Cameleon calcium reporters to study Ca²⁺ spiking in *Medicago* root hairs during rhizobial infection

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Introduction
The interaction of legume roots with soil-living nitrogen fixing rhizobia 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-oligosaccharide Nod-factors, by a distinct calcium spiking response. This early response has been previously 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.

Discontinuous Ca²⁺ spiking during initial stages of root hair 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.

Strategy for performing *in vivo* studies of infection

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Cytosolic YC 3.60 allows detection of Ca²⁺ spiking for several hours

Fig. 1: Ca²⁺ 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 repeated observations over several days and weeks. Additionally we found that Nod factors applied in small quantities trigger transient spiking, which can be re-induced with repeated treatment.

Fig. 5: Insufficient fluorescence signal of both cytoplasmic cameleons (shown here: YC 3.60) is a major problem when following Ca²⁺ 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).

**Cytoplasmic cameleons: sub-cellular distribution and spatial confinement of Ca²⁺ spiking**

**Cytoplasmic YC 2.1 localizes predominantly in the cytoplasm, and is locally detected in the nucleus**

**Cytoplasmic YC 3.60 allows detection of Ca²⁺ spiking for several hours**

**Summary and Perspectives**

- Our results show that the cytoplasmic cameleon YC 3.60 is a very useful tool to study peri-nuclear Ca²⁺ oscillations in Medicago root hairs, especially for long-term observations.
- Ca²⁺ 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 show spikes 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 Ca²⁺ 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.

**Limitations of cytoplasmic cameleons**