the secretion of hydrolytic enzymes (Fig. 2 *A–D* and *SI Appendix*, Fig. S2). Thus, after biotrophic colonization of the outermost cell layer (3–5 dpi), on its route toward the endodermis, the fungus progresses inter- and intracellularly by digestion of barley cortical cell walls, which became most evident at 30 dpi (Fig. 2*C*), but without visible root necrosis at the macroscopic level (10, 17). Host cell wall appositions, named papillae, often are visible beneath the site of fungal penetration attempts of living host barley cells (8), and at later colonization stages, hyphal contact with the endodermis, which is not penetrated (10), results in a strong autofluorescence of the host cell wall, indicating activation of plant defense upon attempted penetration of the root vasculature (*SI Appendix*, Fig. S3). Despite a predominant saprotrophic lifestyle, the presence of *P. indica* in barley leads to beneficial effects (6, 17).

Colonization of *Arabidopsis* by *P. indica* results in growth promotion (5) (*SI Appendix*, Fig. S4) and is characterized by a long-term feeding relationship with living host cells via the production of thicker bulbous invasive hyphae in epidermal cells and no sign for papillae formation upon penetration (Fig. 2 *F* and *G* and *SI Appendix*, Fig. S5). We show here that these intracellular, non–WGA-stainable multilobed invasive hyphae are present throughout the *Arabidopsis* colonization process, leading to a nondestructive progression within the epidermis and cortex layers, as demonstrated by colonized living root cells capable of endocytosis also at later time points (Fig. 2*F*). Consistent with this observation is the reduced expression of *P. indica* genes involved in host cell wall and lipid degradation at 3 and 14 dpi in this host compared with the situation in barley (Fig. 2*D* and *SI Appendix*, Fig. S2). The occurrence of a long-term biotrophic nutrition in *Arabidopsis* and of a switch to a saprotrophic nutrition in barley is supported further by comparative expression analyses of fungal genes involved in primary metabolism and nutrient transport. Whereas in colonized *Arabidopsis* fungal transcripts for amino acid biosynthetic processes and glycolysis are abundant, they show lower expression values in colonized barley at 14 dpi (*SI Appendix*, Fig. S6 and Dataset S4*A*). Conversely, at this time point, transcripts encoding fungal carbohydrate and nitrogen transporters are strongly induced during colonization of barley (Fig. 2*E* and *SI Appendix*, Fig. S7 and Dataset S4*B*). In particular, the high-affinity ammonium transporter PiAmt1 (PIIN_02036), whose transcripts are accumulating upon nitrogen starvation in axenic culture (*SI Appendix*, Fig. S8*A*), is induced during the late saprotrophic phase in colonized barley but to a much lower extent in colonized *Arabidopsis* (*SI Appendix*, Fig. S9 *A* and *B*), strongly indicating that in barley, but not in *Arabidopsis*, a status of nitrogen depletion is reached.

Transcripts for *P. indica* ABC transporters and other transporters most probably implicated in detoxification processes are well represented in both hosts, but to a greater extent in colonized *Arabidopsis* at 14 dpi (*SI Appendix*, Figs. S2 and S10). ABC transporters are efflux pumps that have been implicated in resistance to antifungal compounds in various host–fungal interactions, but little is known about their substrate specificities (18–20). Their host-dependent expression profiles therefore might represent a fungal stress response matched to distinct phytoalexins and other antifungal compounds produced by different hosts (21).

The Switch to Saprotrophic Nutrition in Barley Is Affected by Nitrogen Availability in a PiAMT1-Dependent Manner.

To address whether nitrogen availability is one of the key switches to saprotrophic nutrition *in planta*, *P. indica* RNAi strains carrying a silencing
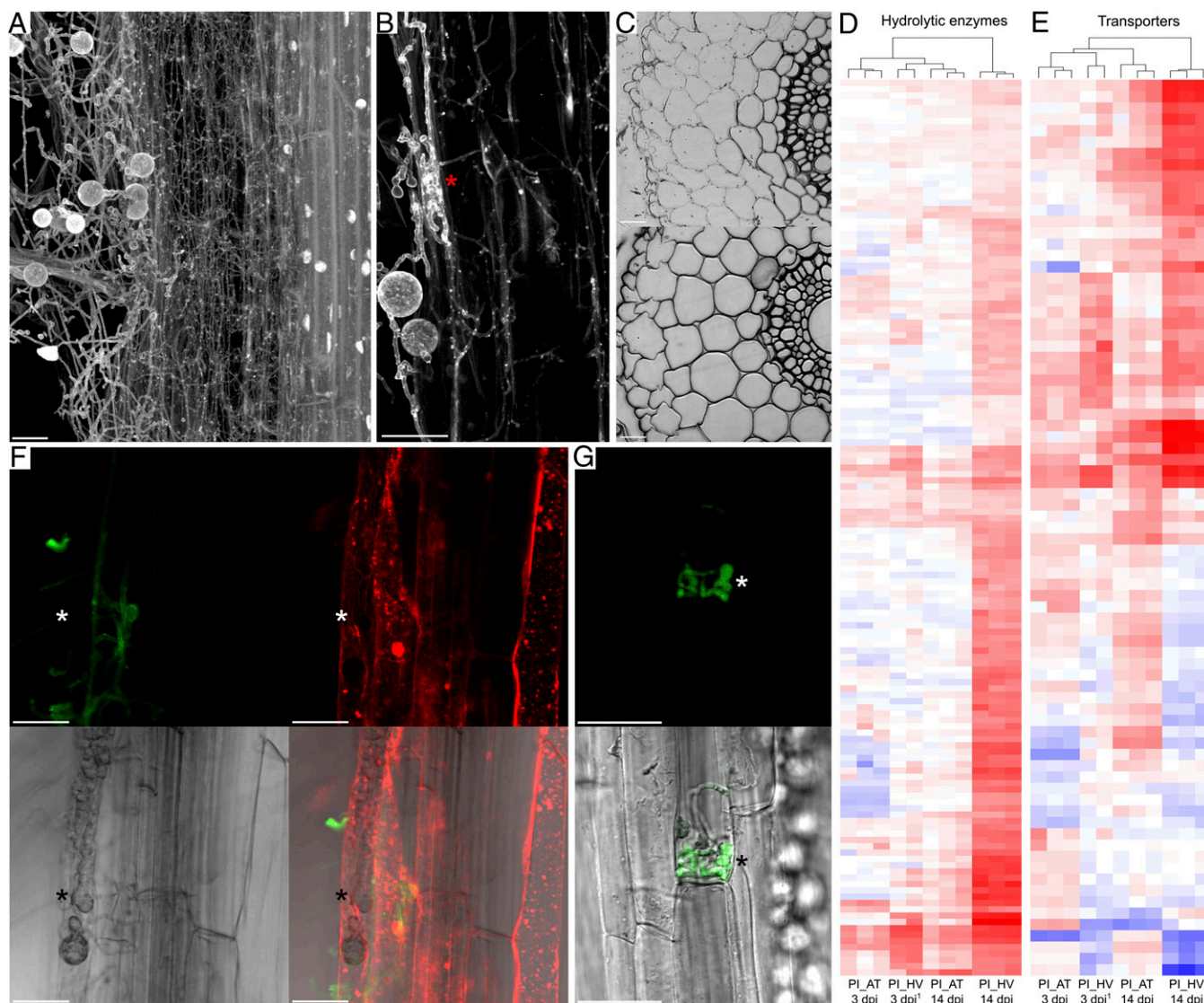
Fig. 2. Colonization patterns and *P. indica* gene expression polymorphisms correlate with extended biotrophy in *Arabidopsis* (AT) and with a switch from biotrophic to saprotrophic nutrition during colonization of barley (HV). (*A*) Maximum projection of a barley root colonized by *P. indica* at 30 dpi. Broad extraradical hyphae are visible at the boundary of the epidermis, whereas thin secondary hyphae are filling the cortical cells. Host nuclei are absent in the cortex cells while the cylinder is undamaged and preserves intact nuclei. The root was stained with acid fuchsine. (*B*) Closer view of biotrophic broad invasive hyphae within a barley epidermal cell and secondary hyphae in cortical cells (*). (*C*) Transverse 4-μm sections of barley roots inoculated with *P. indica* (30 dpi), stained with toluidine blue. (*Upper*) Heavily colonized cortex cells. (*Lower*) A noncolonized part of the root. (*D* and *E*) Heat map showing log$_2$-fold expression changes of *P. indica* genes encoding (*D*) hydrolytic enzymes and (*E*) transporters. Significant (*t* test, $P < 0.05$) log$_2$-fold expression changes were calculated vs. PNM control. A consistent divergence is observed at 14 dpi, where a strong induction is visible for most of these genes during colonization of barley only (for a detailed overview, see Dataset S1). [1]Raw expression data for barley 3 dpi were retrieved from ref. 8. Color coding indicates up-regulation (red) and down-regulation (blue) of genes. (*F*) Biotrophic broad invasive hyphae (white *) in *Arabidopsis* epidermal cell at 14 dpi. In contrast to extracellular hyphae, invasive hyphae are not stainable with WGA-AF488 (green) because of the presence of a plant-derived membrane. Endomembrane structures stained by FM4-64 (red) are visible inside the plant cells, indicating cell viability. (*G*) *Arabidopsis* epidermal cell with biotrophic invasive hyphae (white *) of *P. indica* GFP strain (14 dpi). The scale bars represent 25 μm.

construct targeting the high-affinity ammonium transporter *PiAMT1* were generated. The success of transformation for selected *P. indica* RNAi strains was confirmed by Southern blot, and the efficiency of silencing was verified by quantitative PCR (qPCR) experiments (Fig. 3*A* and *SI Appendix*, Fig. S11*A*). PiAmt1 displays strong homology (75% amino acid residues identity) to the high-affinity ammonium transporters AMT1 and AMT2 from *Hebeloma cylindrosporum* and to the high-affinity ammonium permease MEP2 from *Saccharomyces cerevisiae* (*SI Appendix*, Fig. S8*B*). These transporters were proposed to function as ammonium sensors, generating downstream signals in response to nitrogen starvation (22, 23). In yeast, MEP2 is

required for pseudohyphal growth, which is the outgrowth of nuclear-free hyphae to better forage the medium at low N availability (22, 24, 25). The ammonium import function of PiAmt1 was verified by yeast complementation (*SI Appendix*, Fig. S8*C*), and the predicted topological structure of the *P. indica* Amt1 polypeptide demonstrated the presence of a long cytoplasmic tail at the C-terminus, which might be involved in downstream signaling (*SI Appendix*, Fig. S8*D*). To verify that ammonium uptake by the *P. indica* RNAi strains is reduced relative to wild-type (WT) and empty-vector (EV) controls, these strains were analyzed for growth on minimal medium with a low concentration of ammonium as the sole nitrogen source. Their growth on complex
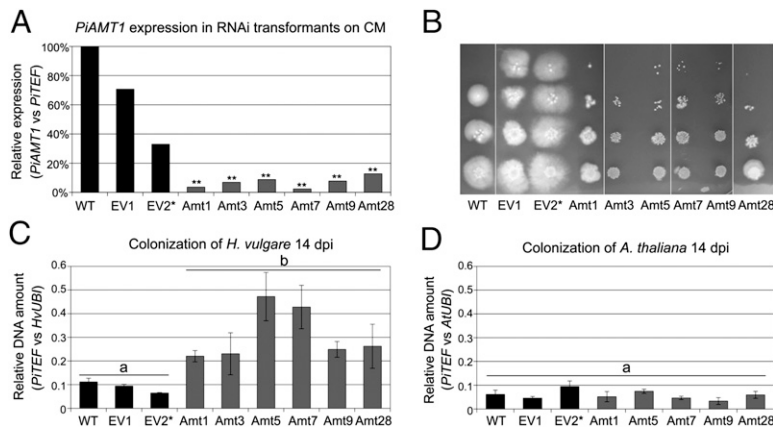
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Fig. 3. Silencing of *PiAMT1* affects *P. indica* colonization of barley but not of *Arabidopsis*. (*A*) Relative expression of *PiAMT* in *P. indica* WT and EV controls and RNAi strains grown in liquid complete medium (CM) for 7 d. **ANOVA, *P* < 0.01. (*B*) Colony phenotype of *P. indica* WT and transformants on yeast nitrogen base medium supplemented with 2 mM NH_4Cl as the sole nitrogen source, 14 dpi. (*C* and *D*) Relative amount of fungal DNA in (*C*) barley and (*D*) in *Arabidopsis* roots colonized by *P. indica* WT or transformants at 14 dpi. Plants were grown on 1/10 PNM. Error bars represent SE of the mean from three independent biological repetitions. Grouping was done by ANOVA. *Southern blot analyses showed that all transformed strains had one to two integrations of the plasmids, with the exception of EV2, which had multiple integrations (*SI Appendix*, Fig. S11).

media and media supplied with large amounts of ammonium was not affected (*SI Appendix*, Fig. S11*B*), whereas hyphal growth at low N was suppressed (Fig. 3*B*). The RNAi strains were investigated further for altered root colonization. Despite a substantial reduction in growth under a low supply of ammonium as the sole nitrogen source and the absence of any evidence for a compensatory up-regulation of the second ammonium transporter *PiAMT2* (*SI Appendix*, Fig. S9*C*), the RNAi strains displayed a significantly increased colonization of barley roots at 14 dpi compared with the WT and EV controls, calculated as the ratio of fungal DNA to plant DNA (elogation factor *PiTEF*/ubiquitin *HvUBI*) (Fig. 3*C*) or as the ratio of *PiTEF* to *HvUBI* transcripts (*SI*
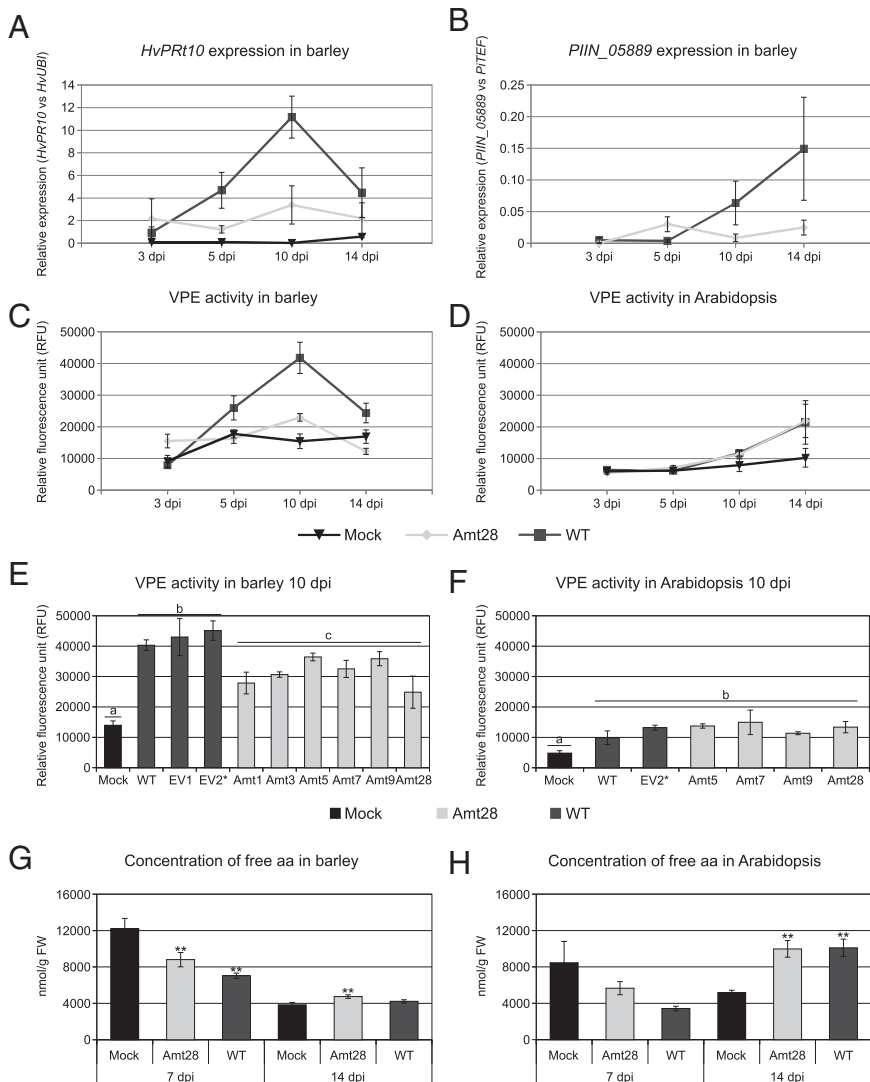

Fig. 4. Gene expression profiles, VPE-like enzymatic activity, and amino acid levels in barley roots suggest extended biotrophy in *P. indica* AMT1-RNAi strains compared with WT. (*A*) Relative expression of the plant defense-related gene *PR10* and (*B*) of the *P. indica* gene PIIN_05889 encoding a putative xylanase, during colonization of barley by the *P. indica* WT and RNAi strain Amt28 at different time points. Expression data are standardized relative to *HvUBI* or to *PiTEF*. SEs are calculated from three independent biological repetitions. (*C*) VPE-like enzymatic activities during biotrophic (≤5 dpi) and cell death-associated colonization (≥10 dpi) of barley roots colonized by *P. indica* WT and RNAi strain Amt28 or mock treated. For the assay, the fluorescent VPE-specific substrate Ac-ESEN-MCA was added to the root extracts for spectrophotometric determination of enzymatic activities (for details, see *SI Appendix*). (*D*) VPE-like enzymatic activities during early (3 and 5 dpi) and late (10 and 14 dpi) colonization of *Arabidopsis* roots. (*E*) VPE-like enzymatic activities at the onset of saprotrophy (10 dpi) in barley roots and (*F*) in *Arabidopsis* roots. (*G*) Concentrations of free amino acids in the roots of barley noncolonized or colonized by *P. indica* WT and RNAi strain Amt28 at 7 and 14 dpi. (*H*) Concentrations of free amino acids in the roots of *Arabidopsis* at 7 and 14 dpi. Error bars represent SEM from three to four independent biological repetitions. **ANOVA, *P* < 0.01. Columns not sharing a letter are significantly different (ANOVA, *P* < 0.01).

Appendix, Fig. S9D). The temporal expression pattern for transcripts encoding the barley pathogenesis-related gene PR10 was examined to determine the plant's response to colonization by the *P. indica* WT and the RNAi strain Amt28. A decreased transcript accumulation for the PR gene in roots colonized by the RNAi strain compared with the WT strain at the onset of saprotrophy was observed (Fig. 4*A*). Consistent with this, expression of the *P. indica* saprotrophic marker gene, PIIN_05889 (encoding a putative xylanase), was reduced in the RNAi strain compared with *P. indica* WT (Fig. 4*B*). This indicates an extended biotrophic phase of the RNAi strain Amt28, which likely contributes to the enhanced colonization of barley roots at 14 dpi. To verify this hypothesis, we measured the activity of the vacuolar processing enzyme (VPE). This is a cysteine protease with caspase-like activity responsible for the maturation of various vacuolar proteins in higher plants and reported to be induced in dying cells (9, 26). VPE-like enzymatic activity increased significantly in barley roots colonized by *P. indica* WT compared with barley mock treated and barley roots colonized by the RNAi strain Amt28, especially at the onset of saprotrophy (Fig. 4*C*). Analyses of the VPE-like enzymatic activity performed at 10 dpi with the other RNAi strains and EV controls confirmed this finding (Fig. 4*E*). Remarkably, in the RNAi strains, elicitation of growth promotion was not affected compared with the WT situation (*SI Appendix*, Fig. S12). This indicates that an extended biotrophic phase does not seem to influence *P. indica*'s beneficial effects on barley.

Consistent with low expression of the *PiAMT1* gene in *Arabidopsis* at 14 dpi, analyses of the *P. indica* RNAi strains impaired in ammonium uptake and possibly ammonium sensing displayed no difference in their colonization patterns compared with the WT and EV controls (Fig. 3*D* and *SI Appendix*, Fig. S9E). Similarly, no differences in the VPE-like enzymatic activity were observed in *Arabidopsis* roots colonized by *P. indica* WT or RNAi strain Amt28, in which both displayed moderate increased activity compared with mock control at later stages (Fig. 4 *D* and *F*). We therefore concluded that PiAmt1 is not required for biotrophic growth but is involved in the lifestyle switch of *P. indica* to saprotrophic growth as observed in barley.

Quantification of free amino acid levels in barley roots by ultra-pressure reversed-phase chromatography (see *SI Appendix* for details on this method) showed that amino acid concentrations decreased significantly in colonized roots by *P. indica* WT compared with roots colonized by the RNAi strain Amt28 and control roots at the onset of the saprotrophic stage/late biotrophic stage (Fig. 4*G* and *SI Appendix*, Fig. S13). At 14 dpi, the concentrations of free amino acids were remarkably lower than at 7 dpi in the older/basal root zone of barley, irrespective of colonization (Fig. 4*G* and *SI Appendix*, Fig. S13). In particular, asparagine and glutamine decreased by approximately five- and sevenfold, respectively, in control roots (*SI Appendix*, Fig. S13). This might be the result of reallocation of nitrogen to younger root zones in response to developmental RCD (16) and is in agreement with the microarray data, which indicate limited nitrogen availability to *P. indica* in the basal root zone of barley at 14 dpi. In *Arabidopsis*, colonization by *P. indica* WT decreased amino acid concentrations 7 dpi (Fig. 4*H* and *SI Appendix*, Fig. S13), whereas the level of free amino acids increased significantly 14 dpi (Fig. 4*H* and *SI Appendix*, Fig. S13). The altered organic nitrogen allocation upon *P. indica* colonization at 14 dpi was mainly the result of changes in asparagine and glutamine (*SI Appendix*, Fig. S13) and suggests that nitrogen supply to the fungus is not limited in this phase. Biotrophic and hemibiotrophic fungi have been shown to induce plant nitrogen mobilization and accumulation at the site of infection. In particular, nitrogen-rich amino acids such as glutamine and asparagine have been identified as the major forms of nitrogen reallocation during infection of different plant hosts (27–29). Changes in free amino acid pools during biotrophy have been speculated to reflect the demand for organic nitrogen by the fungus or to be required to launch defense responses by the host (27, 30).

Asparagine and glutamine are the preferred nitrogen source of *P. indica* in axenic culture, and when grown on these amino acids as the sole nitrogen source, *P. indica* produces enlarged hyphae that resemble the multilobed biotrophic hyphae *in planta* (*SI Appendix*, Fig. S14). On nitrate-containing medium, known to be a poor nitrogen source for *P. indica* (8), or on medium without nitrogen, *P. indica* hyphae are thin and less branched, similar to the secondary hyphae found in barley at later colonization stages (*SI Appendix*, Fig. S14). Asparagine and glutamine therefore may represent a ready source of organic nitrogen during biotrophy.

Taken together, these results strongly suggest that host-related nutritional cues affect *P. indica*'s lifestyle and that the switch from biotrophic to saprotrophic nutrition during colonization of barley is affected by PiAmt1 and by nitrogen availability.

Discussion

Establishment of biotrophy during colonization of *Arabidopsis* and barley by *P. indica* is an important feature of the symbioses with both plant hosts, and it implies a strong interdependence between host metabolism and fungal nutrient uptake in this endophyte. Transcriptional profiling revealed that far fewer genes need to be induced to maintain biotrophy compared with those necessary for coordinating the switch to saprotrophy and the manipulation of RCD. Evolution of obligate biotrophy was shown to correlate, among others, with the loss of genes involved in nitrate metabolism, and it is speculated that host plants provide a ready source of organic nitrogen in the form of amino acids (15). In the draft genome of *P. indica*, no nitrate transporters or reductases were found (8), indicating that nitrate is not essential for this symbiont. *P. indica* can grow on ammonium and on glutamine and asparagine, which are its favorite nitrogen sources. The low expression of the *P. indica* high-affinity ammonium transporter in *Arabidopsis* at later colonization stages suggests that
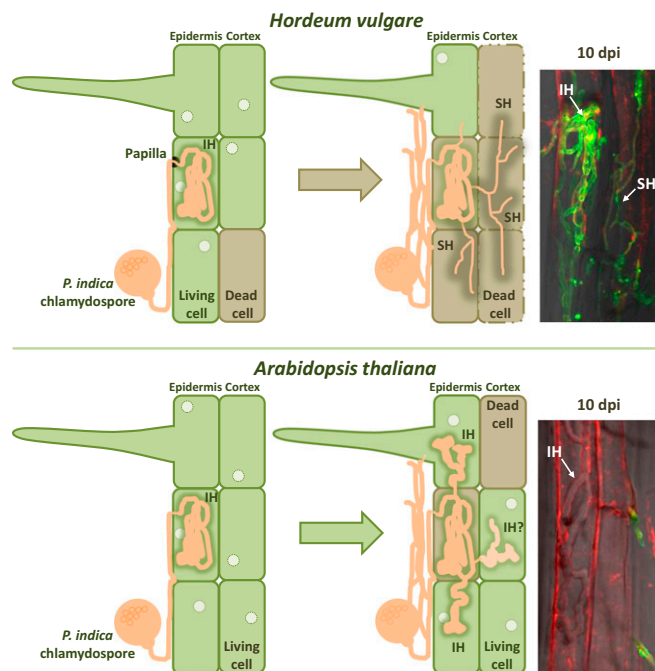

Fig. 5. Schematic representation of *P. indica* colonization strategies at different symbiotic stages in barley and in *Arabidopsis*. (*Upper*) Invasive hyphae (IH) and secondary thin hyphae (SH) of *P. indica* in barley dead cells (10 dpi). (*Lower*) *P. indica* non–WGA-stainable biotrophic broad invasive hyphae in an *Arabidopsis* epidermal cell (10 dpi). Fungal structures were stained with WGA-AF488 (green); membranes were stained with FM4-64 (red). Staining of fungal structures by WGA-AF488 is enhanced in dead host cells. In *H. vulgare* roots, cell death is initiated a few days after germination by developmental RCD (16) and is increased by *P. indica* colonization.

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an adequate source of nitrogen is provided by this host during colonization. Increased concentrations of free amino acids, principally of glutamine and asparagine upon *P. indica* colonization of this host at 14 dpi, strongly support this conclusion. The induction of *PiAMT1* and other fungal nitrogen transporters in barley leads to the assumption that this plant cannot provide *P. indica* with sufficient organic nitrogen during the onset of the RCD program and that nitrogen depletion therefore may function as a trigger for the *in planta* expression of fungal genes encoding hydrolytic enzymes and for the activation of the saprotrophic program. Silencing of the high-affinity ammonium transporter *PiAMT1* by RNAi resulted in reduced expression of fungal xylanase, barley defense response, and VPE activity in colonized roots, supporting the hypothesis that PiAmt1 is involved in sensing the N status and in downstream signaling upon nitrogen depletion in *P. indica*. Because biotrophic growth of *P. indica* in *Arabidopsis* was independent of PiAmt1, we conclude that expression and a signaling function of PiAmt1 are needed for the switch of *P. indica*'s lifestyle to saprotrophy. Loss or suppression of the expression of *PiAMT1* would represent a step toward a progressively more intimate biotrophic association with its hosts. The maintenance of this gene at the expense of biotrophy might benefit *P. indica* during prolonged saprotrophic growth on decaying plant material, making this fungus also able to survive in the absence of living hosts or on dying host cells.

Plant-associated fungi are either specialists, which are adapted to one or a few distinct hosts, or generalists that can thrive in highly variable host environments. Specialists and their hosts are in an evolutionary arms race that leads to the development of fungal tools and colonization strategies that are efficiently tailored to the respective host. Conversely, broad-host range species must evolve adaptations to cope with a plethora of different host-associated signals and host-specific defense mechanisms. The evolutionary force, in this case, possibly drives the expansion and diversification of the fungal toolkit and the host-adapted gene expression to better suit different plants. Recently it was shown that the obligate biotrophic ascomycete powdery mildew pathogen, *Blumeria graminis* f. sp. *hordei*, which can grow and reproduce only on living cells of its natural host, barley, displays a conserved transcriptional program during early pathogenesis on barley and on immunocompromised *Arabidopsis* (31). Although we cannot exclude that at early time points (<2 dpi, prepenetration stage) a certain conservation of the transcriptional program may be present, our data show that the broad-host range fungal root symbiont *P. indica* responds differently to divergent hosts, especially at later time points during establishment and maintenance of the intracellular biotrophic interaction.

In conclusion, the transcriptional and phenotypic plasticity of *P. indica* during symbioses (summarized in Fig. 5) establishes a highly adaptive capacity in a root endophyte with broad compatibility that can reconfigure itself and its lifestyle in response to different environmental and host signals.

Material and Methods

Microarray Analyses. Microarray experiments were performed with total RNA extracted from *P. indica*-inoculated barley and *Arabidopsis* roots. As a control, total RNA from *P. indica* grown on 1/10 PNM–agar was used. Root samples from three independent biological replicates were labeled and hybridized according to Agilent's One-Color Microarray-Based Gene Expression Analysis Low Input Quick Amp Labeling protocol. For details, see *SI Appendix*.

RNAi Vector Construction and *P. indica* Transformation. A 570-bp fragment of the *PiAMT1* gene was amplified by PCR (Dataset S5) from cDNA and inserted in the EcoRV site of the convergent dual-promoter vector pPiRNAi (17). *P. indica* was transformed with vector pPiRNAi-AMT1 and the EV control as described in ref. 17. For details, see *SI Appendix*.

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