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Calcium signaling in pathogenic and beneficial plant microbe interactions

What can we learn from the interaction between *Piriformospora indica* and *Arabidopsis thaliana*

Jyothilakshmi Vadassery[1] and Ralf Oelmüller[2,*]

[1]Department of Plant Biology; Cornell University; Ithaca, NY USA; [2]Institut für Allgemeine Botanik und Pflanzenphysiologie; Jena, Germany

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Elevation of intracellular calcium levels in a plant cell is an early signaling event in many mutualistic and pathogenic plant/microbe interactions. In pathogenic plant/fungus interactions, receptor-mediated cytoplasmic calcium elevations induce defense genes via the activation of ion fluxes at the plasma membrane, an oxidative burst and MAPK activation. Mycorrhizal and beneficial endophytic plant/fungus interactions result in a better plant performance through sequencial cytoplasmic and nuclear calcium elevations. The specificity of the calcium responses depends on the calcium signature, its amplitude, duration, frequency and location, a selective activation of calcium channels in the diverse cellular membranes and the stimulation of calcium-dependent signaling components. Arabidopsis contains more than 100 genes for calcium-binding proteins and channels and the response to pathogens and beneficial fungi relies on a highly specific activation of individual members of these protein families. Genetic tools are required to understand this complex response patterns and the cross talks between the individual calcium-dependent signaling pathways. The beneficial interaction of Arabidopsis with the growth-promoting endophyte *Piriformospora indica* provides a nice model system to unravel signaling events leading to mutualistic or pathogenic plant/fungus interactions.

Introduction

Plant roots interact with a wide array of microorganisms in soil, resulting in mutualistic (beneficial for both partners), commensalistic (beneficial for the host, but not invader) or pathogenic (harmful for the host or both partners) interactions.[1-3] Microbes release various factors which are necessary for their recognition by plant cells.[4-6] In pathogenic plant/fungi interactions, chitins, glucans, lipids, fatty acids, (glycol-)proteins or peptides activate defense gene expression in the plant cells.[5,6] In contrast, symbiotic interactions lead to a close physical association of the microorganism with the plant, which is beneficial for both organisms.[7,8]

*Correspondence to: Ralf Oelmüller; Email: b7oera@hotmail.de
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Establishment of such a beneficial symbiosis is complex, the interaction is not always as harmonious as it appears, and a rejection of the invading symbiont can occur at any stage of the infection.[9,10] For successful infection a molecular dialogue between the partners is essential. In rhizobial symbiosis plant roots produce flavonoids while the bacteria releases nodulation (Nod) factors (lipochitooligosaccharide),[11,12] which initiate signaling in the host cells. In arbuscular mycorrhizal symbiosis plants release strigolactones which act as branching factors for fungal hyphae, while the fungi release unidentified mycorrhizal (MYC) factors.[13] Besides mycorrhiza, beneficial interactions also occur with endophytic fungi[8] and plant-growth-promoting bacteria[14] which colonize the host's root. The mode of recognition and early signaling steps are crucial in understanding how a plant can differentiate between a beneficial and a detrimental microbe. As far as we know, a very early event in the interaction of pathogenic, mycorrhizal or endophytic microbes with a plant cell is an increase in the intracellular calcium (Ca^{2+}) levels within seconds or minutes after the recognition of the two partners[15-18] which raises the question of how this information is decoded into the appropriate responses in the plant cell.

Ca^{2+} Signaling in Recognition of Microbes

The Ca^{2+} ion is a second messenger in numerous plant signaling pathways, coupling extracellular stimuli to intracellular and whole-plant responses.[19] The cellular Ca^{2+} level is tightly regulated and even a small change in its concentration provides information for protein activation and signaling. Ca^{2+} cannot be synthesized or degraded, thus, its concentration at a given time and location depends on the balance between entry and efflux processes. In eukaryotic cells, various stimuli mobilize different pools of Ca^{2+} to trigger characteristic changes in the cytoplasmic Ca^{2+} ($[Ca^{2+}]_{cyt}$). Ca^{2+} channels have been detected in the plasma membrane, vacuolar membrane, ER, chloroplast, mitochondria and nuclear membranes of plant cells.[20]

The Ca^{2+} signature of a given signal, characterized by its amplitude, duration, frequency and location, was shown to encode a message that, after decoding by downstream effectors, contributes to the specific physiological response. This explains the presence of the large number of Ca^{2+} sensors in plant cells to decode different

incoming stimuli.[18,21] $[Ca^{2+}]_{cyt}$ elevation may be caused by an uptake of Ca^{2+} from the extracellular medium, or by Ca^{2+} mobilization from internal stores such as the ER or ER-derived membrane systems or organelles. The origin of Ca^{2+} signals is important in the physiological response.[22,23] Specificity in the Ca^{2+}-signaling system results from a multifactorial decision process ranging from a specific Ca^{2+} signature to the availability of a specific set of Ca^{2+} sensors and their target proteins that are coupled to downstream components at a give place.

Many Ca^{2+} signaling studies in plant cells are performed using the aequorin technology based on bioluminescence.[24] Aequorin is a Ca^{2+} binding photoprotein found in jellyfish composed of an apoprotein (apoaequorin) and a prosthetic group, a luciferin molecule, coelenterazine. In the presence of molecular oxygen the functional holoprotein aequorin reconstitutes spontaneously. The protein contains three EF-hand Ca^{2+} binding sites. When these sites are occupied by Ca^{2+}, aequorin undergoes a conformational change and behaves as an oxygenase that converts coelenterazine into excited coelenteramide, which is set free together with carbon dioxide. As the excited coelenteramide relax to the ground state, blue light (λ = 469 nm) is emitted. This emitted light can be easily detected with a luminometer.[25]

In most cases, potential pathogens activate basal defense responses in the plant cell through receptor-mediated recognition of pathogen/microbe-associated molecular patterns (PAMPs/MAMPs) and downstream signaling to activate innate immune responses. Basal defense does not prohibit pathogen colonization but controls or slows down pathogen spread. Downstream of receptor activation, the signal chain of events leading to defense-related gene activation and phytoalexin accumulation consists of ion fluxes at the plasma membrane (H^+/Ca^{2+} influxes, K^+/Cl^- effluxes), an oxidative burst and MAPK activation.[26] During compatible interactions, pathogen-derived effector/virulence molecules suppress PAMP-induced defense responses, and enable the pathogen to overcome basal resistance and to successfully infect the plant.[27-29] Ca^{2+} elevations have been reported, for instance, for the oligopeptide elicitor pep-13 in parsley cell cultures,[26] flg22 in Arabidopsis leaf discs, β-glucan fragments in soybean cell cultures[30] and for many proteinacoeus elicitors (like cryptogein) and oligosaccharide elicitors.[20] Rapid and transient elevations in $[Ca^{2+}]_{cyt}$ were shown to be induced by diffusible molecules released by AM fungi.[31]

In the vast majority of plant/fungus interactions on earth, the microbes do not cause diseases. More than 80% of all land plants form mycorrhiza and Ca^{2+} signaling plays a dual role in these interactions. During early phases of the establishment of the interaction, i.e., before the fungus delivers nutrient to the roots and is accepted as a beneficial partner, the plant responds to the microbe by activating a mild defense response. The signaling events leading to the defense appear to be similar to those in pathogenic interactions[26,32] and might activate the same or similar signaling pathways. However fungus-derived MAMPs and early signaling events in the plant cell leading to defense responses in beneficial plant/microbe interactions are mostly unknown. In addition, both $[Ca^{2+}]_{cyt}$ and nuclear Ca^{2+} elevations are also crucial in establishing the benefits for the plants. Early signal transduction during rhizobacteria-mediated nodule and mycorrhiza formations in legumes is associated with ion fluxes across different membrane systems of the host cell.[33-35] Nod factors trigger $[Ca^{2+}]_{cyt}$ influx at the root hair tip within 1 min,[34,36] perinuclear Ca^{2+} oscillations with a delay of 10 to 30 min[33] and the transcription of symbiosis-related plant genes.[33,34,37] While $[Ca^{2+}]_{cyt}$ elevation is likely to be caused by an uptake from cell-external stores, Ca^{2+} oscillations around and in the nucleus appear to depend on Ca^{2+} stores in cisterns of the endoplasmic reticulum and the nuclear envelope.[38] In pea root hair cells, perinuclear Ca^{2+} oscillations are induced by Nod factor-like molecules without induction of Ca^{2+} influx, suggesting that the Ca^{2+} influx across the plasma membrane and the perinuclear Ca^{2+} spiking are two distinct responses.[39] The *Lotus japonicus* mutants *castor* and *pollux*, which are unable to form both bacterial and fungal symbioses, are impaired in the perinuclear Ca^{2+} spiking but retain the Ca^{2+} influx at the root hair tip.[35] Thus, more than one Ca^{2+}-dependent process appears to be involved in mycorrhiza formation.

Ca^{2+} is also a major player in the interaction between the endophytic fungus *Piriformospora indica* and the model plant Arabidopsis. One of the earliest signaling events during the recognition of the two symbionts is a rapid induction of $[Ca^{2+}]_{cyt}$ elevation, which is followed by a nuclear Ca^{2+} response.[40] In this beneficial interaction, the fungus promotes growth, seed yield and biomass production and confers resistance against biotic and abiotic stresses.[1,41] Since several mutants which do not respond to *P. indica* with regard to growth promotion and higher biomass production are also impaired in $[Ca^{2+}]_{cyt}$ elevation, both processes must be linked in these mutants. $[Ca^{2+}]_{cyt}$ elevation can be induced by an autoclaved cell wall extract (CWE) from *P. indica*, which also promote growth of Arabidopsis and other plant species. Thus root colonization by the living fungus is not required for this response. The autoclaved CWE induces $[Ca^{2+}]_{cyt}$ elevation preferentially in the roots, which is consistent with the observation that the endophyte is a root-colonizing fungus. The $[Ca^{2+}]_{cyt}$ response to the CWE shows a maximum response at 2 minutes, followed by gradual decline and reaches background levels in 40 minutes. The same CWE induces a slightly different Ca^{2+} signature in tobacco roots hinting at the possibility of species-specific plant responses.[40] Ca^{2+} influx is prevented by the general serine/threonine protein kinase inhibitor staurosporine indicating that phosphorylation changes may be involved upstream of the Ca^{2+} response probably at or around the receptor level. The involvement of receptors is further proved by the refractive nature. Plant cells lose their capacity to respond a second time to the same type of elicitor (refractive behavior), but remain sensitive to another type of elicitor perceived by another receptor. This characteristic feature of the Ca^{2+} response has first been described for pathogenic plant/microbe interactions, but is also found in beneficial interactions.[31,40] The refractory nature and the inhibition of $[Ca^{2+}]_{cyt}$ elevations in the presence of kinase inhibitors suggest the involvement of a receptor upstream of the Ca^{2+}response, whereas other stimuli, e.g., H_2O_2, show no such behavior when applied after the CWE.

Downstream Events in Ca^{2+} Signaling in Plant/Microbe Interaction

Post-translational modification of proteins by reversible phosphorylation is a key process regulating many functions in plants,

including defence responses downstream of the elicitor-induced Ca^{2+} influx.[42] Protein phosphorylation changes are observed for MAPK after the applications of pathogen-derived PAMPs, such as flg22, or the CWE from *P. indica*.[40] In the latter case, phosphorylation changes are more pronounced in the roots, while flg22 is more effective in shoots. Silencing of *mpk6* compromised both gene-for-gene and basal resistance in Arabidopsis[43,44] and hence is a common element in plant resistance. More recently, the involvement of MAPK3 and -6 in the beneficial interaction between *P. indica* and Arabidopsis has been shown by the analysis of *mpk3* and *mpk6* knock out lines:[40] Ca^{2+} inhibitors such as $LaCl_3$ and BAPTA abolished MAPK phosphorylation and the *mpk6* knock-out lines does not respond to *P. indica*.[40] It suggests both mutualistic and pathogenic interactions share some common signaling components such as MAPKs.

The occurrence of a nuclear Ca^{2+} elevation in response to *P. indica*, measured with tobacco BY-2 cultures,[40] hints to the involvement of an additional Ca^{2+} response, similar to the observations in legumes.[16] The maximum $[Ca^{2+}]_{cyt}$ elevation occurs after 2 min, while the response in the nucleus is only detectable after 6 min, suggesting a sequential response. Measuring the Ca^{2+} response by the fluorescence resonance energy transfer (FRET)-based Ca^{2+}-indicator cameleon[35] would be helpful for the analysis of single cells. This, in combination with the identification of the active component in the CWE from *P. indica*, would be a helpful tool to unravel the signaling pathways activated by Ca^{2+} in this symbiosis. Unfortunately, at present, no transgenic line with nuclear-localized aequorin is available, which would be a helpful tool for genetic screens. The importance of nuclear Ca^{2+} in signaling processes is underlined by the existence of Ca^{2+} effectors in the plant nucleus, including calmodulin (CaM), CaM-binding protein, CDPKs and Ca^{2+}-CaM-regulated protein phosphatases.[45-47] For instance, DMI3 functions downstream of Ca^{2+} spiking and is a chimeric Ca^{2+}/CaM-dependent protein kinase in the nucleus.[48,49] The *dmi3* mutant provides strong genetic corroboration for an essential role of Ca^{2+} signaling in initiating symbiotic interactions.[15,50,51]

Differences between the Role of Ca^{2+} in Nodule/Mycorrhiza Formation in Legumes and the Beneficial Interaction between *P. indica* and Arabidopsis

In legumes, three *DMI* genes have been identified which are essential for rhizobial Nod factor signal transduction and for the symbiosis with arbuscular mycorrhizal fungi. Mutations in these genes fail to allow nodule formation and the entry of the fungus into the cortex of the root.[50] One of the *dmi* genes codes for DMI3, a CCaMK. However, in case of the *P. indica*/Arabidopsis interaction, the fungus itself is not required for establishing growth promotion, and can be replaced by a CWE. Thus, root colonization per se is not required for the establishment of the benefits for the plants, but maybe required for fungal propagation and thus continuous delivery of the fungus-derived effector molecule(s). Furthermore, genes required for nodule and mycorrhiza formation in legumes are not present in the Arabidopsis genome. This suggests that the *P. indica*-induced benefits in Arabidopsis are mediated by Ca^{2+}-dependent processes which differ from those in legumes. This is also supported by the observation that a homolog of the legume *dmi1* that encodes an ion channel and is required for mycorrhiza formation, does not affect the beneficial interaction between *P. indica* and Arabidopsis. DMI1 is a single-copy gene in Arabidopsis which is mainly expressed in roots and slightly upregulated in the presence of *P. indica*.[52] However, microarray analysis uncovered that several calmodulin like genes are targets of signals from *P. indica* in Arabidopsis roots. Whether they are required for defence responses or involved in the mutualistic interaction between the two symbionts, or for both, is currently under investigation.

Interestingly, the legume *Lotus japonicus* is also a host for *P. indica*, promotes its growth and confers efficient resistance against biotic and abiotic stress (Oelmüller R, et al. unpublished). This is consistent with our previous observations that *P. indica* has a wide host range.[53] Analysis of the Lotus mutants impaired in mycorrhiza formation for their response to *P. indica* will help to understand, whether the same Ca^{2+} components are required for both interactions. It is also conceivable that *P. indica* uses species-specific signaling processes to achieve its goal: strengthen the plant to live in a better environment.

Future Strategies

The complexity of Ca^{2+} signaling, the huge number of Ca^{2+}-decoding Ca^{2+}-binding proteins and the specificity of the response to incoming signals makes it difficult to assign a specific Ca^{2+}-dependent signaling process to a specific response. Therefore, the most obvious strategy to unravel this complexity requires genetic tools. For model organisms, mutants can be isolated and the mutated genes identified. Many pathogens interact with Arabidopsis and many mutants are available which are impaired in their response to these pathogens. They can be tested in the *P. indica*/Arabidopsis system. Although a massive defense response has never been observed in Arabidopsis plants exposed to *P. indica*, a constitutive, long-lasting mild defense response might be required for restricting root colonization.[54]

In contrast to our knowledge about Ca^{2+} signaling, much less is known about beneficial interactions of microbes/fungi with Arabidopsis. The role of Ca^{2+} in mutualistic interactions of plant species which do not form mycorrhiza is unknown at present. *P. indica* provides a nice model system for those studies. Testing of available mutants, and generating new mutants, which are impaired in inducing $[Ca^{2+}]_{cyt}$ and/or nuclear Ca^{2+}elevations in response to signals from the fungus might help to understand the complexity of plants interacting with friendly symbionts. The *P. indica*/Arabidopsis system might help to understand the dual role of Ca^{2+} in beneficial and non-beneficial traits in beneficial plant/fungus interactions. Ca^{2+} might activate two independent signaling pathways leading to defense gene activation and the establishment of a beneficial interaction, there might be a cross-talk between these two pathways, or the pathways might overlap and recruit the same Ca^{2+}-dependent signaling compounds. It is conceivable that a sophisticated balance between defense responses and beneficial responses is required, and that imbalances shift the mode of interaction from mutualism to parasitism or vice versa. The initial characterization

of Ca^{2+} mutants from Arabidopsis, which have been isolated in our laboratory, uncovered a so far unexpected complexity in microbe-induced signaling events in plant cells, in which Ca^{2+}-dependent processes could represent an important switchpoint.

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