Piriformospora indica released factors and its role in the molecular interaction with *Arabidopsis thaliana*

Dissertation

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Jyothilakshmi Vadassery

geboren am 01. 06. 1979 in Kerala, India

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Manuscript Overview

Manuscript I

A cell wall extract from the endophytic fungus *Piriformospora indica* promotes growth of *Arabidopsis* seedlings and induces intracellular calcium elevation in roots

Jyothilakshmi Vadassery, Stefanie Ranf, Corinna Drzewiecki, Axel Mithöfer, Christian Mazars, Dierk Scheel, Justin Lee, Ralf Oelmüller
The Plant Journal (2009) *In Press*

This manuscript describes the isolation of a growth-promoting factor from the cell wall of *P. indica*. The factor or cell wall extract induces a transient cytosolic Ca^{2+} ($[Ca^{2+}]_{cyt}$) elevation in the *Arabidopsis* and tobacco roots expressing the Ca^{2+} bioluminescent indicator aequorin. We demonstrate that cellular $[Ca^{2+}]$ elevations are early events in the interaction between the plant growth-promoting fungus *P. indica* and *A. thaliana* and are crucial for growth promotion. The extract and the fungus also induce a similar set of genes in *Arabidopsis* roots, among them are genes with Ca^{2+} signalling-related functions. Nuclear Ca^{2+} transients were also observed in tobacco BY-2 cells. Inhibition of the Ca^{2+} response by staurosporine and the refractory nature of the Ca^{2+} elevation suggest that a receptor may be involved. The CWE does not stimulate H_2O_2 production and the activation of defence gene expression, although it led to phosphorylation of mitogen-activated protein kinases (MAPKs) in a Ca^{2+}-dependent manner. Thus, Ca^{2+} is likely to be an early signalling component in the mutualistic interaction between *P. indica* and *A. thaliana*.

Ralf Oelmüller and I designed all the experiments. Axel Mithöfer, Justin Lee and Dierk Scheel co-supervised the experiments. I isolated the cell wall extract from *P. indica*, measured cytosolic Ca^{2+} elevation, and performed all Ca^{2+} experiments including expression and microarray analysis. Christian Mazars performed the nuclear Ca^{2+} measurement. Stefanie Ranf and Justin Lee did MAPK phosphorylation assay and Corinna Drzewiecki did the MAPK6 growth assay. Ralf Oelmüller and I wrote the manuscript. All the authors read the manuscript and provided their suggestions.

Manuscript II

The Role of Auxins and Cytokinins in the Mutualistic Interaction between *Arabidopsis* and *Piriformospora indica*

Jyothilakshmi Vadassery, Claudia Ritter, Yvonne Venus, Iris Camehl, Ajit Varma, Bationa Shahollari, Ondrej Novák, Miroslav Strnad, Jutta Ludwig-Müller, and Ralf Oelmüller
Molecular Plant Microbe Interaction (2008), 21(10), 1371-83.

In this manuscript we explore the role of phytohormones, auxin and cytokinin, in the interaction between *Piriformospora indica* and *Arabidopsis thaliana*. The endophytic fungus *P. indica* stimulates *A. thaliana* growth and reproduction. The fungus produces low amounts of auxins, but the auxin levels and the expression of auxin-regulated genes are not altered in colonized roots. However, the fungus rescues the dwarf phenotype of the auxin over-producer *sur1-1* by converting free auxin into conjugates, which also results in the downregulation of the auxin-induced *IAA6* gene. The fungus produces relatively high levels of cytokinins, and the cytokinin levels are higher in colonized roots compared with the uncolonized controls. *trans*-Zeatin cytokinin biosynthesis and the CRE1/AHK2 receptor combination are crucial for *P. indica*–mediated growth stimulation, while mutants lacking *cis*-zeatin, impaired in other cytokinin receptor combinations, or containing reduced cytokinin levels respond to the fungus. Since root colonization is not affected in the cytokinin mutants, we propose that cytokinins are required for *P. indica*–induced growth promotion. Finally, a comparative analysis of the phytohormone mutants allows the conclusion that the response to *P. indica* is independent of the architecture and size of the roots.

Ralf Oelmüller and I designed the experiments to analyse the function of cytokinin. Ondrej Novák and Miroslav Strnad measured the cytokinin by LC-MS. I did all the cytokinin experiments. Claudia Ritter, Yvonne Venus, Iris Camehl, Ajit Varma, Bationa Shahollari and Jutta Ludwig-Müller were associated with the auxin part of the story.

Manuscript III

A leucine-rich repeat protein is required for growth promotion and enhanced seed production mediated by the endophytic fungus *Piriformospora indica* in *Arabidopsis thaliana.*

Bationa Shahollari, Jyothilakshmi Vadassery, Ajit Varma and Ralf Oelmuller.
The Plant Journal (2007) 50(1), 1-13.

In this manuscript we identified a previously unknown function of a leucine rich protein (LRR2). We used cellular and molecular responses initiated during the establishment of the interaction between *P. indica* and *Arabidopsis* roots to isolate mutants that fail to respond to the fungus. An ethyl-methane sulfonate mutant (*Piriformospora indica*-insensitive-2; *pii-2*), and a corresponding insertion line, are impaired in a leucine-rich repeat protein (At1g13230). The protein *pii-2*, which contains a putative endoplasmic reticulum retention signal, is also found in Triton X-100-insoluble plasma membrane microdomains, suggesting that it is present in the endoplasmic reticulum/plasma membrane continuum in *Arabidopsis* roots. The microdomains also contain an atypical receptor protein (At5g16590) containing leucine-rich repeats, the message of which is transiently upregulated in *Arabidopsis* roots in response to *P. indica*. This response is not detectable in At1g13230 mutants, and the protein is not detectable in the At1g13230 mutant microdomains. Partial deactivation of a gene for a sphingosine kinase, which is required for the biosynthesis of sphingolipid found in plasma membrane microdomains, also affects the *A. thaliana*/*P. indica* interaction. Thus, pii-2, and presumably also At5g16590, two proteins present in plasma membrane microdomains, appear to be involved in *P. indica*-induced growth promotion.

I conducted the analysis of sphingokinase genes and its role in interaction. I identified homozygous knock out lines and conducted growth promotion assays. Ralf Oelmuller and Bationa Shahollari designed the experiments and Bationa Shahollari conducted all the other experiments.

1. Introduction

Plant roots interact with a wide array of micro organisms in soil, with interactions being beneficial or pathogenic to the plants. In its broadest sense, symbiosis refers to organisms living together, whether the interaction is mutualistic, commensal or parasitic. Plant endosymbioses are characterised by the penetration of living plant cells by a microbial symbiont, followed by a period during which the symbiont lives partially or entirely within plant cells (Parniske, 2000). Endosymbiotic interactions play a significant role in agriculture and natural ecosystems. The evolution of plant-fungal symbiosis 460-480 million years ago is one of the key innovation that enabled plants to colonize the land (Heckman *et al.,* 2001). Most of the land plants (80-90%) have established a symbiotic association with arbuscular mycorrhizal (AM) fungi, which is an intricate association of plant roots with fungi belonging to the order Glomales of the Zygomycotina. The AM fungi are obligate symbionts and assist plants with acquisition of mineral nutrients, particularly phosphorus. In return, up to 20% of plant-fixed carbon is transferred to the fungus (Parniske, 2008). A more recent symbiosis between rhizobial bacteria and legumes evolved 60 million years ago and results in nitrogen fixation. One obstacle in the molecular analyses of beneficial plant/microbe interactions is the lack of genomic information for most plant species that form either bacterial or fungal symbiosis. *Arabidopsis thaliana*, a common model to study plant development at the molecular level, does not belong to the hosts of mycorrhizal fungi or rhizobial bacteria but can interact with *Piriformospora indica*.

1.1 *Piriformospora indica* – a plant growth promoting fungus

Piriformospora indica (*P. indica*) is a root-interacting endophytic fungus discovered in the Indian Thar desert in close association with the spores of AM fungi, *Glomus mosseae* (Varma *et al.,* 1999). Analysis of taxonomic position by molecular methods, based on 18S rRNA sequences and electron microscopy, suggests that this fungus belongs to the group of Sebacinaceous fungi related to the Hymenomycetes of the Basidiomycota (Verma *et al*., 1998). Sebacinales, the most basal Basidiomycota group with known mycorrhizal members are ubiquitously distributed and are found on all continents in temperate and subtropical climates associated with orchids, liverwort thalli and Ericaceae as ectomycorrhizal and endomycorrhizal fungi (Selosse *et al.,* 2007). AM fungi are obligate biotrophs and cannot be cultured without the plant while ericoid and ectomycorrhizal fungi can be grown in pure

culture, but their host spectrum is restricted to the Ericaceae or woody plants. In contrast, *P. indica* can be easily cultivated in axenic culture where it produces chlamydospores (Peškan-Berghöfer *et al*., 2004; Shahollari *et al*., 2005, 2007). The fungus is able to associate with the roots of various plant species including *A. thaliana*, *Nicotiana* sp., barley, rice, wheat and promotes plant growth and seed production (Peškan-Berghöfer *et al*., 2004, Barazani *et al.,* 2005, 2007, Waller *et al*., 2005, Varma *et al.,* 1999). The lack of a species specificity in the fungal host selection points to the ancient origin of the interaction. The fungus is also involved in providing systemic resistance against powdery mildew fungi, *Blumeria graminis* f.sp. *hordei*, root rot fungi, *Fusarium culmorum* in barley and *Golovinomyces orontii* in *A. thaliana* (Schäfer *et al.,* 2007). The role of *P. indica* in conferring drought and salt tolerance is also reported (Sherameti *et al.,* 2008, Baltruschat *et al.,* 2008).

1.2 Molecular basis of the interaction between *P. indica* and *A. thaliana*

The mutualistic *A. thaliana* – *P. indica* association is a new model system for the elucidation of the molecular mechanisms responsible for host recognition, root colonization and subsequent beneficial activities accompanied by microbial plant symbiosis (Fig. 1). The symbiosis results in morphological, physiological and molecular changes in host plants (Peskan-Berghöfer *et al*., 2004). *P. indica* colonization is seen in the root epidermal and cortical tissue and grows inter- and intracellularly forming pear shaped spores. Unlike the AM symbiosis, the growth promoting effect initiated by *P. indica* is accompanied by a co-regulated stimulation of enzymes involved in nitrate and starch metabolisms (Sherameti *et al.,* 2005). The introduction of proteomic approaches combined with ethyl-methane sulfonate (EMS) mutagenesis has led to the identification of several *P. indica* responsive *Arabidopsis* proteins like a MATH [meprin and tumor necrosis factor receptor-associated factor (TRAF) homology] domain containing protein (Oelmüller *et al*., 2005), a leucine-rich repeat protein LRR2 (Shahollari *et al*., 2005, 2007) and PYK10, a β-glucosidase located in the endoplasmic reticulum (Sherameti *et al.,* 2008). These proteins are expressed during early interaction stages and are crucial for growth promotion response. Unlike the plant signalling pathway nothing is known about the fungal released factors and the role they play in this interaction. The current thesis seeks to identify such factors that are released by the fungus, signalling pathways they activate and the role they play in the interaction.

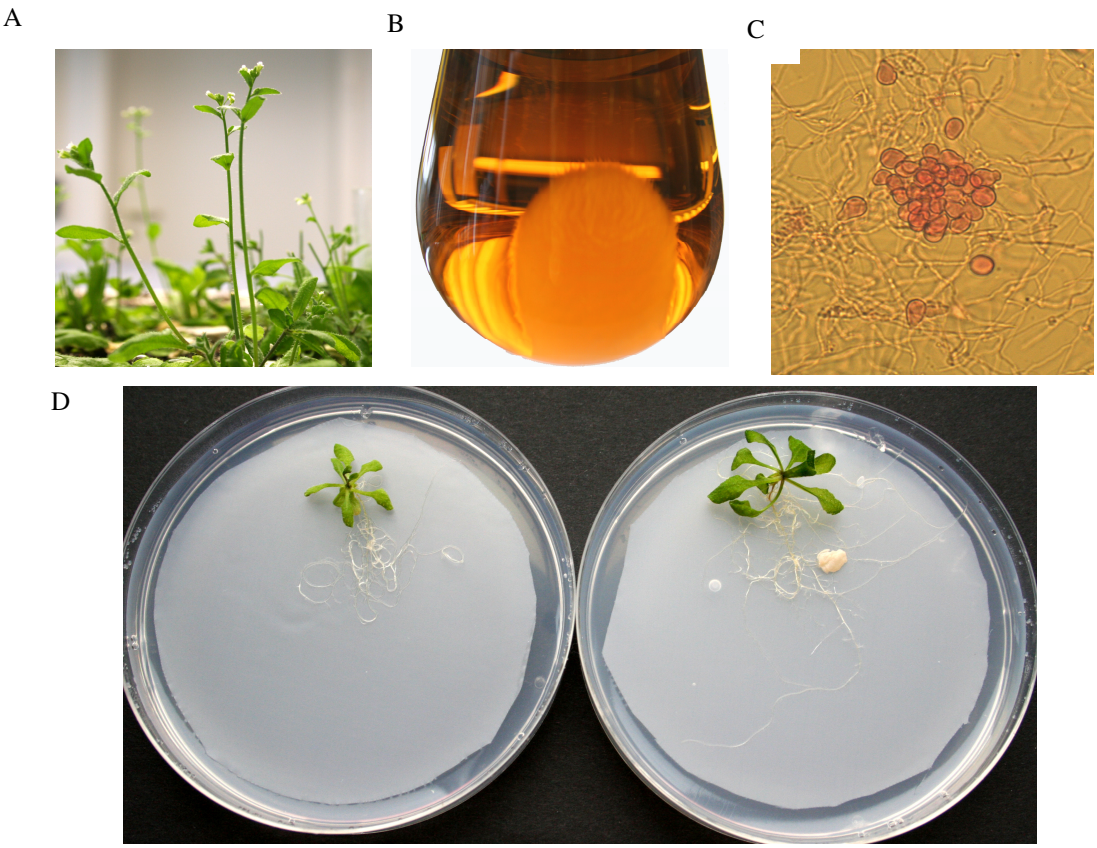


Figure 1. *A. thaliana* and the growth promoting fungus *P. indica*

A. *A. thaliana*

B. *P. indica* axenic culture in KM medium

C. Pear shaped spores of *P. indica*

D. Molecular interaction between *A. thaliana* and *P. indica* resulting in growth promotion.

1.3 Plant recognition of microbial factors

Microbes release various factors necessary for its recognition by plant cells. In pathogenic fungi they are chitin, glucan or protein by nature, which activates defence gene expression on recognition by plant cells. In contrast to this struggle between plants and pathogens, the course of a symbiotic interaction is less contentious and leads to a close physical association of symbiotic micro organism and plants. However, the interaction is not as harmonious as it superficially appears, and a rejection of the invading symbiont can occur at any stage of the infection. For successful, infection a molecular dialogue is essential which is a two way process. In rhizobial symbiosis plant roots produce flavonoids while the bacteria

releases nodulation (Nod) factors (lipochito-oligosaccharide), which initiate signalling. In AM symbiosis plants release strigolactones, which acts as branching factor for fungal hyphae, while the fungus releases the unidentified MYC factor.

In most cases, interaction between plants and microbes do not cause disease. The basal defense of plants against potential pathogens is activated in most cases through receptor-mediated recognition of PAMPs/MAMPs (Pathogen / Microbe Associated Molecular Patterns) and downstream signalling to activate innate immune responses. Basal defence does not prohibit pathogen colonization but only controls its spread and is temporally slower and of lower amplitude and would be activated in most of the interactions, be it pathogenic or symbiotic. (Belkhadir *et al.,* 2004). Downstream of the receptor, the signal chain of events leading to defense-related gene activation and phytoalexin accumulation consists of ion fluxes at the plasma membrane (H^+/Ca^{2+} influxes, K^+/Cl^- effluxes), an oxidative burst and MAPK activation (Blume *et al*., 2000). During compatible interactions, pathogen-derived effector/virulence molecules suppress PAMP-induced defense responses, and enable the pathogen to overcome basal resistance and to successfully infect the plant (Espinosa *et al*., 2003; Kim *et al*., 2005; He *et al*., 2006).

1.4 Calcium signalling – a versatile cellular second messenger

The cellular calcium (Ca^{2+}) levels are tightly regulated and even a small change in the cytosolic concentration provides information for protein activation and signalling. One of the earliest responses of a plant cell to incoming stimuli is the activation of the Ca^{2+} response and Ca^{2+} ion is a second messenger in numerous plant signalling pathways, coupling extracellular stimuli to intracellular and whole-plant responses (Sanders *et al*., 2002). Changes in cytosolic free Ca^{2+} ($[Ca^{2+}]_{cyt}$) occur in response to many biotic and abiotic signals, such as light (Lewis *et al.*, 1997; Sai *et al.*, 2002; Baum *et al.*, 1999), low and high temperature (Pleith *et al.*, 1999), touch (Knight *et al.*, 1991), or drought (Knight *et al*., 1997). The biotic signals include phytohormones such as abscisic acid and gibberellins (Gilroy and Jones, 1992; McAinsh *et al.*, 1992), fungal/oomycete elicitors (Knight *et al*., 1991; Mithöfer *et al.*, 1999; Blume *et al.,* 2000; Lecourieux *et al.*, 2002) or Nod factors (Ehrhardt *et al.*, 1996; Müller *et al.*, 2000). The Ca^{2+} signature of a given signal, characterized by its amplitude, duration, frequency, and location, was shown to encode a message that, after decoding by downstream effectors, contributes to the specific physiological response. This explains the presence of increased

number of Ca^{2+} sensors in plant cells to decode different incoming stimuli. $[Ca^{2+}]_{cyt}$ elevation may be caused by an uptake of Ca^{2+} from the extracellular medium, or by Ca^{2+} mobilization from organelles, and/or by both. The origin of Ca^{2+} signals is important in the physiological response (Kiegle *et al*., 2000; van der Luit *et al.,* 1999).

Most Ca^{2+} signalling studies in plant cells are performed using the aequorin technology based on bioluminescence. Aequorin is a Ca^{2+} binding photoprotein found in jellyfish composed of an apoprotein (apoaequorin) and a prosthetic group, 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 into excited coelenteramide, which is set free together with carbon dioxide. As the excited coelenteramide relax to the ground state, blue light (λ = 469 nm) is emitted. This emitted light can be easily detected with a luminometer (Mithöfer & Mazars, 2002).

1.5 Ca^{2+} as a secondary messenger in symbiotic signalling

Ca^{2+} is an important signalling component that is also activated by incoming symbionts. Nod factors are bacterial lipochito-oligosaccharide signals that play an important role in the early stages of nodule development (Dénarié *et al*., 1996). An early event in this recognition of diffusible Nod factor is triggering of Ca^{2+} elevation. In *Medicago truncatula* it occurs in two phases, the first phase consists of a rapid spike followed by a sustained $[Ca^{2+}]_{cyt}$ increase or plateau that lasts for 3–4 min. Approximately 10 min later, the second phase occurs which consists of "Ca^{2+} spiking" in the nuclear region. A typical spike in *M. sativa* or *M. truncatula* consists of a rapid increase of $[Ca^{2+}]_{cyt}$ (approximately 500 nM) followed by a more gradual return to resting levels. This produces an asymmetric peak with a sharp rising phase and a slower recovery (Ehrhardt *et al.*, 1996). Similar Ca^{2+} elevation has also been found to be crucial for the initiation of mycorrhizal symbiosis. Rapid and transient elevations in $[Ca^{2+}]_{cyt}$ were shown to be induced by diffusible molecules released by AM fungi, indicating that they are perceived by host plant cells through a similar Ca^{2+}-mediated signalling.(Navazio *et al*., 2007)

Large-scale mutant screens have enabled the isolation of genes, which are pre-requisite for rhizobial symbiosis. The most prominent among them are the *Does Not make Infection* (*DMI*) genes. The Nod factor is perceived by a receptor that is a heterodimer of two classes of receptor-like kinases that contain LysM domains in the extracellular region (Limpens *et al*., 2003; Madsen *et al*., 2003; Radutoiu *et al*., 2003). Functioning downstream of these LysM receptor-like kinases are DMI1, a putative cation channel, and DMI2/ NORK/SYMRK/, another receptor-like kinase with Leucine-rich repeat domains in the extracellular portion. DMI3 functions downstream of Ca^{2+} spiking and is a chimeric Ca^{2+}/calmodulin-dependent protein kinase (CCaMK). The identity of DMI1 and DMI3 shows the relevance of Ca^{2+} signalling in symbiotic interactions with its possible role in Ca^{2+} signal generation and decodification. The three *DMI* genes that are essential for rhizobial Nod factor signal transduction are also required for the symbiosis with AM fungi and are referred as *SYM* genes, and mutations in all these genes fail to allow entry of the fungus into the cortex (Catoira *et al.*, 2000). The CCaMK mutations that disrupt symbiotic interactions with bacteria and mycorrhizal fungi are the first loss-of-function mutant phenotypes identified, and these studies provide strong genetic corroboration for an essential role of Ca^{2+} signalling in initiating symbiotic interactions (Fig. 2; Oldroyd *et al.,* 2005; Harper *et al.*, 2005).

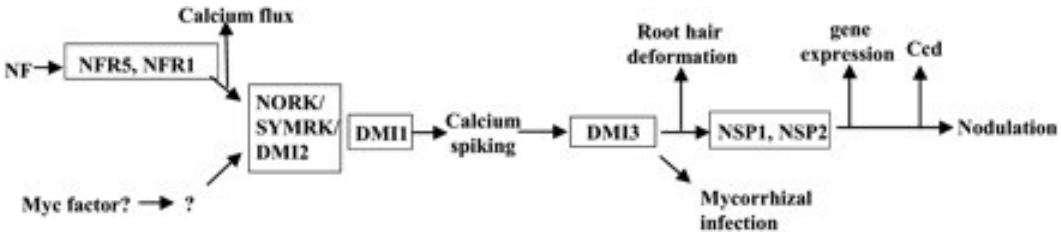


Figure 2. The Nod factor and mycorrhizal signalling pathways. This signalling pathway has been defined through genetics in the model legumes *M. truncatula* and *L. japonicus* and *Medicago sativa*. The genes identified are defined in boxes. Mutations in all these genes have been characterized for calcium spiking except SYMRK. In addition, it has been shown that mutations in *NFR5* and *NFR1* lack the calcium flux, whereas mutations in *DMI1* and *DMI2* show the first phase of the flux response. Components of the Nod factor (NF) signaling pathway are conserved with mycorrhizal signalling (Oldroyd *et al.,* 2005).

1.6 Cytokinin signalling in plants

Cytokinins (CKs) are essential plant hormones that are master regulators of plant growth and development. They are adenine derivatives carrying either an isoprene-derived or an aromatic side chain at the N^6 terminus. CK plays a crucial role in regulating the proliferation and differentiation of plant cells, and also controls various processes in plant growth and development, such as delay of senescence, control of shoot/root balance, transduction of nutritional signals, and increased crop productivity (Sakakibara**,** 2006). Root apical meristems are the major sites of CK synthesis in plants and are transported to shoots *via* xylem. Cytokinin oxidase degrades CK irreversibly and plays a role in regulation of hormone levels. Both isoprenoid and aromatic CKs are naturally occurring, with the former more frequently found in plants and in greater abundance than the latter. Common natural isoprenoid CKs are *N*6- (2-isopentenyl)-adenine (iP), *trans*-zeatin (tZ), *cis*-zeatin (cZ), and dihydrozeatin (DZ). Among them, the major derivatives generally are tZ and iP as well as their sugar conjugates, but there are variations depending on plant species, tissue, and developmental stage. For instance, tZ- and iP-type CKs are the major forms in *Arabidopsis*, whereas substantial amounts of cZ-type CKs are found in maize, rice, and chickpea. Two classes of isopentenyl transferases (IPT) catalyse the first steps in CK biosynthesis (Kakimoto, 2001; Takei *et al*., 2001; Miyawaki *et al.*, 2006; Sakakibara, 2006): ATP/ADP IPTs are responsible for isopentenyladenine (iP) and *trans*-type zeatin synthesis, and tRNA-IPTs are required for *cis*-type zeatin synthesis (Sakakibara**,** 2006). Isopentenyl adenine (iP) and *trans*-zeatin (tZ) predominantly originate from the methylerythritol phosphate (MEP) pathway, whereas a large fraction of the *cis*-zeatin (cZ) side chain is derived from the mevalonate (MVA) pathway. cZ and tZ can be enzymatically interconverted by zeatin *cis-trans* isomerase.*Trans*-isomers are physiologically active in plants, while *cis*-zeatins have been less studied, although they also appear to be active (*cf.* Suttle and Banowetz, 2000; Yonekura-Sakakibara *et al.*, 2004). The *Arabidopsis* CK signal transduction pathway is a multistep two-component pathway in which hybrid histidine protein kinases (AHKs) serve as cytokinin receptors and histidine phosphotransfer proteins (AHPs) transmit signal from AHKs to nuclear response regulators (ARRs),which activate or repress transcription.In *Arabidopsis* three genes encode cytokinin receptors: AHK2, AHK3, and CRE1/AHK4.

1.7 CK signaling in symbiotic interaction

Phytohormones, which are key endogenous plant signals are known to be involved in symbiosis. Shaul-Keinan *et al*. (2002) have shown that plants possessing mycorrhiza accumulate high concentrations of iP-like compounds in roots and shoots. Differences between mycorrhizal and non-mycorrhizal plants in total ZR-like compounds have also been reported (van Rhijn *et al*., 1977; Ginzberg *et al*., 1998). CKs act as suppressive factors of chitinase activity (Shinshi *et al*., 1987), and some authors suggest that elevated levels of CKs in mycorrhizal roots could suppress the induction of some PR-protein genes, specifically chitinase and glucanase genes (Spanu *et al*., 1989; Shaul *et al*., 2000). More recently, the central role of CKs for rhizobacteria-induced nodule formation in legumes has been demonstrated for *Medicago truncatula* and *Lotus japonicus* (Murray *et al*., 2007; Tirichine *et al*., 2007). Some plant associated bacteria and fungi produce and secrete CK and/or cause the plants to synthesise them. Infection of plants with these microbes can induce tissues to divide and in some cases form special structures like galls

Murray *et al.* (2007) and Tirichine *et al.* (2007) demonstrated that activation of a CK receptor, *Lotus* histidine kinase 1 (LHK1), is both necessary and sufficient for nodule organogenesis (Figure 3). This paves way for an important function of cytokinin receptors in nodule development. Perception of Nod factor by the plant is one of the first steps during the interaction between root cells and rhizobial bacteria. Because Nod factor accumulates in cell walls, it is highly unlikely that it can traverse the epidermis to induce responses in root cortical cells. It is possible that the localized production of CKs follows Nod factor perception, acting as a mechanism to coordinate epidermal and cortical responses during nodule formation. In this model, activation of Nod factor signalling at the epidermis leads to increased localized production of CKs. CKs are then transported to cortical cells where they are perceived by LHK1 at the cell surface. This initiates cell division, leading to formation of the nodule primordium. This model predicts that rhizobial bacteria induce CK production in legume roots or redirect CK transport, and also predicts that CCaMK-induced spontaneous nodulation should require LHK1 (Oldroyd, 2007).

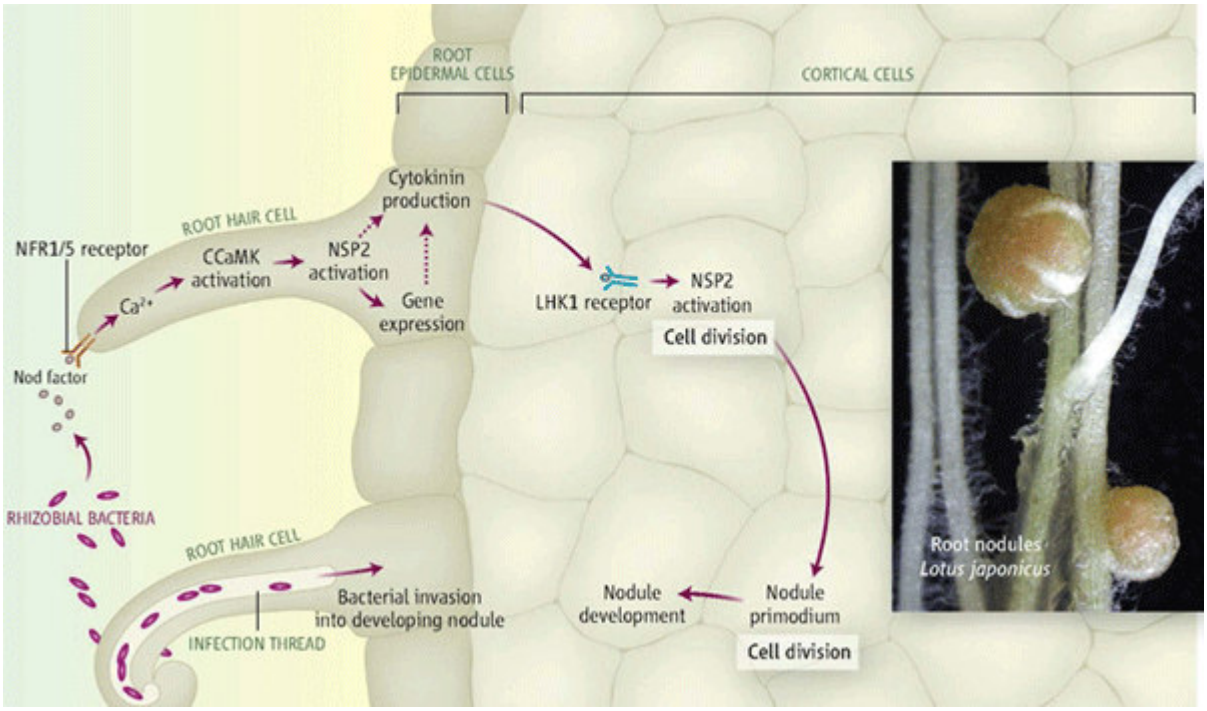


Fig 3. Nod factor, which is released by rhizobial bacteria, is perceived by the plant root epidermis. This triggers a calcium-dependent signalling pathway and the production of the hormone CK. Localized increase in CK may activate cortical cell division, leading to formation of the nodule primordium. Rhizobial bacteria infect the nodule through infection threads that are initiated in root hair cells and invade the developing nodule. From Oldroyd (2007). Copyright © by the American Association for the Advancement of Science.

These results suggest that both Ca^{2+} and CK signalling might play a role in symbiotic interactions and I investigated their involvement in the *P. indica – A. thaliana* symbiosis.

Sphingokinases in *P.indica – A. thaliana* interaction

Sphingolipids are ubiquitous and essential components of eukaryotic cells that were classically viewed as structural components of the membrane. However, it is now clear that sphingolipids and their metabolites are also dynamic regulators of many cellular processes. In particular, studies have shown that sphingolipids control crucial events in mammalian cells that determine normal development and fate of living organisms, including proliferation, differentiation and apoptosis. Far less is known about sphingolipid functions in plants, but recent studies indicate that they have important signalling roles in plants as well. For example, sphingosine-1-phosphate plays a role in Ca^{2+}-mediated guard cell closure, a sphingosine transfer protein is involved in programmed cell death, and plant resistance to fungal toxins is mediated by a plant orthologue of a yeast gene involved in ceramide synthesis (Dunn *et al.*,

2004). Triton-X-100-insoluble plasma membrane microdomains contain sphingolipids and their metabolite sphingosine-1-phosphate is a potent lipid mediator in animal, yeast and plant cells, and is generated by the enzyme sphingosine kinase.

In animals, sphingosine-1-phosphate (S1P) has well-defined roles in cell signalling, especially in the control of growth and survival. It is synthesized from sphingosine by sphingosine kinase (SPHK), and is broken down by S1P phosphatase (SPPASE), the activity of which produces sphingosine. A range of extracellular signals increase SPHK activity with consequent increases in intracellular S1P levels. There is evidence that once S1P is produced it can bring about direct intracellular responses, including an increase in cytosolic calcium concentration $[Ca^{2+}]_{cyt}$. It can also be exported to elicit responses at the extracellular surface of the cell in which it was synthesized, or in other cells located nearby. It does this by binding to a family of receptors (S1P receptors) that are part of the G protein-coupled receptor (GPCR) superfamily (Worrall *et al*., 2008).

The objectives of this thesis were to

1) Identify factors released by *P. indica* and their recognition by root cells,
2) Study the role of $[Ca^{2+}]_{cyt}$ elevation in the *P. indica – A. thaliana* interaction,
3) Test whether CKs of either fungal or plant origin are involved in growth promotion, and
4) To define the role of sphingosine kinase in the *P. indica*-induced growth response.

Manuscript 1 describes how a cell wall extract from *P. indica* promotes plant growth and induces $[Ca^{2+}]_{cyt}$ elevation in roots. Mutants, which do not respond to the fungus, also do not respond to the cell wall extract. We propose that $[Ca^{2+}]_{cyt}$ elevation in root cells might be a key response during early phases of recognition between the two symbionts.

Manuscript 2 describes the role of phytohormones cytokinin (CK) and auxin in the molecular interaction between *A. thaliana* and *P. indica*. We demonstrate that *P. indica* produces CK, however it appears that tZ produced by *Arabidopsis* is crucial in the interaction.

Manuscript 3 describes that a leucine-rich repeat protein is crucial in growth promotion and seed production. We demonstrate that leucine-rich proteins are located in lipid rafts and that sphingosine kinase is required for the growth response.

2.1 **Manuscript I**

The Plant Journal (2009) *In Press*

A cell wall extract from the endophytic fungus *Piriformospora indica* promotes growth of *Arabidopsis* seedlings and induces intracellular calcium elevation in roots

Jyothilakshmi Vadassery[1], Stefanie Ranf[2], Corinna Drzewiecki[1], Axel Mithöfer[3], Christian Mazars[4], Dierk Scheel[2], Justin Lee[2], Ralf Oelmüller[1]

[1]Friedrich-Schiller-Universität Jena, Institut für Allgemeine Botanik und Pflanzenphysiologie, Dornburger Str. 159, D-07743 Jena, Germany

[2]Leibniz-Institut für Pflanzenbiochemie, Abt. Stress- und Entwicklungsbiologie, Weinberg 3, D-06120 Halle/Saale, Germany

[3]Max Planck Institute for Chemical Ecology, Department Bioorganic Chemistry, Hans-Knöll-Straße 8, D-07745 Jena, Germany

[4]Signaux et Messages Cellulaires chez les Végétaux, UMR CNRS/UPS 5546; BP 42617 Auzeville, 31326 Castanet-Tolosan; France

Key words: *Arabidopsis*, *Piriformospora indica*, plant/microbe interaction, calcium, aequorin

Correspondence author
Dr.Ralf Oelmüller
Phone: 49-(0)3641-949231
Fax: 49-(0)3641-949232
Email: b7oera@uni-jena.de

Abstract

Calcium (Ca^{2+}), as a second messenger, is crucial for signal transduction processes during many biotic interactions. We demonstrate that cellular [Ca^{2+}] elevations are early events in the interaction between the plant growth-promoting fungus *Piriformospora indica* and *Arabidopsis thaliana*. A cell wall extract (CWE) from the fungus promotes growth of wild-type seedlings, but not of seedlings from *P. indica*-insensitive mutants. The extract and the fungus also induce a similar set of genes in *Arabidopsis* roots, among them genes with Ca^{2+} signalling-related functions. The CWE induces a transient cytosolic Ca^{2+} ($[Ca^{2+}]_{cyt}$) elevation in the roots of *Arabidopsis* and tobacco plants, as well as in BY-2 suspension cultures expressing the Ca^{2+} bioluminescent indicator aequorin. Nuclear Ca^{2+} transients were also observed in tobacco BY-2 cells. The Ca^{2+} response was more pronounced in roots than in shoots and involved Ca^{2+} uptake from the extracellular space as revealed by inhibitor studies. Inhibition of the Ca^{2+} response by staurosporine and the refractory nature of the Ca^{2+} elevation suggest that a receptor may be involved. The CWE does not stimulate H_2O_2 production and the activation of defence gene expression, although it led to phosphorylation of mitogen-activated protein kinases (MAPKs) in a Ca^{2+}-dependent manner. The involvement of MAPK6 in the mutualistic interaction was shown for an *mpk6* line, which did not respond to *P. indica*. Thus, Ca^{2+} is likely to be an early signalling component in the mutualistic interaction between *P. indica* and *Arabidopsis* or tobacco.

Introduction

An important aspect in plant survival is early detection of and rapid response to specific stimuli. Ca^{2+} signalling, which can be activated within sec (or min) in response to quite diverse sets of stimuli (Harper and Harmon, 2005; Carafoli, 2002), is fitting as part of the early signalling system. Changes in cytosolic free $[Ca^{2+}]_{cyt}$ occur in response to many biotic and abiotic signals (Sanders *et al.*, 2002), such as light (Lewis *et al.*, 1997; Sai *et al.*, 2002; Baum *et al.*, 1999), low and high temperature (Pleith *et al.*, 1999), touch (Knight *et al.*, 1991), or drought (Knight *et al*., 1997). The biotic signals include phytohormones such as abscisic acid (ABA) and gibberellins (Gilroy and Jones, 1992; McAinsh *et al.*, 1992), fungal/oomycete elicitors (Knight *et al*., 1991; Mithöfer *et al.*, 1999; Blume *et al.,* 2000; Lecourieux *et al.*, 2002) or nodulation (Nod) factors (Ehrhardt *et al.*, 1996; Müller *et al.*, 2000). Ca^{2+} acts as a secondary messenger in plant cells and links different input signals to many diverse and

specific responses. The specificity in the Ca^{2+} signalling system is based on multifactorial processes, which start with a specific Ca^{2+} signature and the availability of a specific set of Ca^{2+} sensors and ends with target genes and proteins that activate precise downstream events (Sanders *et al*., 2002; Lecourieux *et al.*, 2006). The source of the Ca^{2+} contributing to the $[Ca^{2+}]_{cyt}$ rise (apoplasts or internal stores or both) is thought to be important for the physiological response (Knight *et al.*, 1991, 1997; van der Luit *et al*., 1999).

In symbiotic interactions a rapid Ca^{2+} influx and an oscillation in nuclear Ca^{2+} levels ($[Ca^{2+}]_{nuc}$), termed Ca^{2+} spiking, are one of the earliest responses to Nod factors (Shaw and Long, 2003; Ehrhardt *et al.*, 1996) and mutant analyses have demonstrated that the Ca^{2+} signalling is central to rhizobial and arbuscular mycorrhizal symbiosis (Harrison, 2005). Genetic studies have demonstrated that a single Ca^{2+}/calmodulin dependent protein kinase, DMI3/CCaMK is responsible for decoding both Nod and possible Myc-factor-induced Ca^{2+} oscillations (Lévy *et al*., 2004; Mitra *et al*., 2004; Kanamori *et al*., 2006). It is located in or at the nucleus, suggesting that besides changes in the cytosol, also nuclear Ca^{2+} levels are important for a proper response in the symbiosis (Oldroyd and Downie, 2006).

The endophyte *Piriformospora indica* colonizes the roots of many plant species including *Arabidopsis thaliana* and promotes their growth, development and seed production. The fungus stimulates nutrient uptake and confers resistance to various biotic and abiotic stresses (cf. Verma *et al.*, 1998; Varma *et al.*, 1999, 2001; Sahay *et al.*, 1999; Pham *et al.*, 2004; Peškan-Berghöfer *et al.*, 2004; Shahollari *et al.*, 2005, 2007; Sherameti *et al.*, 2005, 2008a and b; Waller *et al.*, 2005). *P. indica* is a cultivable fungus and can grow on synthetic or complex media without a host (Varma *et al.*, 2001; Peškan-Berghöfer *et al*., 2004). Since *P. indica* can colonize the roots of many plant species including trees, agri- and horticultural and medicinal plants, mono- and dicots and even mosses (Varma *et al.*, 2001; Glen *et al*., 2002; Peškan-Berghöfer *et al*., 2004; Weiss *et al.*, 2004; Barazani *et al.*, 2005, 2007; Shahollari *et al*., 2005, 2007; Sherameti *et al*., 2005; Waller *et al*., 2005), the interaction between the symbiotic partners should be based on general recognition and signalling processes.In this paper, we report the isolation of a fraction from the cell wall (CW) of *P. indica*, which can mimic the presence of the fungus in the initial stages and induce growth promotion. This fraction is also able to induce a $[Ca^{2+}]_{cyt}$ elevation in plant roots stably expressing the Ca^{2+} bioluminescent indicator aequorin. This may point to a role of Ca^{2+} in the early signalling events between *P.*

indica and *Arabidopsis thaliana*, similar to the well-characterized legume-rhizobia and arbuscular mycorrhizal symbiosis.

Results

A cell wall extract (CWE) from *P. indica* promotes growth in *Arabidopsis* seedlings

We have previously reported that the endophytic fungus *P. indica* interacts with *Arabidopsis* roots and promotes growth and seed production (Shahollari *et al.*, 2007). Here we demonstrate that components extracted from the fungal cell wall (CW) are also effective in stimulating growth in *Arabidopsis* seedlings. The active fraction was obtained from *P. indica* grown in liquid medium. Fungal CW was isolated and autoclaved to release CW-associated factors. A heat-stable fraction is able to stimulate root and shoot growth when applied to the roots (**Fig. 1a-c)**. The overall growth promotion after 10 days was in the range of 18±1%, and root growth (21±1%) was more promoted by the extract than shoot growth (15±1%). The stimulatory effect on growth is weaker when compared to the growth promotion induced by the fungus, which is 36±1% (**Fig. 1c**). Furthermore, 12 days after the application of the CWE, no more growth stimulation can be detected, while the fungus still promotes growth (Supplemental Fig. 1). A second application of 50 µl CWE 6 days after the first application resulted in prolonged growth promotion for 14 days and more (**Fig. 1b**) but could not reach the fungal growth promotion levels.

The transcript levels for a leucine-rich repeat protein (LRR1; Shahollari *et al.,* 2007) and for the 2-nitro-propane-dioxygenase (NPDO; Sherameti *et al.,* 2005) are up-regulated in colonized *Arabidopsis* roots compared to the non-colonized controls. The same genes are also up-regulated after the application of the CWE (**Fig. 1d**). Furthermore, microarray analysis with roots exposed to either the fungus or the CWE for 2 days demonstrated that a subset of the regulated genes responded to both treatments. This includes *MIOX* for an enzyme involved in ascorbate biosynthesis (Kanter *et al*., 2005; Lorence *et al.,* 2004) and *CIPK13* (for a Ca^{2+} sensor) **(Fig. 1d)**. To further test the specificity of the growth promoting effect of the CWE, the *P. indica-insensitive* mutants (*pii*)-3 and -4, which grow like uncolonized plants in the presence of the fungus, were tested for their response to the CWE. The two mutants also failed to respond to the CWE (**Fig. 1e and f**) and the *P. indica* inducible genes were not upregulated (Supplemental Fig. 2). This suggests that the molecular mechanism for CWE-mediated growth promotion is similar to that obtained during *P. indica* colonization.

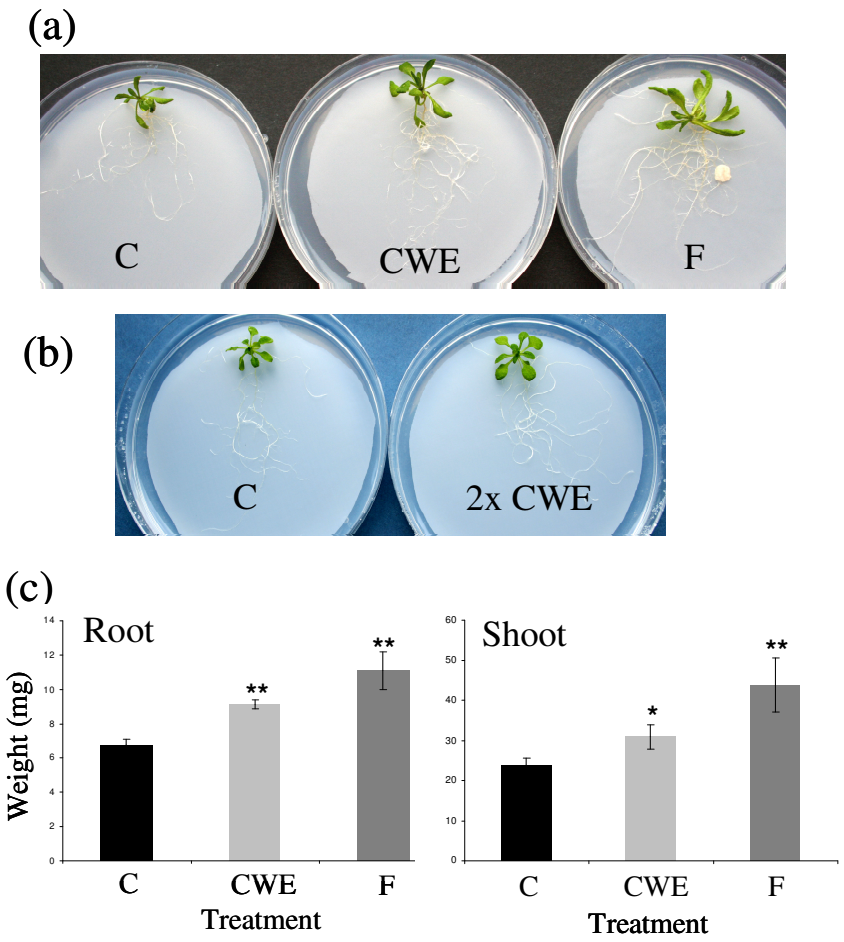


Figure 1

The effect of a CWE from *P. indica* on *Arabidopsis* growth and gene expression.

(a) The effect of *P. indica*, co-cultivated with *Arabidopsis* seedlings for 10 days (F) or of 65 µl CWE, applied to the seedlings for 10 days (CWE), on growth. (C), untreated control.

(b) The effect of two applications (50 µl each) of the CWE at day 1 and 6 on growth (2x CWE). (C), untreated control.

(c) Quantified data for shoots and roots. 65 µl of CWE or a fungal plug (F) were added to the seedlings after 1 day of growth on PNM medium. The figure shows root and shoot fresh weights from seedlings 10 days after the treatments. They were identical in sizes and weights at the beginning of the experiments (i.e. 0 days). Bars represent SE. *, **; statistically significant differences between treated and non-treated plants (unpaired student *t test*, *, $P<0.05$, **, $P<0.01$).

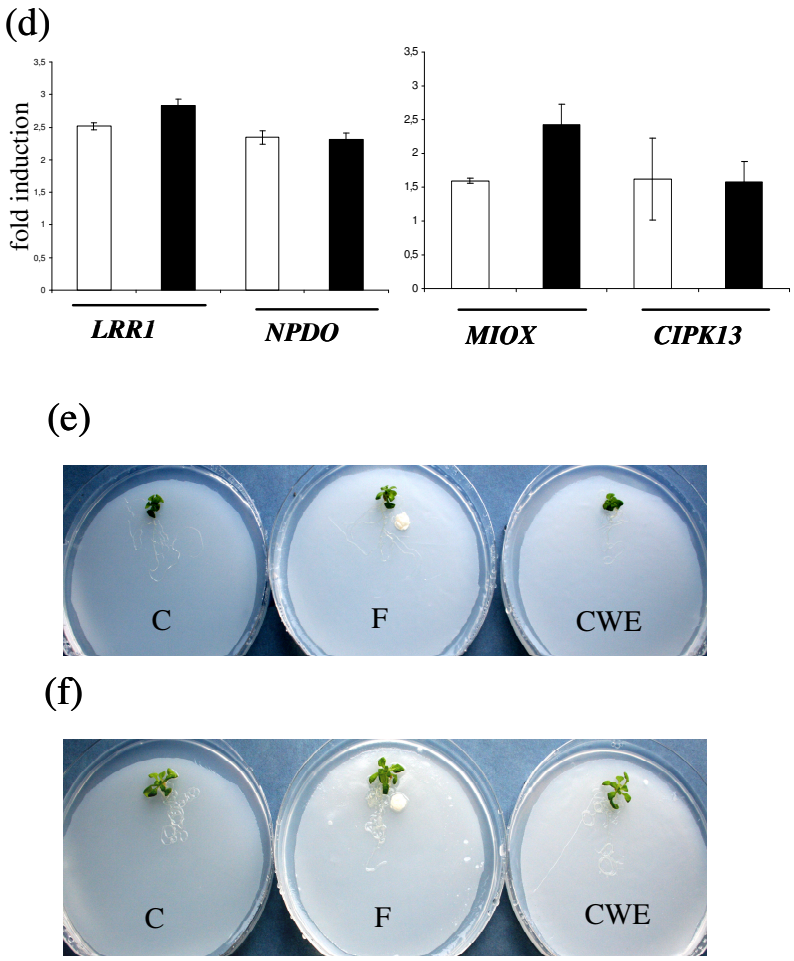


Figure 1 The effect of a CWE from *P. indica* on *Arabidopsis* growth and gene expression.

(d) The response of various genes to treatments with *P. indica* or the CWE in *Arabidopsis* roots. *Arabidopsis* seedlings were grown in the absence or presence of the fungus, or treated with the CWE for 2 days on PNM medium. Relative transcript abundance in roots was determined by real-time PCR analysis and normalised to the plant *GAPDH* mRNA level. The graph shows x-fold induction of the mRNA levels by the fungus (white) or CWE (black) relative to the levels in the untreated control roots.

(e, f) The *P. indica-insensitive* mutants *pii-3* (e) and *pii-4* (f) do not respond to the CWE. For details, cf. (a).

The CWE from *P. indica* induces cytosolic Ca^{2+} elevation in *Arabidopsis* roots

In transgenic *Arabidopsis* plants expressing the Ca^{2+} sensor aequorin, changes in $[Ca^{2+}]_{cyt}$ can be monitored by aequorin-mediated light emission (Knight *et al.*, 1997). A resting $[Ca^{2+}]_{cyt}$ of 80±10 nM was measured in transgenic *Arabidopsis* lines. When the CWE was added to *Arabidopsis* roots after 1 min of measurement, a rapid and transient elevation of the intracellular Ca^{2+} concentration was observed (**Fig. 2a**). After a lag phase of 20±5 sec, the level of $[Ca^{2+}]_{cyt}$ begins to rise and a sharp Ca^{2+} peak of 207±14 nM at 55±5 sec was observed. This was followed by a sustained increase that declines gradually and showed a $[Ca^{2+}]_{cyt}$ of 100±4 nM at 20 min and reaches background values at 40 min after the application of the CWE (**Fig. 2c**). The $[Ca^{2+}]_{cyt}$ response is concentration-dependent: increasing the amount of the CWE also increases the Ca^{2+} peak (**Fig. 2b**). No response was observed with water or 4 nM *cis*-zeatin, which was used as controls. *cis*-Zeatin was used because *P. indica* produces this phytohormone in large amounts, although this has no direct role in growth promotion (Vadassery *et al.,* 2008). Surprisingly, the $[Ca^{2+}]_{cyt}$ response was mainly observed in *Arabidopsis* roots. Only a minor response was observed with leaf disc or hypocotyl and the $[Ca^{2+}]_{cyt}$ increased only to 100±21 nM for leaf discs (**Fig. 2a**). This response in leaves can be slightly raised by increasing the amount of CWE (100 µl) but it is still less compared to the root response (Supplemental Fig. 3).

Previously, we have shown that *N. tabacum* is an excellent host for *P. indica* (Sherameti *et al*., 2005). Transgenic aequorin tobacco seedlings showed the same response to the CWE as *Arabidopsis* seedlings: the increase in the $[Ca^{2+}]_{cyt}$ was high in roots (**Fig. 2c**) and only marginal in leaf discs (not shown). Thus, the CWE contains a signal that is recognised by at least two *P. indica*-responsive plant species. However, the Ca^{2+} signature obtained with tobacco CWE differs from that of *Arabidopsis* in that two peaks can be detected, the first after 60 sec and the second after 355 sec, and the response does not reach background levels within 40 min (**Fig. 2c**). Navazio *et al.* (2007) reported that a diffusible signal from arbuscular mycorrhizal fungi elicits a transient $[Ca^{2+}]_{cyt}$ elevation in cell suspension cultures from soybean. Using the same culture, we did not observe any Ca^{2+} response with our CWE (**Fig. 2c**). Since the culture was generated from green hypocotyls, these results are consistent with the observation that the response induced by the CWE from *P. indica* is predominantly root-specific.

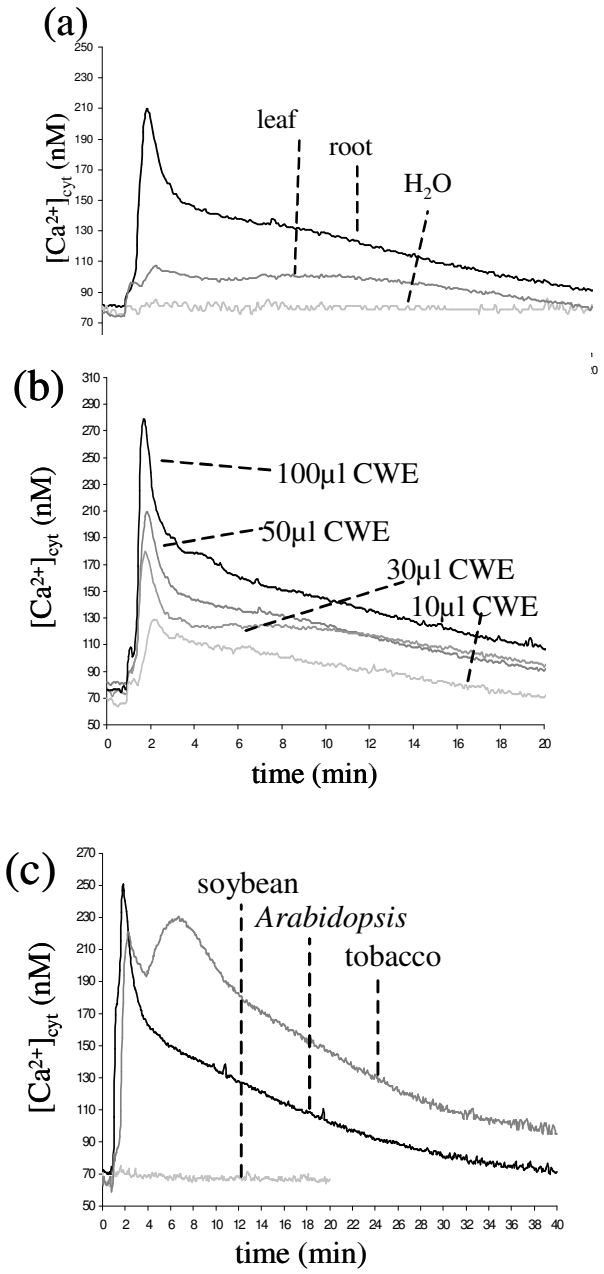


Figure 2

P. indica CWE induces changes in $[Ca^{2+}]_{cyt}$ in apoaequorin-transformed *Arabidopsis* seedlings.

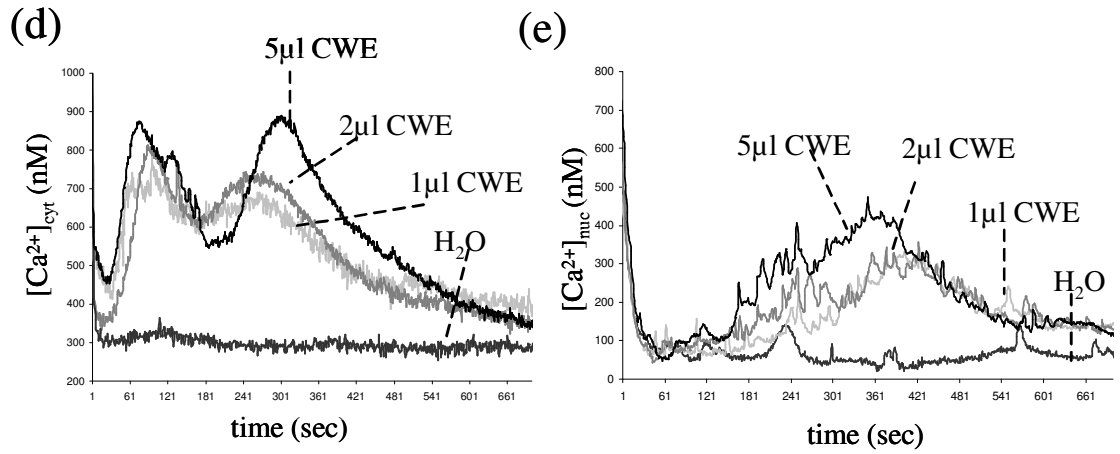
(**a**) Application of 50 µl CWE to *Arabidopsis* roots and leaves with H_2O on roots as control. $[Ca^{2+}]_{cyt}$ was calculated from the RLU measured in various tissues at 5 sec integration time for 20 min. In all the experiments, water was used as control and gave background readings. The values represent mean of 3 independent experiments.

(**b**) Application of various doses [10 µl, 30 µl, 50 µl, and 100 µl] of CWE to the roots.

(**c**) Effect of 50µl CWE on *Arabidopsis* or tobacco roots or soybean cell cultures derived from green hypocotyls. The relative aequorin luminescence was measured for 40 min for *Arabidopsis* and tobacco until the signal returns to background level.

Nuclear Ca^{2+} elevation induced by the *P. indica* CWE

Ca^{2+}-dependent signalling is distributed between different cell organelles (Pauly *et al.,* 2001). Nuclear Ca^{2+} ($[Ca^{2+}]_{nuc}$) elevations are important in symbiotic signalling: for instance the nuclear localized DMI3 is a sensor for Ca^{2+} signals of the Nod and postulated Myc factors (Kalo *et al*., 2005, Smit *et al*., 2005). Since nuclear localised *Arabidopsis* aequorin plants are not available, the effect of the *P. indica* CWE on $[Ca^{2+}]_{nuc}$ was tested using BY-2 tobacco cell cultures expressing apoaequorin either in the cytoplasm or the nucleus (Pauly *et al.,* 2001). The $[Ca^{2+}]_{cyt}$ response with the tobacco cell cultures resembles that shown with tobacco plants (**Fig. 2d**). The CWE also gave a specific Ca^{2+} signature in the nucleus with $[Ca^{2+}]_{nuc}$ reaching a peak at 6 min (**Fig. 2e**). A much lower concentration of the CWE was sufficient to induce these responses in the BY-2 cell cultures, when compared to the transgenic plant/seedlings.



(**2d, e**) *P. indica* CWE induces changes in $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{nuc}$ in apoaequorin transformed tobacco BY 2 cells. (d) Application of various doses of the CWE (1 µl, 2 µl and 5 µl) to BY-2 cell cultures to measure $[Ca^{2+}]_{cyt}$ elevation. (e) $[Ca^{2+}]_{nuc}$ elevation analyzed with different doses of *P. indica* CWE.

Source for Ca^{2+} elevation

Inhibitors were used to study the source of Ca^{2+} influx. Lanthanum chloride ($LaCl_3$) is used to inhibit Ca^{2+} import across the plasma membrane and addition of 1 mM $LaCl_3$ to the roots 15 min prior to the application of the CWE completely blocked the $[Ca^{2+}]_{cyt}$ elevation (both the rapid peak and the gradual decline) (**Fig. 3a**). Exogenous application of the Ca^{2+} chelator BAPTA also blocked the $[Ca^{2+}]_{cyt}$ elevation completely (**Fig. 3a**). Application of staurosporine, a protein kinase inhibitor, also abolishes *P. indica* induced Ca^{2+} elevation (**Fig. 3a**). Cells often do not respond to a consecutive treatment with the same stimulus within a certain time frame ("refractory behaviour"), but remain sensitive to a different stimulus

(Müller *et al.*, 2000). To test if the *P. indica* derived extract follows this rule, it was applied to the roots at the beginning of the experiment and 20 min later when the $[Ca^{2+}]_{cyt}$ induced by the first application is on its descent. A second application of the CWE did not induce a strong $[Ca^{2+}]_{cyt}$ elevation in roots **(Fig. 3b**). In contrast, a different stimulus, 10 mM H_2O_2, applied instead of the second CWE, induced a much stronger $[Ca^{2+}]_{cyt}$ elevation indicating that the system is still competent. 10 mM H_2O_2 applied alone also produced a peak of similar intensity (Supplemental Fig. 4). Thus, both stimuli use different input pathways and probably activate two different receptors and/or channels **(Fig. 3b**). The CWE-desensitised roots regained Ca^{2+} responsiveness again after 2 h and the Ca^{2+} signature was comparable to the one recorded after the first Ca^{2+} application, although the overall signal intensities were lower **(Fig. 3c)**

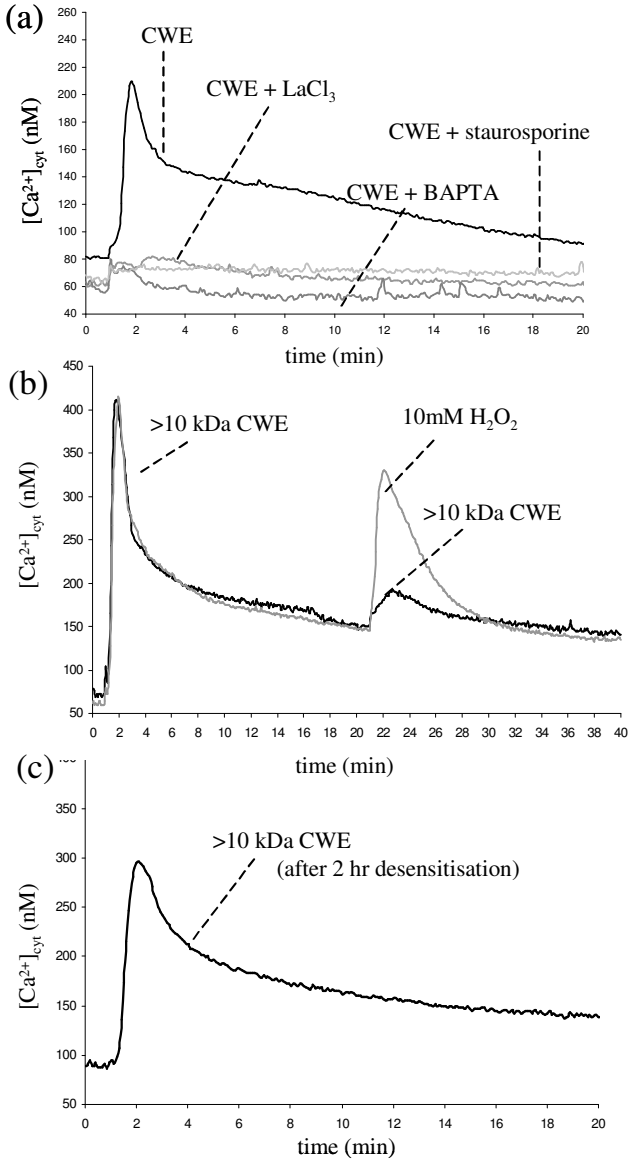


Figure 3

Properties of CWE induced $[Ca^{2+}]_{cyt}$ elevation in *Arabidopsis* roots.

(**a**) The effect of inhibitors blocking Ca^{2+} influx across the plasma membrane and protein phosphorylation in CWE-induced cellular responses. 1 mM $LaCl_3$ and 4 mM BAPTA dissolved in water was added 15 min prior to measurement. 10 µM stauroporine was preincubated with the roots 15 min prior to the measurement.

(**b**) CWE induced Ca^{2+} change is refractory to consecutive application of CWE. a) 50 µl of a >10 kDa CWE subfraction was applied twice to a single root after 1 min and 20 min and luminescence measured over 40 min at 5 sec integration time. Black: application of CWE followed by 10 mM H_2O_2; grey: a second application of water did not induce a response.

(**c**) Application of a >10kDa CWE subfraction, after 2 hours of initial application (desensitisation).

P. indica CWE neither induces H_2O_2 production nor activate defence responses in roots

Interaction with pathogens or MAMPs often induces the production of H_2O_2, which contributes to the activation of defence genes and the hypersensitive response (Grant *et al.*, 2000). Application of the chitotetraose (CH4, 1 µM) derived from pathogens, for instance, induced H_2O_2 production in the roots. The same was observed for the flg22 elicitor in the leaves (data not shown). However, the CWE of *P. indica* did not induce H_2O_2 accumulation that was stronger than observed for the control (**Fig. 4a**). Staining can only be observed at the tips of the main and lateral roots, for which H_2O_2 production has been shown previously (Dunand *et al.,* 2007). Furthermore, stimulation of defence responses by pathogenic fungus is associated with a rapid up-regulation of the mRNA levels for many defence-related genes such as *phenylalanine ammonia lyase* (*PAL*), *pathogenesis-related protein-1* (*PR-1)*, *enhanced disease susceptibility* (*EDS1*) and *lipoxygenase* (*LOX*) (Schenk *et al.*, 2000). Transcript levels for these four genes did not increase in response to the *P. indica* CWE after 2-day treatment when *P. indica*-responsive genes like *LRR1* and *MIOX* are upregulated (**Fig. 4b**).

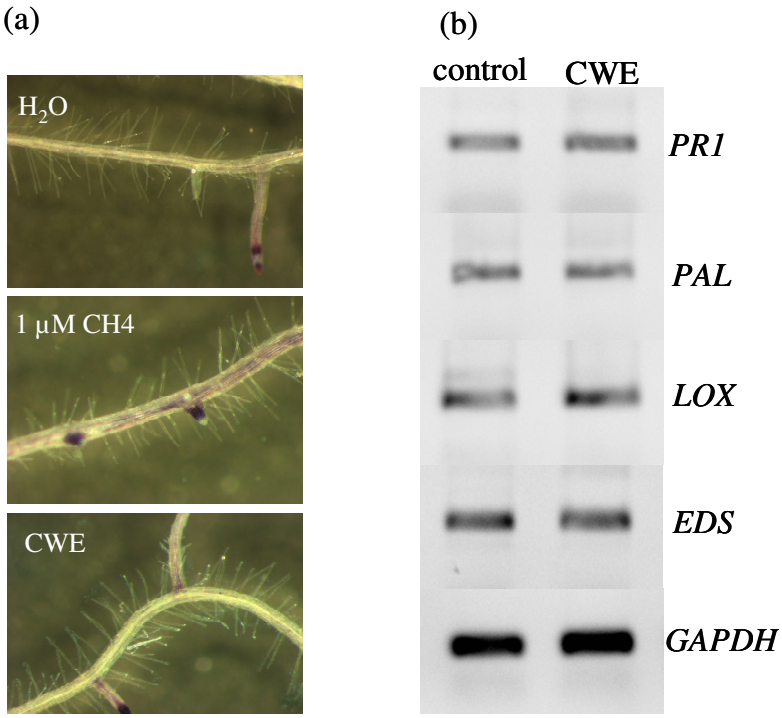


Figure 4

Detection of H_2O_2 production in *Arabidopsis* roots and defence gene expression upon CWE treatment.

(a) NBT staining was performed with 9-day old seedlings treated for 20 min with 1 µM chitotetraose-4 (CH4), or 60 µl *P. indica* CWE. H_20, untreated control. Note that the root tips are stained in all cases.

(**b**) RT-PCR analysis of defence gene expression on treatment of roots with 60 µl CWE for 48 h. *GAPDH* was used as control.

Ca^{2+} signalling genes are regulated by the *P. indica* CWE

Microarray data of *Arabidopsis* roots treated with the CWE for 1 hour was performed to uncover the early signalling events. They were compared with roots co-cultivated with the fungus for 2 days. We observed a substantial overlap among genes associated with Ca^{2+} signal transduction. Regulation of a few of them was confirmed by real-time PCR: a glutamate receptor gene, *GLR2.5*, was up-regulated 2-3 fold by both treatments, the *cyclic nucleotide gated channel* (*CNGC*)*-13* and *-10* genes were upregulated 5.6- and 2.4-fold, respectively, by the CWE (**Fig. 5**). Interestingly, *CNGC2* and *CNGC11,* which are implicated in defence signalling (Ali *et al.,* 2007) and *CNGC20* with unknown function were downregulated by the CWE treatment. Moreover, 15 of the 46 calmodulin-like genes (*CML*) present in *Arabidopsis* were upregulated by both stimuli. The strongest response was observed for *CML42*, followed by *CML30* and *CML38***.** Finally, a calmodulin binding protein, *CBP20,* was upregulated by both treatments (**Fig. 5).**

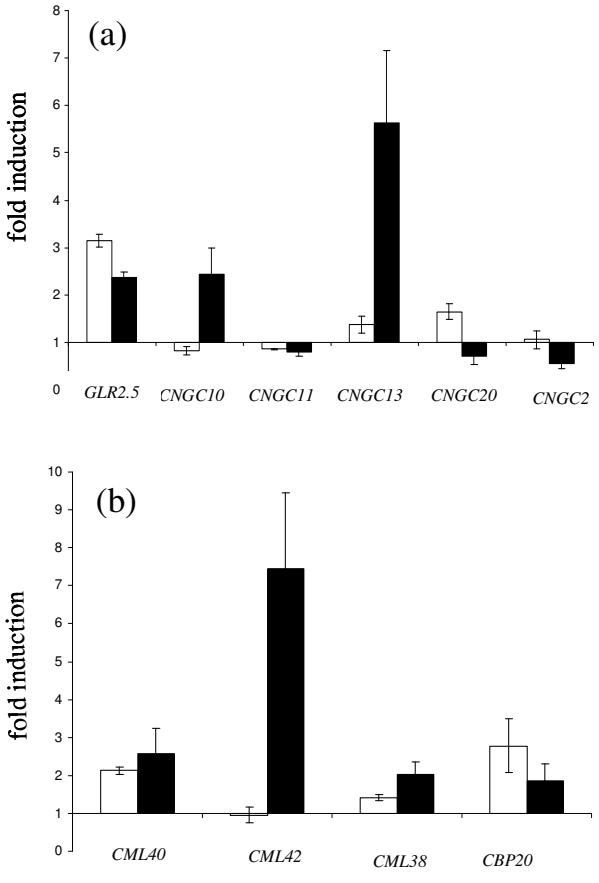


Figure 5

The response of Ca^{2+} signalling genes on treatment with *P. indica* and CWE in *Arabidopsis* roots. *Arabidopsis* seedlings were grown in the absence or presence of the fungus for 2 days on PNM medium, or treated with the CWE for 1 hour. Relative transcript abundance in roots was determined by real-time PCR analysis and normalised to the plant *GAPDH* mRNA level. The graph shows x-fold induction of the mRNA levels by the fungus (white) or CWE (black) relative to the levels in the untreated control roots.

(**a**) The Ca^{2+} permeable channel (*cyclic nucleotide gated channel*, *CNGC*) and glutamate receptor (*GLR*) genes.

(**b**) Ca^{2+} sensor (calmodulin like gene, *CML*) and calmodulin binding protein (*CBP*) genes

Ca^{2+}-dependent MAPK activation and its role in the interaction

Many pathogen-derived elicitors and MAMPs, including flg22, activate MAPKs to regulate defence responses (Asai *et al*., 2002). Activation of three MAPKs (most likely including the flg22-activated MAPK3 and MAPK6) was also observed after the application of the CWE from *P. indica* to the roots, which is much stronger than the activation of MAPKs by flg22 in roots (**Fig. 6a**). In contrast, the reciprocal results were obtained in leaves, where MAPK activation is much stronger when treated with flg22 compared with CWE (**Fig. 6b**). This indicates that MAPK activation is either involved in both beneficial and pathogenic responses, or defence responses downstream of MAPK activation are repressed by other components, present in the CWE. Furthermore, the crude CWE does not contain high levels of MAMPs that can activate MAPKs in leaves.

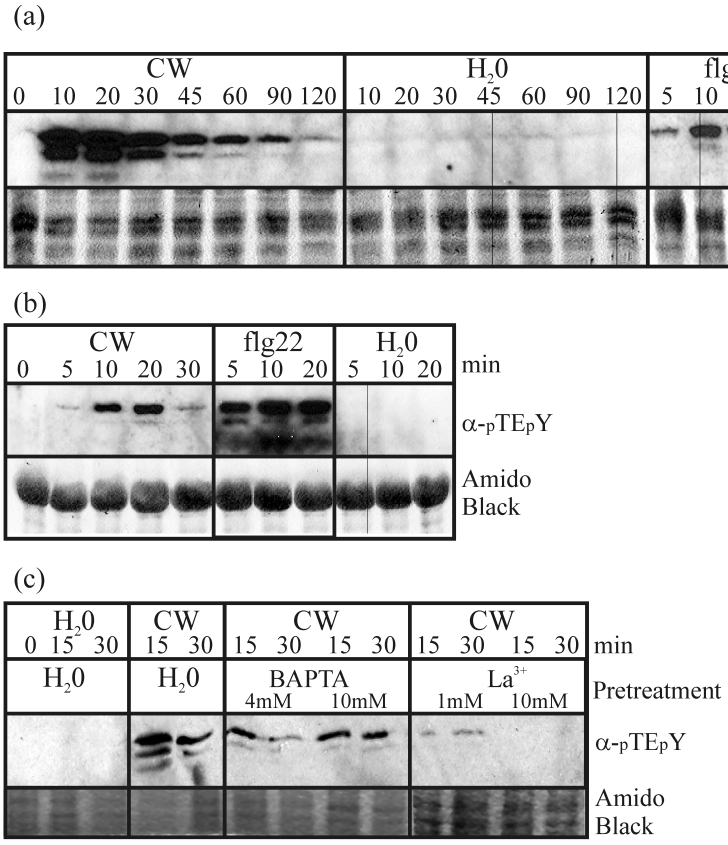
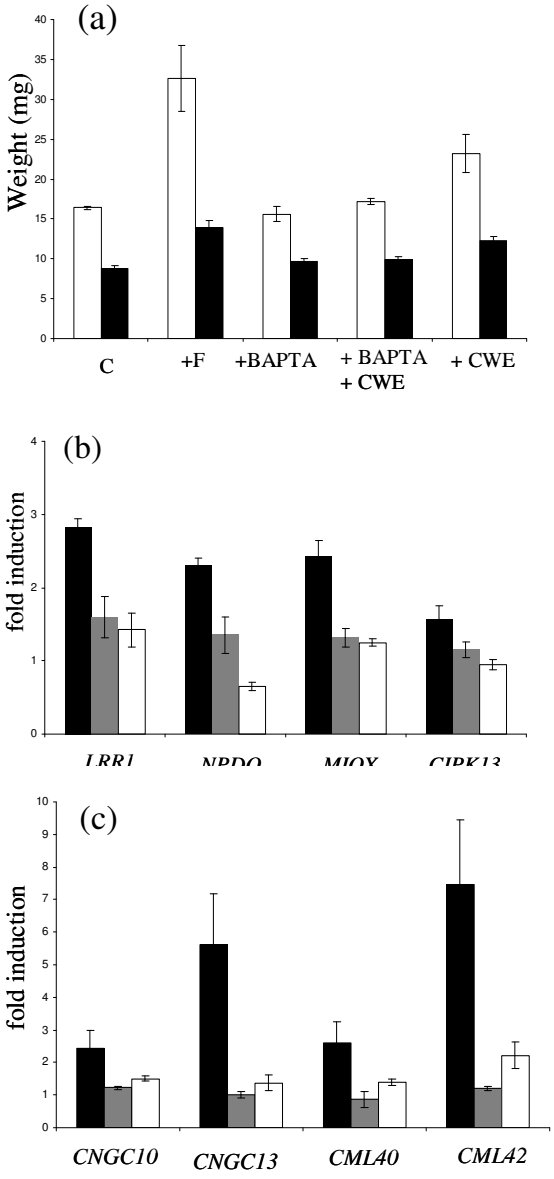


Figure 6

MAPK activation was monitored by western blot with antibodies that recognises the dual phosphorylations of the activation loop of MAPKs (α-$_pTE_pY$), in roots (**a**) or in leaves (**b**). Amido black staining was used to gauge equal loading of proteins in each sample. (**c**) Pretreatment with Ca^{2+} chelator (BAPTA) or antagonist ($LaCl_3$) was used to show the requirement of Ca^{2+} transients for MAPK activation in roots.

To test if MAPK activation in response to the CWE is Ca^{2+}-dependent, roots were pre-treated with the Ca^{2+} chelator, BAPTA (4 and 10mM), or the Ca^{2+} antagonist, $LaCl_3$ (1 and 10 mM), prior to the application of the CWE. $LaCl_3$ and BAPTA blocked or strongly reduced MAPK activation, respectively (**Fig. 6c**). Application of BAPTA (4mM) to the seedlings also blocks the growth promotion response (**Fig. 7a**), the induction of *P. indica* marker genes (**Fig. 7b**) and Ca^{2+} signalling genes (**Fig. 7c**). $LaCl_3$ was toxic in these long-term assays (data not shown). Finally, a *MAPK6* knock-out mutant fails to respond to *P. indica*: while the fungus stimulated growth of wild-type roots by >60%, no significant difference between colonized and uncolonized *MAPK6* roots could be detected (**Fig. 7d**). Taken together, these results suggest that MAPK and their activation *via* Ca^{2+} signals play a role in the mutualistic interaction.



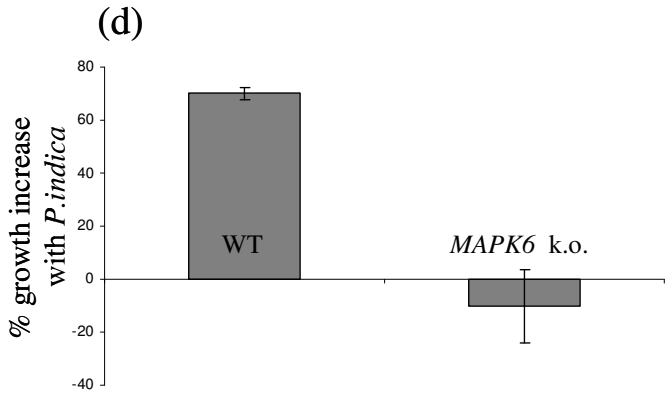


Figure 7

The effect of inhibitors blocking Ca^{2+} influx and MAPK6 on the *P. indica*/*Arabidopsis* interaction.

(a) Shoot (white) and root (black) weights from seedlings 10 days after the treatments with the fungus (F), BAPTA (4 mM) and/or the CWE. (C, untreated control). Bars represent SE.

(b) The response of the *LRR1*, *NPDO*, *MIOX* and *CIPK13* mRNA levels to treatments with the CWE (black), BAPTA (4mM, grey) and BAPTA + CWE (white) in *Arabidopsis* roots. Relative transcript abundance in roots was determined by real-time PCR analysis and normalised to the plant *GAPDH* mRNA level. The graph shows x-fold induction of the mRNA levels relative to the levels in the untreated control roots two days after the treatments. Bars represent SE.

(c) The response of the *CNGC10, -13 CML40, -42* mRNA levels to a 1h treatment with the CWE. For details, cf. (b).

(d) % increase in fresh weight of wild-type and MAPK6 knock out roots, after co-cultivation with *P. indica* for 8 days. Bars represent SE.

Characterisation of the chemical nature of the CWE derived active compound

The growth promoting extract isolated from the fungal CW was heat stable (20 min - 3 h at 121°C) and autoclaving appears to be required for the release of the factor(s) from the CW preparation. Fungal CWs are mainly composed of polysaccharides (chitin, glucan) and proteins (Montesano *et al.,* 2003). Thus, initial size-based separations and enzyme treatments of the CWE were performed to learn more about the nature of the active compound(s) in the CWE.Separation of the CWE into <10 kDa and >10 kDa fractions demonstrated that the active compound is enriched in the >10 kDa fraction (**Fig. 8a**). The >10 kDa fraction was used for enzyme digestions. Treatment with chitinase (**Fig. 8b**) had no significant effect on the Ca^{2+} signature monitored in the roots. Glucanase treatment slightly affected the second phase of the Ca^{2+}-response but the difference was not significant (at 10 min CWE + inactive glucanase

gave a value 160±19 nM, while CWE + glucanase was 140±3.5 nM; **Fig. 8c**). However, trypsin treatment attenuated the response, with the $[Ca^{2+}]_{cyt}$ peak at 2 min reduced from 218±14 nM to 146±12 nM. The subsequent sustained increase does not change upon trypsin treatment (**Fig. 8d**).

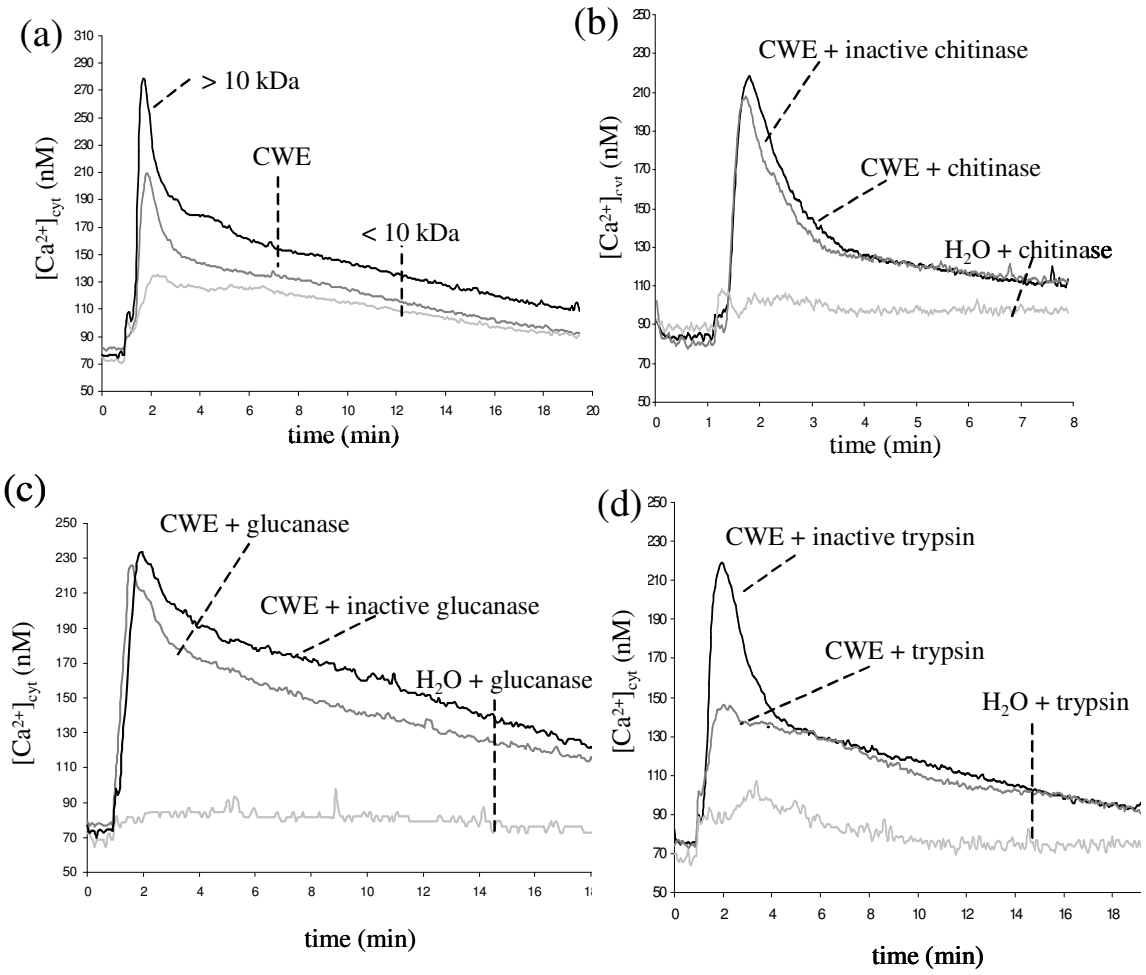


Figure 8

Analysis of the size of the active signal in the CWE and enzyme treatments to determine the chemical nature of the active compound. The luminescence readings were taken for 10 min (b) or 20 min (a, c, d).

(**a**) Separation of the CWE (CWE) into <10 kDa and >10 kDa subfractions. Most of the activity is in the >10 kDa fraction.

(**b-d**) The CWE was treated with chitinase (b) (5 µg/100 µl), glucanase (c) (5 µg/100 µl) or trypsin (d) (1 µg/100 µl) before measuring $[Ca^{2+}]_{cyt}$ elevation in roots. Heat-inactivated enzymes were used as control. The >10 kDa fraction was used for the treatment. All curves represent averages of 3 independent experiments.

Discussion

We report that a CWE from *P. indica* is able to promote root and shoot growth in *A. thaliana*, induces a $[Ca^{2+}]_{cyt}$ increase in root cells and a $[Ca^{2+}]_{nuc}$ increase in tobacco BY-2 cell cultures. To our knowledge, a root cell elevation of intracellular Ca^{2+} levels in *Arabidopsis* induced by a beneficial fungus has not been reported.

P. indica CW-derived molecules trigger Ca^{2+} signalling

Ca^{2+} is a key component of plant signal transduction and the "Ca^{2+} signature" characterised by its amplitude, duration, frequency and location is responsible for the induction of a specific physiological response (McAinsh and Hetherington, 1998; Sanders *et al.,* 2002). We obtained a signature with a transient increase in $[Ca^{2+}]_{cyt}$ after a lag phase of 55 sec in *Arabidopsis* roots. A maximum was observed ~1.5 min after the application of the CWE and dissipated in 40 min. Consistent with the observation that *P. indica* is a root-colonising fungus (Varma *et al.,* 1999) the CWE stimulates only a low response in leaves. This can be attributed to the fact that prolonged incubation stimulated the fungus also to colonise aerial parts of plants as reported for *Populus* (Kaldorf *et al*., 2005). The $[Ca^{2+}]_{cyt}$ elevation observed in tobacco roots has a significantly different signature from the one observed in *Arabidopsis* tissue, since two Ca^{2+} peaks at 60 and 355 sec could be detected. Different Ca^{2+} signatures have been reported depending on the organ, tissue, cell type or developmental stages of cells (McAinsh and Hetherington, 1998; Kiegle *et al.*, 2000; Reddy, 2001; Moore *et al.*, 2002) and the results show how a single stimulus can result in unique Ca^{2+} signatures in two different plant species.

Ca^{2+} elevations have been reported for instance, for the oligopeptide elicitor pep-13 in parsley cell cultures (Blume *et al.*, 2000), flg22 in *Arabidopsis* leaf discs, β-glucan fragments in soybean cell cultures (Mithöfer *et al.,* 1999) and for many proteinacoeus elicitors (like cryptogein) and oligosaccharide elicitors (Lecourieux *et al.*, 2002). The induction of a biphasic response with a second, long lasting plateau seems to be necessary for phytoalexin synthesis (Blume *et al.*, 2000). We also observed a biphasic $[Ca^{2+}]_{cyt}$ elevation in leaf discs with the MAMP lipopolysaccharide (Supplemental Fig. 5). Similar to these pathogenic elicitors, Navazio *et al.* (2007) reported that diffusible factors released by *Gigaspora margarita* (a fungus involved in arbuscular mycorrhizal symbiosis) induce a Ca^{2+} response that dissipates in 30 min. But such an elevation did not activate defence, instead activated the symbiotic *DMI*

genes. In our case, the lag phase (1 min), peak time (~2 min), duration of the Ca^{2+} response (40 min) and tissue specificity differs from other elicitors and this may be one of the factors contributing to its specificity.

Using the Ca^{2+} chelator, BAPTA, and the Ca^{2+} channel blocker, $LaCl_3$, we showed that CWE-induced $[Ca^{2+}]_{cyt}$ increase was strongly reduced. Since $LaCl_3$ and BAPTA pretreatment, prior to CWE addition, were relatively short, it is unlikely that these inhibitors affected endogenous Ca^{2+} pools. Hence, it is likely that the external medium is the primary source for the Ca^{2+} influx. The CWE-induced response also shows a refractive behaviour, since a second application of CWE after the dissipation of a first $[Ca^{2+}]_{cyt}$ elevation results in a strongly reduced response (**Fig. 3b,c**). This is reminiscent of the desensitization of the putative receptors for chitooligosaccharides in tomato and for pep-13 in parsley (Felix *et al*., 1998; Blume *et al.,* 2000). Finally, the sensitivity of the Ca^{2+} elevation to the protein kinase inhibitor staurosporine indicates that protein phosphorylation is involved upstream to the response, probably at the receptor level. Thus, these observations are compatible with the notion that the Ca^{2+} response induced by *P. indica* is receptor-mediated.

Nuclear Ca^{2+} elevation by *P. indica* CWE

Compartmentation of the Ca^{2+} signal is an important parameter in encoding response specificity (Pauly *et al.*, 2001; Walter *et al.*, 2007). $[Ca^{2+}]_{nuc}$ can be regulated separately from $[Ca^{2+}]_{cyt}$ and this can also affect the specificity of the response, or they can also act in co-operation (Allen *et al*., 2001). Using aequorin targeted to the nucleus in a tobacco cell culture system, we could detect an elevation of $[Ca^{2+}]_{nuc}$ with the *P. indica* CWE. The $[Ca^{2+}]_{nuc}$ elevation peaks at 361 sec and $[Ca^{2+}]_{cyt}$ has two peaks at 60 and 355 sec. Since the nuclear signals are delayed by 6 min, they can be independent of the cytoplasmic signals or one signal can follow the other. Because imaging or detecting the Ca^{2+} spikes is not possible with the aequorin system, we cannot rule out that they occur also in our system, as described in other symbiotic interactions. Several Ca^{2+} sensors like CAM and CAM-binding proteins or DMI3 are localised in the nucleus (Oldroyd and Downie, 2006). Hence the $[Ca^{2+}]_{nuc}$ elevation is of importance and *P. indica*-induced $[Ca^{2+}]_{nuc}$ elevation points to a role of the nucleus in the signal processing.

The CWE is involved in growth promotion and not in defence

An interesting observation was that the CWE is capable of promoting plant growth. This indicates that growth promotion can be uncoupled from the colonisation of the roots, although the CWE cannot completely replace the fungus *per se*, since even repeated applications of CWEs do not induce a growth promotion comparable to the fungus. The CWE activates a signalling cascade that initiates growth promotion as blocking Ca^{2+} signalling pathway by BAPTA also blocked growth promotion. Also it is possible that not all factors provided by the fungus are present in the CWE. The staining of the roots for H_2O_2 production and the absence of defence gene activation support the idea that the CWE does not result in prolonged activation of defence responses. We propose that the fungus colonizes and propagates in roots and the amount of the active compound(s) depends on the harmony of the symbiosis and the degree of colonization. In a natural system, the release of MAMPs would be regulated such that defence signalling is not activated *via* controlled colonization. A study with alfalfa roots exposed to both Nod factors and pathogen-derived oligochitin elicitors has shown that activation of defence related reactions might require $[Ca^{2+}]_{cyt}$ elevation above a hypothetical threshold level, which is reached by oligochitin elicitors but not by Nod factors (Felle *et al.,* 2000). Similar results have also been described for *P. indica* and *Arabidopsis* where PYK10 restricts root colonisation, which results in repression of defence responses and increase in mutualistic interaction (Sherameti *et al*., 2008a).

MAPK6 is crucial for the growth promotion response and MAPK activation is Ca^{2+}-dependent

Similar to the activation of the MAPKs, SIMK and SAMK in the roots of *Lupinus albus* after infection by *Bradyrhizobium.* sp., a symbiotic bacterium that forms nodules in lupine (Fernandez-Pascual *et al.*, 2006), the *P. indica*-derived CWE also activated MAPKs. Although signalling events leading to symbiotic and pathogenic interactions should be different, common strategies have been found in the early plant responses, stimulating the idea that pathogenesis and symbiosis are variations on a common theme (Parniske, 2000, 2004; Herouart *et al*., 2002). It has been suggested that general defence responses become activated during early symbiosis events (Carden and Felle, 2003). The MAPK cascade might be involved in plant–pathogen infections as well as in plant–symbiont interactions. The latter is supported by the observation that the *MAPK6* k.o. line does not promote plant growth and

hence is essential for the symbiotic interaction. MAPK6 is activated by various microbial elicitors (Nühse *et al.*, 2000) and synthesis of camalexin, a major phytoalexin in *Arabidopsis*, is regulated by the MAPK3/MAPK6 cascade (Ren *et al.*, 2008). Silencing of MAPK6 compromised both gene-for-gene and basal resistance in *Arabidopsis* (Menke *et al.*, 2004; Takahashi *et al.*, 2007) and hence is a common element in plant resistance. Furthermore, it has been shown that Ca^{2+} influx-dependent activation of MAPK is common in elicitor-treated cells (Lecourieux *et al.*, 2002), comparable to the results shown here (**Fig. 6c**). Thus, activation of MAPK6 and its role in growth promotion are dependent on the $[Ca^{2+}]_{cyt}$ elevation.

Specificity of the response

The temporal and spatial nature as well as the amplitude of the Ca^{2+} signal caused by a given stimulus contributes to the specificity of the response (McAinsh and Hetherington, 1998; Knight *et al.*, 1999). Specificity to the CWE is demonstrated by several observations: the CWE promotes growth of wild type, but not of *pii* seedlings. The CWE and the fungus appear to up-regulate a common set of genes. *P. indica*-specific marker genes are not upregulated by the CWE in *pii* mutants. It induces a transient $[Ca^{2+}]_{cyt}$ elevation in an organ-specific manner. The growth promotion response is blocked when the Ca^{2+} elevation is inhibited by adding BAPTA. In contrast to the common signalling cascade shared by rhizobium and AM symbiosis, inactivation of the *Arabidopsis DMI-1* homolog does not affect the interaction with *P. indica* (Shahollari *et al*., 2007).

Ca^{2+} -regulated genes in the interaction

The cyclic nucleotide-gated ion channels (CNGCs) maintain cation homeostasis essential for a wide range of physiological processes in plant cells. It has been reported that *CNGC10*, which is up-regulated by CWE, codes for a protein that localizes to the plasma membrane and influences growth responses and starch accumulation. *CNGC10* is highly expressed in dividing root cells and controls growth (Christopher *et al.,* 2007; Borsics *et al.,* 2007). *CNGC13* is most responsive to the CWE and is closely related to *CNGC10*. Down-regulation of *CNGC2/DND1*, which is involved in the generation of NO and innate immune response to pathogens (Ali *et al.*, 2007) and *CNGC11*, which is a regulator of resistance against fungal pathogen (Yoshioka *et al.*, 2006), supports the idea that they are suppressed by the CWE to prevent defence gene activation. Many diverse stimuli result in $[Ca^{2+}]_{cyt}$ increases in plant cells and Ca^{2+} sensors play a major role in defining the specificity. The *Arabidopsis* genome

has 7 calmodulin (*CAM*) and 50 *CML* genes that encode potential Ca^{2+} sensors (McCormack *et al.*, 2005). Gene expression analysis revealed that *CML42* is the most up-regulated by the CWE. It is known that a closely related gene, *CML43* is rapidly induced in disease-resistant *Arabidopsis* leaves following inoculation with *Pseudomonas syringae* pv. tomato. Overexpression of *CML43* in *Arabidopsis* accelerated the hypersensitive response (Chiasson *et al*., 2005). The role of the *P. indica*-inducible *CML42, CML40*, *CML38* and *CBP20*, which are upregulated by both the fungus and the CWE is unknown. The up-regulation of Ca^{2+} signalling genes supports the important role of Ca^{2+} in the interaction.

Chemical nature of the CWE

The active compound could be a protein, peptide, glycoprotein, lipid, oligosaccharide (Veit *et al.,* 2001) or other CW components such as chitins or glucans which constitute the main skeletal polysaccharides of basidiomycetes (Wolski *et al.,* 2005). It is known that the Nod factors released by nodule-forming rhizobia in symbiotic interactions are lipochitooligosaccharides and the perception of the Nod factors has evolved from the perception of more general elicitors like LPS or chitins (Boller 1995; Cullimore *et al.,* 2001; Montesano *et al.,* 2003). It is unlikely that the active compound in the *P. indica* CWE is a chitin since corresponding enzyme treatments had no significant effect on the Ca^{2+} response. Observations that the chitin oligomers, CH4 and CH5, did not induce a transient increase in Ca^{2+}_{cyt} in *Arabidopsis* roots support the data. The CWE might contain an active proteinous factor since trypsin treatments partially inhibited the activity of the CWE. But the lack of change in the sustained increase phase of $[Ca^{2+}]_{cyt}$ on trypsin treatment hints at involvement of additional factors.

The role of Ca^{2+} signalling in the *P. indica/Arabidopsis* interaction draws many parallels to the AM symbiosis and rhizobium/legume symbiotic systems. The identification of the active compound in the CWE, its receptor and Ca^{2+} sensor(s) might shed light on this symbiosis.

Experimental Procedures

Growth conditions of plant and fungus

Wild type and mutant (*pii-3* and *pii-4*) *Arabidopsis thaliana* seeds (ecotype Columbia) were surface-sterilized and placed on Petri dishes containing MS nutrient medium (Murashige and

Skoog, 1962). After cold treatment at 4°C for 48 h, plates were incubated for 7 days at 22°C under continuous illumination (100 µmol m^{-2} sec^{-1}). *P. indica* was cultured as described previously (Verma *et al*., 1998; Peškan-Berghöfer *et al*., 2004) on aspergillus-minimal medium. For solid medium 1% (w/v) agar was included.

Preparation of the CWE from P. indica

The CWE was prepared using the protocol of Anderson-Prouty and Albersheim (1975) with modifications. Mycelia from 14-day-old liquid cultures were homogenised using mortar and pestle in 5 ml water per g mycelia. The homogenate was filtered using a coarse sintered glass funnel. The residue was washed three times with water, once with chloroform/methanol (1:1) and finally in acetone. This preparation was air dried for two hours and the mycelial CW material was recovered. Elicitor fractions were prepared from mycelial CWs by suspending 1 g of CW in 100 ml water and autoclaving for 20 min at 121°C. Autoclaving releases the active fraction. The suspension was centrifuged at 14,000 rpm for 10 min, filter-sterilized using a 0.22 µM filter and concentrated to half and used for further assay.

Co-cultivation experiments and estimation of plant growth

Ten days-after the growth of seedlings on MS media, *A. thaliana* seedlings were transferred to nylon disks (mesh size 70 µm) and placed on top of a modified PNM culture medium (Vadassery *et al*. 2008), in Petri dishes. One seedling was used per Petri dish. After 24 h, fungal plugs of approximately 5 mm in diameter were placed at a distance of 1 cm from the roots. 70 µl of the prepared CWE was added directly onto the roots of a seedling. The plates were incubated at 22°C under continuous illumination from the side (80 µmol m^{-2} sec^{-1}). Fresh weights were determined separately for shoots and roots at 6, 10 and 14 day after application of the CWE.

Ca^{2+} measurements

Transgenic *Arabidopsis thaliana* (*Col*) and *Nicotiana tabacum* expressing cytosolic apoaequorin was used for Ca^{2+} measurements (Knight *et al.,* 1996). Plants were grown vertically in Hoagland´s media with 1% agar for 14 days (*Arabidopsis*) or 30 days (*N. tabacum*). For Ca^{2+} measurements the roots were dissected from 14-day-old seedlings and reconstituted in 5 µM coelenterazine (P.J.K. GmbH, Germany) in dark overnight at 21 °C. The luminescence counts obtained with a microplate luminometer (Luminoscan Ascent, version

2.4, Thermo Fischer Scientific, Germany) were calibrated using the equation by Rentel *et al.* (2004). For experimental details, see Supplementary material.

Enzyme treatments

Fractionation of CWE was done using Amicon ultra centifugal devices with a 10 kDa MW cut-off (Millipore, USA) .The CWE was treated with trypsin (1 µg/100 µl), glucanase (5 µg/100 µl) or chitinase (5 µg/100 µl) using the appropriate buffers at 37°C for 30 - 40 min. The reaction with trypsin was stopped using trypsin inhibitor. At the end of all reactions 4 volumes of –20°C cold methanol was added to stop the reactions. The supernatant after centrifugation was lyophilised resuspended in water and used for Ca^{2+} measurements. All enzymes were from Sigma Aldrich, Germany, except Sequencing grade Trypsin (Promega, Germany). Boiled inactive enzymes were used as controls. The buffers used for enzyme treatments were also tested for Ca^{2+} activities.

Inhibitor treatments

Roots were pre-incubated with $LaCl_3$ (1 mM) and BAPTA (4 mM), 15 min prior to the start of the readings. $LaCl_3$ and BAPTA were from Sigma Aldrich, Germany, and dissolved in water. Protein phosphorylation inhibitor, staurosporine (10µM), dissolved in DMSO, was from Alexis (Grünberg, Germany). The final DMSO concentration in the assay was 0.1%.

MAPK assay

Roots from 14 day-old *Arabidopsis* seedlings grown in Hoaglands medium were used. For inhibitor studies, $LaCl_3$ (1 and 10 mM) and BAPTA (4 and 10 mM) were applied 2 h before the experiments and the CWE was applied to the roots at the time points given in the Figure. For growth promotion assays the *mpk6* (At2g43790) k.o line SALK_127507 was used.

H_2O_2 measurements

The whole seedlings were used for H_2O_2 measurements 15 - 20 days after germination. Roots were stained for 5 min in a solution containing 2 mM nitobluetetrazolium (NBT) in 20 mM phosphate buffer pH 6.1. The reaction was stopped by washing the seedlings with water. The elicitor or CW preparations were added for 10-20 min on the seedlings before the staining with NBT. Experiments were repeated 5-times with comparable results. NBT was from Sigma Aldrich. Roots were observed under the stereomicroscope SV 6 with an AxiomCam

HRc color camera (Carl Zeiss, Jena). The chitin oligomer CH4 was obtained from Seikagaku Corporation (Tokyo).

RT-PCR analysis

To analyse the expression of defence genes in response to the CWE, total RNA was isolated from three independent replicates of Arabidopsis roots, treated with the CWE for 24 h. cDNA was synthesised using the Omniscript cDNA synthesis kit (QIAGEN) using 1 µg RNA. Quantitative RT-PCR was performed using 1:2-fold diluted cDNA.

Real time PCR and microarrays

Arabidopsis seedlings, grown as described above, were treated with the CWE for 1 or 48 h. RNA was extracted from 100 mg root materials with the RNeasy Plant Mini Kit (Qiagen, Germany), followed by an On-Column DNAse treatment (Qiagen, Germany). Microarray hybridization was performed with the *Arabidopsis* Genome Array ATH1 from Affymetrix and the data were analysed with the GCOS1.4 software (Affymetrix). The microarrays were performed by KFB, Regensburg. Conditions for real time PCR and all primers are given in the Supplementary material.

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Supplementary material

Ca^{2+} measurements

One root or leaf disc was transferred into a well of a 96 well plate containing 100 µl of reconstitution solution. For leaf measurements, seedlings were grown in MS media for 3 weeks and the leaf disc was incubated overnight with 10 µM coelenterazine. Bioluminescence counts in *Arabidopsis* from roots or shoots were recorded at 5 sec intervals for 20 min, recorded as relative light units (RLU/sec) with a microplate luminometer (Luminoscan Ascent, version 2.4, Thermo Fischer Scientific, Germany). After a 1-min background reading, the CWE was added manually to the well and readings in RLU were taken for 20 min. Calibrations were performed by estimating the amount of aequorin remaining at the end of experiment by discharging all remaining aequorin in 0.1 M $CaCl_2$, 10% ethanol, and the counts were recorded for 10 min. The luminescence counts obtained were calibrated using the equation by Rentel *et al.* (2004) that takes into account double logarithmic relationship between concentration of free Ca^{2+} present in the cell and the remaining aequorin discharged at any point of time. The calibration equation is: pCa = 0.332588(-log $\boldsymbol{k}$) +5.5593, where $\boldsymbol{k}$ is a rate constant equal to luminescence counts per sec divided by total remaining counts. For Ca^{2+} measurements in transgenic tobacco (*N. tabacum* L. cv. BY-2) cell lines expressing the Ca^{2+} sensing protein apoaequorin either in the cytosol or in the nucleus were used and readings were taken at 1 sec interval for 12 min (Mithöfer and Mazars, 2002).

Real time PCR

Real time quantitative RT-PCR was performed using an iCycler iQ real time PCR detection system and iCycler software version 2.2 (Bio-Rad). Total RNA was isolated from three independent replicates of *Arabidopsis* roots, treated with *P. indica* CWE for 1 h or 48 h. cDNA was synthesised using the Omniscript cDNA synthesis kit (QIAGEN) using 1 µg RNA. For the amplification of the RT-PCR products, iQ SYBR Green Supermix (Bio-Rad) was used according to the manufacturer´s protocol in a final volume of 25 µl. The iCycler was programmed to 95°C 2 min, 40 x (95°C 30 sec, 57°C 40 sec, 72°C 45 sec), 72°C 10 min, followed by a melting curve program 55°C to 95°C in increasing steps of 0.5°C. All reactions were performed in triplicate. The mRNA levels for each cDNA probe were normalized with respect to the *GAPDH* mRNA level. Fold induction values of target genes were calculated

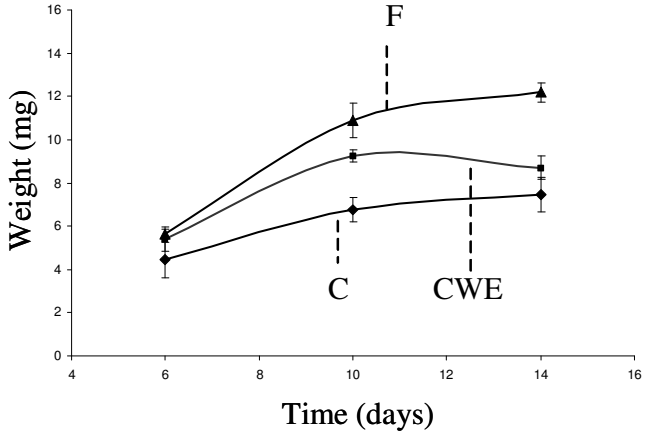
with the ΔΔCP equation of Pfaffl (2001) and related to the mRNA level of target genes in control roots, which were defined as 1.0. The following primer pairs, with product sizes between 150-170 bp were used:

MIOX2 (*At2g19800*)	5'-TTGGCAAGGTTCTCCTTCTG-3'
	5'-TTGGCTGAGTCGAAGGTACA-3'
LRR1 (*At5g16590*)	5'-CTCGTTTCCGTGACATCAGA-3'
	5'- GACCCGACTCACATTGGACT -3'
NPDO (*At5g64250*)	5'-GAAGCAGGTGGGCATGTTAT-3'
	5'-GTGCCTAGACAGACCCCTTG-3'
CIPK13 (*At2g34180*)	5'-GTTGATTCAATGCCGAGACC-3'
	5'-CCAATCCTTCTTCCTCACCA-3'
GAPDH (*At3g04120*)	5'-GAGCTGACTACGTTGTTGAG -3'
	5'-GGAGACAATGTCAAGGTCGG-3'
CNGC10 (*At1g01340*)	5'-GGATCCTGACCACAAGGAAA-3'
	5'-AAGACAGTGCCTTGCGCTAT-3'
CNGC11 (*At2g46440*)	5'-TGTGCTAGGACATCCGACTG-3'
	5'-TAGGACCCCTGACTTCAACG-3'
CNGC13 (*At4g01010*)	5'-GCAGAGCAATGGATGTCTCA-3'
	5'-ATGGCGTTTGATGTCTCTCC-3'
CNGC20 (*At3g17700*)	5'-GCTTTCACTTCCAGGAGTCG-3'
	5'-GGAGGACGTCTCTGAGTTCG-3'
CNGC2 (*At5g15410*)	5'-CATCTTCTGGGGCCTAATGA-3'
	5'-TAACCTCGAGCCAGTTGCTT-3'
GLR2.5 (*At5g11210*)	5'-TTCGGAGGAGAAGAGCTGAA-3'
	5'-GATAGAGCAACGCCGAGTTC-3'
CML40 (*At3g01830*)	5'-TAAGTCTGGCAAACGTGCAG-3'
	5'-TCCTCTCCTTCTTCCACCAA-3'
CML42 (*At4g20780*)	5'-CGTCGAAGAGCTAAGCCAAG-3'
	5'-CCGAAGAAAGAATCGTCGAG-3'
CML38 (*At1g76650*)	5'-TCAGCCGGAGAGATACAACA-3'
	5'-GCAGCTACGGCTTCTTCATC-3'
CBP20 (*At5g26920*)	5'-TCGAAGCTGAGGATGGTTCT-3'

	5’-TAAATCCCTCAACGGTCCAG-3’
PAL (At3g53260)	5’-CAAAAGTGGCGGTTACTACG -3’
	5’-CATGTCTCCTTCGTGTTTCC-3’
EDS1 (At3g48090)	5’-GGAACTGGTACAGTCGATGG-3’
	5’-CGGCTAACTCAGCTCTCTTG-3’
PR-1 (At2g14610)	5’-GCTCAAGATAGCCCACAAGA-3’
	5’-ACACCTCACTTTGGCACATC-3’
LOX1 (At1g55020)	5’-CCGAGTCTTCTTCTCCAACA-3´
	5’-CGTCGAATACAGCCTCAAGT-3’

Supplementary figures

Figure 1



The effect of the CWE on *Arabidopsis* growth.

The kinetics shows the fresh weight of *Arabidopsis* seedlings between 6 and 14 days after the following treatments: (C), untreated control; (F), co-cultivation with a fungal plug; (CWE), application of the CWE. The fungal plug or 65 µl of the CWE were applied at day 0. Bars represent SE.

Figure 2

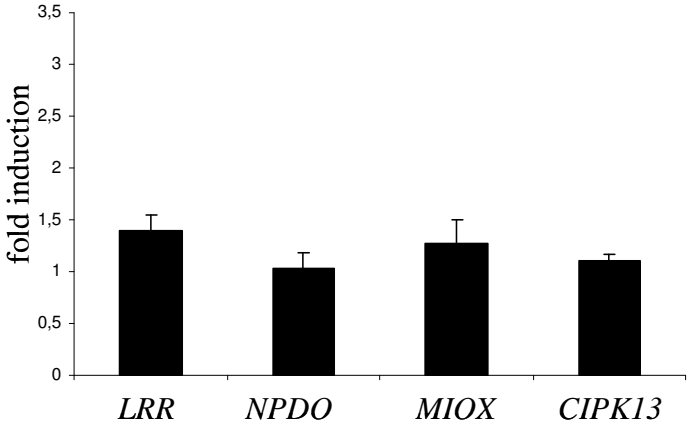


Figure 2

Genes which are upregulated in the wild-type roots after the application of 65 µl of the CWE are not upregulated in the *P. indica-insensitive* mutant *pii-3*.

The graph shows x-fold induction levels of the transcripts in *pii-3* roots on treatment with the CWE relative to the untreated controls. Relative transcript abundance in roots was determined by real-time PCR analysis and normalised to the plant *GAPDH* mRNA levels. Bars represent SE.

Figure 3

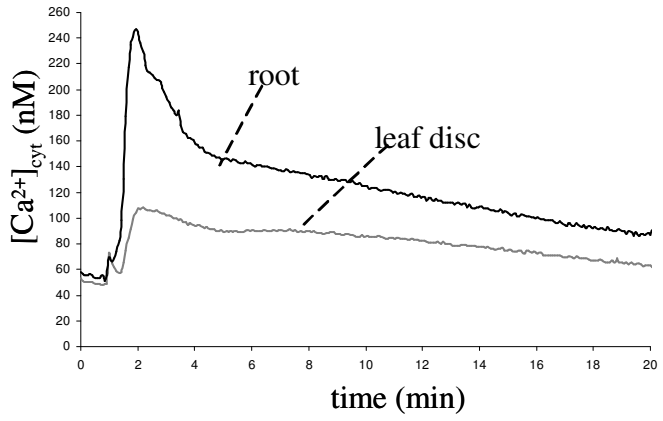


Figure 3

P. indica **CWE induces changes in $[Ca^{2+}]_{cyt}$ in apoaequorin-transformed *Arabidopsis* seedlings.** Application of 100 µl CWE to *Arabidopsis* roots and leaf disc.

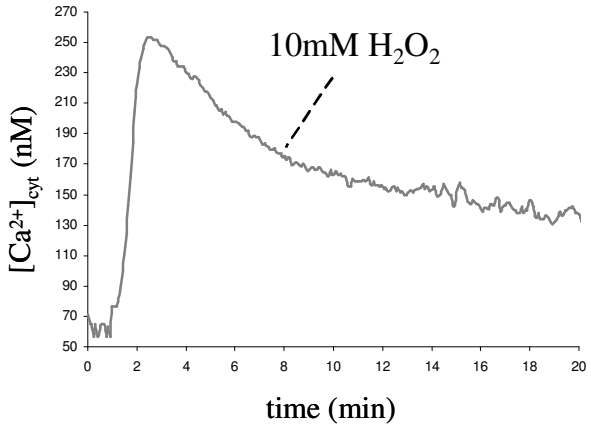


Figure 4

Changes in $[Ca^{2+}]_{cyt}$ in *Arabidopsis* roots on treatment with 10 mM H_2O_2 .

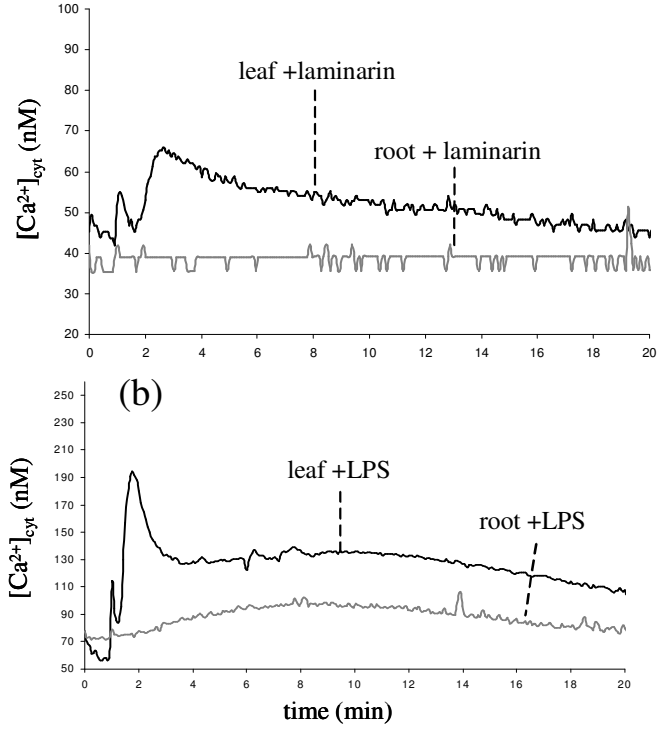


Figure 5

Tissue specific "Ca^{2+} signatures" of various biotic elicitors in *Arabidopsis.*

Leaves and roots were challenged with laminarin (1 mg/ml) (**a**) or LPS (1mg/ml) from *Pseudomonas areuginosa* (**b**).

Comparison of the P. indica *CWE activity with known elicitors*

Laminarin, a linear β-1.3-glucan from the brown algae *Laminaria digitata,* stimulates a $[Ca^{2+}]_{cyt}$ increase and induces defense responses in cell suspension cultures of tobacco (Lecourieux *et al.*, 2002) and grapevine (Aziz *et al.*, 2003). Laminarin (1mg/ml) induces a $[Ca^{2+}]_{cyt}$ elevation in the leaves of *Arabidopsis*, but no response was observed in the roots (Supplemental **Fig. 6a**). Lipopolysaccharides (LPS), a microbe associated molecular pattern (MAMP) found in bacteria, induce a $[Ca^{2+}]_{cyt}$ elevation in the leaves and a much lower response in the roots. The $[Ca^{2+}]_{cyt}$ in leaves reached a maximum at 1 min after application of LPS (1mg/ml), but it induces a second sustained $[Ca^{2+}]_{cyt}$ increase giving a biphasic peak unlike *P. indica* CWE. The response in the roots showed only a weak parabolic increase and thus differs substantially from the signature obtained with the CWE (Supplemental **Fig. 6b**). Pathogen-derived chitin elicitors (chitotetraose, CH4 and chitopentaose, CH5) produced no Ca^{2+} elevation in the roots (data not shown). Thus, *P. indica* CWE is the only elicitor tested that gave a clear response in the roots.

2.2 Manuscript II

Molecular Plant Microbe Interaction (2008), 21(10), 1371-83.

The Role of Auxins and Cytokinins in the Mutualistic Interaction between *Arabidopsis* and *Piriformospora indica*

Jyothilakshmi Vadassery[1], Claudia Ritter[1], Yvonne Venus[1], Iris Camehl[1], Ajit Varma[2], Bationa Shahollari[1], Ondrej Novák[3], Miroslav Strnad[3], Jutta Ludwig-Müller[4], and

Ralf Oelmüller[1]

Institution address

[1]Friedrich-Schiller-Universität Jena, Institut für Allgemeine Botanik und Pflanzenphysiologie, Dornburger Str. 159, 07743 Jena, Germany;

[2]Amity Institute of Herbal and Microbial Studies, Sector 125, Noida 201303, UP, India

[3]Laboratory of Growth Regulators, Palacky University & Institute of Experimental Botany AS CR, Slechtitelu 11, 783 71 Olomouc, Czech Republic

[4]Institut für Botanik, Technische Universität Dresden, Zellescher Weg 20b, 01062 Dresden, Germany

Corresponding Author:

Dr.Ralf Oelmüller

Phone: 49-(0) 3641-949231

Fax: 49-(0) 3641-949232

Email: b7oera@uni-jena.de

Attached as separate pdf

2.3 Manuscript III

The Plant Journal (2007)

A leucine-rich repeat protein is required for growth promotion and enhanced seed production mediated by the endophytic fungus *Piriformospora indica* in *Arabidopsis thaliana.*

Bationa Shahollari, Jyothilakshmi Vadassery, Ajit Varma and Ralf Oelmuller.

The Plant Journal (2007) 50(1), 1-13.

Institution address

[1]Friedrich-Schiller-Universität Jena, Institut für Allgemeine Botanik und Pflanzenphysiologie, Dornburger Str. 159, 07743 Jena, Germany;

[2]Amity Institute of Herbal and Microbial Studies, Sector 125, Noida 201303, UP, India

Corresponding Author:

Dr.Ralf Oelmüller

Phone: 49-(0)3641-949231

Fax: 49-(0)3641-949232

Email: b7oera@uni-jena.de

Attached as separate pdf

3. Discussion

Fungi release a wide array of compounds on its interaction with plant cells. Some of them act as elicitors and activate defence and some as effectors which suppress the pathogen triggered immunity and in symbiotic interactions act as molecular cues for successful recognition. Fungal released factors belong to a wide range of different classes of compounds including oligosaccharides, peptides, proteins and lipids. The tremendous structural diversity of fungal factors suggests that there is no common motif and that plants have evolved an enormous arsenal of perception systems for microbe derived structures. In the current thesis we analyse the impact of two *P. indica* derived factors, the cell wall extract and cytokinins and the signaling pathways they activate during the course of their release.

Calcium signalling in *P. indica-A. thaliana* interaction

It is known that cells can specifically respond to a wide array of biotic and abiotic stimuli through the second messenger Ca^{2+}. Their role in plant fungus signalling is crucial for the activating symbiotic genes. However its role in the *P. indica-A.thaliana* system was unknown until the autoclaved cell wall extract was isolated. An autoclaved cell wall extract (CWE) was isolated from *P. indica*, which can promote growth and also induce root cytosolic Ca^{2+} elevation. Root and shoot growth is promoted by the CWE though it does not completely replace the fungal effect. This may point to the involvement of additional factors released by the fungus. Also the fungus actively grows in the root and releases factors at appropriate stage, whereas the CWE is only applied once at the beginning of the experiment. Hence, the comparision of the two responses can have obvious differences. The promotion of growth by the CWE and the fungus is also accompanied by upregulation of common marker genes for the leucine-rich repeat protein (*LRR1*), and 2-nitro-propane dioxygenase (*NPDO*). Microarray analysis uncovered that *MIOX*, and *CIPK13* are also upregulated by both the fungus and the CWE. *MIOX* is involved in ascorbate biosynthesis and *CIPK13* is a downstream Ca^{2+} responder.

The autoclaved CWE induces a cytosolic Ca^{2+} elevation in *Arabidopsis* roots, which shows a maximum response at 2 minutes, followed by gradual decline and reaches background levels in 40 minutes. It is postulated that every signal has a specific Ca^{2+} signature, which

varies in its amplitude, time and location, and this is one of the determinants of the specificity of Ca^{2+} response (Sanders *et al.,* 2000). The *P. indica*-induced Ca^{2+} signature is observed in roots and is active over a period of 40 minutes consistent with the notation that *P. indica* is a root-colonizing endophyte. This signature is different from many pathogenic elicitors like β-glucan where the initial peak is followed by a second sustained increase or plateau. It has been reported that long lasting Ca^{2+} elevations are responsible for phytoalexin production and HR response in a pathogenic interaction (Lecourieux *et al.,* 2002). However the same extract induces a very different Ca^{2+} signature in tobacco roots hinting at the possibility of species-specific signatures. Ca^{2+} is actively pumped from cytosol to extra cellular spaces or intracellular compartments like endoplasmic reticulum and vacuole to maintain cellular Ca^{2+} at resting levels. Using the Ca^{2+} channel blocker $LaCl_3$ and Ca^{2+} chelator BAPTA it was shown that the source of Ca^{2+} elevation is extra cellular medium and not intracellular organelles and this is crucial in the physiological response. The specificity of the CWE in growth promotion is demonstrated by the use of *P. indica* insensitive lines (*pii*-3, 4), which do not respond to both the fungus and the CWE. They also do not activate the common marker genes like *LRR1*, *NPDO*, *MIOX* and *CIPK13*. To test if the cytosolic Ca^{2+} elevation is crucial for growth promotion and if both are related, Ca^{2+} signalling was blocked by BAPTA application. It was shown that BAPTA also blocks the growth promotion. So the unidentified factor released from the fungus is able to activate Ca^{2+} signalling which is crucial for growth promotion.

Post-translational modification of proteins by reversible phosphorylation is a key process regulating many functions in plants, including defence responses induced by elicitors (Dietrich *et al*., 1990). In several plant cell–elicitor systems, phosphorylation events were described both upstream and downstream of the elicitor-induced Ca^{2+} influx. In our system 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 probably at the receptor level. The involvement of receptors is further proved by the refractive nature. Plant cells lose their capacity to respond to a second time to the same type of elicitor (refractive behaviour), but remain sensitive to another type of elicitor perceived by another receptor. The CWE shows such a refractory nature and hence binds to a single receptor where as a second stimuli, H_2O_2, which bind to a different receptor, shows no such behaviour. Protein phosphorylation changes are also observed downstream the Ca^{2+} response and this is proven by induction of MAPK phosphorylation by the CWE. The phosphorylation changes are more

pronounced in the roots than in the shoots and occur in a time dependent manner. MAPK3 and -6 are phosphorylated as proven from the analysis of *mapk3* and *mapk6* knock out lines (supplementary data 1). MAPK cascades are major components downstream of receptors or sensors that transduce extracellular stimuli into intracellular responses in eukaryotic cells. The use of Ca^{2+} inhibitors like $LaCl_3$ and BAPTA abolished the MAPK response and this indicates the involvement of Ca^{2+} signalling upstream of MAPK pathways and that cytosolic Ca^{2+} elevations are imperative for MAPK phosphorylation. Knock out mutants of MAPK6 also do not respond to *P. indica* and induce no growth promotion. Silencing of MAPK6 compromised both gene-for-gene and basal resistance in *Arabidopsis* (Menke *et al.*, 2004; Takahashi *et al.*, 2007) and hence is a common element in plant resistance. Furthermore, it has been shown that Ca^{2+} influx-dependent activation of MAPK is common in elicitor-treated cells (Lecourieux *et al.*, 2002). Thus, $[Ca^{2+}]_{cyt}$ elevation and subsequent activation of MAPK6 is crucial for the growth promotion response. It also suggests that both mutualistic and pathogenic interactions share some common signalling components although CWE does not induce the expression of H_2O_2 and other common defence genes like phenylalanine-ammonium lyase.

The subcellular localization of the Ca^{2+} signature can also control the specificity of the response in the plant cell. The occurrence of a nuclear Ca^{2+} elevation as measured from tobacco BY-2 cultures hints at the involvement of an additional response. The time lag of maximum activity (2 min for cytosolic response and 6 min for nuclear response) also raises many unanswered questions. The importance of nuclear Ca^{2+} in signalling processes is underlined by the existence of calcium effectors in the plant nucleus, including calmodulin (CaM), CaM-binding protein, CDPKs and Ca^{2+}- CaM-regulated protein phosphatase (Lee *et al*., 2003; Bouche *et al*., 2005; Levy *et al*., 2005). However, in *Arabidopsis* it is not possible to measure nuclear Ca^{2+} changes due to lack of nuclear localized aequorin. Measuring the Ca^{2+} response by fluorescence resonance energy transfer (FRET)-based Ca^{2+}-indicator, cameleon, would be helpful in identification of single cell calcium changes (aequorin system measures the average response of many cells) and shed more light on the specific cell layer in root where such Ca^{2+} responses are activated.

Specificity in the Ca^{2+}-signalling system results from a multifactorial decision process, ranging from a specific Ca^{2+} signature to the availability of a specific set of calcium sensors and their target proteins that are coupled to downstream components in a precise place. In eukaryotic cells, various stimuli mobilize different pools of Ca^{2+} to trigger characteristic

changes in $[Ca^{2+}]_{cyt}$ and Ca^{2+} channels have been detected in the plasma membrane, vacuolar membrane, ER, chloroplast and nuclear membranes of plant cells (Lecourieux *et al.,* 2006). Microarray analysis has uncovered that many Ca^{2+} signaling genes are activated by the CWE. The cyclic nucleotide gated channel genes (*cngc*), and the calmodulin like genes (*cml*) are the most highly regulated. There are 20 *cngc* genes and 46 *cml* genes in the *Arabidopsis* genome. They can have overlapping functions or function in a combination that is unique to each signal and contribute to its specificity. *cngc-13* and *-10* genes are upregulated by the CWE. Interestingly, *cngc2* and *cngc11,* which are implicated in defence signalling (Ali *et al.,* 2007) and *cngc20* with unknown functions were downregulated by the CWE treatment. It has been reported that *cngc10* is localized to the plasma membrane and influences growth responses and starch accumulation and is highly expressed in dividing root cells and controls growth (Christopher *et al.,* 2007; Borsics *et al.,* 2007). *cngc13*, which is most responsive to CWE, is closely related to *cngc10*. Down-regulation of *cngc2/DND1*, which is involved in innate immune response to pathogens (Ali *et al.*, 2007) and *cngc11*, which is a regulator of resistance against fungal pathogen (Yoshioka *et al.*, 2006), shows that the CWE might prevent defence gene activation. Loss-of-function mutations in CNGC2 and CNGC4 prevent plant HR to avirulent pathogens (Clough *et al*., 2000; Balagué *et al*., 2003; Jurkowski *et al*., 2004). Calmodulin is a highly conserved and broadly distributed Ca^{2+}-binding protein, which acts as a multifunctional intermediary connecting Ca^{2+} signals to the activation of other cellular components. Calmodulin like (CML) proteins shares sequence similarity to these CaM proteins. The CNG channels may be regulated by Ca^{2+}-CaM, as suggested by the existence of a high-affinity CaM-binding site overlapping the cyclic nucleotide-binding domain (Arazai *et al*., 2000). 15 of the 46 calmodulin-like genes (*cml*) present in *Arabidopsis* were upregulated by both the CWE and the fungus. The strongest response was observed for *cml42*, followed by *cml30* and *cml38***.** Finally, a calmodulin binding protein, CBP20*,* was upregulated by both treatments. We analyse the T-DNA knock out mutants in these gene families to uncover the specific sensor. When Ca^{2+} inhibitor BAPTA was added it also resulted in the non-regulation of many of these channel and sensor genes in accordance with the fact that growth promotion is also inhibited.

In spite of many similarities between our system and other symbiotic interactions, there are also very obvious differences. Deactivating the *Arabidopsis* single copy gene *DMI-1* that encodes an ion channel required for mycorrhiza formation of legumes does not affect the

beneficial interaction between the two partners (Shahollari *et al.,* 2007). Also the protein responsible for decoding the Ca^{2+} spiking response in legume rhizobial and AM symbiosis, DMI3/CcaMK which is a Ca^{2+} calmodulin dependent protein kinase is absent in *Arabidopsis*. All these results point to the fact that *P. indica* probably activates another similar pathway and not the common *SYM* (symbiosis) gene pathway as in the other two forms of symbiosis. This is also in accordance to the fact that *P. indica* has a wide host range and hence more ancient of the other two symbiotic interactions, which probably means that the signalling pathways activated are also more general. It has been proven from many reports that the interaction between *P. indica* and *Arabidopsis* involves a fine balance between defence pathways as proved from PYK10 (Sherameti *et al*., 2008) and from the MAPK6 data. The signal components such as calcium fluxes, MAPK activation all seem to be not unique to a single signalling pathway but also to many growth and developmental process and probably the initial recognition of microbes by plants involves a common signalling pathway that diverges at a later stage to defence or growth based on fungal colonization and possible nutrient accumulation.

Cytosolic Ca^{2+} response can help in mapping the genes located upstream and downstream of the response. The identity of the CWE is still under investigation. It is clear from trypsin treatment that proteins or peptides could be one of the active factors, but not the only one as none of the proteinase treatments could abolish the response. Further characterization of the CWE by LC-MS using cytosolic Ca^{2+} elevation as a marker has revealed that the active factor probably is more than one component (unpublished data). The identification of the active factor in the CWE is important for identifying the fungal released factor chemistry and any conservation of such factors between symbiotic interactions. Also identification of genes upstream the calcium response could shed light on the involvement of possible receptors and its role in symbiotic signalling.

Role of cytokinins in the *P. indica-A. thaliana* interaction

Microbes are known to produce auxin and cytokinin and full-scan mass spectrometry showed that *P. indica* hyphae grown in liquid medium produce free IAA and relatively high levels of cytokinins. Isopentenyladenine (iP), an active cytokinin form, *cis* zeatin (cZ), cZ riboside (cZR), and its nucleotide cZ riboside-5′-monophosphate (cZRMP) are released into the culture. The levels of the phytohormones on colonization of plant roots were also measured to investigate which of the forms of auxin and cytokinin are produced under the

symbiotic growth promoting conditions. No significant differences in free and conjugated auxin levels were detected in uncolonized and colonized roots. In contrast, the cytokinin levels in colonized roots differ from the control. While the amounts of iP, tZ, its riboside, and glycoside are comparable, those of the *cis* forms are 2- (cZ), 6- (cZR), or 10-times (cZ riboside-*O*-glycoside [cZROG]) higher in colonized roots. *P. indica* itself produces both iP and cZ-type cytokinins, but the cZ and its isoforms are found both in colonized roots and axenic cultures and are more important for the interaction. In order to investigate if cytokinins like cZ, cZR or iP released by *P. indica* are the growth promoting factors, they were applied to roots exogenously. Although both isomers are able to stimulate *ARR5* gene expression in the roots, we could not demonstrate a specific effect of cZ isomers and iP on growth promotion. This shows that they are not the primary cause of growth promotion. In plants, it has been shown that *cis*-cytokinins have only low activity and the most biologically relevant cytokinins have a *trans*-hydroxylated N^6 side-chain. However, existence of a zeatin *cis-trans* isomerase has proved that *cis*-type cytokinins could contribute to the synthesis of *trans*-type cytokinins (Bassil *et al*., 1993).

The cytokinin-responsive *ARR5* gene is highly expressed in the base of the lateral root primordia and at the root tip during lateral root development of *Arabidopsis* (Lohar *et al*., 2004), and the *ARR5* mRNA level in *P. indica*–treated roots is higher than in control roots. A similar stimulation is obtained for the GUS activity in the roots of transgenic *ARR5*::*uidA* lines. This implicates the role of cytokinin signaling in the growth promotion response together with the data on fungal released cytokinins. In order to determine if the fungal released cytokinins or plant derived ones are important for growth promotion we analysed the mutants defective in biosynthetic pathway. In *A. thaliana* the *trans*-type cytokinins are synthesized in plastids from DMAPP precursors originating from the plastidic methylerythritol (MEP) pathway, conversely the *cis*-type cytokinins are synthesized through prenylation of tRNA molecules in the cytosol using DMAPP precursors originating from the cytosolic mevalonate (MVA) pathway. The *atipt1 atipt3 atipt5 atipt7* quadruple mutant possesses decreased levels of iP, tZ type cytokinins, and their metabolites and slightly increased levels of cZ (Miyawaki *et al*., 2006). Neither the roots nor the shoots of the *atipt1 atipt3 atipt5 atipt7* seedlings respond to *P. indica*. In contrast, roots and shoots of the *atipt2 atipt9* double mutant, which contains reduced levels of iP and cZ-type cytokinins, respond to the fungus, comparable

to the wild type. Thus, biosynthesis of tZ- but not cZ-type cytokinins in planta appears to be required for *P. indica*–induced growth promotion in *Arabidopsis*.

cZ, cZR, cZROG (an inactive stable storage form of cytokinin that can be deglycosylated and reversibly converted into cZR) and tZ can activate the cytokinin responsive *ARR5*::*uidA* gene expression in wild-type roots, although cZ is less effective than tZ, which has also been reported earlier (Spichal *et al*., 2004). cZ isomers produced by the fungus cannot replace the missing tZ isomers in symbiotic roots of the quadruple mutant. This suggests that cZ cytokinins are not efficiently converted to tZ cytokinins, although a *cis-trans* isomerase has been identified in *Phaseolus* spp. (Bassil *et al*., 1993). Alternatively, the available amount of cZ of either fungal or plant origin in the quadruple mutant is too low or ineffective to stimulate growth in the symbiosis. It is known that cZ is less effective than tZ in activating the cytokinin receptors, both in yeast and *in planta* (Inoue *et al*., 2001; Spichal *et al*., 2004), which is consistent with our data on *ARR5* regulation in roots. Another reason for the lack of a response to cZ can be that enzymatic activities deriving from one of the four inactivated genes in the quadruple mutant *atipt1*, *atipt3*, *atipt5,* or *atipt 7* may be required for the beneficial interaction. Finally, the dwarf phenotype of the aerial part of the quadruple mutant may be responsible for the lack of the response, although this appears to be unlikely, since the aerial parts of the *ahk2 ahk3* receptor mutant have a similar phenotype and respond to the fungus. Furthermore, the roots of the quadruple mutant resemble those of the wild type, although their growth is not stimulated by *P. indica*. All this points to the fact that tZ biosynthesis is crucial for the growth promotion response.

Three cytokinin receptors, CRE1, AHK2 and AHK3, have been described (Kyozuka 2007) and all of them are expressed in roots. To analyse which of the receptors are crucial in the interaction we tested the T-DNA knock out mutants for all the genes. Mutations in any of the receptor genes had no effect on the response to *P. indica*. All single mutants showed normal growth promotion like the wild type. Therefore, double mutants for different receptor combinations are investigated. The double mutant *cre1ahk2* shows almost no growth response to *P. indica*. In particular, the roots do not respond to *P. indica*, while some response (3.2 ± 0.6%) is observed for the shoot. This indicates that the CRE1 AHK2 receptor combination is crucial for response to *P. indica*. It has been shown that CRE1 and AHK2 function together in primary root elongation, root response to exogenous cytokinin, leaf cell formation and root branching (Reifler *et al.,* 2006). However, besides CRE1 and AHK2, other receptors exist in

the roots that perceive signals from *P. indica*. A rapid increase in the cytosolic Ca^{2+} level in root cells which activates calcium signalling occurs also in the *cre1 ahk2* mutant (Supplementary Figure 2) suggesting that the two receptors are located downstream the cytosolic Ca^{2+} elevation. The importance of tZ biosynthesis and the CRE1 AHK2 receptor combination for the beneficial interaction between the two symbiotic partners is further supported by the observation that the message levels for *LRR1* and *germin*, two genes that are upregulated in wild-type roots in response of *P. indica*, are not upregulated in colonized roots of the two mutants. While the *LRR1* mRNA level is comparable to the level in uncolonized wild-type seedlings that of *germin* is reduced. Finally, this is not caused by differences in the root colonization as revealed from the colonization level measured with fungal gene, *Pitef1* mRNA levels which shows that colonization of root is unaffected by the mutations. The role of CK genes in the interactions is summarized in Fig 4.

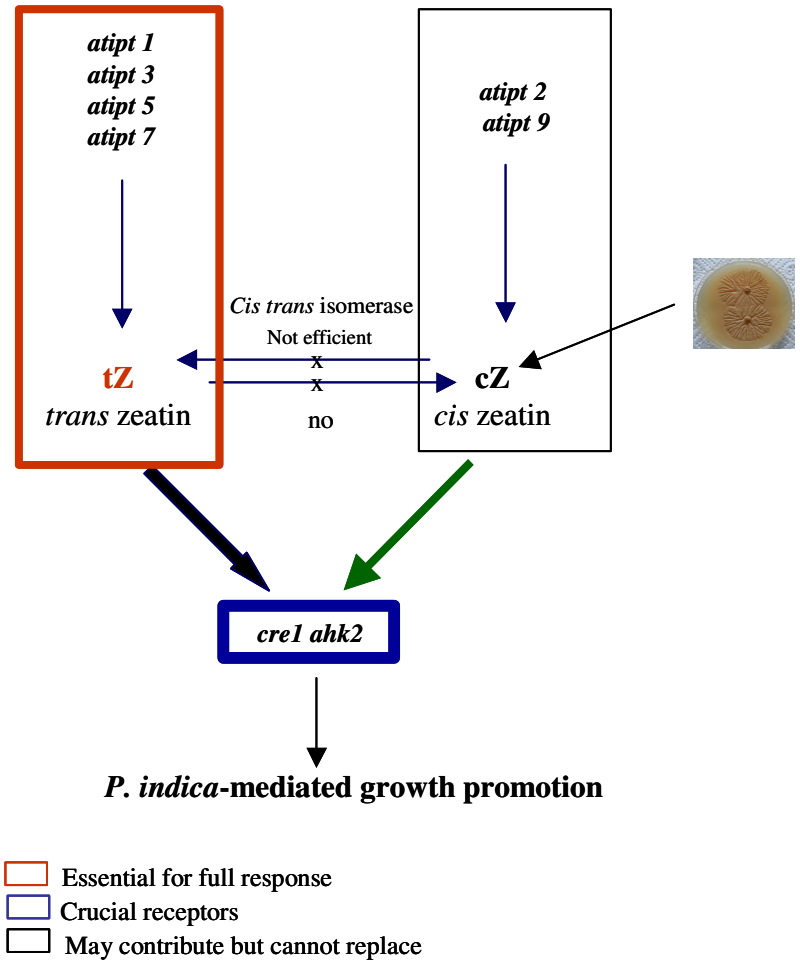


Figure 4: Role of cytokinin from *P. indica* and *Arabidopsis* in the growth promotion response. The *trans* zeatin biosynthetic pathway is essential for the growth promotion response and the CRE1 AHK2 receptor combination is crucial for sensing the response. The fungal released *cis* zeatin cannot replace the missing *trans* zeatin in quadruple mutant as it is not efficiently converted to *trans* zeatin. *cis* zeatin pathway in *Arabidopsis* is not directly involved in the growth promotion response.

The cytokinin oxidase and dehydrogenase overproducers 35S::*AtCKX1* and 35S::*AtCKX2* show reduced cytokinin levels due to an increased cytokinin degradation. The lines have stunted and bushy shoots and their roots are bigger than the wild type (Werner *et al.*, 2003). Growth of both overproducers is clearly promoted by *P. indica*. This indicates that the reduced levels of cytokinins in 35S::*CKX1* and 35S::*CKX2* and the different root architectures do not affect the response to *P. indica*. Many of the mutants analyzed in this study have root architectures, lengths, or root to shoot ratios, which differ substantially from the wild type. For instance, *ahk2ahk3* has long roots relative to the aerial parts, the *35S::CKX1* and *35S::CKX2* lines have stunted and bushy roots (Werner *et al.*, 2003), phenotypes of *atip1 atip3 atip5 atip7* and *ahk2ahk3* are similar under our growth conditions, and both mutants have smaller shoots and longer roots than the wild type, however *atip1 atip3 atip5atip7* does not respond to *P. indica*, while *ahk2ahk3* does. Also, *atipt2 atip9* has a reduced root growth, but its response to *P. indica* is comparable to that of the wild type. This is further supported by the comparative analysis of the cytokinin receptor double mutants. The *ahk2 ahk3* mutant has an increased root system, while that of the other combinations are comparable to the wild type. However, *cre1 ahk2* fails to respond to *P. indica*, while the other mutant combinations do. Taken together, it appears that the size and architecture of the roots and its colonization does not correlate with the growth response induced by *P. indica*.

Colonization of the *cre1 ahk2* and *trans*-zeatin quadruple mutant roots is comparable to that of the wild type, suggesting that recognition of the two symbiotic partners is independent of the phytohormones. We propose that downstream processes such as initiation of cell division, elongation, or both may require the phytohormones. CKs can act as suppressive factors of chitinase activity (Shinshi *et al*., 1987), and some authors suggest that elevated levels of CKs in mycorrhizal roots could suppress the induction of some PR-protein genes, specifically chitinase and glucanase genes (Spanu *et al*., 1989; Shaul *et al*., 2000). Thus fungal released CKs may help *P. indica* in colonization and does not play a direct role in growth promotion. However, the incoming fungus alters the phytohormone levels in

Arabidopsis. It can be to manipulate the auxin to cytokinin ratio when the ratio does not support growth stimulation. Since both root and shoot growth is promoted by *P. indica*, it is likely that the fungus exerts different effects on the phytohormones or their ratios, or both, in the two organs. The involvement in *P. indica* colonized roots of increased cytokinin could also act as developmental signals. Changes in the ratios of phytohormones have been shown to affect different stages in plant development like plant growth, root proliferation and shoot development. All these developmental changes are also associated with *P. indica* interaction and maybe necessary for the plant to regulate the fungal colonization or could be also caused by developmental changes brought about by invading fungus.

Role of sphingokinase in the interaction

In animals, yeast, and, more recently, also in plants, sphingolipids have been shown to be responsible for organizing membrane-localized signaling components (Coursol *et al*., 2005; Hannun and Obeid, 1997; Worrall *et al*., 2003). Their metabolite sphingosine-1-phosphate is a potent lipid mediator in animal, yeast and plant cells, and is generated by the enzyme sphingosine kinase. A leucine-rich repeat protein, pii-2, is required for *P. indica*-mediated growth promotion in *Arabidopsis*. None of the normally observed beneficial responses of *Arabidopsis* mediated by *P. indica* were detectable in *pii-2*, a mutant this protein. Thus, pii-2 appears to be a crucial target protein for *P. indica* in *Arabidopsis*. The pii-2 protein was purified from *Arabidopsis* Triton X-100-insoluble plasma membrane microdomains (Shahollari *et al*., 2007). Triton X-100-insoluble plasma membrane microdomains contain high amounts of phosphosphingolipids, and one sphingolipid-based signaling component is sphingosine-1-phosphate (Ng *et al*., 2001), which is synthesized by sphingosine kinases. We showed that insertion lines for one of the putative four genes encoding sphingosine kinase in *Arabidopsis* show a growth response to *P. indica* that differs from that of the wild type. Two SALK lines (SALK_000250 and SALK_022916) containing insertions in two different intron positions of the *At4g21540* (SPHK1) gene showed a reduced growth response to *P. indica*. This suggests that phospholipids and/or phospholipids-derived components play a role in the interaction between *Arabidopsis* and *P. indica*. It has been shown that SPHK1 activity is required in ABA-induced stomatal movements and in the control of germination. SPHK1 also functions in plant cell signaling as it phophorylates sphingosine, phytosphingosine and, to a lesser extent, other long-chain sphingoid bases (Worall *et al*., 2008). Sphingosine kinases are

required for proper lipid composition and topology of the plasma membrane, which is presumably required for optimal localization of the leucine-rich repeat proteins involved in the *P. indica*/*A. thaliana* interaction.

4. Summary

The interaction between *P. indica* and *A. thaliana* results in growth promotion and enhanced seed production. I studied the role of various *P. indica*-released factors in the interaction and the signalling pathways they activate. In the first manuscript we describe the isolation of a growth-promoting factor from the cell wall of *P. indica*. The cell wall extract (CWE) induces a transient cytosolic Ca^{2+} elevation in the roots of *Arabidopsis* and tobacco plants expressing the Ca^{2+} bioluminescent indicator aequorin. The CWE and the fungus induce a similar set of genes in *Arabidopsis* roots, among them are genes with Ca^{2+} signalling-related functions. The CWE does not promote growth of *P. indica* insensitive lines and the growth promotion is abolished if the Ca^{2+} inhibitor BAPTA is added. The cellular Ca^{2+} elevations and are thus crucial for growth promotion, and Ca^{2+} signalling pathways are activated by incoming fungus. Nuclear Ca^{2+} transients were also observed in tobacco BY-2 cells. Inhibition of the Ca^{2+} response by staurosporine and the refractory nature of the Ca^{2+} elevation suggest that a receptor may be involved. The CWE does not stimulate H_2O_2 production and the activation of defence gene expression, although it led to phosphorylation of mitogen-activated protein kinases (MAPKs) in a Ca^{2+}-dependent manner. Thus, Ca^{2+} is likely to be an early signalling component in the mutualistic interaction between *P. indica* and *A. thaliana* similar to other interactions like AM symbiosis.

P. indica releases isopentenyl-adenine (iP) and *cis*-Zeatin (cZ)-type cytokinins into axenic cultures, but elevated levels of *cis* Zeatin (cZ) isomers accumulate in colonized roots. Although both isomers are able to stimulate *ARR5* gene expression in the roots, we could not demonstrate a specific effect of cZ isomers on growth promotion. However, our data clearly demonstrate that *trans*-zeatin (tZ) biosynthesis in *Arabidopsis* is required for the beneficial interaction as knocking out *atipt1 atipt3 atipt5 atipt7* genes required for (tZ) biosynthesis results in no response to the fungus. cZ isomers produced by the fungus cannot replace the missing tZ isomers in symbiotic roots of the quadruple mutant. Three cytokinin receptors, CRE1, AHK2 and AHK3, have been described. To analyse which of the receptors are crucial in the interaction we tested the T-DNA knock out mutants for all the genes. Single receptor mutants had no effect on the response to *P. indica*.

The double mutant *cre1ahk2* shows no growth response to *P. indica*. It also does not activate common marker genes like *LRR1* and g*ermin* although the colonization is similar to wild type. The CRE1 AHK2 receptor combination is thus crucial for the response to *P. indica*.

Ethyl-methane sulfonate mutant (EMS) mutants which fail to respond to *P. indica* were isolated. One such mutant *P. indica*-insensitive-2 (*pii-2*), and a corresponding insertion line, is impaired in a leucine-rich repeat protein (At1g13230). The protein pii-2, which contains a putative endoplasmic reticulum retention signal, is also found in Triton X-100-insoluble plasma membrane microdomains, suggesting that it is present in the endoplasmic reticulum/plasma membrane continuum in *Arabidopsis* roots. Partial deactivation of a gene for a sphingosine kinase (SPHK1), which is required for the biosynthesis of sphingolipids found in plasma membrane microdomains, also affects the *Arabidopsis*/*P. indica* interaction. Sphingosine kinase (SPHK1) is essential for the growth promotion response and in its absence, *Arabidopsis* growth is not stimulated. Sphingosine kinases are required for proper lipid composition and topology of the plasma membrane, which is presumably required for optimal localization of the leucine-rich repeat proteins involved in the *P. indica*/*A. thaliana* interaction.

5. Zusammenfassung

Die Interaktion von *P. indica* mit *A. thaliana* führt zu einer Wachstumsförderung und einer Zunahme des Saatgutes von *A. thaliana*. In der vorliegenden Arbeit wurden die Funktionen verschiedener, von *P. indica* freigesetzten Faktoren analysiert, die für die Interaktion und die Aktivierung von Signalwegen von Bedeutung sind. Im ersten Manuskript beschreiben wir die Isolation eines wachstumsfördernden Faktors aus der Zellwand von *P. indica*. Dieser Zellwandextrakt bewirkt eine vorübergehende Erhöhung des cytosolischen Ca^{2+}- Spiegels in *Arabidopsis*-Wurzeln und Tabakpflanzen, welche das Ca^{2+} biolumineszente Indikatorprotein Aequorin exprimieren. Zellwandextrakt (ZWE) und Pilz induzieren in *Arabidopsis*-Wurzeln eine ähnliche Gruppe von Genen, unter diesen befinden sich auch Gene mit Ca^{2+}-Signaltransduktion ähnlichen Funktionen. Der ZWE führt bei gegenüber *P. indica* unempfänglichen Linien zu keiner Wachstumsförderung, ebenso wie eine Förderung des Wachstums durch den Ca^{2+}-Inhibitor BAPTA verhindert wird. Wir zeigen, das eine Erhöhung des Ca^{2+}-Spiegels ein frühes Ereignis ist in der Interaktion des wachstumsfördernden Pilzes *P. indica* mit *A. thaliana* und entscheidend für eine Förderung des Wachstums. Eine Erhöhung des Ca^{2+}- Spiegels wurde auch in Zellkernen von BY-2-Tabakzellen festgestellt. Die Hemmung der Ca^{2+}- Antwort (Ausschüttung) durch Staurosporin und die Beständigkeit der Ca^{2+} Erhöhung sprechen für die mögliche Beteiligung eines Rezeptors. Der ZWE stimuliert weder die Produktion von H_2O_2 noch die Aktivierung von Genen der Abwehr, obgleich es zu einer Ca^{2+}- abhängigen Phosphorylierung von Mitogen-aktivierten Proteinkinasen (MAPK) kommt. So ist anzunehmen, das Ca^{2+} zu den frühen Signalkomponenten der mutualistischen Interaktion zwischen *P. indica*, *A. thaliana* und Tabak gehört.

P. indica setzt Isopentenyl-adenin (iP) und *cis*-Zeatin (cZ)- ähnliche Zytokinine in das Kulturmedium frei. In kolonisierten Wurzeln wurde jedoch eine erhöhte Akkumulation von cis-Zeatin (cZ) Isomeren gefunden. Beide Isomere können die Expression des Gens *ARR5* stimulieren, obgleich wir einen spezifischen Effekt von cZ auf die Wachstumsförderung nicht demonstrieren können. Dennoch zeigen unsere Daten deutlich, dass die *trans*-Zeatin- (tZ) Biosynthese in *Arabidopsis* für eine Interaktion mit dem Pilz erforderlich ist, da *atipt1*, *atipt3*, *atipt5* und *atipt7* " knock-out" – Mutanten, welche für die tZ- Biosynthese notwendigen Gene

kodieren, nicht mehr auf den Pilz reagieren. Durch den Pilz produzierte cZ- Isomere können nicht die fehlenden tZ-Isomere in besiedelten Wurzel der 4-fach Mutante ersetzen.

Um zu analysieren, welcher der bekannten Rezeptoren, CRE1, AHK2 und AHK3, ausschlaggebend ist für eine Interaktion, wurden T-DNA "knock-out" Mutanten für diese Gene untersucht. Mutanten mit nur einem defekten Rezeptor-Gen zeigten keine veränderte Reaktion auf *P. indica*, wohingegen die Doppel-Mutante *cre1ahk2* fast keine Wachstumsförderung mehr erkennen lies. Auch Marker Gene wie *LRR1* und *germin* wurden nicht aktiviert, obwohl die Besiedelung durch den Pilz dem Wildtyp vergleichbar war. Demzufolge sind die Rezeptoren CRE1/AHK2 entscheidend für eine Antwort auf *P. indica*. Es wurden Ethylmethansulfonat (EMS) Mutanten isoliert, die keine Reaktion auf *P. indica* zeigten. Eine dieser Mutanten, *Piriformospora indica*-insensitive-*2 (pii-2)* und die entsprechende Insertionslinie, sind beeinträchtigt in einem Leucin-reichen Repeat Protein (At1g13230). Das Protein pii-2, welches ein putatives Signal zur Lokalisation für das endoplasmatische Retikulum enthält und in Triton X-100 unlöslichen Plasmamembran-Mikrodomänen gefunden wurde, schlägt vor, dass dieses Protein im Endoplasmatischen Retikulum/ Plasmamembran-Kontinuum in *Arabidopsis*-Wurzel zu finden ist. Die partielle Deaktivierung eines Genes, welches für eine Sphingosinkinase kodiert (SPHK1) und erforderlich ist für die Biosynthese von Sphingolipiden in Plasmamembran-Mikrodomänen, beeinflußt ebenfalls die *A. thaliana*/*P. indica* Interaktion. Die Sphingosinkinase (SPHK1) ist essentiell für den wachstumsfördernden Effekt und in Abwesenheit des Gens wird das Wachstum von *Arabidopsis* nicht stimuliert.

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8. Declaration of Independent Assignment

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

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

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

Jena, January 12th 2009 ……

Jyothilakshmi Vadassery

Curriculum Vitae

Personal Details

Name: Jyothilakshmi Vadassery

Date of birth: 01.06.1979

Place of birth: Kerala, India

Nationality: Indian

Address: Neugasse 26, Jena

Email: jvadassery@ice.mpg.de

Education:

2006-present: Ph.D. student at Institute of Plant Physiology, Friedrich Schiller University, Jena

2003-2005: Master of Genetics and Plant Breeding from Indian Agricultural Research Institute (IARI), New Delhi, India

1998-2002: Bachelor of Agricultural Sciences, Kerala Agricultural University, India.

Publications:

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

Vadassery, J., Ritter, C., Venus, Y., Camehl, I., Varma, A., Shahollari, B., Novák, O., Strnad, M., Ludwig-Müller, J., Oelmüller, R**.** (2008). The role of auxins and cytokinins in the mutualistic interaction between the *Arabidopsis and Piriformospora indica. Mol. Plant Microb. Interact.* 21(10), 1371-83.

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

Vadassery, J., Tripathi, S., Prasad, R., Verma, A., Oelmüller, R. (2008). Ascorbate, monodehydroascorbate reductase3 and 2 are crucial for the mutualistic interaction between *Piriformospora indica* and *Arabidopsis*. *Journal of Plant Physiology* (In press).

Jyothilakshmi, V., Singh, A., Gaikwad, K., Vinod, K., Singh,N.K., and S.M.S.Tomar.(2008) RNA editing in CMS wheat: Influence of nuclear background leads to differential editing on *orf256.Indian J. Genet*., 68(4), 1-8.

Scientific presentations:

1) Presented a poster in International meeting on "Calcium based signalling systems in plants", at Royal Dublin Society (Dec 2007), Ireland on the topic "Cytosolic calcium elevation by growth promoting fungus, *Piriformospora indica* in Arabidopsis roots".

2) Presented a poster entitled "Role of *Piriformospora indica* released cytokinins" in International meeting on "Communication in plants and their response to the environment", Halle, Germany (May 2007).

3) Presented a poster in 6th Biannual IMPRS Symposium, Altes Schloss Dornburg, Germany (March 2007) on the topic, Hormones and symbiosis: Role of *Piriformospora indica* released factors in interaction with *Arabidopsis*.

4) Gave a talk in South Deutschland Plant physiology meeting, Leipzig, Germany on Recognition of elicitors of *Piriformospora indica* by *Arabidopsis thaliana*.

5) Gave a talk in 5th Biannual IMPRS Symposium / MPI for Chemical Ecology, Jena, Germany on, Cytosolic calcium elevation in response to a cell wall extract from *P. indica* in *Arabidopsis.*

6) Presented a poster in 4th Biannual IMPRS Symposium / MPI for Chemical Ecology, Jena, Germany (March 2006) on the topic, Fungal signals in plant – fungus collaboration.

Supplementary data

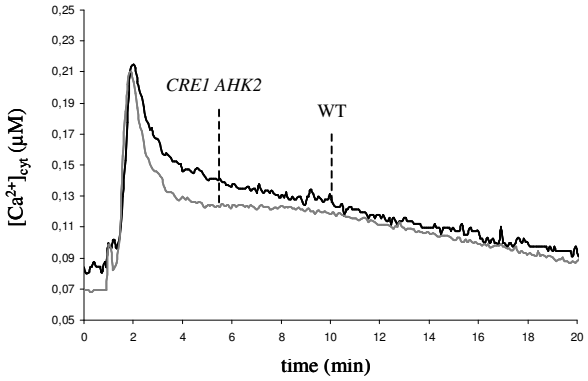

Figure 1: *P. indica* CWE induces similar changes in $[Ca^{2+}]_{cyt}$ in apoaequorin-transformed *Arabidopsis* WT seedlings and *CRE1 AHK2* double mutant. 50 µl CWE was applied to *Arabidopsis* roots and measured for 20 min. The values represent mean of 3 independent experiments.

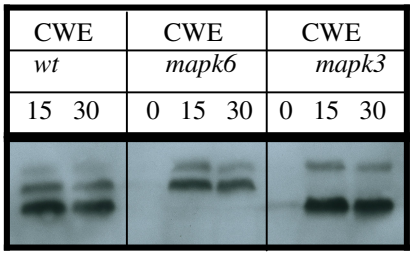

Figure 2: MAPK activation monitored by western blot with antibodies that recognises dual phosphorylation of the activation loop of MAPKs in roots. Wild type (WT) and knock out mutants of *MAPK6* and *MAPK3* were treated with *P. indica* cell wall extract (CWE) to identify which of the bands correspond to MAPK6 and MAPK3.The lowest of three bands corresponds to MAPK6 and second to MAPK3.