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Insight

Ca^{2+} signatures in symbiosis: another level of dynamism for this key messenger

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This article comments on:

Binci F, Offer E, Crosino A, Sciascia I, Kleine-Vehn J, Genre A, Giovannetti M, Navazio L. 2024. Spatially and temporally distinct Ca^{2+} changes in *Lotus japonicus* roots orient fungal-triggered signalling pathways towards symbiosis or immunity. Journal of Experimental Botany **75,** 605–619.

Ca^{2+}, as a key secondary messenger, is known to be important in plant–microbe interactions, and recent work has elucidated details of the role it plays in modulating symbiotic outcome. Using an aequorin-based calcium indicator, Binci *et al.* (2024) successfully quantified Ca^{2+} signaling in the cytoplasm and nucleus when *Lotus japonicus* plants were treated with different chitin-derived fungal elicitors. Their study shows that fungal elicitors trigger biphasic Ca^{2+} signaling, which has previously been overlooked.

Plant roots are constantly exposed to a variety of signals from soil microorganisms, including pathogenic and symbiotic microbes. Plants must discern these signals and integrate them into their developmental programs in order to cope with different microbes. Ca^{2+} is a key second messenger that is essential in a wide range of plant–microbe interactions (e.g. Zipfel and Oldroyd, 2017; Tian *et al.*, 2020). Spatially and temporally distinct calcium signals (Ca^{2+} signature) not only reflect different input stimuli, but also lead to specific outputs (Jiang and Ding, 2023). The Ca^{2+} signature involved in the immune response in the cytoplasm has been quantitatively and qualitatively studied at both the whole-tissue and single-cell level (Keinath *et al.*, 2015; Köster *et al.*, 2022). A well-known Ca^{2+} signature displayed during the establishment of arbuscular mycorrhizal (AM) symbiosis and rhizobial symbiosis is nuclear calcium spiking (oscillations), the study of which is mainly based on imaging observations at the cellular level (Oldroyd and Downie, 2006). In addition to nuclear calcium spiking, a rapid change in the cytoplasmic calcium concentration in response to symbiotic factor (Nod factor) treatment was also observed using GECO-based dual color sensors (Kelner *et al.*, 2018). In contrast to our deep understanding of the immune response, temporal and spatial quantification of Ca^{2+} signals in the cytoplasm and nucleus at the tissue level during symbiosis has been missing. Recently, Binci *et al.* (2024) provided a set of detailed quantitative analyses of Ca^{2+} signaling in response to treatment with chitin-associated oligomers (CO4, CO8, and mycLCO) via an aequorin-based Ca^{2+} sensor, which brought new insights into understanding how the plant root system distinguishes between different fungal molecules in order to engage in symbiotic or immune responses.

Characterizing the subcellular temporal extent of Ca^{2+} elevation

AM fungi can release chitin-associated oligomers, such as CO4, CO8, and mycLCO. CO4 and mycLCOs are required for AM fungi to establish symbiosis with host plants, while CO8, one of the key components of fungal cell walls, acts as an effective pathogen-associated molecular pattern (PAMP) to elicit immune responses in plants (Liu *et al.*, 2012; Zhang *et al.*, 2021). Binci *et al.* (2024) used an aequorin-based calcium reporter and revealed that all three tested chitin-derived oligomers can trigger the calcium changes in both the cytoplasm and nucleus in *L. japonicus* roots. Surprisingly, the calcium changes induced by CO4, mycLCOs, or CO8 exhibit comparable patterns in both the cytoplasm and nucleus. The calcium changes could

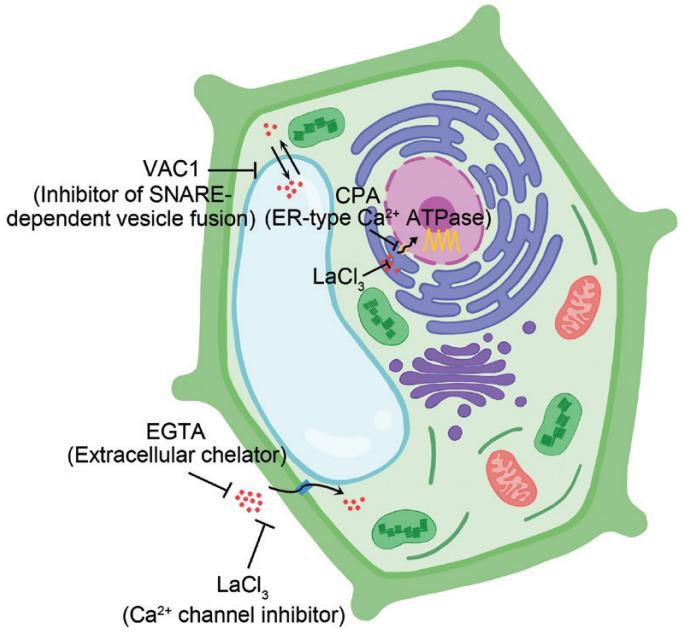

Fig. 1. Dissecting the Ca^{2+} pool for cytosolic and nuclear Ca^{2+} increases by applying pharmacological experiments. Treatment with CO4, CO8, and mycLCO induces Ca^{2+} increases in both the cytoplasm and nucleus. The Ca^{2+} channel inhibitor $LaCl_3$ inhibits the activity of all Ca^{2+} channels, resulting in no Ca^{2+} increases in either the cytoplasm or the nucleus. The extracellular chelator EGTA specifically prevents Ca^{2+} influx from the outside of the cell into the cytoplasm; CPA (cyclopiazonic acid), an inhibitor of endoplasmic reticulum (ER)-type Ca^{2+} ATPase, leads to depletion of Ca^{2+} in the ER lumen, and, as a consequence, the Ca^{2+} increase in the nucleus is lost; VAC1, an inhibitor of SNARE-dependent vesicle fusion, disrupts Ca^{2+} homeostasis in the cytoplasm by interfering with Ca^{2+} storage capacity, which significantly influences the cytoplasmic Ca^{2+} increase (illustration by Qianqian Li).

be further dissected into two temporal phases, namely phase I, characterized by a steep concentration peak in the first 8 min, and phase II with a broader shoulder between 8 min and 30 min. To dissect the calcium pools for the cytoplasm and nucleus Ca^{2+} changes after the stimulation, the authors conducted a series of pharmacological experiments (Fig. 1). EGTA, an extracellular Ca^{2+} chelator, strongly reduced cytosolic and nuclear calcium elevations, and the inhibitor of endoplasmic reticulum-type Ca^{2+} ATPases (CPA) specifically abolished nuclear elevations. Of note, a significant elevation of Ca^{2+} concentration in the cytoplasm was observed after VAC1 (an inhibitor of SNARE-dependent vesicle fusions) treatment, suggesting that vacuoles play a crucial role in Ca^{2+} homeostasis in the cytoplasm.

Identifying the dynamic dependency of Ca^{2+} elevation on the common symbiotic signaling pathway

Among the new findings, Binci *et al.* (2024) proposed that Ca^{2+} influx in phase I is an immune-like response. To analyze the regulatory control of Ca^{2+} elevation in phase I and phase II, the authors resolved the Ca^{2+} quantification profiles in different mutant backgrounds, including the common symbiotic signaling pathway (CSSP) component mutants *Ljsymrk* (Stracke *et al.*, 2002) and *Ljcastor* (Charpentier *et al.*, 2008), and the immune-responsive mutant *Ljcerk6* (Bozsoki *et al.*, 2017). The results showed that in the two CSSP mutants, the three tested fungal molecules still triggered wild-type-like rapid Ca^{2+} elevation corresponding to phase I, whereas CO4- and mycLCO-induced Ca^{2+} changes in nuclear phase II were abolished, suggesting that Ca^{2+} changes in nuclear phase II but not in phase I are dependent on the CSSP. This phenomenon is also consistent with the previous observation of the lack of nuclear and perinuclear Ca^{2+} spikes in CSSP gene mutants (Genre *et al.*, 2013; Feng *et al.*, 2019). Furthermore, the authors demonstrated that aequorin-based profiles of Ca^{2+} elevation in whole organs was comparable with the sum of chameleon-based Ca^{2+} spike signals from individual cells, offering a novel methodological key to correlate the results from two of the most common methods for live Ca^{2+} tracking. In contrast, in the *Ljcerk6* mutant, the changes in phase I Ca^{2+} concentration were blocked, but phase II Ca^{2+} changes were not significantly affected. Moreover, the combination of treatment with different concentrations of fungal molecules and evaluation of several marker genes associated with the immune response using quantitative reverse transcription–PCR (RT–qPCR) showed that the rapid Ca^{2+} influx in the cytoplasm (phase I) was identical to that in plant immunization (Binci *et al.*, 2024).

In addition, Binci *et al.* (2024) observed Ca^{2+} increases in the cytoplasm within 8 min after CO4, CO8, and mycLCO treatments. They suggest that this is an immune-related Ca^{2+} influx, consistent with the Ca^{2+} influx triggered by PAMPs during immune responses. This claim can be supported by the finding that some immunity-related genes are indeed induced during the early stages of symbiotic establishment (Lohar *et al.*, 2006). Notably, during the establishment of rhizobial symbiosis, the influx of Ca^{2+} into the cytoplasm is reduced in *Sinorhizobium meliloti nodL* mutants, which did not affect Ca^{2+} oscillations in the nucleus, but significantly affected rhizobial infection (Morieri *et al.*, 2013), suggesting that Ca^{2+} influx (phase I) plays other important roles rather than merely being a sign of an immune response during establishment of symbiosis. The different amplitudes may determine different outputs, as the expression of immune response marker genes was correlated with the amplitude of the phase I Ca^{2+} changes. However, the specific function of the phase I Ca^{2+} increases during symbiosis still needs to be investigated.

What remains to be achieved in the future?

Extracellular Ca^{2+} is the pool for phase I Ca^{2+} (Binci *et al.*, 2024). Several cyclic nucleotide gated channel (CNGC) family proteins (CNGC2/4 in Arabidopsis and CNGC9 in rice) have

been shown to be involved in regulating Ca^{2+} influx (phase I) in response to immunity (Dietrich *et al.*, 2020; Tian *et al.*, 2020). Hence, it remains to be explored which Ca^{2+} transporters are involved in CO8- and mycLCOs/SmLCOs-mediated Ca^{2+} influx. Although CO8 could induce nuclear calcium oscillations, the frequency of calcium oscillations was different from that induced by mycLCO, which could determine specific outputs (Feng *et al.*, 2019; Binci *et al.*, 2024). This idea can be supported by a study of the Lotus *nfre* mutant, which has altered frequency of calcium oscillations in the nucleus, with consequent severe effects on the establishment of symbiosis (Murakami *et al.*, 2018). In addition, to distinguish the Ca^{2+} signals triggered by different stimuli, specific proteins must be activated to decode the different input signals, such as CCaMK/CYCLOPS, as a decoding protein complex for symbiotic signals (Tirichine *et al.*, 2006; Yano *et al.*, 2008; Charpentier, 2018). Therefore, understanding which proteins can decode CO8-triggered signals and how these proteins are recruited by different input Ca^{2+} signals remains a pressing challenge. Pursuing these lines of investigation will greatly improve our understanding of the establishment of symbiotic relationships between plants and microbes.

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Conflict of interest

The authors declare no conflict of interest.

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