The Mycorrhiza Fungus *Piriformospora indica* Induces Fast Root-Surface pH Signaling and Primes Systemic Alkalinization of the Leaf Apoplast Upon Powdery Mildew Infection

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Submitted 11 December 2008. Accepted 14 May 2009.

We analyze here, by noninvasive electrophysiology, local and systemic plant responses in the interaction of barley (*Hordeum vulgare* L.) with the root-colonizing basidiomycete *Piriformospora indica*. In the short term (seconds, minutes), a constant flow of *P. indica* chlamydospores along primary roots altered surface pH characteristics; whereas the root-hair zone transiently alkalinized—a typical elicitor response—the elongation zone acidified, indicative of enhanced H⁺ extrusion and plasma membrane H⁺ ATPase stimulation. Eight to 10 min after treating roots with chlamydospores, the apoplastic pH of leaves began to acidify, which contrasts with observations of an alkalinization response to various stressors and microbe-associated molecular patterns (MAMPs). In the long term (days), plants with *P. indica*-colonized roots responded to inoculation with the leaf-pathogenic powdery mildew fungus *Blumeria graminis* f. sp. *hordei* with a leaf apoplastic pH increase of about 2, while the leaf apoplast of noncolonized barley responded to *B. graminis* f. sp. *hordei* merely with a pH increase of 0.8. The strong apoplastic pH response is reminiscent of *B. graminis* f. sp. *hordei*-triggered pH shifts in resistance gene-mediated resistant barley leaves or upon treatment with a chemical resistance inducer. In contrast, the MAMP N-acetylchito-octaose did not induce resistance to *B. graminis* f. sp. *hordei* and did not trigger the primed apoplastic pH shift. We speculate that the primed pH increase is indicative of and supports the potentiated systemic response to *B. graminis* f. sp. *hordei*-induced by *P. indica* in barley.

Piriformospora indica is the model species for the recently delineated fungal order *Sebacinales* (phylum *Basidiomycota*) that comprises a great variety of root-colonizing mycorrhiza fungi with a broad host range among mono- and dicotyledonous plants (Schäfer and Kogel 2009; Schäfer et al. 2007; Verma et al. 1998). The symbiotic fungus bears an attractive agronomic prospect, since root colonization of host plants confers resistance to root as well as shoot diseases and tolerance to abiotic stress (Baltruschat et al. 2008; Deshmukh et al. 2006; Stein et al. 2008). In barley, a significant increase in grain yield was observed under outdoor conditions when seedlings were inoculated with the fungus (Waller et al. 2005).

Colonization of barley roots with *P. indica* involves chlamydospore germination and extracellular colonization of the root within 1 to 2 days after inoculation (dai), penetration of single rhizodermal cells, and formation of the first intercellular hyphae 3 dai. At 7 dai, large areas of the root are colonized by inter- and intracellular hyphae, and from about 10 dai onward, fungal sporulation is frequently observed. The fungus does not enter vascular tissue nor is it detectable in stems or leaves. Instead, it shows a preference for developmentally ‘older’ tissue, since it largely colonizes the root-hair zone rather than the elongation and meristematic zone proximal to the root tip (Deshmukh et al. 2006). Despite an extensive colonization by *P. indica*, roots do not show any macroscopic evidence for tissue impairment or necrotization. Instead, root and shoot biomass is considerably enhanced by fungal colonization (Waller et al. 2005).

Among the benefits transferred to cereal hosts are resistance against necrotrophic root and stem base pathogens, such as *Fusarium* spp. and *Pseudocercospora herpotrichoides*, as well as systemic resistance against the leaf powdery mildew pathogen *Blumeria graminis* (Deshmukh and Kogel 2007; Serfling et al. 2007; Waller et al. 2005). The molecular mechanism of systemic resistance conferred by *P. indica* to powdery mildew was studied in *Arabidopsis thaliana*. Genetic dissection of the resistance reaction revealed requirement of an operative jasmonate pathway for systemic responses to powdery mildew fungus *Golovinomyces orontii* (Stein et al. 2008), reminiscent of the induced systemic resistance (ISR) pathway activated by rhizobacteria (Van Wees et al. 2008). In barley, gene expression profiling of leaves from *P. indica*-colonized plants showed few systemic changes in gene expression (Waller et al. 2008), which is consistent with an ISR-like resistance mechanism (Van Loon et al. 1998). On the other hand, gene expression profiling of colonized barley roots at 1, 3, and 7 dai with *P. indica* revealed differential regulation of about 1,300 genes out of approximately 40,000 probe sets present on a microarray (Schäfer et al. in press).

Successful colonization of roots by *P. indica* requires plant defense suppression, although the fungus, by the same time, induces local and systemic resistance to fungal pathogens once the symbiosis is established. Hence, we were interested in analyzing, preferably early, local and systemic responses of the plant root and leaf to an encounter with the symbiotic fungus. Using established electrophysiological techniques, we investigated the immediate effects of the first encounter of barley

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roots with *P. indica* chlamydospores and the consecutive transfer of signals from roots to leaves. Since protons are multifunctional in that they i) provide the basic condition in which biochemical reactions take place, ii) are consumed or are produced in such reactions, iii) are part of the transmembrane driving force, and iv) represent a signaling compound, the knowledge of their activity is indispensable for a more comprehensive understanding of host-microbe interactions. Leaf apoplastic proton activity has been visualized by noninvasive microprobe techniques in the interaction of barley with powdery mildew fungus *Blumeria graminis* f. sp. *hordei* (Felle et al. 2004). These investigations revealed a close correlation between pathogen-induced local pH changes and defense responses conferred by resistance (*R*) genes. Here, we demon-

strate that the barley root responds within seconds to the first contact with chlamydospores of *P. indica* with specific shifts in the surface pH and that signals evoked there are rapidly transferred to the leaves. In the long term (days), the contact of the root with chlamydospores primes leaves for a strong apoplastic alkalization and disease resistance in response to challenge inoculation with the powdery mildew fungus. This alkalization reaction is reminiscent of the apoplastic pH changes induced in barley both by treatment with the chemical resistance inducer benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester (BTH) or in resistance (*R*) gene-mediated powdery mildew resistance.

RESULTS

Short-term effects of *P. indica* chlamydospores on surface pH of barley roots.

The surface pH of plant primary roots is a sensitive parameter for detecting metabolic changes activated or elicited by microbe-associated molecular patterns (MAMPs) and other elicitors. Roots of *Medicago sativa* have been demonstrated to respond specifically to Nod factors and to chitin fragments with a variety of ion fluxes (Felle et al. 1998). The surface pH is not uniform along the root but displays a typical pattern along the different zones, which seems to be generated by different transport activities (Felle 1998; Monshausen et al. 1996; Weisenseel et al. 1979). In order to test for a response of the barley root to chlamydospores, we recorded the pH profile over the different root zones along the first 7 mm from the tip of the primary root before (control) and 30 min after the addition of chlamydospores (Fig. 1A). At the elongation zone (EZ) of 3-day-old seedlings, the surface pH was less acidic than the medium bulk pH, while it was more acidic in the root hair zone (RHZ). After addition of chlamydospores (2×10^4 to 5×10^4 spores ml^{-1}), the difference of the outer pH around the different zones was less pronounced, i.e., the pH in EZ was more acidic (mean $\Delta\text{pH } 0.22 \pm 0.09$ standard error [SE]; $n = 5$), while the pH in RHZ was less acidic (mean $\Delta\text{pH } 0.12 \pm 0.05$ SE) compared with untreated roots. As shown by a kinetic analysis in the range of 140 min after inoculation, the response of RHZ to chlamydospores was a delayed transient alkalization peaking about 5 min after the first encounter (Fig. 1B, mean of maximal pH change 0.17 ± 0.04 SE; $n = 8$). Removing the chlamydospores had no significant effect. Consistently, a second succeeding inoculation of the roots with chlamydospores 2 h after the first encounter yielded only a minor pH change indicating that the barley root responded refractory (Fig. 1B). Such a behavior has been found to be typical for symbiotic interactions, e.g., for the responses of root hairs (depolarization, surface pH) to Nod factors (Felle et al. 1998). Tests with *N*-acetylchito-octaose (Glc8) and dead chlamydo-

Fig. 1. Surface pH responses of barley roots to the addition of *Piriformospora indica* chlamydospores, measured with a pH-sensitive microelectrode at a distance of 5 μm from the surface. **A**, Surface profiles were taken before (control) and 30 min after the addition of *P. indica* chlamydospores. Bulk pH denotes the pH in the medium at least 5 mm away from the root surface. C = calyptra, MZ = meristematic zone, EZ = elongation zone, and RHZ = root hair zone. Representative example of 5 equivalent tests. **B**, Immediate surface pH response of a barley root to the addition of active or dead *P. indica* chlamydospores or 0.1 μM *N*-acetylchito-octaose (Glc8), as indicated. The pH electrode was positioned within the root-hair zone (arrow of inset). Once a stable pH baseline was attained during flow-through (3 to 4 ml of medium per min), the medium (with the spores or Glc8) was added and the pH response was measured. After removing the active spores from the solution (W), spores were added again after about 100 min. Response time was extrapolated after the experiment by adding a solution of pH 7 (not shown). Kinetics are representative of eight equivalent tests (active spores) and three tests with dead spores or Glc8. **C**, As in B, except the electrode position is within the elongation zone (arrow of inset). Spores were added after a stable pH baseline was attained. Kinetics are representative of four equivalent tests each.

Fig. 2. Short-term response of the barley leaf apoplast to root encounter with *Piriformospora indica* chlamydospores (P.i., three independent experiments) or to 0.1 μM *N*-acetylchito-octaose (Glc8). Apoplastic pH was measured with microelectrodes positioned in the cavities of open stomata. The distance from the root to the leaf was about 24 cm.

spores yielded similar kinetics, whereby the roots responded more strongly to the chitin fragments and more weakly to the dead spores as compared with the active spores (Fig. 1B). The immediate pH response of EZ to chlamydospores was different. As shown in Figure 1C, after a delay of 10 to 15 s, surface pH first decreased and then turned into a damped oscillation. Whereas the roots responded with a similar oscillation to dead spores, addition of the chitin fragments caused a transient pH increase, like the one observed in the RHZ (Fig. 1C). This indicates that the pH response to chlamydospores of *P. indica* is not simply based on elicitor-active chitin from chlamydospore walls.

Short-term direct effects of root inoculation with *P. indica* on barley leaf apoplastic pH.

Chlamydospores added to the roots of intact 3-week-old barley plants caused an acidification of the leaf apoplast (Fig. 2). At 5 to 8 min after flushing the chlamydospores along the root, leaf apoplastic pH started to decrease and, after peaking after about 60 min, partly recovered. The maximal pH change was between 0.5 and 1.1 (mean 0.86 ± 0.21 SE, $n = 5$). This data demonstrates that the mycorrhiza fungus induces an immediate strong and systemic response in the leaf well before hyphal colonization of the root tissue. The chitin oligomer Glc8, which transiently increases the root surface pH (Fig. 1B and C), did not affect leaf apoplastic pH.

Priming of leaf apoplast pH by *P. indica* root colonization.

Apoplastic pH increase is a typical response of barley leaves to an inoculation with conidia of the powdery mildew fungus *B. graminis* f. sp. *hordei*. Notably, the magnitude and the kinetic profile of alkalization were shown to be associated with the defense phenotype conferred by specific *R* genes (Felle et al. 2004, 2005). In a compatible interaction with *B. graminis* f. sp. *hordei*, barley leaves display merely a minor pH response to fungal inoculation, while incompatible interactions governed by *R* gene *Mlg* or *Mla12*, in contrast, result in a massive pH increase of over 2 pH units (Felle et al. 2004). Consistently, an increase in leaf apoplastic pH was also seen in barley challenged by *B. graminis* when plants were pretreated with the resistance inducer BTH (Felle et al. 2008). Since it has also been shown that root colonization by *P. indica* results in induced systemic resistance to powdery mildew (Waller et al. 2005), we addressed the question of whether *P. indica* mimics a chemical resistance inducer or *R* genes on the level of leaf

Fig. 3. Apoplastic long-term pH response of barley, which is susceptible to powdery mildew, to various treatments. Pretreated = two examples of the apoplastic pH response of a plant (susceptible to *Blumeria graminis* f. sp. *hordei* A6) after root inoculation with *Piriformospora indica* and inoculation with *B. graminis* f. sp. *hordei* A6 conidia (mean maximal pH increase of 1.82 ± 0.24 standard error [SE]; $n = 5$). Control-1 = apoplastic pH of a plant inoculated with *B. graminis* f. sp. *hordei* A6 (mean maximal pH increase of 0.81 ± 0.16 SE; $n = 5$). Control-2 = apoplastic pH response of a plant after root inoculation with *P. indica* (mean maximal pH increase of 0.27 ± 0.05 SE; $n = 5$). Glc8 = apoplastic pH response of a plant after root treatment with 0.1 μ M *N*-acetylchito-octaose.

apoplastic pH changes. To this end, we compared apoplastic leaf pH changes in barley cv. Ingrid, either root-inoculated or not inoculated with *P. indica*, upon challenge with the virulent powdery mildew race *B. graminis* f. sp. *hordei* A6. In *P. indica*-inoculated plants, a strong increase in leaf pH of >2 was recorded in the time range of about 25 to 55 h, with a peak at about 40 h after inoculation (hai). Root-inoculation with *P. indica* without subsequent challenge with *B. graminis* f. sp. *hordei* A6 conidia or *B. graminis* f. sp. *hordei* challenge of leaves from *P. indica*-free plants did not cause this leaf apoplastic pH increase, neither did 0.1 μ M Glc8 added to the roots (Fig. 3).

Effect of chlamydospores and Glc8 on systemic powdery mildew resistance.

In order to substantiate in the above experimental setup that root inoculation with *P. indica* chlamydospores leads to enhanced resistance against powdery mildew, we assessed colonization of barley leaves with *B. graminis* f. sp. *hordei* A6 in a leaf segment test. At 3 days after treatment of roots of hydroponically grown plants with chlamydospores or 0.1 μ M Glc8, leaves were detached and inoculated with powdery mildew conidia. After 6 days, the number of powdery mildew pustules was determined (Fig. 4). Compared with mock-treated control plants, root inoculation with chlamydospores led to a 22% reduction of powdery mildew pustules. In contrast, treatment of Ingrid roots with Glc8 did not result in a reduction of *B. graminis* f. sp. *hordei* colonies, while BTH (40 mg/liter) used as a positive control exhibited a strong inhibitory effect (44% reduction) on *B. graminis* f. sp. *hordei* development.

DISCUSSION

Here, we demonstrate local and systemic effects of *P. indica* chlamydospores on root surface pH and on apoplastic pH in leaves of barley before and after challenge-inoculation with the powdery mildew fungus. The surface pH of a root represents the extension of its apoplast (Felle 1998). Since the un-

Fig. 4. Development of powdery mildew pustules on leaves of barley plants after root inoculation with different factors. Roots of hydroponically grown barley plants were inoculated with benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester (40 mg per milliliter), *Piriformospora indica* chlamydospores (150,000 spores per milliliter), or *N*-acetylchito-octaose (Glc8). Three days after treatment, the youngest fully expanded leaves were detached and inoculated with powdery mildew spores, and development of powdery mildew pustules was determined 5 days after inoculation. The number of pustules for mock-treated control plants was set to 1 and relative values for the different treatments are shown. Values are means of three or four independent experiments, with each set consisting of 24 plants. Error bars represent standard error of the mean values, asterisks indicate a significance level of $P < 0.05$ (Student's *t*-test of respective treatment compared to control).

disturbed surface-water layer extending into the bulk is rather thin, changes in ion concentration or transport activity induced by elicitors is an indicator of an altered (H^+ –) signaling arising from an activation or deactivation of the plasma membrane H^+ ATPase and, thereby, mediated H^+ cotransport. Thus, changes in surface H^+ activity are characteristic for symbiotic responses, as shown for the Nod factor–legume interactions in *Medicago sativa* (Ehrhardt et al. 1992; Felle et al. 1998) and *Lotus japonicus* (Radutoiu et al. 2003) for stress reactions in which apoplastic pH has been demonstrated to be a sensitive major variable (Felle and Hanstein 2002; Felle et al. 2005; Wilkinson 1999) as well as for the activation of defense responses (Felle et al. 2004).

Differential responses at the root surface.

The surface pH measured along primary roots clearly showed areas with a pH less acidic than the bulk in the EZ but more acidic in the RHZ and at the calyptra. About 30 min after flushing the primary root with *P. indica* chlamydo spores, the differential pH profile changed and appeared less pronounced. However, spot kinetics taken in EZ (Fig. 1C) and RHZ (Fig. 1B) reveal that the immediate responses are transiently stronger than the steady-state values in Figure 1A.

Responses of plant cells to MAMPs, i.e., fungal chitin or fragments thereof, are well known and reported as a distinct, mostly transient pH increase (Baureithel et al. 1994; Felix et al. 1993). These earlier findings are corroborated and extended in that an electrode placed between root hairs displayed an alkalization in intact RHZ tissue in response to *P. indica*, a basidiomycota that contains chitin in its hyphae (Deshmukh et al. 2006) (Fig. 1B). This notion is supported by the observation that 0.1 μ M Glc8 caused a similar pH response. Compared with the pH changes found by Felix and associates (1993) and Baureithel and associates (1994), the pH increase observed here is relatively small and reaches only about 0.2 to 0.3. This may be due to the experimental design; the root's contact with the chlamydo spores is not very tight and the actual amount of one or more MAMP elicitors that are released by the fungal hyphae thereby coming into contact with the putative MAMP receptors may be low. Moreover, since there is a constant flow of medium, part of the effect may have been diluted out, so that the actual pH increase experienced by the root during a natural colonization would have been more substantial. Also, dead chlamydo spores caused a transient alkalization, which supports the notion that we are observing a typical chitin response in the RHZ.

The relatively short pH transient is also reminiscent of the reaction of legume (*Medicago sativa*) roots to Nod factors secreted by nitrogen-fixing bacteria and is in this way associated with the establishment of a root symbiosis (Felle et al. 1998). However, as the plant's response to Nod factors and chitin fragments was found indistinguishable in terms of timing and amplitude, it is not possible to decide whether the root response to *P. indica* was a defense reaction or a response critical at the onset of the symbiosis. The fact that PR genes like *pathogenesis-related 1* are expressed at slightly higher levels in *P. indica*–inoculated barley roots during the first 3 dai (Deshmukh and Kogel 2007) would argue in favor of a transiently enhanced defense response. In a previous study by Felle and associates (1998) as well as in our analysis, two distinct characteristics were observed in response of roots to either treatment, the Nod factor and the chlamydo spores: i) a lag-phase that excludes a direct membrane effect but, rather, points to launching of an intracellular signal cascade and ii) in support of this, a refractory behavior.

Whereas the alkalization effect in the RHZ could have been expected (although acidification following elicitor appli-

cation has been reported in tomato cell suspensions [Vera-Estrella et al. 1994]), the response within the EZ was not. In this zone an initial *P. indica*–induced acidification turned into a damped oscillation. Whereas dead spores were responded to with similar kinetics, as found in the RHZ, chitin fragments caused the typical transient alkalization. This indicates that there is more (specific) information in the spores than just chitin.

Whatever about the oscillation, which probably is a regulatory phenomenon, the observation that most of the kinetics is on the acidification side is very likely indicative of an activation of H^+ export, e.g., by the plasma membrane H^+ ATPase. Such an activation of the EZ could indicate faster cell growth, an effect actually measured as root biomass increase, induced by *P. indica* (Achatz 2006; Waller et al. 2005). In addition, the observed induction of resistance by *P. indica* but not by inoculation with chitin (Figs. 3 and 4) indicates that mechanisms distinct from elicitor-mediated responses or different elicitors than chitin lead to systemic resistance against powdery mildew. This finding is consistent with a recent report showing that a cell-wall extract from *P. indica* promotes growth of *Arabidopsis* seedlings and induces a transient cytosolic Ca^{++} elevation in roots but does not stimulate defense responses such as H_2O_2 production and defense gene activation (Vadassery et al. 2009).

Signal transfer to the leaves and its implications.

It has been demonstrated that a variety of stimuli (e.g., salt stress, abscissic acid, low oxygen) applied to barley roots are rapidly transferred to the leaves (Felle et al. 2005). Most interestingly, all of these stimuli were somehow transformed into a pH signal (alkalinization) that reached the leaf apoplast within minutes. Since the velocity of signal transfer depended on the transpiration rate, the xylem was assumed the most likely transducing system. We show here that chlamydo spores are sensed by the roots and responded to with a pH shift (Figs. 1 to 3), which is subsequently transmitted within minutes as a pH signal to the leaf apoplast. However, in contrast to other stimuli applied to barley roots (Felle et al. 2005), the response to chlamydo spores of *P. indica* was an acidification rather than an alkalization. Although an apoplastic acidification could have more than one cause, an activation of the plasma membrane ATPase (H^+ pump) that leads to activation of a number of transporters is likely. These transporters include, for instance, proton cotransporters, of which activation is essential for growth stimulation. Leaf apoplastic acidification instead of alkalization also suggests that the root's encounter with *P. indica* is not sensed as stress, otherwise the apoplastic response should have been a pH increase (Felle et al. 2005; Wilkinson 1999).

Effect of *P. indica* root colonization on the leaf response to pathogen inoculation.

Leaf apoplast pH changes have been shown to be associated with specific interaction phenotypes in the barley–*B. graminis* pathosystem. We detected an apoplastic pH increase that commences about 24 hai of barley leaves with conidia of *B. graminis* f. sp. *hordei* race A6, which is virulent on cv. Ingrid (Fig. 3) (Felle et al. 2004). This pH increase was much more pronounced in leaves from plants pretreated with *P. indica*, indicating that the symbiotic fungus primes barley leaves for a stronger alkalization response. This effect was demonstrated 5 days after inoculating the root system with chlamydo spores, indicating that the primed status is stable and can be judged as a long-term effect. These data are consistent with earlier results showing that a pronounced alkalization induced by powdery mildew fungus is specifically associated with increased barley defense reactions governed either by the specific *R* genes

Mla12 and *Mlg* or by chemical resistance inducers (Felle et al. 2004). There is neither a pH response to treatment with *P. indica* spores in the absence of a *B. graminis* f. sp. *hordei* challenge nor is there a pH response to root treatment with the MAMP Glc8 irrespective of a *B. graminis* f. sp. *hordei* challenge (Fig. 3). Together these data suggest that the alkalization response is a consequence of a successful defense reaction. Consequently, we observed in this experimental setup that root inoculation with *P. indica* chlamydospores—as well as root treatment with the chemical resistance inducer BTH—led to enhanced resistance of the leaves against powdery mildew fungus already 3 dai (Fig. 4), as previously observed in barley leaves 3 weeks after inoculation of the roots with *P. indica* (Waller et al. 2005).

The defense phenotypes mediated by the R genes *Mla12* and *Ml*, as well as those induced by the chemical BTH or by *P. indica* are all associated with a hypersensitive reaction and, thus, with epidermal single-cell death that may result in a local apoplastic pH increase (Felle et al. 2004; Waller et al. 2005). On the other hand, alkalization could serve the plant to inhibit fungal growth, e.g., by shifting the proton concentration far away from the optima of fungal effectors, e.g., those enzymes released for cell-wall softening.

In sum, we here confirm earlier results showing that *P. indica*, which is restricted to the root rhizodermis and to cortical cells (Deshmukh et al. 2006; Stein et al. 2008), induces in the monocotyledonous barley a systemic response against powdery mildew fungus. According to the growth pattern and short term of the transfer, it is feasible that *P. indica* generates one or more signals in the root outer tissue that reach the leaf epidermis via the xylem after a few minutes and that possibly reprogram barley leaves for a strong primed alkalization response upon challenge inoculation with the powdery mildew fungus.

MATERIALS AND METHODS

Plant and fungal material and general growth conditions.

For root experiments, individual plants of *Hordeum vulgare* L. cv. Ingrid (Deutsche Saatveredelung, Lippstadt, Germany) were grown from seed under a 12-h light and 12-h dark regime at 20 to 25°C. Seeds were surface-sterilized by soaking them for 3 to 4 h in diluted hypochlorite and were then rinsed four times in sterile tap water. Seedlings were then grown for 3 days on wet filter paper in a damp chamber. For measurements, intact seedlings were fixed to the bottom of a Plexiglas flow-through chamber and were incubated for 1 to 2 h in the respective basic solution (1 mM KCl, 0.1 mM NaCl, 0.1 mM CaCl₂, and 0.5 mM MES/TRIS buffer) mixed to the bulk pH given in the figures.

For apoplast experiments, barley was grown under sterile conditions by placing sterilized seeds in 2.5-liter glass containers (Weck GmbH+Co KG, Wehr, Germany) filled with filter paper stacks soaked in autoclaved medium containing CaCl₂ at 0.05 g/liter, NaCl 0.025 at g/liter, KH₂PO₄ at 0.05 g/liter, (NH₄)₂HPO₄ at 0.025 g/liter, MgSO₄ × 7 H₂O at 0.15 g/liter, FeCl₃ at 0.012 g/liter, and thiamine HCl at 0.1 mg/liter, at pH 5.6.

For powdery mildew resistance tests, barley plants were grown for 10 days in hydroponic culture as described. Inoculation with BTH (40 mg ml⁻¹), *P. indica* chlamydospores (150,000 spores ml⁻¹), or 0.1 μM Glc8 was performed by immersing roots in the respective solutions for 10 min and returning the plants into hydroponic cultures. Chlamydospores were applied in a 0.05% Tween solution, which was also used as the mock treatment (control plants). Three days after treatment, the youngest fully expanded leaves were detached and inocu-

lated with *B. graminis* f. sp. *hordei* conidia (8 to 12 spores per square millimeter). The number of *B. graminis* f. sp. *hordei* pustules was determined 6 dai.

P. indica (DSM11827 from Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany), was propagated on agar plates prepared with modified *Aspergillus* minimal medium (Pham et al. 2004) kept at 25°C for 3 to 4 weeks. Chlamydospores were isolated from plates using a 0.05% Tween-20 solution collected at 500 × g for 10 min and were resuspended in basic solution (discussed above) three times, resulting in a solution containing *P. indica* chlamydospores at the rate of 5 × 10⁵ ml⁻¹. Inoculation with powdery mildew fungus was performed by shaking barley leaves heavily infected with *Blumeria graminis* f. sp. *hordei* A6 (Wiberg 1974) in an inoculation tower about 1 m above the leaves and manually circulating the air to ensure equal distribution of the spores. Inoculation density was counted using a counting plate of defined size placed beside the leaves.

Recording surface pH.

The flow-through Plexiglas chamber permitted a horizontal approach of one or two microelectrodes. pH-sensitive microelectrodes were positioned at the root surface using a micro-manipulator (Narishige, Tokyo). Surface pH profiles were recorded as previously described (Felle 1998). By moving the electrodes from the bulk of the medium towards the surface at 90°, the difference between the two positions was attained. The electrode was then moved a certain distance along the root and the same procedure was repeated, and so on. For point measurements, pH microelectrodes were positioned at a certain root area and left there for the entire experiment. Before adding the spores, a constant flowthrough (3 to 4 ml min⁻¹) of the medium was adjusted until a stable baseline was recorded. The spore suspension was added without changing the velocity of the flow.

Apoplastic pH measurements.

Apoplastic pH measurements were carried out as described (Felle et al. 2004). Intact barley plants were used throughout the experiments. Plants were kept in an Erlenmeyer flask,

Fig. 5. Basic setup to measure apoplastic pH on an intact barley plant. Root system was submersed in a solution of 1 mM KCl, 0.1 mM NaCl, and 0.1 mM CaCl₂.

which permitted continuous fluid exchange around the root system (Fig. 5). Roots were adapted to a basic solution of 1 mM KCl, 0.1 mM NaCl, and 0.1 mM CaCl₂. One leaf (target leaf) was fixed with double-adhesive tape on a Plexiglas surface. Under optical control, the electrodes were positioned inside cavities of open stomata at an angle of approximately 45°, using a 20× long-distance objective. The earth-reference electrode was positioned at the end of the target leaf. Electrical contact was attained by bringing the electrodes into contact with the apoplastic fluid, which was signaled by the digital response of the amplifier.

pH-sensitive microelectrodes.

These have been fabricated and used as described before (Felle and Zimmermann 2007; Felle 1998). Briefly, all microprobes were fabricated from 1.5-mm glass tubing (Hilgenberg, Malsfeld, Germany). Capillaries were pulled on a two-stage puller (List-L/P-3P-A; Darmstadt, Germany) to tips of 2 to 5 µm, which were heat-polished over a platinum wire. These capillaries were filled with 0.5 M KCl to be used as apoplastic voltage reference electrodes. To prepare pH-sensitive electrodes, capillaries were heated to 200°C in an oven for 1 h and were silanized internally by repeatedly dipping the rear end of the hot capillaries into a 0.02% tributylsilane and chloroform solution. The capillaries were kept at 200°C for approximately 1 h before the silanization procedure was repeated. Silanized capillaries (cooled to room temperature) were backfilled with the respective sensor mixture (Fluka #95297; Fluka Chemical Corp., Milwaukee, WI, U.S.A.) and were topped up with the reference solution (100 mM morpholineethanesulfonic acid-Tris, adjusted to pH 6, 0.5 M KCl). Electrodes were stored for several days before use. Ready-to-use electrodes were connected through a suitable electrode holder (World Precision Instruments, Sarasota, FL, U.S.A.) with a high-impedance (10¹⁵ ohm) amplifier (WPI-FD223; World Precision Instruments). Kinetics were recorded on a chart recorder (Linseis-L 2200; Linseis, Selb, Germany).

ACKNOWLEDGMENTS

The authors gratefully acknowledge financial support for this study by the Deutsche Forschungsgemeinschaft (FOR 666; Fe 213/16-1, 16-2, and WA 2478/1-1).

LITERATURE CITED

Achatz, B. 2006. Untersuchungen zum Einfluss des Wurzelendophyten *Piriformospora indica* auf das Wachstum von *Hordeum vulgare*, die Resistenz gegen *Blumeria graminis* f. sp. *hordei* und die Genexpression in den Blättern. Marburg, Germany. Ph.D. thesis. Philipps-University Marburg, Germany.

Baltruschat, H., Fodor, J., Harrach, B. D., Niemczyk, E., Barna, B., Gullner, G., Janeczko, A., Kogel, K. H., Schäfer, P., Schwarczinger, I., Zuccaro, A., and Skoczowski, A. 2008. Salt tolerance of barley induced by the root endophyte *Piriformospora indica* is associated with a strong increase in antioxidants. *New Phytol.* 180:501-510.

Baureithel, K., Felix, G., and Boller, T. 1994. Specific, high affinity binding of chitin fragments to tomato cells and membranes. Competitive inhibition of binding by derivatives of chitoooligosaccharides and a Nod factor of *Rhizobium*. *J. Biol. Chem.* 269:17931-17938.

Deshmukh, S., and Kogel, K.-H. 2007. *Piriformospora indica* protects barley from root rot disease caused by *Fusarium graminearum*. *J. Plant Dis. Prot.* 114:263-268.

Deshmukh, S., Hüchelhoven, R., Schäfer, P., Imani, J., Sharma, M., Weiss, M., Waller, F., and Kogel, K.-H. 2006. The root endophytic fungus *Piriformospora indica* requires host cell death for proliferation during mutualistic symbiosis with barley. *Proc. Natl. Acad. Sci. U.S.A.* 103:18450-18457.

Ehrhardt, D. W., Atkinson, E. M., and Long, S. R. 1992. Depolarization of alfalfa root hair membrane potential by *Rhizobium meliloti* Nod factors. *Science* 256:998-1000.

Felle, H. H. 1998. The apoplastic pH of *Zea mays* root cortex as measured with pH-sensitive microelectrodes: Aspects of regulation. *J. Exp. Bot.* 49:987-995.

Felle, H. H., and Hanstein, S. 2002. The apoplastic pH of the substomatal cavity of *Vicia faba* leaves and its regulation responding to different stress factors. *J. Exp. Bot.* 53:73-82.

Felle, H. H., Kondorosi, É., Kondorosi, Á., and Schultze, M. 1998. The role of ion fluxes in nod factor signaling in *Medicago sativa*. *Plant J.* 13:455-463.

Felle, H. H., Herrmann, A., Hanstein, S., Hüchelhoven, R., and Kogel, K.-H. 2004. Apoplastic pH signaling in barley leaves attacked by the powdery mildew fungus *Blumeria graminis* f. sp. *hordei*. *Mol. Plant-Microbe Interact.* 17:118-123.

Felle, H. H., Herrmann, A., Hüchelhoven, R., and Kogel, K.-H. 2005. Root-to-shoot signaling: Apoplastic alkalisation, a general stress signal and defence factor in barley (*Hordeum vulgare*). *Protoplasma* 227:17-24.

Felle, H. H., and Zimmermann M. R. 2007. Systemic signaling in barley through action potentials. *Planta* 226:203-214.

Felle, H. H., Herrmann, A., Schäfer, P., Hüchelhoven, R., and Kogel, K.-H. 2008. Interactive signal transfer between host and pathogen during successful infection of barley leaves by *Blumeria graminis* and *Bipolaris sorokiniana*. *J. Plant Physiol.* 165:52-59.

Felix, G., Regenass, M., and Boller, T. 1993. Specific perception of sub-nanomolar concentrations of chitin fragments by tomato cells: Induction of extracellular alkalization, changes in protein phosphorylation, and establishment of a refractory state. *Plant J.* 4:307-316.

Monshausen, G. B., Zieschang, H. E., and Sievers, A. 1996. Differential proton secretion in the apical elongation zone caused by gravi-stimulation is induced by a signal from the root cap. *Plant Cell Environ.* 19:1408-1414.

Pham, G. H., Kumari, R., Singh, A., Malla, R., Prasad, R., Sachdev, M., Kaldorf, M., Buscot, F., Oelmüller, R., Hampp, R., Saxena, A. K., Rexer, K. H., Kost, G., and Varma, A. 2004. Axenic culture of symbiotic fungus *Piriformospora indica*. Pages 593-612 in: *Plant Surface Microbiology*. A. Varma, L. Abbot, D. Werner, and R. Hampp, eds. Springer, Berlin.

Radutoiu, S., Madsen, L. H., Madsen, E. B., Felle, H. H., Umehara, Y., Grønlund, M., Sato, S., Nakamura, Y., Satoshi, T., Sandal, N., and Stougaard, J. 2003. Plant recognition of symbiotic bacteria requires two LysM receptor-like kinases. *Nature* 425:585-592.

Schäfer, P., Khatabi, B., and Kogel, K.-H. 2007. Recent advances in the study of *Piriformospora indica*, challenges and perspectives. *FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Lett.* 275:1-7.

Schäfer, P., and Kogel, K.-H. 2009. The Sebacinoid fungus *Piriformospora indica*: An orchid mycorrhiza with a robust endophytic potential for reprogramming host plants for increased reproduction and fitness. Pages 99-112 in: *The Mycota Vol. V, Plant Relationships*. 2nd ed. H. B. Deising, ed. Springer, Berlin.

Schäfer, P., Piffli, S., Voll, L.M., Zajic, D., Chandler, P. M., Waller, F., Scholz, U., Pons-Kühnemann, J., Sonnewald, S., Sonnewald, U., Kogel, K. H. Gibberellin contributes to compatibility of barley roots to the mycorrhiza fungus *Piriformospora indica*. *Plant J.* In press.

Serfling, A., Wirsal, S. G. R., Lind, V., and Deising, H. B. 2007. Performance of the biocontrol fungus *Piriformospora indica* on wheat under greenhouse and field conditions. *Phytopathology* 97:523-531.

Stein, E., Molitor, A., Kogel, K.-H., and Waller, F. 2008. Systemic resistance in *Arabidopsis* conferred by the mycorrhiza fungus *Piriformospora indica* requires jasmonic acid signaling and the cytoplasmic function of NPR1. *Plant Cell Physiol.* 49:1747-1751.

Vadassery, J., Ranf, S., Drzewiecki, C., Mithöfer, A., Mazars, C., Scheel, D., Lee, J., and Oelmüller, R. 2009. A cell wall extract from the endophytic fungus *Piriformospora indica* promotes growth of *Arabidopsis* seedlings and induces intracellular calcium elevation in roots. *Plant J.* 59:193-206.

Van Loon, L. C., Bakker, P. A. H. M., and Pieterse, C. M. J. 1998. Systemic resistance induced by rhizosphere bacteria. *Annu. Rev. Phytopathol.* 36:453-83.

Van Wees, S. C. M., Van der Ent, S., and Pieterse, C. M. J. 2008. Plant immune responses triggered by beneficial microbes. *Curr. Opin. Plant Biol.* 11:443-448.

Vera-Estrella, R., Barckla, B. J., Higgins, V. J., and Blumwald, E. 1994. Plant defense response to fungal pathogens. I. Activation of host plasma membrane H⁺-ATPase by elicitor-induced enzyme depolarization. *Plant Physiol.* 104:209-215.

Verma, S., Varma, A., Rexer, K.-H., Hassel, A., Kost, G., Sarabhoj, A., Bisen, P., Bütenhorn, B., and Franken, P. 1998. *Piriformospora indica*, gen. nov. sp. nov., a new root-colonizing fungus. *Mycologia* 90:896-903.

Waller, F., Achatz, B., Baltruschat, H., Fodor, J., Becker, K., Fischer, M., Heier, T., Hüchelhoven, R., Neumann, C., von Wettstein, D.,

- Franken, P., and Kogel, K.-H. 2005. The endophytic fungus *Piriformospora indica* reprograms barley to salt-stress tolerance, disease resistance, and higher yield. Proc. Natl. Acad. Sci. U.S.A. 102:13386-13391.
- Waller, F., Mukherjee, K., Deshmukh, S. D., Achatz, B., Sharma, M., Schäfer, P., and Kogel, K.-H. 2008. Systemic and local modulation of plant responses by *Piriformospora indica* and related Sebaciniales species. J. Plant Physiol. 165:60-70.
- Weisenseel, M. H., Dorn, A., and Jaffe, L. F. 1979. Natural H⁺ currents traverse growing roots and root hairs of barley (*Hordeum vulgare* L.). Plant Physiol. 64:512-518.
- Wiberg, A. 1974. Genetical studies of spontaneous sources of resistance to powdery mildew in barley. Hereditas 77:89-148.
- Wilkinson, S. 1999. pH as a stress signal. Plant Growth Reg. 29:87-99.