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


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Edited by Diter von Wettstein, Washington State University, Pullman, WA, and approved September 14, 2006 (received for review July 10, 2006)

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.

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 fungi (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

This paper results from the Arthur M. Sackler Colloquium of the National Academy of Sciences, “From Functional Genomics of Model Organisms to Crop Plants for Global Health,” held April 3–5, 2006, at The National Academy of Sciences in Washington, DC. Papers from this Colloquium will be available as a collection on the PNAS web site. The complete program is available on the NAS web site at www.nasonline.org/functionalgenomics.

Author contributions: K.-H.K. designed research; S.D., P.S., J.I., M.S., and M.W. performed research; S.D., R.H., P.S., M.W., and F.W. analyzed data; and R.H., F.W., and K.-H.K. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS direct submission.

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

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

**Results**

**\( P. \text{indica} \) Belongs to the Recently Defined Order Sebacinales.** Based on the nuclear genes coding for the large ribosomal subunit (nuLSU), available strains of the \( \text{Sebacina vermifera} \) species complex (Sebacinales group B) are closely related to \( P. \text{indica} \) (Fig. 1). We addressed the question whether strains of the \( S. \text{vermifera} \) complex exhibit comparable biological activities as \( P. \text{indica} \). To this end, barley seedlings were inoculated with \( P. \text{indica} \) or different isolates of \( S. \text{vermifera} \) 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 \( P. \text{indica} \). 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 \( B. \text{graminis} \) f.sp. \( \text{hordei} \), and powdery mildew pustules were counted after 7 days. We found consistent resistance-inducing activity of all strains of the \( S. \text{vermifera} \) 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. 2a). 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. 2b–e). In young differentiated root tissue, the fungus then often colonizes and completely fills up single cells (Fig. 2f and g) before adjacent cells are colonized, whereas an unrestricted net-like intra- and intercellular colonization pattern is observed in mature parts. Intracellularly growing hyphae show necks at sites where the fungus traverses a cell wall (Fig. 2h). 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. 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, with the protoplasts covered by a dense meshwork of fungal hyphae. Eventually arrays of single spores developed from intracellular hyphal tips (Fig. 3b 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

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 species or cultures are printed in bold. Sebacinales 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; C, Canada; CH, People’s Republic of China; ECU, Ecuador; EST, Estonia; FRA, France; GER, Germany; GUY, Guyana; IND, India; MEX, Mexico; NOR, Norway; and SPA, Spain.

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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 visualize 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 revealed fungal development in intercellular spaces and formation, chlamydospores also were generated in the mycelial mats at the root surface.

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

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.

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. 3f). 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. 3i). 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).
Quantitative PCR analysis of HvBI-1 expression showed it slowly increasing during root development throughout the course of the experiment (Fig. 4a). 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. 4a). 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 website). 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. 4b).

**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.
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 chlamydomospores, 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. 2h). In either case, fungal colonization proceeds by intra- and intercellular infestation of surrounding tissue and gradually increases with tissue matura- tion. 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 the fungus with living tissue contributes to host reprogramming and, finally, cell death.

![Association of fungal structures with living and dead cells of the host tissue.](https://www.pnas.org/cgi/doi/10.1073/pnas.0605697103)

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 fuchsia-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.).]
Therefore, indicates that PCD observed in the interaction with controlled by defense. It remains to be shown what kind of PCD might be different from hypersensitive cell death in pathogen indica. HvBI-1 tionally confirm the role of host PCD and a requirement of significant reduction of invasive growth of P. indica-colonized roots showed a significant reduction of HvBI-1 expressed in barley. All transgenic lines showed enhanced resistance 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 of 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 cavendishoid mycorrhizas (14) with certain hemiepiphytic eri-
The 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 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, seedlings were immersed in an aqueous solution of 0.05% Tween-20 containing 5 × 10^7 ml⁻¹ *P. indica* chlamydomospores or homogenized mycelial solution (1 g/ml) of *S. vermifera*, respectively. Inoculated seedlings were grown in a 2:1 solution of 0.05% Tween-20 containing 5 ml in situ 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 chin-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−ΔΔCt 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 DNAs easy 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 in triplicate. The oligonucleotides used were as a template for quantitative PCR with gene-specific primers. The plant-specific first-strand cDNA were subsequently used as a template for 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 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^ΔCt) 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′-CAGTAGTG-GCGGTGCGAGTG-3′ and 5′-ACCCCTCGCGGACTACAAA-

CAT-3′; P. indica Tef (accession no. AJ249911) 5′-ACCGTCTT-TGGGTTGTATCC-3′ and 5′-TCGCGTGTGCAA-

CAAGATG-3′; Bax inhibitor-1 (accession no. AJ290421) 5′-

GTCCACCTCAAGTCTGTTT-3′ and 5′-ACCCCTGAC-

GAGATGTCT-3′; and P. indica ITS (accession no. AF

019636) 5′-CAACACATGTCAGTCTGAT-3′ and 5′-

CCTATGCTATCAGAACGA-3′.

We thank Valiollah Babaezad for propagating the GFP:: HvBI-1 plants and Dagmar Biedenkopf for technical assistance.