RESEARCH PAPER

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Growth of *Arabidopsis* seedlings on high fungal doses of *Piriformospora indica* has little effect on plant performance, stress, and defense gene expression in spite of elevated jasmonic acid and jasmonic acid-isoleucine levels in the roots

Khabat Vahabi, Iris Camehl, Irena Sherameti, and Ralf Oelmüller*

Institute of Plant Physiology; Friedrich-Schiller-University Jena; Jena, Germany

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The endophytic fungus *Piriformospora indica* colonizes the roots of many plant species including *Arabidopsis* and promotes their performance, biomass, and seed production as well as resistance against biotic and abiotic stress. Imbalances in the symbiotic interaction such as uncontrolled fungal growth result in the loss of benefits for the plants and activation of defense responses against the microbe. We exposed *Arabidopsis* seedlings to a dense hyphal lawn of *P. indica*. The seedlings continue to grow, accumulate normal amounts of chlorophyll, and the photosynthetic parameters demonstrate that they perform well. In spite of high fungal doses around the roots, the fungal material inside the roots was not significantly higher when compared with roots that live in a beneficial symbiosis with *P. indica*. Fifteen defense- and stress-related genes including *PR2*, *PR3*, *PAL2*, and *ERF1* are only moderately upregulated in the roots on the fungal lawn, and the seedlings did not accumulate H_2O_2/radical oxygen species. However, accumulation of anthocyanin in *P. indica*-exposed seedlings indicates stress symptoms. Furthermore, the jasmonic acid (JA) and jasmonic acid-isoleucine (JA-Ile) levels were increased in the roots, and consequently *PDF1.2* and a newly characterized gene for a 2-oxoglurate and Fe^{2+}-dependent oxygenase were upregulated more than 7-fold on the dense fungal lawn, in a JAR1- and EIN3-dependent manner. We conclude that growth of *A. thaliana* seedlings on high fungal doses of *P. indica* has little effect on the overall performance of the plants although elevated JA and JA-Ile levels in the roots induce a mild stress or defense response.

Introduction

Mutualism is a balanced stage of plant/microbe interaction where both partners benefit from each other.[1-3] In the symbiosis between the clavicipitaceous fungal endophyte *Epichloë festucae* and its host ryegrass, the degree of root colonization determines whether the interaction is mutualistic or parasitic.[4] In mycorrhiza, the beneficial interaction is based on the delivery of soil nutrients from the fungus to the plant and reduced carbon from the plant to the fungus.[5] However, environmental changes or mutations can shift these mutualistic interactions to commensalism (when one organism benefits without affecting the other) or parasitism (when one organism benefits while the other is harmed). Crucial for the maintenance of a mutualistic interaction is a balanced growth of the 2 symbionts, which requires a permanent signaling to establish an equilibrium between plant defense gene activation and propagation of the fungus.[1,2,6-13] This balance is also a prerequisite for appropriate reprogramming of the host development in response to endosymbionts.[14] The important role of defense gene activation in symbiotic interactions is shown for rice, where 43% of the genes respond to colonization by both arbuscular mycorrhiza fungi and pathogenic fungi, and many of them are involved in plant defense and stress.[15] Campos-Soriano and Segundo[8] proposed that increased demands for sugars by the fungus might activate host defense responses that will then contribute to the stabilization of root colonization. Plants may restrict carbohydrate flux toward their mycorrhizal partners to avoid fungal parasitism.[7] In tomato mycorrhiza, oxylipin metabolism and signaling may activate host defense responses that will contribute to both the control of fungal spread and the increased resistance to fungal pathogens in mycorrhizal plants.[9] Finally, Barto et al.[16] proposed fungal superhighways

*Correspondence to: Ralf Oelmüller; Email: b7oera@uni-jena.de
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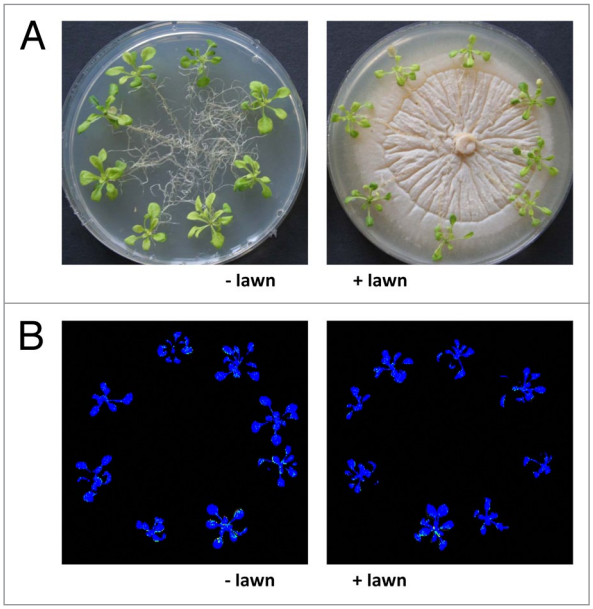

Figure 1. Plants exposed to a dense fungal lawn. (**A**) Nine-day-old Arabidiopsis wild-type seedlings were transferred from MS medium to a plate with KM without *P. indica* (left) or with a dense fungal lawn (right) for 7 d. Growth occurred in continuous light at 80 µmol min^{-2} sec^{-1}. (**B**) False color images of typical seedlings representing F_s/F_m values as described in Methods and Material and ref. 29.

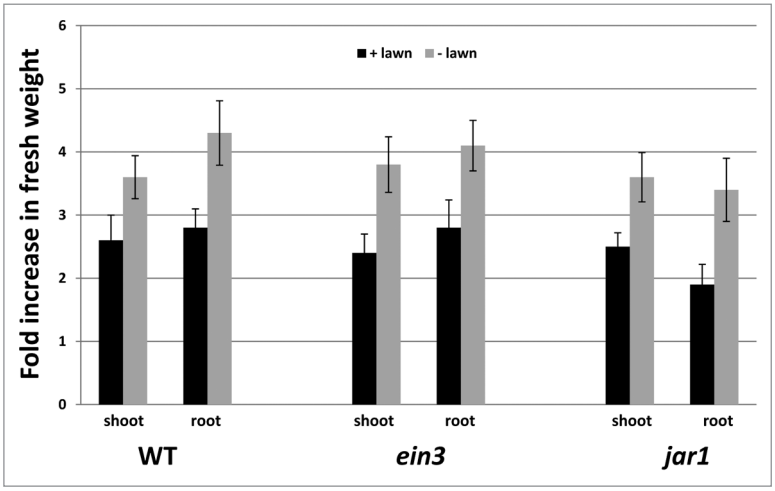

Figure 2. Fold increase in the fresh weight of the shoots and roots during the 7-d growth of the *Arabidopsis* wild-type (WT), *ein3*, and *jar1* seedlings on KM in the presence (+ lawn) or absence (- lawn) of *P. indica*. The values represent [fresh weight $_{9d\ MS\ +\ 7d\ KM\ \pm\ P.\ indica}$/fresh weight$_{9d\ MS}$]. Based on 6 independent experiments with 20 seedlings each, bars represent SEs. Errors are the sum of the individual errors.

that enhance belowground communication. In their model, infochemical transport via common mycorrhizal networks allows chemical defense signaling across plant populations. These examples demonstrate that regulation of plant defense and stress genes is crucial in symbiotic interactions and for the restriction of root colonization. A balanced activation of defense genes from the host is important to control fungal growth.

We study the interaction of *Arabidopsis* roots with the endophytic fungus *Piriformospora indica*, which colonizes the roots of many plant species. Similar to arbuscular mycorrhizal fungi, *P. indica* promotes plant growth, biomass, and seed production[17] and confers resistance to biotic and abiotic stress.[18,19] *P. indica* is a member of Sebacinales, grows inter- and intracellularly and forms pear-shaped spores, which accumulate within the roots and on the root surface.[20] After the establishment of a beneficial interaction, barely any defense or stress genes are activated and no reactive oxygen species (ROS) are produced by the host against *P. indica*.[3,21,22] Like in mycorrhizal symbiosis, the reason for the low level of plant defense against beneficial microbes in unknown. Jacobs et al.[23] proposed that *P. indica* is confronted with a functional plant immune system. It does not evade plant detection but rather suppresses immunity by various microbe-associated molecular patterns. Furthermore, they could show that the ability to suppress host immunity is compromised in the jasmonate mutants *jasmonate-insensitive 1* (*jin1*) and *jasmonate-resistant 1* (*jar1*). We and others have shown that the mutualistic interaction is disturbed in *Arabidopsis* mutants with lesions in specific defense genes or signaling processes leading to defense gene activation.[19,21,23-26] Mutants with lesions in a specific defense response are often unable to restrict growth of *P. indica* hyphae in the roots, and consequently, the roots become overcolonized. The host plant responds to it by activating other defense processes, which are not mutated in the host, to restrict fungal growth and to re-establish a balanced symbiosis of the 2 partners. In contrast to mycorrhizal fungi, *P. indica* can grow on synthetic media without a host.[27] Therefore we addressed the question of how *Arabidopsis* seedlings develop when they are growing on a dense fungal lawn. Do high fungal doses in the environment of the roots also lead to a higher colonization of the roots and, if so, does this affect plants' performance? Do the plants activate defense responses against the high fungal doses that surround the roots?

Results

High doses of *P. indica* inhibit growth but do not have any effect on the efficiency of the photosynthetic electron transport in *Arabidopsis* seedlings. When 9-d-old *Arabidopsis* seedlings were transferred from MS to Kaefer medium (KM) with or without a dense fungal lawn for 7 d (**Fig. 1A**), growth of the seedlings was slower on the medium with the fungus compared with the control. We observed a > 2-fold increase in the shoot and root fresh weights, respectively, for seedlings grown in the presence of the fungus for 7 d, while the increase on media without the fungus was ~3-fold (**Fig. 2**). The 2-fold increase in root and shoot fresh weight clearly indicates that the seedlings can grow on the fungal lawn. The slower growth rate in the presence of the fungus might be caused by an inhibitory effect of the fungus or simply by the fact that the access of the roots to nutrients in the agar medium is reduced (**Figs. 1A and 2**).

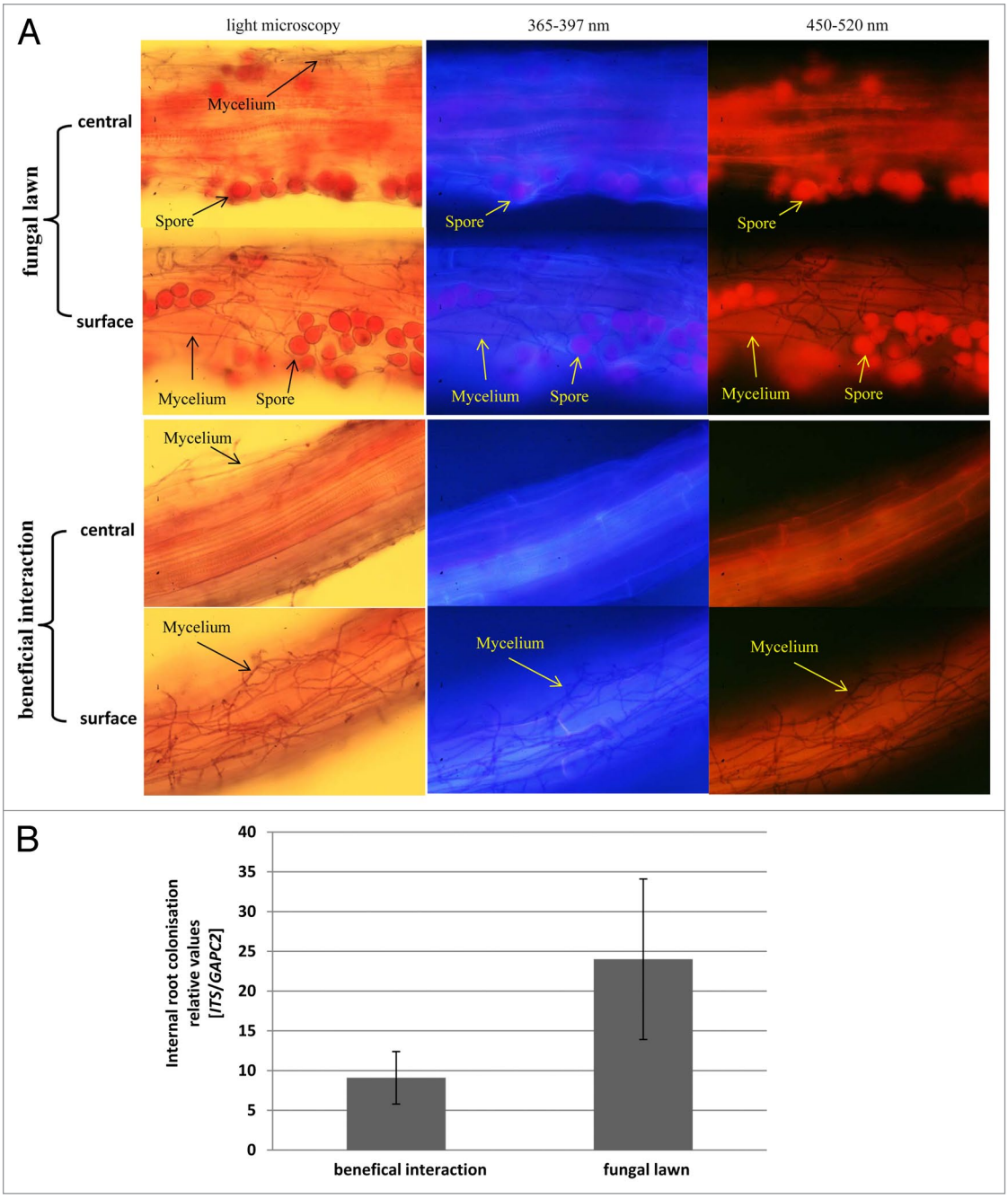

Figure 3. (**A**) Distribution of fungal mycelium and spores on the root surface and in the root material. The seedlings were either co-cultivated with *P. indica* on the fungal lawn for 7 d (upper part, fungal lawn), or co-cultivated with the fungus under beneficial conditions for 7 d (lower part, beneficial interaction). After staining of the fungal material, light microscopic or fluorescent pictures were taken from the root surface or from the central part of the root. Note the high concentration of spores and mycelium on the surface of the roots grown on the fungal lawn, which are not detectable inside of the roots. Under beneficial co-cultivation conditions for 7 d, spores are not yet formed, but the mycelium is detectable on the root surface. (**B**) The amount of the fungal *ITS* cDNA relative to the root *GAPC2* mRNA. *Arabidopsis* seedlings were co-cultivated with *P. indica* under beneficial conditions or on the fungal lawn for 7 d. After extensive washing of the roots, the mRNA was extracted and the amounts of the fungal and plant genes were determined by quantitative RT-PCR.

The efficiency of the photosynthetic electron flow, measured by chlorophyll fluorescence based parameters, is a sensitive parameter for the fitness of a plant.[28-30] After 7 d on KM either with or without the fungal lawn, the seedlings were dark-adapted for 15 min and the chlorophyll fluorescence was measured using a FluorCam 700F. False color images of the seedlings in plates (**Fig. 1A**) are shown in **Figure 1B** (compare ref. 29) and quantified data are presented in **Table 1**. The quantum yield of photosystem II Φ_{PSII}), photochemical (qP) and non-photochemical quenching (NPQ), and maximum quantum yield of PSII

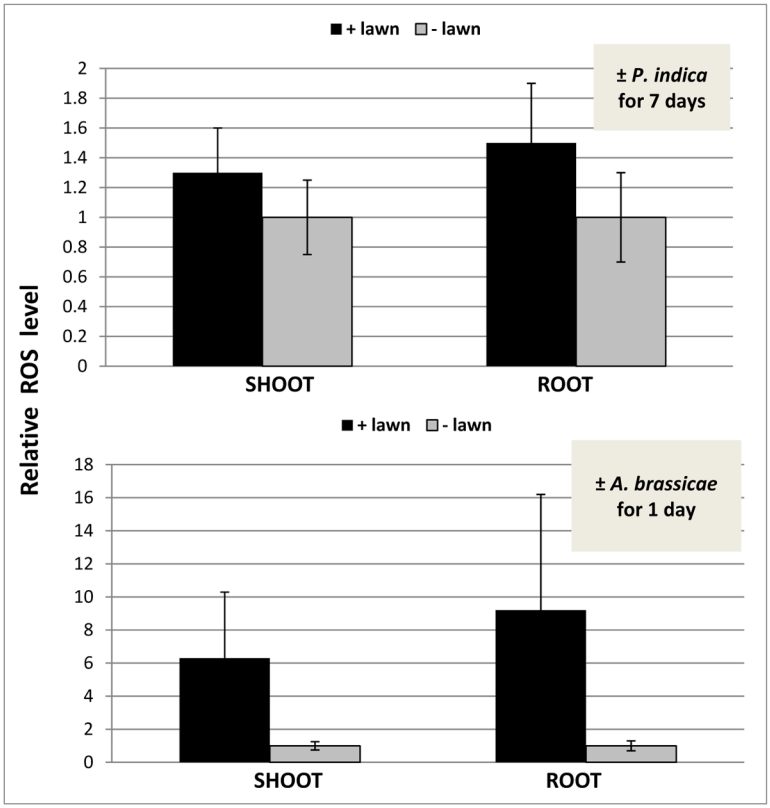

Figure 4. Relative ROS levels in the shoots and roots of *Arabidopsis* seedlings that were kept on KM medium with *P. indica* (+ lawn) or without *P. indica* (- lawn) for 7 d (top) or with *A. brassicae* (+ lawn) or without *A. brassicae* (- lawn) for 24 h (bottom). The ROS levels of the "– lawn" controls were set as 1.0 and the other values expressed relative to them. Based on 6 independent experiments with 20 seedlings each, bars represent SEs.

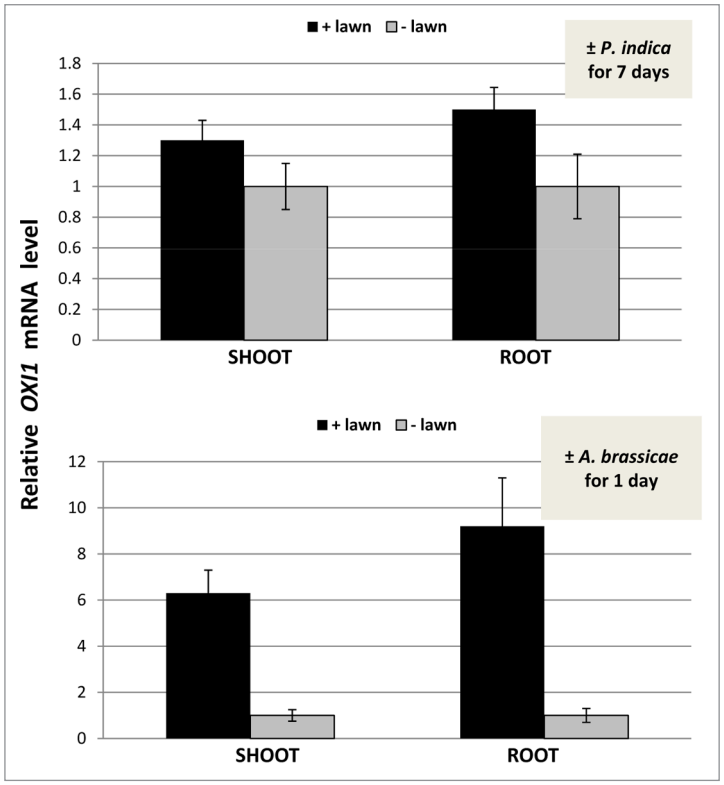

Figure 5. Relative *OXI1* mRNA level in the shoots and roots of *Arabidopsis* seedlings that were kept on KM medium with *P. indica* (+ lawn) or without *P. indica* (- lawn) for 7 d (top) or with *A. brassicae* (+ lawn) or without *A. brassicae* (- lawn) for 24 h (bottom). The *OXI1* mRNA levels in the "– lawn" samples were set as 1.0 and the other values expressed relative to them. Based on 6 independent experiments with 20 seedlings each, bars represent SEs.

(Fv/Fm) were almost identical for seedlings exposed to the dense fungal lawn and the control (**Fig. 1B**; **Table 1**). Thus, the efficiency of the photosynthetic electron transport (Φ_{PSII}, qP), the ability of heat dissipation of photochemical energy (NPQ), and the ratio of function photosystem II to total photosystem II (Fv/Fm) were not impaired by exposure of the seedlings to the dense fungal lawn. Furthermore, the amount of chlorophyll per gram fresh weight was not significantly different between the 2 treatments (**Table 1**). We have previously demonstrated that the efficiency of the photosynthetic electron transport is not affected or even slightly improved when the seedlings are exposed to a low concentration of the fungus and beneficial interaction conditions.[30] We conclude that the overall performance of the seedlings on the dense fungal lawn is quite well and comparable to seedlings that were not exposed to the dense fungal lawn, although their growth rate is reduced.

Intracellular growth of the mycelium on the dense fungal lawn is not significantly higher than under beneficial co-cultivation conditions

Growth of the seedlings on the dense fungal lawn results in a high concentration of mycelium and spores around the roots (**Fig. 3A**). After staining of the fungal material (compare Material and Methods) and microscopic analyses of the distribution of the mycelium and spores on the root surface and in the root material, we observed that the concentration of mycelium and spores inside the roots of seedlings grown on the fungal lawn was not higher than in roots that were grown with the fungus under beneficial co-cultivation conditions (**Fig. 3A**). This demonstrates that growth of the fungus inside the root material is independent of the concentration of the fungal material around the roots. This is further supported by quantitative RT-PCR analyses (**Fig. 3B**): the fungal *ITS* cDNA/plant *GAPC2* cDNA ratio is not significantly different in roots grown under beneficial co-cultivation conditions or on the fungal lawn.

High doses of P. indica do not stimulate H_2O_2/ROS production

Under beneficial co-cultivation conditions, *P. indica* does not induce H_2O_2/ROS production,[22] while exposure of roots to stress or pathogens is often associated with a massive ROS production. NBT staining of roots and shoots did not show any obvious difference between plant material exposed to the *P. indica* lawn and the mock-treatment (data not shown). Therefore, we used a more sensitive assay for quantitative measurement of ROS levels based on the Amplex Red

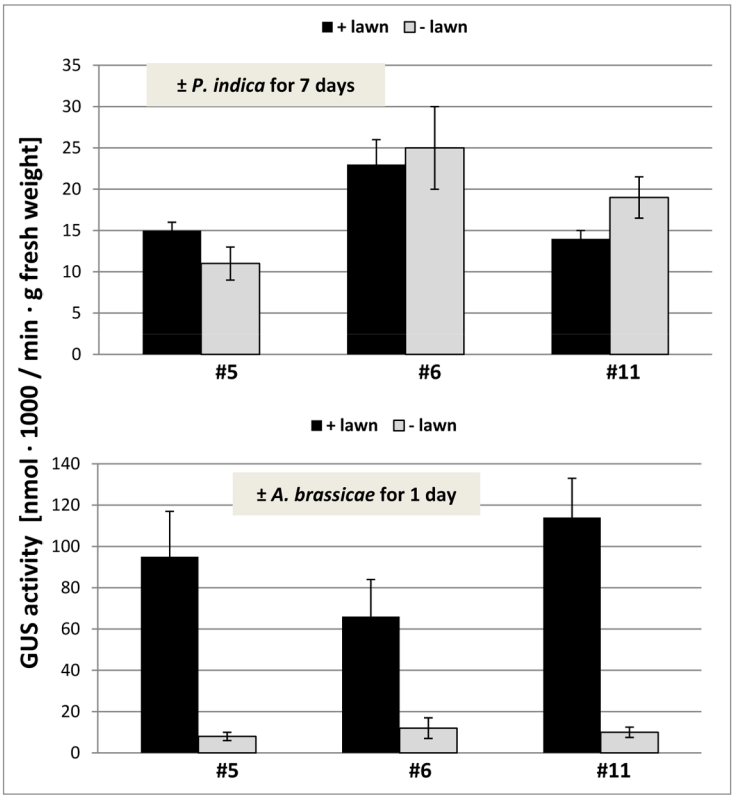

Figure 6. GUS activity in the roots of *Arabidopsis* seedlings that were kept on KM medium with *P. indica* (+ lawn) or without *P. indica* (- lawn) for 7 d (top) or with *A. brassicae* (+ lawn) or without *A. brassicae* (- lawn) for 24 h (bottom). The *uidA* gene was expressed under the control of the *oxi1* promoter (compare ref. 31). Three independent transformants (#5, #6, and #11) were analyzed. Based on 6 independent experiments with 20 seedlings each, bars represent SEs.

peroxidise technology (compare Methods and Material). Within 7 d, the overall ROS production in the roots of seedlings exposed to high fungal doses increased approximately 1.5–2-fold compared with seedlings grown in the absence of the fungus, but the difference was not significant (**Fig.** 4). The differences in the ROS levels in the leaves were even smaller than those in the roots (**Fig.** 4). We conclude that elevated ROS production cannot be observed even by high fungal doses. Furthermore, a ROS-inducible gene, *OXI1*,[31] is not significantly upregulated by *P. indica* in the shoots and roots (**Fig.** 5), and 3 independent *Arabidopsis* lines in which an *OXI1*::*uidA* construct was introduced[31] did not respond to *P. indica* (**Fig.** 6). For comparison, we performed the same cultivation experiment with *Arabidopsis* seedlings on a dense lawn of *Alternaria brassicae*. The measurements were performed after 24 h, since seedlings exposed to *A. brassicae* for 7 d were already dead (data not shown). A strong stimulation of ROS production can be measured within 24 h in the shoots and roots of the pathogen-exposed seedlings (**Fig.** 4). The *OXI1* mRNA level and expression of *OXI1*::*uidA* construct was strongly upregulated after 24 h (**Figs. 5 and 6**). Under these conditions, *OXI1* is induced by H_2O_2/ROS, which accumulate after pathogen attack.[31] The results indicate that exposure of *Arabidopsis* to high doses of *P. indica* over a longer period of time does not lead to the induction of substantial amounts of ROS in roots and shoots.

High doses of *P. indica* induce jasmonic acid and jasmonate-isoleucine levels in roots

Under beneficial co-cultivation conditions for 7 d, *P. indica* exposed and control seedlings of *A. thaliana* did not show differences in the jasmonic acid (JA) and jasmonate-isoleucine (JA-Ile) levels (unpublished). The JA and JA-Ile levels were > 2-fold upregulated in the roots of *Arabidopsis* seedlings grown on the dense fungal lawn for 7 d (**Fig.** 7). Interestingly, the stimulatory effect of the fungus was restricted to the roots and not observed for shoots of the same plant material, and the JA-Ile level in the shoots was even downregulated by the fungus (**Fig.** 7). This suggests a root-specific and not systemic effect of the high fungal doses on JA/JA-Ile levels. Furthermore, we included the *jar1* and *ein3* mutants into the study (compare below). Since JAR1 conjugates JA to Ile,[32-34] the JA-Ile levels are low in the roots and shoots of both *P. indica*-exposed and control *jar1* seedlings (**Fig.** 7). Finally, the JA level in roots of the *jar1* mutant was not upregulated on the dense fungal lawn. Therefore, upregulation of JA by *P. indica* in the roots requires JAR1 (**Fig.** 7).

Table 1. Chlorophyll concentration and photosynthetic electron transfer efficiency in the leaves of *Arabidopsis* seedlings grown on a dense fungal lawn of *P. indica.*

	Fungal Treatment	Chl (M Chl g-1 FW)	ΦPSII	qP	NPQ	Fv/Fm
WT	-	0,14 ± 0,02	0,83 ± 0,04	0,75 ± 0,05	0,33 ± 0,01	0,84 ± 0,02
WT	+	0,13 ± 0,04	0,87 ± 0,06	0,65 ± 0,07	0,34 ± 0,01	0,83 ± 0,01
jar1	-	0,13 ± 0,03	0,86 ± 0,05	0,73 ± 0,06	0,34 ± 0,01	0,83 ± 0,02
jar1	+	0,12 ± 0,04	0,83 ± 0,05	0,66 ± 0,05	0,35 ± 0,02	0,82 ± 0,03
ein3	-	0,13 ± 0,05	0,82 ± 0,07	0,77 ± 0,08	0,33 ± 0,01	0,84 ± 0,01
ein3	+	0,11 ± 0,06	0,89 ± 0,09	0,69 ± 0,09	0,36 ± 0,02	0,82 ± 0,02

Arabidopsis seedlings were kept on Kaefer medium either without (-) or with (+) the dense fungal lawn (compare **Figure 1A**) for 7 d and the chlorophyll content and fluorescence parameters were determined at the end of the experiment. The chlorophyll content per shoot fresh weight, the quantum yield of photosystem II (Φ_{PSII}), photochemical quenching (qP), non-photochemical quenching (NPQ), and the maximum yield of photosystem II (Fv/Fm) were measured for wild-type (WT), *jar1*, and *ein3* seedlings. Data are means ± SEs of 6 independent measurements with n = 5 (chlorophyll measurements) and n = 60 for the chlorophyll parameters.

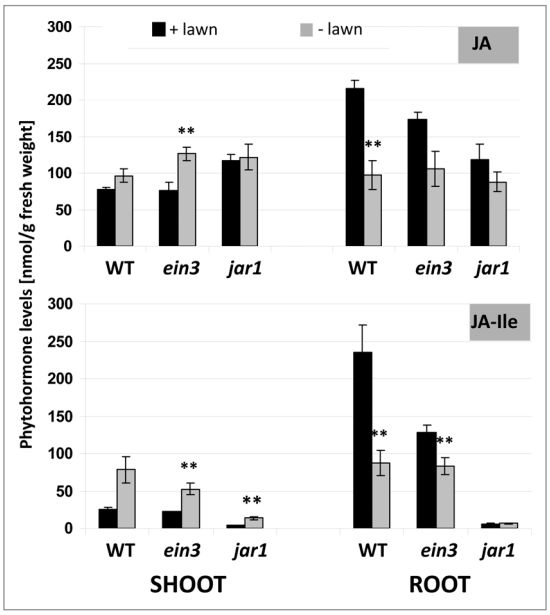

Figure 7. Phytohormone levels (jasmonic acid, JA; jasmonic acid isoleucine, JA-Ile) in the shoots and roots of wild type, *ein3*, and *jar1* seedlings. Nine-day-old seedlings were transferred from MS medium to a plate with KM without *P. indica* or with a dense fungal lawn for 7 d. The phytohormone levels for shoots and roots were analyzed separately. Based on 6 independent experiments with 20 seedlings each, bars represent SEs. **; p £ 0.01.

***jar1* and *ein3* seedlings do not suffer more than the wild-type from high fungal doses**

To test whether JAR1 and EIN3 influence the performance of *Arabidopsis* seedlings on the dense fungal lawn, we compared wild-type, *jar1*, and *ein3* seedlings. However, we did not observe any visible differences in the growth behavior or fitness between the 3 genotypes (**Table 1**). The seedlings of all 3 genotypes grow slower in the presence of the fungus. Also, the chlorophyll content and the photosynthetic parameters were not different from the wild-type seedlings (**Table 1**). ROS production is not stimulated on the dense fungal lawn in the *jar1* and *ein3* mutants, again similar to the wild-type seedlings. We observed a < 2-fold stimulation of ROS production in the roots and shoots of 2 mutants, but these data were not significantly different from the untreated controls. Also the phenotype of their roots (root lengths and root architecture) did not differ when wild-type, *jar1*, and *ein3* seedlings were grown in the presence or absence of the fungus (data not shown). This suggests that JAR1 and EIN3 do not play an important role for the performance of *Arabidopsis* seedlings on a dense fungal lawn, although the JA and JA-Ile levels were increased in the roots.

High doses of *P. indica* Strongly induce *PDF1.2* and the Gene for a 2-oxoglutarate and Fe^{2+}-dependent oxygenase in the roots

PDF1.2 encodes a JA-inducible and ethylene (ET)-responsive plant defensin.[35] Consistent with the observation that the JA and JA-Ile levels were upregulated in the roots, we observed a > 10-fold upregulation of the *PDF1.2* mRNA level in *P. indica*-exposed wild-type roots compared with the mRNA level in roots that were not exposed to the fungus (**Fig. 8**). A ~7-fold stimulation of the *PDF1.2* mRNA level can be detected in the shoots (**Fig. 8**), although the JA and JA-Ile levels did not increase in the shoots on the dense fungal lawn. Therefore, the response in the shoots is systemic. The stimulatory effect in *jar1* and *ein3* seedlings was less than half compared with the wild-type, which indicates that JA/JA-Ile and also ET signaling is involved in *PDF1.2* expression. Interestingly, we identified another gene (*At4g10500*) encoding a 2-oxoglutarate and Fe^{2+}-dependent oxygenase that shows a similar regulation in the 3 genotypes (**Fig. 8**, cf. Discussion).

High doses of *P. indica* induce only a mild upregulation of defense and stress genes

Next, we tested whether defense- and stress-related genes other than *PDF1.2* and *At4g10500* are upregulated on the fungal lawn, since accumulation of anthocyanin in the aerial parts and roots indicates that they are stressed to some extent (**Fig. 9**). We measured an approximately 6-fold upregulation of the anthocyanin level in the roots and 4-fold upregulation in the shoots of wild-type seedlings on the fungal lawn (**Fig. 9**). Comparable results were obtained for *ein3* and *jar1* seedlings (**Fig. 9**), which again confirms that the mutants do not suffer more than the wild-type when exposed to the high fungal doses. The elevated anthocyanin levels are also reflected by a ~2-fold stimulation of the mRNA for the *phenylalanine ammonium lyase* (*PAL*) *2* both in roots and shoots (**Table 2**) after 7 d on the fungal lawn. PAL2 is the main PAL isoform expressed in roots (compare Discussion). Neither the *PAL2* mRNA nor the anthocyanin levels are upregulated in roots or shoots under beneficial interaction conditions. For comparison, after 24 h on an *A. brassicae* lawn, a 22 ± 3-fold increase in the *PAL2* mRNA level was measured in the roots and a 6 ± 1-fold increase in the shoots (data not shown). Similar regulations in response to both *P. indica* for 7 d or to *A. brassicae* for 24 h were observed for the defense-related genes *PR2*, *PR3*, and *ERF1* in roots and shoots: the stimulatory effects by *P. indica* after 7 d were below a factor of 2.5 (**Table 2**), whereas those by *A. brassicae* after 24 h were > 9-fold in the roots and > 5-fold increase in the shoots (data not shown). Again, the expression levels of *PAL2*, *PR2*, *PR3*, and *ERF1* in *P. indica*-exposed or mock-treated *ein3* and *jar1* roots were comparable to those in the wild-type (based on 6 independent experiments with 20 plants each). Taken together, quite different defense and stress genes are only mildly upregulated in the roots and shoots of *Arabidopsis* seedlings even exposed to high doses of *P. indica* for 7 d.

To test whether other genes with stress- and defense-related functions are upregulated in *Arabidopsis* roots exposed to the fungal lawn of *P. indica*, we tested genes that were not upregulated under beneficial co-cultivation conditions of the 2 symbionts, but that responded > 10-fold to unbalances in the symbiontic interaction (Vahabi et al., manuscript in preparation). Interestingly, only *At4g10500* (**Fig. 8**) showed a strong response to the fungal lawn, while all other genes were upregulated less than 2.5-fold (**Table 2**) and thus comparable to *PAL2*, *PR2*, *PR3*, and *ERF1*. This includes genes for P450 enzymes, the calmodulin-binding protein CBP60 g, a chitinase, the stress-related RmlC-like cupins protein At5g38910, the trypsin inhibitor ATT1, a

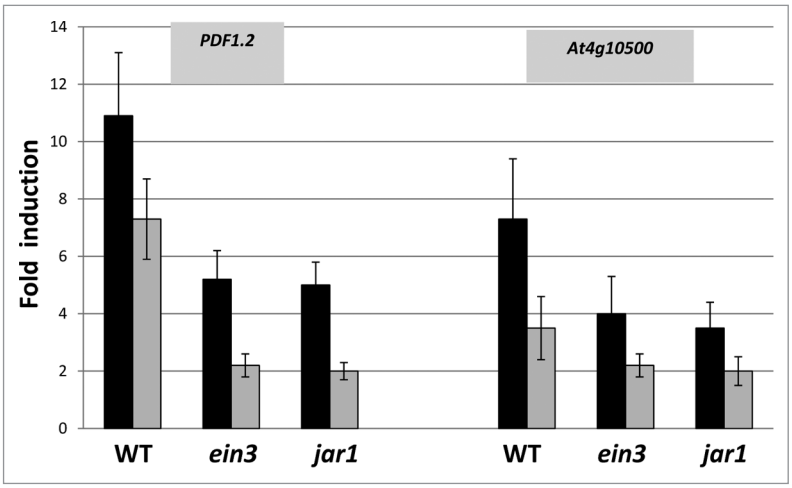

Figure 8. Fold-induction of *PDF1.2* and *At4g10500* transcripts levels in the roots (black) and shoots (gray) of wild-type (WT), *ein3*, or *jar1* seedlings that were either kept on the fungal lawn of *P. indica* or mock-treated. Nine-day-old seedlings were transferred from MS medium to a plate with KM without or with a fungal lawn for 7 d before the RNA was extracted from their roots and shoots. The values indicate fold induction ($mRNA_{+P.\ indica}/mRNA_{-P.\ indica}$). Based on 6 independent experiments with 20 seedlings each, bars represent SEs. Errors are the sum of the individual errors.

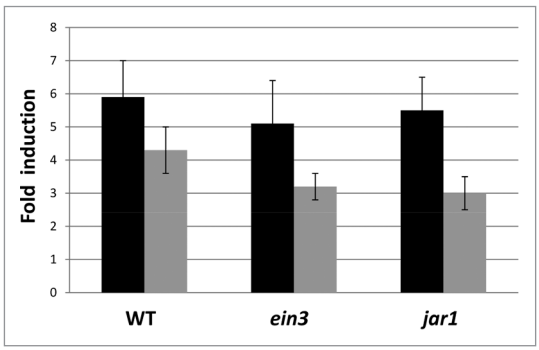

Figure 9. Fold-induction of anthocyanin levels in the roots (black) and shoots (gray) of wild-type (WT), *ein3* and *jar1* seedlings that were either kept on the fungal lawn of *P. indica* or mock-treated. Nine-day-old seedlings were transferred from MS medium to a plate with KM without or with a fungal lawn for 7 d before the anthocyanin level was determined for the roots and shoots. The values indicate fold induction ($A_{530+P.\ indica}/A_{530-P.\ indica}$). For experimental details cf. Material and Methods. Based on 9 independent experiments with 20 seedlings each, bars represent SEs. Errors are the sum of the individual errors.

glutathione-S-transferase (At1g02930), and the GDSL-lipase GLIP1. Several of the genes are involved in JA (CBP60 g, CYP82C2), ABA (CBP60 g), cytokinin (2-oxoglutarate and Fe^{2+}-dependent oxygenase), and ET (GLIP1) signaling (compare Discussion). We conclude that growth on the fungal lawn does not induce a massive stress response in the seedlings (compare Discussion).

Discussion

The overall observation is that growth of *Arabidopsis* seedlings on a dense fungal lawn does not result in a higher fungal concentration in the root material. The performance of the plants, measured on the basis of the efficiency of the photosynthetic electron flow, is not impaired on the dense fungal lawn, compared with plants grown without the fungus or under beneficial co-cultivation conditions. We also did not observe a massive defense or stress response in roots or shoots, although the growth rate of the seedlings is reduced compared with control seedlings grown on the KM without the fungus (**Figs. 1A and 2**). The reduced growth rate may be caused by inhibitory factors from the fungus or by the fact that the seedlings do not have direct access to essential nutrients in the agar. Since accumulation of chlorophyll per gram leaf tissue and the efficiency of the photosynthetic electron transport is not or barely impaired on the fungal lawn (**Table 1**), the plants are fit and do not suffer. We cannot detect significant increases in H_2O_2/ROS production (**Fig. 4**), the stimulation of the H_2O_2 inducible *OXI1* gene (**Fig. 5**), and the activation of the H_2O_2-inducible *oxi1* promoter (**Fig. 6**) in the *P. indica*-exposed tissue, which is consistent with previous observations.[22,36] Furthermore, classical defense genes such as *PR2* (encodes a β-1,3-glucanase), *PR3* (encodes a basic chitinase), and *ERF1* (encodes an ET responsive element binding factor) are only mildly upregulated (**Table 2**). ERF1 has been included in this study because it has previously been demonstrated that this transcription factor gene is involved in the *Arabidopsis*/*P. indica* interaction.[24,25] The elevated anthocyanin and *PAL2* mRNA levels in the *P. indica*-exposed seedlings (**Fig. 9**; **Table 2**) suggest that the seedlings suffer to some extent, although this does not have an effect on the efficiency of the photosynthetic electron transport in the leaves (**Table 1**). Four *PAL* genes are present in the *Arabidopsis* genome,[37] and they respond to a multitude of environmental stress stimuli including pathogen infection, wounding, nutrient depletion, UV irradiation, or extreme temperatures.[38-40] *PAL2* is mainly expressed in roots, and we could show that this gene is only marginally upregulated on the *P. indica* lawn (**Table 2**), when compared with the induction by *A. brassicae*. Furthermore, growth of wild-type, *jar1*, and *ein3* seedlings on the dense fungal lawn was reduced compared with seedlings that were grown without the fungus, but the reduction was similar for the 3 genotypes, and the performance of the seedlings were identical (**Table 1**). Since also stimulation of anthocyanin, and of the *PAL2*, *PR2*, *PR3*, and *ERF1* mRNA levels were comparable for the 3 genotypes (**Table 2**), neither EIN3 nor JAR1 have a significant influence on the performance of the seedlings on the fungal lawn. The lack of massive defense gene activation in the roots exposed to a dense fungal lawn suggests that *P. indica* is either unable to release microbe-associated molecular patterns that activate defense processes, or the fungus activates mechanisms to repress their activation. Jacobs et al.[23] suggested that *P. indica* has established efficient mechanism(s) to bypass or supress host immunity, since the fungus is confronted with a functional root immune system. Either this is also the case for

Table 2. Stress- and defense-related genes and their regulation in the roots (and shoots) after growth of wild-type (WT), *ein3*, or *jar1* seedlings on a dense fungal lawn of *P. indica* for 7 d (**Fig. 1A**)

WT seedlings			
Acc. No.	**Protein**	**Fold stimulation in roots**	**Fold stimulation in shoots**
At3g53260	PAL2	1,6 ± 0,4	1,2 ± 0,3
At3g57260	PR2	1,9 ± 0,4	1,4 ± 0,5
At3g12500	PR3	1,3 ± 0,4	1,5 ± 0,5
At4g17500	ERF1	1,5 ± 0,3	1,2 ± 0,4
***ein3* seedlings**			
Acc. No.	**Protein**	**Fold stimulation in roots**	**Fold stimulation in shoots**
At3g53260	PAL2	1,4 ± 0,4	1,4 ± 0,4
At3g57260	PR2	1,5 ± 0,3	1,5 ± 0,5
At3g12500	PR3	1,5 ± 0,4	1,5 ± 0,7
At4g17500	ERF1	1,2 ± 0,4	1,7 ± 0,3
***jar1* seedlings**			
Acc. No.	**Protein**	**Fold stimulation in roots**	**Fold stimulation in shoots**
At3g53260	PAL2	1,2 ± 0,5	1,7 ± 0,5
At3g57260	PR2	1,8 ± 0,3	1,6 ± 0,4
At3g12500	PR3	1,6 ± 0,5	1,9 ± 0,7
At4g17500	ERF1	1,7 ± 0,4	1,8 ± 0,5
WT seedlings			
Acc. No.	**Protein**	**Fold stimulation in roots**	**References**
At5g26920	Calmodulin-binding protein CBP60 g	1,3 ± 0,3	51–54
At4g31970	P450 enzyme CYP82C2	1,1 ± 0,3	55, 56
At5g38910	RmlC-like cupins protein	1,5 ± 0,4	58 57
At5g25260	Plasmamembrane protein	1,8 ± 0,4	59, 60
At4g11170	Disease response protein	1,3 ± 0,4	61
At3g60270	Cupredoxin protein	1,1 ± 0,2	
At5g57220	P450 enzyme CYP81F2	2,2 ± 0,9	62
At5g40990	GDSL lipase 1	1,5 ± 0,3	63
At1g26390	Berberine protein	1,4 ± 0,4	64–66
At5g39580	Peroxidase	1.6 ± 0,2	67
At2g30750	P450 enzyme CYP1A12	1,3 ± 0,3	68

The values show fold induction relative to the mRNA levels in the roots/shoots of seedlings that were grown on KM medium without the fungus. Based on 3 independent real-time PCR analyses, errors represent SEs. The errors represent the sum of the 2 errors of the original data.

our co-cultivation conditions, or the 2 co-cultivation conditions cannot be compared.

The results shown here open the question why the cultivation of *A. thaliana* on a dense fungal layer does not result in a massive infection (**Fig. 3**). The roots may either have established mechanisms to control fungal invasion for their protection against overcolonization, or intracellular root colonization follows a highly regulated program that is independent of the fungal concentration around the roots and in the rhizosphere. The absence of a massive defense response of the roots against *P. indica* is consistent with the colonization data (**Fig. 3**). For mycorrhizal interaction, empirical studies have shown that mycorrhizal colonization intensity exhibits substantial heritable genetic variation within plant and fungal species and are influenced by plant genotype/fungal genotype interactions, suggesting the potential for ongoing coevolutionary selection.[41] It might be possible that plant genetic traits limit root colonization in the *P. indica*/*Arabidopsis* symbiosis. It has also been discussed

that root colonization maybe controlled by a phosphate/carbon exchange between the 2 partners, and that limitations in any of the nutrients may limit the colonization process.[42] In mycorrhizal interactions, exchange of small molecules from both partners may determine the degree of root colonization.[43] Candidates for small molecules from the fungus that may be important for the control of root colonization are discussed, after the entire *P. indica* genome has been sequenced.[44,45] Finally, JA and salicylic acid signaling during specific stages of root colonization may control a balance between compatibility and defense in mutualistic interactions,[46] and this might be also the reason why the level of the active form of JA increases on the dense fungal lawn. Since the *jar1* mutant does not suffer on the lawn during the 7 d of co-cultivation, it appears that this phytohormone is not required during early phases of the interaction. However, the phytohormone might be required during later phases of the interaction. Finally, Lahmann and Zuccaro[47] and others describe a biphasic colonization strategy of barley and *Arabidopsis* roots by *P. indica* upon penetration of the root: Perturbance of plant hormone homeostasis and secretion of fungal lectins and other small proteins (effectors) may be involved in the evasion and suppression of host defenses at these early colonization steps. At later stages, *P. indica* is found more often in moribund host cells where it secretes hydrolytic enzymes. This strategy of colonizing plants is reminiscent of that of hemibiotrophic fungi, although a defined shift to necrotrophy with massive host cell death is missing. It is reasonable to assume that the plant has established mechanisms that specifically counteract cell-death inducing processes by restricting fungal invasion or growth inside the plant tissue, in particular if the symbiosis is beneficial for the plant.

We identified only 2 genes that were strongly upregulated in response to the fungal lawn. One of these genes is *PDF1.2* (**Fig. 8**). The response is consistent with elevated JA and JA-Ile levels (**Fig.** 7). *PDF1.2* is a marker gene for JA and ET signaling.[35] Since the *PDF1.2* mRNA level is less upregulated in *jar1* and *ein3* roots compared with wild-type roots (**Fig. 8**), it is likely that both phytohormones are involved in the induction. *PDF1.2* is induced both locally at the site of infection by incompatible fungal pathogens and systemically in remote noninoculated regions of the plant.[48] This activation occurs via the JA/ET rather than the SA pathway.[35,48] Necrotrophic fungi can inhibit JA-induced defense gene activation, as recently shown for SSITL, an effector from *Sclerotinia sclerotiorum* that plays a significant role in the suppression of JA/ET signal pathway-mediated resistance at the early stage of infection.[49] Interestingly, *PDF1.2* was also upregulated in the leaves of *P. indica*-exposed seedlings (**Fig. 8**), suggesting a systemic effect, although the JA/JA-Ile levels were not higher in the leaves (**Fig.** 7). Suza et al.[34] have shown that *jar1* has little or no impact on several wound-induced genes. To test for a possible JAR1 role in systemic induction, younger unwounded leaves from the same wounded plants were examined. *PDF1.2* transcripts accumulated in both wounded and unwounded leaves, and *jar1* did not affect the timing or magnitude of accumulation. This provides an example for a JAR1-independent regulation of *PDF1.2*, and might be comparable to the upregulation of *PDF1.2* in leaves where the JA/JA-Ile levels were not elevated, as well as to the upregulation of *PDF1.2* in *jar1* plants on the fungal lawn (**Figs.** 7 and **8**). Finally, *ein3* usually shows a lower *PDF1.2* expression compared with the wild type,[50] consistent with our observations (**Fig. 8**).

Whether *PDF1.2* regulation is a defense response or a general stress response under our co-cultivation conditions is unclear. In barley *P. indica* elicits a non-specific defense reaction by upregulation of a multiplicity of stress responsive genes.[51] If this is also true for the interaction studies described here, the fungus induce only a mild stress response in *Arabidopsis* roots, which has little effect on plant performance and no effect on the efficiency of the photosynthetic electron transport (**Table 1**). Interestingly, the 2-oxoglutarate and Fe^{2+}-dependent oxygenase-encoding gene *At4g10500* shows the same regulation as *PDF1.2* on the fungal lawn (**Fig. 8**). The gene is stimulated in response to calcium stress,[52] senescence,[53] and is involved in cytokinin signaling.[54] The message is also upregulated in *powdery mildew resistant 4* (*pmr4*), a mutant lacking pathogen-induced callose.[55] Further studies are required to understand the role of this protein in the symbiotic interaction.

We also analyzed the expression of a set of less studied defense- and stress-related genes, but all of them were barely or not induced in the roots in response to the dense fungal lawn (**Table 2**). ERF1, a target transcription factor of both JA and ET signaling, is important for *PDF1.2* activation[56,57] and involved in the *Arabidopsis*/*P. indica* interaction.[24,25] CBP60 g is a calmodulin-binding protein that has previously been described to respond to *P. indica* under beneficial co-cultivation conditions in wild-type roots.[58] The protein is a positive regulator of both disease resistance and drought tolerance in *Arabidopsis*.[59] Overexpression of CBP60 g caused elevated SA accumulation, increased expression of the defense genes, enhanced resistance to *Pseudomonas syringae*, hypersensitivity to abscisic acid (ABA), and enhanced tolerance to drought stress.[59] CBP60 g has a partially redundant role with SAD1, which affect defense responses in addition to SA production.[60,61] The P450 protein CYP82C2 (At4g31970) modulates JA-induced root growth inhibition, defense gene expression, and indole glucosinolate biosynthesis.[62] CYP82C2 affects JA-induced accumulation of tryptophan, but not the JA-induced auxin- or pathogen-induced camalexin, and thus acts in the metabolism of tryptophan-derived secondary metabolites under conditions in which JA levels are elevated.[62] The enzyme is also involved in the systemic resistance response induced by the root-colonizing *Pseudomonas fluorescens* strain SS101 against several bacterial pathogens, including *Pseudomonas syringae* pv tomato, and the insect pest *Spodoptera exigua*.[63] The RmlC-like cupins protein At5g38910 is an apoplastic manganese ion binding protein with potential nutrient reservoir activity. The mRNA was induced by cesium stress[64] as well as treatments with 9 other abiotic stresses.[65] The mRNA for the plasma membrane-associated protein At5g25260 is induced in response to geminivirus[66] and *Pseudomonas syringae*[67] infections, and involved in ABA-mediated defense responses.[67] At4g11170 is a defense protein, which responds to ozone and shows a high expression in stems, roots, and stamen.[68] The failure of this gene to respond to the dense fungal lawn is

consistent with the observation that the H_2O_2/ROS levels do not increase. The P450 enzyme CYP81F2 (At5g57220) is involved in glucosinolate biosynthesis and *Arabidopsis* innate immune responses.[69] The GDSL lipase-like 1 (At5g40990) regulates systemic resistance associated with ET signaling[70] and elicits both local and systemic resistance. GLIP1-overexpressors exhibited enhanced resistance against necrotrophic pathogens, including *A. brassicicola* and *Erwinia carotovora*, and the hemibiotrophic pathogen *Pseudomonas syringae*.[70] The FAD-binding berberine protein At1g26390 is an oxidoreductase in the endomembrane system that is upregulated in response to various biotic and abiotic stresses.[71-73] The identified peroxidase At5g39580 is involved in various defense responses.[74] Finally, the P450 enzyme CYP1A12 is involved in camalexin synthesis and thus crucial for defense responses against pathogens and herbivores.[75] All these genes have been identified in previous studies to be upregulated > 10-fold when the symbiotic interaction between *P. indica* and *Arabidopsis* is disturbed (Vahabi et al., unpublished). The lack of regulation of these genes on the dense fungal lawn suggests that the interaction of the 2 symbionts is not harmful. The exact function of these proteins in the *P. indica*/*Arabidopsis* symbiosis under unfavorable co-cultivation conditions is currently under study.

Under beneficial co-cultivation conditions, defense and stress genes including *PDF1.2* are not upregulated in *Arabidopsis* roots.[3,18,19,21,22,24-26] However, *Arabidopsis* mutants, which are impaired in establishing a beneficial interaction or are unable to maintain a long-term harmony between the 2 symbionts, activate a mild defense response against *P. indica*.[18,19,21,22,24-26] In particular, *PDF1.2* has been reported repeatedly as being upregulated under these conditions.[19] Unbalances in the interaction are often accompanied by an increase in root colonization.[19] This has also been observed for other mutualistic interactions, e.g., for mycorrhiza formation[4,46,76,77] and the interaction of plant-growth promoting microbes with roots.[78,79] The results shown here are somewhat surprising since they show that *Arabidopsis* seedlings can be exposed to a high dosis of a beneficial fungus without a significant defense gene activation from the host against the microbe or shift from mutualism to parasitism. The plants perform quite well. The available tools and genes described here allow us to investigate the signaling between the symbionts in greater details. In particular, the results demonstrate that these genes are not simply upregulated if the roots are surrounded by high fungal doses of *P. indica*.

Material and Methods

Growth conditions of plants and fungus

Wild-type or mutant (*jar1*, obtained from Dr. J. Vadassery, Max-Planck-Institute for Chemical Ecology and *ein3*[24]) *Arabidopsis thaliana* seeds were surface-sterilized and placed on petri dishes containing MS nutrient medium.[80] After cold treatment at 4 °C for 48 h, plates were incubated for 7 d at 22 °C under continuous illumination (75 µmol m^{-2} sec^{-1}). *P. indica* was cultured as described previously[20,81,82] on KM modified by Pham et al.[82] For solid medium 1% (w/v) agar was included. *Alternaria brassicae* cultivation has been described in details in Johnson et al.[83]

Nine-day-old wild-type, *ein3*, or *jar1* seedlings were directly transferred from MS medium to a plate with a fungal lawn of *P. indica*.[83] The fungal lawn was obtained by placing a fungal plug on KM and the fungus was allowed to grow for 14 d at 24 °C in the dark, before the seedlings were transferred to the plate. Control seedlings were transferred to KM without the fungus. The plates were incubated for 7 d at 22 °C under continuous illumination (µmol m^{-2} sec^{-1}) from above.[83] The co-cultivation experiments with *A. brassicae* were performed under the same conditions, except that the fungus colonized the plate only for 5 d[83] and co-cultivation was terminated after 24 h. Fresh weights were determined directly after harvest. Beneficial co-cultivation conditions of the 2 symbionts for 7 d was performed on PNM medium, because growth of the fungus on Kaefer medium is too fast for a balanced interaction of *P. indica* and *A. thaliana*.[20] A detailed protocol is given in Johnson et al.[83]

Staining of fungal mycelium and spores

Fuchsin acid and trypan blue staining of fungal hyphae in *Arabidopsis* roots was described in details in Vahabi et al.[84] In brief, *Arabidopsis* roots co-cultivated with *P. indica* were collected and intensively washed with distilled water. After incubation in fuchsin acid solution for 10 min, the material was washed with destilled water for 1 min. They were then stained with trypan blue (0.0001mg/ml) solution for 3 min and washed again for 1 min.

After addition of 50 µl GL solution (glycerol:lactic acid:water, 1:1:1) on a glass slide, the sample was protected with a glass cover, before analysis by light and fluorescent microscopy using different wavelengths. The localization of hyphae and spores on the surface of the roots or in the root material was distinguished by scanning through the plant material.

RNA extraction and cDNA synthesis

RNA was isolated from the roots or shoots with an RNA isolation kit (RNeasy, Qiagen, Hilden, Germany). For quantitative RT-PCR, RNA from *Arabidopsis* mock-treated roots/shoots and roots/shoots treated with a high amount of fungal hyphae were used. Reverse transcription of 1 µg of total RNA was performed with an oligodT Primer. First strand synthesis was performed with a kit from Qiagen (Omniscript RT Kit, Qiagen, Hilden, Germany).

Real-time PCR

Real-time quantitative RT-PCR was performed using the iCycler iQ real time PCR detection system and iCycler software version 2.2 (Bio-Rad, Munich, Germany). For the amplification of the PCR products, iQ SYBR Supermix from Bio-Rad was used according to the manufacturer's instructions in a final volume of 23 µl. The iCycler was programmed to 95 °C 3 min, 40 × (94 °C 30 s, 57 °C 30 s, 72 °C 40 s), 72 °C 10 min followed by a melting curve program (50–85 °C in increasing steps of 0.5 °C). All reactions were repeated at least 4 times. The mRNA levels for each cDNA probe were normalized with respect to the *GAPC2* message level. Fold induction values were calculated with the ΔΔCP equation of Pfaffl.[85] *P. indica* cDNA was detected with primers for the *ITS* region (CAACACATGT GCACGTCGAT;

Box 1. The following *Arabidopsis* primer pairs were used:

Gene	Forward primer	Reverse primer
At5g26920 (CBP60G, CaM-binding protein)	CCGCATTACA GCGGTTAACG ATAG	ACTTCCTTGA AAGTCGATGT GCTG
At5g38910 (RmlC-like cupins superfamily protein)	TATTGCTGAC ACCGTGTTTG GG	ACTTCCTTGA AAGTCGATGT GCTG
At5g39580 (Peroxidase superfamily protein)	GCGATCTCGT CACTCTTGTT GGAG	TAAACCCACA TGCAGCTGTT CCG
At5g40990 (GDSL lipase1)	CCTGATTTCA TCGCGGAGTA CG	TGGCTGTTACC GTTGAATGGT TG
At5g57220 (CYP81F2, cytochrome P450)	TCATCAAAGG GCTCATGCTC AG	GCCATCGCCC ATTCCAATGT TAC
At1g26390 (FAD-binding berberine family protein)	ACGCCACAAT GAGTAGCCTG AG	TCACCACTCG GATTGCTTCC AAC
At3g60270 (Cupredoxin superfamily protein)	TGCAGCCTTG GCATGAAACT CG	ACGGTGGAGG CTCTAATGAA ACG
At5g25260 (PHB domain-containing membrane-associated protein family)	TTGCTAAGAC TAACGCGCTT GC	GTTCTCCACC ATGGTTCCAA ACG
At4g31970 (CYP82C2, cytochrome P450)	ATTAAATCTA CCTGCCTGGC ACTG	GCCCATGTAA GGGTTGATGG TG
At4g11170 (TIR-NBS-LRR class)	AGAAGCTATG GAGTGGAGTT CAGC	AGCTCCACCA AAGACTCACA CC
At5g44420 (PDF1.2)	CTTGTGTGCT GGGAAGACAT A	AGCACAGAAG TTGTGCGAGA A
At4g10500 (2-oxoglutarate and Fe(II)-dependent oxygenase superfamily protein)	TATCGGCGAC CAAATGCAGG TC	ACTACGGCTC TATGGAGCAC AC
At3g57260 (PR2)	TCTTCTCAGC CTTGTAATAG C	TGTTTGTAAA GAGCCACAAC G
At3g12500 (PR3)	TCATGGGGCT ACTGTTTCAA G	TATTGCTCTA CCGCATAGAC C
At4g17500 (ERF1)	TATCCTCAAC GACGCCTTTC	TCTTGACCGG AACAGAATCC
At3g53260 (PAL2)	AGGTACTGAC AGTTACGGAG	CATGTCTCCT TCGTGTTTCC
At3g04120 (GAPC2)	GAGCTGACTA CGTTGTTGAG	GGAGACAATG TCAAGGTCGG

CCAATGTGCA TTCAGAACGA). Root colonization was determined relative to the plant *GAPC2* cDNA levels (See **Box 1**).

Phytohormone measurement

Phytohormones were extracted by homogenizing approximately 100 mg of *Arabidopsis* material and adding 1 ml ethylacetate spiked with internal standards [D6-ABA, D2-JA, D4- SA, and 13C6-JA-Ile (200 ng/each)]. Samples were homogenized twice by reciprocal shaking (FastPrep speed 6.5) for 45 s and centrifuged at 13 000 rpm for 20 min at 4 °C. The supernatant was transferred to a fresh 2 ml microcentrifuge tube and the extraction was repeated by adding 1 ml ethylacetate without internal standards. The organic phases were pooled and evaporated under reduced pressure. The dried sample was dissolved in 500 µl 70% methanol, vortexed, and centrifuged at 13 000 rpm for 10 min. 100 µl of the supernatant were transferred into an HPLC vial with insert and the samples were analyzed by liquid chromatography tandem mass spectormetry system (Varian 1200L Triple-Quadrupol-MS). Ten µl of each sample were injected onto a ProntoSIL column (C18; 5µm, 50 × 2mm). The mobile phase comprised solvent A (0.05% formic acid in water) and solvent B (0.05% formic acid in acetonitrile). Compounds were ionized by electrospray ionization and analyzed in the negative mode by multiple reaction monitoring (MRM).

NBT stain

Arabidopsis seedlings were grown as described before. After 7 d on the fungal lawn, the roots and shoots were stained for 5 min in a solution containing 2 mM nitrobluetetrazolium (NBT; Sigma Aldrich) in water. The reaction was stopped by washing the roots with water. Roots were evaluated under the Axiovert 135 (Carl Zeiss MicroImaging GmbH, Jena, Germany).

ROS measurements

Quantitative ROS measurements from leaves and roots were performed using the Amplex Red hydrogenperoxide/peroxidase assay kit (Molecular Probes) according to the manufacturer's instructions (http://tools.invitrogen.com/content/sfs/manuals/mp 22188.pdf) using the substrate carboxy-H_2DFFDA (Molecular Probes) according to the manufacturer's instructions (https://tools.invitrogen.com/content/sfs/manuals/mp36103.pdf). Leaf sections of 0.5–1 mm width and root sections of 2–3 cm length were incubated in 20 µM carboxy-H_2DFFDA prepared in KRPG buffer for 30 min in the dark. The fluorescence intensity was quantified with a fluorescence microplate reader (TECAN Infinite 200) with an excitation at 485 nm and emission at 530 nm. The reaction mixture without the substrate and plant material served as control.

Anthocyanin measurements

Roots and shoots were ground in liquid nitrogen and extracted with 80% methanol/5% HCl in the dark and incubated overnight at 4 °C. After centrifugation (15.000 g, 20 min) the supernatant was removed and the anthocyanin concentration was determined spectrophotometrically with a Lambda 12 spectrophotometer (Perkin-Elmer Company). The amount of anthocyanin is expressed as A_{530}/mg fresh weight.

GUS assay

Oxi promoter::*uidA* lines[31] were grown on MS media with the appropriate amount of kanamycin for 9 d and were then exposed for 5 d to the treatments. Whole plants were harvested and ground with 500 µl of lysis buffer (100 mM Na_3PO_4, pH 7.0; 500 mM EDTA; 0,1% Triton X-100; 0,1% lauroyl sarcosin; 10 mM β-mercaptoethanol). The homogenate was clarified

by centrifugation and 10 µl of the supernatant was used for assaying GUS activity in a total volume of 100 µl with 1 mM methylumbelliferyl-β-D-glucoside in lysis buffer. The enzyme reaction was performed at 37 °C and stopped after 30 min with 900 µl 100 mM Na_2CO_3. Samples were measured with the fluorometer VersoFluor from Bio-Rad (Bio-Rad, Munich, Germany) after setting the range according to the manufacturer's instructions. The system of reference was an equal amount of fresh weight.

Measurement of chlorophyll and photosynthesis parameters

Measurement of chlorophyll content was performed as described in Porra et al.[86] The *Arabidopsis* seedlings were dark-adapted for 15 min and then the chlorophyll fluorescence was measured using a FluorCam 700F (Photon System Instruments, Czech Republic). Program parameters of FluorCam were set according to Wagner et al.[29] Photosynthesis parameters, quantum yield of PSII (Φ_{PSII}), maximum quantum yield of PSII (Fv/Fm), photochemical quenching (qP), and non-photochemical quenching (NPQ) were calculated according to Maxwell and Johnson.[28] False color images of the seedlings in plates were obtained as described by Wagner et al.[29] Chlorophyll fluorescence images representing F_s/F_m values are shown, whereas blue represents low F_s/F_m values above a threshold of 0.06 and red represents high F_s/F_m values with an upper threshold limit of 0.17.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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