The beneficial fungus *Piriformospora indica* confers tolerance to plants under drought stress and pathogen attack

Dissertation

zur Erlangung des akademischen Grades
doctor rerum naturalium (Dr. rer. nat.)

vorgelegt dem
Rat der Biologisch-Pharmazeutischen Fakultät
der Friedrich-Schiller-Universität Jena

von Master of Science
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geboren am 31.10.1983 in Xinxiang, China

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Tag der öffentlichen Disputation: 28.11.2014

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1. Introduction

Plants live in complex symbiotic associations and they interact with a wide range of microorganisms in ecocommunities. In symbiosis, which means living together, the interrelationships can be divided into three categories: (a) mutualism, when both partners benefit from the relationship, (b) commensalism, when one organism benefits and the other one is not affected, (c) parasitism, when one species benefits while the other is harmed in the process (Kogel *et al.*, 2006; Oelmüller, 2009). In mutualistic interactions, the fungus supports the plant in such a way that the plant benefits to a higher tolerance to biotic and abiotic stress, growth promotion, *etc*. On the other side the microbes also benefits from the mutualistic lifestyle to obtain a continuous carbon supply from the autotrophic plant and avoid most of the biotic stress (Johnson and Oelmüller, 2009). The classical and well-studied symbiotic microbes are arbuscular mycorrhizal fungi (AMF) that promote the uptake of water and minerals, and *Rhizobium* bacteria that fix the atmospheric nitrogen for the plants. The mycorrhizal symbiosis has exsisted for more than 460 million years and it represents one of the oldest evolutionarily beneficial symbioses of plants (Redecker *et al.*, 2000). It is already known that about 80% of all terrestrial plant species form symbiosis with mycorrhizal fungi (van der Heijden *et al.*, 1998). In nutrient-poor soils of the humid tropics, many plants are obligately or ecologically dependent on AM fungi (Gemma *et al.*, 2002). Although the mutualism is relatively stable, it still can jump from mutualism to commensalism or even to parasitism when the surrounding conditions change. This depends on the interacting partners, genetic parameters, environmental conditions and in particular nutrient supply or habitat-specific stress conditions, *etc.* (Johnson and Oelmüller, 2009). To investigate plant/microbe interaction, a proper model system is critical. Since long time, demanding of living host plants to provide nutrients to form the mutualism in AMF limits the study of beneficial interactions. But the appearance of an axenically cultivable plant promoting root endophyte, *Piriformospora indica*, which behaves like a mycorrhizal fungus and can also colonize the roots of *Arabidopsis thaliana*, provides a classic tool for investigating the basal physiological and molecular events in a plant-fungus beneficial interaction.

1.1 *Piriformospora indica* – a plant promoting root endophyte

The fungus *Piriformospora indica* (*P. indica*) was discovered in 1998 while studing the orchid plants in the northwest sand dessert Thar in Rajasthan, India. The fungal spores,

including AM spores, were isolated from colonized soil around the woody shrub roots from *Prosopis juliflora* and *Zizyphus nummulari*. Based on 18S rRNA and microscopy analysis, *P. indica* belongs to the Hymenomycetes of *Basidiomycota*. The fungus colonizes the root cortex and forms typical pear-shaped chlamydospores (Fig. 1D, E_A) without developing arbuscules. The young mycelia are white, thin walled, hyaline, intertwined and they overlap each other (Fig. $1E_B$). In older hyphae, they are irregularly inflated, showing a nodose to coralloid shape (Fig. 1A). The cytoplasm of the chlamydospores is densely packed containing usually 8-25 nuclei. Neither clamp connections nor sexual structures are observed (Verma *et al.*, 1998).

P. indica colonizes the roots of many plant species including Arabidopsis (Peskan-Berghöfer *et al.*, 2004; Oelmüller *et al.,* 2009; Qiang *et al.*, 2012; Lahrmann and Zuccaro, 2012). Like other members of Sebacinales, *P. indica* is found worldwide in association with roots (Selosse *et al.*, 2009) and stimulates growth, biomass (Fig. 1B, C) and seed production of the hosts (Peskan-Berghöfer *et al.*, 2004; Oelmüller *et al.*, 2009; Shahollari *et al.*, 2007; Sherameti *et al.*, 2005, 2008a and b; Vadassery *et al.*, 2009a and b; Waller *et al.*, 2005; Zuccaro *et al.*, 2011). The fungus promotes nitrate and phosphate uptake and metabolism (Sherameti *et al.,* 2005; Shahollari *et al.*, 2005; Yadav *et al.*, 2010). *P. indica* also confers resistance against abiotic (Sherameti *et al.*, 2008a; Baltruschat *et al.*, 2008; Sun *et al.*, 2010) and biotic stress (Oelmüller *et al.*, 2009; Stein *et al.*, 2008). The broad host range of *P. indica* indicates that the beneficial interaction may be based on general recognition and signaling pathways. Enhanced plant growth can be induced by a fungal exudate component (Vadassery *et al.*, 2009a), suggesting the involvement of specific receptors at the plant cell surface. In addition, an atypical receptor kinase with leucine-rich repeats was identified as being required for the growth response in Arabidopsis (Shahollari *et al.*, 2007).

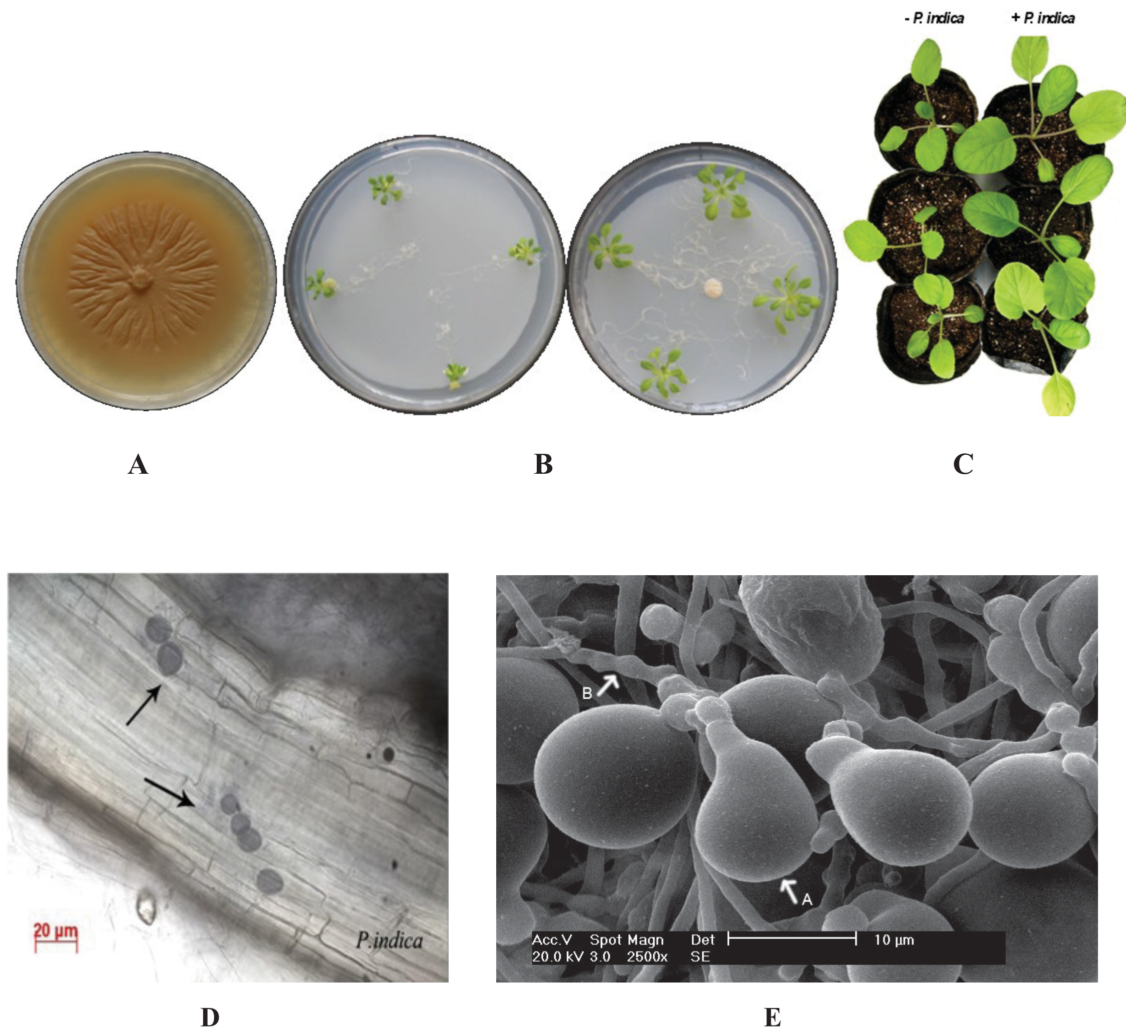

Fig.1. Co-cultivation of *A. thaliana* and Chinese cabbage with *P. indica* on Petri dish or in soil and microscopical view of *P. indica*.

(**A**) *P. indica* hyphae on Kaefer agar plate after 2 weeks; (**B**) Standardized co-cultivation of *P. indica* and Arabidopsis on Petri dish: untreated seedlings (left), seedlings co-cultivated with a fungal plug (right). Growth promotion is visible; (**C**) Growth promotion of Chinese cabbage after 2 weeks co-cultivation with *P. indica* in soil; (**D**) Root of *A. thaliana* colonized with *P. indica* spores (arrows show *P. indica*'s chlamydospores); (**E**) *P. indica* hyphae and chlamydospores under Scanning Electron Microscopy (**A**, chlamydospores; **B**, hyphae).

1.2 Chinese cabbage (*Brassica campestris* L. ssp. *chinensis*) - a close ancestor to *Arabidopsis thaliana* (L.) Heynh

Chinese cabbage (*Brassica chinensis* L.) belongs to *Cruciferae* and is an economically important crop in many parts of the world. One example is the middle and lower region of Changjiang River in China which consumes a huge amount of Chinese cabbage every year. Although the growth period of Chinese cabbage is short and this plant can be easily cultivated under different environmental conditions, it is often exposed to various biotic and abiotic stress. So the growing demand for better quality has emerged since years in the country.

In 2011, using the latest sequencing technology, the researchers of Chinese Academy of Agricultural Sciences in Beijing sequenced more than 98% of the Chinese cabbage genome. They revealed a total of 41,174 genes in the genome, 11,174 more than those found in its close ancestor *Arabidopsis thaliana*. The genome size of the Chinese cabbage was almost three times bigger than that of *A. thaliana*. The finding indicates that many genes of *A. thaliana* have been degenerated during evolution. Majority of the remaining genes are connected to plant growth hormone pathways and supposed to be responded to environmental stress conditions. The genome provides a tool for people to investigate Chinese cabbage evolution and genetic engineering (Duca, 2011).

1.3 Role of *P. indica* in plant tolerance to abiotic and biotic stress

Plants are challenged by different stress conditions, such as drought, low temperature and salinity, which belong to the abiotic stress and, for instance, fungi or harmful insects that cause biotic stress. In the past decade, numerous studies on the tolerance of *P. indica*-colonized plants exposed to abiotic and biotic stress conditions emerged. In both Arabidopsis and Chinese cabagge, the drought stress was relieved in the presence of *P. indica* (Sherameti *et al.*, 2008; Sun *et al.*, 2010). In addition, two ascorbate reductase genes *MDAR2* and *DHAR5* were upregulated in both shoots and roots of colonized seedlings of Arabidopsis under drought conditions (Vadassery *et al.*, 2009b). The higher amount of antioxidants in the fungus-colonized plants also results in stress tolerance (Foyer and Shigeoka, 2011; Baltruschat *et al.*, 2008).

The first indications for the biotic stress protection by *P. indica* were obtained in barley. The fungal colonized plants were more resistant against *Blumeria graminis* infection in shoots and

Fusarium culmoru in roots (Waller *et al.*, 2005). The similar phenomenon was also observed in many other plant species and pathogen isolates, for instance, *Pseudocercosporella herpotrichoides* in *Triticum aestivum*, *Fusarium verticillioides* in *Zea mays*, *Verticillium dahlia*e in *Solanum lycopersicum* and tomato, *etc*. (Serfling *et al.*, 2007; Kumar *et al.*, 2009; Fakhro *et al.*, 2010). Root pathogens could be directly inhibited by antagonistic activities of the endophyte. But the growth of *Pseudocercosporella herpotrichoides* and *Fusarium culmoru* were not inhibited (Waller *et al.*, 2005; Serfling *et al.*, 2007). The plant resistance could be explained by the systemic resistance induced by *P. indica*. In barley, a number of defense-related genes were strongly upregulated in fungus-colonized plants compared to the untreated control challenged by leaf pathogen powdery mildew (Molitor *et al.*, 2011). Meanwhile, the ROS production and the synthesis of antioxidants were also observed during abiotic stress in Barley, Wheat and Maize (Waller *et al.*, 2005; Serfling *et al.*, 2007; Kumar *et al.*, 2009). Stein *et al.*, (2008) showed that JA signaling plays an important role in *P. indica*-induced resistance after examined in *NahG*, *NPR1*, *JAR1 etc*. mutants (Stein *et al.*, 2008). It seems that the mechanisms of *P. indica*-induced resistance are similar to that of the growth-promoting rhizobacteria (Van Wees *et al.*, 2008). Compared to fungal pathogens, Barazani *et al.*, (2005) reported that the endophyte colonized plants were more susceptible to the insect attack. Under low light conditions, the colonized tomato plants showed higher viral spread rate (Fakhro *et al.*, 2010).

1.4 *Verticillium dahliae* - a host specific vascular soil-borne pathogen

The vascular wilt pathogen *Verticillium dahliae* (*Vd*) belongs to the fungal class *deuteromycetes*, Verticillium genus and *V. dahliae* species (Fig. 2A). Verticillium infects more than 200 plant species, among them agriculturally and horticulturally important crops and ornamental plants (Pegg and Brady, 2002; Fradin and Thomma, 2006; Klosterman *et al.*, 2009). Some of disease symptoms are dehydration, chlorosis, necrosis and vein clearing (Fig. 2B). It is estimated that *Vd* infections are responsible for several billions of dollars of annual crop losses worldwide. *Vd* has a broad host range and infects plants from temperate to subtropical climates (Pegg and Brady, 2002). Because of the life cycle of the Verticillium and the formation of microsclerotia (Fig. 2C), this pathogen is major constraint on agricultural production and cannot be controlled by conventional fungicides. Therefore, the isolation of Verticillium-resistant cultivars is an important task for the future (Klosterman *et al.*, 2009). Actually, some resistant genes have already been identified. The *Ve* gene provides resistance

against race 1 isolates of *Vd* in tomato (Kawchuk *et al.*, 2001; Fradin *et al.*, 2009) and the expression of *Ve* gene in Arabidopsis also showed the same function (Fradin *et al.*, 2009). The Brassicaceae-specific *EWR1* (Yadeta *et al.*, 2014), *RabGAP22* (Roos *et al.*, 2014), *GbSTK* (Zhang *et al.*, 2013), *Gbve1* (Zhang *et al.*, 2012), DNA-binding protein gene *AHL19* (Yadeta *et al.*, 2011) are recently identified genes and proteins conferring resistance to plants against Verticillium infection. Moreover, the glucosinolate composition (Witzel *et al.*, 2013), a fungal derived elicitor PevD1 (Bu *et al.*, 2014), cytokinin (Reusche *et al.*, 2013) and plant defensing NaD1 (Gaspar *et al.*, 2014) are also reported to be involved in the disease resistance. However, due to the lack of immune or highly resistant germplasm against the pathogen, since a long time, there is no significant breakthrough in the breeding of resistant cultivars to Verticillium wilt (Cai *et al.*, 2009). Several studies demonstrate that rhizosphere bacteria, for instance, *Pseudomonas putida* B E2, *Ps. chlororaphis* K15 and *Serratia plymuthica* R12 (Berg *et al.*, 2001) or endophytic bacteria isolate HA02 (Li *et al.*, 2012) can be considered as biocontrol agent candidates against *V. dahliae*. The mechanisms of the effects of those bioagents involve mainly antibiosis, parasitism, competition and secretion of enzymes such as glucose oxidase, chitinase and glucanase resulting in the induction of disease resistance in the host (Tjamos *et al.*, 2000, 2005).

Verticillium species are considered as hemibiotroph: a biotrophic phase within root xylem without visible disease symptoms is followed by a necrotrophic phase in shoots. The pathogen can germinate a new mycelium from microsclerotia by taking the exudates from host plants or neighbor plants (Klosterman *et al.*, 2011). The hyphae penetrate the root tip or elongation zone and grow inter- and intracellularly toward the central cylinder of the root (Reusche *et al.*, 2012). After colonizing the xylem vessels, they start to produce conidia and microsclerotia and finally, the water transportation is heavily disturbed and the plants show wilt symptom (Pegg and Brady, 2002). In addition, Verticillium species produce cell wall degrading enzymes and phytotoxins which are sufficient to induce disease symptom development in various species such as cotton, tomato, tobacco, olive tree or Arabidopsis. However, which of these compounds is required for a particular disease phenotype in a given plant species, is largely unknown. The toxin may contain small peptides, amino acids, high molecular weight protein-lipopolysaccharide complexes (PLPCs) and other enzymes (Buchner *et al.*, 1989). Laouane *et al.*, (2011) purified cinnamyl acetate from the crude toxin which might represent a new phytotoxin of *Vd* on olive trees. The crude toxin extract is taken as elicitor to induce the

plant defense and provide a model for investigating the physiological responses (Yao *et al.*, 2012).

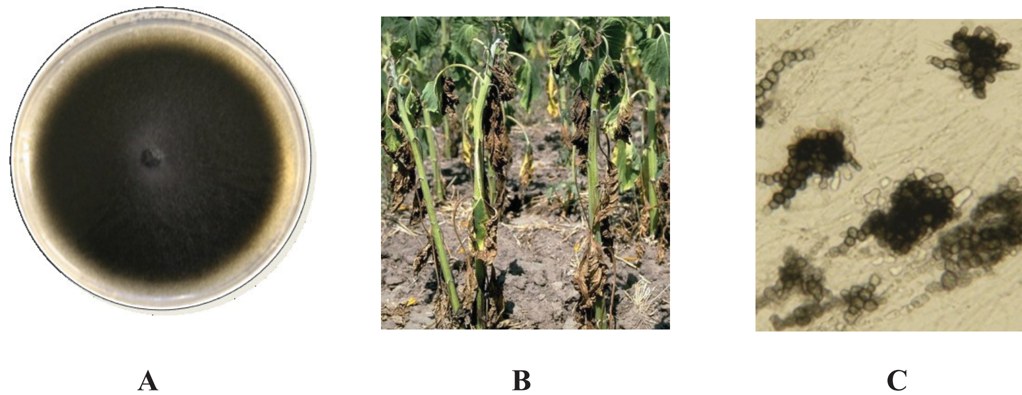

Fig. 2. Typical view of *Vd* on Petri dish and infection in plants caused by Verticilium.

(A) One-month old *Vd* on PDA agar plate; **(B)** Sunflower plants infected by *Vd* in field (picture from internet); **(C)** The microsclerotia produced by *Vd* (picture from internet).

1.5 Role of Calcium signaling in plant-pathogen interaction

Cytoplasmic free Ca^{2+} ($[Ca^{2+}]_{cyt}$) serves as a second messenger in numerous plant signaling pathways, transferring a wide range of environmental and developmental stimuli to appropriate physiological responses. The $[Ca^{2+}]_{cyt}$ level can be regulated under abiotic stimuli, such as touch, cold, wind and drought (Knight *et al.*, 1991, 1992, 1996, 1997), blue light (Lewis *et al.*, 1997) and microbial invasion (Yang *et al.*, 1997; Scheel, 1998). In plant/pathogen interactions, the calcium response performs three patterns: slow spike (min) (Mithöfer *et al.*, 1999, Blume *et al.*, 2000, Grant *et al.*, 2000), sustained elevation (h) and oscillations in $[Ca^{2+}]_{cyt}$ (Lecourieux *et al.*, 2002, Poinssot *et al.*, 2003). The receptor-mediated $[Ca^{2+}]_{cyt}$ elevation is an early event in a signaling cascade that activates immune responses and induces defense gene regulation *via* the activation of ion fluxes at the plasma membrane, an oxidative burst and mitogen-activated protein kinases (MAPKs) (Blume *et al.*, 2000; Vadassery and Oelmüller, 2009). Although several studies were carried out on the plasma membrane binding sites, the molecular mode of fungal pathogen perception in plants remains poor: only one elicitor receptor has been isolated so far, a soybean 70-kD plasma membrane protein that binds *Phytophthora sojae*–derived ß-glucans (Umemoto *et al.*, 1997). In the plant defense against fungal pathogens, a large number of Ca^{2+} sensors, such as calcium-dependent

protein kinases (CDPK), calcium-calmodulin (CaM) and calcineurin B-like (CBL) protein kinases are also involved (Cheng *et al.*, 2002; Lee and Rudd, 2002; Takahashi *et al.*, 2011).

1.6 Role of plant hormones in beneficial plant/fungus interaction

Plant hormones play important roles in regulating the speed and form of plant growth and development. In addition, they also affect the plant cell division, elongation and differentiation. Five classes of plant hormones are already known. They are auxin, cytokinin, ethylene, abscisic acid and gibberellins (Weier and Rost, 1979). Furthermore, jasmonic acid and salicylic acid are considered to be involved in the plant defense responses (Foo *et al.*, 2013a). Each hormone plays multiple functions, depending on the action site and the plant development stage, as well as hormone concentration.

Auxin

Many soilborne fungi directly target at the plant root system, for which the auxin plays important role in plant development. Auxin involves in diverse processes, for instance, tropic responses to light and gravity, general root and shoot architecture, organ patterning, vascular development and growth in tissue culture (Davies, 1995). In AM formation, auxin is supposed to enhance the root branching after fungal infection (Gianiniazzi-Pearson *et al.*, 1996). But the auxin levels depend on the plant species, they are either increased or there is no change in the symbiosis (Jentschel *et al.*, 2007; Campanella *et al.*, 2008). Zsögön *et al.*, (2008) reported that there was no difference in intraradical fungal colonization between an auxin-insensitive *diageotropica* mutant and wild-type infected by *Glomus clarum*. However, a more recent study by Hanlon and Coenen (2011) showed that hyphae could not colonize and even escaped in the cultured *diageotropica* roots. They also found that the enhanced root colonization by *Glomus intraradices* appeared in an auxin hyperactive-polar transport *polycotyledon* mutant. However, this phenomenon was not appeared in intact *polycotyledon* plants, which showed reduced colonization (Hanlon and Coenen, 2011). Independent evidence for the role of auxin in AM infection was supported by Foo (2013b). Foo (2013b) demonstrated that the roots of an auxin-deficient (Symons *et al.*, 2002) *bushy* mutant of pea had decreased amount of mycorrhizal colonization compared to wild-type plants, but the arbuscules appeared normal in roots (Foo, 2013b). Based on the recent findings, it looks that auxin is necessary for the normal AM infection, but not required for post-infection development, such as fungal structures and arbuscule formation. Different studies of *P. indica*-plant interactions showed

that a diffusible factor which could be imitated by indol acetic acid (IAA) or an increased auxin level plays a role in the plant growth in Arabidopsis and Chinese cabbage in *P. indica*-colonized roots (Sirrenberg *et al.*, 2007; Sun *et al.*, 2010). In Chinese cabbage, a growth regulator auxin gene *AUX1* was also considered as the target gene of *P. indica* in the interaction (Sun *et al.*, 2010).

Ethylene, salicylic acid and jasmonic acid

Ethylene (ET), salicylic acid (SA) and jasmonic acid (JA) are considered to respond to both biotic and abiotic stress. In the AMF-plant interaction, up to date, there is no quite clear answer about the effect of ET in AM formation. Both ET-overproducing mutant *epinastic* and ethylene-insensitive mutant *Never ripe* in tomato showed inhibited AM formation (Azcon-Aguiler *et al.*, 1981; Ishii *et al.*, 1996; Geil *et al.*, 2001; Zsögön *et al.*, 2008). In the *epinastic* plant, de Los Santos *et al.*, (2011) confirmed the reduced mycorrhizal root colonization. But interestingly, they also found a contradictory result. They observed an enhanced mycorrhizal colonization in the roots of the *ripening inhibitor* (*rin*) mutant (de Los Santos *et al.*, 2011). Hence, it is difficult to conclude the role of ET in the interaction. However, ET plays a crucial role in the beneficial *P. indica*-Arabidopsis interaction. The growth of *etr1*, *ein2* and *ein3/eil1* mutant plants was not promoted or even inhibited by the fungus (Camehl *et al.*, 2010). In plants, SA responses to pathogen attack. This leads to the question, whether SA is involved in fungal hyphae penetrating plant cells and whether it regulates the AM development. In garlic bulbs, the promoted AM colonization was observed by a lower level of exogenous JA foliar application (Regvar *et al.*, 1996). The modified endogenous JA levels by repeated wounding resulted in an increased AM colonization as well (Landgraf *et al.*, 2012). Tejeda-Sartorius *et al.*, (2008) found a decreased AM colonization in JA-deficient *spr2* mutant in tomato (Li *et al.*, 2003), that it can be recovered by methyl jasmonate application (Tejeda-Sartorius *et al.*, 2008). The above findings suggest a positive role of JA in the AM colonization. However, there are also some results supporting the contradictory conclusions. Ludwig-Müller *et al.*, (2002) concluded that there was a reduction of colonization with higher level of JA in *Tropaeolum majus* and *Carica papaya*. The JA-insensitive *jar-1* mutant in tomato had increased colonization and when the wild-type was treated with methyl jasmonate, reduced colonization was observed (Herrera-Medina *et al.*, 2008). Facing to the inconsistent results, Gutjahr and Paszkowski (2009) assumed that the effects of JA on mycorrhizal colonization depend on the species, hormone dose, timing and nutritional conditions. SA is involved in plant’s defense

against biotrophic pathogens. Herrera-Medina *et al.*, (2003) used the transgenic *NahG* plant which has reduced amount of SA and *CSA* mutant with constitutive SA biosynthesis to compare the AM colonization. They found that the *NahG* plant showed a faster AM colonization than that in *CSA* plants, in spite of the level of colonization was not changed. This suggested that SA level is crucial for early stages of AM colonization (Herrera-Medina *et al.*, 2003; Blilouet *et al.*, 1999).

2. Objectives

The aim of my project was to find out whether the beneficial endophytic fungus *P. indica* can protect plants from abiotic and biotic stress. If yes (the hypothesis is based on the previously published data), what are the mechanisms? I also analysed the role of auxin in the beneficial plant- fungus interaction.

To address these questions, I used Chinese cabbage and the model plant *A. thaliana* as host plants. The vascular soil-borne pathogen *Verticillium dahliae* was taken to investigate the biotic stress condition.

I. Abiotic stress (carried out in Chinese cabbage)
   - Do plants colonized by *P. indica* show better drought resistance compared to the plants without *P. indica* colonization?
   - How are drought-related genes and proteins regulated?
   - What is the role of *P. indica* in the abiotic stress?

II. Biotic stress (carried out with *Arabidopsis thaliana*)
   - Do the plants colonized by *P. indica* show reduced disease symptoms compared to the plants without *P. indica* colonization?
   - Does the sequence of different fungal entering the roots also influence the resistance? Could *P. indica* protect the roots against *Vd* infection when the roots were first exposed to the beneficial fungus?
   - How do *Vd*-induced defense genes and other defense-related factors respond when the roots are first exposed to *P. indica*?
   - How does the calcium signal change?

III. The role of auxin in the beneficial interaction (carried out both in Chinese cabbage and *Arabidopsis thaliana*)
   - Does the growth promotion induced by *P. indica* correlate to auxin effect?
   - Does the auxin play the same role in both host plants?

3. Manuscript Overview

3.1 Manuscript I

***Piriformospora indica* confers drought tolerance in Chinese cabbage leaves by stimulating antioxidant enzymes, the expression of drought-related genes and the plastid-localized CAS protein**

Chao Sun, Joy M. Johnson, Daguang Cai, Irena Sherameti, Ralf Oelmüller, Binggan Lou

Journal of Plant Physiology (2010), 167: 1009-1017

This publication explored whether the endophytic fungus *Piriformospora indica* promotes root and shoot growth and lateral root formation of Chinese cabbage. We used polyethylene glycol to mimic drought stress, and found that the activities of peroxidases, catalases and superoxide dismutases were upregulated within 24 h in the leaves of *P. indica*-colonized plants. The fungus retarded the drought induced-decline in the photosynthetic efficiency and the degradation of chlorophylls and thylakoid proteins. We also found an upregulation of drought-related genes *DREB2A*, *CBL1*, *ANAC072* and *RD29A* in the drought-stressed leaves of colonized plants. The *CAS* mRNA level for the thylakoid membrane associated Ca^{2+}-sensing regulator and the amount of the CAS protein increased. We demonstrate that antioxidant enzyme activities, drought-related genes and CAS are three crucial targets of *P. indica* in Chinese cabbage leaves during the establishment of drought tolerance.

All the experiments are designed by C. S., R. O. and B. L. C. S. investigated all the physiological parameters, including growth parameters, the activities of peroxidases, catalases and superoxide dismutases, catalases and photosynthetic efficiency. The expression of drought-related genes was analysed by J. M. J. The CAS protein was done by I. S. D. C. contributed to the discussion. R. O. and B. L. wrote the paper.

3.2 Manuscript II

Growth promotion of Chinese cabbage and Arabidopsis by *Piriformospora indica* is not stimulated by mycelium-synthesized auxin

Yin-Chen Lee, Joy Michal Johnson, Ching-Te Chien, Chao Sun, Daguang Cai, Binggan Lou, Ralf Oelmüller and Kai-Wun Yeh

Molecular Plant Microbe Interaction (2011), 24(4): 421-431

In this publication, we describe that auxin works differently in *P. indica*-mediated growth promotion in Chinese cabbage and Arabidopsis seedlings. In the roots of *P. indica*-colonized Chinese cabbage, the auxin level was two-fold higher than in uncolonized roots and the genes related to cell wall acidification, intercellular auxin transport carrier proteins and auxin signal proteins were highly upregulated, whereas these responses were not detectable in Arabidopsis roots. The mutants (*aux1*, *aux1*/*axr4* and *rhd6*) that are impaired in auxin synthesis, signaling and transportation responded to *P. indica* in Arabidopsis. The paper also showed that unidentified component(s) from *P. indica* but not auxin induced growth of Chinese cabbage and Arabidopsis seedlings.

All experiments with Chinese cabbage were designed by K. W. Y. and Y. C. L., by R. O. and J. M. J. with Arabidopsis. Y. C. L. and C. T. C. did the overexpression of *Bcaux1* in Arabidopsis. J. M. J. partially purified the cell wall extract and water-diffusible extract. C. S. did the growth promotion assays both in Chinese cabbage and Arabidopsis. D. C. and B. L. contributed to the discussion. R. O. and K. W. Y. wrote the paper.

3.3 Manuscript III

Piriformospora indica **promotes growth of Chinese cabbage by manipulating auxin homeostasis: Role of auxin in** ***P. indica*** **symbiosis**

Joy Michal Johnson, Yin-Chen Lee, Iris Camehl, Chao Sun, Kai-Wun Yeh and Ralf Oelmüller

Piriformospora indica – Sebacinales and their biotechnological applications (2013), *Soil Biology 33: 139-148.* A. Varma *et al.*, (eds.) Berlin-Heidelberg: Springer-Verlag

In this book chapter, we summarized the role of auxin in the beneficial interaction between Chinese cabbage and *P. indica*. We uncovered that the increased auxin level was not from the fungal origin, because both the exogenous auxin application and a preparation of a cell wall-derived fraction from the fungus which does not contain auxin could not replace the growth effect of the fungus. This indicates that *P. indica* activates growth-promoting programs in the roots, which include auxin biosynthesis of plant origin. In addition, the growth promotion is independent of the root architecture in Arabidopsis.

R. O. and J. M. J. wrote the chapter. Y. C. L., I. C., C. S., K. W. Y. participated by giving suggestions.

3.4 Manuscript IV

Balancing defense and growth - Analyses of the beneficial symbiosis between ***Piriformospora indica*** **and** ***Arabidopsis thaliana***

Pyniarlang L. Nongbri, Khabat Vahabi, Anna Mrozinska, Eileen Seebald, Chao Sun, Irena Sherameti, Joy M. Johnson and Ralf Oelmüller

Symbiosis (2012), 58: 17-28

This review paper summarizes the beneficial and non-beneficial physiological responses in a mutualistic interaction between the endophytic and root-colonizing fungus *P. indica* and

Arabidopsis thaliana. *P. indica* confers many benefits to the host symbiont. Colonized Arabidopsis plants produce more biomass and seeds, and are more resistant against biotic and abiotic stress. We have analyzed Arabidopsis mutants defective in defense responses which do not respond to the fungus and do not maintain the symbiotic interaction. However, these mutants launched unspecific defense responses against *P. indica* in order to restrict colonization in roots.

This review was written by R. O. and P. L. N. with the help of K. V., A. M., E. S., C. S., I. S., and J. M. J.

3.5 Manuscript V

The beneficial fungus *Piriformospora indica* protects Arabidopsis from *Verticillium dahliae* infection which requires EIN3 for disease development

Chao Sun, Yongqi Shao, Khabat Vahabi, Jing Lu, Samik Bhattacharya, Sheqin Dong, Kai-Wun Yeh, Irena Sherameti, Binggan Lou, Ian T. Baldwin, Ralf Oelmüller

Submitted to Journal of Experimental Botany (ID: JEXBOT/2014/128512)

In this research paper, we describe the role of *P. indica* under biotic stress conditions. The biomass of *P. indica* pre-treated Arabidopsis seedlings followed by *V. dahliae* infection is similar to that of the control plants. The disease severity and other symptoms are decreased. The total chlorophyll content in Arabidopsis shoots is higher. After *P. indica* treatment the seedlings accumulate less phytohormone, such as JA- Ile and SA. In *P. indica*-pretreated seedlings, there is a bigger number of opened stomata and the expression of defense genes in shoots is inhibited compared to *V. dahliae* treatment alone or in pretreated with *V. dahliae* followed by *P. indica* treatment. The dual culture data and the microscopical identification of microsclerotia confirm that *P. indica* physically inhibits the growth of *V. dahliae* and this could be the main reason for conferring Arabidopsis resistance against *V. dahliae* infection. In addition, we also demonstrate that an exudate preparation induces cytoplasmic calcium

elevation in Arabidopsis roots, and the disease development requires the ethylene-activated transcription factor EIN3.

C. S. designed and carried out most of the experiments. Y. Q. S. prepared the exudates from *V. dahliae*. K. V. did the root microscopy and long term experiment for *ein3* mutant in soil. J. L. contributed the phytohormones (JA, JA-Ile, SA, OPDA and ABA) analysis. S. B. did the ethylene measurement. C. S. and R.O. wrote the manuscript. I. S., B. L. and I. T. B. contributed to the discussion. The project was funded by a project of S. Q. D, K. W. Y. and R.O.

3.6 Manuscript VI

Technical note:
***Piriformospora indica* hyphae and chlamydospores by Scanning Electron Microscopy**

Chao Sun and Ralf Oelmüller
Journal of Endocytobiosis and Cell Research (2010), 20: 34-37

In this technic paper, we described a standard protocol for the visualization of hyphae and spores of *Piriformospora indica* by Scanning Electron Microscopy. We could observe that the hyphae are straight and hyaline, and the surface of the hyphal walls is smooth. The chlamydospores are pear-shaped and have smooth walls.

The protocol was established by C. S. and the paper was written by C. S. and R. O.

4. Manuscripts

4. 1 Manuscript I

Piriformospora indica confers drought tolerance in Chinese cabbage leaves by stimulating antioxidant enzymes, the expression of drought-related genes and the plastid-localized CAS protein

Chao Sun, Joy M. Johnson, Daguang Cai, Irena Sherameti, Ralf Oelmüller, Binggan Lou
Journal of Plant Physiology (2010), 167: 1009-1017

Journal of Plant Physiology 167 (2010) 1009–1017

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.de/jplph

Piriformospora indica confers drought tolerance in Chinese cabbage leaves by stimulating antioxidant enzymes, the expression of drought-related genes and the plastid-localized CAS protein

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ARTICLE INFO

Article history:
Received 9 December 2009
Received in revised form 3 February 2010
Accepted 3 February 2010

Keywords:
Chinese cabbage
Drought tolerance
Antioxidant enzymes
Ca^{2+}-sensing regulator
Piriformospora indica

ABSTRACT

Piriformospora indica, a root-colonizing endophytic fungus of Sebacinales, promotes plant growth and confers resistance against biotic and abiotic stress. The fungus strongly colonizes the roots of Chinese cabbage, promotes root and shoot growth, and promotes lateral root formation. When colonized plants were exposed to polyethylene glycol to mimic drought stress, the activities of peroxidases, catalases and superoxide dismutases in the leaves were upregulated within 24 h. The fungus retarded the drought-induced decline in the photosynthetic efficiency and the degradation of chlorophylls and thylakoid proteins. The expression levels of the drought-related genes *DREB2A*, *CBL1*, *ANAC072* and *RD29A* were upregulated in the drought-stressed leaves of colonized plants. Furthermore, the CAS mRNA level for the thylakoid membrane associated Ca^{2+}-sensing regulator and the amount of the CAS protein increased. We conclude that antioxidant enzyme activities, drought-related genes and CAS are three crucial targets of *P. indica* in Chinese cabbage leaves during the establishment of drought tolerance. *P. indica*-colonized Chinese cabbage provides a good model system to study root-to-shoot communication.
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1. Introduction

The endophytic fungus *Piriformospora indica* colonizes the roots of many plant species, promotes their growth and seed yield and confers tolerance against biotic and abiotic stress (Varma et al., 1999; Verma et al., 1998; Oelmüller et al., 2009). Although the endophytic interaction of *P. indica* with various plant species shares similarities with mycorrhiza (Harrison, 2005; Oldroyd et al., 2005) and the symbiosis between plants and plant-growth promoting rhizobacteria (Stein et al., 2008; Lugtenberg and Kamilova, 2009), relatively little is known about the molecular mechanisms by which the fungus promotes plant performance. Most studies have been performed with model plants such as *Arabidopsis thaliana* (Peškan-Berghöfer et al., 2004; Pham et al., 2004a,b; Oelmüller et al., 2004, 2009; Shahollari et al., 2005, 2007; Sherameti et al., 2005, 2008a,b; Vadassery et al., 2009a,b), barley (Waller et al., 2005, 2008; Baltruschat et al., 2008) or tobacco (Barazani et al., 2005, 2007, for *Sebacina vermifera*). We found that Chinese cabbage (*Brassica campestris* L. ssp. Chinensis) is a good host for *P. indica*, and that the performance of adult plants is strongly promoted by the fungus, particularly under stress conditions. Chinese cabbage is a fast-growing plant and an important vegetable in China, Japan and Korea. Efficient transformation systems have been established (cf. Vanjildorj et al., 2009). Although the plant is closely related to *Arabidopsis*, the genetic programs controlling the development and habitus of Chinese cabbage must be quite different from those of *Arabidopsis*. Therefore, it is of interest to investigate whether the growth response induced by *P. indica* is mediated by the same signaling events in both species. Furthermore, the fungus confers resistance against drought and leaf pathogens (Johnson et al., manuscript in preparation), suggesting efficient information flow from the colonized roots to the leaves. Here, we report that drought tolerance is associated with the activation of antioxidant enzyme activities, the upregulation of mRNA levels for drought-related proteins, and rapid accumulation of the plastid-localized Ca^{2+}-sensing regulator (CAS) protein in the leaves. CAS might function as a regulator of cytoplasmic Ca^{2+} levels in controlling stomata aperture (Nomura et al., 2008). We identified three targets of the fungus that participate in the establishment of *P. indica*-mediated drought tolerance in Chinese cabbage leaves.

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doi:10.1016/j.jplph.2010.02.013

2. Materials and methods

2.1. Growth and cultivation conditions

P. indica was cultured in Petri dishes on a modified Kaefer medium (Sherameti et al., 2005). The plates were placed in a temperature- and light-controlled growth chamber at 25 °C and 80 μmol s^{-1} m^{-2} with a 12 h photoperiod for 2 weeks. The liquid culture was kept at 50 rpm for 15 d at room temperature in the dark.

Chinese cabbage seeds were surface-sterilized and the seedlings were first kept in glass jars containing 1/2 concentration of MS nutrient medium under sterile conditions (Murashige and Skoog, 1962), 25 °C and a 12 h photoperiod. After 5 days, one plug of *P. indica* mycelium was added to infect the roots of the seedlings. The control seedlings were mock-treated (solid medium without *P. indica*). After 15 days of co-cultivation, the seedlings were transferred to soil for 30 days (1 seedling/pot) before further physiological analyses were performed.

2.2. Analysis of root colonization

15 and 30 days after co-cultivation on soil, root colonization was analyzed under the microscope. The roots were washed thoroughly in running tap water to remove the medium and attached mycelium, cut into 1 cm pieces and treated overnight with 10% KOH solution at room temperature. The roots were washed 5 times with distilled water and treated 4 times with 1% HCl before staining with 0.05% trypan blue. The stained root segments were examined microscopically according to Rai et al. (2001).

To compare the colonization of Chinese cabbage roots with that of, *Arabidopsis* the *Pitef1*/*actin* cDNA ratios were determined as described in Sherameti et al. (2008b). RNA was isolated from colonized roots of the two plant species and, after reverse transcription, the following primer pairs were used to amplify the cDNA fragments: *Pitef1*, gagctgactacggttgttgag and ggagacaatgtcaaggtcgg; *Bcactin1* (*Brassica campestris* Pekinensis, FJ969844) and *Atactin2* (*Arabidopsis thaliana*, At3g01015) ggccgaggctgatgacattcaacc and gcctcggtaagaagaaccgggtgc.

2.3. Determination of biomass parameters

Fifteen or 30 days after co-cultivation with *P. indica* in soil, plants, which were either co-cultivated with *P. indica* or mock-treated, were used to assess various biomass parameters including fresh root and shoot weight and root and shoot lengths. The roots were washed with distilled water. The lengths and the weights of the aerial parts and of the roots were analyzed separately. For the determination of the dry weights, the material was dried overnight in an oven at 80 °C.

2.4. Drought-stress experiments

One month after transfer to soil, *P. indica* and mock-treated Chinese cabbage plants were treated with a 20% polyethyleneglycol 4000 (PEG) solution. Water was used for the control plants. The development of PEG-treated and water-control plants either grown in the absence or presence of the fungus was monitored over the next 96 h. The experiments were repeated 4 times.

2.5. Enzyme assays

Leaf material without the midrib (0.3 g) was homogenized in 4 ml 50 mM PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate dibasic, 2 mM potassium phosphate monobasic, pH 7.8) containing 0.2 mM ethylenediaminetetraacetic acid (EDTA) and 1% polyvinylpyrrolidone (PVP). The homogenate was centrifuged at 24,000 × *g* for 20 min and the supernatant was used for the enzyme assays. Superoxide dismutase (SOD) activity was assayed according to Giannopolitis and Ries (1977) by measuring the reduction of nitrotetrazolium blue chloride at 560 nm. The catalase (CAT) activity was determined spectrometrically as described by Gao (2006) by measuring H2O2 consumption at 240 nm, and the peroxidase (POD) activity was determined as described by Cakmak and Marschner (1992). Oxidation of guaiacol in the presence of H_2O_2 was measured by an increase in the absorbance at 470 nm.

2.6. Determination of the malondialdehyde content

The malondialdehyde content was determined according to Zhang and Fan (2007). The component from the supernatant of the extract was precipitated with 0.5% thiobarbituric acid, the suspension was boiled for 10 min and immediately cooled down on ice. After centrifugation at 8000 × *g* for 10 min, the absorbance was measured at 532 and 600 nm.

2.7. Western analysis

Four days after treatment of the *P. indica*-colonized plants with 20% PEG solution, the leaf material was harvested and ground in liquid nitrogen. Proteins were extracted with 50 mM TRIS pH 7.8, 10 mM $MgCl_2$, 5% SDS. The mixture was incubated at 78 °C for 10 min. After centrifugation, the supernatant was used for protein determination according to Lowry et al. (1951). An equal amount of protein (40 μg) was separated on a one-dimensional SDS gel (Laemmli, 1970). After transfer to PVDF membranes (Amersham Pharmacia Biotech), the membranes were incubated with the respective primary antisera, followed by incubation with a goat anti-rabbit secondary antiserum coupled to peroxidase (Sigma–Aldrich Chemie GmbH). Proteins were visualized by enhanced chemiluminescence. The antisera have been described previously: antibodies against D1, the 33 kDa protein of photosystem II, PsaA, PsaD, PetC, nitrate reductase and ferredoxin in Hein et al. (2009), against PsaF and Hcf101 in Stöckel and Oelmüller (2004) and Stöckel et al. (2006), against the 23 kDa protein of photosystem II in Palomares et al. (1993) and against the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase in Oelmüller and Mohr (1986). Equal loading of proteins per lane was confirmed by incubating the membranes with Pouceau S (Sigma–Aldrich, Taufkirchen, Germany) prior to the antibody application.

2.8. Analysis of the thylakoid membranes

Four days after the application of 20% PEG solution (or water as control), chloroplasts were isolated from the leaf material by Percoll gradient centrifugation. After lysis, the membranes were pelleted by high speed centrifugation, washed twice with 0.3 M NaCl, the proteins were dissolved in Laemmli loading buffer, and the suspension was incubated for 10 min at 78 °C. After centrifugation, 50 μg of chlorophyll was loaded on each lane. The chlorophyll concentration was determined before protein extraction according to Lichtenthaler and Wellburn (1983). The 40 kDa band was cut off the gel, extracted, digested with trypsin and analyzed by mass spectrometry, as described by Sherameti et al. (2004).

2.9. Northern analysis

Arabidopsis cDNAs (obtained from the Ohio stock center) were used for the hybridization with RNA from Chinese cabbage. The cDNA fragments were excised from the plasmids according to the instruction from the stock center. RNA was isolated with the RNAeasy extraction kit and equal amounts of RNA (35 μg) were

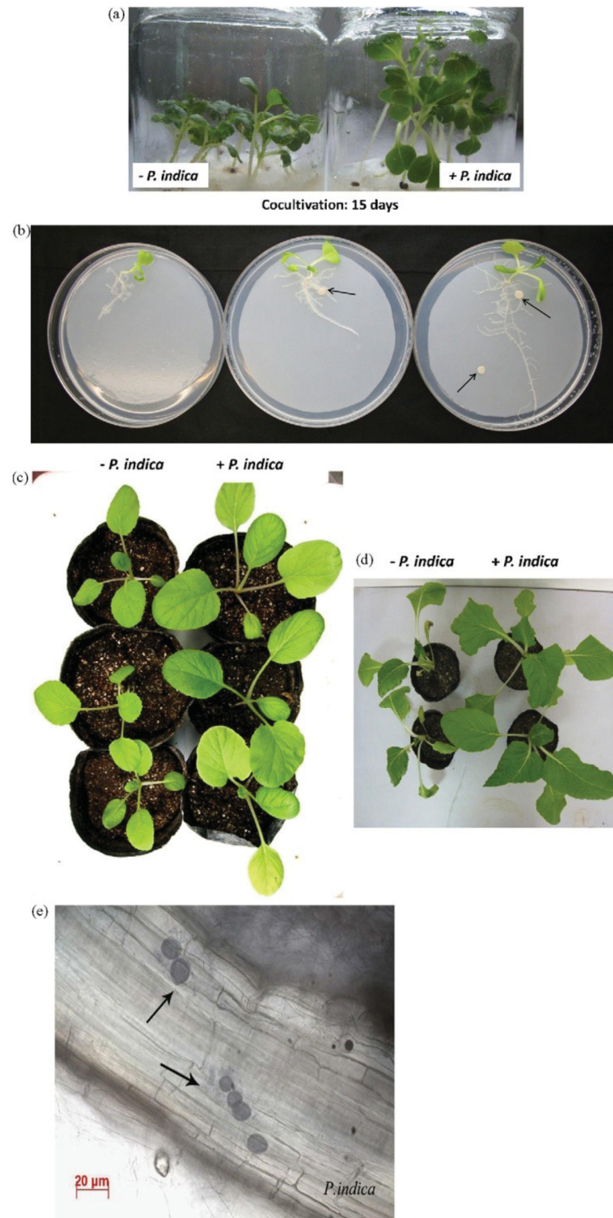

Fig. 1. (A–C) *P. indica* promotes Chinese cabbage growth. (A) The picture shows seedlings kept in jars, which were either co-cultivated with *P. indica* (+ *P. indica*) or mock-treated (− *P. indica*). (B) Sizes of the roots of Chinese cabbage seedlings which were either mock-treated or treated with one or two plaques of *P. indica* (see arrows). The seedlings were co-cultivated with *P. indica* for 7 days in 15 cm-diameter Petri dishes. (C) Colonized and mock-treated plants after transfer to soil for 15 days. (D) Colonized and mock-treated plants after transfer to soil for 20–30 days. For this picture, uncolonized (left) and colonized (right) plants of the same size and weight were chosen and treated once with 200 ml of PEG solution, 4 days before the picture was taken. (E) Spores in the roots. For details cf. Section 2.

separated on a formaldehyde gel. Hybridization occurred at 61 °C for 42 h. Each filter also contained RNA from *Arabidopsis* to confirm that the size of the hybridizing signal corresponds to that of Chinese cabbage RNA. Equal RNA loading was confirmed by hybridization to an actin DNA fragment, which was amplified with the primer pairs described above.

2.10. Statistics

Samples were evaluated with a two sample *t*-test (± *P. indica*, ± drought) and ANOVA analyses (comparison of all data sets). All experiments were repeated three times (RNA and protein data) or four times (enzyme activities and color pictures).

3. Results

3.1. Root colonization stimulates biomass production of Chinese cabbage plants

Growth of Chinese cabbage seedlings, co-cultivated with *P. indica* in jars for 15 days, was strongly promoted compared to mock-treated control seedlings (Fig. 1A). Also, the development of the main and lateral roots was strongly promoted by the fungus (Fig. 1B). After 15 days on soil, the effects were even stronger (Fig. 1C). We observed a 38 ± 5% (n = 20) increase in the root fresh weight and a 46 ± 6% (n = 20) increase in leaf fresh weight. Microscopic analyses revealed that the degree of root colonization was quite high. Spores were detected in almost all areas of the roots, and most of the fungal hyphae could be found in and in the vicinity of the lateral roots (Fig. 1E). Although different plant species are difficult to compare, we determined the amount of the cDNA, generated from RNA of colonized roots, for the translation elongation factor 1 from *P. indica* with the amounts of the *actin* cDNA from Chinese cabbage and *Arabidopsis*. The *Pitef1*/*actin* ratio was at least 5 times higher for Chinese cabbage compared to *Arabidopsis*, which roughly reflects the ratio of spores/lateral root determined under the microscope (7–10 times more spores in Chinese cabbage roots compared to *Arabidopsis* roots). The massive increase in the biomass of the aerial parts of the plant indicates that there must be efficient information flow from the colonized roots to the leaves.

3.2. Chinese cabbage plants co-cultivated with P. indica are more drought-tolerant than the uncolonized control

Due to the fast growth and enormous biomass production of the Chinese cabbage leaves, the plants are quite sensitive to drought, which is a severe problem in agriculture. Therefore, we exposed Chinese cabbage to drought stress. After 4 weeks on soil, colonized and uncolonized Chinese cabbage plants were treated once with 200 ml of a 20% PEG solution (pot size: 10 cm height and 10 cm diameter) and further development of the plants was followed over the next 4 days. After 4 days, PEG-treated plants were severely suffering from drought stress, while no visible stress phenomena could be detected in drought-exposed colonized plants (Fig. 1D).

3.3. P. indica decreases the accumulation of malondialdeyde, a biomarker of oxidative stress in cells

Reactive oxygen species (ROS) degrade polyunsaturated lipids, thereby forming malondialdehyde (MDA, Pryor et al., 1975). The production of this aldehyde is used as a biomarker to measure the level of oxidative stress (Moore and Roberts, 1998; Del Rio et al., 2005). PEG application strongly promoted MDA accumulation in the leaves and the amount of MDA is reduced in colonized plants (Fig. 2). This suggests that the fungus prevents

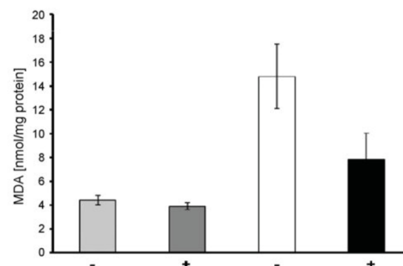

Fig. 2. *P. indica* inhibits MDA accumulation in the leaves of PEG-treated Chinese cabbage plants. Chinese cabbage plants were either treated with 200 ml of water (grey bars) or with 200 ml of PEG solution (black and white bars). −, +: without, with *P. indica*. The measurements were taken 24 h after the PEG/water treatment. Bars represent SEs, based on 4 independent experiments.

ROS formation by retarding the degradation of polyunsaturated lipids.

3.4. Antioxidant enzyme activities are upregulated in colonized drought-exposed leaves

Several reports have demonstrated that antioxidant enzyme activities are crucial for *P. indica*-induced resistance against abiotic stress (Baltruschat et al., 2008, Vadassery et al., 2009b). Therefore, we tested whether *P. indica* influences the enzyme activities of superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) after exposure of the plants to drought. One day after the PEG application, the SOD activity in the leaves of colonized plants was higher than the activity of all other controls (no PEG treatment, no fungus) (Fig. 3A). A similar effect was observed for the POD activity (Fig. 3B). In contrast, the CAT activity was stimulated in colonized and uncolonized leaves after PEG application, although the stimulatory effect was higher in the presence of the fungus (Fig. 3C). We conclude that *P. indica* stimulates antioxidant enzyme activities in the leaves of PEG-treated Chinese cabbage plants, although the effects for the three enzymes tested were quite different.

3.5. P. indica promotes the expression of drought-related genes after PEG treatment

We have previously demonstrated that a large number of drought-induced genes are more quickly and strongly upregulated in drought-exposed *Arabidopsis* leaves when the roots are colonized by *P. indica* (Sherameti et al., 2008a). Using *Arabidopsis* cDNA probes for drought-related genes, we performed a similar experiment with Chinese cabbage plants exposed to PEG treatment. RNA was isolated from the leaves of colonized and uncolonized Chinese cabbage plants 0, 8, 16 and 24 h after PEG treatment. Fig. 4 demonstrates that the mRNA levels for the transcription factors DREB2A and ANAC072, as well as for the drought-related proteins CBL1 and RD29A, responded faster and more strongly to the PEG treatment in colonized plants compared to the uncolonized controls. This confirms our previous results obtained with *Arabidopsis* seedlings and extends them to adult Chinese cabbage plants grown on soil.

3.6. P. indica retards the breakdown of plastid functions after PEG treatment

The PEG treatment has a strong effect on photosynthesis (Flexas et al., 2004; Ramachandra-Reddy et al., 2004, Sherameti et al., 2008a). The *F* variable/*F* maximum (*Fv*/*Fm*) values decreased in

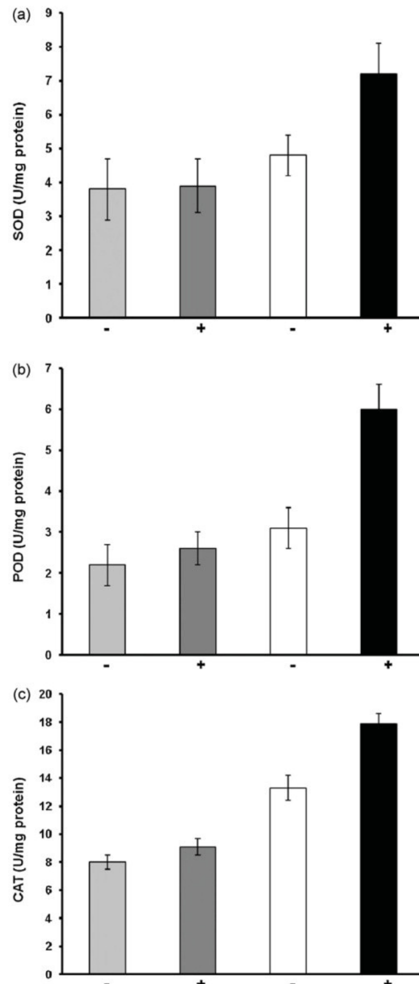

Fig. 3. *P. indica* stimulates antioxidant enzyme activities in the leaves of PEG-treated Chinese cabbage plants. The measurements were taken 24 h after the PEG/water treatment. For details, cf. legend to Fig. 2. (A–C) SOD, POD and CAT activities. Bars represent SEs, based on 4 independent experiments.

the uncolonized, dark-adapted controls within the first 4 days after exposure to drought (from t_{0d} = 0.82 to t_{4d} = 0.61), while no significant difference was observed for the dark-adapted colonized plants (from t_{0d} = 0.82 to t_{4d} = 0.79) (Fig. 5A). *Fv*/*Fm* values around 0.83, measured after dark adaptation, reflect the potential fluorescence quantum efficiency of photosystem II and are sensitive indicators of plant photosynthesis performance (Björkman and Demming, 1987). Values lower than 0.83 indicate that plants are exposed to stress (Maxwell and Johnson, 2000). This clearly demonstrates that root-colonized plants suffer less from drought stress than the uncolonized controls.

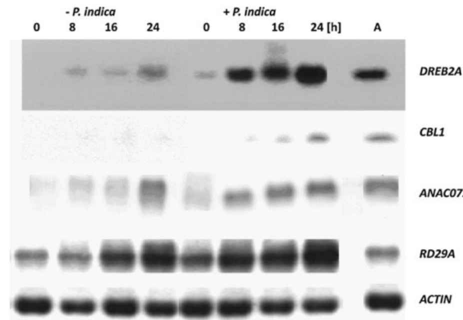

Fig. 4. mRNA levels for drought-related genes (and *ACTIN* as loading control) in the leaves of root-colonized (+ *P. indica*) or mock-treated (− *P. indica*) Chinese cabbage leaves, 0, 8, 16 and 24 h after the PEG treatment. A, RNA from Arabidopsis leaves was used as control. Representative Northern blots from 3 independent experiments are shown.

In addition, 4 days after PEG application, the total chlorophyll level decreased by more than 50% in uncolonized plants, whereas *P. indica*- colonized plants showed only a minor decrease in the total chlorophyll content (Fig. 5B). Furthermore, the decrease in the protein levels of representative components of the thylakoid membrane and of enzymes located in the plastid stroma in PEG-treated plants was retarded in the presence of *P. indica* (Fig. 5C). We analyzed the protein levels of representative components of the photosystem II (D1, 33- and 23-kDa proteins), the photosystem I (PsaA, PsaD, PsaF), the cytochrome-*b6*/*f*-complex (PetC, Rieske protein), the small subunit of ribulose-1,5-bisphosphate (RbcS), nitrate reductase (NR), ferredoxin (Fd) and of a regulatory protein involved in iron-sulfur biosynthesis (Hcf101; Stöckel and Oelmüller, 2004). Analysis of the expression levels of four nuclear and two plastid-encoded genes for thylakoid proteins confirmed that this regulation occurs at the level of transcription (Fig. 5D). The best hybridization signals with the *Arabidopsis* probes on Chinese cabbage RNA filters were obtained for genes encoding the 33-kDa protein of photosystem II, plastocyanin, the subunit III of photosystem I (PsaF), and the subunit δ of the plastid ATP synthase (AtpD), as well as for the two major core proteins of the photosystems II and I, PsbA and PsaA. Therefore, root-colonized Chinese cabbage plants are better protected against drought stress.

3.7. The plastid-localized calcium-sensing receptor CAS is upregulated in colonized leaves exposed to drought

While analyzing the protein composition of the thylakoid membrane, we noted that a protein of approximately 40 kDa was upregulated in *P. indica*- colonized PEG-treated Chinese cabbage leaves (Fig. 6A). The protein was extracted from the gel and analyzed by mass spectrometry. Two fragments corresponded to VFQVVGDALK and AQEAIQSSGFDSEPVFNAAK. A data bank search revealed that both fragments show an almost complete match to the CAS protein from various organisms (cf. Nomura et al., 2008; Weinl et al., 2008). Thus, it is likely that the plastid-localized CAS protein is a target of *P. indica* in drought-stressed Chinese cabbage leaves. Northern analysis with a cDNA probe from *Arabidopsis* demonstrated that the mRNA level follows the pattern observed on the protein level (Fig. 6B). This suggests that the regulation occurs at the level of transcription.

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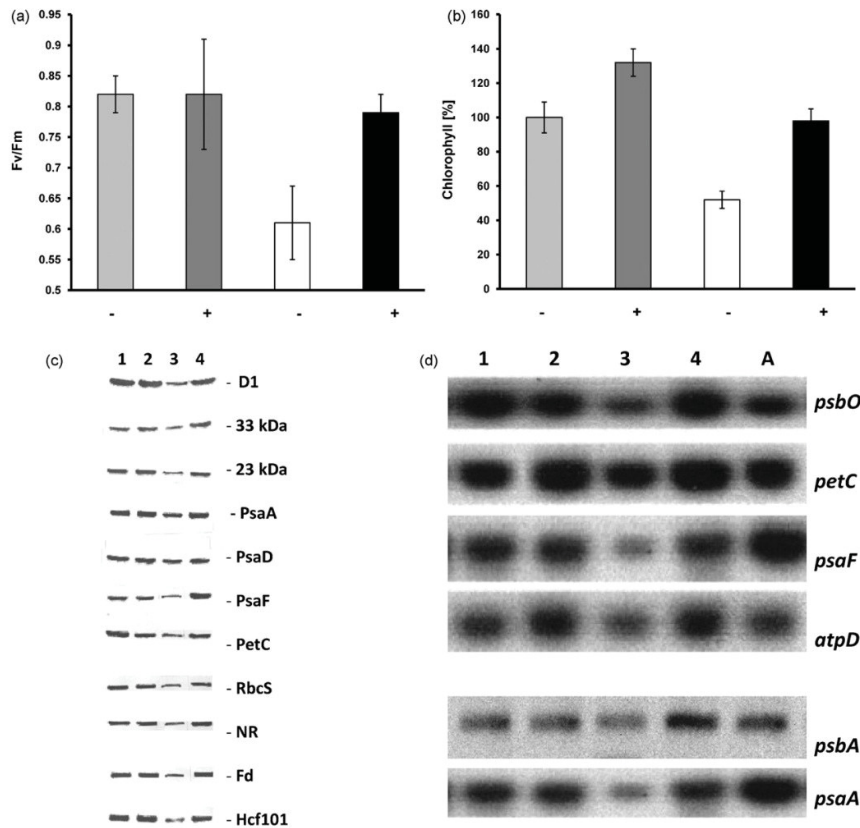

Fig. 5. The effect of *P. indica* on photosynthesis and plastid proteins during drought stress. Chinese cabbage plants were either treated with 200 ml of water (grey bars in figures with bar graphs and lanes 1 and 2 in gel pictures) or with 200 ml of 20% PEG solution (black and white bars in figures with bar graphs and lanes 3 and 4 in gel pictures). (−) and lanes 1 and 3: plants without *P. indica*; (+) and lanes 2 and 4: plants co-cultivated with *P. indica*. (A) Fv/Fm values 24 h after the PEG/water treatment. Bars represent SEs, based on 16 independent experiments. (B) Total chlorophyll content 96 h after the PEG/water treatment. Bars represent SEs, based on 16 independent experiments. The chlorophyll content in the leaves of uncolonized water-treated plants was taken as 100% and the other values are expressed relative to it. (C) Western analyses for plastid proteins, 96 h after PEG/water treatment. D1, reaction center protein of photosystem II; 33 and 23 kDa, two lumenal proteins of photosystem II; PsaA, reaction center protein of photosystem I; PsaD, PsaF, subunits II and III of photosystem I; PetC, the Rieske Fe/S-protein of the cytochrome-*b6*/*f*-complex; RbcS, small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase; NR, nitrate reductase; Fd, ferredoxin; Hcf101, regulatory protein for photosystem I assembly. (D) Northern analyses for representative nuclear-encoded (*psbO*, *petC*, *psaF*, *atpD*) and plastid-encoded (*psbA*, *psaA*) genes. A, RNA from Arabidopsis leaves were used as control. *psbO*, 33 kDa protein of photosystem II; *petC*, the Rieske protein of the cytochrome-*b6*/*f*-complex; *psaF*; subunit III of photosystem I; *atpD*, subunit δ of the ATP synthase; *psbA*, D1 protein; *psaA*, reaction center protein of photosystem I.

4. Discussion

4.1. Chinese cabbage is a good host for P. indica

We noted that *P. indica* strongly interacted with the roots of Chinese cabbage, resulting in efficient colonization (Fig. 1). Hyphae and spores were detected around the roots and root hair, in the extracellular space and within root cells (Fig. 1D). Co-cultivation of both organisms resulted in a rapid increase in root and shoot biomass (Fig. 1A–C). The growth-promoting effect remained visible after transfer of the seedlings to soil (Fig. 1C). Closer inspection of the root of the adult plants revealed that the hyphae continued to propagate in the soil (data not shown). Therefore, once colonized, the symbiosis remains stable and provides the basis for long-term benefits for the plant. Rough estimations suggest that the fungal hyphae propagate much better in Chinese cabbage roots than in *Arabidopsis* roots. Because of the enormous ecological and agricultural importance of Chinese cabbage, we investigated this interaction in greater detail.

4.2. P. indica confers drought tolerance by activating the antioxidant enzyme systems

Accumulation of MDA, a biomarker of oxidative stress, in PEG-treated Chinese cabbage clearly demonstrated that the plants were

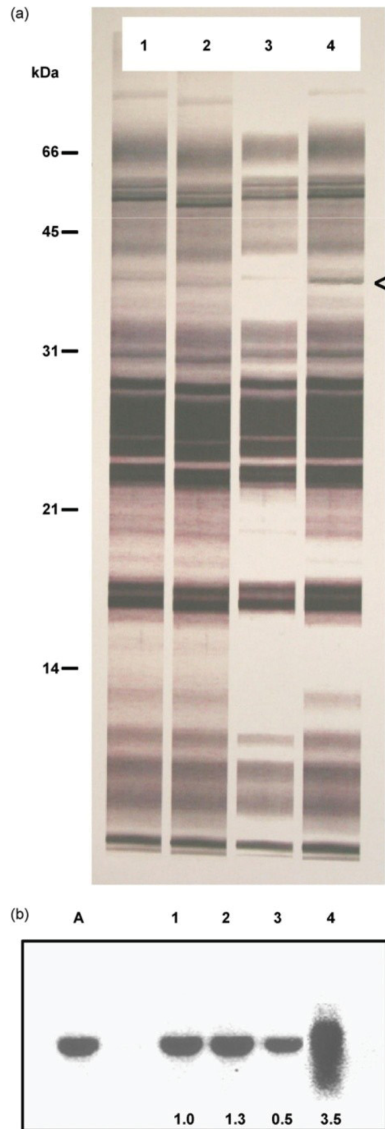

Fig. 6. The plastid-localized CAS is a target of *P. indica* in PEG-treated Chinese cabbage plants. (A) Protein pattern of thylakoid membranes isolated from chloroplasts of Chinese cabbage leaves which were either treated with PEG solution 96 h before plastid isolation (lanes 3 and 4) or mock-treated with water (lanes 1 and 2). Lanes 1 and 3: uncolonized plants; lanes 3 and 4; plants co-cultivated with *P. indica* for 30 days on soil. Proteins corresponding to 50 μg of chlorophyll was loaded per lane. The 40 kDa band that is upregulated in lane 4 is marked. (B) Northern analysis for the *CAS* mRNA. RNA was isolated 96 h after the PEG/water treatment. A, RNA from Arabidopsis leaves was used as control. The numbers under the hybridization signals refer to the amounts of the mRNAs, relative to the level in lane 1.

exposed to stress (Fig. 2). The overall level of MDA was lower in *P. indica*-colonized plants, and thus the fungus could partially counteract this stress response. MDA is mainly formed by the ROS-induced degradation of polyunsaturated lipids (Pryor et al., 1975; Del Rio et al., 2005). *P. indica* could prevent or retard the degradation of these lipids by preventing excess ROS formation under stress conditions. This confirms the results obtained for the antioxidant enzyme activities that become activated in the leaves of colonized plants (Fig. 3A–C). We focused on three classes of enzymes: the SODs catalyze the dismutation of superoxide into oxygen and hydrogen peroxide. In higher plants, SOD isozymes are present in different cell compartments. Mn-SOD is present in mitochondria and peroxisomes, Fe-SOD mainly in chloroplasts, and Cu/Zn-SOD in cytosol, chloroplasts, peroxisomes and the apoplast (Corpas et al., 2001, 2006). In green leaves, the majority of the SOD activity is present in plastids, which also demonstrates the important role of this organelle for the *P. indica*-induced drought tolerance. CATs, peroxisomal enzymes (Alberts et al., 2002), catalyze the decomposition of hydrogen peroxide to water and oxygen (Chelikani et al., 2004). Apparently, antioxidant enzyme activities in the chloroplast-associated peroxisomes are also important targets of *P. indica* in Chinese cabbage leaves.

PODs are a large family of enzymes that detoxify hydrogen peroxide, organic hydroperoxides or lipid peroxides to generate alcohols. PODs contain a heme cofactor in their active sites that is synthesized in the plastid. Heme is also coupled to the iron homeostasis, which may play an important role in plant/microbe interaction (Briat et al., 2007; Stöckel et al., submitted for publication). In addition, PODs contain redox-active cysteine residues, which directly measure the redox potential in the cell or organelle. The most important organelle in a green leaf that controls the redox potential in the cell is the plastid (Mühlenbock et al., 2008; Bräutigam et al., 2009). Whether the fungus interferes with the iron homeostasis and redox potential of the cell and whether this contributes to stress tolerance remains to be investigated. Most of the antioxidant enzymes are regulated posttranslationally in response to oxidative stress. Therefore, we determined the enzyme activities rather than the expression levels of the genes in response to *P. indica*. The data are consistent with previous observations that activation of the antioxidant enzyme systems is a major target of the fungus in leaves (Baltruschat et al., 2008; Vadassery et al., 2009b). In the leaves of colonized *Arabidopsis* plants, monodehydroascorbate reductase and dehydroascorbate reductase, two enzymes of the ascorbate-glutathione cycle that maintain ascorbate in its reduced state, were upregulated. Growth and seed production were not promoted by the fungus in *mdar2* and *dhar5* T-DNA insertion lines. Thus, MDAR2 and DHAR5 are crucial for producing sufficient ascorbate to maintain the interaction between *P. indica* and *Arabidopsis* in a mutualistic state (Vadassery et al., 2009b). It remained to be determined how the information is transferred from the roots to the leaves.

We have previously demonstrated that the mRNA levels for 9 drought-related genes are upregulated in the leaves of *P. indica*-colonized *Arabidopsis* plants (Sherameti et al., 2008a). In addition to transcription factor genes, which stir the proper response of the plant to drought stress (Shinozaki et al., 2003, 2007; Yamaguchi-Shinozaki et al., 2006), *EARLY RESPONSE TO DEHYDRATION (ERD)1* was also strongly regulated by the fungus. ERD1 is a plastid-localized Clp protease regulatory subunit (Nakashima et al., 1997). Here, we show that four of these genes are also upregulated in Chinese cabbage leaves when they are exposed to drought. Although *ERD1* is not included in this study because the heterologous

Arabidopsis probe did not yield reliable data, our results suggest that drought tolerance in the two species might be achieved by similar mechanisms, for instance by establishing reducing conditions in the leaf cells.

4.3. Major targets of P. indica in Chinese cabbage leaves are located in the plastids

Plastids are the major targets of the PEG treatment in Chinese cabbage leaves. In addition to a reduction in photosynthetic efficiency, not only the pigments, but also representative proteins of the photosynthetic machinery and the biosynthetic pathways in the stroma were affected by the PEG treatment (Fig. 5A–D). Interestingly, both nuclear and plastid gene expression were down-regulated (Fig. 5D), indicating that drought stress causes a more general lesion of plastid functions. It is likely that *P. indica* does not target specific photosynthesis genes or proteins to establish drought tolerance, but creates an atmosphere in the cell that prevents a general degradation of plastid functions. Drought stress affects plastid protein degradation to different extents, which is not surprising considering the different stabilities of the thylakoid proteins (cf. Palomares et al., 1993). It is a task for future studies to understand the molecular basis of how the fungus can prevent plastid protein degradation.

4.4. The role of CAS in conferring drought tolerance in Chinese cabbage

Interestingly, the plastid-localized CAS protein appears to be a major target of *P. indica* in the chloroplasts under drought stress (Fig. 6A and B). It has been proposed that the protein – although located in the plastids – controls cytosolic calcium transients and thus stomatal closure (Nomura et al., 2008; Weinl et al., 2008). The CAS protein and *CAS* mRNA levels did not increase after exposure of uncolonized Chinese cabbage to drought stress, although this could ensure better protection of the plant against drought because the stomata could be closed more efficiently. One reason for this observation could be that the severe damage to the plastids after the PEG treatment does not allow a proper regulation of plastid functions. The presence of *P. indica* creates a cellular environment that protects the plastids against severe damage and this allows a proper regulation of *CAS* gene expression. Since drought-stressed *P. indica*-colonized plants clearly showed higher *CAS* mRNA and CAS protein levels in the leaves, it is likely that the fungus counteracts the drought stress by elevating cytoplasmic calcium transients which finally results in stomata closure in the guard cells (Nomura et al., 2008). However, CAS does not only stimulate cytoplasmic calcium elevation in the guard cells, but also in all chloroplast-containing cells of the leaves. This could lead to the activation of a large battery of calcium-induced cellular responses, including plant defense against pathogens. It remains to be determined whether CAS is involved in other stress avoidance responses. The availability of efficient transformation systems for Chinese cabbage will allow us to investigate the role of the CAS protein in greater detail, and to understand the role of this protein in *P. indica*-mediated stress tolerance.

Acknowledgements

This work was supported by a grant (Z3080241) of the Zhejiang provincial natural foundation of China to B.L. and the SFB 605 to R.O.

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4.2 Manuscript II

Growth promotion of Chinese cabbage and Arabidopsis by *Piriformospora indica* is not stimulated by mycelium-synthesized auxin

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Molecular Plant Microbe Interaction (2011), 24(4): 421-431

MPMI Vol. 24, No. 4, 2011, pp. 421–431. doi:10.1094/MPMI-05-10-0110. © 2011 The American Phytopathological Society

e-Xtra*

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

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Submitted 12 May 2010. Accepted 14 December 2010.

***Piriformospora indica*, an endophytic fungus of the order Sebacinales, 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 *rhd6* 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.**

Plants are exposed to a large variety of microorganisms in the rhizosphere, which can be beneficial, neutral, or pathogenic 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 (Dharamasiri 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* [Feraru 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 *rhd6* 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-

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*The ***e*-Xtra** logo stands for "electronic extra" and indicates that a supplementary figure is published online and Figs. 5 and 6 appear in color online.

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 interferes 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 Sebacinales 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

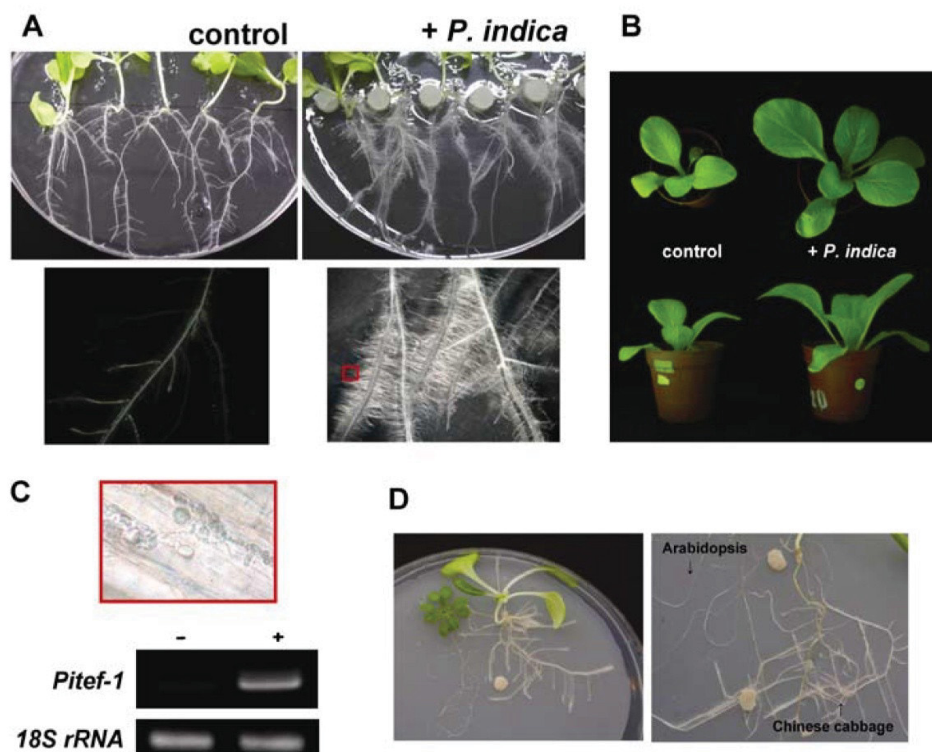

Fig. 1. Interaction of *Piriformospora indica* with Chinese cabbage. **A,** Branching and root hair phenotype of Chinese cabbage at 7 days after fungal co-cultivation with *P. indica.* Five days after seed germination, seedlings were co-cultivated with *P. indica* and grown on $^1/_2$ Murashige-Skoog 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 (Bütehorn 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 ± 6% (*AUX1*) and 44 ± 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 ± 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

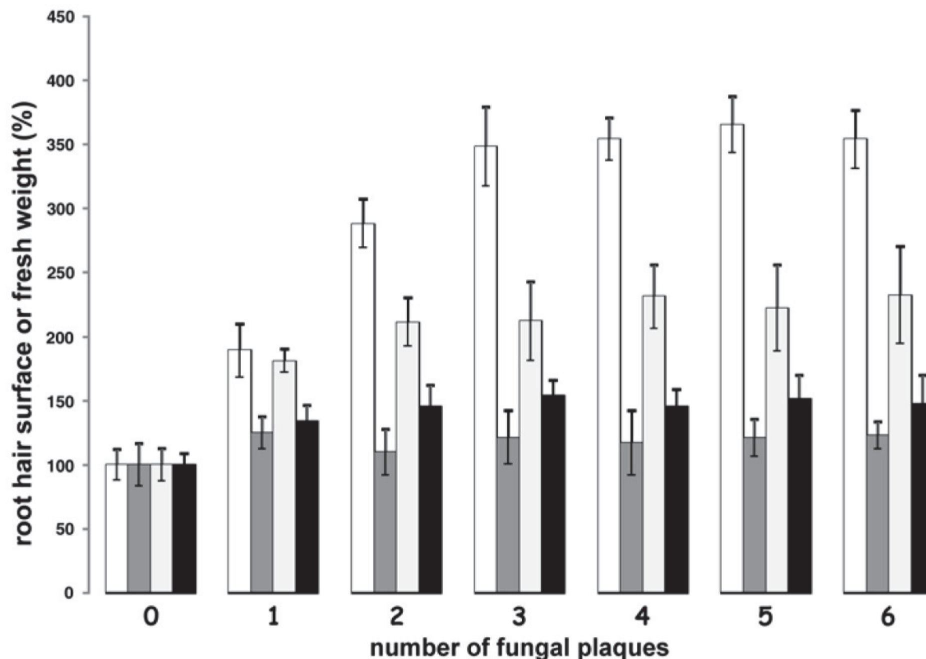

Fig. 2. Root hair surface and fresh weight of Chinese cabbage and *Arabidopsis* seedlings which were co-cultivated with 0 to 6 fungal plaques/seedling for 7 days. One seedling was grown in a petri dish. The 0 values (= control, without *Piriformospora indica*) was taken as 100 and the other values are 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 ± 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.

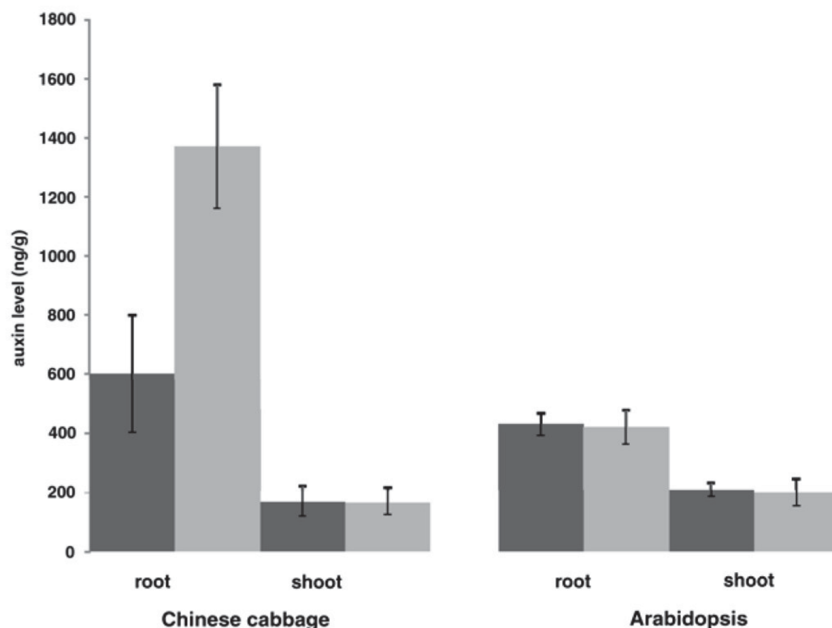

Fig. 3. Auxin levels in root and leaf tissues of Chinese cabbage and *Arabidopsis* seedlings, grown in the presence (light bars) or absence (dark bars) of *Piriformospora indica*. Leaf and root tissues were separately harvested from plants at 7 days after infection by *P. indica*.

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

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

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

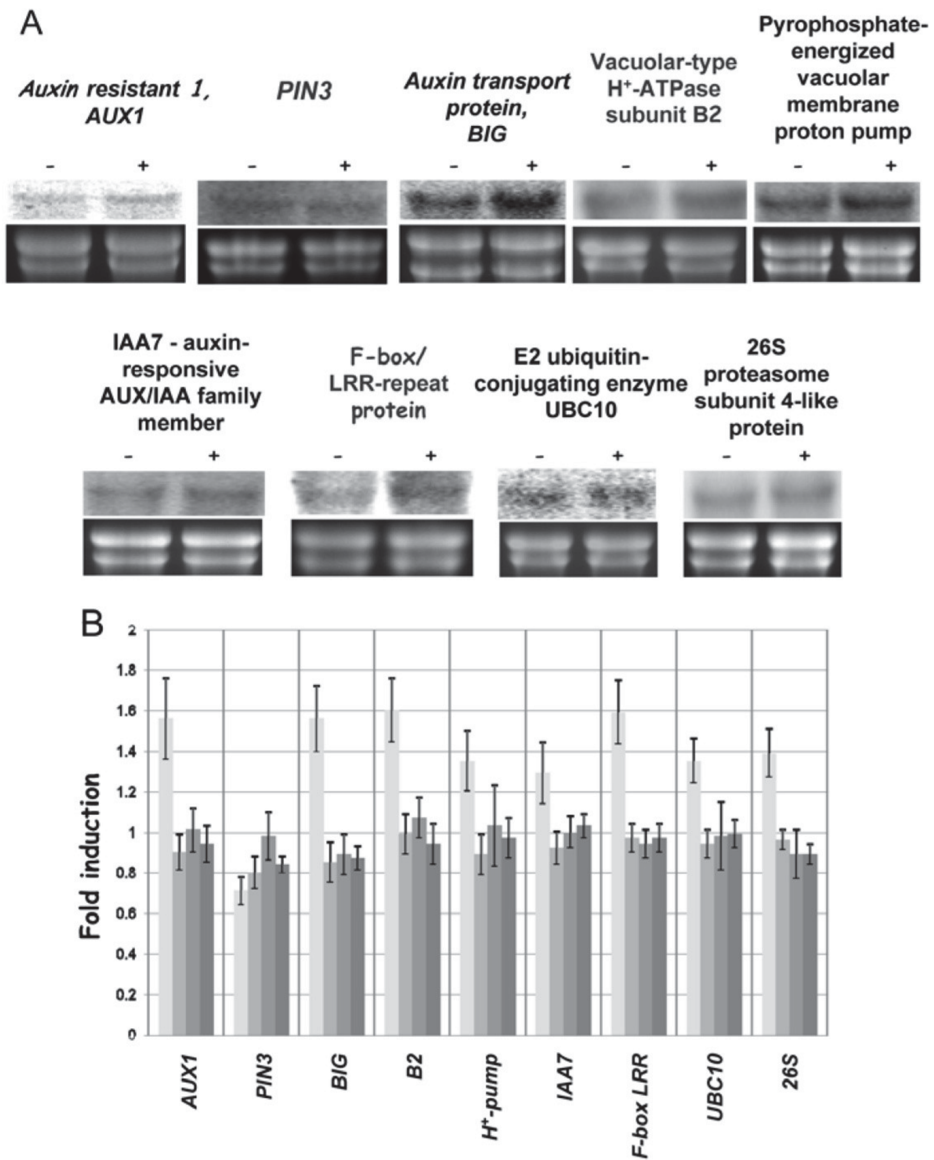

Fig. 4. A, Northern blot analysis for auxin-related genes in Chinese cabbage co-cultivated wit *Piriformospora indica* compared with untreated controls. Root tissues 7 days after infection by *P. indica* were sampled for total RNA extraction. The RNA was resolved on a formaldehyde-denatured agarose gel, 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;

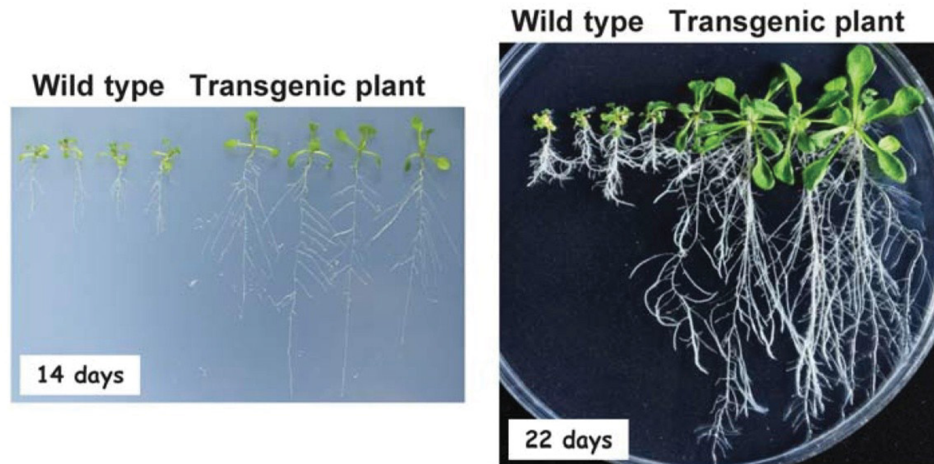

Fig. 5. Root architecture and biomass of T2 transgenic *Arabidopsis* plants overexpressing *BcAUX1*. Shown are 14- and 22-day old seedlings of wild-type (left) and transgenic *Arabidopsis* after germination on 1/2 Murashige-Skoog medium.

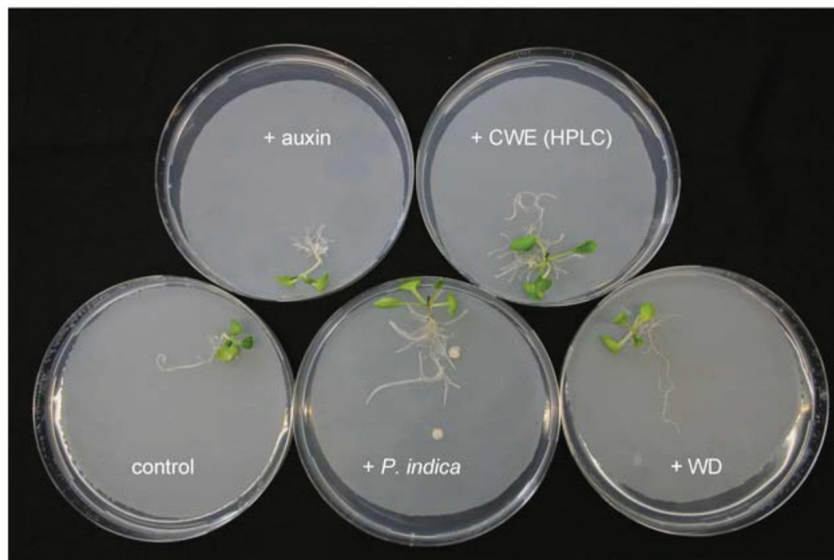

Fig. 6. Chinese cabbage seedlings which were treated with auxin (3 ppm), the high-performance liquid chromatography-purified cell wall extract (HPLC-CWE, 100 µl), a water-diffusible (WD, 100 µl) exudate fraction, or mock-treated (water, control) for 7 days on petri dishes; + *Piriformospora indica* = 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 upregualtion 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 ubiquinated 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 plasmalemma 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 1/2 MS nutrient medium (Murashige and Skoog 1962). Plates were incubated at 22°C under continuous illumination (100 μmol m^{-2} s^{-1}) for seed germination. *Arabidopsis* (ecotype Columbia) seedlings were germinated as described by Vadassery and associates (2009) and grown on petri dishes containing 1/2 MS nutrient medium, identical to the Chinese cabbage seedlings. The homozygote *aux1* (N9583), *aux1/axr4* (N8040), and *rhd6* (N6347) lines were obtained from NASC (Hobbie and Estelle 1995).

Seven days after seed plating on 1/2 MS medium, the growing seedlings were transferred to fresh plates containing 1/2 MS medium. One to six seedlings were used per petri dish and one fungal plague or one agar plague 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 μmol m^{-2} s^{-1}). *P. indica* was cultured as described previously (Peškan-Berghöfer et al. 2004; Verma et al. 1998) on Kaefer medium (Hill and Kaefer 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 [$^{13}C_6$]-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 × 15 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 × 15 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 trimethylsilated 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 Kaefer medium (Hill and Kaefer,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 *Rsa*I 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 µg 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 µl. The iCycler was programmed to 95°C for 2 min; 40 cycles of 95°C for 30 s, 57°C for 40 s, and 72°C for 45 s; 72°C for 10 min; followed by a melting curve program of 55 to 95°C in increasing steps of 0.5°C. All reactions were performed in triplicate. The mRNA levels for each cDNA probe were normalized with respect to the *ACTIN2* mRNA level. Fold induction values of target genes were calculated with the ΔΔCP 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 (Bütehorn 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-µM 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.

ACKNOWLEDGMENTS

We thank the National Science Council of the Republic of China and the Deutscher AkademischerAustausch Dienstof Germany (NSC-DAAD) for their financial support for PPP project NSC-97-2911-1-002-010-2 to K.-W. Yeh and D. Cai. Thanks are also due to the National Taiwan University for partial financial support of international collaboration funding to K.-W. Yeh under project no. 98R0705.

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4.3 Manuscript III

Piriformospora indica promotes growth of Chinese cabbage by manipulating auxin homeostasis: Role of auxin in _P. indica_ symbiosis

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Piriformospora indica – Sebacinales and their biotechnological applications (2013), *Soil Biology 33: 139-148.* A. Varma *et al.*, (eds.) Berlin-Heidelberg: Springer-Verlag

Chapter 8 *Piriformospora indica* Promotes Growth of Chinese Cabbage by Manipulating Auxin Homeostasis: Role of Auxin in *P. indica* Symbioses

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8.1 Introduction: Auxin in Plant–Microbe Interactions

Auxin is the classical phytohormone involved in the development of new organs or promotion of growth. Nodule formation in response to infection with rhizobacteria (Perrine-Walker et al. 2010), the clubroot disease (Ando et al. 2008; Ishikawa et al. 2007; Devos and Prinsen 2006; Devos et al. 2005; Schuller and Ludwig-Müller 2006), or the transformation of root cells into galls/knots by endoparasitic nematodes (Lee et al. 2011a; Grunewald et al. 2009a, b; Wasson et al. 2009) provides examples for microbe-induced developmental reprogramming, in which auxin is involved. Mycorrhizal fungi (Fiorilli et al. 2009; Luo et al. 2009; Ludwig-Müller and Güther 2007; Amiour et al. 2006; Reboutier et al. 2002), plant-growth-promoting rhizobacteria (Contesto et al. 2010; Costacurta and Vanderleyden 1995), or beneficial root-colonizing endophytic fungi (Lee et al. 2011b; Contreras-Cornejo et al. 2009; Felten et al. 2009; Schäfer et al. 2009a, b; Vadassery et al. 2008; Sirrenberg et al. 2007) interfere with auxin metabolism or auxin functions in the plants. Phytohormones including auxins are also involved in plant defense and disease resistance (Kazan and Manners 2009; Bari and Jones 2009; Robert-Seilaniantz et al. 2007). Several microbes produce auxin and release it into the environment or directly into the root, thereby activating auxin signal transduction pathways in the plant cell (cf. Perrine-Walker et al. 2010; Spaepen et al. 2007; Reboutier et al. 2002). However, microbe-induced morphological changes in the roots cannot be caused exclusively by auxin released by the microbe, since

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A. Varma et al. (eds.), *Piriformospora indica*, Soil Biology 33, 139
DOI 10.1007/978-3-642-33802-1_8, © Springer-Verlag Berlin Heidelberg 2013

exogenously applied auxin cannot replace the microbes (cf. Lee et al. 2011b; Felten et al. 2010). The complex developmental programs induced by the microbes require a highly coordinated plant response for which local changes in auxin homeostasis may be initiated by microbial signals.

We study the symbiotic interaction of an endophytic fungus *Piriformospora indica* with different plant species. The ubiquitous fungus promotes growth of all plant species which have been tested so far (Weiß et al. 2011), including the model plant *Arabidopsis thaliana* (Vadassery et al. 2008; 2009) and the agriculturally important crop Chinese cabbage (Sun et al. 2010, Lee et al. 2011b). Growth promotion of these two plant species by *P. indica* involves auxin; however, the role of this phytohormone in the two symbioses is quite different. The comparative analysis of these two symbioses allows interesting conclusions of how *P. indica* controls growth programs on the basis of different genetic backgrounds of individual plant species.

8.2 *P. indica* and Chinese Cabbage

Growth and development of shoots and roots of Chinese cabbage (*Brassica campestris* cv. Chinensis) seedlings is strongly promoted by *P. indica*, and the fresh weight of the seedlings increases approximately twofold (Lee et al. 2011b, Sun et al. 2010). The strong stimulation of root hair development resulted in a bushy root phenotype. Since the auxin level in the infected Chinese cabbage roots was twofold higher compared to the uncolonized controls, the growth-promoting effect in Chinese cabbage clearly involved auxin. We generated a double-subtractive expressed sequence tag library from Chinese cabbage roots grown in the presence or absence of the fungus and isolated genes/gene fragments which are upregulated by *P. indica* in the roots. Many of the identified cDNA fragments are related to auxin metabolism and function: genes for intercellular auxin transport carrier proteins such as AUX1 or PINs, for auxin perception and signal proteins, or for proteins related to cell wall acidification (Lee et al. 2011b). In an initial study, we expressed one of these genes, *BcAUX1,* in *Arabidopsis* and demonstrated that the transgenic lines show a strong promotion in growth and biomass production. This confirms that *BcAUX1* is a target of *P. indica* in Chinese cabbage. However, as expected, exogenous application of auxin could not replace *P. indica*. Thus, several questions arise: (a) What is the origin of auxin in this symbiosis? (b) How does the fungus manipulate the auxin homeostasis in Chinese cabbage roots to induce the growth response? (c) Since we did not observe such a dominant role of auxin in the *P. indica*/*Arabidopsis* symbiosis (Vadassery et al. 2008), what is the difference between the two hosts? (d) Since colonized roots of both plant species are bigger and contain longer roots and root hairs, is the beneficial effect of the plant simply caused by better excess of larger roots to nutrients in the medium/soil?

8.3 Origin of Auxin

P. indica-colonized Chinese cabbage roots contain approximately twice as much auxin as the uncolonized control roots, while the auxin level in the leaves is not affected by the fungus (Lee et al. 2011b). Several lines of evidence indicate that the higher auxin level in the roots is not of fungal origin. First, the bushy root hair phenotype and the longer primary and secondary roots could not be obtained when auxin was applied exogenously to the seedlings. Second, growth promotion of Chinese cabbage roots was also obtained by a preparation of a cell wall-derived fraction from the fungus, which does not contain auxin. This indicates that the fungus activates growth-promoting programs in the roots, which include auxin biosynthesis of plant origin. Third, we have previously demonstrated that *P. indica*-colonized *Arabidopsis thaliana* roots do not contain elevated auxin levels, although their growth is promoted by the fungus. We grew seedlings of both species together with the fungus in the same Petri dish and could confirm that only the roots of Chinese cabbage seedlings contain more auxin (Lee et al. 2011b). Based on these observations, we conclude that the higher auxin level in colonized Chinese cabbage roots is most likely of plant origin. Apparently, *P. indica*-induced growth promotion of Chinese cabbage seedlings is caused by fungal interference with the plant auxin biosynthesis and homeostasis. The difference in the regulation in Chinese cabbage to the regulation in *Arabidopsis* roots must be caused by differences in developmental programs of the two species: *P. indica*-induced growth promotion in *Arabidopsis* is not or less dependent on an overall increase in the auxin level in the root (cf. below).

8.4 Auxin Targets in Chinese Cabbage Roots

The identified auxin-related genes, which are upregulated in colonized Chinese cabbage roots (Lee et al. 2011b), allow conclusions of how *P. indica* induces growth in Chinese cabbage (Fig. 8.1). Upregulation of *TIR1*, the gene for the auxin receptor, is consistent with the idea that the roots are more sensitive to the phytohormone. Stimulation of auxin-induced genes requires the activation of the Aux/IAA degradation machinery, and many components involved in this process have been identified in the differential display analysis (e.g., UBC10, 26S proteasome subunit 4; Lee et al. 2011b). Furthermore, upregulation of the *AUX1* and *BIG* mRNA levels (2 genes for auxin transporters) implies that the fungal interaction results in a more efficient influx of auxin into the root cell. The *PIN3* mRNA level for an auxin efflux regulator is downregulated by the fungus, suggesting that auxin release from the cell is reduced. Auxin responses during plant/microbe interactions are dynamic; therefore, root-associated microbes can actively modify the host's auxin transport (cf. Grunewald et al. 2009a, b). Finally, several isolated cDNA fragments code for components involved in cell wall

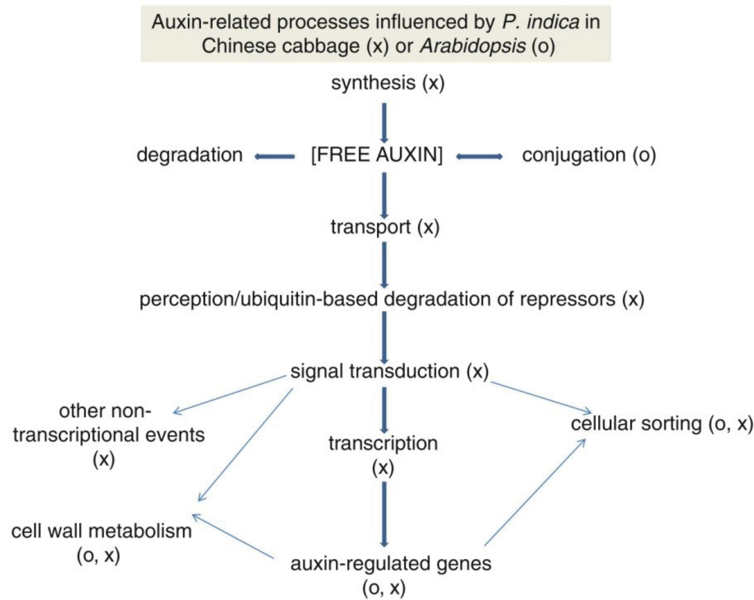

Fig. 8.1 Auxin targets of *P. indica*

acidification. This requires the stimulation of the export machinery for cell wall loosening proteins into the apoplast through the sorting processes in the endoplasmic reticulum. Exocytosis-related proteins in the endoplasmic reticulum are important targets of the fungus in *Arabidopsis* roots (Peškan-Berghöfer et al. 2004). Currently we are characterizing four *P. indica*-responsive genes in Chinese cabbage, which control exocytosis of proteins during endoplasmic reticulum/Golgi passage. Another complex aspect during auxin-controlled growth regulation is connected to the cell volume, which increases by promoting water and ion uptake and by rearranging the cytoskeleton. That both processes are targeted by *P. indica* is supported by the identified cDNA fragments in the screen (cf. Lee et al. 2011b, and unpublished data). In total, the differential expression of these auxin-related genes demonstrates that this phytohormone plays a crucial role in *P. indica*-mediated growth promotion and alteration of root morphology in Chinese cabbage. The bushy root hair phenotype of *P. indica*-colonized Chinese cabbage roots is important for improving the acquisition to water and minerals.

The identified auxin-related target genes/proteins of *P. indica* are upregulated locally, in an organ- or even cell-specific manner. It is important to understand how the fungus can manipulate auxin homeostasis and regulate auxin maxima in a controlled manner. The Chinese cabbage/*P. indica* symbiosis provides us a tool to study this scenario, to identify the underlying mechanism, and to use these tools to manipulate plants for biotechnological applications.

8.5 An Excursion to the *P. indica* Symbiosis with *Arabidopsis* Roots

Large-scale microarray analysis of *Arabidopsis* roots, colonized by *P. indica*, did not reveal many auxin-related genes as a target of the fungus. Expression of *DR5* promoter::ß-glucuronidase gene fusions, which are upregulated by auxin, is not significantly affected by the fungus (Vadassery et al. 2008). Mutants with reduced auxin levels (*ilr1-1*, *nit1-3*, *tfl2*, *cyp79 b2b3*) responded to *P. indica*, which indicated that severe alterations in auxin homeostasis in *Arabidopsis* do not prevent the growth response to *P. indica* (Vadassery et al. 2008). Auxin-related genes which are upregulated in *P. indica*-colonized Chinese cabbage roots are not upregulated in *P. indica*-colonized *Arabidopsis* roots, although growth of both species is promoted by the fungus. However, we obtained clear evidence of the role of auxin in the *P. indica*/*Arabidopsis* symbiosis, by analyzing the *sur1-1* mutant.

sur1-1 lacks a P450-dependent monooxygenase, which catalyzes the N-oxidation of indole-3-acetaldoxime and directs the pathway to indole glucosinolates. The mutant accumulates indole-3-acetaldoxime that is converted to auxin. Thus, *sur1-1* can be considered as an auxin overproducer (Boerjan et al. 1995; Mikkelsen et al. 2004); the seedlings are much smaller than the wild type, and their root length is approximately half the length of the wild type. Growth of *sur1-1* seedlings is strongly stimulated by *P. indica*, and colonized *sur1-1* seedlings are almost as big as wild-type seedlings cocultivated with the fungus. The free auxin level in *sur1-1* roots is reduced in *P. indica*-colonized seedlings, while the conjugated auxin level increases (Vadassery et al. 2008). The complete rescue of the dwarf phenotype of colonized *sur1* seedlings indicates that the fungus interferes with the auxin homeostasis by converting excess auxin into inactive conjugates. Thus, control of local-free auxin levels may be an important target of the fungus in *Arabidopsis*.

8.6 *P. indica*-Mediated Growth Promotion is Independent of the Root Architecture in *Arabidopsis*

Larger roots with numerous secondary roots and root hairs allow better access to water and nutrients in the medium/soil. In nature, it is likely that this contributes substantially to plant performance. However, the question arises whether this is the only reason for better performance of *P. indica*-colonized plants. Since the root architecture is strongly influenced by auxin and cytokinin, we analyzed the response of *Arabidopsis* mutants to *P. indica* with altered root architectures or root/shoot biomass ratios. Interestingly, we noted that the growth response to *P. indica* is not related of the architecture and size of the roots in *Arabidopsis*. For instance, the *ahk2 ahk3* seedlings have long roots relative to the aerial parts (Riefler et al. 2006), the *35S::CKX1* and *35S::CKX2* lines have stunted and bushy

roots (Werner et al. 2003), and the *sur1-1* roots are approximately half as long as the wild type. *tfl2*, in addition to its dwarf phenotype, has fewer lateral roots and shorter root hairs and a greatly reduced root surface (Bennett et al. 2005). All of these mutants respond to *P. indica* suggesting that the root size and architecture have little effect on the growth response induced by the fungus. Furthermore, root hair development is controlled by ROOT HAIR DEFECTIVE2 (RHD2), a plasma membrane localized NADP oxidase (Foreman et al. 2003). *rhd2* mutants have shorter root hairs, but their response to *P. indica* was comparable to the wild type. The *incomplete root hair elongation* (*ire*) mutant is defective in an AGC kinase and exhibits also a short root hair phenotype (Oyama et al. 2002); however, its response to the fungus is also comparable to the wild type (Camehl et al. 2011). Thus, also *Arabidopsis* mutants in which the root architecture is altered by lesions not directly related to phytohormones respond to the *P. indica*. Consequently, better performance of plants in the presence of the fungus is not exclusively caused by the stimulation of root growth.

8.7 Conclusions

We introduced Chinese cabbage as a novel host for *P. indica*. This agriculturally important crop shows a quite different developmental strategy in response to *P. indica* when compared to the model plant *Arabidopsis*. The breeding strategies for Chinese cabbage over the last 200 years are based on the generation of varieties with increased biomass. *Arabidopsis* plants were not exposed to such a selective pressure. Since growth stimulation is most efficiently mediated by manipulating auxin homeostasis, it is reasonable that this phytohormone plays a more dominant role in the symbiosis with Chinese cabbage than in *Arabidopsis*. A comparative analysis of the two symbiotic systems will help to understand how *P. indica* manipulates plant growth programs.

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4.4 Manuscript IV

Balancing defense and growth - Analyses of the beneficial symbiosis between *Piriformospora indica* and *Arabidopsis thaliana*

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Symbiosis (2012), 58: 17-28

Symbiosis (2012) 58:17–28
DOI 10.1007/s13199-012-0209-8

Balancing defense and growth—Analyses of the beneficial symbiosis between *Piriformospora indica* and *Arabidopsis thaliana*

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Received: 16 August 2012 / Accepted: 3 December 2012 / Published online: 15 January 2013
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Abstract The mutualistic interaction between the endophytic and root-colonizing fungus *Piriformospora indica* and *Arabidopsis thaliana* is a nice model system to study beneficial and non-benefical traits in a symbiosis. Colonized Arabidopsis plants are taller, produce more seeds and are more resistant against biotic and abiotic stress. Based on genetic, molecular and cellular analyses, Arabidopsis mutants were identified which are impaired in their beneficial response to the fungus. Several mutants are smaller rather than bigger in the presence of the fungus and are defective in defense responses. This includes mutants with defects in defense-signaling components, defense proteins and enzymes, and defense metabolites. The mutants cannot control root colonization and are often over-colonized by *P. indica*. As a consequence, the benefits for the plants are lost and they try to restrict root colonization by activating unspecific defense responses against *P. indica*. These observations raise the question as to how the plants balance defense gene activation or development and what signaling molecules are involved. *P. indica* promotes the synthesis of phosphatidic acid (PA), which binds to the 3-PHOSPHOINOSITIDE-DEPENDENT-KINASE1 (PDK1). This activates a kinase pathway which might be crucial for balancing defense and growth responses. The review describes plant defense compounds which are necessary for the mutualistic interaction between the two symbionts. Furthermore, it is proposed that the PA/PDK1 pathway may be crucial for balancing defense responses and growth stimulation during the interaction with *P. indica*.

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Keywords Growth · Defense · *Piriformospora indica*

1 Introduction

Mutualistic interaction is a type of symbiosis in which two partners benefit from each other. Mycorrhizae are a classical example: the fungus delivers soil nutrients to the plant and the plant supplies the fungus with carbon compounds. We studied the mutualistic interaction between a root colonizing endophyte, *Piriformospora indica*, and the model plant *Arabidopsis thaliana* (cf. Johnson and Oelmüller 2009). *P. indica*, a cultivable basidiomycete of Sebacinales, colonizes the roots of many plant species including Arabidopsis (Peškan-Berghöfer et al. 2004; Oelmüller et al. 2009; Qiang et al. 2012; Reitz et al. 2012; Lahrmann and Zuccaro 2012). Like other members of Sebacinales, *P. indica* is found worldwide in association with roots (Selosse et al. 2009) and stimulates growth, biomass and seed production of the hosts (Peskan-Berghöfer et al. 2004; Oelmüller et al. 2009; Shahollari et al. 2007; Sherameti et al. 2005, 2008a and b; Vadassery et al. 2009a and b; Waller et al. 2005; Zuccaro et al. 2011). The fungus promotes nitrate and phosphate uptake and metabolism (Sherameti et al. 2005; Shahollari et al. 2004; Yadav et al. 2010). *P. indica* also confers resistance against abiotic (Sherameti et al. 2008a; Baltruschat et al. 2008; Sun et al. 2010) and biotic stress (Oelmüller et al. 2009; Stein et al. 2008). The broad host range of *P. indica* indicates that the beneficial interaction may be based on general recognition and signaling pathways. Enhanced plant growth can be induced by an fungal exudate component (Vadassery et al. 2009a), suggesting the involvement of specific receptors at the plant cell surface. In support of this hypothesis, an atypical receptor kinase with leucine-rich repeats was identified as being required for the growth response in Arabidopsis (Shahollari et al. 2007).

Moreover, a rapid increase in the intracellular calcium concentration in the root cells indicates that an intracellular signaling cascade is triggered early upon plant-fungal interaction (Vadassery et al. 2009a).

Here, one class of mutants is described, for which the interaction is no longer beneficial for the plant. While growth and performance of wild-type plants is promoted by the fungus, colonized mutants are smaller in the presence of the fungus. They produce less seeds and biomass and normally grow slower. These mutants have defects in different and unrelated defense responses, i.e. either in signaling molecules or transcription factors which activate defense genes, or in genes for enzymes which are required for the synthesis of defense compounds. All these mutants have in common that they cannot control root colonization by *P. indica*. Their roots are overcolonized, consequently, the plants show stress symptoms and express stress-related genes. The overcolonized roots try to restrict root colonization by upregulating defense genes which are not impaired by the mutations. Thus, a mild and constitutive defense response is required for establishing or maintaining a beneficial symbiosis between the two partners. Interestingly, the mutated genes code for enzymes involved in quite different and unrelated defense processes. How do the plants balance defense gene activation and development, and how do they distinguish between friends and foes (cf. Johnson and Oelmüller 2009; Paszkowski 2006; Kogel et al. 2006; Tunlid and Talbot 2002)? A model is proposed that describes a balanced activation of defense and growth / development depending on the environment.

2 Ethylene signaling is required for the beneficial interaction between *P. indica* and Arabidopsis

Mutants defective in the ethylene signaling components ETR1 and EIN2 and the ethylene-targeted transcription factors EIN3/EIL1 are unable to establish a beneficial interaction with *P. indica* (Camehl and Oelmüller 2010). Ethylene is perceived by a family of endoplasmatic reticulum-associated two component kinases, one of them is ETR1. The hormone binds to this receptor via a copper co-factor, which results in the inactivation of the receptor function (Hua and Meyerowitz 1998).

ETR1, EIN2 and EIN3/EIL1 are required for *P. indica*-mediated growth promotion of Arabidopsis seedlings (Camehl et al. 2010). Growth promotion by *P. indica* of the corresponding single (*etr1*, *ein2*) and double (*ein3 eil1*) knock-out lines is impaired. Therefore, these ethylene-related genes participate in balancing beneficial and non-beneficial traits in the symbiosis. The signaling compounds are also required for restricting growth of the fungus in the roots, by activating defense genes and other defense responses. The mutant roots are overcolonized which is harmful for the plants. This hypothesis is further supported by the observation that ERF1 over-expressors, which show constitutively activated defense responses, are less colonized. Apparently, manipulation of ethylene-induced defense responses has a strong influence on the degree of root colonization, which in turn determines whether the symbiotic interaction is beneficial or harmful. The fungus does not induce these ethylene-dependent signaling compounds at the transcriptional level, as observed after pathogen infections. It appears that the available amount of these signaling components is sufficient to establish a mild defense response for the restriction of root colonization.

A. thaliana contains 147 ERF (ethylene-responsive element-binding factor) transcription factors with mostly uncharacterized functions. Two of them, ERF9 and ERF14 have been investigated in more details because their mRNA levels are upregulated during early phases of the symbiotic interaction between *P. indica* and Arabidopsis roots. Insertional inactivation of the two genes *ERF9* and *ERF14* has a negative effect on the beneficial interaction between the two symbionts. The mutants are diminished in *P. indica*-induced growth promotion and activate the expression of the *PATHOGENESIS-RELATED1* and −*2* genes. This and additional observation (Camehl and Oelmüller 2010) led to the conclusion that ERF9 and ERF14 represses *PR* gene expression in colonized Arabidopsis roots and that this contributes to the establishment of the beneficial interaction.

Taken together, ethylene signaling components and ethylene-targeted transcription factors are required for restriction of root colonization in wild-type seedlings and adult plants. Since ERF transcription factors can function as transcriptional activators and repressors, they are candidates for establishing a balanced defense response to the fungus without preventing growth and development.

3 WRKY transcription factors are targets of *P. indica* in Arabidopsis roots and leaves

The WRKY transcription factor family plays an important role in the regulation of transcriptional reprogramming of the plants in response to abiotic (Chen et al. 2012) and biotic (Pandey and Somssich 2009) stress. They are involved in various aspects of plant/microbe interactions and plant immunity (Pandey and Somssich 2009). This huge gene family forms a regulatory network, in which the individual members participate in quite different stress responses. In a similar way to the ERFs, they function as positive and negative regulators of gene expression and form complex protein-protein interactions. They interact with MAP kinases, MAP kinase kinases, 14-3-3 proteins, calmodulin, histone deacetylases, resistance proteins and other WRKY transcription factors (Rushton et al. 2010). Most of the studies to date have been performed with leaf tissue, while the role of WRKYs in the roots has been less investigated. WRKY transcription factors also play a central role in controlling leaf senescence in Arabidopsis. One member of this family,

WRKY53, is tightly regulated by unexpected mechanisms and is a convergence node between senescence and biotic and abiotic stress responses (Zentgraf et al. 2010). Interestingly, the *WRKY53* mRNA level is strongly regulated by *P. indica* in Arabidopsis roots (Table 1). As in ERFs, the WRKYs provide another example of a transcription factor family that can integrate diverse internal and environmental signals which allows a rapid and dynamic response to changing environmental conditions. Table 1 presents a summary of the regulation of *WRKY* transcription factor genes in the roots of Arabidopsis seedlings after 2 and 6 days of co-cultivation with *P. indica*. The relatively large number of *WRKY* genes which are differentially regulated in Arabidopsis roots after co-cultivation with *P. indica* suggests that they play a crucial role in the symbiosis. The role of these transcription factor genes in the symbiotic interaction is currently under study.

4 *Cerk* mutants

A fast method for testing root colonization was set up, which allows also the quantification of root colonization (in contrast to methods described previously; cf. McGonigle et al. 1990). The seedlings were kept on PNM medium (Johnson et al. 2011) in the presence of *P. indica* for 14 days. The roots were removed and stained on a glass slide with 100 µl Nile red stain solution (0.005 % Nile red in 75 % glycerol) for 10 min. Microscopy was performed with a Zeiss Oxiovert 135 instrument under the fluorescent channel at 450–520 nm. This staining method results in a high contrast between plant tissue and fungal spores (Figs. 1 and 2) and hyphae (Fig. 2c, d). They can be easily visualized and quantified with the Adobe PhotoshopTM software, by counting pixel ratios. The amount of fungal material can be related to the root area (Fig. 1a, b) or to the root length (Fig. 1c, d). The distribution of fungal material in the entire root is analysed at lower microscopic resolution. Representative sections from different regions of the roots were then analysed in more details to obtain quantitative data. Root colonization is subsequently confirmed by molecular markers, by which the *P. indica TRANSLATION ELONGATION FACTOR1* mRNA or DNA levels are expressed relative to the amount of the plant *GAPC2* mRNA or DNA levels (Bütehorn et al. 2000; Camehl et al. 2011). Although we have not observed many differences between the staining methods and the molecular method, the staining method is faster and allows the localization of the spores and hyphae in the root.

Table 1 Regulation of *WRKY* genes in the roots of Arabidopsis seedlings co-cultivated by *P. indica* for 2 or 6 days on agar plates (cf. Johnson et al. 2011). Based on 3 independent microarray analyses, the values represent fold induction relative to the mock-treated control and are average values of the three hybridizations. The list of the WRKY family members was taken from http://www.arabidopsis.org/browse/genefamily/WRKY-Som.jsp. Only those genes are shown which are regulated > 2-fold at one time point

Protein Name	Genome Locus	TIGR annotation	2 days co-cultivation with *P. indica*	6 days co-cultivation with *P. indica*
Group I				
WRKY25	At2g30250	putative WRKY-type DNA binding protein	2.2 (± 0,31)**	1.7 (± 0,29)**
WRKY33	At2g38470	putative WRKY-type DNA binding protein	5.9 (± 0,98)**	2.9 (± 0,37)**
WRKY45	At3g01970	putative WRKY-like transcriptional regulator protein	4.8 (± 0,88)**	1.2 (± 0,26)
Group II-a				
WRKY40	At1g80840	transcription factor, putative	4.5 (± 0,91)**	0.9 (± 0,15)
WRKY60	At2g25000	putative WRKY-type DNA binding protein	0.4 (± 0,10)**	1.1 (± 0,14)
WRKY6	At1g62300	unknown protein	4.4 (± 0,79)**	1.0 (± 0,19)
Group II-b				
WRKY9	At1g68150	putative DNA binding protein	0.4 (± 0,33)**	1.0 (± 0,17)
WRKY31	At4g22070	putative protein	3.3 (± 0,61)**	2.4 (± 0,42)
WRKY61	At1g18860	hypothetical protein	2.6 (± 0,52)**	1.6 (± 0,33)
Group II-e				
WRKY14	At1g30650	putative DNA-binding protein	0.5 (± 0,11)	1.1 (± 0,23)
Group III				
WRKY38	At5g22570	putative protein	2.0 (± 0,43)	4.1 (± 0,55)**
WRKY53	At4g23810	putative protein	5.3 (± 1,02)**	2.0 (± 0,44)**
WRKY54	At2g40750	hypothetical protein	4.0 (± 0,79)**	5.1 (± 0,96)**
WRKY70	At3g56400	DNA-binding protein-like	4.2 (± 0,80)**	5.0 (± 1,16)**

Errors were calculated as standard errors. Relative errors of the proportion are the sum of the individual relative errors. **, significantly different from the uncolonized control ($p<0.05$)

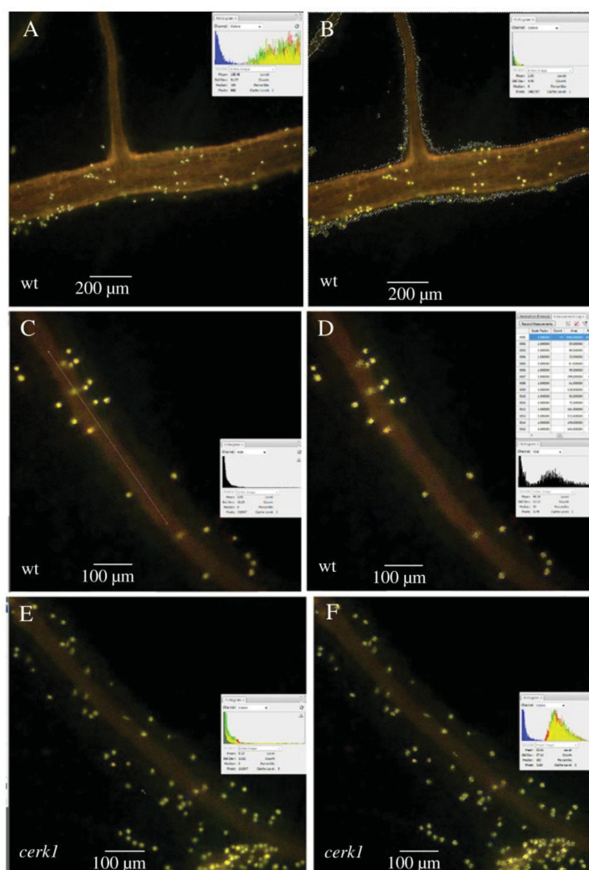

Fig. 1 Root colonization of wild-type (**a**, **b**, **c**, **d**) and *cerk1* (**e**, **f**) seedling grown on PNM media for 2 weeks. Root area was measured by Magic wand tool adjusted with tolerance of 32 for each sample (**b**). The colonization pixels were selected by Adobe Photoshop CS5 Magic wand tool adjusted with tolerance of 50. The signals of the selected pixels were quantified by the Histogram tool (**a**, **d**, **f**). The length of root was measured with the Photoshop Ruler Tool for each sample (**c**). Number of selected spores is available in Measurement Log window (**d**). Spore/root area ratio was calculated on the basis of the whole root. Only wild type and mutant roots of equal size were considered. Root colonization was calculated on the basis of the spore selected pixels relative to root area as [selected pixel/root area]×1000 and root length as [selected pixel/root length]

Using these methods CERK1 was identified as an important component for the beneficial interaction between the two symbionts. CERK1 is activated by chitin, which is the main component of the cell walls of beneficial and pathogenic fungi. Chitin fragments are recognized by plant lysin motif (LysM)-containing proteins, which, in case of pathogens, activate signaling events leading to innate immunity. In Arabidopsis; CERK1 is one of the first LysM-containing receptor-like kinase 1 (LYK1) which was identified as a chitin recognizing protein (Miya et al. 2007; Wan et al. 2008). In rice (*Oryza sativa*) the LysM-containing protein "chitin elicitor binding protein" (CEBiP) is involved in chitin recognition (Kaku et al. 2006). Arabidopsis possesses three *CEBiP*-like genes and five *LYK* genes. Inactivation of *CERK1* results in a reduced induction of chitin-responsive genes (cf. Wan et al. 2012 and references therein for the original publications). *cerk1* is overcolonized by *P. indica*, which has also been demonstrated by Jacobs et al. (2011). Under the assumption that CERK1 in the beneficial *P. indica*/Arabidopsis interaction has a similar function to that in pathogenic interactions, chitin or related compounds from *P. indica* should activate CERK1-dependent defense processes in Arabidopsis roots. *P. indica* might induce a mild defense response via CERK1 activation and this represents an additional facet in the restriction of root colonization.

Microarray analyses suggest that *CERK* and *CEBiP*-like genes are barely regulated by *P. indica* (Table 2). The strongest response was shown for *CERK4*. Therefore, besides CERK1, CERK4 might also be involved in restricting root colonization in Arabidopsis roots (cf. Wan et al. 2012).

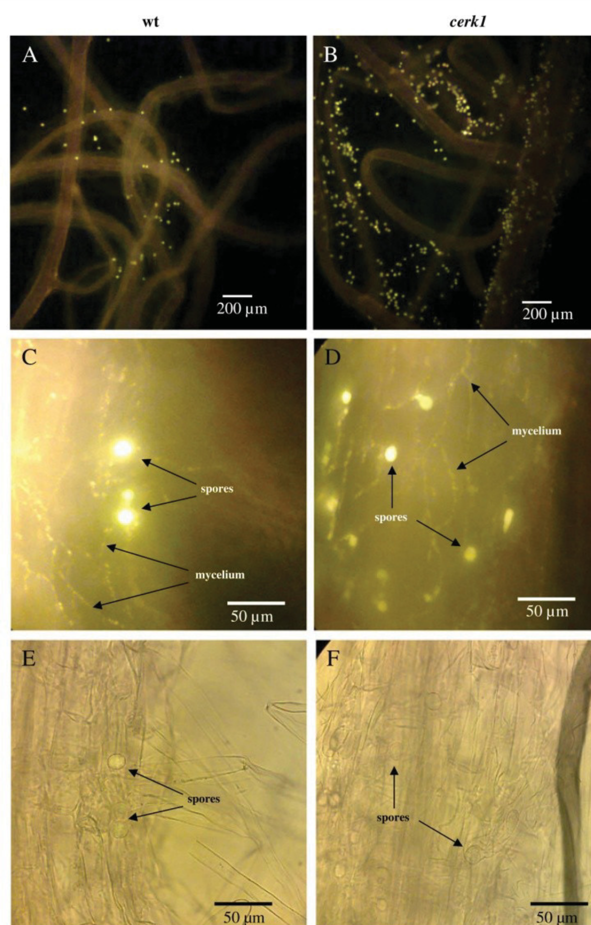

Fig. 2 Root colonization of Arabidopsis wild-type (*left*) and *cerk1* line (*right*). The fungus was stained with Nile red and monitored under the fluorescent channel at 450–520 nm (**a**–**d**) and under visual light (**e**, **f**). Comparison of fluorescent and visual light microscopy in discrimination of spores and mycelium in wt (**c**, **e**) and *cerk1* roots (**d**, **f**)

5 Glucosinolates and enzymes of the glucosinolate metabolism are required to establish or maintain a mutualistic interaction between *P. indica* and Arabidopsis

Members of the order Brassicales synthesize important secondary metabolites such as glucosinolates from tryptophan and methionine. This group of compounds with over 120 different identified chemical structures (Fahey et al. 2001; Sønderby et al. 2010; Janowitz et al. 2009; Piotrowski 2008) and their degradation products provide protection against insect heribivory (McCloskey and Isman 1993; Giamoustaris and Mithen 1995; Müller et al. 2010). The constitutive production of phytoanticipins or phytoalexin is important for plant defense against microbes (Hammerschmidt 1999; Pedras et al. 2007; Bednarek and Osbourn 2009). Upon attack by necrotrophic fungi, Arabidopsis induces the synthesis of the phytoalexin camalexin (Schuhegger et al. 2006; Ferrari et al. 2003). CYP79B2 and CYP79B3 are two functionally redundant cytochrome P450 enzymes which convert tryptophan into indole-3-acetaldoxime (IAOx). This is an intermediate for the biosynthesis of indole glucosinolates (I-GLS), camalexin, other indole compounds such as indole acetonitrile, indole carboxylic acid derivatives, and, under specific conditions, the plant hormone indole-3-acetic acid (IAA). The double *cyp79B2 cyp79B3* mutant lacks I-GLS (Zhao et al. 2002)

Table 2 Fold-induction of the mRNA level for CEBiP and CERK proteins in colonized Arabidopsis roots relative to the mock-treated uncolonized control. Co-cultivation with *P. indica* was performed for 2 or 6 days. Based on 3 independent microarray analyses, the data are averages of the three experiments

Protein Name	Genome Locus	2 days co-cultivation	6 days co-cultivation
CEBiP-like1	At2g17170	n.d.	n.d.
CEBiP-like2	At1g21880	1.01 (± 0,18)	1.31 (± 0,20)
CEBiP-like3	At1g77630	0.96 (± 0,16)	1.33 (± 0,17)
CERK1	At3g21630	1.32 (± 0,22)	1.22 (± 0,20)
CERK2	At3g01840	n.d.	n.d.
CERK3	At1g51940	0.87 (± 0,11)	0.65 (± 0,09)
CERK4	At2g23770	1.94 (± 0,23)	2.22 (± 0,29)
CERK5	At2g33580	0.89 (± 0,13)	1.22 (± 0,21)

Errors were calculated as standard errors. Relative errors of the proportion are the sum of the individual relative errors. Only the CERK4 values 2 and 6 days after co-cultivation are significantly different from the uncolonized control ($p<0.05$). *n.d.*, not detectable

and is unable to induce camalexin synthesis (Glawischnig et al. 2004). Furthermore, it does not accumulate indole-3-carboxylic acid derivatives (Böttcher et al. 2009), i.e. secondary metabolites which are strongly induced by pathogen infections. *P. indica* colonization causes severe growth defects on agar plate-grown *cyp79B2 cyp79B3* seedlings as well as adult plants in soil (Nongbri et al. 2012). This demonstrates that IAOx-derived compounds are essential in the beneficial interaction between Arabidopsis and *P. indica*. PAD3, the last enzyme of camalexin biosynthetic pathway is regulated by a variety of signaling components such as the mitogen-activated protein kinases (MPK) MPK3, MPK6 (Ren et al. 2008) and MPK4 (Qiu et al. 2008). Co-cultivation of Arabidopsis seedlings with *P. indica* on agar plates induced significantly higher levels of camalexin in the roots compared to mock-treated controls (Nongbri et al. 2012). The mRNA levels for CYP79B2, CYP79B3, CYP71A13 (Nafisi et al. 2007), PAD3, and WRK33 (Qiu et al. 2008) are upregulated in colonized wild-type (WT) roots, whereas those for CYP83B1 and SUR1 are not (Nongbri et al. 2012). This demonstrates that the genes for the synthesis of IAOx-derived compounds, including camalexin but not I-GLS, are targets of signals from the fungus. In contrast to the *cyp79B2 cyp79B3* double mutant which is impaired in *P. indica*-mediated growth promotion at seedling and adult stage, the *pad3* mutant is not affected during the initial stage of interaction. However, since growth of adult *pad3* plants is not promoted by *P. indica*, camalexin plays an important role during long term interaction (Nongbri et al. 2012).

5.1 PEN2 (At2g44490)

Screening for Arabidopsis mutants deficient in resistance to barley powdery mildew identified *penetration* (*pen*) mutants. The *PEN2* gene encodes a glycosyl hydrolase which restricts pathogen entry of two powdery mildew fungi into Arabidopsis leaf cells (Lipka et al. 2005). PEN2 localizes to the peroxisomes and acts as a component of an inducible preinvasion resistance mechanism. The *pen3* plants permitted both increased invasion into epidermal cells and initiation of hyphae by *B. hordei*, suggesting that PEN3 contributes to defenses at the cell wall and intracellularly. PEN3 may be involved in exporting toxic materials to attempted invasion sites.

Microarray analysis with *P. indica*-colonized vs. uncolonized Arabidopsis roots demonstrated that all *PEN* genes are expressed in roots and slightly upregulated in response to *P. indica* (Table 3). The strongest response was observed for *PEN2*. A knock-out mutant (kindly obtained from Prof. Schulze-Lefert, MPI Cologne) for PEN2 also showed severe overcolonization of the roots and does not respond properly to the fungus (Seebald et al. unpublished). Similar results have been reported by Jacobs et al. (2011). This indicates that PEN2 participates in the restriction of root colonization and suggests that general mechanisms restrict colonization of plant cells, irrespective of whether they are colonized by pathogens or beneficial microbes. The role of PEN1 and PEN3 is currently under study, however their mRNA levels respond less to *P. indica* colonization in Arabidopsis roots when compared to that for PEN2 (Table 3).

5.2 Pyk10

PYK10 is an abundant protein in the roots of Brassicaceae. Although it appears to be a ß-glucosidases or myrosinases, an enzymatic activity for this protein has not yet been demonstrated. The role of PYK10 in beneficial and pathogenic plant/microbe interactions is not clear. In general, mycrosinases hydrolyze ß-glucosidic bonds of aryl ß-D-glucosides, as well as ß-glucosides with carbohydrate moieties such as cellobiose and other ß-linked oligosaccharides. In particular, the enzymes hydrolyze non-toxic glucosinolates to biologically

Table 3 Fold-induction of the mRNA level for PEN proteins in colonized Arabidopsis roots relative to the uncolonized control. Co-cultivation was performed for 2 or 6 days. Average values based on 3 independent microarray analyses. Errors were calculated as standard errors

Protein Name	Genome Locus	2 days co-cultivation	6 days co-cultivation
PEN1	At3g11820	1.44 (± 0,27)	1.15 (± 0,17)
PEN2	At2g44490	2.25 (± 0,31)	1.07 (± 0,19)
PEN3	At1g59870	1.16 (± 0,22)	1.13 (± 0,14)

Relative errors of the proportion are the sum of the individual relative errors. Only the PEN2 value 2 days after co-cultivation is significantly different from the uncolonized control ($p<0.05$)

active and toxic isothiocyanates, thiocyanates, nitriles and other epithio nitriles and it is believed that the biological function of the myrosinases depends on the nature of the aglycon moieties released from the substrates. To prevent the release of the toxic compounds, myrosinases are present in the endoplasmic reticulum. Release of the enzyme requires damage to the cell. This would mean that the symbiotic interaction between the two symbionts studied here results, at least in part, in cell damage. Alternatively, a minor fraction of the highly abundant protein might also be released from the endoplasmic reticulum due to naturally occurring cell death. This minor fraction of PYK10 might be sufficient to release toxic compounds from conjugates and therefore participates in restriction of root colonization. Since the substrate of PYK10 is not known at present, another explanation might be that the enzyme has an additional function in the cell or that the highly abundant protein catalyzes unspecific and unknown site reactions, which results in the generation of toxic compounds which restrict fungal growth and thus root colonization.

PYK10 is required for the beneficial interaction between Arabidopsis and *P. indica* (Sherameti et al. 2008b). Insertional inactivation of *PYK10* in Arabidopsis results in the loss of the benefits for the plants when the roots are colonized by *P. indica*: growth promotion is no longer visible and for adult plants, the seed production is not enhanced (Sherameti et al. 2008b). Expression of *PYK10* is controlled by the helix-loop-helix containing transcription factor NAI1 and inactivation of this transcription factor gene results in a severe reduction of *PYK10* gene expression. The *nai1* mutant behaves like the *pyk10* mutant in response to the fungus, which confirms the essential role of the myrosinase for the beneficial interaction. Closer inspection of the roots showed that the degree of colonization is significantly higher compared to the wild-type control. This suggests that PYK10 participates in the restriction of root colonization. Like in other mutants, overcolonization of the roots results in a mild activation of defense genes. In particular *PDF1.2* is a very sensitive defense marker gene which is rapidly upregulated when the mutualistic interaction is no longer balanced. In the overcolonized *pyk10* mutant, *PDF1.2* is strongly upregulated (Sherameti et al. 2008b).

PYK10 shares sequence similarities with other family members. One of them is PEN2. Like PEN2, PYK10 belongs to the class of glycosyl hydrolase family 1, both proteins are located in intracellular organellar structures (PYK10 in ER bodies and PEN2 in peroxisomes), and both proteins share a high degree of sequence similarity. The catalytic domains of both proteins contain two conserved nucleophilic glutamates. Lipka et al. (2005) have shown that glutamate[183] is required for PEN2 *function* in vivo, which suggests that PEN2 catalytic activity is required for restricting pathogen entry. Thus, PYK10 might have a similar biological function in our system.

The beneficial traits in the *P. indica*/Araidopsis symbiosis are highly dependent on the density of the hyphae in and around the root (Camehl et al. 2011). Increasing quantities of hyphae resulted in a suboptimal interaction. Furthermore, marker genes for the beneficial interaction were downregulated and those for defense processes, such as *PDF1.2*, were upregulated in the roots in a dose-dependent manner (Oelmüller et al. 2009). Similar response patterns were observed for *PYK10* overexpressor and knockout lines (Sherameti et al. 2008b). In order to maintain a mutualistic interaction with benefits for both partners, the degree of root colonization might be controlled by activating PYK10-dependent defense responses, when too many hyphae colonize the roots and the cells become damaged or wounded by hyphal penetration. In barley, for instance, less-defended root cells undergo cell death after colonization with *P. indica* (Deshmukh et al. 2006).

Figures 3 and 4 summarizes identified plant defense responses which are required for the restriction of Arabidopsis root colonization by *P. indica*. These compounds are involved in signal perception (ETR1, CERK1), in plant signal transduction processes such as the MAPKs, in transcriptional activation such as the ethylene transcription factor members EIN3 and EIL1, ERF1, -9 and -14, as well as members of the WRKY family, defense proteins (PEN2 and probably PYK10), as well as defense metabolites (such as camalexin).

6 Induced systemic resistance: beneficial root-colonizing microbes protect the leaves against pathogens

Induced systemic resistance (ISR) is mediated by beneficial soil-borne microorganisms, such as plant growth promoting rhizobacteria, mycorrhizal fungi or beneficial endophytes. They improve plant performance by inducing systemic defense responses that confer broad-spectrum resistance to plant pathogens and even insect herbivores (van Wees et al. 2008). Different beneficial microbe-associated molecular patterns (MAMPs) are recognized by the plant, which results in a mild, but effective activation of the plant's immune responses in systemic tissues. Systemic resistance induced by different beneficial microbes is regulated by jasmonate-dependent and ethylene-dependent signaling pathways and is associated with priming for enhanced defense (van Wees et al. 2008). A large body of evidence for such a regulatory circuit is described in the literature.

When roots of Arabidopsis seedlings are colonized by *P. indica*, the leaves are much more resistance to *Alternaria brassicae* infections compared with the uncolonized control. This clearly demonstrates root to shoot signaling induced by *P. indica* (cf. also Stein et al. 2006) Several ethylene and jasmonic acid signaling mutants were tested, but the protective function of *P. indica* against *A. brassicae* infection was still evident with these mutants. Therefore, ethylene and jasmonic acid signaling play no or only a minor role in *P. indica*-ISR against *A. brassicae*. However, when the *monodehydroascorbate reductase2* (*mdar2*; SALK_0776335C) and *dehydroascorbate*

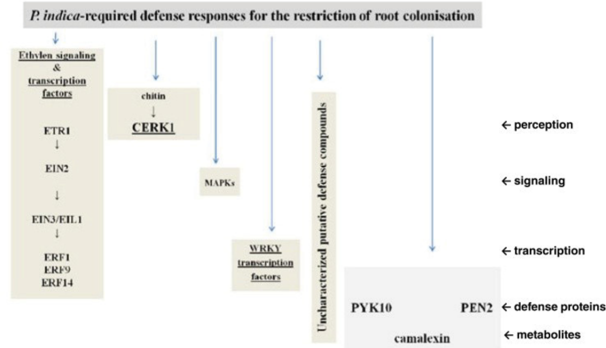

Fig. 3 Defense response components required for restricting Arabidopsis root colonization by *P. indica*. The components are involved in perception, signaling and transcription, or represent defense proteins or secondary metabolites

reductase5 (*dhar5*; SALK_029966C) T-DNA insertion lines (Vadassery et al. 2009c) were studied in the resistance response, the ISR response against *A. brassicae* was lost. MDAR and DHAR are two enzymes of the ascorbate-glutathione cycle that maintain ascorbate in its reduced state. *MDAR2* (At3g09940) and *DHAR5* (At1g19570) expression was upregulated in the roots and shoots of Arabidopsis seedlings co-cultivated with *P. indica* (Vadassery et al. 2009c). It appears that *P. indica* establishes a reduced atmosphere in the roots and leaves which contributes substantially to the ISR response against *A. brassicae* infections in leaves.

7 Novel compounds involved in *P. indica*/plant symbioses

Novel genes/proteins which are required for the restriction of root colonization were also identified. One of these proteins is At2g40000, called HSPRO [an ORTHOLOG OF SUGAR BEET Hs1(pro-1)]. The role of this protein in Arabidopsis is not clear, but recent studies with *Nicotiana attenuata* have shown that HSPRO controls early seedling growth during interaction with *P. indica* (Schuck et al. 2012). *HSPRO* expression was induced during herbivory, when leaves were inoculated with *Pseudomonas syringae* pv tomato DC3000 and roots with *P. indica*. Reduced *HSPRO* expression positively influenced early seedling growth during interaction with *P. indica*; fungus-colonized seedlings with reduced *HSPRO* expression increased their fresh biomass by 30 % compared to the wild type. Grafting experiments demonstrated that reduced *HSPRO* expression in roots was sufficient to induce differential growth promotion in both roots and shoots. This effect was accompanied by changes in the expression of 417 genes in colonized roots, most of which were metabolic genes. The lack of major differences in the metabolic profiles suggested that accelerated metabolic rates were involved. Therefore, HSPRO participates in a whole-plant change in growth physiology when seedlings interact with *P. indica* (Schuck et al. 2012). It would be interesting to see whether the Arabidopsis homolog has a similar function, and whether HSPRO couples growth and defense responses to the metabolic state of the plant.

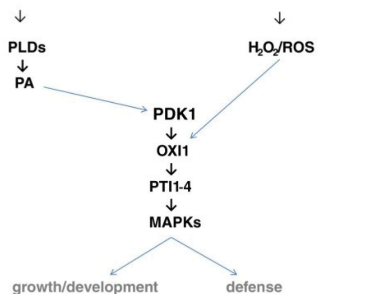

Fig. 4 A model describing the PDK1/OXI1 pathway and its potential involvement in balancing growth/development and defense responses

8 Balancing defense and growth: role of 3-PHOSPHOINOSITIDE-DEPENDENT-KINASE1 (PDK1) and OXIDATIVE-SIGNAL-INDUCIBLE1 (OXI1) in the symbiotic interaction

In natural environments plants either put their energy into growth and development or defense against enemies or pathogens. In a friendly environment, most of the energy is put into growth, and the synthesis of constitutive defense compounds ensures that the plants are protected against mild pathogen attacks. As soon as the plants are exposed to severe attacks by microbes, nematodes, herbivores, etc., the metabolism has to be readjusted or be reprogrammed to

activate induced defense responses. How does the plant balance defense and growth responses?

The roots have to monitor the microbial community in the rhizosphere continuously to establish an appropriate response and to integrate the incoming information from beneficial and pathogenic microbes. Thus, the roots have to identify whether an interacting microbe is a friend or a foe. Mycorrhizal fungi such as *Glomus intraradices* secrete symbiotic signals that are a mixture of sulphated and non-sulphated simple lipochitooligosaccharides (LCOs), which stimulate formation of arbuscular mycorrhizal fungi in plant species of diverse families (Fabaceae, Asteraceae and Umbelliferae) (Maillet et al. 2011). Studies on mycorrhiza have demonstrated that initially, the plant activates a mild defense response against the fungus, before a mutualistic interaction will be established and the microbe is accepted as a friend. Which kind of signals trigger this change is as yet unknown, but it has been proposed that the establishment of a mutualistic interaction starts with the exchange of nutrients between the two symbionts (Harrison 1999). Studies with the Arabidopsis/*P. indica* symbiosis suggest that the PDK1/OXI1 pathway plays a crucial role in this scenario (Camehl et al. 2011; Hirt et al. 2011).

An important second messenger in plant signaling is phosphatidic acid (PA) which can be synthesized either by phospholipase D (Li et al. 2009) or by a phospholipase C pathway which generates diacylglycerol that is phosphorylated to PA via diacylglycerol kinase (Arisz et al. 2009). Both lipases are activated in response to many biotic and abiotic stress signals (Li et al. 2009; Arisz et al. 2009). Although the beneficial fungus *P. indica* stimulates PA synthesis, this does not lead to defense gene activation, but the promotion of growth and plant performance (Camehl et al. 2011; Hirt et al. 2011). Therefore, the PA/PDK1/OXI1 pathway may integrate various external signals in plants to coordinate appropriate downstream responses, such as defense against pathogens and a mutualistic interaction with beneficial microbes. PA binds to PDK1 (Deak et al. 1999). In mammalian systems PDK1 is a master kinase, and more than 100.000 publications have shown that this kinase plays essential roles in cell growth, proliferation, survival, metabolism and apoptosis. Both mammalian and plant PDK1 phosphorylates and thus activates the cAMP-dependent protein kinase A/cGMP-dependent protein kinase G/protein kinase C (AGC) kinases in response to rises in the levels of signaling lipids (Bayascas 2010; Mora et al. 2004). In plants, PDK1 phosphorylates and thus activates the AGC kinase OXI1 in Arabidopsis (Anthony et al. 2004) and in rice (Matsui et al. 2010b) or Adi3 (AvrPto-dependent Pto-interacting protein 3) in tomato (Devarenne et al. 2006). In contrast to mammals, *pdk1* knock-out lines in Arabidopsis and rice are not lethal (Camehl et al. 2011) and OXI1 can still be activated in Arabidopsis PDK1-RNAi knock-down lines.

OXI1 can also be activated independently of PA/PDK1. Important stimuli for PA/PDK1-independent OXI1 activation are H_2O_2 and the pathogen-associated molecular pattern (PAMP) flagellin (Li et al. 2009). H_2O_2 accumulates in plants during pathogen attack, but not after co-cultivation with the beneficial fungus *P. indica*. Therefore, signals from pathogens and beneficial microbes come together at this pathway and it could integrate signals from different microbes in the environment. OXI1 was shown to be required for reactive oxygen species (ROS)-mediated responses in Arabidopsis such as root hair elongation and for disease resistance to biotrophic pathogens (Rentel et al. 2004; Petersen et al. 2009). The kinase activity of OXI1 itself was induced by H_2O_2, wounding, cellulase and various elicitor treatments mimicking pathogen attack (Anthony et al. 2006; Rentel et al. 2004). Furthermore, *oxi1* mutant plants are impaired in the activation of MPK3 and MPK6 in response to cellular injury and oxidative stress (Rentel et al. 2004). OXI1 is an upstream regulator of stress-responsive PTI1 (Anthony et al. 2006; Forzani et al. 2011; Matsui et al. 2010a) and MPKs although the mechanism is still unclear. PTI1 proteins are Ser/Thr protein kinases that share sequence identity to tomato PTI1 (Pto-interacting 1). In tomato, PTI1 is phosphorylated by the Ser/Thr kinase Pto conferring resistance to *P. syringae* expressing the effector AvrPto and positively regulates the cell death response triggered by Pto (Martin et al. 1993; Zhou et al. 1995). In contrast, rice PTI1a inhibits disease resistance and cell death and is negatively regulated by OsPDK1-OsOXI1 signaling cascade in response to ROS and PAMP treatments (Matsui et al. 2010a; Takahashi et al. 2007).

OXI1 is the responsible gene for the growth phenotype induced by *P. indica* (Camehl et al. 2011). OXI1 can be activated by H_2O_2 (and therefore stress signals from pathogens) and by PA/PDK1 (activated by biotic and abiotic stress signals and signals from the beneficial fungus *P. indica*). Root colonization by the fungus stimulates PA synthesis in Arabidopsis plants. These results suggest that *P. indica* stimulates growth by PA-mediated activation of PDK1 which subsequently activates OXI1. ROS production is not stimulated and is even inhibited by the beneficial fungus and thus does not play a role in activating OXI1 (Camehl et al. 2011).

In conclusion, we propose that the PDK1-OXI1 signaling pathway (either directly or by activating downstream components) plays a crucial role in integrating signals from pathogenic and beneficial fungi to induce either defense gene activation or the promotion of growth and development.

9 Conclusions

The data summarized here demonstrate that establishing or maintaining a beneficial symbiotic interaction between *P. indica* and Arabidopsis strongly depend on the defense repertoire of the host. A main function of the host defense is to control hyphal growth in the roots, and consequently genetic

inactivation of specific defense compounds results in uncontrolled fungal growth. It appears that this control mechanism is not associated with a particular defense process, but that the mixture of the different defense strategies available for a particular plant or species is probably crucial for a fined-tuned communication between the beneficial symbionts. Consistent with this observation, we identified genes and proteins which participate in the activation of defense processes at different levels (perception of envionmental signals, plant signal transduction, transcription, defense proteins and compounds; Fig. 3). Interestingly, impairments in a particular defense process often lead to a compensatory upregulation of other, unrelated defense processes to restrict fungal growth. Overall, these defense processes are only mildly activated in roots colonized by the beneficial fungus *P. indica*, and it is conceivable that a strong defense response from the host would result in less root colonisation and consequently a disturbed balance in the symbiosis. Finally, the host has to decide whether it puts its energy and resources into growth or defense. This requires a highly sophisticated sensing of the microbial environment. Any wrong decision has severe consequences for the fitness and survival chance of the plant. Consequently, there must be a crosstalk between signaling events leading to defense and those activating growth and development. The AGC kinases fufill the requirements to integrate signals which are beneficial and non-beneficial for the plant, and have the capability to initiate processes leading to a balanced response between growth, development, defense and cell death (cf. Garcia et al. 2012).

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4.5 Manuscript V

The beneficial fungus *Piriformospora indica* protects Arabidopsis from *Verticillium dahliae* infection which requires EIN3 for disease development

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(*Submitted to Journal of Experimental Botany, ID: JEXBOT/2014/128512*)

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The beneficial fungus *Piriformospora indica* protects Arabidopsis from *Verticillium dahliae* infection which requires EIN3 for disease development

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Running title: *P. indica* protection from *V. dahliae*

Key words: *Piriformospora indica*, *Verticillium dahliae*, jasmonic acid, salicylic acid, ethylene, defense, calcium

Abstract

Verticillium dahliae (*Vd*) is a soil-borne vascular pathogen which causes severe wilt symptoms in a wide range of plants. The microsclerotia produced by the pathogen survive in soil for more than 15 years. We demonstrate that an exudate preparation induces cytoplasmic calcium elevation in Arabidopsis roots, and the disease development requires the ethylene-activated transcription factor EIN3. Furthermore, the beneficial endophytic fungus *Piriformospora indica* (*Pi*) significantly reduced *Vd*-mediated disease development in Arabidopsis. *Pi* inhibited the growth of *Vd* in a dual culture on PDA agar plates and pretreatment of Arabidopsis roots with *Pi* protected the plants from *Vd* infection. The *Pi*-pretreated plants grew better after *Vd* infection and the production of *Vd* microsclerotia was dramatically reduced, all without activating stress hormones and defense genes in the host. We conclude that *P. indica* is an efficient bio-control agent that protects Arabidopsis from *Vd* infection.

Introduction

Verticillium species are wide-spread soil-borne fungi which cause vascular diseases in many plant species and are responsible for devastating diseases for plants that can thwart

agricultural production. The vascular wilt fungus *Verticillium dahliae* (*Vd*), for instance, infects more than 200 plant species, among them agriculturally and horticulturally important crops and ornamental plants (Pegg and Brady, 2002; Fradin and Thomma, 2006; Klosterman *et al.*, 2009). It is estimated that *Vd* infections are responsible for several billions of dollars of annual crop losses worldwide. *Vd* has a broad host range and infects plants from temperate to subtropical climates (Pegg and Brady, 2002). Because of their complex life cycle, their control by classical pesticides or fungicides is difficult; therefore, the identification of Verticillium-resistant cultivars remains the most productive solution to the problem (cf. Clerivet *et al.*, 2000, Cai *et al.*, 2009).

Genetic resistance against Verticillium wilt diseases has been reported for several plant species (Fradin and Thomma, 2006; Pegg and Brady, 2002). The *Ve* gene provides resistance against race 1 isolates of *Vd* in tomato (Kawchuk *et al.*, 2001; Fradin *et al.*, 2009), and the tomato gene is also functional after expression in Arabidopsis (Fradin *et al.*, 2011). Many studies have used Arabidopsis for the isolation of *Vd*-resistant germplasm (Schaible *et al.*, 1951; Veronese *et al.*, 2003) or the identification of novel resistance traits following mutagenesis (Fradin and Thomma, 2006; Pantelides *et al.*, 2010; Tjamos *et al.*, 2005; Veronese *et al.*, 2003; Ellendorff *et al.*, 2009; Johansson *et al.*, 2006). Furthermore, quite recently, a large number of proteins and metabolites from different organisms as well as phytohormones have been described to be involved in establishing partial resistance against Verticillium wilt (Knecht *et al.*, 2010; Tian *et al.*, 2010; Denancé *et al.*, 2012; Ralhan *et al.*, 2012; Iven *et al.*, 2012; Liu *et al.*, 2013; Witzel *et al.*, 2013; Gao *et al.*, 2013; Zhang *et al.*, 2013; Reusche *et al.*, 2013; Hadwiger *et al.*, 2013; Hu *et al.*, 2014; Dracatos *et al.*, 2014; Yadeta *et al.*, 2014; Gaspar *et al.*, 2014; Roos *et al.*, 2014; König *et al.*, 2014; Tran *et al.*, 2014; Bu *et al.*, 2014; Liebrand *et al.*, 2013, 2014).

Like other Verticillium species, *Vd* can overwinter as mycelium in host plants or soil. The fungus can also form seed-like structures called microsclerotia, long-lived survival structures of clusters of melanized cells with thick walls, which survive in the soil without a host plant or in association with plant material for up to 20 years (Agrios, 2005; Klosterman *et al.*, 2011). The microsclerotia germinate in response to stimuli from root exudates (Mol and van Riessen, 1995). The hyphae penetrate and grow inter- and intracellularly through the root cortex toward the central cylinder of the root (Reusche *et al.*, 2012; Zhao *et al.*, 2014). They enter the xylem cells of the root, from where they colonize the xylem of the hypocotyl and leaves.

Ultimately, the water transport is disrupted which results in the wilt phenotype (Pegg and Brady, 2002; Fradin and Thomma, 2006; Klosterman *et al.*, 2009). Verticillium species are considered as hemibiotroph: a biotrophic phase within root xylem without a visible disease phenotype is followed by a necrotrophic phase in the aerial parts of the plant.

The spread of the pathogen occurs primarily by root infections from the soil. Therefore rhizosphere bacterial strains such as *Pseudomonas putida* B E2, *Pseudomonas chlororaphis* K15 or *Serratia plymuthica* R12 (Berg *et al.*, 2001) or bacterial isolates (Li *et al.*, 2012) have been shown to function as efficient biocontrol agents against *Vd* spread. The microbial bioagents induce antibiosis, parasitism, competition and secretion of enzymes such as glucose oxidase, chitinase and glucanase which results in the induction of disease resistance in the hosts (Tjamos *et al.*, 2000, 2005).

To our knowledge, there is no report on endophytic fungi which can be used as biocontrol agent against *Vd*. in Arabidopsis. *Piriformospora indica* (*Pi*), a cultivable basidiomycete of Sebacinales, colonizes the roots of many plant species including Arabidopsis (Peškan-Berghöfer *et al.*, 2004; Oelmüller *et al.*, 2009; Qiang *et al.*, 2012; Reitz *et al.*, 2012; Lahrmann and Zuccaro, 2012). Like other members of Sebacinales, *Pi* is found worldwide in association with roots (Selosse *et al.*, 2009) and stimulates growth, biomass and seed production of the hosts (Peškan-Berghöfer *et al.*, 2004; Oelmüller *et al.*, 2009; Shahollari *et al.*, 2007; Sherameti *et al.*, 2005, 2008a and b; Vadassery *et al.*, 2009a and b; Waller *et al.*, 2005; Zuccaro *et al.*, 2011). The fungus promotes nitrate and phosphate uptake and metabolism (Sherameti *et al.*, 2005; Shahollari *et al.*, 2005; Yadav *et al.*, 2010). *Pi* also confers resistance against abiotic (Sherameti *et al.*, 2008a; Baltruschat *et al.*, 2008; Sun *et al.*, 2010) and biotic stress (Stein *et al.*, 2008; Oelmüller *et al.*, 2009).

Here, we demonstrate that a *Vd*-exudate compound induces cytoplasmic Ca^{2+} ($[Ca^{2+}]_{cyt}$) elevation and that the disease development is dependent on the ethylene-activated transcription factor EIN3. Furthermore, *Pi* strongly inhibits growth of *Vd* both *in vitro* and in Arabidopsis roots and thus confers resistance against *Vd* infection.

Materials and methods

Growth conditions of seedlings and fungi

A. thaliana wild-type (ecotype Columbia-0) seeds, seeds of the *glr2.4*, *glr2.5* and *glr3.3* mutants (obtained from Prof. Laihua Liu) as well as of the *cytoplasmic calcium elevation*

mutant1 (*cycam1*) mutant (Johnson *et al.*, 2014) were surface-sterilized and placed on Petri dishes with MS media (Murashige and Skoog, 1962). After cold treatment at 4°C for 48 h, plates were incubated for 11 days at 22°C under long day conditions (16 h light/8 h dark; 80 µmol m^{-2} sec^{-1}). *Pi* was grown for 3-4 weeks on KM medium as described previously (Hill and Kaefer, 2001). For detailed information see Section A and B in Johnson *et al.* (2011). *Vd* (FSU-343, Jena Microbial Resource Center, Germany) was grown for 2-3 weeks on Potato Dextrose Agar (PDA) medium (Bains and Tewari, 1987; Johnson *et al.*, 2012).

Co-cultivation assays

For co-cultivation assays 13 day-old *A. thaliana* seedlings of equal size were used. Co-cultivation of *A. thaliana* and the fungi *Pi* and/or *Vd* was performed under *in vitro* culture conditions on a nylon membrane on PNM media as described by Johnson *et al.* (2011, Section C1 - Method 1) with a few modifications. *Vd* was grown for 12 days and *Pi* for 10 days on the membrane on top of PNM medium in Petri dishes. 13-day old Arabidopsis seedlings were then transferred to the *Pi* or *Vd* plates, or mock-treated (no fungal mycelium; C). For the shifting experiments, the seedlings were transferred to plates with the other fungus after 4 days (from *Vd* to *Pi* or *vice-versa*). Including the controls, five different treatments were compared: (1) Arabidopsis seedlings grown without *Pi* or *Vd* (control seedlings, C); (2) without *Pi* and with *Vd* (*Vd*); with *Pi* and without *Vd* (*Pi*); (4) with *Pi* for 4 days before transfer to *Vd* plates (*1P2V*); and with *Vd* for 4 days before transfer to *Pi* plates (*1V2P*). The seedlings were harvested between 1 and 21 days after exposure to the first fungus (or mock treatment) for further analysis. A time scheme is shown in Suppl. Figure 1. The light intensity (80 µmol m^{-2} s^{-1}) was quantified weekly. Shoots and roots were harvested separately for DNA and RNA analyses.

Long term co-cultivation in sterile vermiculite

30 g vermiculite was placed into one Magenta box (Sigma-Aldrich, Germany) and autoclaved at 121°C for 30 min. After the addition of 40 ml of sterile liquid PNM medium, Arabidopsis seedlings grown in Petri dishes for 10 days were transferred to the sterile vermiculite boxes (1 plant per box). For each treatment, 16 seedlings were analyzed. After 10 days, the number of survived plants, their biomass and fungal DNA content were determined.

Gene expression analysis

RNA was isolated from shoots and reverse-transcribed for real-time quantitative PCR analysis, using an iCycler iQ real time PCR detection system and iCycler software version 2.2 (Bio-Rad). Total RNA was isolated from 5 independent biological experiments of Arabidopsis shoots. cDNA was synthesized using the Omniscript cDNA synthesis kit (QIAGEN) using 1 µg RNA. For the amplification of the RT-PCR products, iQ SYBR Green Supermix (Bio-Rad) was used according to the manufacturer´s protocol in a final volume of 20 µl. The iCycler was programmed to 95°C 3 min, 40 x (95°C 30 sec, 57°C 15 sec, 72°C 30 sec), 72°C 10 min, followed by a melting curve program 55°C 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 glycerin-aldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA level. The primer pairs are given in Suppl. Table 1.

Quantification of fungal DNAs by PCR

Genomic DNA extraction was conducted with DNeasy Plant Mini Kit from 3 treatments. 12.5 ng DNA was taken for PCR template. The reactions were performed with gene-specific primers, as given in Suppl. Table 1. For details see Camehl *et al.* (2011).

Dual culture of *Pi* and *Vd*

Dual culture of *Pi* and *Vd* on agar plates was performed as described by Johnson *et al.* (2012). A *Pi* plug 5 mm diameter was placed at one end of a PDA plate and a *Vd* plug of the same size at the other end of the plate. The plates were incubated at 22-24°C in dark and 75% relative humidity. Photos were taken after 3 weeks of co-cultivation.

Percentage disease index (PDI) calculation

Disease index was calculated with the following formula

$$PDI= \frac{n_1 x_1 + n_2 x_2 + n_3 x_3 + n_4 x_4 + n_5 x_5}{\text{Total number of leaves observed x maximum grade}} \times 100$$

$n_{1\text{-}5}$ = number of affected leaves of the respective disease severity grade (0-5), $X_{1\text{-}5}$ = disease severity grade based on the percentage of affected leaf area.1, 1%≤ x ≤10%; 2, 10% < x ≤ 20%; 3, 20% < x ≤ 30%; 4, 30% < x ≤ 40%; 5, x > 40%; x100: calculated in percentage scale. Disease severity was estimated on the basis of affected leaf area. 1-5 disease severity grades were described by Naik and Lakkund (1997) (cf. Wheeler 1969).

Quantification of jasmonic acid (JA), JA-isoleucine (JA-Ile), abscisic acid (ABA), salicylic acid (SA), oxophytodinoic acid (OPDA) and ethylene (ET)

Independent samples of 250 mg shoot material were collected from each treatment. Phytohormone extractions (JA, JA-Ile, ABA, SA and OPDA) were performed by adding 1 ml ethyl-acetate containing 200 ng of D_2-JA and 40 ng of D_6-ABA, D_4-SA and JA-$^{13}C_6$-Ile to 100 mg ground tissues. All samples were then vortexed for 10 min and centrifuged at 13,000 rpm for 20 min at 4°C. The supernatants were collected and evaporated to dryness at 30°C using a vacuum concentrator. Residues were resuspended in 500 µl MeOH:H_2O (70:30, v/v) and centrifuged at 13,000 rpm for 10 min. The supernatants were collected and measured with the API 3200 TMLC/MS/MS system (Applied Biosystems, Framingham, USA) as previously described (Vadassery *et al.*, 2012).

For ET measurements, 100 mg shoot material from each treatment was collected into 4 ml vials (Roth, Germany). After 4 h ET accumulation, the measurement was performed with the ETD-300 ethylene detector (Sensor Sense B.V., Nijmegen, The Netherlands).

Chlorophyll content was determined according to Yang *et al.* (2002) and based on g fresh weight.

Quantification of microsclerotia

Roots of Arabidopsis seedlings from the 3 treatments with *Vd* were harvested after 3 weeks of co-cultivation in Petri dishes and transferred to a microscopic glass slide with 80 µl lactic acid / glycerol / H_2O (1:1:1). The number of the microsclerotia formed in the roots was calculated per root visually under the light microscope (200 x magnification). The experiment was performed 3 times independently and for each treatment the roots of 12 seedlings were analysed.

Cytoplasmic Ca^{2+} ($[Ca^{2+}]_{cyt}$) measurement

Aequorin based luminescence measurements were performed using 21-day old individual wild type (WT) plants and mutants grown in Hoagland medium (Vadassery *et al.*, 2009). WT aequorin (pMAQ2) plants served as control (Knight *et al.,* 1991). *GLR* mutants (*glr2.4*, *glr2.5* and *glr3.3* provided by Prof. Laihua Liu) were crossed back to wild-type expressing aequorin. After 2 generation selection based on $[Ca^{2+}]_{cyt}$ responses and RT-PCR of T-DNA insertion

examination, the homozygote seeds were used for the described experiments. Primers used for homozygosity tests are given in Suppl. Table. 1. For $[Ca^{2+}]_{cyt}$ measurements, approximately 70% of the roots per seedling was dissected and incubated overnight in 150 µl of 7.5 µM coelentrazine (P.J.K. GmbH, Germany) in the dark at 20°C in a 96 well plate (Thermo Fischer Scientific, Finland, cat. no. 9502887). Bioluminescence counts from roots were recorded as relative light units (RLU) with a microplate luminometer (Luminoskan Ascent, version 2.4, Thermo Electro Corporation, Finland).

Preparation of exudates from mycelia of *Vd*

A 5 mm *Vd* fungal plug was inoculated in Czapek's medium as described in Zhen *et al.* (2004) and grown for 3 weeks. Then, the fungal culture was filtered through double layers of filter paper and the filtrate was centrifuged at 10000 *g* for 30 min to remove the spores. The supernatant was dialyzed with a dialysis membrane (MWCO) (Spectra/Por® Float-A-lyzer®) in 10 mM phosphate buffer pH 7.0 at 4°C for 24 h. The dialyzed solution was frozen and lyophilized. The powder was dissolved in distilled water and the solution was filtered through a 0.45 µm pore size Millipore filter (Roth, Germany). The resulting filtrate was used as exudate for further experiments.

Statistics

All statistical analyses were performed using Excel or SPSS 17.0 (SPSS Inc., Chicago, IL, USA) for ANOVA.

Results

***Pi* inhibits growth of *Vd* on PDA agar plates**

Pi and *Vd* were co-cultivated as described in Materials and methods on a PDA agar plate for 3 weeks. Figure 1 (A and B) demonstrates that *Pi* strongly inhibits growth of *Vd* hyphae. The *Vd* colony in the dual culture is significantly smaller than the *Vd* colony growing without *Pi*. Furthermore, the number of microsclerotia produced by *Vd* in the dual culture is less than the number of microsclerotia produced by *Vd* growing alone. No obvious inhibition zone can be detected. In contrast, growth of *Pi* is barely affected by the presence of *Vd*. This prompted us to test the role of *Pi* in protecting Arabidopsis plants against *Vd* infection.

Arabidopsis seedlings pretreated with *Pi* are protected against *Vd* infection

To investigate whether *Pi* can protect Arabidopsis for *Vd* infection, we exposed the seedlings first to *Pi* prior to *Vd* infection. Seedlings not exposed to any of the two fungi or to one of the two fungi alone served as controls (cf. Materials and methods). The performance of the seedlings was measured after 10, 14 and 21 days, by visible inspection and measuring the fresh weights. After 10 days of co-cultivation, seedlings treated with *Vd* or *Pi* alone showed ~30% increase in the biomass compared to the untreated control seedlings. A comparable increase in the biomass was observed when the seedlings were first exposed to *Pi* and then to *Vd* or *vice versa* (Fig. 2A). This slight increase in the biomass suggests that both fungi initially form a beneficial interaction with the seedlings, and is consistent with the idea that this phase represents a biotrophic interaction of *Vd* with Arabidopsis roots. On the 14th day, seedlings infected by *Vd* alone or first with *Vd* followed by *Pi* (*1V2P*) showed obviously the disease symptoms. The leaves of these seedlings became paler and the roots browner compared to the seedlings exposed to the other 2 fungal treatments (*Pi* and *1P2V*), although no significant differences in the biomass were observed for the different fungal treatments (Fig. 2A). In contrast, on the 21st day, seedlings exposed to *Vd* alone or exposed to *Vd* prior to exposure to *Pi* (*1V2P*) were severely damaged. Their fresh weights were reduced or no longer measurable. *Pi* treatment alone resulted in a ~30% increase in the fresh weight (Fig. 2A). Interestingly, seedlings which were pretreated with *Pi* and then exposed to *Vd* (*1P2V*) had the same fresh weights as untreated control seedlings, although the visible inspection showed some photo-bleaching (Fig. 2B). This clearly demonstrates that *Pi* protects Arabidopsis seedlings against *Vd*-induced wilt. Therefore, this experimental set-up was used to study the protective function of *Pi* in greater details.

The results were confirmed by calculating the PDI for those seedlings treated with *Vd*. After 10 days of co-cultivation, the PDI for *Vd* and *1V2P* seedlings was ~20%, and after 14 days 40-50%. After 21 days, the PDI was almost 100%. In contrast, seedlings pre-treated with *Pi* prior to exposure to *Vd* (*1P2V*) showed a slow increase in the PDI, which reached ~30% after 21 days (Fig. 3).

Furthermore, the amount of total chlorophyll (Chl) is a sensitive marker for the fitness of a plant. On the 4th day, the shoots of *Vd* and *Pi* treated plants contained slightly higher Chl levels than control seedlings (Fig. 4A). On the 10th day, the Chl content of *Vd* treated seedlings is comparable to that of control seedlings not exposed to the pathogen. Furthermore, while *1P2V* seedlings had the same amount of Chl as *Pi* seedlings, the Chl content in *1V2P*

seedlings was significantly reduced (Fig. 4B). Comparable results were obtained for the 14th day, except that the Chl content for *1P2V* seedlings was reduced compared to *Pi* seedlings (Fig. 4C). On the 21st day, *Pi* seedlings had the highest Chl content, *1P2V* seedlings had the same amount of Chl as control seedlings not exposed to a fungus, while the Chl levels in the *Vd* and *1V2P* plants were strongly decreased (Fig. 4D). This confirms the protective function of *Pi* against *Vd* infection in Arabidopsis leaves.

Pathogenesis and application of pathogen-associated molecular patterns induce stomata closure (Grimmer *et al.*, 2012). In control plants not exposed to any fungus, between 5 and 12% of the stomata were closed. Three days after exposure of the roots to *Vd*, ~25% of the stomata were closed (Fig. 5A), and this increased to ~30% until the 7th day. The *1V2P* treatment showed ~25% stomata closure at the 7th day, and this value is comparable to that for seedlings treated with *Vd* alone. In contrast, exposure of the roots to *Pi* or first to *Pi* followed by *Vd* did not result in stomata closure and these values are comparable to those of the untreated controls (Fig. 5B). This indicates that *Pi* prevents *Vd*-induced stomata closure.

***Pi* represses *Vd*-induced genes in shoots**

Vd induces defense gene expression in shoots. After 1 d, the mRNA levels for *PR1* and *PR2* representing SA-inducible genes and *PDF1.2* for the JA/ET pathway, *ERF1* and *VSP2* for ET pathway were upregulated in the leaves of *Vd*-exposed seedlings. Except for *PR2*, none of the other genes responded to *Pi* exposure (Fig. 6). After 14 d, *Vd*-exposed seedlings showed an even stronger upregulation of the defense genes in the leaves (Fig. 6). Pretreatment of the seedlings with *Pi* prior to *Vd* infection resulted in the repression of defense gene expression compared to seedlings which were not pretreated with *Pi*. This provides additional evidence for the protective function of *Pi* against *Vd* infection. Furthermore, *GLR2.4* (Hammes *et al.*, 2005), *GLR2.5* (Guan and Nothnagel, 2004) and *GLR3.3* (Monzoor *et al.*, 2013) code for putative Ca^{2+} transporters and are involved in defense responses (cf. Discussion). We observed that *GLR2.4* was upregulated in the leaves of *Vd*-exposed seedlings and repressed in the leaves of seedlings which were pretreated with *Pi* prior to *Vd* exposure (Fig. 6 and S2). *RabGAP22* is required for defense to *V. longisporum* and contributes to stomata immunity (Roos *et al.*, 2014). For *Vd*, *RabGAP11* is upregulated after exposure to *Vd* and significantly repressed in seedlings which were pretreated with *Pi* (Fig. 6).

_Pi_ strongly represses _Vd_-induced phytohormone accumulation in shoots

The phytohormones JA, JA-Ile, OPDA, SA, ABA and ET are crucial for the activation of defense responses. Figure 7 demonstrates that these phytohormones accumulated after *Vd* infection in the shoots of Arabidopsis seedlings. The phytohormone levels were also high in the *1V2P* samples, while in all other cases (C, *Pi*, *1P2V*), the phytohormone levels were significantly lower. Thus, *Vd*-induced phytohormone accumulation is repressed if the roots are colonized by *Pi* prior to their exposure to *Vd*. Interestingly, application of *Pi* to roots which were already exposed to *Vd* did not repress the accumulation of the phytohormones in the shoots.

_Pi_ inhibits _Vd_ propagation and microsclerotia formation

Quantification of the amount of *Vd* DNA demonstrated that *Vd* and *1V2P* seedlings contain twice as much pathogen DNA than *1P2V* seedlings in both roots (Fig. 8A and D) and shoots (Fig. 8B and E). Interestingly, the amount of *Pi* DNA in the roots is identical in all *Pi*-treated samples and not affected by a pre-treatment with *Vd* (Fig. 8C and F). Furthermore, microscopic analysis demonstrated that the number of microsclerotia was strongly reduced in root tissue pretreated with *Pi* (Fig. 9). This demonstrates that *Pi* inhibits *Vd* propagation and microsclerotia formation in the roots, while *Vd* does not affect the propagation of *Pi* in Arabidopsis roots.

Long-term experiments confirmed the results obtained for seedlings

In order to study long term interaction, the seedlings were grown according to the 5 regimes on Petri dishes for 10 days before transferred to sterile vermiculite for additional 14 days. All control seedlings (C) and those exposed to *Pi* (*Pi*) were alive. Exposure of *Pi*-pretreated plants to *Vd* resulted in ~20% loss of the plants. However 80% of the plants, which were either exposed to *Vd* alone or first to *Vd* followed by *Pi*, died (Fig. 10A). Furthermore, we measured the fresh weights of the seedlings which survived the treatments. Plants exposed to *Pi* alone showed a ~30% increase in the fresh weight. The fresh weights of *1P2V* plants were comparable to those not exposed to any fungus. *Vd*- and *1V2P*-treated seedlings showed significantly decreased fresh weights compared to all other treatments (Fig. 10B). Finally, the *Vd* DNA amount in both shoots and roots was lower in *1P2V*-treated plants compared to those treated with *Vd* alone or first with *Vd* followed by *Pi* (*1V2P*) (Fig. 10C). Comparable to the

results obtained with seedlings in Petri dishes (Fig. 8), the *Pi* DNA content was the same in all *Pi*-treated roots (Fig. 10C). This confirms that *Pi* inhibits *Vd* growth, but not *vice versa*.

EIN3 is required for full susceptibility of Arabidopsis to *Vd*

The strong upregulation of the phytohormone levels in the leaves of seedlings grown in the presence of *Vd* was further investigated for ET. Pantelides *et al*., (2010) have shown that ET perception *via* ETR1 is required for *Vd* infection in Arabidopsis. We observed a strong requirement of EIN3 for *Vd*-induced disease development in Arabidopsis leaves. *ein3* seedlings which were exposed to *Vd* alone or were first treated with *Vd* before application of *Pi* perform better than wild-type seedlings (Fig. 11A, B and S4). Interestingly, the ET level in *ein3* seedlings is much higher than in wild-type seedlings, even in the absence of *Vd*. Exposure of the seedlings to *Vd* stimulate ET accumulation even further (Fig. 11C and S3). This suggests that *ein3* seedlings try to compensate the lack of EIN3-induced genes by further stimulating ET biosynthesis, in particular after *Vd* infection. Taken together, these data demonstrate that EIN3-induced genes are required for pathogenicity of *Vd* (cf. Discussion).

***Vd* induces $[Ca^{2+}]_{cyt}$ elevation in WT roots, but not in roots of a Ca^{2+} response mutant**

Pathogen-associated molecular pattern-triggered immunity is often initiated by $[Ca^{2+}]_{cyt}$ elevation, which can be induced by exudated compounds from pathogenic fungi (cf. Johnson *et al.*, 2014, and ref. therein). Since the putative plasma membrane-localized Ca^{2+}-transporter gene *GLR2.4* was upregulated by *Vd*, we tested whether exudated compounds from *Vd* can induce $[Ca^{2+}]_{cyt}$ elevation in roots. An exudate preparation from the mycelium was applied to the roots of transgenic pMAQ2 Arabidopsis lines expressing the Ca^{2+}-sensor apoaequorin. Under resting conditions, 21 d-old pMAQ2 lines gave $[Ca^{2+}]_{cyt}$ values of 70 ± 0,6 nM (*n* = 16). A rapid and transient increase in the $[Ca^{2+}]_{cyt}$ concentration is observed 40 sec after the application of *Vd* preparation (Fig. 12A). Discharge at the end of the experiment demonstrates that less than 5% of the reconstituted aequorin was consumed after the stimuli, which ensures that the amount of aequorin in the sample is not limiting for the Ca^{2+} signal (data not shown). The $[Ca^{2+}]_{cyt}$ reached a peak of ~ 400 nM after 90 to 120 sec (Fig. 12A). Subsequently the Ca^{2+} levels steadily decreased. No $[Ca^{2+}]_{cyt}$ elevation is observed with the PBS buffer treatment (Fig. 12A). The magnitude of the $[Ca^{2+}]_{cyt}$ response is dose-dependent (data not shown). Furthermore, an Arabidopsis mutant which does not show $[Ca^{2+}]_{cyt}$ elevation in response to exudate preparation from various pathogenic fungi (*cycam1*, Johnson *et al.*, 2014)

also failed to induce $[Ca^{2+}]_{cyt}$ elevation in response to the *Vd* preparation (Fig. 12B). This indicates that *cycam1* is impaired in the response to exudate preparations from various pathogens (cf. Discussion). Furthermore, we crossed the apoaeqorin gene into the *glr2.4*, *glr2.5* and *glr3.3* knock-out background. Fig. 12B demonstrates that the *Vd* exudate preparation induced $[Ca^{2+}]_{cyt}$ elevation in the knock-out backgrounds, indicating that these putative plasma membrane-localized transporters do not participate in the Ca^{2+} uptake from the extracellular space, although the gene *GLR2.4* was upregulated in *Vd*-infected seedlings (Fig. 6).

To investigate whether $[Ca^{2+}]_{cyt}$ elevation is required for disease development, *cycam1* was infected with *Vd* and the development of the mutant seedlings was compared to that of the WT seedlings. No obvious difference of the disease symptoms in the aerial parts could be detected, which suggests that $[Ca^{2+}]_{cyt}$ elevation is not essential for *Vd* propagation (Fig. S6).

Discussion

Our data demonstrate that *Pi* is a very efficient biocontrol agent for *Vd* wilt in Arabidopsis. *Pi* restricts *Vd* growth both on agar plates (Fig. 1) and in Arabidopsis roots, in particular when they were first colonized by *Pi* prior to infection with *Vd* (Fig. 8). Molecular and biochemical analyses demonstrate that the reduced growth rate of *Vd* in *Pi*-pretreated Arabidopsis roots retards defense gene expression (Fig. 6), the accumulation of defense-related phytohormones (Fig. 7) and stomata closure (Fig. 5). The performance of the seedlings is significantly better (Fig. 2 and 3) and this also continues after shifting the seedlings to vermiculite for a longer period of time (Fig. 10). *Pi* not only inhibits growth of *Vd* mycelium in Arabidopsis roots, but also prevents the spread of the pathogen to the aerial parts of the plant (Fig. 8). Furthermore, microsclerotia formation is strongly reduced (Fig. 9). Previously, several soil-borne bacteria have been identified as biocontrol agents for Verticillium wilt (cf. Li *et al*., 2012; Zhao *et al*., 2012; Maldonado-González *et al*., 2012, Berg *et al*., 2005). *Vd* can induce antimicrobial metabolites such as rutin in potato (El Hadrami *et al*., 2011) or pathogenesis-related proteins in Arabidopsis (Tjamos et al., 2005) which participates in pathogen resistance. Prieto *et al*., (2011) demonstrated that root hair colonization plays an important role in *Pseudomonas* spp.-mediated biocontrol activity against Verticillium wilt in olive roots. Furthermore, the *Bacillus subtilis* strain NCD-2 functions as a biocontrol agent against cotton Verticillium wilt, and the cotton PhoR/PhoP, two component regulatory systems, were involved in the biocontrol

capability of the bacterium (Guo *et al*., 2010). Also quorum sensing is involved in the biocontrol activity of *Serratia plymuthica* against *Vd* (Müller *et al*., 2009). Moderate drought influences the effect of arbuscular mycorrhizal fungi as biocontrol agents against Verticillium-induced wilt in pepper (Garmendia *et al*., 2005). It appears that quite different mechanisms control the fungal spread, probably because of the complicated lifestyle of the pathogen which allows microbial interference at different levels and in different plant tissues. An increasing number of genes were recently identified to be involved in establishing partial resistance to Verticillium wilts (cf. Introduction). Pathogen attack including root colonization by *Vd* is associated with stomata closure as one of the first line of plant defense (Fig. 5). *RabGAP22* is required for defense against *V. longisporum* and contributes to stomatal immunity (Roos *et al.*, 2014). *RabGAP11* gene is upregulated by *Vd* and repressed by *Pi* (Fig. 6). Finally, defensins play a role in the plant defense against *Vd* (Dracatos *et al.*, 2014; Knecht *et al.*, 2010; Gaspar *et al.*, 2014).

Control of microsclerotia formation is crucial for preventing Verticillium spread in nature and agriculture. Our data demonstrate that *Pi* is quite efficient in restricting microsclerotia formation in Arabidopsis roots (Fig. 9), presumable because the pathogen cannot grow fast enough in the presence of *Pi*. Microsclerotia formation is also suppressed by Verticillium itself, i.e. by the fungal transcription activator of adhesion Vta2, and fungi impaired in Vta2 are unable to colonize plants and induces disease symptoms (Tran *et al.*, 2014). Taken together, *Pi* restricts *Vd* growth as well as hyphal and microslerotia propagation, which – in turn – reduces the plant´s defense response against the pathogen at different levels. This is not only important for better performance of individual plants, but has also severe long-term consequences for the control of the *Vd* spread *via* microsclerotia in ecosystems and agricultural areas.

Plant glutamate receptor-like (GRL) homologs are associated with Ca^{2+} influx through the plasma membrane. Figure 6 demonstrates that the mRNA level for *GLR2.4* is upregulated in the leaves of *Vd*-infected Arabidopsis seedlings and these responses are restricted by a pretreatment of the seedlings with *Pi*. GLR3.3 is involved in plant defense and resistance to *Hyaloperonospora arabidopsidis* (Manzoor *et al.*, 2013). The protein also mediates glutathione-triggered cytosolic calcium transients, transcriptional changes, and innate immunity responses in Arabidopsis (Li *et al.*, 2013). *GLR2.5* is upregulated in Arabidopsis cell cultures upon wounding (Guan and Nothnagel, 2004) and *GLR2.4* is induced by

nematodes in Arabidopsis roots (Hammes *et al.*, 2005). GLR2.4, also called AUGMIN subunit 8, is a microtubule plus-end binding protein that promotes microtubule reorientation in hypocotyls (Cao *et al.*, 2013; Farquharson, 2013). Microtubles and microtuble orientation are important for plant defense and immunity (Cheong *et al.*, 2014; Underwood and Sommerville, 2014) and also involved in *Vd* – Arabidopsis interaction. Hu *et al.*, (2014) demonstrated that histone H2B monoubiquitination is involved in regulating the dynamics of microtubules during the defense response to *Vd* toxins in Arabidopsis. Yuan *et al.*, (2006) showed that *Vd* toxins disrupted microfilaments and microtubules in Arabidopsis suspension-cultured cells. Figure 12A shows that exudate compounds from *Vd* induces $[Ca^{2+}]_{cyt}$ elevation in Arabidopsis roots. In order to test whether the $[Ca^{2+}]_{cyt}$ elevation is mediated by one of the three GLRs, we generated transgenic *glr3.3*, *glr2.5* and *glr2.4* knock-out lines in the apoaequorin background and found that the $[Ca^{2+}]_{cyt}$ response is not controlled by the three GLRs (Fig. 12B), although the mRNA level of *glr2.4* is upregulated upon *Vd* infection (Fig. 6). This suggests that GLRs have different functions in the *Vd*-Arabidopsis interaction. However, an ethylmethansulfonate-induced Arabidopsis mutant named *cycam1* which is unable to induce $[Ca^{2+}]_{cyt}$ elevation in response to exudate preparations from *Alternaria brassicae*, *Rhizoctonia solani*, *Phytophthora parasitica* var. *nicotianae* and *Agrobacterium tumefaciens* (Johnson *et al.*, 2014) did not respond to the *Vd* exudate preparation (Fig. 12B). This demonstrates that at least one of the *Vd*-induced signaling events leading the opening of Ca^{2+} channels or the channels themselves are identical to those responding to exudate preparations from other pathogens (Johnson *et al.*, 2014). However, the reduced Ca^{2+} response in the *cycam* mutant does not affect the disease development. It remains to be determined which is the active compound inducing the $[Ca^{2+}]_{cyt}$ response in Arabidopsis roots, and what is the mutated gene in the *cycam1* mutant. Several exudated compounds have been postulated to induce pathogenicity in plants. Klosterman *et al.*, (2009) proposed that based on the sequence information of Verticillium species, pathogenicity may be caused by a cocktail of different compounds and elicitors with different functions in the complex pathogenicity procedure. A Verticillium crude toxin preparation has been often used, although the exact composition of this preparation and the role of the individual compounds are not clear. For instance, recently Yao *et al.*, (2012) have demonstrated that the *Vd* toxin preparation stimulates nitric oxide production in Arabidopsis which serves as a signaling intermediate downstream of H_2O_2 to modulate dynamic microtubule cytoskeleton. This may link the *Vd* toxin function again to GLR2.4, who´s mRNA level is upregulated after *Vd* infection (Fig. 6).

Wang *et al.*, (2012) reported on the purification and characterization of a novel hypersensitive-like response-inducible protein elicitor named PevD1 from *Vd* that induces resistance responses in tobacco. The relationship of the bioactive compound that induces the $[Ca^{2+}]_{cyt}$ response to the toxins which induce disease responses needs to be investigated.

Interestingly, we did not observe a linear relationship between the propagation of *Vd* in the seedlings and the accumulation of defense-related phytohormone levels. For instance, the phytohormone levels were always high when the seedlings were exposed to *Vd*, irrespective of whether they were exposed to *Vd* alone, pre-treated with *Pi* or first with *Vd* followed by *Pi* (Fig. 7), although, growth of *Vd* was strongly reduced by the *Pi* pretreatment (Fig. 1). This suggests that even low infection rates of *Vd* are sufficient to stimulate the accumulation of the defense hormones. This might be a precaution, although propagation of *Vd* is inhibited when the roots were pretreated with *Pi*.

Various reports showed the involvement of plant hormones in the control of Verticillium growth in Arabidopsis. Stabilization of cytokinin levels enhances Arabidopsis resistance against *V. longisporum* (Reusche *et al.*, 2013). The fungus also requires JA-dependent COI1 function in roots to elicit disease symptoms in Arabidopsis shoots (Ralhan *et al.*, 2012). Ethylene perception *via* the receptor ETR1 is required for *Vd* infection in Arabidopsis (Pantelides *et al.*, 2010). Enhanced resistance of *etr1-1* plants, but not in SA-, JA- or other ET-deficient mutants against *Vd* infection indicate a crucial role of ETR1 in defense against this pathogen. We observed a particularly striking resistance of the Arabidopsis *ein3* mutant against *Vd* infection *in vivo* and *vitro* (Fig. S5). This is consistent with the reports by Pantelides *et al.*, (2010) for *etr1*, although they did not observe a significant role of EIN3 in their studies. Our data demonstrate that EIN3 plays an important role in pathogenicity and will provide an important tool to identify EIN3-regulated genes which are required for *Vd* disease development. Furthermore, the ET level in the *ein3* mutant exposed to *Vd* is much higher compared to *Vd*-exposed WT seedlings (Fig. 11C). This suggests a feedback loop by which the lack of EIN3-induced defense responses in the *ein3* mutant results in an additional stimulation of ET synthesis.

In summary, our data demonstrate that *Pi* is a very efficient biocontrol agent for *Vd*. This is mainly caused by the restriction of *Vd* growth in the presence of *Pi*. There appears to be additional mechanisms which prevent pathogenicity of *Vd* in the presence of *Pi*. For instance,

the phytohormone levels accumulate to comparable levels in *Vd* and *1P2V* seedlings, although *Vd* propagation is restricted in the presence of *Pi* (Fig. 1). Since *Pi* pretreatment severely reduces defense gene expression in spite of a comparable phytohormone level in these tissues, additional signals from *Pi* must participate in the regulation of the immune response against *Vd*.

ACKNOWLEDGEMENTS

We like to thank Prof. Laihua Liu for the *glr* mutants; Sarah Mußbach and Claudia Röppischer for their excellent technical assistance. CS was supported by the German Science Foundation and the German Exchange Program (DAAD). RO and KWY are supported by a travel exchange project (DAAD).

Supporting Information

Figure S1. Co-cultivation time scheme

Figure S2. Induction of *GLR* genes in shoots of Arabidopsis seedlings after 1 and 14 days

Figure S3. Phenotype of *ein3-1* and WT after 21 days of co-cultivation following the 5 treatments described in Methods and Material

Figure S4. ET content from 5 different treatments in shoots of *ein3-1* seedlings after 3 weeks

Figure S5. Phenotypes of WT and *ein3-1* after *Vd* spore inoculation *in vivo* and *in vitro*

Figure S6. Phenotype of WT and *cycam1* mutant 21 days after *Vd* inoculation

Figure T1. Primer list for RT-PCR and PCR analysis

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Figure legends

Fig. 1 (A). *Pi* inhibits growth of *Vd* on agar plates. **(B)** Quantification of the colony. The diameter of the *Pi* and *Vd* mycelia on the agar plate is given in cm. Typical plates from 3 independent experiments are shown. Bars represent SDs. Asterisks indicate significant differences, as determined by ANOVA (*** $P \leq 0.001$).

Fig. 2 (A). Fresh weights of seedlings after 10, 14 and 21 days of co-cultivation or mock treatments on Petri dishes. The seedlings were exposed to either *Pi* or *Vd* alone or in combination as described in the Methods and Material and Suppl. Fig. 1. C: seedlings treated without fungi; *Vd*: seedlings treated with *Vd*; *Pi*: seedlings treated with *Pi*; *1P2V*: seedlings first treated with *Pi* for 4 days followed by *Vd*; *1V2P*: *vice-versa* as *1P2V*. n.d: no detectable. The data are based on 3 independent experiments with 16 seedlings each. Bars represent SDs. Asterisks indicate significant differences, as determined by ANOVA (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$). **(B)** The phenotype of typical seedlings on 21st day.

Fig. 3. PDI for seedlings exposed to *Vd*. For treatments, cf. Material and Methods and Suppl. Fig. 1. The data are based on 3 independent experiments with 16 seedlings each. Bars represent SDs.

Fig. 4. Total chlorophyll content (mg/g fresh weight) in shoots. The data were obtained 4, 10, 14 and 21 days after the fungal treatments (cf. Material and Methods, Suppl. Fig. 1 and legend to Fig. 2A). The data are based on 3 independent experiments with 16 seedlings each. Bars represent SDs. Asterisks indicate significant differences to the untreated control (C), as determined by Student's t-test (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$).

Fig. 5. Stomata closure rate in leaves. The data are based on 3 independent experiments with 16 seedlings each. Bars represent SDs. Asterisks indicate significant differences to the untreated control (C), as determined by Student's t-test (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$).

Fig. 6. Induction of defense genes in the shoots of Arabidopsis seedlings 1 and 14 days after the fungal treatments, relative to the untreated control. The data represents fold induction (mRNA level $_{+\ \text{fungal treatments}}$ / mRNA level $_{-\ \text{fungal treatments}}$). (fold of C which is set as 1.0). For experimental details, cf. Material and Methods, Suppl. Fig. 1 and legend to Fig. 2A. The data are based on 5 independent experiments with 16 seedlings each. Bars represent SDs. Asterisks indicate significant differences, as determined by Student's t-test (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$).

Fig. 7. Phytohormone levels in the shoots 21 days after the different fungal treatments. For experimental details, cf. Material and Methods, Suppl. Fig. 1, and legend to Fig. 2A. The data are based on 3 independent experiments with 12 seedlings each. Bars represent SDs. Asterisks indicate significant differences, as determined by Student's t-test (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$).

Fig. 8. The amount of *Vd* DNA in the roots (**A** and **D**) and shoots (**B** and **E**) of Arabidopsis seedlings exposed to the 5 treatments (cf. legend to Fig. 2A). (**C** and **F**) The amount of *Pi* DNA in the roots. For experimental details, cf. Material and Methods and Suppl. Fig. 1. The measurements were performed for the 14th (A, B, C) and 21st (D, E, F) day. The data are based on 3 independent experiments with 12 seedlings each. Bars represent SDs. Asterisks indicate significant differences compared to *Vd* (A,B,D,E) or to *Pi* (**C** and **F**), as determined by Student's t-test (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$).

Fig. 9. *Pi* inhibits the formation of *Vd* micosclerotia in roots, irrespective of whether the roots were first exposed to *Pi* (*1P2V*) or first to *Vd* (*1V2P*). The analysis was performed 21 days

after infection. Left: microscopy of root sections with microslerotia (black spots). Right: Quantification of the number of microsclerotia. The data are based on 3 independent experiments with 12 seedlings each. Bars represent SDs. Asterisks indicate significant differences to *Vd*, as determined by Student's t-test (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$).

Fig. 10. Confirmation of the results for adult plants, grown in sterile vermiculite. After exposure of the seedlings to the 5 treatments in Petri dishes for 10 days (cf. legend to Fig. 2A), they were transferred to Magenta boxes with sterile vermiculite for 14 days. (**A**) Number of survived seedlings. (**B**) Fresh weight of seedlings. (**C**) Fungal DNA content in roots and shoots. The data are based on 3 independent experiments with 16 seedlings each. Bars represent SDs. Asterisks indicate significant differences to *Vd*, as determined by Student's t-test (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$).

Fig. 11. (A) EIN3 is required for full susceptibility of Arabidopsis to *Vd*. The representative picture (3 independent experiments with 32 plants each) was taken after 21 days inoculation with *Vd*. **(B)** Number of survived seedlings. **(C)** Ethylene levels in WT and *ein3* seedlings after exposure to *Vd*. Bars represent SDs. Asterisks indicate significant differences, as determined by Student's t-test (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$).

Fig. 12. (A) *Vd* exudate preparation induces $[Ca^{2+}]_{cyt}$ elevation in *A. thaliana* seedlings expressing cytosolic aequorin. Roots of 21-day old pMAQ2 in Col-0 seedlings were dissected and incubated overnight in 7.5 µM coelenterezine. The roots were challenged with 50 µl of the *Vd* preparations. $[Ca^{2+}]_{cyt}$ level was calculated from the relative light unit (RLU) at 5 s integration time for 10 min. The arrow indicates the time (60 s) of addition of the stimuli/PBS buffer. For all experiments, 10 mM phosphate buffer (PBS, PH 7.0) was used as control and gave background readings. All curves and values represent average of five independent experiments with eight replications in each experiment. **(B)** *Vd* exudate preparation does not induce $[Ca^{2+}]_{cyt}$ elevation in the *cycam1* mutant (Johnson *et al.*, 2014), but induces $[Ca^{2+}]_{cyt}$ elevation in pMAQ2 lines in the *glr2.4*, *glr2.5* and *glr3.3* background.

Figures

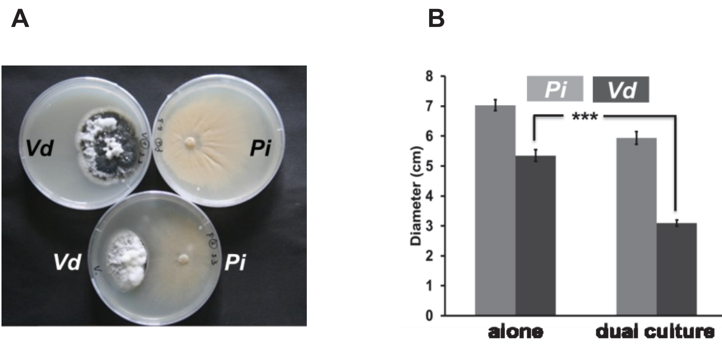
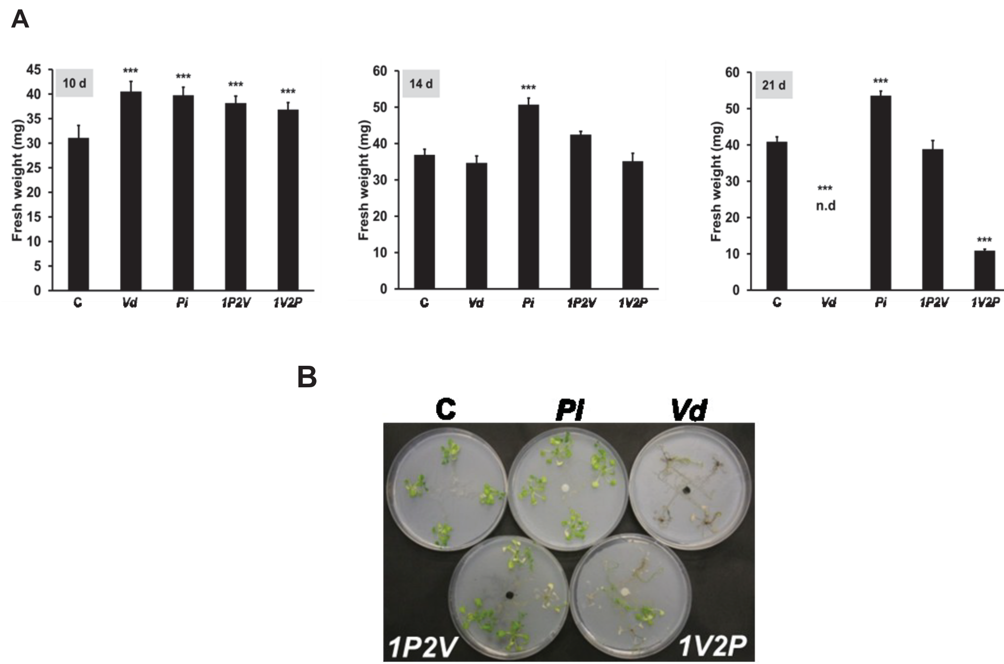


Figure 1



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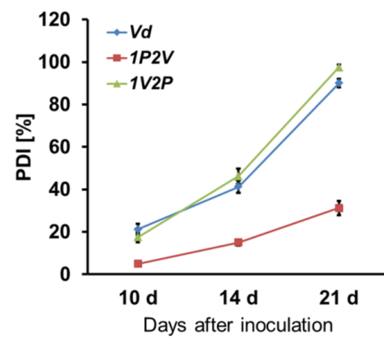

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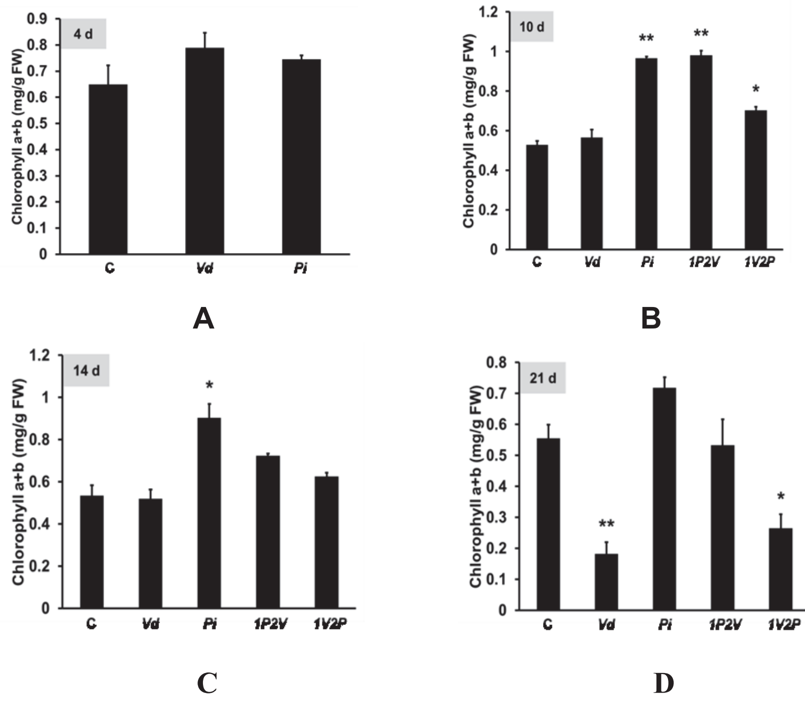

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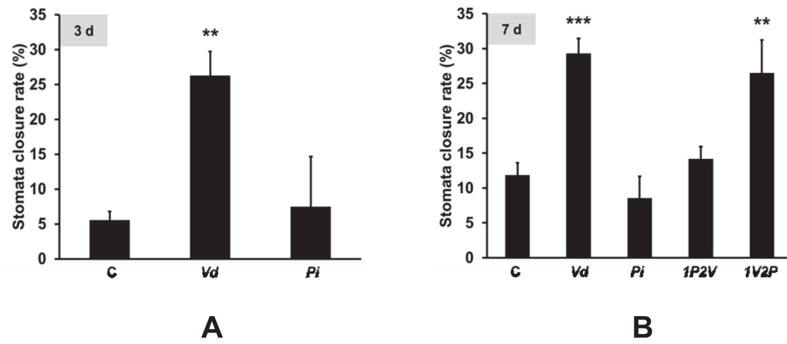
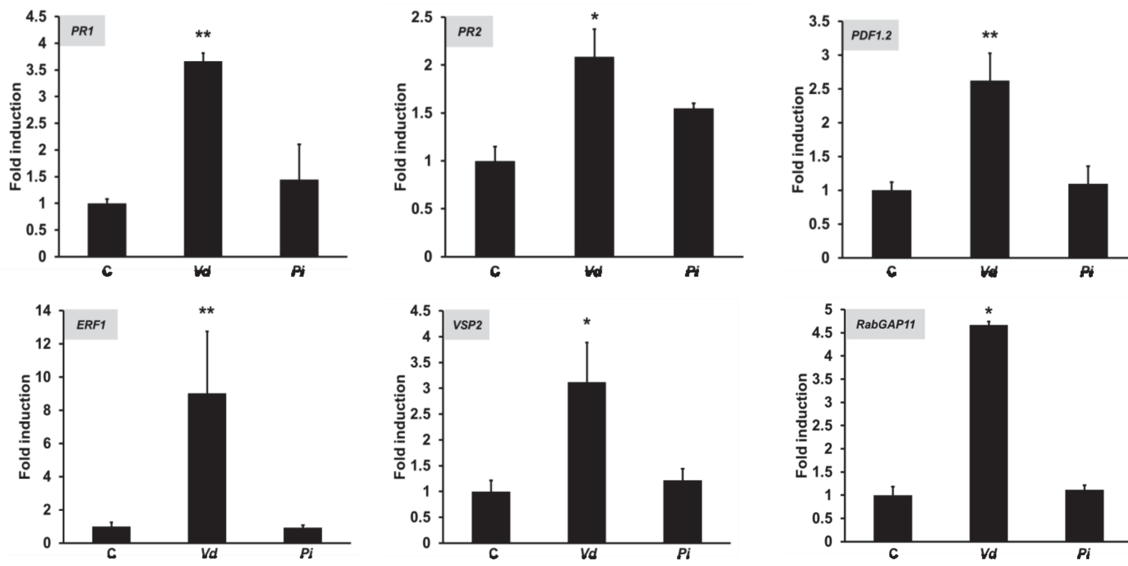
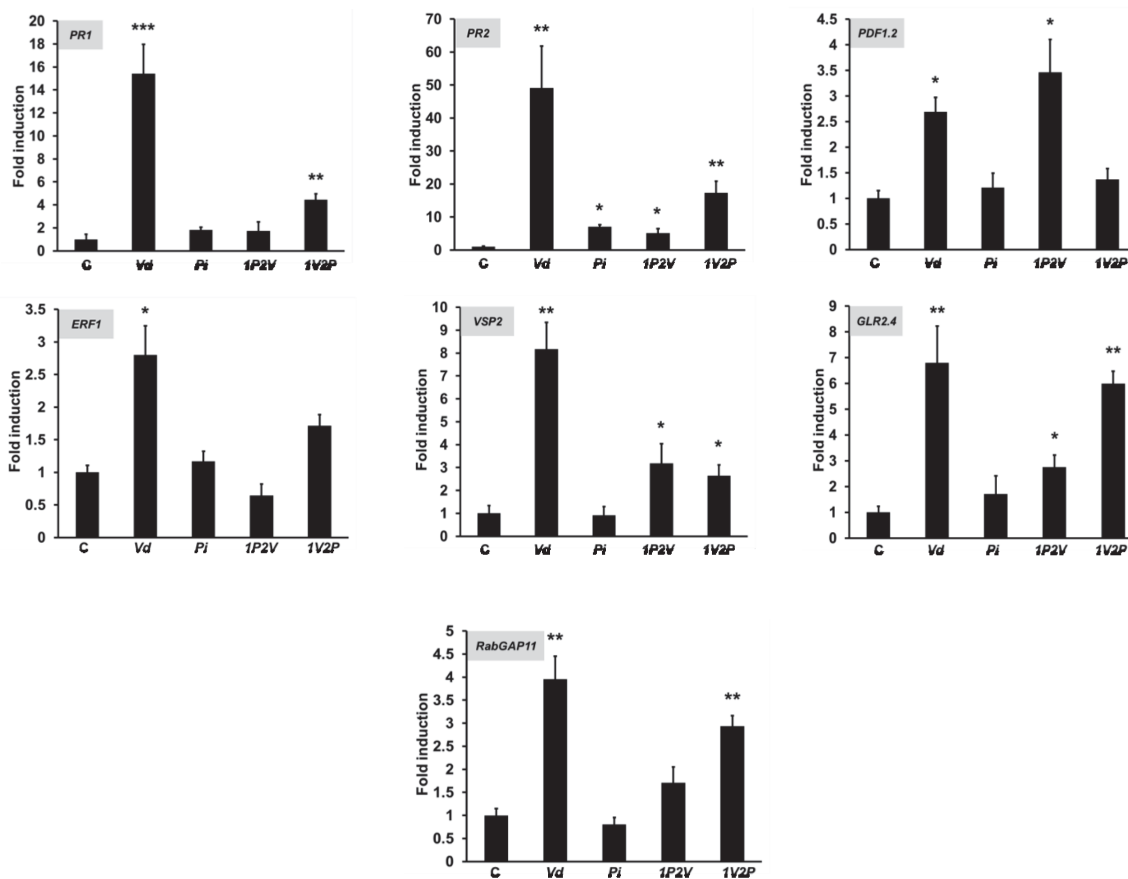


Figure 5

1 d



14d



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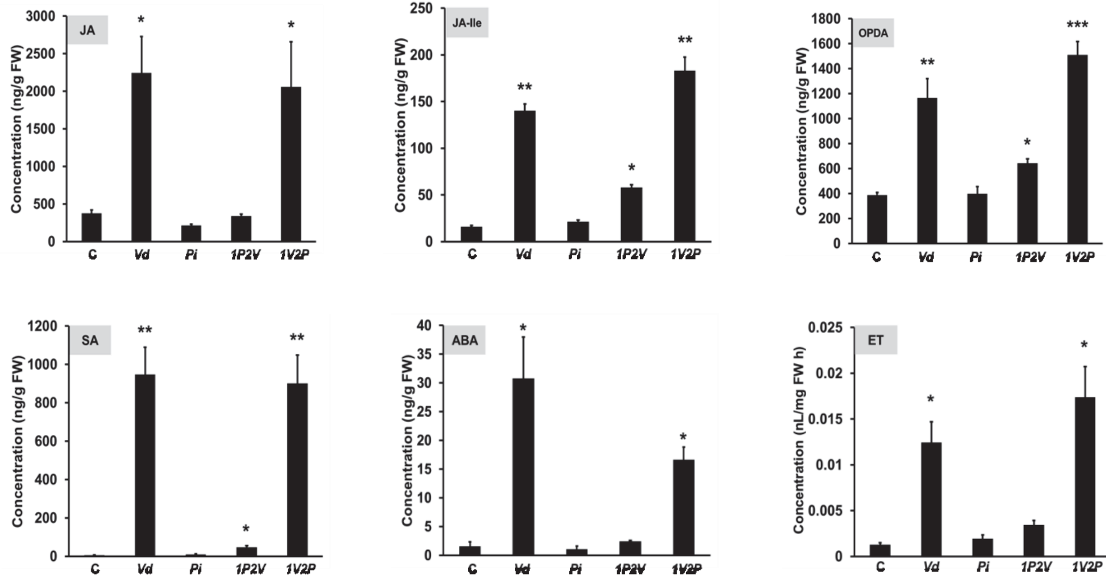
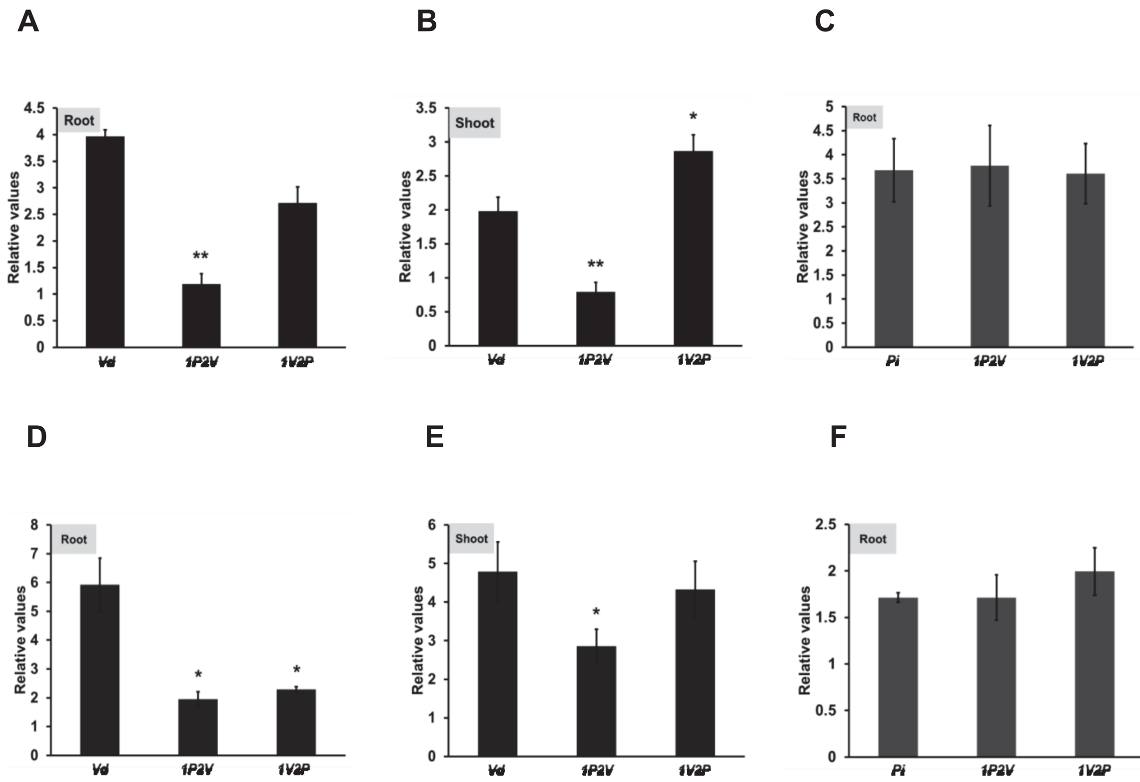


Figure 7



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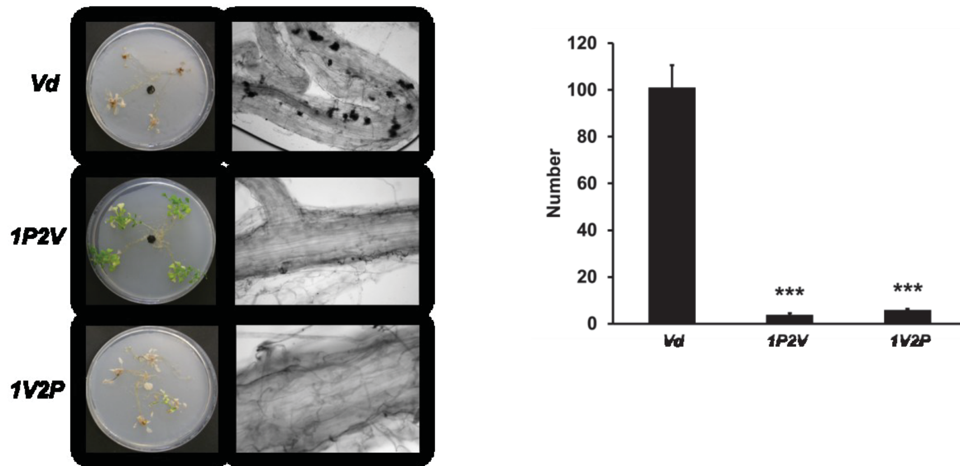

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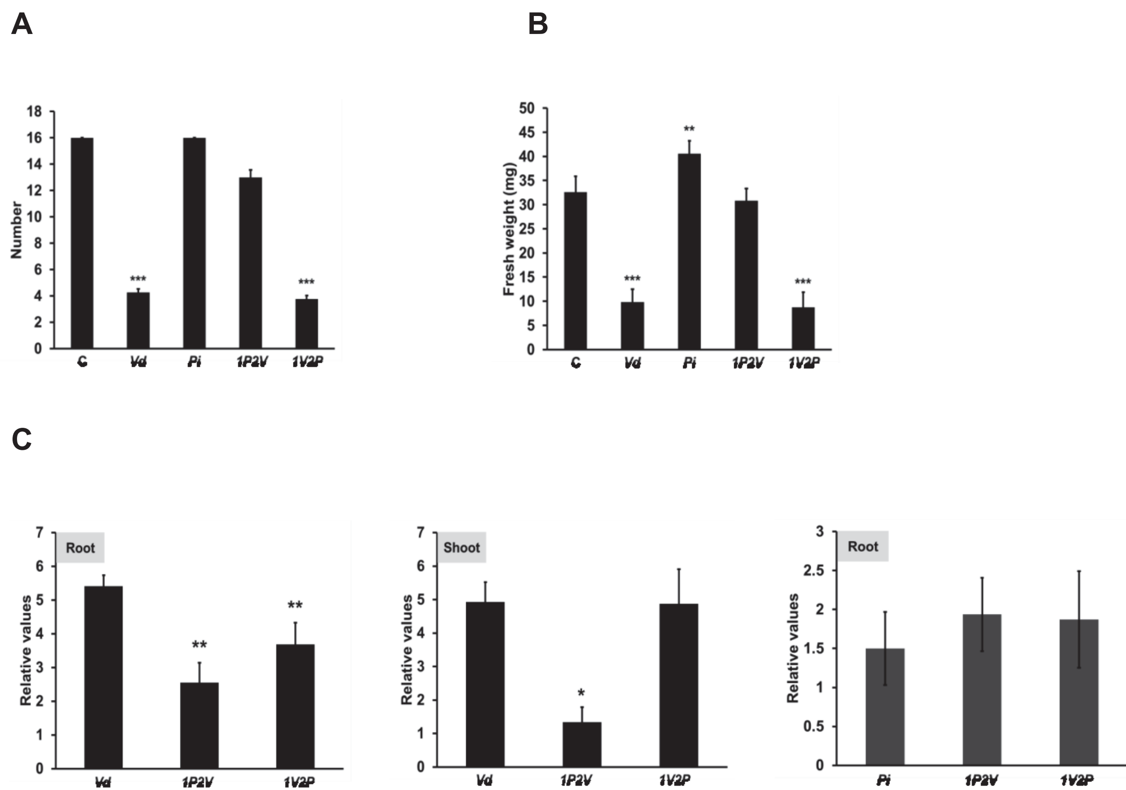

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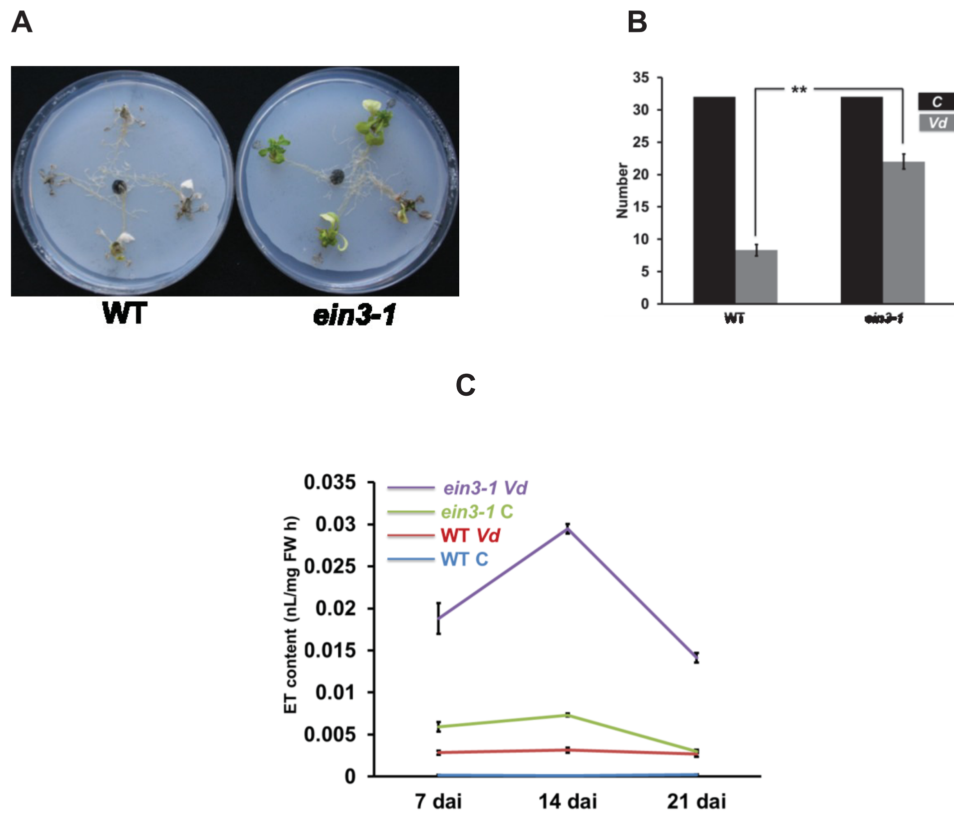


Figure 11

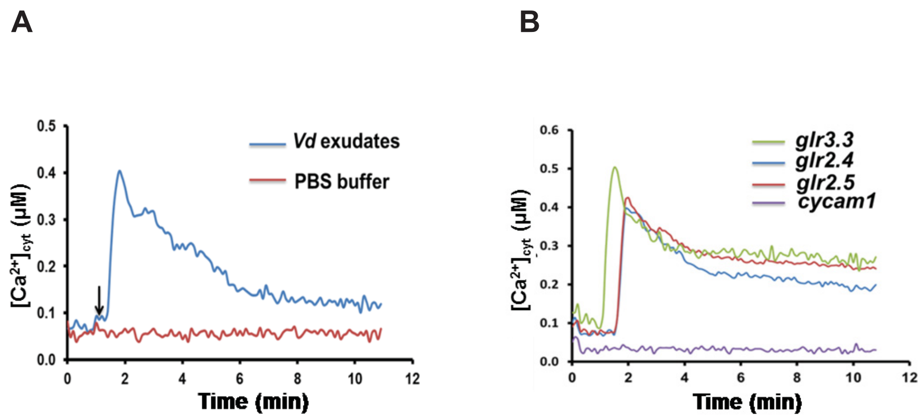


Figure 12

Supplementary Materials

Figure S1. Co-cultivation time scheme. The seeds were first kept at 4°C in the dark for 2 days and were then transferred to a light/dark cycle at 22°C for 9 days. These seedlings were used for the experiments, by either transferring them to a plate with *Vd* or *Pi* (or no fungus, control, C) at day 0. The seedlings were harvested 10, 14 or 21 days later. In case of transfer from *Vd* to *Pi* or *vice versa*, the transfer occurred at day 4.

Figure S2. Induction of *GLR* genes in shoots of Arabidopsis seedlings after 1 and 14 days.

Figure S3. Phenotype of *ein3-1* and WT after 21 days of co-cultivation following the 5 treatments described in Methods and material.

Figure S4. ET content in shoots of *ein3-1* seedlings after 3 weeks.

Figure S5. Phenotypes of WT and *ein3-1* after *Vd* spore inoculation *in vivo* and *in vitro*.

Figure S6. Phenotype of WT and *cycam1* mutant 21 days after *Vd* inoculation.

Figure T1. Primer list for RT-PCR.

Supplementary figures

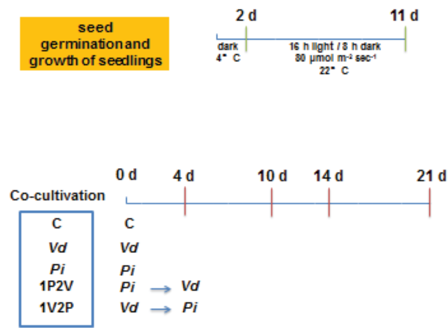
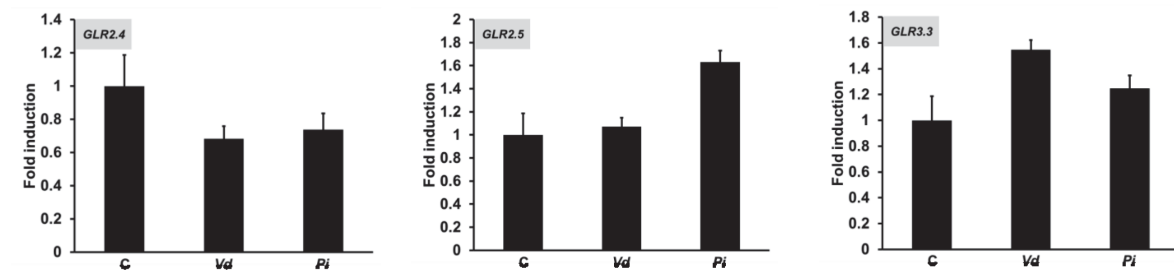


Figure S1

1 d



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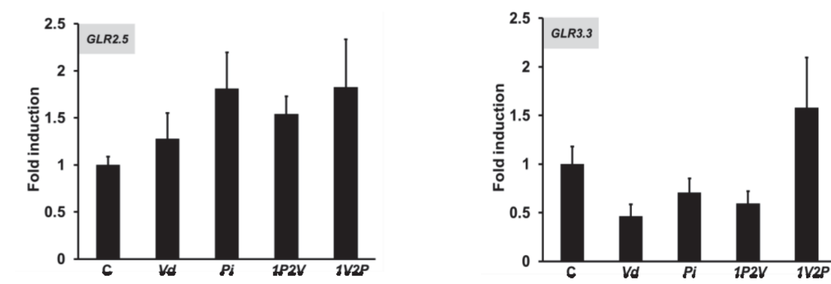


Figure S2

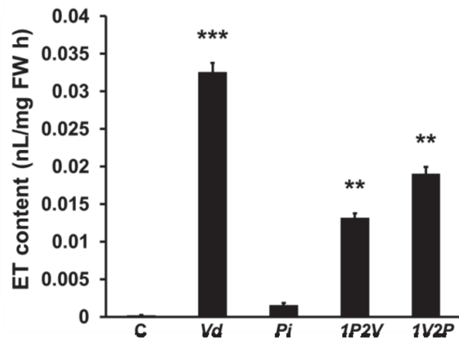
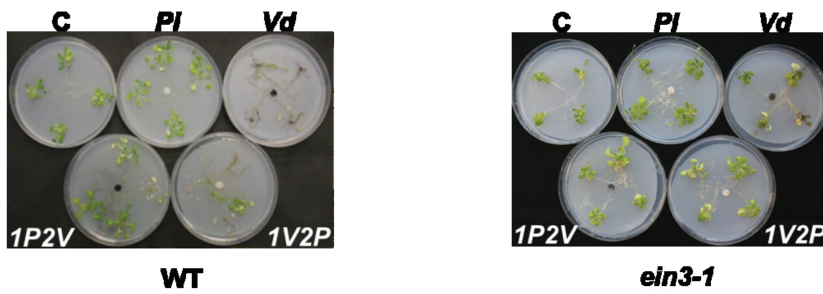


Figure S3



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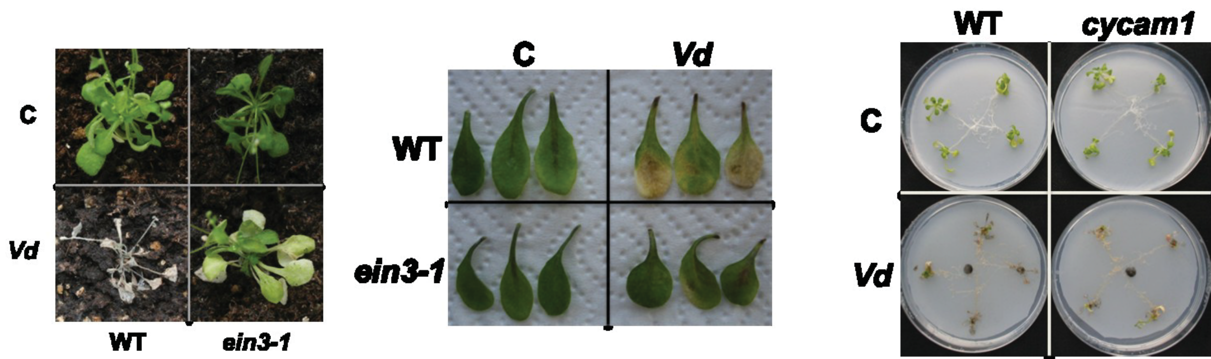

Figure S5 **Figure S6**

Supplementary Table 1

Primer pairs used for real-time PCR and PCR analysis.

PR1	At2g14610	5´-GCTCAAGATAGCCCACAAGA-3´ 5´-ACACCTCACTTTGGCACATC-3´
PDF1.2	At5g44420	5´-CTTGTGTGCTGGGAAGACATA-3´ 5´-AGCACAGAAGTTGTGCGAGAA-3´
PR-2	At3g57260	5´-TCTTCTCAGCCTTGTAATAGC-3´ 5´-TGTTTGTAAAGAGCCACAACG-3´
ERF1	At3g16770	5´-GGGGAAACGGAGGAAGAGG-3´ 5´-GCGATGACGGCGGAGGAGTAT-3´
VSP2	At5g24770	5´- GAGCTGACTACGTTGTTGAG-3´ 5´- GGAGACAATGTCAAGGTCGG-3´
GLR2.4 (for Salk line)	At4g31710	5´-CTTCTCGTCCAGAATCTCGGACC-3´ 5´-GTCAAGGAAAGGGCTCGTTGCAG-3´
GLR2.5 (for Salk line)	At5g11210	5´-CCATGTGTCTCACTGTTGGTCG-3´ 5´-GCAGATGAGGAGTTCAGGGAACAG-3´
GLR3.3 (for Salk line)	At1g42540	5´-ACGGCTGCAGTGAATCTTTT-3´ 5´-AGAAAGCAACAACCCGTGAC-3´
RabGAP11		5´-GCACCTCCCACTGATGATTT-3´ 5´-AACTGCCCTGCCATACTTTG-3´
GLR2.4 (for RT-PCR)	At4g31710	5'-AGGGAAAACATGTGATTGTGC-3' 5'-TCCAATAATGCCCTTGTCAAG-3'
GLR2.5 (for RT-PCR)	At5g11210	5'-CCTTTTAGGTGTTCAAAGGGG-3' 5'-CAGCAGAAGAGAGGTACACCG-3'
GLR3.3 (for RT-PCR)	At1g42540	5'-GATGCTGCATATGGTTGTGTG-3' 5'-GTTGAACGATAAGCTTGCGAG-3'
P. indica	elongation factor	5'-CGCAGAATACAAGGAGGCC-3' 5'-CGTATCGTAGCTCGCCTGC-3'
V. dahliae		5'CCGCCGGTCCATCAGTCTCTCTGTTTATAC-3' 5'-CGCCTGCGGGACTCCGATGCGAGCTGTAAC-3'

4.6 Manuscript VI

Technical note:

***Piriformospora indica* hyphae and chlamydospores by Scanning Electron Microscopy**

Chao Sun and Ralf Oelmüller

Journal of Endocytobiosis and Cell Research (2010), 34-37

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Journal of Endocytobiosis and Cell Research

Technical note:
Piriformospora indica hyphae and chlamydospores by scanning electron microscopy

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Piriformospora indica **is an endophytic fungus with a wide host range. The hyphae of the fungus can penetrate into the root cortex and form chlamydospores. After culturing of *P. indica* on synthetic media we performed scanning electron microscopy to analyze the form and structure of the hyphae and chlamydospores. The hyphae are straight and hyaline, and the surface of the hyphal walls is smooth. The chlamydospores are pear-shaped and have smooth walls. We provide a protocol for the visualization of fungal hyphae and spores by Scanning Electron Microscopy.**

Journal of Endocytobiosis and Cell Research (2010) 34-37
Category: technical note
Keywords: Piriformospora indica, scanning electron microscopy

Received: 29 October 2010; Accepted: 22 November 2010

Introduction

The endophytic fungus *Piriformospora indica* interacts with the roots of many different species and promotes growth and performance of the hosts. *Oryza sativa, Triticum sativum, Hordeum vulgare, Zea mays, Setaria italica, Sorghum vulgare* (Varma et al. 1999, 2000); *Spinacia oleracea* (Kumari et al. 2003), *Glycine max*, *Cicer arietinum*, *Solanum melongena*, *Nicotiana tabacum*, *Petroselinum crispum*, *Artemisia annua*, *Bacopa monniera*, *Cymbidium spp.* (Varma et al. 2000), *Spilanthes calva, Withania somnifera, Adhatoda vasica Nees* (Varma et al. 1999, 2000; Kumari et al. 2003), *Arabidopsis thaliana* (Shahollari et al. 2005) and *Brassica chinensis* (Sun et al. 2010) have been investigated so far. *P. indica* colonizes the roots, grows inter- and intracellularly, forms pear-shaped spores within the cortex, but does not invade the endodermis and aerial parts of the plants. The endophytic fungus also promotes nutrient uptake, allows plants to survive under water, temperature and salt stress, and confers tolerance to toxin, heavy metal ions and pathogenic organisms (Oelmüller et al. 2009). For beneficial interactions, the degree of root colonization is very critical. Over- and under-colonization impairs the benefits for both interaction partners (Sherameti et al. 2008). Here, we describe a protocol for the visualisation of the ultrastructure of the fungus, and present first results.

Until now, the morphology of *P. indica* hyphae and spores in colonized roots is mainly analysed by staining methods. Others studies used epifluorescense (Verma et al. 1998) or confocal microscopy (Stein et al. 2008, Waller et al. 2005). Those methods allow the analysis of the morphology of the hyphae and spores only in two dimensions. We used scanning electron microscopy (SEM) to analyse *P. indica* spores and hyphae since SEM is a useful technique to study morphology and surface microstructure of various biological specimens in three dimensions (Bricelj 2006).

The Method

SEM (Figure 1) is a type of electron microscopy that images the sample surface by scanning it with a high-energy beam of electrons in a raster scan pattern. The electrons interact with the atoms and produce signals about the sample's external morphology, texture, crystalline structure and orientation of surface-bound structures (Vernon-Parry 2000). The method is outlined in the Method box –buffers and solution- and box –protocol-.

Results

The *P. indica* hyphae are straight. The surface of the hyphal walls is smooth and almost hyaline, but in older cultures the hyphae become irregularly inflated. After 15 days of cultivation of the fungus on PDA medium (Figure 2), the pear-shaped chlamydospores are formed from vesicles at the tips of the hyphae. They appear singly or in clusters. The walls of chlamydospores are smooth.

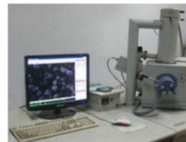

Figure 1: Scanning electron microscope XL30E, Philips (NL)

Figure 2: *P. indica* grown for 15 days on plates with PDA medium

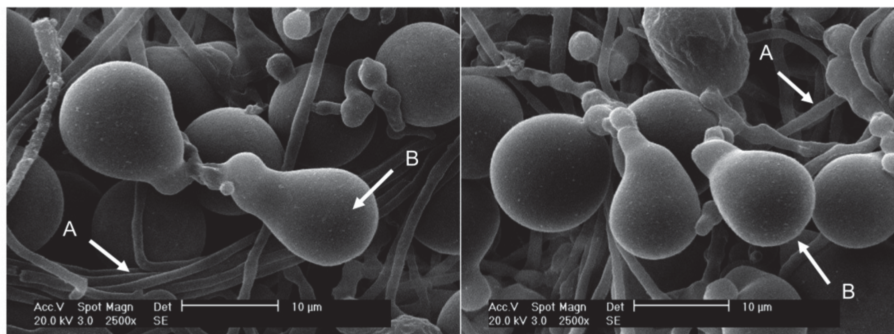

Figure 3: The hyphae and chlamydospores of *P. indica* under SEM (magnification times 2500). The fungus grown for 15 days on PDA medium. (A) Hyphae, (B) chlamydospore.

Conclusion

Under SEM, the morphology of the hyphae and spores of *P. indica* can be seen in three dimensions. The hyphae are smooth, straight and closely attached to each other (Figure 3). The chlamydospores are pear-shaped. They have smooth walls. These results are in agreement with the observations by others (Verma et al. 1998, Waller et al. 2005). As an endophytic fungus, the hyphae of *P. indica* penetrate into the root cortex and form coils, branches or round bodies (Varma et al. 1999). A comparative analysis of the hyphal structure in different hosts may help to understand differences in the interactions and the benefits for both symbionts.

Achnowledgements

The authors like to thank Dr. Irena Sherameti, Iris Camehl, Joy Michal Johnson and Pyniarlang Nongbri for critically reading the manuscript and Binggan Lou and Jie Ma for help. They also like to thank the Analysis and Test Center of Zhejiang University for their help with the SEM protocol.

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Box - Buffers and Solutions

(1) 0.1 mol/L phosphate buffer
Solution A: 0.2 mol/L disodium hydrogen phosphate solution
Na_2HPO_4 28.40 g
Solution B: 0.2 mol/L sodium dihydrogen phosphate solution
Na_2HPO_4 24.00 g
Mix 61.0 ml of solution A and 39.0 ml of solution B, add 100 ml of distilled water.

(2) 2.5% glutaraldehyde solution (for 100 ml)

25% glutaraldehyde solution	10 ml
0.2 mol/L phosphate buffer	50 ml

Add distilled water to 100 ml

(3) 1% buffered osmium tetroxide (for 10 ml)

2% osmium tetroxide aqueous solution	5 ml
0.2 mol/L phosphate buffer	5 ml

Box – Protocol

Step 1	Cultivate *P. indica* on Potato Dextrose Agar (PDA) medium for 15 days. 3mm×2mm fungal plugs from the middle of the colony are taken as samples
Step 2	Fix the sample at 4°C in 2.5% glutaraldehyde solution overnight
Step 3	Remove the fixing solution and rinse the samples 3 times (15 min each) with 0.1M phosphate buffer (pH 7.0)
Step 4	Fix the samples 1-2h in 1% buffered osmium tetroxide at the room temperature
Step 5	Remove 1% buffered osmium tetroxide and rinse the samples 3 times (15 min each) with 0.1M phosphate buffer (pH 7.0)
Step 6	Dehydrate the samples in ethanol grads (70, 80, 90, 95%) for 15 min at each concentration. Finally, wash the samples in 100% ethanol twice (20 min each)
Step 7	Put the samples in ethanol/isoamyl acetate solution (v:v = 1:1) for 30 min. Then treat the samples with isoamyl acetate alone for 1-2h
Step 8	Critical point drying is processed with a HCP-2 critical point dryer. (Hitachi, Tokyo, Japan). Carbon dioxide is used as the transitional fluid
Step 9	Coat the samples with gold
Step 10	Observe the samples under SEM and take photos.

5. Discussion

Plant roots are surrounded by huge amount of microorganisms and always challenged by severe abiotic and biotic stress. To avoid stress attack and survive in this diverse microbial environment, plants have developed precised detection systems to percieve and respond to the abiotic conditions and distinguish pathogenic microorganisms from beneficial microorganisms. Since long time, researchers are focused on mycorrhizal fungi and how they help plants. We introduced a new model with the endophytic beneficial fungus, *Piriformospora indica*, to study the mechanisms of tolerance to stress.

My work investigated (1) the role of antioxidant enzymes, the expression of drought-related genes and the plastid-localized Ca^{2+}-sensing regulator (CAS) protein in the beneficical interaction between Chinese cabbage and *P. indica* under drought condition, (2) the performance of stress-related phytohormones (ET, JA, JA-Ile, OPDA, ABA and SA), the role of the pathogen defense pathway, and Ca^{2+}-related genes after the interaction of *A. thaliana* with *P. indica* and/or the pathogenic fungus *V. dahliae*, (3) the different effects of auxin in Chinese cabbage / *A. thaliana* and *P. indica* interaction.

I propose: (1) the antioxidant enzymes and drought-related genes are upregulated and the CAS protein level is increased in *P. indica*-colonized Chinese cabbage. (2) The stress-related phytohormones accumulate much less and the pathogen defense pathway and Ca^{2+}-related genes are highly suppressed in *P. indica*-pretreated plants after *V. dahliae* infection and the pathogenecity of *V. dahliae* development needs EIN3. (3) Auxin plays the target role in the Chinese cabbage / *P. indica* interaction, but not in the *A. thaliana* / *P. indica* interaction.

5.1 Role of *P. indica* in the Chinese cabbage / *P. indica* interaction under abiotic stress

The abiotic stress is defined as the negative impact of non-living factors on the living organisms in a specific environment. The non-living variable must influence the environment beyond its normal range of variation to adversely affect the population performance or individual physiology of the organism in a significant way (Vinebrooke, *et al.*, 2004). In plants, abiotic stresses include salinity, drought, flooding, heavy metals, temperature, gases and nutrient deficiency or excess (Nadeem *et al.*, 2014). It is essentially unavoidable and disturbs the growth and productivity of crops worldwide severly (Gao *et al.*, 2007). Hormonal and nutritional imbalance, physiological disorders such as abscission and senescence, and susceptibility to diseases are considered to be a few of the common impacts of the stress on

plant growth (Ashraf, 1994; El-Iklil *et al.*, 2000; Nadeem *et al.*, 2010b; Niu *et al.*, 1995; Zhu *et al.*, 1997).

Antioxidant enzymes

Plants live in a variable environment where they are often challenged by different abiotic stress factors. Thus, they have developed efficient mechanisms that allow them to detect and respond to complex stress conditions, overcoming the negative impact of stress either alone or with the help of plant growth promoting rhizobacteria (PGPR) and mycorrhizal fungi (Belimov *et al.*, 2001; Glick 2010; Ma *et al.*, 2011; Mayak *et al.*, 2004b; Nadeemet *et al.*, 2007; Sandhya *et al.*, 2009; Zahir *et al.*, 2008; Adewole *et al.*, 2010, Bhosale and Shinde, 2011; Sannazzaro *et al.*, 2006; Selvakumar and Thamizhiniyan, 2011; Shinde *et al.*, 2013). The most common response of plants under stress conditions is overproducing reactive oxygen species (ROS), such as superoxide, hydrogen peroxide and hydroxyl radical, which cause cellular damage by oxidating lipids and proteins, chlorophyll bleaching, harm to nucleic acids, finally resulting in cell death (Apel and Hirt, 2004; Ashraf, 2009; del Rio *et al.*, 2003; Herbinger *et al.*, 2002). To abolish the over-amount of ROS, the plants can produce antioxidant enzymes like superoxide dismutase (SOD), peroxidase (POD), ascorbate peroxidase (APX), glutathione reductase (GR), glutathione S-transferase (GST) and catalase (CAT) (Abdel Latef, 2011; Ashraf, 2009; Ashraf and Ali, 2008). It is already reported for many plant species that the activities of above antioxidants can be highly stimulated by the colonization of mycorrhizal fungi alone or under salt, drought, heat stresses (Lambais *et al.*, 2003, Wu *et al.*, 2006, He *et al.*, 2007, Arcía *et al.*, 2006, Lee *et al.*, 2012, Maya *et al.*, 2013, García-Sánchez *et al.*, 2014, Alguacil *et al.*, 2006). The similar phenomenon was also observed in barley/*P. indica* interaction. Baltruschat *et al.*, (2008) found that under salt stress, the decline of the activities of CAT, APX, dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDHAR) and GR was delayed and less pronounced in the roots of *P. indica*-colonized salt-sensitive barley cultivar Ingrid and in the salt-tolerant cv. California Mariout, although enzyme activities decreased after an initial induction in both salt-sensitive and -tolerant plants (Baltruschat *et al.*, 2008). In plant/*P. indica* interaction studies, most of the investigations on antioxidants were carried out under biotic stress contiditions. My findings filled the gaps.

Our experiment designed drought stress conditions in Chinese cabbage by applying a 20% polyethylene glycol (PEG) solution. We observed a strong dehydration phenotype in uncolonized plants, but not in the colonized ones. In the leaves, the antioxidant enzymes were also examined. Without PEG application, the activities of SOD, POD and CAT did not show significantly changes with/without fungal colonization. But after 1 day PEG application, all the three enzymes were highly stimulated in colonized drought-exposed leaves. SODs are identified as an essential component in defense mechanism of plants against oxidative stress. They are metal containing enzymes that catalyze the dismutation reaction of superoxide into H_2O_2 and O_2 and generally occur in three different molecular forms as Cu/Zn-SOD, Mn-SOD and Fe-SOD (Bowler *et al.*, 1992). In higher plants, SOD isozymes are present in different cell organs. Mn-SOD is present in mitochondria and peroxisomes, Fe-SOD mainly in chloroplasts, and Cu/Zn-SOD in cytosol, chloroplasts, peroxisomes and the apoplast (Corpas *et al.*, 2001, 2006). CATs are tetrameric heme-containing enzymes. They are found in all aerobic organisms and serve to rapidly degrade H_2O_2. They have multiple enzymatic isozymes. Under stress conditions, CAT is considered to be one of the most rapidly turned over proteins in leaf cells. Meanwhile, leaf CAT activities decline (Hertwig *et al.*, 1992). A large gene family encodes PODs which are heme-containing glycoproteins in plants. PODs participate in various physiological processes where they catalyze the conversion of hydrogen peroxide to water. PODs are involved in lignification, cross-linking of cell wall structure proteins and recovery of cell membrane damage under heat stress (Gulen and Eris, 2004). Our results showed elevated antioxidant enzyme levels (SOD, POD, CAT) in the protected leaves of the fungus-colonized plants which suggests that the antioxidants could be a main target of *P. indica* in the drought tolerance. In Arabidopsis, two enzymes MDHAR and DHAR which maintain ascorbate in the reduced state in ascorbate-glutathione cycle were upregulated in the leaves of *P. indica* colonized plants. In addition, two Arabidopsis mutants, *mdar2* and *dhar5* did not respond to the endophyte (Vadassery *et al.*, 2009b). These data suggested that MDHAR and DHAR play an important role for *P. indica* to establish a mutulistic interaction with plants.

Photosynthesis

Plants respond to abiotic stress through various biochemical and physiological processes. Photosynthesis, the basic physiological process in plants, can be severely interrupted in all its

phases by stress. Photosynthetic capacity is influenced by many different factors, such as photosynthetic pigments and photosystems, the electron transport system, and CO_2 reduction pathways (Ashraf and Harris, 2013). Under drought stress, not only photosynthetic pigments are damaged, but also the thylakoid membranes (Huseynova *et al.*, 2009, Anjum *et al.*, 2011, Kannan and Kulandaivelu, 2011). In the meantime, decreased Chlorophyll content is also observed (Bijanzadeh and Emam, 2010, Mafakheri *et al.*, 2010, Din *et al.*, 2011). Stress factors destroy the photosystem *via* damaging photosynthetic pigments and result in a reduced light-absorbing efficiency of both photosystems (PSI and PSII) and hence a reduced photosynthetic capacity which can be indicated by the Fv/Fm ratio (Geissler *et al.*, 2009, Zhang *et al.*, 2011). The Fv/Fm ratio determines the maximum quantum efficiency of PSII and measures the rate of linear electron transport (Jamil *et al.*, 2007, Tang *et al.*, 2007, Balouchi, 2010). Usually, the Fv/Fm value is around 0.8 in healthy plant leaves. Thus, a lower value often is obtained under stress conditions indicating that the damaged PSII reaction centers result in the photoinhibition (Baker and Rosenqvist, 2004; Zlatev, 2009; Vaz and Sharma, 2011). Our data demonstrate the decreased Chlorophyll content, Fv/Fm ratio and down-regulation of genes and proteins which are involved in the phosynthetic machinery and biosynthetic pathways, for instance, *psbO*, *psbA*, D1 and PsaA in the drought stress-challenged Chinese cabbage leaves. But with *P. indica*-colonized plants, these phenomenons were not observed. Instead, the fungus protected the plants from drought stress and kept the photosynthesis capability similar to the control which was not served with PEG. It looks that *P. indica* creates an atmosphere in the cell that prevents a general degradation of chloroplast function and also transfers a protect signal from root to shoot.

In the cells of plants and algae, plastids are the major organelles where the photosynthesis takes place. They also store products, such as starch, fatty acids and terpenes, which can be used as energy resource, as well as the raw material for synthesizing other molecules (Bräutigam *et al.*, 2009). Interestingly, the plastid-localized Ca^{2+}-sensing refulator CAS was upregulated both at the protein and mRNA level in *P. indica*-colonized Chinese cabbage under drought stress. The CAS protein controls cytosolic calcium transients and stomata closure (Nomura *et al.*, 2008; Weinl *et al.*, 2008). Thus, the elevated CAS protein and *CAS* mRNA level can be an efficient strategy of *P. indica* to protect the plants from dehydration. The Chinese cabbage / *P.indica* interaction provides a model system to investigate the role of the CAS protein under stress conditions in greater detail.

5.2 Role of *P. indica* in *A. thaliana*/*P. indica* interaction under biotic stress

Biotic stress is defined as that the living organisms, such as bacteria, viruses, fungi, parasites, and insects cause the damage to plants. Because of the easy spread from one plant to another, biotic stress is more difficult to be controlled (Flynn, 2003). The main tasks now and in the future are to generate stress resistant cultivars and biocontrol agents. The biocontrol agents can perform direct or indirect antagonism, for instance, producing antibiotics and lytic enzymes (Pal *et al.*, 2006).

As a promising biocontrol agent, *P. indica* has showed the strength in protecting plants from pathogen attack. The fungus confers systemic disease resistance in different plant species such as barley, wheat, Arabidopsis, tomato, against leaf-, root- and stem-based pathogens, for instance, *Blumeria graminis* f. sp. *tritici*, *Fusarium culmorum*, *Fusarium verticillioides*, *Pseudocercosporella herpotrichoides, Golovinomyces orontii* (Molitor *et al.*, 2011; Stein *et al.*, 2008; Kumar *et al.*, 2009; Serfling *et al.*, 2007; Fakhro *et al.*, 2010). Our results confirmed that *P. indica* is a strong inhibitor against the vascular root colonizing pathogen *V. dahliae* in Arabidopsis seedlings. We pretreated the roots of Arabidopsis with *P. indica* and then infected them with *V. dahliae* and *vice-versa*. We observed that in the *P. indica*-pretreated seedlings and adult plants survived much better than that of in *V. dahliae*-pretreatment. The seedlings kept the biomass and total chlorophyll as the mock-treated control, surpressed pathogenesis-related (*PR*) gene expression, increased opening stomata, inhibited the spread of *V. dahliae* hyphae both in shoots and roots and reduced the stress-related phtohormone accumulation. We did not or only partially observe these phenomenons in plants which were first exposed to *V. dahliae*. This is probably caused by the restriction of *V. dahliae* growth in the roots. An efficient biocontrol agent must activate plant defense mechanisms on various levels. *Trichoderma* spp. has been long studied and widely used as biological control agent against abiotic stress and phytopathogens attack. The metabolites they produed can inhibit spores germination, kill the cells or modify the rhizosphere by acidifying the soil, resulting in preventing the growth of the pathogen (Benítez *et al.*, 2004). A typical antagonistic mechanism of Trichoderma strains against pathogens involves sensing of the host pathogen, attraction, attachment, coiling around and lysis by hydrolytic enzymes, in most common cases, the secondary metabolites like glucose oxidase, chitinase and glucanase also play important roles in the antagonism (Mukherjee *et al.*, 2012; Tjamos, *et al.*, 2000, 2005). We observed that *P. indica* strongly inhibites the growth of *V. dahliae* hyphae both on

PDA plates and inside the plants and also the formation of microsclerotia. The antagonistic effect of *P. indica* was also observed in other pathogens in dual culture (Serfling *et al.*, 2007; Johnson *et al.*, 2013). This may rely on the enzymes or metabolites secreted by *P. indica*. While the physical conflict of the hyphae of the two fungi is not yet understood. *Trichoderma* spp. can also induce the host plant hypersensitive response (HR), systemic acquired resistance (SAR), and induced systemic resistance (ISR) by activating enzyme related defensive mechanisms (Harman *et al.*, 2004).

Xylanase and peptaibols (peptaibiotics with high content of alpha amino isobutyric acid) like alamethicin and trichovirin II which are produced by *Trichoderma* spp. were also reported to elicit an immune response in plants (Leitgeb *et al.*, 2007; Viterbo *et al.*, 2007; Luo *et al.*, 2010; Druzhinina *et al.*, 2011). *P. indica* has many common defense mechanisms with *Trichoderma* strains. *P. indica* induces the whole plant systemic disease resistance, plant defense (*PDF*) genes and activates antioxidants like DHA, DHAR, CAT, SOD, GR and GST (Waller *et al.*, 2005; Kumar *et al.,* 2009). ET is necessary for *P. indica* to establish the beneficial interaction with plants (Camehl *et al.*, 2010) and also cyclophilin proteins which are found in almost all eukaryotic and prokaryotic organisms and are localized in the cytosol and in nucleus (Wang and Heitman, 2005) to induce the stress responses (Ansari *et al.*, 2013).

5.3 *V. dahliae* induces the upregulation of *GLR* genes and cytosolic calcium ($[Ca^{2+}]_{cyt}$) elevation

In the Arabidopsis genome, the glutamate receptor-like (*GLR*) gene family contains 20 identified gene members which encode non-selective cation channels (NSCCs) which are proposed to be associated with Ca^{2+} influx through the plasma membrane. From the gene family, 3 genes were studied because they participate in plant defense. *GLR2.4* is induced by nematodes in Arabidopsis roots (Hammes *et al.*, 2005). The histone H2B monoubiquitination regulates microtubules and GLR2.4 promotes their reorientation in hypocotyls (Cao *et al.*, 2013; Farquharson, 2013) during the defense response against *V. dahliae* in Arabidopsis (Hu *et al.*, 2014). Both *GLR2.5* and *GLR3.3* are required for cell and plant defense in wounding (Guan and Nothnagel, 2004; Mousavi *et al.*, 2013). *GLR3.3* induced resistance to *Hyaloperonospora arabidopsidis* and the protein also mediates Arabidopsis immunity responses by inducing glutathione-triggered cytosolic calcium transients and transcriptional changes (Li *et al.*, 2013). The *GLR2.4* responded strongly to *V. dahliae* infection but was

suppressed in the shoots of the seedlings when the plants were pre-treated with *P. indica*. The data suggested that Ca^{2+}-related genes are involved in plant defense. A fraction of *V. dahliae* preparation induced a $[Ca^{2+}]_{cyt}$ elevation in WT Arabidopsis roots. We used the three *glr* mutants which are in the apoaequorin background to test whether the $[Ca^{2+}]_{cyt}$ elevation is mediated by the *GLRs*. Although the mRNA level of *glr2.4* was highly upregulated by *V. dahliae* infection, we did not observe differences of the calcium response compared to the wild-type. These data suggests that *GLRs* play different roles in the interaction. In addition, the *cycam1* mutant which does not have any $[Ca^{2+}]_{cyt}$ response to different elicitors (Johnson *et al.*, 2014) remained the same after applying the *V. dahliae* exudate preparation. It is already known that the complex crude exudates of *Verticillium* spp. which are responsible for multiple disease symptoms such like wilting, dehydration, chlorosis and necrosis contain cell-wall-degrading enzymes and phytotoxins. They are high-molecular weight protein-lipopolysaccharide complexes (PLPCs), glycoproteins, 11-amino acid peptides, 25.9 kDa and 18.5 kDa proteins (Buchner *et al.*, 1989, 1982; Nachmias *et al.*, 1985, 1987; Clovis, 2006). Proteomic analysis uncovered that the identified sequences have roles in stress responses, colonization, melanin biosynthesis, microsclerotia formation, antibiotic resistance, and fungal penetration (El-Bebany *et al.*, 2010). Nowadays, the *V. dahliae* crude exudates are always taken to elicit plant defense and to study physiological responses of the plants. For instance, it can induce H_2O_2 and NO production in Arabidopsis (Jiang *et al.*, 2005; Yao *et al.*, 2012). Although the *V. dahliae* toxin has been long studied, the exact components and their function in the disease pathogenicity development and disease symptoms remained to be investigated.

5.4 *V. dahliae* requires EIN3 for pathogenicity development

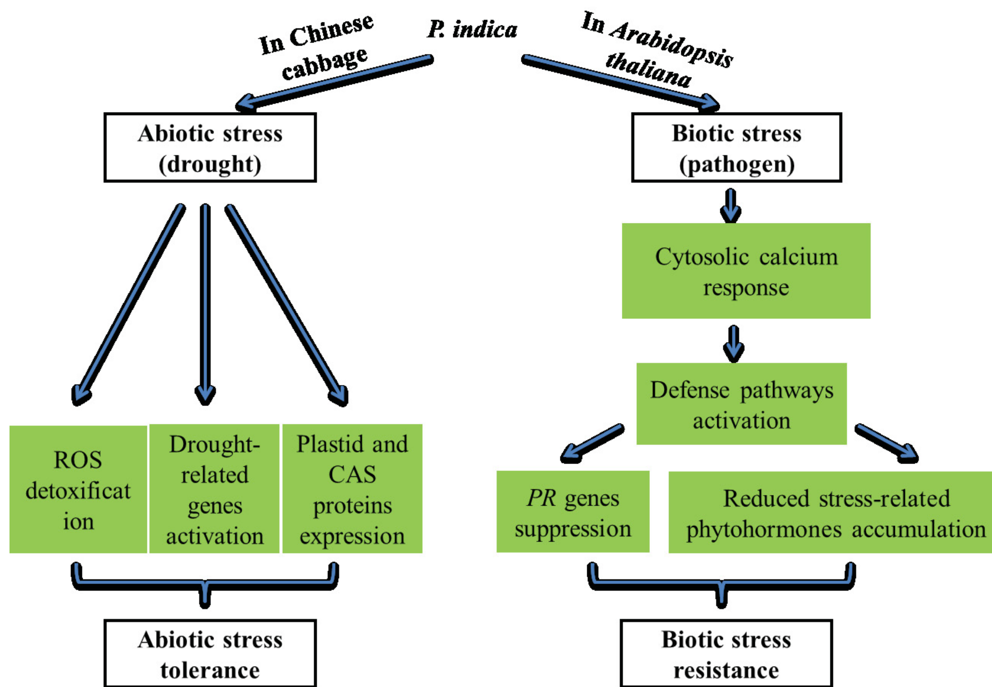
V. dahliae is a very aggressive pathogen and even low amount of infection can result in destroying the whole plant. In the presence of *P. indica*, the formation of microsclerotia was highly inhibited in both *1P2V* and *1V2P* treatments. But the amount of stress phytohormones was no significantly different in *1V2P* and plants treated with *V. dahliae* alone. Even in the shoots of *1P2V*, there was still much higher accumulation compared to the mock treatment. These results suggested that the complex life circle and infection sites is important for preventing the hyphae spread. Since long time, researchers have focused on disease resistant genes and are looking for biocontrol agents against *V. dahliae*. In several plant species, including alfalfa (*Medicago sativa*), cotton (*Gossypium hirsutum*), potato (*Solanum tuberosum*), strawberry (*Fragaria vesca*), sunflower (*Helianthus annuus*), and tomato

(*Solanum lycopersicum*), sources of genetic resistance to Verticillium have been described (Schaible *et al.*, 1951; Lynch *et al.*, 1997, Bae *et al.*, 2008). Posttranscriptional gene silencing is thought to be crucial for the basal defense against Verticillium in Arabidopsis (Ellendorff *et al.*, 2009). In tomato, the *Ve* gene confers resistance against race 1 isolate of *V. dahliae* (Kawchuk *et al.*, 2001; Fradin *et al.*, 2009) and also plays a role in transgenic Arabidopsis plants (Fradin *et al.*, 2011). Overexpression of plant defensin NaD1 also showed field resistance against *V. dahliae* (Gaspar *et al.*, 2014). Biological control agents like *Trichoderma* spp., endophytic bacterial isolate (HA02) and *Pseudomonas fluorescens* PICF7 were also efficient against *V. dahliae* infection (Khiareddine *et al.*, 2009; Maldonado-González *et al.*, 2012; Li *et al.*, 2012). Here, we demonstrate that EIN3 plays an important role in the development of pathogenicity. We observed a delayed disease symptoms and strongly resistance in *ein3* mutant but not in other JA-, SA-deficient mutants. This phenomenon is also approved by the data from Pantelides *et al.*, (2010) that ethylene reception *via* the receptor ETR1 is required for *V. dahliae* infection in Arabidopsis. Another Verticillium species, *Verticillium longisporum*, requires JA-dependent COI1 to elicit the disease symptoms in Arabidopsis shoots (Ralhan *et al.*, 2012). In addition, we also observed the higher ET level in *ein3* mutant than in WT after *V. dahliae* infection. This suggests that the lack of EIN3-induced defense responses in *ein3* mutant results in an additional stimulation of ET synthesis.

5.5 Different roles of auxin in Chinese cabbage and Arabidopsis after the interaction with *P. indica*

Auxin is involved in lateral root growth and root hair development (Fukaki and Tasaka, 2009). Mycorrhizal fungi, plant-growth-promoting rhizobacteria and beneficial root-colonizing endophytic fungi interfere with auxin metabolism or auxin functions in the plants. In 2007, Sirrnberg *et al.*, demonstrated that auxin released from *P. indica* stimulates the plant growth and also changes the root morphology. In our study, the shoots and roots of Chinese cabbage are strongly promoted by *P. indica* and the auxin level is two-fold higher than uncolonized plant roots. The bushy root phenotype confirms that auxin is involved in the interaction. The overexpression of an auxin transporter gene from Brassicaceae *BcAUX1* in Arabidopsis results in a strong biomass increase but not bushy roots. This suggests that *BcAUX1* is a target of *P. indica* in Chinese cabbage. However, the exogenous application of auxin could not replace *P. indica*. An exudate component from *P. indica* which does not contain auxin promotes the growth of both hosts. This demonstrates that auxin signaling instead of auxin

itself is more important for the Chinese cabbage/*P.indica* interaction. Interestingly, we did not observe such an important role of auxin in the interaction as in Chinese cabbage. Many genes which are involved in the auxin biosynthesis, signaling and transportation were not upregulated in fungus-colonized roots. Furthermore, mutants with reduced auxin level still responded normally to *P. indica* (Vadassery *et al.*, 2008; Lee *et al.*, 2011). These results suggest that the promoted growth by *P. indica* in Arabidopsis is not dependent on the auxin released by the fungus and also the auxin biosynthesis and signaling do not play crucial role in the growth promotion.



The last figure summarizes my results in a scheme. I have investigated abiotic and biotic stress responses in the two Brassicaceae species, Chinese cabbage and *Arabidopsis thaliana*. Calcium plays an important role and the $[Ca^{2+}]_{cyt}$ level increases in response to exudated compounds from *P. indica* and the pathogen *V. dahliae*. The different downstream responses leading to *P. indica*-mediated abiotic stress tolerance and biotic stress resistance which were investigated in my thesis are shown in green.

6. Summary

P. indica is a beneficial root endophytic fungus which promotes plant growth and uptake of nitrate and phosphate and induces resistance against abiotic and biotic stress. The wide host range also provides the possibility for invesgating the role of the multiple functions in the symbiosis. In my research, the following 3 aspects were investigated.

1. The role of *P. indica* in Chinese cabbage/*P. indica* interaction under drought stress

P. indica strongly colonizes the roots of Chinese cabbage and promotes lateral root development. Under the mimicked drought stress conditions by applying PEG, the activities of antioxidants were highly upregulated within 24 h in the leaves of fungal-colonized plants. *P. indica* also prevents the degradation of chlorophyll and thylakoid proteins. The biomarker of oxidative stress MDA accumulates less in the drought-exposed leaves of colonized plants. In addition, the drought-related genes were upregulated and the amount of the CAS protein was strongly increased in the leaves of the colonized plant. The drought stress was reliefed in the presence of *P. indica*. Thus, *P. indica*-colonized Chinese cabbage provides a good model system to study root-to-shoot communication.

2. The role of *P. indica* in Arabidopsis/*P. indica* interaction under *V. dahliae* challenge

P. indica reduces *V. dahlia*-mediated disease development and inhibits growth of *V. dahliae* on agar plates as well as in seedlings. The *P. indica*-pretreated plants perform better after *V. dahliae* infection, do not activate stress hormones and defense genes and the number of microsclerotia is dramatically reduced. In addition, the pathogenicity of *V. dahliae* is associated with an increase in $[Ca^{2+}]_{cyt}$ induced by an exudated compound of the pathogen in Arabidopsis roots and requires the ethylene-activated transcription factor EIN3. We propose that *P. indica* is an efficient bio-control agent to protect Arabidopsis from *V. dahliae* infection.

3. Auxin plays a more important role in the interaction of *P. indica* with Chinese cabbage than with Arabidopsis

Both Chinese cabbage and Arabidopsis do not require *P. indica*-derived auxin for the growth response. But the elevated auxin levels in Chinese cabbage suggest that the *P. indica*-induced growth promotion is strongly dependent on auxin.

7. Zusammenfassung

P. indica ist ein Wurzel-kolonisierender endophytischer Pilz. Er fördert das Pflanzenwachstum, die Aufnahme von Nitrat und Phosphat und induziert Resistenz gegen abiotischen und biotischen Stress. Das große Wirtsspektrum ermöglicht die Analyse verschiedener Funktionen in der Symbiose. In meiner Arbeit wurden 3 Aspekte untersucht.

1. Die Rolle von *P. indica* in der Chinakohl/*P. indica* Interaktion unter Trockenstress

P. indica kolonisiert die Wurzeln von Chinakohl und stimuliert das Seitenwurzelwachstum. Unter den vorgetäuschten Trockenstress-Bedingungen nach PEG Applikation waren die Aktivitäten von Antioxidanzien innerhalb von 24 h in den Blättern von pilzinfizierten Pflanzen hochreguliert. *P. indica* verhindert auch den Abbau von Chlorophyll und Thylakoidproteinen. Der Biomarker für oxidativen Stress, MDA, akkumuliert weniger in Trockenstress-exponierten Blättern von kolonisierten Pflanzen. Weiterhin sind Trockenstress-Gene hochreguliert und die Menge des CAS Proteins ist stärker erhöht in Blättern von kolonisierten Pflanzen. Der Trockenstress war verringert in Anwesenheit von *P. indica*. Somit stellt die *P. indica*/Chinakohl Interaktion ein gutes Modellsystem zum Studium der Wurzel-Spross-Kommunikation dar.

2. Die Rolle von *P. indica* in der Arabidopsis/*P. indica* Interaktion nach *V. dahliae* Befall

P. indica reduziert die *V. dahliae*-induzierte Krankheitsentwicklung und verhindert das Wachstum von *V. dahliae* auf Agarplatten und in Keimlingen. *P. indica*-vorbehandelte Keimlinge wachsen besser nach *V. dahliae* Infektion, aktivieren keine Stresshormone und Verteidigungsgene. Die Zahl der Mikrosklerotien ist dramatisch reduziert. Die Pathogenität von *V. dahliae* ist mit einer Erhöhung der $[Ca^{2+}]_{cyt}$ assoziiert, die durch eine Exudatkomponente des Pathogens in Arabidopsis Wurzeln ausgelöst wird. Weiterhin ist die Pathogenität von dem Ethylen-aktivierten Transkriptionsfaktor EIN3 abhängig. Wir schlagen vor, dass *P. indica* ein effizienter *bio-control agent* für den Schutz von Arabidopsis gegen *V. dahliae* Infektion darstellt.

3. Auxin spielt eine wichtigere Rolle in der Interatkion von *P. indica* mit Chinakohl im Vergleich zur Interaktion mit Arabidopsis

Beide Spezien benötigen kein *P. indica*-synthetisiertes Auxin für die Wachstumsstimulation. Der erhöhte Level von Auxin in Chinakohl läßt vermuten, dass seine Wachstumsstimulation auf erhöhte Auxin-Level zurückzuführen ist.

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9. Appendix

9.1 Declaration of Independent Assignment

I declare in accordance with the conferral of the degree of doctor from the School of Biology and Pharmacy of Friedrich Schiller University Jena that the submitted thesis was written only with the assistance and literature cited in the text.

People who assisted in the experiments, data analysis and writing of the manuscripts are listed as co-authors of the respective manuscripts. I was not assisted by a consultant for doctorate thesis.

The thesis has not been previously submitted to the Friedrich-Schiller-University Jena or to any other university.

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Jena, 19 May 2014

9.2 Curriculum vitae

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9.3 List of publications and presentations

Publications

2014

Sun, C., Shao, Y., Vahabi, K., Lu, J., Bhattacharya, S., Dong, S., Yeh, K.-W., Sherameti, I., Lou, B., Baldwin, I.-T., Oelmüller, R. (2014). The beneficial fungus *Piriformospora indica* protects Arabidopsis from *Verticillium dahliae* infection by downregulation plant defense responses. BMC Plant Biology, 14, 268.

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Michal Johnson, J., Lee, Y.-C., Camehl, I., **Sun, C.**, Yeh, K.-W., Oelmüller, R. (2013).

Piriformospora indica promotes growth of Chinese cabbage by manipulating auxin homeostasis - Role of auxin in symbiosis. In A. Varma (Ed.), *Piriformospora indica*, Soil Biology 33. Berlin-Heidelberg: Springer-Verlag.

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Presentations

Oral Presentation

Sun, C. (2014). The beneficial fungus *Piriformospora indica* protects Arabidopsis from *Verticillium dahliae* infection. 13th IMPRS Symposium, MPI for Chemical Ecology, Dornburg, Germany.

Poster Presentations

Sun, C. (2013). The beneficial fungus *Piriformospora indica* protects Arabidopsis from *Verticillium dahliae* infection. 12th IMPRS Symposium, MPI for Chemical Ecology, Jena, Germany.

Sun, C., Oelmüller R. (2011). Role of Glutamate Receptors (GLRs) and Cation/H^+ Exchanger (CAXs) in microbe - Arabidopsis interaction. Botanikertagung, German Botanical Society, Freie Universität Berlin, Berlin, Germany.

Sun, C. (2011). Role of cytoplasmic calcium elevation in plant/microbe interactions. 10th IMPRS Symposium, MPI for Chemical Ecology, Dornburg, Germany.

9.4 Acknowledgement

It is a great honor for me to thank all of the people who helped me during my PhD period. First, I greatly appreciate my supervisor Prof. Dr. Ralf Oelmüller for providing me the chance to do my PhD in his lab, as well as the last half year of my master's period. Without his supervision, encouragement and patience, this thesis would not have been finished.

I would like to express my gratitude to my co-supervisor Prof. Dr. Ian T. Baldwin and also my master's supervisor Prof. Dr. Binggan Lou who recommended and helped me to come here for half a year exchange programm during my master's studies.

I must give my special thanks to Dr. Irena Sherameti who helped me really a lot not only on scientific questions but also privately in Jena. She is always ready to help when I need.

This thesis would not have been possible without the support and cheers of my former and current lab mates especially Dr. Iris Camehl, Dr. Joy Michal Johnson, Dr. Pyniarlang Lyngdoh Nongbri, Dr. Jyothi Vadassery, Eileen Seebald, Sindy Schedel, Doreen Meichsner, Anatoli Ludwig, Khabat Vahabi, Anna Mrozinska and Madhunita Bakshi.

I am particularly grateful for the assistance given by our technicians Claudia Röppischer and Sarah Mußbach.

I would also like to thank Dr. Klaus Appenroth, Prof. Dr. Severin Sasso, Dr. Thomas Pfannschmidt, Dr. Jeannette Pfalz, Ute, Lars, Sebastian, Björn, Moni, Matthias, Natalie and the members of the Department of Plant Physiology for helping me in many ways in the lab.

Of course, I must say thank you very much, my parents! Thank you for your understanding and all the support. You always stand by me and help me to overcome difficult situations. I feel really warm, happy and lucky to be your daughter.

Thanks also go to my friends in Germany and China, including Dr. Juanjuan Wang, Dr. Shipeng Lu, Dr. Yongqi Shao, Dr. Jing Lu, Dr. Samik Bhattacharya and Boqing Hu. With their help and encourage, I carried my PhD to the end.

This work was supported by German Research Foundation (DFG) and German Academic Exchange Service (DAAD) in cooperation with Prof. Sheqin Dong and Prof. Dr. Kai-Wun Yeh.