# The role of glucosinolates in the Arabidopsis/*Piriformospora indica* interaction


seit 1558

**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

**Pyniarlang Lyngdoh Nongbri, MSc**

geboren am 21. 01. 1983 in Shillong, India

Gutachter

1…….

2…….

3…….

Tag der öffentlichen Verteidigung

……

**Table of content**

## 1. Introduction

Although vascular plants are considered to be autonomous in their functions which enable them to carry out the normal growth and development, in reality they live in diverse communities with microorganisms. The symbiotic association of the roots with mycorrhizal fungi provides multiple benefits for the plants, such as enhanced photosynthetic efficiency, nutrient mobilization and water use, tolerance to abiotic stress and protection against pathogen attack. These benefits create ecologically sustainable crops for human consumption, fiber and biofuels production. The intimate associations between different species are widespread in nature and probably the most dominant plant/microbe interaction on earth (de Bary 1887). Already de Bary (1887) used the term 'symbiosis' to define such associations without distinguishing between beneficial and non-beneficial interactions. However, depending on the influence that the microbial partner has in the interaction, the symbiosis can range from mutualism which benefits both partners to parasitism, in which the plant host does not benefit from the interaction and can eventually die (Read *et al*. 2000). Interestingly, none of the symbiotic interactions is stable and influenced by a large range of genetic and environmental factors (Vogelsang *et al*. 2006; Ahonen-Jonnarth *et al*. 2003; Heath 2010; Heath *et al*. 2012; Thompson 1997; Aguilar *et al*. 2004; Lie *et al*. 1987; Lambers *et al*. 2009; Smith and Goodman 1999). Nevertheless, both beneficial and pathogenic interactions share many similarities. For instance, the initial steps in the recognition processes involve plant cell surface receptors signals, which in case of mycorrhiza and the legume/rhizobia symbiosis are LysM-receptor-like kinase (RLK) (Arrighi *et al*. 2006; Radutoiu *et al*. 2007). They resemble recognition mechanisms for pathogens with the chitin elicitor-binding protein, CEBiP (Kaku *et al*. 2006) and a receptor-like kinase, LYK1/CERK1 (Miya *et al*. 2007; Wan *et al*. 2008; Shimizu *et al*. 2010) in rice and Arabidopsis, respectively. The recognition of chitin and nodulation (Nod) factors follow a similar classical mode of response. In Arabidopsis, chitin has been proposed to be recognised by LYK1/CERK1, followed by activation of the mitogen-activated protein kinase (MPK) pathway, leading to the activation of several transcription factors which ultimately results to the induction of genes involved in pathogen defense (Wan *et al*. 2004, 2008; Libault *et al*. 2007). Rhizobial Nod-factor signaling is characterized by the

two-way signal exchange. Host recognition is mediated by NodD proteins which interact with specific flavonoids or isoflavonoids exuded from host roots (Spaink *et al*. 1989). Flavonoid-activated NodD promotes transcription of bacterial *nod* genes involved in synthesis and secretion of lipochitin-oligosaccharides, called Nod factors, required for initiation of nodulation (Lerouge *et al*. 1990; Spaink *et al*. 1991).

Furthermore, common features are also visible during the processes that facilitate infections by beneficial and pathogenic microbes. Establishing of the nitrogen-fixing nodules, endo-mycorrhizal structures and pathogenic infection tubes are accompanied by an initial induction of host defense responses which is followed by a subsequent suppression of this response (Salzer and Boller 2000; Sherameti *et al*. 2008a; Lopez-Gomez *et al*. 2012; Camehl *et al*. 2010b). These processes are required for the establishment of compatible pathogenic (Peleg-Grossman *et al*. 2009) or symbiotic interactions (Stacey *et al*. 2006; Peleg-Grossman *et al*. 2009; Yang *et al*. 2010) and the induction of the morphological changes in the host cells (Hahn *et al*. 1989; Collinge *et al*. 1994; Benhamou *et al*. 1996, 1997; Hammond-Kosack and Jones 1996; Van Peer *et al*. 1991; de Ruijter *et al*. 1998; van Batenburg *et al*. 1986; Radutoiu *et al*. 2003; Amor *et al*. 2003; Gapper and Dolan 2006; Brewin 2004; Cebolla *et al*. 1999). While pathogenic interactions can be studied with the plant model organism *Arabidopsis thaliana*, it does not form symbiotic interactions with mycorrhizal fungi or rhizobacteria. Therefore, comparative analysis of plant/beneficial microbe and plant/pest interactions is rare, although recent attempts to establish a legume model system may facilitate those studies in the future (Offre *et al*. 2007; Ikeda *et al*. 2008). Legumes such as *Lotus japonicus* and *Medicago truncatula* provide an alternative because of their ability to interact with pathogens as well as beneficial microbes and endosymbionts. Drawbacks resulting from the lack of comparative analyses of beneficial and non-beneficial interaction include our knowledge about common processes which progressively change during the establishment of one or the other type of microbial interactions. This is particularly interesting for plant defense processes, which play essential roles in beneficial and non-beneficial interactions.

## 1.1 Symbiotic plant/fungus interaction

Although arbuscular mycorrhizal fungi have a wide host range and can colonize the roots of about 80% of the vascular plants including the majority of crop plants, they are obligate biotrophs and cannot be cultured without host (Newman and Reddel 1987). Furthermore, rhizobial interactions are restricted to a narrow host range of plant species. An alternative can be provided by *Piriformospora indica*, a root colonizing Basidiomycete fungus of Sebacinales which was isolated a decade ago (Varma *et al*. 1999) from the Indian Thar desert. Unlike mycorrhizal fungi, *P. indica* can be cultured in synthetic medium and can colonize Arabidopsis roots (Figure 1.1). The interaction is beneficial for both partners and shares similiarites with mycorrhiza (Peškan-Berghöfer *et al*. 2004). Spores propagate and multiply in the dermal and cortical regions of the roots (Peškan-Berghöfer *et al*. 2004; Oelmüller *et al*. 2009; Lahrmann and Zuccaro 2012). Sebacinales including *P. indica* can be found worldwide in association with roots of many different plant species (Selosse *et al*. 2009). This results in growth stimulation, increased biomass and higher seed yield (Peškan-Berghöfer *et al*. 2004; Oelmüller *et al*. 2009; Verma and Varma 1998; Shahollari *et al*. 2007; Sherameti *et al*. 2005, 2008a, 2008b; Vadassery *et al*. 2009a, 2009b; Deshmukh *et al.* 2006; Lahrmann and Zuccaro 2012). The fungus also promotes nitrate and phosphate uptake and metabolism (Sherameti *et al*. 2005; Shahollari *et al*. 2004; Yadav *et al*. 2010). Besides, *P. indica* also confers tolerance against abiotic (Sherameti *et al*. 2008a; Baltruschat *et al*. 2008; Sun *et al*. 2010) and resistance to biotic stress (Oelmüller *et al*. 2009; Stein *et al*. 2008). Since *P. indica* has such a broad host range, it is likely that the interaction must employ general recognition and signaling pathway for establishing the mutualistic symbiosis. The involvement of the fungal components has been observed but not much is yet known about the molecular steps leading to *P. indica*-induced growth promotion. Vadassery *et al*. (2009a) could show that a component from the fungal cell wall can also induce plant growth, which suggests the involvement of specific receptors at the plant cell surface. This idea could also be supported by the discovery of an atypical receptor kinase with leucine-rich repeats that is essential for the growth response (Shahollari *et al*. 2007). Moreover, a fungal cell wall extract induces a rapid increase in the intracellular calcium concentration in the root, but not leaf cells. This suggests an involvement of a calcium-dependent intracellular signaling cascade during initial stages of the interaction

(Vadassery *et al*. 2009a). Interestingly, a similar mechanism (activation of a membrane-bound receptor, which induces a rapid increase in the intracellular calcium level) has been well established for pathogenic plant/microbe interactions (Frei dit Frey *et al*. 2012; Cho *et al*. 2009; Kwaaitaal *et al*. 2011; Michard *et al*. 2011; Vatsa *et al*. 2011; Stracke *et al*. 2002; Ehrhardt *et al*. 1996; Wais *et al*. 2000).

In addition, phosphatidic acid (PA), synthesized by one of the six phospholipase Ds in Arabidopsis roots, has been proposed to be a second messenger involved in the establishment of the beneficial interaction (Camehl *et al*. 2011; Hirt *et al*. 2011). PA binds to 3-phosphoinositide-dependent protein kinase (PDK), a phospholipid-activated kinase which has been well studied in mammalian systems and identified as a central growth regulator. Camehl *et al*. (2011) proposed that PDK1 might have a similar function in the Arabidopsis/*P. indica* symbiosis, in which it promotes growth and development, but does not stimulate defense gene activation. This is in contrast to an increasing number of reports which demonstrate the contribution of PA in defense processes (Li *et al*. 2009; Crawford 1995; Walch-Liu *et al*. 2006; Hirel *et al*. 2007; Li *et al*. 2006a, 2006b; Cruz-Ramirez *et al*. 2006; Anthony *et al*. 2004; Zhang *et al*. 2004; Fan *et al*. 1997; Testerink *et al*. 2007; Wang *et al*. 2000; Romanov *et al*. 2002). Thus, besides calcium-, also PA-dependent signaling events participate in both beneficial and pathogenic interactions.

Interestingly, signaling downstream of PDK1 starts with the PDK1-independent OXIDATIVE SIGNAL-INDUCIBLE1 (OXI1) kinase, which is induced by $H_2O_2$ in Arabidopsis both transcriptionally and at the posttranscriptional level (Rentel *et al*. 2004; Anthony *et al*. 2004, 2006). The inducibility by $H_2O_2$ links this pathway again to pathogenic interaction, in which the plants often increase the reactive oxygen species (ROS) levels in response to pathogen (in particular necrotroph) attacks.

In summary, these examples highlight two important aspects which can be addressed in a comparative analysis of symbiotic and pathogenic interaction with one model plant system: How can a plant establish either a mutualistic or a pathogenic interaction by using the same second messengers (calcium, PA) and signaling components (PDK1, OXI1)? These components can be involved in separate processes. Alternatively, they are exclusively

involved in defense gene activation, and this process is also required for establishing mutualistic interactions.

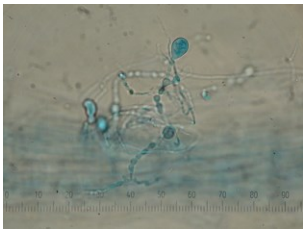
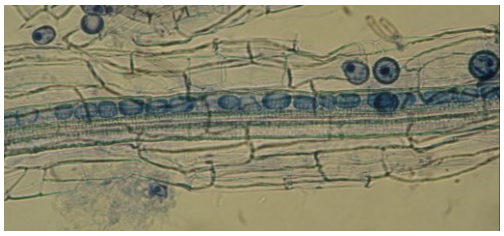

(a)

(b)

Figure 1.1: Fungal mycelia and pear-shaped spores of *P. indica.* (a) spores and mycelia stained with cotton blue (0.05%); magnification 40x. (b) spores in the root cortical region; magnification 40x.

## 1.2 Plant/pathogenic fungus interaction and the role of beneficial microbes in plant protection

Necrotrophic fungi are the largest class of fungal plant pathogens which pose a serious economic problem to crop production. One such group of necrotrophs belongs to *Alternaria brassicae* which severely affects cruciferous plants of the Brassicaceae family including Arabidopsis. Plants respond to necrotrophic fungi by employing effective strategies such as scavenging toxic effects of ROS produced by the pathogen, by the production of antimicrobial metabolites, such as phytoalexins, papillae formation, induction of phytohormone-regulated signaling pathways, mainly jasmonic acid (JA) and cell death control (Thomma *et al*. 1998; Zhou *et al*. 1999; van Wees and Glazebrook 2003; Glazebrook 2005). In general, salicylic acid (SA) signaling and systemic acquired resistance (SAR) are essential defense responses against biotrophic microbes. However, these pathways have also been reported to be involved in pathogenic responses. Ferrari *et al*. (2003) and Govrin and Levine (2002) have shown that *Botrytis cinerea* infection induced SA-related *PATHOGENESIS-RELATED* (*PR-1*, *PR-5*) genes in Arabidopsis while *NahG* plants, which carry the bacterial salicylate hydroxylase gene are susceptible to *Botrytis cinerea.* The infection process for most necrotrophic fungi starts with the production of a penetration peg from fungal appressoria that breaches the

surface of the plant cell and enables the pathogen to colonize host tissues (Mendgen *et al*. 1996; Dehpour *et al*. 2007; Caracuel-Rios and Talbot 2007; Gourgues *et al*. 2004; Nesher *et al*. 2008). Penetration process is initiated by the build up of high turgor pressure inside the appressorium (Park *et al*. 2009) and/or released enzymes, such as cutinases and lipases, which contribute to the degradation of the host cell wall (Kikot *et al*. 2008).

In recent years few research groups have reported the protective role of *P. indica* against pathogens. Waller *et al*. (2005) have noticed that *P. indica*-infested barley plants are more resistant to *Fusarium culmorum* root infection and also induced a systemic resistance (ISR) in leaves against biotrophic fungus *Blumeria graminis* f.sp. *hordei* that caused barley powdery mildew. Modulation of *PR* defense genes by *P. indica* protects barley against root rot pathogen *Fusarium graminearum.* Deshmukh and Kogel (2007) showed that *P. indica* suppresses *F. graminearum*-induced expression of *ß-1, 3-glucanase*, *PR-1B* and *PR-5* in roots which diminished the accumulation of PR protein. They proposed that the reduced *PR* gene expression could be the reason for lower amounts of the pathogenic fungus in these roots, however the mechanism by which *P. indica* mediates this *PR* gene suppression remains unknown. We studied the interaction between Arabidopsis and *A. brassicae* and also explored the role of *P. indica* in suppression of *A. brassicae*-induced infection by modulating the chemical defense response mediated by aliphatic glucosinolates.

### 1.3 Activation of defense responses by *P. indica* in Arabidopsis

It is long known that defense processes are activated in the roots during early phases of the mycorrhizal symbiosis. This includes the stimulation of ROS production (Pozo and Azcón-Aguilar 2007; Salzer *et al*. 1999), defense gene activation (Blilou *et al*. 2000; Molina and García-Olmedo 1993; García-Olmedo *et al.* 1995; Li *et al*. 2006c; Pozo *et al*. 1999; Harrison and Dixon 1993; Morandi 1996; Larose *et al*. 2002), and callose deposition (Cordier *et al*. 1998; Hamiduzzaman *et al*. 2005). During later phases of the symbiosis, defense gene expression is downregulated (David *et al*. 1998; Gianinazzi-Pearson *et al*. 1996; Harrison and Dixon 1993; Kapulnik *et al*. 1996; Lambais and Mehdy 1993; Volpin *et al*. 1995; Harrison 2005). The reason for this is unclear; it can be triggered by signals from the fungus or from

the plant itself. It has been hypothesized that defense gene activation declines once nutrient exchange starts between the two symbiotic partners (Harrison, 1999). Defense genes and their expression also play an important and essential role in the Arabidopsis/*P. indica* symbiosis. Arabidopsis mutants having lesions in different and unrelated defense responses do not respond to the fungus (cf. Camehl and Oelmüller 2010a; Camehl *et al*. 2010b; Sherameti *et al*. 2008a; Nongbri *et al*. 2012; Jacobs *et al*. 2011). While the wild-type Arabidopsis recognizes the fungus as friendly partner and improves its growth and performance, colonized mutants are smaller thereby indicating the loss of beneficial traits in the presence of the fungus. These genes belong either to signaling molecules or transcription factors involved in defense processes, or in genes of enzymes of defense compounds (Fujimoto *et al*. 2000; Ohta *et al*. 2001; Camehl *et al*. 2010b; Thomma *et al*. 1998, 1999; Pieterse *et al*. 1998; van Wees *et al*. 2008). All these mutants have in common the inability to control root colonization by *P. indica*. Uncontrolled over-colonization of roots consequently exerts stress symptoms in plants and induces the expression of stress-related genes. While overcolonized wild-type roots restrict root colonization by upregulating defense genes, such responses are impaired in the corresponding mutants. Therefore, a mild and (maybe) constitutive defense response is required for establishing or maintaining a beneficial symbiosis between the two partners. Because of the different and unrelated defense processes required for the establishment of the beneficial interaction, it was proposed that plants integrate/discriminate fungal signals to balance defense gene activation and development, therefore distinguishing between friends and foes (Camehl and Oelmüller 2010a; Camehl *et al*. 2010b; Sherameti *et al*. 2008a; Nongbri *et al*. 2012). The purpose of my study was to investigate the role of glucosinolates in the beneficial interaction between the two symbionts (cf. below). The sulfur (S)-containing glucosinolates will be introduced in the next chapter.

The PDK1/OXI1 pathway is crucial in Arabidopsis/*P. indica* symbiosis. Signals from abiotic stress, biotic stress and beneficial microbes are perceived by plants which lead to the activation of phospholipids and PA. Therefore PA integrates these external signals and subsequently activates appropriate downstream signal molecules which could bifurcate into defense pathway for pathogenic interaction or symbiotic pathway for beneficial microbes.

PDK1 is the master regulator that binds signaling lipids including PA (Deak *et al*. 1999). Binding leads to phosphorylation of PDK1 and thus activates the AGC kinase OXI1 (Anthony *et al*. 2004). Signals other than PA/PDK1 can also independently activate OXI1 which include $H_2O_2$ and the pathogen-associated molecular pattern (PAMP) flagellin (Li *et al*. 2009). $H_2O_2$ accumulates during pathogen attack (Liu *et al.* 2012; Soylu *et al.* 2005), whereas the beneficial fungus *P. indica* does not induce $H_2O_2$ (Vadassery *et al.* 2009a). Therefore, signals from different microbes in the environment whether pathogens or beneficial microbes come together at this pathway. OXI1 was shown to be required for 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). OXI1 kinase activity is inducible by $H_2O_2$, wounding, cellulase and various elicitor treatments mimicking pathogen attack (Anthony *et al*. 2006; Rentel *et al*. 2004).

Camehl *et al*. (2011) confirmed that the *OXI1* gene is responsible for the growth phenotype induced by *P. indica*. Thus, OXI1 can be activated by pathogen *via* $H_2O_2$ and abiotic and biotic signals *via* PA/PDK1. 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 even inhibited by the beneficial fungus and thus does not play a role in activating OXI1 (Camehl *et al*. 2011).

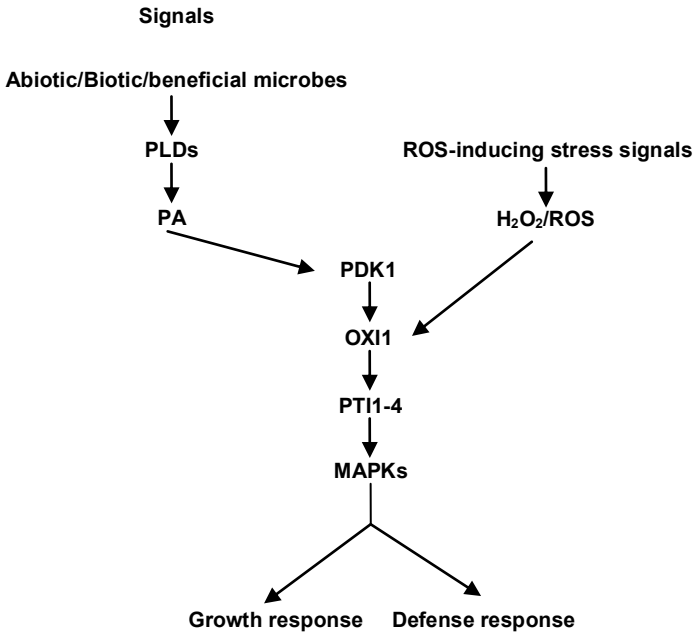



Figure 1.3: PDK1/OXI1 pathway and its potential role in balancing growth/development and defense responses.

### 1.4 Sulfur metabolism in plant/fungus interaction

Sulfur (S) is an essential element for plant performance and productivity. Plants can assimilate soil inorganic sulfate and subsequently reduced and metabolized it into S-containing compounds (Leustek *et al*. 2000; Saito 2004). Animals are unable to reduce sulfate and therefore have dietary requirement for S-containing amino acids and proteins. Therefore, sulfate assimilation by plants is essential for all life on earth. Besides the presence of S in amino acids, it is found in many plant metabolites including vitamins, coenzymes, volatiles and defense compounds (Grubb and Abel 2006; Halkier and Gershenzon 2006; Leustek *et al*. 2000; Saito 2004). The presence of S in many redox mediators also highlights its importance for signaling processes.

When plants encounter S limiting conditions, mechanisms are activated to increase the amount and activity of sulfate uptake and transport systems (Clarkson *et al*. 1983; Deane-Drummond 1987; Smith *et al*. 1995; Smith *et al*. 1997). High-affinity sulfate transporters are located in the plasma membrane of roots (Shibagaki *et al*. 2002; Takahashi *et al*. 2000; Vidmar *et al*. 2000; Yoshimoto *et al*. 2003), transporters for vascular transport (Kataoka *et al*. 2004; Takahashi *et al*. 1997; Yoshimoto *et al*. 2003) and the release of sulfate from the vacuole (Kataoka *et al*. 2004) coordinate the cellular response during initial stages of S limitation. As a second strategy, there is a downregulation of synthesis of S-containing metabolites and storage compounds, and S is released from these compounds through active breakdown processes (Hirai *et al*. 2004, 2005; Kutz *et al*. 2002). In Brassicales, up to 30% of the S is stored in glucosinolates which are S-rich metabolites playing important roles in plants defense against pests and pathogens (Falk *et al*. 2007). Under S deficiency, there occurs an activation of sulfate acquisition and repression of glucosinolate production in parallel (Hirai *et al*. 2003, 2004, 2007; Maruyama-Nakashita *et al*. 2003, 2006). Thus, glucosinolates serve as potential sources of S for other metabolic processes under S limitation (Falk *et al*. 2007; Grubb and Abel 2006; Halkier and Gershenzon 2006). Several enzymes involved in hydrolysis and release of active defense compounds from glucosinolates play important functions in plant/microbe interactions. PEN2, for instance, a myrosinase analysed by Bednarek *et al*. (2009), restricts pathogen entry into leaf cells. Interestingly, there is a striking sequence similarity between PEN2 and a highly abundant enzyme in roots, PYK10 that restricts root colonization by *P. indica* (Sherameti *et al*. 2008a). It was demonstrated that PYK10 is required for the beneficial interaction between Arabidopsis and *P. indica* and speculated that the presence of high amounts of PYK10 in Brassicaceae roots helps to protect them against soil-borne fungi. Very recently, glucosinolate metabolism has been implicated to play an important role in antifungal defense and innate immune response by two groups (Bednarek *et al*. 2009; Clay *et al*. 2009). Certain stress situations promote breakdown of indolic glucosinolates and generate auxins in roots, which may participate in the stimulation of root development for sulfate uptake.

Investigation of the processes that control S metabolism is important for agriculture, horticulture and medicine. The productivity of crops is heavily affected under S deficiency and breakdown of endogenous S-containing glucosinolates affects plant fitness (Walker and Booth 2003; Svanem *et al*. 1997; Schnug and Haneklaus 1993; Schnug *et al*. 1995; Maruyama-Nakashita *et al*. 2003; Nikiforova *et al*. 2003). Optimization of the volatile composition plays a major role in horticulture. Furthermore, many S-containing secondary metabolites are used as cancer-preventives in diets (Talalay and Fahey 2001).

Many processes involved in S metabolism are coordinately regulated under S limitations. However, the mechanism is not fully known yet. In last few years, transcription factors and signaling proteins involved in indole glucosinolate biosynthesis have been characterized in Arabidopsis (Celenza *et al*. 2005; Levy *et al*. 2005; Skirycz *et al*. 2006). Maruyama-Nakashita *et al*. (2006) identified a central transcriptional regulator of plant S responses and metabolism in Arabidopsis, named SULFUR LIMITATION1 (SLIM1). Plants carrying a mutation in *SLIM1* failed to induce transcripts for the high affinity transporter SULTR1;2 under low S conditions. Sulfate uptake and plant growth under S starvation were significantly reduced in *slim1* and SLIM1 finely controlled both sulfate acquisition and degradation of glucosinolates under S limitations. SLIM1 belongs to ETHYLENE-INSENSITIVE-LIKE (EIL) transcription factor family and is identical to EIL3 (Chao *et al*. 1997; Guo and Ecker 2003; Solano *et al*. 1998).

Enzymes participating in S assimilation in Arabidopsis are mainly encoded by multigene families. Their members can have redundant functions (e.g. serine acetyltransferases; Watanabe *et al*. 2008), or are highly specialized and expressed in different tissues (e.g. branched-chain aminotransferases; Schuster *et al*. 2006), or they are incorporated into complex signaling pathways (e.g. adenosine 5′-phosphosulfate reductase; Koprivova *et al*. 2008; Vauclare *et al*. 2002). Several members of these gene families respond to S starvation, while others do not (Maruyama-Nakashita *et al*. 2003). There is increasing evidence that these genes and the activities of their products are regulated by posttranscriptional regulatory circuits and feedback loops.

There is evidence that *P. indica* affects the S metabolism in Arabidopsis. S uptake and metabolism play an important role in plant/microbe interactions. Compared to phosphorus and nitrogen which has been extensively studied in beneficial plant/microbe interactions, not much is known about S regulation with symbiotic soil-borne microorganisms. Mansouri-Bauly *et al*. (2006) have shown that *Laccaria bicolor* enhances the supply of sulfate to the plant by extended sulfate uptake while in return the plant provides the ectomycorrhizal fungus with reduced S. These S-containing components have been shown to play crucial roles in the pathogen's resistance response and in abiotic stress tolerance (Hildebrandt *et al*. 2006; Rausch and Wachter 2005), *via* accumulation in concentration or induced *via* jasmonic acid and/or other signals (cf. Hilpert *et al*. 2001; Mikkelsen *et al*. 2003; Nibbe *et al*. 2002; Xiang and Oliver 1998). The expression of sulfate transporters and several genes of the S-assimilation pathway are controlled by a signaling metabolite cysteine precursor *O*-acetylserine (OAS) which remains highly upregulated (Hirai *et al*. 2003). Glutathione (GSH) which acts as a major redox buffer against ROS is upgraded with increasing sulfate supply and might be involved in defense. Evidently, when γ-glutamylcysteine ligase 1 (GSH1), the rate-limiting enzyme for GSH synthesis is knocked out (Ball *et al*. 2004), defence reactions against pathogens are impaired. GSH has been shown to be involved in *P. indica* induced resistance of barley plants against pathogens (Waller *et al*. 2005; Baltruschat *et al*. 2008). Vadassery *et al*. (2009b) could also show that monodehydroascorbate reductase 2 (MDAR2) and dehydroascorbate reductase 5 (DHAR5) play crucial roles in the mutualistic interaction between *P. indica* and Arabidopsis under drought stress. Many glutathione S-transferases take part in detoxification mechanisms, and GSH is the precursor of phytochelatins, cysteine-rich peptides synthesized *via* phytochelatin synthase (Cobbett and Goldsbrough 2002). Genes for the latter examples are rapidly upregulated when Arabidopsis roots are exposed to *P. indica*.

SLIM1/EIL3 is an important signal in plant immune responses triggered by beneficial microbes (van Wees *et al*. 2008). Plant growth promoting rhizobacteria and mycorrhizal fungi can improve plant performance by ISR-mediated defense responses that confer resistance to pathogens and insects (van Loon 2007; van Wees *et al*. 2008; Yang *et al*. 2009; Pineda *et al*. 2010; Pozo *et al*. 2008; van der Ent *et al*. 2009; Conrath *et al*. 2006). Recognition of microbe-

associated-molecular patterns (MAMPs) from beneficial microbes leads to the activation of the transcription factor gene *MYB72* and the protein interacts with SLIM1/EIL3 to induce a jasmonic acid/ethylene-dependent signaling pathway which primes the aerial parts of the plant for enhanced expression of jasmonic acid/ethylene-dependent genes (Van der Ent *et al*. 2008).

## 1.5 The role of secondary metabolites in Arabidopsis/fungi interaction

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 are employed as protective compounds against insect heribivory (McCloskey and Isman 1993; Giamoustaris and Mithen 1995; Müller *et al*. 2010) by plants. 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).

### 1.5.1 Indole-3-acetaldoxime (IAOx)-derived compounds restrict overcolonization during beneficial Arabidopsis/*P. indica* interaction

CYP79B2 and CYP79B3 are two functionally redundant cytochrome P450 enzymes which convert tryptophan into indole-3-acetaldoxime (IAOx), an intermediate for the biosynthesis of indole glucosinolates (I-GLS), camalexin, other indole compounds such as indole acetonitrile, indole carboxylic acid derivatives, and, under specific conditions, the plant hormone indole-3-acetic acid (IAA). The double *cyp79B2 cyp79B3* mutant lacks I-GLS (Zhao *et al*. 2002) and is unable to induce camalexin synthesis (Glawischnig *et al*. 2004). Furthermore, it does not accumulate indole-3-carboxylic acid derivatives (Böttcher *et al*. 2009), secondary metabolites which are strongly induced by pathogen infections. 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). In this thesis, I address the question whether co-cultivation of Arabidopsis seedling

roots with *P. indica* is affected by camalexin. I investigated the expression levels for CYP79B2, CYP79B3, CYP71A13 (Nafisi *et al*. 2007), PAD3, and WRK33 (Qiu *et al*. 2008) in colonized wild-type roots, and compared them to those for CYP83B1 and SUR1. CYP83B1 catalyzes the first committed step in indole glucosinolate biosynthesis (Bak *et al.* 2001) while SUR1 controls the biosynthesis of both aliphatic and indole glucosinolates (Mikkelsen *et al.* 2004). I tested whether camalexin and camalexin-synthesizing enzymes/genes affect the symbiosis between *P. indica* and Arabidopsis (cf. Manuscript I).

### 1.5.2 Methionine-derived aliphatic glucosinolates

The R2R3 MYB transcription factors MYB28, MYB29 and MYB76 were identified as positive regulators of the aliphatic glucosinolate biosynthesis (Hirai *et al*. 2007; Gigolashvili *et al*. 2007b; Sønderby *et al*. 2007, 2010), whereas MYB29 and MYB76 regulate short-chain aliphatic glucosinolate biosynthesis (Sønderby *et al*. 2007; Beekwilder *et al*. 2008; Gigolashvili *et al*. 2008) and MYB28 plays a major role in controlling long- as well as short-chain aliphatic glucosinolates (Hirai *et al*. 2007; Sønderby *et al*. 2007; Beekwilder *et al*. 2008; Gigolashvili *et al*. 2009). The *myb28 myb29* double knockout mutant is almost devoid of aliphatic glucosinolates, presumably through epistatic effects (Sønderby *et al*. 2007; Beekwilder *et al*. 2008). Interestingly, there was no change in the level of I-GLS when *MYB28*, *MYB29* and *MYB76* were inactivated (Sønderby *et al*. 2007) which therefore demonstrates the exclusive role of *MYB28*, *MYB29* and *MYB76* in aliphatic glucosinolate pathway. *MYB28* is regulated by glucose (Gigolashvili *et al*. 2007b) and wounding (Levy *et al*. 2005; Gigolashvili *et al*. 2007a, 2007b). Gigolashvili *et al*. (2008) reported that exogenous methyl-jasmonate induces *MYB29* while salicylic acid exerts a negative effect, and *MYB76* is induced by wounding. Glucosinolate hydrolysis products and isothiocyanate inhibit bacterial and fungal growth *in vitro* (Mithen *et al*. 1986; Manici *et al*. 1997; Brader *et al*. 2001; Tierens *et al*. 2001; Mari *et al*. 2002; Smolinska *et al*. 2003). Necrotrophic pathogens cause tissue damage (Glazebrook 2005) which results in the release of glucosinolates and myrosinases from their cellular compartments and the generation of the toxic cleavage products (Lambrix *et al*. 2001). *Sclerotinia sclerotiorum*, an aggressive fungal pathogen with a wide host range (Bolton *et al*. 2006), facilitates host cell damage (Kim *et al*. 2008; Guo and Stotz 2010) and

subsequently glucosinolate cleavage. A protective role of aliphatic glucosinolate against *S. sclerotiorum* has been demonstrated using the *myb28* knockout mutant (Stotz *et al*. 2011).

In my thesis, I investigated the role of MYB28 and MYB29 and thus aliphatic glucosinolates for the beneficial interaction between *P. indica* and Arabidopsis (Manuscript VI). Consistent with published data from other pathosystems, I could show that the *A. brassicae*/Arabidopsis interaction is associated with the up-regulation of *MYB28*, *MYB29* and *MYB76* in the respective organs. Furthermore, infection of roots with the pathogen causes severe disease symptom development: the roots become brown and the shoots develop chlorosis. I showed that *P. indica* protects the roots and leaves of Arabidopsis seedlings against *A. brassicae* infections, comparable to the ISR induced by plant-growth promoting bacteria (Van Loon *et al.* 1998). Therefore, I tested whether MYB28/MYB29 is required for establishing resistance in the leaves after *A. brassicae*-infection of the roots, and whether aliphatic glucosinolates play a role in the beneficial symbiosis between *P. indica* and Arabidopsis.

## 2. Goal of my studies

The aim of this project was to investigate the role of host plants' sulfur-containing secondary metabolites in the symbiotic interaction system between the model plant *A. thaliana* and the beneficial endophytic fungus *P. indica.* Are S-containing gluosinolates preferentially involved in defense processes against the beneficial fungus *P. indica* (as suggested from many pathosystems), and if so, what is their role in the beneficial interaction? To address this question, I also examined the involvement of S-containing glucosinolates in *P. indica*-mediated resistance against *A. brassicae*.

I. I investigate the role of indole-3-acetaldoxime (IAOx)-derived compounds in the beneficial interaction between Arabidopsis and *P. indica* with the aim to answer the following questions:
   - Are IAOx-deficient mutant plants still able to respond to the fungus?
   - Is defense response mediated by IAOx indispensible for establishing a beneficial interaction between the two symbionts?

II. Are aliphatic glucosinolates involved in Arabidopsis/*P. indica* symbiosis?
   - Does Arabidopsis require aliphatic glucosinolates for establishing a beneficial symbiosis with *P. indica*?
   - Do aliphatic glucosinolates participate in *P. indica*-mediated resistance of Arabidopsis to *A. brassicae*?

## 3. Manuscript Overview

### 3.1 Manuscript I

**Indole-3-acetaldoxime-derived compounds restrict root colonization in the beneficial interaction between Arabidopsis roots and the endophyte *Piriformospora indica***

Pyniarlang L. Nongbri, Joy M. Johnson, Irena Sherameti, Erich Glawischnig, Barbara Ann Halkier and Ralf Oelmüller

*Molecular Plant Microbe Interaction (2012), 25(9): 1186-1197.*

In this publication we demonstrate the role of indole glucosinolate and camalexin in beneficial interaction between *P. indica* and Arabidopsis. The *cyp79B2 cyp79B3* mutant which lacks indole glucosinolate and camalexin has severe lesion in the growth response to *P. indica* at the seedling stage. Furthermore, *pad3* mutant defective in camalexin biosynthetic pathway did not respond to *P. indica*-mediated growth enhancement during long term interaction in soil. Higher degree of root colonization was observed in these mutants compared to wild-type. We propose that wild-type levels of glucosinolate and camalexin are important to restrict fungal growth in a symbiotic interaction.

All the experiments are designed by R. O; P. L. N. investigated the knock out *cyp79B2 cyp79B3* and *pad3* mutants. J. M. J. analyzed the role of calcium signaling *cam* mutant. E. G. measured camalexin levels. B. A. H. created the *cyp79B2 cyp79B3* double mutant. I. S. coordinated the project.

## 3.2 Manuscript II

**Role of *Piriformospora indica* in Sulfur Metabolism in *Arabidopsis thaliana***

Pyniarlang L. Nongbri and Ralf Oelmüller

*accepted, A. Varma et al. (eds.), Piriformospora indica, Soil Biology 33,*
*DOI 10.1007/978-3-642-33802-1_18, © Springer-Verlag Berlin Heidelberg 2013*

This book chapter highlights sulfur metabolism as an important target by *P. indica* in Arabidopsis. Genes for sulfur metabolic pathway and transcription factors controlling sulfur and glucosinolate metabolism are regulated by *P. indica* as revealed by expression profiling. We demonstrated how *P. indica* regulates S metabolism, glucosinolate biosynthesis and the resistance against pathogens *via* S-containing components. Controlling of sulfur metabolism in Arabidopsis therefore ensures availability of S-containing components for plant growth and development, especially under S limitation, and sufficient S-containing defense components maintain beneficial interaction by preventing over-colonization of the roots.

This book chapter was written by P. L. N. and R. O.

## 3.3 Manuscript III

**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

*accepted, Symbiosis*

This review article summarizes the beneficial and non-beneficial physiological responses in a mutualistic interaction between the endophytic and root-colonizing fungus *P. indica* and Arabidopsis. *P. indica* confers many benefits to the host symbiont. Colonized Arabidopsis plants produce more biomass, seed production 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 no longer maintain 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.

## 3.4 Manuscript IV

**Aliphatic glucosinolates participate in *Piriformospora indica*-induced resistance of Arabidopsis against *Alternaria brassicae***

Pyniarlang L. Nongbri, Joy M. Johnson, Barbara Ann Halkier, Ralf Oelmüller

*in preparation*

In Arabidopsis, R2R3 MYB transcription factors MYB28, MYB29 and MYB76 regulate the biosynthesis of aliphatic glucosinolates from methionine. A *myb28 myb29* mutant is almost devoid of aliphatic glucosinolates. *P. indica* restricts the growth of a necrotrophic fungus *A. brassicae*. Priming with *P. indica* activates resistance in wild-type seedlings which protects against Alternaria infection in roots; however *myb28 myb29* seedlings are less protected. Induced systemic resistance is also activated in wild-type but not in *myb28 myb29* leaves. We propose that *P. indica* protects the seedlings against *A. brassicae* infections by inhibiting growth of the pathogen in the roots and induces root-to-shoot signaling for systemic resistance against *A. brassicae* leaf infections.

P. L. N. and R. O. designed the experiments. P. L. N. performed the interaction studies of the *myb28 myb29* mutant with *P. indica* and gene expression studies. P. L. N. carried out the root inoculation with *P. indica* for priming the resistance against *A. brassicae* root and leaf infections. J. M. J. developed and established the *A. brassicae* pathogenic interaction with Arabidopsis and *A. brassicae*/*P. indica* interaction. B. A. H. created the *myb28 myb29* mutant and analyzed its chemical phenotype.

## 4. 1 Manuscript I

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

Pyniarlang L. Nongbri, Joy Michal Johnson, Irena Sherameti, Erich Glawischnig, Barbara Ann Halkier, and Ralf Oelmüller

*Molecular Plant Microbe Interaction (2012), 25(9), 1186–1197*

MPMI Vol. 25, No. 9, 2012, pp. 1186–1197. http://dx.doi.org/10.1094/MPMI-03-12-0071-R. © 2012 The American Phytopathological Society e-Xtra*

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

**Pyniarlang L. Nongbri,[1] Joy Michal Johnson,[1] Irena Sherameti,[1] Erich Glawischnig,[2] Barbara Ann Halkier,[3] and Ralf Oelmüller[1]**

[1]Institute of General Botany and Plant Physiology, Friedrich-Schiller-University Jena, Dornburger Str. 159, 07743 Jena, Germany; [2]Lehrstuhl für Genetik, Technische Universität München, Emil-Ramann-Str.8, 85350 Freising, Germany; [3]VKR Research Centre Pro-Active Plants, Department of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Copenhagen, Thorvaldsensvej 40, 1871 Frederiksberg C, Denmark

Submitted 29 March 2012. Accepted 17 May 2012.

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

Important secondary metabolites in members of order Brassicales are synthesized from tryptophan. Two functionally redundant cytochrome P450 enzymes (CYP79B2 and CYP79B3) convert tryptophan into indole-3-acetaldoxime (IAOx), an intermediate for the biosynthesis of indole glucosinolates (I-GLS), camalexin, other indole compounds such as indole acetonitrile, indole carboxylic acid derivatives, and, under specific conditions, the plant hormone indole-3-acetic acid (IAA). Whereas camalexin is a true phytoalexin synthesized in the plant in response to pathogen infection or exposure to stress, I-GLS are both phytoanticipins present prior to induction as well as phytoalexins induced upon infection. The first committed step in I-GLS biosynthesis is the enzyme CYP83B1/SUR2 (Bak et al. 2001; Barlier et al. 2000; Hansen et al. 2001). The major IAOx-metabolizing enzyme in camalexin biosynthesis is CYP71A13, which catalyzes the formation of the intermediate indole acetonitrile (Nafisi et al. 2007), supposedly together with the homolog CYP71A12 required for camalexin exudation from roots (Millet et al. 2010). The thiazole ring of camalexin derives from the cysteine moiety of glutathione, which is conjugated with indole acetonitrile after activation (Böttcher et al. 2009; Geu-Flores et al. 2011; Parisy et al. 2007; Su et al. 2011). The single-copy gene *PAD3/CYP71B15* encodes for an enzyme that catalyzes the final two steps in the camalexin pathway (Böttcher et al. 2009; Schuhegger et al. 2006).

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

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

P. L. Nongbri and J. M. Johnson contributed equally to the work.

Corresponding author: R. Oelmüller; E-mail: b7oera@uni-jena.de

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

pathways. Crucial components are the mitogen-activated protein kinases (MPK) MPK3, MPK6 (Ren et al. 2008), and MPK4 (Qiu et al. 2008). MPK4 becomes activated in response to infection and phosphorylates MPK4 substrate 1 (MKS1) and the transcription factor WRKY33 in the nucleus, which ultimately allows WRKY33 to activate camalexin biosynthetic genes (Qiu et al. 2008). Upstream of MPK, the 3-phosphoinositide-dependent protein kinase 1 (PDK1)/oxidative signal-inducible 1 (OXI1) pathway is activated in response to various pathogens (Anthony et al. 2004, 2006; Rentel et al. 2004). OXI1 is required for full activation of MPK3 and MPK6 in response to many microbial pathogens or elicitors (Rentel et al. 2004; van der Luit et al. 2000; Yamaguchi et al. 2005). OXI1 phosphorylates and, thus, activates the downstream serine/threonine kinase PTI1-2 and also controls root hair growth (Rentel et al. 2004). OXI1 is activated by $H_2O_2$ and phospholipid signals via PDK1 (Anthony et al. 2006). PDK1 binds to different signaling lipids, including the second messenger phosphatidic acid, which accumulates in response to microbial infections or elicitor treatments (Deak et al. 1999; Hirt et al. 2011). Phosphatidic acid is mainly produced by phospholipase $\alpha$1 in *Arabidopsis* roots, and a corresponding mutant contains severely reduced levels of this plant second messenger (Devaiah et al. 2006). PDK1 phosphorylates and, thus, activates OXI1 in *Arabidopsis* (Anthony et al. 2004) and rice (Matsui et al. 2010) or AvrPto-dependent Pto-interacting protein 3 (Adi3) in tomato (Devarenne et al. 2006).

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

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

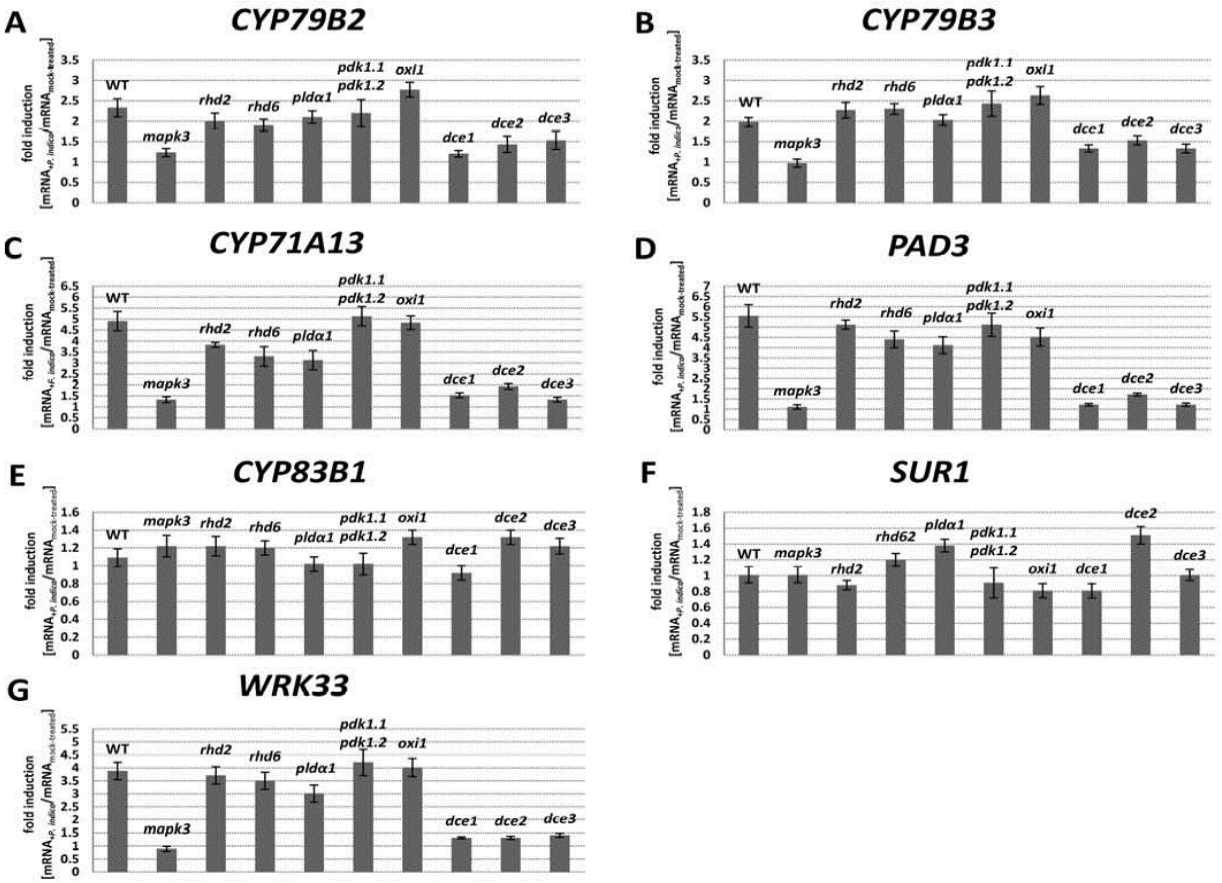



**Fig. 1.** Fold-induction of the mRNA levels for **A,** *CYP79B2*; **B,** *CYP79B3*; **C,** *CYP71A13*; **D,** *PAD3*; **E,** *CYP83B1*; **F,** *SUR1*; and **G,** *WRKY33* by *Piriformospora indica* in *Arabidopsis* roots. RNA was isolated from the roots of wild-type (WT, Col 0) seedlings and different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

action between the endophytic fungus *P. indica* and *Arabidopsis* requires a balanced activation of defense processes, and mutants defective in specific defense-related genes are often unable to restrict fungal growth (Camehl and Oelmüller 2010; Camehl et al. 2010; Johnson and Oelmüller 2009; Sherameti et al. 2008). Here, we demonstrate that IAOx-derived indole compounds are crucial for the beneficial interaction between the two symbionts, and that these compounds restrict root colonization. We also demonstrate that *P. indica*-induced cytoplasmic calcium elevation and MPK3 but not $H_2O_2$, RHD2 and RHD6, and the PDK1/OXI1 pathway are required for the activation.

## RESULTS

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

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

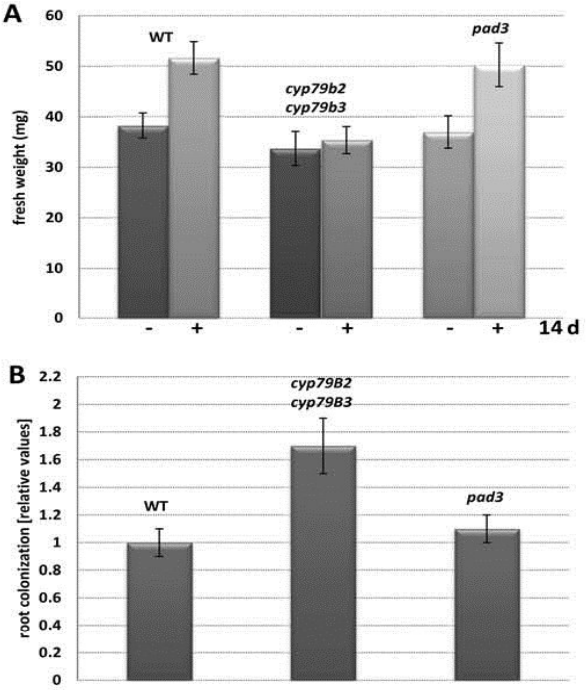



**Fig. 2. A,** Fresh weight of wild type (WT), *cyp79B2 cyp79B3*, and *pad3* seedlings after 14 days of co-cultivation with *Piriformospora indica* or mock treatment. **B,** Increase of root colonization based on quantitative reverse-transcription polymerase chain reaction of *cyp79B2 cyp79B3* and *pad3*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

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

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

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

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

### Cytoplasmic $Ca^{2+}$ elevation and MPK3 but not RHD2, RHD6, PLDα1, PDK1, or OXI1 are involved in the *P. indica*-induced upregulation of the mRNAs for CYP79B2, CYP79B3, CYP71A13, PAD3, and WRKY33.

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

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

To identify upstream components in the signaling cascade leading to *PAD3*, *CYP79B2*, *CYP79B3*, *CYP71A13*, and *WRKY33* mRNA induction by *P. indica* in *Arabidopsis* roots, mutants of MPK3, the ROS-producing RHD2, the phosphatidic-acid-producing phospholipase Dα1, the OXI-activating PDK1s, and ROS-activated OXI1 were compared with the WT after co-cultivation with *P. indica* (Fig. 1A to G). The *rhd6* mutant was included to investigate the role of root hairs for the induction of the genes by the fungus. Furthermore, we included three genetically distinct *Arabidopsis* ethyl-methane-sulfonate mutants, called *deficient in cytoplasmic calcium elevation* (*dce*)*1* to *dce3* which are impaired in inducing cytoplasmic $Ca^{2+}$ elevation in response to the fungus and a fungal cell wall preparation (Fig. 5) (Vadassery et al. 2009). Because these mutants do not respond to the fungus in terms of growth promotion (*unpublished data*), the rapid increase in cytoplasmic $Ca^{2+}$ elevation appears to be required for establishing the beneficial symbiosis.

*PAD3* (Fig. 1D), *CYP79B2* (Fig. 1A), *CYP79B3* (Fig. 1B), *CYP71A13* (Fig. 1C), and *WRKY33* (Fig. 1G) were downregulated by *P. indica* in all mutants impaired in cytoplasmic $Ca^{2+}$ elevation and in the *mpk3* mutant, compared with WT, *rhd2*, *rhd6*, the *pdk1.1 pdk1.2* double knock-out line, and *oxi1* mutants (Fig. 1A to D and G) but CYP83B1 (Fig. 1E) and SUR1 (Fig. 1F) were not affected. Although phosphatidic acid might be a second messenger in *P. indica*-mediated growth promotion (Camehl et al. 2011) and phospholipase Dα1 is a main producer of phosphatidic acid in *Arabidopsis* roots (Devaiah et al. 2006), the *PAD3*, *CYP79B2*, *CYP79B3*, *CYP71A13,* and *WRKY33* mRNA levels are not significantly different in a mutant lacking phospholipase Dα1 compared with the WT control (Fig. 1A to D and G). Furthermore, we could not detect an increase in the ROS level in infected WT and mutant roots (Camehl et al. 2011; Vadassery et al. 2009). This suggests that *P. indica* stimulates the expression of these genes through a $Ca^{2+}$- and MPK3-dependent pathway but does not require production of ROS through the NADH oxidase RHD2 and the activity of the PDK1 and OXI1 (discussed below).

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

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

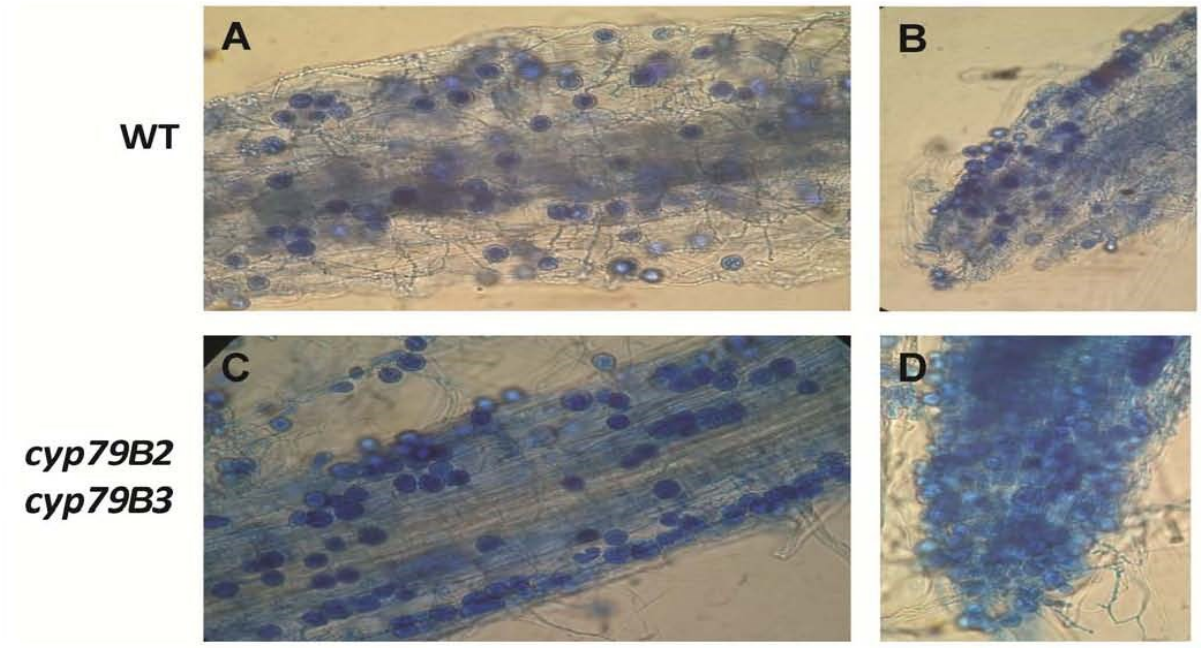



**Fig. 3.** Distribution of fungal spores in *Arabidopsis* **A** and **C,** root segments (1 to 2 cm away from the root tip) or **B** and **D,** at the root tip of **A** and **B,** the wild type (WT) and **C** and **D,** *cyp79B2 cyp79B3*. Representative pictures for seedlings after 14 days of co-cultivation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

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

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

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

After 42 days on soil (after 42 days, the vegetative phase was over, most of the plants flowered, and there was not any more camalexin increase), the camalexin level in the colonized WT roots was not higher than in the mock-treated control (0.75 ± 0.46 μg $g^{-1}$ fresh weight; mock-treated control: 1.17 ± 0.58 μg $g^{-1}$ fresh weight). However, when compared with seedlings, the camalexin levels in the roots of adult plants were five- to 10-fold higher. Thus, the overall amount of camalexin per fresh weight increased in the WT roots during the 42 days on soil but the small stimulatory effect of *P. indica* observed for the seedlings was no longer detectable for adult plants. Also the *PAD3*, *CYP79B2*, *CYP79B3*, *CYP71A13*, and *WRKY33* mRNA levels were no longer upregulated by the fungus in WT roots (Supplementary Table S2): in none of the experiments did we observe a significant stimulation by the fungus. Furthermore, pathogenesis-related (*PR*) genes (*PR-1*, *PDF1.2*, *phenylalanine ammonia lyase* [*PAL*], and *germin*) which are highly upregulated in colonized roots of *cyp79B2 cyp79B3* seedlings (Fig. 4A and B) were also upregulated in the roots of colonized adult *cyp79B2 cyp79B3* and *pad3* plants but at a lower scale (Fig. 7B1 and B2). The ROS-related *RHD2* and the root-hair-controlling *RHD6* genes are not regulated by *P. indica* (Fig. 7B3). We also could not detect ROS production in the roots of adult WT, *cyp79B2 cyp79B3*, and *pad3* plants (Supplementary Table S3). Taken together, defense processes in the colonized mutant and WT roots of adult plants are not much activated by the fungus compared with the situation in the seedlings.

However, root colonization was much higher in the mutant plants compared with the WT (Fig. 7C). After 42 days in soil, the *Pitef1* DNA or cDNA/*AtGAPC2* DNA or cDNA ratios for the *cyp79B2 cyp79B3* and *pad3* plants were 11 and 5 times higher, respectively, than the ratio for WT plants (Fig. 7C). Kinetic analysis demonstrates that these ratios increased gradually with longer co-cultivation times on soil (Supplementary Table S4). Microscopic inspection of the colonization did not discover significant differences to the colonization pattern found for seedlings (Fig. 3): younger root sections were more colonized than older regions of the roots and spores were mainly found in association with lateral roots. Thus, the low level of defense gene activation in combination with the absence of IAOx-derived indole compounds promotes fungal

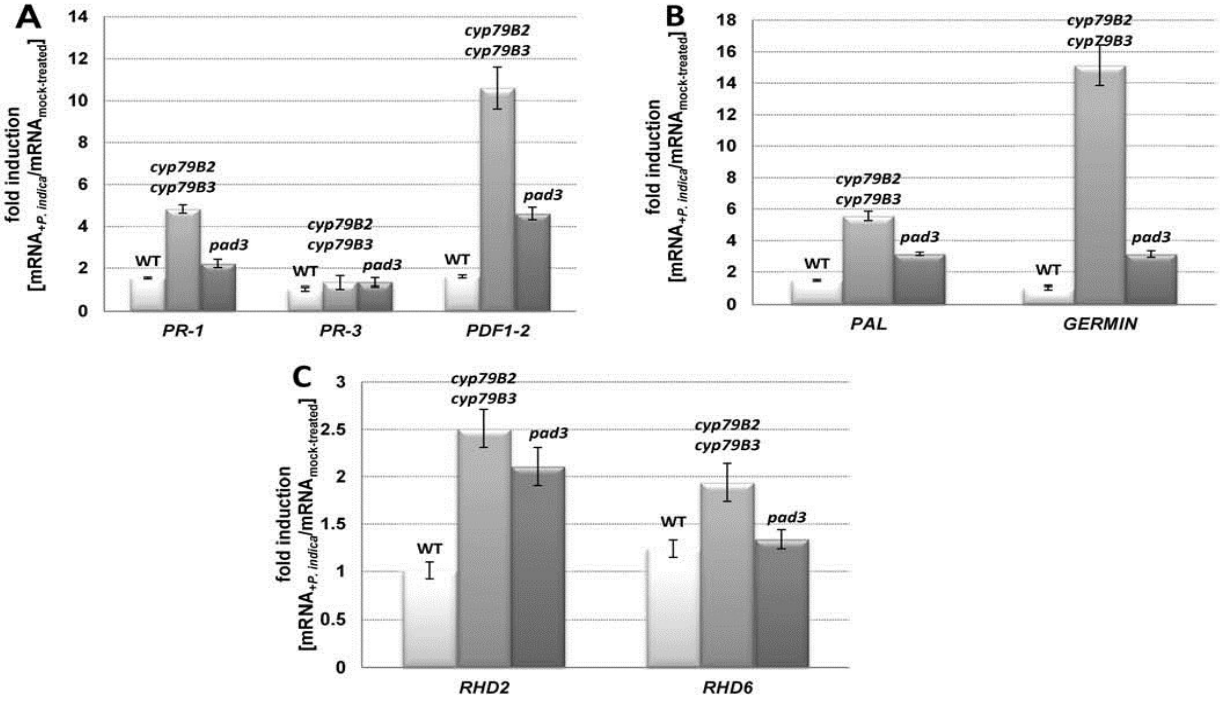



**Fig. 4.** Fold induction of the mRNA levels for pathogenesis-related (*PR*) genes **A,** *PR-1*, *PR-3*, and *PDF1.2*; **B,** phenylalanine ammonia lyase (*PAL*) and *germin*; and **C,** root-hair-deficient (*RHD*) genes *RHD2* and *RHD6* by *Piriformospora indica* in *Arabidopsis* roots. RNA was isolated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

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

## DISCUSSION

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

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

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

The higher degree of fungal colonization in the roots of *cyp79B2 cyp79B3* seedlings (Fig. 2B) and adult *cyp79B2 cyp79B3* and *pad3* plants (Fig. 7C) and the initial activation of defense genes against the fungus in the roots of the *cyp79B2 cyp79B3* seedlings suggest that IAOx and its derived compounds, including camalexin, participate in the control of fungal growth. Initially, the roots responded to the lack of IAOx and its derived compounds by activating other defense genes such as *PR-1, PDF1.2, PAL*, and *germin* (Fig. 4A and B). However, this mechanism appears to be restricted to early phases of the symbiosis and is no longer active in adult plants (Fig. 7B1 and B2). In the roots of adult mutants, expression of these defense genes is much lower when compared with the expression in seedling's roots, either because the plant recognizes the microbe as a friendly symbiont and no longer activates defense genes or because the symbiont actively represses the host's defense response (Jacobs et al. 2011). The low level of defense might explain the high colonization rate and the loss of benefits for the adult mutants.

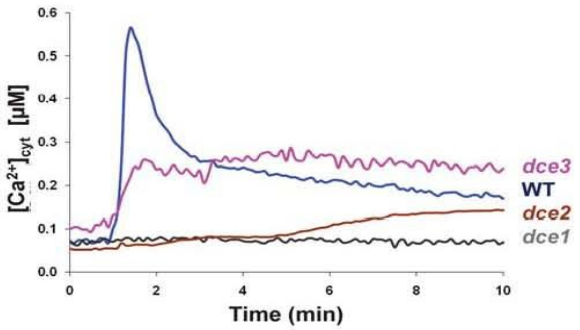



**Fig. 5.** *Piriformospora indica* cell wall extract (CWE) induces changes in cytosolic $Ca^{2+}$ ($[Ca^{2+}]_{cyt}$) levels in the roots of apoaequorin-transformed *Arabidopsis* wild-type (WT) seedlings or in the roots of three *Arabidopsis* mutants, *deficient in cytoplasmic calcium elevation* (*dce*)*1* to *dce3*. $[Ca^{2+}]_{cyt}$ elevation was induced by the application of 50 µl of CWE to *Arabidopsis* roots. The $[Ca^{2+}]_{cyt}$ was calculated from the relative light units (RLU) measured at 5-s integration times for 10 min. In all the experiments, water was used as control and gave background readings. The four calcium signatures are representative of several hundred measurements (Vadassery and associates 2009).

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

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

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

strong induction of tryptophan and camalexin biosynthetic genes (Schuhegger et al. 2006, 2007). It is believed that lesions of the infected tissue are a prerequisite for camalexin induction in nature, although an autoclaved yeast suspension (Raacke et al. 2006) and peptidoglucan preparations (Gust et al. 2007) can induce the phytoalexin synthesis in leaves without observed cell death. Deshmukh and associates (2006) and Jacobs and associates (2011) have demonstrated that root regions undergoing cell death are more colonized by *P. indica* than other regions. Therefore, the interaction occurs preferentially at sides with injured root cells. We observed that, other than *P. indica*, a cell wall preparation from the fungus that promotes plant growth (Vadassery et al. 2009) induces *PAD3*, *CYP79B2*, *CYP79B3*, *CYP71A13*, and *WRKY33* mRNA levels in a dose- and time-dependent manner in the roots of *Arabidopsis* seedlings (data not shown). Therefore, the response does not necessarily require local injuries of individual root cells (e.g., by hyphal penetration) but can also be induced by a *P. indica*-released microbe-associated molecular pattern (MAMP) that is present in the cell wall preparation. Our data suggest that the *P. indica*-induced response occur locally, although Truman and associates (2010) provided evidence for a role of indole-derived compounds in the establishment and maintenance of systemic immunity. Pathogens and pathogenic MAMPs often induce ROS production via NADH oxidases. Because ROS can function as a rapid, long-distance, auto-propagating signal (Mittler et al. 2011) and because *P. indica* and the *P. indica*-derived cell wall extract does not induce ROS production (Camehl et al. 2011; Vadassery et al. 2009), we may observe only a local induction of the *PAD3*, *CYP79B2*, *CYP79B3*, *CYP71A13*, and *WRKY33* mRNA levels.

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

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

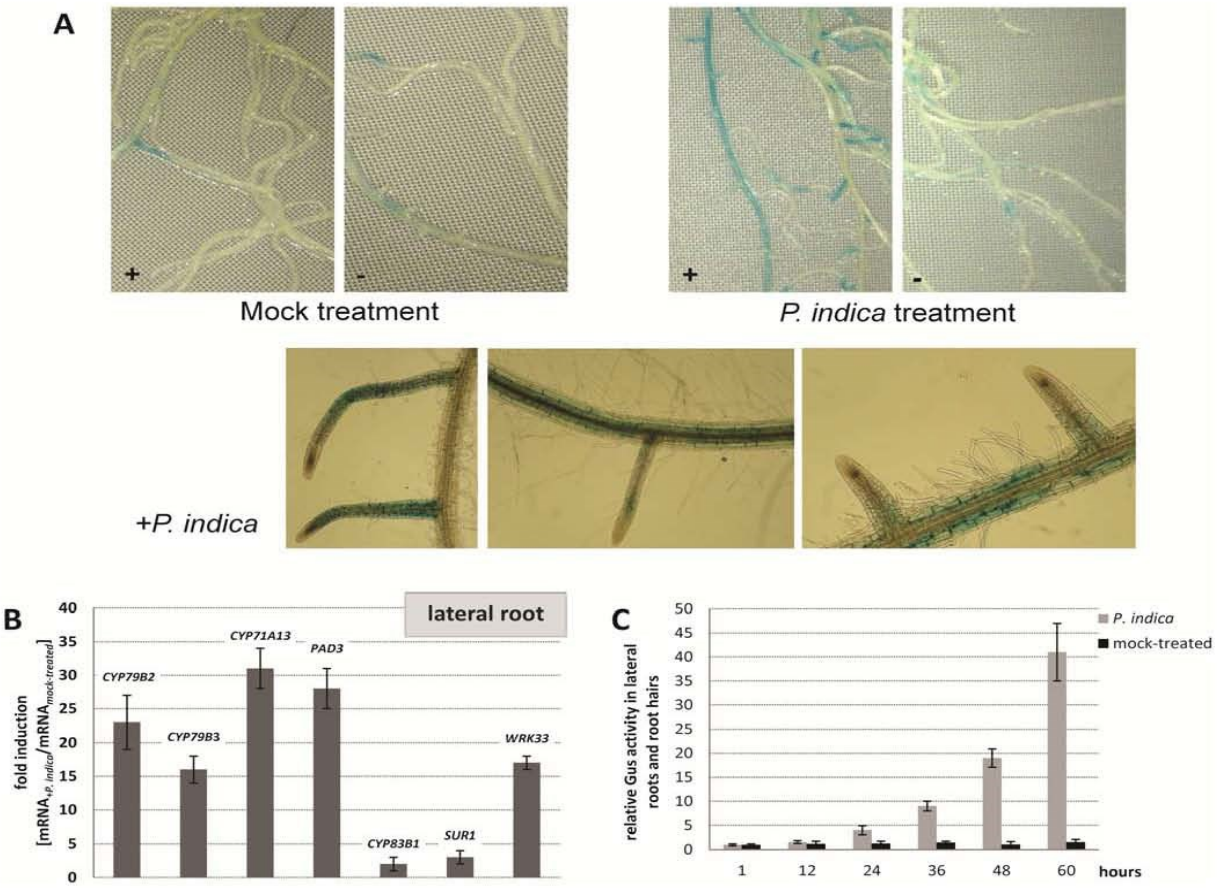



**Fig. 6. A,** β-Glucuronidase (GUS) staining of *pad3::uidA* roots in split petri dishes. Roots were not exposed (–) or exposed (+) to fungal hyphae. Mock treatment: without hyphae; *Piriformospora indica* treatment: plug was at the (+) site of the split petri dish. **A,** Bottom pictures show enlargement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

localized receptor, followed by intracellular $Ca^{2+}$ elevations, $H_2O_2$ production, and MPK3 or MPK6 phosphorylation. In pathogenic interactions, cytoplasmic $Ca^{2+}$ elevation proceeds ROS production and MPK3 activation (Blume et al. 2000; Jabs et al. 1997; Kroj et al. 2003; Lee et al. 2004; Ligterink et al. 1997; Nürnberger et al. 1994; Zimmermann et al. 1997). Induction of the cytochrome P450 genes under study required MPK3 but appeared to be independent of $H_2O_2$-dependent processes leading to defense gene activation via the PDK1/OXI1 pathway (Fig. 1). Interestingly, three genetically distinct ethyl-methanesulfonate mutants that are impaired in inducing cytoplasmic $Ca^{2+}$ elevation in response to *P. indica* and a cell wall extract from this fungus also fail to induce camalexin synthesis genes. Therefore, cytoplasmic $Ca^{2+}$ elevation is required for *P. indica*-induced stimulation of the genes for the P450 enzymes and WRK33, similar to the signaling in pathosystems.

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

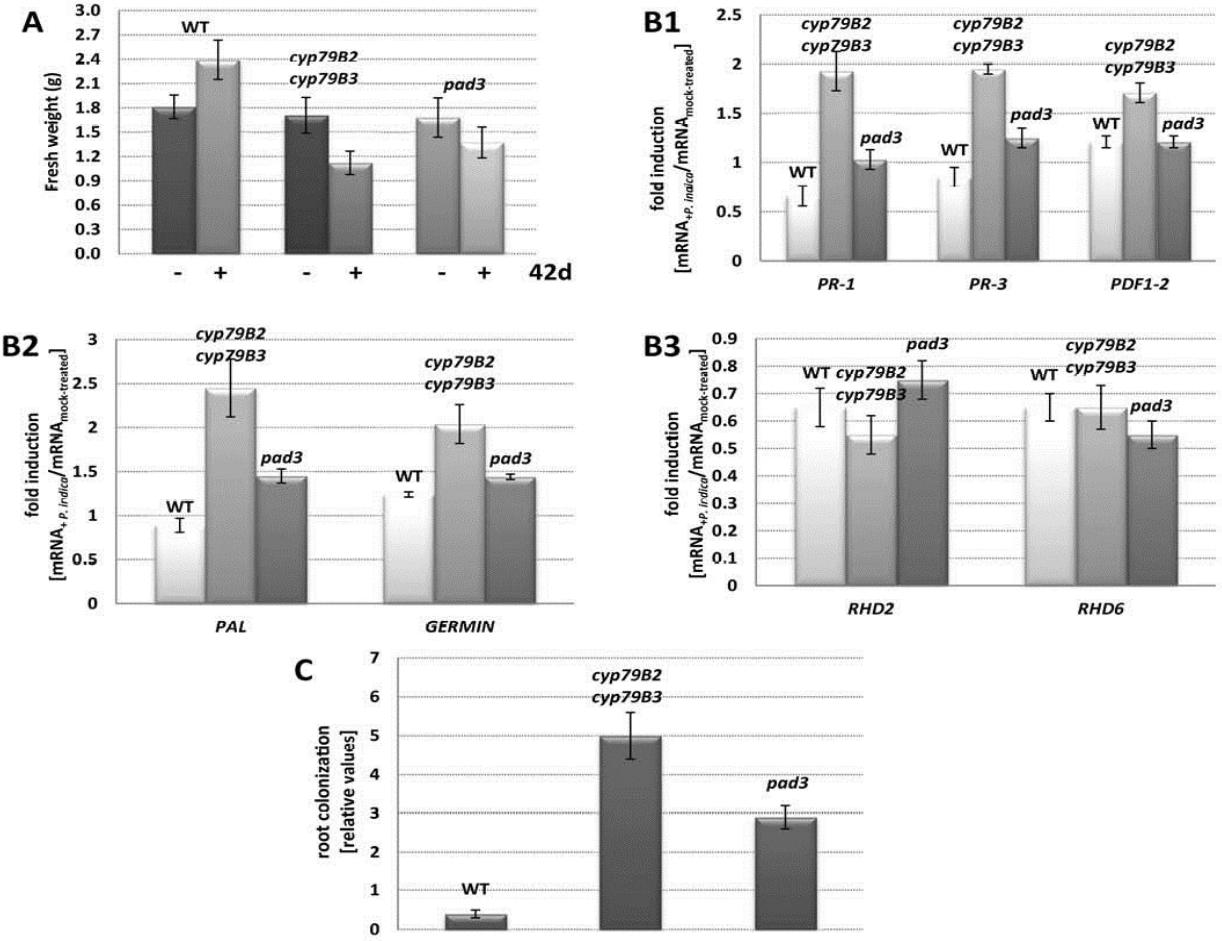



**Fig. 7. A,** Shoot fresh weight of the wild type (WT), *cyp79B2 cyp79B3*, and *pad3* of adult plants after 42 days in soil. WT and mutants seedlings were either co-cultivated with *Piriformospora indica* for 14 days or mock treated on PNM plates before transfer to soil. Standard errors (SE) are based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

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

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

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

## MATERIALS AND METHODS

**Growth conditions of plant and fungus.**

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

**Co-cultivation experiments and estimation of plant growth.**

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

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

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

**Experiments on soil.**

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

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

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

**Gene expression.**

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

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

**Root colonization.**

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

**GUS assays for *pad3::uidA* lines.**

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

**Camalexin determination.**

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

**$Ca^{2+}$ measurements, isolation of $Ca^{2+}$ mutants.**

Transgenic apoaequorin *A. thaliana* (Col-0) lines (Knight et al. 1997) were used for $Ca^{2+}$ measurements. The roots were dissected from 14-day-old seedlings and reconstituted in 5 µM coelenterazine (P.J.K. GmbH, Kleinblittersdorf, Germany) in the dark overnight at 21°C. The luminescence counts obtained with a microplate luminometer (Luminoscan Ascent, version 2.4; Thermo Fisher Scientific, Schwerte, Germany) were calibrated using the equation by Rentel and Knight (2004) (Johnson et al. 2011a; Vadassery et al. 2009, appendix S1). The mutants were obtained after ethyl methanesulfonate mutagenesis. Approximately 450,000 individuals were screened with the cell wall extract from *P. indica* (J. M. Johnson and R. Oelmüller, *unpublished*). The three mutant lines used here were propagated on soil and M5 seed were used for these analyses. Crosses between the lines confirmed that they are not allelic.

**Data analysis.**

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

## ACKNOWLEDGMENTS

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

## LITERATURE CITED

Anderson-Prouty, A. J., and Albersheim, P. 1975. Host–pathogen interactions. VIII. Isolation of a pathogen-synthesized fraction rich in glucan that elicits a defense response in the pathogens host. Plant Physiol. 56:286-291.

Anthony, R. G., Henriques, R., Helfer, A., Mészáros, T., Rios, G., Testerink, C., Munnik, T., Deák, M., Koncz, C., and Bögre, L. 2004. A protein kinase target of a PDK1 signalling pathway is involved in root hair growth in *Arabidopsis*. EMBO (Eur. Mol. Biol. Organ.) J. 23:572-581.

Anthony, R. G., Khan, S., Costa, J., Pais, M. S., and Bögre, L. 2006. The *Arabidopsis* protein kinase PTI1-2 is activated by convergent phosphatidic acid and oxidative stress signaling pathways downstream of PDK1 and OXI1. J. Biol. Chem. 281:37536-37546.

Bak, S., Tax, F. E., Feldmann, K. A., Galbraith, D. W., and Feyereisen, R. 2001. CYP83B1, a cytochrome P450 at the metabolic branch point in auxin and indole glucosinolate biosynthesis in *Arabidopsis*. Plant Cell 13:101-111.

Barlier, I., Kowalczyk, M., Marchant, A., Ljung, K., Bhalerao, R., Bennett, M., Sandberg, G., and Bellini, C. 2000. The SUR2 gene of *Arabidopsis thaliana* encodes the cytochrome P450 CYP83B1, a modulator of auxin homeostasis. Proc. Natl. Acad. Sci. U.S.A. 97:14819-14824.

Blume, B., Nürnberger, T., Nass, N., and Scheel, D. 2000. Receptor-mediated rise in cytoplasmic free calcium required for activation of pathogen defence in parsley. Plant Cell 12:1425-1440.

Böttcher, C., Westphal, L., Schmotz, C., Prade, E., Scheel, D., and Glawischnig, E. 2009. The multifunctional enzyme CYP71B15 (PHYTOALEXIN DEFICIENT3) converts cysteine-indole-3-acetonitrile to camalexin in the indole-3-acetonitrile metabolic network of *Arabidopsis thaliana*. Plant Cell 21:1830-1845.

Burow, M., Halkier, B. A., and Kliebenstein, D. J. 2010. Regulatory networks of glucosinolates shape *Arabidopsis thaliana* fitness. Curr. Opin. Plant Biol. 13:348-353.

Bütehorn, B., Rhody, D., and Franken, P. 2000. Isolation and characterization of Pitef1 encoding the translation elongation factor EF-1α of the root endophyte *Piriformospora indica*. Plant Biol. 2:687-692.

Camehl, I., and Oelmüller, R. 2010. Do ethylene response factors 9 and 14 repress *PR* gene expression in the interaction between *Piriformospora indica* and *Arabidopsis*? Plant Signal Behav. 9:5-8.

Camehl, I., Sherameti, I., Venus, Y., Bethke, G., Varma, A., Lee, J., and Oelmüller, R. 2010. Ethylene signalling and ethylene-targeted transcription factors are required to balance beneficial and nonbeneficial traits in the symbiosis between the endophytic fungus *Piriformospora indica* and *Arabidopsis thaliana*. New Phytol. 185:1062-1073.

Camehl, I., Drzewiecki, C., Vadassery, J., Shahollari, B., Sherameti, I., Forzani, C., Munnik, T., Hirt, H., and Oelmüller, R. 2011. The OXI1 kinase pathway mediates *Piriformospora indica*-induced growth promotion in *Arabidopsis*. PLoS Pathog. 7:e1002051.

Contreras-Cornejo, H. A., Macías-Rodríguez, L., Cortés-Penagos, C., and López-Bucio, J. 2009. *Trichoderma virens*, a plant beneficial fungus, enhances biomass production and promotes lateral root growth through an auxin-dependent mechanism in *Arabidopsis*. Plant Physiol. 149:1579-1592.

Deak, M., Casamayor, A., Currie, R. A., Downes, C. P., and Alessi, D. R. 1999. Characterisation of a plant 3-phosphoinositide-dependent protein kinase-1 homologue which contains a pleckstrin homology domain. FEBS (Fed. Eur. Biochem. Soc.) Lett. 451:220-226.

de Hoff, P. L., Brill, L. M., and Hirsch, A. M. 2009. Plant lectins: the ties that bind in root symbiosis and plant defence. Mol. Genet. Genomics 282:1-15.

Deshmukh, S., Hückelhoven, R., Schäfer, P., Imani, J., Sharma, M., Weiss, M., Waller, F., and Kogel, K. H. 2006. . The root endophytic fungus *Piriformospora indica* requires host cell death for proliferation during mutualistic symbiosis with barley. Proc. Natl. Acad. Sci. U.S.A. 103:18450-18457.

Devaiah, S. P., Roth, M. R., Baughman, E., Li, M., Tamura, P., Jeannotte, R., Welti, R., and Wang X. 2006. Quantitative profiling of polar glycerolipid species from organs of wild-type *Arabidopsis* and a phospholipase Dalpha1 knockout mutant. Phytochemistry 67:1907-1924.

Devarenne, T. P., Ekengren, S. K., Pedley, K. F., and Martin, G. B. 2006. Adi3 is a Pdk1 interacting AGC kinase that negatively regulates plant

cell death. EMBO (Eur. Mol. Biol. Organ.) J. 25:255-265.
Fester, T., and Hause, G. 2005. Accumulation of reactive oxygen species in arbuscular mycorrhizal roots. Mycorrhiza 15:373-379.
Foreman, J., Demidchik, V., Bothwell, J. H., Mylona, P., Miedema, H., Torres, M. A., Linstead, P., Costa, S., Brownlee, C., Jones, J. D., Davies, J. M., and Dolan, L. 2003. Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. Nature 422:442-446.
Geu-Flores, F., Møldrup, M. E., Böttcher, C., Olsen, C. E., Scheel, D., and Halkier, .BA. 2011. Cytosolic γ-glutamyl peptidases process glutathione conjugates in the biosynthesis of glucosinolates and camalexin in *Arabidopsis*. Plant Cell 23:2456-2469.
Glawischnig, E. 2007. Camalexin. Phytochemistry 68:401-406.
Glawischnig, E., Hansen, B. G., Olsen, C. E., and Halkier, B. A. 2004. Camalexin is synthesized from indole-3-acetaldoxime, a key branching point between primary and secondary metabolism in *Arabidopsis*. Proc. Natl. Acad. Sci. U.S.A. 101:8245-8250.
Gust, A. A., Biswas, R., Lenz, H. D., Rauhut, T., Ranf, S., Kemmerling, B., Götz, F., Glawischnig, E., Lee, J., Felix, G., and Nürnberger, T. 2007. Bacteria-derived peptidoglycans constitute pathogen-associated molecular patterns triggering innate immunity in *Arabidopsis*. J. Biol. Chem. 282:32338-32348.
Gutjahr, C., and Paszkowski, U. 2009. Weights in the balance: jasmonic acid and salicylic acid signaling in root-biotroph interactions. Mol. Plant-Microbe Interact. 22:763-772.
Hansen, C. H., Du, L., Naur, P., Olsen, C. E., Axelsen, K. B., Hick, A. J., Pickett, J. A., and Halkier, B. A. 2001. CYP83b1 is the oxime-metabolizing enzyme in the glucosinolate pathway in *Arabidopsis*. J. Biol. Chem. 276:24790-24796.
Herre, E. A., Mejía, L. C., Kyllo, D. A., Rojas, E., Maynard, Z., Butler, A., and Van Bael, S. A. 2007. Ecological implications of anti-pathogen effects of tropical fungal endophytes and mycorrhizae. Ecology 88:550-558.
Hill, T. W., and Kaefer, E. 2001. Improved protocols for *Aspergillus* medium: trace elements and minimum media salt stock solutions. Fungal Genet. Newl. 48:20-21.
Hirt, H., Garcia, A. V., and Oelmüller, R. 2011. AGC kinases in plant development and defense. Plant Sign. Behavior 6:1030-1033.
Jabs, T., Tschöpe, M., Colling, C., Hahlbrock, K., and Scheel, D. 1997. Elicitor-stimulated ion fluxes and $O_2^-$ from the oxidative burst are essential components in triggering defence gene activation and phytoalexin synthesis in parsley. Proc. Natl. Acad. Sci. U.S.A. 94:4800-4805.
Jacobs, S., Zechmann, B., Molitor, A., Trujillo, M., Petutschnig, E., Likpa, V., Kogel, K. H., and Schäfer, P. 2011. Broad-spectrum suppression of innate immunity is required for colonization of *Arabidopsis* roots by the fungus *Piriformospora indica*. Plant Physiol. 156:726-740.
Johnson, J. M., and Oelmüller, R. 2009. Mutualism and parasitism: life in an unstable continuum. What can we learn from the mutualitic interaction between *Piriformospora indica* and *Arabidopsis thaliana*? Endocyt. Cell Res. 19:81-111.
Johnson, J. M., Nongbri, P. L., Sherameti, I., and Oelmüller, R. 2011a. Calcium signaling and cytosolic calcium measurements in plants. Endocyt. Cell Res. 21:64-76. http://zs.thulb.uni-jena.de/receive/jportal_jparticle_00231945. Published online.
Johnson, J. M., Sherameti, I., Ludwig, A., Nongbri, P.L., Sun, C., Lou, B., Varma, A., and Oelmüller, R. 2011b. Protocols for *Arabidopsis thaliana* and *Piriformospora indica* cocultivation—A model system to study plant beneficial traits. Endocyt. Cell Res. 21:101-113. http://zs.thulb.uni-jena.de/receive/jportal_jparticle_00247947. Published online.
Kennedy, P. 2010. Ectomycorrhizal fungi and interspecific competition: species interactions, community structure, coexistence mechanisms, and future research directions. New Phytol. 187:895-910.
Kliebenstein, D. J., Rowe, H. C., and Denby, K. J. 2005. Secondary metabolites influence *Arabidopsis/Botrytis* interactions: variation in host production and pathogen sensitivity. Plant J. 44:25-36.
Knight, H., Trewavas, A. J., and Knight, M. R. 1997. Calcium signalling in *Arabidopsis thaliana* responding to drought and salinity. Plant J. 12:1067-1078.
Kroj, T., Rudd, J. J., Nürnberger, T., Gabler, Y., Lee, J., and Scheel, D. 2003. Mitogen-activated protein kinases play an essential role in oxidative burst-independent expression of pathogenesis-related genes in parsley. J. Biol. Chem. 278:2256-2264.
Lee, J., Rudd, J. J., Macioszek, V. K., and Scheel, D. 2004. Dynamic changes in the localization of MAPK cascade components controlling pathogenesis-related (PR) gene expression during innate immunity in parsley. J. Biol. Chem. 279:22440-22448.
Ligterink, W., Kroj, T., zur Nieden, U., Hirt, H., and Scheel, D. 1997. Receptor-mediated activation of a MAP kinase in pathogen defence of plants. Science 276:2054-2057.
Ljung, K., Hull, A. K., Celenza, J., Yamada, M., Estelle, M., Normanly, J., and Sandberg, G. 2005. Sites and regulation of auxin biosynthesis in *Arabidopsis* roots. Plant Cell 17:1090-1104.
Martin, F., Kohler, A., and Duplessis, S. 2007. Living in harmony in the wood underground: ectomycorrhizal genomics. Curr. Opin. Plant Biol. 10:204-210.
Masucci, J. D., and Schiefelbein, J. W. 1994. The *rhd6* mutation of *Arabidopsis thaliana* alters root-hair initiation through an auxin- and ethylene-associated process. Plant Physiol. 106:1335-1346.
Matsui, H., Miyao, A, Takahashi, A., and Hirochika, H. 2010. Pdk1 kinase regulates basal disease resistance through the OsOxi1–OsPti1a phosphorylation cascade in rice. Plant Cell Physiol. 51:2082-2091.
Mikkelsen, M. D., Fuller, V. L., Hansen, B. G., Nafisi, M., Olsen, C. E., Nielsen, H. B., and Halkier, B. A. 2009. Controlled indole-3-acetaldoxime production through ethanol-induced expression of CYP79B2. Planta 229:1209-1217.
Millet, Y. A., Danna, C. H., Clay, N. K., Songnuan, W., Simon, M. D., Werck-Reichhart, D., and Ausubel, F. 2010. Innate immune responses activated in *Arabidopsis* roots by microbe-associated molecular patterns. Plant Cell 22:973-990.
Mittler, R., Vanderauwera, S., Suzuki, N., Miller, G., Tognetti, V. B., Vandepoele, K., Gollery, M., Shulaev, V., and Van Breusegem, F. 2011. ROS signaling: the new wave? Trends Plant Sci. 16:300-309.
Murashige, T., and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-497.
Nafisi, M., Sønderby, I. E., Hansen, B. G., Geu-Flores, F., Nour-Eldin, H. H., Nørholm, M. H. H., Jensen, N. B., Li, J., and Halkier, B. A. 2006. Cytochromes P450 in the biosynthesis of glucosinolates and indole alkaloids. Phytochem. Rev. 5:331-346.
Nafisi, M., Goregaoker, S., Botanga, C. J., Glawischnig, E., Olsen, C. E., Halkier, B. A., and Glazebrook, J. 2007. *Arabidopsis* cytochrome P450 monooxygenase 71A13 catalyzes the conversion of indole-3-acetaldoxime in camalexin synthesis. Plant Cell 19:2039-2052.
Nürnberger, T., Nennstiel, D., Jabs, T., Sacks, W. R., Hahlbrock, K., and Scheel, D. 1994. High affinity binding of a fungal oligopeptide elicitor to parsley plasma membranes triggers multiple defence responses. Cell 78:449-460.
Oelmüller, R., Sherameti, S., Tripathi, S., and Varma, V. 2009. *Piriformospora indica*, a cultivable root endophyte with multiple biotechnological applications. Symbiosis 49:1-17.
Parisy, V., Poinssot, B., Owsianowski, L., Buchala, A., Glazebrook, J., and Mauch, F. 2007. Identification of PAD2 as a γ-glutamylcysteine synthetase highlights the importance of glutathione in disease resistance of *Arabidopsis*. Plant J. 49:159-172.
Peškan-Berghöfer, T., Shahollari, B., Giong, P. H., Hehl, S., Markert, C., Blanke, V., Varma, A. K., and Oelmüller, R. 2004. Association of *Piriformospora indica* with *Arabidopsis thaliana* roots represents a novel system to study beneficial plant-microbe interactions and involves early plant protein modifications in the endoplasmatic reticulum and at the plasma membrane. Physiol. Plant. 122:465-477.
Pfaffl, M. W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29:45.
Pozo, M. J., and Azcón-Aguilar, C. 2007. Unraveling mycorrhiza-induced resistance. Curr. Opin. Plant Biol. 10:393-398.
Purin, S., and Rillig, M. C. 2008. Parasitism of arbuscular mycorrhizal fungi: reviewing the evidence. FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Lett. 279:8-14.
Qiu, J. L., Fiil, B. K., Petersen, K., Nielsen, H. B., Botanga, C. J., Thorgrimsen, S., Palma, K., Suarez-Rodriguez, M. C., Sandbech-Clausen, S., Lichota, J., Brodersen, P., Grasser, K. D., Mattsson, O., Glazebrook, J., Mundy, J., and Petersen, M. 2008. *Arabidopsis* MAP kinase 4 regulates gene expression through transcription factor release in the nucleus. EMBO (Eur. Mol. Biol. Organ.) J. 27:2214-2221.
Qutob, D., Kemmerling, B., Brunner, F., Küfner, I., Engelhardt, S., Gust, A. A., Luberacki, B., Seitz, H. U., Stahl, D., Rauhut, T., Glawischnig, E., Schween, G., Lacombe, B., Watanabe, N., Lam, E., Schlichting, R., Scheel, D., Nau, K., Dodt, G., Hubert, D., Gijzen, M., and Nürnberger, T. 2006. Phytotoxicity and innate immune responses induced by Nep1-like proteins. Plant Cell 18:3721-3744.
Raacke, I. C., Rad, U. v., Mueller, M. J., and Berger, S. 2006. Yeast increases resistance in *Arabidopsis* against *Pseudomonas syringae* and *Botrytis cinerea* by salicylic acid-dependent as well as -independent mechanisms. Mol. Plant-Microbe Interact. 19:1138-1146.
Rauhut, T., and Glawischnig, E. 2009. Evolution of camalexin and structurally related indolic compounds. Phytochemistry 70:1638-1644.
Rauhut, T., Luberacki, B., Seitz, H. U., and Glawischnig, E. 2009. Inducible expression of a Nep1-like protein serves as a model trigger system of camalexin biosynthesis. Phytochemistry 70:185-189.
Ren, D., Liu, Y., Yang, K. Y., Han, L., Mao, G., Glazebrook, J., and Zhang, S. 2008. A fungal-responsive MAPK cascade regulates phytoalexin biosynthesis in *Arabidopsis*. Proc. Natl. Acad. Sci. U.S.A. 105:5638-5643.
Rentel, M. C., and Knight, M. R. 2004. Oxidative stress-induced calcium signalling in *Arabidopsis*. Plant Physiol. 135:1471-1479.

Rentel, M. C., Lecourieux, D., Ouaked, F., Usher, S. L., Petersen, L., Okamoto, H., Knight, H., Peck, S. C., Grierson, C. S., Hirt, H., and Knight, M. R. 2004. OXI1 kinase is necessary for oxidative burst-mediated signalling in *Arabidopsis*. Nature 427:858-861.

Rogers, E. E., Glazebrook, J., and Ausubel, F. M. 1996. Mode of action of the *Arabidopsis thaliana* phytoalexin camalexin and its role in *Arabidopsis*–pathogen interactions. Mol. Plant-Microbe Interact. 9:748-757.

Sanchez-Vallet, A., Ramos, B., Bednarek, P., López, G., Piślewska-Bednarek, M., Schulze-Lefert, P., and Molina, A. 2010. Tryptophan-derived secondary metabolites in *Arabidopsis thaliana* confer non-host resistance to necrotrophic *Plectosphaerella cucumerina* fungi. Plant J. 63:115-127.

Schlaeppi, K., Abou-Mansour, E., Buchala, A., and Mauch F. 2010. Disease resistance of *Arabidopsis* to *Phytophthora brassicae* is established by the sequential action of indole glucosinolates and camalexin. Plant J. 62:840-851.

Schuhegger, R., Nafisi, M., Mansourova, M., Petersen, B. L., Olsen, C. E., Svatos, A., Halkier, B. A., and Glawischnig, E. 2006. CYP71B15 (PAD3) catalyzes the final step in camalexin biosynthesis. Plant Physiol. 141:1248-1254.

Schuhegger, R., Rauhut, T., and Glawischnig, E. 2007. Regulatory variability of camalexin biosynthesis. J. Plant Physiol. 164:636-644.

Sherameti, I., Nakamura, M., Yamamoto, Y. Y., Pfannschmidt, T., Obokata, J., and Oelmüller, R. 2002. Polyribosome loading of spinach mRNAs for photosystem I subunits is controlled by photosynthetic electron transport. Plant J. 32:631-639.

Sherameti, I., Venus, Y., Drzewiecki, C., Tripathi, S., Dan, V. M., Nitz, I., Varma, A., Grundler, F. M., and Oelmüller, R. 2008. PYK10, a beta-glucosidase located in the endoplasmatic reticulum, is crucial for the beneficial interaction between *Arabidopsis thaliana* and the endophytic fungus *Piriformospora indica*. Plant J. 54:428-439.

Siemens, J., Glawischnig, E., and Ludwig-Müller, J. 2008. Indole glucosinolates and camalexin do not influence the development of the clubroot disease in *Arabidopsis thaliana*. J. Phytopathol. 156:322-337.

Stone, J. M., Heard, J. E., Asai, T., and Ausubel, F. M. 2000. Simulation of fungal-mediated cell death by fumonisin B1 and selection of fumonisin B1-resistant (fbr) *Arabidopsis* mutants. Plant Cell 12:1811-1822.

Strack, D., Fester, T., Hause, B., Schliemann, W., and Walter, M. H. 2003. Arbuscular mycorrhiza: biological, chemical, and molecular aspects. J. Chem. Ecol. 29:1955-1979.

Su, T., Xu, J., Li, Y., Lei, L., Zhao, L., Yang, H., Feng, J., Liu, G., and Ren, D. 2011. Glutathione-indole-3-acetonitrile is required for camalexin biosynthesis in *Arabidopsis thaliana*. Plant Cell 23:364-380.

Sugawara, S., Hishiyama, S., Jikumaru, Y., Hanada, A., Nishimura, T., Koshiba, T., Zhao, Y., Kamiya, Y., and Kasahara, H. 2009. Biochemical analyses of indole-3-acetaldoxime-dependent auxin biosynthesis in *Arabidopsis*. Proc. Natl. Acad. Sci. U.S.A. 106:5430-5435.

Truman, W. M., Bennett, M. H., Turnbull, C. G., and Grant, M. R. 2010. *Arabidopsis* auxin mutants are compromised in systemic acquired resistance and exhibit aberrant accumulation of various indolic compounds. Plant Physiol. 152:1562-1573.

Vadassery, J., Ranf, S., Drzewiecki, C., Mithöfer, A., Mazars, C., Scheel, D., Lee, J., and Oelmüller, R. 2009. A cell wall extract from the endophytic fungus *Piriformospora indica* promotes growth of *Arabidopsis* seedlings and induces intracellular calcium elevation in roots. Plant J. 59:193-206.

Van Breusegem, F., Bailey-Serres, J., and Mittler, R. 2008. Unraveling the tapestry of networks involving reactive oxygen species in plants. Plant Physiol. 147:978-984.

van der Luit, A. H., Piatti, T., van Doorn, A., Musgrave, A., Felix, G., Boller, T., and Munnik, T. 2000. Elicitation of suspension-cultured tomato cells triggers the formation of phosphatidic acid and diacylglycerol pyrophosphate. Plant Physiol.123:1507-1516.

Yamaguchi, T., Minami, E., Ueki, J., and Shibuya, N. 2005. Elicitor-induced activation of phospholipases plays an important role for the induction of defense responses in suspension-cultured rice cells. Plant Cell Physiol. 46:579-587.

Zhao, Y., Hull, A. K., Gupta, N. R., Goss, K. A., Alonso, J., Ecker, J. R., Normanly, J., Chory, J., and Celenza, J. L. 2002. Trp-dependent auxin biosynthesis in *Arabidopsis*: involvement of cytochrome P450s CYP79B2 and CYP79B3. Genes Dev. 16:3100-3112.

Zimmermann, S., Nürnberger, T., Frachisse, J. M., Wirtz, W., Guern, J., Hedrich, R., and Scheel, D. 1997. Receptor-mediated activation of a plant $Ca^{2+}$-permeable ion channel involved in pathogen defence. Proc. Natl. Acad. Sci. U.S.A. 94:2751-2755.

## 4.2 Manuscript II

**Role of *Piriformospora indica* in Sulfur Metabolism in *Arabidopsis thaliana***

Pyniarlang L. Nongbri and Ralf Oelmüller

*A. Varma et al. (eds.), Piriformospora indica, Soil Biology 33,*
*DOI 10.1007/978-3-642-33802-1_18,* 

# Chapter 18
# Role of *Piriformospora indica* in Sulfur Metabolism in *Arabidopsis thaliana*

**Pyniarlang L. Nongbri and Ralf Oelmüller**

## 18.1 Introduction

Plant performance and productivity are highly dependent on sulfur (S), which is mainly taken up from the soil as sulfate before reduction and metabolism into S-containing compounds (Leustek et al. 2000; Saito et al. 2004). Animals are unable to reduce sulfate and thus require S-containing amino acids or proteins as diet. Therefore, sulfate assimilation by plants is essential for all life on earth. Beside the presence of S in amino acids, it is found in many plant metabolites including vitamins, coenzymes, volatiles, and defense compounds (Grubb and Abel 2006; Halkier and Gershenzon 2006; Leustek et al. 2000; Saito et al. 2004). The presence of S in many redox mediators also highlights its importance for signaling processes.

Plants respond to S-limiting conditions by increasing the amount and activity of sulfate uptake and transport systems (Clarkson et al. 1983; Deane-Drummond 1987; Smith et al. 1995, 1997). High-affinity sulfate transporters located in the plasma membrane of roots (Shibagaki et al. 2002; Takahashi et al. 2000; Vidmar et al. 2000; Yoshimoto et al. 2003), transporters for vascular transport (Kataoka et al. 2004a, b; Takahashi et al. 1997; Yoshimoto et al. 2003), and the release of sulfate from the vacuole (Kataoka et al. 2004a, b) coordinate the cellular response during initial stages of S limitation. As a second strategy, synthesis of S-containing metabolites and storage compounds is downregulated, and S is released from these compounds through active breakdown processes (Hirai et al. 2004, 2005; Kutz et al. 2002). In Brassicales, up to 30 % of the S is stored in glucosinolates, S-rich metabolites that function in the defense of plants against pests and pathogens (Falk et al. 2007). Under S deficiency, there is a general downregulation of glucosinolate biosynthesis genes which accompanies an upregulation of genes

P.L. Nongbri • R. Oelmüller (✉)
Institut für Pflanzenphysiologie, Friedrich-Schiller-Universität Jena, Dornburger Str, 159, 07743 Jena, Germany
e-mail: pyndrop@gmail.com; b7oera@uni-jena.de

A. Varma et al. (eds.), *Piriformospora indica*, Soil Biology 33,
DOI 10.1007/978-3-642-33802-1_18, 

controlling glucosinolate breakdown. Activation of sulfate acquisition and repression of glucosinolate production may occur in parallel in response to S limitation (Hirai et al. 1995, 2003, 2004, 2005, 2007; Maruyama-Nakashita et al. 2003, 2005). Thus glucosinolates may be considered a potential source of S for other metabolic processes under S limitation (Falk et al. 2007; Grubb and Abel 2006; Halkier and Gershenzon 2006). The active defense compounds are released from the glucosinolates after enzymatic cleavage. Several enzymes for these reactions play important functions in plant/microbe interactions. PEN2, for instance, a myrosinase analyzed by Bednarek et al. (2009), restricts pathogen entry into leaf cells. PEN2 exhibits striking sequence similarities to PYK10, a highly abundant enzyme in the roots that restricts root colonization by *P. indica* (Sherameti et al. 2008). We could show that PYK10 is required for the beneficial interaction between the two symbionts and speculated that Brassicaceae contain such high amounts of this enzyme in the roots to protect them against soil-borne fungi. Very recently, the importance of the glucosinolate metabolism for antifungal defense and innate immune response has been reported by two groups (Bednarek et al. 2009; Clay et al. 2009). Breakdown of indolic glucosinolates also generates auxins in roots under certain stress conditions, which may participate in the stimulation of root development for sulfate uptake. One of the central transcription factors that regulate aliphatic glucosinolate biosynthesis in *Arabidopsis* is MYB28 (Gigolashvili et al. 2008; Hirai et al. 2007; Sønderby et al. 2007). The aliphatic glucosinolates derive from methionine and are synthesized by two P450 enzymes, CYP79F1 and CYP79F2 (Chen et al. 2003). The *myb28 myb29* double mutant completely lacks aliphatic glucosinolates, and the glucosinolate biosynthesis genes are drastically downregulated (Beekwilder et al. 2008).

Processes controlling S metabolism are important for agriculture, horticulture, and medicine. The qualities and biomass production of crop plant species are severely impaired under S starvation and breakdown of endogenous S compounds such as glucosinolates affects plant fitness and reproductivity. Optimization of the volatile composition plays a major role in horticulture. Furthermore, many S-containing secondary metabolites are used as cancer preventives in diets (Talalay and Fahey 2001).

Although the network of S metabolism is coordinately regulated under S limitations, many of these processes are not fully understood yet. Several transcription factors and signaling proteins involved in indole glucosinolate biosynthesis have been characterized in *Arabidopsis* (Celenza et al. 2005; Levy et al. 2005; Skirycz et al. 2006). In 2006, Maruyama-Nakashita et al. identified sulfur limitation 1 (SLIM1) as a central regulator of plant S responses and metabolism in *Arabidopsis*. The identified mutant *slim1* was unable to induce transcripts for the high-affinity transporter SULTR1;2 under low S conditions. Sulfate uptake and plant growth under S starvation were significantly reduced in *slim1*, and SLIM1 controlled both the activation of sulfate acquisition and degradation of glucosinolates under S limitations. SLIM1 is a member of the ethylene-insensitive-like (EIL) transcription factor family and identical to EIL3 which is crucial for ethylene signal transduction (Chao et al. 1997; Guo and Ecker 2003; Solano et al. 1998). The EIL family consists

of six transcription factors (EIN3, EIL1-5; Guo and Ecker 2003). Interestingly, overexpression of *SLIM1*, but not of the other *EIL* genes from *Arabidopsis*, restored the wild-type (WT) phenotype of *slim1* mutants, suggesting uniqueness of SLIM1 in the EIL group as S response regulators. A short domain from SLIM1 ($S_{162}$–$G_{288}$) recognizes double-stranded DNA with the conserved EIL- and EIN3-binding motif AYGWAYCT (Kosugi and Ohashi 2000; Solano et al. 1998; Yamasaki et al. 2005). Furthermore, MYB72 has been identified as interaction partner of SLIM1 in the yeast-two-hybrid (Y2H) system (Van der Ent et al. 2008). MYB72 is required in early signaling steps of rhizobacteria-induced systemic resistance (ISR) in *Arabidopsis* (Van Wees et al. 2008).

Enzymes involved in S assimilation are often encoded by multigene families in *Arabidopsis*. Their members can have redundant functions (e.g., serine acetyltransferases; Watanabe et al. 2008) or are highly specialized and expressed in different tissues (e.g., branched-chain aminotransferases; Schuster et al. 2006), or they are imbedded into complex signaling pathways (e.g., adenosine 5′-phosphosulfate reductase; Koprivova et al. 2008; Vauclare et al. 2002). Several members of these gene families respond to S starvation, while others do not (Maruyama-Nakashita et al. 2003). There is increasing evidence that posttranscriptional regulatory circuits and feedback loops play important roles in the regulation of the activities of these enzymes.

We found that *Piriformospora indica* strongly affects the S metabolism in the symbiotic interaction with *Arabidopsis*. *P. indica* is an endophytic fungus of the Basidiomycetes which colonizes the roots of many plant species including mono- and dicots and mosses, many of which are of forestry, agricultural, horticultural, and medicinal importance. Root colonization is accompanied by the promotion of growth and higher seed yield, and the plants are more resistant to various biotic and abiotic stresses (Shahollari et al. 2007; Sherameti et al. 2008; Vadassery et al. 2009a, b).

S uptake and metabolism play an important role in plant/microbe interactions. However, in contrast to phosphorus and nitrogen, little is known about S regulation in beneficial interactions of roots with soilborne microorganisms. Mansouri-Bauly et al. (2006) have shown that *L. bicolor* increases sulfate supply to the plant by extended sulfate uptake and the plant provides the ectomycorrhizal fungus with reduced S. Many S-containing components play a crucial role in defense against pathogens and in biotic and abiotic stress (Hildebrandt et al. 2006; Rausch and Wachter 2005), when they are either upgraded or induced *via* jasmonic acid and/or other signals (cf. Hilpert et al. 2001; Mikkelsen et al. 2003; Nibbe et al. 2002; Xiang and Oliver 1998). While the cysteine content is tightly regulated, the cysteine precursor *O*-acetylserine (OAS) is rapidly upregulated and functions as a signaling metabolite by controlling the expression of sulfate transporters and several genes of the S-assimilation pathway (Hirai et al. 2003). Glutathione (GSH) is upgraded with increasing sulfate supply and may establish resistance to stress. GSH is a major redox buffer and protects the cell against reactive oxygen species. An *Arabidopsis* mutant lacking the gamma-glutamylcysteine ligase 1 (GSH1), the rate-limiting enzyme for GSH synthesis (Ball et al. 2004), is impaired in defense reactions

against pathogens. Waller et al. (2005) and Baltruschat et al. (2008) have shown that GSH plays a crucial role in *P. indica*-induced resistance of barley plants against pathogens. In the ascorbate–GSH cycle, the function of GSH is linked to ascorbic acid, and the electron flow from NADPH (Foyer and Noctor 2005; Rausch and Wachter 2005). Dehydroascorbate reductase (DHAR) and glutathione reductase activities are stimulated by *P. indica* in salt-stress barley (Baltruschat et al. 2008). Monodehydroascorbate reductase 2 and DHAR 5 are crucial for a mutualistic interaction between *P. indica* and *Arabidopsis* under drought stress (Vadassery et al. 2009a). Many glutathione *S*-transferases (GST) are crucial for detoxification mechanisms, and GSH is the precursor of phytochelatins, cysteine-rich peptides synthesized *via* phytochelatin synthase (Cobbett and Goldsbrough 2002). Genes for the latter examples are rapidly upregulated when *Arabidopsis* roots are exposed to *P. indica*.

GSH may also be responsible for the activation of the nonexpressor of PR genes (NPR1). NPR1 affects transcription of salicylic acid-induced genes for pathogenesis-related proteins (Dong 2004). In the oxidized, non-induced plants, NPR1 is cross-linked by intermolecular disulfide bridges and localized in aggregated form in the cytosol. Upon infection, NPR1 becomes reduced, and the monomers are translocated to the nucleus. Inactivation of *NPR1* does not affect the beneficial interaction at the seedling's stage; however, adult plants become over-colonized, and thus, the interaction shifts to parasitism (unpublished).

SLIM1/EIL3 also plays an important role in plant immune responses triggered by beneficial microbes (Van Wees et al. 2008). Plant growth-promoting rhizobacteria and mycorrhizal fungi can improve plant performance by ISR-mediated defense responses that confer resistance to pathogens and insects. Recognition of microbe-associated molecular patterns from beneficial microbes leads to the activation of the transcription factor gene *MYB72*, and the protein interacts with SLIM1/EIL3 to induce a jasmonic acid/ethylene-dependent signaling pathway which primes the aerial parts of the plant for enhanced expression of jasmonic acid/ethylene-dependent genes (Van der Ent et al. 2008).

Here, we summarize recent results which demonstrate that quite diverse aspects of the S metabolism in *Arabidopsis* are affected by the root-colonizing beneficial fungus *P. indica*.

## 18.2 *P. indica* Becomes More Important for Plant Performance Under S Limitations

The analysis of the interaction of *P. indica* with *Arabidopsis* roots uncovered that genes involved in S uptake and metabolism are targets of the fungus. We therefore tested how plant performance is influenced under S limitations. Reduction of the S level in the growth medium strongly enhanced the beneficial effect induced by *P. indica*, and the differences in the growth rates between untreated and treated WT

18 Role of *Piriformospora indica* in Sulfur Metabolism in *Arabidopsis thaliana*

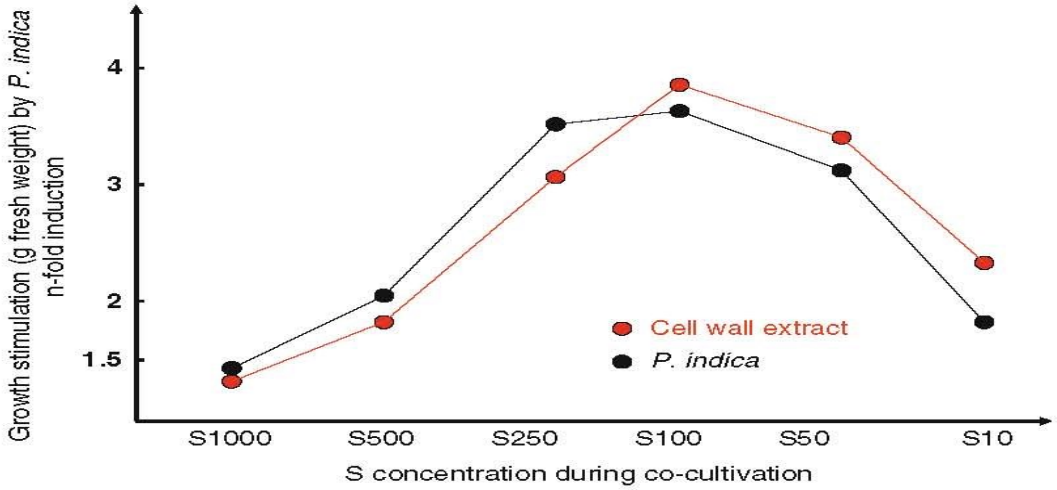



**Fig. 18.1** Growth stimulation by *P. indica* (expressed as *n*-fold increase in fresh weight of *P. indica*-colonized vs. control seedlings after 10 days) on different sulfate concentration in the medium. Cocultivation occurred either with the fungus (*black*) or a cell wall extract from the fungus (*red*), cf. below

seedlings increased greater than threefold on media with 10 μM S compared to the full medium (cf. Vadassery et al. 2009a, b; Fig. 18.1). Interestingly, stimulation of plant performance under S limitations is not only promoted by the fungus itself but also observed if a cell wall extract of the fungus is applied to Arabidopsis roots. This suggests that microbe-associated molecular patterns present in the cell wall extract trigger processes in the roots cells which promote plant performance. Thus, better plant performance is not exclusively caused by a more efficient transfer of S nutrients *via* the hyphae to the roots. These observations in combination with the results outlined below support the idea that the S metabolism is a major target of the fungus in Arabidopsis and that apparently signals from the fungus trigger processes in the roots which strongly affect the S metabolism.

## 18.3 Transcription Factors Regulating S Metabolism and Genes Involved in S Metabolism

SLIM1 is a regulatory transcription factor controlling many downstream genes involved in S metabolism (Maruyama-Nakashita et al. 2006). Expression of *slim1* is barely regulated in Arabidopsis roots. However, we observed an approximately 50 % increase in the *SLIM1* mRNA levels in *P. indica*-colonized Arabidopsis roots grown under S limitations. Since this result is confirmed when a cell wall extract from the fungus was applied to the roots, we postulate that *P. indica*-derived

components trigger signaling events in the roots that activate the S metabolism by activating *slim1* gene expression.

Genes involved in S metabolism, which are regulated by SLIM1 (Maruyama-Nakashita et al. 2006), are also regulated by *P. indica* during early phases of cocultivation (i.e., within 1 h under sufficient S supply; 1,000 μM). This is particularly striking for *ASP1*, *ASP3*, and *ASP4* and demonstrates that the ATP sulfurylases are specific targets of the fungus even under sufficient S supply. However, some of them are even strongly upregulated under S starvation. Examples are *GSH1*, *GSH2*, *BCAT2*, *BCAT4*, *MAM3*, three *GST* genes, *PHYTOCHELATIN SYNTHASE*, and *NPR1*. This again confirms that *P. indica* controls S metabolism, in particular under S-limiting conditions.

## 18.4 Glucosinolates

The message levels for enzymes involved in glucosinolate biosynthesis and cleavage are upregulated by *P. indica* under sufficient S conditions which raises many questions. A mutant which shows a reduced response to *P. indica* has a lesion in *myb28*. We thus analyzed *myb28 myb29* seedlings in greater details and found that adult plants grow much slower compared to the uncolonized controls. These results suggest that glucosinolates are targets of the fungus and that long-term harmony between the two symbionts requires aliphatic glucosinolate biosynthesis (Fig. 18.2).

Furthermore, we have recently demonstrated that PYK10, a myrosinase abundantly expressed in roots, is required for the beneficial interaction between *P. indica* and *Arabidopsis* (Sherameti et al. 2008). Inactivation of *PYK10* results in an uncontrolled growth of fungal hyphae, presumably because the enzyme is required to release an aglycone from a so far unidentified glucosinolate substrate. PYK10 exhibits striking sequence similar to PEN2, a glycosyl hydrolase, which restricts pathogen entry of two ascomycete powdery mildew fungi into *Arabidopsis* leaf cells (Lipka et al. 2005). 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 (cf. also Bednarek et al. 2009). Thus, PYK10 might have a similar biological function in our system. This indicates that cleavage of certain glucosinolates might be important for the beneficial interaction of the two symbionts. We postulated that PYK10 as an abundant myrosinase in Arabidopsis roots controls the release of a toxic compound required to control root colonization. Partial inactivation of PYK10 results in over-colonized roots.

We established a hydroponic system in which Arabidopsis growth is strongly promoted when the roots were infected with fungal spores prior to transfer to the

18 Role of *Piriformospora indica* in Sulfur Metabolism in *Arabidopsis thaliana*

**Fig. 18.2** WT (*top*) and *myb28 myb29* (*bottom*) plants grown in the presence of *P. indica*. Without *P. indica*, no difference between the two genotypes can be detected

liquid medium. This allowed us to expose the roots of wild-type and mutant seedlings to liquid media with defined combinations of beneficial and pathogenic microbes. Growth of the individual microbes can be followed over time using qRT-PCR. Mutations affecting glucosinolate biosynthesis have a very strong influence on the growth rate of the individual microbes in the hydroponic media, consequently the microbial community changes. We often observe that changes in the glucosinate pattern results in one or a few very dominant microbes, while others barely grow or cannot survive at all. Interestingly, *P. indica* belongs to the class of microbes with a great potential to dominate the others, in particular in mutants impaired in the biosynthesis of certain glucosinolates. Currently, we are trying to understand how changes of the composition of the S-containing secondary metabolites can affect the microbial community in the root environment.

## 18.5 S as Antioxidant

S plays a crucial role as antioxidant, and ascorbate is a major antioxidant and radical scavenger. Monodehydroascorbate reductase (MDAR) and dehydroascorbate reductase (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 cocultivated with *P. indica* (Vadassery et al. 2009a). Since root colonization does not only affect the redox situation in the roots but also in the leaves, an efficient information flow from the roots to the leaves has to be postulated. Furthermore,

a cell wall extract or a culture filtrate from the fungus is sufficient to establish a more reduced atmosphere in roots and leaves. This again demonstrates that *P. indica*-associated molecular patterns are sufficient to trigger signaling processes in roots, which affect the redox potential in both roots and shoots. The important role of MDAR2 and DHAR5 for the symbiotic interaction can be demonstrated by using knockout lines. While wild-type seedlings are taller and perform better in the presence of the fungus when compared to the uncolonized control, growth, plant performance, and seed production were not promoted in *mdar2* (SALK_0776335C) and *dhar5* (SALK_029966C) T-DNA insertion lines. After exposure to drought stress, the two insertion lines suffered severely in the presence of the fungus, because the roots of the drought-stressed insertion lines were colonized more heavily than were the wild-type plants. Upregulation of the message for the antimicrobial PDF1.2 protein in drought-stressed insertion lines indicated that MDAR2 and DHAR5 are crucial for producing sufficient ascorbate to maintain the interaction between *P. indica* and *Arabidopsis* in a mutualistic state. Since not only these two genes for antioxidants are upregulated in *P. indica*-colonized *Arabidopsis* roots, we hypothesize that many more proteins involved in maintaining the redox homeostasis in the cell are involved in establishing and maintaining a mutualistic interaction between the two symbionts.

## 18.6 Summary

Based on the preliminary results obtained so far, we propose the following hypothesis (Fig. 18.3): *P. indica* stimulates the S metabolism, in particular under S-limiting conditions, which results in better plant performance. One of the primary targets might be SLIM1/EIL3 as a central regulator of the entire S pathway. SLIM1 stimulates sulfate reduction by controlling the expression of several enzymes of the S metabolism including those that promote glucosinolate biosynthesis. The availability of higher levels of reduced S is beneficial for plant growth, whereas stimulation of glucosinolate biosynthesis provides the plant with sufficient defense compounds which are required to control the degree of root colonization. The glucosinolates are also involved in the defense against pathogens. Furthermore, the secondary metabolites function as an S storage which can be used under S-limiting conditions. The balance between these two processes might be important for a long-term harmony. The expression of genes regulating the S metabolism is not only controlled by the fungus itself but also by a cell wall extract from the fungus. Apparently *P. indica*-derived components are sufficient to trigger responses in the root cells which control S metabolism. We propose a "master switch" in the signal transduction pathway that determines whether signals from *P. indica* direct the S metabolism towards the primary S metabolism or glucosinate biosynthesis. This "master switch" should integrate biotic and abiotic signals for optimal adaption of the plant to the environment. Finally, based on two-hybrid screens, it has been proposed that SLIM1 interacts with MYB72 in the roots and induces ISR

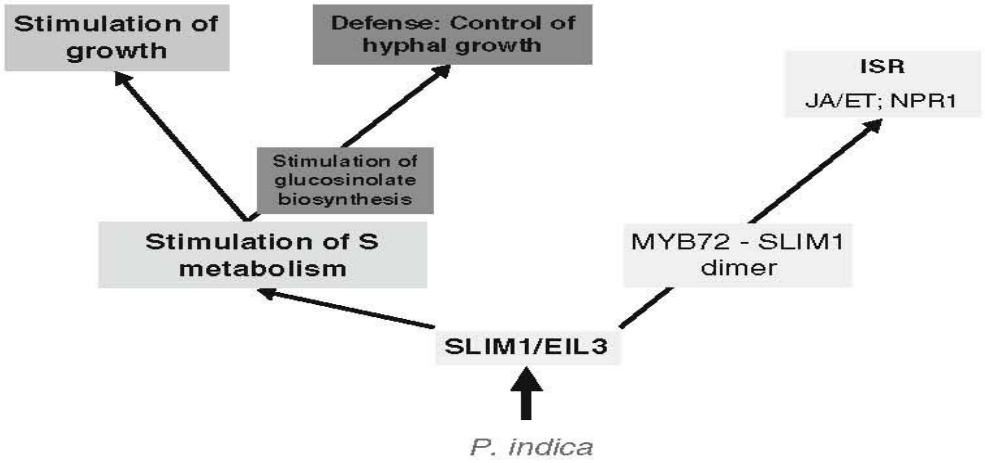



**Fig. 18.3** A scheme describing the working hypothesis for this proposal. *P. indica* required SLIM1 to activate the S metabolism for the stimulation of growth and development of the plants. This also results in the stimulation of the glucosinolate metabolism to control hyphal growth and pathogen attack (under sufficient S conditions). Simultaneously, SLIM1, together with MYB72, triggers ISR.

which primes the aerial parts of the plant to respond more efficiently to pathogenic attack. If this hold true for the *in vivo* situation, ISR against pathogen attack in the leaves is also linked to the S metabolism.

## References

Ball L, Accotto GP, Bechtold U, Creissen G, Funck D, Jimenez A, Kular B, Leyland N, Mejia-Carranza J, Reynolds H, Karpinski S, Mullineaux PM (2004) Evidence for a direct link between glutathione biosynthesis and stress defense gene expression in *Arabidopsis*. Plant Cell 16:2448–2462

Baltruschat H, Fodor J, Harrach BD, Niemczyk E, Barna B, Gullner G, Janeczko A, Kogel KH, Schäfer P, Schwarczinger I, Zuccaro A, Skoczowski A (2008) Salt tolerance of barley induced by the root endophyte *Piriformospora indica* is associated with a strong increase in antioxidants. New Phytol 180:501–510

Bednarek P, Pislewska-Bednarek M, Svatos A, Schneider B, Doubsky J, Mansurova M, Humphry M, Consonni C, Panstruga R, Sanchez-Vallet A, Molina A, Schulze-Lefert P (2009) A glucosinolate metabolism pathway in living plant cells mediates broad-spectrum antifungal defense. Science 323:101–106

Beekwilder J, van Leeuwen W, van Dam NM, Bertossi M, Grandi V, Mizzi L, Soloviev M, Szabados L, Molthoff JW, Schipper B, Verbocht H, de Vos RC, Morandini P, Aarts MG, Bovy A (2008) The impact of the absence of aliphatic glucosinolates on insect herbivory in *Arabidopsis*. PLoS One 30:e2068

Celenza JL, Quiel JA, Smolen GA, Merrikh H, Silvestro AR, Normanly J, Bender J (2005) The *Arabidopsis* ATR1 Myb transcription factor controls indolic glucosinolate homeostasis. Plant Physiol 137:253–262

Chao Q, Rothenberg M, Solano R, Roman G, Terzaghi W, Ecker JR (1997) Activation of the ethylene gas response pathway in *Arabidopsis* by the nuclear protein ETHYLENE-INSENSITIVE3 and related proteins. Cell 89:1133–1144

Chen S, Glawischnig E, Jorgensen K, Naur P, Jorgensen B, Olsen CE, Hansen CH, Rasmussen H, Pickett JA, Halkier BA (2003) CYP79F1 and CYP79F2 have distinct functions in the biosynthesis of aliphatic glucosinolates in *Arabidopsis*. Plant J 33:923–937

Clarkson DT, Smith FW, Van den Berg PJ (1983) Regulation of sulfate transport in a tropical legume. *Macroptilium atropurpureum* cv. Siratro. J Exp Bot 34:1463–1483

Clay NK, Adio AM, Denoux C, Jander G, Ausubel FM (2009) Glucosinolate metabolites required for an *Arabidopsis* innate immune response. Science 323:95–101

Cobbett C, Goldsbrough P (2002) Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis. Annu Rev Plant Biol 53:159–182

Deane-Drummond CE (1987) The regulation of sulfate uptake following growth of *Pisum sativum* L. seedlings in S nutrient limiting conditions. Interaction between nitrate and sulphate transport. Plant Sci 50:27–35

Dong X (2004) NPR1, all things considered. Curr Opin Plant Biol 7:547–552

Falk KL, Tokuhisa JG, Gershenzon J (2007) The effect of sulfur nutrition on plant glucosinolate content: physiology and molecular mechanisms. Plant Biol 9:573–581

Foyer CH, Noctor G (2005) Redox homeostasis and antioxidant signaling: a metabolic interface between stress perception and physiological responses. Plant Cell 17:1866–1875

Gigolashvili T, Engqvist M, Yatusevich R, Müller C, Flügge UI (2008) HAG2/MYB76 and HAG3/MYB29 exert a specific and coordinated control on the regulation of aliphatic glucosinolate biosynthesis in *Arabidopsis thaliana*. New Phytol 177:627–642

Grubb C, Abel S (2006) Glucosinolate metabolism and its control. Trends Plant Sci 11:89–100

Guo H, Ecker JR (2003) Plant responses to ethylene gas are mediated by $SCF^{EBF1/EBF2}$-dependent proteolysis of EIN3 transcription factor. Cell 115:667–677

Halkier BA, Gershenzon J (2006) Biology and biochemistry of glucosinolates. Annu Rev Plant Biol 57:303–333

Hildebrandt U, Ouziad F, Marner FJ, Bothe H (2006) The bacterium *Paenibacillus validus* stimulates growth of the arbuscular mycorrhizal fungus *Glomus intraradices* up to the formation of fertile spores. FEMS Microbiol Lett 254:258–267

Hilpert B, Bohlmann H, op den Camp RO, Przybyla D, Miersch O, Buchala A, Apel K (2001) Isolation and characterization of signal transduction mutants of *Arabidopsis thaliana* that constitutively activate the octadecanoid pathway and form necrotic microlesions. Plant J 26:435–446

Hirai MY, Fujiwara T, Chino M, Naito S (1995) Effects of sulfate concentrations on the expression of a soybean seed storage protein gene and its reversibility in transgenic *Arabidopsis thaliana*. Plant Cell Physiol 36:1331–1339

Hirai MY, Fujiwara T, Awazuhara M, Kimura T, Noji M, Saito K (2003) Global expression profiling of sulfur-starved *Arabidopsis* by DNA macroarray reveals the role of *O*-acetyl-L-serine as a general regulator of gene expression in response to sulfur nutrition. Plant J 33:651–663

Hirai MY, Yano M, Goodenowe DB, Kanaya S, Kimura T, Awazuhara M, Arita M, Fujiwara T, Saito K (2004) Integration of transcriptomics and metabolomics for understanding of global responses to nutritional stresses in *Arabidopsis thaliana*. Proc Natl Acad Sci USA 101:10205–10210

Hirai MY, Klein M, Fujikawa Y, Yano M, Goodenowe DB, Yamazaki Y, Kanaya S, Nakamura Y, Kitayama M, Suzuki H, Sakurai N, Shibata D, Tokuhisa J, Reichelt M, Gershenzon J, Papenbrock J, Saito K (2005) Elucidation of gene-to-gene and metabolite-to-gene networks in *Arabidopsis* by integration of metabolomics and transcriptomics. J Biol Chem 280:25590–25595

Hirai MY, Sugiyama K, Sawada Y, Tohge T, Obayashi T, Suzuki A, Araki R, Sakurai N, Suzuki H, Aoki K, Goda H, Nishizawa OI, Shibata D, Saito K (2007) Omics-based identification of

*Arabidopsis* Myb transcription factors regulating aliphatic glucosinolate biosynthesis. Proc Natl Acad Sci USA 104:6478–6483

Kataoka T, Hayashi N, Yamaya T, Takahashi H (2004a) Root-to-shoot transport of sulfate in *Arabidopsis*: evidence for the role of SULTR3;5 as a component of low-affinity sulfate transport system in the root vasculature. Plant Physiol 136:4198–4204

Kataoka T, Watanabe-Takahashi A, Hayashi N, Ohnishi M, Mimura T, Buchner P, Hawkesford MJ, Yamaya T, Takahashi H (2004b) Vacuolar sulfate transporters are essential determinants controlling internal distribution of sulfate in *Arabidopsis*. Plant Cell 16:2693–2704

Koprivova A, North KA, Kopriva S (2008) Complex signaling network in regulation of adenosine 5′-phosphosulfate reductase by salt stress in *Arabidopsis* roots. Plant Physiol 146:1408–1420

Kosugi S, Ohashi Y (2000) Cloning and DNA binding properties of a tobacco Ethylene-Insensitive3 (EIN3) homolog. Nucleic Acids Res 28:960–967

Kutz A, Müller A, Hennig P, Kaiser WM, Piotrowski M, Weiler EW (2002) A role for nitrilase 3 in the regulation of root morphology in sulphur-starving *Arabidopsis thaliana*. Plant J 30:95–106

Leustek T, Martin MN, Bick JA, Davies JP (2000) Pathways and regulation of sulfur metabolism revealed through molecular and genetic studies. Annu Rev Plant Physiol Plant Mol Biol 51:141–165

Levy M, Wang Q, Kaspi R, Parrella MP, Abel S (2005) *Arabidopsis* IQD1, a novel calmodulin-binding nuclear protein, stimulates glucosinolate accumulation and plant defense. Plant J 43:79–96

Lipka V, Dittgen J, Bednarek P (2005) Pre- and postinvasion defenses both contribute to nonhost resistance in *Arabidopsis*. Science 310:1180–1183

Mansouri-Bauly H, Kruse J, Sýkorová Z, Scheerer U, Kopriva S (2006) Sulfur uptake in the ectomycorrhizal fungus *Laccaria bicolor* S238N. Mycorrhiza 16:421–427

Maruyama-Nakashita A, Inoue E, Watanabe-Takahashi A, Yamaya T, Takahashi H (2003) Transcriptome profiling of sulfur-responsive genes in *Arabidopsis* reveals global effect on sulfur nutrition on multiple metabolic pathways. Plant Physiol 132:597–605

Maruyama-Nakashita A, Nakamura Y, Watanabe-Takahashi A, Inoue E, Yamaya T, Takahashi H (2005) Identification of a novel *cis*-acting element conferring sulfur deficiency response in *Arabidopsis* roots. Plant J 42:305–314

Maruyama-Nakashita A, Nakamura Y, Tohge T, Saito K, Takahashi H (2006) *Arabidopsis* SLIM1 is a central transcriptional regulator of plant sulfur response and metabolism. Plant Cell 18:3235–3251

Mikkelsen MD, Petersen BL, Glawischnig E, Jensen AB, Andreasson E, Halkier BA (2003) Modulation of CYP79 genes and glucosinolate profiles in *Arabidopsis* by defense signaling pathways. Plant Physiol 131:298–308

Nibbe M, Hilpert B, Wasternack C, Miersch O, Apel K (2002) Cell death and salicylate- and jasmonate-dependent stress responses in *Arabidopsis* are controlled by single *cet* genes. Planta 216:120–128

Rausch T, Wachter A (2005) Sulfur metabolism: a versatile platform for launching defence operations. Trends Plant Sci 10:503–509

Saito S, Soga T, Nishioka T, Tomita M (2004) Simultaneous determination of the main metabolites in rice leaves using capillary electrophoresis mass spectrometry and capillary electrophoresis diode array detection. Plant J 40:151–163

Schuster J, Knill T, Reichelt M, Gershenzon J, Binder S (2006) Branched-chain aminotransferase4 is part of the chain elongation pathway in the biosynthesis of methionine-derived glucosinolates in *Arabidopsis*. Plant Cell 18:2664–2679

Shahollari B, Vadassery J, Varma A, Oelmüller R (2007) A leucine-rich repeat protein is required for growth promotion and enhanced seed production mediated by the endophytic fungus *Piriformospora indica* in *Arabidopsis thaliana*. Plant J 50:1–13

Sherameti I, Shahollari B, Venus Y, Altschmied L, Varma A, Oelmüller R (2005) The endophytic fungus *Piriformospora indica* stimulates the expression of nitrate reductase and the starch-degrading enzyme glucan-water dikinase in tobacco and *Arabidopsis* roots through a

homeodomain transcription factor which binds to a conserved motif in their promoters. J Biol Chem 280:2641–2647

Sherameti I, Venus Y, Drzewiecki C, Tripathi S, Dan VM, Nitz I, Varma A, Grundler FM, Oelmüller R (2008) PYK10, a β-glucosidase located in the endoplasmatic reticulum, is crucial for the beneficial interaction between *Arabidopsis thaliana* and the endophytic fungus *Piriformospora indica*. Plant J 54:428–439

Shibagaki N, Rose A, Mcdermott JP, Fujiwara T, Hayashi H, Yoneyama T, Davies JP (2002) Selenate-resistant mutants of *Arabidopsis thaliana* identify SULTR1;2, a sulfate transporter required for efficient transport of sulfate into roots. Plant J 29:475–486

Skirycz A, Reichelt M, Burow M, Birkemeyer C, Rolcik J, Kopka J, Zanor MI, Gershenzon J, Strnad M, Szopa J, Mueller-Roeber B, Witt I (2006) DOF transcription factor AtDof1.1 (OBP2) is part of a regulatory network controlling glucosinolate biosynthesis in *Arabidopsis*. Plant J 47:10–24

Smith FW, Ealing PM, Hawkesford MJ, Clarkson DT (1995) Plant members of a family of sulfate transporters reveal functional subtypes. Proc Natl Acad Sci USA 92:9373–9377

Smith FW, Hawkesford MJ, Ealing PM, Clarkson DT, Vanden Berg PJ, Belcher AR, Warrilow AGS (1997) Regulation of expression of a cDNA from barley roots encoding a high affinity sulfate transporter. Plant J 12:875–884

Solano R, Stepanova A, Chao Q, Ecker JR (1998) Nuclear events in ethylene signaling: A transcriptional cascade mediated by ETHYLENE-INSENSITIVE3 and ETHYLENE-RESPONSE-FACTOR1. Genes Dev 12:3703–3714

Sønderby IE, Hansen BG, Bjarnholt N, Ticconi C, Halkier BA, Kliebenstein DJ (2007) A systems biology approach identifies a R2R3 MYB gene subfamily with distinct and overlapping functions in regulation of aliphatic glucosinolates. PLoS One 2:e1322

Takahashi H, Yamazaki M, Sasakura N, Watanabe A, Leustek T, Engler JA, Engler G, Van Montagu M, Saito K (1997) Regulation of sulfur assimilation in higher plants: a sulfate transporter induced in sulfate starved roots plays a central role in *Arabidopsis thaliana*. Proc Natl Acad Sci USA 94:11102–11107

Takahashi H, Watanabe-Takahashi A, Smith FW, Blake-Kalff M, Hawkesford MJ, Saito K (2000) The role of three functional sulfate transporters involved in uptake and translocation of sulfate in *Arabidopsis thaliana*. Plant J 23:171–182

Talalay P, Fahey JW (2001) Phytochemicals from cruciferous plants protect against cancer by modulating carcinogen metabolism. J Nutr 131:3027S–3033S

Vadassery J, Tripathi S, Prasad R, Varma A, Oelmüller R (2009a) Monodehydroascorbate reductase 2 and dehydroascorbate reductase 5 are crucial for a mutualistic interaction between *Piriformospora indica* and *Arabidopsis*. J Plant Physiol 166(12):1263–1274

Vadassery J, Ranf S, Mithöfer A, Mazars C, Scheel D, Lee J, Oelmüller R (2009b) A cell wall extract from the endophytic fungus *Piriformospora indica* promotes growth of *Arabidopsis* seedlings and induces intracellular calcium elevation in roots. Plant J 59:193–206

Van der Ent S, Verhagen BW, Van Doorn R, Bakker D, Verlaan MG, Pel MJ, Joosten RG, Proveniers MC, Van Loon LC, Ton J, Pieterse CM (2008) MYB72 is required in early signaling steps of rhizobacteria-induced systemic resistance in *Arabidopsis*. Plant Physiol 146:1293–1304

Van Wees SC, Van der Ent S, Pieterse CM (2008) Plant immune responses triggered by beneficial microbes. Curr Opin Plant Biol 11:443–448

Vauclare P, Kopriva S, Fell D, Suter M, Sticher L, von Ballmoos P, Krähenbühl U, den Camp RO, Brunold C (2002) Flux control of sulphate assimilation in *Arabidopsis thaliana*: adenosine 5′-phosphosulphate reductase is more susceptible than ATP sulphurylase to negative control by thiols. Plant J 31:729–740

Vidmar JJ, Tagmount A, Cathala N, Touraine B, Davidian J-CE (2000) Cloning and characterization of a root specific high-affinity sulfate transporter from *Arabidopsis thaliana*. FEBS Lett 475:65–69

18 Role of *Piriformospora indica* in Sulfur Metabolism in *Arabidopsis thaliana*

Waller F, Achatz B, Baltruschat H, Fodor J, Becker K, Fischer M, Heier T, Hückelhoven R, Neumann C, von Wettstein D, Franken P, Kogel KH (2005) The endophytic fungus *Piriformospora indica* reprograms barley to salt-stress tolerance, disease resistance, and higher yield. Proc Natl Acad Sci USA 102:13386–13391

Watanabe M, Mochida K, Kato T, Tabata S, Yoshimoto N, Noji M, Saito K (2008) Comparative genomics and reverse genetics analysis reveal indispensable functions of the serine acetyltransferase gene family in *Arabidopsis*. Plant Cell 20:2484–2496

Xiang C, Oliver DJ (1998) Glutathione metabolic genes coordinately respond to heavy metals and jasmonic acid in *Arabidopsis*. Plant Cell 10:1539–1550

Yamasaki K, Kigawa T, Inoue M, Yamasaki T, Yabuki T, Aoki M, Seki E, Matsuda T, Tomo Y, Terada T, Shirouzu M, Tanaka A, Seki M, Shinozaki K, Yokoyama S (2005) Solution structure of the major DNA-binding domain of *Arabidopsis thaliana* Ethylene-insensitive3-like3. J Mol Biol 348:253–264

Yoshimoto N, Inoue E, Saito K, Yamaya T, Takahashi H (2003) Phloem-localizing sulfate transporter, Sultr1;3, mediates re-distribution of sulfur from source to sink organs in *Arabidopsis*. Plant Physiol 131:1511–1517

## 4.3 Manuscript III

**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

*accepted, Symbiosis*

**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, Ralf Oelmüller *

Institute of General Botany and Plant Physiology, Friedrich-Schiller-University Jena, Dornburger Str. 159, 07743 Jena, Germany

*Author for correspondence: Ralf Oelmüller, b7oera@uni-jena.de

**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*.

**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 (Peskan-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.

**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 et al., 2010a and b). 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., 2010a). 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 overexpressors, 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 et al., 2010b) 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.

**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.

Tab. 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. 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 ($p < 0.05$).

| 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)**** |

***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.

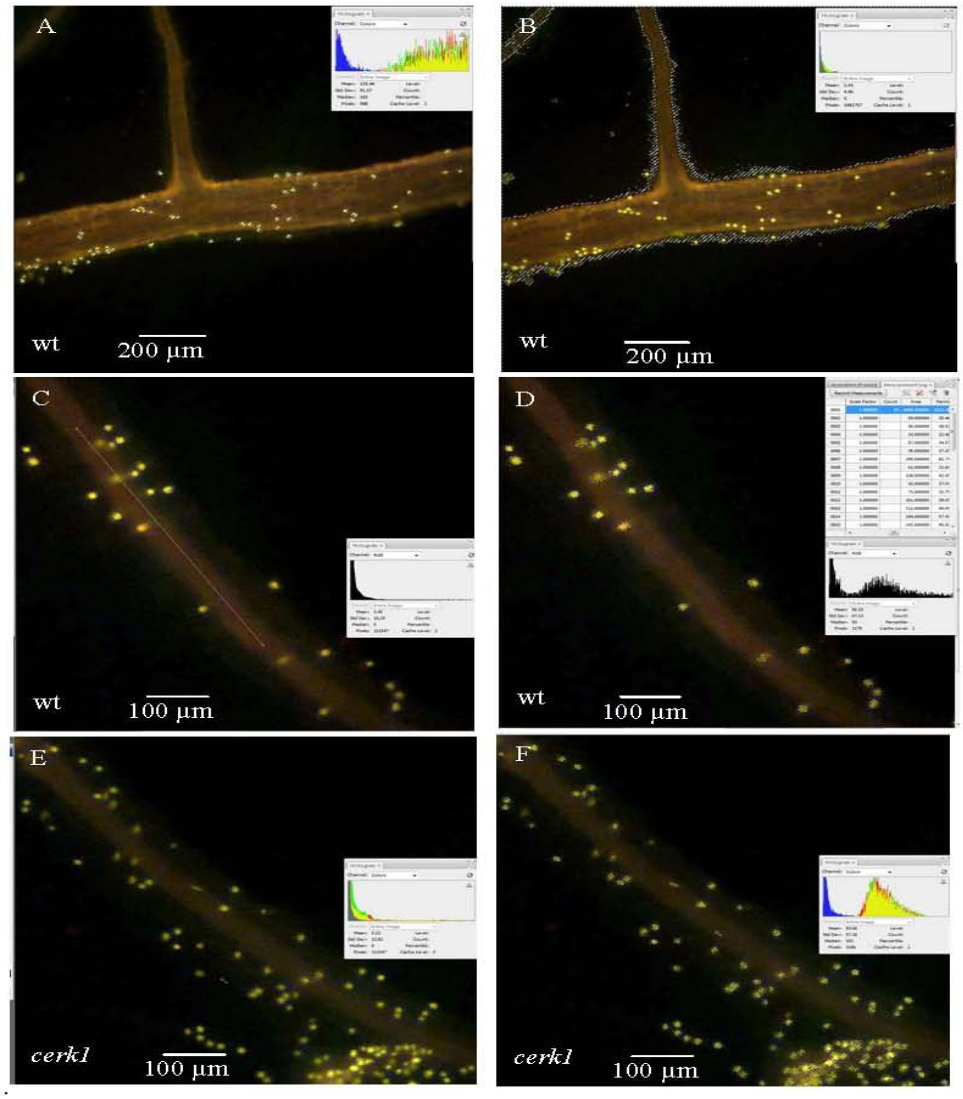



Fig. 1. Root colonization of wild-type (A, B, C, D) and *cerk1* (E, F) seedling grown on PNM media for two weeks. Root area was measured by Magic wand tool adjusted with tolerance of 32 for each sample (B). The colonization pixels were selected by Adobe Photoshop CS5 Magic wand tool adjusted with tolerance 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].

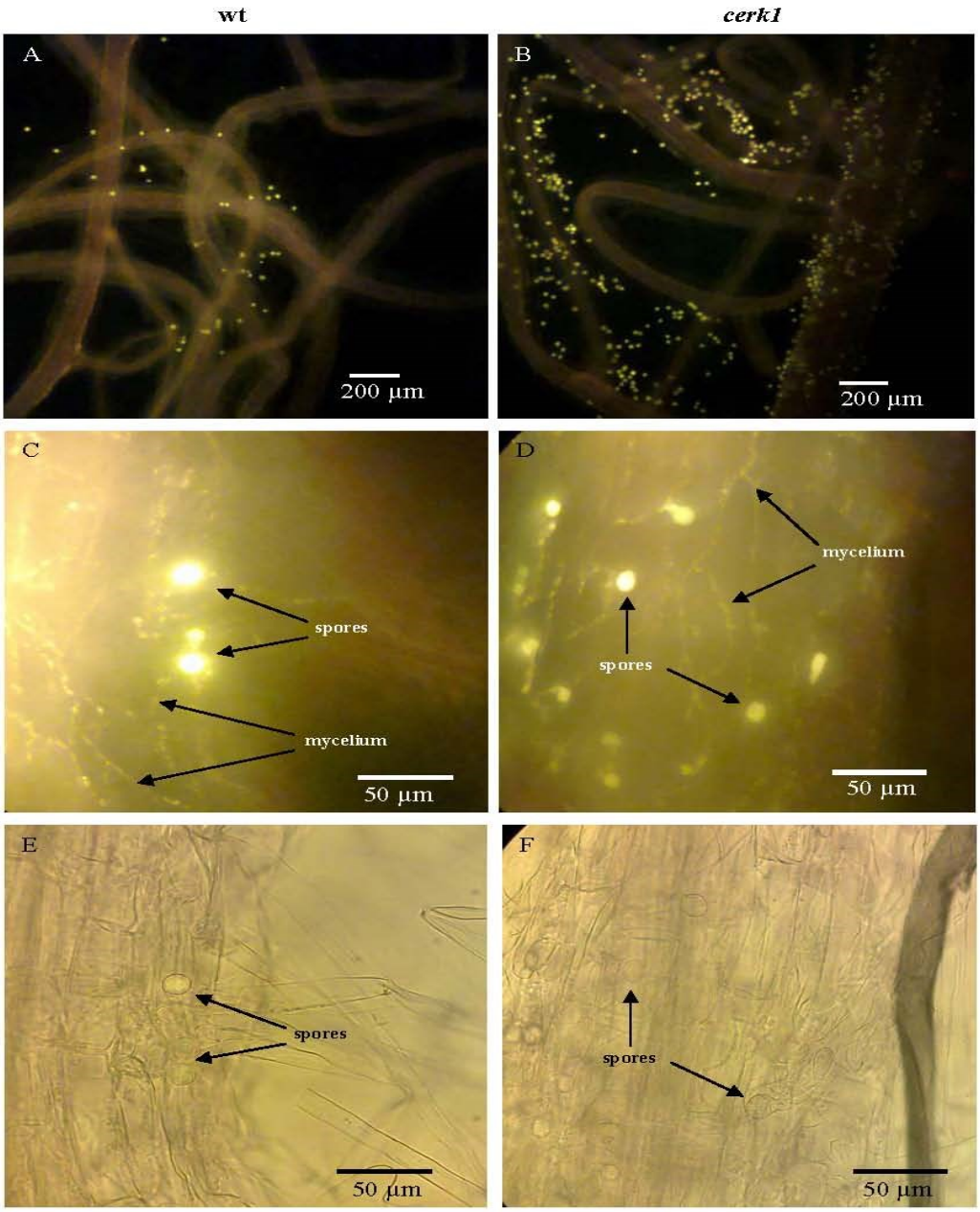



Fig. 2 Root colonization of Arabidopsis wild-type (left) and *cerk1* line (right). The fungus was stained with Nile red and monitored under the fluorescent channel at 450-520 nm (A-D) and under visual light (E, F). Comparison of fluorescent and visual light microscopy in discrimination of spores and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

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* (Tab. 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).

Tab. 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. 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.

| 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)** |

**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) 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).

*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*

(Tab. 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 (Tab. 3).

Tab. 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. 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 ($p < 0.05$).

| 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)** |

*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 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).

Figure 3 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).

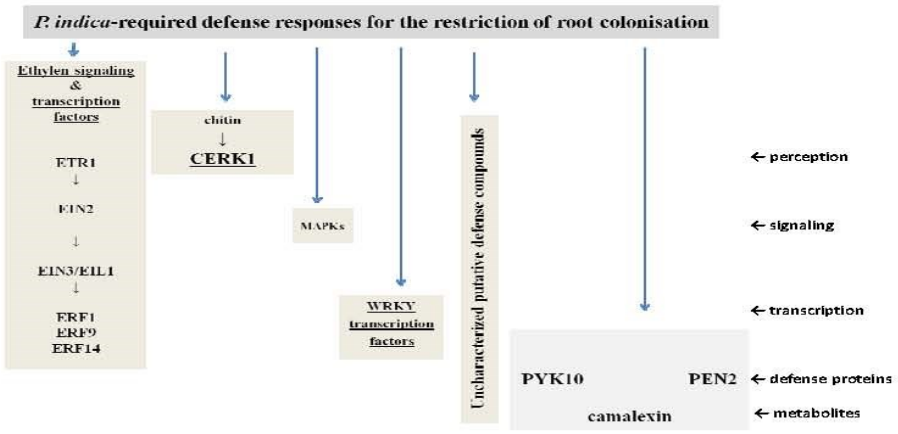



Fig. 3: Defense response components required for restricting Arabidopsis root colonization by *P. indica*. The components are involved in perception, signaling and transcription, or represent defense proteins or secondary metabolites.

**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 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.

**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.

**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.

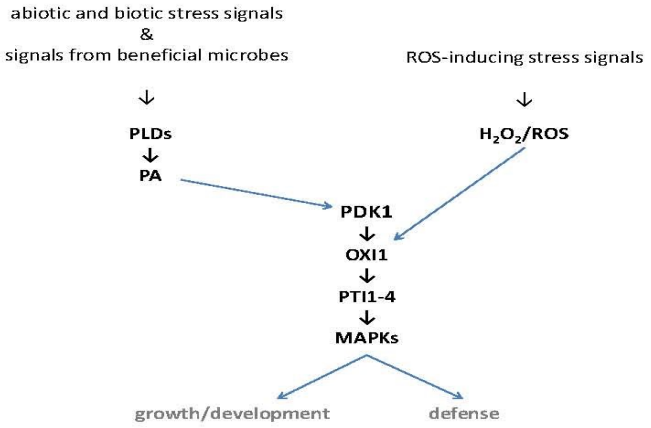



Fig. 4: A model describing the PDK1/OXI1 pathway and its potential involvement in balancing growth/development and defense responses.

**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).

**References**

Anthony, R. G., Khan, S., Costa, J., Pais, M. S., and Bögre, L. 2006. The Arabidopsis protein kinase PTI1-2 is activated by convergent phosphatidic acid and oxidative stress signaling pathways downstream of PDK1 and OXI1. *Journal of Biological Chemistry* **281(49)**:37536-37546.

Anthony, R. G., Henriques, R., Helfer, A., Mészáros, T., Rios, G., Testerink, C., Munnik, T., Deák, M., Koncz, C., and Böqre, L. 2004. A protein kinase target of a PDK1 signaling pathway is involved in root hair growth in Arabidopsis. *EMBO Journal* **23(3):**572-581.

Arisz, S. A., Testerink, C., and Munnik, T. 2009. Plant PA signaling *via* diacylglycerol kinase. *Biochimica et Biophysica Acta* **1791(9)**:869-875.

Baltruschat, H., Fodor, J., Harrach, B. D., Niemczyk, E., Barna, B., Gullner, G., Janeczko, A., Kogel, K-H., Schäfer, P., Schwarczinger, I., Zuccaro, A., and Skoczowski, A. 2008. Salt tolerance of barley induced by the root endophyte *Piriformospora indica* is associated with a strong increase in antioxidants. *New Phytologist* **180(2)**:501-510.

Bayascas, J. R. 2010. PDK1: The major transducer of PI 3-kinase actions. *Current Topics in Microbiology and Immunology* **346**:9-29.

Bednarek, P., and Osbourn, A. 2009. Plant-microbe interactions: chemical diversity in plant defense. *Science* **324:**746-748.

Böttcher, C., Westphal, L., Schmotz, C., Prade, E., Scheel, D., and Glawischnig, E. 2009. The multifunctional enzyme CYP71B15 (PHYTOALEXIN DEFICIENT3) converts cysteine-indole-3-acetonitrile to camalexin in the indole-3-acetonitrile metabolic network of *Arabidopsis thaliana*. *Plant Cell* **21(6)**:1830-1845.

Bütehorn, B., Rhody, D., and Franken, P. 2000. Isolation and characterization of *Pitef*1 encoding the translation elongation factor EF-1α of the root endophyte *Piriformospora indica*. *Plant Biology* **2**:687-692.

Camehl, I., Drzewiecki, C., Vadassery, J., Shahollari, B., Sherameti, I., Forzani, C., Munnik, T., Hirt, H., and Oelmüller, R. 2011. The OXI1 kinase pathway mediates *Piriformospora indica*-induced growth promotion in Arabidopsis. *PLoS Pathogens* **7(5)**:e1002051.

Camehl, I., Sherameti, I., Venus, Y., Bethke, G., Varma, A., Lee, J., and Oelmüller, R. 2010a. Ethylene signalling and ethylene-targeted transcription factors are required to balance beneficial and non-beneficial traits in the symbiosis between the endophytic fungus *Piriformospora indica* and *Arabidopsis thaliana*. *New Phytologist* **185(4)**:1062-1073.

Camehl, I., and Oelmüller, R. 2010b. Do ethylene response factors-9 and -14 repress PR gene expression in the interaction between *Piriformospora indica* and Arabidopsis? *Plant Signaling and Behavior* **5(8)**:932-936.

Chen, L., Song, Y., Li, S., Zhang, L., Zou, C., and Yu, D. 2012. The role of WRKY transcription factors in plant abiotic stresses. *Biochimica et Biophysica Acta* **1819(2)**:120-128.

Deak, M., Casamayor, A., Currie, R. A., Downes, C. P., and Alessi, D. R. 1999. Characterisation of a plant 3-phosphoinositide-dependent protein kinase-1 homologue which contains a pleckstrin homology domain. *FEBS Letters* **451(3)**:220-226.

Deshmukh, S., Hückelhoven, R., Schäfer, P., Imani, J., Sharma, M., Weiss, M., Waller, F. and Kogel, K. H. 2006. The root endophytic fungus *Piriformospora indica* requires host cell death for proliferation during mutualistic symbiosis with barley. *Proceedings of the National Academy of Sciences USA* **103(49)**:18450-18457.

Devarenne, T. P., Ekengren, S. K., Pedley, K. F., and Martin, G. B. 2006. Adi3 is a Pdk1-interacting AGC kinase that negatively regulates plant cell death. *EMBO Journal* **25(1)**:255-265.

Fahey, J. W., Zalcmann, A. T., and Talalay, P. 2001. The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. *Phytochemistry* **56**:5-51.

Ferrari, S., Plotnikova, J. M., De Lorenzo, G., and Ausubel, F. M. 2003. Arabidopsis local resistance to *Botrytis cinerea* involves salicylic acid and camalexin and requires EDS4 and PAD2, but not SID2, EDS5 or PAD4. *Plant Journal* **35:**193-205.

Forzani, C., Carreri, A., de la Fuente van Bentem, S., Lecourieux, D., Lecourieux, F., and Hirt, H. 2011. The Arabidopsis protein kinase Pto-interacting 1–4 is a common target of the oxidative signal-inducible 1 and mitogen-activated protein kinases. *FEBS Journal* **278(7)**:1126-1136.

Garcia, A.V., Al-Yousif, M., and Hirt, H. 2012. Role of AGC kinases in plant growth and stress responses. *Cell Mol Life Sci*. **69**:3259-3267.

Giamoustaris, A., and Mithen, R. 1995. The effect of modifying the glucosinolate content of leaves of oilseed rape (*Brassica napus* ssp. *oleifera*) on its interaction with specialist and generalist pests. *Annals of Applied Biology* **126:**347-363.

Glawischnig, E., Hansen, B. G., Olsen, C. E., and Halkier, B. A. 2004. Camalexin is synthesized from indole-3-acetaldoxime, a key branching point between primary and secondary metabolism in Arabidopsis. *Proceedings of the National Academy of Science USA* **101(21)**:8245-8250.

Hammerschmidt, R. 1999. Phytoalexins: what have we learned after 60 years? *Annual Review of Phytopathology* **37**:285-306.

Harrison, M. J. 1999. Molecular and cellular aspects of the arbuscular mycorrhiza symbiosis. *Annual Review of Plant Physiology and Plant Mololecular Biology* **50**: 361-389.

Hirt, H., Garcia, A. V., and Oelmüller, R. 2011. AGC kinases in plant development and defense. *Plant Signaling and Behavior* **6(7)**:1030-1033.

Hua, J., and Meyerowitz, E. M. 1998. Ethylene responses are negatively regulated by a receptor gene family in *Arabidopsis thaliana*. *Cell* **94**:261-271.

Jacobs, S., Zechmann, B., Molitor, A., Trujillo, M., Petutschnig, E., Lipka, V., Kogel, K-H., and Schäfer, P. 2011. Broad-spectrum suppression of innate immunity is required for colonization of Arabidopsis roots by the fungus *Piriformospora indica*. *Plant Physiology* **156**:726-740.

Janowitz, T., Trompetter, I., and Piotrowski, M. 2009. Evolution of nitrilases in glucosinolate-containing plants. *Phytochemistry* **70**:1680-1686.

Johnson, J. M., Sherameti, I., Ludwig, A., Nongbri, P. L., Sun, C., Lou, B., Varma, A., and Oelmüller, R. 2011. Protocols for *Arabidopsis thaliana* and *Piriformospora indica* co-

cultivation – A model system to study plant beneficial traits. *Journal of Endocytobiosis and Cell Research* **21**:101-113.

Johnson, J. M., and Oelmüller, R. 2009. Mutualism or parasitism: life in an unstable continuum. What can we learn from the mutualistic interaction between *Piriformospora indica* and *Arabidopsis thaliana*? *Journal of Endocytobiosis and Cell Research* **19**:81-111.

Kaku, H., Nishizawa, Y., Ishii-Minami, N., Akimoto-Tomiyama, C., Dohmae, N., Takio, K., Minami, E., and Shibuya, N. 2006. Plant cells recognize chitin fragments for defense signaling through a plasma membrane receptor. *Proceedings of the National Academy of Sciences* **103(29)**:11086-11091.

Kogel, K. H., Franken, P., and Hückelhoven, R. 2006. Endophyte or parasite - what decides? *Current Opinion in Plant Biology* **9**:358-363.

Lahrmann, U., and Zuccaro, A. 2012. Opprimo ergo sum - evasion and suppression in the root endophytic fungus *Piriformospora indica*. *Molecular Plant Microbe Interaction* **25**:727-737.

Li, M., Hong, Y., and Wang, X. 2009. Phospholipase D- and phosphatidic acid-mediated signaling in plants. *Biochimica et Biophysica Acta* **1791(9)**:927-935.

Lipka, V., Dittgen, J., Bednarek, P., Bhat, R., Wiermer, M., Stein, M., Landtag, J., Brandt, W., Rosahl, S., Scheel, D., Llorente, F., Molina, A., Parker, J., Somerville, S., and Schulze-Lefert, P. 2005. Pre- and postinvasion defenses both contribute to nonhost resistance in Arabidopsis. *Science* **310**:1180-1183.

Maillet, F., Poinsot, V., André, O., Puech-Pagès, V., Haouy, A., Gueunier, M., Cromer, L., Giraudet, D., Formey, D., Niebel, A., Martinez, E.A., Driguez, H., Bécard, G., and Dénarié, J. 2011. Fungal lipochitooligosaccharide symbiotic signals in arbuscular mycorrhiza. *Nature* **469**:58-63.

Martin, G. B., Brommonschenkel, S. H., Chunwongse, J., Frary, A., Ganal, M. W., Spivey, R., Wu, T., Earle, E. D., and Tanksley, S. D. 1993. Map-based cloning of a protein kinase gene conferring disease resistance in tomato. *Science* **262(5138)**:1432-1436.

Matsui, H., Miyao, A., Takahashi, A., and Hirochika, H. 2010a. Pdk1 kinase regulates basal disease resistance through the OsOxi1-OsPti1a phosphorylation cascade in rice. *Plant and Cell Physiology* **51(12)**:2082-2091.

Matsui, H., Yamazaki, M., Kishi-Kaboshi, M., Takahashi, A., and Hirochika, H. 2010b. AGC kinase OsOxi1 positively regulates basal resistance through suppression of OsPti1a-mediated negative regulation. *Plant and Cell Physiology* **51(10)**:1731-1744.

McGonigle T. P., Miller M. H., Evans D. G., Fairchild G. L., and Swan J. A. 1990. A new method which gives an objective measure of colonization of roots by vesicular-arbuscular mycorrhizal fungi. *New Phytologist* **115**, 495-501.

McCloskey, C., and Isman, M. B. 1993. Influence of foliar glucosinolates in oilseed rape and mustard on feeding and growth of the Bertha Armyworm, *Mamestra configurata* Walker. *Journal of Chemical Ecology* **19**:249-266.

Miya, A., Albert, P., Shinya, T., Desaki, Y., Ichimura, K., Shirasu, K., Narusaka, Y., Kawakami, N., Kaku, H., and Shibuya, N. 2007. CERK1, a LysM receptor kinase, is essential for chitin elicitor signaling in Arabidopsis. *Proceedings of the National Academy of Sciences USA* **104(49)**:19613-19618.

Mora, A., Komander, D., van Aalten D. M. F., and Alessi, D. R. 2004. PDK1, the master regulator of AGC kinase signal transduction. *Seminars in Cell and Development Biology* **15**:161-170.

Müller, R., de Vos, M., Sun, J. Y., Sønderby, I. E., Halkier, B. A., Wittstock, U., Jander, G. 2010. Differential effects of indole and aliphatic glucosinolates on lepidopteran herbivores. *Journal of Chemical Ecology* **36(8)**:905-13.

Nafisi, M., Goregaoker, S., Botanga, C. J, Glawischnig, E., Olsen, C. E., Halkier, B. A., and Glazebrook, J. 2007. Arabidopsis cytochrome P450 monooxygenase 71A13 catalyzes the conversion of indole-3-acetaldoxime in camalexin synthesis. *Plant Cell* **19(6)**:2039-2052.

Nongbri, P. L., Johnson, J. M., Sherameti, I., Glawischnig, E., Halkier, B. A., and Oelmüller, R. 2012. Indole-3-acetaldoxime-derived compounds restrict root colonization in the beneficial interaction between Arabidopsis roots and the endophyte *Piriformospora indica*. *Molecular Plant Microbe Interaction* 25(9):1186-1197.

Oelmüller, R., Sherameti, I., Tripathi, S., and Varma, A. 2009. *Piriformospora indica*, a cultivable root endophyte with multiple biotechnological applications. *Symbiosis* **49**:1-17.

Pandey, S. P., and Somssich, I. E. 2009. The role of WRKY transcription factors in plant immunity. *Plant Physiology* **150(4)**:1648-1655.

Paszkowski, U. 2006. Mutualism and parasitism: the yin and yang of plant symbioses. *Current Opinion in Plant Biology* **9**:364-370.

Pedras, M. S., Jha, M., Minic, Z., and Okeola, O. G. 2007. Isosteric probes provide structural requirements essential for detoxification of the phytoalexin brassinin by the fungal pathogen *Leptosphaeria maculans*. *Bioorganic and Medicinal Chemistry* **15**:6054-6061.

Peškan-Berghöfer, T., Shahollari, B., Giong, P. H., Hehl, S., Markert, C., Blanke, V., Kost, G., Varma, A., and Oelmüller, R. 2004. Association of *Piriformospora indica* with *Arabidopsis thaliana* roots represents a novel system to study beneficial plant-microbe interactions and involves early plant protein modifications in the endoplasmic reticulum and at the plasma membrane. *Physiologia Plantarum* **122:**465-477.

Petersen, L. N., Ingle, R. A., Knight, M. R., and Denby, K. J. 2009. OXI1 protein kinase is required for plant immunity against *Pseudomonas syringae* in Arabidopsis. *Journal of Experimental Botany* **60(13)**:3727-3735.

Piotrowski, M. 2008. Primary or secondary? Versatile nitrilases in plant metabolism. *Phytochemistry* 69: 2655-2667.

Qiang, X., Zechmann, B., Reitz, M. U., Kogel, K. H., and Schäfer, P. 2012. The mutualistic fungus *Piriformospora indica* colonizes Arabidopsis roots by inducing an endoplasmic reticulum stress-triggered caspase-dependent cell death. *Plant Cell* **24**:794-809.

Qiu, J. L., Fiil, B. K., Petersen, K., Nielsen, H. B., Botanga, C. J., Thorgrimsen, S., Palma, K., Suarez-Rodriguez, M. C., Sandbech-Clausen, S., Lichota, J., Brodersen, P., Grasser, K. D., Mattsson, O., Glazebrook, J., Mundy, J., and Petersen, M. 2008. Arabidopsis MAP kinase 4 regulates gene expression through transcription factor release in the nucleus. *EMBO Journal* **27(16)**:2214-2221.

Reitz, M. U., Bissue, J. K., Zocher, K., Attard, A., Hückelhoven, R., Becker, K., Imani, J., Eichmann, R., and Schäfer, P. 2012. The subcellular localization of tubby-like proteins and participation in stress signaling and root colonization by the mutualist *Piriformospora indica*. *Plant Physiology* 160:349-364.

Ren, D., Liu, Y., Yang, K-Y., Han, L., Mao, G., Glazebrook, J., and Zhang, S. 2008. A fungal-responsive MAPK cascade regulates phytoalexin biosynthesis in Arabidopsis. *Proceedings of the National Academy of Science USA* **105(14)**:5638-5643.

Rentel, M. C., Lecourieux, D., Ouaked, F., Usher, S. L., Petersen, L., Okamoto, H., Knight, H., Peck, S. C., Grierson, C. S., Hirt, H., and Knight, M. R. 2004. OXI1 kinase is necessary for oxidative burst-mediated signalling in Arabidopsis. *Nature* **427**:858-861.

Rushton, P. J., Somssich, I. E., Ringler, P., and Shen Q. J. 2010. WRKY transcription factors. *Trends in Plant Science* **15(5)**:247-258.

Schuck, S., Camehl, I., Gilardoni, P. A., Oelmüller, R., Baldwin, I. T., and Bonaventure, G. 2012. HSPRO controls early *Nicotiana attenuata* seedling growth during interaction with the fungus *Piriformospora indic*a. *Plant Physiology* **160**:929-943.

Schuhegger, R., Nafisi, M., Mansourova, M., Petersen, B. L., Olsen, C. E., Svatos, A., Halkier, B. A., and Glawischnig, E. 2006. CYP71B15 (PAD3) catalyzes the final step in camalexin biosynthesis. *Plant Physiology* **141**:1248-1254.

Selosse, M. A., Dubois, M. P., and Alvarez, N. 2009. Do Sebacinales commonly associate with plant roots as endophytes? *Mycological Research* **113**:1062-1069.

Shahollari, B., Vadassery, J., Varma, A., and Oelmüller, R. 2007. A leucine-rich repeat protein is required for growth promotion and enhanced seed production mediated by the endophytic fungus *Piriformospora indica* in *Arabidopsis thaliana*. *Plant Journal* **50(1)**:1-13.

Shahollari, B., Peškan-Berghöfer, T., and Oelmüller, R. 2004. Receptor kinases with leucine-rich repeats are enriched in Triton X-100 insoluble plasma membrane microdomains from plants. *Physiologia Plantarum* **122(4)**:397-403.

Sherameti, I., Tripathi, S., Varma, A., and Oelmüller, R. 2008a. The root-colonizing endophyte *Piriformospora indica* confers drought tolerance in Arabidopsis by stimulating the expression of drought stress-related genes in leaves. *Molecular Plant Microbe Interaction* **21(6)**:799-807.

Sherameti, I., Venus, Y., Drzewiecki, C., Tripathi, S., Dan, V. M., Nitz, I., Varma, A., Grundler, F. M., and Oelmüller, R. 2008b. PYK10, a beta-glucosidase located in the endoplasmatic reticulum, is crucial for the beneficial interaction between *Arabidopsis thaliana* and the endophytic fungus *Piriformospora indica*. *Plant Journal* **54(3)**:428-439.

Sherameti, I., Shahollari, B., Venus, Y., Altschmied, L., Varma, A., and Oelmüller, R. 2005. The endophytic fungus *Piriformospora indica* stimulates the expression of nitrate reductase and the starch-degrading enzyme glucan-water dikinase in tobacco and Arabidopsis roots through a homeodomain transcription factor that binds to a conserved motif in their promoters. *Journal of Biological Chemistry* **280(28)**:26241-26247.

Sønderby, I. E., Geu-Flores, F., and Halkier, B. A. 2010. Biosynthesis of glucosinolates- gene discovery and beyond. *Trends in Plant Sci*ence **15(5)**: 283-290.

Stein, E., Molitor, A., Kogel, K-H., and Waller, F. 2008. Systemic resistance in Arabidopsis conferred by the mycorrhizal fungus *Piriformospora indica* requires jasmonic acid signaling and the cytoplasmic function of NPR1. *Plant Cell Physiology* **49(11)**:1747-1751.

Stein, M., Dittgen, J., Sanchez-Rodriguez, C., Hou, B-H., Molina, A., Schulze-Lefert, P., Lipka, V., and Somerville, S. 2006. Arabidopsis PEN3/PDR8, an ATP binding cassette transporter, contributes to nonhost resistance to inappropriate pathogens that enter by direct penetration. *Plant Cell* **18(3)**:731-746.

Sun, C., Johnson, J. M., Cai, D., Sherameti, I., Oelmüller, R., and Lou, B. 2010. *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. *Journal of Plant Physiology* **167(12)**:1009-1017.

Takahashi, A., Agrawal, G. K., Yamazaki, M., Onosato, K., Miyao, A., Kawasaki, T., Shimamoto, K., and Hirochika, H. 2007. Rice Pti1a negatively regulates RAR1-dependent defense responses. *Plant Cell* **19(9)**:2940-2951.

Tunlid, A., and Talbot, N. J. 2002. Genomics of parasitic and symbiotic fungi. *Current Opinion in Microbiology* **5**: 513-519.

Vadassery, J., Ranf, S., Drzewiecki, C., Mithöfer, A., Mazars, C., Scheel, D., Lee, J., and Oelmüller, R. 2009a. A cell wall extract from the endophytic fungus *Piriformospora indica* promotes growth of Arabidopsis seedlings and induces intracellular calcium elevation in roots. *Plant Journal* **59(2)**:193-206.

Vadassery, J., and Oelmüller, R. 2009b. Calcium signaling in pathogenic and beneficial plant microbe interactions: what can we learn from the interaction between *Piriformospora indica* and *Arabidopsis thaliana*. *Plant Signaling and Behavior* **4(11)**:1024-1027.

Vadassery, J., Tripathi, S., Prasad, R., Varma, A., and Oelmüller, R. 2009c. Monodehydroascorbate reductase 2 and dehydroascorbate reductase 5 are crucial for a mutualistic interaction between *Piriformospora indica* and Arabidopsis. *Journal of Plant Physiology* **166:** 1263-1274.

Van Wees, S. C., Van der Ent, S., and Pieterse, C. M. J. 2008. Plant immune responses triggered by beneficial microbes. *Current Opinion in Plant Biology* **11(4)**:443-448.

Waller, F., Achatz, B., Baltruschat, H., Fodor, J., Becker, K., Fischer, M., Heier, T., Hückelhoven, R., Neumann, C., von Wettstein, D., Franken, P., and Kogel, K-H. 2005. The endophytic fungus *Piriformospora indica* reprograms barley to salt-stress tolerance, disease resistance, and higher yield. *Proceedings of the National Academy of Sciences USA* **102(38)**:13386-13391.

Wan, J., Tanaka, K., Zhang, X-C., Son, G. H., Brechenmacher, L., Nguyen, T. H. N., and Stacey, G. 2012. LYK4, a lysin motif receptor-like kinase, is important for chitin signaling and plant innate immunity in Arabidopsis. *Plant Physiology* **160**: 396-406.

Wan, J., Zhang, X. C., Neece, D., Ramonell, K. M., Clough, S., Kim, S. Y., Stacey, M. G., and Stacey, G. 2008. A LysM receptor-like kinase plays a critical role in chitin signaling and fungal resistance in Arabidopsis. *Plant Cell* **20**:471-481.

Yadav, V., Kumar, M., Deep, D. K., Kumar, H., Sharma, R., Tripathi, T., Tuteja, N., Saxena, A. K., and Johri, A. K. 2010. A phosphate transporter from the root endophytic fungus *Piriformospora indica* plays a role in phosphate transport to the host plant. *Journal of Biological Chemistry* **285(34)**:26532-26544.

Zentgraf, U., Laun, T., and Miao, Y. 2010. The complex regulation of *WRKY53* during leaf senescence of *Arabidopsis thaliana*. *European Journal of Cell Biology* **89**:133-137.

Zhao, Y., Hull, A. K., Gupta, N. R., Goss, K. A., Alonso, J., Ecker, J. R., Normanly, J., Chory, J., and Celenza, J. L. 2002. Trp-dependent auxin biosynthesis in Arabidopsis: involvement of cytochrome P450s CYB79B2 and CYP79B3. *Genes and Development* **16:**3100-3112.

Zhou, J., Loh, Y. T., Bressan, R. A., and Martin, G. B. 1995. The tomato gene Pti1 encodes a serine/threonine kinase that is phosphorylated by Pto and is involved in the hypersensitive response. *Cell* **83(6)**:925-935.

Zuccaro, A., Lahrmann, U., Güldener, U., Langen, G., Pfiffi, S., Biedenkopf, D., Wong, P., Samans, B., Grimm, C., Basiewicz, M., Murat, C., Martin, F., and Kogel, K-H. 2011. Endophytic life strategies decoded by genome and transcriptome analyses of the mutualistic root symbiont *Piriformospora indica*. *PLoS Pathogens* **7(10)**:e1002290.

## 4.4 Manuscript IV

Aliphatic glucosinolates participate in *Piriformospora indica*-induced resistance of Arabidopsis against *Alternaria brassicae*

Pyniarlang L. Nongbri, Joy M. Johnson, Barbara Ann Halkier, Ralf Oelmüller
*in preparation*

# Aliphatic glucosinolates participate in *Piriformospora indica*-induced resistance of Arabidopsis against *Alternaria brassicae*

**Pyniarlang L. Nongbri[1], Joy M. Johnson[1], Barbara Ann Halkier[2], Ralf Oelmüller[1*]**

[1]Institute of General Botany and Plant Physiology, Friedrich-Schiller-University Jena, Dornburger Str. 159, 07743 Jena, Germany

[2]VKR Research Centre Pro-Active Plants, Department of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Copenhagen, Thorvaldsensvej 40, 1871 Frederiksberg C, Denmark

**Key words:** *Alternaria brassicae*; glucosinolates; induced systemic resistance; MYB28; MYB29; *Piriformospora indica*

**Running title:** aliphatic glucosinolates in *Alternaria brassicae* resistance

[*]Author for correspondence**:** Ralf Oelmüller, b7oera@uni-jena.de

## ABSTRACT

In Arabidopsis, biosynthesis of methionine-derived aliphatic glucosinolates is regulated by the R2R3 MYB transcription factors MYB28, MYB29 and MYB76. A *myb28 myb29* double mutant is almost devoid of aliphatic glucosinolates. Here we demonstrate that *myb28 myb29* seedlings are more susceptible to the necrotrophic fungus *Alternaria brassicae* than wild-type seedlings. The beneficial root-colonizing fungus *Piriformospora indica* restricts growth of *A. brassicae* in wild-type and *myb28 myb29* roots and the restriction is the same for both genotypes. *P. indica*-pretreated wild-type seedlings perform better after *A. brassicae* infection of roots than *myb28 myb29* seedlings which are caused by the restriction of *A. brassicae*

growth in *P. indica*-pretreated roots. Furthermore, root colonization by *P. indica* induces systemic resistance against *A. brassicae* infections in wild-type leaves, and this process is severely impaired in the *myb28 myb29* mutant. We propose that *P. indica* protects the seedlings against *A. brassicae* infections in two ways: it inhibits growth of the pathogen in the roots and induces root-to-shoot signaling for systemic resistance against *A. brassicae* leaf infections. The latter process requires MYB28 and MYB29.

## INTRODUCTION

The glucosinolates are organic compounds which contain sulfur and nitrogen and occur as secondary metabolites in *Arabidopsis thaliana*, agriculturally important *Brassica* plants and almost all the plants of order Brassicales. Over 120 different chemical structures of glucosinolates have been identified in plants (Fahey et al. 2001; Sønderby et al. 2010; Janowitz et al. 2009; Piotrowski 2008). The basic core structure of all glucosinolates contains a β-thioglucose, a N-hydroxyiminosulphate group and a structurally diverse aglycone side-chain. Hydrolysis by myrosinases cleaves the glucose moiety from glucosinolates which ultimately release toxic nitriles and isothiocyanates (Kelly et al. 1998). Glucosinolates are stable and non-toxic and sequestered in the vacuoles of plant cells (Koroleva et al. 2000) whereas myrosinases are localized separately but adjacent in guard cells and phloem idioblasts (Husebye et al. 2002). However, tissue damages due to herbivory or pathogen infection (Glazebrook 2005) release the glucosinolates and myrosinases from their respective compartments which results in the generation of the toxic products (Lambrix et al. 2001).

Over 36 different glucosinolates with mostly indolic or aliphatic side-chains are identified in Arabidopsis (Brown et al. 2003; Reichelt et al. 2002). Indolic glucosinolates derive from tryptophan while aliphatic glucosinolates derive from methionine. *Arabidopsis thaliana* contains high concentrations of methylsulfinylalkyl side-chained aliphatic glucosinolates in the leaves, of which the alkyl group varies from 3 to 8 carbons in length (Kliebenstein et al. 2001; Burow et al. 2010). Genetic and biochemical approaches in the last years have lead to near-complete elucidation of the core biosynthetic pathway of glucosinolates in Arabidopsis (Halkier and Gershenzon 2006; Chen and Andreasson 2001; Grubb and Abel 2006). Five

glucosinolate-biosynthesizing gene products, CYP79A2, CYP79B2, CYP79B3, CYP79F1 and CYP79F2, catalyze the enzymatic conversion of phenylalanine (Wittstock and Halkier 2000), tryptophan (Hull et al. 2000; Mikkelsen et al. 2000) or short-chain and long-chain elongated methionine substrates (Hansen et al. 2001; Chen et al. 2003; Hull et al. 2000; Mikkelsen et al. 2000; Wittstock and Halkier 2000) to the corresponding aldoximes. The aldoximes are further metabolized to form intact glucosinolates (Halkier and Gershenzon 2006). The R2R3 MYB transcription factors MYB28, MYB29 and MYB76 were identified as positive regulators of the aliphatic glucosinolate biosynthesis (Hirai et al. 2007; Gigolashvili et al. 2007b, 2008; Sønderby et al. 2007, 2010), whereas MYB29 and MYB76 regulate short-chain aliphatic glucosinolate biosynthesis (Sønderby et al. 2007; Beekwilder et al. 2008; Gigolashvili et al. 2008) and MYB28 plays a major role by controlling long- as well as short-chain aliphatic glucosinolates (Hirai et al. 2007; Sønderby et al. 2007; Beekwilder et al. 2008; Gigolashvili et al. 2009). The *myb28 myb29* mutant is almost devoid of aliphatic glucosinolates, presumably through epistatic effects (Sønderby et al. 2007; Beekwilder et al. 2008). Interestingly, there was no change in the level of indole glucosinolates when *MYB28*, *MYB29* and *MYB76* were inactivated (Sønderby et al. 2007). *MYB28* is regulated by glucose (Gigolashvili et al. 2007b) and wounding (Levy et al. 2005; Gigolashvili et al. 2007a, b). Gigolashvili et al. (2008) reported that exogenous methyl-jasmonate induces *MYB29* while salicylic acid (SA) exerts a negative effect and *MYB76* is induced by wounding.

Glucosinolates and their degradation products play an important role on plant protection against insect herbivory (McCloskey and Isman 1993; Giamoustaris and Mithen 1995; Agrawal et al. 2003; Kliebenstein et al. 2002; Müller et al. 2010) but their role in pathogen resistance is less studied. *myb28*, an aliphatic glucosinolate biosynthesis mutant, is hypersusceptible to *Sclerotinia sclerotiorum* (Stotz et al. 2011). Glucosinolate hydrolysis products such as isothiocyanate inhibit bacterial and fungal growth *in vitro* (Mithen et al. 1986; Manici et al. 1997; Brader et al. 2001; Tierens et al. 2001; Mari et al. 2002; Smolinska et al. 2003). Constitutive production of phytoanticipins or phytoalexins is important for plant defense against microbes (Hammerschmidt 1999; Tierens et al. 2001; Pedras et al. 2007; Bednarek and Osbourn 2009). Arabidopsis responds to necrotrophic fungal attacks by synthesizing the phytoalexin camalexin (Schuhegger et al. 2006; Thomma et al. 1999; Ferrari

et al. 2003). *S. sclerotiorum*, an aggressive fungal pathogen with a wide host range (Bolton et al. 2006), facilitates host cell damage (Kim et al. 2008; Guo and Stotz 2010) and subsequently glucosinolate cleavage. Elevated levels of p-hydroxybenzyl glucosinolate, isopropyl glucosinolate, 1-methylpropyl glucosinolate or benzyl glucosinolate in Arabidopsis lines overexpressing the respective biosynthesis genes revealed altered disease resistance against the pathogens *Alternaria brassicicola*, *Pseudomonas syringae* and *Erwinia carotovora* (Brader et al. 2006; Bak et al. 1999; Mikkelsen and Halkier 2003; Petersen et al. 2001; Wittstock and Halkier 2000). *pad3* and *cyp79b2 cyp79b3* mutants are hypersensitive to *B. cinerea* infections (Kliebenstein et al. 2005), demonstrating a protective role of camalexin and indole glucosinolates against necrotrophic fungi infections. Finally, the level of indole glucosinolates increases upon exogenous application of jasmonic acid (JA) while aliphatic glucosinolates are not induced (Bodnaryk 1994; Oughty et al. 1995).

Non-pathogenic microorganisms can induce systemic resistance (ISR) in plants against pathogens. Different signaling pathways are proposed to explain this resistance response. *Pseudomonas fluorescens* WCS417r induces systemic resistance independently of SA accumulation and *PATHOGENESIS-RELATED* (*PR*) expression against *Pseudomonas syringae* (Pieterse et al. 1996). The beneficial fungus *Piriformospora indica* which promotes growth and confers resistance against biotic and abiotic stress (Oelmüller et al. 2009; Lahrmann and Zuccaro 2012) mediates also a strong resistance response against the growth of *Golovinomyces orontii* conidiophores *via* JA signaling and the cytoplasmic function of NPR1 (Stein et al. 2008). Rhizobacteria confer protection of Arabidopsis against DC3000 *via* SA- and ethylene (ET)-dependent pathways (Rudrappa et al. 2010). *MYB72*, also a member of R2R3-MYB-like transcription factor gene family, is required for rhizobacteria-mediated ISR against a broad-spectrum of pathogens (Van der Ent et al. 2008). *MYB72* transcripts are induced in the roots upon colonization by *P. fluorescens* WCS417r but not systemically induced in the leaves (Verhagen et al. 2004). The transcriptional regulator MYB72 is one of the essential components necessary for mounting ISR response. MYB72 physically interacts *in vitro* with the ETHYLENE INSENSITIVE3 (EIN3)-LIKE3 transcription factor EIL3, linking MYB72 function to the ET response pathway. Therefore it is postulated that MYB72

is required in early ISR signaling upstream of JA and ET (Segarra et al. 2009; Van der Ent et al. 2008).

Here, we demonstrate that colonization of Arabidopsis roots by *P. indica* restricts growth of the pathogenic fungus *Alternaria brassicae*. This fungus causes Alternaria black spots which are responsible for significant losses of Brassica crops (Bains and Tewari 1987; Conn et al. 1990; Tewari and Bains 1997). Consequently, *P. indica* retards and diminishes *A. brassicae*-mediated disease development in the roots and leaves of the host plant. The *P. indica*-colonized seedlings are also more resistant against spore infection of the leaves indicating the existence of roots-to-shoot signaling events which lead to ISR. The *myb28 myb29* mutant is more susceptible to *A. brassicae* infection than the wild-type. Since the *P. indica*-mediated resistance response is reduced in *myb28 myb29*, aliphatic glucosinolates are required for the establishment of full *P. indica*-induced resistance.

## RESULTS

### MYB28/MYB29 participates in the resistance of Arabidopsis against *A. brassicae* infection.

Root infection of Arabidopsis seedlings with the necrotrophic fungus *A. brassicae* is associated with the up-regulation of a major regulator of aliphatic glucosinolate synthesis, *MYB28* (Fig. 1A). Furthermore, infection of roots with the pathogen causes severe disease symptom development: the roots become brown and the shoots develop chlorosis within 10 days after root infection. While the development of the disease phenotype is similar for wild-type and *myb28 myb29* roots (Fig. 1B), the chlorotic phenotype in the shoots developed faster and was stronger in the mutant compared to the wild-type control (Fig. 5A). This indicates that MYB28/MYB29 participates in establishing resistance in the leaves after *A. brassicae*-infection of the roots.

In contrast, the transcript level for *MYB28* did not respond to root colonization by the beneficial fungus *P. indica* (Fig. 1A). This suggests that MYB28 and MYB29 and aliphatic glucosinolates do not play an important role in the beneficial symbiosis. Consistent with this

observation, co-cultivation of *myb28 myb29* seedlings with *P. indica* for 14 days on PNM agar plates resulted in the increase of biomass of 23% (mock-treated: 36.4±1.9; *P. indica* treated: 44.7±1.8; n=6). The effect was less, but not significant different from the growth response observed for the wild-type (40%; mock-treated: 38.2±2.2; *P. indica* treated: 53.7±2.8; n=6).

**_P. indica_ restricts _A. brassicae_ growth in roots.**

*A. brassicae* rapidly colonizes the roots of Arabidopsis seedlings and induce severe disease symptoms in roots and shoots (Fig. 1B). Therefore, we tested whether *P. indica* can protect the seedlings against *A. brassicae*-induced disease development. Wild-type and *myb28 myb29* roots were infected with *P. indica* for three days or mock-treated before *A. brassicae* spore infection. Brownishing of the roots and development of the chlorotic phenotype in the shoots were strongly retarded in the presence of *P. indica* (Fig. 2A). Quantification of the mRNA levels using specific primers for the *A. brassicae* ATP-binding cassette transporter *AbreAtr1* (Guillemette et al. 2004; Fig 2B) demonstrated that propagation of the pathogen was strongly inhibited in wild-type and no change in *myb28 myb29* roots in the presence of *P. indica* (Fig. 2C). *P. indica* also inhibits growth of *A. brassicae* in the dual culture on PDA agar plates (Fig. 4C). We conclude that *P. indica* protects Arabidopsis roots against *A. brassicae* by restricting growth of the pathogen in the roots.

**MYB28/MYB29 is required for _P. indica_-mediated resistance against _A. brassicae_ infection in shoots.**

Since colonization of Arabidopsis roots by *A. brassicae* is severely retarded after a pretreatment with *P. indica*, it is not surprising that disease development induced by the pathogen in the wild-type is reduced compared to seedlings which were not pretreated with the beneficial fungus (Fig. 2A). The fresh weight of *A. brassicae*-infected wild-type leaves pretreated with *P. indica* is much higher than that of seedlings which were not pretreated with *P. indica* (Fig. 3). However, the fresh weight of *A. brassicae* infected *myb28 myb29* leaves pretreated with *P. indica* is comparable to that which was not pretreated with *P. indica* (Fig. 3). Since restriction of *A. brassicae* growth by *P. indica* is identical in wild-type and in *myb28*

*myb29* roots (Fig. 2C), MYB28/MYB29 participates in the *P. indica*-induced resistance against *A. brassicae* infection in Arabidopsis shoots.

**_P. indica_ represses _A. brassicae_-induced _PDF1.2_ expression in wild-type but not _myb28 myb29_ roots.**

*PDF1.2* is a sensitive marker gene for imbalances in the *P. indica*/Arabidopsis symbiosis (Camehl et al. 2010, 2011).

In wild-type and *myb28 myb29* roots, *PDF1.2* is up-regulated by *A. brassicae*, but not by *P. indica* (Fig. 4A). Pretreatment of wild-type roots with *P. indica* prior to *A. brassicae* infection severely reduced *PDF1.2* expression in the roots (Fig. 4B). Pretreatment of *myb28 myb29* roots with *P. indica* prior to *A. brassicae* infection had very little effect on *PDF1.2* expression (Fig. 4B). This indicates that the double mutant is partially impaired in efficient suppression of the host innate immune response by the beneficial fungus (cf. Jacobs et al. 2011).

**_P. indica_ and MYB28/MYB29 promote systemic resistance against _A. brassicae_ infections in shoots.**

The data suggest so far that *P. indica* restricts *A. brassicae* growth in the roots of wild-type and *myb28 myb29* (Fig. 2C) seedlings. Furthermore, the beneficial fungus protects wild-type seedlings against *A. brassicae* infection, and this protection is almost lost for *myb28 myb29* seedlings (Fig. 3). To test whether *myb28 myb29* participates in *P. indica*-induced systemic resistance and root-to-shoot signaling, we infected the leaves of *P. indica*-colonized or mock-treated wild-type and *myb28 myb29* seedlings with *A. brassicae* spores. Figure 5A demonstrates that *P. indica* protects the leaves of wild-type seedlings against infection with *A. brassicae* spores. *A. brassicae* growth in the leaves of wild-type seedlings is reduced after *P. indica* pre-treatment, while this reduction was less for the double knock out as shown for the level of *AbreAtr1*(Fig. 5B). Consequently, the mock-treated wild-type seedlings showed a strong chlorotic phenotype four days after *A. brassicae* infection, while the *P. indica*-treated seedlings were still green (Fig. 5A). The protective function of *P. indica* is not or less visible for the *myb28 myb29* mutant (Fig. 5A). We observed a mild induction of

*PDF1.2* in leaves when *P. indica* was pretreated in roots of both wild-type and *myb28 myb29* (Fig. 5C). This demonstrates that there is root-to-shoot signal transfer by *P. indica*. In contrast, leaf infection of unprimed seedlings with *A. brassicae* strongly activates *PDF1.2* in wild-type and *myb28 myb29* mutant (Fig. 5C). The comparable induction of *PDF1.2* in wild-type and *myb28 myb29* mutant by priming the roots with *P. indica* prior to infection of leaves with *A. brassicae* spores suggests that defense response mediated is independent of MYB28, MYB29 and MYB72. We conclude that *P. indica*-induced systemic resistance against *A. brassicae* infection in leaves is severely reduced in the *myb28 myb29* mutant.

## DISCUSSION

We have recently demonstrated that camalexin is required for the beneficial interaction between Arabidopsis and *P. indica*. In contrast to the results shown here, the beneficial fungus stimulates the expression of *CYP79B2* and *CYP79B3* in the roots and simultaneous inactivation of both genes prevents the beneficial interaction (Nongbri et al. 2012). The *cyp79B2 cyp79B3* roots become over-colonised which results in defense gene activation against *P. indica*. Also *CYP71A13* and *PAD3* which code for enzymes catalyzing the conversion of IAOx to camalexin and *WRKY33* for the camalexin-inducing transcription factor were upregulated by *P. indica* in Arabidopsis roots. Apparently, aliphatic and indole glucosinolates play different roles in the symbiotic interaction.

Priming the Arabidopsis roots with beneficial symbiont, *P. indica* leads to improved growth performance of Arabidopsis seedlings against subsequent challenge by the tested necrotrophic fungus *A. brassicae.* This resulting phenotype could be suggested to occur due to two reasons- there could be a competition between *P. indica* and *A. brassicae* for the resources as well as for the host roots to enable to establish themselves and eventually exert their effect, or *P. indica* being a beneficial symbiont activated the basal defense response as a result of which plants are better prepared for reacting to pathogen attack. Here, we proposed the involvement of *MYB28* and *MYB29* in the onset of *P. indica*-mediated defense to necrotrophic fungus *A. brassicae.*

Aliphatic glucosinolate synthesis is not an exclusive requirement for imparting *P. indica*-mediated growth promoting effect. There is a reduced growth promotion (23%) in *myb28 myb29* mutant seedlings compared to wild-type (40%), but the response is not dramatic so as to attribute the functions of *myb28* and *myb29* genes to interaction with *P. indica.* However, aliphatic glucosinolate synthesis is essential as defense compound against pathogens as well as insect including the tested necrotroph *A. brassicae.* While the role of *P. indica* in the priming process leading to protection against *A. brassicae* infection is prominent in wild-type, inability of *myb28 myb29* mutant to respond to *P. indica*-mediated priming resulted into the loss of protection.

Root colonization requires suppression of host innate defense system (Jacobs et al., 2011) to enable intracellular *P. indica* propagation of root tissue which eventually delivers benefits to the host by modulating its metabolic pathways.

It was also reported previously that *PDF1.2* gene was systemically induced in Arabidopsis leaves upon infection with *A. brassicicola* which is closely related to *A. brassicae* (Penninckx et al. 1996). JA and ethylene (ET) hormonal signaling mediates defense gene activation against necrotrophic pathogens such as *B. cinerea* (Thomma et al. 1998, 1999) and *S. sclerotiorum* (Guo and Stotz 2007) and activate *PDF1.2* expression. A JA receptor mutant *coi1,* which is highly susceptible to *S. sclerotiorum* also produces significantly less aliphatic and indole glucosinolates after infection (Stotz et al. 2011). *PDF1.2* is also a sensitive marker gene for imbalances in *P. indica*/Arabidopsis interaction (Camehl et al. 2010).

## MATERIALS AND METHODS

### Growth conditions of *Arabidopsis thaliana* and fungi

*Arabidopsis thaliana* WT (ecotype Columbia-0) and mutant seeds were surface-sterilized and placed on Petri dishes containing MS nutrient medium (Murashige and Skoog 1962). The *myb28 myb29* line was obtained from Barbara A. Halkier (University of Copenhagen, Denmark). Homozygozity of the seeds was confirmed with gene-specific primers as published previously. After cold treatment at 4°C for 48 h, plates were incubated for 10 days at 22°C under continuous illumination (100 µmol $m^{-2}$ $sec^{-1}$). *Piriformospora indica* was cultured for

three to four weeks at 22-24°C on *Aspergillus*-minimal medium (Hill and Kaefer 2001; Johnson et al. 2011: Section A, B). *Alternaria brassicae* was cultured for 2 weeks on Potato Dextrose Agar medium (PDA) at 22-24°C under 12/12 h light/dark illumination and 75% relative humidity. For details see Johnson et al. (2011, 2012).

**Co-cultivation with *P. indica* and assessment of plant growth**

Twelve day-old (cold treatment for 48 h and 10 days illumination) Arabidopsis wild-type (WT) and mutant seedlings of equal sizes which were grown on MS media under continuous illumination (100 µmol $m^{-2}$ $sec^{-1}$) at 22°C were selected for co-cultivation experiments with three to four week-old *P. indica* or mock-treated. Co-cultivation was done on PNM media with a fungul plug 5 mm in diameter as described by Johnson et al. (2011, Section C1-Method 1). Light intensity (80 µmol $m^{-2}$ $sec^{-1}$) was checked every third day to ensure that both the *P. indica* and mock-treated seedlings receive equal amount of light. Weights of seedlings were determined at 14 day after co-cultivation. For DNA and RNA isolation and gene expression studies only roots were used.

**Root and leaf infection with *A. brassicae***

Root infection with *A. brassicae* was done on PNM media as described for the co-cultivation with *P. indica* (Johnson et al. 2011) using a fungal plug 5 mm in diameter from a two week-old culture (Johnson et al. 2012). Leaf infection was done on PNM media by adding 8-10 µl spore suspension ($10^5$-$10^6$ spores/ml) of Alternaria directly to the plant leaves (Johnson et al. 2012). Six to eight leaves per seedling were used. Plates were incubated at 22-24°C under 12/12 h dark/light illumination. For preparation of Alternaria spores a fungal plug 5 mm in diameter from a 2 week-old culture was inoculated to Potato dextrose broth and incubated at 22-24°C under 12/12 h light/dark illumination and 75% relative humidity. After 2 weeks spores were harvested by homogenizing the mycelia in distilled water and filtered through 4 layers of sterilized-nylon membrane. The spore concentration was adjusted to $10^5$-$10^6$/ml.

**Dual culture experiment of *P. indica* and *A. brassicae***

Dual culture experiment was done as described by Johnson et al. (2012). A *P. indica* plug 5 mm in diameter was placed at one end of the PDA plate and an *A. brassicae* plug of the same size was placed at the other end. The plate was incubated at 22-24°C under 12/12h light/dark illumination and 75% relative humidity. The inhibition zone was observed after 5 days.

**Priming with *P. indica* and quantification of *A. brassicae* in roots**

After growing on MS media for 12 days (as described above) Arabidopsis seedlings were co-cultivated with *P. indica* for three days on PNM media or mock-treated (Johnson et al. 2011; Section C1 - Method1). For priming with *P. indica* WT and mutant seedlings of equal size were co-cultivated with a *P. indica* plug 5 mm in diameter on PNM plates. Accordingly, seedlings of equal size of that for *P. indica* treatment were taken for mock-treated control. Plates were incubated at 22°C under continuous illumination (80 µmol $m^{-2}$ $s^{-1}$). Three days after priming, a plug of *A. brassicae* 5 mm in diameter was used to challenge the *P. indica*-primed and mock-treated control by replacing the *P. indica*/KM plug with *A. brassicae* plug. The fresh weight was measured at sixth and tenth day after *A. brassicae* infection. The concentration of *A. brassicae* in roots was quantified by PCR for the *AbreAtr1* gene marker (Guillemette et al. 2004).

**Induced systemic resistance (ISR) and quantification of *A. brassicae* in leaves**

Co-cultivation of Arabidopsis seedlings with *P. indica* in order to prime the shoot for ISR was done as described by Johnson et al. (2011, Section C1 - Method 2). Arabidopsis seedlings and *P. indica* plug were co-cultivated for seven days on PNM media. The same was done for the mock-treated control. In this case a KM plug was used. After seven days, the primed seedlings were inoculated with *A. brassicae* spores suspension ($10^6$ spores/ml) on 6-8 leaves per plant. After five days disease phenotype was observed and the leaves were harvested for RNA isolation and gene expression analysis. The concentration of *A. brassicae* was quantified by PCR for the *AbreAtr1* gene marker (Guillemette et al. 2004).

### Gene expression

Total RNA was isolated from both roots and shoots of WT and mutant seedlings after five, six and ten days of co-cultivation with/without *P. indica*/*A. brassicae* using RNeasy Mini Kit (Qiagen). After reverse-transcription, cDNA was used for semi-quantitative PCR analysis. Total RNA was isolated from three independent biological experiments. cDNA was synthesised 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 25 µl. The iCycler was programmed to 95°C 2 min, 40 x (95°C 30 sec, 57°C 40 sec, 72°C 45 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 *GAPC2* 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 mock-treated roots, which were defined as 1.0. All the primers used for this study are given in the supplementary Table 1.

### Data analysis

Data points represent the mean of three to six independent biological experiments with 12-24 seedlings per treatment per experiment. Samples were evaluated with a two sample t-test (+*P. indica*/mock-treated) and ANOVA analyses (comparison of all data sets).

### ACKNOWLEDGEMENT

Work was supported by the Jena School for Microbial Communication. We thank Sarah Muβbach and Claudia Röppischer for excellent technical assistance and our colleges for the mutant lines used for these experiments.

## REFERENCES

Agrawal, A.A. and Kurashige, N.S. (2003) A role for isothiocyanates in plant resistance against the specialist herbivore *Pieris rapae*. *J Chem Ecol.* 29: 1403-1415.

Bak, S., Olsen, C.E., Petersen, B.L., Müller, B.L. and Halkier, B.A. (1999) Metabolic engineering of p-hydroxybenzylglucosinolate in Arabidopsis by expression of the cyanogenic CYP79A1 from *Sorghum bicolor*. *Plant J*. 20: 663-671.

Bains, P.S. and Tewari, J.P. (1987) Purification, chemical characterization and host-specificity of the toxin produced by *Alternaria brassicae*. *Physiol. Mol. Plant Pathol*. 30: 259-271.

Bednarek, P. and Osbourn, A. (2009) Plant-microbe interactions: chemical diversity in plant defense. *Science* 324: 746-748.

Beekwilder, J., van Leeuwen, W., van Dam, N.M., Bertossi, M., Grandi, V., Mizzi, L., Soloviev, M., Szabados, L., Molthoff, J.W., Schipper, B., Verbocht, H., de Vos, R.C., Morandini, P., Aarts, M.G. and Bovy, A. (2008) The impact of the absence of aliphatic glucosinolates on insect herbivory in Arabidopsis. *PLoS One* 3: e2068.

Bolton, M.D., Thomma, B.P.H.J. and Nelson, B.D. (2006) *Sclerotinia sclerotiorum* (Lib.) de Bary: biology and molecular traits of a cosmopolitan pathogen. *Mol. Plant Pathol*. 7: 1-16.

Bodnaryk, R.P. (1994) Potent effect of jasmonates on indole glucosinolates in oilseed rape and mustard. *Phytochemistry* 35: 301-305.

Brader, G., Tas, E. and Palva, E.T. (2001) Jasmonate-dependent induction of indole glucosinolates in Arabidopsis by culture filtrates of the nonspecific pathogen *Erwinia carotovora. Plant Physiol.* 126: 849-860.

Brader, G., Mikkelsen, M.D, Halkier, B.A. and Palva, E.T. (2006) Altering glucosinolate profiles modulates disease resistance in plants. *The Plant J.* 46: 758-767.

Brown, P.D., Tokuhisa, J.G., Reichelt, M. and Gershenzon, J. (2003) Variation of glucosinolate accumulation among different organs and developmental stages of *Arabidopsis thaliana*. *Phytochem.* 62: 471-481.

Burow, M., Halkier, B.A. and Kliebenstein, D.J. (2010) Regulatory networks of glucosinolates shape *Arabidopsis thaliana* fitness. *Curr. Opin. Plant Biol*. 13: 348-53.

Camehl, I., Sherameti, I., Venus, Y., Bethke, G., Varma, A., Lee, J. and Oelmüller, O. (2010) Ethylene signalling and ethylene-targeted transcription factors are required to balance

beneficial and nonbeneficial traits in the symbiosis between the endophytic fungus *Piriformospora indica* and *Arabidopsis thaliana*. *New Phytol.* 185: 1062-1073.

Camehl, I., Drzewiecki, C., Vadassery, J., Shahollari, B., Sherameti, I., Forzani, C., Munnik, T., Hirt, H. and Oelmüller, R. (2011) The OXI1 kinase pathway mediates *Piriformospora indica*-induced growth promotion in Arabidopsis. *PLoS Pathogens* 7: e1002051.

Chen, S. and Andreasson, E. (2001) Update on glucosinolate metabolism and transport. *Plant Physiol. Biochem*. 39: 743-758.

Chen, S., Glawischnig, E., Jorgensen, K., Naur, P., Jorgensen, B., Olsen, C.E., Hansen, C.H., Rasmussen, H., Pickett, J.A. and Halkier, B.A. (2003) CYP79F1 and CYP79F2 have distinct functions in the biosynthesis of aliphatic glucosinolates in Arabidopsis. *Plant J*. 33: 923-937.

Conn, K.L., Tewari, J.P. and Awasthi, R.P. (1990) A disease assessment key for Alternaria blackspot in rapeseed and mustard. *Can. Plant Dis. Surv*. 70: 19-22.

Fahey, J.W., Zalcmann, A.T. and Talalay, P. (2001) The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. *Phytochem.* 56: 5-51.

Ferrari, S., Plotnikova, J.M., De Lorenzo, G. and Ausubel, F.M. (2003) Arabidopsis local resistance to *Botrytis cinerea* involves salicylic acid and camalexin and requires EDS4 and PAD2, but not SID2, EDS5 or PAD4. *Plant J*. 35: 193-205.

Giamoustaris, A. and Mithen, R. (1995) The effect of modifying the glucosinolate content of leaves of oilseed rape (*Brassica napus* ssp. *oleifera*) on its interaction with specialist and generalist pests. *Ann. Appl. Biol.* 126: 347-353.

Gigolashvili, T., Yatusevich, R., Rollwitz, I., Humphry, M., Gershenzon, J. and Flügge, U-I. (2009) The plastidic bile acid transporter 5 is required for the biosynthesis of methionine-derived glucosinolate in *Arabidopsis thaliana*. *Plant Cell* 21: 1813-1329.

Gigolashvili, T., Engqvist, M., Yatusevich, R., Müller, C. and Flügge, U-I. (2008) HAG2/MYB76 and HAG3/MYB29 exert a specific and coordinated control on the regulation of aliphatic glucosinolate biosynthesis in *Arabidopsis thaliana. New Phytol.* 177: 627-642.

Gigolashvili, T., Berger, B., Mock, H.P., Muller, C. and Weisshaar, B. (2007a) The transcription factor HIG1/MYB51 regulates indolic glucosinolate biosynthesis in *Arabidopsis thaliana*. *Plant J.* 50: 886-901.

Gigolashvili, T., Yatusevich, R., Berger, B., Müller, C. and Flügge, U-I. (2007b) The R2R3-MYB transcription factor HAG1/MYB28 is a regulator of methionine-derived glucosinolate biosynthesis in *Arabidopsis thaliana*. *The Plant J.* 51: 247-261.

Glazebrook, J. (2005) Contrasting mechanisms of defense against biotrophic and necrotrophic

pathogens. *Annu. Rev. Phytopathol*. 43: 205-227.

Grubb, C.D. and Abel, S. (2006) Glucosinolate metabolism and its control. *Trends Plant Sci*. 11: 89-100.

Guillemette, T., Sellam, A. and Simoneau, P. (2004) Analysis of a nonribosomal peptide synthetase gene from *Alternaria brassicae* and flanking genomic sequences. *Curr. Genet.* 45: 214-224.

Guo, X. and Stotz, H.U. (2007) Defense against *Sclerotinia sclerotiorum* in Arabidopsis is dependent on jasmonic acid, salicylic acid, and ethylene signaling. *Mol. Plant-Microbe Interact*. 20: 1384-1395.

Guo, X. and Stotz, H.U. (2010) ABA signaling inhibits oxalate-induced production of reactive oxygen species and protects against *Sclerotinia sclerotiorum*. *Eur. J. Plant Pathol*. 128: 7-19.

Halkier, B.A. and Gershenzon, J. (2006) Biology and biochemistry of glucosinolates. *Annu. Rev. Plant Biol*. 57: 303-333.

Hammerschmidt, R. (1999) Phytoalexins: what have we learned after 60 years? *Annu. Rev. Phytopathol.* 37: 285-306.

Hansen, C.H., Du, L., Naur, P., Olsen, C.E., Axelsen, K.B., Hick, A.J., Pickett, J.A. and Halkier, B.A. (2001) CYP83B1 is the oxime-metabolizing enzyme in the glucosinolate pathway in Arabidopsis. *J. Biol. Chem*. 276: 24790-24796.

Hill, T.W. and Kaefer, E. (2001) Improved protocols for Aspergillus medium: trace elements and minimum media salt stock solutions. *Fungal Genet. Newl.* 48: 20-21.

Hirai, M.Y., Sugiyama, K., Sawada, Y., Tohge, T. and Obayashi, T. (2007) Omics-based identification of Arabidopsis Myb transcription factors regulating aliphatic glucosinolate biosynthesis. *Proc. Natl. Acad. Sci. USA.* 104: 6478-6483.

Hull, A.K., Vij, R. and Celenza, J.L. (2000) Arabidopsis cytochrome P450s that catalyze the first step of tryptophandependent indole-3-acetic acid biosynthesis. *Proc. Natl. Acad. Sci. USA*. 97: 2379-2384.

Husebye, H., Chadchawan, S., Winge, P., Thangstad, O.P. and Bones, A.M. (2002) Guard cell- and phloem idioblast-specific expression of thioglucoside glucohydrolase 1 (myrosinase) in Arabidopsis. *Plant Physiol*. 128: 1180-1188.

Jacobs, S., Zechmann, B., Molitor, A., Trujillo, M., Petutschnig, E., Lipka, V., Kogel, K,-H. and Schäfer, P. (2011) Broad-spectrum suppression of innate immunity is required for colonization of Arabidopsis roots by the fungus *Piriformospora indica*. *Plant Physiol.* 156: 726-740.

Janowitz, T., Trompetter, I. and Piotrowski, M. (2009) Evolution of nitrilases in glucosinolate-containing plants. *Phytochem*. 70: 1680-1686.

Johnson, J.M., Sherameti, I., Nongbri, P.L. and Oelmüller, R. (2012) Standardized conditions to study beneficial and non-beneficial traits in the *Piriformospora indica*/Arabidopsis interaction. *In: Varma, A., Kost, G. and Oelmüller, R. (eds) Piriformospora indica*: Sebacinales and their biotechnological application. *Springer, Heidelberg, Vol. 33* (in press).

Johnson, J.M., Sherameti, I., Ludwig, A., Nongbri, P.L., Sun, C., Lou, B., Varma, A. and Oelmüller, R. (2011) Protocols for *Arabidopsis thaliana* and *Piriformospora indica* cocultivation - A model system to study plant beneficial traits. *Endocyt. Cell Res*. 101-113. (http://zs.thulb.uni-jena.de/receive/jportal_jparticle_00247947).

Kelly, P.J., Bones, A. and Rossiter, J.T. (1998) Sub-cellular immunolocalization of the glucosinolate sinigrin in seedlings of *Brassica juncea*. *Planta* 206: 370-377.

Kim, K.S., Min, J.Y. and Dickman, M.B. (2008) Oxalic acid is an elicitor of plant programmed cell death during *Sclerotinia sclerotiorum* disease development. *Mol. Plant-Microbe Interact*. 21: 605-612.

Kliebenstein, D.J., Kroymann, J., Brown, P., Figuth, A. and Pedersen, D. (2001) Genetic control of natural variation in Arabidopsis glucosinolate accumulation. *Plant Physiol.* 126: 811-825.

Kliebenstein, D., Pedersen, D., Barker, B. and Mitchell-Olds, T. (2002) Comparative analysis of quantitative trait loci controlling glucosinolates, myrosinase and insect resistance in *Arabidapsis thaliana*. *Genetics* 161: 325-332.

Kliebenstein, D.J., Rowe, H.C. and Denby, K.J. (2005) Secondary metabolites influence Arabidopsis/Botrytis interactions: variation in host production and pathogen sensitivity. *Plant J*. 44: 25-36.

Koroleva, O.A., Davies, A., Deeken, R., Thorpe, M.R., Tomos, D. and Hedrich, R. (2000) Identification of a new glucosinolate-rich cell type in Arabidopsis flower stalk. *Plant Physiol*. 124: 599-608.

Lahrmann, U. and Zuccaro, A. (2012) Opprimo ergo sum - Evasion and suppression in the root endophytic fungus *Piriformospora indica*. *Mol. Plant-Microbe Interact*. 25: 727-737.

Lambrix, V., Reichelt, M., Mitchell-Olds, T., Kliebenstein, D.J. and Gershenzon, J. (2001) The Arabidopsis epithiospecifier protein promotes the hydrolysis of glucosinolates to nitriles and influences *Trichoplusia ni* herbivory. *Plant Cell* 13: 2793-2807.

Levy, M., Wang, Q.M., Kaspi, R., Parrella, M.P. and Abel, S. (2005) *Arabidopsis* IQD1, a novel calmodulin-binding nuclear protein, stimulates glucosinolate accumulation and plant defense. *Plant J.* 43: 79-96.

Manici, L.M., Lazzeri, L. and Palmieri, S. (1997) *In vitro* fungitoxic activity of some glucosinolates and their enzyme-derived products toward plant pathogenic fungi. *J. Agric. Food Chem.* 45: 2768-2773.

Mari, M., Leoni, O., Iori, R. and Cembali, T. (2002) Antifungal vapour-phase activity of allyl-isothiocyanate against *Penicillium expansum* on pears. *Plant Pathol.* 51: 231-236.

McCloskey, C. and Isman, M.B. (1993) Influence of foliar glucosinolates in oilseed rape and mustard on feeding and growth of the Bertha Armyworm, *Mamestra configurata* Walker. *J. Chem. Ecol.* 19: 249-266.

Mikkelsen, M.D., Hansen, C.H., Wittstock, U. and Halkier, B.A. (2000) Cytochrome P450 CYP79B2 from Arabidopsis catalyzes the conversion of tryptophan to indole-3-acetaldoxime, a precursor of indole glucosinolates and indole-3-acetic acid. *J. Biol. Chem*. 275: 33712-33717.

Mikkelsen, M.D. and Halkier, B.A. (2003) Metabolic engineering of valine- and isoleucine-derived glucosinolates in Arabidopsis expressing CYP79D2 from Cassava. *Plant Physiol*. 131: 773-779.

Mithen, R.F., Lewis, B.G. and Fenwick, G.R. (1986) *In vitro* activity of glucosinolates and their products against *Leptosphaeria maculans*. *Trans. Br. Mycol. Soc*. 87: 433-440.

Müller, R., de Vos, M., Sun, J.Y., Sønderby, I.E., Halkier, B.A., Wittstock, U. and Jander, G (2010) Differential effects of indole and aliphatic glucosinolates on lepidopteran herbivores. *J. Chem. Ecol.* 36: 905-913.

Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant*. 15: 473-497.

Nongbri, P.L., Johnson, J.M., Sherameti, I., Glawischnig, E., Halkier, B.A. and Oelmüller, R. (2012) Indole-3-acetaldoxime-derived compounds restrict root colonization in the beneficial interaction between Arabidopsis roots and the endophyte *Piriformospora indica*. *Mol. Plant Microbe-Interact*. 25: 1186-1197.

Oelmüller, R., Sherameti, I., Tripathi, S. and Varma, A. (2009) *Piriformospora indica*, a cultivable root endophyte with multiple biotechnological applications. *Symbiosis* 48: 1-17.

Oughty, K.J., Kiddle, G,A., Pye, B.J., Wallsgrove, R.M. and Pickett, J.A. (1995) Selective induction of glucosinolates in oilseed rape leaves by methyl jasmonate. *Phytochem.* 38: 347-350.

Pedras, M.S., Jha, M., Minic, Z. and Okeola, O.G. (2007) Isosteric probes provide structural requirements essential for detoxification of the phytoalexin brassinin by the fungal pathogen *Leptosphaeria maculans*. *Bioorg. Med. Chem*. 15: 6054-6061.

Penninckx, I.A.M.A., Eggermont, K., Terras, F.R.G., Thomma, B.P.H.J., De Samblanx, G.W., Buchala, A., Métraux, J-P., Manners, J.M. and Broekaert, W.F. (1996) Pathogen- induced systemic activation of a plant defensin gene in Arabidopsis follows a salicylic acid-independent pathway. *Plant Cell* 8: 2309-2323.

Petersen, B.L., Andreasson, E., Bak, S., Agerbirk, N. and Halkier, B.A. (2001) Characterization of transgenic *Arabidopsis thaliana* with metabolically engineered high levels of p-hydroxybenzylglucosinolate. *Planta* 212: 612-618.

Pfaffl, M.W. (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucl. Acids Res.* 29: 2002-2007.

Piotrowski, M. (2008) Primary or secondary? Versatile nitrilases in plant metabolism. *Phytochem.* 69: 2655-2667.

Pieterse, C.M.J., van Wees, S.C.M., Hoffland, E., van Pelt, J.A. and van Loon, L.C. (1996) Systemic Resistance in Arabidopsis induced by biocontrol bacteria is independent of salicylic acid accumulation and pathogenesis-related gene expression. *The Plant Cell* 8: 1225-1237.

Reichelt, M., Brown, P.D., Schneider, B., Oldham, N.J., Stauber, E. (2002) Benzoic acid glucosinolate esters and other glucosinolates from *Arabidopsis thaliana. Phytochem.* 59: 663-671.

Rudrappa, T., Biedrzycki, M.L., Kunjeti, S.G., Donofrio, N.M., Czymmek, K.J., Pare, P.W. and Bais, H.P. (2010) The rhizobacterial elicitor acetoin induces systemic resistance in *Arabidopsis thaliana. Communicative & Integrative Biology* 3: 130-138.

Schuhegger, R., Nafisi, M., Mansourova, M., Petersen, B.L., Olsen, C.E., Svatos, A., Halkier, B.A. and Glawischnig, E. (2006) CYP71B15 (PAD3) catalyzes the final step in camalexin biosynthesis. *Plant Physiol*. 141: 1248-1254.

Segarra, G., Van der Ent, S., Trillas, I. and Pieterse, C.M.J. (2009) MYB72, a node of convergence in induced systemic resistance triggered by a fungal and a bacterial beneficial microbe. *Plant Biol*. 11: 90-96.

Smolinska, U., Morra, M.J., Knudsen, G.R. and James, R.L. (2003) Isothiocyanates produced by *Brassicaceae* species as inhibitors of *Fusarium oxysporum*. *Plant Dis.* 87: 407-412.

Sønderby, I.E., Geu-Flores, F. and Halkier, B.A. (2010) Biosynthesis of glucosinolates- gene discovery and beyond. *Trends Plant Sci*. 15: 283-290.

Sønderby, I.E., Hansen, B.G., Bjarnholt, N., Ticconi, C., Halkier, B.A. and Kliebenstein, D.J. (2007) A systems biology approach identifies a R2R3 MYB gene subfamily with distinct and overlapping functions in regulation of aliphatic glucosinolates. *PLoS One* 2: e1322.

Sønderby, I.E., Burow, M., Rowe, H.C., Kliebenstein, D.J. and Halkier, B.A. (2010) A complex interplay of three R2R3 MYB transcription factors determines the profile of aliphatic glucosinolates in Arabidopsis. *Plant Physiol*. 153: 348-363.

Stein, E., Molitor, A., Kogel, K-H. and Waller, F. (2008) Systemic resistance in Arabidopsis conferred by the mycorrhizal fungus *Piriformospora indica* requires jasmonic acid signaling and the cytoplasmic function of NPR1. *Plant Cell Physiol*. 49: 1747-1751.

Stotz, H.U., Sawada, Y., Shimada, Y., Hirai, M.Y., Sasaki, E., Krischke, M., Brown, P.D., Saito, K. and Kamiya, Y. (2011) Role of camalexin, indole glucosinolates, and side chain modification of glucosinolate-derived isothiocyanates in defense of Arabidopsis against *Sclerotinia sclerotiorum*. *The Plant J.* 67: 81-93.

Tewari, J.P. and Bains, P.S. (1997) Phytotoxins produced by *Alternaria brassicae* and bioassay of destruxin B. *In: Rajeev, K. and Mukerji, K. G. (eds)*. Toxins in plant disease development and evolving biotechnology. *Science Publisher, Enfield, NH, pp. 21-35.*

Thomma, B.P.H.J., Nelissen, I., Eggermont, K. and Broekaert, W.F. (1999) Deficiency in phytoalexin production causes enhanced susceptibility of *Arabidopsis thaliana* to the fungus *Alternaria brassicicola*. *Plant J*. 19: 163-171.

Thomma, B.P.H.J., Eggermont, K., Penninckx, I.A.M.A., Mauch-Mani, B., Vogelsang, R., Cammue, B.P.A. and Broekaert, W.F. (1998) Separate jasmonate-dependent and salicylate-dependent defense response pathways in Arabidopsis are essential for resistance to distinct microbial pathogens. *Proc. Natl. Acad. Sci. USA*. 95: 15107-15111.

Tierens, K.F., Thomma, B.P.H.J., Brouwer, M., Schmidt, J., Kistner, K., Porzel, A., Mauch-Mani, B., Cammue, B.P. and Broekaert, W. F. (2001) Study of the role of antimicrobial glucosinolate-derived isothiocyanates in resistance of Arabidopsis to microbial pathogens. *Plant Physiol*. 125: 1688-1699.

Van der Ent, S., Verhagen, B.W.M., Van Doorn, R., Bakker, D., Verlaan, M.G., Pel, M.J.C., Joosten, R.G., Proveniers, M.C.G., Van Loon, L.C., Ton, J. and Pieterse, C.M.J. (2008) MYB72 is required in early signaling steps of rhizobacteria-induced systemic resistance in Arabidopsis. *Plant Physiol*. 146: 1293-1304.

Verhagen, B.W.M., Glazebrook, J., Zhu, T., Chang, H.-S., Van Loon, L.C. and Pieterse, C.M.J. (2004) The transcriptome of rhizobacteria-induced systemic resistance in Arabidopsis. *Mol. Plant–Microbe Interact*. 17: 895-908.

Wittstock, U. and Halkier, B.A. (2000) Cytochrome P450 CYP79A2 from *Arabidopsis thaliana* L. catalyzes the conversion of L-phenylalanine to phenylacetaldoxime in the biosynthesis of benzylglucosinolate. *J. Biol. Chem*. 275: 14659-14666.

Yan, X.F. and Chen, S.X. (2007) Regulation of plant glucosinolate metabolism. *Planta* 226: 1343-1352.

Zimmermann, P., Hirsch-Hoffmann, M., Hennig, L. and Gruissem, W. (2004) GENEVESTIGATOR. Arabidopsis microarray database and analysis toolbox. *Plant Physiol.* 136: 2621-2632.

## FIGURE LEGENDS

**Figure 1. A,** mRNA levels for *MYB28* in the roots of wild-type (WT) seedlings colonized by either *P. indica* and *A. brassicae*. The seedlings were either co-cultivated with *P. indica/A. brassicae* for 6 days or mock-treated. mRNA levels for *MYB28* were induced only by *A. brassicae* infection and not by *P. indica*. *GAPC2* was used as a control house keeping gene. MC: mock-treated control; *P.i*: *P. indica*-treated; *A.b*: *A. brassicae*-treated. **B,** Root infection of *A. thaliana* WT and *myb28 myb29* seedlings by *A. brassicae*. Browning of roots and chlorotic symptoms in shoots were recorded after 10 days of root infection. MC: mock-treated control; *A.b*: *A. brassicae*-treated.

**Figure 2. A,** Priming with *P. indica* leads to improved growth performance of Arabidopsis WT seedlings after Alternaria spore infection. *P. indica* retards *A. brassicae*-induced brownishing of roots and chlorotic phenotype in the shoots as compared to the mock-treated controls. Representative pictures of five independent biological experiments taken after 8 days of Alternaria infection are shown. ± *P.i*: ± *P. indica*-treated; ± *A.b*: ± *A. brassicae*-treated. **B,** mRNA levels for *AbreAtr1* in the Arabidopsis roots. RNA was extracted from WT roots treated with *P. indica* (*P.i*) or *A. brassicae* (*A.b*) for 6 days. The figure shows the specificity of the primers for *A. brassicae*. **C,** Fold induction of *AbreAtr1* in the roots of WT and *myb28 myb29* seedlings after 10 days of *A. brassicae* infection. The $mRNA_{+P.\ indica}/mRNA_{mock\text{-}treated}$ ratio was calculated. Data are based on five independent biological experiments with three replications each. Error bars were calculated as standard errors.

**Figure 3**. Seedlings fresh weight (mg) of WT and *myb28 myb29* seedlings after root infection with *A. brassicae* for 10 days. Seedling were either primed with *P. indica* (+*P.i*) for 3 days or not primed (-*P.i*) before infection with Alternaria (+*A.b*) for 10 days. Data are based on five independent experiments with 12 plants per treatment. Bars represent SEs.

**Figure 4. A,** mRNA levels for *PDF1.2* in WT and *myb28 myb29* seedlings treated with *P. indica* and/or *A. brassicae*. RNA was isolated from the roots of wild type (WT) and *myb28 myb29* Arabidopsis seedlings which were co-cultivated with *P. indica/A. brassicae* for 6 days or mock-treated. mRNA levels for *PDF1.2* are regulated by *A. brassicae*, but not *P. indica*. *GAPC2* was used as a control house keeping gene. MC: mock-treated control; *P.i*: *P. indica*-treated; *A.b*: *A. brassicae*-treated. **B,** Fold change of *PDF1.2* in the roots of WT and *myb28 myb29*. Seedlings were primed with *P. indica* for 3 days or not primed before Alternaria infection for 10 days. Data are $PDF1.2_{+P.\ indica\ \text{pretreatment}}/PDF1.2_{-P.\ indica\ \text{pretreatment}}$. Bars represent SEs. **C,** Interaction between *P. indica* and *A. brassicae* on Potato Dextrose Agar (PDA) medium. A clear zone of inhibition (see arrow) was observed. Representative picture taken after 10 days of interaction.

**Figure 5. A,** Systemic resistance (ISR) in Arabidopsis. Both WT and *myb28 myb29* mutant were primed with *P. indica* (+*P.i*) for 5 days or not primed (-*P.i*) before Alternaria spores infection for 5 days. **B,** Fold induction of *AbreAtr1* in the leaves of WT and *myb28 myb29* seedlings after 5 days of *A. brassicae* spores infection. Data are based on three independent biological experiments with three replications. Error bars were calculated as standard errors. **C,** Fold induction of *PDF1.2* in the leaves of WT and *myb28 myb29* after *P. indica* (+*P.i*) or *A. brassicae* (+*A.b*) infection for 5 days. Data are based on three independent biological experiments with three replications. Error bars were calculated as standard errors.

## FIGURES

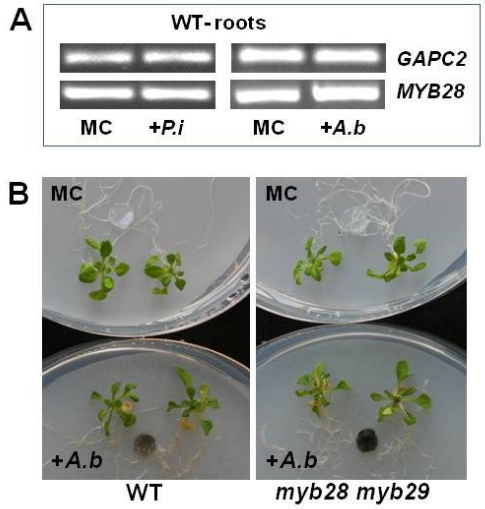



Figure 1

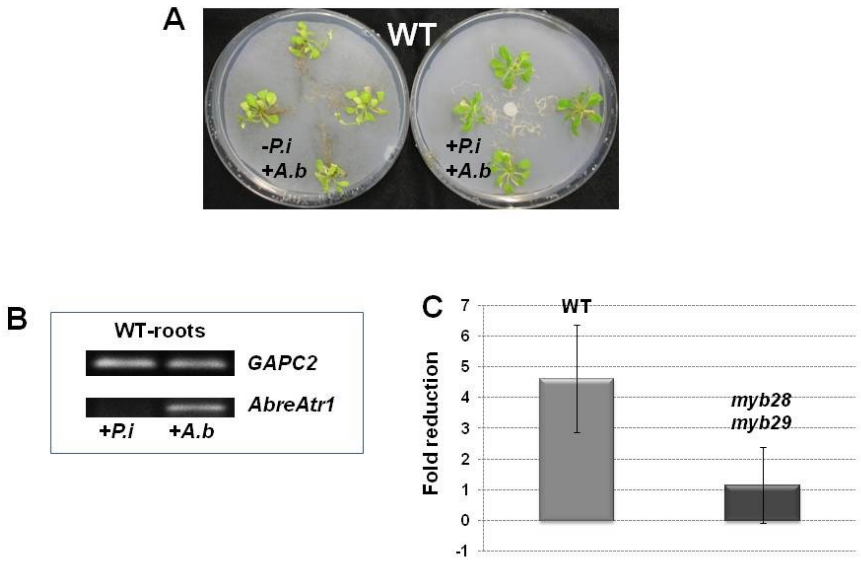



Figure 2

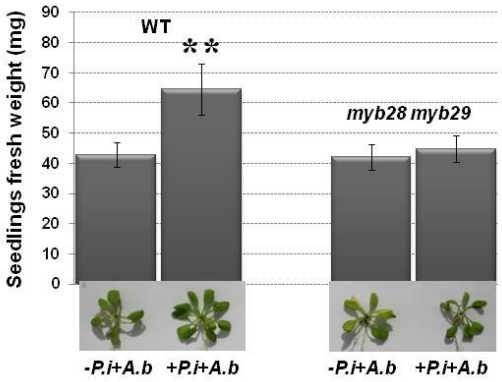



Figure 3

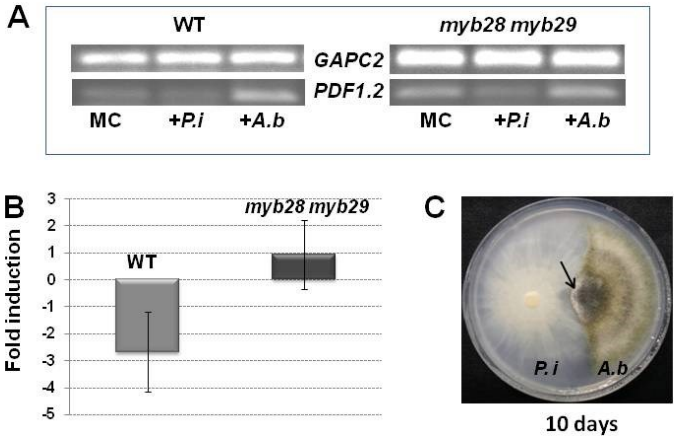



Figure 4

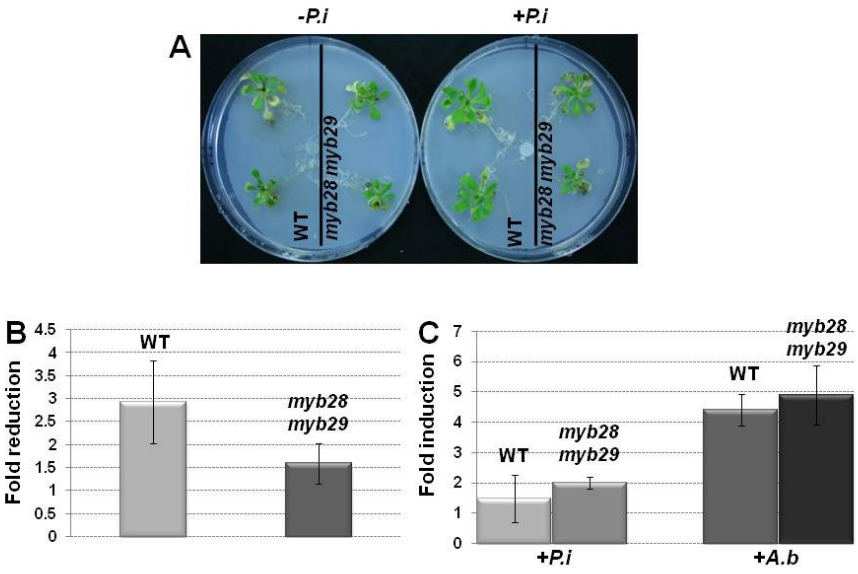

A
-P.i
+P.i
WT
myb28 myb29
WT
myb28 myb29
B
Fold reduction
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
WT
myb28
myb29
C
Fold induction
7
6
5
4
3
2
1
0
WT
myb28
myb29
WT
myb28
myb29
+P.i
+A.b

Figure 5

Supplementary Tab. 1

Primer pairs used in this study.

| | | |
|---|---|---|
| *MYB28* (At5g61420) | GTGGTCGGTCATAGCGAGAC | GCGAGTCTGAGTCGGTGTCA |
| MYB29(5g07690) | TACAACGGTCGTCTACCACA | TCATCCGGGTTTGAGTCATA |
| *MYB76*(At5g07700) | TTGAGCCCATGAAGTTCGA | CGATCATGTACTCATATGATTG |
| *PDF1.2* (At5g44420) | CTTGTGTGCTGGGAAGACATA | AGCACAGAAGTTGTGCGAGAA |
| *GAPC2* (At3g04120) | GAGCTGACTACGTTGTTGAG | GGAGACAATGTCAAGGTCGG |
| *AbreAtr1*(Guillemette et al. 2004) | ACCCGCATTCCTCGCCAAA | AAGTCAAGGATTGTGTCGAGCTT |
| *PR-3* (At3g12500) | TCATGGGGCTACTGTTTCAAG | TATTGCTCTACCGCATAGACC |
| *PR-1* (At2g14610) | TGTATGAGTCTGCAGTTGCC | CAACTGCAGACTCATACA |

# 5. Discussion

Sulfur is one of the limiting nutrients in agricultural productions. Plants respond to sulfur deficiency by activating root uptake systems (Gutierrez-Marcos *et al*. 1996; Takahashi *et al*. 1997, 2000; Vidmar *et al*. 2000; Yoshimoto *et al*. 2002) and remobilization of internal sulfur sources (Blake-Kalff *et al*. 1998; Hirai *et al*. 2004, 2005; Schnug and Haneklaus 1993; Schnug *et al*. 1995; Maruyama-Nakashita *et al*. 2003; Nikiforova *et al*. 2003). Furthermore, the status of sulfur in the plants is crucial for growth as well as the plant´s response against biotic stresses. I could demonstrate that Arabidopsis synthesizes sulfur-containing secondary metabolites which play an important role in the symbiotic interaction with *P. indica*. Furthermore, *P. indica* prevents pathogen infection of roots and activates a systemic resistance response in leaves against the pathogenic fungus *A. brassicae*, and this response requires the sulfur-containing glucosinolates.

I investigated the role of the two enzymes CYP79B2 and CYP79B3 (involved in indole glucosinolate and camalexin biosynthesis) and of PAD3 (which catalyzes the last step of camalexin biosynthesis) in the roots of Arabidopsis during the symbiotic interaction with *P. indica*. I presented evidence that inactivation of CYP79B2 and CYP79B3 has severe consequences for the beneficial interaction which is associated with an uncontrolled growth of *P. indica* in the roots. I also examined the role of aliphatic glucosinolates in the symbiotic interaction to *P. indica*, by exposing the *myb28 myb29* double mutant, which is devoid of aliphatic glucosinolates, to the fungus. My data demonstrate that aliphatic glucosinolates are crucial for *P. indica*-induced systemic resistance of Arabidopsis seedlings against leaf infections by the pathogenic fungus *A. brassicae*. This highlights the importance of the root-colonizing fungus for systemic resistance responses in the leaves.

## 5.1 Antimicrobial indole-3-acetaldoxime (IAOx)-derived compounds in Arabidopsis/*P. indica* interaction

Plants constitute an immune system which responds to microbial infection by the biosynthesis of antimicrobial secondary metabolites. IAOx-derived compounds belong to a group of secondary metabolites which are synthesized in roots and leaves. These secondary

metabolites, which for many years were believed to lack physiological significance (Hartmann 2008), were found to have multiple important functions in plant interactions with the environment, in forming beneficial interactions with root-colonizing microbes and as deterrence to potential enemies (Hartmann 2007, 2008).

Plant chemical defenses depend on the constitutive production of phytoanticipins (VanEtten *et al*. 1994) and induced production of phytoalexins (Bednarek and Osbourn 2009). Thomma *et al*. (1999) and Ferrari *et al*. (2003) have demonstrated the importance of the Arabidopsis phytoalexin camalexin (Schuhegger *et al*. 2006) as a significant defense component against necrotrophic fungal pathogens. These phytoanticipins derived from glucosinolates in Brassicaceae after myrosinase-catalyzed hydrolysis and are inhibitory to the growth of various pathogens *in vitro* (Tierens *et al*. 2001). Stotz *et al*. (2011) have shown the accumulation of glucosinolates in systemic leaves of *S. sclerotiorum*-challenged Arabidopsis, whereas camalexin are induced at local infection sites. The mRNA levels for *CYP79B2*, *CYP79B3* and *PAD3* are also induced by *P. indica* in colonized roots. I could show that indole glucosinolates and camalexin are necessary for the beneficial interaction between *P. indica* and *A. thaliana*. The *cyp79B2 cyp79B3* double mutant, which lacks indole glucosinolates and camalexin, does not respond to *P. indica* with growth promotion, both at the seedling´s stage and adult plant stage, whereas growth of the *pad3* mutant, which does not synthesize camalexin, is not promoted by *P. indica* under long term interaction in soil (Nongbri *et al*. 2012). Camalexin and other IAOx-derived compounds including indole glucosinolates may be involved in independent processes. We, therefore, suggested that IAOx-derived compounds are important for establishing a beneficial interaction between *P. indica* and Arabidopsis during the initial steps, while camalexin is essential in the later phase during long term interaction. Additional mutants defective in the synthesis of specific IAOx-derived secondary metabolites need to be investigated to understand the precise role of these secondary metabolites in the beneficial symbiosis. Schlaeppi *et al*. (2010) have noticed that both indole glucosinolate and camalexin are crucially important for the resistance of Arabidopsis to *Phytophthora brassicae* while lacking either one of them has little effect on the disease resistance. They observed a wild-type response of *pad3* to *P. brassicae* infection, while the *cyp79B2 cyp79B3* double mutant was highly susceptible. In their studies, CYP79B2 and

CYP79B3 participate in the control of the penetration rate of *P. brassicae* hyphae. Unlike the Arabidopsis/*P. brassicae* pathosystem, which also revealed the wild-type level of classical defenses responses in the *cyp79B2 cyp79B3* mutant, the beneficial fungus *P. indica* initially induces defense genes in the roots of the mutant seedlings but this response disappears in adult plants. Furthermore, *cyp79B2 cyp79B3* are higher colonized at seedling´s and adult plant stages compared to wild-type plants, while *pad3* adult plants do not respond to *P. indica* with growth promotion and likewise have higher fungal colonization. The overcolonized *cyp79B2 cyp79B3* roots responded to the deficiency of IAOx and its derived compounds by activating other defense genes such a *PR-1*, *PDF1.2*, *PAL* and *germin* in the early phase of interaction and this defense response is restricted to seedlings and no longer visible for adult plants. Infection with pathogens strongly activates the plants' defense genes (Govrin and Levine 2002), while the symbiotic interaction of various investigated plant species with *P. indica* or mycorrhiza requires a relatively mild activation of defense responses (De Hoff *et al*. 2009; Fester and Hause 2005; Gutjahr and Paszkowski 2009; Herre *et al*. 2007; Martin *et al*. 2007; Pozo and Azcón-Aguilar 2007; Purin and Rillig 2008; Strack *et al*. 2003; Camehl and Oelmüller 2010a; Camehl *et al*. 2010b). Therefore, IAOx and its derived compounds, including camalexin, may be considered as another example for a defense process that restricts growth of fungi in beneficial root symbioses.

We observed that there is an inverse relationship between the *P. indica*-mediated growth phenotype and the degree of colonization in the roots (cf. Camehl *et al*. 2010b; Sherameti *et al*. 2008a). During symbiosis, the interacting Arabidopsis receives several benefits such as nutrient exchange and enlarged surface area of their roots, and at the same time, the plant prevents the fungus from over-colonizing the roots. The loss of benefits for the plants due to overcolonization is consistent with my observation that adult *cyp79B2 cyp79B3* plants are five times and *pad3* plants three times higher colonized than wild-type plants. This fluctuation in the degree of colonization during the different stages of interaction has also been observed for mycorrhizal fungi (Kennedy 2010). We observed that the mutants tested are highly penetrated and over-colonized and this high degree of fungal growth in the roots probably creates an imbalance in the interaction. This situation appears to be unfavorable for the mutants leading to a shift from mutualism to parasitism which results in a reduced growth rate. I measured the

degree of colonization of Arabidopsis roots by quantifying the level of expression of the *elongation factor 1*(*Pitef1*) DNA and cDNA of *P. indica*.

Expression of camalexin synthesis genes against biotrophic and necrotrophic microbes has been shown to be specifically upregulated at the site of infection (Kliebenstein *et al*. 2005; Schuhegger *et al*. 2007). I observed a similar response to *P. indica*. Interestingly, determination of *pad3::uidA* expression in a split-root-system revealed increased GUS activity in *P. indica*-treated lateral roots and root hairs. This response is strictly local as I could not observe any stimulation of GUS activity in the uninfected region as well as in mock-treated roots. It was previously believed that camalexin induction requires a lesion in the infected tissues. However, in the recent years it has been reported that phytoalexin synthesis can also be induced by autoclaved yeast suspension (Raacke *et al*. 2006) and a peptidoglycan preparation (Gust *et al*. 2007) in leaves which did not undergo cell death. We also observed that a cell wall extract from *P. indica*, that promotes growth (Vadassery *et al*. 2009a), also stimulates the expression of *PAD3*, *CYP79B2*, *CYP79B3*, *CYP71A13* and *WRKY33* mRNA in *P-indica*-exposed roots. This indicates that *P. indica*-released MAMPs can also induce a response without making physical injuries by hyphal penetration of the individual root cells. Generally, pathogens and pathogenic MAMPs can induce a rapid, long-distance, auto-propagating signal through ROS production *via* NADH oxidases (Mittler *et al*. 2011). Since *P. indica* and a *P. indica*-derived cell wall extract do not induce ROS production (Camehl *et al*. 2011; Vadassery *et al*. 2009a), the local response is probably not transmitted to neighboring cells.

### 5.2 Aliphatic glucosinolates in plant defense

The role of glucosinolates has been largely studied as insect deterrent or attractant for oviposition (Halkier and Gershenson 2006; Gabrys and Tjallingii 2002; Mewis *et al*. 2002; Miles *et al*. 2005; Rojas 1999). The involvement of aliphatic glucosinolates in insect resistance has been revealed by producing elevated levels of aliphatic glucosinolates by the over-expression of MYB28 in Arabidopsis plants; these plants are highly resistant to feeding by *Spodoptera exigua* larvae (Gigolashvili *et al*. 2007b). Furthermore, the relevance of aliphatic glucosinolates in defense responses has been demonstrated by Beekwilder *et al.*

(2008) who were the first to identify the *myb28 myb29* double mutant lacking aliphatic glucosinolates as susceptible to the generalist Lepidopteran insect *Mamestra brassicae*. Antimicrobial properties of aliphatic glucosinolates were demonstrated by Stotz *et al.* (2011) with respect to resistance to the necrotrophic fungus *Sclerotinia sclerotiorum*. Genes contributing to the aliphatic glucosinolate biosynthetic pathway as well as for the camalexin and sulfur metabolic pathway were induced in leaves following infection with *S. sclerotiorum.*

I also investigated the role of aliphatic glucosinolates in the symbiotic interaction of *P. indica* with Arabidopsis. I focused on the double knockout mutant *myb28 myb29* which is an ideal mutant candidate since it lacks aliphatic glucosinolates. The transcript level of *MYB28*, coding for the major regulator of aliphatic glucosinolate biosynthesis, is not upregulated in roots colonized by *P. indica*. Since *myb28 myb29* still responds to *P. indica*, aliphatic glucosinolates do not play an important role in the beneficial symbiosis. However, the necrotrophic fungus *A. brassicae* induces *MYB28*. The pathogen causes severe disease symptoms in infected wild-type and *myb28 myb29* roots, turning the roots into brownish color. The induction of chlorosis in shoots is higher in the *myb28 myb29* mutant compared to the wild-type. This demonstrates root-to-shoot signaling and an involvement of aliphatic glucosinolates. The protective role of *P. indica* in barley from root rot caused by *Fusarium graminearum* has been reported by Deshmukh and Kogel (2007). I investigated if *P. indica* can protect Arabidopsis seedlings against *A. brassicae*-induced infection. When the roots were pretreated with *P. indica* for three days prior to *A. brassicae* infection, we observed that brownishing of roots and chlorosis development in shoots were strongly retarded compared to the mock-treated control. Furthermore, the fresh weight of *A. brassicae*-infected seedlings pretreated with *P. indica* is higher than that without *P. indica*-pretreatment in the wild-type. Interestingly, the *myb28 myb29* mutant did not show such an increase in the fresh weight after *P. indica*-pretreatment. This demonstrates an involvement of aliphatic glucosinolates in the *P. indica*-induced resistance against *A. brassicae* infections in leaves. We therefore proposed a dual role of *P. indica* in the protection of Arabidopsis seedlings against *A. brassicae*. First, the beneficial fungus protects the roots against *A. brassicae* infection. This might simply be a competition between the two microbes in the root environment. We could also observe an

antagonistic effect in a dual culture plate experiment where *P. indica* clearly inhibits or restricts the growth of *A. brassicae*. Reduced colonization of the pathogen inside the roots improved the growth performance of the seedling. The amount of fungal material was determined by quantifying the level of expression of *AbreAtr1* of *A. brassicae*. Second, *P. indica* protects the aerial parts of the plant against *A. brassicae* infection and this response requires MYB28 and MYB29.

*P. indica* does not induce *PDF1.2* in the wild-type, but the defense gene is often upregulated in *P. indica*-colonized mutants (Camehl *et al*. 2010b). Therefore, *PDF1.2* has been shown to be a marker gene for the imbalances in Arabidopsis/*P. indica* symbiosis. *PDF1.2* is upregulated after infections with the pathogen *A. brassicae*. However, pretreatment by *P. indica* suppresses *A. brassicae*-induced expression of *PDF1.2* in wild-type roots. In contrast, there is very little effect on the *PDF1.2* expression of the pretreated *myb28 myb29* roots which indicates that the innate immune response is impaired in the double mutant. Deshmukh and Kogel (2007) have also reported that *P. indica* suppresses *F. graminearum*-induced expression of *PR* genes in barley roots. We could show that pretreatment of the roots by *P. indica* activates resistance (ISR) in the shoot against *A. brassicae* spore infection. The development of chlorotic symptoms is delayed in the *P. indica*-pretreated wild-type leaves compared to the mock-treated control leaves, while there is no or less visible protection in the *myb28 myb29* mutant. We observed a mild induction of *PDF1.2* in the leaves of both wild-type and *myb28 myb29* when their roots were colonized by *P. indica*. This suggests that the beneficial fungus is involved in root-to-shoot signal transfer. In contrast, the *PDF1.2* mRNA level in the leaves after *A. brassicae* infection is strongly upregulated in wild-type and the *myb28 myb29* mutant. We therefore propose the involvement of MYB28 and MYB29 in defense against *A. brassicae* infection in leaves. Furthermore, pretreatment by *P. indica* in the roots strongly inhibits the growth of *A. brassicae* in the leaves of wild-type when compared to *myb28 myb29* mutant.

### 5.3 Role of *P. indica* in Sulfur Metabolism in *A. thaliana*

Amongst the macroelements that are found in plants, sulfur is the least abundant. Unlike, carbon and nitrogen which are incorporated as structural component in biomolecules, sulfur

forms components that are involved in catalytic or electrochemical functions. Metabolism of sulfur is very important for living organisms because higher plants are dependent on inorganic sulfate present in soil to meet their nutritional sulfur requirement. Sulfate is a major anionic solute. Following uptake from the soil, sulfate is either deposited in the vacuole or it is assimilated into organic compounds. Plants growing under sulfur deficiency conditions activate sulfate acquisition and transport processes by upregulating the expression of sulfate transporter genes in roots and vacuoles. Simultaneous to the efficient uptake and transport strategy, there is a parallel repression of the synthesis of the S-containing glucosinolates and regulation of their breakdown to release sulfur. Breaking down of glucosinolates during sulfur deficiency helps to replenish the nutrient requirement, as 30% of sulfur is stored in glucosinolates in Brassicales (Falk *et al*. 2007).

Glucosinolates are important defense compounds against insect herbivores and pathogens (Falk *et al*. 2007). The biologically active compounds are released from the glucosinolates after enzymatic hydrolysis with myrosinase enzymes which in turn play important functions in plant/microbe interactions. Two glycosyl hydrolase family 1 members, PEN2 (Bednarek *et al*. 2009) and PYK10, a highly abundant enzyme in the roots (Sherameti *et al*. 2008a) have been reported to be involved in restricting pathogen entry in the leaves and roots, respectively. Although PYK10 has been demonstrated to be involved in maintaining beneficial interaction between Arabidopsis and *P. indica*, the high abundance of this enzyme in roots was proposed to protect Brassicaceae against soil-borne fungi. The importance of the glucosinolate metabolism for antifungal defense and innate immune response has been reported by two groups (Bednarek *et al*. 2009; Clay *et al*. 2009). The auxin pool in roots accumulates as a result of breakdown of indolic glucosinolates under certain stress conditions (Searle *et al*. 1982; Zhao *et al.* 2002). Since auxin is a plant growth hormone that stimulate root development which therefore contributes in efficient uptake of sulfate.

Maruyama-Nakashita *et al*. (2006) identified a central regulator of plant S responses and metabolism in Arabidopsis under sulfur limitation. Mutation in *SULFUR LIMITATION1* (*SLIM1*) inhibits the induction of transcripts for the high-affinity transporter *SULTR1; 2* under low sulfur conditions. The *slim1* mutant has reduced sulfate uptake and growth due to the fact that the mutant could not regulate the activation of sulfate acquisition and degradation of

glucosinolates under sulfur limitations as the functional SLIM1 protein controls both these processes.

The study of sulfur uptake, transport and metabolism has been of great interest in the recent years because these processes affect plant/microbe interactions. The ectomycorrhizal fungus *L. bicolor* increases sulfate uptake and the plant provides the fungus with reduced sulfur in return (Mansouri-Bauly *et al*. 2006). Glutathione (GSH) is a major redox buffer and protects the cell against reactive oxygen species. GSH plays a crucial role in *P. indica*-induced resistance of barley plants against pathogens (Waller *et al*. 2005; Baltruschat *et al*. 2008). It is interesting to note that the genes regulated by SLIM1 (Maruyama-Nakashita *et al*. 2006), are also regulated by *P. indica* during early phases of cocultivation. The three ATP sulfurylase genes *APS1*, *APS3* and *APS4* are targeted by the fungus during sufficient S supply, while *GSH1*, *GSH2*, *BCAT2*, *BCAT4*, *MAM3*, three *GST* genes, *phytochelatin synthase*, and *NPR1* are strongly upregulated under sulfur starvation. This again confirms that *P. indica* controls S metabolism, in particular under S-limiting conditions.

Sulfur also plays an important role in the antioxidant and radical scavenger system. Ascorbate-glutathione cycle is one such system that maintains ascorbate in its reduced state. Vadassery *et al*. (2009b) have shown that the two enzymes of the ascorbate-glutathione cycle monodehydroascorbate reductase 2 (MDAR2) and dehydroascorbate reductase 5 (DHAR5) are upregulated in the roots and shoots of Arabidopsis seedlings cocultivated with *P. indica*. This indicates that the fungus induces the flow of signal information from root to shoot. The role of MDAR2 and DHAR5 in symbiotic interaction with *P. indica* has been uncovered by the loss of growth promotion in *mdar2* and *dhar5* mutants. Moreover, the mutants are heavily colonized by the fungus and suffered severely upon exposure to drought stress. Furthermore, these two genes are upregulated in *P. indica*-colonized Arabidopsis roots. Therefore, it is likely that more proteins controlling the redox homeostasis in the cell are involved in establishing and maintaining a mutualistic interaction between the two symbionts.

## 6. Summary

*P. indica* is a symbiotic partner in the interaction with the host Arabidopsis. The host plant benefits from the interaction by increased growth and better protection against pathogens. The following two aspects were investigated.

1 - *P. indica* controls the accumulation of IAOx-derived compounds in the roots of Arabidopsis. The phytoanticipin indole glucosinolate has an essential role in the initial phase of the interaction between the two symbionts, while camalexin, a phytoalexin, is required in the later phase of the interaction. The IAOx biosynthetic genes, *CYP79B2* and *CYP79B3* as well as the camalexin biosynthesis gene *PAD3* are induced by *P. indica*. They are essential for establishing a beneficial symbiosis by restricting fungal colonization.

2 - I discovered the involvement of *MYB28* and *MYB29* in the resistance response of Arabidopsis against *A. brassicae*. The *myb28 myb29* double mutant, which lacks aliphatic glucosinolates, responds to *P. indica* with growth promotion similar to the wild-type. MYB28 and MYB29 have been shown to be important in insect herbivory resistance and resistance against nonhost pathogenic bacteria and necrotrophic fungi. I found that *P. indica* protects Arabidopsis against root infection by *A. brassicae*. Furthermore, I propose that MYB28 and MYB29 play a role in induced systemic resistance. *P. indica* activates an induced systemic resistance response against *A. brassicae* leaf infection in Arabidopsis. This response is strongly reduced in the *myb28 myb29* mutant. Hence, *P. indica*-induced systemic resistance in the leaves against *A. brassicae* infection requires MYB28/MYB29 and therefore aliphatic glucosinolates.

## 7. Zusammenfassung

*P. indica* ist ein symbiotischer Partner in der Interaktion mit dem Wirt Arabidopsis. Die Wirtspflanze profitiert von der Interaktion durch verbessertes Wachstum und besseren Schutz gegen Pathogene. Folgende zwei Aspekte wurden untersucht.

1 – *P. indica* kontrolliert die Akkumulation von IAOx-gesteuerten Verbindungen in der Wurzel von Arabidopsis. Das Phytoanticipin Indol-Glucosinolat besitzt eine wesentliche Rolle in der ersten Phase der Interaktion zwischen den beiden Symbionten, während Camalexin, ein Phytoalexin, in der späteren Phase der Interaktion erforderlich ist. Die IAOx-Biosynthesegene, *CYP79B2* und *CYP79B3*, sowie das Camalexin-Biosynthesegen *PAD3* werden durch *P. indica* induziert. Die Genprodukte verhindern eine Überkolonisierung der Wurzel und sind somit wichtig für die Etablierung einer vorteilhaften Symbiose.

2 – MYB28 und MYB29 sind an der Resistenzantwort von Arabidopsis gegen *A. brassicae* Infektion beteiligt. Die Doppelmutante *myb28 myb 29*, welcher aliphatische Glucosinolat fehlen, reagiert auf *P. indica* bezüglich der Wachstumsförderung wie der Wildtyp. MYB28 und MYB29 sind wichtig für die Resistenz gegen Insektenfrass, die Resistenz gegen nicht wirtsspezifischer pathogener Bakterien und nekrotropher Pilze. Ich fand heraus, dass *P. indica* Arabidopsis gegen Wurzelinfektion durch *A. brassicae* schützt. Weiterhin konnte ich zeigen, dass MYB28 und MYB29 eine Rolle bei der induzierten systemischen Resistenz spielen. *P. indica* aktiviert eine induzierte systemische Resistenz Reaktion gegen *A. brassicae* in Arabidopsis Blätten. Diese Antwort ist in der *myb28 myb29* Mutante stark reduziert. Folglich benötigt die *P. indica*-induzierte Systemresistenz in den Blättern gegen eine *A. brassicae* Infektion MYB28/MYB29 und demzufolge aliphatische Glucosinolat.

## 8. Literature cited

**Aguilar, O.M., Riva, O. and Peltzer, E.** (2004) Analysis of *Rhizobium etli* and of its symbiosis with wild *Phaseolus vulgaris* supports coevolution in centers of host diversification. *Proc Natl Acad Sci USA*, **101**, 13548-13553.

**Ahonen-Jonnarth, U., Göransson, A. and Finlay, R.D.** (2003) Growth and nutrient uptake of ectomycorrhizal *Pinus sylvestris* seedlings treated with elevated Al concentrations. *Tree Physiol*, **23**, 157-167.

**Amor, B.B., Shaw, S.L., Oldroyd, G.E., Maillet, F., Penmetsa, R.V., Cook, D., Long, S.R., Dénarié, J. and Gough, C.** (2003) The NFP locus of *Medicago truncatula* controls an early step of Nod factor signal transduction upstream of a rapid calcium flux and root hair deformation. *Plant J*, **34**, 495-506.

**Anthony, R.G., Khan, S., Costa, J., Pais, M.S. and Bögre, L.** (2006) The Arabidopsis protein kinase PTI1-2 is activated by convergent phosphatidic acid and oxidative stress signaling pathways downstream of PDK1 and OXI1. *J Biol Chem*, **281**, 37536-37546.

**Anthony, R.G., Henriques, R., Helfer, A., Mészáros, T., Rios, G., Testerink, C., Munnik, T., Deák, M., Koncz, C. and Bögre, L.** (2004) A protein kinase target of a PDK1 signaling pathway is involved in root hair growth in Arabidopsis. *EMBO J*, **23**, 572-581.

**Arrighi, J.F., Barre, A., Amor, B.B., Bersoult, A., Soriano, L.C., Mirabella, R., de Carvalho-Niebel, F., Journet, E-P., Ghérardi, M., Huguet, T., Geurts, R., Dénarié, J., Rougé, P. and Gough, C.** (2006) The *Medicago truncatula* lysine motif-receptor-like kinase gene family includes NFP and new nodule-expressed genes. *Plant Physiol*, **142**, 265-279.

**Bak, S., Tax, F.E., Feldmann, K.A., Galbraith, D.W. and Feyereisen, R.** (2001) CYP83B1, a cytochrome P450 at the metabolic branch point in auxin and indole glucosinolate biosynthesis in Arabidopsis. *Plant Cell*, **13**, 101-111.

**Ball, L., Accotto, G.P., Bechtold, U., Creissen, G., Funck, D., Jimenez, A., Kular, B., Leyland, N., Mejia-Carranza, J., Reynolds, H., Karpinski, S. and Mullineaux, P.M.** (2004) Evidence for a direct link between glutathione biosynthesis and stress defense gene expression in Arabidopsis. *Plant Cell*, **16**, 2448-2462.

**Baltruschat, H., Fodor, J., Harrach, B.D., Niemczyk, E., Barna, B., Gullner, G., Janeczko, A., Kogel, K.-H., Schäfer, P., Schwarczinger, I., Zuccaro, A. and Skoczowski, A.** (2008) Salt tolerance of barley induced by the root endophyte *Piriformospora indica* is associated with a strong increase in antioxidants. *New Phytol*, **180**, 501-510.

**Bednarek, P. and Osbourn, A.** (2009) Plant-microbe interactions: chemical diversity in plant defense. *Science*, **324**, 746-748.

**Bednarek, P., Pislewska-Bednarek, M., Svatos, A., Schneider, B., Doubsky, J., Mansurova, M., Humphry, M., Consonni, C., Panstruga, R., Sanchez-Vallet, A., Molina, A. and Schulze-Lefert, P.** (2009) A glucosinolate metabolism pathway in living plant cells mediates broad-spectrum antifungal defense. *Science*, **323**, 101-106.

**Beekwilder, J., van Leeuwen, W., van Dam, N.M., Bertossi, M., Grandi, V., Mizzi, L., Soloviev, M., Szabados, L., Molthoff, J.W., Schipper, B., Verbocht, H., de Vos, R.C., Morandini, P., Aarts, M.G. and Bovy, A.** (2008) The impact of the absence of aliphatic glucosinolates on insect herbivory in Arabidopsis. *PLoS One*, **3**, e2068.

**Benhamou, N., Rey, P., Chérif, M., Hockenhull, J. and Tirilly, Y.** (1997) Treatment with the mycoparasite *Pythium oligandrum* triggers induction of defense-related reactions in tomato roots when challenged with *Fusarium oxysporum* f. sp. *radicis-lycopersici*. *Phytopathol*, **87**, 108-122.

**Benhamou, N., Kloepper, J.W., Quadt-Hallman, A. and Tuzun, S.** (1996) Induction of defense-related ultrastructural modifications in pea root tissues inoculated with endophytic bacteria. *Plant Physiol*, **112**, 919-929.

**Blake-Kalff, M.M.A., Harrison, K.R., Hawkesford, M.J., Zhao, F.J. and McGrath, S.P.** (1998) Distribution of sulfur within oilseed rape leaves in response to sulfur deficiency during vegetative growth. *Plant Physiol*, **118**, 1337-1344.

**Blilou, I., Ocampo, J.A. and Garcia-Garrido, J.M.** (2000) Induction of Ltp (Lipid transfer protein) and Pal (Phenylalanine ammonia-lyase) gene expression in rice roots colonized by the arbuscular mycorrhizal fungus *Glomus mosseae*. *J Exp Bot*, **51**, 1969-1977.

**Bolton, M.D., Thomma, B.P.H.J. and Nelson, B.D.** (2006) *Sclerotinia sclerotiorum* (Lib.) de Bary: biology and molecular traits of a cosmopolitan pathogen. *Mol Plant Pathol*, **7**, 1-16.

**Böttcher, C., Westphal, L., Schmotz, C., Prade, E., Scheel, D. and Glawischnig, E.** (2009) The multifunctional enzyme CYP71B15 (PHYTOALEXIN DEFICIENT3) converts cysteine-indole-3-acetonitrile to camalexin in the indole-3-acetonitrile metabolic network of *Arabidopsis thaliana*. *Plant Cell*, **21**, 1830-1845.

**Brader, G., Tas, E. and Palva, E.T.** (2001) Jasmonate-dependent induction of indole glucosinolates in Arabidopsis by culture filtrates of the nonspecific pathogen *Erwinia carotovora*. *Plant Physiol*, **126**, 849-860.

**Brewin, N.J.** (2004) Plant cell wall remodelling in the Rhizobium-legume symbiosis. *Crit Rev Plant Sci*, **23**, 293-316.

**Camehl, I. and Oelmüller, R.** (2010a) Do ethylene response factors 9 and 14 repress PR gene expression in the interaction between *Piriformospora indica* and Arabidopsis? *Plant Signal Behav*, **9**, 5-8.

**Camehl, I., Sherameti, I., Venus, Y., Bethke, G., Varma, A., Lee, J. and Oelmüller, R.** (2010b) Ethylene signaling and ethylene-targeted transcription factors are required to balance beneficial and nonbeneficial traits in the symbiosis between the endophytic fungus *Piriformospora indica* and *Arabidopsis thaliana. New Phytol*, **185**, 1062-1073.

**Camehl, I., Drzewiecki, C., Vadassery, J., Shahollari, B., Sherameti, I., Forzani, C., Munnik, T., Hirt, H. and Oelmüller, R.** (2011) The OXI1 kinase pathway mediates *Piriformospora indica*-induced growth promotion in Arabidopsis. *PLoS Pathogens* **7**, e1002051.

**Caracuel-Rios, Z. and Talbot, N.** (2007) Cellular differentiation and host invasion by the rice blast fungus *Magnaporthe grisea*. *Curr Opin Microbiol*, **10**, 339-345.

**Cebolla, A., Vinardell, J.M., Kiss, E., Olah, B., Roudier, F., Kondorosi, A. and Kondorosi, E.** (1999) The mitotic inhibitor ccs52 is required for endoreduplication and ploidy-dependent cell enlargement in plants. *EMBO J*, **18**, 4476-4484.

**Celenza, J.L., Quiel, J.A., Smolen, G.A., Merrikh, H., Silvestro, A.R., Normanly, J. and Bender, J.** (2005) The Arabidopsis ATR1 Myb transcription factor controls indolic glucosinolate homeostasis. *Plant Physiol*, **137**, 253-262.

**Chao, Q., Rothenberg, M., Solano, R., Roman, G., Terzaghi, W. and Ecker, J.R.** (1997) Activation of the ethylene gas response pathway in Arabidopsis by the nuclear protein ETHYLENE-INSENSITIVE3 and related proteins. *Cell*, **89**, 1133-1144.

**Cho, D., Kim, S.A., Murata, Y., Lee, S., Jae, S.K., Nam, H.G. and Kwak, J.M.** (2009) Deregulated expression of the plant glutamate receptor homolog AtGLR3.1 impairs long-term $Ca^{2+}$-programmed stomatal closure. *Plant J*, **58**, 437-449.

**Clarkson, D.T., Smith, F.W. and Van den Berg, P.J.** (1983) Regulation of sulfate transport in a tropical legume, *Macroptilium atropurpureum* cv. Siratro. *J Exp Bot*, **34**, 1463-1483.

**Clay, N.K., Adio, A.M., Denoux, C., Jander, G. and Ausubel, F.M.** (2009) Glucosinolate metabolites required for an Arabidopsis innate immune response. *Science*, **323**, 95-101.

**Cobbett, C. and Goldsbrough, P.** (2002) Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis. *Annu Rev Plant Biol*, **53**, 159-182.

**Collinge, D.B., Gregersen, P.L. and Thordal-Christensen, H.** (1994) The induction of gene expression in response to pathogenic microbes. Pages 391-433 *in: Mechanisms of Plant Growth and Improved Productivity: Modern Approaches and Perspectives*. A. S. Basra, ed. Marcel Dekker, *New York*.

**Conrath, U., Beckers, G.J.M., Flors, V., Garcia-Agustin, P., Jakab, G., Mauch, F., Newman, M.A., Pieterse, C.M.J., Poinssot, B., Pozo, M.J., Pugin, A., Schaffrath, U., Ton, J., Wendehenne, D.,**

**Zimmerli, L. and Mauch-Mani, B.** (2006) Priming: getting ready for battle. *Mol Plant Microbe Interact*, **19**, 1062-1071.

**Cordier, C., Pozo, M.J., Barea, J.M., Gianinazzi, S. and Gianinazzi-Pearson, V.** (1998) Cell defense responses associated with localized and systemic resistance to *Phytophthora parasitica* induced in tomato by an arbuscular mycorrhizal fungus. *Mol Plant Microbe Interact*, **11**, 1017-1028.

**Crawford, N.M.** (1995) Nitrate: nutrient and signal for plant growth. *Plant Cell*, **7**, 859-868.

**Cruz-Ramírez, A., Oropeza-Aburto, A., Razo-Hernández, F., Ramírez-Chávez, E. and Herrera-Estrella, L.** (2006) Phospholipase DZ2 plays an important role in extraplastidic galactolipid biosynthesis and phosphate recycling in Arabidopsis roots. *Proc Natl Acad Sci USA*, **103**, 6765-6770.

**David, R., Itzhaki, H., Ginzberg, I., Gafni, Y., Galili, G. and Kapulnik, Y.** (1998) Suppression of tobacco basic chitinase gene expression in response to colonization by the arbuscular mycorrhizal fungus *Glomus intraradices*. *Mol Plant Microbe Interact*, **11**, 489-497.

**de Bary, A.** (1887). Comparative morphology and biology of the fungi, Mycetozoa and Bacteria. English Translation. *Oxford: Clarendon press, London & New York*.

**De Hoff, P.L., Brill, L.M. and Hirsch, A.M.** (2009) Plant lectins: the ties that bind in root symbiosis and plant defence. *Mol Genet Genomics*, **282**, 1-15.

**de Ruijter, N.C.A., Rook, M.B., Bisseling, T. and Emons, A.M.C.** (1998) Lipochito-oligosaccharides re-initiate root hair tip growth in *Vicia sativa* with high calcium and spectrin-like antigen at the tip. *Plant J*, **13**, 341-350.

**Deak, M., Casamayor, A., Currie, R.A., Downes, C.P. and Alessi, D.R.** (1999) Characterisation of a plant 3-phosphoinositide-dependent protein kinase-1 homologue which contains a pleckstrin homology domain. *FEBS Lett,* **451**, 220-226.

**Deane-Drummond, C.E.** (1987) The regulation of sulfate uptake following growth of *Pisum sativum* L. seedlings in S nutrient limiting conditions. Interaction between nitrate and sulphate transport. *Plant Sci*, **50**, 27-35.

**Dehpour, A.A., Alavi, S.V. and Majd, A.** (2007) Light and scanning electron microscopy studies on the penetration and infection processes of *Alternaria alternata*, causing brown spot on *Minneola tangelo* in the West Mazandaran—Iran. *World App Sci J*, **2**, 68-72.

**Deshmukh, S.D. and Kogel, K.-H.** (2007) *Piriformospora indica* protects barley from root rot caused by *Fusarium graminearum*. *J Plant Dis Protect*, **114**, 263-268.

**Deshmukh, S., Hückelhoven, R., Schäfer, P., Imani, J., Sharma, M., Weiss, M., Waller, F. and Kogel, K.-H.** (2006) The root endophytic fungus *Piriformospora indica* requires host cell death for proliferation during mutualistic symbiosis with barley. *Proc Natl Acad Sci USA*, **103**, 18450-18457.

**Ehrhardt, D.W., Wais, R. and Long, S.R.** (1996) Calcium spiking in plant root hairs responding to Rhizobium nodulation signals. *Cell*, **85**, 673-681.

**Fahey, J.W., Zalcmann, A.T. and Talalay, P.** (2001) The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. *Phytochem*, **56**, 5-51.

**Falk, K.L., Tokuhisa, J.G. and Gershenzon, J.** (2007) The effect of sulfur nutrition on plant glucosinolate content: physiology and molecular mechanisms. *Plant Biol*, **9**, 573-581.

**Fan, L., Zheng, S. and Wang, X.** (1997) Antisense suppression of phospholipase Dα retards abscisic acid- and ethylene-promoted senescence of post-harvest Arabidopsis leaves. *Plant Cell*, **9**, 2183-2196.

**Ferrari, S., Plotnikova, J.M., De Lorenzo, G. and Ausubel, F.M.** (2003) Arabidopsis local resistance to *Botrytis cinerea* involves salicylic acid and camalexin and requires EDS4 and PAD2, but not SID2, EDS5 or PAD4. *Plant J*, **35**, 193-205.

**Fester, T. and Hause, G.** (2005) Accumulation of reactive oxygen species in arbuscular mycorrhizal roots. *Mycorrhiza*, **15**, 373-379.

**Frei dit Frey, N., Mbengue, M., Kwaaitaal, M., Nitsch, L., Altenbach, D., Häweker, H., Lozano-Duran, R., Njo, M.F., Beeckman, T., Huettel, B., Borst, J.W., Panstruga, R. and Robatzek, S.** (2012) Plasma membrane calcium ATPases are important components of receptor-mediated signaling in plant immune responses and development. *Plant Physiol*, **159**, 798-809.

**Fujimoto, S.Y., Ohta, M., Usui, A., Shinshi, H. and Ohme-Takagi, M.** (2000) Arabidopsis ethylene-responsive element binding factors act as transcriptional activators or repressors of GCC box-mediated gene expression. *Plant Cell*, **12**, 393-404.

**Gabrys, B. and Tjallingii, W.F.** (2002) The role of sinigrin in host plant recognition by aphids during initial plant penetration. *Entomol Exp Appl*, **104**, 89-93.

**Gapper, C. and Dolan, L.** (2006) Control of plant development by reactive oxygen species. *Plant Physiol*, **141**, 341-345.

**García-Olmedo, F., Molina, A., Segura, A. and Moreno, M.** (1995) The defensive role of nonspecific lipid-transfer proteins in plant. *Trends Microbiol*, **3**, 72-74.

**Giamoustaris, A. and Mithen, R.** (1995) The effect of modifying the glucosinolate content of leaves of oilseed rape (*Brassica napus* ssp. *oleifera*) on its interaction with specialist and generalist pests. *Annal Appl Biol*, **126**, 347-353.

**Gianinazzi-Pearson, V., Dumas-Gaudot, E., Gollotte, A., Tahiri-Alaoui, A. and Gianinazzi, S.** (1996) Cellular and molecular defence-related root responses to invasion by arbuscular mycorrhizal fungi. *New Phytol*, **133**, 45-57.

**Gigolashvili, T., Berger, B. and Flügge, U.-I.** (2009) Specific and coordinated control of indolic and aliphatic glucosinolate biosynthesis by R2R3-MYB transcription factors in *Arabidopsis thaliana*. *Phytochem Rev*, **8**, 3-13.

**Gigolashvili, T., Engqvist, M., Yatusevich, R., Müller, C. and Flügge, U.-I.** (2008) HAG2/MYB76 and HAG3/MYB29 exert a specific and coordinated control on the regulation of aliphatic glucosinolate biosynthesis in *Arabidopsis thaliana. New Phytol*, **177**, 627-642.

**Gigolashvili, T., Berger, B., Mock, H.P., Muller, C. and Weisshaar, B.** (2007a) The transcription factor HIG1/MYB51 regulates indolic glucosinolate biosynthesis in *Arabidopsis thaliana*. *Plant J*, **50**, 886-901.

**Gigolashvili, T., Yatusevich, R., Berger, B., Müller, C. and Flügge, U.-I.** (2007b). The R2R3-MYB transcription factor HAG1/MYB28 is a regulator of methionine-derived glucosinolate biosynthesis in *Arabidopsis thaliana*. *Plant J*, **51**, 247-261.

**Glawischnig, E., Hansen, B.G., Olsen, C.E. and Halkier, B.A.** (2004) Camalexin is synthesized from indole-3-acetaldoxime, a key branching point between primary and secondary metabolism in Arabidopsis. *Proc Natl Acad Sci USA*, **101**, 8245-8250.

**Glazebrook, J.** (2005) Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annu Rev Phytopathol*, **43**, 205-227.

**Gourgues, M., Brunet-Simon, A., Lebrun, M-H. and Levis, C.** (2004) The tetraspanin BcPls1 is required for appressorium-mediated penetration of *Botrytis cinerea* into host plant leaves. *Mol Microbiol*, **51**, 619-629.

**Govrin, E.M. and Levine, A.** (2002) Infection of Arabidopsis with a necrotrophic pathogen, *Botrytis cinerea*, elicits various defense responses but does not induce systemic acquired resistance (SAR). *Plant Mol Biol*, **48**, 267-276.

**Grubb, C. and Abel, S.** (2006) Glucosinolate metabolism and its control. *Trends Plant Sci*, **11**, 89-100.

**Guo, H. and Ecker, J.R.** (2003) Plant responses to ethylene gas are mediated by $SCF^{EBF1/EBF2}$-dependent proteolysis of EIN3 transcription factor. *Cell*, **115**, 667-677.

**Guo, X. and Stotz, H.U.** (2010) ABA signaling inhibits oxalate-induced production of reactive oxygen species and protects against *Sclerotinia sclerotiorum* in *Arabidopsis thaliana*. *Eur J Plant Pathol*, **128**, 7-19.

**Gust, A.A., Biswas, R., Lenz, H.D., Rauhut, T., Ranf, S., Kemmerling, B., Götz, F., Glawischnig, E., Lee, J., Felix, G. and Nürnberger, T.** (2007) Bacteria-derived peptidoglycans constitute pathogen-associated molecular patterns triggering innate immunity in Arabidopsis. *J Biol Chem*, **282**, 32338-32348.

**Gutierrez-Marcos, J.F., Roberts, M.A., Campbell, E.I. and Wray, J.L.** (1996) Three members of a novel small gene-family from *Arabidopsis thaliana* able to complement functionally an *Escherichia coli* mutant defective in PAPS reductase activity encode proteins with a thioredoxin-like domain and 'APS reductase' activity. *Proc Natl Acad Sci USA*, **93**, 13377-13382.

**Gutjahr, C. and Paszkowski, U.** (2009) Weights in the balance: jasmonic acid and salicylic acid signaling in root-biotroph interactions. *Mol Plant Microbe Interact*, **22**, 763-772.

**Hahn, M.G., Bucheli, P., Cervone, F., Doares, S.H., O'Neill, R.A., Darvill, A. and Albersheim, P.** (1989) Roles of cell wall constituents in plant-pathogen interactions. Pages 131-181 *in: Plant-Microbe Interactions: Molecular and Genetic Perspectives, 3*. T. Kosuge and E. W. Nester, eds. *Macmillan, New York*.

**Halkier, B.A. and Gershenzon, J.** (2006) Biology and biochemistry of glucosinolates. *Annu Rev Plant Biol*, **57**, 303-333.

**Hammerschmidt, R.** (1999) Phytoalexins: what have we learned after 60 years? *Annu Rev Phytopathol*, **37**, 285-306.

**Hammond-Kosack, K.E. and Jones, J.D.G.** (1996) Resistance gene-dependent plant defense responses. *Plant Cell*, **8**, 1773-1791.

**Hamiduzzaman, M.M., Jakab, G., Barnavon, L., Neuhaus, J.M. and Mauch-Mani, B.** (2005) β-Aminobutyric acid-induced resistance against downy mildew in grapevine acts through the potentiation of callose formation and jasmonic acid signaling. *Mol Plant Microbe Interact*, **18**, 819-829.

**Harrison, M.J.** (2005) Signaling in the arbuscular mycorrhizal symbiosis. *Annu Rev Microbiol*, **59**, 19-42.

**Harrison, M.J. and Dixon, R.A.** (1993) Isoflavonoid accumulation and expression of defense gene transcripts during the establishment of vesicular arbuscular mycorrhizal associations in roots of *Medicago truncatula*. *Mol Plant Microbe Interact*, **6**, 643-659.

**Harrison, M.J. (1999)** Molecular and cellular aspects of the arbuscular mycorrhizal symbiosis. *Annu Rev Plant Physiol Plant Mol Biol*, **50**, 361-389.

**Hartmann, T.** (2007) From waste products to ecochemicals: Fifty years research of plant secondary metabolism. *Phytochem*, **68**, 2831-2846.

**Hartmann, T.** (2008) The lost origin of chemical ecology in the late 19th century. *Proc Natl Acad Sci USA*, **105**, 4541-4546.

**Heath, K.D.** (2010) Intergenomic epistasis and coevolutionary constraint in plants and Rhizobia. *Evolution*, **64**, 1446-1458.

**Heath, K.D., Burke, P.V. and Stinchcombe, J.R.** (2012) Coevolutionary genetic variation in the legume-Rhizobium transcriptome. *Mol Ecol*, **21**, 4735-4747.

**Herre, E.A., Mejía, L.C., Kyllo, D.A., Rojas, E., Maynard, Z., Butler, A. and Van Bael, S.A.** (2007) Ecological implications of anti-pathogen effects of tropical fungal endophytes and Mycorrhizae. *Ecology*, **88**, 550-558.

**Hildebrandt, U., Ouziad, F., Marner, F.J. and Bothe, H.** (2006) The bacterium *Paenibacillus validus* stimulates growth of the Arbuscular Mycorrhizal fungus *Glomus intraradices* up to the formation of fertile spores. *FEMS Microbiol Lett*, **254**, 258-267.

**Hilpert, B., Bohlmann, H., op den Camp, R., Przybyla, D., Miersch, O., Buchala, A. and Apel, K.** (2001) Isolation and characterization of signal transduction mutants of *Arabidopsis thaliana* that constitutively activate the octadecanoid pathway and form necrotic microlesions. *Plant J*, **26**, 435-446.

**Hirai, M.Y., Sugiyama, K., Sawada, Y., Tohge, T. and Obayashi, T.** (2007) Omics-based identification of Arabidopsis Myb transcription factors regulating aliphatic glucosinolate biosynthesis. *Proc Natl Acad Sci USA*, **104**, 6478-6483.

**Hirai, M.Y., Klein, M., Fujikawa, Y., Yano, M., Goodenowe, D.B., Yamazaki, Y., Kanaya, S., Nakamura, Y., Kitayama, M., Suzuki, H., Sakurai, N., Shibata, D., Tokuhisa, J., Reichelt, M., Gershenzon, J., Papenbrock, J. and Saito, K.** (2005) Elucidation of gene-to-gene and metabolite-to-gene networks in Arabidopsis by integration of metabolomics and transcriptomics. *J Biol Chem*, **280**, 25590-25595.

**Hirai, M.Y., Yano, M., Goodenowe, D.B., Kanaya, S., Kimura, T., Awazuhara, M., Arita, M., Fujiwara, T. and Saito, K.** (2004) Integration of transcriptomics and metabolomics for understanding of global responses to nutritional stresses in *Arabidopsis thaliana*. *Proc Natl Acad Sci USA*, **101**, 10205-10210.

**Hirai, M.Y., Fujiwara, T., Awazuhara, M., Kimura, T., Noji, M. and Saito, K.** (2003) Global expression profiling of sulfur-starved Arabidopsis by DNA macroarray reveals the role of *O*-acetyl-L-serine as a general regulator of gene expression in response to sulfur nutrition. *Plant J*, **33**, 651-663.

**Hirel, B., Le Gouis, J., Ney, B. and Gallais, A.** (2007) The challenge of improving nitrogen use efficiency in crop plants: towards a more central role for genetic variability and quantitative genetics within integrated approaches. *J Exp Bot*, **58**, 2369-2387.

**Hirt, H., Garcia, A.V. and Oelmüller, R.** (2011) AGC kinases in plant development and defense. *Plant Signal Behav*, **6**, 1030-1033.

**Ikeda, S., Rallos, L.E.E., Okubo, T., Eda, S., Inaba, S., Mitsui, H. and Minamisawa, K.** (2008) Microbial community analysis of field-grown soybeans with different nodulation phenotypes. *Appl Environ Microbiol*, **74**, 5704-5709.

**Jacobs, S., Zechmann, B., Molitor, A., Trujillo, M., Petutschnig, E., Lipka, V., Kogel, K.-H. and Schäfer, P.** (2011) Broad-spectrum suppression of innate immunity is required for colonization of Arabidopsis roots by the fungus *Piriformospora indica*. *Plant Physiol*, **156**, 726-740.

**Janowitz, T., Trompetter, I. and Piotrowski, M.** (2009) Evolution of nitrilases in glucosinolate-containing plants. *Phytochem*, **70**, 1680-1686.

**Kaku, H., Nishizawa, Y., Ishii-Minami, N., Akimoto-Tomiyama, C., Dohmae, N., Takio, K., Minami, E. and Shibuya, N.** (2006) Plant cells recognize chitin fragments for defense signaling through a plasma membrane receptor. *Proc Natl Acad Sci USA*, **103**, 11086-11091.

**Kapulnik, Y., Volpin, H., Itzhaki, H., Ganon, D., Galili, S., David, R., Shaul, O., Elad, Y., Chet, I. and Okon, Y.** (1996) Suppression of defence responses in mycorrhizal alfalfa and tobacco roots. *New Phytol*, **133**, 59-64.

**Kataoka, T., Hayashi, N., Yamaya, T. and Takahashi, H.** (2004) Root-to-shoot transport of sulfate in Arabidopsis: Evidence for the role of SULTR3;5 as a component of low-affinity sulfate transport system in the root vasculature. *Plant Physiol*, **136**, 4198-4204.

**Kennedy, P.** (2010) Ectomycorrhizal fungi and interspecific competition: species interactions, community structure, coexistence mechanisms, and future research directions. *New Phytol*, **187**, 895-910.

**Kikot, G.E., Hours, R.A. and Alconada, T.M.** (2008) Contribution of cell wall degrading enzymes to pathogenesis of *Fusarium graminearum*: a review. *J Basic Microbiol*, **48**, 1-11.

**Kim, K.S., Min, J.Y. and Dickman, M.B.** (2008) Oxalic acid is an elicitor of plant programmed cell death during *Sclerotinia sclerotiorum* disease development. *Mol Plant Microbe Interact*, **21**, 605-612.

**Kliebenstein, D.J., Rowe, H.C. and Denby, K.J.** (2005) Secondary metabolites influence Arabidopsis/Botrytis interactions: variation in host production and pathogen sensitivity. *Plant J*, **44**, 25-36.

**Koprivova, A., North, K.A. and Kopriva, S.** (2008) Complex signaling network in regulation of adenosine 5'-phosphosulfate reductase by salt stress in Arabidopsis roots. *Plant Physiol*, **146**, 1408-1420.

**Kutz, A., Müller, A., Hennig, P., Kaiser, W.M., Piotrowski, M. and Weiler, E.W.** (2002) A role for nitrilase 3 in the regulation of root morphology in sulphur-starving *Arabidopsis thaliana*. *Plant J*, **30**, 95-106.

**Kwaaitaal, M., Huisman, R., Maintz, J., Reinstädler, A. and Panstruga, R.** (2011) Ionotropic glutamate receptor (iGluR)-like channels mediate MAMP-induced calcium influx in *Arabidopsis thaliana*. *Biochem J*, **440**, 355-365.

**Lambais, M.R. and Mehdy, M.C.** (1993) Suppression of endochitinase, β-1,3-endoglucanase, and chalcone isomerase expression in bean vesicular-arbuscular mycorrhizal roots under different soil phosphate conditions. *Mol Plant Microbe Interact*, **6**, 75-83.

**Lambers, H., Mougel, C., Jaillard, B. and Hinsinger, P.** (2009) Plant-microbe-soil interactions in the rhizosphere: an evolutionary perspective. *Plant Soil*, **321**, 83-115.

**Lambrix, V., Reichelt, M., Mitchell-Olds, T., Kliebenstein, D.J. and Gershenzon, J.** (2001) The Arabidopsis epithiospecifier protein promotes the hydrolysis of glucosinolates to nitriles and influences *Trichoplusia ni* herbivory. *Plant Cell*, **13**, 2793-2807.

**Lahrmann, U. and Zuccaro, A.** (2012) Opprimo ergo sum-evasion and suppression in the root endophytic fungus *Piriformospora indica. Mol Plant Microbe Interact*, **25**, 727-737.

**Larose, G., Chenevert, R., Moutoglis, P., Gagne, S., Piche, Y. and Vierheilig, H.** (2002) Flavonoid levels in roots of *Medicago sativa* are modulated by the developmental stage of the symbiosis and the root colonizing arbuscular mycorrhizal fungus. *J Plant Physiol*, **159**, 1329-1339.

**Lerouge, P., Roche, P., Faucher, C., Maillet, F., Truchet, G., Prome, J.C. and Denarie, J.** (1990) Symbiotic host-specificity of *Rhizobium meliloti* is determined by a sulphated and acylated glucosamine oligosaccharide signal. *Nature*, **344**, 781-784.

**Leustek, T., Martin, M.N., Bick, J.-A. and Davies, J.P.** (2000) Pathways and regulation of sulfur metabolism revealed through molecular and genetic studies. *Annu Rev Plant Physiol Plant Mol Biol*, **51**, 141-165.

**Levy, M., Wang, Q.M., Kaspi, R., Parrella, M.P. and Abel, S.** (2005) Arabidopsis IQD1, a novel calmodulin-binding nuclear protein, stimulates glucosinolate accumulation and plant defense. *Plant J*, **43**, 79-96.

**Li, M., Hong, Y. and Wang, X.** (2009) Phospholipase D- and phosphatidic acid-mediated signaling in plants. *Biochim et Biophysic Acta*, **1791**, 927-935.

**Li, M., Welti, R. and Wang, X.** (2006a) Quantitative profiling of Arabidopsis polar glycerolipids in response to phosphorus starvation. Roles of phospholipases Dζ1 and ζ2 in phosphatidylcholine hydrolysis and digalactosyldiacylglycerol accumulation in phosphorus-starved plants. *Plant Physiol*, **142**, 750-761.

**Li, M., Qin, C., Welti, R. and Wang, X.** (2006b) Double knockouts of phospholipases Dζ1 and Dζ2 in Arabidopsis affect root elongation during phosphate-limited growth but do not affect root hair patterning. *Plant Physiol*, **140**, 761-770.

**Li, H.Y., Yang, G.D., Shu, H.R., Yang, Y.T., Ye, B.X., Nishida, I. and Zheng, C.C.** (2006c) Colonization by the arbuscular mycorrhizal fungus *Glomus versiforme* induces a defense response against the root-knot nematode *Meloidogyne incognita* in the grapevine (*Vitis amurensis* Rupr.), which includes transcriptional activation of the class III chitinase gene VCH3. *Plant Cell Physiol*, **47**, 154-163.

**Libault, M., Wan, J., Czechowski, T., Udvardi, M. and Stacey, G.** (2007) Identification of 118 Arabidopsis transcription factor and 30 ubiquitin-ligase genes responding to chitin, a plant-defense elicitor. *Mol Plant Microbe Interact*, **20**, 900-911.

**Lie, T.A., Goktan, D., Engin, M., Pijnenborg, J. and Anlarsal, E.** (1987) Co-evolution of the legume-Rhizobium association. *Plant Soil*, **100**, 171-181.

**Liu, J., Macarisin, D., Wisniewski, M., Sui, Y., Droby, S., Norelli, J. and Hershkovitz, V.** (2012) Production of hydrogen peroxide and expression of ROS-generating genes in peach flower petals in response to host and non-host fungal pathogens. *Plant Pathol*, Doi: 10.1111/j.1365-3059.2012.02683.x

**Lopez-Gomez, M., Sandal, N., Stougaard, J. and Boller, T.** (2012) Interplay of flg22-induced defence responses and nodulation in *Lotus japonicus*. *J Exp Bot*, **63**, 393-401.

**Manici, L.M., Lazzeri, L. and Palmieri, S.** (1997) *In vitro* fungitoxic activity of some glucosinolates and their enzyme-derived products toward plant pathogenic fungi. *J Agri Food Chem*, **45**, 2768-2773.

**Mansouri-Bauly, H., Kruse, J., Sýkorová, Z., Scheerer, U. and Kopriva, S.** (2006) Sulfur uptake in the ectomycorrhizal fungus *Laccaria bicolor* S238N. *Mycorrhiza*, **16**, 421-427.

**Mari, M., Leoni, O., Iori, R. and Cembali, T.** (2002) Antifungal vapour-phase activity of allyl-isothiocyanate against *Penicillium expansum* on pears. *Plant Pathol*, **51**, 231-236.

**Martin, F., Kohler, A. and Duplessis, S.** (2007) Living in harmony in the wood underground: ectomycorrhizal genomics. *Curr Opin Plant Biol*, **10**, 204-210.

**Maruyama-Nakashita, A., Nakamura, Y., Tohge, T., Saito, K. and Takahashi, H.** (2006) Arabidopsis SLIM1 is a central transcriptional regulator of plant sulfur response and metabolism. *Plant Cell*, **18**, 3235-3251.

**Maruyama-Nakashita, A., Inoue, E., Watanabe-Takahashi, A., Yamaya, T. and Takahashi, H.** (2003) Transcriptome profiling of sulfur-responsive genes in Arabidopsis reveals global effect on sulfur nutrition on multiple metabolic pathways. *Plant Physiol*, **132**, 597-605.

**McCloskey, C. and Isman, M.B.** (1993) Influence of foliar glucosinolates in oilseed rape and mustard on feeding and growth of the Bertha Armyworm, *Mamestra configurata* Walker. *J Chem Ecol*, **19**, 249-266.

**Mendgen, K., Hahn, M. and Deising, H.** (1996) Morphogenesis and mechanisms of penetration by plant pathogenic fungi. *Annu Rev Phytopathol*, **34**, 367-386.

**Mewis, I.Z., Ulrich, C. and Schnitzler, W.H.** (2002) The role of glucosinolates and their hydrolysis products in oviposition and host-plant finding by cabbage webworm, *Hellula undalis*. *Entomol Exp Appl*, **105**, 129-139.

**Michard, E., Lima, P.T., Borges, F., Silva, A.C., Portes, M.T., Carvalho, J.E., Gilliham, M., Liu, L.H., Obermeyer, G. and Feijó, J.A.** (2011) Glutamate receptor-like genes form $Ca^{2+}$ channels in pollen tubes and are regulated by pistil D-serine. *Science*, **332**, 434-437.

**Mikkelsen, M.D., Naur, P. and Halkier, B.A.** (2004) Arabidopsis mutants in the *C-S* lyase of glucosinolate biosynthesis establish a critical role for indole-3-acetaldoxime in auxin homeostasis. *Plant J*, **37**, 770-777.

**Mikkelsen, M.D., Petersen, B.L., Glawischnig, E., Jensen, A.B., Andreasson, E. and Halkier, B.A.** (2003) Modulation of *CYP79* genes and glucosinolate profiles in Arabidopsis by defense signaling pathways. *Plant Physiol*, **131**, 298-308.

**Miles, C.I., del Campo, M.L. and Renwick, J.A.A.** (2005) Behavioral and chemosensory responses to a host recognition cue by larvae of *Pieris rapae*. *J Comp Phys A*, **191**, 147-155.

**Mithen, R.F., Lewis, B.G. and Fenwick, G.R.** (1986) *In vitro* activity of glucosinolates and their products against *Leptosphaeria maculans*. *Trans Br Mycol Soc*, **87**, 433-440.

**Mittler, R., Vanderauwera, S., Suzuki, N., Miller, G., Tognetti, V.B., Vandepoele, K., Gollery, M., Shulaev, V. and Van Breusegem, F.** (2011) ROS signaling: the new wave? *Trends Plant Sci*, **16**, 300-309.

**Miya, A., Albert, P., Shinya, T., Desaki, Y., Ichimura, K., Shirasu, K., Narusaka, Y., Kawakami, N., Kaku, H. and Shibuya, N.** (2007) CERK1, a LysM receptor kinase, is essential for chitin elicitor signaling in Arabidopsis. *Proc Natl Acad Sci USA*, **104**, 19613-19618.

**Molina, A. and García-Olmedo, F.** (1993) Developmental and pathogen-induced expression of three barley genes encoding lipid-transfer protein. *Plant J*, **4**, 983-991.

**Morandi, D.** (1996) Occurence of phytoalexins and phenolic compounds on endomycorrhizal interactions and their potential role in biological control. *Plant Soil*, **185**, 241-251.

**Müller, R., de Vos, M., Sun, J.Y., Sønderby, I.E., Halkier, B.A., Wittstock, U. and Jander, G.** (2010) Differential effects of indole and aliphatic glucosinolates on lepidopteran herbivores. *J Chem Ecol*, **36**, 905-913.

**Nafisi, M., Goregaoker, S., Botanga, C.J., Glawischnig, E., Olsen, C.E., Halkier, B.A. and Glazebrook, J.** (2007) Arabidopsis cytochrome P450 monooxygenase 71A13 catalyzes the conversion of indole-3-acetaldoxime in camalexin synthesis. *Plant Cell*, **19**, 2039-2052.

**Nesher, I., Barhoom, S. and Sharon, A.** (2008) Cell cycle and cell death are not necessary for appressorium formation and plant infection in the fungal plant pathogen *Colletotrichum gloeosporioides*. *BMC Biol*, **6:**9. doi:10.1186/1741-7007-6-9.

**Newman, E.L. and Reddel, P.** (1987) The distribution of mycorrhizas among families of vascular plants. *New Phytol*, **106**, 745-751.

**Nibbe, M., Hilpert, B., Wasternack, C., Miersch, O. and Apel, K.** (2002) Cell death and salicylate- and jasmonate-dependent stress responses in Arabidopsis are controlled by single *cet* genes. *Planta*, **216**, 120-128.

**Nikiforova, V., Freitag, J., Kempa, S., Adamik, M., Hesse, H. and Hoefgen, R.** (2003) Transcriptome analysis of sulfur depletion in *Arabidopsis thaliana*: interlacing of biosynthetic pathways provides response specificity. *Plant J*, **33**, 633-650.

**Nongbri, P.L., Johnson, J.M., Sherameti, I., Glawischnig, E., Halkier, B.A. and Oelmüller, R.** (2012) Indole-3-acetaldoxime-derived compounds restrict root colonization in the beneficial interaction between Arabidopsis roots and the endophyte *Piriformospora indica*. *Mol Plant Microbe Interact*, **25**, 1186-1197.

**Nongbri, P.L. and Oelmüller, R.** (accepted) Role of *Piriformospora indica* in Sulfur Metabolism in *Arabidopsis thaliana*. *In*: Varma, A., Kost, G. and Oelmüller, R. (eds) *Piriformospora indica: Sebacinales and their biotechnological application. Springer, Heidelberg, Vol. 33*

**Nongbri, P.L., Vahabi, K., Mrozinska, A., Seebald, E., Sun, C., Sherameti, I., Johnson, J.M. and Oelmüller, R.** (accepted) Balancing defense and growth-Analyses of the beneficial symbiosis between *Piriformospora indica* and *Arabidopsis thaliana*. *Symbiosis.*

**Nongbri, P.L., Johnson, J.M., Halkier, B.A. and Oelmüller, R.** (in prep.) Aliphatic glucosinolates participate in *Piriformospora indica*-induced resistance of Arabidopsis against *Alternaria brassicae*.

**Oelmüller, R., Sherameti, I., Tripathi, S. and Varma, A.** (2009) *Piriformospora indica*, a cultivable root endophyte with multiple biotechnological applications. *Symbiosis*, **49**, 1-17.

**Offre, P., Pivato, B., Siblot, S., Gamalero, E., Corberand, T., Lemanceau, P. and Mougel, C.** (2007) Identification of bacterial groups preferentially associated with mycorrhizal roots of *Medicago truncatula*. *Appl Environ Microbiol*, **73**, 913-921.

**Ohta, M., Matsui, K., Hiratsu, K., Shinshi, H. and Ohme-Takagi, M.** (2001) Repression domains of class II ERF transcriptional repressors share an essential motif for active repression. *Plant Cell*, **13**, 1959-1968.

**Park, J.-Y., Jin, J., Lee, Y.-L., Kang, S. and Lee, Y.-H.** (2009) Rice blast fungus (*Magnaporthe oryzae*) infects *Arabidopsis thaliana via* a mechanism distinct from that required for the infection of rice. *Plant Physiol*, **149**, 474-486.

**Pedras, M.S., Jha, M., Minic, Z. and Okeola, O.G.** (2007) Isosteric probes provide structural requirements essential for detoxification of the phytoalexin brassinin by the fungal pathogen *Leptosphaeria maculans*. *Bioorg Medicinal Chem*, **15**, 6054-6061.

**Peleg-Grossman, S., Golani, Y., Kaye, Y., Melamed-Book, N. and Levine, A.** (2009) NPR1 protein regulates pathogenic and symbiotic interactions between Rhizobium and legumes and non-legumes. *PLoS One*, **4**, e8399.

**Peškan-Berghöfer, T., Shahollari, B., Giong, P.H., Hehl, S., Markert, C., Blanke, V., Kost, G., Varma, A.K. and Oelmüller, R.** (2004) Association of *Piriformospora indica* with *Arabidopsis thaliana* roots

represents a novel system to study beneficial plant-microbe interactions and involves early plant protein modifications in the endoplasmatic reticulum and at the plasma membrane. *Physiol Plant*, **122**, 465-477.

**Petersen, L.N., Ingle, R.A., Knight, M.R. and Denby, K.J.** (2009) OXI1 protein kinase is required for plant immunity against *Pseudomonas syringae* in Arabidopsis. *J Exp Bot*, **60**, 3727-3735.

**Pieterse, C.M.J., van Wees, S.C.M., van Pelt, J.A., Knoester, M., Laan, R., Gerrits, H., Weisbeek, P.J. and van Loon, L.C.** (1998). A novel signaling pathway controlling induced systemic resistance in Arabidopsis. *Plant Cell*, **10**, 1571-1580.

**Pineda, A., Zheng, S.-J., van Loon, J.J.A., Pieterse, C.M.J. and Dicke, M.** (2010) Helping plants to deal with insects: the role of beneficial soil-borne microbes. *Trends Plant Sci*, **15**, 507-514.

**Piotrowski, M.** (2008) Primary or secondary? Versatile nitrilases in plant metabolism. *Phytochem*, **69**, 2655-2667.

**Pozo, M.J., van der Ent, S., van Loon, L.C. and Pieterse, C.M.J.** (2008) Transcription factor MYC2 is involved in priming for enhanced defense during rhizobacteria-induced systemic resistance in *Arabidopsis thaliana*. *New Phytol*, **180**, 511-523.

**Pozo, M.J. and Azcón-Aguilar, C.** (2007) Unraveling mycorrhiza-induced resistance. *Curr Opin Plant Biol*, **10**, 393-398.

**Pozo, M.J., Azcon-Aguilar, C., Dumas-Gaudot, E. and Barea, J.M.** (1999) β-1,3-glucanase activities in tomato roots inoculated with Arbuscular Mycorrhizal fungi and/or *Phytophthora parasitica* and their possible involvement in bioprotection. *Plant Sci*, **141**, 149-157.

**Purin, S. and Rillig, M.C.** (2008) Parasitism of arbuscular mycorrhizal fungi: reviewing the evidence. *FEMS Microbiol Lett*, **279**, 8-14.

**Qiu, J.L., Fiil, B.K., Petersen, K., Nielsen, H.B., Botanga, C.J., Thorgrimsen, S., Palma, K., Suarez-Rodriguez, M.C., Sandbech-Clausen, S., Lichota, J., Brodersen, P., Grasser, K.D., Mattsson, O., Glazebrook, J., Mundy, J. and Petersen, M.** (2008) Arabidopsis MAP kinase 4 regulates gene expression through transcription factor release in the nucleus. *EMBO J*, **27**, 2214-2221.

**Raacke, I.C., Rad, U.V., Müller, M.J. and Berger, S.** (2006) Yeast increases resistance in Arabidopsis against *Pseudomonas syringae* and *Botrytis cinerea* by salicylic acid-dependent as well as -independent mechanisms. *Mol Plant Microbe Interact*, **19**, 1138-1146.

**Radutoiu, S., Madsen, L.H., Madsen, E.B., Jurkiewicz, A., Fukai, E., Quistgaard, E.M.H., Albrektsen, A.S., James, E.K., Thirup, S. and Stougaard, J.** (2007) LysM domains mediate lipochitin-oligosaccharide recognition and *Nfr* genes extend the symbiotic host range. *EMBO J*, **26**, 3923-3935.

**Radutoiu, S., Madsen, L.H., Madsen, E.B., Felle, H.H., Umehara, Y., Grønlund, M., Sato, S., Nakamura, Y., Tabata, S., Sandal, N. and Stougaard, J.** (2003) Plant recognition of symbiotic bacteria requires two LysM receptor-like kinases. *Nature*, **425**, 585-592.

**Rausch, T. and Wachter, A.** (2005) Sulfur metabolism: a versatile platform for launching defence operations. *Trends Plant Sci*, **10**, 503-509.

**Read, D.J., Duckett, J.G., Francis, R., Ligrone, R. and Russell, A.** (2000) Symbiotic fungal associations in 'lower' land plants. *Phil Trans R Soc Lond B*, **355**, 815-831.

**Ren, D., Liu, Y., Yang, K.-Y., Han, L., Mao, G., Glazebrook, J. and Zhang, S.** (2008) A fungal-responsive MAPK cascade regulates phytoalexin biosynthesis in Arabidopsis. *Proc Natl Acad Sci USA*, **105**, 5638-5643.

**Rentel, M.C., Lecourieux, D., Ouaked, F., Usher, S.L., Petersen, L., Okamoto, H., Knight, H., Peck, S.C., Grierson, C.S., Hirt, H. and Knight, M.R.** (2004) OXI1 kinase is necessary for oxidative burst-mediated signaling in Arabidopsis. *Nature*, **427**, 858-861.

**Romanov, G.A., Kieber, J.J. and Schmülling, T.** (2002) A rapid cytokinin response assay in Arabidopsis indicates a role for phospholipase D in cytokinin signaling. *FEBS Lett*, **515**, 39-43.

**Rojas, J.C.** (1999) Electrophysiological and behavioral responses of the cabbage moth to plant volatiles. *J Chem Ecol*, **25**, 1867-1883.

**Saito, K.** (2004) Sulfur assimilatory metabolism. The long and smelling road. *Plant Physiol*, **136**, 2443-2450.

**Salzer, P., Corbiere, H. and Boller, T.** (1999) Hydrogen peroxide accumulation in *Medicago truncatula* roots colonized by the Arbuscular Mycorrhiza-forming fungus *Glomus intraradices*. *Planta*, **208**, 319-325.

**Salzer, P. and Boller, T.** (2000) Elicitor-induced reactions in Mycorrhizae and their suppression. *In*: *Podila GK, Douds DD, eds.* Current advances in mycorrhizae research. *Minnesota, USA: The American Phytopathogical Society*, 1-10.

**Schlaeppi, K., Abou-Mansour, E., Buchala, A. and Mauch, F.** (2010) Disease resistance of Arabidopsis to *Phytophthora brassicae* is established by the sequential action of indole glucosinolates and camalexin. *Plant J*, **62**, 840-851.

**Schnug, E. and Haneklaus, S.** (1993) Physiological backgrounds of different sulphur utilization in *Brassica napus* varieties. *Aspects Appl Biol*, **34**, 235- 242.

**Schnug, E., Haneklaus, S., Borchers, A. and Polle, A.** (1995) Relations between sulphur supply and glutathione and ascorbate concentrations in *Brassica napus*. *Zeitschrift für Pflanzenernährung und Bodenkunde*, **158**, 67-69.

**Schuhegger, R., Rauhut, T. and Glawischnig, E.** (2007) Regulatory variability of camalexin biosynthesis. *J Plant Physiol*, **164**, 636-644.

**Schuhegger, R., Nafisi, M., Mansourova, M., Petersen, B.L., Olsen, C.E., Svatos, A., Halkier, B.A. and Glawischnig, E.** (2006) CYP71B15 (PAD3) catalyzes the final step in camalexin biosynthesis. *Plant Physiol*, **141**, 1248-1254.

**Schuster, J., Knill, T., Reichelt, M., Gershenzon, J. and Binder, S.** (2006) Branched-chain aminotransferase4 is part of the chain elongation pathway in the biosynthesis of methionine-derived glucosinolates in Arabidopsis. *Plant Cell*, **18**, 2664-2679.

**Searle, L.M., Chamberlain, K., Rausch, T. and Butcher, D.N.** (1982) The conversion of 3-indolylmethylglucosinolate to 3-indolylacetonitrile by myrosinases, and its relavance to the clubroot disease of the Cruciferiae. *J Exp Bot*, **33**, 935-942.

**Selosse, M-A., Dubois, M-P. and Alvarez, N.** (2009) Do Sebacinales commonly associate with plant roots as endophytes? *Mycol Res*, 1062-1069.

**Shahollari, B., Vadassery, J., Varma, A. and Oelmüller, R.** (2007) A leucine-rich repeat protein is required for growth promotion and enhanced seed production mediated by the endophytic fungus *Piriformospora indica* in *Arabidopsis thaliana*. *Plant J*, **50**, 1-13.

**Shahollari, B., Peškan-Berghöfer, T. and Oelmüller, R.** (2004) Receptor kinases with leucine-rich repeats are enriched in Triton X-100 insoluble plasma membrane microdomains from plants. *Physiol Plant*, **122**, 397-403.

**Sherameti, I., Venus, Y., Drzewiecki, C., Tripathi, S., Dan, V.M., Nitz, I., Varma, A., Grundler, F.M. and Oelmüller, R.** (2008a) PYK10, a beta-glucosidase located in the endoplasmatic reticulum, is crucial for the beneficial interaction between *Arabidopsis thaliana* and the endophytic fungus *Piriformospora indica*. *Plant J*, **54**, 428-439.

**Sherameti, I., Tripathi, S., Varma, A. and Oelmüller, R.** (2008b) The root-colonizing endophyte *Piriformospora indica* confers drought tolerance in Arabidopsis by stimulating the expression of drought stress-related genes in leaves. *Mol Plant Microbe Interact*, **21**, 799-807.

**Sherameti, I., Shahollari, B., Venus, Y., Altschmied, L., Varma, A. and Oelmüller, R.** (2005) The endophytic fungus *Piriformospora indica* stimulates the expression of nitrate reductase and the starch-degrading enzyme glucan-water dikinase in tobacco and Arabidopsis roots through a homeodomain transcription factor that binds to a conserved motif in their promoters. *J Biol Chem*, **280**, 26241-26247.

**Shibagaki, N., Rose, A., Mcdermott, J.P., Fujiwara, T., Hayashi, H., Yoneyama, T. and Davies, J.P.** (2002) Selenate-resistant mutants of *Arabidopsis thaliana* identify SULTR1;2, a sulfate transporter required for efficient transport of sulfate into roots. *Plant J*, **29**, 475-486.

**Shimizu, T., Nakano, T., Takamizawa, D., Desaki, Y., Ishii-Minami, N., Nishizawa, Y., Minami, E., Okada, K., Yamane, H., Kaku, H. and Shibuya, N.** (2010) Two LysM receptor molecules, CEBiP and OsCERK1, cooperatively regulate chitin elicitor signaling in rice. *Plant J*, **64**, 204-214.

**Skirycz, A., Reichelt, M., Burow, M., Birkemeyer, C., Rolcik, J., Kopka, J., Zanor, M.I., Gershenzon, J., Strnad, M., Szopa, J., Mueller-Roeber, B. and Witt, I.** (2006) DOF transcription factor AtDof1.1 (OBP2) is part of a regulatory network controlling glucosinolate biosynthesis in Arabidopsis. *Plant J*, **47**, 10-24.

**Smith, K.P. and Goodman, R.M.** (1999) Host variation for interactions with beneficial plant-associated microbes. *Annu Rev Phytopathol*, **37**, 473-491.

**Smith, F.W., Hawkesford, M.J., Ealing, P.M., Clarkson, D.T., Vanden Berg, P.J., Belcher, A.R. and Warrilow, A.G.S.** (1997) Regulation of expression of a cDNA from barley roots encoding a high affinity sulfate transporter. *Plant J*, **12**, 875-884.

**Smith, F.W., Ealing, P.M., Hawkesford, M.J. and Clarkson, D.T.** (1995) Plant members of a family of sulfate transporters reveal functional subtypes. *Proc Natl Acad Sci USA*, **92**, 9373-9377.

**Smolinska, U., Morra, M.J., Knudsen, G.R., and James, R.L.** (2003) Isothiocyanates produced by *Brassicaceae* species as inhibitors of *Fusarium oxysporum*. *Plant Dis*, **87**, 407-412.

**Solano, R., Stepanova, A., Chao, Q. and Ecker, J.R.** (1998) Nuclear events in ethylene signaling: A transcriptional cascade mediated by ETHYLENE-INSENSITIVE3 and ETHYLENE-RESPONSE-FACTOR1. *Genes Dev*, **12**, 3703-3714.

**Sønderby, I.E., Geu-Flores, F. and Halkier, B.A.** (2010) Biosynthesis of glucosinolates - gene discovery and beyond. *Trends Plant Sci*, **15**, 283-290.

**Sønderby, I.E., Hansen, B.G., Bjarnholt, N., Ticconi, C., Halkier, B.A. and Kliebenstein, D.J.** (2007) A systems biology approach identifies a R2R3 MYB gene subfamily with distinct and overlapping functions in regulation of aliphatic glucosinolates. *PLoS One*, **2**, e1322.

**Soylu, S., Brown, I. and Mansfield, J.W.** (2005) Cellular reactions in Arabidopsis following challenge by strains of *Pseudomonas syringae*: From basal resistance to compatibility. *Physiol Mol Plant Pathol*, **66**, 232-243.

**Spaink, H.P., Okker, R.J.H., Wijffelman, C.A., Tak, T., Goosen-De Roo, L., Pees, E., van Brussel, A.A.N. and Lugtenberg, B.J.J.** (1989) Symbiotic properties of rhizobia containing a flavonoid-independent hybrid nodD product. *J Bacteriol*, **171**, 4045-4053.

**Spaink, H.P., Sheeley, D.M., van Brussel, A.A., Glushka, J., York, W.S., Tak, T., Geiger, O., Kennedy, E.P., Reinhold, V.N. and Lugtenberg, B.J.J.** (1991) A novel highly unsaturated fatty acid moiety of lipo-oligosaccharide signals determines host specificity of Rhizobium. *Nature*, **354**, 125-130.

**Stacey, G., McAlvin, C.B., Kim, S.-Y., Olivares, J. and Soto, M.J.** (2006) Effects of endogenous salicylic acid on nodulation in the model legumes *Lotus japonicus* and *Medicago truncatula*. *Plant Physiol*, **141**, 1473-1481.

**Stein, E., Molitor, A., Kogel, K.-H. and Waller, F.** (2008) Systemic resistance in Arabidopsis conferred by the mycorrhizal fungus *Piriformospora indica* requires jasmonic acid signaling and the cytoplasmic function of NPR1. *Plant Cell Physiol*, **49**, 1747-1751.

**Stotz, H.U., Sawada, Y., Shimada, Y., Hirai, M.Y., Sasaki, E., Krischke, M., Brown, P.D., Saito, K. and Kamiya, Y.** (2011) Role of camalexin, indole glucosinolates, and side chain modification of glucosinolate-derived isothiocyanates in defense of Arabidopsis against *Sclerotinia sclerotiorum*. *Plant J*, **67**, 81-93.

**Stracke, S., Kistner, C., Yoshida, S., Mulder, L., Sato, S., Kaneko, T., Tabata, S., Sandal, N., Stougaard, J., Szczyglowski, K. and Parniske, M.** (2002) A plant receptor-like kinase required for both bacterial and fungal symbiosis. *Nature*, **417**, 959-962.

**Strack, D., Fester, T., Hause, B., Schliemann, W. and Walter, M.H.** (2003) Arbuscular mycorrhiza: biological, chemical, and molecular aspects. *J Chem Ecol*, **29**, 1955-1979.

**Sun, C., Johnson, J.M., Cai, D., Sherameti, I., Oelmüller, R. and Lou, B.** (2010) *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. *J Plant Physiol*, **167**, 1009-1017.

**Svanem, P.J., Bones, A.M. and Rossiter, J.T.** (1997) Metabolism of [α-$^{14}$C]-desulphophenylethylglucosinolate in *Nasturtium officinale*. *Phytochem*, **44**, 1251-1255.

**Takahashi, H., Watanabe-Takahashi, A., Smith, F.W., Blake-Kalff, M., Hawkesford, M.J. and Saito, K.** (2000) The role of three functional sulfate transporters involved in uptake and translocation of sulfate in *Arabidopsis thaliana*. *Plant J*, **23**, 171-182.

**Takahashi, H., Yamazaki, M., Sasakura, N., Watanabe, A., Leustek, T., Engler, J.A., Engler, G., Van Montagu, M. and Saito, K.** (1997) Regulation of sulfur assimilation in higher plants: A sulfate transporter induced in sulfate starved roots plays a central role in *Arabidopsis thaliana*. *Proc Natl Acad Sci USA*, **94**, 11102-11107.

**Talalay, P. and Fahey, J.W.** (2001) Phytochemicals from cruciferous plants protect against cancer by modulating carcinogen metabolism. *J Nutr*, **131**, 3027S-3033S.

**Testerink, C., Larsen, P.B., van der Does, D., van Himbergen, J.A. and Munnik, T.** (2007) Phosphatidic acid binds to and inhibits the activity of Arabidopsis CTR1. *J Exp Bot*, **58**, 3905-3914.

**Thomma, B.P.H.J., Nelissen, I., Eggermont, K. and Broekaert, W.F.** (1999) Deficiency in phytoalexin production causes enhanced susceptibility of *Arabidopsis thaliana* to the fungus *Alternaria brassicicola*. *Plant J*, **19**, 163-171.

**Thomma, B.P.H.J., Eggermont, K., Penninckx, I.A.M.A., Mauch-Mani, B., Vogelsang, R., Cammue, B.P.A. and Broekaert, W.F.** (1998) Separate jasmonate-dependent and salicylate-dependent defense-response pathways in Arabidopsis are essential for resistance to distinct microbial pathogens. *Proc Natl Acad Sci USA*, **95**, 15107-15111.

**Thompson, J.N.** (1997) Evaluating the dynamics of coevolution among geographically structured populations. *Ecology*, **78**, 1619-1623.

**Tierens, K.F., Thomma, B.P., Brouwer, M., Schmidt, J., Kistner, K., Porzel, A., Mauch-Mani, B., Cammue, B.P. and Broekaert, W.F.** (2001) Study of the role of antimicrobial glucosinolate-derived isothiocyanates in resistance of Arabidopsis to microbial pathogens. *Plant Physiol*, **125**, 1688-1699.

**Vadassery, J., Ranf, S., Drzewiecki, C., Mithöfer, A., Mazars, C., Scheel, D., Lee, J. and Oelmüller, R.** (2009a) A cell wall extract from the endophytic fungus *Piriformospora indica* promotes growth of Arabidopsis seedlings and induces intracellular calcium elevation in roots. *Plant J*, **59**, 193-206.

**Vadassery, J., Tripathi, S., Prasad, R., Varma, A. and Oelmüller, R.** (2009b) Monodehydroascorbate reductase 2 and dehydroascorbate reductase 5 are crucial for a mutualistic interaction between *Piriformospora indica* and Arabidopsis. *J Plant Physiol*, **166**, 1263-1274.

**van Batenburg, F.H.D., Jonker, R. and Kijne, J.W.** (1986) Rhizobium induces marked root hair curling by redirection of tip growth: a computer simulation. *Physiol Plant*, **66**, 476-480.

**Van der Ent, S., Verhagen, B.W., Van Doorn, R., Bakker, D., Verlaan, M.G., Pel, M.J., Joosten, R.G., Proveniers, M.C., Van Loon, L.C., Ton, J. and Pieterse, C.M.J.** (2008) MYB72 is required in early signaling steps of rhizobacteria-induced systemic resistance in Arabidopsis. *Plant Physiol*, **146**, 1293-1304.

**Van der Ent, S., van Hulten, M., Pozo, M.J., Czechowski, T., Udvardi, M.K., Pieterse, C.M.J. and Ton, J.** (2009) Priming of plant innate immunity by rhizobacteria and beta-aminobutyric acid: differences and similarities in regulation. *New Phytol*, **183**, 419-431.

**VanEtten, H.D., Mansfield, J.W., Bailey, J.A. and Farmer, E.E.** (1994) Two classes of plant antibiotics: Phytoalexins versus ‘‘phytoanticipins’’. *Plant Cell*, **6**, 1191-1192.

**Van Loon, L.C.** (2007) Plant responses to plant growth-promoting rhizobacteria. *Eur J Plant Pathol*, **119**, 243-254.

**Van Loon, L.C., Bakker, P.A.H.M. and Pieterse, C.M.J.** (1998) Systemic resistance induced by rhizosphere bacteria. *Annu Rev Phytopathol*, **36**, 453-483.

**Van Peer, R., Niemann, G.J. and Schippers, B.** (1991) Induced resistance and phytoalexin accumulation in biological control of fusarium wilt of carnation by Pseudomonas sp. strain WCS417r. *Phytopathol*, **81**, 728-734.

**van Wees, S.C., Van der Ent, S. and Pieterse, C.M.** (2008) Plant immune responses triggered by beneficial microbes. *Curr Opin Plant Biol*, **11**, 443-448.

**van Wees, S.C. and Glazebrook, J.** (2003) Loss of non-host resistance of Arabidopsis NahG to *Pseudomonas syringae* pv. *phaseolicola* is due to degradation products of salicylic acid. *Plant J*, **33**, 733-742.

**Varma, A., Verma, S., Sudha Sahay, N., Britta, B. and Franken, P.** (1999) *Piriformospora indica* – a cultivable plant growth promoting root endophyte with similarities to arbuscular mycorrhizal fungi. *Appl Environ Microbiol USA*, **65**, 2741-2744.

**Vatsa, P., Chiltz, A., Bourque, S., Wendehenne, D., Garcia-Brugger, A. and Pugin, A.** (2011) Involvement of putative glutamate receptors in plant defence signaling and NO production. *Biochimie*, **93**, 2095-2101.

**Vauclare, P., Kopriva, S., Fell, D., Suter, M., Sticher, L., von Ballmoos, P., Krähenbühl, U., den Camp, R.O. and Brunold, C.** (2002) Flux control of sulphate assimilation in *Arabidopsis thaliana*: Adenosine 5'-phosphosulphate reductase is more susceptible than ATP sulphurylase to negative control by thiols. *Plant J*, **31**, 729-740.

**Verma, S. and Varma, A.** (1998) *Piriformospora indica*, gen. et sp. nov., a new root-colonizing fungus. *Mycologia*, **90**, 896-903.

**Vidmar, J.J., Tagmount, A., Cathala, N., Touraine, B. and Davidian, J.-C.E.** (2000) Cloning and characterization of a root specific high-affinity sulfate transporter from *Arabidopsis thaliana*. *FEBS Lett*, **475**, 65-69.

**Vogelsang, K.M., Reynolds, H.L. and Bever, J.D.** (2006) Mycorrhizal fungal identity and richness determine the diversity and productivity of a tallgrass prairie system. *New Phytol*, **172**, 554-562.

**Volpin, H., Phillips, D.A., Okon, Y. and Kapulnik, Y.** (1995) Suppression of an isoflavonoid phytoalexin defense response in mycorrhizal alfalfa roots. *Plant Physiol*, **108**, 1449-1454.

**Wais, R.J., Galera, C., Oldroyd, G., Catoira, R., Penmetsa, R.V., Cook, D., Gough, C., Dénarié, J. and Long, S.R.** (2000) Genetic analysis of calcium spiking responses in nodulation mutants of *Medicago truncatula*. *Proc Natl Acad Sci USA*, **97**, 13407-13412.

**Walker, K.C. and Booth, E.J.** (2003) Sulphur nutrition and oilseed quality. *In Sulphur in Plants* (Abrol, Y. P. and Ahmad, A., eds.), *Dordrecht: Kluwer Academic Publishers*, pp. 323-339.

**Waller, F., Achatz, B., Baltruschat, H., Fodor, J., Becker, K., Fischer, M., Heier, T., Hückelhoven, R., Neumann, C., von Wettstein, D., Franken, P. and Kogel, K.-H.** (2005) The endophytic fungus *Piriformospora indica* reprograms barley to salt-stress tolerance, disease resistance, and higher yield. *Proc Natl Acad Sci USA*, **102**, 13386-13391.

**Walch-Liu, P., Ivanov, I.I., Filleur, S., Gan, Y., Remans, T. and Forde, B.G.** (2006) Nitrogen regulation of root branching. *Ann Bot*, **97**, 875-881.

**Wan, J., Zhang, S. and Stacey, G.** (2004) Activation of a mitogen-activated protein kinase pathway in Arabidopsis by chitin. *Mol Plant Pathol*, **5**, 125-135.

**Wan, J., Zhang, X.C., Neece, D., Ramonell, K.M., Clough, S., Kim, S.Y., Stacey, M.G. and Stacey, G.** (2008) A LysM receptor-like kinase plays a critical role in chitin signaling and fungal resistance in Arabidopsis. *Plant Cell*, **20**, 471-481.

**Wang, C., Zien, C.A., Afitlhile, M., Welti, R., Hildebrand, D.F. and Wang, X.** (2000) Involvement of phospholipase D in wound-induced accumulation of jasmonic acid in Arabidopsis. *Plant Cell*, **12**, 2237-2246.

**Watanabe, M., Mochida, K., Kato, T., Tabata, S., Yoshimoto, N., Noji, M. and Saito, K.** (2008) Comparative genomics and reverse genetics analysis reveal indispensable functions of the serine acetyltransferase gene family in Arabidopsis. *Plant Cell*, **20**, 2484-2496.

**Xiang, C. and Oliver, D.J.** (1998) Glutathione metabolic genes coordinately respond to heavy metals and jasmonic acid in Arabidopsis. *Plant Cell*, **10**, 1539-1550.

**Yadav, V., Kumar, M., Deep, D.K., Kumar, H., Sharma, R., Tripathi, T., Tuteja, N., Saxena, A.K. and Johri, A.K.** (2010) A phosphate transporter from the root endophytic fungus *Piriformospora indica* plays a role in phosphate transport to the host plant. *J Biol Chem*, **285**, 26532-26544.

**Yang, S., Tang, F., Gao, M., Krishnan, H.B. and Zhu, H.** (2010) R gene-controlled host specificity in the legume-Rhizobia symbiosis. *Proc Natl Acad Sci USA*, **107**, 18735-18740.

**Yang, J., Kloepper, J.W. and Ryu, C.-M.** (2009) Rhizosphere bacteria help plants tolerate abiotic stress. *Trends Plant Sci*, **14**, 1-4.

**Yoshimoto, N., Inoue, E., Saito, K., Yamaya, T. and Takahashi, H.** (2003) Phloem-localizing sulfate transporter, Sultr1;3, mediates re-distribution of sulfur from source to sink organs in Arabidopsis. *Plant Physiol*, **131**, 1511-1517.

**Yoshimoto, N., Takahashi, H., Smith, F.W., Yamaya, T. and Saito, K.** (2002) Two distinct high-affinity sulfate transporters with different inducibilities mediate uptake of sulfate in Arabidopsis roots. *Plant J*, **29**, 465-473.

**Zhang, W., Qin, C., Zhao, J. and Wang, X.** (2004) Phospholipase Dα1-derived phosphatidic acid interacts with ABI1 phosphatase 2C and regulates abscisic acid signaling. *Proc Natl Acad Sci USA*, **101**, 9508-9513.

**Zhao, Y., Hull, A.K., Gupta, N.R., Goss, K.A., Alonso, J., Ecker, J.A., Normanly, J., Chory, J. and Celenza, J.L.** (2002) Trp-dependent auxin biosynthesis in Arabidopsis: involvement of cytochrome P450s CYB79B2 and CYP79B3. *Genes Dev*, **16**, 3100-3112.

**Zhou, N., Tootle, T.L. Glazebrook, J.** (1999) Arabidopsis PAD3, a gene required for camalexin biosynthesis, encodes a putative cytochrome P450 monooxygenase. *Plant Cell*, **11**, 2419–2428.

# 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 theses.

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

…….....

Pyniarlang Lyngdoh Nongbri

Jena, December 10, 2012

## 9.2 Curriculum Vitae

### Personal Details


| | |
|---|---|
| Name: | Pyniarlang Lyngdoh Nongbri |
| Date of birth: | 21.01.1983 |
| Place of birth: | Shillong, India |
| Nationality: | Indian |
| Address: | Neugasse 26, 07743 Jena, Germany |
| Email: | pyndrop@gmail.com |


### Education:

| | |
|---|---|
| 2008-present: | Ph.D. student. Institute of Plant Physiology, Friedrich-Schiller Universität Jena, Germany |
| 2003-2005: | M.Sc. in Life Sciences. School of Life Sciences, Jawaharlal Nehru University, New Delhi, India |
| 2000-2003: | B.Sc. in Biotechnology, St. Anthony's College, North Eastern Hill University, Shillong, India |

### Research experience:

2005-2008: National Institute of Plant Genome Research, New Delhi

-Plant-microbe interaction and nutritional genomics.

## 9.3 List of publications and presentations

### Publications

**Nongbri, P.L., Johnson, J.M., Sherameti, I., Glawischnig, E., Halkier, B.A. and Oelmüller, R.** (2012) Indole-3-acetaldoxime-derived compounds restrict root colonization in the beneficial interaction between Arabidopsis roots and the endophyte *Piriformospora indica*. *Mol Plant Microbe Interact*, **25**, 1186-1197.

**Nongbri, P.L. and Oelmüller, R.** (2012) Role of *Piriformospora indica* in Sulfur Metabolism in *Arabidopsis thaliana*. *In: Varma, A., Kost, G. and Oelmüller, R. (eds) Piriformospora indica: Sebacinales and their biotechnological application. Springer, Heidelberg, Vol. 33* (**accepted**).

**Trebicka, A., Oelmüller, R., Sherameti, I., Nongbri, P.L. and Johnson, J.M.** (2012) Utilization of root-colonizing fungi for improved performance of agricultural crops. *Albanian J Agri Sci*, **11**, 9-16.

**Johnson, J.M., Sherameti, I., Nongbri, P.L. and Oelmüller, R.** (2012) Standardized conditions to study beneficial and non-beneficial traits in the *Piriformospora indica*/Arabidopsis interaction. *In: Varma, A., Kost, G. and Oelmüller, R. (eds) Piriformospora indica: Sebacinales and their biotechnological application. Springer, Heidelberg, Vol. 33* (**accepted**).

**Nongbri, P.L., Vahabi, K., Mrozinska, A., Seebald, E., Sun, C., Sherameti, I., Johnson, J.M. and Oelmüller, R.** (2012) Balancing defense and growth-Analyses of the beneficial symbiosis between *Piriformospora indica* and *Arabidopsis thaliana*. (**accepted, Symbiosis**).

**Johnson, J.M., Sherameti, I., Ludwig, A., Nongbri, P.L., Sun, C., Lou, B., Varma, A. and Oelmüller, R.** (2011) Protocols for *Arabidopsis thaliana* and *Piriformospora indica* cocultivation-A model system to study plant beneficial traits. *Endocyt Cell Res*, 101-113.

**Johnson, J.M., Nongbri, P.L., Sherameti, I. and Oelmüller, R.** (2011) Calcium signaling and cytosolic calcium measurements in plants. *Endocyt Cell Res*, 64-76.

**Sherameti, I., Johnson, J.M., Nongbri, P.L. and Oelmüller, R.** (2010) The central roles of iron and calcium for plant/microbe interaction and shaping microbial communities in the soil. *Albanian J Agri Sci*, **9**, 1-24.

**Talks**

**Nongbri, P.L., Oelmüller, R.** (2011) S metabolism and glucosinolates in Arabidopsis/*P. indica* interaction. *Südostdeutsche Pflanzenphysiologie Tagung*, Leipzig, Germany.

**Nongbri, P.L., Oelmüller, R.** (2011) Indole-3-acetaldoxime-derived compounds and aliphatic glucosinolates are essential for beneficial interaction between Arabidopsis and *Piriformospora indica. MiCom2011- 2nd International Student Conference on Microbial Communication*, Jena, Germany.

**Posters**

**Nongbri, P.L., Oelmüller, R.** (2009) Role of *Piriformospora indica* in sulfur metabolism in *Arabidopsis thaliana. Joint Symposium of JSMC and ILRS*, Jena, Germany.

**Nongbri, P.L., Oelmüller, R.** (2010) The role of Indole-glucosinolate and Camalexin in *Piriformospora indica*/Arabidopsis interaction. *Micom2010-PhD Conference on Microbial Communication*, Jena, Germany.

**Nongbri, P.L., Johnson, J.M., Sherameti, I., Glawischnig, E., Halkier, B.A., Oelmüller, R.** (2012) Different roles of indole-3-acetaldoxime-derived compounds and aliphatic glucosinolate in *Arabidopsis thaliana* and *Piriformospora indica* symbiosis. *MiCom2012- 3rd International Student Conference on Microbial Communication*, Jena, Germany.

## 9.4 Acknowledgement

It is with immense gratitude that I acknowledge the support and help of my supervisor Prof. Dr. Ralf Oelmüller for his valuable and constructive suggestions during the planning and development of this research work. His willingness to give his time during the preparation of the manuscripts so generously has been very much appreciated. Special thanks are addressed to Prof. Dr. Erika Kothe, my co-supervisor for her support during the work.

Advice given by Joy Michal Johnson has been a great help in completion of the project. Thank you very much Joy for your help and the wonderful time we had together in Jena. I cannot find words to express my gratitude to Dr. Irena Sherameti for her willingness to lend her helping hands when I needed.

I am particularly grateful for the assistance given by our technicians Sarah Mußbach, Claudia Röppischer, and Heide for all the technical help.

This thesis would not have been possible without the support and cheers of my former and current lab mates especially, Dr. Jyothi Vadassery, Dr. Matsuo Matsuhiro, Dr. Iris Camehl, Dr. Corinna Drzewiecki, Ute Holtzegel, Eileen Seebald, Chao Sun, Sindy Schedel, Qerimann Bakii, Doreen Meischner, Anatoli Ludwig, Khabat Vahabi, Anna Mrozinska, Dr. Dahai Yang and Madhunita Bakshi.

I would also like to thank Dr. Klaus Appendroth, Dr. Thomas Pfannschmidt, Yvonne, Lars, Sebastian, Bjorn, Hannah, Matthias, Moni and all the members of the Department of Plant Physiology for helping me in many ways in the lab.

Thank you Carmen Galambos, Ulrich Weber and Ulrich Möller for all the technical help related to my work in the Institute.

I extend my gratitude to Dr. Carsten Thoms and Mrs. Ulrike Schleier for their help in various ways during my PhD in Jena and the Jena School for Microbial Communication for funding. Thanks to all the people at the Friedrich-Schiller-University involved in the dissertation procedure.

It was great fun getting started my life in Jena with Yamuna, Somya, Tohir, Dr. Ravi and Dr. Biplab Kumar in Am Herrenberg 11.

It is worth mentioning the warm feeling of home away from home in the company of Pyndap, Michael, Larissa and Meban in Leipzig.

These names would never get away no matter how great the distance is; Pynskhem, Raplang, Lamgin, Kipgen, Macmillan, Moses, Badaplin, Ferdinand, Wandon, Arban, Banlum, Teibor, Wanshai and Gean.

Most especially to my Mei, Pa, Hymmen bad Para who have given me their unequivocal emotional and moral support and unconditional love throughout, as always, for which my mere expression of thanks likewise does not suffice. My special love to late sister Merryparadise, you were there at the beginning of my Ph.D.

And to Almighty 'GOD', who made all things possible.

***''KHUBLEI''***