Growth Promotion of Chinese Cabbage and *Arabidopsis* by *Piriformospora indica* Is Not Stimulated by Mycelium-Synthesized Auxin

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Piriformospora indica, an endophytic fungus of the order Sebaciales, interacts with the roots of a large variety of plant species. We compared the interaction of this fungus with Chinese cabbage (*Brassica campestris* subsp. *chinensis*) and *Arabidopsis* seedlings. The development of shoots and roots of Chinese cabbage seedlings was strongly promoted by *P. indica* and the fresh weight of the seedlings increased approximately twofold. The strong stimulation of root hair development resulted in a bushy root phenotype. The auxin level in the infected Chinese cabbage roots was twofold higher compared with the uncolonized controls. Three classes of auxin-related genes, which were upregulated by *P. indica* in Chinese cabbage roots, were isolated from a double-subtractive expressed sequence tag library: genes for proteins related to cell wall acidification, intercellular auxin transport carrier proteins such as AUX1, and auxin signal proteins. Overexpression of *B. campestris* *BcAUX1* in *Arabidopsis* strongly promoted growth and biomass production of *Arabidopsis* seedlings and plants; the roots were highly branched but not bushy when compared with colonized Chinese cabbage roots. This suggests that *BcAUX1* is a target of *P. indica* in Chinese cabbage. *P. indica* also promoted growth of *Arabidopsis* seedlings but the auxin levels were not higher and auxin genes were not upregulated, implying that auxin signaling is a more important target of *P. indica* in Chinese cabbage than in *Arabidopsis*. The fungus also stimulated growth of *Arabidopsis aux1* and *aux1/axr4* and *rh6* seedlings. Furthermore, a component in an exudate fraction from *P. indica* but not auxin stimulated growth of Chinese cabbage and *Arabidopsis* seedlings. We propose that activation of auxin biosynthesis and signaling in the roots might be the cause for the *P. indica*-mediated growth phenotype in Chinese cabbage.

genic for the host. Beneficial microbes often stimulate plant growth and biomass production, which result from a better nutrient exchange between the two symbionts and the activation of signaling pathways controlling plant development and performance (Morgan et al. 2005). The direct target of soilborne microorganisms is the plant root system, for which auxin is a crucial growth regulator. Auxins stimulate lateral root growth and root hair development (Fukaki and Tasaka 2009), similar to many beneficial microbes (Gupta et al. 2000; Montesinos et al. 2002; Morgan et al. 2005). Inhibition of auxin transport reduces root branching (Casimiro et al. 2001; Reed et al. 1998) and prevents the effect of beneficial microbes. In *Arabidopsis*, a number of auxin-resistant mutants and auxin overproducers with altered root and root hair development have been identified. Mutants *axr1* and *axr2* show a reduced number of lateral roots (Estelle and Somerville 1987), whereas *rooty* mutants such as *alf1* or *superroot1* (*sur1*) with elevated auxin levels show an increased development of lateral roots (Boerjan et al. 1995; Celenza et al. 1995; King et al. 1995). Furthermore, the *aux1* mutant is impaired in auxin influx into the cell and auxin acropetal and basipetal transport in the root tip (Swarup et al. 2001). AXR4 is required for proper trafficking and location of AUX1 via the lateral root cap to the elongating epidermal cells in the roots (Dharmasiri et al. 2006). Other auxin mutants are impaired in the correct localization of auxin transporters (such as *doc1/big*) (Gil et al. 2001), in the basipetal auxin transport (*eir1/pin2*) (Luschnig et al. 1998), or in an efflux carrier (*pin1* [Ferarú and Friml 2008; Paponov et al. 2005] and *pin2*, *pin3* [Friml 2003]). PIN3 is localized symmetrically in columella cells and mediates lateral auxin distribution to all sides of the root cap. After the root is turned by 90 degrees, PIN3 rapidly relocates to the bottom side of columella cells and, thus, probably regulates auxin flux to the lower side of the root (Friml 2003). Auxin is further transported through lateral root cap and epidermis cells basipetally by a PIN2-dependent route. This basipetal transport also requires AUX1-dependent auxin influx into the cell. AUX1 is present in the same cells as PIN3 and PIN2 (Friml 2003). Root biomass production can be stimulated by the promotion of longitudinal growth of the primary and lateral roots or the initiation of additional lateral root primordia. RHD6 is required for root hair initiation and the *rh6* mutation can be rescued by auxin (Masucci and Schiefelbein 1994) or auxin-producing microbes. Auxin-induced increase in root biomass can be monitored by the stimulation of auxin-re-

Plants are exposed to a large variety of microorganisms in the rhizosphere, which can be beneficial, neutral, or patho-

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sponsive promoters fused to reporter genes. The best-characterized examples are transgenic *Arabidopsis* lines harboring *DR5::uidA* (Ulmasov et al. 1995, 1997) or *BA3::uidA* (Oono et al. 1998) constructs.

Little is known about the molecular basis of root growth promotion by beneficial microbes or fungi (Pieterse and Dicke 2007). It is obvious that microbe-induced stimulation of root growth or changes in root architecture involve auxins or interfere with the auxin metabolism or signaling in the roots (Contreras-Cornejo et al. 2009; Schäfer et al. 2009; Sirrenberg et al. 2007; Vadassery et al. 2008). Microbes often release plant-growth-regulating compounds, including auxin, into the medium or the rhizosphere. More recently, it became obvious that auxin also plays an important role in plant defense (Kazan and Manners 2009). These processes can occur in many ways: by elevating auxin synthesis, releasing auxin from stores or conjugates, stimulating its transport, or activating auxin-induced genes in various tissues which are required for cell growth or proliferation (Ludwig-Müller 1999, 2000; Ludwig-Müller et al. 2005). The auxin-induced signaling pathway involves the F-box protein TIR1 receptor. Its activation leads to ubiquitination-based degradation of transcriptional repressors and complex transcriptional reprogramming (Vanneste and Friml 2009). In addition, several examples demonstrate that auxin or auxin-related compounds can be synthesized by the microbe which can trigger the plant auxin signaling pathway (Contreras-Cornejo et al. 2009). Like exogenous application of auxin, beneficial microbes can suppress the root hair formation defects of *RHD6* by synthesizing auxin-related compounds (Contreras-Cornejo et al. 2009).

We studied the molecular mechanisms by which the endophyte *Piriformospora indica* promotes growth and biomass production of various plant species. *P. indica* belongs to the new order of Sebaciales and can be easily grown on various complex and minimal substrates even without a host plant (Oelmüller et al. 2004, 2009; Sahay and Varma 1999). Because it colonizes the roots of many plant species, including mono- and dicots, mosses, and all tested crop plants, we propose that the fungus should target general growth-promoting programs in the plants. Recently, we challenged the fungus with a major crop in Asia, *Brassica campestris*, the Chinese cabbage. We confirmed the beneficial effects of *P. indica* on growth and biomass production (Sun et al. 2010). The comparative analysis of *P. indica* on Chinese cabbage and *Arabidopsis* growth revealed that auxin synthesis and signaling is a more important target of the fungus in Chinese cabbage than it is in *Arabidopsis*.

RESULTS

Infection of Chinese cabbage by *P. indica* promotes growth and stimulates root hair development.

Seedlings of Chinese cabbage (*B. campestris* subsp. *Chinensis*) and *Arabidopsis* (as control), aseptically germinated on 1/2 Murashige-Skoog (MS) medium (Murashige and Skoog 1962) for 5 days, were transferred to a fresh medium and co-cultivated with *P. indica* by depositing mycelial discs (or agar discs for control plants) next to the root tissue (Fig. 1A, upper panel, for Chinese cabbage). Seven days after infection, numerous lateral roots were visible on the Chinese cabbage roots. We also observed a strong increase in root hair development, resulting in a bushy phenotype (Fig. 1A, lower panel). Both the length and the number of the lateral roots and root hairs increased in the presence of the fungus (Figs. 1 and 2). After 7 days of co-cultivation, the size and fresh weight of the infected Chinese cabbage roots (and shoots; data not shown) was at least twofold higher compared with the uninfected controls. An increase of the number of fungal plaques per seedling

caused only a marginal additional effect (Fig. 2). Growth promotion extended to adult plants in pots (Fig. 1B). Microscopic analysis confirmed that the mycelium not only grew around the root system but also penetrated into the intracellular space of the Chinese cabbage root tissue (Fig. 1C). In several root cells, arthrospores of *P. indica* were visible. These results suggest that the changes in root morphology and the increase in biomass are caused by efficient root colonization. Quite similar effects have previously been reported for *Arabidopsis* seedlings (Peškan-Berghöfer et al. 2004). However, comparison of the two species demonstrated that root hair development was much more strongly stimulated in colonized Chinese cabbage than in *Arabidopsis*. We never observed a bushy root phenotype in *Arabidopsis* (Figs. 1D and 2). Furthermore, the increase of the fresh weight of *Arabidopsis* seedlings (approximately 50%) after 7 days of co-cultivation with *P. indica* was less than half of the increase observed for Chinese cabbage, and the growth response of *Arabidopsis* seedlings was less dependent on the number of fungal plaques which were applied to the roots (Fig. 2).

Auxin levels were determined for root and leaf tissues of Chinese cabbage and *Arabidopsis* seedlings, co-cultivated separately or together in petri dishes with and without *P. indica*. The auxin level of the infected Chinese cabbage roots was twofold higher compared with the uninfected controls, whereas no difference was observed for leaf tissues (Fig. 3). This suggests that the strong root branching, the bushy phenotype, and the increase in biomass of the infected Chinese cabbage seedlings are caused by higher auxin levels. In contrast, no significant difference could be detected for the auxin level in *Arabidopsis* roots and shoots, even if the two types of seedlings were grown in the same petri dish in the presence of *P. indica* (Fig. 3). This clearly demonstrates differences in the auxin levels in the colonized roots of the two species.

Isolation of *P. indica* target genes from a double-subtracted expressed sequence tag cDNA library.

In order to identify genes which are targeted by *P. indica* in Chinese cabbage roots, a subtractive expressed sequence tag (EST) cDNA library was constructed from cDNAs of infected plants as tester and cDNAs from both control root tissues and *P. indica* mycelium as drivers. The nucleotide sequences of 265 clones with an average length of 900 bp from the double-subtracted EST library were determined. Their annotation uncovered 193 protein-coding cDNAs. In all, 13 EST genes encoding auxin-related proteins were selected for this analysis. These proteins can be grouped into three categories (Table 1): (i) proteins involved in cell wall (apoplast) acidification, such as the H⁺-ATPase; (ii) proteins involved in auxin transport, such as AUX1 and BIG; and (iii) proteins involved in auxin signal transduction, such as TIR/AFB (auxin receptor), AUX/indole-acetic acid (IAA) (auxin repressor), and E2-ubiquitin conjugating enzymes for auxin degradation (Table 1). Thus, auxin-related proteins might be involved in growth promotion and the establishment of the highly branched and bushy root architecture in colonized Chinese cabbage seedlings.

Characterization of auxin-related genes activated by *P. indica* in Chinese cabbage.

Northern blot analyses (Fig. 4A) and quantitative reverse-transcription polymerase chain reaction (qRT-PCR) (Fig. 4B) confirmed that all isolated auxin-related genes were upregulated in *P. indica*-infected Chinese cabbage roots, although to different extents. First, upregulation of the *AUX1* and *BIG* mRNA levels implies that the fungal interaction results in a more efficient influx of auxin into the root cell. Because the *PIN3* mRNA level is slightly downregulated by the fungus, auxin release from the

cell could be reduced. Second, the higher mRNA levels for the vacuolar-type H⁺-ATPase subunit B2 and the pyrophosphate-energized vacuolar membrane H⁺ pump (Fig. 4A and B) in infected Chinese cabbage roots indicate that cell wall acidification is enhanced, as proposed for homologues of these two proton pumps in other plant species which participate in auxin-mediated growth promotion. Finally, crucial genes for auxin-signaling components are also upregulated in *P. indica*-infected Chinese cabbage. *TIR1* coding for an F-box and leucine-rich-repeat protein, an auxin receptor in the nucleus, is upregulated in response to the fungus (Fig. 4A and B). The F-box protein was first discovered as a component of the Skp1-cullin-F box protein (SCF) E3 ubiquitin ligase complex (Vanneste and Friml 2009). *IAA7* is a member of the auxin-responsive *AUX/IAA* family. *AUX/IAA* proteins are important negative regulators of auxin-regulated genes. They regulate gene transcription indirectly by binding to the DNA-associated ARF protein (Gray et al. 2001). *UBC10* codes for an E2 ubiquitin-conjugating enzyme, one of three enzymes in the ubiquitin-protein conjugation pathway that

conjugates ubiquitin to the SCF complex. Another *P. indica*-responsive gene encodes the 26S proteasome subunit 4, which is involved in the degradation of *AUX/IAA* proteins (Fig. 4A and B). In total, the differential expression of these auxin-related genes demonstrates that they play a crucial role in *P. indica*-mediated growth promotion and alteration of root morphology in Chinese cabbage.

The *Arabidopsis* homologues of *P. indica*-responsive and auxin-related genes from Chinese cabbage are not upregulated in colonized *Arabidopsis* roots.

Because of the difference in the root hair phenotypes and auxin levels in *P. indica*-colonized Chinese cabbage and *Arabidopsis* roots, we tested whether the *Arabidopsis* homologues of the auxin-related genes identified in Chinese cabbage are upregulated in colonized *Arabidopsis* roots. The experiments were performed with RNA isolated from *Arabidopsis* seedlings grown in a petri dish together with Chinese cabbage seedlings (Fig. 1D). None of the tested auxin-related genes was upregulated in

Fig. 1. Interaction of *Piriformospora indica* with Chinese cabbage. **A**, Branching and root hair phenotype of Chinese cabbage at 7 days after fungal co-cultivation with *P. indica*. Five days after seed germination, seedlings were co-cultivated with *P. indica* and grown on $\frac{1}{2}$ Murashige-Skoog medium for 7 days. Left panel: mock treatment; right panel: co-cultivated by *P. indica*. Lower panels enlarge the roots. For this picture, five seedlings and five fungal plaques were used. **B**, Biomass of Chinese cabbage promoted by *P. indica*. Plants co-cultivated with *P. indica* in petri dishes for 7 days (as in A) were moved to pots and further cultivated for an additional 30 days. **C**, Microscopic structures of roots of Chinese cabbage showing the hyphal penetration by *P. indica*. Top: dissected root tissue stained with lactophenol or cotton blue. Hyphae and arthrospores are observed. Bottom: molecular identification of the marker gene *Pitef-1* by polymerase chain reaction, indicating fungal colonization of the root tissue (Bütehörn et al. 2000). **D**, *Arabidopsis* and Chinese cabbage seedlings (left) and roots (right) co-cultivated with *P. indica* in the same petri dish. Notice the difference in the root architecture. One or two fungal plaques are visible.

Arabidopsis roots and many of them were even downregulated (Fig. 4B), although these results were not significantly different. This implies that auxin is less important for the beneficial interaction between *P. indica* and *Arabidopsis* than it is for the interaction with Chinese cabbage, although seedlings of both species were taller in the presence of the fungus.

Overexpression of BcAUX1 in *Arabidopsis* mimics the *P. indica* effects but does not induce root hair development.

AUX1 is a permease-like auxin influx carrier which functions in the short-distance cell-to-cell transport of the hormone and facilitates active auxin uptake into the cell (Raven 1975). Loss of AUX1 function results in growth inhibition and a reduced response to gravity (Maher and Martindale 1980). To confirm the importance of AUX1 in *P. indica*-mediated growth promotion and alteration in the root morphology in Chinese cabbage, a full-length *BcAUX1* cDNA was isolated by 5' and 3' rapid amplification of cDNA ends (RACE). A 1,650-bp-long cDNA fragment was obtained which contained a 1,479-bp-long open reading frame coding for a 493-amino-acid-long protein with a predicted molecular mass of 59 kDa (accession no. GU191828). This cDNA was subsequently delivered into *Arabidopsis* by *Agrobacterium tumefaciens*-mediated transformation. Transgenic T2 *Arabidopsis* seedlings and plants overexpressing *BcAUX1* under the control of 35S promoter were significantly

bigger than the untransformed controls (Fig. 5). These results demonstrate that *BcAUX1* plays a key role in the response of Chinese cabbage to *P. indica* and heterologous expression of this gene induces similar growth responses in *Arabidopsis*, even in the absence of the fungus. However, although *P. indica* and overexpression of *BcAUX1* promote root growth and lateral root development in *Arabidopsis*, a bushy root hair phenotype was not detectable.

AUX1, AUX1/AXR4, and RHD6 seedlings respond to *P. indica*.

Chinese cabbage AUX1 is an important growth regulator in *Arabidopsis*, although the endogenous *AUX1* gene does not seem to be a target of *P. indica* in this species (Figs. 4 and 5). Consistent with previous observations (Maher and Martindale 1980), we observed that loss-of-function *AUX1* and *AUX1/AXR4* seedlings are smaller than wild-type seedlings (data not shown). However in the presence of the fungus, the loss-of-function mutants showed a $45 \pm 6\%$ (*AUX1*) and $44 \pm 5\%$ (*AUX1/AXR4*) (n for all assays = 10 independent experiments with 20 seedlings each) increase in fresh weights, which is comparable with the wild type ($41 \pm 8\%$; $n = 10$). This indicates that AUX1-independent auxin transport is sufficient to trigger *P. indica*-induced growth promotion in *Arabidopsis*. As expected from the results in Figures 1D and 2, also *RHD6* seedlings which are defected in root hair initiation responded

Fig. 2. Root hair surface and fresh weight of Chinese cabbage and *Arabidopsis* seedlings which were co-cultivated with 0 to 6 fungal plaques/seedling for 7 days. One seedling was grown in a petri dish. The 0 values (= control, without *Piriformospora indica*) was taken as 100 and the other values are expressed relative to it. Left two bars: root hair surface of Chinese cabbage and *Arabidopsis* seedlings, respectively, as measured by pixel numbers on an electronic photograph. Right two bars: fresh weights of Chinese cabbage and *Arabidopsis* roots, respectively. Bars represent standard errors, based on six independent experiments.

to the fungus ($41 \pm 7\%$; $n = 10$). Thus, *P. indica*-induced growth promotion of *Arabidopsis* seedlings is independent of AUX1 and RHD6.

A fungal exudate fraction, but not auxin, promotes growth of *Arabidopsis* and Chinese cabbage seedlings.

As reported previously, a cell wall extract from the fungus stimulates growth of *Arabidopsis* seedlings (Vadassery et al. 2009). We observe the same for Chinese cabbage seedlings (Fig. 6). The cell wall extract also stimulated growth of *AUX1*, *AUX1/AXR4*, and *RHD6* seedlings, while two previously identified *P. indica*-insensitive (*pii*) mutants, called *pii-3* and *pii-4*,

did not respond (Vadassery et al. 2009; data not shown). The active compounds in the cell wall extract were further enriched by high-performance liquid chromatography (HPLC). The resulting fraction had the same growth-promoting features as the cell wall extract and contained no auxin (below detection limits, at least 10^5 -fold less auxin than in the cell wall extract before HPLC fractionation). Growth promotion of Chinese cabbage and *Arabidopsis* seedlings could not be achieved by auxin exogenously applied to the roots (data not shown). These results demonstrate that *P. indica*-induced growth promotion in Chinese cabbage and *Arabidopsis* seedlings is not caused by fungus-released auxin.

Fig. 3. Auxin levels in root and leaf tissues of Chinese cabbage and *Arabidopsis* seedlings, grown in the presence (light bars) or absence (dark bars) of *Piriformospora indica*. Leaf and root tissues were separately harvested from plants at 7 days after infection by *P. indica*.

Table 1. Annotated expressed sequence tag sequences related to auxin function

Class, gene annotation ^a	Reference organism	GI number	E value	Frequency
Class 1				
AUX1 (AUXIN RESISTANT 1); amino acid transmembrane transporter/transport	<i>Arabidopsis thaliana</i>	18404642	1.00E-47	1
Efflux carrier, pin3	<i>Brassica juncea</i>	15485155	2.00E-96	1
Auxin transport protein; BIG	<i>A. thaliana</i>	21779966	2.00E-41	1
Class 2				
Vacuolar-type H ⁺ -ATPase subunit B2	<i>A. thaliana</i>	62321641	9.00E-66	1
Pyrophosphate-energized vacuolar membrane proton pump	<i>Thellungiella salsuginea</i>	60476796	3.00E-111	2
Class 3				
Indole-acetic acid 7 (IAA7)-auxin-responsive AUX and IAA family member	<i>Zea mays</i>	195635917	1.00E-38	1
F-box and leucine-rich repeat protein 15	<i>A. thaliana</i>	124007179	9.00E-110	1
E2 ubiquitin-conjugating enzyme UBC10	<i>B. napus</i>	183013548	2.00E-59	1
Ubiquitinating enzyme	<i>A. thaliana</i>	66354424	9.00E-71	1
26S proteasome subunit 4-like protein	<i>B. napus</i>	11045086	2.00E-102	1
26S proteasome AAA-ATPase subunit RPT4a	<i>A. thaliana</i>	6652884	3.00E-62	1
26S proteasome regulatory complex subunit p42D, putative	<i>A. thaliana</i>	15219503	9.00E-61	1
Auxin-regulated protein-like protein	<i>Populus trichocarpa</i>	109676318	6.00E-05	1

^a Class 1: auxin transport carrier; class 2: cell wall acidification; class 3: auxin signal transduction.

Fig. 4. A, Northern blot analysis for auxin-related genes in Chinese cabbage co-cultivated with *Piriformospora indica* compared with untreated controls. Root tissues 7 days after infection by *P. indica* were sampled for total RNA extraction. The RNA was resolved on a formaldehyde-denatured agarose gel, and hybridized to the indicated cDNA probes. **B**, Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analyses for auxin-related genes from Chinese cabbage and *Arabidopsis* roots, co-cultivated with *P. indica* in the same petri dish. Lightest bar: qRT-PCR analysis for the same genes in Chinese cabbage roots, as shown in A. Based on three independent experiments. Bars represent standard errors (SE). Second lightest bar: qRT-PCR for the *Arabidopsis* homologs of the auxin-related Chinese cabbage genes in *Arabidopsis* roots. Based on three independent experiments. Bars represent SE. Dark gray bars: qRT-PCR of the auxin-related genes in Chinese cabbage roots after the application of a cell wall extract (lighter bars) or a water-diffusible fraction (darker bars) from *P. indica* to the roots. Based on three independent experiments. Bars represent SE.

DISCUSSION

Auxin plays a crucial role in Chinese cabbage-*P. indica* interaction.

Auxins play multiple roles in plant-microbe interactions (Spaepen et al. 2007); for example, by the formation of new

patterns and organs (Devos et al. 2005; Grunewald et al. 2009; Tanaka et al. 2006), growth (Contreras-Cornejo et al. 2009), or defense responses (Bari and Jones 2009; Kazan and Manners 2009). Plant-growth-promoting microbes, including *P. indica*, produce auxins, which may be active in plants (Contreras-Cornejo et al. 2009; Devos et al. 2005; Sirrenberg et al. 2007;

Fig. 5. Root architecture and biomass of T2 transgenic *Arabidopsis* plants overexpressing *BcAUX1*. Shown are 14- and 22-day old seedlings of wild-type (left) and transgenic *Arabidopsis* after germination on $\frac{1}{2}$ Murashige-Skoog medium.

Fig. 6. Chinese cabbage seedlings which were treated with auxin (3 ppm), the high-performance liquid chromatography-purified cell wall extract (HPLC-CWE, 100 μ l), a water-diffusible (WD, 100 μ l) exudate fraction, or mock-treated (water, control) for 7 days on petri dishes; + *Piriformospora indica* = treatment with a fungal plaque.

Splivallo et al. 2009). Microbes also interfere with the plant auxin synthesis, metabolism, signaling, and transport (Grunewald et al. 2009; Vadassery et al. 2008) or they affect the phytohormone balance (Splivallo et al. 2009). Infection of Chinese cabbage by *Plasmodiophora brassicae* leads to a stimulation of plant growth: The auxin increase, together with an increased xyloglucan endotransglucosylase/hydrolase action, results in wall loosening and, consequently, cell expansion (Devos et al. 2005). The different distribution of the hormone within plant tissues and the establishment of gradients are important for developmental processes induced by auxins or root-colonizing microbes (Sorefan et al. 2009). Here, we demonstrate that *Piriformospora indica*-induced growth promotion in Chinese cabbage is associated with severe alterations in the root morphology (Fig. 1; Table 1) such as the promotion of adventitious root formation and root branching, processes which are controlled by auxins (Druege et al. 2007; Sirrenberg et al. 2007). Therefore, it is not surprising that co-cultivation of the two symbionts results in an increased auxin level and the upregulation of auxin-related genes in the roots (Fig. 4).

The distribution of auxin is crucial for lateral root initiation and root development in *Arabidopsis* (Lucas et al. 2008). The polarized transport of auxin into and out of cells allows the control of cellular auxin levels and the generation of auxin gradients. Cellular auxin levels are controlled by influx and efflux carriers. AUX1 efficiently regulates auxin uptake and the expression of AUX1 is upregulated in colonized Chinese cabbage roots (Fig. 4). PIN3, one of the efflux carriers of auxin, which is downregulated at the mRNA level in this symbiosis (Fig. 4), acts as a negative regulator of root hair growth, and overexpression of *pin3* in tobacco represses root hair development (Lee and Cho 2006). Therefore, regulation of AUX1 and PIN3 by *P. indica* should result in a higher auxin level in the root cell, which leads to the activation of the SCF^{TIR1/AFBs} cascade genes. Consequently, a variety of physiological responses are induced, such as root growth, branching, and root hair emergence. The highly branched root phenotype of the *Arabidopsis BcAUX1* overexpressor (Fig. 5) also suggests that the morphology in colonized Chinese cabbage roots is caused by the higher AUX1 mRNA level. Elevation of the cellular hormone level stabilizes the interaction between TIR1/AFBs and AUX/IAA proteins. In association with SCF^{TIR1/AFBs}, AUX/IAA become ubiquitinated by the 26S proteasome for proteolysis, which results in ARF derepression and modulation of transcription of auxin-regulated genes. Many loss-of-function mutations in the components of SCF assembly in *Arabidopsis* are impaired in various auxin responses (Mockaitis and Estelle 2008). Furthermore, the expression of genes for the vacuolar-type H⁺-ATPase subunit B2 and the pyrophosphate-energized vacuolar membrane proton pump was induced in infected Chinese cabbage plants (Fig. 4A and B). It has been shown for maize seedlings that even low concentrations of exogenously applied IAA (10⁻¹⁰ and 10⁻¹⁵ M) present in the soil of a field could activate a vacuolar H⁺-ATPase and an H⁺-pyrophosphatase, which promotes lateral root formation (Zandonadi et al. 2007). The concerted activation of the plasma-membrane and tonoplast H⁺-pumps by auxins and other environmental stimuli plays a key role in root cell expansion by generating an H⁺ electrochemical gradient which maintains the osmotic pressure of the vacuole sufficiently high enough for water uptake and vacuolation (Maeshima et al. 1996; Smart et al. 1998). According to the acid growth theory, maintenance of the vacuolar turgor is the driving force for volume expansion and, thus, cell elongation (Cosgrove 2000). Therefore, the upregulation of genes involved in proton pumping in the infected Chinese cabbage is not surprising.

Although the auxin-related genes isolated from the double-subtracted EST library are not complete to cover all aspects,

they clearly define a central role of auxin in the *P. indica*–Chinese cabbage interaction. Here, we focus on those genes which are related to auxin functions. EST related to other hormone functions such as cytokinin signaling could not be identified. Because *P. indica* produces auxin and releases small amounts into the medium, it is tempting to speculate that this auxin is responsible for these morphological changes and growth promotion. However, several observations prompted us to investigate this in greater detail.

Vadassery and associates (2008) performed similar co-cultivation experiments with *Arabidopsis* and *P. indica* and came to the conclusion that neither the auxin levels nor auxin-related genes were significantly upregulated in colonized roots. Why is the response of the two species toward *P. indica* different? The aerial parts of Chinese cabbage cultivars are optimized to produce large amounts of biomass within a short period of time, which can best be facilitated by optimal water and nutrient uptake from the soil. Therefore, *P. indica* may increase the active auxin level to enlarge the root surface and promote root hair development, in particular in response to growth-promoting stimuli. Compared with Chinese cabbage, growth stimulation is less important and not a primary developmental strategy of *Arabidopsis*. What is the origin of the additional auxin in the Chinese cabbage roots? Because exogenously applied auxin does not stimulate growth of Chinese cabbage seedlings, whereas a fungal exudate which does not contain auxin induces growth, auxin synthesized by the fungus is probably not responsible for the plant response. A comparable situation has been described for Chinese cabbage (*B. rapa* L.) roots infected by the clubroot disease-inducing *Plasmodiophora brassicae*. Upon infection by the obligate biotroph, the auxin level in the roots increases. This auxin is presumably synthesized by the plant nitrilase. Ando and associates (2008) have shown that transcriptional regulation of one gene for the nitrilase from *B. rapa*, *BrNIT2*, is involved in auxin overproduction during clubroot development.

Fungal exudates trigger plant growth.

Because an exudate fraction from *Piriformospora indica* without detectable auxin promotes growth of *Arabidopsis* and Chinese cabbage seedlings, a living fungus is not required for growth promotion. The HPLC-purified exudate fraction contains no sucrose or other sugars; therefore, those metabolites as growth-promoting substances can also be excluded. It remains to be determined which components in the fungal exudates are responsible for the plant response and what are their targets in the root cell. We propose that *P. indica*-associated molecular patterns promote growth of Chinese cabbage by interfering with the auxin homeostasis. Higher auxin levels in colonized Chinese cabbage roots indicate that the fungal signals induce auxin biosynthesis or its release from conjugates. Regulation of auxin transporter genes also suggests that auxin homeostasis may also be influenced through interference with its transport.

AUX1 is a growth regulator.

We identified AUX1 as a target gene of *P. indica* in Chinese cabbage roots. Overexpression of *BcAUX1* in *Arabidopsis* demonstrates that this protein is an important growth regulator in *Arabidopsis*, although it is not targeted by the fungus in this species. Growth promotion by *P. indica* or fungal exudates in *Arabidopsis* seedlings is not dependent on AUX1, because *aux1* and *aux1/axr4* deletion lines respond to the fungus. Similarly, Splivallo and associates (2009) have shown that truffles promote root growth of *aux1-7* seedlings by fungus-derived metabolites. Furthermore, *rhd6* seedlings which are impaired in root hair initiation also responded to the fungus, consistent with the results that root hair development and, apparently, nu-

trient uptake from agar plates via the root hairs is not limiting for the response to *P. indica*.

The AUX1 protein sequences of both species differ only in their extreme N and C termini while the middle part is almost completely conserved. Therefore, it is likely that the growth-promoting function of AUX1 is not restricted to the Chinese cabbage protein. Because the stimulatory effect cannot be achieved by exogenous application of auxin, AUX1 appears to fulfill rate-limiting functions in auxin transport or signaling in both species. The larger root system, including the increase in lateral roots in the overexpressor line, may provide advantages for the plants when grown under nutrient limitations, and it could affect plant performance, depending on the microbial communities in the rhizosphere. How the growth response of the aerial parts is related to the manipulation of AUX1 remains to be investigated. Finally, comparison of *P. indica*- and AUX1-mediated effects on plant performance may help to identify target genes useful for biotechnological applications.

MATERIALS AND METHODS

Growth conditions of plants and fungus, co-cultivation experiments, and estimation of plant growth.

Seed of Chinese cabbage (*B. campestris* subsp. *chinensis*) were donated from the Ming-Hong Seed Company, Feng-Yuan City, Taiwan. Seeds were surface-sterilized with 75% alcohol for 10 min, then placed on a petri dish containing $\frac{1}{2}$ MS nutrient medium (Murashige and Skoog 1962). Plates were incubated at 22°C under continuous illumination ($100 \mu\text{mol m}^{-2} \text{s}^{-1}$) for seed germination. *Arabidopsis* (ecotype Columbia) seedlings were germinated as described by Vadassery and associates (2009) and grown on petri dishes containing $\frac{1}{2}$ MS nutrient medium, identical to the Chinese cabbage seedlings. The homozygote *aux1* (N9583), *aux1/auxr4* (N8040), and *rhb6* (N6347) lines were obtained from NASC (Hobbie and Estelle 1995).

Seven days after seed plating on $\frac{1}{2}$ MS medium, the growing seedlings were transferred to fresh plates containing $\frac{1}{2}$ MS medium. One to six seedlings were used per petri dish and one fungal plaque or one agar plaque without fungus of 5 mm in diameter per seedling was placed at a distance of 1 cm from the roots. If more than one seedling was used in the co-cultivation experiment in one petri dish, this is mentioned in the figure legend or visible in the figure. For the results shown in Figure 2, the number of fungal or agar plaques or seedlings was varied. For direct comparison of Chinese cabbage and *Arabidopsis* seedlings, both seedlings were grown in the same petri dish, with a fungal or agar plaque positioned 1 cm away from each seedling. However, we did not observe any difference to seedlings which were grown on separate plates. For experiments with fungal-derived extracts, 100 μl of the extracts was applied once to the roots instead of the fungal plaques at the same position and time. IAA from 1.5 to 15 ppm served as positive control and was applied in the same way as the fungal exudates. The plates were incubated at 22°C under continuous illumination from the side ($80 \mu\text{mol m}^{-2} \text{s}^{-1}$). *P. indica* was cultured as described previously (Peškan-Berghöfer et al. 2004; Verma et al. 1998) on Kaefel medium (Hill and Kaefel 2001). Seedlings were removed from the plates at 7 days after co-cultivation for auxin measurements, analysis of the morphology, determination of the fresh weight, and RNA extraction. Alternatively, the seedlings were transferred to pots and grown in a walk-in growth chamber, as described previously (Vadassery et al. 2009).

Measurement of auxin concentration.

The auxin level of the plant tissues was analyzed by gas chromatography-mass spectrometry-selected ion monitoring (GC-MS-SIM) as described previously (Chen et al. 2008). The tissues

were sampled 7 days after co-cultivation with *P. indica*. The tissue was ground in liquid N_2 with a mortar and pestle. Internal standards of 100 ng of [$^{13}\text{C}_6$]-IAA were added to each sample after grinding. Tissue extraction was carried out overnight with 15 ml of 80% (vol/vol) methanol containing 0.4 mg of butylated hydroxytoluene and 2 mg of ascorbate at 5°C. The methanol extracts were combined and reduced to 1 to 2 ml with a rotary vacuum evaporator and SpeedVac (Savant Instruments, Hicksville, NY, U.S.A.). The concentrate was adjusted to pH 8.5 with 0.05 M potassium phosphate buffer and passed through a polyvinyl polypyrrolidone column (5 g). The eluate was partitioned with ethyl acetate ($3 \times 15 \text{ ml}$). The aqueous fraction was then adjusted to pH 3.0 with 0.5 M potassium phosphate (pH 2.0) and partitioned with ethyl acetate ($3 \times 15 \text{ ml}$). The pooled ethyl acetate fraction was completely dried with a SpeedVac. The pellet was dissolved in 0.05 M potassium phosphate buffer (3 g). The ODS-silica column was washed three times with double-distilled H_2O in 0.1% acetic acid and eluted with 80% aqueous methanol containing 0.1% acetic acid.

After drying under vacuum, the sample was dissolved in 30% aqueous methanol containing 0.1% acetic acid and injected into a Beckman System Gold HPLC with a LiChrosphere RP-18 column (250 by 4 mm in diameter by 5- μm particle size (Merck, Darmstadt, Germany). The fraction of IAA from this column was dried under vacuum and derivatized by adding ethereal diazomethane, then dried with N_2 . IAA was further trimethylsilylated and the derivatized samples were analyzed using Agilent Technologies 6890N GC and 5973 MSD with a DB-1 capillary column (30 by 0.25 mm in diameter, 0.25- μm film thickness (J&W Scientific, Folsom, CA, U.S.A.).

Construction of subtractive EST library and analysis of EST clones.

A previously published method for pine tree (Chang et al. 1993) was used to extract total RNA from Chinese cabbage and fungal mycelium. Roots of Chinese cabbage, which was co-cultivated with *P. indica* for 7 days, were freshly sampled. Mycelium of *P. indica* cultured on Kaefel medium (Hill and Kaefel, 2001) for 3 weeks was employed for total RNA extraction. mRNA was purified from total RNA with an Oligotex mRNA Kit (Qiagen, Chatsworth, CA, U.S.A.) and cDNA was synthesized by using the PCR-selected TMcDNA subtraction kit following the manufacturer's instruction (Clontech, Mountain View, CA, U.S.A.). cDNA was digested by *RsaI* and then ligated with PCR adaptors. The double-subtracted hybridization was performed by using cDNAs of infected plant roots as tester. cDNAs of uninfected plant roots and of fungal mycelium were used together as drivers. According to the manufacturer's instructions, the subtracted cDNA mixture was amplified by PCR once and the products were cloned into the pGEM-T easy vector (Promega Corp., Madison, WI, U.S.A.) with blue and white selection in *Escherichia coli* XL1-Blue. Three hundred white clones were randomly selected and cultured in Luria-Bertani medium at 37°C overnight. Plasmid DNA was extracted and the insertions were sequenced. The EST sequences were assembled to obtain contigs and singletons. To annotate the clusters and singles, sequence alignment was performed by BlastX (Altschul et al. 1977) with the nonredundant protein sequence database in GenBank (National Center for Biotechnology Information) with an *E* value threshold of $E-10$.

Analysis of gene expression in Chinese cabbage roots by Northern blot hybridization.

Total RNA (10 μg each) from infected root tissues and control tissue was loaded on 1% agarose/formaldehyde gels and transferred onto nylon membranes (Amersham Bioscience, Piscataway, NJ, U.S.A.). The EST insert fragments, randomly

labeled with α - 32 P-dCTP (Rediprime II Kit; Amersham Bioscience), were employed as probe to detect the expression pattern. The membrane hybridization and fluorescent signal detection (Typhoon 9400; Amersham Bioscience) were carried out following standard molecular protocols. The *ACTIN2* gene from *Arabidopsis* was used as loading control (data not shown).

Analysis of gene expression by qRT-PCR.

RNA was isolated from roots of Chinese cabbage and *Arabidopsis* seedlings with an RNA isolation kit (RNeasy, Qiagen, Hilden, Germany) and reverse-transcribed for real-time qPCR analysis using an iCycler iQ real-time PCR detection system and iCycler software (version 2.2; Bio-Rad, Munich). Total RNA was isolated from three independent replicates of the roots. cDNA was synthesized using the Omniscript cDNA synthesis kit (Qiagen, Hilden, Germany) using 1 μ g of RNA. For the amplification of the RT-PCR products, iQ SYBR Green Supermix (Bio-Rad, Hilden, Germany) was used according to the manufacturer's protocol in a final volume of 25 μ l. 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 *ACTIN2* mRNA level. Fold induction values of target genes were calculated with the $\Delta\Delta$ CP equation of Pfaffl (2001) and related to the mRNA level of target genes in control roots, which were defined as 1.0. The primer pairs for product sizes between 150 and 170 bp are given in Supplementary Figure 1.

Detection of fungal RNA in Chinese cabbage roots has been performed as described previously (Bütehörn et al. 2000).

5' and 3' RACE for the generation of the full-length AUX1 gene from Chinese cabbage.

The RACE experiments were performed according to the manufacturer's instructions (InVitroGene, Karlsruhe, Germany).

A. tumefaciens transformation.

Arabidopsis transformation was performed according to Bechtold and associates (1993).

Preparation of a water-diffusible fraction from *P. indica*.

P. indica mycelium, propagated in liquid culture medium for 2 weeks, was filtered through eight layers of nylon membrane, collected in a funnel, and intensively washed seven times with distilled water. After air drying of the mycelium, it was resuspended in distilled water. After 48 h, the mycelium was removed from the water by high-speed centrifugation and the supernatant was used for the experiments (=water-diffusible fraction).

Preparation of an extract from the cell-wall fraction from *P. indica*.

The cell wall extract was prepared using the protocol of Anderson-Prouty and Albersheim (1975) with modifications. Mycelia from 14-day-old liquid cultures were homogenized using a Waring blender in 5 ml of water per gram of mycelia. The homogenate was filtered through eight layers of nylon membrane and a coarse-sintered glass funnel. The residue was washed three times with water, twice with chloroform/methanol (1:1), and, finally, twice 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 of cell wall material in 100 ml of water and autoclaving for 30 min at 121°C. Autoclaving releases the active fraction. After filtration through nylon membranes, the suspension was centrifuged at 14,000 rpm for 10 min and filter-sterilized using a 0.22- μ m filter. The extract was further

purified by passing it through a reverse-phase Superclean LC-18 Cartridge (Sigma-Aldrich, Taufkirchen, Germany). The active fractions were collected and concentrated to half.

Statistics.

Statistics were performed by one-way analysis of variance, and the experiments were repeated four times, with values represented by standard errors.

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