Chapter 14
Role of Defense Compounds in the Beneficial Interaction Between *Arabidopsis thaliana* and *Piriformospora indica*

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14.1 Defense in Beneficial Plant/Microbe Interactions: An Introduction

In nature, plants are exposed to a large number of quite diverse microorganisms, which can be beneficial (mutualistic interaction), harmful (pathogenic interaction), or neutral (commensalistic interaction) for plant performance. Root-colonizing rhizobacteria, mycorrhizal fungi, or beneficial endophytes often promote plant growth and biomass production and establish tolerance against biotic and abiotic stresses. They compete with soil-borne microbes which are harmful for the plant (Johnson and Oelmüller 2009). These two types of symbiotic interactions are extremes. Therefore, it is not surprising that the mode of interaction between two symbionts is never stable, and all types of transitions have been observed in nature, depending on environmental conditions and genetic programs. Genetic studies have uncovered that single gene loci in both plants and microbes determine the mode of interaction and manipulation of crucial genes may cause severe alterations in the symbiosis (Johnson and Oelmüller 2009 and references therein).

We study the beneficial interaction between the growth- and biomass-promoting endophyte *Piriformospora indica* and plant roots. The endophytic fungus, a basidiomycete of the Sebacinaceae family, interacts with many plant species, including *Arabidopsis*. Like other members of the Sebacinales, *P. indica* colonizes the roots, grows inter- and intracellularly, and forms pear-shaped spores in the roots as well as on the root surface. The endophyte promotes nutrient uptake, allows plants to survive under abiotic (water and salt) stress, confers resistance to toxins, heavy metal ions, and pathogenic organisms, and stimulates growth and seed production

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(cf. Oelmüller et al. 2004, 2005, 2009; Peškan-Berghöfer et al. 2004; Pham et al. 2004; Sahay and Varma 1999; Shahollari et al. 2005, 2007; Sherameti et al. 2005; Varma et al. 1999, 2001). *P. indica* is a cultivable fungus and can grow on synthetic media without a host (Peškan-Berghöfer et al. 2004; Varma et al. 2001; Verma et al. 1998). The host range includes trees, agricultural, horticultural, and medicinal plants, monocots, dicots, and mosses (Barazani et al. 2005; Glen et al. 2002; Peškan-Berghöfer et al. 2004; Shahollari et al. 2005, 2007; Sherameti et al. 2005; Varma et al. 2001; Waller et al. 2005; Weiss et al. 2004), suggesting that the interaction is based on general recognition and signaling processes.

Using a genetic approach, we identified genes and proteins which are required for the beneficial interaction between the two symbionts (Oelmüller et al. 2004; Shahollari et al. 2005, 2007; Sherameti et al. 2008; Camehl et al. 2010). For this screen, we used independent plant responses which are induced by *P. indica* in *Arabidopsis* roots and leaves (e.g. growth promotion, seed yield, resistance against drought or leaf pathogens, marker gene expression, protein phosphorylation pattern, spore germination) and identified those mutants in which these responses are not induced by the fungus. We isolated two classes of mutants: those which do not respond to *P. indica* and grow like uncolonized plants in the presence of the fungus and those for which *P. indica* was pathogenic (Camehl et al. 2010). For some of these mutants, the genes were identified by map-based cloning strategies. Interestingly, several of these mutants were defective in defense compounds. Their analysis uncovered that mutualism depends on a balanced activation of defense mechanisms. For some of the defense compounds, we also discovered novel functions in this beneficial symbiosis.

Defense responses have been intensively studied in beneficial microbial and mycorrhizal communications. As long as the microbial partner is not recognized as a friend (e.g. during early phases of mycorrhizal interaction, when the plants have not yet benefited from the fungus and nutrient exchange has not yet started), the plant often initiates defense responses against the symbiont. Güimil et al. (2005) have shown that over 40% of the genes in the roots of rice seedlings respond to beneficial and non-beneficial fungi and many of them are involved in plant defense. When the establishment of a beneficial symbiosis proceeds and the plant recognizes the microbial partner as a friend, the expression of defense genes becomes downregulated. The molecular mechanism which causes the shutdown of defense responses is unclear at present. It can be an active process initiated by either the plant or the fungus or simply a passive process since defense activating compounds are no longer present in the beneficial symbiosis (Harrison 2005).

Plant hormones such as salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) play major roles in regulating plant defense responses. Simplified, SA is involved in the reaction against biotrophic and hemi-biotrophic pathogens while JA and ET are associated with defense against necrotrophic pathogens and herbivorous insects. The two main defense mechanisms in plants are the systemic acquired resistance (SAR) where SA is essential and the induced systemic resistance (ISR) which is mainly based on ET and JA signaling. ISR results from colonization of roots by certain nonpathogenic bacteria (van Loon et al. 1998) but

also friendly fungi such as *Trichoderma* spp. (Shoresh et al. 2010) or *P. indica* (Stein et al. 2008).

Furthermore, reactive oxygen species (ROS), in particular H_2O_2, are important signaling compounds in plants dealing with pathogenic microorganisms (Apel and Hirt 2004) but also with rhizobia (Puppo et al. 2005) or arbuscular mycorrhizal fungi (Fester and Hause 2005). Tanaka et al. (2006) have shown the important role of ROS in regulating the mutualistic interaction between a clavicipitaceous fungal endophyte, *Epichloë festucae*, and its grass host, *Lolium perenne*. *E. festucae* grows systemically in intercellular spaces of leaves as infrequently branched hyphae parallel to the leaf axis. A fungal mutant defective in a NADPH oxidase gene (*noxA*) altered the interaction from mutualistic to antagonistic. Plants infected with the *noxA* mutant lose apical dominance, become severely stunted, show precocious senescence, and eventually die. The fungal biomass in these associations is increased dramatically. ROS accumulation was detected cytochemically in the endophyte extracellular matrix and at the interface between the extracellular matrix and host cell walls of meristematic tissue in wild-type but not in *noxA* mutant associations. These results demonstrate that not only plant-synthesized ROS but also fungal ROS production is critical in mutualistic fungus–plant interactions, presumably by restricting root colonization.

These examples demonstrate that defense gene activation is an important aspect in beneficial symbioses, which is further highlighted by the identification of genes involved in defense processes in the *P. indica*/*Arabidopsis* screen outlined above. Here, we discuss four defense-related processes which are required for the beneficial interaction between *P. indica* and *Arabidopsis*.

14.2 PYK10

Glucosinolate biosynthesis plays an important role in plant/pathogen interactions (Halkier and Gershenzon 2006). Many genes for glucosinolate biosynthesis and/or degradation are upregulated in *P. indica*-colonized *Arabidopsis* roots. Several of them appear to be involved in establishing a mild defense response against the fungus. One of the genes codes for the putative myrosinase PYK10 (Nitz et al. 1999), an abundant protein in the roots of Brassicaceae. The putative β-glucosidase of 65 kDa is located in so-called endoplasmic reticulum (ER) bodies and contains the ER-retention signal KDEL (Matsushima et al. 2003b). ER bodies are spindle-shaped structures of ~10 μm in length and ~1 μm in width (cf. Matsushima et al. 2003a; Haseloff et al. 1997; Hawes et al. 2001; Hayashi et al. 1999; Ridge et al. 1999) which have been found in more than 50 plant species (Behnke and Eschlbeck 1978; Bones et al. 1989; Bonnett and Newcomb 1965; Gunning 1998; Iversen 1970). ER bodies are surrounded by ribosomes (Hayashi et al. 1999) and are highly enriched in the roots of young seedlings (Matsushima et al. 2002). Interestingly, ER bodies can also be induced in rosette leaves by JA (McConn et al. 1997), and the JA-insensitive *coronatine insensitive1* (*coi1*; Xie et al. 1998) mutant does not form

ER bodies (Matsushima et al. 2002). This suggests that PYK10 might be involved in JA-induced defense responses.

PYK10 has been identified as a target of *P. indica* in *Arabidopsis* roots (Peškan-Berghöfer et al. 2004). Within minutes after the contact of the roots with the fungus, a shift in the electrophoretic mobility of PYK10 can be observed on two-dimensional gels, suggesting that the protein becomes modified in response to signals from the fungus. We identified an EMS and a T-DNA insertion line, which are defective in *PYK10* expression. These mutants do not respond to *P. indica,* indicating that PYK10 is required for the establishment of the beneficial interaction between the two symbionts (Sherameti et al. 2008). This observation is further supported by an independent mutant with a lesion in the transcription factor NAI1. The basic helix-loop-helix domain-containing transcription factor NAI1 is responsible for *PYK10* expression. Closer inspection of plants with altered PYK10 levels uncovered that the putative myrosinase controls the degree of root colonization: lower *PYK10* mRNA levels result in higher root colonization, while plants overexpressing *PYK10* under the control of the 35S promoter are less colonized. Although the physiological role and natural substrate(s) of PYK10 are unknown at present, these observations suggest that enzymatic activities associated with PYK10 may restrict root colonization. Apparently, the beneficial interaction between *Arabidopsis* and *P. indica* is based on a highly sophisticated balance between the two symbiotic partners. It is conceivable that increasing quantities of fungal hyphae lead to a degree of root colonization that provokes plant defense responses and represses beneficial responses, whereas decreasing quantities of hyphae in the root environment result in suboptimal exchanges of information and nutrients between the two partners. This resembles mycorrhizal symbioses, in which initially activated defense responses against the symbiont are reduced during later phases of the interaction or are even actively repressed (cf. Pozo and Azcón-Aguilar 2007). Although not studied in detail, Zeng et al. (2003) have also shown that myrosinase activity controls the growth of ectomycorrhiza fungi.

β-glucosidases and myrosinases hydrolyze β-glucosidic bonds of aryl and alkyl β-D-glucosides, as well as glucosides with carbohydrate moieties such as cellobiose and other β-linked oligosaccharides (Esen 1993). In particular, myrosinases hydrolyze nontoxic glucosinolates to biologically active isothiocyanates, thiocyanates, nitriles, or epithionitriles (cf. Bones and Rossiter 1996; Poulton 1990; Rask et al. 2000; Wittstock and Halkier 2002), and the biological function of a myrosinase depends upon the nature of the aglycon moieties released from the substrates. A well-studied role of these agylcons is their involvement in plant defense against herbivores and microbes (Rask et al. 2000; Stotz et al. 1999, 2000; Tierens et al. 2001; Sanchez-Vallet et al. 2010). PYK10 is released from the endosomal system and reacts with PBP1, forming a multimeric complex. Thus, the substrate(s) of PYK10 is likely to be separated from the enzyme through membranes (cf. references in Nagano et al. 2005), and destruction of the cell and cellular compartments is required to bring these components together. One might speculate that this occurs during root colonization after the two organisms come into contact

with each other. Overcolonization might result in more damage to the root cells and thus more activation of glucosinolate-based defense responses.

Although the role of PYK10 in the interaction between *Arabidopsis* and *P. indica* is unclear at present, the observation that *Arabidopsis* lines with reduced PYK10 protein levels are more susceptible to fungal colonization/association supports the idea that the enzyme is involved in defending the root cells against an excess of invading hyphae, which could result in a disturbance of the balanced mutualistic interaction. PYK10 exhibits striking sequence similarities 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. Thus, PYK10 might have a similar biological function in the *P. indica*/*Arabidopsis* system.

An important task for the future will be to understand the function of PYK10, one of the most abundant protein in Brassica roots. It is not known whether the enzyme has myrosinase activity. Furthermore, the appropriate substrate(s) and product(s) need to be identified.

14.3 ET Signaling Components

ET and JA often function synergistically in plant defense response. Defense genes such as *PLANT DEFENSIN 1.2* (*PDF1.2*) and *PATHOGENESIS-RELATED PROTEIN* (*PR*)*-3* encoding the basic chitinase are activated against necrotrophic fungi primarily by the ET/JA pathway. Both hormones are also required for the ISR which is triggered by beneficial rhizobacteria and fungi (Pieterse et al. 1998; van Wees et al. 2008). In contrast, biotrophic pathogens are more efficiently countered by SA-controlled defense mechanisms (Thomma et al. 1998, 1999) and the activation of *PR-1*, *PR-2*, and *PR-5*.

We identified *Arabidopsis* mutants which are smaller in the presence of the fungus compared to the uncolonized control. This suggests that these mutants consider *P. indica* as a foe and that the interaction is shifted from mutualism to parasitism. Several mutated genes were identified as components of the ET signaling pathway (Camehl et al. 2010).

ET is perceived by a family of membrane-associated two-component systems at the ER, including ETR1/ETR2, ET response sensor (ERS) 1 and 2, and EIN4 in *Arabidopsis* (Chang et al. 1993; Hua et al. 1995; Hua and Meyerowitz 1998; Sakai et al. 1998). EIN2, EIN3, EIN5, and EIN6 are positive regulators of ET responses, acting downstream of CTR1. CTR1 derepresses EIN2, and this leads to the activation of EIN3 and EIN3-like (EIL) transcription factors. EIN2 is an integral

membrane protein of unknown function with similarities to NRAMP metal transporters (Alonso et al. 1999). Growth of *etr1*, *ein2*, and *ein3*/*eil1* plants is not promoted or even inhibited by the fungus. The plants produce less seeds, and the roots are more colonized compared to the wild type roots. This results in a mild activation of defense responses. These results clearly demonstrate that restriction of fungal growth by ET signaling components is required for the beneficial interaction between the two symbionts. Furthermore, overexpression of the *ETHYLENE RESPONSE FACTOR1* (ERF1) constitutively activates defense responses, which also abolishes the benefits for the plants. Therefore, ET signaling components and ET-targeted transcription factors are required for balancing beneficial and non-beneficial traits in the symbiosis. Manipulation of signaling components of this pathway, including crucial target transcription factors, results in an unstable symbiosis which is no longer beneficial for the plant (Camehl et al. 2010).

Several questions remain unanswered. The exact target genes of the ET signaling pathway, which are required for the establishment of the beneficial interaction, are still unknown. Furthermore, it is interesting to note that growth promotion is abolished at the seedlings level. Thus, ET signaling is already required during early phases of the interaction. Again, a link between ET signaling and the control of the growth response is unknown. Finally, we and others have demonstrated that leaves of *P. indica*-colonized plants are more resistant against leaf pathogens. It is likely that the information flow from the roots to the leaves is mediated by a mechanism that resembles an ISR response (Pieterse and Van Loon 2004). Stein et al. (2008) have shown that *P. indica* SAR in *Arabidopsis* requires JA signaling and the cytoplasmic function of NPR1. Since the ISR in *Arabidopsis* depends on ET and JA signaling, a putative role of ET in the *P. indica*-induced ISR needs to be defined.

14.4 OXI1/PDK1

One of the mutants which do not respond to *P. indica* has a lesion in *OXI1*. OXI1 is a serine/threonine kinase which is necessary for oxidative burst-mediated signaling in *Arabidopsis* roots (Anthony et al. 2004; Rentel et al. 2004). The enzyme is a member of the AGC protein kinase family and was originally identified because its expression was induced by H_2O_2 in vivo (Rentel et al. 2004). OXI1 is required for full activation of the two MITOGEN-ACTIVATING PROTEIN KINASES 3 and 6 after treatment with ROS or elicitors and for different ROS-mediated processes including basal resistance to *Peronospora parasitica* infection and root hair growth (Rentel et al. 2004). Besides ROS, OXI1 is also activated by the PHOSPHOLIPID-BINDING KINASE (PDK)1 (Anthony et al. 2004). The main phospholipid in plants is phosphatidic acid (PA) which functions as a second messenger in many stress response pathways. The active OXI1 phosphorylates and thus activates the downstream serine/threonine kinase PTI1-2 in response to ROS and PA signals (Anthony et al. 2006), and many of these signals derive from microbial pathogens

or elicitors, such as cell wall fragments or specific protein factors released by pathogens (van der Luit et al. 2000; Yamaguchi et al. 2005).

P. indica does not induce ROS production in *Arabidopsis* roots (Vadassery et al. 2009a), while the PA level is stimulated. Furthermore, under beneficial, growth-promoting cocultivation conditions, defense genes are downregulated in *Arabidopsis*. Genetic studies established the PA-activated PDK1-OXI1 pathway as a novel signaling event which is crucial for a beneficial interaction between the two symbionts. Thus, like in mammalian systems, this pathway is required for *P. indica*-induced growth promotion and proliferation rather than activation of defense processes. Even under non-beneficial cocultivation conditions of the two symbionts, activation of defense genes is independent of the PA-PDK1-OXI1 pathway. This novel function of the originally identified defense pathway and the role of AGC kinase in beneficial plant/microbe interactions may be of general importance (cf. Pislariu and Dickstein 2007).

14.5 AtHSPRO2

In our screen for *Arabidopsis* mutants which recognizes *P. indica* as a pathogen rather than a beneficial fungus, we identified *hspro2*. HSPRO2 is required for basal resistance against the bacterial pathogen *Pseudomonas syringae* pv. *tomato*. The *Arabidopsis* protein exhibits striking sequence similarities to a nematode resistance protein from *Beta procumbens* (Cai et al. 1997). HSPRO2 appears to function downstream of SA and is negatively regulated by signaling through JA and ET (Murray et al. 2007). We are only at the beginning to understand the role of this protein in pathogenic and beneficial plant–microbe interactions.

14.6 Conclusions

The initially activated defense response of a plant against beneficial microbes resembles that against pathogenic microbes. The same genes and signaling pathways are activated by microbe-associated molecular patterns from pathogens and beneficial fungi. We show that defense gene activation plays a crucial role in the beneficial symbiosis between *Arabidopsis* and *P. indica*. These defense responses appear to function at different levels (Fig. 14.1). Glucosinolates and enzymes involved in their breakdown appear to be activated only after cell damage. This may occur, at least to some extent, if the fungus enters the plant cell. It is conceivable that this defense strategy becomes important if uncontrolled hyphal growth occurs in the roots. Reduced levels of PYK10, for instance, allows overcolonization of the root cells, presumably because the roots cannot restrict hyphal growth. Quite similar, ET signaling controls the interaction between the two symbionts at early stages. Inactivation of components of the ET pathway has a

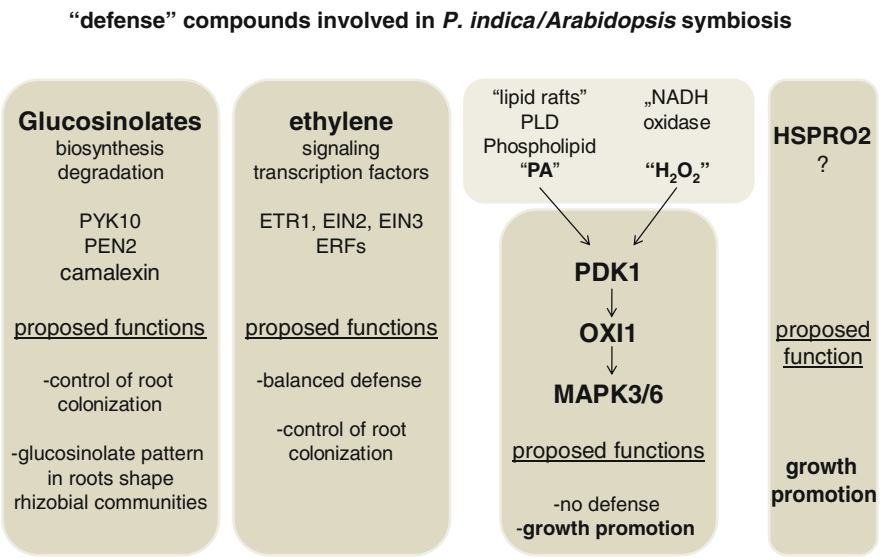

Fig. 14.1 Plant defense compounds identified in the beneficial interaction between *P. indica* and *Arabidopsis*

severe impact on root colonization, whereas higher ET signaling represses and lower ET signaling promotes hyphal growth. Overcolonization of the roots is associated with a mild defense response for the restriction of fungal growth. On the other hand, overexpression of *ERF1* induces a mild defense response, which also restricts root colonization. As a consequence, the roots are less colonized as wild-type roots, which—in turn—is less beneficial for the plant. These two examples support classical concepts developed for mycorrhizal symbiosis that some defense strategies are necessary for long-term harmony between symbionts.

In contrast, the PDK1/OXI1 pathway, previously identified to activate defense responses against pathogens, appears to have a different function in beneficial interactions. The kinases are required for long-term harmony between the two symbionts, and they are not involved in defense gene activation against or restriction of hyphal growth of *P. indica*. Finally, the role of HSPRO2 in the *P. indica*/*Arabidopsis* interaction is unclear. All available information suggests that HSPRO2 has another/additional function than activating defense processes against *P. indica*.

Taken together, a sophisticated network of defense responses which need to be active in a time- and space-dependent manner is required to maintain a beneficial *P. indica*/*Arabidopsis* symbiosis. The relative large number of mutants which have been identified in our screen demonstrates that defense components are crucial for this beneficial symbiosis. While some of them activate mild defense responses, the function of other is not yet understood.

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