Molecular analyses of the interaction between *Arabidopsis thaliana* and the endophytic fungus *Piriformospora indica*

Thesis

in order to receive the academic degree doctor rerum naturalium (Dr.rer.nat.)

submitted to the

Rat der Biologisch-Pharmazeutischen Fakultät

Friedrich Schiller University Jena

by

Bationa Shahollari

Jena, Mai 2006

Referees:

1. Prof. Dr. Jutta Ludwig-Müller
2. PD. Dr. Thomas Pfannschmidt
3. Prof. Dr. Ralf Oelmüller

Date of the *Rigorosum*: 14.06.06

Date of the public defence: 26.06.06

Manuscript I

Association of *Piriformospora indica* with *Arabidopsis thaliana* roots represents a novel system to study beneficial plant–microbe interactions and involves early plant protein modifications in the endoplasmic reticulum and at the plasma membrane

Tatjana Peškan-Berghöfer, Bationa Shahollari, Pham Huong Giong, Solveig Hehl, Christine Markert, Verena Blanke, Gerhard Kost, Ajit Varma and Ralf Oelmüller

Physiologia Plantarum (2004) **122**:4, 465-477

This manuscript describes, for the first time, the newly established system to study the beneficial interaction between P. indica, an endophytic fungus of the Sebacinaceae family, and A. thaliana.

Dr. Tatjana Peškan-Berghöfer and Prof. Ralf Oelüller planned the experiments. Most of the experiments were performed by Dr. Tatjana Peškan-Berghöfer. Fluorescence measurements were performed by Verena Blanke in the Department of Professor Gerhard Kost. Christine Markert helped us with the mass spectrometry. Pham Huong Giong introduced us into the techniques required for the experiments on soil.
I repeated all experiments performed by Dr. Tatjana Peškan-Berghöfer. In addition, I extracted the spots from the two-dimensional gels and identified the proteins by mass spectrometry. The quantification of the data was performed by myself. I also performed the cultivation of plants and inoculation with fungus, and all experiments on soil.

Manuscript II

Receptor kinases with leucine-rich repeats are enriched in Triton X-100 insoluble plasma membrane microdomains from plants

Bationa Shahollari, Tatjana Peskan-Berghöfer and Ralf Oelmüller

Physiologia Plantarum (2004) **122**: 397-403

This manuscript describes the protein pattern of Triton X-100 insoluble plasma membrane microdomains. The protein composition is enriched in signalling components: receptor kinases with leucine-rich repeats, 10 other kinases, the ß subunit of heterotrimeric G-proteins and five small GTP-binding proteins. Thus, specific signalling components are highly enriched in plant plasma membrane microdomains while others are excluded.

I isolated plasma membrane microdomains from A. thaliana and mustard cotyledons, separated the protein in one and two dimensional gels and determined the protein composition of the plasma membrane microdomains by mass spectrometry. The experiments in this manuscript were designed by me, Dr. Tatjana Peskan-Berghöfer and Prof. Ralf Oelmüller and performed by myself.

Manuscript III

Expression of a receptor kinase in *Arabidopsis* roots is stimulated by the basidiomycete *Piriformospora indica* and the protein accumulates in Triton X-100 insoluble plasma membrane microdomains

Bationa Shahollari, Ajit Varma, Ralf Oelmuller.

J Plant Physiol. (2005) **162**(8):945-58.

Manuscript III describes the accumulation of the mRNA for a receptor kinase in A. thaliana roots, which were co-cultivated with P. indica. This represents one of the earliest events of a plant root in response to a fungus. During the recognition period of both organisms, the mRNA for a receptor kinase with leucine-rich repeats (LRR1) is transiently upregulated. The kinase is located in Triton X-100-insoluble plasma membrane microdomains. P. indica promotes growth of A. thaliana, and this promotion was accompanied by a massive transfer of phosphate from the media to the aerial parts of the seedlings.

All experiments, with the exception of the phosphate uptake experiment, were designed by me and Prof. Oelmüller and performed by myself. The phosphate uptake studies were performed in the laboratory of Professor Ajit Varma.

Manuscript IV

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, Ajit Varma, Ralf Oelmüller

(revised version, submitted to *The Plant Journal*).

Manuscript IV describes *A. thaliana* mutants which fail to respond to the *P. indica*. An ethylmethane sulfonate (EMS) and the corresponding insertion line are impaired in a leucine-rich repeat protein (LRR2) and are blind to *P. indica.*
Inactivation of the *A. thaliana* single-copy gene *DMI1*, which codes for an ion carrier required for mycorrihiza formation in Legumes, does not affect the beneficial interaction between the two symbiotic partners.

All experiments were designed by me and Prof. Oelmüller and performed by me . The original EMS mutant was isolated by Prof. Ajit Varma. Also the Fluorescence measurements are done by Prof. Ajit Varma.

Manuscript V

MATH domain proteins represent a novel protein family in *Arabidopsis thaliana*, and at least one member is modified in roots during the course of a plant–microbe interaction

Ralf Oelmüller, Tatjana Peškan-Berghöfer, Bationa Shahollari, Artan Trebicka, Irena Sherameti, Ajit Varma

Physiologia Plantarum (2005) **124:**152-166

A MATH [meprin and TRAF (tumour necrosis factor receptor-associated factor) homology] domain-containing protein in the plasma membrane of *A. thaliana* roots becomes transiently modified in response to *P. indica*. Since nothing is known about MATH proteins in plants, we analysed the fifty nine genes present in the *A. thaliana* genome.

The experiment described here and the design of the manuscript was planned by me and Prof. Oelmüller. I performed the isolation of microsomes, the separation of proteins by two-dimensional gel electrophoresis and analysed the proteins by mass spectrometry. Dr. Artan Trebicka and Dr. Irena Sherameti isolated the chloroplasts and plastid RNA. I generated the phylogenetic tree for the MATH proteins, with some help from Dr. Artan Trebicka.

The modification of the MATH protein in A. thaliana roots in response to P. indica was originally discovered by Dr. Peskan-Berghöfer. I continued with the analysis of the MATH proteins in P. indica insensitive mutants, by running two-dimensional gels from plasma membrane microsomes from roots. I analysed the proteins by mass spectrometry. I also participated in the analysis of the gene family in A. thaliana.

Manuscript VI

Molecular analyses of the interaction between *Arabidopsis* roots and the growth-promoting fungus *Piriformspora indica*

Ralf Oelmüller, Bationa Shahollari, Tatjana Peškan-Berghöfer, Artan Trebicka, Pham Huong Giong, Irena Sherameti, Menno Oudhoff, Yonne Venus, Lothar Altschmied, Ajit Varma.

Endocytobiosis Cell Res. (2004) **15** (2), 504-517.

This review proposes a working hypothesis for early phases of the recognition between P. indica and A. thaliana. A MATH protein with homology to metalloproteases is transiently modified in the plasma membrane of the roots during the recognition period of both organisms. Furthermore, the mRNA for two receptor kinases are transiently upregulated. Biochemical studies uncovered that the receptor kinases co-purify with a small GTP-binding protein. Inactivation of one of the receptor kinases strongly retards the interaction between both organisms. Finally, recognition of both organisms appear to depend on a lipid-signalling pathway, since inactivation of AGC2-1, a protein kinase activated by the 3´-phosphoinositide-dependent kinase PDK1, completely abolishes the growth promoting effect induced by P. indica.

Manuscript VII

The endophytic fungus *Piriformospora indica* stimulates the expression of nitrate reductase and the starch-degrading enzyme glucan-water dikinase in tobacco and *Arabidopsis* roots through a homeodomain transcription factor that binds to a conserved motif in their promoters

Irena Sherameti, Bationa Shahollari, Yvonne Venus, Lothar Altschmied, Ajit Varma, Ralf Oelmüller

J. Biol. Chem. (2005) **280**(28):26241-26247.

The growth-promoting effect initiated by *P. indica* is accompanied by a co-regulated stimulation of enzymes involved in nitrate and starch metabolisms. *P. indica* stimulates nitrogen accumulation and the expression of the genes for nitrate reductase and the starch-degrading enzyme glucanwater dikinase (SEX1) in tobacco roots. *P. indica* also stimulates the expression of the *uidA* gene under the control of the *A. thaliana* nitrate reductase (*Nia2*) promoter in transgenic tobacco seedlings. These responses are mediated by a homeodomain transcription factor.

I generated the homeodomain transcription factor knock out line in *A. thaliana* and analysed the mutant in details. Yvonne Venus performed the root staining and Dr. Lothar Altschmied performed the microarray analysis.

1. Introduction

***Piriformospora indica*, a growth-promoting fungus of the Sebacinaceae family**

In nature, most plants live in symbiotic association with soil fungi (Smith and Read 1997; Harrison 1999; Kistner and Parniske 2002; Strack *et al.* 2003; Parniske 2004). Plants perform photosynthesis and deliver photoassimilates to the fungi, while the fungi provide inorganic nutrients to the plants due to better access of the hyphae to soil minerals (Smith and Read 1997; Harrison 1999; Kistner and Parniske 2002; Strack *et al.* 2003; Jia *et al.* 2004; Karandashov *et al.* 2004; Sherameti *et al.* 2005). The cooperation of plant roots and the mycelium of fungi thus leads to an optimized usage of nutrient resources. The interaction between the two symbiotic organisms, called mycorrhiza, is poorly understood at the molecular level. Only recently, substantial progress has been made with the help of mutants (cf. below).

Concerning the interaction with plants, fungi can be divided into three groups: Parasitic, saprophytic and symbiotic or mycorrhizal fungi. Parasitic fungi live at the expense of a host. Many parasitic species are only able to infect diseased or weakened host plants. Saprophytic fungi are able to digest and thus recycle dead organic material. Most of them are also capable of decomposing lignin and cellulose. Mycorrhizal fungi form beneficial interactions with plants. This association is usually considered to be mutualistic due to the highly beneficial relationships between the two partners, where the fungal partner belongs to the Basidiomycetes, Ascomycetes or Zygomycetes. They form association with most vascular plants (Harley and Smith 1983; Brundrett 1991; Kendrick 1992). At least seven different types of mycorrhizal associations have been described so far. They include different taxonomic groups of fungi and host plants with distinct morphology patterns: ectomycorrhiza, orchid mycorrhiza, ectendomycorrhiza, arbutoid mycorrhiza, monotropoid mycorrhiza, ericoid mycorrhiza, vesicular-arbuscular mycorrhiza and the special mycorrhiza formed with the Australian lily *Thysanotus*.

Arbuscular mycorrhiza fungi from the order Glomales of the Zygomycota are the most widespread and probably most ancient symbionts in the world. Glomales are found in many ecosystems and interact with many different plant species (cf. Schüssler and Kluge 2001). However, it is generally assumed that fungi do not form any beneficial interactions with the model plant *Arabidopsis thaliana*, whereas the Basidiomycete *Piriformospora indica* fungus (Fig. 1), is able to colonise *A. thaliana* roots. *P. indica* is an endophytic fungus of the Sebacinaceae family, and the interaction is different in many aspects from mycorrhizal interactions. Although endophytic interactions between plants and fungi have already been described in the 19th century, endophytic microorganisms received considerable attention only during the last 20 years, when their capacity to protect their hosts against insects, pathogens or herbivores was recognized. Beside an increased nutrient accommodation, endophytic fungi also confer other important properties to plants, such as resistance to stress, alteration in physiological properties, production of phytohormones as well as the production of compounds of biotechnological interest.

The aim in the present investigations was to use the genetically well characterized model plant *A. thaliana* to study its interaction with *P. indica* at the molecular level.

Figure 1. *P. indica* grown in liquid Käfers medium (left) and on agar with Käfers medium (right).

P. indica promotes growth and seed production of *A. thaliana* plants (Peskan *et al.* 2004; Shahollari *et al.* 2005). This interaction is comparable to mycorrhiza. The molecular analysis of mycorrhizal interactions is often difficult to study, because either one of the symbiotic partners or both of them are not well characterized at the molecular level. Furthermore, in almost all cases the fungus can only grow in the

presence of plant roots. If *A. thaliana* can be used as interaction partner, the availability of genetic tools provides enormous advantages over the natural host plants of this fungus. *P. indica* was isolated from the rhizosphere of desert plants in Rajasthan, India and possesses a wide spectrum of host plants. All arbuscular mycorrhiza plants so far tested showed positive response to *P. indica.* Additionally, this fungus has been shown to act as a growth promoting symbiont of terrestrial orchids (Blechert *et al.* 1999) and non-mycorrhizal plants, such as *A. thaliana* (L.) Heynh. (Brassicaceae) or *Spinacia oleracea* L. (Pham *et al.* 2004; Kaldorf *et al.* 2005). *P. indica* forms chlamydospores containing 8 to 25 nuclei (Verma *et al.* 1998). Stages of a sexual life cycle have not been observed. Analysis of the taxonomic position by molecular methods based on 18S rRNA sequences and by electron microscopy suggest that *P. indica* is related to the Hymenomycetes of the Basidiomycota (Varma *et al.* 1999). *P. indica* belongs to the Sebacinaceae family (Fig. 2).

Originally, it was believed that members of the Sebacinaceae family form exclusively saprophytic or parasitic interactions with plant roots. However, more recently, also a broad diversity of beneficial associations of various members of the heterobasidiomycetous Sebacinaceae fungi have been observed (Varma *et al.* 1999; Sahay and Varma 1999; Selosse *et al.* 2002; Glen *et al.* 2002; Urban *et al.* 2003; Weiss *et al.* 2004; Peškan-Berghöfer *et al.* 2004; Shahollari *et al.* 2005; Kaldorf *et al.* 2005; Barazani *et al.* 2005; Waller *et al.* 2005; Sherameti *et al.* 2005). Since most of the more basal taxa of Basidiomycetes consist of predominantly mycoparasitic and phytoparasitic fungi, it appears that Sebacinaceae is the most basal group of Basidiomycetes which contains mycorrhiza-forming taxa. Mycorrhizal taxa of Sebacinaceae include mycobionts of ectomycorrhizas, orchid mycorrhizas, ericoid mycorrhizas, and jungermannioid mycorrhizas. Such a wide spectrum of mycorrhizal types in one fungal family is unique (Weiss *et al.* 2004).

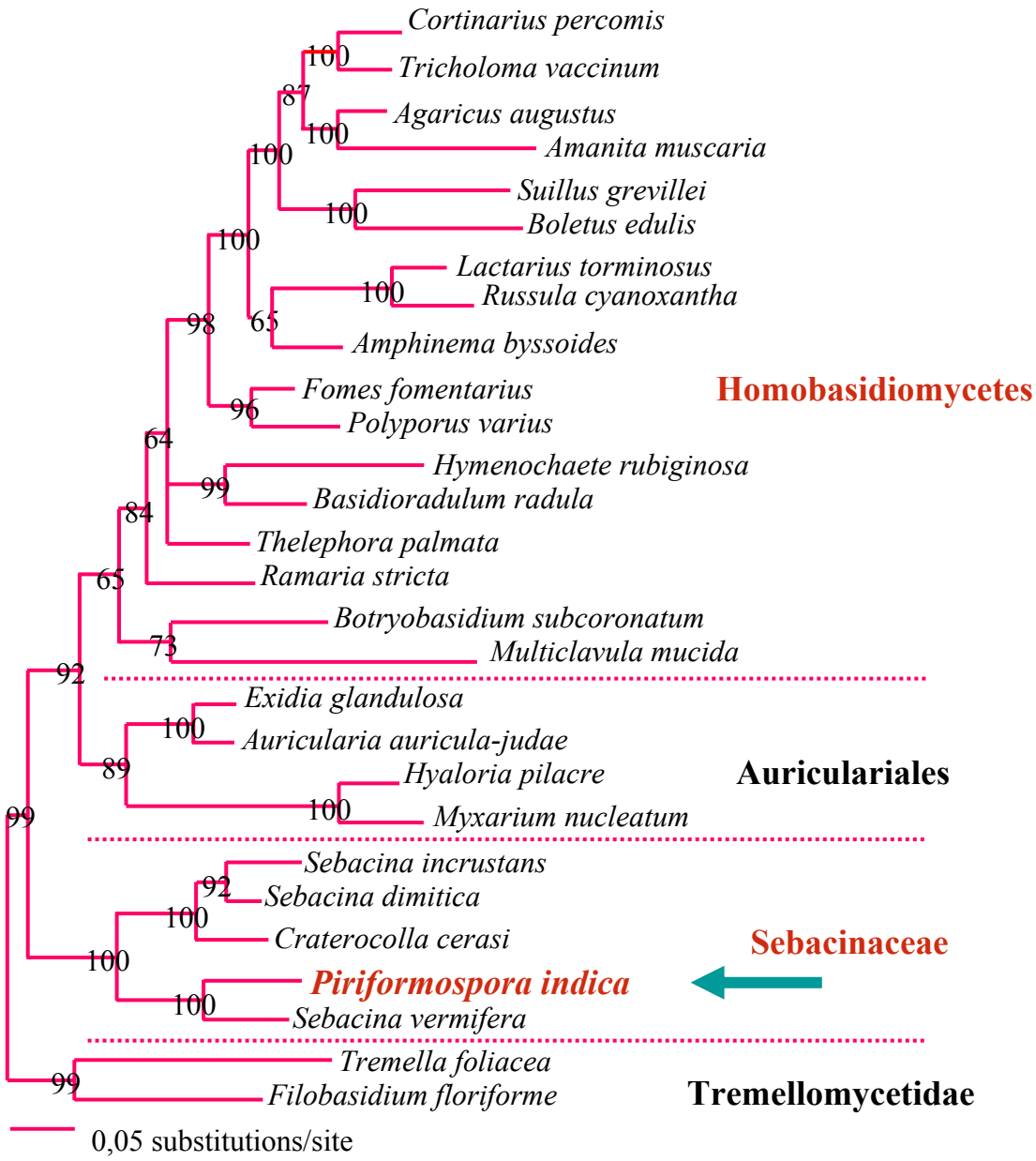
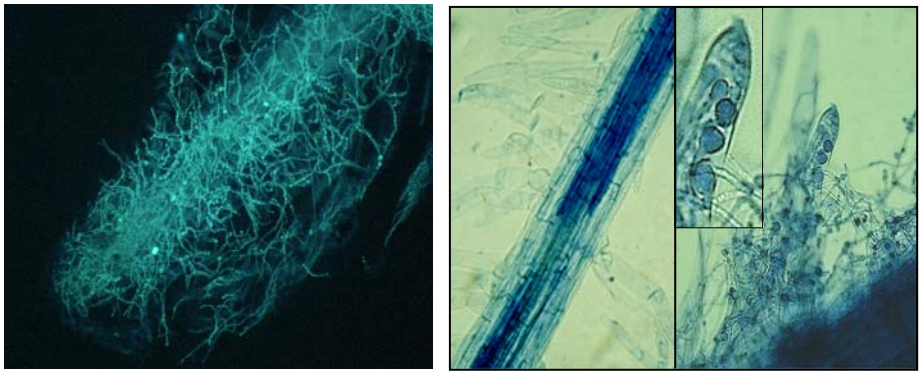


Figure 2. The phylogenetic position of *P. indica*. The phylogenetic position of *P. indica* in the Sebacinaceae family within the Basidiomycetes is suggested from molecular phylogenetic analyses based on nuclear rDNA. From Weiss *et al.* (2004).

Most studies with root endophytes were carried out with plants of alpine and subalpine regions (Read and Haselwandter 1981; Stoyke and Currah 1990; O'Dell and Trappe 1992). In several cases, a positive influence on plant growth has been shown (cf. Haselwandter and Read 1982; Sneh *et al.* 1986; Dewan and Sivasithamparan 1988; Mucciarelli *et al.* 1995; Gasoni and Stegman de Gurfinkel 1997; Varma 1999). *P. indica* forms inter- and intracellular hyphae in the root cortex, often differentiating into dense hyphal coils and chlamydospores (Varma *et al.* 2001). As in typical mycorrhizal symbioses, hyphae never traverse the endodermis (cf. Fig. 3). In contrast to arbuscular mycorrhizal fungi, *P. indica* can be easily cultivated in axenic culture where it produces chlamydospores (Peskan *et al.* 2004; Pham *et al.* 2004; Shahollari e*t al.* 2004). The fungus is able to interact with the roots of various plants species including trees, agri- and horticultural and medicinal plants, mono- and dicots and mosses (Varma *et al.* 1999, 2001; Kumari *et al.* 2003; Shahollari *et al.* 2004; Peškan-Berghöfer *et al.* 2004; Sherameti *et al.* 2005; Waller *et al.* 2005). Upon successful establishment of the interaction in the roots, *P. indica* reprograms barley to salt stress tolerance, resistance to diseases and causes higher yield of seeds (Waller *et al.* 2005). Hence, it provides a promising model organism for the investigations of beneficial plant-microbe interaction and enables the identification of compounds, which may improve plant growth, productivity and fertility. The observation that growth and development of *A. thaliana* is stimulated by *P. indica*, and that the presence of the fungus has a strong impact on the number of siliques and seeds per plant is consistent with the interaction patterns observed for other plant species.



A B C

Figure 3. ***P. indica* colonises *A. thaliana* roots.** *P. indica* hyphae in *A. thaliana* root tip (A), roots of *A. thaliana* not colonized by *P. indica* (B), *P. indica* chlamydospores within the root cells of *A. thaliana* (C), under fluorescent light microscopy. From Peskan-Berghöfer, (unpublished).

Leucine-rich repeat containing proteins and lipid rafts in plants play important roles in early recognition events

In the present project two leucine-rich repeat (LRR) containing proteins were identified in *A. thaliana*, which might be involved in early recognition events between the two symbiotic partners. One of them (LRR2) is crucial for the establishment of this beneficial interaction. Silencing of the gene encoding LRR2 abolishes the response of the plants to *P. indica*. This could be confirmed by two independent mutations, an EMS (ethylmethane sulfonate) mutant and a T-DNA knock out line, respectively (Tab. 1). The other LRR protein (LRR1) is an atypical receptor kinase, and the mRNA for this protein is transiently upregulated during the recognition period of both organisms. Biochemical analysis allowed me to identify these two LRR proteins in Triton X-100-insoluble plasma membrane microdomains. I will first describe here the LRR proteins and then the lipid rafts.

	Name of the gene	Name of the Protein	Type of mutation
1	At5g16590	LRR1	T-DNA
2	At5g16590	Pi-2	EMS
3	At1g13230	LRR2	T-DNA

Table 1. Mutant used for this work are T-DNA lines or EMS mutant. At5g1659 and At1g13230 are two genes involved in *P. indica* and *A. thaliana* interaction, encoding LRR1 and LRR2 protein, respectively.

LRR proteins

The majority of plant proteins with extracellular LRR motifs belong to the LRR-receptor kinase (RK) family. Transmembranal RKs are implicated in all aspects of plant biology from early embryogenesis to disease resistance. They mediate signal transduction pathways leading to cell proliferation, growth and differentiation. Plant RKs comprise a monophyletic group related to animal RKs (Torii 2004). Only a small number of RK ligands (both endogenous and exogenous) have been identified in plants so far. This is probably attributed to three facts. First, differences in the biochemistry of extracellular matrices in animals and plants lead to differences in extracellular domains of the RK, and probably also in their ligands. Second, technical problems associated with plant extracellular matrix make direct physical identification of ligands difficult. And third, probable genetic redundancy between genes that encode at least some endogenous ligands (Vanoosthuyse *et al.* 2001; Cock and Cormick 2001) makes genetic identification difficult (Kim *et al.* 2005).

Plant receptor kinase genes constitute the largest family of plant kinases, with more than 600 members in *A. thaliana* (Shiu *et al.* 2004). They are classified into several groups based on the structure of the extracellular domains. RKs containing an extracellular LRR motif comprise by far the largest subfamily of plant receptor kinases, with approximately 222 members in the *A. thaliana* genome (Shiu and Bleecker 2001). The RK signature motifs include a N-terminal signal peptide, an extracellular LRR domain, a single membrane-spanning region and a cytoplasmic protein kinase domain (Torii 2004; cf. Fig. 4). Their overall structure suggests a role of the extracellular domain in the perception of an extracellular ligand and signal transduction through the intracellular kinase domain (Yoshida and Parniske 2005).

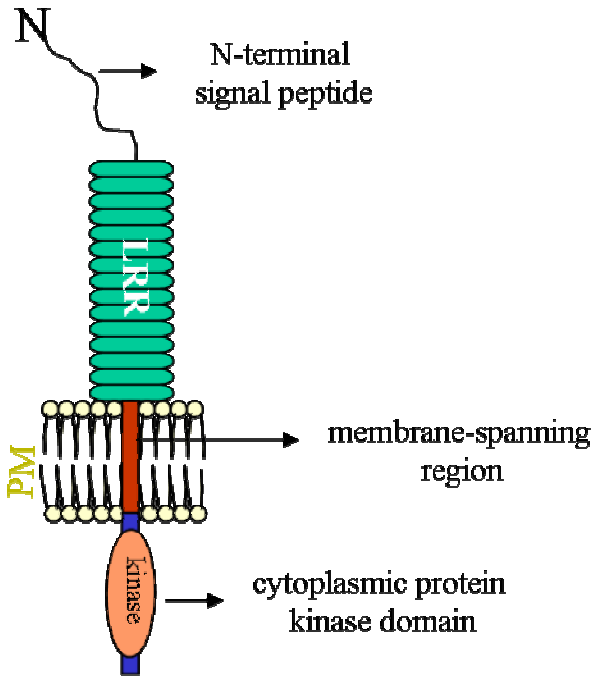


Figure 4. **Schematic representation of RK.** RK signature motifs include an N-terminal signal peptide, an extracellular LRR domain, a single membrane-spanning region, and a cytoplasmic protein kinase domain (Torii 2004). PM: Plasma membrane.

LRR-RKs play fundamental roles in development, steroid hormone response, stress response, desease resistance, symbiosis and pathogen recognition (Jones and Jones 1997; Torii 2000; Torii and Clark 2000; Bioshop and Koncz 2002; Kistner and Parniske 2002; Gomez-Gomez and Boller 2002). Almost all plant LRR-RKs analyzed to date possess serine/threonine kinase activities (cf. below). The phenotypes associated with mutations in various LRR-RKs show that they play roles in diverse processes during growth and development (Diévart and Clark 2004).

For the entry of both mycorrhizal fungi and nodule-forming bacteria into root epidermal or cortical cells, are at least seven components required: a receptor kinase (Endré *et al.* 2002; Stracke *et al.* 2002), a calmodulin-dependent protein kinase (Levy *et al.* 2004), the two plastid localized proteins CASTOR and POLLUX (Imaizumi-Anraku *et al.* 2005) and a predicted ion channel (DMI; *does not make infection*; Ané *et al.* 2004). Based on our results, a receptor kinase, seems to be also required for the *A. thaliana*/*P. indica* symbiosis (cf. below).

Microdomains or lipid rafts

Plant plasma membrane microdomains are highly enriched in signalling compounds such as receptor kinases, monomeric and heterotrimeric G proteins and signalling kinases (Shahollari e*t al.* 2004). A large number of signalling molecules are concentrated within these rafts which have been proposed to function as signalling centres capable of facilitating efficient and specific signal transduction (Peskan e*t al.* 2000; cf. below).

Lipids and proteins in plasma membrane are often organized into domains or rafts enriched in sphingolipid and cholesterol. Sphingolipid microdomains float in the phospholipid bilayer, leading to the term 'lipid rafts". Cholesterol preferentially partitions into the liquid-ordered phase rather than the liquid-disordered phospholipid bilayer and is essential for the maintenance of the two phases. It seems that the main forces enabling the formation of rafts are lipid–lipid interactions. Sphingolipids are able to associate with each other through interactions between their carbohydrate heads and their long, predominantly saturated, lipid hydrocarbon chains, while cholesterol molecules are supposed to serve as spacers to fill voids between sphingolipids (Simons and Ikonen 1997). It was also suggested that the molecular composition of lipid rafts further differs from the remainder of the plasma membrane by hosting a specific subset of integral and membrane-associated proteins including glycosylphosphatidylinositol (GPI)-anchored polypeptides, while excluding others (Fig. 5).

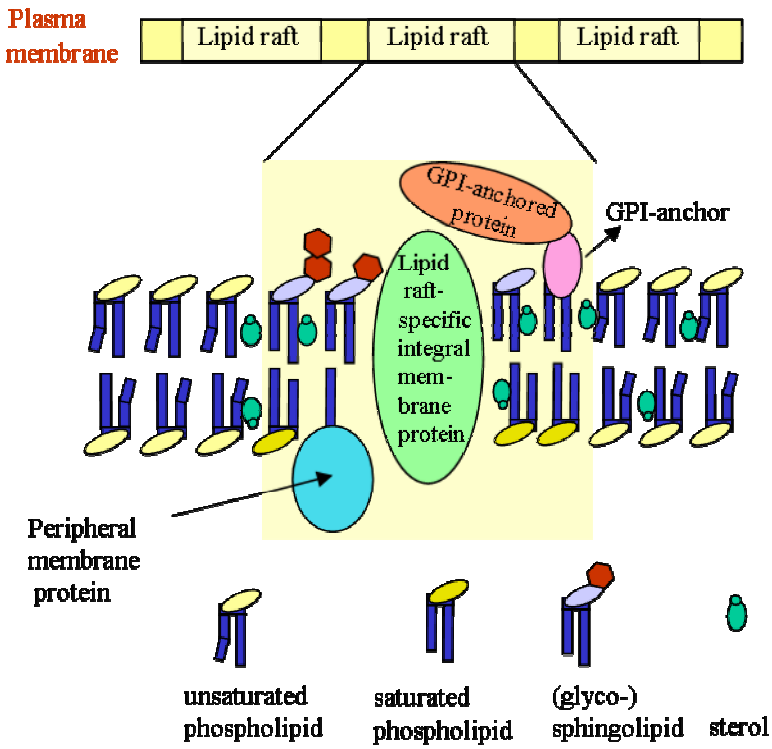


Figure 5. **Schematic representation of presumed lipid raft organization.** According to the lipid raft hypothesis, rafts are plasma membrane (PM) patches characterized by a particular molecular composition. They are supposed to be enriched in saturated phospholipids, sterols, and sphingolipids and assumed to harbor a subset of membrane-associated polypeptides, including GPI-anchored proteins, peripheral and integral PM proteins. Modified from Riyaz *et al.* (2005).

In eukaryotes, lipids fulfil numerous roles: they form indispensable hydrophobic barriers for cellular compartments, functions as energy store, signalling molecules, defence compounds and they are also employed for post-translational protein modification. In plants, lipids additionally represent essential components of cutins and waxes that protect the plant against the various environmental factor (Riyaz *et al.* 2005). Furthermore, a range of biochemical, immunological and biophysical methods provide evidence for the existence of plasma membrane microdomains. These sphingolipid/cholesterol-rich liposomes were found to be insoluble in mild nonionic detergents such as Triton X-100 at 4°C (Fig. 6). Such detergent-insoluble low density membrane fractions are thought to reflect the *in vivo* composition of lipid microdomains (Brown and Rose 1992; Schroeder *et al.* 1994; Simons and Ikonen 1997; Brown and London 1998).

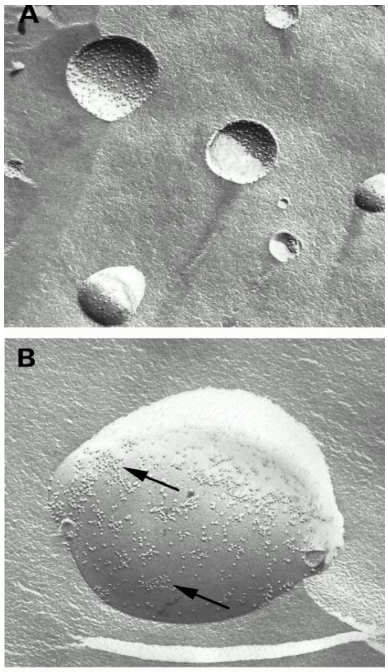


Figure 6. **Freeze-fracture electron microscopy.** A: Tobacco plasma membrane vesicles, B: Low-density plasma membrane subfractions after Triton X-100 solubilization. From Peskan *et al.* (2000).

A MATH protein in the plasma membrane of *A. thaliana* roots is transiently modified in response to *P. indica*.

The interaction between *A. thaliana* and *P. indica* is also accompanied by the modification of a plasma membrane protein called MATH [Meprin and TRAF (tumor necrosis receptor associated factor) Homology] domain-containing protein (Peskan-Berghöfer *et al.* 2004). This modification is considered to be independent of other responses of *A. thaliana* to *P. indica*, because it is a transient and posttranscriptional modification at the plasma membrane of roots. This modification was used as one of the markers to monitor the interaction between wild-type and mutants of *A. thaliana* to *P. indica* in my studies. In general MATH proteins are involved in nodule formation in *Medicago* and protein degradation in the *A. thaliana* cytosol. They exhibit sequence similarities to meprins, extracellular peptidases which cleave (signal) peptides, and to TRAFs, intracellular proteins which interact with receptor kinases at the plasma membrane. Fifty nine genes for MATH proteins are present in the *A. thaliana* genome. Members of this protein family are predicted to be found in the endoplasmatic

reticulum–plasma membrane–extracellular space continuum, in the nucleus–cytosol compartment and in organelles. It has been shown that the MATH protein is modified even before visible physical contact between both organisms (Oelmüller *et al.* 2005). Furthermore, this modification is no longer detectable in an *A. thaliana* mutant which does not respond to *P. indica*. Comparable with the results obtained for the mRNA of the receptor kinase LRR1, the modification of the MATH protein is only transiently and no longer detectable once the interaction between both organisms has been established (Oelmüller *et al.* 2005). This implies that a functional link exists between the recognition of the fungus and the modification of the protein in the plasma membrane.

A homeodomain transcription factor is a downstream target for *P. indica* in *A. thaliana*.

Often, nitrogen is the limiting factor for plant growth and development. It is recruited by plants either as nitrate or ammonium, or, for a few species, by nitrogen fixation with the help of rhizobia (Esseling and Emons 2004; Gage 2004). Mycorrhizal fungi also play an important role in delivering either nitrate or ammonium to the root cells. It is assumed that mycorrhizal fungi recruit preferentially ammonium rather than nitrate from the soil, and that amino acids represent the major compounds transfering nitrogen to the host plant (Boukcim and Plassard 2003; Guescini *et al.* 2003). By analysing the interaction of *P. indica* with tobacco plants we found that the fungus promotes growth of tobacco seedlings and is accompanied by an enormous requisition of nitrogen from the environment. In contrast to mycorrhizal associations, *P. indica* stimulates nitrogen accumulation and the expression of the genes encoding nitrate reductase (*Nia2*) and the starch-degrading enzyme glucan water dikinase (*SEX1*) in roots. A homeodomain transcription factor responds to the fungus and binds to promoter regions of the *P. indica* responsive *Nia2*, *SEX1* and *2-nitropropane dioxygenase* genes (Sherameti *et al.* 2005). The mRNA for this transcription factor is upregulated by *P. indica* as well. These results suggest that the expression of *P. indica*-responsive target genes may be controlled by common regulatory elements and *trans*-factors. The absence of the response of the homeodomain transcription factor was used as a marker to characterize *P. indica*-insensitive mutants.

In the present project the following questions were addressed:

1. What are the molecular mechanisms underlying the early recognition processes of interaction between *A. thaliana* and *P. indica*?

2. Which genes are responsible for the recognition of the two symbiotic partners?

3. Which is the protein composition of Triton X-100 insoluble plasma membrane vesicles of *A. thaliana*?

2.1 Manuscript I

Association of *Piriformospora indica* with *Arabidopsis thaliana* roots represents a novel system to study beneficial plant–microbe interactions and involves early plant protein modifications in the endoplasmic reticulum and at the plasma membrane.

Tatjana Peškan-Berghöfer, Bationa Shahollari, Pham Huong Giong, Solveig Hehl, Christine Markert, Verena Blanke, Gerhard Kost, Ajit Varma and Ralf Oelmüller

Physiologia Plantarum (2004) **122**:4, 465-477

PHYSIOLOGIA PLANTARUM 122: 465–477. 2004
Printed in Denmark – all rights reserved

doi: 10.1111/j.1399-3054.2004.00424.x

Association of *Piriformospora indica* with *Arabidopsis thaliana* roots represents a novel system to study beneficial plant–microbe interactions and involves early plant protein modifications in the endoplasmic reticulum and at the plasma membrane

Tatjana Peškan-Berghöfer[a], Bationa Shahollari[a], Pham Huong Giong[b], Solveig Hehl[c], Christine Markert[a], Verena Blanke[d], Gerhard Kost[d], Ajit Varma[b] and Ralf Oelmüller[a,*]

[a]*Institute of General Botany and Plant Physiology, Friedrich-Schiller-University Jena, Dornburgerstr. 159, 07743 Jena, Germany*
[b]*School of Life Sciences, Jawaharlal Nehru University, New Delhi-110 067, India*
[c]*Advanced Imaging Microscopy, Carl-Zeiss Jena GmbH, D-07745 Jena, Germany*
[d]*Fachbereich Biologie, Spezielle Botanik und Mykologie, Karl-von-Frisch-Straße, 35032 Marburg, Germany*
**Corresponding author, e-mail: b7oera@uni-jena.de*

Received 28 April 2004; revised 4 August 2004

***Piriformospora indica*, an endophytic fungus of the Sebacinaceae family, colonizes the roots of a wide variety of plant species and promotes their growth, in a manner similar to arbuscular mycorrhizal fungi. The results of the present study demonstrate that the fungus interacts also with the non-mycorrhizal host *Arabidopsis thaliana* and promotes its growth. The interaction is detectable by the appearance of a strong autofluorescence in the roots, followed by the colonization of root cells by fungal hyphae and the generation of chlamydospores. Promotion of root growth was detectable even before noticeable root colonization. Membrane-associated proteins from control roots and roots after cultivation with *P. indica* were separated by two-dimensional gel-electrophoresis and identified by electrospray ionization mass spectrometry and tandem mass spectrometry. Differences were found in the expression of glucosidase II, beta-glucosidase PYK10, two glutathione-*S*-transferases and several so-far uncharacterized proteins. Based on conserved domains present in the latter proteins their possible roles in plant–microbe interaction are predicted. Taken together, the present results suggest that the interaction of *Arabidopsis thaliana* with *P. indica* is a powerful model system to study beneficial plant–microbe interaction at the molecular level. Furthermore, the successful accommodation of the fungus in the root cells is preceded by protein modifications in the endoplasmatic reticulum as well as at the plasma membrane of the host.**

Introduction

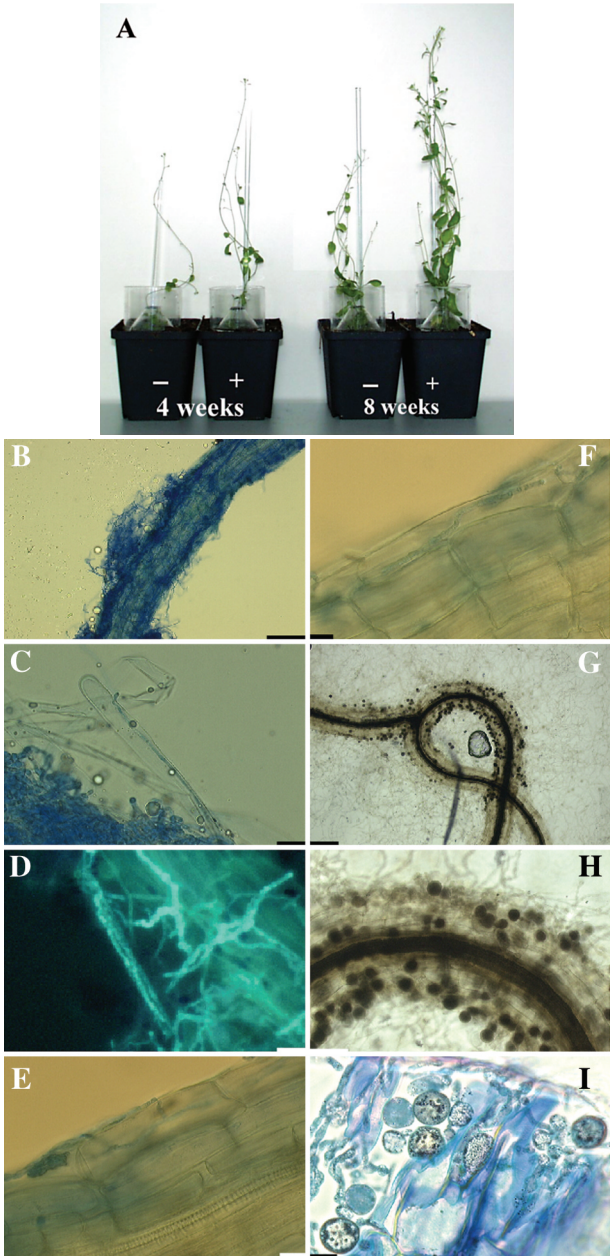
Plants are involved in mutualistic or parasitic interactions with a variety of micro-organisms, which has a strong impact on ecosystems, agriculture and forestry (cf. Smith and Read 1997, Harrison 1999, Parniske 2000, Strack et al. 2003). Most of the studies on beneficial plant–microbe interactions have been focused on the symbiosis of plants with rhizobia and arbuscular mycorrhizal fungi. Whereas rhizobial interactions have a narrow range of host plant species, arbuscular mycorrhizal symbioses are widely spread and involve the majority of higher plants in ecosystems throughout the world (cf. Smith and Read 1997). The benefits in mycorrhizal associations arise from the nutrient transport between the plant roots and fungal hyphae. The carbon source is transported from the plant to the fungus, whereas fungal hyphae serve as a fine link between the roots and the rhizosphere and improve the supply of the plant with inorganic nutrients (Harrison 1999 and ref. therein; cf. Harrison et al. 2002, Bucking and Heyser 2003, Karandashov et al. 2004).

Although the importance of arbuscular mycorrhizal associations was recognized a long time ago, the knowledge

Abbreviations – ESI-MS, electrospray ionization mass spectrometry; MS-MS, tandem mass spectrometry.

about the mechanisms leading to the establishment and functioning of this symbiosis is still limited (cf. Limpens and Bisseling 2003, Breuninger and Requena 2004, Marx 2004, Parniske 2004). Three components of a plant signalling network, a receptor-like kinase (Endré et al. 2002, Stracke et al. 2002), a predicted ion-channel (Ané et al. 2004) and a calmodulin-dependent protein kinase (Levy et al. 2004) have been identified. Besides the complexity of the interaction between the plant and fungal partners, additional obstacles reside in the application of molecular techniques. *Arabidopsis thaliana*, a common model to study plant development at the molecular level, is not among the hosts of mycorrhizal fungi. On the other side, arbuscular mycorrhizal fungi, which colonize the roots of 80% of vascular plants, including the majority of crop plants, are obligate biotrophs and cannot be cultured without hosts (Newman and Reddel 1987). *Piriformospora indica* is a recently isolated root-interacting fungus, related to the Hymenomycetes of the Basidiomycota (Varma et al. 1999). In contrast to arbuscular mycorrhizal fungi, it can be easily cultivated in axenic culture where it produces spores (Pham et al. 2004). The fungus is able to associate with the roots of various plant species in a manner similar to arbuscular mycorrhizal fungi and promotes plant growth (Varma et al. 1999, 2001, Pham et al. 2003, Singh et al. 2003, Shahollari et al. 2004). Hence, it provides a promising model organism for the investigations of beneficial plant–microbe interaction and enables the identification of compounds, which may improve plant growth and productivity.

Genetic factors from the plant are necessary for the interaction of plants with micro-organisms (Marsh and Schultze 2001, Kistner and Parniske 2002, Mellersh and Heath 2003). Depending on the interaction partners, they determine whether the micro-organism will be rejected or accommodated by the plant host (Parniske 2000, Gadkar et al. 2001). For instance, a receptor-like kinase has been identified which is absolutely necessary for the formation of nodules in response to rhizobactria and which supports the infection of roots with arbuscular mycorrhizal fungi (Endré et al. 2002, Stracke et al. 2002). We challenged *P. indica* and the conventional non-mycorrhizal host *A. thaliana* in axenic culture and observed that the fungus colonized the roots and promoted growth. We used this system to elucidate processes which occur early during the recognition process prior to the establishment of this interaction.



Materials and methods

Growth conditions of plant and fungus

Wild-type *Arabidopsis thaliana* (ecotype Columbia) seeds 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} s^{-1}). *Piriformospora indica* was cultured as described previously (Varma et al. 1998) on *Aspergillus* minimal medium modified by Pham et al. (2004). For solid medium 1% (w/v) agar was included.

Co-cultivation experiments and estimation of plant growth

Nine-day-old *A. thaliana* seedlings were transferred to nylon discs (mesh size 70 µm) placed on top of a modified MMN culture medium (MMN medium with a 1/10 of nitrogen and phosphorus and no carbohydrate; Marx 1969), in 90 mm 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 3 cm from the roots. Plates were incubated at

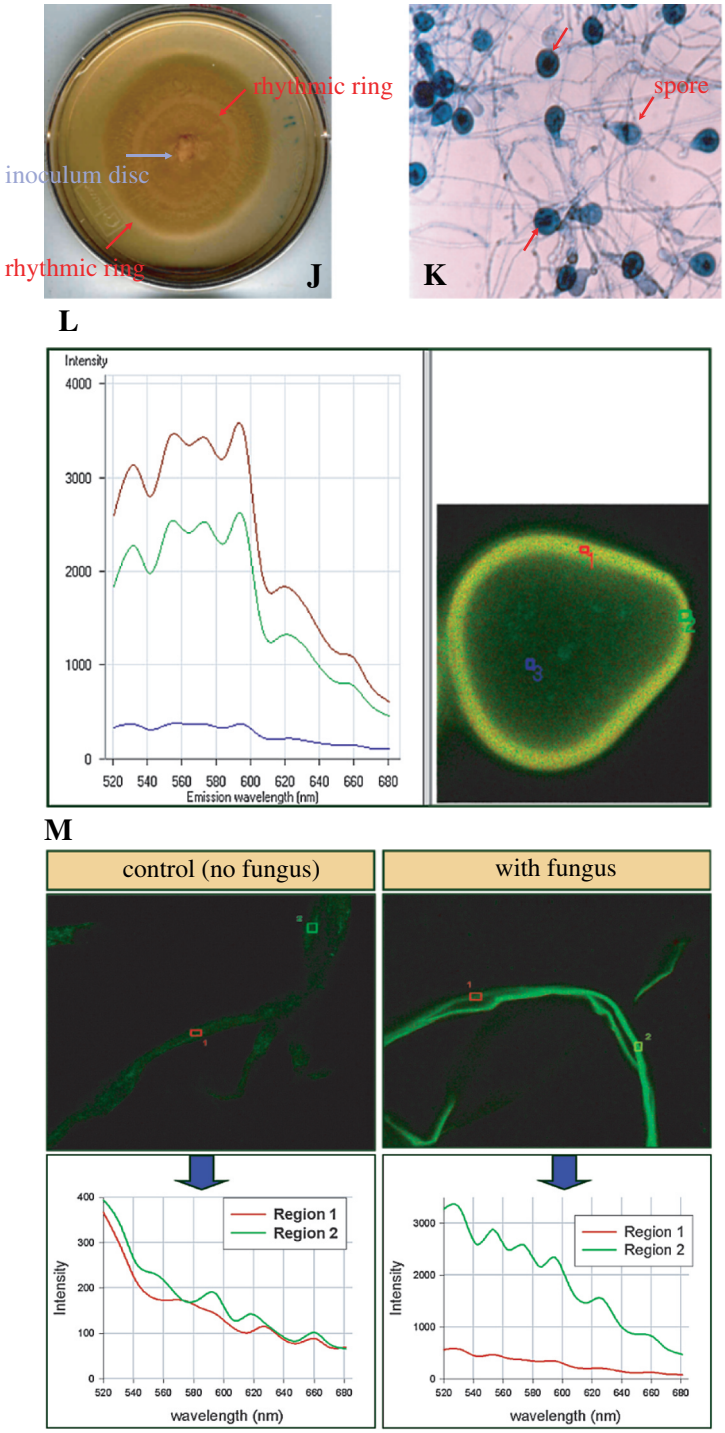


Fig. 1. Interaction of *Arabidopsis* with *P. indica*. (A) 4- and 8-week-old *Arabidopsis* plants grown in the absence (–) or presence (+) of *P. indica*. B–F, 10 days after inoculation, panels G–I, 31 days after inoculation. (B) Mycelium covering the surface of the root, stained with cotton blue; bar = 100 µm. (C) Hypha penetrating a root hair, stained with cotton blue; bar = 10 µm. (D) Mycelium covering the root surface and growing inside a root hair, stained with aniline blue (epifluorescence); bar = 20 µm. (E, F) Hypha penetrating into a rhizodermis cell (arrow), the fungus forms a very narrow penetration neck, stained with cotton blue; bar = 10 µm. (G) *Arabidopsis* root embedded in historesin, *P. indica* has formed chlamydospores; stained with OsO_4; bar = 100 µm. (H) Same root as in (E) at higher magnification, chlamydospores were formed on the surface and in the cortex of the root; bar = 50 µm. (I) Tangential section of an *Arabidopsis* root; mycelium and chlamydospores were formed inside and outside the cells of the cortex; stained with OsO_4 and toluidin blue; bar = 10 µm. (J, K) Growth of *P. indica* on modified *aspergillus* medium incubated at 28°C for 7 days. Incubation was conducted in dark. A typical rhythmic growth is seen. For broth medium agar was not included and grown under constant shaking condition (150 r.p.m., GFL 3.19). Typical pear-shaped spores appeared after 10 days. (L, M) Auto-fluorescence in the fungal chlamydospore (L) and the developing root hairs (M) as a result of co-culture with *P. indica*. Emission spectra are given for the section indicated in the figure.

22°C under continuous illumination from the side (max. 80 µmol $m^{-2} s^{-1}$).

Roots of control plants and of inoculated plants were stained with cotton-blue before examination under a light microscope (Zeiss Axioplan model MC 100: Carl-Zeiss Jena GmbH, Jena, Germany). Dry weight of the shoots was determined after incubation of the tissue at 105°C for 16 h. Photosynthesis parameters were determined as described previously (Pfannschmidt et al. 2001).

Experiments on soil

For the experimens on soil, *Arabidopsis* seedlings were germinated on MS medium before transfer to sterile soil. The soil was mixed carefully with the fungus (1%, w/v). The fungal mycelium was obtained from liquid cultures after removal of the medium and washed with an excess of distilled water. Cultivation occurred in multi-trays with Aracon tubes in a temperature-controlled growth chamber at 22°C under continuous illumination (max. 80 µmol $m^{-2} s^{-1}$) and long-day conditions.

The roots of *A. thaliana* inoculated with *P. indica* were collected after 10 and 31 days. The root fragments were cleared in KOH (Vierheilig et al. 1998). The cleared root fragments were stained with cotton blue (Vierheilig et al. 1998, Sime et al. 2002) and analysed with the help of a Zeiss Axiophot (bright field) or were stained with aniline blue (Hood and Shew 1996) and analysed with epi-fluorescence. For the preparation of sections, the roots were fixed with glutaraldehyde for 2 min, post-fixed in OsO_4 in the dark for 1 h and embedded in Historesin according to the instructions of the manufacturer (Leica Microsystems, Nussloch, Germany). Sections were taken with a Ultramicrotome OmU2 (Reichert, Vienna, Austria) and stained with toluidine blue (0,5% w/v in aqua dest.).

Fluorescence measurements

Auto-fluorescence in the spores and the developing root hairs as a result of co-culture with *P. indica* (Fig. 1) were detected with the confocal microscope Meta-450 (Carl-Zeiss).

Protein extraction

Arabidopsis thaliana roots were ground with mortar and pestle on ice in homogenization buffer containing 50 m*M* Tris-HCl, pH 7.4; 330 m*M* sucrose; 3 m*M* EDTA and 10 m*M* 1,4-dithiothreitol. The homogenate was filtered through four layers of cheesecloth and centrifuged at 10 000 *g* for 15 min. The supernatant was centrifuged at 50 000 *g* for 1 h to pellet the microsomes. The pellet was re-suspended in a buffer containing 50 m*M* Tris-HCl, pH 7.4 and 1 *M* NaCl, incubated on ice for 30 min and centrifuged as before. Pelleted membranes were re-suspended in 50 m*M* Tris-HCl, pH 7.4; 3 m*M* EDTA and 1 m*M* 1,4-dithiothreitol and kept at −80°C until protein analysis.

Two-dimensional gel electrophoresis

Membrane proteins were solubilized with 1% Triton X-100 and precipitated with methanol/chloroform according to Wessel and Flügge 1983). Two-dimensional protein separation and silver-staining of the gels were performed according to Hippler et al. (2001). Staining of the gels was performed with colloidal Coomassie stain Roti-Blue (Roth, Karlsruhe, Germany) according to the manufacturer's instructions. Quantification of signals was performed using the Image Master VDS system (Amersham-Pharmacia, Freiburg, Germany). A protein dilution standard was used to confirm that the values were in the linear range.

Mass spectrometry

In-gel trypsin digestion of excised protein spots and elution of the peptides from the gel matrix was performed according to Hippler et al. (2001). Peptide analysis by coupling liquid chromatography with electrospray ionization mass spectrometry (ESI-MS) and tandem mass spectrometry (MS-MS) has been described previously (Stauber et al. 2003).

Protein identification

The measured MS-MS spectra were matched with the amino-acid sequences of tryptic peptides from the *A. thaliana* database in FASTA format. Cys modification by carbamidomethylation (+57 Da) was taken into account and known contaminants were filtered out. Raw MS-MS data were analysed by the Finnigan Sequest/Turbo Sequest software (revision 3.0; ThermoQuest, San Jose, CA). The parameters for the analysis by the Sequest algorithm were set according to Stauber et al. (2003). The similarity between the measured MS-MS spectrum and the theoretical MS-MS spectrum, reported as the cross-correlation factor (X_{corr}) was equal or above 1.5, 2.5 and 3.5 for singly, doubly or triply charged precursor ions, respectively. In order to identify corresponding loci, identified protein sequences were subjected to BLAST search at NCBI (http://www.ncbi.nlm.nih.gov/) and FASTA searches by using the AGI protein database at TAIR (http://www.arabidopsis.org/). Identification of conserved domains and signal peptides was performed by using SMART (Schultz et al. 1998) and SIGNALP (Nielsen et al. 1997), respectively.

Results

Interaction of *P. indica* with *Arabidopsis*

We first characterized the interaction of *P. indica* with *Arabidopsis thaliana* seedlings. For all experiments described, we used the Columbia ecotype. Although other ecotypes of *A. thaliana* tested also responded to *P. indica* treatments, the response pattern differed, mainly in the time course of the establishment of the

interaction. A growth-promoting effect could be observed during the whole life time of the plant: When *Arabidopsis* seedlings were inoculated with the fungus and transferred to soil, the rosette leaves were slightly larger and bolding occurred earlier. Consequently, the plants grew faster, contained more leaves and started to flower earlier (Fig. 1A). The overall habitus of the plants looks bigger and stronger when co-cultivated with *P. indica* (Fig. 1A). We observed a higher seed yield per plant, however, seed ripening occurred earlier in comparison with uninoculated plants. Moreover, physiological experiments with *Arabidopsis* seedlings kept on MS medium in Petri dishes revealed that they appear to be more resistant to stressors in the presence of *P. indica*. We optimized conditions in which *Arabidopsis* seedlings co-cultivated with *P. indica* tolerate elevated cadmium concentrations (200 µm in MS medium) which normally lead to death. We could also demonstrate that recruitment of iron is substantially enhanced in the presence of the fungus (Oelmüller et al., unpublished data).

Additional experiments were performed to identify fungal components which mediate the growth-promoting effect. Under the physiological conditions relevant for these studies we could not detect a growth-promoting effect with heat-treated *P. indica* or extracts from the fungus, either heat-treated or untreated. However, growth promotion was observed in the presence of a large excess of heat-inactivated fungal mycelium.

Figure 1B demonstrates that after 10 days of co-cultivation of both organisms in soil, the mycelium covers the surface of the roots. The hyphae also penetrate the root hair (Fig. 1C) and form a net both around the root cells and inside a root hair (Fig. 1D). The hyphae also penetrate into a rhizodermis cell and the fungus forms a very narrow penetration neck (Fig. 1E and F). Ultimately, this results in the formation of many *c*hlamydospores (Fig. 1G–I; pictures taken after 31 days of co-cultivation in soil). *Piriformospora indica* also form spores on plates (Fig. 1J) and in liquid cultures (Fig. 1K). Finally, the outer layer of the spore generates an intensive autofluorescence, which disappears after germination (Fig. 1L). This autofluorescence appears again after the co-cultivation of *P. indica* with *Arabidopsis* root hair. Since the fluorescence is not detectable in control root hairs, establishment of a successful interaction between both organisms can be monitored by the fungus-derived autofluorescence (Fig. 1M).

Analysis of the growth-promoting effect of *P. indica* on *A. thaliana* roots

Nine-day-old *A. thaliana* plantlets were transferred to MMN1/10 medium and inoculated with *P. indica*. The fungal inoculum was placed more than 3 cm away from the roots to avoid initial physical contact. MMN 1/10 medium was chosen since it contains low concentrations of phosphate and nitrate and no carbon source – conditions known to promote the interaction between plants and symbiotic fungi. The fungus grew slowly on the co-cultivation medium and produced only a few spores. We could not observe any difference in root growth within the first 2 days of co-cultivation. After 3 days, stimulation of root growth became visible, whereas after 7 days, intensive and uniform root proliferation in form of extended and branched lateral roots was detectable for all inoculated plants (Fig. 2A and C). At this time, an approximately 1 cm wide ring of fine fungal mycelium had been built. Interestingly, the growing mycelium ring did not yet physically contact the root surface and microscopic inspections confirmed that the roots were depleted of hyphae. Nevertheless, an increase in root fresh weight was measurable already after 5 days of co-cultivation with the fungus (Fig. 2D, cf. also Shahollari et al. 2004). We also inoculated plants with the ectomycobiont *Pisolithus tinctorius*, since it was shown that hypaphorine, a major indolic compound from this fungus has an impact on *Arabidopsis* root growth (Reboutier et al.

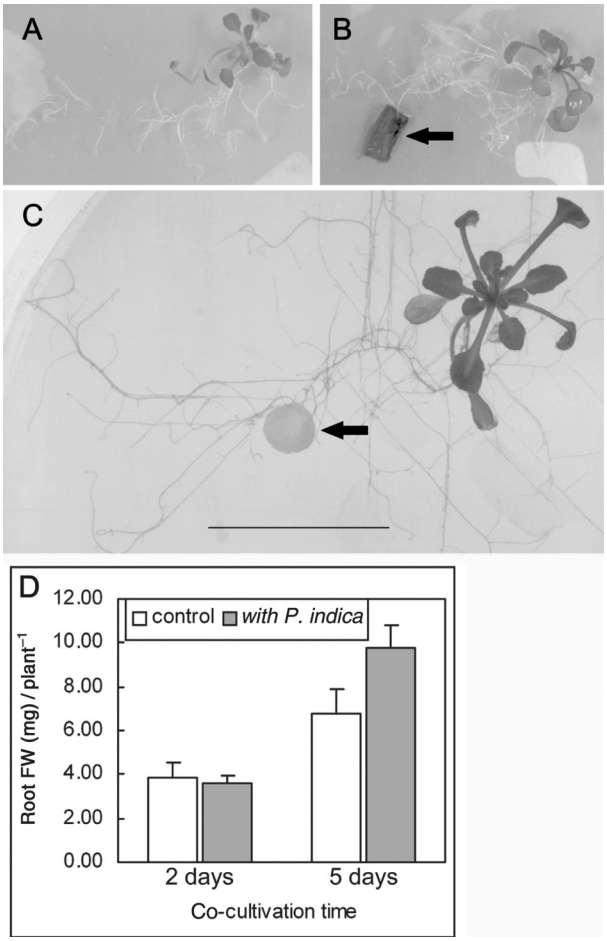


Fig. 2. *Arabidopsis thaliana* root development after 9 days of co-culture with *P. indica*. Nine-day-old *A. thaliana* plantlets were transferred to MMN1/10 medium and cultivated without fungi (A), or were inoculated with *P. tinctorius* (B) or *P. indica* (C). The arrows show the location of the inoculum. The fresh weights (FW) of the roots were determined after 2 or 5 days of co-cultivation and represent the mean of four independent experiments with 64 plants each. Bar represents 3 cm for (A–C). (D). Differences in FW after 5 days were significant according to Student's *t*-test.

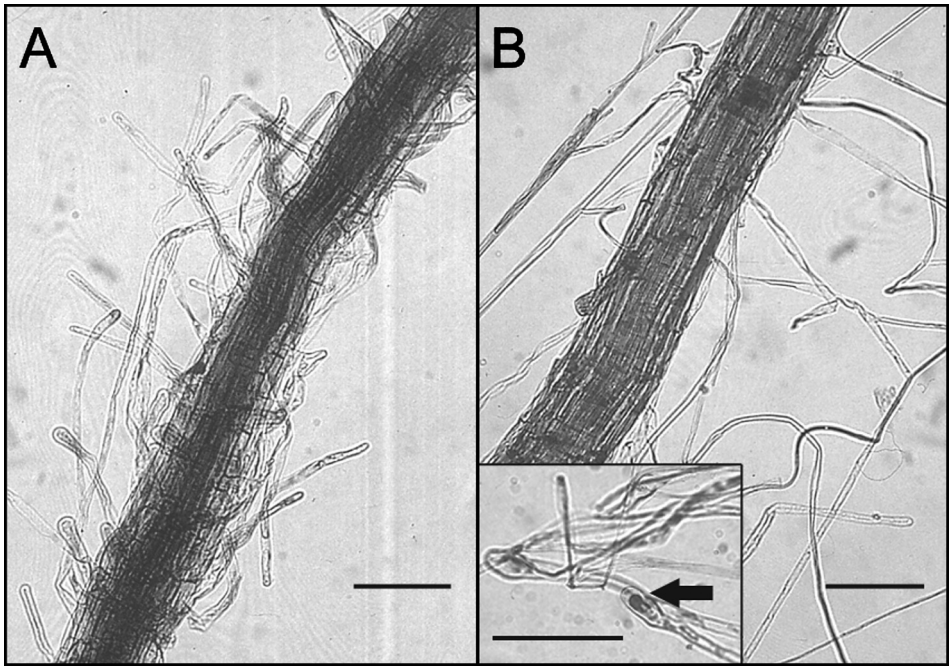


Fig. 3. Root hairs of *A. thaliana* after co-cultivation with or without *P. indica* for 7 days. The roots of control (A) and inoculated (B) plants were stained with cotton-blue and examined under the light microscope (Zeiss Axioplan model MC 100). A magnified view of an infested root hair is shown in the inset. Bars = 20 µm.

2002). However, plants inoculated with *P. tinctorius* did not differ from the uninoculated control plants (Fig. 2B).

Inoculation of *Arabidopsis* roots with *P. indica* was accompanied by changes in the morphology of the root hairs, which were visible even before the root proliferation became obvious (Fig. 3). The root hairs of inoculated plants grew longer and were thinner in comparison with those of the controls. After 2 weeks of co-cultivation the fungal hyphae surrounded the root surface and grew inside the root hairs (Fig. 3, inset). Accommodation of the fungus inside the root cells did not harm the plants and no damage could be detected even after longer co-cultivation (cf. Fig. 1). The chlorophyll content was comparable in leaves of inoculated and control plants. Furthermore, the chlorophyll fluorescence parameters confirmed that the fungus did not exert a negative impact on photosynthesis (Fig. 4A). After 8 days of co-cultivation a significant increase in shoot dry weight as a result of the interaction with *P. indica* was detectable (Fig. 4B). The observation that the interaction of these organisms exhibit features similar to those observed for arbuscular mycorrhizal fungi further support the idea that it might be a suitable model system to study plant–microbe interaction at a molecular level.

Differences in root protein pattern as a result of interaction with the fungus

We analysed changes in the protein pattern of roots during early phases of the interaction between the organisms. Therefore, we utilized a protein preparation which gave the most differences in the protein pattern in our hands. We found that further fractionation of this preparation into purer fractions caused substantial losses of many proteins which responded to the fungal infection. Our protein preparation was enriched in membrane-bound and membrane–associated proteins and proteins from the cell surface. Plants were cultivated with and without the fungus for 5 days. At this time point, no physical interaction between the organisms was detectable and we never observed any hyphae growing on or inside the roots. Furthermore, the root growth started to

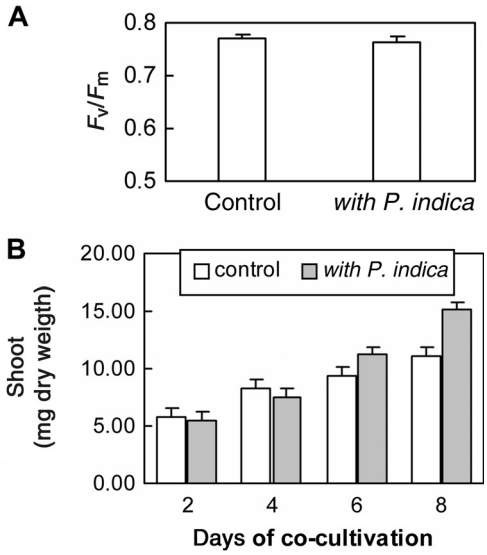


Fig. 4. Shoot growth as a result of interaction with *P. indica*. (A) Chlorophyll *a* fluorescence was measured on plantlets cultivated for two weeks on MMN1/10 medium with or without the fungus. The fluorescence parameter F_v/F_m reflects the efficiency of photosynthesis at photosystem II. (B) Increase in shoot dry weight for control and inoculated plants. The shoots of six plantlets were pooled for one measurement and the weights of three independent samples were estimated for each time period. After 6 days of co-cultivation, the differences were significant according to the Student's *t*-test.

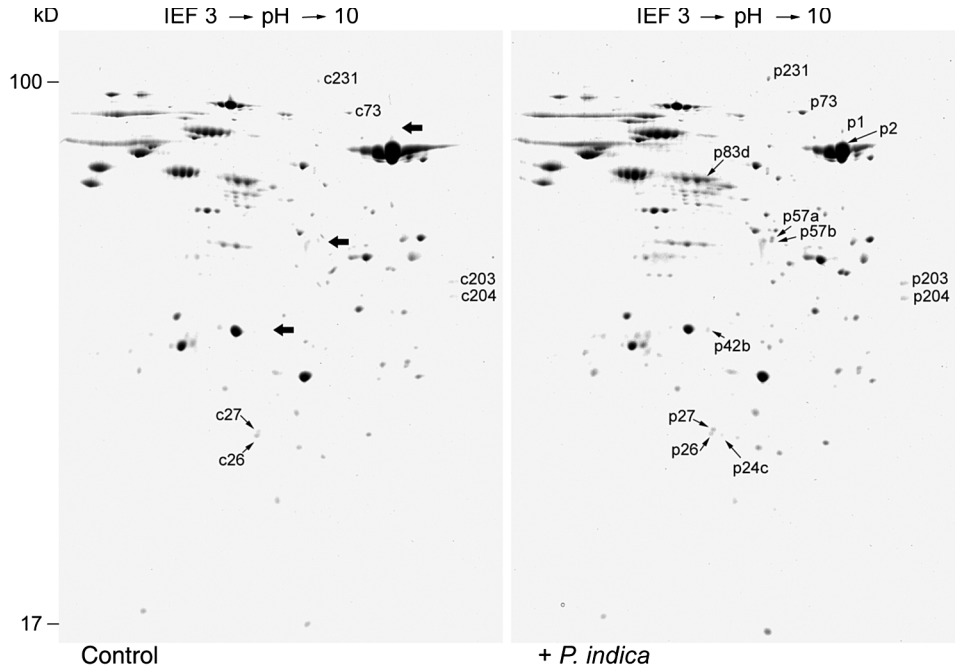


Fig. 5. Protein patterns of *A. thaliana* roots after 5 days of cultivation with and without *P. indica*. After separation of the proteins by 2D-PAGE, the gels were stained with Coomassie blue. Protein spot labels are placed to the right of the corresponding spots, except when spots are marked by arrows. Thick arrows indicate spots on the control gel which are missing on the gel with protein extracts from *A. thaliana* co-cultivated with *P. indica*.

be significantly different between the inoculated and the control plants, which also reflected reproducible differences in the patterns of our protein preparations. During this pre-contact phase, the communication between both organisms should occur through the medium.

When protein of our preparation were analysed by two-dimensional gel electrophoresis, an average of 150–200 protein spots could be visualized after staining of the gels with Coomassie blue (Fig. 5). Although almost all results discussed here are based on spots which are easily detectable on Coomassie-stained gels, subsequent silver staining uncovered more details and allowed a more accurate analysis of weakly expressed proteins, as shown in Fig. 6. Differentially expressed proteins were excised from the gels and analysed by mass spectrometry, after digestion with trypsin. The most prominent proteins which responded to the fungal co-cultivation, are summarized in Table 1.

To confirm that the changes observed for the protein spots presented in Table 1 are caused by the fungus, we

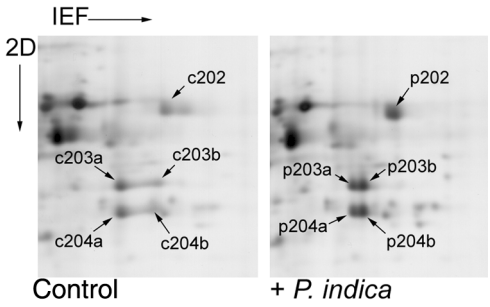


Fig. 6. Regions from silver-stained two-dimensional (2D) gels showing modifications of a protein containing MATH-domains (p203, p204). After separation of the proteins by 2D-polyacrylamide gel electrophoresis, the gels were stained with silver.

compared these spots with those in their environment on the two-dimensional gels. Figure 7 demonstrates that the 'responsive' protein spots are always surrounded by spots which do not respond at all to the fungal treatment. We consider these spots as controls. The amounts of several other protein spots on the gels also differ although to a lesser extent. The reason for this is not clear. This might be caused by the fungus, by the normal variation of the biological material or by the extraction procedure. However, the comparative quantitative analysis of the protein patterns of the gels suggests that the proteins presented in Table 1 are the most likely candidates for a specific response to the fungus.

For six proteins (p1/2, p57a, p57b, p202, p203/204 and p231; Table 1) signal peptides for the vesicular pathway were predicted. One protein (p83; Table 1) appears to be located in mitochondria, whereas the subcellular localization of the residual four (p24c, p26/7, p42b, p73) is unclear. Although the proteins without a predicted signal peptide do not possess transmembrane domains, it cannot be excluded that they are attached to membranes via interaction with other proteins (cf. below).

One of the identified proteins is the α-subunit of glucosidase II (p231, RSW3, Table 1). Although a biological function for this enzyme has been partially elucidated (Burn et al. 2002), nothing is known about its possible role in plant–microbe interaction (cf. Discussion). The biological functions of the residual polypeptides can only be predicted from conserved domains and expression patterns. We also report, for the first time, their putative involvement in plant–microbe interactions and/or regulation of root growth.

Furthermore, several spots which are up-regulated after fungal infection, are located in close vicinity to others (p1/2, p26/27, p203/204). The analysis uncovered

Table 1. Proteins related to plant–fungus interaction, identified by mass spectrometry. [a]The peptides obtained by database searching, used for the protein identification. [b]Total number of different peptides identified for a matched protein, with the X_{corr} values above 1.5, 2.5 and 3.5 for singly, doubly, or triply charged precursor ions, respectively. [c]Calculated pI and molecular weight (MW) in kDa. [d]Signal peptide, predicted by SignalP.

Spot ID	Sequence [a]	N [b]	Database match	Accession number (gi)	pI/MW of matched protein [c]	S [d] (Locus)
p1	SGYEAYLVTHNLLISHAEAVEAYR IGIAHSPAWFEAHDLADSQDGASIDR PLTAALNVYSR	9	Glycosyl hydrolase family 1, PYK10, PSR3.1	1363489 (At3g09260)	6.45/59.7	+
p2	GVSQAGVQFYHDLIDELIK EYADFVFQEYGGK WMQDSLITWESK	7	Glycosyl hydrolase family 1, PYK10, PSR3.1	1363489 (At3g09260)	6.45/59.7	+
p24c	FANFSIESEVPK VTEFVSELR NPILPSDPYLR	3	glutathione *S*-transferase, GST8	18411929 (At1g78380)	5.80/25.6	–
p26	LEAVLDVYEAQULSK IPVLVDGDYK	2	Putative glutathione *S*-transferase, ERD13	15224582 (At2g30870)	5.49/42.2	–
p27	LAEVLDVYEAQLSK QPEYLAIQPFGK IPVLVDGDYK	3	Putative glutathione *S*-transferase, ERD13	15224582 (At2g30870)	5.49/24.2	–
p42b	KVYVGQAQDGISAVK IFGSDGSVITMLR QTSPPFGLEAGTVFELK	7	Putative lectin, similar to myrosinase-binding proteins gi:1711296 and gi: 1883005	15228198 (At3g16420)	5.46/32.1	–
p57a	PLSLESQTIEFVK LYDNSAMENLLK SVYLSFVPDEEIGGHDGAEK	8	Aminoacylase, putative, similar to aminoacylase-1	30691729 (At4g38220)	5.93/47.7	+
p57b	FAEINNAYEVLSDEEKR SYYDVLQVPK	2	Expressed protein, contains DnaJ-chaperone domain	15228802 (At3g62600)	5.93/39.1	+
p73	ALAGQTNESFFTANADALSSR ALGVETVPVLVGPVSYLLLSK	13	Methionine synthase-related	14532772 (At3g03780)	6.09/84.6	–
p83d	FTQANSEVSALLGR VLNTGAPITVPVGR TIAMDGTEGLVR	5	H^+-transporting ATPsynthase, beta chain, mitochondria	18415911 (At5g08690)	6.18/59.7	–
p202	NNPNADASTQQAFVTSVTNK LKNDISLLYSSGASK AANWNDDFVKK	5	Lipase/Acylhydrolase with GDSL-motif family, similar to myrosinase-associated protein gi: 1769969	4587542 18404748 (At1g54010)	6.11/47.7	+
p203a	NSYLSEVFSIGGR SWNIQINPSGLGTGEGK TMWGFSQVLPIDTFK	7	Expressed protein, contains MATH domain	18402593 (At3g20370)	6.16/43.4	+
p203b	SWNIQINPSGLGTGEGK YFTIQDTDVWK	4	Expressed protein, contains MATH domain	18402593 (At3g20370)	6.16/43.4	+
p204a	FYIFNK ALNQLNLSNIER KYFTIQDTDVWK	5	Expressed protein, contains MATH domain	18402593 (At3g20370)	6.16/43.4	+
p204b	SELFSVTENFLNPR MESFNTLLK FYIFNK	7	Expressed protein, contains MATH domain	18402593 (At3g20370)	6.16/43.4	+
p231	WNYKDEEDVAQVDSK MDAPEESIPAFQK FQVPDVVVSEFEEK	7	Alpha-subunit of glucosidase II, RSW3	15237538 (At5g63840)	5.86/104.2	+

that the newly appearing spots are modified forms of proteins which are already present in the absence of the fungus. Thus protein modifications in addition to de novo protein synthesis appear to be crucial for early events in the establishment of plant–microbe interactions.

Among them, the most abundant protein spot (p2, Fig. 5) corresponded to the β-glucosidase PYK10. This protein was found to be a major constituent of ER bodies in *A. thaliana* (Matsushima et al. 2003) and exhibits a hypocotyl- and root-specific expression pattern (Nitz et al. 2001). Inoculated roots contain an additional protein spot located above this β-glucosidase spot (p1, Fig. 5). MS-MS-analysis showed that both spots originate from the same protein. Both forms differ in their electrophoretic mobility in the second dimension, since they focus at the same pH (Fig. 5). This is consistent with observations by Matsushima et al. (2003) who showed that the electrophoretic mobility of PYK10 on gels can be modified by N-linked oligosaccharides.

The protein spots p26 and p27 were both identified as a putative glutathione-*S*-transferase (ERD13, Table 1). The two proteins differ in both, pI and relative mobility, suggesting the existence of complex modifications, probably on p27 (Fig. 5). Although both polypeptide forms are present in roots, only p27 became more apparent after co-cultivation with the fungus.

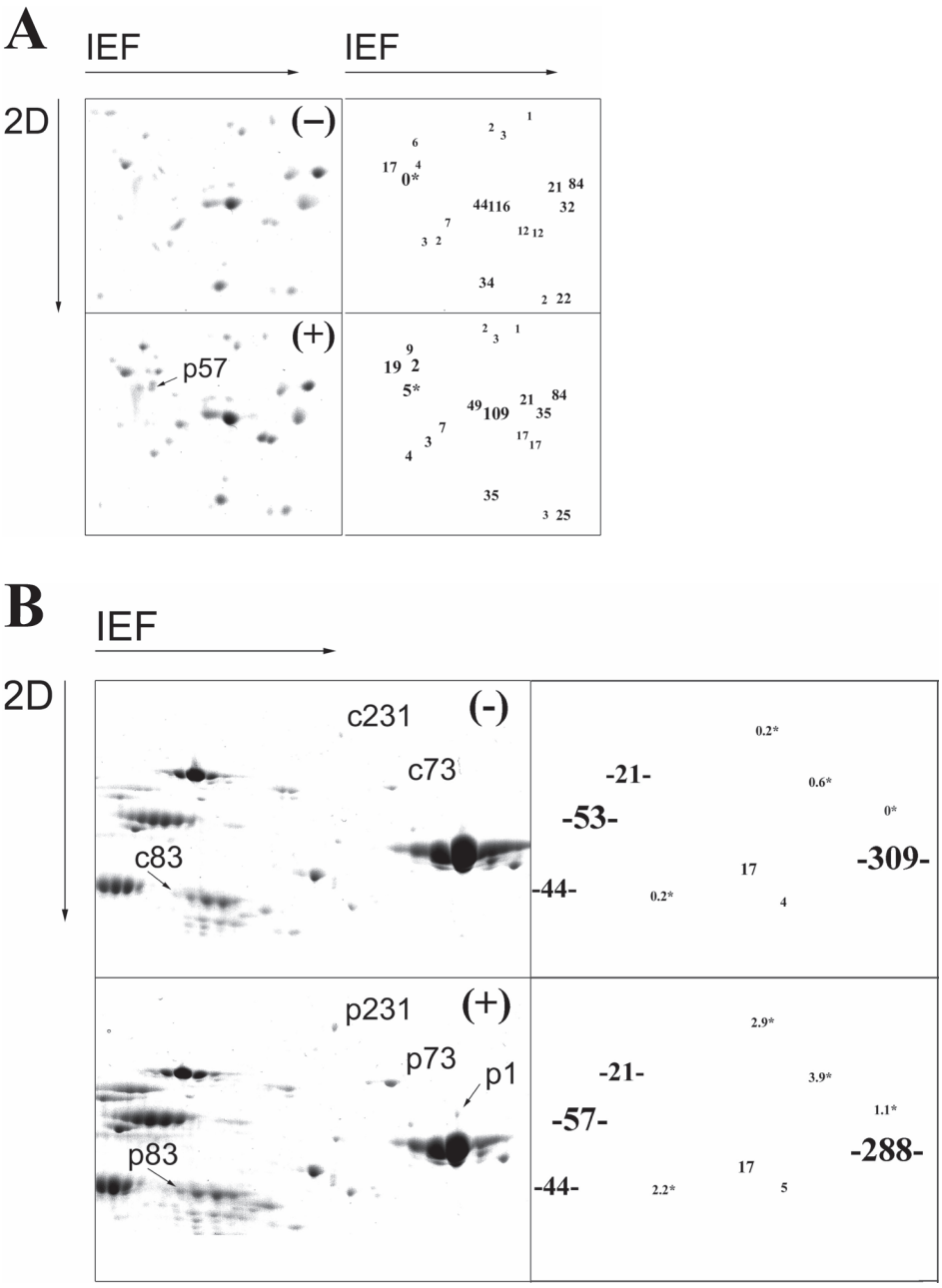
The most intriguing modification was observed for the protein spots 203 and 204 (Fig. 6). The intensity of the protein spots 203a and 204a were similar under both cultivation conditions, whereas the spots p203b and p204b appeared only after interaction with the fungus. MS-MS-analysis of the four spots 203a, 203b, 204a and 204b under both conditions showed that they derived from the same, so far uncharacterized *Arabidopsis* protein (Table 1, cf. Discussion). It seems that two native forms of the protein with slightly different pIs occur in roots (a and b). Both native forms are composed of the high and low mobility polypeptides, which become apparent after separation in the second dimension (c,p203a,b and c,p204a,b; Fig. 6). The polypeptides p203b and p204b seem to be specific for the interaction with the fungus.

Discussion

Co-cultivation of *A. thaliana* with *P. indica* promotes plant growth. The effects of the fungus on root proliferation and the morphology of the root hairs are particularly striking. Growth promotion occurred before the fungal hyphae grew around or inside the roots. Therefore, this effect must be initiated by early signalling events from the fungus. Promotion of plant growth before the establishment of symbiosis is unusual for many arbuscular mycorrhiza systems, since plants respond to the fungus only after the symbiosis is established, as a result of a better supply with nutrients (Harrison 1999). Promotion of plant growth before the establishment of symbiosis was reported previously for the interaction between *Quercus robur* and the ectomycorrhizal fungus *Piloderma croceum* (Herrmann et al. 1998, 2004, Krüger et al. 2004). Although the nature of *A. thaliana*/*P. indica* interaction is necessarily different from an ectomycorrhizal symbiosis on trees, the early mechanism of recognition between the organisms and the signals released from *P. indica* or from an ectomycorrhizal fungus might be similar. It appears that the changes on the roots provoked by the micro-organism ensure successful accommodation of the fungus and prevent its rejection from the plant.

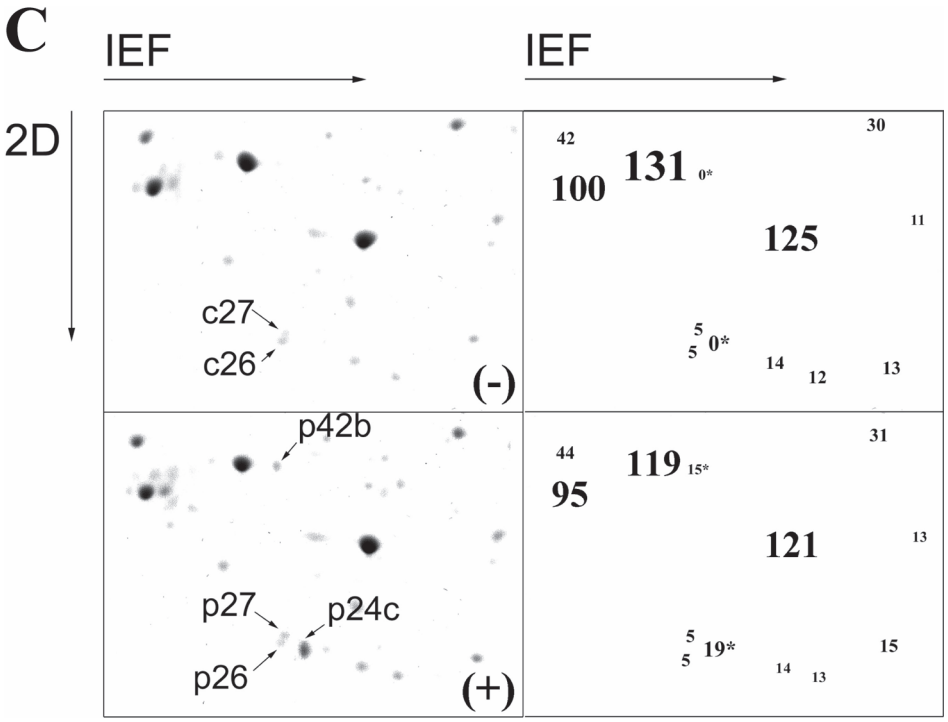
Promotion of root growth after cultivation with the fungus correlates with the up-regulation of the glucosidase II α subunit, which belongs to the family of glycosyl hydrolases. The *A. thaliana* mutant *rsw3* with a lesion in the glucosidase II α subunit gene has radially swollen, short roots and is deficient in cellulose, the major structural polysaccharide of higher plant cell walls. RSW3 possesses a predicted N-terminal signal peptide for the ER, but lacks ER-retention signals at its C-terminus, consistent with the features of the mammalian glucosidase II α subunit. In mammals, glucosidase II is resident in ER, where it contributes to the processing of N-linked glycans on newly synthesized glycoproteins. The holoenzyme is kept in the ER by an ER-retention signal of the β subunit (Trombetta et al. 1996) and associated with membranes due to its interaction with other transmembrane proteins (Arendt and Ostergaard 1997). The mechanism by which the putative ER-enzyme regulates plant development and synthesis of the cell wall is still intriguing. However, the identification of this protein in our studies is consistent with the idea that cell wall synthesis is stimulated during early phases of the recognition between *Arabidopsis* roots and *P. indica*. The shortened root system in mutants deficient in the glucosidase II α subunit is consistent with the observation of an expansion of the root system upon co-cultivation with the fungus when the protein is up-regulated. However, up- or down-regulation of cell wall synthesis cannot explain all growth defects in the mutant and all stimulatory effects observed after fungal co-cultivation. The enzyme appears also to be involved in regulating plant development (Burn et al. 2002). Glucosidase II also plays a crucial role in the N-glycosylation/quality control pathway (Taylor et al. 2000). In concert with glucosidase I, the enzyme catalyses the fist steps in N-linked glycan trimming and processing which occur in the ER. The role of glycans in the early secretory pathway is related to protein folding, quality control and protein sorting (reviewed in Helenius and Aebi 2001). Removal of terminal glucosyl residues from improperly folded proteins enables binding of chaperones and other proteins that control protein folding. *Arabidopsis thaliana* mutants lacking glucosidase I are lethal, whereas those lacking glucosidase II exhibit growth defects, suggesting that the ER quality-control mechanism is crucial for the synthesis of the glycoproteins involved in plant growth processes (Boisson et al. 2001, Vitale 2001). Up-regulation of glucosidase II upon cultivation with the fungus indicates that the fungus modulates plant growth at the level of protein processing and folding in ER. This is further supported by the identification of another up-regulated protein with a DnaJ chaperone domain (p57b, Table 1). The predicted N-terminal signal peptide suggests that the latter protein is also directed into the ER. DnaJ is a chaperone associated with the Hsp70 heat-shock system, involved in protein folding and renaturation after stress (Caplan et al. 1993).

Another glycosyl hydrolase (p1/2) that emerged as a result of the interaction with the fungus was identified as a modified form of the β-glucosidase PYK 10, the most abundant protein in roots. The function of PYK 10 appears to be related to ER-bodies (Matsushima et al. 2003). These ER-derived structures are present in the epidermal cells of healthy *A. thaliana* seedlings and in roots of mature plants. In rosette leaves, which are normally depleted of these structures, the formation of ER-bodies can be induced by stress, wounding or methyl jasmonate (Matsushima et al. 2002). Hayashi et al. 2001) described ER-bodies as a part of a proteinase-sorting system that is involved in processes leading to



cell death under stress conditions. The modification on PYK 10 after co-cultivation with the fungus might change the protein function and/or subcellular localization. This suggests that the fungus utilizes PYK 10 to cope with stress responses in the plant.

One of the identified proteins (p202, Table 1) exhibits sequence similarities to a myrosinase-associated protein (gi:1769969) from *Brassica napus* (identity: 61.8%). The protein contains a lipase/acylhydrolase domain with a GDSL motif and a predicted signal peptide for sorting via the vesicular pathway, but its physiological function or expression patterns are unknown. The protein identified as a putative lectin (p42b, Table 1) also exhibits similarities to myrosinase-binding proteins. Myrosinase hydrolyses thioglucoside bonds in glucosinolates that results in the production of compounds involved in defense reactions against micro-organisms and insects (Bones and Rossiter 1996 and ref. therein). Although myrosinase enzymes have been considered as defence-related enzymes in *Brassicaceae*, the role of glucosinolates seems to exceed defense responses. It has been hypothesized that the indole glucosinolate pool may act as a sink for the production of indole acetic acid and therefore regulates plant growth processes (Bones and Rossiter 1996). Zeng et al. (2003) showed that degraded compounds of indole glucosinolates may play a role in growth stimulation of ectomycorrhizal fungi. Moreover, the function of myrosinase-associated proteins is unknown at present, except that certain myrosinase-binding proteins were



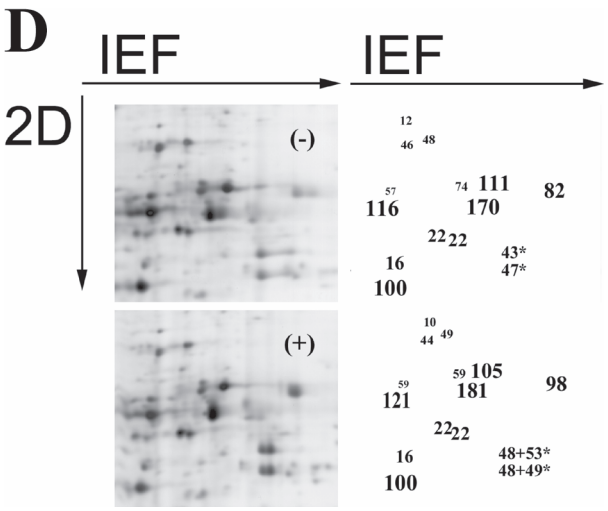


Fig. 7. Regions from Coomassie (A–C)- and silver (D)-stained gels with protein extracts from *Arabidopsis* seedlings grown in the absence (–) or presence (+) of *P. indica*. Left: protein gels, right: quantification of protein spots. The designations of the spots on the gels refer to those in Figs. 5 and 6 and Table 1. The amounts of the protein spots are given in relative values and can only be compared within a panel. One representative gel is shown in the left panels, the quantified data in the right panels are average values based on the analyses of three independent experiments and gel sets. Not all spots on the gels are quantified. *, spots relevant for this study.

found to be important for complex formation of myrosinase isoenzymes in *Brassica napus* (Eriksson et al. 2002).

Furthermore, the identification of methionine synthase as an up-regulated protein upon co-cultivation with *P. indica* indicates that the methyl cycle, leading to the synthesis of methionine and *S*-adenosyl-methionine is influenced by the fungus. Methionine synthase catalyses the last step in methionine synthesis by transferring the methyl group to homocysteine. Methionine is the immediate precursor of *S*-adenosyl-methionine, the major methyl-group donor in transmethylation reactions. *S*-adenosyl-methionine plays a crucial role in the biosynthesis of polyamines and ethylene, components implicated to be involved in plant–microbe interactions (Ravanel et al. 1998).

Two proteins with homology to glutathione-*S*-transferases were identified (p26/27 and p24c, cf. Table 1). ERD13 (p26/27, cf. Table 1) is up-regulated and possibly modified in response to the fungus and belongs to a class of dehydration-inducible genes in *A. thaliana* (Kiyosue et al. 1993). GST8 (p24, cf. Table 1) was found to be up-regulated at transcriptional level in response to dehydration, oxidative stress and high levels of auxin or cytokinin (Bianchi et al. 2002). Glutathione-*S*-transferases participate in detoxification of reactive electrophylic compounds and have been often associated with plant–microbe interactions (Mauch and Dudler 1993, Strittmatter et al. 1996, Bestel-Corre et al. 2002, Wulf et al. 2003). Production of reactive oxygen species occurs

during growth and defence processes (Kawano 2003). It is likely that the production of reactive oxygen species increase after co-cultivation with the fungus, either as a result of intensified growth, or as a response to fungal elicitors, or as both.

The spots 203 and 204 correspond to a protein of unknown function that contains two MATH domains. Upon cultivation with the fungus the protein is up-regulated and modified, which results in two additional protein forms. Four potential N-glycosylation sites for the MATH domain-containing protein are predicted, which might account for the different electrophoretic mobilities on gels, however, the precise nature of the protein modifications could not be identified yet. MATH-domains are common for meprins (mammalian tissue-specific metalloproteases) and TRAFs (tumour necrosis factor receptor-associated factors). TRAF proteins interact with tumour necrosis factor receptors and promote cell survival by the activation of downstream protein-kinases and transcription factors (Chung et al. 2002 and references therein). In plants, a tumour necrosis factor-like receptor kinase was shown to be involved in maize epidermal differentiation, but it is not known whether it interacts with MATH domain-containing proteins (Becraft et al. 1996). More than 50 genes for MATH-domain proteins are present in the *Arabidopsis* genome (Oelmüller, unpublished) and for almost all of them, knock-out lines are available. Unfortunately, the MATH-domain protein identified in this study does not belong to this group.

For the majority of the proteins identified in this screen, an involvement in plant–microbe interactions has not been described before. For many of them, a connection to any physiological function is new. We showed that *P. indica* stimulate growth of the plant, possibly by influencing N-linked glycosylation in ER, and has an impact on plant stress response by modulating β-glucosidase PYK10 and/or by up-regulation of glutathione-*S*-transferases. The interaction of *P. indica* with *A. thaliana* roots provides an ideal tool to study the molecular events by which the fungus recognizes the host, colonizes the roots and ultimately stimulates plant growth.

Acknowledgements – A.V. and C.M. were supported by the Federal State of Thuringia. We thank Karl-Heinz Rexer, François Buscot and Susann Klemm for their support and advice.

References

Ané JM, Kiss GB, Riely BK, Penmetsa RV, Oldroyd GE, Ayax C, Levy J, Debelle F, Baek JM, Kalo P, Rosenberg C, Roe BA, Long SR, Denarie J, Cook DR (2004) *Medicago truncatula* DMI1 required for bacterial and fungal symbioses in legumes. Science 303: 1364–1367

Arendt CW, Ostergaard HL (1997) Identification of the CD45-associated 116-kDa and 80-kDa proteins as the α- and β-subunits of α-glucosidase II. J Biol Chem 272: 13117–13125

Becraft PW, Stinard PS, McCarty DR (1996) CRINKLY4: a TNFR-like receptor kinase involved in maize epidermal differentiation. Science 273: 1406–1409

Bestel-Corre G, Dumas-Gaudot E, Poinsot V, Dieu M, Dierick J-F, van Tuinen D, Remacle J, Gianinazzi-Pearson V, Gianinazzi S (2002) Proteome analysis and identification of symbiosis-related proteins from *Medicago truncatula* Gaertn. by two-dimensional electrophoresis and mass spectrometry. Electrophoresis 23: 122–137

Bianchi MW, Roux C, Vartanian N (2002) Drought regulation of GST8, encoding the *Arabidopsis* homologue of ParC/Nt107 glutathione transferase/peroxidase. Physiol Plant 11: 96–105

Boisson M, Gomord VV, Audran C, Berger N, Dubreucq B, Granier F, Lerouge P, Faye L, Caboche M, Lepiniec L (2001) Arabidopsis glucosidase I mutants reveal a critical role of N-glycan trimming in seed development. EMBO J 20: 1010–1019

Bones AM, Rossiter JT (1996) The myrosinase-glucosinolate system, its organisation and biochemistry. Physiol Plant 97: 194–208

Breuninger M, Requena N (2004) Recognition events in AM symbiosis: analysis of fungal gene expression at the early appressorium stage. Fungal Genet Biol 41: 794–804

Bucking H, Heyser W (2003) Uptake and transfer of nutrients in ectomycorrhizal associations: interactions between photosynthesis and phosphate nutrition. Mycorrhiza 13: 59–68

Burn JE, Hurley UA, Birch RJ, Arioli T, Cork A, Williamson RE (2002) The cellulose-deficient Arabidopsis mutant rsw3 is defective in a gene encoding a putative glucosidase II, an enzyme processing N-glycans during ER quality control. Plant J 32: 949–960

Caplan AJ, Cyr DM, Douglas MG (1993) Eukaryotic homologues of *Escherichia coli* dnaJ: a diverse protein family that functions with hsp70 stress proteins. Mol Biol Cell 4: 555–563

Chung JY, Park YC, Ye H, Wu H (2002) All TRAFs are not created equal: common and distinct molecular mechanisms of TRAF-mediated signal transduction. J Cell Sci 115: 679–688

Endré G, Kereszt A, Kevei Z, Mihacea S, Kalo P, Kiss GB (2002) A receptor kinase gene regulating symbiotic nodule development. Nature 417: 962–966

Eriksson S, Andreasson E, Ekborn B, Graner G, Pontoppidan B, Taipalensuu J, Zhang J, Rask L, Meijer J (2002) Complex formation of myrosinase isoenzymes in oilseed rape seeds are dependent on the presence of myrosinase-binding proteins. Plant Physiol 129: 1592–1599

Gadkar V, David-Schwatz R, Kunik T, Kapulnik Y (2001) Arbuscular mycorrhizal fungal colonization. Factors involved host recognition. Plant Physiol 127: 1493–1499

Harrison MJ (1999) Molecular and cellular aspects of the arbuscular mycorrhizal symbiosis. Annu Rev Plant Physiol Plant Mol Biol 50: 361–389

Harrison MJ, Dewbre GR, Liu J (2002) A phosphate transporter from *Medicago truncatula* involved in the acquisition of phosphate released by arbuscular mycorrhizal fungi. Plant Cell 14: 2413–2429

Hayashi Y, Yamada K, Shimada T, Matsushima R, Nishizawa NK, Nishimura M, Hara-Nishimura I (2001) A proteinase-storing body that prepares for cell death or stress in the epidermal cells of Arabidopsis. Plant Cell Physiol 42: 894–899

Helenius A, Aebi M (2001) Intracellular functions of N-linked glycans. Science 291: 2364–2369

Herrmann S, Munch J-C, Buscot F (1998) A gnotobiotic culture system with oak microcuttings to study specific effects of mycobionts on plant morphology before, and in the early phase of, ectomycorrhiza formation by *Paxillus involutus* and *Piloderma croceum*. New Phytol 138: 203–212

Herrmann S, Oelmüller R, Buscot F (2004) Manipulation of the onset of ectomycorrhiza formation with indolic acetic acid, activated charcoal or relative humidity in the association between oak microcuttings and *Piloderma croceum*: influence on plant development and photosynthesis. J Plant Physiol 161: 509–517

Hippler M, Klein J, Fink A, Allinger T, Hoerth P (2001) Towards functional proteomics of membrane protein complexes: analysis of thylakoid membranes from *Chlamydomonas reinhardtii*. Plant J 28: 595–606

Hood ME, Shew HD (1996) Applications of KOH-aniline blue fluorescence in the study of plant–fungal interactions. Phytopathology 86: 704–708

Karandashov V, Nagy R, Wegmüller S, Amrhein N, Bucher M (2004) Evolutionary conservation of a phosphate transporter in the arbuscular mycorrhizal symbiosis. Proc Natl Acad Sci USA 101: 6285–6290

Kawano T (2003) Roles of the reactive oxygen species-generating peroxidase reactions in plant defense and growth induction. Plant Cell Rep 21: 829–837
Kistner C, Parniske M (2002) Evolution of signal transduction in intracellular symbiosis. Trends Plant Sci 7: 511–518
Kiyosue T, Yamaguchi-Shinozaki K, Shinozaki K (1993) Characterization of two cDNAs (ERD11 and ERD13) for dehydration-inducible genes that encode putative glutathione *S*-transferases in *Arabidopsis thaliana* L. FEBS Lett 335: 189–192
Krüger A, Peškan-Berghöfer T, Frettinger P, Herrmann S, Buscot F, Oelmüller R (2004) Identification of premycorrhiza-related plant genes in the association between *Quercus robur* and *Piloderma croceum*. New Phytol 163: 149–157
Levy J, Bres C, Geurts R, Chalhoub B, Kulikova O, Duc G, Journet EP, Ané JM, Lauber E, Bisseling T, Denarie J, Rosenberg C, Debelle F (2004) A putative Ca^{2+} and calmodulin-dependent protein kinase required for bacterial and fungal symbioses. Science 303: 1361–1364
Limpens E, Bisseling T (2003) Signaling in symbiosis. Curr Opin Plant Biol 6: 343–350
Marsh JF, Schultze M (2001) Analysis of arbuscular mycorrhizas using symbiosis-defective plant mutants. New Phytol 150: 525–532
Marx DH (1969) The influence of ectotrophic mycorrhizal fungi on the resistance of pine roots to pathogenic infections. I. Antagonism of mycorrhizal fungi to root pathogenic fungi and soil bacteria. Phytopathology 59: 153–163
Marx J (2004) The roots of plant-microbe collaborations. Science 304: 234–239
Matsushima R, Hayashi Y, Kondo M, Shimada T, Nishimura M, Hara-Nishimura I (2002) An endoplasmic reticulum-derived structure that is induced under stress conditions in Arabidopsis. Plant Physiol 130: 1807–1814
Matsushima R, Kondo M, Nishimura M, Hara-Nishimura I (2003) A novel ER-derived compartment, the ER body, selectively accumulates a β-glucosidase with an ER-retention signal in Arabidopsis. Plant J 33: 493–502
Mauch F, Dudler R (1993) Differential induction of distinct glutathione-*S*-transferases of wheat by xenobiotics and by pathogen attack. Plant Physiol 102: 1193–1201
Mellersh DG, Heath MC (2003) An investigation into the involvement of defense signaling pathways in components of the non-host resistance of *Arabidopsis thaliana* to rust fungi also reveals a model system for studying rust fungal compatibility. Mol Plant Microbe Interact 16: 398–404
Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15: 473–497
Newman EL, Reddel P (1987) The distribution of mycorrhizas among families of vascular plants. New Phytol 106: 745–751
Nielsen H, Engelbrecht J, Brunak S, von Heijne G (1997) A neural network method for identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Int J Neural Syst 8: 581–599
Nitz I, Berkefeld H, Puzio PS, Grundler FM (2001) Pyk10, a seedling and root specific gene and promoter from *Arabidopsis thaliana*. Plant Sci 161: 337–346
Parniske M (2000) Intracellular accommodation of microbes by plants: a common development program for symbiosis and disease? Curr Opin Plant Biol 3: 320–328
Parniske M (2004) Molecular genetics of the arbuscular mycorrhizal symbiosis. Curr Opin Plant Biol 7: 414–421
Pfannschmidt T, Schütze K, Brost M, Oelmüller R (2001) A novel mechanism of nuclear photosynthesis gene regulation by redox signals from the chloroplast during photosystem stoichiometry adjustment. J Biol Chem 276: 36125–36130
Pham GH, Kumari R, Singh An, Sachdev M, Prasad R, Kaldorf M, Buscot F, Oelmüller R, Peskan T, Weiss M, Hampp R, Varma A (2004) Axenic cultures of *Piriformospora indica*. In: Varma A, Abbott L, Werner D, Hampp R (eds) Plant Surface Microbiology. Springer-Verlag, Germany, pp 593–616
Ravanel S, Gakiere B, Job D, Douce R (1998) The specific features of methionine biosynthesis and metabolism in plants. Proc Natl Acad Sci USA 95: 7805–7812
Reboutier D, Bianchi M, Brault M, Roux C, Dauphin A, Rona JP, Legue V, Lapeyrie F, Bouteau F (2002) The indolic compound hypaphorine by ectomycorrhizal fungus interferes with auxin action and evokes early responses in nonhost *Arabidopsis thaliana*. Mol Plant Microbe Interact 15: 932–938
Schultz J, Milpetz F, Bork P, Ponting CP (1998) SMART, a simple modular architecture research tool: identification of signaling domains. Proc Natl Acad Sci USA 95: 5857–5864
Shahollari B, Varma A, Oelmüller R (2004) Expression of a receptor kinase in *Arabidopsis* roots is stimulated by the basidiomycete *Piriformospora indica* and the protein accumulates in Triton X-100 insoluble plasma membrane microdomains. J Plant Physiol, in press
Sime AD, Abbott LL, Abbott SP (2002) A mounting medium for use in indoor air quality spore-trap analyses. Mycologia 94: 1087–1088
Singh An, Singh Ar, Kumari M, Rai MK, Varma A (2003) Importance of *Piriformospora indica*-A novel symbiotic mycorrhiza-like fungus: an overview. Ind J Biotechnol 2: 65–75
Smith SE, Read DJ (1997) Mycorrhizal Symbiosis, 2nd edn. Academic Press, San Diego, CA, USA
Stauber EJ, Fink A, Markert C, Kruse O, Johanningmeier U, Hippler M (2003) Proteomics of *Chlamydomonas reinhardtii* light-harvesting proteins. Eukaryotic Cell 2: 978–994
Strack D, Fester T, Hause B, Schliemann W, Walter MH (2003) Arbuscular mycorrhiza: biological, chemical, and molecular aspects. J Chem Ecol 29: 1955–1979
Stracke S, Kistner C, Yoshida S, Mulder L, Sato S, Kaneko T, Tabata S, Sandal N, Stougaard J, Szczyglowski K, Parniske M (2002) A plant receptor-like kinase required for both bacterial and fungal symbiosis. Nature 417: 959–962
Strittmatter G, Cheysen G, Gianinazzi-Pearson V, Hahn K, Niebel A, Rohde W, Tacke E (1996) Infections with various types of organisms stimulate transcription from a short promoter fragment of the potato *gst1* gene. Mol Plant Microbe Interact 9: 68–73
Taylor MA, Ross HA, McRae D, Stewart D, Roberts I, Duncan G, Wright F, Miliam S, Davies HV (2000) A potato α-glucosidase gene encodes a glycoprotein-processing α-glucosidase II-like activity. Demonstration of enzyme activity and effects of down-regulation in transgenic plants. Plant J 24: 305–316
Trombetta ES, Simons JF, Helenius A (1996) Endoplasmic reticulum glucosidase II is composed of a catalytic subunit, conserved from yeast to mammals, and a tightly bound noncatalytic HDEL-containing subunit. J Biol Chem 271: 27509–27516
Varma A, Singh A, Sudha Sahay N, Sharma J, Roy A, Kumari M, Rana D, Thakran S, Deka D, Bharti K, Franken P, Hurek T, Blechert O, Rexer K-H, Kost G, Hahn A, Hock B, Maier W, Walter M, Strack D, Kranner I (2001) *Piriformospora indica*: a cultivable mycorrhiza-like endosymbiotic fungus. In: Mycota IX. Springer Series, Heidelberg, Germany, pp. 123–150
Varma A, Verma S, Sudha Sahay N, Britta B, Franken P (1999) *Piriformospora indica* – a cultivable plant growth promoting root endophyte with similarities to arbuscular mycorrhizal fungi. Appl Environ Microbiol, USA 65: 2741–2744
Vierheilig H, Coughlan AP, Wyss U, Piche Y (1998) Ink and vinegar, a simple staining technique for arbuscular-mycorrhizal fungi. Appl Environ Microbiol 64: 5004–5007
Vitale A (2001) Uncovering secretory secrets: inhibition of endoplasmic reticulum (ER) glucosidases suggests a critical role for ER quality control in plant growth and development. The Plant Cell 13: 1260–1262
Wessel D, Flügge UI (1983) A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. Anal Biochem 138: 141–143
Wulf A, Manthey K, Doll J, Perlick AM, Linke B, Bekel T, Meyer F, Franken P, Kuster H, Krajinski F (2003) Transcriptional changes in response to arbuscular mycorrhiza development in the model plant *Medicago truncatula*. Am Phytopath Soc 16: 306–314
Zeng RS, Mallik AU, Setliff E (2003) Growth stimulation of ectomycorrhizal fungi by root exudates of Brassicaceae plants: role of degraded compounds of indole glucosinolates. J Chem Ecol 29: 1337–1355

Edited by J. K. Schjørring

2.2 Manuscript II

Receptor kinases with leucine-rich repeats are enriched in Triton X-100 insoluble plasma membrane microdomains from plants

Bationa Shahollari, Tatjana Peskan-Berghöfer and Ralf Oelmüller

Physiologia Plantarum (2004) **122**: 397-403

PHYSIOLOGIA PLANTARUM 122: 397–403. 2004
Printed in Denmark – all rights reserved

doi: 10.1111/j.1399-3054.2004.00414.x

Receptor kinases with leucine-rich repeats are enriched in Triton X-100 insoluble plasma membrane microdomains from plants

Bationa Shahollari, Tatjana Peskan-Berghöfer and Ralf Oelmüller*

Institute for General Botany and Plant Physiology, Friedrich-Schiller-University Jena, Dornburger Str. 159, D-07743 Jena, Germany
**Corresponding author, e-mail: b7oera@uni-jena.de*

Received 28 April 2004; revised 30 June 2004

In mammals, signalling components at the cell surface are clustered in Triton X-100 insoluble plasma membrane microdomains. We isolated plasma membrane microdomains from *Arabidopsis* and mustard cotyledons and determined their protein composition by mass spectrometry. Although the protein composition of the plant vesicles differ from the composition of the animal vesicles, they are also enriched in signalling components. We identified at least seven receptor kinases with leucine-rich repeats, 10 other kinases, the β subunit of heterotrimeric G-proteins and five small GTP-binding proteins. Thus, specific signalling components are highly enriched in plant plasma membrane microdomains while others are excluded.

Introduction

Cells communicate with their environment through signals which are perceived at the plasma membrane. In animals, plasma membranes contain lipid rafts, namely membrane subdomains with a unique protein and lipid composition (cf. Simons and Toomre 2000; Galbiati et al. 2001; Munro 2003). A large number of signalling molecules are concentrated within these rafts which have been proposed to function as signalling centres capable of facilitating efficient and specific signal transduction. Components for different signalling pathways appear to be compartmentalized within these microdomains which, depending on the isolation conditions and physiological parameters, are connected to downstream events. Although cell signalling in plant cells should be similar, little is known about the structure, lipid and protein composition of comparable microdomains from plant plasma membranes (cf. Peskan et al. 2000). We isolated plasma membrane microdomains from the cotyledons of *Arabidopsis* and mustard seedlings and determined their protein composition by mass spectrometry. Although the protein composition differs from that of mammals, the plant microdomains are highly enriched in signalling compounds such as receptor kinases, small and heterotrimeric G proteins and signalling kinases.

Materials and methods

Growth conditions

Wild-type *Arabidopsis thaliana* (ecotype Columbia) seeds 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 21 days at 22°C under continuous illumination (100 μmol $m^{-2} s^{-1}$). *Sinapsis alba* seedlings were grown on vermiculite in temperature-controlled growth chambers at 20°C for 7 days.

Isolation of Triton X-100 insoluble plasma membrane microdomains

One hundred grams of cotyledons from *Arabidopsis* or mustard seedlings were used to isolate microsomes. The cotyledons were homogenized in a Warring Blender

Abbreviations – ESI-MS, electrospray ionization mass spectrometry; MS-MS, tandem mass spectrometry.

(seven times for 5 s) in a buffer containing 50 m*M* Tris/HCl pH 7.4, 330 m*M* sucrose, 3 m*M* EDTA, 1 m*M* 1,4-dithiothreitol and 4% (w/v) polyvinylpolypyrrolidone. The homogenate was filtered through four layers of cheesecloth and centrifuged for 20 min at 10 000 *g*. The supernatant was then centrifuged at 50 000 *g* for 60 min in order to pellet the microsomes. Plasma membranes were prepared from microsomes by two-phase partitioning with 6.4% (w/w) dextrane T-500 and 6.4% (w/w) polyethylene glycol (average molecular weight 3350) as described by Peskan et al. (2000), Larsson et al. (1987) and Briskin et al. (1987). The plasma membranes were re-suspended in a buffer containing 50 m*M* Tris/HCl pH 7,4; 3 m*M* EDTA and 1 m*M* 1,4-dithiothreitol. After treatment with 1% (v/v) Triton X-100 at 4°C for 30 min, membranes were mixed with 60% (w/w) sucrose to the final concentration of 48% (w/w), placed at the bottom of a centrifuge tube and overlaid with a continuous sucrose gradient (15–45%, w/w). Gradients were centrifuged at 250 000 *g* for 20 h in a swinging bucket rotor (SW 40; Beckman, Palo Alto, CA).

Alternatively, membranes were sonicated seven times for 10 s (Sonoplus HD70 with tip SH70; Bandelin Electronic, Berlin, Germany; power 20 W) without addition of Triton X-100 and purified on a continuous sucrose gradient. All fractions (microsomes, plasma membranes, vesicles from Triton X-100 and sonicated plasma membranes) from both organisms were routinely used for the identification of their protein patterns by mass spectrometry.

For one-dimensional gel electrophoresis, the buffer system of Schägger and von Jagow (1987) was used. Coomassie-staining of the gels was performed with colloidal Coomassie stain Roti-Blue (Roth, Karlsruhe, Germany) according to the manufacturer's instructions.

Mass spectrometry

Proteins extracted from membrane fractions were further purified by two rounds of methanol precipitation before digestion with trypsin. In-gel trypsin digestion of excised protein spots and elution of the peptides from the gel matrix was performed according to Sherameti et al. (2004). Peptide analysis by coupling liquid chromatography with electrospray ionization mass spectrometry (ESI-MS) and tandem mass spectrometry (MS-MS) has been described previously (Stauber et al. 2003).

Protein identification

The measured MS-MS spectra were matched with the amino-acid sequences of tryptic peptides from the *A. thaliana* database in FASTA format. Cys modification by carbamidomethylation (+ 57 Da) was taken into account and known contaminants were filtered out. Raw MS-MS data were analysed by using the Finnigan Sequest/Turbo Sequest software (revision 3.0; ThermoQuest, San Jose, CA). The parameters for the analysis by the Sequest algorithm were set according to (Stauber et al. 2003). The similarity between the measured MS-MS spectrum and the theoretical MS-MS spectrum, reported as the cross-correlation factor (X_{corr}) was above 2.95 and 3.85 for doubly or triply charged precursor ions, respectively. In order to identify corresponding loci, identified protein sequences were subjected to BLAST search at NCBI (http://www.ncbi.nlm.nih.gov/) and FASTA searches by using the AGI protein database at TAIR (http://www.arabidopsis.org/).

Western analysis was performed as described (Peskan et al. 2000).

Results

Following standard protocols established for mammalian cells, we purified microsomal fractions from *Arabidopsis* and mustard cotyledons. They were then further used to isolate purified plasma membranes. The membranes were either sonicated or treated with Triton X-100 to obtain plasma membrane microdomains (Fig. 1; Peskan et al. 2000). As described previously for tobacco (Peskan et al. 2000), the protein patterns of plasma membrane microdomains from both organisms obtained after sonication or after Triton X-100 treatment were identical on Coomassie-stained gels (cf. Figs. 1 and 2). This is not surprising since vesicles from both preparations exhibits an identical mobility during

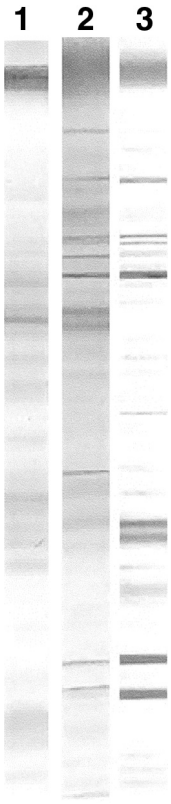


Fig. 1. Protein pattern of microsomes (lane 1), plasma membranes (lane 2) and Triton-X-100-insoluble plasma membrane vesicles (lane 3) from *Arabidopsis* cotyledons. Ten µg of protein was loaded per lane.

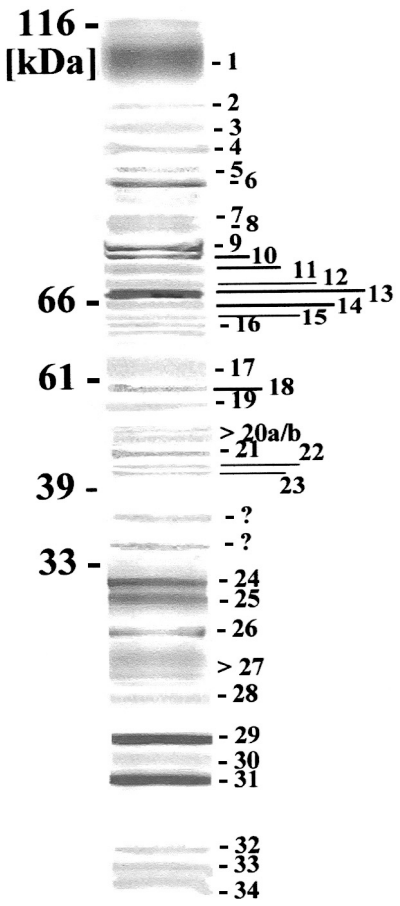


Fig. 2. Protein composition of Triton X-100 insoluble plasma membrane vesicles from *Arabidopsis* leaves. Numbers refer to the proteins in Table 1. Identical protein patterns were obtained from *Arabidopis* plasma membrane preparations obtained after sonication (Peskan et al. 2000) and mustard plasma membrane preparation treated with Triton X-100 or obtained after sonication. The protein bands were identified by mass spectrometry. The correlation factors (X_{corr}) for doubly (triply) charged precursor ions were >2.95 (3.85). Only those peptides are given in the list which were identified in at least three independent Triton X-100 insoluble plasma membrane microdomain preparations from *Arabidopsis*. Thirty-five μg of protein was loaded on the gel.

sucrose gradient centrifugations (cf. Peskan et al. 2000). We isolated the bands from the *Arabidopsis* preparation and identified the proteins by mass spectrometry (Figs. 1 and 2 and Table 1). In addition to plasma membrane ATPases and aquaporins/membrane-intrinsic proteins (MIPs), seven different receptor kinases can be identified in the region >60 kDa (At1g13230, At2g26730, At3g02880; At3g17840, At5g16590, At3g08680, At3g14350). The microdomains contain several small GTP-binding proteins (e.g. a Ras-like protein, Rab1c, Rab2, SAR1), several kinases, Ca^{2+}-binding proteins involved in signalling processes, a Sec14 homolog, phospholipase D δ, a pectinesterase, remorin, an unknown protein and several proteins which have not yet been characterized (cf. Table 1 and Discussion).

Silver-staining of the gel shown in Fig. 2 uncovered additional bands which were not detectable on the Coomassie-stained gels (data not shown). We have previously demonstrated that plasma membrane microdomains from tobacco leaves contain the β subunit of heterotrimeric G proteins (Gβ) (Peskan et al. 2000). Western analysis confirmed that Gβ is also present in the *Arabidopsis* preparations although we have not identified Gβ by mass spectrometry (Fig. 3). This demonstrates that additional proteins are present in our Triton X-100 insoluble plasma membrane preparations.

Discussion

Low density Triton X-100 insoluble plasma membrane microdomains are well characterized in animals and yeast; however, little is known about their role and protein composition in plants. In animals, they contain resident integral membrane proteins such as caveolin, stomatin, and flotillin, extracellular proteins with glycophosphatidylinositol anchors and cytoplasmic proteins modified by myristoylation/palmitoylation (Simons and Toomre 2000, Nebl et al. 2002, Munro 2003). These microdomains are less than 50 μm in diameter and can recruit different signalling components depending on their cellular signalling functions and receptor activation. We isolated Triton X-100 insoluble vesicles from plants, namely from the cotyledons of *Arabidopsis* and mustard seedlings, using two well-established protocols for animal cells (Larsson et al. 1987, Peskan et al. 2000). The protein patterns of both preparations were identical. There was also no difference in the protein composition of micodomains from the two Brassicaceae *Arabidopsis* and mustard. We found that the number of proteins in these vesicles, which can be detected on Coomassie-stained gels, is very similar to that from animals. However, they contain many proteins which are not present in or not characteristic for mammalian microdomains. The plasma membrane ATPase, aquaporins and membrane-intrinsic proteins are major constituents of the plant vesicles and normally not detected in animal microdomains. We also found that receptor kinases with leucine-rich repeats are highly enriched in these vesicles. This suggests that they either interact with each other or are organized in similar plasma membrane environments or share similar biochemical purification features. Six of these receptor kinases appear to be integral membrane proteins, whereas one protein (At1g13230) exhibits unusual features. Its apparent molecular weight on the denaturing SDS gel differs from the calculated size and the protein does not contain a predicted transmembrane segment. Thus, this receptor kinase might be located at the inner plasma membrane site. We also find typical plasma membrane proteins such as phospholipase D δ or a cAMP-dependent kinase, several, so far uncharacterized protein kinases, Ca^{2+}- and small GTP-binding proteins, heterotrimeric G-protein subunits and putative downstream signalling compounds (cf. CBL- interacting kinase 8 and 9; a 14-3-3 protein). Protein/protein interaction studies between these proteins and the analyses of

Table 1. Proteins identified in Triton X-100 insoluble plasma membrane microdomains from *Arabidopsis* cotyledons. Numbers refer to the protein bands in Fig. 1. The identified loci and peptides are given. For details, see Materials and methods and legend to Fig. 1. The cross-correlation factor (X_{corr}) was above 2.95 and 3.85 for doubly or triply charged precursor ions, respectively.

No.	Protein	Gene no.	Peptides
Predicted integral membrane proteins			
1	Plasma membrane ATPase	At1g17260	KADIGIAVADATDAAR
			ADGFAGVFPEHK
		At1g80660	WSEQEAAILVPGDIISIK
			QGELEAVVIATGVHTFFGK
			EAQWAQAQR
			MITGDQLAIGK
		At2g18960	IPIEEVFQQLK
			WSEQEAAILVP GDIVSIK
			VDQSALTGESLPVTK
			AAHLVDST NQVGHFQ
			GVEKDQVLLFAAMASRVEN QDAIDAAMVG MLADPK
			EVHFLPFNPVDKRTALTYIDSDGNWHR
			VSKGAPEQILDLANARPDLR
			ESPGGPWEFVGLLPLFDPPR
			LGMGTNMYPSAALLGTDKDSNIASIP VEELIEK
			KADIGIAVADATDAAR
			GASDIVLTEPGLSVIISAVLTSR
			AWASLFDNR
			ELSEIAEQAK
			HIVGMTGDGVNDAPALK
		At2g24520	IDQSSLTGESIPVTK
			IENQDAIDAAIVGMLADPK
			ADGFAGVFPEHK
			KADIGIAVVDATDAAR
			GASDIVLTEPGLSVIISAVLTSR
		At2g07560	KADIGIAVDDATDAAR
		At4g30190	TALTYIDGSGNWHR
			ESPGAPWEFVGLLPLFDPPR
			LGMGTNMYPSSALLGTHK
			KADIGIAVADATDAAR
		At3g47950	WGEQDAAILVPGDIISIK
			KADIGIAVADATDAAR
			LENQDAIDAAIVGMLADPK
		At5g62670	ETVDLENVPIEEVFESLR
			ESAGGPWQFMGLMPLFDPPR
			KADIGIAVADATDAAR
			THFNELSQMAEEAK
			EVHFLPFNPTDK
			SLAVAYQEVPEGTK
2	Kinase	At3g59420	NSNIISSSLVDCWGYNMTR
			ARVFTYEELEK
			GSFSCVYK
			VTIAVQAAR
3	Receptor kinase (S-Locus) ARK3	At4g21380	SPLVAELLDNGNFVLR
			ENNTDDLELPLMEFEEVAMATNNFSNANK
			IIGSSIGVSVLLLLSFIIFFLWK
6	Kinase	At1g16760	GTRNGSVAIAIDK
			YSVQEIEEGTANFAESR
			HKTANTPALPK
7	ERD4 protein	At1g30360	QVDSIEYYTELINESVAK
			EAWYPGDLSYATR
9	LRR-RK-1	At3g17840	LNLAENEFSGEISSGFK
			GLDYLHSQDPLSSHGNVK
			VSDFGLAQLVSASSTTPNR
			SSNILLTNSHDAR
10	LRR-RK-2	At5g16590	LATLYLQDNQLTGPIPEIK
			SPLNWETR
			GSLSALLHGNK
11	LRR-RK-3	At2g26730	QALLTFLQQIPHENR
			LPGTGLVGQIPSGSLGR
			SLYLQHNEFSGEFPTSFTQLNNLIR
			LLVFDFMPTGSLSALLHGSR
			AVLEEGTTVVVK
12	LRR-RK-4	At3g02880	FNSLSGPIPSDFSNLVLLR
			ASFEHGLVVAVK
			GSLSAILHGNK
			TPLNWETR
			SPTHQQLNEEGVDLPR
			AISYLHSR

Table 1. Continued.

No.	Protein	Gene no.	Peptides
13	Pectinesterase	At4g12420	LDEGLLLHWNGIQQR
			DQIGSFFYFPSLHFQR
			ASGGFGSFVVNPR
			DLGMPDGVL
			VSNVGISTSLNFR
			TENLDSWYLGQETYVR
14	LRR-RK-5	At1g13230	PELFELKHLRSLSFFNCFISPMVIAK
15	LRR-RK-6	At3g08680	AEEFGSGVQEAEK
			TTGHEEVVDLPK
			LPGSGLYGPLPEK
16	LRR-RK-7	At3g14350	YLNLAHNQLK
			SFDDDDSTMRK
			IAHLDHENVTK
25	cAMP-dependent kinase	At2g20040	NGLKWEAISNR
			FTICGNADYLAPEIVQGK
			LLEVDENLR
26	Aquaporin, intrinsic protein	At4g35100	DYVDP PPAPLLDMGELKSWSFYR
			TPYNTLGGG ANTVADGYSK
			SFGAAVIYNNEK
27	Intrinsic proteins, aquaporins	At1g01620	VGANKFPER
		(PIP 1C)	GSGLGAEIIGTFVLVYTVFSATDAK
			SLGAAIIYNK
		At2g45960	QP IGTSAQSDKDYKEPPPAPLF EPGELASWSFWR
		(PIP 1B)	QYQALGGGANTIAHGYTK
			GSGLGAEIIGTFVLVYTVFSATDAK
			SLGAAIIFNK
		At2g16850	SFGAAVIYNNEK
		At2g39010	SFGAAVIYNNQK
		At3g53420	DVEAVPGEGFQTRDYQDPPPAPFIDGAELK
		(PIP 2A)	AFQSSYYTR
		At4g23400	DYKEPPPAPFFEPGELK
			GFQPGLYQTNGGGANVVAHGYTK
Predicted peripheral proteins			
4/5	EF hand protein	At1g05150	AVSLLGAGETEEAK
			DNDVPVSYSGSGGPTK
			TYDDGAGDVDR
			EEAFDGHMAIGR
		At2g32450	ILSVLDDSGSGR
			VELHDAVSHLK
8	Phospholipase D δ	At4g35790	VITSDPYVTVVVPQATLAR
			ILHDLDTVFK
			HSSVICVLSPR
17	Calcium-dependent kinase	At4g04720	VIAESLSEEEIK
			GGEAPDKPIDSAVLSR
18	Calnexin-like protein	At5g07340	SEGHDDYGLLVSEK
			WSSPLIDNPAYK
			GNLLSAEDFEPPLIPSK
19	Kinase	At4g35230	AATNNFSSDNIVSESGEK
			LLVAEFMPNDTLAK
			VLFDEDGDPR
			VTPESVTYSFGTVLLDLLSGK
			DDEGTNELSFQEWTQQMK
			LNMNTDAADMLNEAAQLEEK
20a	CBL-interacting protein kinase 8	At4g24400	FAQNTETGESVAMK
			PANVVLSSMEVVSQSMGFK
			IYIILEYITGGELFDK
20b	CBL-interacting protein kinase 9	At1g01140	LKEDEARRYFQQLINAVDYCHSR
			ILEPNPITR
			PENLIL DANGVLK
21	Ankyrin kinase IV	At1g14000	NVLLVNSSADHLK
			RTPLHVASLHGWIDVVK
			CDWEIEPAELDFSNAAMIGK
			KLRHPNIVQFLGAVTER
			YMAPEVFKHR
22	Kinase	At1g63500	LLVAEFMPNETLAK
			VALHIAQALEYCTGK
23	Kinase	At3g01490	GIYDGQDVAVK
			SLDEQLQR
			SLSDGEDNVNNTR

Table 1. Continued.

No.	Protein	Gene no.	Peptides
24	Hypersensitive-induced proteins (band 7 family proteins)	At5g62740	AMNEINAAAR
			LLLDDVFEQK
			AVEEELEK
			SSAVFIPHGPGAVR
			YLSGLGIAR
		At3g01290	VLNPGLQFVPWVIGDYVAGTLTLR
			TKDNVFVTVVASIQYR
			AYVFDVIR
			LNLDDVFEQK
			DSVLGFAGNVPGTSAK
			DVLDMVMMTQYFDMR
			SSAVFIPHGPGAVSDVAAQIR
		At1g69840	ALAESAQDAFYK
			LDLDSTFEQK
			NQIQAYVFDVIR
			AMNEINAASR
			DVMDMVLVTQYFDTLK
			RAEGEAESK
28	14-3-3 protein	At5g10450	QAFEEAIAELDTLGEESYK
			AAQDIAAADMAPTHPIR
			NLLSVAYK
29	GTP-binding proteins (ras-rel.)	At3g09910	ILLIGDSGVGK
		At4g18430	AQLWDTAGQER
			STIGVEFATR
			DHTDANVVIMLVGNK
		At1g56330	LQLWDTAGQER
			ELNVMFIETSAK
		At1g02130	LQIWDTAGQER
30	Rab1c	At4g17530	FADDSYLDSYISTIGVDFK
			TITSSYYRGAHGIIVTYDVTDLESFNNVK
31	Remorins	At2g45820	VDVESPAVLAPAK
			ISDVHAWENSK
			EPTPAPVEVADEK
			DVILADLEK
		At3g48940	RGEDVLKAEEMAAK
32	Rab2-like GTP-binding protein	At4g17170	TAQNVEEAF
			FQPVHDLTIGVEFGAR
33	SAR1B-homolog	At1g56330	VDAVVYLVDAYDKER
			ILFLGLDNAGK
34	SAR1A-homolog	At1g02620	VDALVYLVDAYDGER
			ELDALLSDESLATVPFLILGNK

knock-out line will help to elucidate the function of these proteins in signalling processes across the plasma membrane. It is also interesting that the microdomains contain several protein involved in stress responses (At1g30360, At5g62740). Further transduction of activating signals depends on the presence of proteins associated at the inner site of the plasma membrane. Our list of proteins provides several candidates. The list contains also proteins involved in protein trafficking through the secretory pathway (e.g. Sar1, Rab1c and Rab2). Since

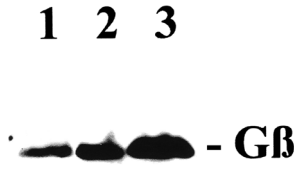


Fig. 3. Western analyses with protein extracts from Trition X-100 insoluble plasma membrane preparations from *Arabidopsis* leaves with an antibody against the β subunit of heterotrimeric G proteins (Peskan et al. 2000). Lane 1: microsomal fraction, lane 2, plasma membrane preparation, lane 3, Triton X-100 vesicles.

the organization of a plant cell with its huge vacuole differs substantially from that of animal cells, membrane trafficking between the ER and the plasma membrane might be different.

Recently, Wienkoop and Saalbach (2003) analysed the proteom of the plasma membrane-derived peribacteroid membrane from *Lotus japonicus* root nodules. Many of the proteins identified in our microdomains are also present in their peribacteroid membrane system. In particular, both membrane preparations contain ATPases, aquaporins, GTP-binding proteins, proteins involved in signalling processes, receptor kinases, 14-3-3 proteins and pathogen-related proteins. Wienkoop and Saalbach (2003) also found proteins which are expected to be localized in other plant endomembranes, comparable with the results reported here. Likewise, Marmagne et al. (2004) analysed the proteom of soluble and insoluble plasma membrane fractions from suspension-cultured *Arabidopsis* cells and found several transporter proteins, receptors, GTP-binding proteins, proteins involved in various trafficking processes as well as stress-related proteins. Taken together, the high degree of overlap of

the identified proteins in these membrane preparations provides a solid basis for future studies.

Acknowledgements – Research was supported by the SFB 604. B.S. was supported by a fellowship from the DAAD (Bonn, Germany).

References

Briskin DP, Leonard RT, Hodges TK (1987) Isolation of the plasma membrane: membrane markers and general principles. Meth Enzymol 148: 542–548

Galbiati F, Razani B, Lisanti MP (2001) Emerging themes in lipid rafts and caveolae. Cell 106: 403–411

Larsson C, Widell S, Kjellbom P (1987) Preparation of high-purity plasma membranes. Meth Enzymol 148: 588–568

Marmagne A, Rouet MA, Ferro M, Rolland N, Alcon C, Joyard J, Garin J, Barbier-Brygoo H, Ephritikhine G (2004) Identification of new intrinsic proteins in Arabidopsis plasma membrane proteome. Mol Cell Proteomics 2004 April 1 [Epub ahead of print]

Munro S (2003) Lipid rafts: elusive or illusive? Cell 115: 377–388

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

Nebl T, Pestonjamasp KN, Leszyk JD, Crowley JL, Oh SW, Luna EJ (2002) Proteomic analysis of a detergent-resistant membrane skeleton from neutrophil plasma membranes. J Biol Chem 277: 43399–43409

Peskan T, Westermann M, Oelmüller R (2000) Identification of low-density triton X-100-insoluble plasma membrane microdomains in higher plants. Eur J Biochem 267: 6989–6995

Schägger H, von Jagow G (1987) Tricine-sodium dodecyl sulphate polyacrylamide gel electrophoresis for the separation of proteins in the range of 1–100 kDa. Anal Biochem 166: 368–379

Sherameti I, Shahollari B, Landsberger M, Westermann M, Cherepneva G, Kusnetsov V, Oelmüller R (2004) Cytokinin stimulates polyribosome loading of nuclear-encoded mRNAs for the plastid ATP synthase in etioplasts of *Lupinus luteus*: the complex accumulates in the inner envelope membrane with the CF_1 moiety located towards the stromal space. Plant J 38: 578–593

Simons K, Toomre D (2000) Lipid rafts and signal transduction. Nat Rev Mol Cell Biol 1: 31–39

Stauber EJ, Fink A, Markert C, Kruse O, Johanningmeier U, Hippler M (2003) Proteomics of *Chlamydomonas reinhardtii* light-harvesting proteins. Eurkaryotic Cell 2: 978–994

Wienkoop S, Saalbach G (2003) Proteome analysis. Novel proteins identified at the peribacteroid membrane from *Lotus japonicus* root nodules. Plant Physiol 131: 1080–1090

Edited by P. Nissen

2.3 Manuscript III

Expression of a receptor kinase in *Arabidopsis* roots is stimulated by the basidiomycete *Piriformospora indica* and the protein accumulates in Triton X-100 insoluble plasma membrane microdomains

Bationa Shahollari, Ajit Varma, Ralf Oelmuller.

J Plant Physiol. (2005) **162**(8):945-58.

Journal of Plant Physiology **162** (2005) 945—958

Available online at www.sciencedirect.com

SCIENCE DIRECT®

JOURNAL OF PLANT PHYSIOLOGY

www.elsevier.de/jplph

Expression of a receptor kinase in *Arabidopsis* roots is stimulated by the basidiomycete *Piriformospora indica* and the protein accumulates in Triton X-100 insoluble plasma membrane microdomains

Bationa Shahollari[a], Ajit Varma[b], Ralf Oelmüller[a,*]

[a]*Institute for General Botany and Plant Physiology, Friedrich-Schiller-University Jena, Dornburger Str. 159, D-07743 Jena, Germany*
[b]*School of Life Sciences, Jawaharlal Nehru University, New Delhi-110 067, India*

Received 3 May 2004; accepted 12 August 2004

KEYWORDS
Arabidopsis;
Receptor kinase;
Mycorrhiza;
Plasma membrane;
Plasma membrane microdomains;
Plant-microbe-interaction

Summary

Piriformospora indica, an endophytic fungus of the Sebacinaceae family, colonises the roots of a wide variety of plant species and promotes their growth, in a manner similar to mycorrhizal fungi. We demonstrate that the fungus also interacts with the non-mycorrhizal host *Arabidopsis thaliana*. Promotion of root growth was detectable even before noticeable root colonization, and was accompanied by a massive transfer of phosphate from the media to the aerial parts of the seedlings. During the recognition period of both organisms, the message for a receptor kinase with leucine-rich repeats is transiently upregulated. The kinase is located in Triton X-100-insoluble plasma membrane microdomains. Thus, this is one of the earliest events of a plant root in response to a fungus reported to date.

Introduction

The majority of land plants live in symbiotic relationships with fungi called mycorrhiza (Smith and Read, 2001). While the plant delivers photo-assimilates to the fungus, the fungus promotes root access to nutrients in the soil. The establishment of mycorrhizal symbioses occurred early in evolution

*Corresponding author. Institute of General Botany and Plant Physiology, Friedrich-Schiller-University Jena, Dornburger Str. 159, D-07743 Jena, Germany. Tel.: ++49 3641 949230; fax: ++49 3641 949232.
E-mail address: b7oera@uni-jena.de (R. Oelmüller).

0176-1617/$ - see front matter © 2005 Elsevier GmbH. All rights reserved.
doi:10.1016/j.jplph.2004.08.012

of land plants (cf. Heckman et al., 2001). Many plant genes and proteins which have been identified to respond to fungi are involved in later phases of the interactions (cf. Harrison 1999; Voiblet et al., 2001; Bestel-Corre et al., 2002, Wulf et al., 2003, Krüger et al., 2004). Only recently, Endré et al. (2002) and Stracke et al. (2002) have shown that a receptor kinase is required for both bacterial and fungal recognition in legume roots. The exact role of the identified kinase in mycorrhizal symbiosis is not clear. However, it appears that it defines an ancient signalling pathway that evolved in the context of arbuscular mycorrhiza, and has been recruited subsequently for endosymbiosis with bacteria (Parniske, 2000; Kistner and Parniske, 2002). Ané et al. (2004) identified a protein with similarities to a ligand-gated cation channel domain of archaea, which is required for bacterial and fungal symbioses in legumes. Since this protein is highly conserved in angiosperms and ancestral to land plants, it may be involved in establishing mycorrhizal associations. Furthermore, a putative Ca^{2+} and calmodulin-dependent protein kinase may be involved in transducing bacterial and fungal signals to downstream events (Levy et al., 2004).

To further elucidate these mechanisms and to identify genes and proteins which are involved in early steps of plant/fungus recognition, we studied the interactions between *Arabidopsis* roots and the basidiomycete *Piriformospora indica*. The fungus interacts with many plant species, mimics an arbuscular mycorrhiza and promotes plant growth and development (Varma et al., 1999, 2001; Peškan-Berghöfer et al., 2004). A stimulatory effect of the fungus on plant growth and development can be detected before physical contact between the organisms is established. We have shown recently that a modification of a MATH protein in the plasma membrane of *Arabidopsis* roots belongs to one of the earliest events which can be monitored in response to the fungus (Peškan-Berghöfer et al., 2004). Here, we demonstrate that the message for a receptor kinase is also upregulated during early phases of co-cultivation. Both responses are transient and disappear as soon as a stable interaction between both organisms is established. This implies that they are connected to early steps in the recognition between *Arabidopsis* roots and *P. indica*. Furthermore, biochemical studies have demonstrated that the receptor kinase is located in Triton X-100 insoluble plasma membrane microdomains, together with other receptor kinases and signalling components.

Materials and methods

Growth conditions of plants and fungus

Wild type *Arabidopsis thaliana* (ecotype Columbia) seeds 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 ($100\,\mu mol\,m^{-2}\,s^{-1}$). *Piriformospora indica* was cultured as described previously (Varma et al., 1999).

Co-cultivation experiments and estimation of plant growth, uptake of phosphate

Seven day-old *Arabidopsis thaliana* seedlings were transferred to nylon disks (mesh size 70 μm) placed on top of a modified MMN culture medium (MMN medium with a 1/10 of nitrogen and phosphorus and no carbohydrate (Marx 1969), in 90 mm Petri dishes. After 24 h, fungal plugs of 5 mm in diameter were placed at a distance of 3 cm from the roots. Plates were incubated at 22 °C under continuous illumination from the side (max. $80\,\mu mol\,m^{-2}\,s^{-1}$). Fresh weight of the shoots and roots was determined at the time point indicated in Fig. 1.

For phosphate uptake studies, the nylon net with the *Arabidopsis* seedlings in the presence or absence of *Piriformospora indica*, was transferred to a fresh Petri dish with agar medium containing 1.5×10^7 counts per minute (cpm) ^{32}P-labelled orthophosphate (without HCl, Amersham, Freiburg). The radioactivity was determined by liquid scintillation counting. Ten cotyledons were harvested with a candlewick cutter and homogenized in 1 ml 100 mM Tris/HCl pH 7.0, and aliquots of trichloro acetic acid-precipitable material were used to determine the radioactivity in the cotyledons. The results presented here are based on eight independent experiments.

Array analysis

A dot blot filter with 96 putative cDNAs for signal transduction components from *Arabidopsis thaliana* was used for hybridization. The cDNAs were obtained either from stock centers, amplified from RT-PCR, or from an *Arabidopsis* cDNA library. The filter contained genes for the following proteins:

G protein-coupled receptor: At1g48270;
G protein α subunit: At2g26300;
G protein β subunit: At4g34460.

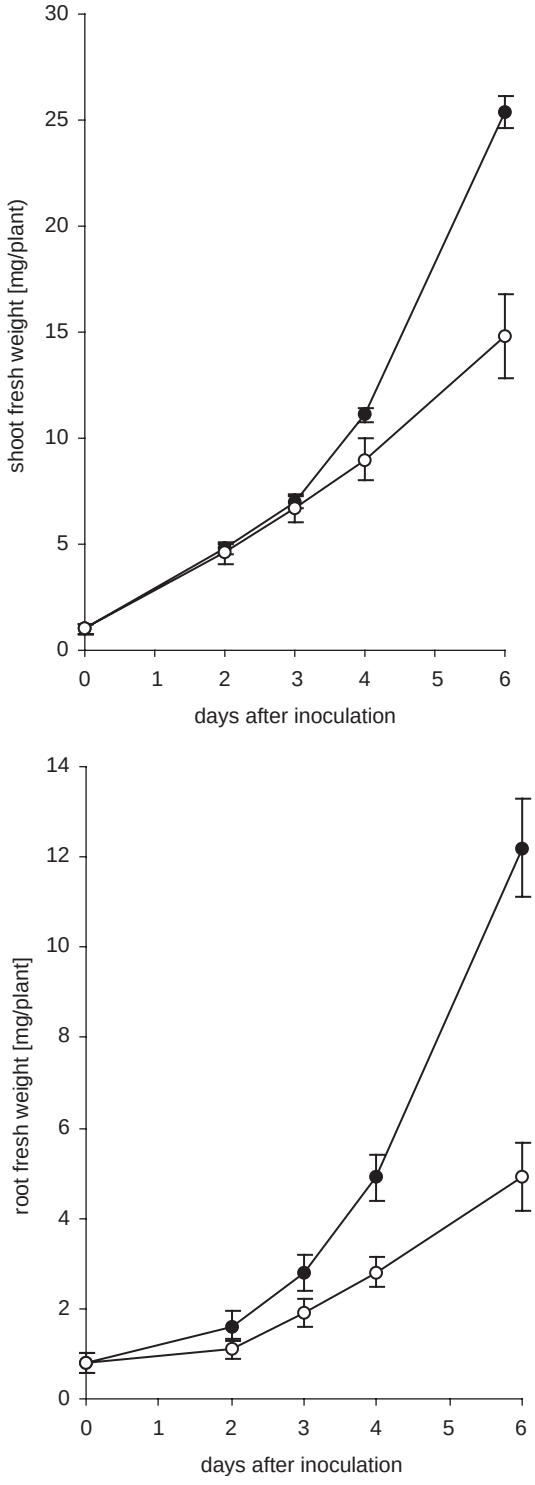


Figure 1. *Piriformospora indica* stimulates the fresh weight of *Arabidopsis* shoots (top) and roots (bottom). At day 0, 7-day-old *Arabidopsis* seedlings were transferred to new media and the fresh weight was determined in the presence and absence of the fungus. ●, with fungus and ○, control (without fungus). Based on eight independent experiments, bars represent SEs.

Receptor protein kinases (with LRR motifs): At1g49100; At1g51820; At1g07550; At1g51910; At2g19230; At2g28990; At3g46330; At4g20790; At2g02780; At3g46370; At3g46410; At4g22130; At5g41180; At5g65710; At5g16590; At3g17840, At2g26730; At3g02880.

MLO homologs: At1g61560; At4g24250; At5g65970; At2g17480.

Small GTP-binding proteins: At4g18800; At1g06400; At4g17170; At1g49300; At5g45130; At5g10260; At4g35950; At5g45970; At1g02440; At5g67560.

Phosphatidyl-inositol-3-kinase: At1g60490.

MATH-domain-containing proteins: At3g20360; At5g26280;At3g28220; At3g20380; At3g20370; At2g04170; At4g09770; At5g26290; At5g26300; At5g26320.

DMI1 protein homolog: At5g49960.

Response regulator genes: At4g16110; At1g59940; At1g10470; At1g19050; At2g41310; At1g67710; At2g25180; At2g40670.

Sensor histidine kinases: At2g40940; At1g27320; At1g66340; At2g17820.

Histidine phosphotransfer protein: At1g03430; At5g39340.

Calmodulin: At5g37780; At1g66410.

Calcineurin B-like calcium sensor proteins: At1g64480; At5g47100.

CBL-interacting serine-threonine protein kinases: At5g45810; At5g57630; At4g14580; At5g07070.

Calmodulin-binding proteins: At2g15760; At2g26530.

Immunophilin/FKBP-type peptidyl-prolyl *cis*–*trans* isomerase family protein: At4g19830.

Phospholipase D δ: At4g35790.

14-3-3-proteins: At1g35160; At1g22300; At5g65430; At1g78220.

MAP kinase pathway: At4g36450; At3g45640; At5g66850; At4g08480; At5g55090; At1g01560; At1g10210; At2g18170; At4g01370; At2g43790; At4g11330.

Disease resistance locus: At3g04220.

Band 7 protein: At1g69840.

Other proteins: At1g04560; At4g14480; At3g15220; AT3g18590; AT5g17520; AT4g28050.

Insertions from plasmids were amplified with vector-specific primers. Gene-specific primers were used to amplify fragments from RT-PCRs. The primers were designed such that the DNA fragments contained the entire coding region with four nucleotides upstream of the ATG codon, and three nucleotides downstream of the stop codon. A cDNA library from *Arabidopsis* roots was constructed by using the SMART™ cDNA Library Construction Kit (Biosciences, Palo Alto). The genes were amplified from the library by using the Expand™ High Fidelity

PCR System (Roche, Mannheim, Germany) with one library-specific (5′ λ TriplEx2) and one gene-specific primer. The identities of the DNA fragments were confirmed by sequence analyses. A molar excess (>100-fold) of the amplified fragments was spotted onto a nylon membrane (Hybond N, Amersham Biosciences, Freiburg, Germany) and hybridized under high stringency conditions with the radioactively labelled cDNA probes from control roots and roots co-cultivated with *Piriformospora indica* (Sambrook et al., 1989). The ^{32}P-cDNA probes (10^6 cpm/ml) were prepared from 1 μg of total RNA from the control or inoculated roots with Superscript II reverse transcriptase (Invitrogen, Karlsruhe, Germany). After hybridization and washing of the membranes, they were exposed to Hyperfilm™ ECL™ (Amersham Biosciences, Freiburg, Germany). The intensities of the hybridization signals were quantified with the ImageQuant Software (Amersham Biosciences, Freiburg, Germany). The intensities of the hybridization signals from control roots were set as 1.0, and the signal from the inoculated material expressed relative to it.

RNA was isolated with an RNA isolation kit (RNeasy, Qiagen, Hilden, Germany). For Northern analysis total RNA (15 μg) was separated on 1.2% (w/v) agarose gels containing formaldehyde, and transferred to a positively charged nylon membrane (Roche, Mannheim, Germany). Blots were probed with in vitro transcribed antisense RNAs of the cloned genes. For RT-PCR, two primer pairs were simultaneously used for the reaction in order to confirm the specificity of the results. The following primers were used: At5g16590: TTCGGATCAATCTCGCCCAG and CCTTGTACGAAGATCCGAAC, At3g17840: GATTCTCCGGTGAGATTCCG and ACTTCCACAGCTTTCACTGC; At2g26730: GGTGGTCCGTTAAAGCCTTG and CAATCTAACGGAGTTCGCCC; At3g02880: TCTGCCGACCCGGTTGGTTAC and CCTTTCCCAAGAACCTCAGC.

Isolation of microsomes, plasma membranes and Triton X-100 insoluble plasma membrane microdomains from *Arabidopsis* roots; two-dimensional gel electrophoresis

Root material from *Arabidopsis* seedlings was used to isolate microsomes. Roots were homogenized in a Warring Blender (11 times for 5 s) in a buffer containing 50 mM Tris/HCl pH 7.4, 330 mM sucrose, 3 mM EDTA, 1 mM 1,4-dithiothreitol and 5% (w/v) polyvinylpolypyrrolidone. The homogenate was filtered through four layers of cheesecloth and centrifuged for 20 min at 10,000*g*. The supernatant was then centrifuged at 50,000*g* for 60 min in order to pellet the microsomes. The pellet was resuspended in a buffer containing 50 mM Tris-HCl, pH 7.4 and 1 M NaCl, incubated on ice for 30 min and centrifuged as before. Pelleted membranes were resuspended in 50 mM Tris-HCl, pH 7.4; 3 mM EDTA and 1 mM 1,4-dithiothreitol and kept at −80 °C until protein analysis (see below) or isolation of plasma membranes.

Plasma membranes were prepared from microsomes as described previously for green material (Peškan et al., 2000; Larsson et al., 1987), washed with an excess of a buffer containing 50 mM Tris/HCl pH 7.4; 100 mM NaCl, 3 mM EDTA and 1 mM 1,4-dithiothreitol and resuspended in 50 mM Tris/HCl pH 7.4; 3 mM EDTA and 1 mM 1,4-dithiothreitol. For the two-dimensional gel electrophoreses shown in Fig. 4, protein extracts from 10 g of *Arabidopsis* roots cultivated with or without *P. indica* for 3 days were used.

For the isolation of plasma membrane microdomains (Fig. 5), 100 g of Arabidopsis roots without the fungus was used and the washing step for the plasma membrane with 50 mM Tris/HCl pH 7.4; 100 mM NaCl, 3 mM EDTA and 1 mM 1,4-dithiothreitol was omitted.

After treatment with 1% (v/v) Triton X-100 at 4 °C for 30 min, membranes were mixed with 60% (w/w) sucrose to the final concentration of 48% (w/w), placed at the bottom of a centrifuge tube and overlaid with a continuous sucrose gradient (15–45%, w/w). Gradients were centrifuged at 250,000*g* for 20 h in a swinging bucket rotor (SW 40, Beckman, Palo Alto, USA).

Alternatively, membranes were sonicated seven times for 10 s (Sonoplus HD70 with tip SH70, Bandelin electronic, Berlin, Germany; power 20 W) without addition of Triton X-100 and purified on a continuous sucrose gradient. All fractions (microsomes, plasma membranes, vesicles from Triton X-100 and sonicated plasma membranes) were routinely used for the identification of their protein patterns by mass spectrometry (cf. below).

Two-dimensional gel electrophoresis was performed as described in Sherameti et al. (2004).

For the second dimension, the buffer system of Schägger and von Jagow (1987) was used. Coomassie-staining of the gels was performed with colloidal Coomassie stain Roti-Blue (Roth, Karlsruhe, Germany) according to the manufacturer's instructions, and silver-staining according to standard protocols.

Mass spectrometry and identification of proteins

Proteins extracted from membrane fractions were further purified by two rounds of methanol

precipitations before digestion with trypsin. In-gel trypsin digestion of excised protein spots, elution of the peptides from the gel matrix, peptide analysis by coupling liquid chromatography with electrospray ionization mass spectrometry (ESI-MS), and tandem mass spectrometry (MS-MS) were performed as previously described (Sherameti et al., 2004).

The measured MS-MS spectra were matched with the amino-acid sequences of tryptic peptides from the *Arabidopsis thaliana* database in FASTA format. Cys modification by carbamidomethylation (+57 Da) was taken into account, and known contaminants were filtered out. Raw MS-MS data were analysed by the Finnigan Sequest/Turbo Sequest software (revision 3.0; ThermoQuest, San Jose, USA). The parameters for the analysis by the Sequest algorithm were set according to Stauber et al. (2003). The similarity between the measured MS-MS spectrum and the theoretical MS-MS spectrum, reported as the cross-correlation factor (X_{corr}), was above 2.95 and 3.85 for doubly or triply charged precursor ions, respectively; these values are presented in Table 1. In order to identify corresponding loci, identified protein sequences were subjected to BLAST search at NCBI (http://www.ncbi.nlm.nih.gov/) and FASTA searches by using the AGI protein database at TAIR (http://www.arabidopsis.org/).

Results

Figure 1 demonstrates that co-cultivation of *Arabidopsis* seedlings with *P. indica* caused an increase in fresh weight of the roots and shoots. The growth-promoting effect was first visible for the roots before physical contact between the hyphae and the root surface could be detected by light microscopy. Under the conditions used for these studies, the increase in root fresh weight was mainly caused by a stimulation of lateral root growth, while growth of the primary root was not affected by the fungus, or even slightly reduced when compared to the controls (data not shown). Stimulation of growth was accompanied by a massive uptake of phosphate from the agar plate, as determined by the accumulation of radiolabel in the cotyledons. After 6 days of co-cultivation, the radioactivity in the aerial parts of seedlings co-cultivated with *P. indica* had more than doubled (16,200 cpm vs. 41,500 cpm) (Fig. 2A). We also tested short-term uptake of phosphate into control seedlings and seedlings co-cultivated with *P. indica*, and found that the uptake was stimulated more than three-fold within 6 h by the fungus, although there was no measurable increase in the fresh weight of the aerial parts of the seedlings. Furthermore, comparison of Figs. 1 and 2 also demonstrates that the increase in phosphate uptake cannot be explained by differences in the fresh weights for roots. Taken together, these results suggest that *P. indica* stimulates *Arabidopsis* growth in a fashion similar to that described for mycorrhizal symbioses (cf. Smith and Read, 2001).

After 3 days of co-cultivation, control roots and roots from co-cultivated material were harvested for RNA extraction and hybridization to arrays with putative cDNAs for signalling components from *Arabidopsis* (cf. Materials and methods). In three independent experiments, only two from 96 messages were significantly upregulated in material co-cultivated with *P. indica*. The most prominent response was observed for a gene which codes for a receptor kinase with leucine-rich repeat (LRR) motifs (At5g16590). The second cDNA which responded to the fungus codes for a second, so far uncharacterized, receptor kinase (data not shown). For the other genes on the array filter, no more than a 10% increase in the mRNA level could be detected. Therefore, we consider them as controls (data not shown, however three genes (At3g17840, At2g26730 and At3g02880) were used as control in the Northern experiments shown in Fig. 3). Northern analyses and RT-PCRs confirmed that the message for At5g16590 responds to the fungus (Fig. 3A and B). Under our culture conditions, the message level increased approximately two days after the co-cultivation of *Arabidopsis* roots with *P. indica*. The strongest, approximately 2.8-fold stimulation was observed 3 days after co-cultivation. On day 5, the level had dropped again to that seen in control plants (Fig. 3). Three control mRNAs for other plasma membrane-localized receptor kinases with LRR sequences (At3g17840, At2g26730, At3g02880) did not respond to *P. indica*. Furthermore, the stimulatory effect on the responsive mRNA appears to be specific for *P. indica*, since no stimulation could be observed when the infection was performed with *P. croceum* (Fig. 3C). This is consistent with the observation that the latter fungus does not cause promotion of root growth in *Arabidopsis* (Peškan-Berghöfer et al., 2004). Thus, the stimulatory effect of the fungus on At5g16590 expression is one of the earliest events that occurs during the recognition of roots and fungi. To our knowledge, this is the first report that a receptor kinase increases transiently during early phases of plant/microbe interactions.

Mass spectrometry uncovered that At5g16590, together with at least six other LRR receptor kinases, is present in microsomal fractions from

Table 1. Proteins identified in microsomes, plasma membrane preparations, and the top fraction, as well as the Triton X-100 insoluble plasma membrane microdomain fraction of the sucrose gradient used to fractionate plasma membranes after Triton X-100 treatments or sonication

No.	Protein	Gene no.	Peptides	X_{corr}	Microsome	Pl. memb.	Top of grad.	Microdomain
1	Plasma membrane ATPase	At2g18960	IPIEEVFQQLK	5.22	+	+		+
			WSEQEAAILVP GDIVSIK	4.93	+	+		
			VDQSALTGESLPVTK	4.33		+		+
			AAHLVDST NQVGHFQ	5.17				+
			GVEKDQVLLFAAMASRVEN	3.92	+	+		
			QDAIDAAMVG MLADPK	3.44				+
			EVHFLPFNPVDKRTALTYIDSDGNWHR	5.01	+	+		+
			VSKGAPEQILDLANARPDLR	4.44	+			+
			ESPGGPWEFVGLLPLFDPPR	5.19	+	+	+	+
			LGMGTNMYPSAALLGTDKDSNIASIP	4.41				+
			VEELIEK	3.77	+	+		+
			KADIGIAVADATDAAR	4.32	+	+		+
			GASDIVLTEPGLSVIISAVLTSR	3.59	+	+		
			AWASLFDNR	4.09	+	+		+
			ELSEIAEQAK	5.08				+
			HIVGMTGDGVNDAPALK	4.44	+	+		+
2	Phospholipase D δ	At4g35790	VITSDPYVTVVVPQATLAR	3.93	+	+		+
			ILHDLDTVFK	4.54	+	+		+
			HSSVICVLSPR	4.33	+	+		+
3	LRR-RK-1	At3g17840	LNLAENEFSGEISSGFK	3.45	+			+
			GLDYLHSQDPLSSHGNVK	4.44	+	+		+
			VSDFGLAQLVSASSTTPNR	4.21	+	+		+
			SSNILLTNSHDAR	4.03		+		+
4	**LRR-RK-2**	**At5g16590**	**LATLYLQDNQLTGPIPEIK**	**3.39**	+	+		+
			SPLNWETR	**4.02**	+	+		+
			GSLSALLHGNK	**4.53**	+	+		+
5	LRR-RK-3	At2g26730	QALLTFLQQIPHENR	5.02	+			+
			LPGTGLVGQIPSGSLGR	3.99	+			+
			SLYLQHNEFSGEFPTSFTQLNNLIR	3.78	+	+		+
			LLVFDFMPTGSLSALLHGSR	3.69		+		+

			AVLEEGTTVVVK	4.36		+		+
6	LRR-RK-4	At3g02880	FNSLSGPIPSDFSNLVLLR	4.18		+		+
			ASFEHGLVVAVK	4.02		+		+
			GSLSAILHGNK	3.97	+	+		+
			TPLNWETR	3.88	+			+
			SPTHQQLNEEGVDLPR	3.91	+			+
			AISYLHSR	3.19	+			+
7	Pectinesterase	At4g12420	LDEGLLLHWNGIQQR	5.22	+	+		
			DQIGSFFYFPSLHFQR	5.01		+		+
			ASGGFGSFVVNPR	4.56		+		+
			DLGMPDGVL	4.62		+		
			VSNVGISTSLNFR	4.90				+
			TENLDSWYLGQETYVR	3.78				+
8	Intrinsic proteins, aquaporins	At2g45960 (PIP A1B)	QP IGTSAQSDKDYKEPPPAPLF EPGELASWSFWR	5.03	+	+		+
			QYQALGGGANTIAHGYTK	5.31	+	+		+
			GSGLGAEIIGTFVLVYTVFSATDAK	5.11	+	+		+
			SLGAAIIFNK	5.02	+	+		+
				4.43	+	+		+
9	GTP-binding proteins (ras-rel.)	At3g09910	ILLIGDSGVGK					
		At4g18430	AQLWDTAGQER	5.11	+		+	+
			STIGVEFATR	3.29		+		+
			DHTDANVVIMLVGNK	4.53	+	+		+
		At1g56330	LQLWDTAGQER	4.37	+	+		+
			ELNVMFIETSAK	4.03		+		+
		At1g02130	LQIWDTAGQER	3.74	+	+		+
				3.86	+			+
10	18.3 kDa protein of photosystem II		YNEAVYSSA	3.78	+			
			ADAFEYADQVLE	3.92	+			
11	Disease resistance locus	At3g04220	QSIHETGQRQFLVDATDIR	4.90	+	+	+	
			LQLQQRFLSQITNQENVQIPHLGVAQERL	4.61	+	+	+	
			NLEWLDLTCS	3.56	+	+	+	
12	LRR-RK	At3g51740	KLSLHNNVIAGSVTRSLGYLK	4.54	+		+	
			KTVSAGVAGTASAGGE	4.21	+	+	+	
13	MATH protein	At3g20370	SWNIQINPSGLGTGEGK	5.12	+			
			NSYLSEVFSIGGR	4.04	+		+	
			TMWGFSQVLPIDTFK	4.16	+	+	+	
			FYIFNK	4.33		+		
			KYFTIQDTDVWK	3.43	+	+	+	
			YFTIQDTDVWK	3.54	+	+		
			MESFNTLLK	3.02		+	+	
14	ATPase, mitochondrial	At5g08690	TIAMDGTEGLVR	3.87	+			

Table 1. (*continued*)

No.	Protein	Gene no.	Peptides	X_{corr}	Microsome	Pl. memb.	Top of grad.	Microdomain
			VLNTGAPITVPVGR	4.32	+			
			FTQANSEVSALLGR	3.97	+			
15	band 7 proteins	At1g69840	LDLDSTFEQK	4.22	+			
			AMNEINAASR	4.20	+	+		+
			DVMDMVLVTQYFDTLK	3.55	+	+		+
			RAEGEAESK	3.59	+	+		+
16	CBL-interactging protein kinase 9	At1g01140	PENLILDANGVLK	4.04	+	+		+
			ILEPNPITR	3.97	+	+		+
17	Protein kinase	At3g25250	PPYIPAPDDGGDKGTDVNTK	3.22	+	+		
			PPYIPAPDDGGDK	3.54	+			
			PDNVMIQENGHLMLIDFDLSTNLAPR	3.91		+		

Membranes were isolated from *Arabidopsis* roots, proteins precipitated as described, digested with trypsin and directly used for mass spectrometry. A few identified loci and peptides, which characterize the plasma membrane microdomain purification process, are given. For more information about the protein, see text or information in the Database under the given accession numbers. For experimental details, cf. Materials and methods. LRR-RK, leucine-rich repeat receptor kinase. At5g16590, relevant for this study, is given in bold. +indicates the presence of the peptide in the fraction.

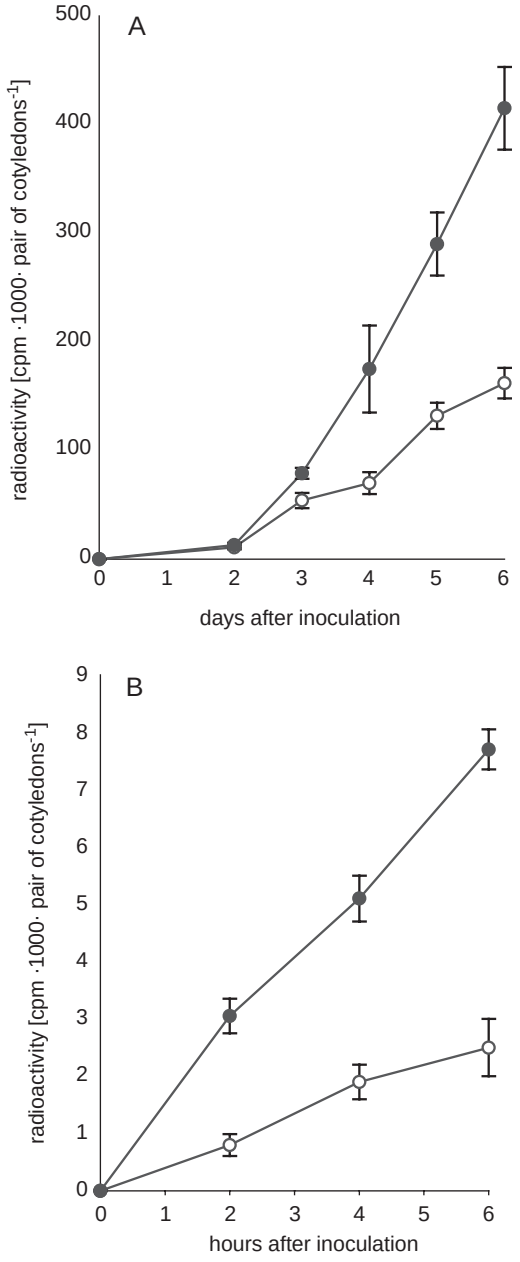


Figure 2. Uptake of ^{32}P-phosphate from the media into the cotyledons of *Arabidopsis* seedlings. (A) From day 0 on, *Arabidopsis* seedlings were co-cultivated with *P. indica* and simultaneously transferred to ^{32}P-phosphate-containing media (●). After 2, 4 and 6 days, the cotyledons were harvested for determination of radioactivity. (○), control, *Arabidopsis* seedlings without fungus. (B) Four days after co-cultivation with (●) or without (○) *P. indica*, ^{32}P-phosphate was added and the uptake of radioactivity into the cotyledons was monitored over a period of 6 h. (○), control, *Arabidopsis* seedlings without fungus. Based on 8 independent experiments, bars represent SEs.

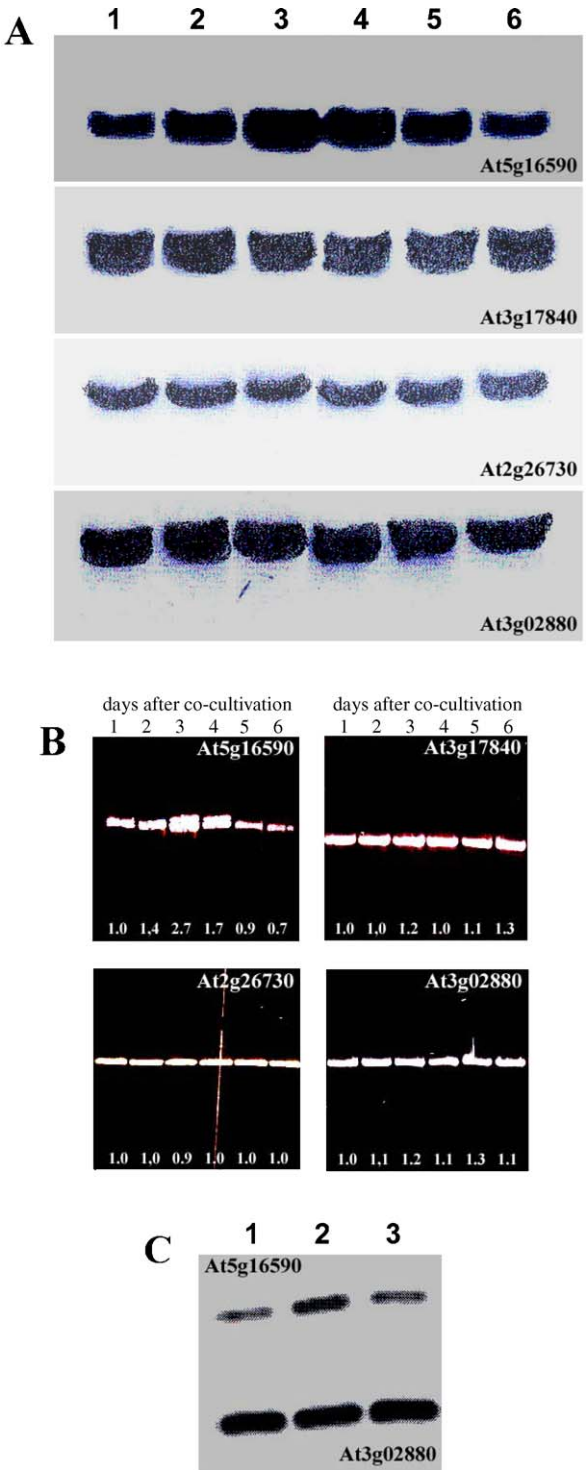


Figure 3. Expression analyses of LRR receptor kinase genes. *Arabidopsis* seedlings were co-cultivated with *P. indica* and RNA extracted from the roots at the time points indicated. (A) Northern analyses with LRR receptor kinase-specific probes. (B) Quantitative RT-PCR. (C) Northern analyses for At5g16590 with RNA from control roots (lane 1) and roots which were co-cultivated with *P. indica* (lane 2) or *P. croceum* (lane 3) for 3 days.

Arabidopsis roots (Table 1, no. 4). These microsomes were then used to purify plasma membranes.

Figure 4 shows two-dimensional gels with plasma membrane proteins from *Arabidopsis* roots cultivated without or with *P. indica*. The same LRR receptor kinases, including At5g16590, could also be detected in these preparations. Individual spots for LRR-receptor kinases (including At5g16590) could not be identified on the gels. However, the six LRR receptor kinases are located in the gel regions marked by the squares, as determined by in-gel trypsin digestion of the excised gel region, elution of the peptides from the gel matrix and peptide analyses (Fig. 4).

Comparison of the plasma membranes from control roots with roots co-cultivated with *P. indica* for 3 days did not reveal significant differences. However, three protein spots are repeatedly detectable in membrane preparations isolated after 3 days of co-cultivation: two of them could not be identified by our means, and thus, might not be encoded by the *Arabidopsis* genome. Spot 1 represents the protein kinase At3g25250 (Fig. 4). This kinase exhibits strong sequence similarities to members of the AGC protein kinase family (cf. Bogre et al., 2003 and discussion).

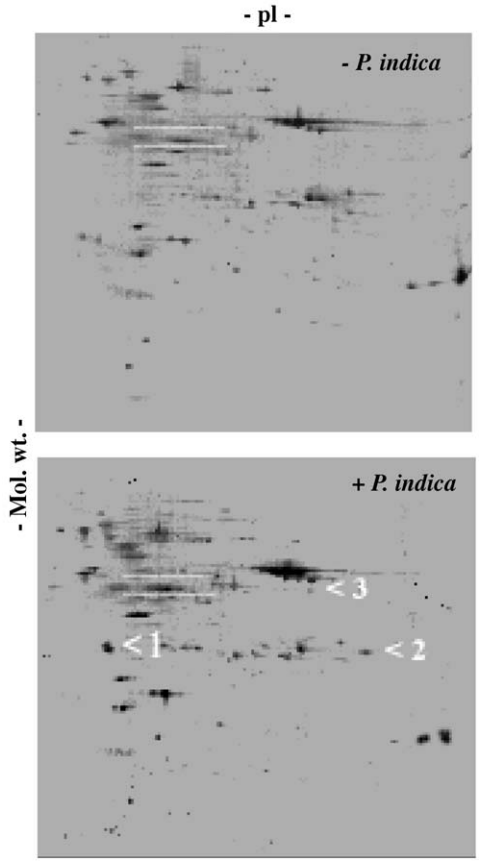


Figure 4. Protein patterns of washed plasma membrane preparations of *A. thaliana* roots after 3 days of cultivation with and without *P. indica*. After separation of the proteins by two-dimensional gel electrophoresis, the gels were stained with silver. The boxed region was used for protein extraction and MS/MS analysis. 1, 2 and 3 represent protein spots which appear after co-cultivation with *P. indica*.

Plasma membrane microdomains are well characterized in mammalian systems, and are highly enriched in signalling components (cf. Discussion). Therefore, 10 times more root material (100 g) was used to purify plasma membrane microdomains from plasma membrane preparations in order to identify minor polypeptides on two-dimensional gels (cf. below). The plasma membrane preparations were then either sonicated or treated with Triton X-100 to obtain plasma membrane microdomains (Peškan et al., 2000). At5g16590 was clearly detectable in microdomain preparations obtained with both protocols.

Table 1 shows the distribution of several proteins during the purification of *Arabidopsis* plasma membrane microdomains. These proteins, identified by mass spectrometry, were chosen because they represent specific membranes and because they were identified by more than one peptide and with high correlation factors (X_{corr}, cf. Material and methods). The 18.3 kDa protein (no. 10) from the plastids and the mitochondrial ATP synthase subunit (no. 14) are major contaminations of the microsomal fractions. Although the 18.3 kDa protein is supposed to be a component of photosystem II, we found it as a major component in our microsome preparations from roots. However, both polypeptides are no longer detectable in the plasma membrane preparations. The plasma membrane microdomains contained several receptor kinases, including Atg16590 (nos. 3–6). Characteristic for these microdomains are also the plasma membrane ATPase (no. 1), the pectinesterase (no. 7), aquaporins (no. 8), several ras-related small GTP-binding proteins (no. 9), phospholipase D δ (no. 2), several band 7 proteins related to a hypersensitive response (no. 15) and the CBL-interacting kinase 9 (no. 16) (Shahollari et al., 2004). In contrast, microdomains do not contain a recently identified MATH protein associated with plasma membranes (no. 13), which also responds to *P. indica* (Peškan-Berghöfer et al., 2004), and two other receptor kinases. One appears to be involved in disease resistance (no. 11), and the other belongs to the class of LRR proteins (compare no. 12 with nos. 3–6). The latter three proteins are present in microsomal and plasma membrane preparations, but remained on top of the sucrose gradient during the microdomain isolation procedure (Table 1, top of gradient).

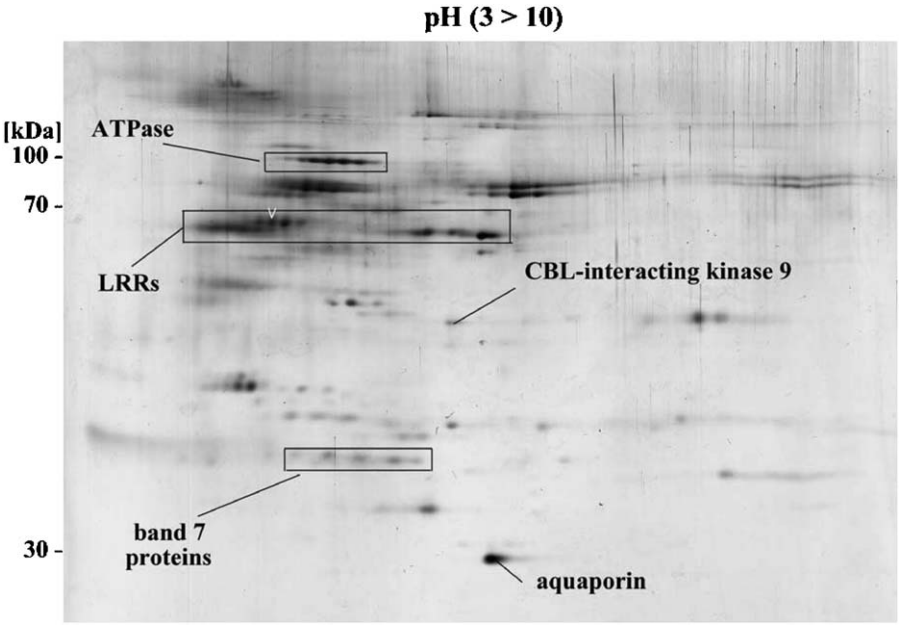


Figure 5. Protein composition of Triton X-100 insoluble plasma membrane vesicles from *Arabidopsis* roots. Identical protein patterns were obtained from *Arabidopis* plasma membrane microdomains obtained after sonication of plasma membranes. The protein bands were identified by mass spectrometry. The correlation factors (X_{corr}) for doubly (triply) charged precursor ions are given in Table 1. The accession number of the proteins of the identified fragments are also given in Table 1. Four LRR-receptor kinases (At5g16590, At3g17840, At2g26730, At3g02880) were identified in the indicated region. The white arrow points to At5g16590. The proteins are stained with Comassie.

To test whether the receptor kinase At5g16590 could be identified as an individual protein in plasma membrane microdomains, we separated the protein extracts on two-dimensional gels and identified several protein spots by mass spectrometry (Fig. 5). As expected, major constituents of these vesicles were the ATPase, aquaporins, and several band 7 proteins, i.e. hypersensitive-induced proteins. At5g16590, together with other receptor kinases, is located in the very same region on the gel, and had already been identified on gels with protein extracts from plasma membrane preparations (cf. Figs. 4 and 5). The arrowhead in the LRR box demonstrates that At5g16590 can now be identified as an individual protein spot (Fig. 5).

Discussion

Figures 1 and 2 demonstrate that growth and development of *Arabidopsis* seedlings is substantially promoted by the endophytic fungus *P. indica*. Promotion of root and shoot growth on agar plates was accompanied by a substantial uptake of minerals, including phosphorus, into the aereal parts of the fungus (cf. Fig. 2; Smith and Read, 2001). Although the exact nature of the growth-promoting effect of the fungus on *Arabidopsis* seedlings is not yet clear, it provides an excellent model system to study plant/microbe interaction. We are interested in early recognition events, preferentially events which occur before physical contact between the two organisms is detectable. To identify genes and proteins which are involved in this scenario, we performed a series of experiments and identified several components which are among the earliest to respond to the presence of the fungus. Recently, we demonstrated that a plasma membrane-associated MATH protein is transiently modified in response to the fungus (Peškan-Berghöfer et al., 2004). MATH proteins belong to a new class of plant proteins (cf. Conserved Domain Database cd 00121.2, MATH at www.ncbi.nlm.nih.gov) which may contain members with extracellular metalloprotease activities (cf. Sunnerhagen et al., 2002). Up-regulation of the receptor kinase described here occurs within the same time period of co-cultivation of both organisms. Analysis of knock-out lines will allow us to define the role of these proteins in greater detail. In addition, the presence of the receptor kinase in Triton X-100 insoluble plasma membrane microdomains provides us with a subset of plasma membrane proteins or proteins associated with the plasma membrane, which may directly interact with the identified kinase.

Receptor kinases with LRR motifs are commonly used as sensors for signals from the outside of the cell (cf. Shiu and Bleecker, 2001, 2003; Diévart and Clark, 2004). Characterization of LRR proteins based on the composition of their domain structures (Torii, 2004) revealed that they represent one of the largest groups of receptor kinases, with 216 members in *Arabidopsis* (Arabidopsis Genome Initiative, 2000). LRR proteins are involved in plant/microbe interactions, pathogen resistance (Kistner and Parniske, 2002; Diévart and Clark, 2004; Song et al., 1995; van der Biezen et al., 2002; Kevei et al., 2002; Scheer and Ryan, 2002; Grant et al., 2003), flagelin sensing (Gomez-Gomez and Boller, 2000), meristem proliferation (Clark et al., 1997), abscission (Jinn et al., 2000) and regulation of organ size (Torii et al., 1996). A LRR-type receptor kinase, which is required for both fungal and bacterial recognition, has been identified (Endre et al., 2002, Stracke et al., 2002). The protein At5g16590 identified in this study exhibits sequence similarities to a LRR protein which is upregulated in response to salicylic acid (Ohtake et al., 2000), to an atypic receptor kinase with a defective kinase domain (Llompart et al., 2003), to several LRR proteins reported to be involved in pollen-tube development (Muschietti et al., 1998; Kim et al., 2002), to the peptide hormone phytosulfokine (Matsubayashi et al., 2002) and the brasssinolide receptor kinase BRI1 (Li and Chory, 1997, cf. Bishop and Koncz, 2002). Up-regulation of the At5g16590 message appears to be specific for the growth promoting fungus *P. indica*, and is not observed for other fungi, or for the other LRR protein messages tested (Fig. 3). Finally, the stimulatory effect is transient and detectable before a physical contact between fungal hyphae and *Arabidopsis* roots can be detected, suggesting that this protein might be involved in early recognition events (Fig. 3). Analysis of knock-out lines is crucial for the understanding of the protein function in plant/microbe interaction, however at present, no knock-out line with an insertion in an exon of *At5g16590* is available to us.

Little is known about downstream events of LRR protein signalling in plants. In the Clavata signalling pathway, CLV1, a LRR-receptor kinase, and CLV2, a LRR receptor-like protein, form a heterodimer and bind a rho GTPase-related protein at the cytoplasmic site of the plasma membrane (Trotochaud et al., 1999). BRI1, the brassinolide receptor kinase, might bind to Bin2, an *Arabidopsis* ortholog of the human glycogen synthase kinase β and the *Drosophila* SHAGGY protein kinase (Li and Nam, 2002). Activation of the Drosophila TOLL receptor leads to phosphorylation and recruitment of the adaptor protein TUBE and activation of the PELLE kinase (Morisato and Anderson, 1995). At5g16590 is localized in Triton X-100 insoluble plasma membrane microdomains (cf. Anderson, 1998; Simons and Toomre, 2000; Munro, 2003; Galbiati et al., 2001), together with other LRR proteins and potential candidates for downstream signalling events. These are potential candidates for protein/protein interactions to initiate downstream signalling events.

Interestingly, although our plasma membrane preparations were washed with a buffer containing 100 mM NaCl, which removes most of the peripheral proteins, we identified a protein kinase of the AGC protein family which is upregulated in response to *P. indica* (Fig. 4). The *Arabidopsis* AGC kinases contain sequence motifs for the docking of a protein kinase called PDK1, which becomes activated by 3-phosphoinositide. Thus, PDK1 could couple lipid signals to the activation of downstream protein kinases of the so-called AGC kinase family. Lipid-derived signals are central to regulating a multitude of cellular processes in plants, including growth (cf. Bogre et al., 2003 for detailed information). Since specific members of the AGC kinases appear to be involved in key growth signalling pathways, they might be good candidates for *P. indica* induced root (hair) elongation. The fact that this protein is present in our plasma membrane preparation indicates that it becomes recruited to the membrane in response to signals from *P. indica*.

Acknowledgements

This research was supported by the SFB 604. BS was supported by a fellowship from the DAAD (Bonn, Germany) and AV by the Federal State of Thuringia. We thank François Buscot and Susann Klemm for helpful suggestions.

References

Anderson RGW. The caveolae membrane system. Annu Rev Biochem 1998;67:199–225.

Ané JM, Kiss GB, Riely BK, Penmetsa RV, Oldroyd GE, Ayax C, Levy J, Debelle F, Baek JM, Kalo P, Rosenberg C, Roe BA, Long SR, Denarie J, Cook DR. Medicago truncatula DMI1 required for bacterial and fungal symbioses in legumes. Science 2004;303:1364–7.

Arabidopsis Genome Initiative. Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature 2000;408:796–815.

Bestel-Corre G, Dumas-Gaudot E, Poinsot V, Dieu M, Dierick J- F, van Tuinen D, Remacle J, Gianinazzi-

Pearson V, Gianinazzi S. Proteome analysis and identification of symbiosis-related proteins from *Medicago truncatula* Gaertn. by two-dimensional electrophoresis and mass spectrometry. Electrophoresis 2002;23:122–37.

Bishop GJ, Koncz C. Brassinosteroids and plant steroid hormone signaling. Plant Cell 2002:97–110.

Bogre L, Okresz L, Henriques R, Anthony RG. Growth signalling pathways in Arabidopsis and the AGC protein kinases. Trends Plant Sci 2003;8: 424–31.

Clark SE, Williams RW, Meyerowitz EM. The CLAVATA1 gene encodes a putative receptor kinase that controls shoot and floral meristem size in *Arabidopsis*. Cell 1997;89:575–87.

Diévart A, Clark SE. LRR-containing receptors regulating plant development and defense. Development 2004;131:251–61.

Endré G, Kereszt A, Kevei Z, Mihacea S, Kalo P, Kiss GB. A receptor kinase gene regulating symbiotic nodule development. Nature 2002;417:962–6.

Galbiati F, Razani B, Lisanti MP. Emerging themes in lipid rafts and caveolae. Cell 2001;106:403–11.

Gomez-Gomez L, Boller T. FLS2: An LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in *Arabidopsis*. Mol Cell 2000;5: 1003–11.

Grant JJ, Chini A, Basu D, Loake GJ. Targeted activation tagging of the *Arabidopsis* NBS-LRR gene, ADR1, conveys resistance to virulent pathogens. Mol Plant Microbe Interact 2003;16:669–80.

Harrison MJ. Molecular and cellular aspects of the arbuscular mycorrhizal symbiosis. Annu Rev Plant Physiol Plant Mol Biol 1999;50:361–89.

Heckman DS, Geiser DM, Eidell BR, Stauffer RL, Kardos NL, Hedges SB. Molecular evidence for the early colonization of land by fungi and plants. Science 2001;293:1129–33.

Jinn TL, Stone JM, Walker JC. HAESA, an *Arabidopsis* leucine-rich repeat receptor kinase, controls floral organ abscission. Genes Dev 2000;14:108–17.

Kevei Z, Vinardell JM, Kiss GB, Kondorosi A, Kondorosi E. Glycine-rich proteins encoded by a nodule-specific gene family are implicated in different stages of symbiotic nodule development in Medicago spp. Mol Plant Microbe Interact 2002;15:922–31.

Kim HU, Cotter R, Johnson S, Senda M, Dodds P, Kulikauska R, Tang W, Ezcura I, Herzmark P, McCormick S. New pollen-specific receptor kinases identified in tomato, maize and Arabidopsis: the tomato kinases show overlapping but distinct localization patterns on pollen tubes. Plant Mol Biol 2002;50:1–16.

Kistner C, Parniske M. Evolution of signal transduction in intracellular symbiosis. Trends Plant Sci 2002; 7:511–8.

Krüger A, Peškan-Berghöfer T, Frettinger P, Herrmann S, Buscot F, Oelmüller R. Identification of pre-mycorrhiza related plant genes in the association between *Quercus robur* and *Piloderma croceum*. New Phytol 2004;163:149–57.

Larsson C, Widell S, Kjelbom P. Preparation of high-purity plasma membranes. Methods Enzymol 1987;148: 558–68.

Levy J, Bres C, Geurts R, Chalhoub B, Kulikova O, Duc G, Journet EP, Ane JM, Lauber E, Bisseling T, Denarie J, Rosenberg C, Debelle F. A putative Ca^{2+} and calmodulin-dependent protein kinase required for bacterial and fungal symbioses. Science 2004;303:1361–4.

Li JM, Chory J. A putative leucine-rich repeat receptor kinase involved in brassinosteroid signal transduction. Cell 1997;90:929–38.

Li J, Nam KH. Regulation of brassinosteroid signaling by a GSK3/SHAGGY-like kinase. Science 2002;295: 1299–301.

Llompart B, Castells E, Río A, Roca R, Ferrando A, Stiefel V, Puigdomènech P, Casacuberta JM. The direct activation of MIK, a germinal center kinase (GCK)-like kinase, by MARK, a maize atypical receptor kinase, suggests a new mechanism for signaling through kinase-dead receptors. J Biol Chem 2003;278: 48105–11.

Marx DH. The influence of ectotrophic mycorrhizal fungi on the resistance of pine roots to pathogenic infections. I. Antagonism of mycorrhizal fungi to root pathogenic fungi and soil bacteria. Phytopathology 1969;59:153–63.

Matsubayashi Y, Ogawa M, Morita A, Sakagami Y. An LRR receptor involved in perception of a peptide plant hormone, phytosulfokine. Science 2002;296:1470–2.

Morisato D, Anderson KV. Signaling pathways that establish the dorsal-ventral pattern of the Drosophila embryo. Annu Rev Genet 1995;29:371–99.

Munro S. Lipid rafts: elusive or illusive? Cell 2003; 115:377–88.

Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 1962;15:473–97.

Muschietti J, Eyal Y, McCormick S. Pollen tube localization implies a role in pollen-pistil interactions for the tomato receptor-like protein kinases LePRK1 and LePRK2. Plant Cell 1998;10:319–30.

Ohtake Y, Takahashi T, Komeda Y. Salicylic acid induces the expression of a number of receptor-like kinase genes in *Arabidopsis thaliana*. Plant Cell Physiol 2000;41:1038–44.

Parniske M. Intracellular accommodation of microbes by plants: a common development program for symbiosis and disease? Curr Opin Plant Biol 2000;3: 320–8.

Peškan T, Westermann M, Oelmüller R. Identification of low-density Triton X-100-insoluble plasma membrane microdomains in higher plants. Eur J Biochem 2000;267:6989–95.

Peškan-Berghöfer T, Markert C, Varma A, Oelmüller R. Association of *Piriformospora indica* with *Arabidopsis thaliana* roots represents a novel system to study beneficial plant-microbe interactions and involves early plant protein modifications in the endoplasmatic reticulum and at the plasma membrane. Physiol. Plant. 2004;122:465–77.

Sambrook J, Fritsch F, Maniatis T. Molecular cloning. A laboratory manual. New York: Cold Spring Harbor Laboratory Press; 1989.

Schägger J, von Jagow G. Tricine-sodium dodecyl sulphate polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal Biochem 1987;166:368–79.

Scheer JM, Ryan Jr. CA. The systemin receptor SR160 from Lycopersicon peruvianum is a member of the LRR receptor kinase family. Proc Natl Acad Sci USA 2002;99:9090–2.

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

Sherameti I, Shahollari B, Landsberger M, Westermann M, Cherepneva GN, Kusnetsov V, Oelmüller R. Cytokinin stimulates polyribosome loading of nuclear-encoded mRNAs for the plastid ATP synthase in etioplasts of *Lupinus luteus*: the complex accumulates in the inner envelope membrane with the CF1 moiety located towards the stromal space. Plant J 2004;38:578–93.

Shiu SH, Bleecker AB. Receptor-like kinases from Arabidopsis form a monophyletic gene family related to animal receptor kinases. Proc Natl Acad Sci USA 2001;98:10763–8.

Shiu SH, Bleecker AB. Expansion of the receptor-like kinase/Pelle gene family and receptor-like proteins in *Arabidopsis*. Plant Physiol 2003;132:530–43.

Simons K, Toomre D. Lipid rafts and signal transduction. Nat Rev Mol Cell Biol 2000;1:31–9.

Smith SE, Read DJ. Mycorrhizal symbiosis. Cambridge: University Press; 2001.

Song WY, Wang GL, Chen LL, Kim HS, Pi LY, Holsten T, Gardner J, Wang B, Zhai WX, Zhu LH, Fauquet C, Ronald P. A receptor kinase-like protein encoded by the rice disease resistance gene, Xa21. Science 1995;270:1804–6.

Stauber EJ, Fink A, Markert C, Kruse O, Johanningmeier U, Hippler M. Proteomics of *Chlamydomonas reinhardtii* light-harvesting proteins. Eukaryot Cell 2003;2:978–94.

Stracke S, Kistner C, Yoshida S, Mulder L, Sato S, Kaneko T, Tabata S, Sandal N, Stougaard J, Szczyglowski K, Parniske M. A plant receptor-like kinase required for both bacterial and fungal symbiosis. Nature 2002;417:959–62.

Sunnerhagen M, Pursglove S, Fladvad M. The new MATH: homology suggests shared binding surfaces in meprin tetramers and TRAF trimers. FEBS Lett 2002;530:1–3.

Torii KU. Leucine-rich repeat receptor kinases in plants: structure, function, and signal transduction pathways. Int Rev Cytol 2004;234:1–46.

Torii KU, Mitsukawa N, Oosumi T, Matsuura Y, Yokoyama R, Whittier RF, Komeda Y. The *Arabidopsis* ERECTA gene encodes a putative receptor protein kinase with extracellular leucine-rich repeats. Plant Cell 1996; 8:735–46.

Trotochaud AE, Hao T, Wu G, Yang ZB, Clark SE. The CLAVATA1 receptor-like kinase requires CLAVATA3 for its assembly into a signalling complex that includes KAPP and a Rho-related protein. Plant Cell 1999; 11:393–405.

van der Biezen EA, Freddie CT, Kahn K, Parker JE, Jones JD. Arabidopsis RPP4 is a member of the RPP5 multigene family of TIR-NB-LRR genes and confers downy mildew resistance through multiple signalling components. Plant J 2002;29:439–51.

Varma A, Singh A, Sudha, Sahay N, Sharma J, Roy A, Kumari M, Rana D, Thakran S, Deka D, Bharti K, Franken P, Hurek T, Blechert O, Rexer K-H, Kost G, Hahn A, Hock B, Maier W, Walter M, Strack D, Kranner I. *Piriformospora indica*: a cultivable mycorrhiza-like endosymbiotic fungus. In: Mycota IX, Springer Series, Germany, 2001. p. 123–50.

Varma A, Verma S, Sudha, Sahay N, Britta B, Franken P. *Piriformospora indica*—a cultivable plant growth promoting root endophyte with similarities to arbuscular mycorrhizal fungi. Appl Environ Microbiol USA 1999;65:2741–4.

Voiblet C, Duplessis S, Encelot N, Martin F. Identification of symbiosis-regulated genes in Eucalyptus globulus-Pisolithus tinctorius ectomycorrhiza by differential hybridization of arrayed cDNAs. Plant J 2001;25: 181–91.

Wulf A, Manthey K, Doll J, Perlick AM, Linke B, Bekel T, Meyer F, Franken P, Kuster H, Krajinski F. Transcriptional changes in response to arbuscular mycorrhiza development in the model plant *Medicago truncatula*. Amer Phytopath Soc 2003;16:306–14.

2.4 Manuscript IV

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, Ajit Varma, Ralf Oelmüller

(revised version, submitted to *The Plant Journal*).

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[1], Ajit Varma[2], Ralf Oelmüller[1]

[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

Key words: Sebacinaceae, receptor kinase, *Arabidopsis*, plant/microbe interaction; growth promotion, *Piriformospora indica*

Abstract

P. indica, a basidiomycete of the Sebacinaceae family, promotes growth, development and seed production of a variety of plant species including *Arabidopsis thaliana*. *Arabidopsis* plants colonized with the fungus produce approximately 22% more seeds than uncontrolled plants. Inactivation of the *Arabidopsis* single-copy gene *DMI1*, which codes for an ion carrier required for mycorrihiza formation in Legumes, does not affect the beneficial interaction between the two symbiotic partners. Based on cellular and molecular responses which are initiated during the establishment of the interaction between *P. indica* and *Arabidopsis* roots, we have isolated mutants which fail to respond to the fungus. An ethylmethane sulfonate mutant (and a corresponding insertion line) is impaired in a leucine-rich repeat protein (At1g13230) with striking similarities to Cf from tomato. The protein contains a putative endoplasmatic reticulum (ER) retension signal, but is also found in Triton X-100 insoluble plasma membrane microdomains, suggesting that it is present in the ER/plasma membrane continuum in *Arabidopsis* roots. The microdomains contain also a leucine-rich repeat domain-containing atypical receptor kinase (At5g16590), the message of which is transiently upregulated in *Arabidopsis* roots in response to *P. indica*. This response is not detectable in the *At1g13230* mutants and the protein is not detectable in the *At1g13230* mutant microdomains. Thus, Atg13230 (and presumably also At5g16590) appear to be involved in *P. indica*-induced growth promotion and enhanced seed production in *Arabidopsis thaliana.*

Introduction

The majority of land plants lives in mycorrhizal symbioses with fungi (Smith and Read, 1997; Harrison, 1999, 2005; Kistner and Parniske, 2002; Strack *et al.*, 2003; Parniske, 2004, Hause and Fester, 2005, Oldroyd *et al*., 2005), in which the plant delivers photoassimilates

to the fungus, and the fungus promotes access of the roots to nutrients in the soil (cf. Jia *et al*., 2004; Karandashov *et al*., 2004; Karandashov and Bucher, 2005; Sherameti *et al*., 2005). Mycorrhizal interactions also enhance plant resistance to various toxins and pathogens (Marx, 1969; Smith and Read, 1997; Harrier and Watson, 2004). Although the importance of mycorrhiza for agri- and horticulture has been recognized long time ago, its application is limited by the lack of knowledge about the molecular basis of the interaction between the two symbiotic partners. In legumes, at least seven components including receptor kinases (Endré *et al.*, 2002; Stracke *et al.*, 2002) , a predicted ion channel (Ané *et al*., 2004), a calmodulin-dependent protein kinase (Levy *et al*., 2004) and the two plastid localized proteins CASTOR and POLLUX (Imaizumi-Anraku *et al*., 2005) are required for the entry of both mycorrhizal fungi and nodule-forming bacteria into root epidermal or cortical cells (Kistner and Parniske, 2002; cf. also Kistner *et al.*, 2005). Thus, it is believed that nitrogen fixing root nodule symbiosis with bacteria developed from a more ancient mycorrhizal symbiosis (cf. Kistner *et al*., 2005).

Since symbiotic interactions between photosynthetic organisms and fungi have already been observed during the colonization of land (Heckman *et al*. 2001), beneficial interactions between the symbiotic partners must have been established early during evolution. We study an endophytic interaction between the model plant *Arabidopsis* and *Piriformospora indica*, a basidiomycete of the Sebacinaceae family. Reminescent to mycorrhizal interactions, colonization of *Arabidopsis* roots by the endophyte promotes nutrient uptake, confers resistance to toxins and pathogenic organisms and ultimately leads to growth promotion and enhanced seed production (Varma *et al*., 1998, 1999; Barazani *et al*., 2005; Sherameti *et al*., 2005; Waller *et al.*, 2005). However, in contrast to most mycorrhiza fungi, *P. indica* is a cultivable fungus and can grow on synthetic or complex media without hosts (Varma *et al.*, 2001; Peškan-Berghöfer *et al*., 2004). The fungus can colonize the roots of many plant

species including trees, agri- and horticultural and medicinal plants, mono- and dicots and mosses (Varma *et al.*, 2001, Sherameti *et al*., 2005, Peškan-Berghöfer *et al*., 2004, Waller *et al*., 2005). The apparent lack of species specificity suggests that this beneficial symbiosis might be based on general recognition and signalling processes.

P. indica belongs to the Sebacinaceae, an ancient Basidiomycete family. Originally, it was believed that members of the Sebacinaceae family form exclusively saprophytic or parasitic interactions with plant roots. However, more recently, also a broad diversity of beneficial associations of variouis members of the heterobasidiomycetous Sebacinaceae fungi have been observed (Varma *et al*., 1999, Sahay and Varma, 1999; Selosse *et al*., 2002a and b; Glen *et al*., 2002; Urban *et al*., 2003, Weiss *et al*., 2004, Peškan-Berghöfer *et al*., 2004, Shahollari *et al.*, 2005, Kaldorf *et al*., 2005; Barazani *et al*., 2005, Waller *et al*., 2005; Sherameti *et al*., 2005). Since most of the more basal taxa of basidiomycetes consist of predominantly mycoparasitic and phytoparasitic fungi, it appears that Sebacinaceae is the most basal group of Basidiomycetes which contains mycorrhiza-forming taxa. Mycorrhizal taxa of Sebacinaceae include mycobionts of ectomycorrhizas, orchid mycorrhizas, ericoid mycorrhizas, and jungermannioid mycorrhizas. Such a wide spectrum of mycorrhizal types in one fungal family is unique (Weiss *et al*., 2004).

Here we describe an *Arabidopsis* mutant with a lesion in a leucine-rich repeat protein which does not recognize the presence of *P. indica.* We present evidence that the expressiona and location of a second leucine-rich repeat containing protein, a plasma membrane localized atypical receptor kinase responds to the fungus in *Arabidopsis* roots. Finally, we demonstrate that the *Arabidopsis* DMI1 protein, a membrane-spanning ion channel-like protein (Ané *et al*., 2004; Imaizumi-Anraku *et al*., 2005), which is required for the establishment of a symbiosis with bacterial and fungi partners in legumes, is not required for the interaction between *Arabidopsis* and *P. indica*.

Results

P. indica colonizes the roots of *Arabidopsis* plants and promotes its growth and development (Peškan-Berghöfer *et al*., 2004; Shahollari *et al*., 2004, 2005; Sherameti *et al*., 2005). Under our growth conditions in Petri dishes on MS medium, we observe a 21.3±0.4% increase in fresh weight after 14 days of co-cultivation. If seedlings with colonized roots are transferred to soil, seed production is enhanced by 22±1.5% (n= 50 plants) compared to the uncolonized controls. This prompted us to screen for mutants, which do not respond to the fungus. One of the mutant, called *Piriformospora indica*-insensitve (Pi)-2, is described here.

In wild-type *Arabidopsis* seedlings, root colonization by *P. indica* can be monitored by a strong autofluorescene in the root cells (Peškan-Berghöfer *et al*., 2004). This fluorescence originates presumably from the fungal hyphae growing in and around the plant root, because the fluorescence can also be detected in germinating fungal spores which were cultivated without a host (Peškan-Berghöfer *et al.*, 2004). In colonized wild-type *Arabidopsis* roots, mycelia are not only spread on the surface of the aerial parts, but also invaded the cortical tissues. Hyphae and spores can be found inter- and intracellularly. The autofluorescence in the roots of *Pi-2* is comparable to that in the wild-type. We also observe normal root colonisation and spore formation in *Pi-2* roots. Thus, the absence of the growth response is not caused by the inability of the fungus to colonize the plant roots.

The root morphology of *Pi-2* did not differ from the wild-type. Also the root fresh weight, the ratio between lateral and main roots, as well as the number of the root hair were comparable to the control (data not shown). However, we did not observe any growth promotion in response to *P indica* (Fig. 1). When colonized *Pi-2* seedlings were transfered to soil, they grow like uncolonized control plants. Also the seed production was not stimulated

by the fungus and comparable to the amount produced by the uncolonized control (data not shown). This indicates that *Pi-2* might be blind to stimulatory signals from *P. indica*.

In order to identify *Arabidopsis* genes which respond early to *P. indica*, we performed substractive hybridizations with root RNAs from seedlings, which were either co-cultivated or not co-cultivated with *P. indica* for 2 to 5 days. Several transcripts were further analysed by RT-PCR. Recognition of both organisms is accompanied by the up-regulation of the messages for the receptor kinase At5g16590 (Shahollari *et al*., 2005), the homeodomain transcription factor At2g35940, the 2-nitropropane dioxygenase (*NPDO*; At5g64250), the glucan-water dikinase (*SEX1*; At1g10760) and for nitrate reductase (*Nia2*; At1g37130) in the roots (Sherameti *et al.*, 2005). The message levels for all these proteins are not stimulated by *P. indica* in *Pi-2* and a knock-out line with an insertion in the same gene (Fig. 2, and cf. below). Figure 2 (bottom) also shows a kinetic for the message of the receptor kinase At5g16590, which is transiently upregulated after the co-cultivation with *P. indica* in wild-type seedlings. This response is not detectable in *Pi-2*, the corresponding knock-out line (cf. below) and another *P. indica* insensitive mutant, called *Pi-1* (Oelmüller *et al.*, 2005).

Colonization of *Arabidopsis* roots with *P. indica* is accompanied by the modification of a MATH protein, which is located in the plasma membrane from roots (Peškan-Berghöfer et al., 2004). We have previously shown that *Pi-1* does not show this posttranslational modification (Oelmüller *et al.*, 2005). The nature of this modification is unknown at present. This modification is also not detectable in *P. indica*-colonized *Pi-2* roots (Fig. 3). Taken together, multiple *P. indica* induced responses are not induced in *Pi-2*. The mutated gene was mapped on chromosome 1 using SSLP and CAPS markers (cf. Experimental Procedures).

The inability of *P. indica* to modify the MATH protein in the plasma membrane preparations from roots prompted us to analyse these membranes in greater details. The receptor kinase At5g16590, for which the message is upregulated in response to *P. indica* (Fig. 2) is a dominant component of Triton-X-100 insoluble plasma membrane microdomains from *Arabidopsis* (Shahollari *et al.*, 2004). We prepared low density Triton X-100-insoluble plasma membrane microdomains (Peškan *et al*., 2000, Shahollari *et al*., 2004) from *Pi-2* and analysed the protein composition by mass spectrometry. While the microdomain vesicles from wild-type seedlings contain 7 dominant leucine-rich repeat proteins (Table 1, Shahollari *et al*., 2004), two of them are not detectable in the mutant. The message for one of these LRR proteins, At5g16590, was upregulated in wild-type roots during the first few days of co-cultivation with *P. indica* and this response was not observed for *Pi-2* (Fig. 2). The other protein, At1g13230, is encoded by a gene located on chromosome 1 and codes for a small LRR-contain protein with striking sequence similarities to parts of Cf-2 from tomato. The RT-PCR product for *At1g13230* from the *Pi-2* mutant was longer than expected. Sequence analysis demonstrated that the only intron was not spliced out because of a G to A conversion at the only exon/intron junction in the gene (CAG/GGT to CAA/GGT). We also isolated a full-length cDNA from wild-type roots which contained the predicted nucleotide sequence. After amplification of the corresponding genomic region of *At1g13230* from *Pi-2* we confirmed the G to A conversion in the mutant genome (Fig. 4). Comparable G to A conversions have often been observed at intron/exon junctions and result in unspliced messages (cf. Stöckel and Oelmüller, 2004).

To confirm that the mutation in At1g13230 is responsible for the observed phenotype, we analysed an independent knock-out line SALK_079723 (cf. Experimental Procedures). No

At1g13230 transcripts could be detected in the homozygote seedlings (Fig. 5). The seedlings did not grow taller and the plants did not produce more seeds when co-cultivated with *P. indica* (data not shown). Furthermore, the transcript levels for the above tested genes were identical those found in the EMS mutant line grown under the same conditions (Fig. 2). We also could not detect the modified MATH protein in the microsomal preparations from roots (Fig. 3). This suggests that *P. indica*-mediated growth promotion and enhanced seed production in *Arabidopsis* is dependent on At1g13230.

Besides At1g13230, also At5g16590 was missing in the microdomains of *Pi-2*. Sequence analysis revealed that the gene for the latter leucine-rich repeat protein was not altered in *Pi-2* (data not shown). Fig. 2 demonstrates that the *At5g16590* transcript level, which is transiently upregulated in *Arabidopsis* roots in response to *P. indica* (Shahollari *et al*., 2004) remains unaltered in *Pi-2*. These results suggest that At5g16590 is involved in early recognition processes of the two symbiotic partners. At5g16590 is a plasma-membrane localized atypical receptor kinase (cf. Llompart *et al*., 2003). To gain insight into the role of At5g16590 for this symbiotic interaction, we analysed the SALK_053366 line with an insertion in the promoter region. However, homozygote knock-out lines contained *At5g16590* transcripts and responded to *P. indica* (data not shown). Biochemical studies demonstrated that longer incubation of the isolated microdomains with Triton X-100 at 4°C (60 min instead of 30 min during the incubation procedure) released only At1g13230, while the other six LRR proteins including At5g16590 remained in the microdomains (Tab. 1). This suggests that At1g13230 is only loosely associated with the microdomains. Subsequent treatment of the microdomains with saponine released also At5g16590 (Tab. 1). The looser association of At1g13230 with the membranes is consistent with the observation that this protein lacks a predictable transmembrane domain, while At5g16590 appears to be an

integral plasmamembrane protein. At1g13230 might be associated with either the endoplasmatic reticulum or the plasma membrane or both in the roots (cf. Discussion). The presence of a KKxx motif (Thomas *et al*., 1998) suggests that the processed At1g13230 is retrieved to the endoplasmatic reticulum (cf. Discussion). Taken together, we can detect two LRR proteins in Triton X-100 insoluble plasma membrane microdomains, which are related to an interaction between the two symbiotic partners.

DMI1 is not required for *P. indica* mediated growth promotion in *Arabidopsis.*

DMI1 (does not make infections), a highly conserved protein from angiosperms with similarities to ligand-gated cation channels is required for bacterial and fungal symbioses in legumes (Ané *et al*., 2004). DMI1 is a candidate for mediating early ion fluxes across the plasma membrane, although its location is not clear at present (cf. Hogg et al., 2005). *At5g49960* encodes the closest *Arabidopsis* homolog of the *Medicago truncatula* gene *DMI1* (Ané *et al*., 2004). We analysed a knock out line for this gene (SALK_066135). No *At5g49960* transcripts can be detected in the roots of the homozygote lines, consistant with the observation that the insertion is located in an exon region (data not shown). However, the response to *P. indica* was normal (Fig. 6). Since At5g49960 is a single-copy gene in *Arabidopsis* and expressed in roots (data not shown, cf. also Ané *et al*., 2004), it appears that this channel protein is not required for the interaction of *Arabidopsis* with *P. indica*.

Discussion

We study the interaction between *Arabidopsis thaliana* and the primitive basidiomycete *P. indica* to identify genes and processes, which promote plant growth and seed production. *P. indica* belongs to the Sebacinaceae family with members which form pathogenic, saprophytic and mycorrhizal interactions with plants (Weiss *et al.*, 2004). Since *P. indica*

interacts with many plant species (Varma *et al.*, 1999, Waller *et al*., 2005, Peškan-Berghöfer *et al*., 2004), the mechanism of interaction between the roots and the fungus might be conserved and of ancient phylogenetic origin. Basic mechanisms for mycorrhizal interactions have been established when plants first came to land (Heckman *et al*., 2001). In Legumes, mycorrhiza formation by fungi and nodule formation by nitrogen-fixing bacteria require common signalling components, because the entry mechanism of both organisms into the root cell is similar (Kistner and Parniske 2002; Parniske, 2004; Udvardi *et al.,* 2005; Harrison, 2005, Geurts *et al.*, 2005). One of the crucial components that are required for both interactions in legumes is DMI1 (Ané *et al*., 2004). Since inactivation of the DMI1 homolog in *Arabidopsis*, a single copy gene expressed preferentially in roots, does not eliminate the interaction with *P. indica*, the recognition and probably also the entry mechanism of the fungus into the *Arabidopsis* root cell appears to differ from the entry of fungi and bacteria into Legumes root cells. This raises the question whether Legume mycorrhiza utilize genetic programs, which differ from those in primitive plant-fungi interactions.

We identified a leucine-rich repeat protein, At1g13230, which is required for *P. indica*-mediated growth promotion in *Arabidopsis*. None of the normally observed responses of *Arabdiopsis* to *P. indica* were detectable in *Pi-2*, a mutant defective in At1g13230. This includes the growth response and the enhanced production of seeds. Thus, At1g13230 appears to be a crucial target protein for *P. indica* in *Arabidopsis*.

At1g13230 can be purified with *Arabidopsis* Triton X-100 insoluble plasma membrane microdomains (Shahollari *et al.*, 2004). The protein lacks a predictable transmembrane segment and can easily be removed from the microdomains by longer detergent treatments. Thus, At1g13230 appears to be a soluble protein which is loosely attached to the membrane. The predicted signal sequence suggests that At1g13230 is sorted *via* the secretory pathway.

Based on the KKxx endoplasmatic reticulum retardation sequence in the C terminal part of the protein, At1g13230 might remain in the endoplasmatic reticulum. Since we detect this protein in our Triton X-100 insoluble plasma membrane microdomains, this fraction is either contaminated with membranes from the endoplasmatic reticulum, or both membrane systems form a continuum, which cannot be separated from root extracts with our means. Although significantly shorter, At1g13230 exhibits striking similarities to Cf-2/4/5/9s, tomato transmembrane proteins which confer resistance to *Cladosporium fulvum* (Dixon *et al.*, 1996; de Wit *et al.*, 1999, 2002; Rivas and Thomas, 2005). Comparison of the tomato *Cf* proteins with At1g13230 makes it unlikely that At1g13230 is the primary target site for fungal elicitors (cf. Rivas and Thomas, 2005; Rooney *et al*., 2005). In particular, the C and N-terminal domains present in Cf2 proteins are not found in At1g13230, suggesting that the protein alone cannot receive signals directly and transfer them to downstream components. Thus, the conserved LRR domain in At1g13230 might be crucial for its function, presumably by establishing and/ or controlling protein/protein interactions required for the beneficial interaction between the two symbiotic organisms.

A putative disease resistance protein from rice (XP_549876) appears to be the closest homolog of At1g13230 in another plant species. The leucine-rich repeat sequence of At1g13230 exhibits also striking simililarities to TOO MANY MOUTHS, a transmembrane leucine-rich repeat receptor kinase. The protein appears to function in a position-dependent signaling pathway that controls the plane of patterning divisions as well as the balance between stem cell renewal and differentiation in stomatal and epidermal development (cf. Nadeau and Sack, 2002, 2003; Shpak *et al*., 2005). Similar to At1g13230, TOO MANY MOUTHS also lacks a recognizable intracellular domain for downstream signalling. Thus both components might require interaction partners with co-receptor kinase activities.

Triton X-100 insoluble plasma membrane microdomains contain at least 7 LRR-containing proteins, and only two of them, At1g13230 and At5g16590, are released from these domains by detergent treatments. The message for *At5g16590* is transiently upregulated in response to *P. indica* (Shahollari *et al.*, 2005). We also observed that At1g16590 is not detectable in microdoamins isolated from *Pi-2*. Although the reason is unclear at present and requires further investigations, this observation provides additional evidence that At1g16590 is involved in the interaction. At5g16590 appears to be an atypical receptor kinase, which transduces signals by phosphorylation-independent mechanisms (Kroiher *et al.*, 2001). Although the intracellular domains of atypical receptor kinases such as At5g16590 contain conserved Ser/Thr kinase domains, some of the highly conserved amino acids within these domains are altered. For instance, an aspartic acid in the subdomain IVb which is assumed to be part of the kinase-active site (Knighton *et al.*, 1993) is replaced by an asparagines (position 468) and the phenylalanine and glycine within the DFG activation loop are replaced by a tyrosine and a cysteine (positions 487 and 488) in At5g16590. The crucial role of the phenylalanine reside in the DFG triplet has been demonstrate for H-Ryk (Katso *et al.,*1999). Identical or similar amino acid substitutions leading to the loss of autophosphorylation *in vitro* have also been observed for MARK, a maize atypical receptor kinase (Llompart *et al.*, 2003). The intracellular domain of MARK interacts with the regulatory domain of MIK, a germinal center kinase-like kinase, and strongly induces MIK kinase activity. Llompart et al. (2003) proposed that MIK could represent a novel component for signalling through atypical receptor kinases in plants. Interestingly, an atypical receptor kinase of the LysM type is also involved in legume perception or rhizobial signals (Madson *et al*., 2003).

Experimental procedures

Growth conditions of plant and fungus

Wild type *Arabidopsis thaliana* seeds (ecotype Columbia), EMS mutant seeds (Lehle, San Diego, USA) and seeds from the homozygote T-DNA insertion lines 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 (100µmol 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.

Co-cultivation experiments and estimation of plant growth

Nine day-old *A. thaliana* seedlings were transferred to nylon disks (mesh size 70 µm) placed on top of a modified PMN culture medium ((5 mM KNO_3, 2 mM $MgSO_4$, 2 mM $Ca(NO_3)_2$, 0.01 µM $FeSO_4$, 70 µM H_3BO_3, 14 µM $MnCl_2$, 0.5 µM $CuSO_4$, 1 µM $ZnSO_4$, 0,2 µM Na_2MoO_4, 0.01 µM $CoCl_2$, 10.5 g l^{-1} agar, pH 5.6), in 90 mm 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 3 cm from the roots. Plates were incubated at 22°C under continuous illumination from the side (max. 80 µmol m^{-2} sec^{-1}).

Fresh weights were determined directly after removal of the seedlings from the Petri dishes.

Experiments on soil

For the experiments on soil, *Arabidopsis* seedlings were germinated on MS medium before transfer to sterile soil. Co-cultivation with the fungus was initiated on the Petri dishes as described above. For experiments with the fungus, the soil was mixed carefully with the fungus (1%, w/v). The fungal mycelium was obtained from liquid cultures after removal of

the medium and washed with an excess of distilled water. Before transfer to soil, the roots were examined under the microscope to ensure that hyphae and spores have been developed within the roots. Cultivation occurred in multi-trays with Aracon tubes in a temperature-controlled growth chamber at 22°C under long-day conditions (light intensity: max. 80 µmol m^{-2} sec^{-1}). The size of the plants was monitored between 4 and 8 weeks.

For the mutant screen, the heights of EMS mutant plants grown in the presence of *P. indica* were compared to those of control plants (- *P. indica*; + *P. indica*). Seeds were collected from those EMS mutated plants grown in the presence of *P. indica* which were comparable in height to control plants grown in the absence of *P. indica*. The absence of the response to *P. indica* was confirmed in the next two generations. The physiological results for *Pi-2* presented here were obtained from the M_3 and M_4 generations.

Seed production (g seeds/plant) was monitored by collecting seeds from individual plants grown under the standardized conditions in Aracon tubes as described above.

Staining of fungal hyphae and spores

Roots from seedlings which were co-cultivated with *P. indica* were transferred to 10% KOH and boiled for 10 min. After washing with water for 1 min, the roots were put into a 0,01% acid fuchsin-lactic acid solution and boiled again for 10 min. Excess dye was removed with water prior to microscopy.

Fluorescence measurements

Auto-fluorescence in the developing root hairs as a result of co-culture with *P. indica* were detected with the LSM 510 META microscope (Carl-Zeiss Jena GmbH). Relative values (550 nm) were obtained for the emission spectra (cf. Peškan-Berghöfer *et al*., 2004).

Isolation of Triton X-100 insoluble plasma membrane microdomains

100 g of *Arabidopsis* seedlings were used to isolate microsomes. The material was homogenized in a Warring Blender (seven times for 5 sec) in a buffer containing 50 mM Tris/HCl pH 7.4, 330 mM sucrose, 3 mM EDTA, 1 mM 1,4-dithiothreitol and 5% (w/v) polyvinylpolypyrrolidone. The homogenate was filtered through four layers of cheesecloth and centrifuged for 20 min at 10.000 *x g*. The supernatant was then centrifuged at 50.000 *x g* for 60 min in order to pellet the microsomes. Plasma membranes were prepared from microsomes by two-phase partitioning with 6.4% (w/w) dextrane T-500 and 6.4% (w/w) polyethylene glycol (average molecular weight 3350) (Larsson *et al*., 1987; Briskin *et al*., 1987; Peškan *et al*., 2000). The plasma membranes were resuspended in a buffer containing 50 mM Tris/HCl pH 7,4; 3 mM EDTA and 1 mM 1,4-dithiothreitol. After treatment with 1% (v/v) Triton X-100 at 4°C for 30 (or 60) min, membranes were mixed with 60% (w/w) sucrose to the final concentration of 48% (w/w), placed at the bottom of a centrifuge tube and overlayed with a continuous sucrose gradient (15-45%, w/w). Gradients were centrifuged at 250.000 *x g* for 20 h in a swinging bucket rotor (SW 40, Beckman, Palo Alto, USA).

Alternatively, membranes were sonicated seven times for 10 s (Sonoplus HD70 with tip SH70, Bandelin electronic, Berlin, Germany; power 20W) without addition of Triton X-100 and purified on a continuous sucrose gradient. All fractions (microsomes, plasma membranes, vesicles from Triton X-100 and sonicated plasma membranes) were routinously used for the identification of their protein patterns by mass spectrometry.

For saponine treatment, microdomains were washed with 0,3 % saponine, dissolved in 1 x PBS buffer for 30 min at 4°C. The solubilized proteins were separated from the microdomains by centrifugation (15 min;10, 000 *x g*). The protein composition of the pellet and of the supernatant was checked by mass spectrometry.

Mass spectrometry

Aliquots of the eluted protein fractions were used for mass spectrometry. Trypsin digestion of protein mixtures was performed according to Sherameti *et al*. (2004). Peptide analysis by coupling liquid chromatography with electrospray ionization mass spectrometry (ESI-MS) and tandem mass spectrometry (MS-MS) was described previously (Stauber *et al.*, 2003; Sherameti *et al.*, 2004; Shahollari *et al*., 2004). Experimental details and identified peptides are given in Shahollari *et al.* (2004). For the identification of the MATH protein from gels, cf. Peškan-Berghöfer *et al.* (2004) and Oelmüller *et al.* (2005).

Protein identification

The measured MS-MS spectra were matched with the amino-acid sequences of tryptic peptides from the *A. thaliana* database in FASTA format. Cys modification by carbamidomethylation (+57 Da) was taken into account and known contaminants were filtered out. Raw MS-MS data were analysed by the Finnigan Sequest/Turbo Sequest software (revision 3.0; ThermoQuest, San Jose, USA). The parameters for the analysis by the Sequest algorithm were set according to Stauber *et al.* (2003). The similarity between the measured MS-MS spectrum and the theoretical MS-MS spectrum, reported as the cross-correlation factor (X_{corr}) was equal or above 1.5, 2.5 and 3.5 for singly, doubly or triply charged precursor ions, respectively. In order to identify corresponding loci, identified protein sequences were subjected to BLAST search at NCBI (http://www.ncbi.nlm.nih.gov/) and FASTA searches by using the AGI protein database at TAIR (http://www.arabidopsis.org/). Identification of conserved domains and signal peptides was performed by using SMART (Schultz *et al*., 1998).

RNA analysis

RNA was isolated with an RNA isolation kit (RNeasy, Qiagen, Hilden, Germany). For quantitative RT-PCR, RNA from *Arabidopsis* roots grown in the absence or presence of *P. indica* was used with gene-specific (cf. below) and several control primer pairs (Sambrook *et al*.,1989). Two primer pairs were simultaneously used for the reaction in order to confirm the specificity of the results. RT-PCR was performed by reverse transcription of 5 :g of total RNA with gene-specific reverse primers. First strand synthesis was performed with a kit (#K1631) from MBI Fermentas (St. Leon-Roth, Germany). After 20 PCR cycles, the products were analysed on 1.5% agarose gels, stained with ethidium bromide and visualized bands were quantified with the Image Master Video System (Amersham Pharmacia Biotech, Uppsala, Sweden). The following primer pairs were used:

At5g16590: “gtgatcggctgtttcgtcttg“ and „ggacaaaggacctggtgaagc”; At1g13230: “gaaaagctattgattcccaacgacc” or “cgtgaagccttgtactctgcgattc” and “gccctaaactcaaatccgtgaca”.

For Northern analysis, gene-specific primers were designed to amplify four DNA fragments from our cDNA library (Sherameti *et al.*, 2004): *Nia2* (At1g37130), *SEX1* (At1g10760), and the genes for the homeodomain transcription factor (At2g35940) and for 2-nitropropane dioxygenase (At5g64250). The primers were designed such that they amplify the entire coding region including 4 nucleotides up- and downstream of the genes. Northern analyses were preformed with purified PCR products.

Suppression substractive hybridisation was performed by using the SMART PCR cDNA Synthesis Kit and the Clontech RCR-Select cDNA Substraction Kit (BD Biosciences, Palo Alto, CA) according to the manufacturer´s instructions. RNA was isolated from roots two, three, four and five days after the co-cultivation with *P. indica*, and from control roots. Several genes were further analysed by quantitative RT-PCR. For experimental details, cf. Krüger *et al.* (2004).

Miscellenous

DNA extraction and sequence analysis was performed according to standard protocols (Stöckel and Oelmüller, 2004). For cloning of PCR products, the PCR cloning kit from Quiagen was used.

To assign the mutant *Pi-2* locus to one of the *Arabidopsis* chromosomes, a segregating F_2 progeny was generated by crosses of male pollen donor plants to homozygote lines of *Pi-2*. Restriction fragment length polymorphism analyses of the F_2 plants were performed with the pARMS set (Schäffner, 1996).

Acknowledgements

We thank the Salk Institute Genomic Analysis Laboratory for providing the sequence-indexed Arabidopsis T-DNA insertion mutants. Work was supported by the SFB 604, a grant from the DFG (Oe133/19-1), the BMBF (IND 03/013) and the Friedrich-Schiller-University Jena.

REFERENCES

Ané, J.M., Kiss, G.B., Riely, B.K., Penmetsa, R.V., Oldroyd, G.E., Ayax, C., Levy, J., Debelle, F., Baek, J.M., Kalo, P., Rosenberg, C., Roe, B.A., Long, S.R., Denarie, J. and Cook, D.R. (2004) Medicago truncatula DMI1 required for bacterial and fungal symbioses in legumes. *Science* **303**, 1364-1367.

Barazani O, **Benderoth, M**, **Groten, K**, **Kuhlemeier, C**. **and Baldwin, I.T**. (2005) *Piriformospora indica* and *Sebacina vermifera* increase growth performance at the expense of herbivore resistance in *Nicotiana attenuata*. *Oecologia 146*, 234-243.

Briskin, D.P., Leonard, R.T. and Hodges, T.K. (1987) Isolation of plasma membrane: membrane markers and general principles. *Methods Enzymol.* **148**, 542-568.

de Wit PJ, Brandwagt BF, van den Burg HA, Cai X, van der Hoorn RA, de Jong CF, van Klooster J, de Kock MJ, Kruijt M, Lindhout WH, Luderer R, Takken FL, Westerink N, Vervoort JJ and Joosten MH (2002) The molecular basis of co-evolution between *Cladosporium fulvum* and tomato. *Antonie Van Leeuwenhoek*. **81**, 409-412.

de Wit PJ and Joosten MH (1999) Avirulence and resistance genes in the *Cladosporium fulvum*-tomato interaction. *Curr Opin Microbiol*. **2**, 368-373.

Dixon, M.S., Jones, D.A., Keddie, J.S., Thomas, C.M., Harrison, K. and Jones, J.D. (1996) The tomato Cf-2 disease resistance locus comprises two functional genes encoding leucine-rich repeat proteins. *Cell* **84**, 451-459.

Endré, G., Kereszt, A., Kevei, Z., Mihacea, S., Kalo, P. and Kiss, G.B. (2002) A receptor kinase gene regulating symbiotic nodule development. *Nature* **417**, 962-966

Glen, M., Tommerup, I.C., Bougher, N.L. and O'Brien, P.A. (2002) Are Sebacinaceae common and widespread ectomycorrhizal associates of Eucalyptus species in Australian forests? *Mycorrhiza* **12**, 243-247.

Geurts R, Fedorova E and Bisseling T. (2005) Nod factor signaling genes and their function in the early stages of *Rhizobium* infection. *Curr Opin Plant Biol***. 8**, 346-352.

Hause, B. and Fester, T. (2005) Molecular and cell biology of arbuscular mycorrhizal symbiosis. *Planta* **221**, 184-196.

Harrier, L.A. and Watson, C.A. (2004) The potential role of arbuscular mycorrhizal (AM) fungi in the bioprotection of plants against soil-borne pathogens in organic and/or other sustainable farming systems. *Pest Manag Sci* **60**, 149-157.

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

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

Heckman, D.S., Geiser, D.M., Eidell, B.R., Stauffer, R.L., Kardos, N.L. and Hedges, S.B. (2001) Molecular evidence for the early colonization of land by fungi and plants. *Science*. **293**, 1129-1133.

Hogg, B.V., Cullimore, J.V., Ranjeva and R., Bono, J.-J. (2005) The DMI1 and DMI2 early symbiotic genes *of Medicago truncatula* are required for a high affinity nodulation factor-binding site associated to a particulate fraction of roots. *Plant Physiol.* **140**, 365-373.

Imaizumi-Anraku, H., Takeda, N., Charpentier, M., Perry, J., Miwa, H., Umehara, Y., Kouchi, H., Murakami, Y., Mulder, L., Vickers, K., Pike, J., Allan Downie, J., Wang, T., Sato, S., Asamizu, E., Tabata, S., Yoshikawa, M., Murooka, Y., Wu, G.J., Kawaguchi, M., Kawasaki, S., Parniske, M. and Hayashi, M. (2005) Plastid proteins crucial for symbiotic fungal and bacterial entry into plant roots. *Nature* **433**, 527-531.

Jia Y, Gray VM, and Straker CJ. (2004) The influence of *Rhizobium* and arbuscular mycorrhizal fungi on nitrogen and phosphorus accumulation by *Vicia faba*. *Ann Bot (Lond).* **94,** 251-258.

Kaldorf, M., Koch, B., Rexer, K.H., Kost, G. and Varma, A. (2005) Patterns of interaction between *Populus* Esch5 and *Piriformospora indica*: a transition from mutualism to antagonism. *Plant Biol.* **7**:210-218.

Karandashov V, and Bucher M. (2005) Symbiotic phosphate transport in arbuscular mycorrhizas. *Trends Plant Sci* **10**, 22-29.

Karandashov, V., Nagy, R., Wegmuller, S., Amrhein, N. and Bucher, M. (2004) Evolutionary conservation of a phosphate transporter in the arbuscular mycorrhizal symbiosis. *Proc. Natl. Acad. Sci. USA* **101**, 6285-6290.

Katso, R.M., Russell, R.B. and Ganesan, T.S. (1999) Functional analysis of H-Ryk, an atypical member of the receptor tyrosine kinase family. *Mol. Cell. Biol.* 19, 6427-40.

Kistner, C. and Parniske, M. (2002) Evolution of signal transduction in intracellular symbiosis. *Trends in Plant Science* **7**, 511-518.

Kistner, C., Winzer. T., Pitzschke, A., Mulder, L., Sato, S., Kaneko, T., Tabata, S., Sandal, N., Stougaard, J., Webb, K.J., Szczyglowski, K. and Parniske, M..(2005) Seven Lotus japonicus genes required for transcriptional reprogramming of the root during fungal and bacterial symbiosis. *Plant Cell* **17**, 2217-2229.

Knighton D.R., Cadena D.L., Zheng J., Ten Eyck, L.F., Taylor, S.S., Sowadski, J.M. and Gill, G.N. (1993) Structural features that specify tyrosine kinase activity deduced from homology modeling of the epidermal growth factor receptor. *Proc. Natl. Acad. Sci. USA* **90**, 5001-5005.

Kroiher M., Miller M.A. and Steele RE. (2001) Deceiving appearances: signaling by "dead" and "fractured" receptor protein-tyrosine kinases. *Bioassays* **23**, 69-76.

Krüger, A., Peškan-Berghöfer, T., Frettinger, P., Herrmann, S., Buscot, F. and Oelmüller, R. (2004) Identification of premycorrhiza-related plant genes in the association between *Quercus robur* and *Piloderma croceum*. *New Phytol*. **163**, 149-157.

Larsson, C., Widell, S. and Kjellbom, P. (1987**)** Preparation of high-purity plasma membranes. *Methods Enzymol.* **148**, 558-568.

Levy, J., Bres, C., Geurts, R., Chalhoub, B., Kulikova, O., Duc, G., Journet, E.P., Ane, J.M., Lauber, E., Bisseling, T., Denarie, J., Rosenberg, C. and Debelle, F. (2004) A putative Ca^{2+} and calmodulin-dependent protein kinase required for bacterial and fungal symbioses. *Science* **303**, 1361-1364.

Llompart, B., Castells, E., Rio, A., Roca, R., Ferrando, A., Stiefel, V., Puigdomenech, P. and Casacuberta, J.M. (2003) The direct activation of MIK, a germinal center kinase (GCK)-like kinase, by MARK, a maize atypical receptor kinase, suggests a new mechanism for signaling through kinase-dead receptors. *J. Biol. Chem.* **278**, 48105-48111.

Madsen, E.B., Madsen, L.H., Radutoiu, S., Olbryt, M., Rakwalska, M., Szczyglowski, K., Sato, S., Kaneko, T., Tabata, S., Sandal, N. and Stougaard, J. (2003) A receptor kinase gene of the LysM type is involved in legume perception of rhizobial signals. *Nature* **425**, 637-640.

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

Nadeau, J.A. and Sack, F.D. (2002) Control of stomatal distribution on the Arabidopsis leaf surface. *Science* **296**, 1697-1700.

Nadeau, J.A. and Sack, F.D. (2003) Stomatal development: cross talk puts mouths in place. *Trends Plant Sci.* **8**, 294-299.

Oelmüller, R., Peškan-Berghöfer, T., Shahollari, B., Sherameti, I. and Varma, A. (2005) MATH-domain containing proteins represent a novel gene family in *Arabidopsis thaliana* and are involved in plant/microbe interactions. *Phys. Plant* **124**, 152-166.

Oelmüller, R., Shahollari, B., Peškan-Berghöfer, T., Trebicka, A., Giong, P.H., Sherameti, I., Oudhoff, M., Venus, Y., Altschmied, L. and Varma, A. (2004)

Molecular analyses of the interaction between Arabidopsis roots and the growth-promoting fungus Piriformospora indica. *Endocytobiosis Cell Res.* **15**, 504-517.

Oldroyd G.E., Harrison M.J. and Udvardi M.. (2005) Peace talks and trade deals. Keys to long-term harmony in legume-microbe symbioses. *Plant Physiol*. **137**, 1205-1210.

Otero JT, Ackerman JD, and Bayman P. (2004) Differences in mycorrhizal preferences between two tropical orchids. *Mol Ecol.* **13**, 2393-2404.

Parniske, M. (2004) Molecular genetics of the arbuscular mycorrhizal symbiosis. *Curr. Op. Plant Biol.* **7**, 414-421.

Pawson T and Nash P (2000) Protein-protein interactions define specificity in signal transduction. *Genes Dev.* **14**, 1027-1047.

Peskan, T., Westermann, M., and Oelmüller, R. (2000) Identification of low-density Triton X-100 insoluble plasma membrane microdomains in higher plants. *Eur. J. Biochem*. **267**, 6989-6995.

Peškan-Berghöfer, T., Shahollari, B., Giang, P.H., Hehl, S., Markert, C., Blanke, V., Varma, A.K. and Oelmüller, R. (2004) Association of *Piriformospora indica* with *Arabidopsis thaliana* roots represents a novel system to study beneficial plant-microbe interactions and involves early plant protein modifications in the endoplasmatic reticulum and at the plasma membrane. *Physiol. Plant* **122**, 465-477.

Pham, G.H., Kumari, R., Singh, An., Sachdev, M., Prasad, R., Kaldorf, M., Buscot, F., Oelmüller, R., Peškan, T., Weiss, M., Hampp, R. and Varma, A. (2004) Axenic cultures of *Piriformospora indica.* In: Plant Surface Microbiology (eds: Varma A, Abbott L, Werner D and Hampp R), Springer-Verlag, Germany pp. 593-616.

Rivas, S. and Thomas, C.M. (2005) Molecular interactions between tomato and the leaf mold pathogen *Cladosporium fulvum. Annual Review of Phytopathology* **43**, 395-436.

Rooney, H.C.E., van't Klooster, J.W., van der Hoorn, R.A.L., Joosten, M.H.A.J., Jones, J.D.G. and de Wit, P.J.G.M.(2005) Cladosporium Avr2 Inhibits Tomato Rcr3 Protease Required for *Cf-2*-Dependent Disease Resistance. *Science* **308**, 1783 – 1786.

Sahay, N.S. and Varma, A. (1999) *Piriformospora indica*: a new biological hardening tool for micropropagated plants. *FEMS Microbiol Lett*. **181**, 297-302.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular cloning. A Laboratory Manual, Second Edition. Cold Spring Harbor Laboratory, New York.

Schägger, H., and von Jagow, G. (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range of 1 to 100 kDa. *Anal. Biochem.* **166**, 368-379.

Schultz, J., Milpetz, F., Bork, P. and Ponting, C.P. (1998) SMART, a simple modular architecture research tool: identification of signaling domains. *Proc. Natl. Acad. Sci. USA* **95**, 5857-5864.

Selosse, M.-A., Bauer, R.. and Moyersoen, B. (2002a) Basal hymenomycetes belonging to the Sebacinaceae are ectomycorrhizal on temperate deciduous trees. *New Phytologist* **155**, 183-195.

Selosse, M.A., Weiss, M., Jany, J.L. and Tillier, A. (2002b) Communities and populations of sebacinoid basidiomycetes associated with the achlorophyllous orchid Neottia nidus-avis (L.) L.C.M. Rich. and neighbouring tree ectomycorrhizae. *Mol Ecol*. **11**, 1831-1844.

Schäffner, **A.R**. (1996) pARMS, multiple marker containing plasmids for easy RFLP analysis in Arabidopsis thaliana. *Plant Mol. Biol. Rep*. **14**, 11-16.

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

Shahollari, B., Varma, A. and Oelmüller, R. (2005) Expression of a receptor kinase in *Arabidopsis* roots is stimulated by the basidiomycete *Piriformospora indica* and the protein accumulates in Triton X-100 insoluble plasma membrane microdomains. *J. Plant Physiol.* **162**, 945-958

Sherameti, I., Shahollari, B., Landsberger, M., Westermann, M, Cherepneva, G., Kusnetsov, V. and Oelmüller, R. (2004) Cytokinin stimulates polyribosome loading of nuclear-encoded mRNAs for the plastid ATP synthase in etioplasts of *Lupinus luteus*: the complex accumulates in the inner-envelope membrane with the CF_1 moiety located towards the stromal space. *Plant J.* **38**, 578-593.

Sherameti I, Shahollari B, Venus Y, Altschmied L, Varma A and Oelmüller R (2005) The endophytic fungus *Piriformospora indica* stimulates the expression of nitrate reductase and the starch-degrading enzyme glucan-water dikinase in tobacco and *Arabidopsis* roots through a homeodomain transcription factor which binds to a conserved motif in their promoters. *J. Biol. Chem*.**280**, 2641-2647.

Shpak, E.D., McAbee, J.M., Pillitteri, L.J. and Torii, K.U. (2005) Stomatal patterning and differentiation by synergistic interactions of receptor kinases. *Science* **309**, 290-293.

Smith, S.E. and Read, D.J. (1997) Mycorrhizal symbiosis. 2nd edition, San Diego USA, Academic Press.

Stauber, E.J., Fink, A., Markert, C., Kruse, O., Johanningmeier, U. and Hippler, M. (2003) Proteomics of Chlamydomonas reinhardtii Light-Harvesting Proteins. *Eukaryotic Cell* **2**, 978-994.

Stöckel, J. and Oelmüller, R. (2004) A novel protein for photosystem I biogenesis. *J. Biol. Chem*. **279**, 10243-10251.

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

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

Thomas, C.M., Dixon, M.S., Parniske, M., Goldstein, C. and Jones, J.D.G. (1998) Genetic and molecular analysis of tomato *Cf* genes for resistance *to Cladosporium fulvum*. *Phil. Trans. R: Soc: London Ser. B*. **353**, 1413-1424.

Torii KU (2004) Leucine-rich repeat receptor kinases in plants: structure, function, and signal transduction pathways. *Int Rev Cytol*. **234**, 1-46.

Udvardi, M.K., Tabata, S., Parniske, M. and Stougaard, J. (2005) *Lotus japonicus*: legume research in the fast lane. *Trends Plant Sci.* **10**, 222-228.

Urban, A., Weiss, M. and Bauer, R. (2003) Ectomycorrhizas involving sebacinoid mycobionts. *Mycol Res.* **107**, 3-14.

Varma, A., Singh, A., Sudha, Sahay, N., Sharma, J., Roy, A., Kumari, M., Rana, D., Thakran, S., Deka, D., Bharti, K., Franken, P., Hurek, T., Blechert, O., Rexer, K.-H., Kost, G., Hahn, A., Hock, B., Maier, W., Walter, M., Strack, D. and Kranner, I. (2001) *Piriformospora indica*: A cultivable mycorrhiza-like endosymbiotic fungus. In: Mycota IX, Springer Series, Germany, pp. 123-150.

Varma, A., Verma, S., Sudha, Sahay, N.S., Butehorn, B. and Franken, P. (1999) *Piriformospora indica*, a cultivable plant growth promoting root endophyte. *Applied and Environ. Microbiol*. **65**, 2741-2744.

Verma, S.A., Varma, A., Rexer, K.-H., Hassel, A., Kost, G., Sarbhoy, A., Bisen, P., Bütehorn, B. and Franken, P. (1998) *Piriformospora indica*, gen. et sp. nov., a new root-colonizing fungus. *Mycologia* **90**, 898-905.

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

Weiss, M., Selosse, M.A., Rexer ,K.H., Urban, A. and Oberwinkler, F. (2004). Sebacinales: a hitherto overlooked cosm of heterobasidiomycetes with a broad mycorrhizal potential. *Mycol Res.* **108**, 1003-1010.

Figure legends

Figure 1:

Wild-type (Col) and *Pi-2* seedlings, which were grown in the absence (-) or presence (+) of *P. indica* for 8 days. Representative for 16 independent experiments.

Figure 2:

Northern analysis with RNA from roots of *Arabidopsis* seedlings which were grown in the absence (lanes 1, 3, 5) or presence (lanes 2, 4, 6) of *P. indica*. (**Top panel**): Northern analysis with root RNA after 8 days of co-cultivation. Lanes 1 and 2, wild-type; lanes 3 and 4, *Pi-2*, and lanes 5 and 6, the knock-out line SALK_079723. 25 µg total root RNA was loaded per lane, the actin probe is used as control. **(Bottom panel**): Time course for the message of the atypical receptor kinase At5g16590. Root RNA from wild-type (WT) and *Pi-2* seedlings as well as seedlings from the knock-out line SALK_079723 was extracted 2-12 days after the onset of co-cultivation. The *Pi-1* seedlings (Oelmüller *et al.*, 2004) were used as control. 25 µg total root RNA was loaded per lane.

Figure 3:

A MATH protein is modified in response to *P. indica* in plasma membrane preparations from *Arabidopsis* roots and this modification is not detectable in *Pi-2* and the knock-out line SALK_079723. A section from a two-dimensional gel with protein spots from root microsomes shows various modifications of a MATH protein (cf. Peškan-Berghöfer *et al.*, 2004). The upper panel shows the situation in wild-type protein extracts after co-cultivation with *P. indica* for 3 days, the middle (lower) panels show the corresponding spots from *Pi-2* (and the SALK_079723 line) protein extracts. The spots were identified by mass

spectrometry, all of them correspond to the protein At3g20370. For experimental details and the regulation of the MATH protein in response to *P. indica*, cf. Peškan-Berghöfer *et al.* (2004) and Oelmüller *et al.* (2005).

Figure 4:

Sequencing gel showing the intron/exon junction of *At1g13230* in the EMS mutant *Pi-2* (*Pi-2*) and the wild-type Columbia (Col). Note the G>A transition in the mutant at the intron/exon junction.

Figure 5:

Analysis of the *At1g13230* knock out line. The *At1g13230* message is not detectable in the SALK_079723 line. PCR was performed with reverse transcribed wild-type RNA (lane 1), wild-type DNA (lane 2) and reverse transcribed mutant RNA (lane 3).

Figure 6:

The SALK_066135 line with an insertion in the *Arabidopsis* DMI1 homolog shows normal response to *P. indica*. (A) and (B): wild-type seedlings; (C) and (D): the SALK_066135 line. Seedlings shown in (A) and (C) were co-cultivated with *P. indica* for 6 days.

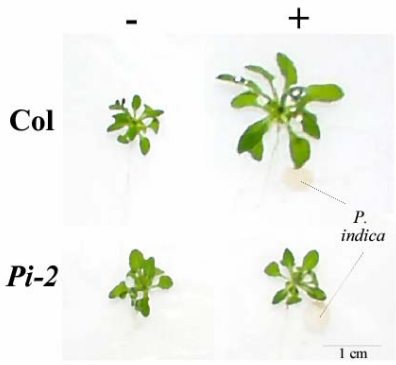


Figure 1

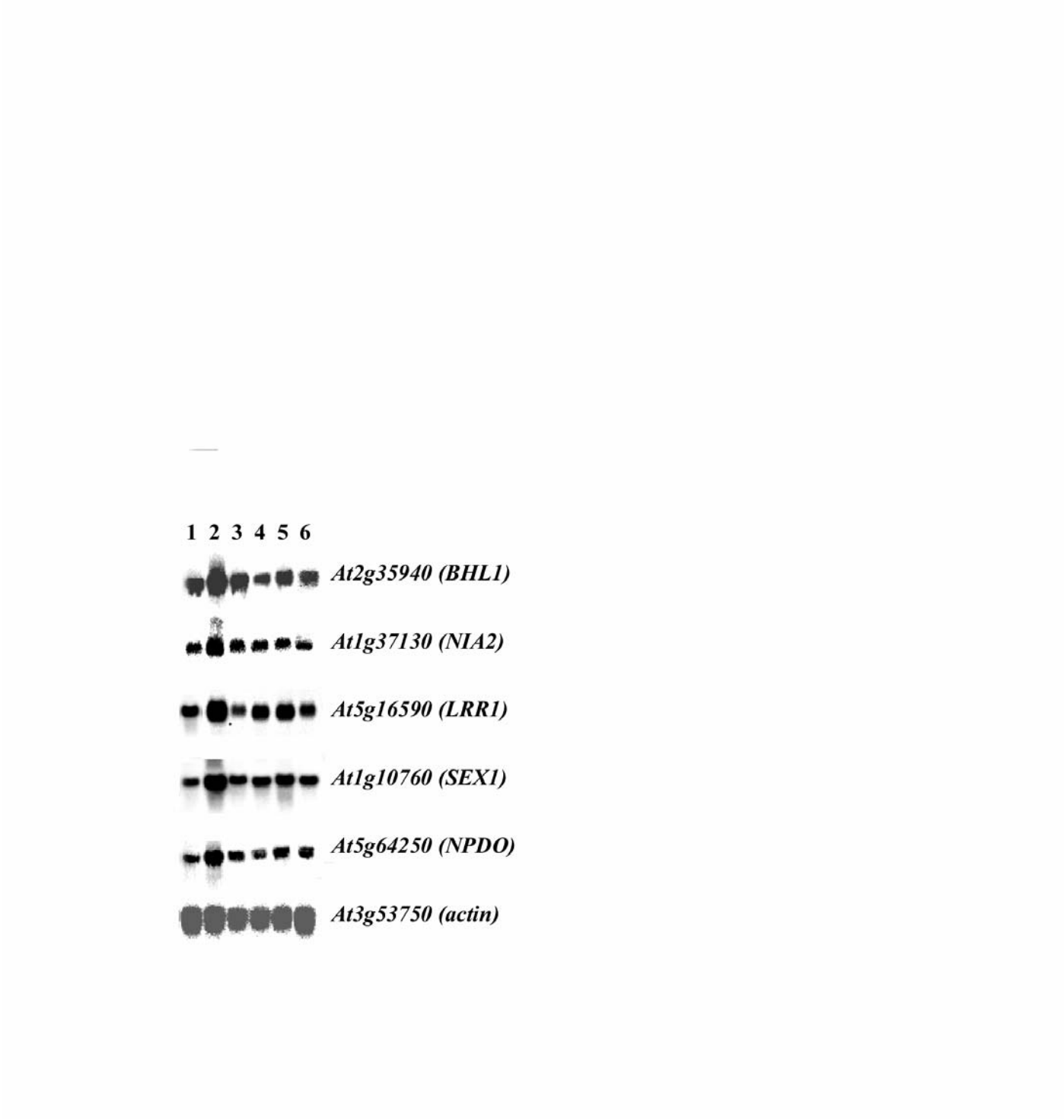


Figure 2 (top panel)

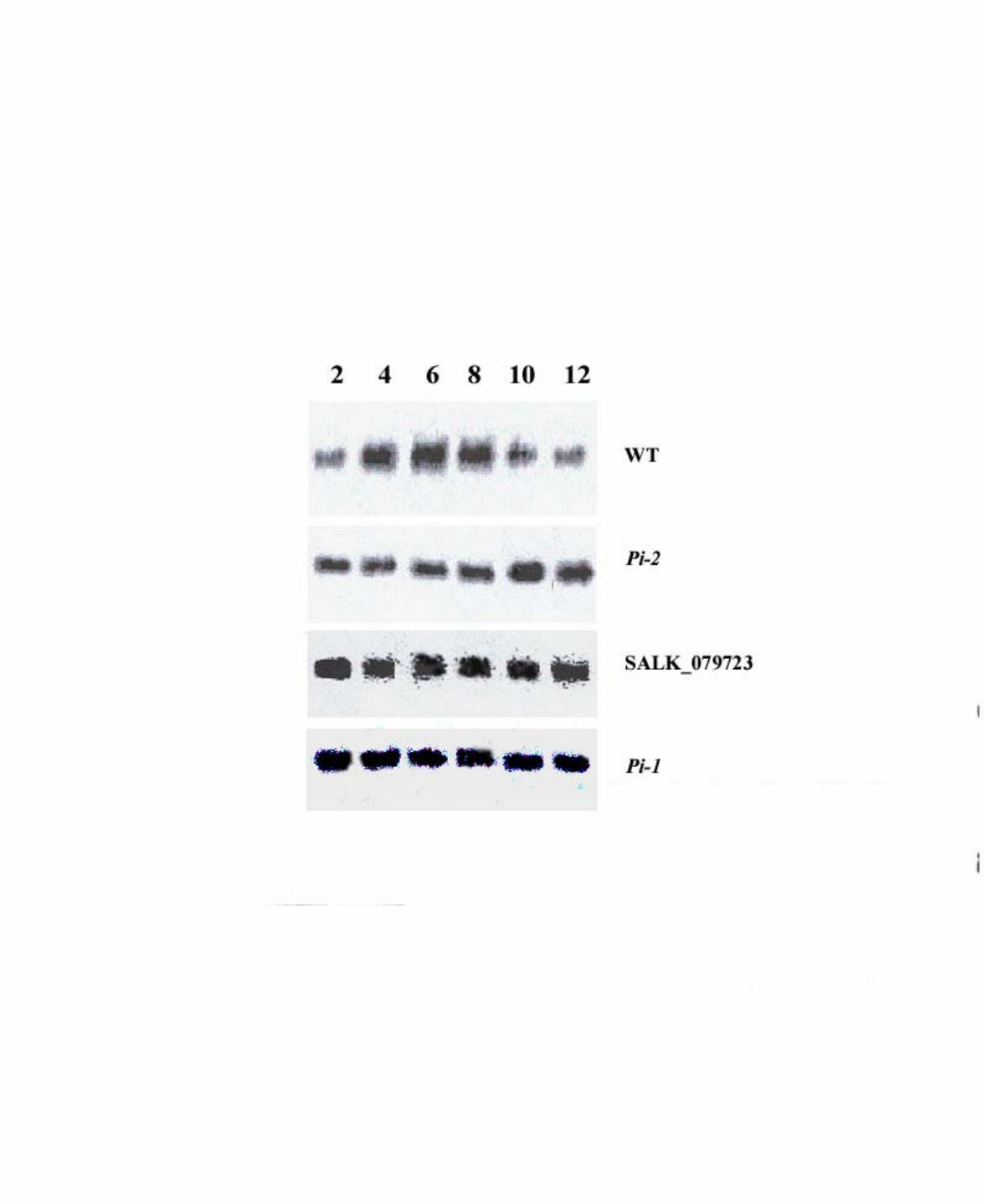


Figure 2 (bottom panel)

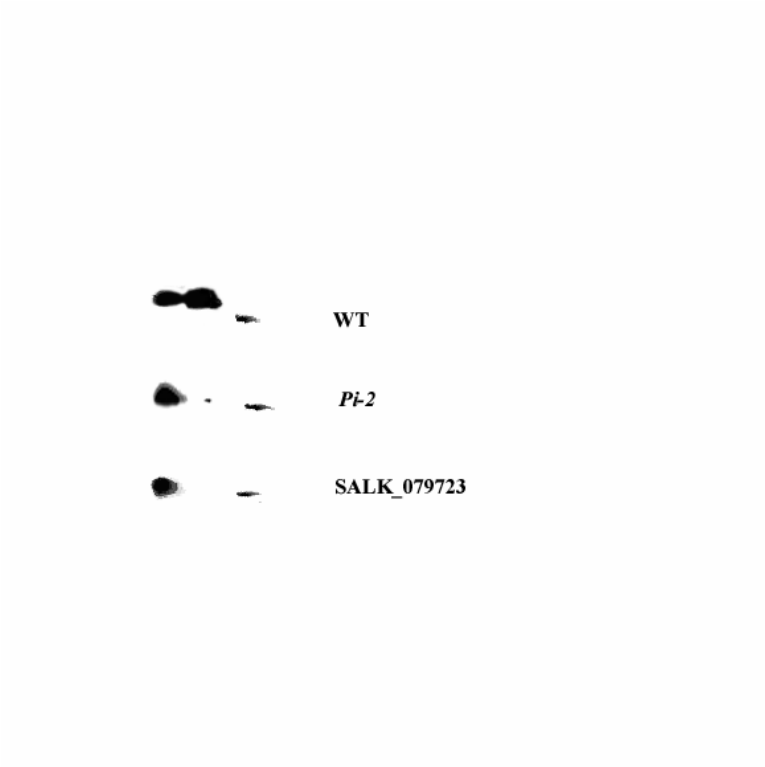


Figure 3

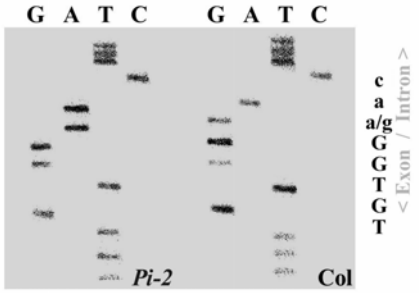


Figure 4

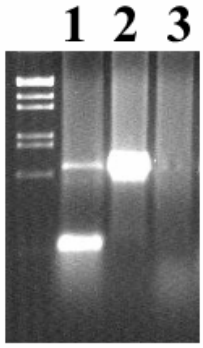


Figure 5

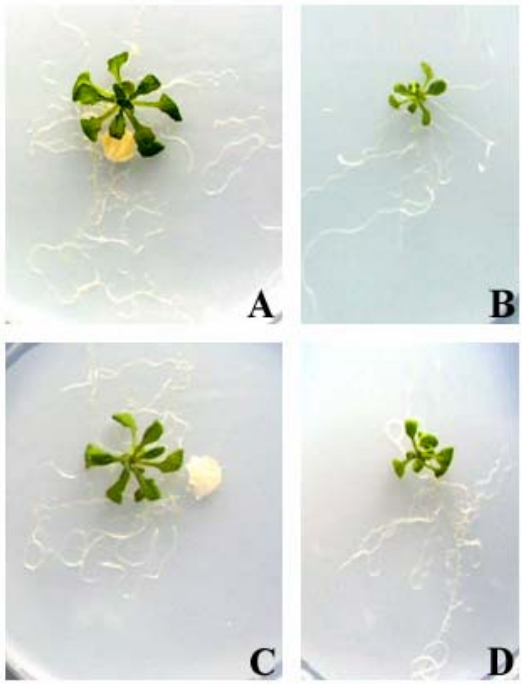


Figure 6

Table 1:

At1g13230 and At5g16590 are components of the Triton X-100 insoluble plasma membrane microdomain fraction from wild-type *Arabidopsis* seedlings. List shows major components of this fraction. Longer incubation of the microdomains with Triton X-100 (60 min instead of 30 min during the isolation procedure) releases At1g13230. Subsequent treatment with saponine releases also At5g16590. Both components are below detectability in microdomains isolated from *Pi-2* seedlings. For experimental details, gels and minor components, cf. Shahollari *et al.* (2004).

Plasma membrane ATPases	At2g18960, At1g17260, At2g24520, At3g47950, At5g62670
ERD4 protein	At1g30360
Pectinesterase	At4g12420
cAMP-dependent kinase	At2g20040
Aquaporin, intrinsic protein	At4g35100
Phospholipase D	At4g35790
Calcium-dependent kinase	At4g04720
Ankyrin kinase	At1g14000
Calnexin-like protein	At5g07340
Band 7 proteins	At5g62740
CBL-interacting kinases	At4g24400, At1g01140
Remorin	At2g45820
LRR-protein-1	At3g17840
LRR-protein 2	**At5g16590**
LRR-protein 3	At2g26730
LRR-protein 4	At3g02880
LRR-protein 5	**At1g13230**
LRR-protein 6	At3g08680
LRR-protein 7	At3g14350

Manuscript V

MATH domain proteins represent a novel protein family in *Arabidopsis thaliana*, and at least one member is modified in roots during the course of a plant–microbe interaction.

Ralf Oelmüller, Tatjana Peškan-Berghöfer, Bationa Shahollari, Artan Trebicka, Irena Sherameti, Ajit Varma

Physiologia Plantarum (2005) **124:**152-166

MATH domain proteins represent a novel protein family in *Arabidopsis thaliana*, and at least one member is modified in roots during the course of a plant–microbe interaction

Ralf Oelmüller[a]*, Tatjana Peškan-Berghöfer[a], Bationa Shahollari[a], Artan Trebicka[b], Irena Sherameti[a] and Ajit Varma[c]

[a]Institute of General Botany and Plant Physiology, Friedrich-Schiller-University Jena, Dornburgerstr. 159, 07743 Jena, Germany
[b]Tirana University, Faculty of Natural Sciences, Department of Biology, Tirana, Albania
[c]School of Life Sciences, Jawaharlal Nehru University, New Delhi-110 067, India and Amity Institute of Herbal and Microbial Studies, E-27 Defence Colony, New Delhi 110024, India

Correspondence
*Corresponding author,
e-mail: b7oera@uni-jena.de

Received 3 January 2005; revised 31 January 2005

doi: 10.1111/j.1399-3054.2005.00505.x

The basidiomycete *Piriformospora indica* interacts with *Arabidopsis* roots and mimics an arbuscular mycorrhiza. A MATH [meprin and TRAF (tumour necrosis factor receptor-associated factor) homology] domain-containing (MATH) protein at the plasma membrane of *Arabidopsis* roots is one of the first components to respond to the presence of this fungus. MATH proteins are involved in nodule formation in *Medicago* and protein degradation in the *Arabidopsis* cytosol. They exhibit sequence similarities to meprins, extracellular peptidases which cleave (signal) peptides, and to TRAFs, intracellular proteins which interact with receptor kinases at the plasma membrane. Fifty-nine genes for MATH proteins are present in the *Arabidopsis* genome. Members of this protein family are predicted to be found in the ER–plasma membrane–extracellular space continuum, in the nucleus–cytosol compartment and in organelles. In this article, we describe this novel class of plant genes. We also use MS-MS analyses to identify the subcellular localization of individual members of the MATH protein family in *Arabidopsis thaliana*.

Introduction

The majority of land plants live in mycorrhizal interaction with fungi, a symbiosis which has a strong impact on ecosystems, agriculture, flori-horticulture and forestry (Sanders 2003, Bidartondo et al. 2004, Koide and Mosse 2004, Pennisi 2004). The benefits of mycorrhizal associations arise from the nutrient transport between the plant roots and fungal hyphae. The carbon source is transported from the plant to the fungus, whereas fungal hyphae serve as a fine link between the roots and the rhizosphere and improve the supply of the plant with inorganic nutrients (Harrison 1999, Rausch and Bucher 2002, Bucking and Heyser 2003, Herrmann et al. 2004, Koide and Mosse 2004). Although the importance of mycorrhizal associations has been recognized for a

Abbreviations – BTB, BRC, ttk and bab domain; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio] propanesulfonate; ESI-MS, electrospray ionization-mass spectrometry; MATH, meprin and TRAF (tumour necrosis factor receptor-associated factor) homology; MMN, modified Melin-Norkrams; MS-MS, tandem mass spectrometry; PMSF, phenylmethylsulphonyl fluoride; POZ, pox virus and zinc finger; RT-PCR, reverse transcriptase-polymerase chain reaction; SSH, suppression subtractive hybridization.

long time, knowledge about the mechanisms leading to the establishment and functioning of this symbiosis is still limited. Substantial progress has been made in recent years, when it became apparent that bacterial (rhizobial) and fungal (mycorrhizal) interactions with plant roots share common signalling components (Marx 2004, Parniske 2004). During the establishment of the arbuscular mycorrhizal interaction, fungal hyphae grow throughout root epidermal, exodermal and cortical cell layers to reach the inner cortex where the arbuscles develop. Three essential components of a plant signalling network, a receptor-like kinase (Endré et al. 2002, Stracke et al. 2002), a predicted ion channel (Ané et al. 2004) and a calmodulin-dependent protein kinase (Levy et al. 2004), have been identified. Furthermore, two plastid-localized proteins, named CASTOR and POLLUX, are indispensable for microbial admission into plant cells and act upstream of intracellular calcium spiking, one of the earliest plant responses to symbiotic stimulation (Imaizumi-Anraku et al. 2005). Finally, evidence of a diffusible fungal signalling factor that triggers gene activation in the root has recently been obtained (Marx 2004, Parniske 2004; and references cited therein).

One obstacle in the molecular analysis of beneficial plant–microbe interactions is the lack of genomic information for most plant species which form either bacterial or fungal symbioses. *Arabidopsis thaliana*, a common model for studying plant development at the molecular level, is not a host of mycorrhizal fungi or rhizobial bacteria. Moreover, arbuscular mycorrhizal fungi, which colonize the roots of 80% of vascular plants, including the majority of crop plants, are obligate biotrophs and cannot be cultured without hosts (Newman and Reddel 1987, Varma et al. 1999).

Piriformospora indica is a recently isolated root-interacting fungus, related to the Hymenomycetes of the Basidiomycota (Verma et al. 1998). In contrast with arbuscular mycorrhizal fungi, it can be easily cultivated in axenic culture where it produces chlamydospores (Oelmüller et al. 2004, Peškan-Berghöfer et al. 2004, Pham et al. 2004, Shahollari et al. 2004a). The fungus is able to associate with the roots of various plant species in a manner similar to mycorrhiza and promotes plant growth (Varma et al. 1999, 2001, Singh et al. 2002, 2003, Kumari et al. 2003, Oelmüller et al. 2004, Peškan-Berghöfer et al. 2004, Pham et al. 2004, Shahollari et al. 2004a). A comprehensive molecular phylogenetic analysis using the nuclear gene for the ribosomal large subunit shed light on the ecology and evolution of the group of Sebacinaceous fungi whose striking biodiversity and ecological importance has only recently been recognized (Weiß and Oberwinkler 2001, Glen et al. 2002, Kottke et al. 2003). Hence, it provides a promising model organism for the investigation of beneficial plant–microbe interactions, and enables the identification of compounds which may improve plant growth, productivity and maintain soil fertility.

To elucidate these mechanisms and to identify the genes and proteins involved in early recognition processes, we co-cultivated *Arabidopsis* seedlings with the fungus and analysed changes in the roots within the first few days after the onset of the experiments. RNA and protein patterns were analysed before physical contact of both organisms, and a growth-promoting effect of the fungus on *Arabidopsis* seedlings was observed (Peškan-Berghöfer et al. 2004, Shahollari et al. 2004a). One of the earliest *genes* to respond to the fungus encodes a plasma membrane-bound receptor kinase (Shahollari et al. 2004a). The message for this kinase is transiently upregulated during the recognition period of both organisms and downregulated again once the interaction has been established (Shahollari et al. 2004a). Furthermore, the receptor kinase is located in Triton X-100-insoluble plasma membrane microdomains, together with other signalling components (Shahollari et al. 2004b). One of the earliest plasma membrane *proteins* to respond to the fungus is a MATH [meprin and TRAF (tumour necrosis factor (TNF) receptor-associated factor) homology] domain-containing (MATH) protein (Peškan-Berghöfer et al. 2004). Here, we demonstrate that this protein becomes modified before physical contact between both organisms is visible. Furthermore, this modification is no longer detectable in an *Arabidopsis* mutant which does not respond to *P. indica*. Comparable with the results obtained for the message of the receptor kinase, the modification of the MATH protein is only transient and no longer detectable once the interaction between both organisms has been established. This implies that a functional link exists between the recognition of the fungus and the modification of the protein in the plasma membrane. As nothing is known about MATH proteins in plants, we analysed this novel protein family in greater detail.

Materials and methods

Growth conditions of plant and fungus

Wild-type *A. thaliana* (ecotype Columbia) seeds were surface sterilized and placed on Petri dishes containing Murashige and Skoog (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} s^{-1}). *P. indica* was

cultured on a modified aspergillus minimal medium (Verma et al. 1998, Pham et al. 2004). For solid medium, 1% (w/v) agar was included.

Co-cultivation experiments and estimation of plant growth

Nine-day-old *A. thaliana* seedlings were transferred to nylon discs (mesh size, 70 μm) placed on top of a modified Melin-Norkrans culture medium (MMN medium with 1/10 of nitrogen and phosphorus and no carbohydrate; Marx 1969) in 90 mm 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 3 cm from the roots. Plates were incubated at 22°C under continuous illumination from the side (maximum, 80 μmol m^{-2} s^{-1}). The growth conditions have been described in detail in Peškan-Berghöfer et al. (2004). Between 20 and 500 plates were used to isolate the RNA and protein fractions.

Protein extraction

Microsomes from roots

A. thaliana roots were ground with a mortar and pestle on ice in homogenization buffer containing 50 m*M* Tris-HCl, pH 7.4, 330 m*M* sucrose, 3 m*M* EDTA and 10 m*M* 1,4-dithiothreitol. The homogenate was filtered through four layers of cheesecloth and centrifuged at 10 000 *g* for 15 min. The supernatant was centrifuged at 50 000 *g* for 1 h to pellet the microsomes. The pellet was resuspended in a buffer containing 50 m*M* Tris-HCl, pH 7.4, and 1 *M* NaCl, incubated on ice for 30 min and centrifuged as before. Pelleted membranes were resuspended in 50 m*M* Tris-HCl, pH 7.4, 3 m*M* EDTA and 1 m*M* 1,4-dithiothreitol, and kept at −80°C until protein analysis.

Soluble root proteins

Roots were ground in 50 m*M* Tris-HCl, pH 7.4, 10 m*M* $MgCl_2$, 3 m*M* EDTA and 10 m*M* 1,4-dithiothreitol, and the slurry was clarified by centrifugation (20 min, 40 000 *g*). The supernatant was adjusted to 50% $(NH_4)_2SO_4$ and the protein pellet was again collected by centrifugation (20 min, 40 000 *g*). After resolution and dialysis against the above-mentioned buffer, the proteins were precipitated with ice-cold acetone (80%) and used directly for trypsin digestion as described previously (Sherameti et al. 2004).

Nuclei

Nuclei and nuclear protein extracts were prepared from *Arabidopsis* roots as described previously (Oelmüller et al. 1993, Shahollari et al. 2004a). The material was homogenized in a Waring Blender with extraction buffer [25 m*M* MES, pH 6.0, 250 m*M* sucrose, 5 m*M* EDTA, 10 m*M* KCl, 0.5 m*M* dithioerythritol, 0.5 m*M* spermidine, 0.3% Triton X-100 and 0.5 m*M* phenylmethylsulphonyl fluoride (PMSF)] and the homogenate was filtered through five layers of cheesecloth. Nuclei were pelleted by centrifugation (650 *g*, 4°C, 10 min), washed once with extraction buffer with 2 m*M* PMSF and without Triton X-100, and resuspended in the same buffer. After sonication for 5 s, the material was shaken on ice for 12 h. After removal of the subcellular debris (100 000 *g*, 4°C, 1 h), the proteins were precipitated from the supernatant by $(NH_4)_2SO_4$ (0.35 g ml^{-1}, w/v). The salt was removed by dialysis against 25 m*M* HEPES-KOH, pH 7.8, 50 m*M* KCl, 14 m*M* mercaptoethanol, 0.1 m*M* EDTA and 2 m*M* PMSF, and the proteins were precipitated with methanol. After resuspension in 500 ml of 50 m*M* ammonium bicarbonate, they were used directly for trypsin digestion (Sherameti et al. 2004).

Chloroplasts

Highly purified chloroplasts were isolated per Percoll gradient centrifugation from the cotyledons (Stöckel and Oelmüller 2004). Chloroplasts were washed twice with isolation medium (0.3 *M* sorbitol, 5 m*M* $MgCl_2$, 5 m*M* EGTA, 5 m*M* Na_2EDTA, 20 m*M* HEPES-KOH, pH 8, and 10 m*M* $NaHCO_3$) and disrupted in breaking buffer (50 m*M* HEPES-KOH, pH 8, and 10 m*M* $MgCl_2$). The stromal and membrane fractions were separated by centrifugation (20 000 *g* for 20 min). The soluble proteins from the supernatant were precipitated with trichloroacetic acid and resuspended in 500 ml of 50 m*M* ammonium bicarbonate before digestion with trypsin (Sherameti et al. 2004).

Two-dimensional gel electrophoresis

Membrane proteins were solubilized with 1% Triton X-100 and precipitated with methanol–chloroform according to Wessel and Flügge (1983) and Hippler et al. (2001). In brief, 180 g of protein in 100 ml of extraction buffer (50 m*M* Tris-HCl, pH 7.4, 3 m*M* EDTA and 10 m*M* 1,4-dithiothreitol) was precipitated with methanol, dried and resuspended in 380 ml of sample buffer [8 *M* urea, 2 *M* thiourea, 30 m*M* dithioerythritol, 4% (w/v) 3-[(3-cholamidopropyl)-dimethylammonio] propane-sulphate (CHAPS), 20 m*M* Tris base, 0.5% bromophenol blue, 0.5% IPE buffer (pH 3–10, Amersham Pharmacia, Freiburg, Germany) and 0.05% dodecyl-β-D-maltoside]; 350 ml of the supernatant was added to 1.75 ml of 0.5% (v/v) IPE buffer for isoelectric focusing (Amersham Pharmacia). For the second dimension, the gel system of Schägger and von Jagow (1987) was used. Gels were stained with silver.

Mass spectrometry

Silver-stained gel spots were excised and the proteins were extracted into 500 ml of 50 m*M* ammonium bicarbonate, supplemented with 60 ng ml^{-1} trypsin. After lyophilization, the pellet was resuspended in 5 ml of water–acetonitrile–formic acid (95 : 5 : 0.1) prior to LC-MS analysis. Peptide analyses, analyte sampling, chromatography and acquisition of data were performed on a liquid chromatograph (Famos-Ultimate; LC-Packings, Sunnyvale, USA) coupled with an LCQ Deca XP ITMS, according to the manufacturer's instructions (Sherameti et al. 2004). Peptide analysis by coupling liquid chromatography with electrospray ionization mass spectrometry (ESI-MS) and by tandem mass spectrometry (MS-MS) has been described previously (Stauber et al. 2003).

Protein identification

The measured MS-MS spectra were matched with the amino acid sequences of tryptic peptides from the *A. thaliana* database in FASTA format. Cys modification by carbamidomethylation (+ 57 Da) was taken into account and known contaminants were filtered out. Raw MS-MS data were analysed by the Finnigan Sequest/Turbo Sequest software (revision 3.0; ThermoQuest, San Jose, CA). The parameters for the analysis by the Sequest algorithm were set according to Stauber et al. (2003). The similarity between the measured MS-MS spectrum and the theoretical MS-MS spectrum, reported as the cross-correlation factor (X_{corr}), was equal or above 1.5, 2.5 and 3.5 for singly, doubly or triply charged precursor ions, respectively. In order to identify corresponding loci, identified protein sequences were subjected to BLAST search at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) and FASTA searches using the AGI protein database at the *Arabidopsis* Information Resource (TAIR) (http://www.arabidopsis.org/). Identification of conserved domains and signal peptides was performed using SMART (Schultz et al. 1998) and SignalP (Nielsen et al. 1997), respectively.

Data analysis

Sequence analyses were performed at http://www.ncbi.nlm.nih.gov/. The *Arabidopsis* homepage (http://www.arabidopsis.org/) was used for further analyses. For subcellular localization of proteins, the following websites were consulted: http://www.psort.org/; http://www.cbs.dtu.dk/services/ChloroP/; http://ihg.gsf.de/ihg/mitoprot.html; http://www.cbs.dtu.dk/services/TargetP/; and http://www.sbc.su.se/~miklos/DAS/. Multiple sequence alignments were performed at the Institut National de la Recherche Agronomique (INRA) (http://prodes.toulouse.inra.fr/multalin/multalin.html). For MATH domain sequence analyses, the following homepage was used: http://www.ebi.ac.uk/interpro/IEntry?ac=IPR002083. The phylogenetic tree (http://www.genebee.msu.su/services/phtree_reduced.html) was generated with an approximately 130-amino-acid-long segment of all *Arabidopsis* MATH proteins, which exhibits the highest degree of sequence similarities to the consensus MATH domain sequence 'KFTWKIKNFSQLKKEEKIYSPPFYVGGYKWRLKVYPNGNGNGRGNHLSLYLHVADSESLPLGWKRYAKFTLTVLNQKSDKRKEVIHTFSAKKSSEKNRGWGFPKFIPLSKLEDSSKGFLVNDTLKIEVEV' generated from all *known* MATH proteins (NCBI, Conserved Domain Database; CD 00121.2). Phylogenetic trees generated on the basis of conserved regions within the 130-amino-acid-long segment (either with or without gaps) did not differ significantly from the tree shown here. For proteins with more than one MATH domain, the one with the highest degree of sequence similarity to the consensus sequence was used for the analysis.

RNA analysis

RNA was isolated with an RNA isolation kit (RNeasy, Qiagen, Hilden, Germany). For quantitative reverse transcriptase-polymerase chain reaction (RT-PCR), RNA from *Arabidopsis* roots grown in the absence or presence of *P. indica* was used with gene-specific (At3g20370) and several control primer pairs (Shahollari et al. 2004a). Two primer pairs were simultaneously used for the reaction in order to confirm the specificity of the results. RT-PCR was performed by reverse transcription of 5 mg of total RNA with gene-specific reverse primers. First strand synthesis was performed with a kit (#K1631) from MBI Fermentas (St. Leon-Roth, Germany). After 20 PCR cycles, the products were analysed on 1.5% agarose gels stained with ethidium bromide, and visualized bands were quantified with the Image Master Video System (Amersham Pharmacia Biotech, Uppsala, Sweden).

Results

A MATH protein in the plasma membrane of *Arabidopsis* roots recognizes the presence of the basidiomycete *P. indica*

We have previously demonstrated that a crude plasma membrane preparation from *Arabidopsis* roots contains nine polypeptides which respond very early to the presence of the basidiomycete *P. indica* (Peškan-Berghöfer

et al. 2004). Of these, two spots on a two-dimensional gel corresponded to the same MATH protein, At3g20370. The reason for the presence of two forms of this protein in our membrane preparation is not clear at present; however, MATH proteins contain phosphorylation and glycosylation sites which are targets for protein modifications in animals (see below). On cultivation with the fungus, the overall amount of the protein was upregulated, and part of the protein appeared to be modified, resulting in two additional protein forms with different isoelectric mobilities on two-dimensional gels (Fig. 1A). Modification of the MATH protein was only transient. While two forms of At3g20370 were found in control seedlings, two additional forms appeared 5 days after co-cultivation of the roots with the fungus. This also correlated with an increase in the overall amount of the protein. After 7 days, i.e. the time point at which the interaction between the two organisms had been established (Shahollari et al. 2004a) and the first growth-promoting effects were measured, a sharp decrease in the amount of the modified form of the protein was detected, and, after 9 days, the starting situation was re-established (Fig. 1A). Furthermore, the modification of the MATH protein in root membrane extracts was not observed for the *Arabidopsis* mutant *P. indica-insensitive-1* (Pi-1) (Fig. 2), which was isolated in a screen for plants which were blind to *P. indica* (B. Shahollari, 2004, unpublished observations). This mutant showed wild-type growth and development when co-cultivated with *P. indica* (Fig. 2A), and did not develop a strong autofluorescence in the root hairs, a typical symptom observed for *Arabidopsis* seedlings interacting with the fungus (Peškan-Berghöfer et al. 2004). Thus, the modification of this MATH protein is one of the earliest plant responses observed so far in the establishment of a beneficial plant–microbe interaction. The transient modification implies that the MATH protein may be involved in the recognition process rather than the interaction itself.

Quantitative PCR with sequence-specific primers for At3g20370 uncovered only a minor increase in the amount of the message (Fig. 1B). It is worth noting that the very same gene was also identified in our suppression subtractive hybridization (SSH) studies (see below), although the overall stimulation of the mRNA level was only low. It is concluded that *P. indica* induces primarily

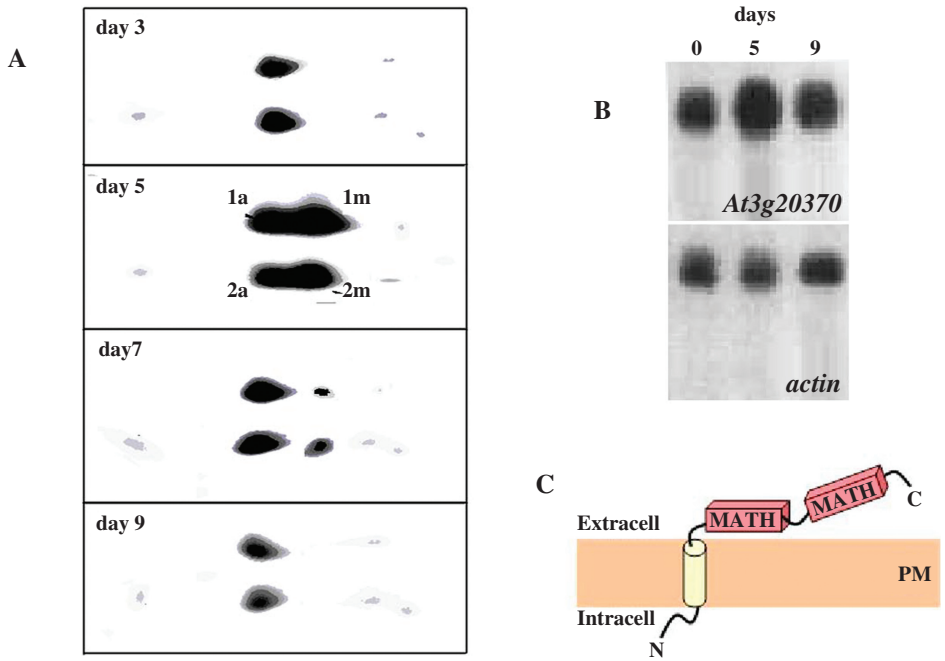


Fig. 1. Regions (1.0 cm × 0.5 cm) from silver-stained two-dimensional gels showing modifications of a protein containing MATH domains (At3g20370). (A) After separation of the proteins by two-dimensional PAGE, the gels were stained with silver. The major spots correspond to At3g20370, as determined by mass spectrometry. 1a and 2a, protein spots also present in control extracts; 1m and 2m, modified versions appearing in response to *Piriformospora indica*. Extracts were prepared 3, 5, 7 and 9 days after the onset of co-cultivation; equal protein loading was confirmed by comparing other spots on the gel (see Peškan-Berghöfer et al. 2004 for more information). (B) Northern blot for the *At3g20370* message. Twenty-five mg of RNA extracted from *Arabidopsis* roots grown in the presence of *P. indica* for 0, 5 or 9 days were loaded per lane and hybridized to *At3g20370* and actin probes. (C) Model of the protein: extracell, extracellular region; intracell, intracellular region; MATH, MATH domain; PM, plasma membrane.

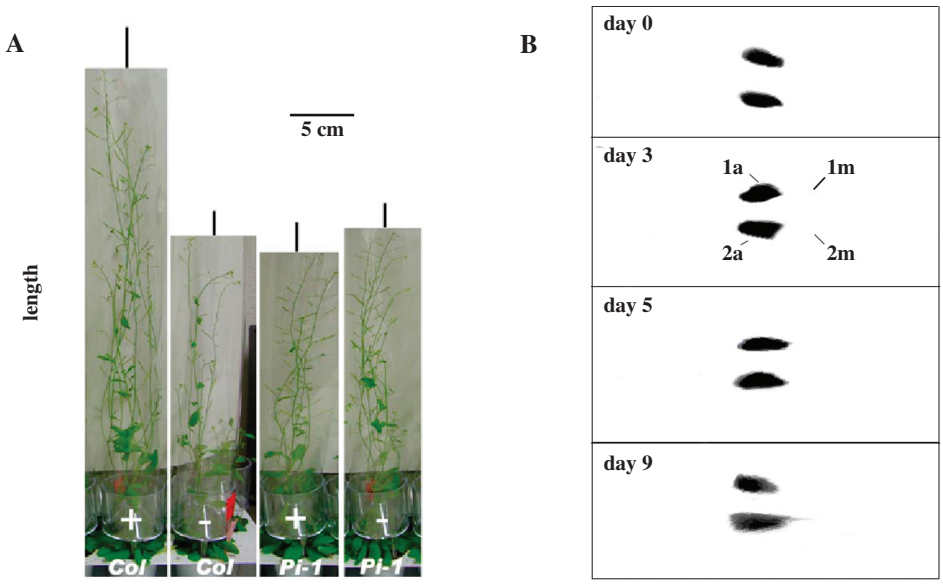


Fig. 2. The *Arabidopsis* mutant *Piriformospora indica*-insensitive-1 (Pi-1) is blind to *P. indica*. (A) Seven-week-old *Arabidopsis* plants (wild-type, Pi-1) grown in the presence (+) or absence (–) of *P. indica*. Typical plants are shown. Bar represents 5 cm. Error bars on top of the plants represent SE of 25 independent plants analysed. (B) At3g20370 is not modified in the *Arabidopsis* mutant Pi-1. Proteins were extracted from the root membranes from seedlings before (0) and 3, 5 or 9 days after the beginning of co-cultivation. For experimental details, see legend to Fig. 1.

a modification of the MATH protein rather than the stimulation of *At3g20370* mRNA accumulation.

The *Arabidopsis* genome contains 59 genes for MATH proteins

To understand more about MATH proteins in plants and to integrate At3g20370 with other members of this protein family, the *Arabidopsis* genome was searched for *MATH* genes. Fifty-nine genes code for (potential) MATH proteins in the *Arabidopsis* genome (Table 1). To our knowledge, apart from the protein identified in our studies (Peškan-Berghöfer et al. 2004), only two other plant MATH proteins have been mentioned in the literature: At5g06600 is the ubiquitin-specific protease 12 (Yan et al. 2000), and a homologue of At3g58200 in *Medicago* has been identified in an SSH screen because it is induced during root nodule development (Gamas et al. 1996). Members of this new gene family are predicted to be located in different cellular compartments. Computer analyses of the *Arabidopsis* sequences allow the following conclusions.

(1) Several MATH proteins are predicted to be located in the ER–Golgi–plasma membrane continuum (Table 1). Their sorting occurs via the secretory pathway. From them, at least seven MATH proteins, including At3g20370, appear to be plasma membrane-anchored extracellular proteins. Their N-terminal regions are lipophilic and their C-terminal segments contain two MATH domains orientated towards the extracellular space (e.g. At1g58270, At3g20360, At3g20370, At3g20380, At5g26280, At5g26290, At5g26300, At5g26320; Table 1 and Fig. 1C).

(2) At least 11 MATH proteins are predicted to be located in the cytosol (Table 1). All have a single MATH domain at the N-terminal part of the protein. In some cases, the MATH domains are followed by either a UCH domain (ubiquitin carboxyl-terminal hydrolase; At5g06600, At3g11910) or a BRC, ttk and bab (BTB) domain [pfam00651.11, BTB; the BTB/POZ (pox virus and zinc finger) domain mediates homomeric dimerization and, in some instances, heteromeric dimerization; At3g03740]. Thus, the UCH domain-containing MATH proteins might be involved in protein degradation processes. The only MATH protein from plants for which a function has been assigned is the ubiquitin-specific protease 12 (At5g06600) (Yan et al. 2000).

(3) For several MATH proteins, a nuclear localization is predicted (Table 1). Whether these proteins are exclusively located in the nucleus or shuttle between the nucleus and the cytoplasm is unknown at present. All contain an N-terminal MATH domain and their modular structures are very similar to those of the cytoplasmic proteins. Three proteins contain an additional second MATH domain located in the central part of the protein (At2g38920, At2g42470, At4g09770).

(4) MATH proteins predicted to be located in microbodies/peroxisomes are dominated by their MATH domain(s). They contain a single (At1g65050, At1g03580, At5g52330), two (At2g32870, At2g32880, At4g00780, At1g65370, At3g17380, At1g65150, At2g04190, At2g15710) or more than two (At3g22080) MATH domains.

(5) Several MATH proteins are predicted to be located in plastids (At2g25330, At3g43700, At5g19000, At5g43560). Of these, At2g25330 contains four MATH domains.

Table 1. List of MATH proteins in *Arabidopsis* and the predicted cellular localization. The longest predicted protein is underlined.

Genes	Proteins	Predicted localization	Location of MATH domain(s)	Other conserved domains
At1g03580	Q8RWZ4, Q9LR66, Q9FWZ4	Microbody, cytoplasm	13-93	
At1g04300	P93826	Mitochondrium (?)	0-116	
At1g31390	Q9SHE2, Q9C870	Cytoplasm	8-123	
At1g31400	Q9C869	Cytoplasm	8-131	
At1g58270	Q8L7C1, Q9SLV3, Q84WH6	Golgi apparatus, plasma membrane, ER	112-231 254-373	
At1g65050	Q9SS54	Microbody	27-133	
At1g65150	Q9S9J5	Microbody	27-133 162-283	
At1g65370	O80808, Q940M1	Microbody, cytoplasm	1-80 (?) 97-217	
At1g69650	Q9C9K9, Q9FWZ3	Plastid stroma, microbody (?)	24-125 158-282	
At2g01790	Q9ZUA7	Microbody, cytoplasm	8-126	
At2g04170	Q9SHS6	Plasma membrane, microbody	133-261 283-409	
At2g04190	Q9SHS5	Microbody	123-252 274-400	
At2g05420	Q9SHT2	Nucleus, cytoplasm	9-139	
At2g15710	Q9ZQE3	Microbody	100-230 252-361	
At2g25320	Q9SIR1	(No information)	75-200 237-368 404-527 559-681	Mitotic check-point protein; Smc, SbcC, UCH, myosin-like
At2g25330	Q9SIR0	Plastid stroma (?)	50-175 204-335 359-493 524-646	
At2g32870	Q8GUK0, O48778, Q84WY0	Microbody	137-244 277-407	
At2g32880	8GX90, O48777	Microbody, ER (?)	32-144 178-309	
At2g38920	Q8GWI0	Cytoplasm, nucleus	Weak homology	RING, SPX
At2g39760	O22286, Q8L977	Cytoplasm	26-158	POZ or BTB
At2g42460	Q8GXS4, Q9SLB4	Nucleus	8-129	
At2g42470	Q9SLB3	Nucleus (plastid)	33-148 492-607	Myosin tail
At2g42480	Q9SLB2	Microbody, nucleus	8-131 392-508	
At3g03740	Q8LGI9, Q9SRV1	Cytoplasm, plastid stroma (?)	48-180	POZ or BTB
At3g06190	Q9M8J9	Nucleus	34-166	POZ or BTB
At3g11910	Q9M134, Q9SF08, Q84WU2	Cytoplasm	55-170	UCH
At3g17380	Q9LUT3	Microbody	21-151 175-300	
At3g20360	Q8W0Z1, Q9LTQ7	Outside, plasma membrane	68-201 223-352	
At3g20370	Q9LTQ5	Outside, plasma membrane	68-201 223-352	
At3g20380	Q9LTQ4	Outside, plasma membrane	88-219 242-363	
At3g22080	Q8LF44, Q9LRJ6, Q9LRJ7	Microbody, ER	20-107 152-260 302-438 460-582	

Table 1. Continued

Genes	Proteins	Predicted localization	Location of MATH domain(s)	Other conserved domains
At3g27040	Q9LSD2	Microbody	9-76 (?) 96-220	
At3g28220	Q9LHA6	Plasma membrane	82-216 239-359	
At3g29580	Q9LJB5	Nucleus (?)	8-132	
At3g43700	Q9M2B6	Plastid stroma	37-169	POZ or BTB
At3g46190	Q9LX76	Microbody, cytoplasm	34-111 162-269	
At3g58200	Q9M2J6, Q8LB51	Cytoplasm	8-121	
At3g58210	Q9M2J5	Nucleus	8-133	
At3g58220	Q9M2J4	Cytoplasm	69-170	
At3g58250	Q9M2J1	ER, plasma membrane	10-124	
At3g58260	Q9M2J0	Plasma membrane	8-124	
At3g58270	Q9M2I9	Cytoplasm	8-131	
At3g58350	Q9M2I1	Cytoplasm	64-194	
At3g58360	Q9M2I0	Cytoplasm	8-132	
At3g58410	Q9M2H5, Q9M2I2	ER	29-135	
At3g58440	Q9M2H2	Golgi apparatus, plasma membrane	10-131	
At4g00780	O23098, Q8VZ38, Q8LAS4	Microbody, mitochondrium (?)	21-142 166-290	
At4g01390	O04596	Microbody (?)	13-134 160-282	
At4g09770	O81493, Q9SZ93	Nucleus	11-138 160-282	
At4g09780	Q9SZ94	Mitochondrium/microbody	64-208 230-360	
At5g06600	Q9C5K1, Q9FG10, Q9FPT1, Q9FU99	Cytoplasm	55-178	UCH
At5g19000	Q8L765	Plastid stroma	36-167	POZ or BTB
At5g21010	Q8LFW4, Q94B33	Plasma membrane, ER	30-162	POZ or BTB
At5g26280	Q93Z83, O81494, Q8LEX1	Outside, microbody	61-191 213-339	
At5g26290	O81496	Outside	56-181 203-322	
At5g26300	O81497	Outside	61-190 212-338	
At5g26320	O81499	Outside	61-193 215-340	
At5g43560	Q8RY18, Q9FIY4	Plastid stroma (?)	70-191	
At5g52330	Q9FHC7	Microbody	20-141	

Phylogenetic analyses of the MATH domain regions of the *Arabidopsis* proteins

The MATH domains in the 59 MATH proteins exhibit the highest degree of similarity (Table 1), and they are also highly conserved with the proposed 130-amino-acid-long consensus MATH motif present in the database (NCBI, CD 00121.2, MATH). Therefore, these regions were used for multiple sequence alignment (Fig. 3). If a MATH protein contained more than one MATH domain, the one with the highest degree of similarity to the consensus sequence was used for the alignment. Fig. 3 demonstrates that the N- and C-terminal regions within the MATH domains contain the most conserved amino acids, while the middle parts are less conserved. Three amino acids (red in the consensus sequence of Fig. 3) are highly conserved in all MATH proteins from *Arabidopsis* and are also present at an identical position in the proposed consensus sequence for all MATH proteins present in the databases (data not shown).

A phylogenetic tree (Fig. 4) revealed that the predicted subcellular localization was not related to the origin of

Fig. 3. Sequence alignment of the MATH domains of the 59 MATH proteins from *Arabidopsis thaliana*. The 130-amino-acid-long MATH consensus domain (see 'Materials and methods') was used to identify MATH domains in the *Arabidopsis* genome. For proteins with more than one MATH domain, the one with the greatest similarity to the consensus sequence was used for alignment. Red, identical amino acids; blue, conserved amino acids. For more information, see 'Materials and methods'.

the protein (Table 1 and Fig. 4). MATH proteins predicted to be located in the cytosol were preferentially found in the middle part of the tree, while those predicted to be sorted via the secretory pathway were positioned on different branches (Table 1 and Fig. 4). In addition, polypeptides predicted to be located in plastids were found on different branches of the tree, although a few appeared to cluster (At3g43700 and At5g19000). In contrast, all speckle-type POZ domain (NCBI, pfam00651.11, BTB)-related proteins were located on a single branch of the tree, although they were found in different cellular compartments. The *P. indica*-responsive At3g20370 clustered together with two other highly homologous MATH proteins (At3g20360 and At3g20380), and all three proteins were predicted to be located in the plasma membrane (Fig. 4). Several peptides for the *P. indica*-responsive protein (Fig. 2), which were identified by mass spectrometry, matched only to At3g20370 and not to the other two proteins (e.g. IRQITDDLKT was not present in At3g20360, MESFNT was not found in At3g2080 and TMWGFSQVLPIDTFK was not observed in At3g2060 and At3g20380). Thus, the response to *P. indica* appears to be highly specific for At3g20370. No significant differences were observed when the tree was generated under higher stringency (data not shown). The position of a protein with more than one MATH domain did not change significantly in the tree if one MATH domain was replaced by another. Taken together, the tree reflects the involvement of MATH domains in proteins with different functions.

Proteomics uncovers MATH proteins in several subcellular compartments

To obtain more information on the subcellular localization of MATH proteins and to provide an experimental basis for the localization of individual members of the MATH protein family, we checked for these proteins in plasma membrane preparations, soluble protein fractions, nuclei and plastids. After trypsin digestion of the protein extracts from these fractions (see 'Materials and methods'), the

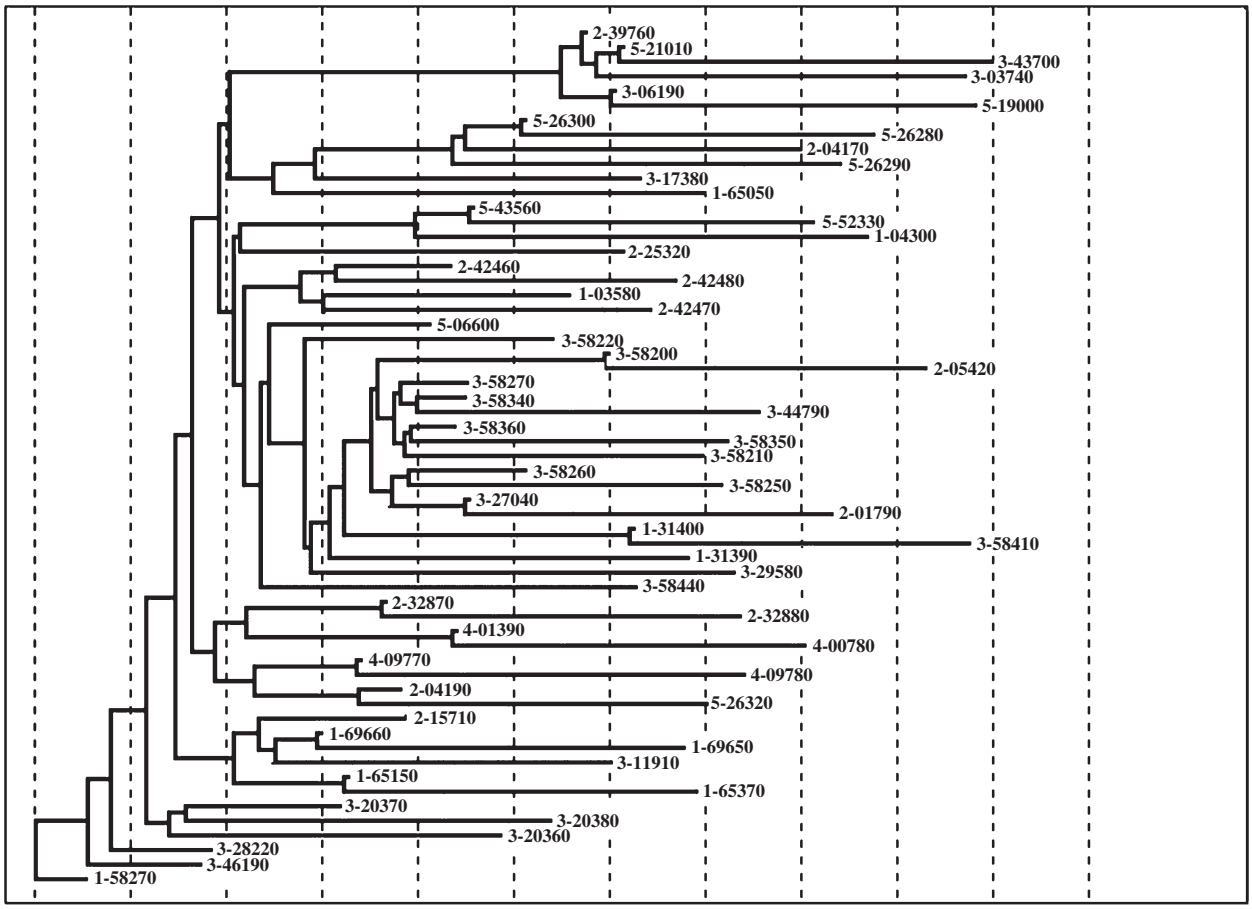


Fig. 4. Phylogenetic tree of the MATH domains from the 59 MATH proteins from *Arabidopsis*. 3-20370, At3g20370 protein identified in this study. For details, see 'Materials and methods'.

polypeptides were identified by mass spectrometry. Each fraction contained at least one MATH protein which could not be identified in any of the other fractions (Table 2). At3g20370 appeared to be a prominent protein in plasma membranes. All together, we identified 17 different peptides which corresponded to 78% of the entire peptide sequence; however, not all of them were specific for At3g20370, because they also matched to At3g20360 and At3g20380. At3g58210 was only detected in the nuclear extract, while At2g05420 was found in the nuclear as well as the cytosolic fraction. At least two peptides in the cytosolic fraction matched to At3g58360 and two peptides in plastid protein preparations matched to At1g69650. More detailed studies are under way to identify other members of this protein family by mass spectrometry.

Discussion

We identified a modification of a MATH protein in plasma membrane preparations of *Arabidopsis* roots after co-cultivation with *P. indica*. As this protein modification is one of the earliest plant responses to a fungus, as it does not occur in an *Arabidopsis* mutant which is blind to *P. indica*, and as MATH proteins exhibit similarities to extracellular proteases which are known to be involved in the perception of fungal signals (see below), we investigated MATH proteins in *Arabidopsis* in more detail.

Table 2. MATH proteins in different cellular subfractions. The protein numbers, subcellular localizations and identified peptides are given.

At3g20370	(plasma membrane)
	IRQITDDLKT
	MESFNT
	FYIFNK
	ERKYFTIQDTDVWK
	KYFTIQDTDVWK
	TMWGFSQVLPIDTFK
	SELFSVTENFLNPR
	NSYLSEVFSIGGRSWNIQINPSGLGTGEGK
	ALNQLNLSNIER
At3g58210	(nuclear protein extract)
	ACMNFLLSLMETLCQPPQK
	LDVPEESEEETQVTQPMK
	LDVPEESEEETQVTQPMKK
	SLPPGWSR
At2g05420	(nuclear protein extract and cytoplasm)
	FRPK
	FRPKNPYLK
	TAYMNVLLSLTQTTICQSPGELSNDDLSDSGAALAYLR
At3g58360	(cytoplasm)
	VDLAEGYVALR
	CLDLEAQLEK
	ITWAIENFSSLHSK
At1g69650	(plastid)
	YLSVFLYLADNETLK
	TYLDK
	EDTLMIEAEFEVVSATK

The function of MATH proteins in general is unclear at present; however, the modular organization and domain structure of MATH proteins, together with their homology and structural similarity to meprins and TRAFs (see below), suggest that they may be involved in processes such as protein degradation and protein or peptide cleavage and/or activation, or in transferring signals to intracellular signalling pathways. Meprins and TRAFs may provide clues to the function of MATH proteins in plants. The characteristic features of the MATH protein which responds to *P. indica* are the two MATH domains predicted to be located in the extracellular space.

Meprins belong to the astacin protein family of zinc-dependent metalloendopeptidases (Dumermuth et al. 1991, Bertenshaw et al. 2003, Norman et al. 2003a, Kruse et al. 2004). Mature meprins are composed of evolutionarily related α and β subunits which exist as homo- and heterooligomeric complexes. Homooligomers of α subunits are secreted into the extracellular space. They are zymogens that form high-molecular-mass complexes of 1–6 MDa with up to 100 molecules (Bertenshaw et al. 2003). The extent of oligomerization is strongly dependent on the activation state of the enzyme and various regulatory factors in the extracellular environment. Oligomers containing the β subunit are plasma membrane associated. In contrast with the β subunit, the C-terminal membrane anchor of the α subunit is cleaved off in the ER, which leads to secretion of the protein (Marchand et al. 1995).

Extracellular meprins are amongst the largest extracellular proteases identified thus far in animal systems (Villa et al. 2003a). A TNF-associated enzyme has been identified as an activating component of meprins, and the enzyme itself becomes activated via a protein kinase C-dependent mechanism (Hahn et al. 2003, Villa et al. 2003b). Thus, meprins provide examples of novel ways of concentrating proteolytic activity at the cell surface and in defined areas in the extracellular milieu.

Meprins cleave a large variety of bioactive peptides including growth factors, cytokines, factors required for morphogenesis and extracellular matrix proteins (Wolz and Bond 1995, Chestukhin et al. 1997, Becker et al. 2003; and references cited therein). Disruption of the meprin allele in mice affects embryonic viability and the distribution of meprin in kidney and intestine (Norman et al. 2003b). These tissue-specific proteinases are also involved in developmental processes and pathogenic responses (Bond and Beynon 1995, Dietrich et al. 1996).

Four potential glycosylation sites for the MATH protein have been predicted (Leuenberger et al. 2003). Although the nature of the modification of the MATH protein described here is not clear, the modification occurs in response to fungal signals. The activated MATH protein may have the potential to cleave a peptide that, in turn, activates an intracellular signalling pathway, as proposed for the *Drosophila* receptor kinase TOLL system (De Gregorio et al. 2002). In *Drosophila*, this mechanism is activated by pathogenic fungi. In addition, plant–fungus interactions depend on extracellular proteinase activities. For instance, a tomato cysteine protease is required for Cf-2-dependent disease resistance and suppression of autonecrosis (Krüger et al. 2002).

TRAFs are the major signal transducers for the TNF receptor superfamily and the interleukin-1 receptor/Toll-like receptor superfamily (Inoue et al. 2000, Wajant and Scheurich 2001, Chung et al. 2002). TNF exerts its functions by interaction with the death domain-containing TNF-receptor 1 (TNF-R1) and the non-death domain-containing TNF-receptor 2 (TNF-R2). Several TRAFs participate in the apoptotic pathway and the signalling cascades leading to the activation of NF-KB and JNK (Jun N-terminal kinase; Rothe et al. 1994, Park et al. 1999, Ye et al. 1999). TRAF proteins promote cell survival by the activation of downstream protein kinases and transcription factors (Chung et al. 2002; and references cited therein). Of special interest for this study are TRAF 1 and 2, which define a novel group of proteins involved in signal transduction by many members of the TNF receptor family, including the TOLL-like receptor proteins. TOLL proteins belong to the same class of receptor kinases which, in plants, are involved in plant–microbe interactions (Kistner and Parniske 2002). In plants, a TNF-like receptor kinase has been shown to be involved in maize epidermal differentiation, but it is not known whether it interacts with MATH proteins (Becraft et al. 1996).

Extracellular meprins and intracellular TRAFs share a conserved region, called the MATH (meprin and TRAF homology) domain (Sunnerhagen et al. 2002). The importance of MATH proteins is also apparent from *Caenorhabditis elegans* (The C. elegans Sequencing Consortium 1998), which contains approximately 100 genes for these proteins. Databank analyses revealed that MATH proteins were not exclusively found in plasma membranes and/or the extracellular space, but also in the ER, Golgi apparatus, cytosol, nucleus and organellar membranes, preferentially peroxisomes. This also appears to be true for *Arabidopsis* MATH proteins. The phylogenetic tree does not relate the phylogenetic classifications to cellular localizations. Those proteins predicted to be located in plasma membranes differ substantially in length and in their N- and C-terminal regions.

Although the overall number of genes for MATH proteins is similar in *C. elegans* and *Arabidopsis*, almost nothing is known about their function in plants. The high degree of similarity in all eukaryotic signalling systems, and the identification of MATH proteins in two independent screens for plant–microbe interactions (Gamas et al. 1996, Peškan-Berghöfer et al. 2004), indicate that this protein family should be analysed in greater detail.

References

Ané JM, Kiss GB, Riely BK, Penmetsa RV, Oldroyd GE, Ayax C, Levy J, Debelle F, Baek JM, Kalo P, Rosenberg C, Roe BA, Long SR, Denarie J, Cook DR (2004) *Medicago truncatula* DMI1 required for bacterial and fungal symbioses in legumes. Science 303: 1364–1367

Becker C, Kruse MN, Slotty KA, Kohler D, Harris JR, Rosmann S, Sterchi EE, Stocker W (2003) Differences in the activation mechanism between the alpha and beta subunits of human meprin. J Biol Chem 384: 825–831

Becraft PW, Stinard PS, McCarty DR (1996) CRINKLY4: a TNFR-like receptor kinase involved in maize epidermal differentiation. Science 273: 1406–1409

Bertenshaw GP, Norcum MT, Bond JS (2003) Structure of homo- and hetero-oligomeric meprin metalloproteases. Dimers, tetramers, and high molecular mass multimers. J Biol Chem 278: 2522–2532

Bidartondo MI, Burghardt B, Gebauer G, Bruns TD, Read DJ (2004) Changing partners in the dark: isotopic and molecular evidence of ectomycorrhizal liaisons between forest orchids and trees. Proc R Soc London Ser B 271: 1799–1806

Bond JS, Beynon RJ (1995) The astacin family of metalloendopeptidases. Protein Sci 4: 1247–1261

Bucking H, Heyser W (2003) Uptake and transfer of nutrients in ectomycorrhizal associations: interactions between photosynthesis and phosphate nutrition. Mycorrhiza 13: 59–68

Chestukhin A, Litovchick L, Muradov K, Batkin M, Shaltiel S (1997) Unveiling the substrate specificity of meprin beta on the basis of the site in protein kinase A cleaved by the kinase splitting membranal proteinase. J Biol Chem 272: 3153–3160

Chung JY, Park YC, Ye H, Wu H (2002) All TRAFs are not created equal: common and distinct molecular mechanisms of TRAF-mediated signal transduction. J Cell Sci 115: 679–688

De Gregorio E, Spellman PT, Tzou P, Rubin GM, Lemaitre B (2002) The Toll and Imd pathways are the major regulators of the immune response in *Drosophila*. EMBO J 21: 2568–2579

Dietrich JM, Jiang W, Bond JS (1996) A novel meprin beta´ mRNA in mouse embryonal and human colon carcinoma cells. J Biol Chem 241: 2271–2278

Dumermuth E, Sterch EE, Jiang W, Wolz RL, Bond JS, Flannery AV, Beynon RJ (1991) The astacin family of metalloendopeptidases. J Biol Chem 266: 21 381–21 385

Endré G, Kereszt A, Kevei Z, Mihacea S, Kalo P, Kiss GB (2002) A receptor kinase gene regulating symbiotic nodule development. Nature 417: 962–966

Gamas P, Niebel FC, Lescure N, Cullimore J (1996) Use of a subtractive hybridization approach to identify new *Medicago truncatula* genes induced during root nodule development. Mol Plant–Microbe Interact 9: 233–242

Glen M, Tommerup IC, Bougher NL, O'Brien PA (2002) Are Sebacinaceae common and widespread ectomycorrhizal associates in *Eucalyptus* species in Australian forests? Mycorrhiza 12: 243–247

Hahn D, Pischitzis A, Roesmann S, Hansen MK, Leuenberger B, Luginbuehl U, Sterchi EE (2003) Phorbol 12-myristate 13-acetate-induced ectodomain shedding and phosphorylation of the human meprin beta metalloprotease. J Biol Chem 278: 42 829–42 839

Harrison MJ (1999) Molecular and cellular aspects of the arbuscular mycorrhizal symbiosis. Annu Rev Plant Physiol Plant Mol Biol 50: 631–689

Herrmann S, Oelmüller R, Buscot F (2004) Manipulation of the onset of ectomycorrhiza formation by indole-3-acetic acid, activated charcoal or relative humidity in the association between oak microcuttings and *Piloderma croceum*: influence on plant development and photosynthesis. J Plant Physiol 161: 509–517

Hippler M, Klein J, Fink A, Allinger T, Hoerth P (2001) Towards functional proteomics of membrane protein complexes: analysis of thylakoid membranes from *Chlamydomonas reinhardtii*. Plant J 28: 595–560

Imaizumi-Anraku H, Takeda N, Charpentier M, Perry J, Miwa H, Umehara Y, Kouchi H, Murakami Y, Mulder L, Vickers K, Pike J, Allan Downie J, Wang T, Sato S, Asamizu E, Tabata S, Yoshikawa M, Murooka Y, Wu GJ, Kawaguchi M, Kawasaki S, Parniske M, Hayashi M (2005) Plastid proteins crucial for symbiotic fungal and bacterial entry into plant roots. Nature 433: 527–531

Inoue J, Ishida T, Tsukamoto N, Kobayashi N, Naito A, Azuma S, Yamamoto T (2000) Tumor necrosis factor receptor-associated factor (TRAF) family: adapter proteins that mediate cytokine signaling. Exp Cell Res 254: 14–24

Kistner C, Parniske M (2002) Evolution of signal transduction in intracellular symbiosis. Trends Plant Sci 7: 511–518

Koide RT, Mosse B (2004) A history of research on arbuscular mycorrhiza. Mycorrhiza 14: 145–163

Kottke I, Beiter A, Weiß M, Haug I, Oberwinkler F, Nebel M (2003) Heterobasidiomycetes from symbiotic associations with hepatics: *Jungermanniales* have sebacinoid mycobionts while *Aneura pinguis* (*Metzgeriales*) is associated with a *Tulasnella* species. Mycol Res 107: 957–968

Krüger J, Thomas CM, Golstein C, Dixon MS, Smoker M, Tang S, Mulder L, Jones JDG (2002) A tomato cysteine protease required for *Cf-2*-dependent disease resistance and suppression of autonecrosis. Science 296: 744–747

Kruse MN, Becker C, Lottaz D, Kohler D, Yiallouros I, Krell HW, Sterchi EE, Stocker W (2004) Human meprin alpha and beta homo-oligomers: cleavage of basement membrane proteins and sensitivity to metalloprotease inhibitors. Biochem J 378: 383–389

Kumari R, Yadav HK, Bhoon YK, Varma A (2003) Colonization of cruciferous plants by *Piriformospora indica*. Curr Sci 85: 1672–1674

Leuenberger B, Hahn D, Pischitzis A, Hansen MK, Sterchi EE (2003) Human meprin beta: O-linked glycans in the intervening region of the type I membrane protein protect the C-terminal region from proteolytic cleavage and diminish its secretion. Biochem J 369: 659–665

Levy J, Bres C, Geurts R, Chalhoub B, Kulikova O, Duc G, Journet EP, Ane JM, Lauber E, Bisseling T, Denarie J, Rosenberg C, Debelle F (2004) A putative Ca^{2+} and calmodulin-dependent protein kinase required for bacterial and fungal symbioses. Science 303: 1361–1364

Marchand P, Tang J, Johnson GD, Bond JS (1995) COOH-terminal proteolytic processing of secreted and membrane forms of the alpha subunit of the metalloprotease meprin A. Requirement of the I domain for processing in the endoplasmic reticulum. J Biol Chem 270: 5449–5456

Marx DH (1969) The influence of ectotrophic mycorrhizal fungi on the resistance of pine roots to pathogenic infections. I. Antagonism of mycorrhizal fungi to root pathogenic fungi and soil bacteria. Phytopathology 59: 153–163

Marx J (2004) The roots of plant–microbe collaborations. Science 304: 234–236

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

Newman EL, Reddel P (1987) The distribution of mycorrhizas among families of vascular plants. New Phytol 106: 745–751

Nielsen H, Engelbrecht J, Brunak S, von Heijne G (1997) A neural network method for identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Int J Neural Syst 8: 581–599

Norman LP, Jiang W, Han X, Saunders TL, Bond JS (2003b) Targeted disruption of the meprin beta gene in mice leads to underrepresentation of knockout mice and changes in renal gene expression profiles. Mol Cell Biol 23: 1221–1230

Norman LP, Matters GL, Crisman JM, Bond JS (2003a) Expression of meprins in health and disease. Curr Top Dev Biol 54: 145–166

Oelmüller R, Bolle C, Tyagi AK, Niekrawietz N, Breit S, Herrmann RG (1993) Characterization of the promoter from the single-copy gene encoding ferredoxin-$NADP^+$-oxidoreductase from spinach. Mol Gen Genet 237: 261–272

Oelmüller R, Shahollari B, Peškan-Berghöfer T, Trebicka A, Giang PH, Sherameti I, Oudhoff M, Altschmied L, Varma A (2004) Molecular analyses of the interaction between *Arabidopis* roots and the growth-promoting fungus *Piriformospora indica*. Endocyt Cell Res 15: 639–652

Park YC, Burkitt V, Villa AR, Tong L, Wu H (1999) Structural basis for self association and receptor recognition of human TRAF2. Nature 398: 533–538

Parniske M (2004) Molecular genetics of the arbuscular mycorrhizal symbiosis. Curr Opin Plant Biol 7: 414–421

Pennisi E (2004) The secret life of fungi. Science 304: 1620–1622

Peškan-Berghöfer T, Shahollari B, Giang PH, Hehl S, Markert C, Blanke V, Kost G, Varma A, Oelmüller R (2004) Association between *Piriformospora indica* and *Arabidopsis thaliana*: a novel system to study beneficial plant–microbe interactions. Physiol Plant 122: 465–477.

Pham GH, Kumari R, Singh An, Sachdev M, Prasad R, Kaldorf M, Buscot F, Oelmüller R, Peškan T, Weiss M, Hampp R, Varma A (2004) Axenic cultures of *Piriformospora indica*. In Varma A, Abbott L, Werner D, Hampp R (eds) Plant Surface Microbiology. Springer-Verlag, Berlin, pp 593–616

Rausch C, Bucher M (2002) Molecular mechanisms of phosphate transport in plants. Planta 216: 23–37

Rothe M, Henzel WJ, Wong SC, Goeddel DV (1994) A novel family of putative signal transducers associated with the cytoplasmic domain of the 75 kDa tumor necrosis factor receptor. Cell 78: 681–692

Sanders IR (2003) Preference, specificity and cheating in the arbuscular mycorrhizal symbiosis. Trends Plant Sci 8: 143–145

Schägger H, von Jagow G (1987) Tricine-Sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 KDa. Anal Biochem 166: 368–379

Schultz J, Milpetz F, Bork P, Ponting CP (1998) SMART, a simple modular architecture research tool: identification of signaling domains. Proc Natl Acad Sci USA 95: 5857–5864

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

Shahollari B, Varma A, Oelmüller R (2004a) Expression of a receptor kinase in *Arabidopsis* roots is stimulated by the basidiomycete *Piriformospora indica* and the protein accumulates in Triton X-100 insoluble plasma membrane microdomains. J Plant Physiol in press.

Sherameti I, Shahollari B, Landsberger M, Westermann M, Cherepneva G, Kusnetsov V, Oelmüller R (2004) Cytokinin stimulates polyribosome loading of nuclear-encoded mRNAs for the plastid ATP synthase in etioplasts of *Lupinus luteus*: the complex accumulates in the inner envelope membrane with the CF_1 moiety located towards the stromal space. Plant J 38: 578–593

Singh An, Singh Ar, Kumari M, Rai MK, Varma A (2003) Importance of *Piriformospora indica*, a novel symbiotic mycorrhiza-like fungus: an overview. Plant biotechnology. Indian J Biotechnol 2: 65–75

Singh Ar, Singh An, Varma A (2002) *Piriformospora indica – in vitro* raised leguminous plants: a new dimension in establishment and phyto-promotion. Indian J Biotechnol 1: 372–376

Stauber EJ, Fink A, Markert C, Kruse O, Johanningmeier U, Hippler M (2003) Proteomics of *Chlamydomonas reinhardtii* light-harvesting proteins. Eukaryotic Cell 2: 978–994

Stöckel J, Oelmüller R (2004) A novel protein for photosystem I biogenesis. J Biol Chem 279: 10 243–10 251

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

Sunnerhagen M, Pursglove S, Fladvad M (2002) The new MATH: homology suggests shared binding surfaces in meprin tetramers and TRAF trimers. FEBS Lett 530: 1–3

The C. elegans Sequencing Consortium (1998) Genome sequence of the nematode C. elegans: a platform for investigating biology. Science 282: 2012–2018

Varma A, Singh A, Sudha S, Sahay N, Sharma J, Roy A, Kumari M, Rana D, Thakran S, Deka D, Bharti K, Franken P, Hurek T, Blechert O, Rexer K-H, Kost G, Hahn A, Hock B, Maier W, Walter M, Strack D, Kranner I (2001) *Piriformospora indica*: a cultivable mycorrhiza-like endosymbiotic fungus. In Mycota IX. Hock, B., ed. Springer, Berlin, pp 123–150

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

Verma S, Varma A, Rexer K-H, Hassel A, Kost G, Sarbhoy A, Bisen P, Bütehorn B, Franken P (1998) *Piriformospora indica*, gen. Nov. Sp. Nov., a new root-colonizing fungus. Mycologia 90: 898–905

Villa JP, Bertenshaw GP, Bond JS (2003b) Critical amino acids in the active site of meprin metalloproteinases for substrate and peptide bond specificity. J Biol Chem 278: 42 545–42 550

Villa JP, Bertenshaw GP, Bylander JE, Bond JS (2003a) Meprin proteolytic complexes at the cell surface and in extracellular spaces. Biochem Soc Symp 70: 53–63

Wajant H, Scheurich P (2001) Tumor necrosis factor receptor-associated factor (TRAF) 2 and its role in TNF signaling. Int J Biochem Cell Biol 3: 19–32

Weiß M, Oberwinkler F (2001) Phylogenetic relationships in *Ariculariales* and related groups – hypotheses derived from nuclear ribosomal DNA sequences. Myol Res 105: 403–415

Wessel D, Flügge UI (1983) A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. Anal Biochem 138: 141–143

Wolz RL, Bond JS (1995) Meprins A and B. Methods Enzymol 248: 325–345

Yan N, Doelling JH, Falbel TG, Durski AM, Vierstra RD (2000) The ubiquitin-specific protease family from Arabidopsis. AtUBP1 and 2 are required for the resistance to the amino acid analog canavanine. Plant Physiol 124: 1828–1843

Ye H, Park YC, Kreishman M, Kieff E, Wu H (1999) The structural basis for the recognition of diverse receptor sequences by TRAF2. Mol Cell 4: 321–330

Edited by J. K. Schjørring

Manuscript VI

Molecular analyses of the interaction between *Arabidopsis* roots and the growth-promoting fungus *Piriformspora indica*

Ralf Oelmüller, Bationa Shahollari, Tatjana Peškan-Berghöfer, Artan Trebicka, Pham Huong Giong, Irena Sherameti, Menno Oudhoff, Yonne Venus, Lothar Altschmied, Ajit Varma.

Endocytobiosis Cell Res. (2004) **15** (2), 504-517.

Endocytobiosis Cell Res. 15 (2), 504-517 (2004)

Molecular analyses of the interaction between *Arabidopsis* roots and the growth-promoting fungus *Piriformospora indica*

R Oelmüller[1*], B Shahollari[1], T Peškan-Berghöfer[1], A Trebicka[2], PH Giong[3], I Sherameti[1], M Oudhoff[1], Y Venus[1], L Altschmied[4], A Varma[3]

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

[2] *Tirana University, Faculty of Natural Sciences, Department of Biology, Tirana, Albania*

[3] *School of Life Sciences, Jawaharlal Nehru University, New Delhi-110 067, India*

[4]*IPK Gatersleben, Corrensstraße 3, 06466 Gatersleben, Germany*

** corresponding author: email: b7oera@uni-jena.de, Tel: 0049-3641-949-231, Fax: 0049-3641-949232*

Abstract

The basidiomycete *Piriformospora indica* interacts with *Arabidopsis* roots and mimics an arbuscular mycorrhiza. In order to identify components which are involved in early phases of recognition, we isolated mRNAs and proteins from *Arabidopsis* roots which respond to the fungus. A MATH protein with homology to metalloproteases is transiently modified in the plasma membrane of the roots during the recognition period of both organisms. Furthermore, the messages for two receptor kinases are transiently upregulated. Biochemical studies uncovered that the receptor kinases co-purify with a small GTP-binding protein of the Rab-family. Inactivation of one of the receptor kinases strongly retards the interaction between both organisms. Finally, recognition of both organisms appear to depend on a lipid-signalling pathway, since inactivation of AGC2, a protein kinase activated by the 3´-phosphoinositide-dependent kinase PDK1, completely abolishes the growth promoting effect induced by *P. indica*. Based on the available data we propose a working hypothesis which describes early phases of the recognition between both organisms on the basis of the MATH protein, membrane-bound receptor kinases and downstream signaling compoments such as small GTP-binding proteins and AGC2.

Introduction

Piriformospora indica – *a growth promoting fungus of the Sebacinaceae family*

The majority of land plants live in mycorrhizal interaction with fungi, a symbiosis which has a strong impact on ecosystems, agriculture, flori-horticulture and forestry. The benefits of mycorrhizal associations arise from the nutrient transport between the plant roots and fungal hyphae. The carbon source is transported from the plant to the fungus, whereas fungal hyphae serve as a fine link between the roots and the rhizosphere and improve the supply of the plant with inorganic nutrients (Harrison 1999 and references therein). Although the importance of mycorrhizal associations has been recognised long time ago, the knowledge about the mechanisms leading to the establishment and functioning of this sym-

biosis is still limited. Substantial progress has been made in the last years when it became apparent that bacterial (rhizobial) and fungal (mycorrhizal) interactions with plant roots share common signalling components (cf. Marx 2004; Kistner and Parniske 2002; Parniske 2000, 2004). During the establishment of the arbuscular mycorrhizal interaction, fungal hyphae grow throughout root epidermal, exodermal and cortical cell layers to reach the inner cortex where the arbuscles develop. Three essential components of a plant signalling network, a receptor-like kinase (Endré et al. 2002; Stracke et al. 2002), a predicted ion-channel (Ané et al. 2004) and a calmodulin-dependent protein kinase (Levy et al. 2004) have been identified. Furthermore, evidence of a diffusible fungal signalling factor that triggers gene activation in the root has recently been obtained (Marx 2004; Parniske 2004, and references therein).

One obstacle in the molecular analyses of benefical plant/microbe interactions is the lack of genomic information for most plant species which form either bacterial or fungal symbioses. *A. thaliana*, a common model to study plant development at the molecular level, does not belong to the hosts of mycorrhizal fungi or rhizobial bacteria. On the other side, arbuscular mycorrhizal fungi, which colonize the roots of 80% of vascular plants, including the majority of crop plants, are obligate biotrophs and cannot be cultured without hosts (Newman and Reddel 1987; Varma 1999).

P. indica is a recently isolated root-interacting fungus of the group of Sebacinaceous fungi: related to the Hymenomycetes of the Basidiomycota (Verma et al. 1998). A comprehensive molecular phylogenetic analysis using the nuclear gene for the ribosomal large subunit (nrLSU) shed light on the ecology and evolution of the group of Sebacinaceous fungi whose striking biodiversity and ecological importance has only recently to be recognized (Glen et al. 2002; Kottke et al. 2003; Weiss and Oberwinkler 2001). In contrast to arbuscular mycorrhizal fungi, it can be easily cultivated in axenic culture where it produces chlamydospores (Peškan-Berghöfer et al. 2004; Pham et al. 2004; Shahollari et al. 2004a). The fungus is able to associate with the roots of various plant species in a manner similar to mycorrhiza and promotes plant growth (Kumari et al. 2003; Pham et al. 2004; Singh et al. 2003; Varma et al. 1999, 2001; Shahollari et al. 2004a). Hence, it provides a promising model organism for the investigations of beneficial plant-microbe interaction and enables the identification of compounds, which may improve plant growth, productivity and maintain soil fertility. The observation that growth and development of *Arabidopsis* is dramatically stimulated by *P. indica* and that the presence of the fungus has also a strong impact on the number of siliques and seeds per plant is consistent with observations for other plant species which interact with this fungus. Thus, identification of components which are involved in this interaction might be benefical for the understanding of the interaction at the molecular level as well as for agricultural applications.

Results and Discussion

Strategies to identify genes and proteins involved in early phases of the recognition of P. indica *and* Arabidopsis thaliana

To identify genes and proteins which are involved in early recognition processes, we co-cultivated *Arabidopsis* seedlings with the fungus and analysed changes in the roots within the first few days after the

onset of the experiments. RNA and protein patterns were analysed before a physical contact of both organisms and a growth-promoting effect of the fungus on *Arabidopsis* seedlings became visible (cf. Shahollari et al. 2004a; Peškan-Berghöfer et al. 2004). For these studies, microarray analyses with RNAs from roots grown in the presence or absence of *P. indica* were performed to identify genes which are either up- or down-regulated in response to the fungus. As expected the number of regulated genes decreases, the earlier the interaction was studied. In parallel, suppression subtractive hybridizations (SSH) libraries were generated. For these experiments root RNA (± fungus) was extracted 2, 4, 5, 6, 7, 10 and 14 days after the beginning of the co-cultivation experiment. This covers early phases of recognition to a fully established interaction (which can be monitored by the cellular responses shown in Fig. 1, cf. below). They confirmed the regulation of several of the genes which were already identified by the microarray analyses. Five days after the onset of co-cultivation, approximately 50% of the isolated genes code for proteins involved in metabolic processes (such as nitrate assimilation, sulfate reduction or carbohydrate metabolism), 30% of them encode proteins involved in signalling processes (such as kinases, GTP and Ca^{2+}-binding proteins, enzymes involved in osmoregulation or ion fluxes across the plasma memembrane) and the residual 20% for proteins with unknown functions. Genes which were up- or down-regulated two days after co-cultivation have either not yet been characterized for *Arabidopsis* (for instance genes which code for kinases) or code for signalling components. We are currently analysing 31 knock-out lines of these genes to understand their role in the interaction between *P. indica* and *Arabidopsis* roots in greater details. In summary, these approaches let to the identification of several candidate genes which code for receptor kinases, signalling components at the plasma membrane, and putative downstream signalling molecules which might play specific functions during early phases of the recognition between *P. indica* and *Arabidopsis* (cf. below).

In parallel, we isolated (crude or highly purified) plasma membranes from *Arabidopsis* roots grown in the presence and absence of *P. indica*. After two-dimensional gel electrophoresis, differentially regulated protein spots were excised, digested with trypsin and the fragments were analysed by mass spectrometry. Again, the number of proteins which respond to the fungus decrease, the earlier the experiment was performed. We could identify nine protein spots which appear to be specific for the recognition period between both organisms (Peškan-Berghöfer et al. 2004). Again, their importance for the recognition between both organisms is currently be analysed in knock-out lines. Some of the proteins correspond to those identified in our mRNA screens, indicating that the response to *P. indica* can be detected at the transcript and protein levels. The other proteins identified in this screen have either not yet been studied or are signalling components (cf. below).

These three approaches (microarray analyses, SSH, proteomics of *Arabidopsis* plasma membranes from roots) were supplemented by a screen for *Arabidopsis* mutants which fail to interact with *P. indica*. A number of lines were identified in an EMS population and in insertion lines, however, only one of them has been characterized so far at the molecular level (cf. below).

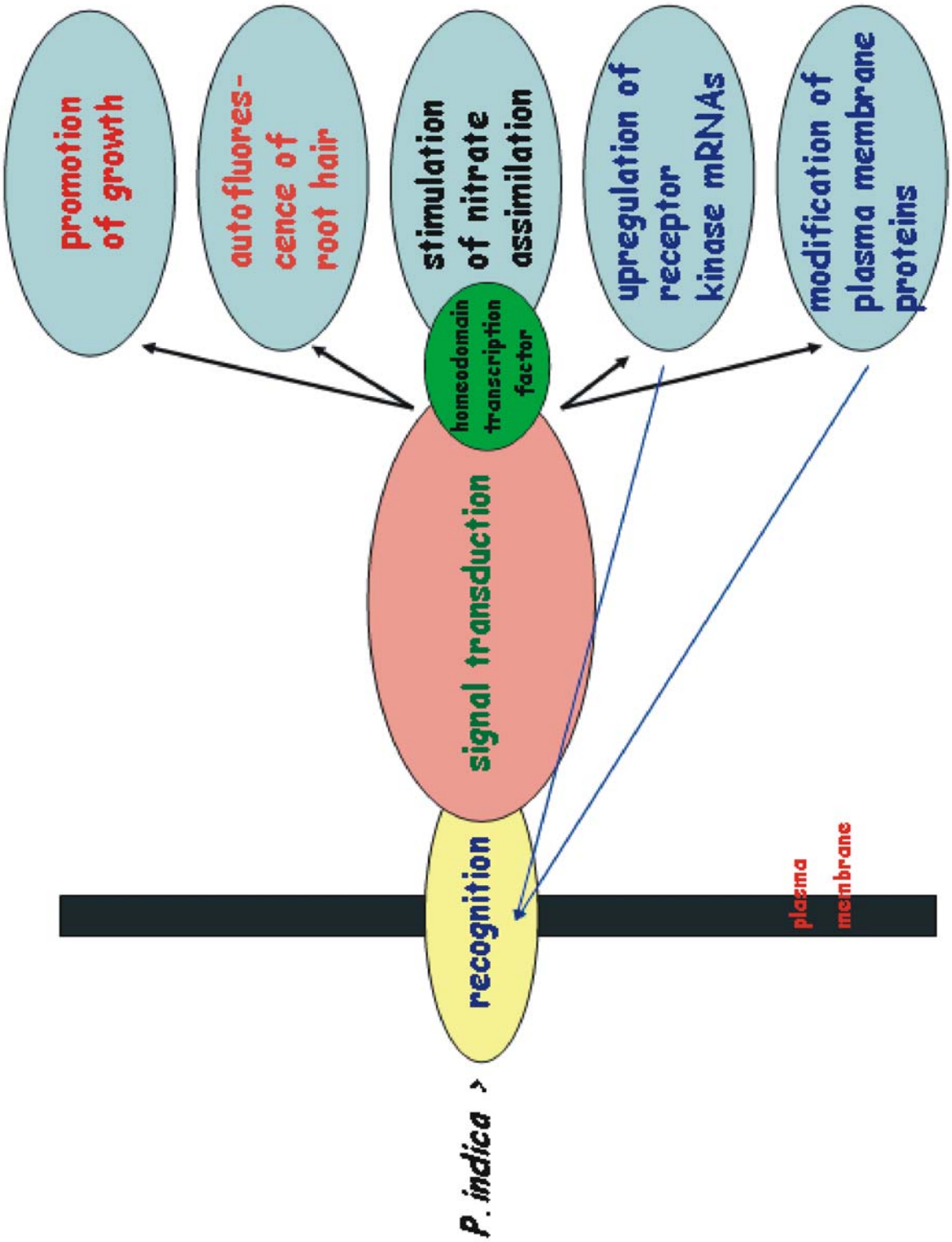


Figure 1: A scheme which describes five cellular functions which become activated in *Arabidopsis* roots after co-cultivation with *P. indica*.

The screen for mutants is based on at least five independent parameters which become apparent after the establishment of a positive interaction between both organisms (Fig. 1). First, we can monitor a strong autofluorescence in the root hairs. This fluorescence cannot be detected if both organisms grow together on the same Petri dish, but do not interact with each other (Peškan-Berghöfer et al. 2004). Thus, the initial screen focused on those lines which fail to produce the autofluorescence in the presence of the fungus. The nature of the autofluorescence is unknown at present. Second, we observe a growth-promoting effect. This can first be observed for the *Arabidopsis* roots (approx. 4-5 days after co-cultivation on Petri dishes) and later also for the aerial parts of the seedlings (approx. 5-6 days after co-cultivation on Petri dishes). Growth promotion is visible throughout the whole life, even after transfer of the seedlings to soil, and results in a faster growth and a larger seed yield (Peškan-Berghöfer et al. 2004). Third, between 3-9 days after co-cultivation, the fungus induces a modification of a plasma membrane protein, At3g20370, in the *Arabidopsis* roots which can easily be monitored on two dimensional gels (cf. below). This modification is only transient and disappears again, as soon as the interaction between both organisms is established. Fourth, as mentioned above, *P. indica* stimulates the expression of several genes in the roots. For the mutant screen, we monitor the transcript level for a receptor kinase, At5g16590, because the stimulatory effect of *P. indica* on this mRNA level is also only transient during early phases of the recognition. Fifth, growth promotion is associated with a stimulatory effect on nitrate assimilation in the roots. We monitor this effect by measuring nitrate reductase activity (cf. below).

Genes and proteins involved in early recognition events between P. indica *and* Arabidopsis thaliana

The information which we gathered over the last few years shed light on early steps during the recognition between both organisms (cf. Fig. 2). We first observed a modification of a MATH protein (At3g20370) which resulted in a different electrophoretic mobility of this protein on two-dimensional gels (Peškan-Berghöfer et al. 2004). This modification was only transient and disappeared again, as soon as the interaction between both organisms was established (Oelmüller et al. submitted). The nature of this modification is not known at present, however MATH proteins contain phosphorylation and glycosylation sites which might be responsible for the observed alteration in the electrophoretic mobility. The fungus did not only induce a modification of the MATH protein, but also stimulated its overall amount, consistent with the observation that a slight increase in the amount of the MATH protein mRNA in *Arabidopsis* roots was observed after the co-cultivation with *P. indica*. 59 genes for MATH proteins are present in the *Arabidopsis* genome, however the function of the proteins has not yet been studied so far (cf. Sunnerhagen et al. 2002; Oelmüller et al. submitted). Some members of this protein family exhibit strong sequence similarities to extracellular metalloproteases. It remains to be determined whether such an activity might be required for the recognition of signals from the fungus. It is also worth noting that MATH proteins are involved in nodule formation in *Medicago* (Gamas et al. 1996) and protein degradation in the *Arabidopsis* cytosol (Yan et al. 2000).

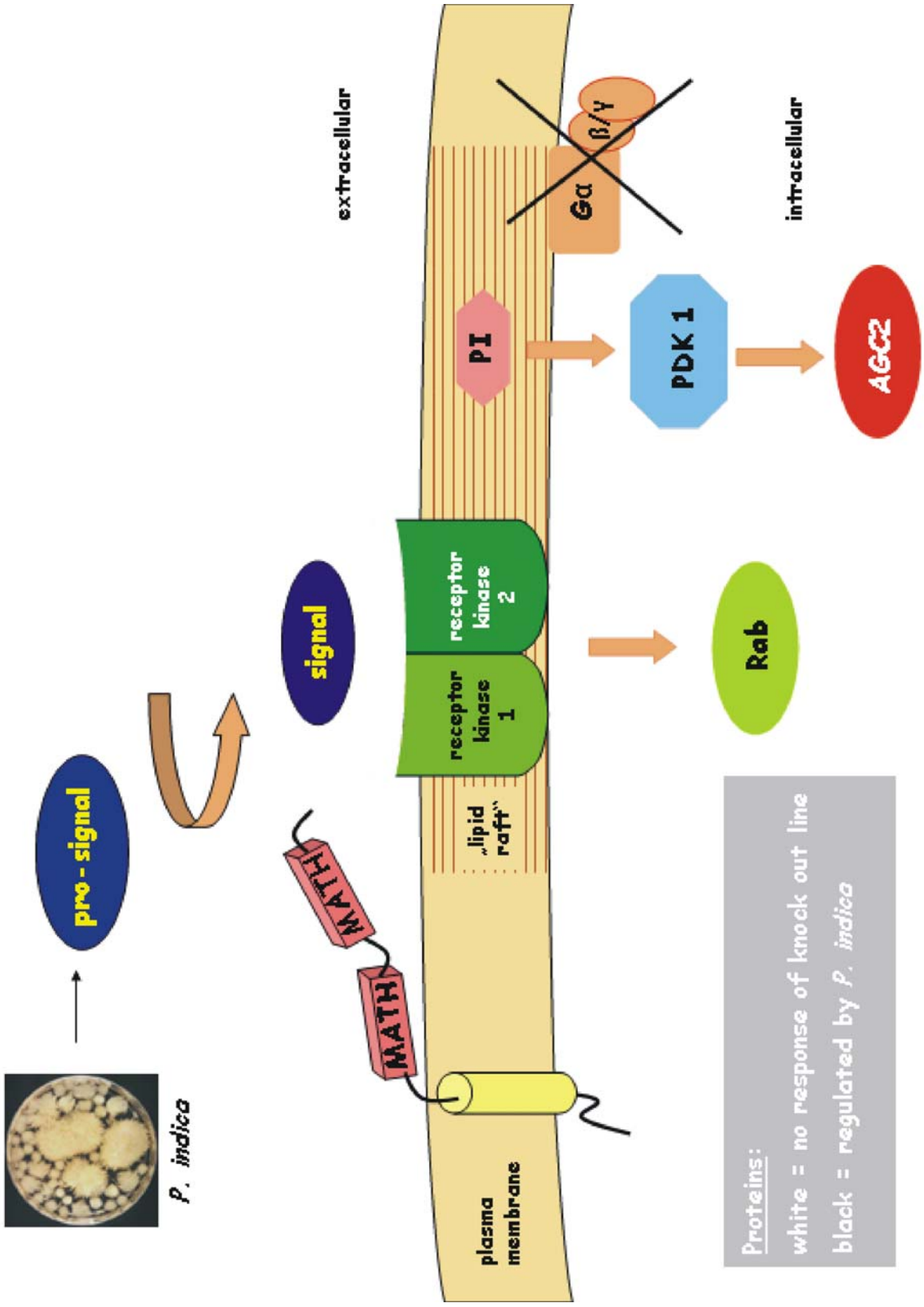


Figure 2: A model which describes putative proteins and signalling components in early steps of the recognition between *Arabidopsis* roots and *P. indica*. For details, see text. MATH, MATH domain in MATH proteins; PI, phosphoinositides; PDK1, 3'-phosphoinositide dependent kinase 1; AGC2, AGC-type kinase; Gα; Gβ, Gγ, α, β, γ subunit of heterotrimeric G proteins; Rab, small GTPase

Analyses of gene expression profiles uncovered that the message for a plasma membrane-localized receptor kinase (At5g16590) is transiently upregulated in RNA preparations from *Arabidopsis* roots during early phases of the interaction between both organisms (Shahollari et al. 2004a). Since the messages for 29 other receptor kinases did not respond to the fungus, we have analysed this gene in greater details. We first observed that the increase in the mRNA level was observed only during a period of approximately 72 h, i.e. the time period, when the interaction between *Arabidopsis* roots and *P. indica* became established. This suggests that the protein might be specifically involved in early recognition events between both organisms. Using mass spectrometry, we could also identify this protein in plasma membrane preparations from roots. Further fractionation of plasma membrane preparations uncovered that the receptor kinase is present in a subfraction with similarities to lipid rafts from mammals (Shahollari et al. 2004b). The observation that the receptor kinase can easily be detected by mass spectrometry suggests further that it is present in relatively large amounts in the plasma membrane from *Arabidopsis* roots.

We have then analysed the plasma membrane subfraction in greater details. It contains 34 polypeptides and all of them were identified by mass spectrometry (Shahollari et al. 2004b). Seven of these polypeptides are receptor kinases. It was also apparent that signalling components are highly enriched in these vesicles, among them we found several small GTP-binding proteins (Shahollari et al. 2004b). Dis-integration of these vesicles by sonication at 30°C in the presence of elevated levels of detergents and subsequent separation of the vesicles on sucrose gradients uncovered that most of the polypeptides remained on the top of the gradient. However, one fraction contained at least three polypeptides: two receptor kinases, At5g16590 and a so far uncharacterized receptor kinase and a small GTP-binding protein. A knock out line for the so far uncharacterized receptor kinase revealed that the mutant is strongly retarded in its response to *P. indica*. Again, the message for this receptor kinase also responds to the fungus. Thus, we propose that the two receptor kinases are involved in the recognition of *P. indica* at the plasma membrane of *Arabidopsis* roots.

The small GTP binding protein, which co-purifies with the two receptor kinases after solubilzation of plasma membrane microdomains belongs to the Rab-family and might be a good candidate for transducing the activating fungal signal to downstream events. The involvement of small GTP-binding proteins in plant/microbe interaction is not new. At least 93 genes for small GTP-binding proteins are present in the *Arabidopsis* genome (Fig. 3a, b; cf. also Vernoud et al. 2003). The similarities between several GTP-binding proteins of one subfamily suggest that they have overlapping functions. We are currently trying to understand the role of individual GTP-binding proteins in the interaction between *Arabidopsis* and *P. indica* in greater details.

Besides growth promoting effects, the interaction between both organisms can be followed by other parameters, one of them is the strong autofluorescence in the root hairs mentioned above (cf. above, Peškan-Berghöfer et al. 2004). We have used these parameters (cf. Fig. 1) to screen for mutants which fail to respond to *P. indica*. One of the mutants, which show normal plant development, but fails to exhibit growth promotion in response to *P. indica*, has a lesion in the kinase

Figure 3a: A neighbour-joining tree of *Arabidopsis* small GTP-binding proteins. The protein sequences of 93 small GTPases genes of *A. thaliana* were aligned using *MultAlin* (Corpet 1988). This procedure resulted in the elimination of the Attg07410 gene, one of the 57 Rab GTPases (Vernoud et al. 2003). Afterwards the alignment was edited manually to point out the conserved protein sequences. A phylogenetic tree prediction with 100 bootstrap replicates was made with *TreeTop* (Yushmanov and Chumakov 1988; Brodsky et al. 1995). The resulting dataset established a basis to compile an unrooted tree diagram using *Phylip* (Felsenstein 1989).

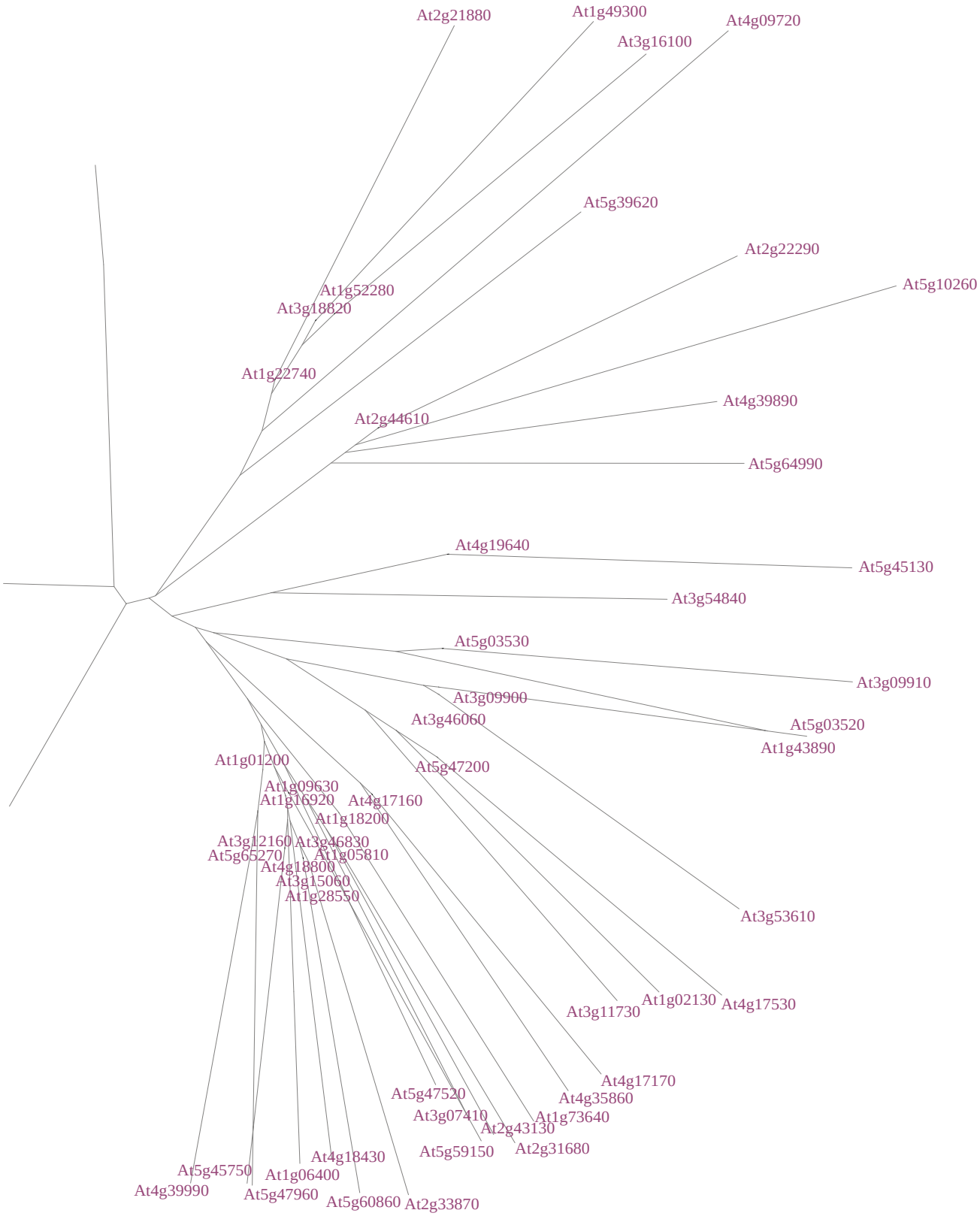


Figure 3b: A neighbour-joining tree of *Arabidopsis* Rab GTPases. Enlargement of the Rab GTPases branch shown in Fig. 3a.

AGC2 (At3g25250). This kinase has recently been identified to be required for proper root hair development (Anthony et al. 2004). AGC2 interacts with 3´-phosphoinositide-dependent kinase-1 (PDK1), which, in turn, becomes activated by phosphatidic acid (PA) at the plasma membrane (Anthony et al. 2004, and references therein). In animals, PDK1 is a central integrator for many different signalling events downstream of various receptors. Among other processes, the animal PDK1 is crucial for growth, cell division and apoptosis (cf. Alessi 2001). The functional homolog in *Arabidopsis* (Storz and Toker 2002) might be involved in similar signalling events, although the enzyme lacks two conserved amino acid residues in the C-terminal Peckstrin Homology (PH) domain that interacts with high affinity with the PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 second messangers and with low affinity to PtdIns(4,5)P_2. The *Arabidopsis* PDK1 is expressed in all plant tissues analysed so far, whereas expression of its interaction partner, AGC2, which was identified in our screen, is abundant in fast growing organs and dividing cells (Anthony et al., 2004). AGC2 is highly dynamic in root hairs, confined to root hair tips and nuclei and knock out mutations result in a reduction of root hair length, suggesting a role for this kinase in root hair growth and development (Anthony et al. 2004). Interesting, the first interactions that can be detected during the co-cultivation of *P. indica* with *Arabidopsis* roots were observed at the root hairs. We are currently analysing different AGC2 knock-out lines in greater details. The data obtained so far allow already the conclusions, that recognition of *P. indica* by *Arabidopsis* roots involves PA, PDK1 and downstream signalling kinases of the AGC type.

Mastoporan is a known activator of heterotrimeric G proteins and triggers PA signalling *via* phospholipase C and D activation (Munnik et al., 1995). Since Mas7, but not its non-functional analogue Mas17 rapidly activates AGC2 *in vitro*, Anthony et al. (2004) suggested that a G-protein-coupled receptor upstream of PA might activate PDK1 in *Arabidopsis*, leading to subsequent PDK1-dependent activation of AGC2. However, studies with transgenic tobacco plants overexpressing an antisense construction for the β subunit of the heterotrimeric G protein revealed that a more than 95% reduction of this subunit does not affect the growth - promoting effect induced by *P. indica* (Peškan-Berghöfer et al. 2004; Sherameti et al. submitted). This implies that heterotrimeric G proteins are not required for the recognition of *P. indica* in tobacco roots, and that AGC2 might be activated *via* other signalling cascades. This also implies that AGC2 is activated by a different upstream component.

Deak et al. (1999) and Anthony et al. (2004) have shown that the PH domain of the *Arabidopsis* PDK1 binds PI(4,5)P_2 and PA. Binding of these lipids increase the activity of PDK1, whereas AGC2 activity is only regulated by PA in a PDK1-dependent manner. PA controls many signalling events in animals. For instance, it regulates membrane trafficking, and it is involved in activation of the enzyme NADPH oxidase, which functions as part of the defence mechanism against infection and tissue damage during inflammation. Formation of PA has also been linked to a variety of responses in plants, such as biotic or abiotic stresses, dormancy, formation of nodules and root hairs (Meijer and Munnik 2003). In animals, PA can be generated from diacylglycerols by the action of a diacylglycerol kinase. However, a more important route

is *via* hydrolysis of other phospholipids by the enzyme phospholipase D (or by a family of related enzymes of this kind). 12 members of the phospholipase D family are present in the *Arabidopsis* genome, but it is still unknown how these PA-generating enzymes are coupled to downstream signalling events. Downstream candidates are MAP kinases, however unravelling of the complete signalling events require more detailed analyses of many mutants, which fail to respond to *P. indica*. Protein/protein interaction studies are one way of defining signalling networks in biological systems. Our approach clearly puts AGC2 into a physiological context, in that it defines a specific role of this enzyme in plant/microbe interaction.

Although the data obtained so far are only the beginning of the understanding of the interaction between *P. indica* and *Arabidopsis* roots, several observations provide a solid basis for further analyses. The specific and transient alterations of the MATH protein in the plasma membrane and of the mRNA level for the receptor kinase At5g16590 during the early phases of the co-cultivation of both organisms are likely to be functionally related to recognition events. Inactivation of the receptor kinase At1g13230, which can be copurified with At5g16590, strongly retards the interaction between both organisms during early phases of the recognition. A screen for mutants with lesions in the recognition machinery identifies a protein that is involved in root hair development (Anthony et al. 2004). Figure 2 presents a working hypothesis for our future studies. In analogy to the Toll system from *Drosophila* (cf. De Gregorio et al. 2002) and similar to the recognition of rhizobia by legumes (cf. Introduction) fungal signals might be recognized by the extracellular MATH domains of the MATH protein. Upon activation of the fungal signals, one or more receptor kinases can be activated. The physical contact of the two receptor kinases (which respond to the presence of *P. indica*) to a small GTP binding protein suggests that they might be involved in downstream signalling events. Growth promotion requires AGC2. How this kinase is integrated into the signalling events is unknown at present, however, Anthony et al. (2004) have shown that AGC2 become activated by PDK1 and PA. Finally, it appears that heterotrimeric G proteins are not involved in this scenario.

In order to couple the recognition processes to downstream events, we analysed nitrate assimilation in the roots of *Arabidopsis* after infection with *P. indica* (Sherameti et al. submitted). Nitrate reductase activity increases in the presence of the fungus. Western analyses confirmed that the increase is caused by an elevated level of the protein rather than an activation of pre-existing enzyme molecules. Northern analyses suggests that the fungus stimulates nitrate reductase by increasing the amount of its message. We also analysed the *Nia2* promoter from *Arabidopsis* in transgenic tobacco and found that it also responds to *P. indica* in the tobacco background. This suggests that the recognition mechanisms are conserved among different plant species. A crucial *cis* element in this promoter binds to a homeodomain protein. Surprisingly, also the message for this transcription factor is upregulated by *P. indica*. This suggests that the homeodomain transcription factor plays a crucial role in stimulating nitrate reductase gene expression in response to *P. indica*.

Acknowledgement

Research was supported by the Deutsche Forschungsgemeinschaft (SFB 604, to R.O.), the Deutscher Akademischer Austauschdienst (to B.S.), the International Max-Planck Research School (to M.O.), the Alexander-von-Humboldt-Foundation (to A.V.) and the Friedrich-Schiller-University of Jena.

References

ALESSI DR (2001) Discovery of PDK1, one of the missing links in insulin signal transduction. Colworth Medal Lecture. Biochem Soc Trans 29, 1-14

ANÉ JM, KISS GB, RIELY BK, PENMETSA RV, OLDROYD GE, AYAX C, LEVY J, DEBELLE F, BAEK JM, KALO P, ROSENBERG C, ROE BA, LONG SR, DENARIE J, COOK DR (2004) Medicago truncatula DMI1 required for bacterial and fungal symbioses in legumes. Science 303, 1364-1367

ANTHONY RG, HENRIQUES R, HELFER A, MESZAROS T, RIOS G, TESTERINK C, MUNNIK T, DEAK M, KONCZ C, BÖGRE L (2004) A protein kinase target of a PDK1 signalling pathway is involved in root hair growth in Arabidopsis. EMBO J 23, 572-581

BRODSKY LI, IVANOV VV, KALAIDZIDIS YL, LEONTOVICH AM, NIKOLAEV VK, FERANCHUK SI, DRACHEV VA (1995) GeneBee-NET: Internet-based server for analyzing biopolymers structure. Biochem 60, 923-928

CORPET F (1988) Multiple sequence alignment with hierarchical clustering. Nucl Acids Res 16, 10881-10890

DEAK M, CASAMAYOR A, CURRIE RA, DOWNES CP, ALESSI DR (1999) Characterisation of a plant 3-phosphoinositide-dependent protein kinase-1 homologue which contains a pleckstrin homology domain. FEBS Lett 451, 220-226

DE GREGORIO E, SPELLMAN PT, TZOU P, RUBIN GM, LEMAITRE B (2002) The Toll and Imd pathways are the major regulators of the immune response in Drosophila. EMBO J 21, 2568-2579

ENDRÉ G, KERESZT A, KEVEI Z, MIHACEA S, KALO P, KISS GB (2002) A receptor kinase gene regulating symbiotic nodule development. Nature 417: 962-966

FELSENSTEIN J (1989) PHYLIP – Phylogeny inference package (Version 3.2). Cladistics 5, 164-166

GAMAS P, NIEBEL FC, LESCURE N, CULLIMORE J (1996) Use of a subtractive hybridization approach to identify new Medicago truncatula genes induced during root nodule development. Mol Plant Microbe Interact 9, 233-242

GLEN M, TOMMERUP IC, BOUGHER NL, O´BRIEN PA (2002) Are Sebacinaceae common and widespread ectomycorrhizal associates of Eucalyptus species in Australian forests? Mycorrhiza 12, 243-247

HARRISON MJ (1999) Molecular and cellular aspects of the arbuscular mycorrhizal symbiosis. Ann Rev Plant Physiol Plant Mol Biol 50, 631-689

KISTNER C, PARNISKE M (2002) Evolution of signal transduction in intracellular symbiosis. Trends Plant Sci 7, 511-518

KOTTKE I, BEITER A, WEIß M, HAUG I, OBERWINKLER F, NEBEL M (2003) Heterobasidiomycetes form symbiotic associations with hepatics: Jungermanniales have sebacinoid mycobionts while Aneura pinguis (Metzgeriales) is associated with a Tulasnella species. Mycol Res 107, 957-968

KUMARI R, YADAV HK, BHOON YK, VARMA A (2003) Colonization of cruciferous plants by *Piriformospora indica.* Curr Sci 85, 1672-1674

LEVY J, BRES C, GEURTS R, CHALHOUB B, KULIKOVA O, DUC G, JOURNET EP, ANE JM, LAUBER E, BISSELING T, DENARIE J, ROSENBERG C, DEBELLE F (2004) A putative Ca^{2+} and calmodulin-dependent protein kinase required for bacterial and fungal symbioses. Science 303, 1361-1364

MARX J (2004) The roots of plant-microbe collaborations. Science 304, 234-236

MEIJER HJ, MUNNIK T (2003) Phospholipid-based signaling in plants. Annu Rev Plant Biol 54, 265-305

MUNNIK T, ARISZ SA, DE VRIJE T, MUSGRAVE A (1995) G protein activation stimulates phospholipase D signaling in plants. Plant Cell 7, 2197-2210

NEWMAN EL, REDDEL P (1987) The distribution of mycorrhizas among families of vascular plants. New Phytol 106, 745-751

OELMÜLLER R, PEŠKAN-BERGHÖFER T, SHAHOLLARI B, TREBICKA A, SHERAMETI I, VARMA A (2004) MATH-domain proteins represent a novel protein family in *Arabidopsis thaliana* and at least one member is involved in plant/microbe interaction. Submitted

PARNISKE M (2000) Intracellular accommodation of microbes by plants: a common development program for symbiosis and disease? Curr Op Plant Biol 3, 320-328

PARNISKE M (2004) Molecular genetics of the arbuscular mycorrhizal symbiosis. Curr Op Plant Biol 7, 414-421

PEŠKAN-BERGHÖFER T, SHAHOLLARI B, GIONG PH, HEHL S, MARKERT C, BLANKE V, KOST G, VARMA A, OELMÜLLER R (2004) Association between *Piriformospora indica* and *Arabidopsis thaliana*: a novel system to study beneficial plant-microbe interactions Physiol Plant (in press)

PHAM G H, KUMARI R, SINGH AN, SACHDEV M, PRASAD R, KALDORF M, BUSCOT F, OELMÜLLER R, PESKAN T, WEISS M, HAMPP R, VARMA A (2004) Axenic cultures of *Piriformospora indica.* In: Plant Surface Microbiology (eds: Varma A, Abbott L, Werner D and Hampp R), Springer-Verlag, Germany pp. 593-616

SHAHOLLARI B, PEŠKAN-BERGHÖFER T, OELMÜLLER R (2004b) Receptor kinases with leucine-rich repeats are enriched in Triton X-100 insoluble plasma membrane microdomains from plants. Physiol. Plant (in press)

SHAHOLLARI B, VARMA A, OELMÜLLER R (2004a) Expression of a receptor kinase in *Arabidopsis* roots is stimulated by the basidiomycete *Piriformospora indica* and the protein accumulates in Triton X-100 insoluble plasma membrane microdomains. J Plant Physiol (in press)

SINGH AN, SINGH AR, KUMARI M, RAI MK, VARMA A (2003) Importance of *Piriformospora indica* - A novel symbiotic mycorrhiza-like fungus: An Overview. Ind J of Biotechnol 2, 65-75

STORZ P, TOKER A (2002) 3'-phosphoinositide-dependent kinase-1 (PDK-1) in PI 3-kinase signaling. Front Biosci 7, 7886-7902

STRACKE S, KISTNER C, YOSHIDA S, MULDER L, SATO S, KANEKO T, TABATA S, SANDAL N, STOUGAARD J, SZCZYGLOWSKI K, PARNISKE M (2002) A plant receptor-like kinase required for both bacterial and fungal symbiosis. Nature 417, 959-962

SUNNERHAGEN M, PURSGLOVE S, FLADVAD M (2002) The new MATH: homology suggests shared binding surfaces in meprin tetramers and TRAF trimers. FEBS Lett 530, 1-3

VARMA A., SINGH A, SUDHA, SAHAY N, SHARMA J, ROY A, KUMARI M, RANA D, THAKRAN S, DEKA D, BHARTI K, FRANKEN P, HUREK T, BLECHERT O, REXER K-H, KOST G, HAHN A, HOCK B, MAIER W, WALTER M, STRACK D, KRANNER I (2001) *Piriformospora indica*: A cultivable mycorrhiza-like endosymbiotic fungus. In: Mycota IX, Springer Series, Germany, pp. 123-150

VARMA A, VERMA S, SUDHA, SAHAY N, BRITTA B, FRANKEN P (1999) *Piriformospora indica* – a cultivable plant growth promoting root endophyte with similarities to arbuscular mycorrhizal fungi. Applied and Environmental Microbiology USA 65, 2741-2744

VARMA A, SINGH A, SUDHA, SAHAY N, SHARMA J, ROY A, KUMARI M, RANA D, THAKRAN S, DEKA D, BHARTI K, FRANKEN P, HUREK T, BLECHERT O, REXER K-H, KOST G, HAHN A, HOCK B, MAIER W, WALTER M, STRACK D, KRANNER I (2001) *Piriformospora indica*: a cultivable mycorrhiza – like endosymbiotic fungus. In: Mycota IX, Springer Series, Germany, pp. 123-150

VERMA SA, VARMA A, REXER K-H, HASSEL A, KOST G, SARBHOY A, BISEN P, BÜTEHORN B, FRANKEN P (1998) *Piriformospora indica*, gen. et sp. nov., a new root-colonizing fungus. Mycologia 90: 898-905

VERNOUD V, HORTON AC, YANG Z, NIELSEN E (2003) Analysis of the small GTPase gene superfamily of Arabidopsis. Plant Physiol 131, 1191-1208

WEIß M, OBERWINKLER F (2001) Phylogenetic relationships in *Ariculariales* and related groups – hypotheses derived from nuclear ribosomal DNA sequences. Myol Res 105, 403-415

YAN N, DOELLING JH, FALBEL TG, DURSKI AM, VIERSTRA RD (2000) The ubiquitin-specific protease family from Arabidopsis. AtUBP1 and 2 are required for the resistance to the amino acid analog canavanine. Plant Physiol 124, 1828-1843

YUSHMANOV SV, CHUMAKOV KM (1988) Algorithms of the maximum topological similarity phylogenetic trees construction. Mol Genet Microbiol Virusol (Russian) 3, 9-15

Received: 17. 10. 2004
Accepted: 03. 11. 2004

Manuscript VII

The endophytic fungus *Piriformospora indica* stimulates the expression of nitrate reductase and the starch-degrading enzyme glucan-water dikinase in tobacco and *Arabidopsis* roots through a homeodomain transcription factor that binds to a conserved motif in their promoters.

Irena Sherameti, Bationa Shahollari, Yvonne Venus, Lothar Altschmied, Ajit Varma, Ralf Oelmüller

J. Biol. Chem. (2005) **280**(28):26241-26247.

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 280, No. 28, Issue of July 15, pp. 26241–26247, 2005

Printed in U.S.A.

The Endophytic Fungus *Piriformospora indica* Stimulates the Expression of Nitrate Reductase and the Starch-degrading Enzyme Glucan-water Dikinase in Tobacco and *Arabidopsis* Roots through a Homeodomain Transcription Factor That Binds to a Conserved Motif in Their Promoters*

Received for publication, January 13, 2005, and in revised form, February 14, 2005
Published, JBC Papers in Press, February 14, 2005, DOI 10.1074/jbc.M500447200

Irena Sherameti‡, Bationa Shahollari‡, Yvonne Venus‡, Lothar Altschmied§, Ajit Varma¶, and Ralf Oelmüller‡‖**

From the ‡Institute of General Botany and Plant Physiology, Friedrich Schiller University Jena, 07743 Jena, Germany, §Institut für Pflanzengenetik und Kulturpflanzenforschung (IPK), D-06466 Gatersleben, Germany, and ¶School of Life Sciences, Jawaharlal Nehru University, New Delhi 110067, India

***Piriformospora indica*, an endophytic fungus of the Sebacinaceae family, promotes growth of *Arabidopsis* and tobacco seedlings and stimulates nitrogen accumulation and the expression of the genes for nitrate reductase and the starch-degrading enzyme glucan-water dikinase (*SEX1*) in roots. Neither growth promotion nor stimulation of the two enzymes requires heterotrimeric G proteins. *P. indica* also stimulates the expression of the *uidA* gene under the control of the *Arabidopsis* nitrate reductase (*Nia2*) promoter in transgenic tobacco seedlings. At least two regions (−470/−439 and −103/−89) are important for *Nia2* promoter activity in tobacco roots. One of the regions contains an element, ATGATAGATAAT, that binds to a homeodomain transcription factor *in vitro*. The message for this transcription factor is up-regulated by *P. indica*. The transcription factor also binds to a CTGATAGATCT segment in the *SEX1* promoter *in vitro*. We propose that the growth-promoting effect initiated by *P. indica* is accompanied by a co-regulated stimulation of enzymes involved in nitrate and starch metabolisms.**

Often nitrogen is the limiting source for plant growth and development. It is recruited by plants either as nitrate or ammonium or for a few species by nitrogen fixation with the help of rhizobia (1, 2). Mycorrhizal fungi also play an important role in delivering either nitrate or ammonium to the root cells. It is believed that mycorrhizal fungi preferentially recruit ammonium rather than nitrate from the soil and that amino acids represent the major compounds that serve to transfer nitrogen to the host plant (*cf.* Refs. 3 and 4). We studied *Piriformospora indica*, an endophytic fungus of the Sebacinaceae family, which colonizes the roots of a wide variety of plant species and promotes their growth (5–10). The interaction of the endophytic fungus with plant roots is accompanied by an enormous requisition of nitrogen from the environment. By analyzing the interaction of *P. indica* with *Arabidopsis* and tobacco roots we found that in contrast to mycorrhizal associations, nitrate reduction in the roots is stimulated by *P. indica*. A homeodomain transcription factor responds to the fungus and binds to promoter regions of the *P. indica*-responsive *Nia2*, *SEX1*, and 2-nitropropane dioxygenase genes. These results suggest that the expression of *P. indica*-responsive target genes may be controlled by common regulatory elements and *trans*-factors.

MATERIALS AND METHODS

Transgenic Tobacco—Transgenic seeds of *Nicotiana tabacum* L., var. Samsun NN were obtained from greenhouse-grown plants (6). They were sterilized and germinated on Murashige-Skoog medium (11) supplemented with 2% (w/v) sucrose and 0.8% (w/v) agar in temperature-controlled (25 °C) growth chambers under a 16-h light/8-h dark cycle. 80 μg of ml^{-1} (w/v) kanamycin was added to the medium. Four-week-old plantlets were transferred to soil to obtain seeds for the physiological experiments. The antisense lines for the heterotrimeric G protein subunit β were described previously (12).

Growth Conditions of Plant and Fungus—For physiological experiments in Petri dishes, transgenic or wild-type tobacco or *Arabidopsis* seeds were surface-sterilized and placed on Petri dishes containing Murashige and Skoog (11) nutrient medium. After cold treatment at 4 °C for 48 h, plates were incubated for 10 days (*Arabidopsis thaliana*) or 14 days (*Nicotiana tabacum*) at 22 °C under continuous illumination (100 μmol m^{-2} sec^{-1} photosynthetic active radiation). *P. indica*, a cultivable plant growth-promoting root endophyte (10), and *Pisolithus tinctorius* were cultured as described previously (6, 13).

Co-cultivation Experiments, Determination of Fresh and Dry Weight, Protein Content, and Nitrate Uptake—14-day-old tobacco (or 10-day-old *Arabidopsis*) seedlings were transferred to nylon disks (mesh size 70 μm) and placed on top of a modified MMN[1] culture medium ($MMN_{1/10}$ medium with a 1/10 ratio of nitrogen and phosphorus and no carbohydrate) (14) in 90-mm Petri dishes. After 24 h, fungal plugs of 5 mm in diameter were placed at a distance of 1 cm from the roots. Plates were incubated at 22 °C under continuous illumination from the side (maximum 80 μmol m^{-2} s^{-1} photosynthetic active radiation).

Root length was measured with a ruler. The fresh weight of the roots and aerial parts was determined directly. Proteins were extracted into a 5-ml extraction buffer (50 mM Tris-HCl, pH 8.0, 0.1% SDS), precipitated with trichloroacetic acid, and the protein concentration was determined according to Lowry. Dry weight of the roots and aerial parts was determined after incubation of the tissue at 105 °C for 16 h.

For pot experiments, 9-day-old sterile tobacco seedlings were transferred to sterile soil in pots (25-cm diameter). Aliquots of the soil were

* This work was supported by Grant SFB604 from the Deutsche Forschungsgemeinschaft. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** Present address: Amity Institute of Herbal and Microbial Studies, Sector 125, Noida 201303, Uttar Pradesh, India.

‖ To whom correspondence should be addressed: Inst. of General Botany and Plant Physiology, Friedrich Schiller University Jena, Dornburger Strasse 159, 07743 Jena, Germany. Tel.: 49-3641-949230; Fax: 49-3641-949232; E-mail: b7oera@hotmail.com.

[1] The abbreviations used are: MMN, Marx-Melin-Norkrans; RT, reverse transcription; NR, nitrate reductase; MS-MS, tandem mass spectrometry; Gβ, β-subunit of heterotrimeric GTP-binding proteins; GUS, β-glucuromidase.

mixed with 1% (w/v) fungus and put into preformed holes surrounding the roots of the seedling. Growth was followed in temperature-controlled (25 °C) growth chambers under a 16-h light/8-h dark cycle.

Analysis of the Nia2 Promoter—Some transgenic tobacco lines harboring *Nia2*-promoter::*uidA* gene fusions were described previously (15). For the analyses performed here, the *Nia2* 5′-upstream region −1088/−1 (relative to the ATG start codon) of *At1g37130* was fused to the *uidA* reporter gene. Manipulations of the −1088/−1 fragment occurred in pBSC$^+$ (Stratagene, San Diego, CA).

Starting from the −1088/−1 *Nia2* fragment in pBSC$^+$, 5′-deletions were obtained with exonuclease III digestions. After religation, the clones were sequenced. Further analyses were performed with the following fragments: −1088/−1, −650/−1, −628/−1, −470/−1, −438/−1, −361/−1, −310/−1, −111/−1, and −89/−1. Although seeds from all plants were analyzed in parallel, only those plants that gave important information for this study are mentioned under "Results."

Site-directed mutagenesis was performed according to Mikaelian and Sergeant (16) and specified in Ref. 17. The 5′-end of the promoter segment, obtained after PCR with genomic DNA from *A. thaliana*, ecotype Landsberg, was located toward the 3′-end of the vector. The mutagenized DNA was obtained by three successive PCRs. An oligonucleotide with the mutagenized region and 12 authentic nucleotides on each side and the T7 primer were used for the first reaction. The second reaction was performed with the T3 primer and an oligonucleotide (5′-AAAAAACCGCTCTAGAACTAGTG-3′), which primes 20 bp 3′ of the T7 primer. This oligonucleotide contains a 5′-mismatched end (*cf.* Ref. 16). The amplified fragments were purified on agarose gels, and 10 ng of each of them was used for the third PCR reaction with the T3 and T7 primers. The final products were ligated into pBSC$^+$ and sequenced before transfer to pBI101 (18) as BamHI/SalI fragments. After triparental mating (19) and plant transformation, 20 independent lines per construct were generated. For physiological experiments with the seeds of the F_1 generation, detailed analyses were performed only for those constructs relevant for this study. The transcription start site of the *Nia2* promoter was determined with *Arabidopsis* root RNA and primer extension analysis.

For GUS staining, seedlings were harvested and immediately put into 5-bromo-4-chloro-3-indolyl β-D-glucopyranoside (X-Glc) solution (50 mg of X-Glc, 1 ml of dimethylformamide, 4.9 ml of 50 mM sodium phosphate, pH 7.0, 250 μl of Me_2SO, 500 μl of potassium hexacyanoferrate (III) (100 mM), 500 μl of potassium hexacyanoferrate (II) (100 mM)) and incubated overnight at 37 °C. After washing with water, the seedlings were incubated in 70% ethanol and stored at 4 °C. For GUS staining of root hairs, seeds were germinated and seedlings were grown in liquid Murashige and Skoog medium to avoid hair damage.

RNA Preparation and Quantitative RT-PCR—Total RNA from root material was isolated with the TRIzol reagent (Invitrogen). RT-PCR analysis was performed by reverse transcription of 5 μg of total RNA with gene-specific reverse primers (see below). First strand synthesis was performed with a kit (K1631) from MBI Fermentas (St. Leon-Roth, Germany). After 20 PCR cycles, the products were analyzed on 1.5% agarose gels and stained with ethidium bromide; visualized bands were quantified with the Image Master Video System (Amersham Biosciences). For Northern analysis, gene-specific primers were designed to amplify four DNA fragments from our cDNA library (20): *Nia2* (At1g37130), *SEX1* (At1g10760), and the genes for the homeodomain transcription factor (At2g35940) and for 2-nitropropane dioxygenase (At5g64250). The primers were designed such that they amplified the entire coding region including 4 nucleotides up- and downstream of the genes.

Gel Mobility Shift Assays—Gel mobility assays were performed with a fraction enriched in root nuclei proteins from *A. thaliana*. Approximately 10 g of *Arabidopsis* roots was used to isolate a fraction enriched in nuclei (21). The extracted proteins were further purified on heparin-Sepharose (Amersham Biosciences) columns. After elution with 700 mM KCl, the protein fraction was dialyzed against NEB buffer (25 mM HEPES, pH 7.8, 50 mM KCl, 0.1 mM EDTA, 14 mM β-mercaptoethanol) and concentrated using spin columns (Amicon, Witten, Germany). These protein factions were used for gel mobility shift assays and the filter binding assay.

Five pairs of oligonucleotides (Nia2-a, ATGATAGATAAT, Nia2-b, ATTATCTATCAT; Nia2mu-a, ATGATGCATAAT; Nia2mu-b, ATTATGCATCAT; SEX1-a, CTGATAGATCT; SEX1-b, AGATCTATCAG; SEX1mu-a, CTGATGCATCT; SEX1mu-b, AGATGCATCAG; NpdO-a, AGGATCGATGA; NpdO-b, TCATCGATCCT) were annealed and cloned into the SmaI site of pBSC$^+$. After restriction of the recombinant plasmid DNA with EcoRI and XbaI, the recessive ends were filled in with Klenow enzyme and radiolabeled nucleotides, and the insert was isolated by polyacrylamide gel electrophoresis. For the filter binding assay, the fragments were excised from the plasmid with XbaI and EcoRI and purified on polyacrylamide gels (5%).

Enzyme Assays—The nitrate reductase (NR) and GUS assays were described earlier (15, 17). In both instances the system of reference was an equal amount of fresh weight.

Mass Spectrometry—Proteins extracted from membrane fractions were further purified by two rounds of methanol precipitation before digestion with trypsin (6). Alternatively, silver-stained gel spots from the gels were excised and the proteins extracted into 500 μl of 50 mM ammonium bicarbonate supplemented with 60 ng/μl trypsin. After lyophilization, the pellet was resuspended in 5 μl of water/acetonitrile/formic acid (95:5:0.1) prior to liquid chromatography-MS analysis. Peptide analyses, analyte sampling, chromatography, and acquisition of data were performed on a LC (Famos-Ultimate; LC-Packings) coupled with an LCQ Deca XP ion trap mass spectrometer according to the manufacturer's instructions.

The measured MS-MS spectra were matched with the amino acid sequences of tryptic peptides from the *A. thaliana* data base in FASTA format. Cys modification by carbamidomethylation (+57 Da) was taken into account, and known contaminants were filtered out. Raw MS-MS data were analyzed by the Finnigan Sequest/Turbo Sequest software (revision 3.0; ThermoQuest, San Jose, CA). The parameters for the analysis by the Sequest algorithm were set according to Stauber *et al.* (22). The similarity between the measured MS-MS spectrum and the theoretical MS-MS spectrum, reported as the cross-correlation factor (x_{corr}) was above 2.95 and 3.85 for doubly or triply charged precursor ions, respectively. To identify corresponding loci, identified protein sequences were subjected to BLAST search at NCBI (www.ncbi.nlm.nih.gov/) and FASTA searches by using the AGI protein data base at The Arabidopsis Information Resource (www.arabidopsis.org/).

Macroarray Analyses—RNA was isolated from *Arabidopsis* roots 2 and 5 days after co-cultivation with *P. indica*. The macroarray filters used for the hybridization and the hybridization conditions have been described previously (23). Genes identified in these studies and those that responded to *P. indica* were further analyzed in separate RT-PCR studies. Only those genes that are relevant for this study are mentioned here.

Miscellaneous—DNA extraction was performed according to standard protocols (24). Western analyses with polyclonal antibodies raised against nitrate reductase (gift from Dr. K.-J. Appenroth) and the β-subunit of heterotrimeric G proteins from tobacco (25) were performed according to Stöckel and Oelmüller (26). Denaturing polyacrylamide gel electrophoresis was performed with the buffer system from Laemmli (27). Determination of total nitrogen was performed with Kjeldahl equipment and protocols provided by "behr Labor-Technik" (Düsseldorf, Germany). Roots of control and inoculated plants were stained with cotton (blue) before examination under the light microscope (Zeiss Axioplan model MC 100).

RESULTS

Co-cultivation of Tobacco Seedlings with P. indica—14-Day-old tobacco seedlings were transferred to $MMN_{1/10}$ medium and inoculated with *P. indica*. The fungal inoculum was placed 1 cm away from the roots. $MMN_{1/10}$ medium was chosen because it contains low concentrations of phosphate and nitrate and no carbon source, conditions known to promote the interaction between plants and symbiotic fungi. The fungus grew slowly on the co-cultivation medium and produced only a few spores. A difference in root growth was not observed within the first 2 days of co-cultivation. After 5 days, a stimulatory effect of *P. indica* on root growth became visible. After 7 days, the inoculated seedlings were significantly larger and heavier when compared with control seedlings, and after 10 days the size of the tobacco seedlings was substantially larger (*cf.* Fig. 1*A*, *a* and *b*, *cf.* also *B–D*). We also inoculated plants with the ectomycobiont *P. tinctorius* because it was shown that hypaphorine, a major indolic compound from this fungus, has an impact on *Arabidopsis* root growth (28). However, seedlings inoculated with *P. tinctorius* did not differ from the uninoculated controls (Fig. 1*A*, *c–f*). The stimulatory effect of *P. indica* on the growth of tobacco was still detectable 6 weeks after transfer of the seedlings to soil, and inoculated plants were much bigger compared with their controls (Fig. 1*E*). This is not

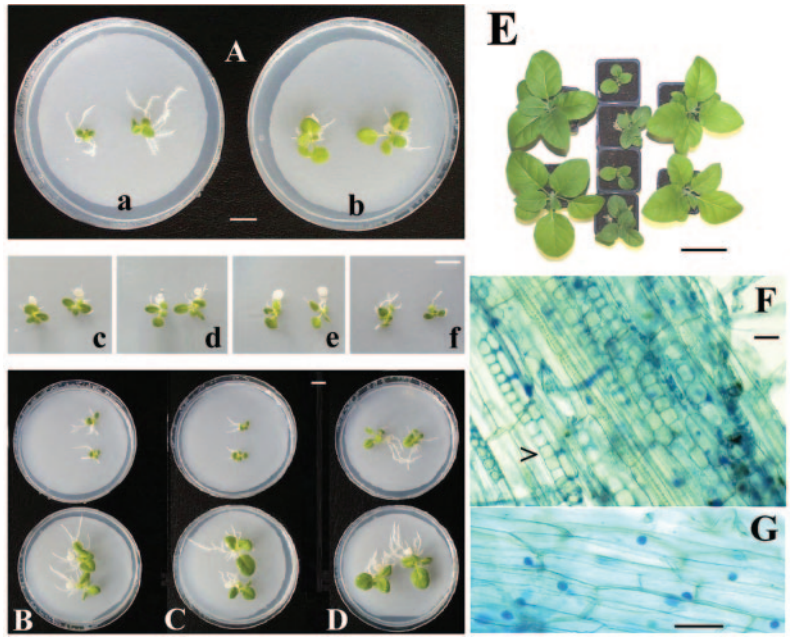


FIG. 1. **Tobacco plants grown in the presence or absence of *P. indica* or *P. tinctorius*.** *A*, 24-day-old tobacco seedlings grown either in the absence (*a* and *f*) or presence of *P. indica* (*b*) or *P. tinctorius* (*c–e*). *Bars* represent 1 cm. *B–D*, 24-day-old tobacco seedlings overexpressing an antisense block for the β-subunit of heterotrimeric GTP-binding proteins. *B*, line 14-4; *C*, line 15-4; *D*, line 15-6, representing independent primary transformants. *Top*, without *P. indica*; *bottom*, with *P. indica*. *E*, tobacco plants 6 weeks after transfer of the seedlings to pots. *Middle*, without *P. indica*; *left* and *right*, with *P. indica*. *Bar* represents 10 cm. *F*, the outer cell layers of inoculated roots under the light microscope (Zeiss Axioplan model MC 100). *Arrowhead*, fast dividing fungal cells, which are in focus. *Bar* represents 100 μm. *G*, root epidermal cells stained with cotton (*blue*). *Bar* represents 100 μm.

surprising considering the extensive colonization of the outer cell layers of the roots by the fungal hyphae (Fig. 1, *F* and *G*). After the initial lateral growth (Fig. 1*F*), the fungus produced spores, which could be detected in almost all outer root cells (Fig. 1*G*). It can be concluded that under the given conditions, growth of tobacco seedlings is substantially stimulated by *P. indica*, similar to previous observations with *A. thaliana* seedlings[2] (6, 30, 31).

P. indica Stimulates Nitrate Uptake and Nitrate Reductase Gene Expression—After 10 days of co-cultivation with *P. indica* the fresh weight of the aerial parts of the tobacco seedlings was enhanced by 42.2 ± 3.8%, the dry weight by 41.0 ± 4.0%, and the total protein content by 42.2 ± 3.1% (n = 480 seedlings). This was accompanied by substantial recruitment of nitrogen from the agar medium. Based on equal amounts of fresh weight, the nitrogen content in the aerial parts of the seedlings increased by 21.4 ± 4.4%. Thus, considering the larger size of the cotyledons, ~60% more nitrogen must have been transferred from the medium to the aerial parts of the seedlings. This prompted us to investigate the effect of *P. indica* on nitrate assimilation in more detail. We focused on seedlings grown on agar because quantitative analyses were much easier when compared with adult plants grown on soil.

Co-cultivation of tobacco seedlings with *P. indica* causes a 50.2 ± 4.2% increase in the plant-specific NADH-dependent NR activity in the roots. Western analysis with antibodies against plant NR confirmed that the higher NADH-dependent enzyme activity correlated with an increase in the amount of the root enzyme (Fig. 2). Equal loading of root protein extracts was confirmed with an antibody against the β-subunit of heterotrimeric GTP-binding proteins (25). Thus, in contrast to mycorrhizal symbioses, the endophytic fungus *P. indica* stimulates the assimilatory enzyme NR. The stimulatory effect of *P. indica* on NR is predominantly found in roots and to a much lesser extent in the shoots (12.2 ± 1.2%). Thus, the higher nitrogen level in the aerial parts of the seedlings after co-cultivation with *P. indica* must be caused by more efficient nitrate assimilation in the roots. In principle, the same results were obtained for *Arabidopsis* seedlings except that the overall stimulation of NR activity in the roots was only 29.8 ± 3.1%.

[2] Oelmüller, R., Peškan-Berghöfer, T., Shallohari, B., Trebicka, A., Sherameti, I., and Varma, A. (2005) *Physiol. Plant.* **124,** 152–166.

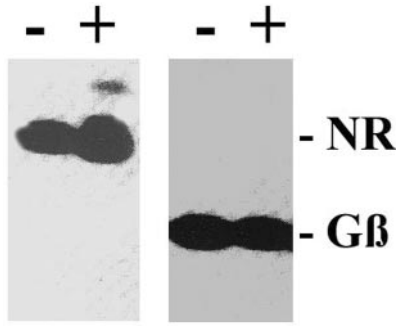


FIG. 2. **Western analyses with antibodies against nitrate reductase (*left*) and the Gβ (*right*, control).** Protein extracts from 24-day-old tobacco roots were either grown in the absence (−) or presence (+) of *P. indica*. 25 μg of protein was loaded per lane.

The *Arabidopsis Nia2* 5′-upstream region −1088/−1 (relative to the ATG codon, the transcription start site is located 88 nucleotides upstream of the ATG codon) fused to the β-glucuronidase gene (*uidA*) was introduced into *Nicotiana*. When co-cultivated with *P. indica*, a stimulatory effect can also be monitored at the level of transgene expression (Table I). Comparable with the results obtained for NR activity, *P. indica* stimulated *uidA* gene expression in tobacco roots by ~50%. Again, a much lower stimulatory effect was observed in shoots (~10–15%).

Deletions of the promoter from −1088 to −470 had no significant effect on *uidA* expression, whereas further deletion to −438 caused an ~60% decrease in *uidA* expression in roots but not in shoots. Thus, the region −470/−439 appears to be important for the promoter activity in roots.

Replacement of AG by GC in the ATGATAGATAAT sequence (−459/−448) within this region causes the same decrease in GUS activity as deletion from −470/−1 to −438/−1 (Table I). Furthermore, although the −111/−1 segment is active in roots and shoots, deletion to −89 completely abolishes the promoter activity. Thus, an additional crucial *cis*-element(s) for *Nia2* expression appears to be located in the region between −111 and the transcription start site at position −88. Random site-directed mutagenesis in the latter region in the context of the −1088/−1 fragment followed by expression analysis in transgenic tobacco revealed that the two GT nucleotides directly

TABLE I

GUS activity in 24-day-old transgenic tobacco seedlings, grown in either the presence (+) or absence (−) of P. indica

The *uidA* gene was expressed under the control of the *Arabidopsis Nia2* promoter fragment −1088/−1, −470/−1, −438/−1 or Mu (−470/−1, in which AG at position −454/−453 was replaced by GC). Experimental details, cultivation of the seedlings, and the enzyme assays are described under "Materials and Methods." The enzyme activity was determined separately for shoots and roots. For each value, 490 individual plants were analyzed. The system of reference is fresh weight. The protein content in the shoots and roots increased linearly with the fresh weight. Errors are given as S.E.s.

	GUS activity							
	−1088/−1		−470/−1		−438/−1		Mu	
	−	+	−	+	−	+	−	+
	nmol/g^{-1} *min*$^{-1}$							
Shoot	87.1 ± 4.1	102.2 ± 4.1	89.2 ± 3.4	98.7 ± 4.1	89.5 ± 6.2	107.3 ± 6.3	91.7 ± 3.9	103.4 ± 5.1
Root	11.3 ± 0.2	21.3 ± 0.1	10.0 ± 0.2	20.1 ± 0.2	1.7 ± 0.2	8.9 ± 0.3	1.5 ± 0.1	8.3 ± 0.8

upstream of the transcription start site and the CA nucleotides at position −97/−96 play a crucial role in the promoter activity *in vivo* because replacement of GT by AA and of CA by GC completely abolished gene expression. The close vicinity of the CA and GT motifs to the transcription start site makes it unlikely that this region functions as a TATA box element. Taken together, the −470/−439 and −97/−89 regions upstream of the ATG codon are essential for *Nia2* promoter activity in transgenic tobacco.

GUS staining revealed that the promoter was active in almost all of the living cells of the roots (Fig. 3). High GUS activity is found in the living cells of the vascular tissue. In the larger cells surrounding the vascular tissue, the stain is mainly detectable in the narrow cytoplasmic tubes attached to the plasma membrane. Most of the GUS staining was observed in the cytoplasm of the root hair (Fig. 3, *upper panel*, *cf.* also Ref. 15). Semiquantitative analysis revealed that the stimulatory effect of *P. indica* on *uidA* gene expression in the root hairs is at least 2-fold, *i.e.* significantly higher than the effect observed for the entire root (data not shown). When grown in the presence of *P. indica*, a significant stimulation of *uidA* expression in the roots was detected for all active fragments tested. However, the extent of the stimulatory effect in the roots declines dramatically when the promoter is deleted from −470 to −438 (Table I). Thus, in addition to its specific role for *Nia2* promoter activity in roots, the −470/−439 region also functions as a *P. indica*-responsive element in the *Nia2* promoter. A stimulatory effect in response to *P. indica* was still measurable for the −110/−1 promoter fragment (24 ± 2%), although to a lesser extent. Thus sequences within this segment in combination with or in addition to the *P. indica*-responsive element are involved in *P. indica*-mediated *Nia2* expression. A fusion of the −470/−439-bp region to the −90-bp cauliflower mosaic virus minimal promoter did not respond to *P. indica* (data not shown).

A double-stranded nucleotide from the −459/−448 segment ATGATAGATAAT shows a retarded band in gel mobility shift assays with nuclear extracts from *Arabidopsis* roots (Fig. 4). No retardation is detectable with the double-stranded mutant oligonucleotide ATGATGCATAAT. Furthermore, the binding can be competed with an excess of the original but not with mutant oligonucleotide (Fig. 4). These and other results (*cf.* below) indicate that root nuclei from *Arabidopsis* contain a protein(s), which binds to the *P. indica*-responsive element in the *Nia2* promoter.

The Nia2 Promoter Binds a Homeodomain Transcription Factor in Vitro, and the Message for the Transcription Factor Is Up-regulated in Response to P. indica—The *Nia2* (and the mutant) double-stranded promoter segments were immobilized on nylon membranes. The membranes were then incubated with nuclear extract from *Arabidopsis* roots for ~60 h at 6 °C under continuous shaking. The filters were washed five times

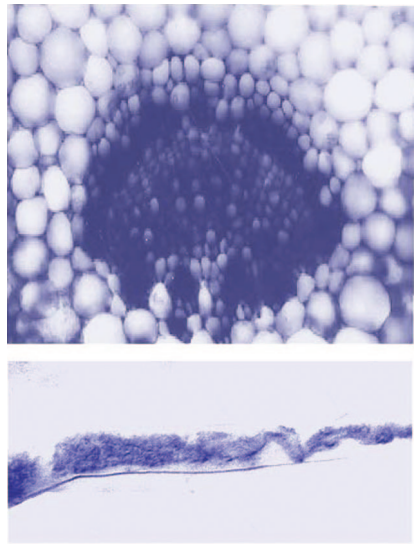


FIG. 3. *Upper panel*, cross-section through a root from a 24-day-old tobacco seedling stained for GUS. *Lower panel*, a growing root hair; GUS staining is visible in the cytoplasm. The seedling expresses the *uidA* gene under the control of the −1088/−1 bp *Nia2* promoter fragment from *A. thaliana*.

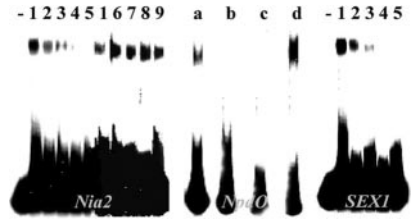


FIG. 4. **Gel mobility shift assays with the *Nia2* promoter fragment ATGATAGATAAT, the *NpdO* promoter fragment AGGATCGATGA, and the *SEX1* fragment CTGATAGATCT with nuclear extract from *Arabidopsis* roots.** The figure also shows cross-competition experiments between the binding activities to these fragments. −, lanes without nuclear extract. *Lane 1*, retardation assay with ATGATAGATAAT (*Nia2)*; *lanes 2–5*, competition with increasing concentrations of cold fragment (*Nia2)*; *lanes 6–9*, competition with increasing concentrations of cold ATGATGCATAAT fragment (*Nia2)*. *Lanes a* and *d*, retardation assay with AGGATCGATGA (*NpdO*); *lane b*, competition with excess *Nia2* fragment ATGATAGATAAT (*NpdO*); *lane c*, competition with excess SEX1 fragment CTGATAGATCT (*NpdO*). *Lane 1*, retardation assay with CTGATAGATCT (*SEX1*); *lanes 2–5*, competition with increasing concentrations of the *Nia2* fragment ATGATAGATAAT (*SEX1*). For experimental details, *cf.* "Materials and Methods."

with 90 mM NaCl (10 min each, first at 6 °C, then at 18 °C). Finally, DNA-bound proteins were solubilized by boiling the membranes in loading buffer, and the extracted proteins were analyzed on SDS-polyacrylamide gels. Two dominant protein bands of ~75 and 160 kDa, which are not detectable in protein

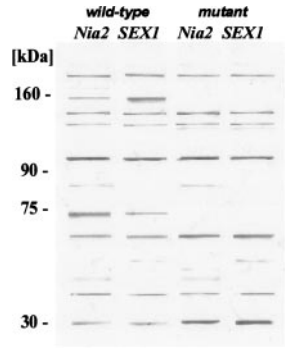


FIG. 5. **Silver-stained gel with proteins extracted from filter-bound oligonucleotides.** Wild-type Nia2, ATGATAGATAAT; wild-type SEX1, CTGATAGATCT; mutant Nia2, ATGATGCATAAT; mutant SEX1, CTGATGCATCT. *Left*, sizes in kDa.

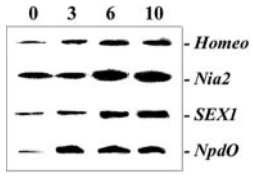


FIG. 6. **Northern analyses for the homeodomain transcription factor (*Homeo*), *Nia2*, *SEX1*, and 2-nitropropane dioxygenase (*NpdO*) genes.** RNA was extracted from *Arabidopsis* roots 0, 3, 6, and 10 days after co-cultivation with *P. indica*. An equal amount of RNA (25 μg) was loaded per lane.

extracts from the control oligonucleotide (Fig. 5), can be eluted from the *Nia2* oligonucleotide. The bands were extracted from the gel and analyzed by mass spectrometry after trypsin digestion. The lower band corresponds to the *Arabidopsis* fragments SLGEEDSVSGVGR and TSDETMMQPINADFSSNEK. Searches of the literature show that the upper band correspond to GSCGNDK and PVELGTAER. The four peptides are present in the homeodomain protein At2g35940. In the upper band, we could also identify peptides that correspond to other homeodomain transcription factors; however, the x_{corr} values were too low to allow conclusions. It appears that the upper band contains components of a larger, unresolved complex together with At2g35940 (*cf.* "Discussion").

Northern analyses uncovered that the message for *At2g35940* is up-regulated in roots co-cultivated with *P. indica* (Fig. 6). Stimulation of the homeodomain protein mRNA level was observed before the *Nia2* mRNA level increased.

The Expression-relevant Upstream Region ATGATAGATAAT of the Nia2 Promoter Exhibits Sequence Similarities to a SEX1 Promoter Region for the Starch-degrading Enzyme Glucan-water Dikinase and to a Region in the 2-Nitropropane Dioxygenase (At5g64250) Promoter, and the Messages of All Three Genes Are Up-regulated in Response to P. indica—Computer analyses uncovered that sequences with similarities to the expression-relevant ATGATAGATAAT element are also present in the *SEX1* promoter (−1182, CTGATAGATCT, −1172) and the promoter of the 2-nitropropane dioxygenase (At5g64250) (−238, AGGATCGATGA, −228). A gel shift assay with double-stranded oligonucleotide sequences from these two promoter regions confirmed that they also bind to protein factors from root nuclei extracts (Fig. 4). Because both binding activities competed with the *Nia2* promoter sequence, it is likely that they bind to the same or similar DNA-binding proteins (Fig. 4). Furthermore, filter binding assays with the *SEX1* promoter segment led to the identification of the same two protein bands of 75 and 160 kDa, which also bind to the *Nia2* promoter sequence, although the amounts of the two bands relative to each other differed for the two fragments (Fig. 5). Mass spectrometry identified a peptide that corresponds to PVELGTAER in the lower band and to LSNMLHEVEQR in the upper band; both are present in At2g35940. No reproducible data with this assay could be obtained for the *At5g64250* promoter segment, presumably because the binding activity to this fragment was too low (data not shown). Macroarray and Northern analyses confirmed that the *SEX1* and *At5g64250* messages also respond to *P. indica*. Although the *SEX1* and *Nia2* messages accumulate with similar kinetics (Fig. 6), the *At5g64250* message begins to accumulate earlier, although later than the message for the homeodomain transcription factor (Fig. 6). The enzyme glucan-water dikinase catalyzes the phosphorylation of starch by a dikinase-type reaction in which the β-phosphate of ATP is transferred to either the C-6 or the C-3 position of the glycosyl residue of amylopectin. As a consequence, transitory starch is more rapidly degraded (*cf.* Refs. 32–34). Up-regulation of the message for this enzyme in response to *P. indica* might have two functions. Starch breakdown products released from the root amyloplasts might be required for growth that is promoted by *P. indica* or for the export to the fungus, which is dependent on sucrose supply from the plant. It appears that nitrate assimilation and starch degradation is co-regulated in *Arabidopsis* roots via the same *cis*-elements in their promoters. 2-Nitropropane dioxygenase might have different functions in the interaction. The enzyme incorporates molecular oxygen into 2-nitropropane and releases acetone and nitrite. Thus, the enzyme might detoxify 2-nitropropane, which is generated under stress or as a defensive toxin (35), and generates nitrite, which might serve as an additional nitrogen source. Plants also detoxify 2-nitropropane from the environment because it is used commercially as a solvent, although it is a known mutagen (36, 37).

Growth Promotion and Stimulation of Nitrate Assimilation by P. indica Does Not Require Heterotrimeric G Proteins—We tested whether *P. indica* can stimulate growth and NR in tobacco lines expressing an antisense construct for the β-subunit of heterotrimeric GTP-binding proteins (Gβ). Previously, we have characterized three tobacco lines in which Gβ was severely reduced in green leaves (12). Fig. 7 demonstrates that a comparable reduction of Gβ can also be observed in root. Severely reduced levels of Gβ do not inhibit the stimulatory effect of the fungus on growth (Fig. 1, *B–D*) and NR activity (data not shown). Thus, bulk Gβ is not required for *P. indica* action.

DISCUSSION

Nitrate Assimilation Is Stimulated by P. indica—We demonstrate that co-cultivation of tobacco and *Arabidopsis* seedlings with *P. indica* is accompanied by a massive transfer of nitrogen from the agar plates into the aerial part of the seedlings, an observation that is not surprising considering the growth promotion caused by the fungus. This effect is associated with a stimulation of the NADH-dependent NR, the key enzyme of nitrate assimilation in plants. Whether the stimulation of nitrate assimilation by *P. indica* is the reason for the growth promotion or the result of it remains to be determined. A stimulatory effect of mycorrhizal associations has also been reported for nitrate uptake into tomato root cells (38). However, recruitment of nitrogen in endophytic interactions differs from mycorrhizal interactions in which the fungus preferentially recruits ammonium rather than nitrate from the soil (*cf.* Refs. 3 and 4). Moreover, several studies have demonstrated that after the establishment of ectomycorrhizal symbioses, the fungal NR is increased and the plant enzyme down-regulated (4, 38, 39). Apparently in mycorrhizal symbioses, amino acids represent the major compounds that serve to transfer nitrogen to the host plant (38). We did not study NR in the fungal hyphae,

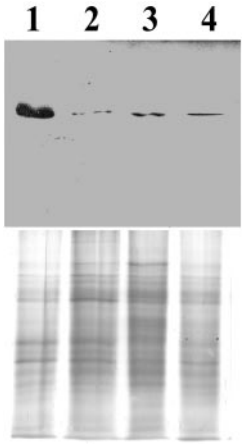


FIG. 7. **Western blot for Gβ in root protein extracts from three antisense plants.** *Lane 1*, wild-type extract; *lanes 2–4*, extract from three antisense lines (14-4, 15-4, and 15-6, representing independent primary transformants). The protein extracts were prepared from the roots of the seedlings shown in Fig. 1, *B–D*. *Bottom*, stained gel to show equal protein loading. 25 μg of protein was loaded per *lane*.

and thus we cannot exclude that the fungal NR also contributes to nitrate assimilation. However, it appears that the fungal NR alone cannot account for the entire nitrate assimilation.

A Homeodomain Transcription Factor Appears to Be Involved in Nitrate Assimilation—P. indica activates uptake of nitrate and induces a signaling pathway, which ultimately leads to a higher transcription of *Nia2*, *SEX1*, and the gene for 2-nitropropane dioxygenase. The ATGATAGATAAT sequence in the *Nia2* promoter was shown to be crucial for the regulation of the expression, and a homeodomain transcription factor binds to this sequence *in vitro*. Binding activity was also observed to a related motif in the *SEX1* promoter. The 2-nitropropane dioxygenase promoter also contains a conserved motif; however, we could not demonstrate conclusively that this element also binds to the same transcription factor. The homeodomain factor was identified by mass spectrometry. Most strikingly, the message for the transcription factor itself was up-regulated by the fungus in *Arabidopsis* roots. This was first discovered by macroarray analyses with 4000 randomly chosen cDNAs from *Arabidopsis*; the homeodomain transcription factor attracted attention because its message was among the few transcription factor messages, which was up-regulated by the fungus in *Arabidopsis* roots.[3] Later, this result was confirmed by RT-PCR and Northern analyses. Because homeodomain proteins are involved in the transcriptional regulation of key eukaryotic developmental processes, we propose that this factor might play a crucial role in coupling the expression of *P. indica*-responsive genes to upstream signaling events. We also propose that the genes identified in this study (for enzymes involved in nitrate assimilation, starch degradation, and detoxification of nitroalkanes) are not the only ones that are regulated by this transcription factor. Homeodomain proteins bind to DNA as monomers or as homo- and/or heterodimers in a sequence-specific manner, and thus their target genes depend on their interaction partners. The upper band on the SDS gel, which contains the homeodomain transcription factor, might therefore represent an unresolved protein-DNA complex, which contains another homeodomain partner(s). The best studied homologs of the protein identified in this study are the BEL1-like transcription factors from potato (40). Seven members of the BEL1 protein family interact with KNOX transcription factors, and they control vegetative development and tuber formation. A rice homolog of the homeodomain protein is inducible by benzothiadiazole, a component that induces disease resistance in *Arabidopsis* and wheat by activation of the systemic acquired resistance signal transduction pathway (41, 42). Benzothiadiazole also activates resistance in sunflower to the root-parasitic weed *Orobanche cumana* (43). This suggests that the identified homeodomain transcription factor might also be involved in signaling pathways related to plant/microbe interaction.

Hoth *et al.* (44) have identified *At2g35940* as one of the genes that are up-regulated in response to abscisic acid. Whether abscisic acid- and *P. indica*-signaling in roots is related to each other is unknown at present.

Heterotrimeric GTP-binding Proteins Are Not Involved in P. indica-induced Growth Promotion and Nitrate Assimilation—Heterotrimeric GTP-binding proteins are tested here because they are involved in many signaling events including those for plant/microbe interactions. However, antisense tobacco lines with severely reduced Gβ protein levels exhibit a normal growth response to *P. indica* and contain elevated levels of NR. This indicates that bulk levels of heterotrimeric G proteins are not involved in *P. indica*-induced signaling events. A similar result has been described for the defense of barley against the powdery mildew fungus (45).

A Comparative Analysis of Promoter Regions Led to the Identification of New Genes with a Similar Response Pattern—We identified four genes that are up-regulated in response to *P. indica*, *i.e.* the gene for the homeodomain transcription factor itself as well as three genes that share a conserved sequence element. For two of these elements (the ATGATAGATAAT motif in the *Nia2* promoter and the CTGATAGATCT motif in the *SEX1* promoter) we could show binding to the homeodomain transcription factor *in vitro*. Comparison of these two sequences with the AGGATCGATGA element in the promoter of the 2-nitropropane dioxygenase suggests that the central GAT(A/C) GAT(C/T) sequence might be crucial for binding. TAGA is also part of the binding site of the homeodomain protein POU3F1 (29). The three enzymes have in common that they are involved in key metabolic processes in roots (nitrate assimilation, starch degradation, and detoxification of nitro compounds). It remains to be determined whether other genes contain similar DNA binding sites and thus might also be regulated by the homeodomain transcription factor.

Acknowledgments—We thank Dr. K.-J. Appenroth for carefully reading the manuscript and for providing the antibodies against NR, Günther Gessner for nitrate determination, Drs. T. Peškan-Berghöfer, Victor Kusnetsov, and Sudhir Sopory for generating the transgenic lines used in these studies, and Martin Rienth and Heidi Renz for the generation of *Nia2* promoter deletions and for the initial studies with the promoter segments, which ultimately lead to the identification of the homeodomain protein.

[3] L. Altschmied and R. Oelmüller, unpublished results.

REFERENCES

1. Esseling, J. J., and Emons, A. M. (2004) *J. Microsc.* (*Oxf.*) **214,** 104–113
2. Gage, D. J. (2004) *Microbiol. Mol. Biol. Rev.* **68,** 280–300
3. Boukcim, H., and Plassard, C. (2003) *J. Plant Physiol.* **160,** 1211–1218
4. Guescini, M., Pierleoni, R., Palma, F., Zeppa, S., Vallorani, L., Potenza, L., Sacconi, C., Giomaro, G., and Stocchi, V. (2003) *Mol. Genet. Genomics* **269,** 807–816
5. Kumari, R., Yadav, H. K., Bhoon, Y. K., and Varma, A. (2003) *Curr. Sci.* **85,** 1672–1674
6. Peškan-Berghöfer, T., Markert, C., Varma, A., and Oelmüller, R. (2004) *Physiol. Plant.* **122,** 465–477
7. Pham, G. H., Singh, A. N., Malla, R., Kumari, R., Prasad, R., Sachdev, M., Rexer, K.-H., Kost, G., Luis, P., Kaldorf, M., Buscot, F., Herrmann, S., Peskan, T., Oelmüller, R., Saxena, A. K., Declerck, S., Mittag, M., Stabentheiner, E., Hehl, S., and Varma A. (2004) in *Plant Surface Microbiology* (Varma, A., Abbott, L., Werner, D., and Hampp, R., eds) pp. 237–265, Springer-Verlag New York Inc., New York
8. Singh, A. N., Singh, A. R., Kumari, M., Rai, M. K., and Varma, A. (2003) *Indian J. Biotech.* **2,** 65–75
9. Singh, A. N., Singh, A.R., Rexer, K.-H., Kost, G., and Varma, A. (2001) *J. Orchid Soc. India* **15,** 89–101
10. Varma, A., Verma, S., Sudha, Sahay, N.S., Butehorn, B., and Franken, P. (1999) *Appl. Environ. Microbiol.* **65,** 2741–2744
11. Murashige, T., and Skoog, F. (1962) *Plant Physiol.* **15,** 473–497

12. Peškan-Berghöfer, T., Neuwirth, J., Kusnetsov, V., and Oelmüller, R. (2005) *Planta* **5,** 737–746
13. Verma, S., Varma, A., Rexer, K.-H., Hassel, A., Kost, G., Sarabhoy, A., Bisen, P., Bütenhorn, B., and Franken, P. (1998) *Mycologia* **90,** 896–903
14. Marx, D. H. (1969) *Phytopathology* **59,** 153–163
15. Sherameti, I., Sopory, S. K., Trebicka, A., Pfannschmidt, T., and Oelmüller, R. (2002) *J. Biol. Chem.* **277,** 46594–46600
16. Mikaelian, I., and Sergeant, A. (1996) *Methods Mol. Biol.* **57,** 193–202
17. Flieger, K., Tyagi, A., Sopory, S., Cseplo, A., Hermann, R. G., and Oelmüller, R. (1993) *Plant J.* **4,** 9–17
18. Jefferson, R. A., Burgess, S. M., and Hirsh, D. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **893,** 8447–8451
19. Bevan, M. (1984) *Nucleic Acids Res.* **12,** 8711–8721
20. Sherameti, I., Shahollari, B., Landsberger, M., Westermann, M., Cherepneva, G., Kusnetsov, V., and Oelmüller, R. (2004) *Plant J.* **38,** 578–593
21. Oelmüller, R., Bolle, C., Tyagi, A. K., Niekrawietz, N., Breit, S., and Herrmann, R. G. (1993) *Mol. Gen. Genet.* **237,** 261–272
22. Stauber, E. J., Fink, A., Markert, C., Kruse, O., Johanningmeier, U., and Hippler, M. (2003) *Eukaryot. Cell* **2,** 978–994
23. Chen, I. P., Haehnel, U., Altschmied, L., Schubert, I., and Puchta, H. (2003) *Plant J.* **35,** 771–786
24. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York
25. Peškan, T., and Oelmüller, R. (2000) *Plant Mol. Biol.* **42,** 915–922
26. Stöckel, J., and Oelmüller, R. (2004) *J. Biol. Chem.* **279,** 10243–10251
27. Laemmli, U. K. (1970) *Nature* **227,** 275–280
28. Reboutier, D., Bianchi, M., Brault, M., Roux, C., Dauphin, A., Rona, J. P., Legue, V., Lapeyrie, F., and Bouteau, F. (2002) *Mol. Plant-Microbe Interact.* **15,** 932–938
29. Jeon, J. S., Jang, S., Lee, S., Nam, J., Kim, C., Lee, S. H., Chung, Y. Y., Kim, S. R., Lee, Y. H., Cho, Y. G., and An, G. (2000) *Plant Cell* **12,** 871–884
30. Oelmüller, R., Shahollari, B., Peškan-Berghöfer, T., Trebicka, A., Giong, P.-H., Sherameti, I., Oudhoff, M., Venus, Y., Altschmied, L., and Varma, A. (2004) *Endocyt. Cell Res.* **15,** 504–517
31. Shahollari, B., Varma, V., and Oelmüller, R. (2005) *J. Plant Physiol.*, in press
32. Reimann, R., Hippler, M., Machelett, B., and Appenroth, K. J. (2004) *Plant Physiol.* **135,** 121–128
33. Ritte, G., Lloyd, J. R., Eckermann, N., Rottmann, A., Kossmann, J., and Steup, M. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 7166–7171
34. Ritte, G., Scharf, A., Eckermann, N., Haebel, S., and Steup M. (2004) *Plant Physiol.* **135,** 2068–2077
35. Hanawa, F., Tahara, S., and Towers, G. H. (2000) *Phytochemistry* **53,** 55–58
36. Fiala, E. S., Conaway, C. C., and Mathis, J. E. (1989) *Cancer Res.* **49,** 5518–5522
37. Hite, M., and Skeggs, H. (1979) *Environ. Mutagen.* **1,** 383–389
38. Hildebrandt, U., Schmelzer, E., and Bothe, H. (2002) *Physiol. Plant.* **115,** 125–136
39. Kaldorf, M., Schmelzer, E., and Bothe, H. (1998) *Mol. Plant-Microbe Interact.* **11,** 439–448
40. Chen, H., Rosin, F. M., Prat, S., and Hannapel, D. J. (2003) *Plant Physiol.* **132,** 1391–1404
41. Gorlach, J., Volrath, S., Knauf-Beiter, G., Hengy, G., Beckhove, U., Kogel, K. H., Oostendorp, M., Staub, T., Ward, E., Kessmann, H., and Ryals, J. (1996) *Plant Cell* **8,** 629–643
42. Lawton, K. A., Friedrich, L., Hunt, M., Weymann, K., Delaney, T., Kessmann, H., Staub, T., and Ryals, J. (1996) *Plant J.* **10,** 71–82
43. Sauerborn, J., Buschmann, H., Ghiasvand Ghiasi, K., and Kogel, K.-H. (2002) *Phytopathology* **92,** 59–64
44. Hoth, S., Morgante, M., Sanchez, J.-P., Hanafey, M. K., Tingey, S. V., and Chua, N. H. (2002) *J. Cell Sci.* **115,** 4891–4900
45. Kim, M. C., Panstruga, R., Elliott, C., Muller, J., Devoto, A., Yoon, H. W., Park, H. C., Cho, M. J., and Schulze-Lefert, P. (2002) *Nature* **416,** 447–451

3. Discussion

Role of receptor kinases in cellular signalling processes.

Plant/microbe recognition is often mediated *via* receptor kinases (RKs) which exhibit a high degree of variation in their receptor domains and, after ligand binding and receptor oligomerization, activate an intracellular kinase domain (Torii 2004). The kinase domains play important roles in signalling, because they provide docking sites for substrates that are implicated in activating downstream phosphorylation cascades. Additionally, proteins that are involved in the regulation of receptor activity could directly bind to the kinase domain (Johnson and Ingram 2005).

An important mechanism for regulating plant RK signalling at the subcellular level is realized by restriction of their localisation to microdomains or lipid rafts. This mechanism is probably mediated by cytoplasmic interactions and was shown to be an important mechanism for the spatial control of signalling in animal systems (Foster *et al.* 2003). Formation of microdomain-like structures that contain specific plasma membrane proteins at sites of infection has recently been shown to be important in disease resistance (Bhat *et al.* 2005).

Studies of animal receptor kinases revealed that a ligand binds to the extracellular LRR domain and causes receptor dimerization, which triggers the subsequent activation of the intracellular kinase domain. The activated kinases then phosphorylate substrate proteins within the cell, resulting in the transduction of the signals (Hunter 1995). Recently, a number of RKs with this characteristic architecture have been identified also in plants. The sequence homology and structural similarity of animal and plant RKs suggests similar mechanisms of action of these signal transduction pathways (Zhang 1998).

Many RKs contain extracellularly located leucine-rich repeats (LRR), which might be involved in the perception of microbial signal molecules (Hause and Fester 2005). It is not clear whether ligand binding induces dimerization and/or phosphorylation of LRR-RKs. Likewise, it is not known whether forcing dimerization can trigger constitutively active signal transduction of plant LRR-RKs. In the majority of LRR-RKs, the LRR domain seems to play a role as receptor heterodimer, followed by the activation of the intracellular kinase domain. Mutations in the kinase domain inactivate the signalling

process, as shown for BRI1, CLV1, ERECTA, FLS2, and SYMRK/NORK, and confer severe phenotypes (Clark *et al.* 1997; Gomez-Gomez *et al.* 2001; Lease *et al.* 2001; Stracke *et al.* 2002; Endré *et al.* 2002). Several studies indicate that LRR-RKs act as dimers, and some may form a receptor complex with an LRR-domain that lacks the cytoplasmic kinase region (Torii 2004). Some LRR-RKs, including CLV1 and BRI1, may form a core receptor dimer in the absence of corresponding ligands, to which other compounds become associated after activation. For instance, CLV1 and CLV2 exist as a disulfide-linked core receptor dimer (Trotochaud *at al.* 1999). In yeast cells it was found that BRI1 and BAK1 constitute a ligand–independent dimer, because they associate with each other in the absence of the activating ligand (Li *et al.* 2002; Nam and Li 2002).

The identified ligands for LRR-RKs are structurally diverse, from steroids to peptides and secreted proteins. Studies of CLV1, BRI1, and FLS2 signaling pathways revealed that downstream components of LRR-RKs also differ substantially, although the logic of signal transduction, such as activation or repression of the downstream components *via* phosphorylation/dephosphorylation, appears universal (Torii 2004).

SYMRK, a RK involved in mycorrhiza and nodule formation in Legumes, favours a model in which the extracellular domain is involved in the perception of an extracellular ligand, and the signal is then transduced through the intracellular kinase domain. Whether the LRRs of SYMRK are required for the direct interaction with a signalling ligand, or they play a role in the assembly of a receptor complex, is still not known. It may be possible that bacterial and fungal signal molecules are sufficiently similar to be recognized by the same receptor, or alternatively, additional specific components are involved which perceive either the rhizobial Nod factors or an as yet hypothetical signal emanating from the arbuscular mycorrhiza fungus (Kistner and Parniske 2002). The fact that SYMRK has kinase activity supports furthermore the idea that the signal is transduced *via* a phosphorylation event (Yoshida and Parniske 2005). A popular conceptual model proposes that SYMRK/DMI2 acts upstream of DMI1 and regulates channel activity, probably by phosphorylation (Parniske 2004; Riely 2004). DMI1 is a candidate for mediating early ion fluxes across the plasma membrane, although its location is not clear at present (Hogg *et al.* 2006). In Legumes, mycorrhiza formation by fungi and nodule formation by nitrogen-fixing bacteria require common signalling components, due to the same components required for entry mechanism of both organisms into the root cell (Kistner and Parniske 2002;

Parniske 2004; Udvardi *et al.* 2005; Harrison 2005; Geurts *et al.* 2005). One of the crucial components required for both interactions in Legumes is DMI1 (Ané *et al.* 2004). DMI1 does not cause calcium spiking, and the early calcium flux into the root hairs is intact in the DMI1 mutant (Shaw and Long 2003). The calcium spiking response occurs in the DMI3 mutant, and therefore DMI3 is placed downstream of DMI1, SYMRK/DMI2 and the calcium spiking response (Wais *et al.* 2000). On the basis of the identification of DMI3 as a calcium-calmodulin-dependent kinase, it is anticipated that DMI3 perceives and transduces the calcium spiking signal, leading to the activation of downstream responses including the expression of early nodulation genes (Levy *et al.* 2004; Mitra *et al.* 2004). It has been shown in this study that inactivation of the DMI1 homolog in *A. thaliana*, a single copy gene expressed exclusively in roots, does not eliminate the interaction with the fungus *P. indica*. This means that the entry mechanism of the fungus into the *A. thaliana* root cell appears to differ from the entry of fungi and bacteria into Legume root cells. This raises the question whether Legume mycorrhiza utilize genetic programs, which differ from those in primitive plant-fungi interactions (Shahollari *at al.* 2006). More recently, Kost´s group (Marburg, personal communication) has provided evidence that *P. indica* can colonize root and root hair cells, but these cells are either dead or weakened. Although these studies were not confirmed for *A. thaliana*, they support our results that the colonization of root cells by *P. indica* differs from that of mycorrhiza fungi.
For the rhizobium-legume interaction, the input signal is defined and activation of the pathway occurs via the Nod factor receptors NFR1/LYK3 and NFR5 (Limpens *et al.* 2003; Madsen *et al.* 2003; Radutoiu *et al.* 2003). For the arbuscular mycorrhiza, the input and, consequently, the beginning of the pathway are not yet clear. There might be additional receptors, or the pathway might begin with the SYMRK/DMI2 receptor kinase (Harrison 2005).

LRR1 is an atypical receptor kinase involved in the interaction between *P. indica* and *A. thaliana* roots.

Based on differential display and microarray analysis, the mRNA for a LRR-RK called LRR1 was isolated in the present research. No functional knock out line for this protein was available in the *A. thaliana* databases. I, therefore, characterized a knock

out line with an insertion in the promoter region. However the *LRR1* gene was expressed in this line and therefore, the response to *P. indica* was normal. Thus, a direct proof for the involvement of LRR1 in the interaction between *A. thaliana* and *P. indica* is still missing. However, two lines of evidence suggest that LRR1 should be involved in the interaction. First, the *LRR1* mRNA is transiently upregulated in roots during the recognition process of the two symbiotic partners. Second, in a knock out line for LRR2, the LRR1 protein (together with LRR2) fails to accumulate in the Triton X-100 insoluble plasma membrane microdomains. LRR1 (Fig. 7) exhibits sequence similarities to a LRR protein which is upregulated in response to salicylic acid (Ohtake *et al.* 2000), to an atypical receptor kinase with a defective kinase domain (Llompart *et al.* 2003), to several LRR proteins reported to be involved in pollen-tube development (Muschietti *et al.* 1998; Kim *et al.* 2002), to the peptide hormone phytosulfokine receptor (Matsubayashi *et al.* 2002) and the brassinosteroide insensitive receptor kinase BRI1 (Li and Chory 1997, cf. Bishop and Koncz 2002). Upregulation of the mRNA for LRR1 appears to be specific for *P. indica* and is not observed for other fungi and also not for a set of other LRR protein mRNA tested (Shahollari *et al.* 2005). Finally, mutants which fail to respond to *P. indica*, also do not upregulate the *LRR1* mRNA during the recognition period.

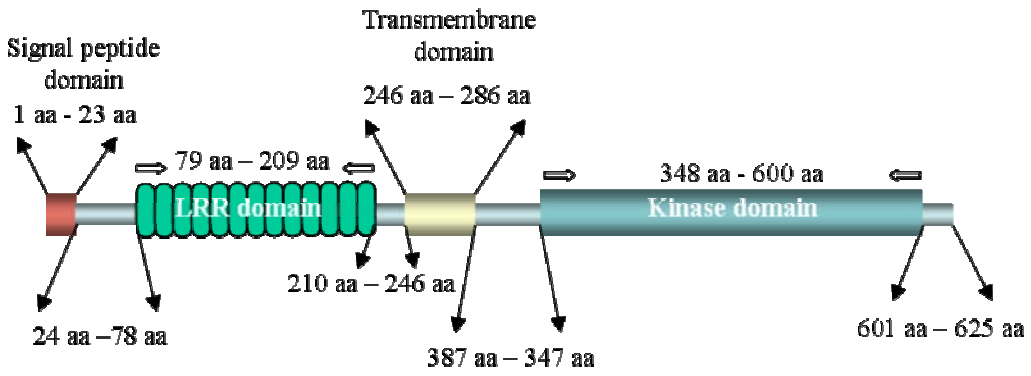


Figure 7. Schematic representation of the LRR1 protein. LRR1 signature motifs include a signal peptide, an extracellular LRR domain, a transmembrane domain and an atypical kinase domain (aa amino acids).

LRR1 appears to be an atypical receptor kinase (Fig. 8), which transduces signals by phosphorylation-independent mechanisms (Kroiher *et al.* 2001). Although the intracellular domains of atypical receptor kinases such as LRR1 contain conserved serine/threonine kinase domains, some of the highly conserved amino acids within these domains are altered. For instance, an aspartic acid in the subdomain IVb which is assumed to be part of the kinase-active site (Knighton *et al.* 1993) is replaced by asparagine (position 468) and the phenylalanine and glycine within the DFG activation loop are respectively replaced by tyrosine and cysteine (positions 487 and 488) in LRR1. The crucial role of the phenylalanine in the DFG triplet has been demonstrated for H-Ryk (Katso *et al.* 1999).

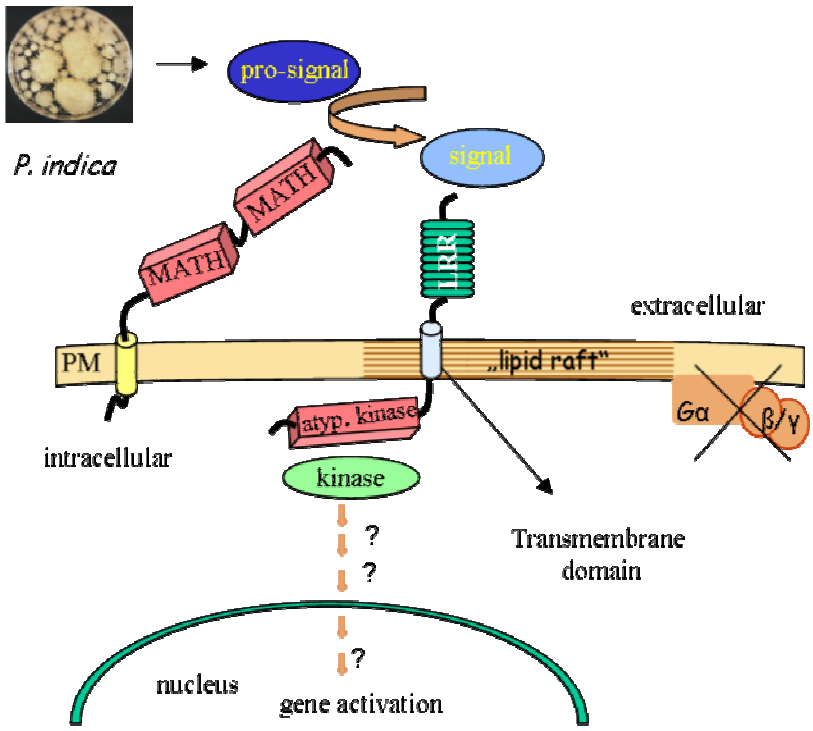


Figure 8. Model how the LRR1 and MATH proteins can be involved in signalling pathways. This model describes the mechanism how the LRR1 and MATH proteins can be involved in signalling pathways in early steps of the recognition between *A. thaliana* and *P. indica.* The MATH protein might be involved in peptide cleavage from pro-signal to the signal. This signal is then recognised by the LRR domain. Since the LRR1 protein has an atypical kinase domain, it needs an interaction partner, like kinase proteins to induce the signal. G proteins (αβγ) do not play a role in the interaction.

Identical or similar amino acid substitutions have also been observed for MARK, a maize atypical receptor kinase which does not autophosphorylate *in vitro* (Llompart *et al.* 2003). The intracellular domain of MARK interacts with the regulatory domain of

MIK, a germinal center kinase-like kinase, and strongly induces MIK kinase activity. Llompart *et al.* (2003) proposed that MIK could represent a novel component for signalling through atypical receptor kinases in plants. Interestingly, an atypical receptor kinase of the LysM type is also involved in legume perception or rhizobial signals (Madsen *et al.* 2003). One might speculate that both kinases can activate the same downstream signalling path.

A second leucine-rich repeat protein, LRR2, is essential for the stimulatory effect of *P. indica* on *A. thaliana* growth and enhanced seed production.

It could be shown here that another leucine-rich repeat containing protein, called LRR2 (cf. Tab.1), is required for the interaction between *A. thaliana* and *P. indica.* In a *P. indica* insensitive (Pi)-EMS mutant, called Pi-2, none of the normally observed responses of *Arabdiopsis* to *P. indica* were detectable. This includes the growth response and the enhanced production of seeds (Shahollari e*t al.* 2006). The EMS mutant is defective in the gene encoding LRR2. A conversion of G to A at an exon/intron junction prevents intron splicing, leading to the accumulation of a non-functional *LRR2* mRNA. A T-DNA knock out line for the same gene has also no responses to *P. indica*. Thus, LRR2 appears to be crucial for the interaction between *P. indica* and *A. thaliana*.

LRR2 was purified with *A. thaliana* Triton X-100 insoluble plasma membrane microdomains (Shahollari *et al.* 2004). The protein lacks a predictable transmembrane segment (Fig. 9) and can easily be removed from the microdomains by extended detergent treatments. Thus, LRR2 appears to be a soluble protein which is loosely attached to these membrane preparations. The predicted signal sequence suggests that LRR2 is sorted *via* the secretory pathway. Based on the KKxx endoplasmic reticulum retardation sequence in the C terminal part of the protein, LRR2 appear to remain in the endoplasmic reticulum. Although significantly shorter, LRR2 exhibits striking similarities to the Cf-2/4/5/9s, tomato transmembrane proteins which confer resistance to *Cladosporium fulvum* (Dixon *et al.* 1996; de Wit *et al.* 1999, 2002; Rivas and Thomas 2005). Comparison of the LRR2 protein with tomato Cf proteins makes it unlikely that LRR2 is the primary target site for fungal elicitors (Rivas and Thomas 2005; Rooney *et al.* 2005).

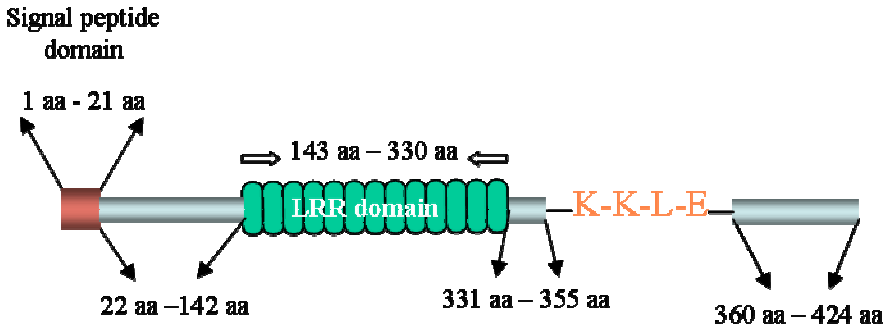


Figure 9. Schematic representation of the LRR2 protein. LRR2 signature motifs include a signal peptide, a LRR domain and a KKLE sequence (aa amino acids).

In particular, the C and N-terminal domains present in Cf2 proteins are not found in LRR2, suggesting that the protein alone cannot receive signals directly and transfer them to downstream components. Thus, the conserved LRR domain in the LRR2 protein might be crucial for its function, presumably by establishing and/or controlling protein/protein interactions required for the beneficial interaction between the two symbiotic organisms. The LRR sequence of LRR2 exhibits also striking similarities to TOO MANY MOUTHS (TMM), a transmembrane LRR-RK. The protein appears to function in a position-dependent signaling pathway that controls the plane of patterning divisions as well as the balance between stem cell renewal and differentiation in stomatal and epidermal development (Nadeau and Sack 2002, 2003; Shpak *et al.* 2005). Similar to LRR2, TMM also lacks a recognizable intracellular domain for downstream signalling. Thus, both components might require interaction partners with co-receptor kinase activities.

LRR1, LRR2 and additional five LRR proteins are localised in Triton X-100 insoluble plasma membrane microdomains in *A. thaliana*.

The observation that LRR1 and LRR2 are present in Triton X-100 insoluble plasma membrane microdomains offers the opportunity to identify interacting signalling components with biochemical means. Proteins, presumably interacting with or residing in lipid rafts are therefore often enriched and identified based on their ability to float *in vitro* on gradients with detergent-resistant membranes (DRMs) that were derived from

cell lysates treated with Triton X-100 (Mongrand *et al.* 2004; Shahollari *et al.* 2004). Despite the wealth of information concerning presumptive lipid raft composition and structure, the precise functional roles of these microdomains are still subject to considerable debate (Shin and Abraham 2001). Many cellular tasks have been ascribed to sterol-rich lipid microdomains, including such diverse processes as signal transduction, polarized secretion, membrane transport, transcytosis across epithelial monolayers, cytoskeletal organization, apoptosis, generation of cell polarity and the entry of infectious organisms in living cells (Simons and Ikonen 1997; Rosenberger *et al.* 2000; Bagnat and Simons 2002). In this project Triton X-100 insoluble plasma membrane microdomains were isolated from *A. thaliana* and mustard seedlings, using established protocols for animal cells (Larsson *et al.* 1987; Peškan *et al.* 2000). Low density Triton X-100 insoluble plasma membrane microdomains are well characterized in animals and yeast; however, little is known about their role and their protein composition in plants. In animals, they contain resident integral membrane proteins such as caveolin, stomatin, and flotillin, extracellular proteins with glycophosphatidylinositol anchors and cytoplasmic proteins modified by myristoylation/palmitoylation (Simons and Toomre 2000; Nebl *et al.* 2002; Munro 2003). These microdomains are less than 50 nm in diameter and can recruit different signalling components depending on their cellular signalling functions and receptor activation. The protein patterns of microdomain preparations from roots grown in the presence or absence of the fungus were identical. There was also no difference in the protein composition of micodomains from the two Brassicaceae *A. thaliana* and mustard. We found that the number of proteins in these vesicles, which can be detected on two-dimensional Coomassie-stained gels, is very similar to that from animals. However, they contain many proteins which are not present in or not characteristic for mammalian microdomains. The plasma membrane ATPase, aquaporins and membrane-intrinsic proteins are major constituents of the plant vesicles. We also found that receptor kinases with LRRs are highly enriched in these vesicles. This suggests that they either interact with each other or are organized in similar plasma membrane environments or share similar biochemical purification features. Six of these receptor kinases appear to be integral membrane proteins, whereas one protein (LRR2), exhibits unusual features. Its apparent molecular weight on the denaturing SDS gel differs from the calculated size and the protein does not contain a predicted transmembrane segment. We also found typical plasma membrane proteins such as phospholipase Dδ

or a cAMP-dependent kinase, several so far uncharacterized protein kinases, Ca^{2+}- and small GTP-binding proteins, heterotrimeric G-protein subunits and putative downstream signalling compounds (cf. the CBL-interacting protein kinases 8 and 9). Protein/protein interaction studies between these proteins and the analyses of knock out lines will help in future to elucidate the function of these proteins in signalling processes across the plasma membrane. It is also interesting that the microdomains contain several proteins involved in stress responses (At1g30360, At5g62740). Further transduction of activating signals depends on the presence of proteins associated at the inner site of the plasma membrane. Our list of proteins provides several candidates. The list contains also proteins involved in protein trafficking through the secretory pathway (e.g. Sar1, Rab1c and Rab2). Since the organization of a plant cell with its huge vacuole differs substantially from that of animal cells, membrane trafficking between the ER and the plasma membrane might be different. Recently, Wienkoop and Saalbach (2003) analysed the proteome of the plasma membrane-derived peribacteroid membrane from *Lotus japonicus* root nodules. Many of the proteins identified in our microdomains are also present in their peribacteroid membrane system. In particular, both membrane preparations contain ATPases, aquaporins, GTP-binding proteins, proteins involved in signalling processes, receptor kinases, 14-3-3 proteins and pathogen-related proteins. Wienkoop and Saalbach (2003) also found proteins that are expected to be localized in other plant endomembranes, comparable with the results reported here. Likewise, Marmagne *et al.* (2004) analysed the proteome of soluble and insoluble plasma membrane fractions from suspension-cultured *A. thaliana* cells and found several transporter proteins, receptors, GTP-binding proteins, proteins involved in various trafficking processes as well as stress-related proteins. The high degree of overlapping of the identified proteins in these membrane preparations provides a solid basis for future studies. The increasing awareness of lipid rafts is probably fuelled by the broad range of essential cellular tasks that are attributed to these plasma membrane microdomains (Bhat *et al.* 2005).

Triton X-100 insoluble plasma membrane microdomains contain at least 7 LRR-containing proteins, and only two of them, LRR1 and LRR2, are released from these domains by detergent treatments. This suggests that they are only loosely associated with these vesicles. LRR1 is clearly a RK with a plasma membrane-localized transmembrane domain. Its release from the vesicles might indicate that LRR1 is in a

lipid environment which differs from that of the rafts. In contrast, LRR2 might be located in the endoplasmic reticulum. The presence of this protein in the microdomain preparation is consistent with our observation that also other proteins normally found in the endoplasmic reticulum are present in the microdomain preparation. This indicates that either the plasma membrane microdomain preparations used are contaminated with endoplasmic reticulum, or both membrane systems are so similar in plants that they cannot be separated with the classical protocols established for mammalian membranes.

A MATH (meprin and TRAF homology) protein is modified during early recognition events between *P. indica* and *A. thaliana.*

P. indica induces a modification of a plasma membrane protein, a MATH protein in the *A. thaliana* roots which can easily be monitored on two dimensional gels (Peškan-Berghöfer *et al.* 2004). This modification is only transient and disappears as soon as the interaction between both organisms is established (Oelmüller *et al.* 2004). The MATH protein modification is one of the earliest plant responses to a fungus and does not occur in the *A. thaliana* mutant Pi-2 which fails to recognise *P. indica*. MATH proteins exhibit similarities to extracellular proteases which are known to be involved in the perception of fungal signals. The function of MATH proteins in general is unclear at present. However, the modular organization and domain structure of MATH proteins suggest that they may be involved in processes such as protein degradation and protein or peptide cleavage and/or activation. The characteristic features of the MATH protein which responds to *P. indica* are the two MATH domains predicted to be located in the extracellular space (Fig. 8). These domains exhibit sequence similarities to zinc dependent metallo-endopeptidases (Dumermuth *et al.* 1991, Bertenshaw *et al.* 2003, Norman *et al.* 2003a, Kruse *et al.* 2004) and are amongst the largest extracellular proteases so far identified in animal systems (Villa *et al.* 2003a). It has been postulated that they provide examples of novel ways of concentrating proteolytic activity at the cell surface and in defined areas in the extracellular milieu. The catalytic domains of these proteins cleave a large variety of bioactive peptides including growth factors, cytokinins, factors required for morphogenesis and extracellular matrix proteins (cf. Wolz and Bond 1995; Chestukhin *et al.* 1997; Becker

et al. 2003). These tissue-specific proteinases are also involved in developmental processes and pathogenic responses (Bond and Beynon 1995; Dietrich *et al.* 1996). Thus, MATH proteins may have the potential to cleave a peptide that in turn activates an intracellular signalling pathway, as proposed for the *Drosophila* receptor kinase TOLL system (De Gregorio *et al.* 2002). Another example provides the tomato cysteine protease which is required for Cf-2-dependent disease resistance and suppression of autonecrosis (Krüger *et al.* 2002). The identification of MATH proteins in two independent screens for plant–microbe interactions (Gamas *et al.* 1996; Peskan-Berghöfer *et al.* 2004) suggest that they might play a role in plant/microbe interactions.

The growth promoting effect initiated by *P. indica* is accompanied by a co-regulated stimulation of enzymes involved in nitrate and starch metabolisms and requires a homeodomain transcription factor.

By analysing the interaction of *P. indica* with both *A. thaliana* and tobacco roots it was found that in contrast to mycorrhizal associations, nitrate reduction in the roots is stimulated by *P. indica*. The mRNA for a homeodomain transcription factor is upregulated in response to *P. indica* in *A. thaliana* roots and the corresponding protein binds to promoter regions of the *P. indica* responsive *Nia2* and *SEX1* genes. The TTCTAGAGT sequence in the *Nia2* promoter was shown to be crucial for the regulation and the homeodomain transcription factor binds to this sequence *in vitro*. Binding activity was also observed to a related motif in the *SEX1* promoter. The homeodomain factor was identified by mass spectrometry and in an independent experiment by microarray analyses. These results suggest that the expression of *P. indica*-responsive target genes may be controlled by common regulatory elements and *trans*-factors.

Homeodomain proteins bind to DNA as homo- and/or heterodimers in a sequence-specific manner, and thus their target genes depend on their interaction partners. The best studied homologs of the protein identified in this study are the BEL1-like transcription factors from potato (Chen *et al.* 2003). Seven members of the BEL1 protein family interact with KNOX transcription factors and they control vegetative development and tuber formation. A rice homolog of the homeodomain protein is

inducible by benzothiadiazole, a component which induces disease resistance in *A. thaliana* and wheat by activation of the systemic acquired resistance signal transduction pathway (Gorlach *et al.* 1996; Lawton *et al.* 1996). Benzothiadiazole also activates resistance in sunflower to the root-parasitic weed *Orobanche cumana* (Sauerborn *et al.* 2002). This suggests that the identified homeodomain transcription factor might also be involved in signalling pathways related to plant/microbe interaction.

In the present project was a knock out line for the homeodomain transcription factor from *A. thaliana* isolated. It could be shown that this mutant does not respond to *P. indica*. (Fig. 10). This confirms the important role of the transcription factor (TF), originally identified and characterized in tobacco, also for *A. thaliana*.

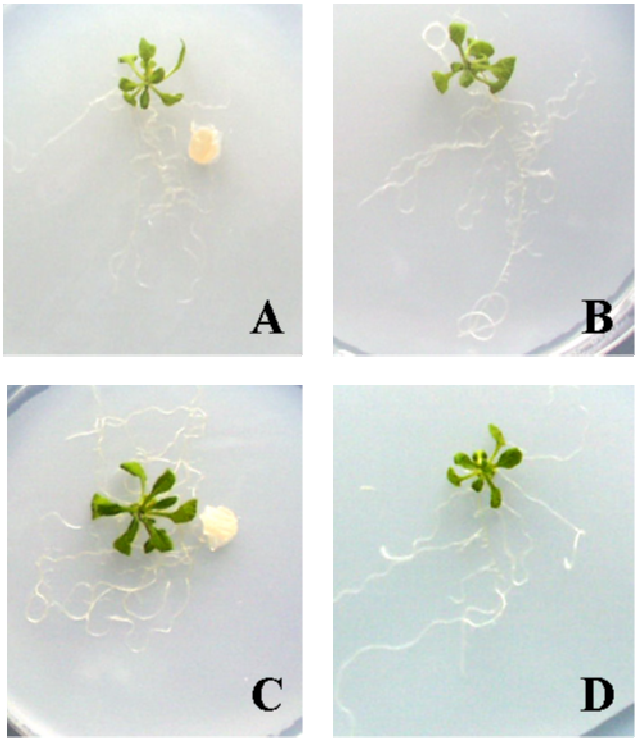


Figure 10. A knock out line for a transcription factor does not respond to *P. indica*. (A) and (B): TF knock out line seedlings with and without fungus, respectively, (C) and (D) wild-type seedlings with and without fungus, respectively. Plants shown in this figure were co-cultivated with *P. indica* for 10 days.

4. Summary

The fungus *Piriformospora indica* promotes growth and seed production of *Arabidopsis thaliana* plants, very similar to what has been observed for other plant species. Using molecular tools and mutant screens, two leucine-rich repeat proteins, LRR1 and LRR2, were identified which are involved in the recognition of the two symbiotic partners.

The mRNA for LRR1 is transiently upregulated in *A. thaliana* roots co-cultivated with *P. indica*. Mutants which do not respond to *P. indica* do not show this upregulation. LRR1 is an atypical receptor kinase located in the plasma membrane. The protein is present in Triton X-100 insoluble plasma membrane microdomains. A mutant with a lesion in another leucine-rich repeat protein, LRR2, also fails to accumulate LRR1 in Triton X-100 insoluble plasma membrane microdomains. The requirement of LRR1 for the symbiotic interaction is unclear, because no functional knock out line is available.

Two mutants with lesions in LRR2 were characterized. Both of them fail to respond to *P. indica* with regard to growth promotion and enhanced seed production. LRR2 appears to be loosely associated with Triton X-100 insoluble plasma membrane microdomains and might be located in the endoplasmatic reticulum.

Triton X-100 insoluble plasma membrane microdomains were isolated from *A. thaliana* and the protein composition of this fraction was determined by mass spectrometry. 34 proteins mainly related to signalling processes are present in this preparation, including seven leucine-rich repeat proteins. Two of them are LRR1 and LRR2.

DMI1 is an essential protein for mycorrhiza formation in legumes. Inactivation of the only homologous gene in *A. thaliana*, a gene that is exclusively expressed in roots does not affect growth promotion and enhanced seed production in *A. thaliana* in response to *P. indica*. Thus, the endophytic interaction between *P. indica* and *A. thaliana* appears to differ from mycorrhiza in Legumes.

The interaction between *A. thaliana* and *P. indica* is accompanied by the modification of a plasma membrane-localized MATH protein (Peskan-Berghöfer *et al.* 2004). This protein modification does not occur in an *Arabidopsis* mutant, which does not respond to *P. indica*. At present the function of MATH proteins is still unclear; however, the modular organization and domain structure of MATH proteins suggest that they may be involved in protein degradation or protein/peptide cleavage.

This homeodomain transcription factor *BHL1* responds to the fungus and binds to promoter regions of the *P. indica* responsive *Nia2*, *SEX1* and *2-nitropropane dioxygenase* genes in tobacco (Sherameti *et al.* 2005). Inactivation of the *BHL1* gene in *A. thaliana* completely abolished the growth promotion and enhanced seed production.

5. Zusammenfassung

Der endophytische Pilz *Piriformospora indica* fördert das Wachstum und die Samenproduktion von *Arabidopsis thaliana* vergleichbar mit Untersuchungen an anderen Pflanzen. Mit Hilfe der Analyse von Mutanten und molekularen Techniken konnten in der vorliegenden Arbeit zwei *leucine-rich repeat* Proteine (LRR1 und LRR2) identifiziert werden, die vermutlich an der Erkennung von *P. indica* durch *A. thaliana* beteiligt sind.

Die Expression von LRR1 ist während der Erkennungsphase beider Organismen vorübergehend gesteigert. Diese Reaktion bleibt in *A. thaliana* Mutanten, die *P. indica* nicht erkennen, aus. LRR1 ist eine atypische Rezeptorkinase, die in der Plasmamembran lokalisiert ist. Das Protein ist in Triton X-100-unlöslichen Plasmamembranvesikeln vorhanden. In einer Mutante mit einem Defekt in einem zweiten *leucine-rich repeat* Protein, LRR2, ist LRR1 hingegen nicht in den Plasmamembranvesikeln nachweisbar. Obwohl die physiologischen Daten einen Bezug von LRR1 zur Interaktion mit *P. indica* vermuten lassen, konnte dieser letztendlich nicht bewiesen werden, da keine funktionsfähige *knock out* Linie für das LRR1 Gen existiert.

Für das LRR2 Protein wurden zwei Mutanten charakterisiert. Beide zeigen in Anwesenheit von *P. indica* keine Wachstumssteigerung und keine erhöhte Samenproduktion. LRR2 ist ebenfalls in Triton X-100 unlöslichen Plasmamembranvesikeln nachweisbar. Im Gegensatz zu LRR1 kann dieses Protein aber leicht abgelöst werden und ist vermutlich im endoplasmatischen Retikulum lokalisiert. LRR2 ist somit eine essentielle Komponente für die Etablierung der Interaktion zwischen *A. thaliana* und *P. indica*.

Die Proteinzusammensetzung der Triton X-100 unlöslichen Plasmamembranvesikel von *A. thaliana* wurde mit Hilfe der Massenspektrometrie bestimmt. 34 Proteine, die vor allem mit Prozessen der Signalweiterleitung in Verbindung stehen, finden sich in dieser Fraktion, dazu gehören sieben *leucine-rich repeat* Proteine. LRR1 und LRR2 sind zwei dieser Proteine.

DMI (*does not make infection*) ist als essentielles Protein für die Mykorrhizabildung in Leguminosen bekannt. Eine *knock out* Linie für das einzige *DMI* Gen in *A. thaliana* wurde analysiert. Die *DMI* mRNA ist wie bei Leguminosen nur in Wurzeln nachweisbar. Die *knock out* Linie reagiert wie der Wildtyp auf *P. indica*. Die endophytische Interaktion zwischen *P. indica* und *A. thaliana* scheint sich demnach von der Mykorrhizabildung bei Leguminosen zu unterscheiden. Das Protein muss bei *A. thaliana* eine andere Funktion haben.

Die Interaktion zwischen *P. indica* und *A. thaliana* verursacht eine Veränderung eines MATH Proteins in der Plasmamembran von Wurzeln (Peškan-Berghöfer *et al.* 2004). Diese Modifikation ist in einer Mutante, die keine Reaktion auf *P. indica* zeigt, nicht nachweisbar. Die Funktion von MATH Proteinen ist unklar, Sequenzanalysen lassen allerdings vermuten, dass es sich bei dem von uns charakterisiertem MATH Protein um eine extrazelluläre Metalloprotease handeln könnte, die in der Plasmamembran verankert ist.

Die mRNA für den Homeodomän-Transkriptionsfaktor *BHL1* wird in Antwort auf *P. indica* in Tabak-Wurzeln verstärkt exprimiert, dieser Transkriptionsfaktor bindet *in vitro* an die Promotoren der Gene von *Nia2, SEX*1 und *2-Nitropropan Dioxygenase* und stimuliert deren Expression (Sherameti *et al.* 2004). In der vorliegenden Arbeit konnte gezeigt werden, dass die Inaktivierung von *BHL1* in *A. thaliana* die Wachstumsstimulation und erhöhte Samenproduktion komplett blockiert. *BHL1* ist folglich essentiell an der Reaktion von *A. thaliana* mit dem endophytischen Pilz *P. indica* beteiligt.

6. Literature Cited

Ané JM, Kiss GB, Riely BK, Penmetsa RV, Oldroyd GE, Ayax C, Levy J, Debelle F, Baek JM, Kalo P, Rosenberg C, Roe BA, Long SR, Denarie J, Cook DR (2004) "*Medicago truncatula* DMI1 required for bacterial and fungal symbioses in legumes" *Science* **303**: 1364–1367.

Bagnat M, Simons K (2002) "Cell surface polarization during yeast mating" *Proc Natl Acad Sci USA* **99**: 14183–14188.

Barazani O, Benderoth M, Groten K, Kuhlemeier C, Baldwin IT (2005) "*Piriformospora indica* and *Sebacina vermifera* increase growth performance at the expense of herbivore resistance in *Nicotiana attenuata*" *Oecologia* **146**: 234-243.

Becker C, Kruse MN, Slotty KA, Kohler D, Harris JR, Rosmann S, Sterchi EE, Stocker W (2003) "Differences in the activation mechanism between the alpha and beta subunits of human meprin" *J Biol Chem* **384**: 825–831.

Bertenshaw GP, Norcum MT, Bond JS (2003) "Structure of homo- and hetero-oligomeric meprin metalloproteases. Dimers, tetramers, and high molecular mass multimers" *J Biol Chem* **278**: 2522–2532.

Bhat RA, Miklis M, Schmelzer E, Schulze-Lefert P, Panstruga R (2005) "Recruitment and interaction dynamics of plant penetration resistance components in a plasma membrane microdomain" *Proc Natl Acad Sci USA* **102**: 3135-3140.

Bishop GJ, Koncz C (2002) "Brassinosteroids and plant steroid hormone signaling" *Plant Cell* **14**: Suppl: 97-110.

Blechert O, Kost G, Hassel A, Rexer RH, Varma A (1999) "First remarks on the symbiotic interactions between *Piriformospora indica* and terrestrial orchid" *in*: Varma A and Hocke B. eds. "Mycorrhizae" 2nd ed. *Springer Verlag,* Germany: 683-688.

Bond JS, Beynon RJ (1995) "The astacin family of metalloendopeptidases" *Protein Sci* **4**: 1247–1261.

Boukcim H, Plassard C (2003) "Juvenile nitrogen uptake capacities and root architecture of two open-pollinated families of Picea abies. Effects of nitrogen source and ectomycorrhizal symbiosis" *J Plant Physiol* **160(10)**: 1211-1218.

Brown DA, Rose JK (1992) "Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell-surface" *Cell* **68**: 533–544.

Brown DA, London E (1998) "Functions of lipid rafts in biological membranes" *Annu Rev Cell Dev Biol* **14**: 111–136.

Brundrett MC (1991) "Mycorrhizas in natural ecosystems" *in*: Macfayden A, Begon M and Fitter AH. eds. "Advances in Ecological Research" Vol. 21. *Academic Press*, London, UK: 171–313.

Chen H, Rosin FM, Prat S, Hannapel DJ (2003) "Interacting transcription factors from the three-amino acid loop extension superclass regulate tuber formation" *Plant Physiol* **132(3)**: 1391-1404.

Chestukhin A, Litovchick L, Muradov K, Batkin M, Shaltiel S (1997) "Unveiling the substrate specificity of meprin beta on the basis of the site in protein kinase A cleaved by the kinase splitting membranal proteinase" *J Biol Chem* **272**: 3153–3160.

Clark SE, Williams RW, Meyerowitz EM (1997) "The CLAVATA1 gene encodes a putative receptor kinase that controls shoot and floral meristem size in Arabidopsis" *Cell* **89**: 575-585.

Cock JM, McCormick S (2001) "A large family of genes that share homology with CLAVATA3" *Plant Physiol* **126**: 939-942.

De Gregorio E, Spellman PT, Tzou P, Rubin GM, Lemaitre B (2002) "The Toll and Imd pathways are the major regulators of the immune response in Drosophila" *EMBO J* **21**: 2568–2579.

Dewan MM, Sivasithamparan K (1988) "A plant growth promoting sterile fungus from wheat and rye grass with potential for suppressing take-all" *Trans Br Mycol Soc* **91**: 687-717.

de Wit PJ, Joosten MH (1999) "Avirulence and resistance genes in the *Cladosporium fulvum*-tomato interaction" *Curr Opin Microbio* **2:** 368-373.

de Wit PJ, Brandwagt BF, van den Burg HA, Cai X, van der Hoorn RA, de Jong CF, van Klooster J, de Kock MJ, Kruijt M, Lindhout WH, Luderer R, Takken FL, Westerink N, Vervoort JJ, Joosten MH (2002) "The molecular basis of co-evolution between *Cladosporium fulvum* and tomato" *Antonie Van Leeuwenhoek* **81**: 409-412.

Dietrich JM, Jiang W, Bond JS (1996) "A novel meprin beta´ mRNA in mouse embryonal and human colon carcinoma cells" *J Biol Chem* **241**: 2271–2278.

Diévart A, Clark SE (2004) "LRR-containing receptors regulating plant development and defense" *Development* **131**: 251-261.

Dixon MS, Jones DA, Keddie JS, Thomas CM, Harrison K, Jones JD (1996) "The tomato Cf-2 disease resistance locus comprises two functional genes encoding leucine-rich repeat proteins" *Cell* **84**: 451-459.

Dumermuth E, Sterch EE, Jiang W, Wolz RL, Bond JS, Flannery AV, Beynon RJ (1991) "The astacin family of metalloendopeptidases" *J Biol Chem* **266(21)**: 381–385.

Endré G, Kereszt A, Kevei Z, Mihacea S, Kalo P, Kiss GB (2002) "A receptor kinase gene regulating symbiotic nodule development" *Nature* **417(6892)**: 962-966.

Esseling JJ, Emons AM (2004) "Dissection of Nod factor signalling in legumes: cell biology, mutants and pharmacological approaches" *J Microsc* **214(2)**: 104-113.

Foster LJ, De Hoog CL, Mann M (2003) "Unbiased quantitative proteomics of lipid rafts reveals high specificity for signaling factors" *Proc Natl Acad Sci USA* **100**: 5813-5818.**Friedrichsen DM, Joazeiro CA, Li J, Hunter T, Chory J** (2000) „Brassinosteroid-insensitive-1 is a ubiquitously expressed leucine-rich repeat receptor serine/threonine kinase" *Plant Physiol* **123**: 1247-1256.

Gage DJ (2004) "Infection and invasion of roots by symbiotic, nitrogen-fixing rhizobia during nodulation of temperate legumes" *Microbiol Mol Biol Rev* **68(2)**: 280-300.

Gamas P, Niebel FC, Lescure N, Cullimore J (1996) "Use of a subtractive hybridization approach to identify new Medicago truncatula genes induced during root nodule development" *Mol Plant–Microbe Interact* **9**: 233–242.

Gasoni L, Stegman de Gurfinkel B (1997) "The endophyte *Cladorrhinum forcundissimum* in cotton roots: phosphorus uptake and host growth" *Mycol Res* **101**: 867-870.

Geurts R, Fedorova E, Bisseling T (2005) "Nod factor signaling genes and their function in the early stages of *Rhizobium* infection" *Curr Opin Plant Biol* **8**: 346-352.

Glen M, Tommerup IC, Bougher NL, O'Brien PA (2002) "Are Sebacinaceae common and widespread ectomycorrhizal associates of Eucalyptus species in Australian forests?" *Mycorrhiza* **12**: 243-247.

Gomez-Gomez L, Bauer Z, Boller T (2001) "Both the extracellular leucine-rich repeat domain and the kinase activity of FSL2 are required for flagellin binding and signaling in Arabidopsis" *Plant Cell* **13**: 1155-1163.

Gomez-Gomez L, Boller T (2002) **"**Flagellin perception: a paradigm for innate immunity" *Trends Plant Sci* **7(6)**: 251-256.

Gorlach J, Volrath S, Knauf-Beiter G, Hengy G, Beckhove U, Kogel KH, Oostendorp M, Staub T, Ward E, Kessmann H, Ryals J (1996) "Benzothiadiazole, a novel class of inducers of systemic acquired resistance, activates gene expression and disease resistance in wheat" *Plant Cell* **8(4)**: 629-643.

Guescini M, Pierleoni R, Palma F, Zeppa S, Vallorani L, Potenza L, Sacconi C, Giomaro G, Stocchi V (2003) "Characterization of the *Tuber borchii* nitrate

reductase gene and its role in ectomycorrhizae" *Mol Genet Genomics* **269(6)**: 807-816.

Harley JL, Smith SE (1983) "Mycorrhizal symbiosis" *Academic Press*, New York.

Harrison MJ (1999) "Molecular and cellular aspects of the arbuscular mycorrhizal symbiosis" *Annu Rev Plant Physiol Plant Mol Biol* **50**: 631–689.

Harrison MJ (2005) "Signaling in the arbuscular mycorrhizal symbiosis" *Annu Rev Microbiol* **59**:19-42.

Haselwandter K, Read DJ (1982) "The significance of a root-fungus association in two *Carex* species of high-alpine plant communities" *Oecologia* **53**: 352-354.

Hause B, Fester T. (2005) "Molecular and cell biology of arbuscular mycorrhizal symbiosis" *Planta* **221(2)**:184-96.

Hogg BV, Cullimore JV, Ranjeva R, Bono JJ (2006) "The DMI1 and DMI2 early symbiotic genes of medicago truncatula are required for a high-affinity nodulation factor-binding site associated to a particulate fraction of roots" *Plant Physiol* **140(1)**: 365-73.

Hunter T (1995) "Protein kinases and phosphatases: the yin and yang of protein phosphory-lation and signaling" *Cell* **80(2)**: 225-236.

Imaizumi-Anraku H, Takeda N, Charpentier M, Perry J, Miwa H, Umehara Y, Kouchi H, Murakami Y, Mulder L, Vickers K, Pike J, Downie JA, Wang T, Sato S, Asamizu E, Tabata S, Yoshikawa M, Murooka Y, Wu GJ, Kawaguchi M, Kawasaki S, Parniske M, Hayashi M (2005) "Plastid proteins crucial for symbiotic fungal and bacterial entry into plant roots" *Nature* **433(7025)**: 527-531.

Jia Y, Gray VM, Straker CJ (2004) "The influence of *Rhizobium* and arbuscular mycorrhizal fungi on nitrogen and phosphorus accumulation by *Vicia fab*" *Ann Bot (Lond)* **94**: 251-258.

Jones DA, Jones JDG (1997) "Advances in Botanical Research incorp" *Advances in Plant Pathology* **24**: 90-167.

Johnson KL, Ingram GC (2005) "Sending the right signals: regulating receptor kinase activity" *Curr Opin Plant Biol* **8(6)**: 648-656.

Kaldorf M, Koch B, Rexer KH, Kost G, Varma A (2005) "Patterns of interaction between *Populus* Esch5 and *Piriformospora indica*: A Transition from mutualism to antagonism" *Plant Biology* **7**: 210-218.

Karandashov V, Nagy R, Wegmuller S, Amrhein N, Bucher M (2004) "Evolutionary conservation of a phosphate transporter in the arbuscular mycorrhizal symbiosis" *Proc Natl Acad Sci USA* **101**: 6285-6290.

Katso RM, Russell RB, Ganesan TS (1999) "Functional analysis of H-Ryk, an atypical member of the receptor tyrosine kinase family" *Mol Cell Biol* **19**: 6427-40.

Kendrick B (1992) "The fifth kingdom" 2nd edition *Mycologue Publications*, Waterloo, Canada.

Kim HU, Cotter R, Johnson S, Senda M, Dodds P, Kulikauska R, Tang W, Ezcura I, Herzmark P, McCormick S (2002) "New pollen-specific receptor kinases identified in tomato, maize and Arabidopsis: the tomato kinases show overlapping but distinct localization patterns on pollen tubes" *Plant Mol Biol* **50(1)**: 1-16.

Kim L, Johnson, Ingram GC (2005) "Sending the right signals: regulating receptor kinase activity" *Plant Biology* **8**: 1-9.

Kistner C, Parniske M (2002) "Evolution of signal transduction in intracellular symbiosis" *Trends Plant Sci* **7(11)**: 511-518.

Knighton DR, Cadena DL, Zheng J, Ten Eyck LF, Taylor SS, Sowadski JM, Gill GN (1993) "Structural features that specify tyrosine kinase activity deduced from homology modeling of the epidermal growth factor receptor" *Proc Natl Acad Sci* **90**: 5001-5005.

Kroiher M, Miller MA, Steele RE (2001) "Deceiving appearances: signaling by "dead" and "fractured" receptor protein-tyrosine kinases" *Bioessays* **23(1)**: 69-76.

Krüger J, Thomas CM, Golstein C, Dixon MS, Smoker M, Tang S, Mulder L, Jones JDG (2002) "A tomato cysteine protease required for Cf-2-dependent disease resistance and suppression of autonecrosis" *Science* **296**: 744–747.

Kruse MN, Becker C, Lottaz D, Kohler D, Yiallouros I, Krell HW, Sterchi EE, Stocker W (2004) "Human meprin alpha and beta homo-oligomers: cleavage of basement membrane proteins and sensitivity to metalloprotease inhibitors" *Biochem J* **378**: 383–389.

Kumari R, Yadav HK, Bhoon YK, Varma A (2003) "Colonization of cruciferous plants by *Piriformospora indica*" *Current Science* **85**: 1672-1674.

Larsson C, Widell S, Kjellbom P (1987) "Preparation of high-purity plasma membranes" *Methods Enzymol* **148**: 558–568.

Lawton KA, Friedrich L, Hunt M, Weymann K, Delaney T, Kessmann H, Staub T, Ryals J (1996) "Benzothiadiazole induces disease resistance in Arabidopsis by activation of the systemic acquired resistance signal transduction pathway" *Plant J* **10(1)**: 71-82.

Lease KA, Wen J, Li J, Doke JT, Liscum E, Walker JC (2001) "A mutant Arabidopsis heterotrimeric G-protein beta subunit affects leaf, flower, and fruit development" *Plant Cell* **13(12)**: 2631-2641.

Levy J, Bres C, Geurts R, Chalhoub B, Kulikova O, (2004) "A putative $Ca2^{+}$ and calmodulin-dependent protein kinase required for bacterial and fungal symbioses" *Science* **303**: 361–64.

Li J, Chory J (1997) "A putative leucine-rich repeat receptor kinase involved in brassinosteroid signal transduction" *Cell* **90(5)**: 929-938.

Li J, Wen J, Lease KA, Doke JT, Tax FE, Walker JC (2002) "BAK1, an Arabidopsis LRR receptor-like protein kinase, interacts with BRI1 and modulates brassinosteroid signaling" *Cell* **110**: 213-222.

Limpens E, Franken C, Smit P, Willemse J, Bisseling T, Geurts R (2003) "LysM domain receptor kinases regulating rhizobial Nod factor-induced infection" *Science* **302**: 630–633.

Llompart B, Castells E, Rio A, Roca R, Ferrando A, Stiefel V, Puigdomenech P, Casacuberta JM (2003) "The direct activation of MIK, a germinal center kinase (GCK)-like kinase, by MARK, a maize atypical receptor kinase, suggests a new mechanism for signaling through kinase - dead receptors" *J Biol Chem* **278**: 48105-48111.

Madsen EB, Madsen LH, Radutoiu S, Olbryt M, Rakwalska M, Szczyglowski K, Sato S, Kaneko T, Tabata S, Sandal N, Stougaard J (2003) "A receptor kinase gene of the LysM type is involved in legume perception of rhizobial signals" *Nature* **425**: 637-640.

Marmagne A, Rouet MA, Ferro M, Rolland N, Alcon C, Joyard J, Garin J, Barbier-Brygoo H, Ephritikhine G (2004) "Identification of new intrinsic proteins in Arabidopsis plasma membrane proteome" *Mol Cell Proteomics* **3(7)**: 675-691.

Matsubayashi Y, Ogawa M, Morita A, Sakagami Y (2002) "An LRR receptor kinase involved in perception of a peptide plant hormone, phytosulfokine" *Science* **296(5572)**: 1470-1472.

Mitra RM, Gleason CA, Edwards A, Hadfield J, Downie JA (2004) "A $Ca2^{+}$/calmodulin-dependent protein kinase required for symbiotic nodule development: gene identification by transcript-based cloning" *Proc Natl Acad Sci USA* **101**: 4701–4705.

Mongrand S, Morel J, Laroche J, Claverol S, Carde JP, Hartmann MA, Bonneu M, Simon-Plas F, Lessire R, Bessoule JJ (2004) "Lipid rafts in higher plant cells - purification and characterization of triton X-100-insoluble microdomains from tobacco plasma membrane" *J Biol Chem* **279**: 36277–36286.

Mucciarelli M, Scannerini S, Bricarello M, Varese C, Sacco T, Maffei M (1995) "*In vitro* growth promoting effects of a fungus (WSF) on the yield of *Mentha X piperita*. A chance for sustainable agriculture" *G Bot Ital* **129:** 121.

Munro S (2003) "Lipid rafts: elusive or illusive?" *Cell* **115**: 377-388.

Muschietti J, **Eyal Y, McCormick S** (1998) "Pollen tube localization implies a role in pollen-pistil interactions for the tomato receptor-like protein kinases LePRK1 and LePRK2" *Plant Cell* **10(3)**: 319-330.

Nadeau JA, Sack FD (2002) "Control of stomatal distribution on the Arabidopsis leaf surface" *Science* **296**: 1697-1700.

Nadeau JA, Sack FD (2003) "Stomatal development: cross talk puts mouths in place" *Trends Plant Sci* **8**: 294-299.

Nam KH, Li J (2002) "BRI1/BAK1, a receptor kinase pair mediating brassinosteroid signaling" *Cell* **110(2)**: 203-212.

Nebl T, Pestonjamasp KN, Leszyk JD, Crowley JL, Oh SW, Luna EJ (2002) "Proteomic analysis of a detergent-resistant membrane skeleton from neutrophil plasma membranes" *J Biol Chem* **277**: 43399-43409.

Norman LP, Matters GL, Crisman JM, Bond JS (2003a) "Expression of meprins in health and disease" *Curr Top Dev Biol* **54**: 145–166.

O'Dell TE, Trappe JM (1992) "Root endophytes of lupine and some other legumes in Northwestern USA" *New Phytol* **122**: 479-485.

Oelmüller R, Shahollari B, Peškan-Berghöfer T, Trebicka A, Giong PH, Sherameti I, Oudhoff M, Venus Y, Altschmied L, Varma A (2004) "Molecular analyses of the interaction between *Arabidopsis* roots and the growth-promoting fungus *Piriformspora indica" Endocyt Cell Res* **15**: 504–517.

Oelmüller R, Peškan-Berghöfer T, Shahollari B, Trebicka A, Sherameti I, Varma A (2005) "MATH domain proteins represent a novel protein family in Arabidopsis thaliana, and at least one member is modified in roots during the course of a plant–microbe interaction. *Physiologia Plantarum* **124:2**: 152-166.

Ohtake Y, Takahashi T, Komeda Y (2000) "Salicylic acid induces the expression of a number of receptor-like kinase genes in *Arabidopsis thaliana*" *Plant Cell Physiol* **41(9)**: 1038-1044.

Parniske M (2004) "Molecular genetics of the arbuscular mycorrhizal symbiosis" *Curr Opin Plant Biol* **7**: 414–421.

Peškan T, Westermann M, Oelmuller R (2000) "Identification of low-density Triton X-100-insoluble plasma membrane microdomains in higher plants" *Eur J Biochem* **267**: 6989–6995.

Peškan-Berghöfer T, Shahollari B, Giang PH, Hehl S, Markert C, Blanke V, Kost G, Varma A, Oelmüller R (2004) "Association between *Piriformospora indica* and *Arabidopsis thaliana*: a novel system to study beneficial plant–microbe interactions" *Physiol Plant* **122**: 465–477.

Pham GH, Kumari R, Singh An, Sachdev M, Prasad R, Kaldorf M, Buscot F, Oelmüller R, Peśkan T, Weiss M, Hampp R, Varma A (2004) "Axenic cultures of *Piriformospora indica*" *in*: Varma A, Abbott L, Werner D, Hampp R eds. "Plant Surface Microbiology" *Springer-Verlag*, Germany: 593–616.

Radutoiu S, Madsen LH, Madsen EB, Felle HH, Umehara Y (2003) "Plant recognition of symbiotic bacteria requires two LysM receptor-like kinases" *Nature* **425**: 585–92.

Read DJ, Haselwandter K (1981) "Observations on the mycorrhizal status of some alpine plant communities" *New Phytol* **88**: 341-352.

Riely BK, Ane JM, Penmetsa RV, Cook DR (2004) "Genetic and genomic analysis in model legumes bring Nod-factor signaling to center stage" *Curr Opin Plant Biol* **7**: 408–413.

Rivas S, Thomas CM (2005) "Molecular interactions between tomato and the leaf mold pathogen *Cladosporium fulvum*" *Annual Review of Phytopathology* **43**: 395-436.

Bhat RA, Panstruga R (2005) "Lipid rafts in plants" *Planta* **223**: 5-19.

Rooney HCE, van't Klooster JW, van der Hoorn RAL, Joosten MHAJ, Jones JDG de Wit PJGM (2005) "Cladosporium Avr2 Inhibits Tomato Rcr3 Protease Required for *Cf-2*-Dependent Disease Resistance" *Science* **308**: 1783–1786.

Rosenberger CM, Brumell JH, Finlay BB (2000) "Microbial pathogenesis: lipid rafts as pathogen portals" *Curr Biol* **10**: R823–R825.

Sahay NS, Varma A (1999) "*Piriformospora indica*: a new biological hardening tool for micropropagated plants" *FEMS Microbiol Lett* **181**: 297-302.

Sauerborn J, Buschmann H, Ghiasvand Ghiasi K. Kogel KH (2002) "Benzothiadiazole Activates Resistance in Sunflower (*Helianthus annuus*) to the Root-Parasitic Weed *Orobanche cumana*" *Phytopathology* **92**: 59-64.

Schroeder R, London E, Brown D (1994) "Interactions between saturated acyl chains confer detergent resistance on lipids and glycosylphosphatidylinositol (GPI)-anchored proteins – GPIanchored proteins in liposomes and cells show similar behavior" *Proc Natl Acad Sci USA* **91**: 12130–12134.

Schüßler A, Kluge M (2001) „*Geosiphon pyriforme*, an endocytosymbiosis between fungus and cyanobacteria, and its meaning as a model system for arbuscular mycorrhizal research" *in*: Hock B ed. "The Mycota IX" *Springer Verlag*, Germany: 151-161.

Selosse MA, Bauer R, Moyersoen B (2002) "Basal hymenomycetes belonging to the Sebacinaceae are ectomycorrhizal on temperate deciduous trees" *New Phytologist* **155**: 183-195.

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

Shahollari B, Varma A, Oelmüller R (2005) "Expression of a receptor kinase in *Arabidopsis* roots is stimulated by the basidiomycete *Piriformospora indica* and the protein accumulates in Triton X-100 insoluble plasma membrane microdomains" *J Plant Physiol* **162**: 945-958.

Shahollari B, Varma A, Oelmüller R (submitted) "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*.

Shaw SL, Long SR (2003) "Nod factor elicits two separable calcium responses in *Medicago truncatula* root hair cells" *Plant Physiol* **131**: 976–984.

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

Shin JS, Abraham SN (2001) "Caveolae as portals of entry for microbes" *Microbes Infect* **3**: 755–761.

Shiu SH, Bleecker AB (2001) "Receptor-like kinases from Arabidopsis form a monophyletic gene family related to animal receptor kinases" *Proc Natl Acad Sci USA* **98**: 10763-10768.

Shiu SH, Karlowski WM, Pan R, Tzeng YH, Mayer KF, Li WH (2004) "Comparative analysis of the receptor-like kinase family in Arabidopsis and rice" *Plant Cell* **16(5)**: 1220–1234.

Shpak ED, McAbee JM, Pillitteri LJ, Torii KU (2005) "Stomatal patterning and differentiation by synergistic interactions of receptor kinases" *Science* **309**: 290-293.

Simons K, Ikonen E (1997) "Functional rafts in cell membranes" *Nature* **387(6633)**: 569-72.

Simons K, **Toomre D** (2000) "Lipid rafts and signal transduction" *Nat Rev Mol Cell Biol* **1**: 31-39.

Smith SE, Read DJ (1997) "Mycorrhizal symbiosis" 2nd edition, *Academic Press*, San Diego USA.

Sneh BM, Zeidan M, Ichielevich-Auster I, Barash I, Koltin Y (1986) "Increased growth responses induced by a nonpathogenic *Rhizoctonia solani*" *Can J Bot* **64**: 2372-2378.

Stoyke G, Currah RS (1990) "Endophytic fungi from the mycorrhizae of alpine ericoid plants" *Can J Bot* **69**: 347-352.

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

Stracke S, Kistner C, Yoshida S, Mulder L, Sato S, Kaneko T, Tabata S, Sandal N, Stougaard J, Szczyglowski K, Parniske M. (2002) "A plant receptor-like kinase required for both bacterial and fungal symbiosis" *Nature* **417(6892)**: 959-962.

Torii KU (2000) "Receptor kinase activation and signal transduction in plants: an emerging Picture"*Curr Opin Plant Biol* **3(5)**: 361-367.

Torii KU, Clark SE (2000) "Receptor-like kinases in plant development" A*dvances in Botanical Research Incorperating Advances in Plant Pathology* **32**: 226-268.

Torii KU (2004) "Leucine-rich repeat receptor kinases in plants: structure, function, and signal transduction pathways" *Int Rev Cytol* **234**: 1-46.

Trotochaud AE, Hao T, Guang W, Yang Z, Clark SE (1999) "The CLAVATA1 receptor-like kinase requires CLAVATA3 for its assembly into a signaling complex that includes KAPP and a Rho-related protein" *Plant Cell* **11**: 393-405.

Udvardi MK, Tabata S, Parniske M, Stougaard J (2005) "*Lotus japonicus*: legume research in the fast lane" *Trends Plant Sci* **10**: 222-228.

Urban A, Weiss M, Bauer R (2003) "Ectomycorrhizas involving sebacinoid mycobionts" *Mycol Res* **107**: 3-14.

Vanoosthuyse V, Miege C, Dumas C, Cock JM (2001) "Two large *Arabidopsis thaliana* gene families are homologous to the Brassica gene superfamily that encodes pollen coat proteins and the male component of the self-incompatibility response" *Plant Mol Biol* **46**: 17-34.

Varma A, Verma S, Sudha, Sahay N, Bütehorn B, Franken P (1999) "*Piriformospora indica*, a cultivable plant-growth-promoting root endophyte" *Appl Environ Microbiol* **65(5**): 2741-2744.

Varma A, Singh A, Sudha Sahay N, Sharma J, Roy A, Kumari M, Rana D, Thakran S, Deka D, Bharti K, Franken P, Hurek T, Blechert O, Rexer KH, Kost G, Hahn A, Hock B, Maier W, Walter M, Strack D, Kranner I (2001) "*Piriformospora indica*: a cultivable mycorrhiza-like endosymbiotic fungus" *in*: "Mycota IX" *Springer Series*, Heidelberg, Germany: 123–150.

Verma S, Varma A, Rexer KH, Hassel A, Kost G, Sarbhoy A, Bisen P, Bütehorn B, Franken P. (1998) "*Piriformospora indica*, gen. et sp. nov., a new root-colonizing fungus" *Mycologia* **90**: 898-905.

Villa JP, Bertenshaw GP, Bylander JE, Bond JS (2003a) "Meprin proteolytic complexes at the cell surface and in extracellular spaces" *Biochem Soc Symp* **70**: 53–63.

Wais RJ, Galera C, Oldroyd G, Catoira R, Penmetsa RV (2000) "Genetic analysis of calcium spiking responses in nodulation mutants of *Medicago truncatula*" *Proc Natl Acad Sci USA* **97**: 13407–13412.

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

Weiss M, Selosse MA, Rexer KH, Urban A, Oberwinkler F (2004) „Sebacinales: a hitherto overlooked cosm of heterobasidiomycetes with a broad mycorrhizal potential" *Mycol Res* **108**: 1003-1010.

Wienkoop S, Saalbach G (2003) "Proteome analysis. Novel proteins identified at the peribacteroid membrane from *Lotus japonicus* root nodules" *Plant Physiol* **131(3)**: 1080-1090.

Wolz RL, Bond JS (1995) "Meprins A and B" *Methods Enzymol* **248**: 325–345.

Yoshida S, Parniske M (2005) "Regulation of Plant Symbiosis Receptor Kinase through Serine and Threonine Phosphorylation" *J Biol Chem* **280(10)**: 9203-9209.

Zhang X (1998) "Leucine-rich Repeat Receptor-like Kinases in Plants" *Plant Mol Biol Reporter* **16**: 301–311.

7.1 List of publications

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

Oelmüller R, Shahollari B, Peškan-Berghöfer T, Trebicka A, Huong-Giong P, Sherameti I, Oudhoff M, Venus Y, Altschmied L, Varma A (2004) **"**Molecular analyses of the interaction between *Arabidopsis* roots and the growth-promoting fungus *Piriformspora indica*" *Endocytobiosis Cell Res* **15(2)**: 504-517.

Peškan-Berghöfer T, Shahollari B, Huong-Giong P, Hehl S, Markert C, Blanke V, Kost G, Varma A, Oelmüller R (2004) "Association of Piriformospora indica with Arabidopsis thaliana roots represents a novel system to study beneficial plant–microbe interactions and involves early plant protein modifications in the endoplasmic reticulum and at the plasma membrane" *Physiologia Plantarum* **122(4)**: 465-477.

Sherameti I, Shahollari B, Landsberger M, Westermann M, Cherepneva G, Kusnetsov V, Oelmüller R (2004) **"**Cytokinin stimulates polyribosome loading of nuclear-encoded mRNAs for the plastid ATP synthase in etioplasts of Lupinus luteus: the complex accumulates in the inner-envelope membrane with the CF(1) moiety located towards the stromal space" *Plant J* 38(4): 578-593.

Shahollari B, Varma A, Oelmuller R (2005) **"**Expression of a receptor kinase in Arabidopsis roots is stimulated by the basidiomycete Piriformospora indica and the protein accumulates in Triton X-100 insoluble plasma membrane microdomains" *J Plant Physiol* **62(8)**: 945-958.

Oelmüller R, Peškan-Berghöfer T, Shahollari B, Trebicka A, Sherameti I, Varma A. (2005) "MATH domain proteins represent a novel protein family in Arabidopsis thaliana, and at least one member is modified in roots during the course of a plant–microbe interaction" *Physiologia Plantarum* **124**: 152-166.

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

Shahollari B, Varma A, Oelmüller R (submitted to *The Plant Journal*) "A leucine rich repeat protein is required for growth promotion and enhanced seed production mediated by the endophytic fungus *Piriformospora indica* in *Arabidopsis thaliana*".

7.2 List of presentations: Talks

February 7, 2004 Second German Middle East Meeting, Jena, Germany
"Co-cultivation of *Arabidopsis* seedlings with the endophytic fungus *Piriformospora indica* strongly promotes growth and development: A model system to study plant/microbe interaction".

September 11-16, 2004 International Society of Endocytobiology (ISE) IX, Jena, Germany
"Early steps in plant/microbe interaction: *Piriformospora indica* meets *Arabidopsis* roots".

September 25, 2004 The First Biannual Symposium of the International Max Planck Research School: The Exploration of Ecological Interactions with Molecular and Chemical Techniques, Jena, Germany
"Early steps in plant/microbe interaction: *Piriformospora indica* meets *Arabidopsis* roots".

March 19-20, 2005 The Second Biannual Symposium of the International Max Planck Research School: The Exploration of Ecological Interactions with Molecular and Chemical Techniques, Jena, Germany
"Interaction between *Piriformospora indica* and *Arabidopsis thaliana*: Signal perception and final responses".

July 1-3, 2005 Yearly meeting of the German Section of the International Society for Endocytobiology, Bremerhaven, Germany
"Interaction between *Piriformospora indica* and *Arabidopsis thaliana*: Signal perception and final responses".

March 7-10, 2006 Conference of the Molecular Biology of the Plants, Dabringhausen, Germany
"Two leucine-rich repeat proteins, LRR1 and LRR2 are involved in the interaction between *Piriformospora indica* and *Arabidopsis thaliana*".

March 20-21, 2006 The Fourth Biannual Symposium of the International Max Planck Research School: The Exploration of Ecological Interactions with Molecular and Chemical Techniques, Jena, Germany
"Two leucine-rich repeat proteins, LRR1 and LRR2 are involved in the interaction between *Piriformospora indica* and *Arabidopsis thaliana*".

List of presentations: Posters

2003, SFB project 604 meeting, Jena Gemany

Shahollari B, Peškan T, Oelmüller, R
"Protein composition of plant Triton X-100-insoluble plasma membrane domains".

March 18, 2005 Evaluation of the 604 SFB project, Jena, Germany

Oelmüller R, Institut für Allgemeine Botanik & Pflanzenphysiologie FSU Jena
"Recognition of the growth- promoting fungus *Piriformospora indica* by *Arabidopsis thaliana* roots".

November 4-5, 2005 The Third Biannual Symposium of the International Max Planck Research School: The Exploration of Ecological Interactions with Molecular and Chemical Techniques, Jena, Germany

Shahollari B, Venus Y, Drzewiecki C, Oelmüller R
"Recognition of the growth-promoting fungus*Piriformospora indica* by *Arabidopsis thaliana* roots".

7.3 Acknowledgment

I would like to thank my "Doktorvater" **Ralf Oelmüller** for his support and advices. He was always patient with my questions, open for new ideas regarding the project and he was always available for discussion.

A special thank is for **Tatjana Peškan-Berghöfer**. She was my superviser during my first year. I was fealing lucky that I had the possibility to work with her. I could use what I learnt from her during all the time of my Ph.D.

I would also like to thank **Irena Sherameti** and **Staver Bezhani** for the support during the hard time at the beginning of my Ph.D. Without them I would have had more difficulties with my English and my "German". Irena gave me many times the strength to go on.

I also thank the youngest member of our institutes fungi-group: **Die Yvonne**. She was for me like "health insurance", with her around I was fealing better ☺.

I thank **Barbara**, **Meta**, **Gabi** and **Heide** for their help in the lab. They helped me with lot of patience to advance in German speaking. Our Meta never forgot to bring walnuts and Christmas cookies every year, she even told Santa Claus and the Easter Bunny to bring chocolate to our institute. Heide with her hospitality and her optimism was always there for me.

I would also like to thank **Klaus Appenroth** and **Thomas Pfannschmidt**. They always answered my questions and they were willing to help me anytime.

Furthermore I thank the fungi-group (**Corinna and Jyoti**) and all members of the Institute of Plant Physiology in Jena for their help. Althought my German was "not so good" at the beginning, they were always kind and good helpers in the lab.

I would like to thank all my friends who gave me friendship and made my life outside the laboratory comfortable and lovly. Particularly **Claudia,** I thank her for her patience and her loving support. At possible and impossible times she was there for me. I also thank **Stefan,** for his help and his patience ☺.

I thank my family for the support, my parents and especially my sisters, **Alketa** and **Reida**.

Concluding I would like to thank the **DAAD** that financed three years of my Ph.D.

7.4 Curriculum Vitae

Name: Bationa Shahollari

PERSONAL DATA:

Birthday	May 15, 1979
Place of Birth	Fier
Nationality	Albanian
Citizenship	Albanian
Civil Status	Single

EDUCATION:

September 1993 - June 1997

Secondary school at the Gymnasium "Lefter Talo" Patos, Albania.

October 1997 - July 2002

State University of Tirana; Faculty Natural Sciences; Diploma in the branch **Biology**.

Diploma thesis: Genetic structure of human population of the Fier district, evaluated according to surname distribution.

Evaluation of diploma thesis :Final degree: very good (10 out of 10).

October 2002 - July 2005

DAAD scholarship for Ph.D. studies in Molecular Biology – Institute of General Botany and Plant Physiology, University of Jena, Germany.

From August 2005 I am scientific co-worker in Institute of General Botany and Plant Physiology, University of Jena, Germany.

SUPPLEMENTARY STUDIES:

July, 11th - July 25th 1999

Attended and obtained a certificate in the School of Environment, Tirana, Albania.

July 2001 - October 2001.

Participated in a research project at the Institute of Plant Physiology (Friedrich Schiller University, Jena, Germany), on plant molecular biology techniques.

From October 2004 I participate the International Max Planck Research School in Jena.

LANGUAGES:

Albanian	- Native
English	- Very good
French	- Good
German	- Good

7.5 Ehrenwörtliche Erklärung zur Anfertigung der Dissertation

Entsprechend der Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität erkläre ich hiermit, dass ich die vorliegende Arbeit selbständig und nur unter Verwendung der angegebenen Hilfsmittel und Literatur angefertigt habe.

Personen, die bei der Auswahl und Auswertung des Materials und der Erstellung der Manuskripte behilflich waren sind am Beginn eines jeden Manuskripts angegeben.

Die Hilfe eines Promotionsberaters wurde nicht in Anspruch genommen.
Die vorgelegte Arbeit wurde bislang weder an der FSU, noch an einer anderen Hochschule als Dissertation eingereicht.

Jena, den 3 Mai 2006

Bationa Shahollari