Indole-3-Acetaldoxime-Derived Compounds Restrict Root Colonization in the Beneficial Interaction Between *Arabidopsis* Roots and the Endophyte *Piriformospora indica*

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Submitted 29 March 2012. Accepted 17 May 2012.

The growth-promoting and root-colonizing endophyte *Piriformospora indica* induces camalexin and the expression of *CYP79B2*, *CYP79B3*, *CYP71A13*, *PAD3*, and *WRKY33* required for the synthesis of indole-3-acetaldoxime (IAOx)-derived compounds in the roots of *Arabidopsis* seedlings. Upregulation of the mRNA levels by *P. indica* requires cytoplasmic calcium elevation and mitogen-activated protein kinase 3 but not root-hair-deficient 2, radical oxygen production, or the 3-phosphoinositide-dependent kinase 1/oxidative signal-inducible 1 pathway. Because *P. indica*-mediated growth promotion is impaired in *cyp79B2 cyp79B3* seedlings, while *pad3* seedlings—which do not accumulate camalexin—still respond to the fungus, IAOx-derived compounds other than camalexin (e.g., indole glucosinolates) are required during early phases of the beneficial interaction. The roots of *cyp79B2 cyp79B3* seedlings are more colonized than wild-type roots, and upregulation of the defense genes *pathogenesis-related (PR)-1*, *PR-3*, *PDF1.2*, *phenylalanine ammonia lyase*, and *germin* indicates that the mutant responds to the lack of IAOx-derived compounds by activating other defense processes. After 6 weeks on soil, defense genes are no longer upregulated in wild-type, *cyp79B2 cyp79B3*, and *pad3* roots. This results in uncontrolled fungal growth in the mutant roots and reduced performance of the mutants. We propose that a long-term harmony between the two symbionts requires restriction of root colonization by IAOx-derived compounds.

Important secondary metabolites in members of order Brassicales are synthesized from tryptophan. Two functionally redundant cytochrome P450 enzymes (*CYP79B2* and *CYP79B3*) convert tryptophan into indole-3-acetaldoxime (IAOx), an intermediate for the biosynthesis of indole glucosinolates (I-GLS), camalexin, other indole compounds such as indole acetonitrile, indole carboxylic acid derivatives, and, under specific condi-

tions, the plant hormone indole-3-acetic acid (IAA). Whereas camalexin is a true phytoalexin synthesized in the plant in response to pathogen infection or exposure to stress, I-GLS are both phytoanticipins present prior to induction as well as phytoalexins induced upon infection. The first committed step in I-GLS biosynthesis is the enzyme *CYP83B1/SUR2* (Bak et al. 2001; Barlier et al. 2000; Hansen et al. 2001). The major IAOx-metabolizing enzyme in camalexin biosynthesis is *CYP71A13*, which catalyzes the formation of the intermediate indole acetonitrile (Nafisi et al. 2007), supposedly together with the homolog *CYP71A12* required for camalexin exudation from roots (Millet et al. 2010). The thiazole ring of camalexin derives from the cysteine moiety of glutathione, which is conjugated with indole acetonitrile after activation (Böttcher et al. 2009; Geu-Flores et al. 2011; Parisy et al. 2007; Su et al. 2011). The single-copy gene *PAD3/CYP71B15* encodes for an enzyme that catalyzes the final two steps in the camalexin pathway (Böttcher et al. 2009; Schuëgger et al. 2006).

The double *cyp79B2 cyp79B3* mutant lacks I-GLS (Zhao et al. 2002) and is unable to induce camalexin synthesis (Glawischnig et al. 2004). Furthermore, it does not accumulate indole-3-carboxylic acid derivatives (Böttcher et al. 2009), secondary metabolites which are strongly induced by pathogen infections. IAA levels were unchanged in seedlings of this mutant (Sugawara et al. 2009), although some reduction was observed under heat stress (Zhao et al. 2002) and in root tips (Ljung et al. 2005). In summary, IAOx has an important role as a metabolic branch point regulating flux into I-GLS, camalexin, other secondary indole compounds, and IAA biosynthesis (Burrow et al. 2010; Mikkelsen et al. 2009), and diversion of IAOx into one of the pathways may occur at the expense of the others (Bak et al. 2001; Glawischnig et al. 2004; Nafisi et al. 2006).

Production of camalexin is induced in response to a variety of exposures such as plant pathogens, including bacteria, fungi, and oomycetes (Glawischnig 2007; Rauhut and Glawischnig 2009), pathogen-associated molecular patterns (PAMPs), toxins (Gust et al. 2007; Qutob et al. 2006; Rauhut et al. 2009; Stone et al. 2000), and reactive oxygen species (ROS)-inducing abiotic stress (Van Breusegem et al. 2008). Camalexin exhibits cytotoxicity, particularly against eukaryotic pathogens (Rogers et al. 1996). Expression of the last enzyme of the camalexin biosynthetic pathway, *PAD3*, is regulated by a variety of signaling

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*The *e-Xtra* logo stands for “electronic extra” and indicates that four supplementary tables and one supplementary figure are published online and that Figure 5 appears in color online.

pathways. Crucial components are the mitogen-activated protein kinases (MPK) MPK3, MPK6 (Ren et al. 2008), and MPK4 (Qiu et al. 2008). MPK4 becomes activated in response to infection and phosphorylates MPK4 substrate 1 (MKS1) and the transcription factor WRKY33 in the nucleus, which ultimately allows WRKY33 to activate camalexin biosynthetic genes (Qiu et al. 2008). Upstream of MPK, the 3-phosphoinositide-dependent protein kinase 1 (PDK1)/oxidative signal-inducible 1 (OXI1) pathway is activated in response to various pathogens (Anthony et al. 2004, 2006; Rentel et al. 2004). OXI1 is required for full activation of MPK3 and MPK6 in response to many microbial pathogens or elicitors (Rentel et al. 2004; van der Luit et al. 2000; Yamaguchi et al. 2005). OXI1 phosphorylates and, thus, activates the downstream serine/threonine kinase PTI1-2 and also controls root hair growth (Rentel et al. 2004). OXI1 is activated by H₂O₂ and phospholipid signals via PDK1 (Anthony et al. 2006). PDK1 binds to different signaling lipids, including the second messenger phosphatidic acid, which accumulates in response to microbial infections or elicitor treatments (Deak et al. 1999; Hirt et al. 2011). Phosphatidic acid is mainly produced by phospholipase α 1 in *Arabidopsis* roots, and a corresponding mutant contains severely reduced levels of this plant second

messenger (Devaiah et al. 2006). PDK1 phosphorylates and, thus, activates OXI1 in *Arabidopsis* (Anthony et al. 2004) and rice (Matsui et al. 2010) or AvrPto-dependent Pto-interacting protein 3 (Adi3) in tomato (Devarenne et al. 2006).

Root-hair-deficient 2 (RHD2) is crucial for ROS production in roots, and inactivation of *RHD2* compromises the expansion of root hair cells. Furthermore, Ca²⁺ influx is required for the cell elongation in roots, and *rhd2* mutants are defective in Ca²⁺ uptake. As a consequence, *rhd2* mutants have short root hairs and stunted roots (Foreman et al. 2003). RHD6 controls root hair initiation and the *RHD6* mutation can be rescued by auxin (Masucci and Schiefelbein 1994) or microbes producing auxin-related compounds (Contreras-Cornejo et al. 2009). The observation that ROS induction and MPK3, MPK4, and MPK6 signaling activates camalexin biosynthesis suggests that ROS-producing enzymes and the PDK1/OXI1 pathway may be upstream in the signaling pathway to induce genes for IAOx-derived compounds.

Piriformospora indica colonizes the roots of many plant species, thereby promoting growth and biomass production and conferring resistance against biotic and abiotic stress (Oelmüller et al. 2009; Peškan-Berghöfer et al. 2004). The beneficial inter-

Fig. 1. Fold-induction of the mRNA levels for **A**, *CYP79B2*; **B**, *CYP79B3*; **C**, *CYP71A13*; **D**, *PAD3*; **E**, *CYP83B1*; **F**, *SUR1*; and **G**, *WRKY33* by *Piriformospora indica* in *Arabidopsis* roots. RNA was isolated from the roots of wild-type (WT, Col 0) seedlings and different mutants as indicated which were either co-cultivated with *P. indica* for 14 days or mock treated. After real-time polymerase chain reaction analysis, the mRNA_{+P.indica}/mRNA_{mock-treated} ratio was calculated. Values are based on five independent biological experiments. Error bars were calculated as standard errors. Relative errors of the proportion are the sum of the individual relative errors.

action between the endophytic fungus *P. indica* and *Arabidopsis* requires a balanced activation of defense processes, and mutants defective in specific defense-related genes are often unable to restrict fungal growth (Camehl and Oelmüller 2010; Camehl et al. 2010; Johnson and Oelmüller 2009; Sherameti et al. 2008). Here, we demonstrate that IAOx-derived indole compounds are crucial for the beneficial interaction between the two symbionts, and that these compounds restrict root colonization. We also demonstrate that *P. indica*-induced cytoplasmic calcium elevation and MPK3 but not H₂O₂, RHD2 and RHD6, and the PDK1/OXI1 pathway are required for the activation.

RESULTS

Role of CYP79B2, CYP79B3, PAD3, and camalexin in the interaction between *P. indica* and *Arabidopsis* seedlings.

Co-cultivation of *Arabidopsis* roots with *P. indica* on agar plates for 14 days resulted in a low but significant (*t* test, *n* = 5, *P* = 0.025) induction of camalexin (127 ± 63 ng g⁻¹ fresh weight) compared with the mock-treated control (50 ± 10 ng g⁻¹ fresh weight), although it is synthesized in approximately two orders of magnitude lower concentrations compared with

roots infected with pathogens such as *Plasmodiophora brassicae* (Siemens et al. 2008). Also, the mRNA levels for CYP79B2, CYP79B3, CYP71A13 (Nafisi et al. 2007), PAD3, and WRK33 (Qiu et al. 2008) are upregulated in colonized wild-type (WT) roots, whereas those for CYP83B1 and SUR1 are not (Fig. 1A to G). This demonstrates that the genes for the synthesis of IAOx-derived compounds, including camalexin but not I-GLS, are targets of signals from the fungus.

In order to test whether IAOx-derived compounds are required for the beneficial interaction, we analyzed the growth response of *cyp79B2 cyp79B3* and *pad3* seedlings to *P. indica*. The weight of WT seedlings co-cultivated with *P. indica* on plant nutrient medium (PNM) plates for 14 days was approximately 35% higher compared with the mock-treated controls (Fig. 2A). The *pad3* seedlings also responded to the fungus and the fresh weight was promoted similar to WT. However, the weight of *P. indica*-colonized *cyp79B2 cyp79B3* seedlings was not significantly different from the mock-treated control (Fig. 2A). This demonstrates that camalexin synthesis is not essential for early processes during *P. indica*-mediated growth promotion and suggests that IAOx-derived compounds other than camalexin are required for the beneficial interaction.

Because camalexin exhibits cytotoxicity (Rogers et al. 1996), we tested whether it influences root colonization by *P. indica*. Therefore, the *P. indica* translation elongation factor 1 (*Pitef1*) DNA and mRNA levels relative to the plant glyceraldehyde-3-phosphate dehydrogenase C2 (*AtGAPC2*) DNA and mRNA levels were determined in colonized WT, *cyp79B2 cyp79B3*, and *pad3* plants. Colonization of the *cyp79B2 cyp79B3* but not *pad3* mutant roots was higher than that of WT roots (Fig. 2B). The data shown for *pad3* colonization are consistent with those published by Jacobs and associates (2011). Furthermore, they showed that *pen2-1* (defective in another enzyme linked to IAOx metabolism) displayed higher colonization. Because the *Pitef1/AtGAPC2* ratios were almost identical when the polymerase chain reactions (PCRs) were performed with DNA or with cDNA synthesized from mRNA of the colonized roots, the fungus is alive in the roots and expresses the *Pitef1* gene. We also stained fungal spores in the roots and found the same colonization pattern for the WT and the mutants (Fig. 3). Counting of the spores in a 1-cm segment (1 cm from root tip) confirmed the data from Figure 2B. Analysis of 120 seedlings showed that *cyp79B2 cyp79B3* roots (Fig. 3C and D) contain 59 ± 8 spores/cm, WT roots contain 39 ± 4 spores/cm, and *pad3* roots contain 37 ± 5 spores/cm. In all seedlings, spores are often associated with the root tips (Fig. 3B and D).

In colonized mutant roots, we observed a moderate but significant upregulation of plant defense genes (Fig. 4A to C), and this was more pronounced in *cyp79B2 cyp79B3* than in *pad3* plants. In particular, *PDF1.2* and *germin* were strongly upregulated in *cyp79B2 cyp79B3* roots (Fig. 4A and B). Comparison of Figures 2 and 4 demonstrates that higher root colonization rates correlate with the activation of defense genes and the loss of benefits for the plants. This suggests that metabolites synthesized by the CYP79B enzymes restrict growth of *P. indica*.

Cytoplasmic Ca²⁺ elevation and MPK3 but not RHD2, RHD6, PLDα1, PDK1, or OXI1 are involved in the *P. indica*-induced upregulation of the mRNAs for CYP79B2, CYP79B3, CYP71A13, PAD3, and WRKY33.

Camalexin is induced by many plant pathogens in the roots and shoots of *Arabidopsis*. PAD3 activation requires MPK3 (Ren et al. 2008) and is stimulated by biotic and abiotic stresses leading to the formation of ROS (Van Breusegem et al. 2008). ROS production activates OXI1 which, in turn, is required for

Fig. 2. A, Fresh weight of wild type (WT), *cyp79B2 cyp79B3*, and *pad3* seedlings after 14 days of co-cultivation with *Piriformospora indica* or mock treatment. **B,** Increase of root colonization based on quantitative reverse-transcription polymerase chain reaction of *cyp79B2 cyp79B3* and *pad3* seedlings with respect to WT after 14 days of co-cultivation with *P. indica*. Root colonization was calculated as outlined in the text. Here, mRNA-based data are shown; however, they are not significantly different from DNA-based data, which are not shown. The value for WT was set as 1.0 and the other values are expressed relative to it. Bars represent standard errors, based on six independent biological experiments with 12 seedlings per treatment per experiment. Relative errors of the proportion ($\text{mRNA}_{+P. \text{indica}} / \text{mRNA}_{\text{mock-treated}}$) in B are the sum of the individual relative errors.

full activation of MPK3 and MPK6 (Rentel et al. 2004). In addition to ROS, OXI1 is also activated by the PDK1, after binding to the second messenger phosphatidic acid (Camehl et al. 2011).

To identify upstream components in the signaling cascade leading to *PAD3*, *CYP79B2*, *CYP79B3*, *CYP71A13*, and *WRKY33* mRNA induction by *P. indica* in *Arabidopsis* roots, mutants of MPK3, the ROS-producing RHD2, the phosphatidic-acid-producing phospholipase D α 1, the OXI-activating PDK1s, and ROS-activated OXI1 were compared with the WT after co-cultivation with *P. indica* (Fig. 1A to G). The *rhb6* mutant was included to investigate the role of root hairs for the induction of the genes by the fungus. Furthermore, we included three genetically distinct *Arabidopsis* ethyl-methane-sulfonate mutants, called *deficient in cytoplasmic calcium elevation (dce)1* to *dce3* which are impaired in inducing cytoplasmic Ca²⁺ elevation in response to the fungus and a fungal cell wall preparation (Fig. 5) (Vadassery et al. 2009). Because these mutants do not respond to the fungus in terms of growth promotion (*unpublished data*), the rapid increase in cytoplasmic Ca²⁺ elevation appears to be required for establishing the beneficial symbiosis.

PAD3 (Fig. 1D), *CYP79B2* (Fig. 1A), *CYP79B3* (Fig. 1B), *CYP71A13* (Fig. 1C), and *WRKY33* (Fig. 1G) were downregulated by *P. indica* in all mutants impaired in cytoplasmic Ca²⁺ elevation and in the *mpk3* mutant, compared with WT, *rhb2*, *rhb6*, the *pdcl.1 pdcl.2* double knock-out line, and *oxi1* mutants (Fig. 1A to D and G) but *CYP83B1* (Fig. 1E) and *SUR1* (Fig. 1F) were not affected. Although phosphatidic acid might be a second messenger in *P. indica*-mediated growth promotion (Camehl et al. 2011) and phospholipase D α 1 is a main producer of phosphatidic acid in *Arabidopsis* roots (Devaiah et al. 2006), the *PAD3*, *CYP79B2*, *CYP79B3*, *CYP71A13*, and *WRKY33* mRNA levels are not significantly different in a mutant lacking phospholipase D α 1 compared with the WT con-

trol (Fig. 1A to D and G). Furthermore, we could not detect an increase in the ROS level in infected WT and mutant roots (Camehl et al. 2011; Vadassery et al. 2009). This suggests that *P. indica* stimulates the expression of these genes through a Ca²⁺- and MPK3-dependent pathway but does not require production of ROS through the NADH oxidase RHD2 and the activity of the PDK1 and OXI1 (discussed below).

PAD3 expression is induced locally by *P. indica*.

We analyzed the expression of a *PAD3 promoter::uidA* fusion in mock-treated and colonized transgenic *Arabidopsis* seedlings using the root system in split petri dishes. Stimulation of β -glucuronidase (GUS) was only visible in root sections surrounded by fungal mycelium (Fig. 6A). A lower level of GUS activity was found in mock-treated roots (Fig. 6A). In particular, *P. indica*-induced GUS activity accumulated mainly in lateral roots during the early phase of interaction but, later, we noticed GUS staining in the main roots. To test whether this reflects the regulation of mRNA levels for IAOx-derived compounds, we isolated RNA from the roots and from preparations enriched in lateral roots and root hairs (discussed below) from colonized and uncolonized areas of the seedlings. The RNA extracted from fungus-exposed root areas after 14 days of co-cultivation of the two symbionts contained approximately three times higher mRNA levels for the *P. indica*-inducible *PAD3*, *CYP79B2*, *CYP79B3*, *CYP71A13*, and *WRKY33* genes compared with the root areas which were not exposed to fungal hyphae. When RNA was extracted from the fungus-exposed lateral root areas alone, we observed a much higher stimulation of these mRNA levels (Fig. 6B). Again, *CYP83B1* and *SUR1* were not or much less upregulated compared with the other genes. This suggests that the former genes are induced locally by the fungus, preferentially in lateral roots and root hairs. We also analyzed relative GUS activity in lateral roots

Fig. 3. Distribution of fungal spores in *Arabidopsis* A and C, root segments (1 to 2 cm away from the root tip) or B and D, at the root tip of A and B, the wild type (WT) and C and D, *cyp79B2 cyp79B3*. Representative pictures for seedlings after 14 days of co-cultivation with *Piriformospora indica* on PNM plates. The colonization pattern was the same for WT and mutant seedlings on PNM plates and in soil, except that the spore densities differed.

and root hairs at different time points after *P. indica* treatment (Fig. 6C). We noticed that, from 24 h onward, GUS expression was rapidly increased in *P. indica* co-cultivated lateral roots and root hairs (Fig. 6C).

Role of CYP79B2, CYP79B3, PAD3, and camalexin in the interaction between *P. indica* and *Arabidopsis* plants in soil.

After 14 days of co-cultivation on PNM medium (details below), colonized and mock-treated WT, *cyp79B2 cyp79B3*, and *pad3* seedlings were transferred to soil. After 42 days, *P. indica*-infected WT plants produced $32 \pm 4\%$ more shoot biomass than the mock-treated control. In contrast, the shoot biomass of infected *cyp79B2 cyp79B3* and *pad3* plants was reduced compared with the mock-treated control (Fig. 7A). Thus, long-term harmony between the two symbionts requires CYP79B2/CYP79B3 and PAD3.

After 42 days on soil (after 42 days, the vegetative phase was over, most of the plants flowered, and there was not any more camalexin increase), the camalexin level in the colonized WT roots was not higher than in the mock-treated control ($0.75 \pm 0.46 \mu\text{g g}^{-1}$ fresh weight; mock-treated control: $1.17 \pm 0.58 \mu\text{g g}^{-1}$ fresh weight). However, when compared with seedlings, the camalexin levels in the roots of adult plants were five- to 10-fold higher. Thus, the overall amount of camalexin per fresh weight increased in the WT roots during the 42 days on soil but the small stimulatory effect of *P. indica* observed for the seedlings was no longer detectable for adult plants. Also the *PAD3*, *CYP79B2*, *CYP79B3*, *CYP71A13*, and *WRKY33* mRNA levels

were no longer upregulated by the fungus in WT roots (Supplementary Table S2); in none of the experiments did we observe a significant stimulation by the fungus. Furthermore, pathogenesis-related (PR) genes (*PR-1*, *PDF1.2*, *phenylalanine ammonia lyase* [PAL], and *germin*) which are highly upregulated in colonized roots of *cyp79B2 cyp79B3* seedlings (Fig. 4A and B) were also upregulated in the roots of colonized adult *cyp79B2 cyp79B3* and *pad3* plants but at a lower scale (Fig. 7B1 and B2). The ROS-related *RHD2* and the root-hair-controlling *RHD6* genes are not regulated by *P. indica* (Fig. 7B3). We also could not detect ROS production in the roots of adult WT, *cyp79B2 cyp79B3*, and *pad3* plants (Supplementary Table S3). Taken together, defense processes in the colonized mutant and WT roots of adult plants are not much activated by the fungus compared with the situation in the seedlings.

However, root colonization was much higher in the mutant plants compared with the WT (Fig. 7C). After 42 days in soil, the *Pitefl* DNA or cDNA/*AtGAPC2* DNA or cDNA ratios for the *cyp79B2 cyp79B3* and *pad3* plants were 11 and 5 times higher, respectively, than the ratio for WT plants (Fig. 7C). Kinetic analysis demonstrates that these ratios increased gradually with longer co-cultivation times on soil (Supplementary Table S4). Microscopic inspection of the colonization did not discover significant differences to the colonization pattern found for seedlings (Fig. 3): younger root sections were more colonized than older regions of the roots and spores were mainly found in association with lateral roots. Thus, the low level of defense gene activation in combination with the absence of IAOx-derived indole compounds promotes fungal

Fig. 4. Fold induction of the mRNA levels for pathogenesis-related (PR) genes **A**, *PR-1*, *PR-3*, and *PDF1.2*; **B**, phenylalanine ammonia lyase (*PAL*) and *germin*; and **C**, root-hair-deficient (*RHD*) genes *RHD2* and *RHD6* by *Piriformospora indica* in *Arabidopsis* roots. RNA was isolated from the roots of wild-type (WT) and mutant (*cyp79B2 cyp79B3* and *pad3*) seedlings which were either co-cultivated with *P. indica* for 14 days or mock treated. After real-time polymerase chain reaction analysis, the $\text{mRNA}_{+P. \text{indica}}/\text{mRNA}_{\text{mock-treated}}$ ratio was calculated. Data are based on five independent biological experiments with three replications. Error bars were calculated as standard errors. Relative errors of the proportion are the sum of the individual relative errors.

growth (discussed below). Overcolonization of the roots might be the reason for the reduced benefits for the plants. This confirms that *P. indica*-mediated long-term benefits for the plants require CYP79B2/CYP79B3 and PAD3.

DISCUSSION

Beneficial interaction between *P. indica* and *Arabidopsis* requires IAOx-derived compounds.

We demonstrated that IAOx-derived compounds are essential for initial steps in the beneficial interaction between *P. indica* and *Arabidopsis*, because seedlings of the *cyp79B2 cyp79B3* double mutants do not respond to the fungal infection with growth promotion (Fig. 2A). Because *pad3* seedlings respond to *P. indica* (Fig. 2A), camalexin seems not to be important during the initial phase of interaction. However, PAD3 is required during later stages (Fig. 7A).

Therefore, camalexin (Böttcher et al. 2009) and other IAOx-derived compounds may be involved in independent processes in this symbiosis. Similar observations have been reported in different pathosystems. Schlaeppi and associates (2010) showed that a deficiency in either camalexin or I-GLS accumulation had only a minor effect on the disease resistance of *Arabidopsis* against *Phytophthora brassicae* infection, while *cyp79B2 cyp79B3* was highly susceptible to *P. brassicae*. Sanchez-Vallet and associates (2010) have demonstrated that tryptophan-derived secondary metabolites may have differential contributions in non-host resistance to necrotrophic and biotrophic pathogens. Analysis of additional mutants impaired in the synthesis of specific IAOx-derived secondary metabolites will help to understand their role in this beneficial symbiosis.

The higher degree of fungal colonization in the roots of *cyp79B2 cyp79B3* seedlings (Fig. 2B) and adult *cyp79B2 cyp79B3* and *pad3* plants (Fig. 7C) and the initial activation of defense genes against the fungus in the roots of the *cyp79B2 cyp79B3* seedlings suggest that IAOx and its derived compounds, including camalexin, participate in the control of fungal growth. Initially, the roots responded to the lack of IAOx and its derived compounds by activating other defense genes such as *PR-1*, *PDF1.2*, *PAL*, and *germin* (Fig. 4A and B). However, this mechanism appears to be restricted to early phases of the symbiosis and is no longer active in adult plants (Fig. 7B1 and B2). In

Fig. 5. *Piriformospora indica* cell wall extract (CWE) induces changes in cytosolic Ca^{2+} ($[\text{Ca}^{2+}]_{\text{cyt}}$) levels in the roots of apoaequorin-transformed *Arabidopsis* wild-type (WT) seedlings or in the roots of three *Arabidopsis* mutants, deficient in cytoplasmic calcium elevation (*dce1* to *dce3*). $[\text{Ca}^{2+}]_{\text{cyt}}$ elevation was induced by the application of 50 μl of CWE to *Arabidopsis* roots. The $[\text{Ca}^{2+}]_{\text{cyt}}$ was calculated from the relative light units (RLU) measured at 5-s integration times for 10 min. In all the experiments, water was used as control and gave background readings. The four calcium signatures are representative of several hundred measurements (Vadassery and associates 2009).

the roots of adult mutants, expression of these defense genes is much lower when compared with the expression in seedling's roots, either because the plant recognizes the microbe as a friendly symbiont and no longer activates defense genes or because the symbiont actively represses the host's defense response (Jacobs et al. 2011). The low level of defense might explain the high colonization rate and the loss of benefits for the adult mutants.

Although overall defense gene activation in WT and mutant roots by *P. indica* is relatively mild compared with pathogen infections, previous studies have demonstrated that it is required for the beneficial interaction. Disturbance of ethylene signaling (Camehl and Oelmüller 2010; Camehl et al. 2010) or manipulation of the abundance of the root myrosinase PYK10 (Sherameti et al. 2008) have severe consequences for the beneficial interaction and are associated with a partial shift from mutualism to parasitism. In agreement with these observations, a number of reports have demonstrated that mycorrhiza rely on a mild activation of defense responses (de Hoff et al. 2009; Fester and Hause 2005; Gutjahr and Paszkowski 2009; Herre et al. 2007; Martin et al. 2007; Pozo and Azcón-Aguilar 2007; Purin and Rillig 2008; Strack et al. 2003). IAOx and its derived compounds, including camalexin, may provide another example for a defense process that restricts growth of fungi in beneficial root symbioses.

In this context, comparison of the root colonization of the seedlings (Fig. 2B) and adult plants (Fig. 7C) is interesting. After 14 days of co-cultivation of the seedlings with the fungus on PNM plates, they were transferred to soil for an additional 42 days. Root colonization in adult WT roots was approximately 40% of that of WT seedlings (Figs. 2B and 7C) although, in both cases, the increase in fresh weight is >30% relative to the mock-treated controls (Figs. 2A and 7A). This suggests that the *P. indica*-mediated growth response of the WT seedlings could probably be achieved by even less colonization of roots. Furthermore, the root colonization of adult *cyp79B2 cyp79B3* plants is five times and that of adult *pad3* plants three times higher than that of the WT seedlings in plates. This indicates that the degree of root colonization fluctuates, which has also been observed for mycorrhizal fungi (Kennedy 2010). On the other hand, comparison of these values with the increase in biomass during the 42 days on soil clearly indicates that the fungus propagates in WT and mutant roots and that the propagation is much stronger in mutant than in WT roots. The higher degree of colonization of the fungus in adult mutant plants leads to reduction of biomass, where the balance of interaction is shifted to a low level of parasitism. Finally, the calculated *Pitef1/AtGAPC2* ratios did not change significantly for seedlings and adult plants regardless of whether they are based on cDNA or DNA levels (Supplementary Fig. S1). This suggests that the fungus is viable and expresses the *Pitef1* gene during growth on soil.

The split-root-system experiment in combination with the expression analysis of genes involved in the synthesis of IAOx and its derived products demonstrate that the *P. indica*-induced responses are local. The relative GUS activity was rapidly increased in *P. indica* co-cultivated lateral roots and root hairs (Fig. 6C) at an early phase of interaction (from 24 to 60 h). No stimulatory response has been observed in the uninfected sections of the roots and in mock-treatment. The mRNA levels were much higher in infected lateral roots when compared with the mock treatment (Fig. 6B). Also, Kliebenstein and associates (2005) and Schuhegger and associates (2007) reported that camalexin synthesis genes are typically upregulated locally in proximity to the sites of pathogen infections, and both biotrophic and necrotrophic pathogens can induce camalexin biosynthesis. The local camalexin accumulation corresponds to a

strong induction of tryptophan and camalexin biosynthetic genes (Schuhegger et al. 2006, 2007). It is believed that lesions of the infected tissue are a prerequisite for camalexin induction in nature, although an autoclaved yeast suspension (Raacke et al. 2006) and peptidoglycan preparations (Gust et al. 2007) can induce the phytoalexin synthesis in leaves without observed cell death. Deshmukh and associates (2006) and Jacobs and associates (2011) have demonstrated that root regions undergoing cell death are more colonized by *P. indica* than other regions. Therefore, the interaction occurs preferentially at sides with injured root cells. We observed that, other than *P. indica*, a cell wall preparation from the fungus that promotes plant growth (Vadassery et al. 2009) induces *PAD3*, *CYP79B2*, *CYP79B3*, *CYP71A13*, and *WRKY33* mRNA levels in a dose- and time-dependent manner in the roots of *Arabidopsis* seedlings (data not shown). Therefore, the response does not necessarily require local injuries of individual root cells (e.g., by hyphal penetration) but can also be induced by a *P. indica*-

released microbe-associated molecular pattern (MAMP) that is present in the cell wall preparation. Our data suggest that the *P. indica*-induced response occur locally, although Truman and associates (2010) provided evidence for a role of indole-derived compounds in the establishment and maintenance of systemic immunity. Pathogens and pathogenic MAMPs often induce ROS production via NADH oxidases. Because ROS can function as a rapid, long-distance, auto-propagating signal (Mittler et al. 2011) and because *P. indica* and the *P. indica*-derived cell wall extract does not induce ROS production (Camehl et al. 2011; Vadassery et al. 2009), we may observe only a local induction of the *PAD3*, *CYP79B2*, *CYP79B3*, *CYP71A13*, and *WRKY33* mRNA levels.

Plant signaling events for the *P. indica*-induced camalexin synthesis.

Defense gene activation in pathogenic plant–microbe interactions is often initiated by the activation of a plasma-membrane

Fig. 6. A, β -Glucuronidase (GUS) staining of *pad3::uidA* roots in split petri dishes. Roots were not exposed (–) or exposed (+) to fungal hyphae. Mock treatment: without hyphae; *Piriformospora indica* treatment: plug was at the (+) site of the split petri dish. **A**, Bottom pictures show enlargement of *P. indica*-exposed root sections from split petri dishes. **B**, Fold induction of the mRNA levels for *CYP79B2*, *CYP79B3*, *CYP71A13*, *PAD3*, *CYP83B1*, *SUR1*, and *WRKY33* by *P. indica* in the lateral root and root hair preparation from *Arabidopsis*. RNA was isolated from the root material of wild-type (WT) seedlings which were either co-cultivated with *P. indica* for 14 days or mock treated. After real-time polymerase chain reaction analysis, the mRNA_{+P. indica}/mRNA_{mock-treated} ratio was calculated. Data are based on four independent biological experiments with three replications. Error bars represent standard errors (SE). Relative errors of the proportion are the sum of the individual relative errors. **C**, Relative GUS activity in lateral roots and root hairs of mock-treated and *P. indica*-infected seedlings at the early phase of interaction (from 0 to 60 h) of WT roots. Data are based on four independent biological experiments with 10 replications. Error bars represent SE. Relative errors of the proportion are the sum of the individual relative errors.

localized receptor, followed by intracellular Ca^{2+} elevations, H_2O_2 production, and MPK3 or MPK6 phosphorylation. In pathogenic interactions, cytoplasmic Ca^{2+} elevation proceeds ROS production and MPK3 activation (Blume et al. 2000; Jabs et al. 1997; Kroj et al. 2003; Lee et al. 2004; Ligerink et al. 1997; Nürnberg et al. 1994; Zimmermann et al. 1997). Induction of the cytochrome P450 genes under study required MPK3 but appeared to be independent of H_2O_2 -dependent processes leading to defense gene activation via the PDK1/OXI1 pathway (Fig. 1). Interestingly, three genetically distinct ethyl-methane-sulfonate mutants that are impaired in inducing cytoplasmic Ca^{2+} elevation in response to *P. indica* and a cell wall extract

from this fungus also fail to induce camalexin synthesis genes. Therefore, cytoplasmic Ca^{2+} elevation is required for *P. indica*-induced stimulation of the genes for the P450 enzymes and WRK33, similar to the signaling in pathosystems.

MPK3 inactivation prevents the induction of camalexin-synthesizing genes by *P. indica* almost completely (Fig. 1). This is in agreement with a previous observation that a cell-wall extract from the fungus phosphorylates MPK in a Ca^{2+} -dependent manner (Vadassery et al. 2009). In pathogen-induced camalexin synthesis in leaves, MPK3 has an overlapping function with MPK6 and both kinases are required for full induction of camalexin biosynthesis (Ren et al. 2008). For instance, both

Fig. 7. A, Shoot fresh weight of the wild type (WT), *cyp79B2 cyp79B3*, and *pad3* of adult plants after 42 days in soil. WT and mutants seedlings were either co-cultivated with *Piriformospora indica* for 14 days or mock treated on PNM plates before transfer to soil. Standard errors (SE) are based on six independent biological experiments with 12 plants. B, Fold induction of the mRNA levels for pathogenesis-related (PR) genes 1, PR-1, PR-3, and PDF1.2; 2, phenylalanine ammonia lyase (PAL) and germin; and 3, root-hair-deficient (RHD) genes RHD2 and RHD6 by *P. indica* in the roots of *Arabidopsis* WT, *cyp79B2 cyp79B3*, and *pad3* plants. RNA was isolated from the roots of WT and mutant seedlings which were either co-cultivated with *P. indica* for 14 days or mock treated on PNM plates before transfer to soil for 42 days. After real-time polymerase chain reaction analysis, the $\text{mRNA}_{+P. indica} / \text{mRNA}_{\text{mock-treated}}$ ratio was calculated. Data are based on six independent biological experiments with three replications. Error bars represent SE. Relative errors of the proportion are the sum of the individual relative errors. C, Root colonization (relative values) of WT, *cyp79B2 cyp79B3*, and *pad3* plants, which were either co-cultivated with *P. indica* for 14 days on PNM plates or mock treated before transfer to soil for 42 days. Root colonization was calculated as outlined in the text. Here mRNA-based data are shown; however, they are not significantly different from DNA-based data. To allow comparison of the root colonization data of the adult plants shown here with those of the seedlings shown in Figure 2B, the value for the WT control in this graph is expressed relative to that for WT seedlings in Figure 2B, which was set as 1.0. Bars represent SE, based on six independent biological experiments with three replications.

MPK3 and MPK6 are required for *Botrytis cinerea*-induced camalexin synthesis and subsequent limitation of fungal growth (Ren et al. 2008). Whether this is different for beneficial or root-specific fungi remains to be determined. *CYP79B2*, *CYP79B3*, and *PAD3* expression in response to *P. indica* is relatively low compared with the regulation by pathogens, which might be caused by the involvement of an additional control mechanism (for instance, active gene repression by either the plant or the fungus) to ensure a peaceful co-existence between the two symbionts.

Elevated H_2O_2 levels lead to the activation of OXI1, followed by MPK3 and MPK6 and PTI1-2 phosphorylation and transcriptional activation of defense genes (Anthony et al. 2004, 2006; Rentel et al. 2004). OXI1, in turn, is also activated by pathogens and MAMPs via phospholipid signaling and PDK1 activation (Anthony et al. 2006). Our data indicate that activation of *CYP79B2*, *CYP79B3*, *CYP71A13*, *PAD3*, and *WRKY33* by *P. indica* in roots is independent of H_2O_2 production, H_2O_2 -initiated OXI1 signaling, and phosphatidic acid-activated PDK (Fig. 1). ROS is not produced in *P. indica*-colonized WT roots (Camehl et al. 2011; Vadassery et al. 2009). In agreement with this observation, RHD2, a major H_2O_2 -producing NADH oxidase in roots, was not required for the activation of the P450 enzyme genes and *WRKY33* (Fig. 1). This is in agreement with previous observations that the PDK1-OXI1 cascade is important for the beneficial interaction between *P. indica* and *Arabidopsis* but not involved in defense gene activation (Camehl et al. 2011).

In summary, production of IAOx-derived metabolites is activated by *P. indica* during early phases of the interaction. This requires cytoplasmic Ca^{2+} elevation and MPK3; therefore, the fungus may utilize the classical defense pathway in the host. H_2O_2 production and activation of the H_2O_2 -dependent OXI1 pathway is not involved in this response. After the beneficial symbiosis is established, defense processes, including the activation of genes for IAOx-derived metabolite synthesis, are no longer stimulated. However, for long-term harmony between the two symbionts, WT levels of IAOx-derived compounds are required to restrict root colonization.

MATERIALS AND METHODS

Growth conditions of plant and fungus.

Arabidopsis thaliana WT (ecotype Columbia-0) and mutant seed were surface-sterilized and placed on petri dishes containing Murashige-Skoog (MS) nutrient medium (Murashige and Skoog 1962). The *rhd2* line was obtained from V. Zarsky (Charles University, Prague); the *rhd6* (N6347), *pdk1.1* (N613251), and *pdk1.2* (N820699) lines from the Nottingham Arabidopsis Stock Centre; *mpk3* from D. Scheel (Halle); and *oxi1* from H. Hirt (Paris). The *pdk1.1 pdk1.2* double knock-out lines and all other mutants were described by Camehl and associates (2011). Homozygosity of the seed used for these studies was confirmed with gene-specific primers as previously published elsewhere. After cold treatment at 4°C for 48 h, plates were incubated for 10 days at 22°C under continuous illumination ($100 \mu\text{mol m}^{-2} \text{s}^{-1}$). *P. indica* was cultured as described previously on *Aspergillus* minimal medium (Hill and Kaefer 2001). More detailed information has been published by Johnson and associates (2011b). To ensure that the beneficial effect remain constant during fungal propagation, spores were reisolated from freshly infected *Arabidopsis* roots every 6 months for further propagation of the fungal hyphae (Johnson et al. 2011b, section A2).

Co-cultivation experiments and estimation of plant growth.

Twelve days after the growth of seedlings on MS media, *A. thaliana* seedlings of equal sizes were selected for co-cultiva-

tion experiments with or without *P. indica*. Co-cultivation was done as described by Johnson and associates (2011b, section C1, method 1). Because equal light intensity for each seedling is critical, it was checked weekly. Weights of seedlings were determined at 14 day after co-cultivation. For DNA and RNA isolation and gene expression studies, only roots were used.

Split root system, enrichment of lateral roots with root hairs.

Arabidopsis seedlings were grown on MS medium as described above. Then, plants were transferred on nylon membrane on split plates containing PNM medium. Half of the root system was posed to one-half of the plate with a fungal plug, while the other half was posed on other half of the plate. Mock treatment was done with a Kaefer medium plug. Both petri dishes without lids were kept in a larger petri dish for 14 days under the conditions described above to allow growth of the seedlings. Roots were harvested for RNA extraction. Alternatively, the two sections of the roots were cut separately from the aerial parts with a scalpel and dipped into liquid nitrogen for 1 s. Under this condition, smaller lateral roots and root hairs broke away from the main root and were recovered for RNA extraction.

Experiments on soil.

At 14 days after co-cultivation with *P. indica* or mock treatment on PNM plates, the seedlings were transferred to pots with uninfected soil for an additional 42 days (Johnson et al. 2011b, section C2, method 1). For harvest, the soil was removed from the roots with distilled water prior to RNA extraction. Plant were grown in a temperature (22°C)- and moisture-controlled room with light from the top ($80 \pm 10 \mu\text{mol m}^{-2} \text{s}^{-1}$ at the pot level) under short-day conditions (8 h of light and 16 h of darkness). The light intensity was monitored weekly.

Preparation of the cell wall extract from *P. indica*.

The cell wall extract was prepared using the protocol of Anderson-Prouty and Albersheim (1975), with modifications as described by Johnson and associates (2011a). Mycelia from 14-day-old liquid cultures were homogenized using mortar and pestle in water at 5 ml/g of mycelia. The homogenate was filtered using a coarse-sintered glass funnel. The residue was homogenized three times with water, twice with chloroform/methanol (1:1), and finally in acetone. This preparation was air dried for 2 h and the mycelial cell wall material was recovered. Elicitor fractions were prepared from mycelial cell walls by suspending 1 g in 100 ml of water and autoclaving for 30 min at 121°C. Autoclaving releases the active fraction. The suspension was centrifuged at 14,000 rpm for 10 min, was filter-sterilized using a 0.22- μm filter, and was concentrated to half. This fraction was further purified by passing it through a Reverse Phase Superclean LC-18 Cartridges (Sigma-Aldrich, Taufkirchen, Germany). The active fractions were collected and again concentrated to half (Johnson et al. 2011a).

Gene expression.

RNA was isolated from root tissue, including the lateral roots and the root hairs, and reverse-transcribed for real-time quantitative PCR analysis, using an iCycler iQ real-time PCR detection system and iCycler software (version 2.2; Bio-Rad, Munich). Total RNA was isolated from independent biological experiments of *Arabidopsis* roots. cDNA was synthesized using the Omniscript cDNA synthesis kit (Qiagen, Hilden, Germany) using 1 μg of RNA. For the amplification of the reverse-transcription PCR products, iQ SYBR Green Supermix (Bio-Rad) was used according to the manufacturer's protocol in a final volume of 25 μl . The iCycler was programmed to 95°C for 2 min;

40 cycles of 95°C for 30 s, 57°C for 40 s, and 72°C for 45 s; 72°C for 10 min; followed by a melting curve program of 55 to 95°C in increasing steps of 0.5°C. All reactions were performed in triplicate. The mRNA levels for each cDNA probe were normalized with respect to the *GAPC2* mRNA level. Fold-induction values of target genes were calculated with the $\Delta\Delta C_P$ equation of Pfaffl (2001) and related to the mRNA level of target genes in mock-treated roots, which were defined as 1.0. Primer pairs with product sizes between 150 and 170 bp are given in Supplementary Table S1.

Root colonization.

Roots from plates or soil were harvested and roots from soil were washed intensively with distilled water before RNA or DNA extraction. *P. indica* was monitored with a primer pair for the elongation factor 1 (*Pitef1*) (Büthorn et al. 2000). The *Pitef1* cDNA/DNA levels were expressed relative to the plant *GAPC2* cDNA/DNA levels. In order to ensure that the fungus was alive, the data presented in the manuscript were based on cDNA synthesized from RNA of the colonized roots. The same calculations were performed for PCR products obtained for fungal and root DNA. However, we did not observe significant differences between cDNA-based and DNA-based data. DNA isolation has been described previously (Camehl et al. 2011). Staining of hyphae and spores occurred with Trypan blue (0.05%) prior to light microscopy (Johnson et al. 2011b).

GUS assays for *pad3::uidA* lines.

Two independent *pad3* promoter::*uidA* lines were used for these experiments (Schuhegger et al. 2006). GUS staining was performed by transferring the nylon membrane with the seedlings (and hyphae) to a fresh petri dish prior to the application of the substrates, as described by Sherameti and associates (2002).

Camalexin determination.

The determination of camalexin concentrations has been described previously (Glawischnig et al. 2004). Five biological replicates were analyzed.

Ca²⁺ measurements, isolation of Ca²⁺ mutants.

Transgenic apoaquorin *A. thaliana* (Col-0) lines (Knight et al. 1997) were used for Ca²⁺ measurements. The roots were dissected from 14-day-old seedlings and reconstituted in 5 μ M coelenterazine (P.J.K. GmbH, Kleinblittersdorf, Germany) in the dark overnight at 21°C. The luminescence counts obtained with a microplate luminometer (Luminoscan Ascent, version 2.4; Thermo Fisher Scientific, Schwerte, Germany) were calibrated using the equation by Rentel and Knight (2004) (Johnson et al. 2011a; Vadassery et al. 2009, appendix S1). The mutants were obtained after ethyl methanesulfonate mutagenesis. Approximately 450,000 individuals were screened with the cell wall extract from *P. indica* (J. M. Johnson and R. Oelmüller, unpublished). The three mutant lines used here were propagated on soil and M5 seed were used for these analyses. Crosses between the lines confirmed that they are not allelic.

Data analysis.

Data points represent the mean of six independent biological experiments with 12 to 24 seedlings or plants per treatment per experiment (if not otherwise stated). Samples were evaluated with a two-sample *t* test (+*P. indica* or mock treated) and analyses of variance (comparison of all data sets). Percent promotion is the proportion of *x* (value_{+P. indica}) and *y* (value_{mock-treated}), and the relative error of the proportion is the sum of the individual relative errors (standard errors of the mean).

ACKNOWLEDGMENTS

Work was supported by the Jena School of Microbial Communication and the Deutsche Forschungsgemeinschaft (OE133/28-1). We thank M. Hitzentbichler, S. Mußbach, and C. Röppischer for excellent technical assistance; and our colleges for the mutant and transgenic lines used for these experiments.

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