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Ethylene Supports Colonization of Plant Roots by the Mutualistic Fungus *Piriformospora indica*

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**Abstract**

The mutualistic basidiomycete *Piriformospora indica* colonizes roots of mono- and dicotyledonous plants, and thereby improves plant health and yield. Given the capability of *P. indica* to colonize a broad range of hosts, it must be anticipated that the fungus has evolved efficient strategies to overcome plant immunity and to establish a proper environment for nutrient acquisition and reproduction. Global gene expression studies in barley identified various ethylene synthesis and signaling components that were differentially regulated in *P. indica*-colonized roots. Based on these findings we examined the impact of ethylene in the symbiotic association. The data presented here suggest that *P. indica* induces ethylene synthesis in barley and *Arabidopsis* roots during colonization. Moreover, impaired ethylene signaling resulted in reduced root colonization, *Arabidopsis* mutants exhibiting constitutive ethylene signaling, ethylene synthesis or ethylene-related defense were hyper-susceptible to *P. indica*. Our data suggest that ethylene signaling is required for symbiotic root colonization by *P. indica*.


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**Introduction**

Ethylene plays a prominent role in senescence and plant development [1,2]. In *Arabidopsis thaliana*, ethylene is perceived by five ER membrane-bound receptors (e.g. Ethylene Triple Response 1, ETR1). In the absence of ethylene, the receptors activate a Raf-like kinase (Constitutive Trp Response 1, CTR1), which negatively regulates the downstream ethylene response pathway [3]. Binding of ethylene inactivates the receptors, resulting in the deactivation of CTR1, which allows downstream effectors such as Ethylene Insensitive 2 (EIN2) to function as positive regulators of ethylene signaling [4,5] by activating transcription factors Ethylene Insensitive 3 (EIN3) and EIN3-like 1 (EIL1) [6]. Constitutive ethylene signaling is observed in *ctr1* [3] and *ethylene ocroproducer 1 (eto1)* mutants. ETO1 negatively regulates ethylene synthesis by inactivating and/or degrading 1-aminoacyclopropane-1-carboxylic acid synthase 5 (ACS5) and probably other ACS isoforms such as ACS4, ACS8, and ACS9 [7,8,9]. It has long been known that ethylene supports plant immunity [2]. For instance, EIN3 and EIL1 drive the expression of primary ethylene transcriptional activators, such as *Ethylene Response Factor 1* (ERF1). ERF1 regulates ethylene responsive and defense-related genes (e.g. Pathogenesis-related 3, *Plant Defensin 1.2*) [10] thereby contributing to defense against necrotrophic pathogens [11]. Recent studies underlined the participation of ethylene in very early processes of immune signaling [12,13]. Plant immunity is induced after perception of conserved microbial molecules, so called microbe-associated molecular patterns (MAMPs, e.g. flagellin, chitin), by specific pattern recognition receptors (PRRs) [14]. The recognition of bacterial flagellin by the PRR Flagellin Sensing 2 (FLS2) results in the activation of an array of immune responses summarized as MAMP-triggered immunity (MTI), and includes the rapid production of reactive oxygen species (ROS) as well as ethylene [15]. It has been shown that ethylene signaling is essential for flagellin-triggered ROS production [13,16]. In a model proposed by Boutrot et al. (2010), flagellin recognition by FLS2 results in MAP kinase (MAPK) 3 and 6 phosphorylation that, in turn, phosphorylates and thereby stabilizes ACS2, ACS6, and EIN3 [12,16,17,18]. Consequently, rapid ethylene production is immediately downstream of MAMP recognition, and, due to the transcriptional regulation of FLS2 by EIN3, ethylene mediates a steady-state level of FLS2 at the plasma membrane [13,16]. By contrast, impaired ethylene signaling disturbs FLS2 regulation, subsequent MAPK3/6 phosphorylation and ROS production, processes that are required to stop pathogen invasions. Thus, ethylene has a more complex role in the activation of early and late immune responses.

*Piriformospora indica* is a root-colonizing basidiomycete that colonizes mono- and dicotyledonous plants, including barley (*Hordeum vulgare*) and *Arabidopsis*, in which the fungus increases yield and adaptation to abiotic and biotic stress [19,20,21,22,23]. Cytological and genetic studies have shown that *P. indica* initially
colonizes living cells. This biotrophic growth phase is observed up to 3 days after inoculation [24] and is followed by a second cell death-dependent colonization phase (≥3 dai), which is restricted to colonized cells [24,25,26]. The fungus has an immune suppressing activity, which is essential for biotrophic root colonization, and may particularly explain its remarkably broad host range [24]. DNA microarray-based gene expression analysis of barley roots colonized by P. indica showed the differential expression of genes related to ethylene synthesis and signaling [27]. In the present study, we therefore analyzed the effect of ethylene on the colonization of Arabidopsis and barley roots by P. indica. We demonstrate that P. indica induces 1-aminoacyclopropane-1-carboxylic acid (ACC) synthesis and that ethylene signaling is not detrimental to fungal growth. We discuss the possibility that ethylene is a positive modulator of the mutualistic plant root-P. indica symbiosis.

Results

Impaired ethylene signaling reduces colonization of plant roots by P. indica

Global transcriptome analyses revealed differential regulation of components with putative functions in ethylene synthesis and signaling in barley roots inoculated with P. indica [27]. Among the ethylene synthesis genes were three 1-aminoacyclopropane-1-carboxylic acid (ACC) oxidases (Table 1). Six genes involved in signaling encoded putative transcription factors: ethylene-responsive element binding protein, ethylene insensitive 3-like 2, AP2 domain transcription factor EREBP, a putative RAV2-like DNA binding protein, ethylene-responsive factor, and ethylene-binding protein-like (Table 1). Interestingly, while ethylene synthesis genes were mostly induced, signaling components were generally suppressed during P. indica colonization (Table 1).

The data raised the possibility that ethylene modulates P. indica’s ability to colonize plant roots. Because barley mutants with compromised ethylene biosynthesis and signaling are not available, we conducted tentative pharmacological experiments in order to determine the significance of ethylene at early stages of a successful symbiosis. To this end, two-day-old barley seedlings were transferred to agar plates containing 100 μM of the ethylene precursor 1-aminoacyclopropane-1-carboxylic acid (ACC), or to a jar containing a vial with 1 mM of the ethylene antagonist 1-methylcyclopropene (MCP), which blocks ethylene signaling by interacting with ethylene receptors [28]. Seedlings were inoculated with P. indica (500,000 chlamydospores ml⁻¹) and fungal colonization was determined at 3 and 7 days after inoculation (dai) by quantitative real time PCR (qRT-PCR). While root colonization was unaltered after ACC treatment, MCP treatment resulted in an approximately 50% reduction in the amount of fungal DNA at 7 dai (Students t-test, P<0.05) (Figure 1A). Neither of the compounds had adverse effects on morphology or growth of P. indica in vitro (not shown). We further tested whether ethylene might generally affect colonization of different plant hosts. Upon treatment of Arabidopsis with MCP, P. indica also displayed reduced root colonization, although in contrast to barley, the effect was already detectable at 3 dai (Figure 1B). These data suggest that colonization of barley and Arabidopsis roots by P. indica is supported by ethylene signaling.

ACC levels are increased in P. indica-colonized barley roots

While blockage of ethylene signaling reduced fungal colonization of barley, application of ACC, the immediate precursor of ethylene, had no effect. One explanation could be that ACC levels were high per se in young roots regardless of fungal colonization. If ethylene signaling was indeed saturated, treatment with ACC would not further affect ethylene synthesis and thus fungal root colonization. To test this hypothesis, we determined ACC contents in P. indica-colonized roots. The ACC pool in plants consists of free and malonylated ACC. Malonylation is a mean to inactivate and thereby control the amount of active (free) ACC that might be used for ethylene production. We measured free and malonylated ACC at 1, 3, and 7 dai. Because previous studies showed that P. indica preferentially colonizes the maturation zone of roots [24,25], the upper two centimeters of the root (basal part = maturation zone) were analyzed separately from the remaining apical root tissue (apical part). The amount of malonylated ACC was higher than free ACC indicative of a saturated ACC pool (Figure 2A, B). Significantly elevated amounts of free ACC were found in the apical root part during biotrophic colonization (3 dai) and in the apical as well as basal part during the cell-death associated growth phase (7 dai) (Figure 2A).

MAMP-triggered root oxidative burst is suppressed by P. indica

Global gene expression analysis demonstrated that P. indica hardly induces defense responses in barley roots [27]. Consistent

<table>
<thead>
<tr>
<th>Table 1. List of barley genes differentially regulated by P. indica and involved in ethylene synthesis or signaling.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gene</strong></td>
</tr>
<tr>
<td>1-aminoacyclopropane-1-carboxylate oxidase</td>
</tr>
<tr>
<td>putative 1-aminoacyclopropane-1-carboxylate acid oxidase</td>
</tr>
<tr>
<td>putative 1-aminoacyclopropane-1-carboxylate oxidase</td>
</tr>
<tr>
<td>AP2 domain transcription factor EREBP</td>
</tr>
<tr>
<td>ethylene-binding protein-like</td>
</tr>
<tr>
<td>ethylene insensitive 3-like 2</td>
</tr>
<tr>
<td>ethylene-responsive element binding protein</td>
</tr>
<tr>
<td>ethylene-responsive factor</td>
</tr>
<tr>
<td>Similar to probable RAV2-like DNA binding protein</td>
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1 Gene expression data was published in [27].

doi:10.1371/journal.pone.0035502.t001
with this we showed that *P. indica* suppresses MAMP-triggered responses such as the oxidative burst and defense gene expression in *Arabidopsis* roots [24]. Ethylene signaling is required for MAMP-triggered oxidative burst, one of the earliest innate immune responses [13,16]. Hence, the finding that *P. indica* induces ethylene synthesis genes (Table 1) and ACC synthesis during early (3 dai) and late colonization (7 dai) stages prompted us to assess the fungus’ ability to suppress chitin-induced oxidative burst in barley roots. To this end, we determined chitin-induced root oxidative burst in non-colonized and *P. indica*-colonized roots. In non-colonized roots, a strong accumulation of H2O2 was measured after treatment with the octamer of fungal chitin (1M N-acetyldolichoocose, Figure 3) as was reported for leaves. In contrast, chitin-induced root oxidative burst was almost completely abolished in *P. indica*-colonized roots. This finding corroborates earlier results showing that *P. indica* has a strong capability in suppressing plant defense responses [24,27]. Apparently, the anticipated increase in ethylene production does not result in a colonization-associated MAMP-triggered oxidative burst, or related signaling processes are suppressed by the fungus. To exclude the possibility that the suppressing activity is a general attribute of root colonizing fungi, we also tested the ROS-suppressing activity of *Rhizoctonia solani*, a pathogenic root-colonizing basidiomycete that also displays a broad host range. We found that *R. solani* could not suppress the chitin-induced ROS accumulation, suggesting that ROS-suppressing activity is associated with the symbiotic potential of *P. indica*.

**Colonization-associated induction of ACC synthases in *Arabidopsis* roots**

ACC quantification in barley roots did not allow for cellular resolution of ACC production, nor did it prove an association of ACC synthesis with *P. indica* colonization. Since reporter lines for ACC synthesis are not available for barley, we took advantage of the *Arabidopsis*-*P. indica* system. We used *Arabidopsis*-reporter plants for ACC synthesis that express β-glucuronidase (GUS) fusions with promoters of genes encoding 1-aminoacyclopropane-1-carboxylic acid synthases (ACS). ACS are the rate limiting enzymes in ethylene synthesis [29]. In *Arabidopsis*, nine ACS genes (ACS1, ACS2, ACS4, ACS5, ACS6, ACS7, ACS8, ACS9, and ACS11) have been identified [29]. The respective reporter lines allowed monitoring of the spatio-temporal expression of an individual ACS gene upon *P. indica* colonization. To this end, *Arabidopsis* (reporter) plants were analyzed by fluorescence and bright field microscopy at 3 and 7 dai upon double-staining for GUS activity and for fungal hyphae with WGA-AF 488. Based on the AREX database [30,31], all nine ACS genes are expressed in the meristematic, elongation, and maturation zone, but differ in level and site of expression level as well as site (Table S1). Among all the tested lines, only ACS1::GUS and ACS8::GUS showed a response to *P. indica* (Figure 4, 5). ACS1 was induced by the fungus at primordia and the base of lateral roots at 7 dai (Figure 4). Most obviously and consistent with the ACC accumulation pattern in barley (see Figure 2), both ACS1::GUS and ACS8::GUS plants showed a strong GUS activation at 7 dai at the root tip region of *P. indica*-colonized plants (Figure 5) although the staining pattern slightly differed among both lines. GUS activity in ACS1::GUS was detected in the elongation zone, while GUS accumulated also in the meristem of ACS8::GUS (Figure 5). However, the GUS accumulation pattern did not differ in any line in non-colonized compared to *P. indica*-colonized roots at 3 dai.

**Ethylene signaling enhances colonization of *Arabidopsis* roots by *P. indica* at the cell death-associated interaction stage**

To further confirm in *Arabidopsis* that ethylene affects *P. indica* colonization, we quantified fungal growth in the *Arabidopsis* mutants etr1-3 and ein2-1, which are impaired in ethylene signaling, as well as *ctr1-1*, which shows constitutive ethylene signaling. In addition, the ethylene signaling mutant *eto1-1* was tested. Quantitative real time (qRT)-PCR-based quantification of the amount of fungal DNA at 3 dai (biotrophic colonization) and 14 dai (cell death-dependent colonization) showed higher colonization of mutants that displayed constitutive ethylene signaling (*ctr1-1*) or enhanced ethylene synthesis (*eto1-1*) during cell death-associated colonization. Comparable to MCP treatment of *Arabidopsis* roots (Figure 1B), colonization of *ein2-1* was reduced at 3 dai and reached wild type levels at 14 dai. Colonization of *etr1-3* was not altered as compared to the wild-type (Figure 6A). Subsequently, we analyzed the colonization of 3SS::ERF1 plants. ERF1 is a transcription factor that is central to ethylene-associated defense signaling in *Arabidopsis* [11]. Like *ctr1-1* and *eto1-1*, plants overexpressing ERF1 were significantly more colonized by *P. indica* at 14 dai (Figure 6A). Improved colonization of 3SS::ERF1 is contradictory to a recent study, which demonstrated unaltered colonization of this line at 12 dai [32]. Most probably, the divergent experimental set up resulted in the different outcomes. In our assay, plants were grown on sugar-free medium in square petri dishes to which a defined amount of spores (500,000 spores ml⁻¹) was directly applied to roots, thereby avoiding detachment of seedlings and roots (see Materials and Methods). Detachment of

![Figure 1. Colonization of barley and *Arabidopsis* by *P. indica* in response to ACC and MCP. (A) Two-day-old barley seedlings or (B) two-week-old *Arabidopsis* seedlings were inoculated with *P. indica* and subsequently treated with 500 ppt 1-methylcyclopropene (MCP) as described in Materials and Methods. Barley was also treated with 100 µM 1-aminoacyclopropane-1-carboxylic acid (ACC). MCP inhibited *P. indica* colonization at 3 or 7 dai in *Arabidopsis* or barley, respectively. The values are normalized to colonization in mock-treated roots (set to one). The data are based on three independent biological experiments. Student’s t-test indicates a significant difference in *P. indica*-colonization of MCP-treated roots (*P*<0.05). doi:10.1371/journal.pone.0035502.g001]
roots might result in root injuries and activation of wound-induced stress signaling which might affect root colonization. Consistent with this, when we slightly injured roots with forceps and inoculated these roots one day later, we observed reduced colonization of 35S::ERF1 roots at 3 dai, while colonization was unaltered at 7 dai (Figure 6B).

Discussion

The spatio-temporal events associated with the colonization of barley and Arabidopsis roots by P. indica are very similar, including a biotrophic followed by a cell death-dependent colonization phase [24,25,26,27,33]. Our analyses suggest fungus-induced ethylene production especially in apical root parts of barley and Arabidopsis since we detected enhanced ACC production in barley (Figure 2) and induction of ACS1 and ACS8 in Arabidopsis (Figure 5), ACS1 induction has not been reported in roots [29], but is in accordance with the AREX database prediction (Table S1). The analyses suggest systemic regulation of ACS1 and ACS8 as the fungus was...
not detected at root apices. Notably, ACC is a mobile molecule and not necessarily produced at sites of ethylene action. ACC produced in roots is known to be transported via the xylem to allow ethylene synthesis in distant tissue [34,35,36]. Ethylene, like jasmonic acid (JA) and salicylic acid (SA), effectively sustains MAMP-triggered immune responses against pathogens [2,37,38], and also affects mutualistic symbioses, since ethylene inhibits mycorrhization and rhizobial nodulation of legumes [39,40,41,42]. However, P. indica-induced ACC production is probably not participating in early immune signaling (e.g. oxidative burst). First, based on leaf expression data in Genevestigator [43], ACS1 and ACS8 are not responsive to biotic stress. Secondly, as already reported for Arabidopsis roots [24], P. indica also suppressed MAMP-induced oxidative burst in barley roots (Figure 3). Thirdly, ethylene significantly supported P. indica colonization in both plants (Figure 1, 6). Blockage of ethylene signaling by MCP (Figure 1B) or by the lack of EIN2 resulted in reduced root colonization at 3 dai in Arabidopsis (Figure 6A), while MCP treatment caused reduced colonization of barley at 7 dai (Figure 1A). The temporally different effects of ethylene signaling on compatibility (Figure 1) suggest differences in fungal requirements to colonize both plants and indicates P. indica’s adaptive potential to colonize root cells of different species. It will be interesting to see in future studies which ethylene-regulated processes are influenced by the fungus in both plants. Fourthly, ctr1-1, eto1-1 and 35S:ERF1 plants that display constitutive ethylene signaling, synthesis or defense, respectively, were significantly better colonized by P. indica at 14 dai (Figure 6). The stunted root morphology of eto1-1 and ctr1-1 may contribute, but cannot entirely account for improved colonization, as we also observed increased colonization in 35S:ERF1 plants, which possess an unaltered root phenotype. Notably, lack of etr1 did not affect root colonization as seen in ein2-1 mutants (Figure 6) and suggests redundancy among the five ethylene receptors during P. indica colonization of Arabidopsis roots, which is not observed for the downstream effector EIN2. Consistent with this, blockage of ethylene perception by MCP resulted in reduced colonization at 3 dai as observed for ein2-1 (Figure 1, 6).

Interestingly, the pronounced GUS accumulation in P. indica-colonized ACS8::GUS roots (Figure 5) is reminiscent of its induction in Arabidopsis roots after auxin treatment [29]. Notably, auxin stimulates the activities of several ACS [44,45] and antagonizes

Figure 4. GUS accumulation in roots of ACS1::GUS reporter plants colonized by P. indica. Arabidopsis line ACS1::GUS was harvested at 7 dai and, after GUS and WGA-AF 488 staining, analyzed cytologically. (A, B) P. indica colonization at the base of lateral roots (arrows) or primordia (asterisks) of line ACS1::GUS was associated with enhanced GUS accumulation. P. indica (arrowsheads in A) was visualized by staining with WGA-AF 488. (C) In mock-treated ACS1::GUS, GUS staining was weakly detectable e.g. at the lateral root base. Bars = 60 μm. doi:10.1371/journal.pone.0035502.g004

Figure 5. GUS accumulation in roots of ACS1::GUS and ACS8::GUS reporter plants colonized by P. indica. Arabidopsis lines ACS1::GUS and ACS8::GUS were harvested at 7 dai and, after GUS and WGA-AF 488 staining, analyzed cytologically. GUS staining was more pronounced in root tip regions of colonized roots as compared to mock-treated roots (upper images). At 7 dai, P. indica-colonized roots of both lines showed a significant increase of dark blue tips and a significant reduction in pale blue tips as compared to mock-treated roots. GUS staining did not colocalize with colonization sites of P. indica or extracellular fungal growth. The data base on at least two biological experiments. Asterisks indicate significant differences between control and P. indica-colonized roots according to Students t-test (∗ P<0.05, ** P<0.001).

doi:10.1371/journal.pone.0035502.g005

Figure 6. Colonization of ethylene synthesis and signaling mutants by P. indica. (A) Three-week-old plants were inoculated with P. indica and fungal biomass was determined in ein2-1, etr1-3, eto1-1, ctr1-1, and 35S::ERF1 by qRT-PCR at 3 and 14 dai. (B) Three-week-old 35S::ERF1 plants were injured with forceps and inoculated with P. indica at 1 day after wounding. Fungal biomass was determined by qRT-PCR at 3 and 7 dai. All values were related to Col-0 (set to one). The data are based on at least three independent experiments. Students t-test indicated significant difference in P. indica-colonization (∗ P<0.05, ** P<0.001).

doi:10.1371/journal.pone.0035502.g006
SA-mediated defense [46]. In turn, SA defense restricts P. indica colonization, while JA signaling, in analogy to ethylene signaling, supported root colonization [24]. An antagonistic activity of ethylene to SA-related defense has been demonstrated in 35S::ERF1 seedlings [47], which displayed enhanced susceptibility to P. indica (Figure 6). It is tempting to speculate that auxin might be synthesized by the plant [27], and/or by the fungus as was recently hypothesized [48]. It would be interesting to investigate in future, whether auxin metabolism might be activated during P. indica colonization, thereby regulating ACS8 (and ACS7) expression and impairing SA-related immune processes.

The inconsistency of our results on the colonization of 35S::ERF1 (Figure 6A, B) in comparison to a recent report [32] is most probably explained by the different colonization assays used in the two studies. In addition, we determined fungal colonization in a direct approach by quantifying fungal DNA in relation to plant DNA via qRT-PCR. We found this to be essential for a sensitive quantification of fungal DNA in roots.

Taken together, we demonstrated that ethylene supports colonization of barley and Arabidopsis roots by P. indica. This is in line with the requirement of JA for Arabidopsis root colonization by P. indica [22], considering the synergistic activity of JA and ethylene in plant signaling [44]. Recent studies revealed the significance of ethylene in JA-SA crosstalk. While SA blocked JA signaling, this inhibition did not occur in plants in which JA and ethylene pathways were activated [49]. It is tempting to speculate that P. indica recruits ethylene together with JA in order to outcompete SA immunity. Recent studies demonstrated the effectiveness of SA-related immunity to restrict root colonization by P. indica [22]. Interestingly, JA and ethylene are required for induced systemic resistance (ISR) observed in leaves after colonization of roots by beneficial microbes such as Pseudomonas fluorescens [50,51]. Therefore, recruitment of JA/ethylene during root colonization might be further connected to systemic resistance that is induced in leaves of P. indica-colonized Arabidopsis [52]. Indeed, systemic resistance induced by P. indica was dependent on JA signaling and suggested to be based on ISR [52].

Materials and Methods

Plant material and fungal inoculation

Seeds of Arabidopsis thaliana ecotype Col-0 and mutants eto1-1 (N3072), ctrl-1 (N8057), etr-1 (N3070), 35S::ERF1 (N6142), and ACS::GUS reporter plants (N31379, N31380, N31381, N31392, N31383, N31385, N31386, N31387) were obtained from the European Arabidopsis Stock Centre (NASC). All the Arabidopsis plants and the respective parents were grown on ½ Murashige and Skoog medium on square petri dishes, which were vertically positioned. Plants were grown at 22/18°C day/night cycle under short-day conditions (10 hours light) at 60% relative humidity in a growth chamber. Three-week-old plants were inoculated with 500,000 P. indica chlamydomspores ml⁻¹ (DSM11827 from Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) by adding 1 ml spore suspension per petri dish containing ~ 40 plants and harvested at indicated timepoints. For root injury experiments, 35S::ERF1 plants were grown for three weeks on ½ Murashige and Skoog medium on squared petri dishes and thereafter scratched with a forceps. One day later roots were inoculated with P. indica and harvested at 3 and 7 dai. For barley, all experiments were conducted with cultivar Golden Promise. Golden Promise is a barley line that is no longer commercially available and the seeds used for our studies derive from our own annual propagation. Plants were inoculated as described previously [25]. In brief, barley kernels were sterilized with 6% sodium hypochloride, rinsed in water, and germinated for 2 days. Subsequently, seedling roots were immersed in an aqueous solution of 0.05% Tween-20 containing 500,000 spores ml⁻¹ of P. indica chlamydomspores. Inoculated seedlings were transferred to 1.5 L glass jars containing plant nutrient medium (PNM)/1/10 [27]. Barley root treatment and harvest was performed as described below.

Cyto-histological observations and β-glucuronidase (GUS)-based studies

For cytological examinations, the fungus was stained with wheat germ agglutinin-Alexa Fluor 488 (WGA-AF 488) as previously described [25]. Arabidopsis ACS::GUS plants were harvested at indicated timepoints. GUS staining was performed as described previously [33]. Briefly, roots were stained with staining solution (50 mM phosphate buffer, pH 7.0, 0.5 mM potassium ferrocyanide, 0.2% Triton X-100, 0.5% DMSO, 20% methanol, 2 mM EDTA, 1 mM X-Glu) and incubated overnight at 37°C. The staining reaction was stopped by incubation in 70% ethanol. The roots were analyzed microscopically using an Axioskop 2 microscope (Carl Zeiss, Jena, Germany). WGA-AF 488 was detected at 470/20 nm (excitation) and 505–530 nm (emission).

Application of ACC and 1-methylcyclopropene (MCP)

Two-day-old barley seedlings (cv. Golden Promise) were inoculated with P. indica and transferred to PNM/1/10 [27] supplemented with 100 μM ACC (Sigma-Aldrich, Munich, Germany). ACC was dissolved in water and filter-sterilized prior to its addition to autoclaved plant growth media. For MCP (Rohm and Hasa Company, Philadelphia, USA) treatment, inoculated barley seedlings were transferred to glass jars (volume 1.5 l) in which a vial was placed containing 16 mg MCP (0.14% active ingredient) dissolved in 200 μl water. For Arabidopsis, vials containing MCP were placed inside petri dishes in which plants were grown and inoculated as described above. MCP treatment was conducted immediately after inoculation of roots with P. indica. The final concentration of 1-MCP in the gas phase of the jar and petri dishes was expected to be about 500 ppt [54]. Roots were harvested at 3 and 7 dai, frozen in liquid nitrogen and subjected to DNA isolation (see below).

Determination of ACC content

Two-day-old barley plants (cv. Golden Promise) were inoculated with P. indica or mock treated and transferred to jars containing PNM/1/10 [27]. Roots were harvested at 0, 1, 3, and 7 dai. At 3 and 7 dai, the upper two centimeters (basal part) were harvested separately from the lower apical part. Plant material was ground in liquid nitrogen and extracted according to Langebartels et al. (1991) [55]. Free ACC and total ACC released by acid hydrolysis (2 N HCl for 3 h at 120°C) were determined [55,56]. The amount of conjugated ACC was calculated by subtracting the amount of ACC from total ACC.

Quantitation of P. indica colonization by qRT-PCR

Genomic DNA of wild type and Arabidopsis mutant roots as well as ACC-/MCP- and mock-treated barley roots was extracted from ~100 mg root material using Plant DNeasy Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Ten ng of total DNA served as template for qRT-PCR analyses. Amplifications were performed in 20 μl SYBR green JumpStart Taq ReadyMix (Sigma-Aldrich, Munich, Germany) with 350 nM oligonucleotides, using an Mx3000P thermal cycler with a standard amplification protocol (Stratagene, La Jolla, USA).
Fungal colonization was determined by the 2^{ΔΔCt} method [57] by subtracting the raw threshold cycle (Ct) values of P. indica Internal Transcribed Spacer (ITS) gene from those of Actin/UBQ (Actg62250) or HvUBQ (NIASHv1058N10), respectively, using the ITS-specific primers 5’-CCAGAAGCTTGATCGACGATG-3’ and 5’-CTGGCAGTAATGCAAGGAA-3’ (slope: -3.208, Y-intercept: 30.55, R²: 0.995, efficiency [%]: 104.966), Actin/UBQ-specific primers 5’-CCAAGCGGAGGATCGATG-3’ and 5’-ATCTGCTTCTCTAGACGCTGA-3’ (slope: -3.281, Y-intercept: 27.938, R²: 0.998, efficiency [%]: 101.754), or HvUBQ-specific primers 5’-ACCCTGCGGACTCACCAAT-3’ and 5’-CAG-TAGTGGCGGTGCAAGTG-3’ (slope: -3.212, Y-intercept: 24.559, R²: 0.988, efficiency [%]: 104.783).

Chitin-induced root oxidative burst

Three-day-old barley seedlings were either treated with P. indica, Rhizoctonia solani AG8, or mock-treated. For determination of oxidative burst, roots were cut in 1 cm long pieces (10 mg per assay) at 3 dai and floated in water over night. Roots were transferred to tubes with 20 μM l-homogentisic acid synthase by MPK6, a stress-responsive mitogen-activated protein kinase, which activates the ACC synthase by converting the ethylene precursor acetyl-CoA to ACC [16]. Ethylene-dependent transcription factors EIN3 and EIL1. Pro Natl Acad Sci U S A 96: 1005–1010.

Statistical analysis

All experiments were conducted at least in duplicate and standard errors were calculated for all mean values. Levels of significance were calculated using Student’s t-test.

Supporting Information

Table S1 Regulation of ACC synthase genes in Arabidopsis roots according to the AREX database.

(AOC)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: JD CL DW PS. Performed the experiments: BK AM SP CL PS. Analyzed the data: CL JD DW KHK PS. Wrote the paper: BK KHK PS.

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