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THE CENTRAL ROLES OF IRON AND CALCIUM FOR PLANT/MICROBE INTERACTION AND SHAPING MICROBIAL COMMUNITIES IN THE SOIL

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Abstract

Roots are associated with a large number of different microbes, which can form beneficial, neutral or pathogenic interactions. "Infochemicals" from the microbial community, released into the soil or plants, induce signaling processes in the root cells which determine the fitness of a plant and its response to the environment. The plant integrates the information from the different microbes for an appropriate and balanced response. On the other hand, the microbial community is shaped by signals from the roots. We have established a symbiotic interaction system, in which the information exchange between the model plant *Arabidopsis thaliana* and a beneficial, plant-growth promoting endophytic fungus, called *Piriformospora indica*, can be studied and compared with other root-interacting microbes. Biochemical and molecular-genetic data indicate that two ions, calcium and iron, are major players in determining the fitness of the plant and the response to microbial signals. We will highlight the central role of these two ions in plant/microbe interactions.

Keywords: Arabidopsis, *P. indica*, iron, calcium, siroheme, rhizosphere

1. Introduction

A major determinant for plant growth and performance is the microbial community around the root in the rhizosphere. More than 10 billion microbes (viruses, bacteria, fungi, oomycetes, nematodes, *etc.*) are found in and around the root of a plant. Which of these microbes are present in the soil, and how the microbial community is formed, is dependent on a large number of quite different parameters. Increasing interest in basic science aims to understand of how these communities are shaped. This is not only interesting for basic science, but has an enormous influence on plant productivity, fitness, biomass production and tolerance against biotic and abiotic stresses.

The microbial community in the soil is mainly dependent on the soil itself, the availability of nutrients and water or depositions in the soil, such as toxins. Moreover, in a given environment, exudates from the roots also contribute to the formation of the microbial community in the soil. Roots release exudates which can favor or restrict microbial growth in general or which can target specific microbes within the

community. Furthermore, quorum sensing processes allow the microbes to communicate with each other and to adjust the community to environmental clues. Very little is known about these complex scenarios. Beginning to understand the complex signaling network that shapes the community requires model systems in which specific parameters can be modified in a controlled way, and for which quantitative measuring systems allow to monitor the effects of these changes on the communities. We will discuss a few possible tools for those studies.

The vast majority of all land plants live in a symbiosis with friendly microbes (Harrison 2005). Classical examples are mycorrhizal fungi which colonize the roots of more than 80% of all land plants, but also plant-growth promoting rhizobacteria (Bonfante and Anca 2009). In mutualistic (= friendly) interactions both partners benefit from the symbiosis. In the case of mycorrhiza, the fungi support the roots in having access to the nutrients in the soil. In retreat, they obtain reduced carbon compounds from the photosynthesis of the host. The symbiotic mutualistic interaction has many additional benefits for the plants. For instance, beneficial microbes induce signaling pathways in the plants which establish a better tolerance/resistance against biotic and abiotic stresses.

Many microbes in the soil are also pathogens: Parasitism is a type of symbiotic interaction, where one organism, the parasite, benefits at the expense of the host. Parasites reduce host fitness in many ways, depending on the microbe and the mode of interaction with the host plants. Biotrophic fungi, for instance, benefit from the plants without killing them, whereas necrotrophic fungi induce ultimately host death. A third type of interaction represents commensialism: this is a class of relationship between two organisms where one organism benefits but the other is unaffected (http://en.wikipedia.org/wiki/Commensalis).

1.1 A model fungus: the plant growth-promoting endophyte Piriformospora indica

In order to understand the signaling networks that shape microbial communities in the rhizosphere, we established a model system, that allows to study many aspects of these symbioses with molecular tools. The endophytic fungus *Piriformospora indica* interacts with many plant species including *Arabidopsis thaliana* (cf. below). It colonizes the roots, grows inter- and intracellularly, forms pear-shaped spores within the cortex and extramatrically, and does not invade the endodermis and the aerial parts of the plants. The endophyte promotes nutrient uptake, allows plants to survive under water, temperature and salt stress, confers tolerance to toxins, heavy metal ions and pathogenic organisms and stimulates growth, biomass and seed production (cf. Verma et al., 1998;

Varma et al., 1999, 2001; Sahay and Varma 1999; Oelmüller et al., 2005; Pham et al., 2004a and b; Peškan-Berghöfer et al., 2004; Kaldorf et al., 2005; Shahollari et al., 2005, 2007a, Sherameti et al., 2005, 2008a and b; Vadassery et al., 2008, 2009a, b; Waller et al., 2005, 2008). The host range includes bryophytes (*Aneura pinguis*), pteridophytes (*Pteris ensiormis*), gymnosperms (*Pinus halepensis*), and a large number of angiosperms (Varma et al., 2001; Glen et al., 2002; Selosse et al., 2002a and b; Urban et al., 2003; Peškan-Berghöfer et al., 2004; Weiß et al., 2004; Barazani et al., 2005; Shahollari et al., 2005, 2007a; Sherameti et al., 2005; Waller et al., 2005; Serfling et al., 2007). Most importantly, all known agriculturally important plant species, such as barley, wheat, maize, pea, cotton, Chinese cabbage, sorghum, sunflower *etc.* respond to *P. indica*. Because the fungus has a huge host range, it is likely that the interaction is based on general recognition and signalling processes, and not on processes, which are specific for a particular host.

The fungus was originally isolated from the Indian Thar desert, where it is associated with a number of different xerophytes (Verma et al. 1998; Varma et al. 1999). Later, it was found that *P. indica* lacks host specificity and is cosmopolitan in nature. Sequence analysis of ribosomal DNA (rDNA) regions uncovered that *P. indica* belongs to the family Sebacinaceae, which was recently raised to the order Sebacinales (Weiß et al., 2004; Waller et al. 2008). This basal order of Hymenomycetes (Basidiomycetes) encompasses fungi with longitudinally septae basidia and imperforate parenthesomes (Selosse et al., 2007; Verma et al., 1998; Varma et al., 2001). They also lack cystidia and structures formed during cytokinesis on some basidiomycetous hyphae, the so-called clamp connections. Like other cultivable species of the Sebacinales, *P. indica* forms moniliod hyphae which look like pearls in a chain. Based on this phenotype and rDNA sequence analyses, the endophyte is placed in the polyphyletic genus Rhizoctonia (Selosse et al., 2007).

P. indica is not a unique endophytic fungus with these features. It is believed that approximately half of the members of Sebacinales form beneficial symbioses with different plant species but *P. indica* has obtained attraction because most of the studies were performed with this fungus. Another well characterized member of Sebacinales with similar features is *Sebacina vermifera* (cf. Barazani et al., 2008).

1.2 A model plant Arabidopsis thaliana

In order to understand the molecular basis of the beneficial interaction of *P. indica* with the roots of the different plant species, we have chosen Arabidopsis as an appropriate host. Arabidopsis is not the best host for *P. indica*, since the symbiotic interaction with other plant species is often more beneficial for the plant. However,

Arabidopsis is a model plant in plant physiology. Its entire genome is sequenced, and for almost all genes, knock-out lines are available. This allows an analysis of the role of individual genes/proteins for the interaction with *P. indica*. An incredible amount of molecular and biochemical tools allows to study many aspects of plant development including the interaction with microbes. The international Arabidopsis community has established a dense network that allows the transfer of information and material all over the world (http://www.arabidopsis.org/). Therefore, Arabidopsis provides an ideal model plant to study the beneficial interaction with *P. indica*. The aim of our study is to elucidate general mechanisms, which are required for the establishment of beneficial interactions and which participate in shaping plant communities. Our genetic studies on the model system *P. indica* /Arabidopsis identified two ions, calcium (Ca^{2+}) and iron (Fe = Fe^{2+} and Fe^{3+}), which are crucial for the response of the plant to microbial signals. These ions initiate signalling events in the plant (root) which determine the response of the plant to microbial signals and its interaction with the microbes in the environment. Therefore, the complicated cross-talk between microbes and roots in the rhizosphere can be studied by manipulating specific signalling events in the roots.

2. Iron (Fe)

Everybody is familiar with the fact that Fe is an important ion for live on earth (Briat et al., 2007). Many enzymes require Fe as cofactors, many biochemical pathways are controlled by the availability of Fe in the plant cell, *etc*. Fe deficiency is associated with alterations in several metabolic pathways in both roots and shoots that lead to, among other alterations in metabolic profiles, an increase in CO_2 dark fixation and an accumulation of organic acids in roots, in particular citrate (Thimm et al., 2001; Buckhout and Thimm 2003; Zocchi et al., 2007; López-Millán et al., 2009). The physiological responses to Fe limitation are associated with a shorter lateral root phenotype, or changes in the density, positions and characteristics of root hairs (Landsberg 1986; Schmidt and Schikora 2001). In Arabidopsis, the major morphological response induced by Fe deficiency is the formation of root hairs that are branched at their base (Müller and Schmidt 2004). The important role of Fe for plants is also demonstrated by its requirement for respiration and photosynthesis. Photosystem I has many Fe ions which are required for its activity (Lezhneva et al., 2004; Stöckel and Oelmüller 2004; Schwenkert et al., 2010). Fe limitation restricts photosynthesis, results in a rapid bleaching effect and reduced biomass production. Furthermore, many redox

processes in the plant cell are depending on Fe^{2+}/Fe^{3+} redox reactions. Moreover, reduction of sulfate and nitrate in plants is entirely dependent on the availability of Fe (cf. below). These examples demonstrate that the availability, uptake and distribution of Fe in the plant/cell is crucial and requires elaborated control mechanisms. A major task for the plant is to control Fe homeostasis in the cell, and this is not only essential for the survival, but also fitness, biomass production, fertility, *etc*. of the plant. In addition, our studies demonstrate that Fe homeostasis in the plant cell is an important target of *P. indica* (Figure 1). Microarray analysis of Arabidopsis roots, which were either grown alone or in the presence of *P. indica* have demonstrated that central genes required for Fe homeostasis (Yang et al., 2010) are controlled by *P. indica*. A few of these targets are discussed below.

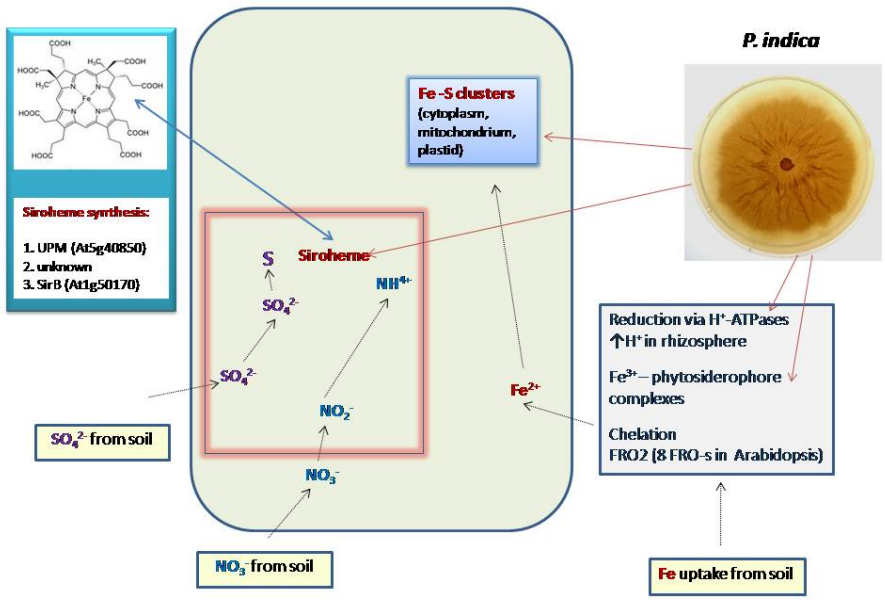

Figure 1. Effects of *P. indica* on iron metabolism and iron-dependent processes in root cells.

2.1 Fe uptake

Understanding plant Fe homeostasis is pivotal for improving crop yields and thus also human nutrition. Plants obtain Fe from the environment by mechanisms which are based on Fe reduction or chelation. Upon Fe deficiency in the root cell, protons are released into the rhizosphere to increase the solubility of Fe, *via* H^+-ATPases of the root plasma membrane. Several Arabidopsis H^+-ATPases are induced in Fe-deficient roots (Santi and Schmidt 2009) and they are targeted by *P. indica*, suggesting that the

fungus participates in or controls Fe uptake under Fe limitation conditions.

After acidification of the soil around the roots, Fe^{3+} is reduced to Fe^{2+} by a membrane-bound ferric reductase oxidase (FRO). Most studies have been performed for FRO2, one of eight FRO proteins in Arabidopsis. The *FRO2* gene is specifically expressed in roots, while others, such as *FRO6* and *FRO7* are preferentially expressed in the aerial parts of the plants. Fe^{2+} is transported into the root by the iron-regulated transporter 1 (IRT1). IRT1 regulation in root cells is quite interesting. The protein is only present under Fe-deficient conditions, even if the gene is expressed at high levels (e.g. in transgenic plants expressing *IRT1* under the control of a strong promoter). Therefore, Fe regulates IRT1 protein biosynthesis posttranscriptionally. Other plants such as grasses have established additional mechanisms to acquire sufficient amounts of Fe. Besides the reduction/chelation process, Fe^{3+} in the apoplast can also be chelated by phytosiderophores, such as mugineic acids, which are released from the root cells into the apoplast. They form Fe^{3+}-phytosiderophore complexes in the apoplast, which are taken up into the roots by an oligopeptide transporter, YELLOW-STRIPE1 (Curie et al., 2001). Although we are only at the beginning to understand how microbes such as *P. indica* control Fe uptake in roots, the available information suggests that beneficial microbes promote Fe uptake.

Regulation of reduction-based Fe deficiency responses was first described for the Fe-inefficient tomato (*Solanum lycopersicum*) mutant *fer* (Brown and Chaney 1971; Brown and Ambler 1974). *fer* plants do not show any of the responses, which are induced by Fe limitations in the wild-type, suggesting that the *fer* mutant is defective in a crucial Fe deficiency regulator. *FER* encodes a basic helix-loop-helix (bHLH) transcription factor that controls the expression of genes with key functions in Fe acquisition, including the tomato orthologs of *IRT1* and *FRO2* (Ling et al., 2002). The Arabidopsis *FER* ortholog, named *FER-LIKE IRON DEFICIENCY-INDUCED TRANSCRIPTION FACTOR* (*FIT*), was shown to control many genes which are regulated under Fe-deficient conditions (Colangelo and Guerinot 2004; Jakoby et al., 2004; Yuan et al., 2005). FIT forms heterodimers with two other bHLH proteins, bHLH38 and bHLH39 (Yuan et al., 2008). All three bHLH genes are Fe responsive, indicating the existence of upstream components in the cascade that mediates the sensing and signaling of Fe deficiency (Wang et al., 2007). There is a huge overlap of Fe-regulated genes with those which are regulated by *P. indica* in Arabidopsis roots, suggesting again that Fe uptake and homeostasis steps are controlled by signals from the beneficial fungus. Here, we described two Fe-containing cofactors, the Fe-S clusters and siroheme, which are

essential for all life on earth and targeted by *P. indica*.

2.2 Fe-sulfur [Fe-S] cluster

Fe-S clusters are prosthetic groups in many proteins involved in respiration and photosynthesis, as well as nitrogen (N) and S metabolism. The cluster is also involved in redox processes, since it can transfer electrons (Leustek et al., 2000; Sato et al., 2004; Kopriva et al., 2007). The biogenesis of the clusters occurs in three steps: (1) release of S from cysteine, (2) co-assembly with Fe on a scaffold protein and (3) transfer of the nascent cluster to the target Fe-S protein. Two types of F-S clusters are known in plants: [2Fe-2S]- and [4Fe-4S]-clusters, for which specific scaffold proteins have been identified. The enzymatic reactions and enzymes involved in Fe-S cluster biogenesis have been best characterized in bacteria and yeast, where at least three different systems for Fe-S cluster biogenesis have been identified. Enzymes involved in Fe-S cluster formation in higher plants are found in the cytoplasm, mitochondrium and plastid. Expression of typical proteins with Fe-S clusters is promoted under beneficial co-cultivation conditions of plants with *P. indica*. This correlates with the observation that restriction of gene expression for those proteins severely inhibits plant growth. As expected, knock-out lines for many genes coding for Fe-S cluster biosynthesis proteins are lethal, therefore a more detailed analysis of the role of these components is difficult. A functional analysis of these proteins is only possible by manipulating their amounts in the cell, and not by deleting them completely. However, EPS measurements and other techniques will help to compare Fe-S clusters in colonized and uncolonized Arabidopsis plants, and to indentify key targets of the fungus in this scenario.

2.3 Siroheme

Siroheme, a tetrapyrolle, is another essential co-factor for many enzymes, including enzymes for N and S metabolism. In higher plants tetrapyrrole synthesis occurs in plastids (Cornah et al., 2003; Raux-Deery et al., 2005), where the final steps are synthesized by three enzymatic reactions: (i) Two methylation steps, (ii) oxidation and (iii) ferrochelatation. The methylation reactions are catalysed by uroporphyrinogen III methyltransferase (UPM, At5g40850, accession number in Arabidopsis). The last step inserts Fe into the center of the tetrapyrrole to form siroheme by sirohydrochlorin ferrochelatase (SirB; At1g50170). The enzyme for the second step is not known in higher plants. Little is known about the regulation of siroheme biosynthesis in higher plants. As expected, knock-out lines for the two known plant enzymes UPM and SirB are lethal. Thus, similar to genes for enzymes of the Fe-S cluster biogeneisis, only manipulation of the *UPM* and *SIRB* expression levels in plants will allow the analysis of the enzymes for siroheme biosynthesis by genetic tools. Interestingly, a

comparative analysis of microarray data from the literature uncovered that many pathogens inhibit and many beneficial fungi promote siroheme biosynthesis, because this is probably central for the control of siroheme-dependent processes described now.

2.4 S and N metabolism depends on siroheme

Live on earth is absolutely dependent on S and N. In plants, S is mainly taken up from the soil as sulphate, the oxidized form of S, before reduction and metabolisation into S-containing compounds (Leustek et al., 2000; Sato et al., 2004; Kopriva et al., 2007). Animals are unable to reduce sulphate and thus require S-containing amino acids (such as cysteine and methionine) or proteins as diet. The S-containing amino acids are distributed to all compartments of the cell, where they can be integrated into proteins or used for the synthesis of other S-containing compounds, such as redox-active peptides or heavy metal-complexing metallothioneins. The presence of S in many redox mediators also highlights its importance for signalling processes (Höfgen et al., 2001; Townsend et al., 2004). Therefore, sulphate assimilation by plants is essential for all life on earth.

Likewise, plants recruit N mainly from the soil as nitrate, which is reduced to ammonium before integration into N-containing compounds. N supply is a limiting factor for plant growth and ultimately for the production of food for heterotrophic organisms (cf. Lawlor 2002; Miller et al., 2007; Leustek et al., 1997; Lillo 2008).

The second steps in the reduction of sulphate and nitrate are mediated by the enzymes sulphite and nitrite reductases (SiR, NiR). Higher plant SiRs and NiRs contain siroheme as prosthetic group which is central to the catalytic activity of the higher plant enzymes (Crane et al., 1997) and catalyze the six electron reduction of sulphite and nitrite, respectively. Thus, assimilation of all inorganic S and the majority of N in the biosphere depends on the availability of siroheme and without siroheme, there would be no reduced S for the synthesis of the amino acids cysteine and methionine and for the biogenesis of Fe-S centers. Interestingly, both SiRs and NiRs also contain Fe-S cofactors (Crane and Getzoff 1996).

S also plays an important role in the biosynthesis of compounds of the secondary S metabolism, such as of glucosinolates. In Brassicaceae, up to 30% of the S can be incorporated into glucosinolates, S-rich plant metabolites that function as S stores or in the defence of plants against pests and pathogens (Halkier and Gershenzon 2006; Bednarek et al., 2009; Clay et al., 2009). It appears that different S-containing antimicrobial substances of the secondary S-metabolism contribute to the resistance against necrotrophic and biotropic fungi, and thus play a central role in shaping microbial communities in the rhizosphere. A role of glucosinolates in beneficial plant/microbe

interactions has also been postulated (Figure 2) (Sherameti et al., 2008). Glucosinolate breakdown products might restrict hyphal growth, which - besides interfering with the spread of pathogens, is also necessary to maintain the interaction of the roots with a beneficial microbe in a beneficial stage.

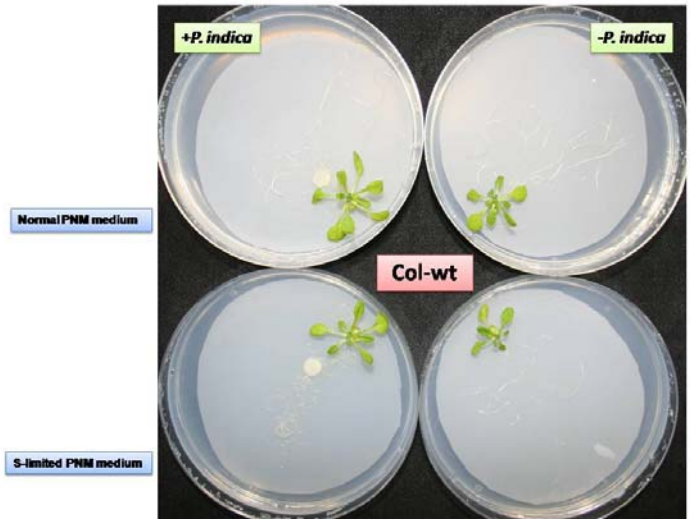

Fig. 2. *Arabidopsis thaliana* Col-wt seeds grown in normal PNM and Sulfur-limitted PNM with/without *P. indica*. After 10 days, seedlings grown in the presence of *P. indica* showed growth promotion response as compared to those grown in the absence of *P. indica*

Recently, the transcription factor SLIM1 has been identified as a central transcriptional regulator of the primary and secondary S metabolism in Arabidopsis (Maruyama-Nakashita et al., 2006). The same gene was identified in a forward screen identified SLIM1 as an essential component in the beneficial interaction between *P. indica* and Arabidopsis (Sherameti et al., unpublished). In the yeast-two hybrid system, SLIM1 also forms dimmers with MYB72, a transcription factor that is required for induced systemic resistance in Arabidopsis (van der Ent et al., 2008; Segarra et al., 2008). Thus, SLIM1 is directly involved in the plant/microbe interaction (cf. Durrant and Dong 2004). It is conceivable, at least for Brassicaceae, that S metabolism is controlled by beneficial microbes through SLIM1 for three reasons: (i) to strengthen plant performance by a better supply of S, (ii) to promote the S-dependent defense machinery for better protection against pathogens and/or to maintain a balanced growth of the beneficial microbe in the host, (iii) and to optimize plant defense against pathogen attack *via* the induced systemic resistance response. Therefore, *P. indica* controls sulfate reduction via the control of Fe-containing cofactors (Fe-S clusters and siroheme) required for SiR, and further metabolism of S via SLIM1.

2.5 Redox regulation and detoxification of the cell require glutathione

One of the primary mechanisms of a cell is to maintain an environmental-dependent balance of the oxidized and reduced form of glutathione to balance the cellular redox state (Meister 1995; Inzé and Montagu 1995). Gutathione is a major S compound in plants and a key component of plant stress responses, since it counteracts oxidative stress or damage. The synthesis of glutathione occurs in two ATP-dependent steps: glutamate-cysteine ligase catalyzes the formation of γ-glutamylcysteine from cysteine and Glu, the rate limiting step in the pathway. Glutathione synthase adds Gly to γ-glutamylcysteine to yield glutathione. The reduced form of glutathione provides a substrate for multiple cellular reactions that yield oxidized glutathione, in which two molecules are linked by a disulphide bridge. Regulation of the glutathione pool is complex and occurs at the transcriptional and post-transcriptional level. Glutathione is also the substrate for metallothioneins, which chelate and detoxify excess heavy metals in the cell. A testable hypothesis could be that beneficial fungi/microbes establish a more reduced atmosphere in a cell (Baltruschat et al., 2008, Vadassery et al., 2008), while pathogens establish a more oxidized atmosphere due to the induction of radical oxygen species. In order to protect the plant cells against these oxidizing agents, they had to develop sophisticated signaling and biochemical pathways.

2.6 Rhizobacteria-mediated N_2 fixation

Fe is also central for rhizobacteria-mediated N_2 fixation in legumes, since leghaemoglobin and the nitrogenase contain huge amounts of Fe. While the Fe-containing heme cofactor of leghaemoglobin is synthesized by the bacterium, the apoprotein is of plant origin. Furthermore, the nitrogenase consists of two subunits: subunit 1 contains MoFe cofactors and subunit 2 Fe cofactors. Thus, the availability of huge amounts of Fe and the proper insertion into the proteins is a prerequisite for the function of a symbiotic nodule.

Taken together, control of Fe homeostasis is central for every organism. If a beneficial fungus manages to control Fe homeostasis, this can be considered as a clever strategy to get major cellular processes in the plant under fungal control. Besides the control of Fe uptake, the two co-factors Fe-S cluster and siroheme are important checkpoints, since many essential downstream reactions depend on them. This only describes the role of Fe within a plant cell: there is an additional level of complexity in the rhizosphere, where Fe functions as a crucial sensor and regulator for the microbial communities. The availability of Fe and the accurate control of its cellular level is probably one of the most important

tasks in a rhizospheral community. Inbalances in Fe homeostasis in the cells (and in the rhizosphere) has severe consequences for all partners in the soil community.

3. Calcium metabolism

Since Ca^{2+} is a second messenger in cells, all living cells have to regulate the intracellular Ca^{2+} concentration very carefully. In contrast to mammalian cells, plant cells have cell wall and contain 1000 - 10.000 times higher Ca^{2+} concentrations than the concentration in the cell. Since many signals from the environment/apoplast/rhizosphere induce cytosolic Ca^{2+} ($[Ca^{2+}]_{cyt}$) elevations, it is obvious that a highly sophisticated regulatory mechanism must exist that couple environmental signals to appropriate intracellular Ca^{2+} responses. Mutant analyses have shown that these processes determine the plant response to environmental signals, and shape the microbial community around the root. We describe molecular tools, which may help to understand the role of Ca^{2+} scenarios in and around a root cell and we will also highly the important role of this second messenger for plant performance.

As mentioned above, plant roots interact with many different microorganisms in the soil, resulting in mutualistic (beneficial for both partners), commensalistic (beneficial for the host, but not invader) or pathogenic (harmful for the host or both partners) interactions. Different interactions could induce $[Ca^{2+}]_{cyt}$ which is one of the earliest physiological response in cells. Microbes release various factors into the soil which are necessary for their recognition by plant cells (Johnson and Oelmüller 2009; Kogel et al., 2006; Paszkowski 2006). In pathogenic interactions, chitins, glucans, lipids, fatty acids, (glycol-)proteins or peptides activate defense gene expression in the plant cells (Cui et al., 2009; Hématy et al., 2009; Zipfel 2009). In beneficial symbiotic interactions a close physical association of the microorganism with the plant induces cytoplasmic Ca^{2+} signals which activate responses beneficial for both organisms (Hématy et al., 2009; Zipfel 2009). Establishment of a beneficial symbiosis is not always as harmonious as it appears, and a rejection of the invading symbiont can occur at many stages (Harrison 2005; Rodriguez et al., 2009). For a mutualistic interaction a molecular dialogue between the partners is essential (Reinhardt 2007; Requena et al., 2007). In rhizobial symbiosis the roots produce flavonoids while the bacteria releases nodulation (Nod) factors (lipochito-oligosaccharide), which initiate signaling in the host cells (Long 1989; Lerouge et al., 1990). In arbuscular mycorrhizal symbiosis plants release strigolactones which act as branching factors for fungal hyphae, while the fungi release so-called mycorrhizal (MYC) factors. Besides mycorrhiza (Akiyama and Hayashi 2006), beneficial interactions also occur with endophytic fungi (Rodriguez et al., 2009) and plant-growth-promoting bacteria (Mantelin and Touraine

2004) which colonize the host´s root. The mode of recognition and early signaling steps in the plants helps them to differentiate between a beneficial and a detrimental microbe. As far as we know, a very early event in the interaction of pathogenic, mycorrhizal or endophytic microbes with a plant cell is an increase in the intracellular Ca^{2+} levels within seconds or minutes after the recognition of the two partners (Harper and Harmon 2005; Charpentier et al., 2008; Mazars et al., 2009; McAinsh and Pittman 2009). This raises the question of how this Ca^{2+} information is decoded into the appropriate responses in the plant cell.

3.1 Ca^{2+} signalling in recognition of microbes

The Ca^{2+} ion is a classical second messenger in many plant signalling pathways and couple extracellular stimuli to intracellular and whole-plant responses. In all living cells, the cellular Ca^{2+} level is tightly regulated (Sanders et al., 2002). Small changes in its concentration provide information for protein activation, signaling and the activation of many plant responses. The Ca^{2+} concentration depends on the balance between influx and efflux processes. In eukaryotic cells, various stimuli mobilize different pools of Ca^{2+} to trigger characteristic changes in the cytoplasmic Ca^{2+} ($[Ca^{2+}]_{cyt}$). Ca^{2+} channels are located in the plasma membrane, vacuolar membrane, ER, chloroplast, mitochondria and nuclear membranes of plant cells. Upon activation of the channels, the Ca^{2+} level can change rapidly in particular cellular positions (Lecourieux et al., 2002), e.g. at the plasma membrane, in the vicinity of the endoplasmatic reticulum (ER), around or in the nucleus, *etc*.

The induced Ca^{2+} signature of a given signal is characterized by its amplitude, duration, frequency and location, and encodes a message that contributes to the specific physiological response. The presence of the large number of Ca^{2+} sensors in plant cells is required to decode different incoming stimuli. $[Ca^{2+}]_{cyt}$ elevation may be caused by an uptake of Ca^{2+} from the apoplast/rhizosphere/extracellular medium, or by Ca^{2+} mobilization from internal stores such as the ER or ER-derived membrane systems or organelles (McAinsh and Pittman 2009; Sanders et al., 2002; Lecourieux et al., 2002; McCormack et al., 2005). The origin of Ca^{2+} signals is important in the physiological response. Specificity in the Ca^{2+}-signalling system results from a multifactorial decision process ranging from a specific Ca^{2+} signature to the availability of Ca^{2+} sensors and Ca^{2+} -binding proteins that are coupled to downstream components (Kiegle et al., 2000; van der Luit et al., 1999).

Changes in Ca^{2+} levels in the cells can be experimentally monitored in many ways. Highly sophisticated techniques have been evolved to determined the level of this ion by

Ca^{2+} -binding dyes, such as fura-2 (Paredes et al., 2008). Many Ca^{2+} signalling studies in plant cells are also performed using the aequorin technology which is based on bioluminescence and allows measurement of the level of the second messenger Ca^{2+}. Aequorin is a Ca^{2+} binding photoprotein found in jellyfish composed of an apoprotein (apoaequorin) and a prosthetic group (Knight et al., 1991), a luciferin molecule, coelenterazine. In the presence of molecular oxygen the functional holoprotein aequorin reconstitutes spontaneously. The protein contains three EF-hand Ca^{2+} binding sites. When these sites are occupied by Ca^{2+}, aequorin undergoes a conformational change and behaves as an oxygenase that converts coelenterazine to excited coelenteramide, which is set free together with carbon dioxide. As the excited coelenteramide relax to the ground state, blue light at a wavelength of 469 nm is emitted. This light can be easily recorded with a luminometer and quantified (Mithöfer and Mazars 2002).

Pathogens activate basal defense responses in the plant cell through receptors that recognize and bind microbe-released elicitors. Downstream signaling activates defense responses for innate immunity. Basal defense does not prohibit initial colonization of pathogen but inhibits its further spread. After receptor activation, the signaling pathway leading to defense activation consists of ion fluxes at the plasma membrane (H^+/Ca^{2+} influxes, K^+/Cl^- effluxes), an oxidative burst and the activation of a mitogen-activated protein kinase (MAPK) pathway. Ca^{2+} elevations can be induced by an oligopeptide elicitor pep-13 in parsley cell cultures (Blume et al., 2000), flg22 in Arabidopsis leaf discs (Blume et al., 2000), ß-glucan fragments in soybean cell cultures (Mithöfer et al., 1999) and by many proteinacoeus elicitors (like cryptogein) or oligosaccharide elicitors. Rapid and transient elevations in $[Ca^{2+}]_{cyt}$ (Lecourieux et al., 2002) were also shown to be induced by diffusible molecules released by arbuscular mycorrhizal fungi (Navazio et al., 2007).

The majority of all plant/fungus interactions are peaceful and the microbes do not cause diseases. More than 85% of all land plants are believed to form mycorrhiza. In this type of beneficial symbiosis, the microbe also induces cytoplasmic Ca^{2+} elevation in the host cell. Therefore, Ca^{2+} signaling may play a dual role in plant/microbe interactions. During early phases of the establishment of a beneficial interaction, i.e. before the fungus delivers nutrient to the roots and is accepted as a beneficial partner (Harrison 2005), the plant responds to the microbe by activating a mild defense response. These events lead to defense processes which are similar in beneficial and pathogenic interactions (Blume et al., 2000; Belkhadir et al., 2004) and probably activated by the same or similar signaling pathways in the root cell. In beneficial plant/microbe interactions, fungus-derived elicitors, often released into the rhizosphere, induce early signaling events in the plant cell which are mostly unknown. In

addition, both $[Ca^{2+}]_{cyt}$ and nuclear Ca^{2+} elevations are crucial in establishing the benefits for the plants. Early signal transduction during rhizobacteria-mediated nodule and mycorrhiza formations in legumes are activated by ion fluxes across different membranes of the host cell. For instance, nodulation (Nod) factors trigger $[Ca^{2+}]_{cyt}$ influx at the root hair tip within 1 min (Ehrhardt et al., 1996; Felle et al., 1999; Miwa et al., 2006), perinuclear Ca^{2+} oscillations with a delay of 10 to 30 min (Ehrhardt et al., 1996) and the transcription of symbiosis-related plant genes (Felle et al., 1999; Cardenas et al., 1999). $[Ca^{2+}]_{cyt}$ elevation is initially caused by an uptake from cell-external stores (Ehrhardt et al., 1996; Felle et al., 1999; Pichon et al., 1992). Subsequent Ca^{2+} oscillations around and in the nucleus appear to depend on Ca^{2+} stores in cisterns of the ER and the nuclear envelope. Genetic studies have demonstrated that perinuclear Ca^{2+} oscillations are induced by Nod factor-like molecules without induction of Ca^{2+} influx (Oldroyd and Downie 2006), suggesting that the Ca^{2+} influx across the plasma membrane and the perinuclear Ca^{2+} spiking are two distinct responses. For instance, the *Lotus japonicus* mutants *castor* and *pollux* are unable to form both bacterial and fungal symbioses and impaired in the perinuclear Ca^{2+} spiking (Walker et al., 2000), but they retain the Ca^{2+} influx at the root hair tip. Thus, more than one Ca^{2+}-dependent process appears to be involved in the formation of beneficial symbiosis between microbes and plants (Miwa et al., 2006).

Ca^{2+} is also a major player in the interaction between *P. indica* and Arabidopsis. One of the earliest signaling events during the recognition of the two symbionts is a rapid induction of $[Ca^{2+}]_{cyt}$ elevation, which is followed by a nuclear Ca^{2+} response. Since several mutants which do not respond to *P. indica* are also impaired in $[Ca^{2+}]_{cyt}$ elevation (Vadassery et al., 2009), the beneficial effects for the plants appear to be linked to Ca^{2+}. $[Ca^{2+}]_{cyt}$ elevation can be induced by a component present in an autoclaved cell wall extract (CWE) from *P. indica*, or by a component in the exudate fraction released into the rhizosphere (Figure 3). Since these components also promote plant growth, they can be considered as active stimuli (Figure 4). This is consistent with the observation that root colonization by the living fungus is not required for the beneficial response in the plant. The $[Ca^{2+}]_{cyt}$ response shows a maximum after 2 minutes. The same exudate preparations also induce a Ca^{2+} response in tobacco roots, which supports the idea that the plant response is not host-specific. Ca^{2+} influx is prevented by the general serine/threonine protein kinase inhibitor staurosporine indicating that phosphorylation changes may be involved upstream of the Ca^{2+} response (Vadassery et al., 2009). The involvement of receptors is further proved by the refractive nature. Plant

cells lose their capacity to respond a second time to the same type of elicitor (refractive behaviour), but remain sensitive to another type of elicitor perceived by another receptor.

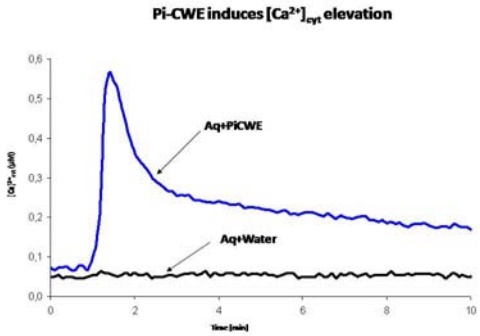

Figure 3. *P. indica*-CWE induces cytosolic Ca^{2+} elevation in apo-aequorin (Ca^{2+} sensor apoprotein) transformed *Arabidopsis thaliana* roots. Transgenic Arabidopsis roots with reconstituted aequorin have a basal $[Ca^{2+}]_{cyt}$ level between 0.04 to 0.06µM. When the CWE was added, a rapid and transient elevation of the $[Ca^{2+}]_{cyt}$ upto 0.6µM was observed in 1-2 minutes after initial lag phase. Then the concentration was declined gradually and reached at its basal level after 80-90 minutes of CWE application. Sterilised double distilled water was used as control.

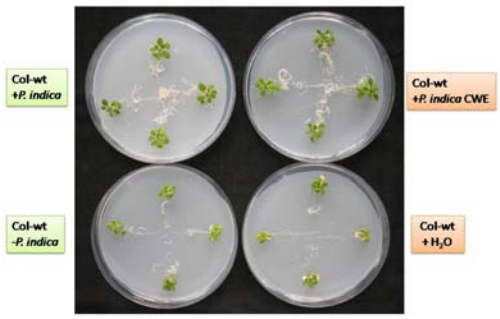

Figure 4. Both *P. indica* and *P. indica*-CWE promotes growth in *Arabidopsis thaliana* seedlings. The CWE was applied directly on the roots. The fractions of *P. indica*-CWE which induced $[Ca^{2+}]_{cyt}$ level in roots could also promote the growth of the plant as the fungus does. Seedlings without the fungus and also treated with sterilised double distilled water served as control.

These characteristic features of Ca^{2+} responses are also known from pathogenic plant/microbe interactions. The refractory nature and the inhibition of $[Ca^{2+}]_{cyt}$

elevations in the presence of kinase inhibitors suggest the involvement of a receptor upstream of the Ca^{2+}response (Navazio et al., 2007; Vadassery et al., 2009). The concept implies that a Ca^{2+} channel is opened after receptor activation and a short signal transduction chain, which may include phosphorylation events around the plasma membrane. This holds true for beneficial and pathogenic interactions as well, which raises the question how the plant decodes the Ca^{2+} signature for appropriate responses.

3.2 Downstream events in Ca^{2+} signaling in plant/microbe interaction

Phosphorylation is a key process in defence responses downstream of the elicitor-induced Ca^{2+} influx. Protein phosphorylation changes are observed for MAPK after the applications of pathogen-derived elicitors, such as flg22 (Dietrich et al., 1990), but also after application of the elicitor fraction from *P. indica*. The occurrence of a nuclear Ca^{2+} elevation in response to *P. indica* (Vadassery et al., 2009) hints to the involvement of an additional Ca^{2+} response, similar to the observations in legumes. The maximum $[Ca^{2+}]_{cyt}$ elevation occurs after 2 min (Charpentier et al., 2008), while the response in the nucleus is only detectable after 6 min, suggesting a sequential response.

3.3 Future strategies for Ca^{2+}-dependent processes in Arabidopsis

The complexity of Ca^{2+} signaling, the huge number of Ca^{2+}-decoding Ca^{2+}-binding proteins and the specificity of the response to incoming signals makes it difficult to assign a specific Ca^{2+}-dependent signaling process to a specific response. Therefore, the most obvious strategy to unravel this complexity requires genetic tools. For model organisms, mutants can be isolated and the mutated genes identified. Many pathogens interact with Arabidopsis and many mutants are available which are impaired in their response to these pathogens. They can be tested in the beneficial *P. indica*/Arabidopsis system. Although a massive defense response has never been observed in Arabidopsis plants exposed to *P. indica*, a constitutive, long-lasting mild defense response might be required for restricting root colonization. Furthermore, many Arabidopsis mutants are also available which are defective in beneficial responses to *P. indica* (Sherameti et al., 2008). Identification of the mutated genes let to the description of novel components and signaling pathways which are involved in beneficial symbioses.

P. indica provides a nice model system for Ca^{2+} studies. Testing of available mutants, and generating new mutants, which are impaired in inducing $[Ca^{2+}]_{cyt}$ and/or nuclear Ca^{2+}elevations in response to signals from pathogenic and beneficial fungi might help to understand the complexity of plants interacting with microbes. The *P. indica*/Arabidopsis system might help to

understand the dual role of Ca^{2+} in beneficial and non-beneficial traits in beneficial plant/fungus interactions. Ca^{2+} might activate two independent signaling pathways leading to defense gene activation and the establishment of a beneficial interaction, there might be a cross-talk between these two pathways, or the pathways might overlap and recruit the same Ca^{2+}-dependent signaling compounds. It is conceivable that a sophisticated balance between defense responses and beneficial responses is required, and that imbalances shift the mode of interaction from mutualism to parasitism or *vice versa*. Furthermore, depending on the response of the plant to the elicitors from different types of microbes in the soil, the roots establish a response pattern, that has an enormous influence of the microbial community in the soil. Deep sequencing techniques are available now to test how biochemical pathways and (secondary) metabolites in roots can influence the microbial community in the rhizosphere, and how this influences plant fitness and biomass production.

4. Conclusion

In conclusion, we propose a model in which the concentration of two ions in the root cells, Fe and Ca, are crucial determinants for the symbiosis of plants and microbes in the rhizosphere. Signals from the microbial environment of the root induce plant responses *via* these two ions, which are important for the fitness of the plant and its performance. The Fe- and Ca^{2+} -induced biochemical changes in the plant result in the production of signals which are released from the plant/root and shape the microbial community in the rhizosphere. Basic science will help to understand the information flow between the different organisms. Besides this task, understanding of these processes allows their manipulation, and this will provide us with tools to improve agriculture under different nature-given conditions.

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