

## REVIEW PAPER

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# Role of fungal biomolecules for establishing mutualistic/pathogenic interactions with Arabidopsis roots: Possibilities to decipher downstream signaling events

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## Abstract

A huge number of beneficial and pathogenic fungi release biomolecules into the rhizosphere which initiate signaling events in the roots leading to mutualistic or pathogenic plant/fungus interactions. The combination and concentration of the individual biomolecules in the rhizosphere is critical for the plant's decision to invest in either growth or defense. These biomolecules activate receptors in the roots and induce cytoplasmic  $\text{Ca}^{2+}$  elevation in a phosphorylation-dependent manner. Furthermore, they stimulate phospholipid signaling and AGC kinase activities which coordinate the balanced response between growth/development and defense, by cross-talking to the  $\text{Ca}^{2+}$  signals. We use transgenic Arabidopsis plants expressing the  $\text{Ca}^{2+}$  sensor aequorin to isolate and identify biomolecules from the exudates of the beneficial root-colonizing fungi *Piriformospora indica* and *Mortierella hyalina*, and from the pathogenic fungi *Alternaria brassicae* and *Verticillium dahliae*. In this brief summary, we will introduce these microorganisms and their specific role in the interaction with roots. Their exudate components induce a rapid (< 90 s) and transient increase in cytoplasmic  $\text{Ca}^{2+}$  levels in the roots. We isolated Arabidopsis non-allelic EMS mutants which do not respond to these biomolecules. Their initial characterization has demonstrated that the  $\text{Ca}^{2+}$  response is necessary for the proper plant response to the fungi, and that these mutants are impaired in abscisic acid (ABA) signaling.

**Key words:** *P. indica*, *M. hyalina*, *A. brassicae*, *V. dahliae*, transgenic Arabidopsis,  $[\text{Ca}^{2+}]_{\text{cyt}}$  elevation, fungal exudates

## 1. Introduction

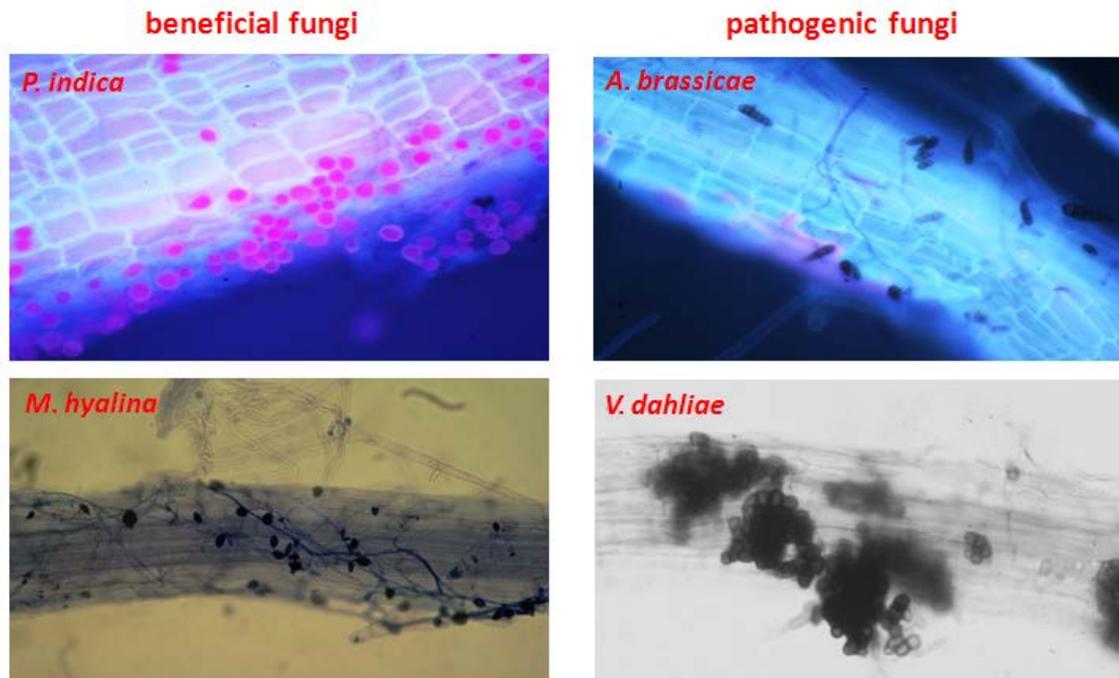
The rhizosphere is probably one of the most complex plant-microbe interaction systems in nature where roots are associated with a wide range of different microorganisms with both detrimental and beneficial outcomes. The relationship between these organisms requires a permanent information exchange during which the roots have to respond to different chemicals from millions of microbes. Central to plant survival is the ability to either limit their growth or intrusion, in the case of pathogens, or promote the association, in the case of symbionts [5, 17]. After perception of the chemical mixture, elaborate communication systems in the roots integrate the information for an appropriate and balanced response which range from defense against pathogens to establishing a mutualistic interaction with beneficial symbionts. A crucial second messenger in the plants with the capability of integrating such diverse chemical information is  $\text{Ca}^{2+}$ . Both pathogenic and beneficial microbes release microbe- (or pathogen-) associated molecular patterns (MAMPs and PAMPs) and/or uncharacterized compounds which induce a rapid and transient increase in cytoplasmic  $\text{Ca}^{2+}$

( $[\text{Ca}^{2+}]_{\text{cyt}}$ ) levels in the root cells upon receptor activation. Immediately downstream of receptor activation, a phosphorylation cascade participates in the establishment of the so-called  $\text{Ca}^{2+}$  signature, i. e. the generation of  $[\text{Ca}^{2+}]_{\text{cyt}}$  transients with different amplitudes, durations, frequencies and  $[\text{Ca}^{2+}]_{\text{cyt}}$  locations, the selective activation of  $\text{Ca}^{2+}$  channels and the stimulation of respective  $\text{Ca}^{2+}$ -dependent signaling components [9, 10]. Changes in  $[\text{Ca}^{2+}]_{\text{cyt}}$  levels and an oscillation in nuclear  $[\text{Ca}^{2+}]$  levels ( $\text{Ca}^{2+}$  spiking) are early responses to Nod factors and central to arbuscular mycorrhizal symbiosis [6]. A  $\text{Ca}^{2+}$ /calmodulin dependent protein kinase, DMI3/CCaMK decodes Nod- and Myc-factor-induced  $\text{Ca}^{2+}$  oscillations [9, 12]. In contrast, PAMP-induced  $[\text{Ca}^{2+}]_{\text{cyt}}$  elevation in pathogenic plant/microbe interactions activate innate immune responses, the production of reactive oxygen species (ROS) and defense gene expression. Pathogenic and symbiotic microbes can also suppress host responses by secretion of extracellular polysaccharides that sequester apoplasmic  $\text{Ca}^{2+}$  [1].  $[\text{Ca}^{2+}]_{\text{cyt}}$  elevation can be achieved by the regulation of plasma membrane bound cation/ $\text{Ca}^{2+}$ -channels/-pumps, but also by the

release from the nucleus, ER, mitochondria or chloroplasts [8].

We study the response of the roots of the model plant *Arabidopsis thaliana* to biomolecules from four root-colonizing fungi in the laboratory and nature (Fig. 1). The four fungi: *Piriformospora indica*, *Mortierella hyalina*, *Alternaria brassicae* and *Verticillium dahliae* have in common that their

exudates contain one or more compounds which induce rapid (within 90 sec)  $[Ca^{2+}]_{cyt}$  elevation in the roots. Genetic studies in *Arabidopsis* have demonstrated that  $[Ca^{2+}]_{cyt}$  elevation is crucial for the quite diverse downstream responses of the plants to these microbes, and that there exist a massive cross-talk between these  $Ca^{2+}$ -dependent downstream responses.



**Figure 1.** Microscopical view of *Arabidopsis thaliana* roots colonized with different fungi (beneficial and pathogenic). *Piriformospora indica*, *Mortierella hyalina*, *Alternaria brassicae*, *Verticillium dahliae*.

## 2. Beneficial and pathogenic fungi

The endophytic fungus *P. indica* colonizes the roots of many plant species. Similar to arbuscular mycorrhizal fungi, *P. indica* promotes plant growth, biomass and seed production and confers resistance to biotic and abiotic stress [3, 21, 22, 24, 25]. *P. indica* is a member of Sebaciniales, grows inter- and intracellularly and forms pear shaped spores, which accumulate within the roots and on the root surface (Fig. 1). After the establishment of a beneficial interaction, barely any defense or stress genes are activated and no reactive-oxygen species (ROS) are produced by the host against *P. indica* [3, 8, 16, 24, 25]. The endophyte releases a trihexose that induces root-specific  $[Ca^{2+}]_{cyt}$  elevation in *Arabidopsis* and *N. tabacum*, which is followed by a nuclear  $Ca^{2+}$  response in *N. tabacum* [24, 25].

Similar benefits for the performance of *Arabidopsis*, *N. tabacum* and other plant species are mediated by the root-colonizing fungus *M. hyaline* [23]. This fungus (Fig. 1) was originally discovered in

association with the roots of grass plants in golf courts. The fungal exudate contains two biomolecules which strongly induce  $[Ca^{2+}]_{cyt}$  elevation in roots and we are actually working to purify one of these compounds (Ludwig et al. manuscript in preparation).

The necrotrophic fungus *A. brassicae* (Fig. 1) is a seed-, air- and soil-borne fungus that penetrates through all plant parts and causes lesions on leaves, stems, siliques and roots [2]. It causes black leaf spot disease in crucifers including *A. thaliana*. The disease progression ultimately results in plant death, mostly caused by host-specific toxins [2, 14, 15, 18, 26] and unknown low molecular weight biomolecules of different chemical classes which induce  $[Ca^{2+}]_{cyt}$  elevations in *Arabidopsis* roots and mimic the fungus in inducing host defense gene activation, ROS production and innate immunity. The two well known phytotoxins destruxin B and sirodesmin PL from *A. brassicae* induce phytoalexin and camalexin biosynthesis in crucifers [18]. The vascular wilt fungus *V. dahlia* has a broad host range and infects more than 200 plant species [10, and references

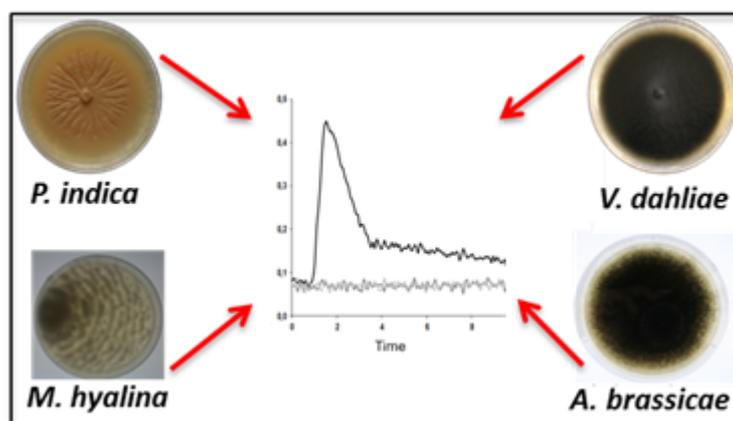
therein]. The fungus forms seed-like long-lived survival structures called microsclerotia, which germinate in response to stimuli from root exudates. The hyphae grow inter- and intracellularly through the root cortex toward the central cylinder of the root (Fig. 1). They enter the xylem cells, proliferate in the vascular system of the root, and at later stages colonize the xylem of the hypocotyl, stem and leaf tissue, where they cause the characteristic symptoms of wilting, by disrupting water transport and ultimately death of aerial tissues [10]. *Verticillium* species are considered as hemibiotroph with a biotrophic life phase within the nutrient-poor environment of the root xylem and a necrotrophic phase in the aerial plant tissues. *V. dahliae* releases components, peptides and proteins, probably a cocktail of them, into the medium/environment, which are sufficient to induce disease symptom development in various species including Arabidopsis [9, and references therein]. This exudate preparation contains low molecular weight compounds which rapidly induce  $[Ca^{2+}]_{\text{cyt}}$  elevations in Arabidopsis root cells. However, which of these compounds is required for a particular disease phenotype is unknown. We concentrate on two of these biomolecules which induces the strongest  $Ca^{2+}$  response in the roots. Finally, we address questions which need to be answered in order to understand how fungal signals can reprogram the root to either establish a mutualistic interaction with beneficial fungi or to activate the plant immune system against pathogen invaders.

### 3. Fungal exudates: Induction of cytosolic $Ca^{2+}$

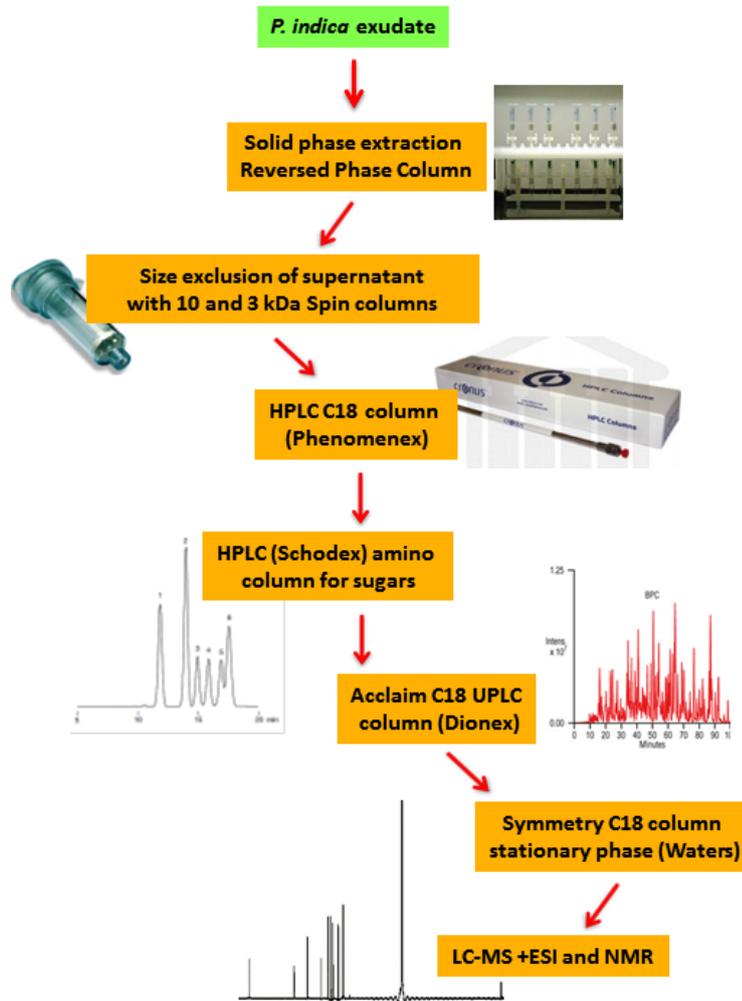
The exudate preparations from germinating fungal spores in distilled water are used to purify the biomolecules which induce  $[Ca^{2+}]_{\text{cyt}}$  elevations in the

roots (Fig. 2). The exudate compounds are separated by chromatographic steps (as shown for the *P. indica* component in Fig. 3) and fractions with the active compound are identified by the  $Ca^{2+}$  assay.

We use transgenic Arabidopsis lines with the aequorin system [11] to measure  $[Ca^{2+}]_{\text{cyt}}$  levels in roots [24, 25]. The Arabidopsis aequorin line was used for ethyl methane sulfonatemutagenesis (EMS). For isolating mutants defective in  $[Ca^{2+}]_{\text{cyt}}$  elevation to the different fungal cell wall extracts (CWE) we performed a  $Ca^{2+}$ -based screen of approximately 150.000 individual plants (with roots from individual 18-day-old  $M_2$  seedlings; for details see [7]). We identified 12 mutants which completely failed to induce  $[Ca^{2+}]_{\text{cyt}}$  elevation in response to the CWE from *A. brassicae*. Later on, we found out that some of these mutants also failed to respond to CWE preparations for other microbes. Under our growth conditions the mutants did not show any visible phenotype compared to wild type (WT) plants [7]. These mutants were named *cytoplasmic  $Ca^{2+}$  elevation mutants (cycam)* [7]. Genetic analyses of crosses uncovered that four *cycam* mutants were allelic and two of them, *cycam1-1* and *cycam1-2*, were randomly chosen for further analyses [7]. The *cycam1-1* and *cycam1-2* roots did not respond to the  $Ca^{2+}$ -inducing extract preparations from mycelia and extract preparation from spores of *A. brassicae* (data not shown) [7]. Since *cycam1* was isolated by a screen in which  $[Ca^{2+}]_{\text{cyt}}$  elevation was impaired in Arabidopsis roots, we further tested exudate preparations from other microbes which interact with roots. This list included *Rhizoctonia solani*, a necrotrophic fungus, *Phytophthora parasitica* var. *nicotianae*, a hemibiotrophicoomycete, and *Agrobacterium tumefaciens*, a tumor-inducing bacterium.



**Figure 2.** Fungal exudate compounds induce  $Ca^{2+}$  responses in root cells.



**Figure 3.** Purification protocol of the *P. indica* compound that induces  $[Ca^{2+}]_{\text{cyt}}$  elevation.

Interestingly, *cycam1* did not respond to the exudate preparations from these fungi as well, and less to a CWE from *A. tumefaciens*, even though these preparations induced  $[Ca^{2+}]_{\text{cyt}}$  elevation in WT. A CWE preparation from the root-colonizing fungus *Mortierella hyalina* [23] induced  $[Ca^{2+}]_{\text{cyt}}$  elevation in the roots of the WT and *cycam1* mutant. Therefore, CYCAM1 is involved in  $[Ca^{2+}]_{\text{cyt}}$  elevations in response to different, but not all microbes. Studies are in progress to decipher whether the  $[Ca^{2+}]_{\text{cyt}}$  elevation responses differ between pathogenic and beneficial root-colonizing microbes.

#### 4. Conclusions and perspectives

Our future goal is to compare the responses of the roots of the model plant *Arabidopsis thaliana* to biomolecules from root-colonizing beneficial and pathogenic fungi. How does a plant distinguish between a beneficial and a pathogenic fungus and how can it mount an appropriate response to signals from

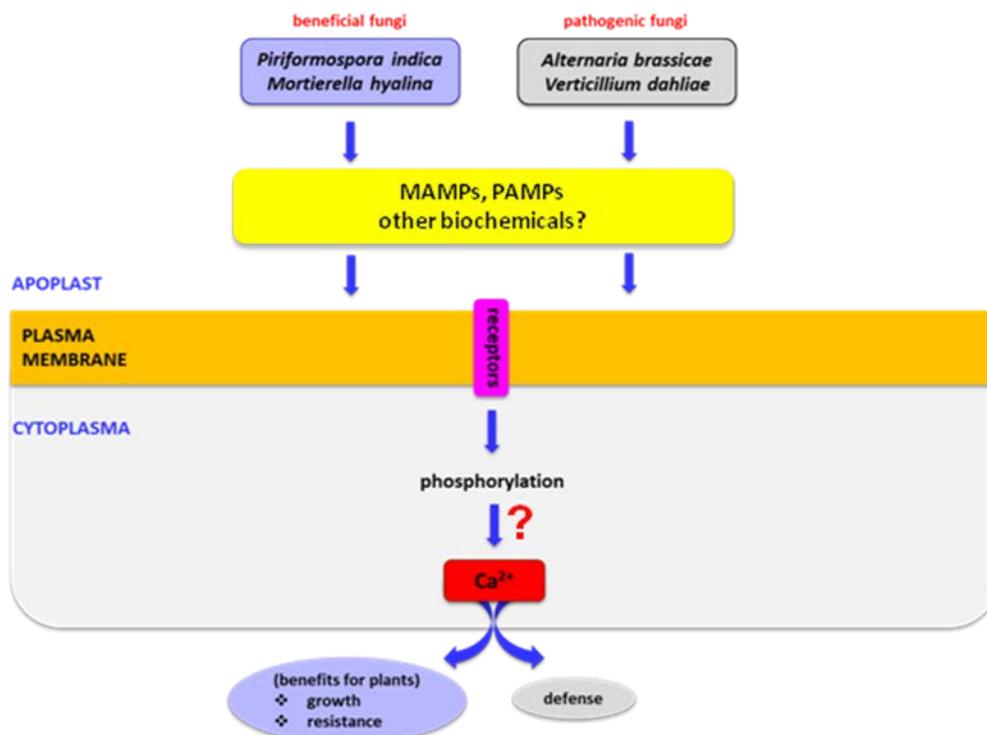
the individual microbes? To address these questions, the bioactive compounds, which induce  $[Ca^{2+}]_{\text{cyt}}$  elevations, must be isolated and characterized in details. The receptors in the roots which become activated after binding of the fungal compound have to be identified. The available mutants which do not show  $[Ca^{2+}]_{\text{cyt}}$  elevation in response to the fungal stimuli are the best tools to identify the responsible genes/protein by map-based cloning strategies and probably Illumina sequencing (next generation sequencing). In this context, it is important to see whether the receptors can be classified into categories which (a) direct beneficial interactions with beneficial microbes and those which (b) activate the plant immune system. The symbiotic signals of mycorrhizal fungi, the Myc factors, and those from rhizobial bacteria, Nod factors, are lipo-chitoooligosaccharides. They are perceived by lysin-motif (LysM) receptors which induce a signaling pathway leading to either mycorrhiza or nodule formation. Myc factors from *Glomus intraradices* reprogram root gene expression,

induce root branching and mycorrhization in *Medicago truncatula* [20, and ref. therein]. Interestingly, LysM receptors are also involved in the perception of chitoooligosaccharides, fungal cell wall compounds that induce defense responses and resistance to pathogens. This raises the question again of how plants discriminate between beneficial and pathogenic microorganisms and their exudated chemical mediators [cf. 4], and further highlights the importance of this question, for instance for agri- and horticulture. It is reasonable to assume that the perception of fungal signals by root receptors is evolutionarily connected. It is also possible that the exudate compounds from beneficial fungi induce plant immune responses (as signals from pathogens do), which are later on balance by the release of elicitors which reduce or restrict the plant defense response against the beneficial fungi. Such mechanisms have often been described to establish a stable mutualistic interaction, for instance by balancing the growth of the microbes in the roots with the demands of the host.

Another important question is the origin of  $\text{Ca}^{2+}$ . It is generally believed that it is of apoplastid origin, but a large body of studies also indicates that internal stores, such as the endoplasmatic reticulum, the vacuole and organelles such as nuclei, plastids and

mitochondria store  $\text{Ca}^{2+}$  that can be released upon stimulating signals. Most importantly, the  $\text{Ca}^{2+}$  channels have to be identified which transport the  $\text{Ca}^{2+}$  into the cytoplasm. Since the  $[\text{Ca}^{2+}]_{\text{cyt}}$  elevation is only transient, there must also be efficient mechanisms which transfer the  $\text{Ca}^{2+}$  ions back to its original place. This is most likely mediated by  $\text{Ca}^{2+}$  exporters in the plasma membrane or the respective membranes that separate the  $\text{Ca}^{2+}$  stores from the cytoplasm.

The  $\text{Ca}^{2+}$  signature is decoded by  $\text{Ca}^{2+}$ -binding proteins, and in Arabidopsis, more than 100 different proteins belonging to different protein classes have been identified. Again, the multitude of proteins involved in transducing the  $\text{Ca}^{2+}$  information to downstream targets maybe important for establishing specificity. Early signaling events also involve reversible phosphorylation events, which can be as fast as seconds after receptor activation. A number of modern mass-spectrometric-based techniques are available now to identify the phosphorylated/dephosphorylated proteins after receptor activation. A comparative analysis of these compounds along the signal transduction pathway may help to decipher events leading to beneficial and pathogenic interactions.



**Figure 4.** A model describing the apoplastic and cytoplasmic events leading from biomolecules in fungal exudates to plant responses.

Finally reprogramming the plant genome requires the activation of specific subsets of genes by transcription factors. These transcription factors need to be identified. However, identification of a specific function associated with a particular transcription factor is often difficult, because they are members of large gene/protein families and thus, individual members of these families often have overlapping functions. Secondly, the vast majorities of transcription factors do not bind DNA as monomers, but form homo- and particularly heterodimers to activate gene expression. Since one transcription factor can be part of different complexes with quite different functions, it is difficult to pinpoint down a particular function for a transcription factor within a given scenario. The story becomes even more complicated since a particular transcription factor can be part of a complex that functions as a transcriptional activator and of another complex that functions as a transcriptional repressor.

Based on our data and those from the literature we propose a model describing the apoplastic and cytoplasmic events leading from biomolecules in fungal exudates to plant responses (Fig. 4).

We are interested to study early phosphorylation events prior to  $[Ca^{2+}]_{cyt}$  elevations, to check whether differences in the phosphorylation pattern can be detected after application of the biomolecules to the roots. For this we initially used a *cerk1* *cerk4* knock-out mutant which completely has lost the ability to respond to chitin oligosaccharides [13]. Chitin, a polymer of  $\beta$ -1, 4-linked N-acetyl-glucosamine, is the main component of the fungal cell wall and chitin oligosaccharides which are released from the cell walls of pathogenic fungi function as fungal MAMPs in plant immunity. In *A. thaliana*, the Chitin Receptor like Kinase CERK1 with three extracellular Lys motifs is responsible for chitin oligosaccharide recognition [7, 19]. CERK1 binds chitin oligomers *in vitro*, for which all three Lys motifs are required [19] and activates defense genes and the production of ROS. Chitohexaose application to roots induce a rapid increase in  $[Ca^{2+}]_{cyt}$  elevations, and this response depends mainly on CERK1, but also CERK4, one of the four receptors expressed in roots (Mrozinska and Oelmüller, unpublished). It is unclear at present why chitin oligosaccharides from fungal pathogens, but not beneficial fungi, function as PAMPs in plant immunity. Chitin signaling is an excellent example, because the signaling events lead to defense gene activation and many steps in chitin-mediated innate immunity have been elucidated in the past. Therefore,

we will use this information and compare it with the information (and signaling molecules) which become activated after the application of a chemical compound from a beneficial fungus. As mentioned above, also their cell walls contain chitin, and thus, also beneficial fungi can release cell wall compounds which activate immune responses in roots. How can an interaction with a beneficial fungus then lead to the repression of the plant defense system against pathogen attack? By comparing early signaling events in roots after stimulation with either a MAMP (from a beneficial fungus) or a PAMP (from a pathogen), we should be able to answer the question whether chemical mediators in exudate preparations from beneficial and pathogenic fungi trigger the same or different signaling events in Arabidopsis roots.

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