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Cameleon calcium reporters to study Ca^{2+} spiking in *Medicago* root hairs during rhizobial infection

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Introduction

The interaction of legume roots with soil-living nitrogen fixing rhizobacteria leads to the formation of new plant organs, the root nodules, in which symbiotic bacterial nitrogen fixation takes place.

The legume root hair plays a crucial role in the recognition and the initial up-take of the bacteria *via* a plant cell-derived membrane invagination called the infection thread (**IT**). Root hairs respond to specific rhizobial signal molecules, the lipochito-oligisaccharide Nod-factors, by a distinct calcium spiking response. This early response has been successfully studied recently* using a cameleon (YC 2.1) reporter and appears to be essential for a signalling pathway leading to the expression of symbiosis-specific early nodulin genes.

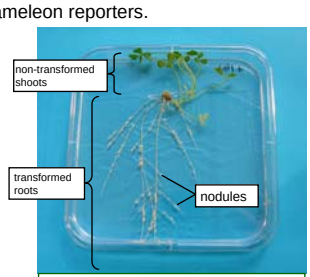
Our aim is to evaluate to what extent calcium spiking responses play a role throughout the rhizobial infection process which follows this initial early response to Nod factors.

For this we have established an experimental system allowing us to follow infection in the root hair *in vivo* using the confocal microscope. We have also investigated the potential of several available cytoplasmic cameleon reporters for these studies including cameleon YC 2.1 and the higher dynamic range variant YC 3.60.

*reference: Miwa et al. (2006) Plant J. **48**: 883-894

Strategy for performing *in vivo* studies of infection

- *Agrobacterium rhizogenes*-mediated transformation of *Medicago truncatula* yields composite plants with transformed root systems expressing cameleon reporters.
- 3-4 weeks are required to obtain transformed roots which can be inoculated by *Sinorhizobium meliloti*.
- A plant culture method has been developed allowing repeated observations over many days with the confocal laser scanning microscope in order to follow the infection process.



Composite *M. truncatula* plant growing in a Petri dish on agarose. This culture method allows inoculation with rhizobia and repeated microscopic observation over several days and weeks.

Cytoplasmic YC 3.60 allows detection of Ca^{2+} spiking for several hours

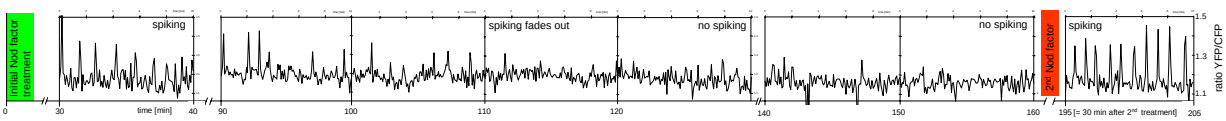


Fig. 1: **Ca^{2+} spiking in the peri-nuclear region of a growing root hair after application of Nod factors in solution. The larger dynamic range of YC 3.60 allows confocal ratio imaging with low resolution, low laser intensities and a fast scanning mode for several hours and yields ratio spikes larger in amplitude than for YC2.1.** To ensure that the observed fading of the spikes is not due to photobleaching we applied Nod factor solution for a second time and could re-induce Ca^{2+} spiking. This shows that YC 3.60 is a well-suited tool to trace cytoplasmic Ca^{2+} spiking over an extended period of several hours under low light conditions. Additionally we found that Nod factors applied in small quantities trigger transient spiking, which can be re-induced with repeated treatment.

Cytoplasmic cameleons: sub-cellular distribution and spatial confinement of Ca^{2+} spiking

YC 2.1 localizes predominantly in the cytoplasm, and is barely detected in the nucleus

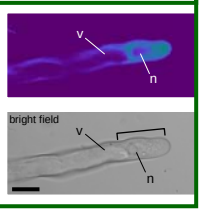


Fig. 2: **Confocal laser scanning microscopy shows that the cytoplasmic cameleon YC 2.1 localizes in abundance in the cytoplasmically dense apical and subapical region of a growing root hair (bracket). Little if any signal can be detected in the nucleus (n).**

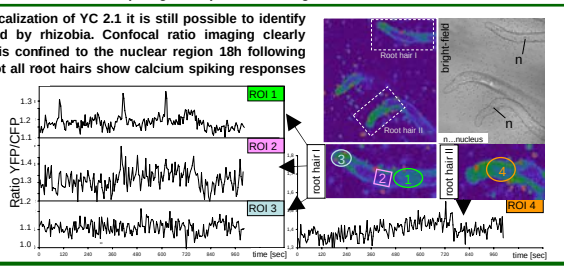
Similar observations were made for YC 3.60 (data not shown). v…vacuole, bar = 20µm.

Cytoplasmic YC 2.1 allows the identification of Ca2+ spiking in the peri-nuclear region after rhizobial inoculation

Fig. 3: Despite the cytoplasmic localization of YC 2.1 it is still possible to identify peri-nuclear Ca^{2+} spiking induced by rhizobia. Confocal ratio imaging clearly shows that in root hairs spiking is confined to the nuclear region 18h following rhizobial inoculation. However, not all root hairs show calcium spiking responses (compare root hair I with hair II).

Region of interest 1 (ROI1): Ca^{2+} spiking in the peri-nuclear region. **ROI 2** (vacuole) and **ROI 3** (apical region): no spiking.

ROI 4: Interestingly, the nuclear region in root hair II – although in direct contact with rhizobia (seen as red dots: *S. meliloti*-GFP) – does not show Ca^{2+} spiking.



Discontinuous Ca^{2+} spiking during initial stages of root hair infection

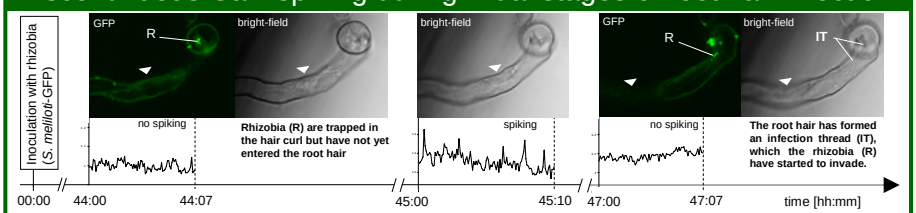


Fig. 4: **Ca^{2+} spiking in the peri-nuclear region of a root hair during infection by rhizobia, followed over time by confocal ratio imaging. Apparently, spiking can be discontinuous during the infection process.** Graphs YFP/CFP ratio; cameleon YC 3.60; rhizobacteria *S.meliloti*-GFP, arrowheads indicate the position of the nucleus.

Limitations of cytoplasmic cameleons

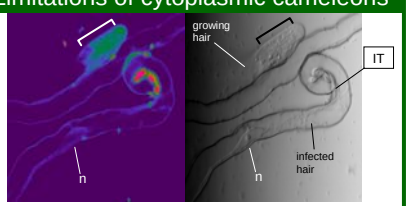


Fig. 5: Insufficient fluorescence signal of both cytoplasmic cameleons (shown here: YC 3.60) is a major problem when following Ca^{2+} spiking in infected root hairs during IT growth. The nucleus (n) in the infected hair is not surrounded by substantial amounts of cytoplasm; hence the signal is insufficient. In contrast, the growing hair has sufficient signal in its cytoplasmically dense tip region (bracket).

Summary and Perspectives

- Our results show that the cytoplasmic **cameleon YC 3.60** is a very useful tool to study peri-nuclear Ca^{2+} oscillations in *Medicago* root hairs, especially for long-term observations.
- Ca^{2+} spiking induced in root hairs following rhizobial inoculation is unpredictable in terms of timing, frequency and amplitude and only a small percentage of root hairs spike at a particular moment after inoculation (lack of synchrony), which is different from spiking induced by purified Nod factors. Furthermore, spiking appears to be discontinuous during early stages of rhizobial infection
- **However**, cytoplasmic cameleons are unfortunately of limited use for studying peri-nuclear Ca^{2+} spiking in stages following initial root hair infection by rhizobia. We are therefore planning to use **cameleons targeted to the cell nucleus** in order to obtain improved signals during infection.