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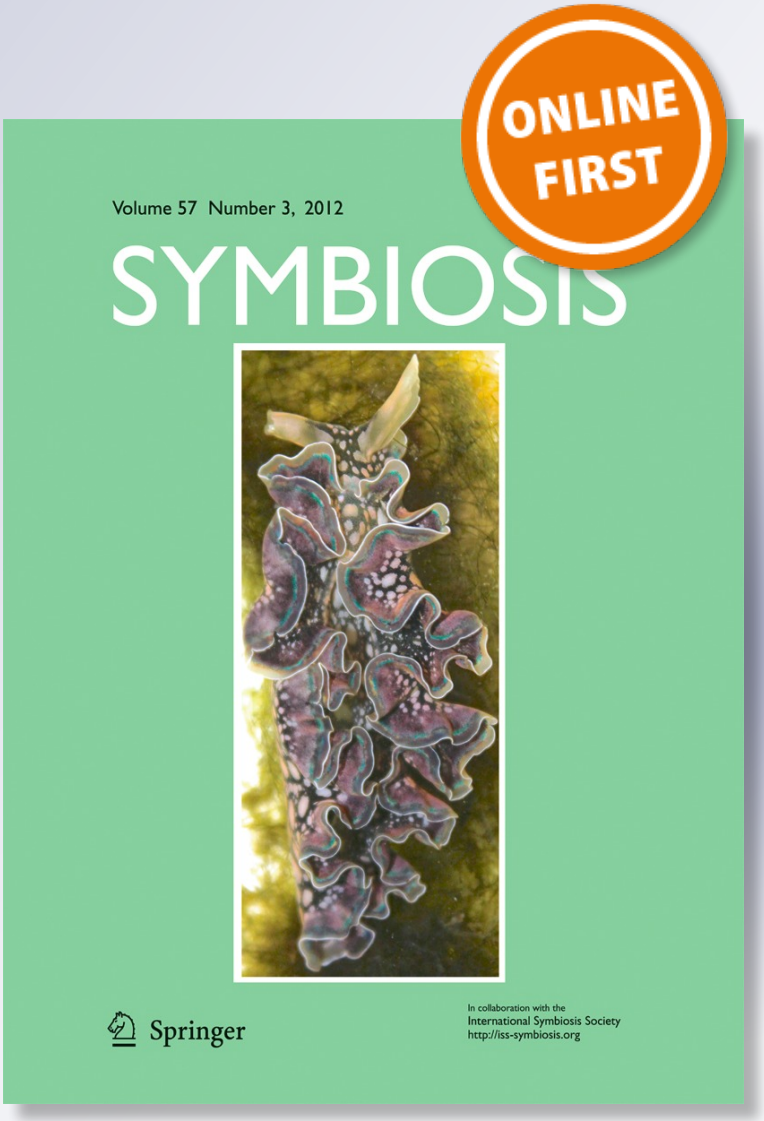

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Balancing defense and growth—Analyses of the beneficial symbiosis between *Piriformospora indica* and *Arabidopsis thaliana*

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

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1 Introduction

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

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

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

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

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

ETR1, EIN2 and EIN3/EIL1 are required for *P. indica*-mediated growth promotion of Arabidopsis seedlings (Camehl et al. 2010). Growth promotion by *P. indica* of the corresponding single (*etr1*, *ein2*) and double (*ein3 eil1*) knockout 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 and Oelmüller 2010) led to the conclusion that ERF9 and ERF14 represses *PR* gene expression in colonized Arabidopsis roots and that this contributes to the establishment of the beneficial interaction.

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

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

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

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

4 *Cerk* mutants

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

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

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

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

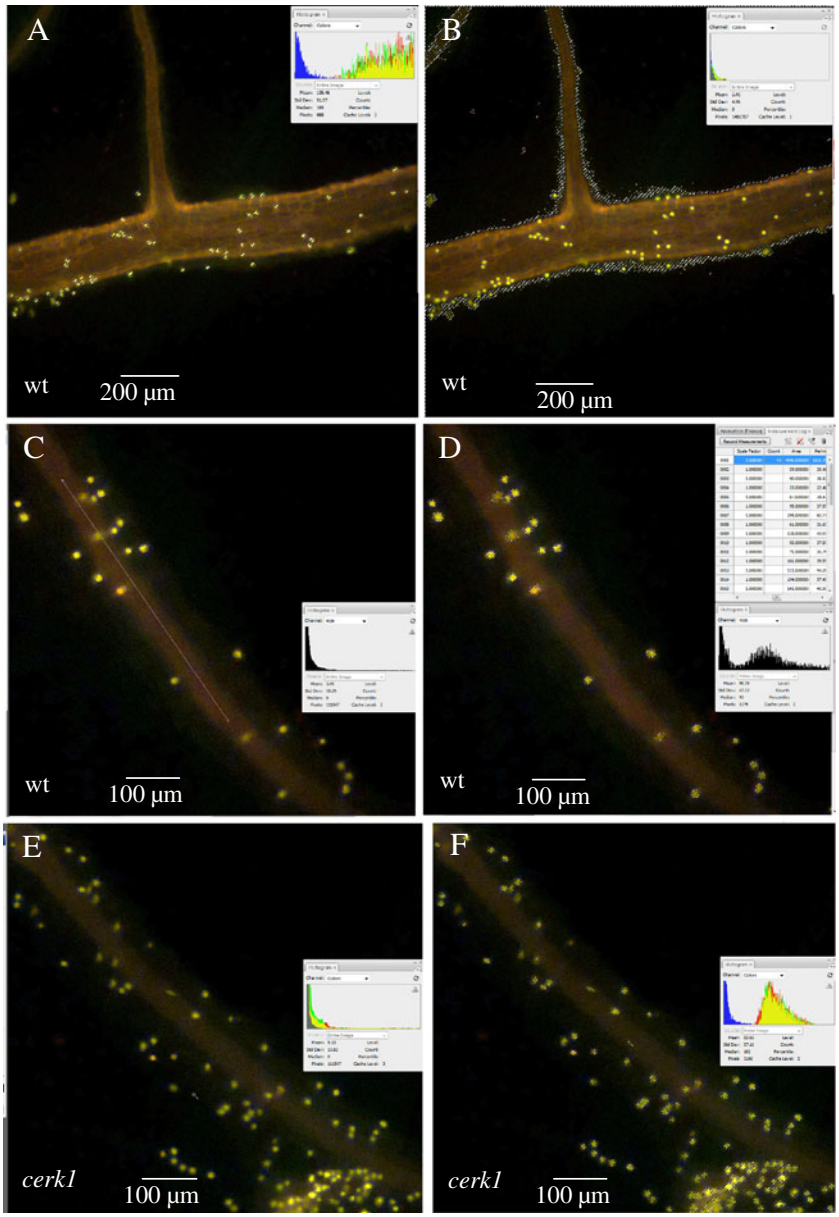

Fig. 1 Root colonization of wild-type (**a**, **b**, **c**, **d**) and *cerk1* (**e**, **f**) seedling grown on PNM media for 2 weeks. Root area was measured by Magic wand tool adjusted with tolerance of 32 for each sample (**b**). The colonization pixels were selected by Adobe Photoshop CS5 Magic wand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

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

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

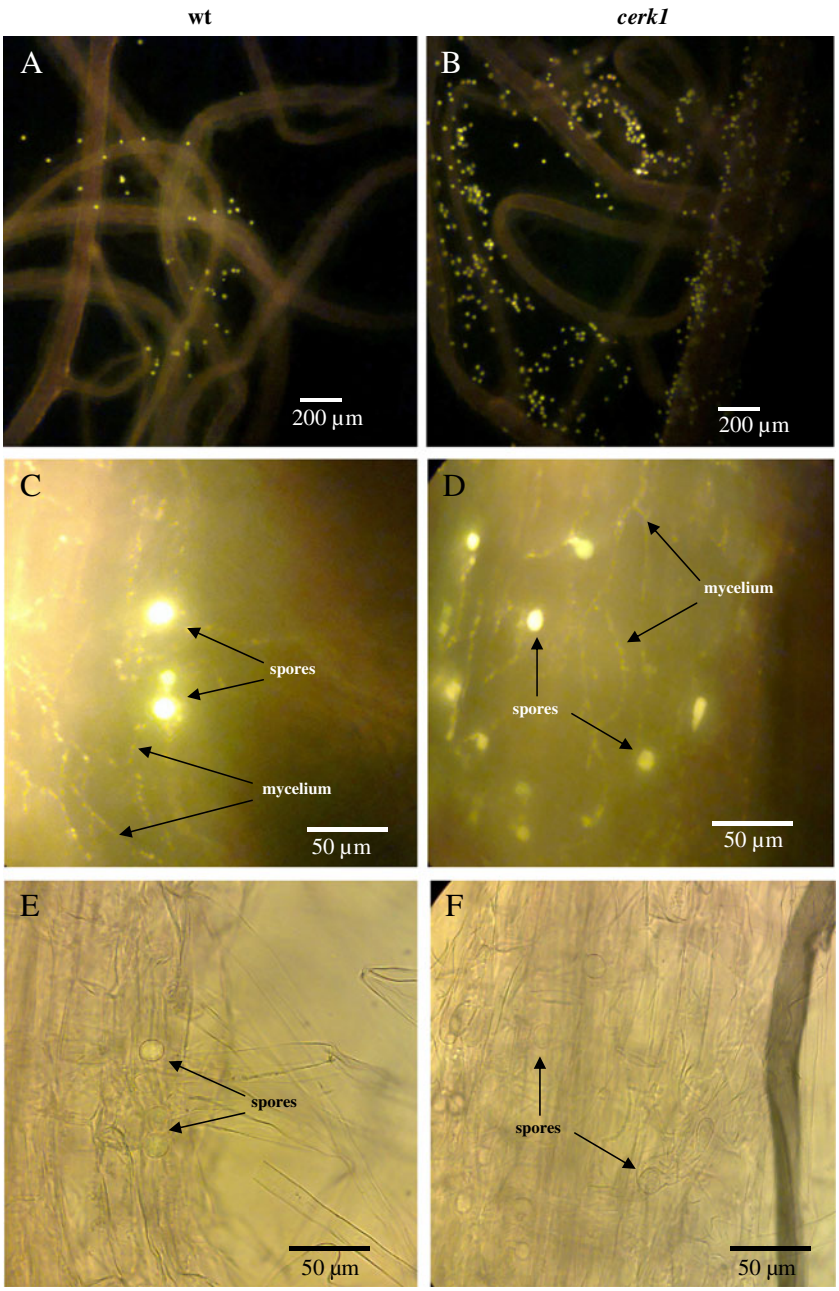

Fig. 2 Root colonization of Arabidopsis wild-type (*left*) and *cerk1* line (*right*). The fungus was stained with Nile red and monitored under the fluorescent channel at 450–520 nm (**a**–**d**) and under visual light (**e**, **f**). Comparison of fluorescent and visual light microscopy in discrimination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

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

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

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

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

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

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

5.1 PEN2 (At2g44490)

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

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

5.2 Pyk10

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

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

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

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

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

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

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

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

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

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

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

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

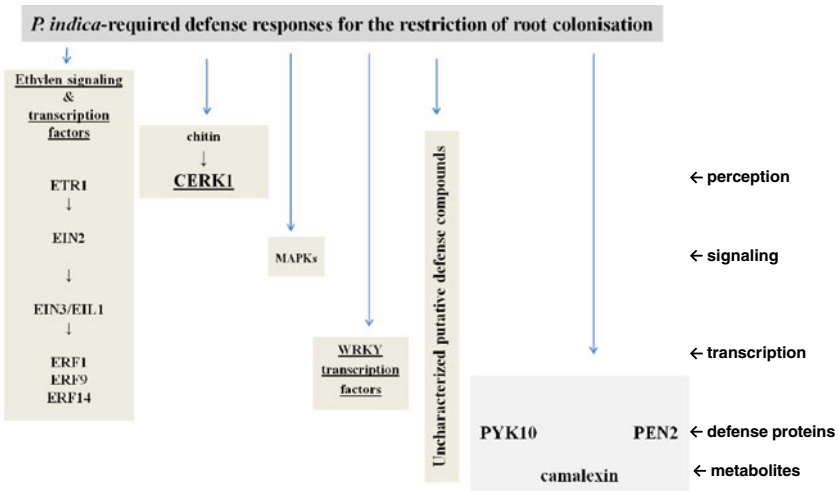

Fig. 3 Defense response components required for restricting Arabidopsis root colonization by *P. indica*. The components are involved in perception, signaling and transcription, or represent defense proteins or secondary metabolites

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

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

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

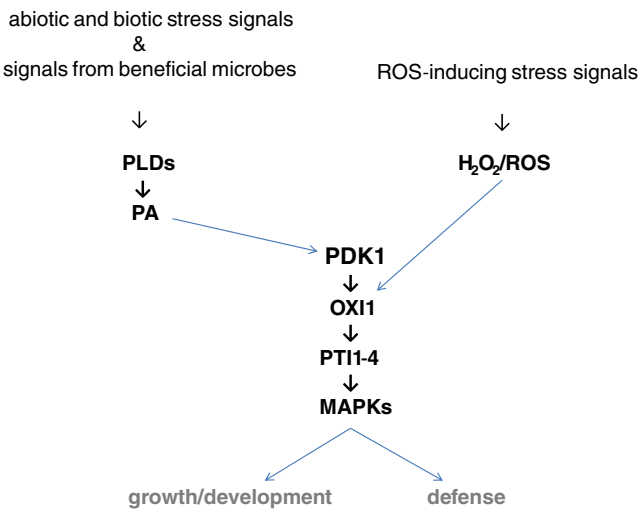

Fig. 4 A model describing the PDK1/OXI1 pathway and its potential involvement in balancing growth/development and defense responses

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

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

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

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

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

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

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

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

9 Conclusions

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

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

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