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Forisome dispersion in Vicia faba is triggered by Ca\textsuperscript{2+} hotspots created by concerted action of diverse Ca\textsuperscript{2+} channels in sieve element

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Remote-controlled Ca$^{2+}$ influx, elicited by electropotential waves, triggers local signaling cascades in sieve elements and companion cells along the phloem of *Vicia faba* plants. The stimulus strength seems to be communicated by the rate and duration of Ca$^{2+}$ influx into sieve elements (SEs). The cooperative recruitment of Ca$^{2+}$ channels results in a graded response of forisome culminating in full sieve-tube occlusion. Several lines of evidence are integrated into a model that links the mode and strength of the electropotential waves (EPWs) with forisome dispersion, mediated by transiently enhanced levels of local Ca$^{2+}$ release dependent on both plasma membrane and ER Ca$^{2+}$ channels.

**Electropotential Waves and Ca$^{2+}$ Influx into Sieve Tubes**

Burning the tip of a *Vicia faba* leaf induces an EPW along the sieve tubes that triggers remote sieve-tube occlusion by dispersion of forisomes and subsequent callose production. It is suspected that both occlusion mechanisms are triggered by influx of Ca$^{2+}$ ions during passage of EPWs. Our recent study highlights (sub)cellular location and activity of Ca$^{2+}$ channels in SEs and their physiological role in *Vicia faba* sieve tubes during injury-triggered EPWs.

**Ca$^{2+}$ Resting Levels and Ca$^{2+}$ Elevations Triggered by Electropotential Waves**

Given the potential importance of Ca$^{2+}$ channels for signal propagation along sieve tubes, we determined the resting Ca$^{2+}$ levels and investigated Ca$^{2+}$ dynamics during passage of EPWs, along with the potential Ca$^{2+}$ stores, and Ca$^{2+}$ channels that might be involved in stimulus-response coupling. Ca$^{2+}$-resting levels of around 100 nM (Fig. 2) were measured in the cytoplasm.
of CCs and close to the mictoplasmic layer of SEs using fluorescent dyes whilst Ca\(^{2+}\) concentrations in sieve-tube sap collected by aphid stylectomy were approximately 50 nM. Measurement of Ca\(^{2+}\) dynamics is technically challenging in intact phloem tissue. However, using fluorescent Ca\(^{2+}\) reporters and 4-D (x, y, z, t) confocal laser scanning microscopy, as well as rapid line-scans to gain higher temporal resolution of fast Ca\(^{2+}\) transients, we observed Ca\(^{2+}\) transients with similar kinetic profiles to the EPWs. The stimulus-dependent elevation of Ca\(^{2+}\) concentration across the SE/CC was unexpectedly low (<1 μM). Furthermore the presence of the dye was sufficient to prevent forisome dispersion.

**Ca\(^{2+}\) Resting Levels and Forisome Dispersion Thresholds**

These modest Ca\(^{2+}\) elevations are considerably lower than the 50 μM Ca\(^{2+}\) threshold measured for forisome dispersion in vitro and in vivo. Therefore, we postulate that forisome dispersion is only initiated at localised Ca\(^{2+}\) hotspots where the critical concentration required to trigger forisome dispersion is reached (Fig. 2). This would be consistent with established precedents from the animal literature (reviewed in refs. 9 and 10: up to 300 μM at the cytosolic membrane surface of plasma membrane) which argue that transient, local Ca\(^{2+}\) peaks up to 100 μM can exist in the vicinity of Ca\(^{2+}\)-channel pores. Furthermore, whilst the mobility of Ca\(^{2+}\) in the cytosol is normally restricted as dictated by cytosolic Ca\(^{2+}\)-binding capacity, the presence of a mobile Ca\(^{2+}\)-buffering dye is sufficient to dissipate the gradients.

**Differential Deployment of Ca\(^{2+}\) Channels within SEs**

The pathway of Ca\(^{2+}\) influx could involve Ca\(^{2+}\) channels operating at either the plasma membrane (PM) or endoplasmic reticulum (ER), but not the tonoplast as sieve elements lack a vacuolar compartment. A fluorescent nifedipine derivative (DM BODIPY-DHP) localizes Ca\(^{2+}\) channels to both ER and plasma membrane. Ca\(^{2+}\)-channel density was highest near sieve plates and pore-plasmodesma units (PPUs) with the lowest densities in central parts of SEs (Fig. 2). Co-localisation studies revealed that the Ca\(^{2+}\)-channel distribution matched the localization of stacks of ER. As the ER density is higher in the vicinity of sieve plates, the absolute number of ER-bound Ca\(^{2+}\) channels is anticipated to be higher there and, hence, the degree of Ca\(^{2+}\) release (Fig. 2). The ER-density is somewhat higher at the CC-side due to the presence of PPUs, where the density of PM-bound Ca\(^{2+}\) channels also tend to be higher (Fig. 2).

**Interplay between Ca\(^{2+}\) Channels Culminates in Successive Steps of Occlusion**

Taken together we suggest that Ca\(^{2+}\) micro-gradients are established in the vicinity of PM- and SER-Ca\(^{2+}\) channels, especially in the unstirred interstices between ER stacks (Fig. 2B). A sudden rise of Ca\(^{2+}\) close to the Ca\(^{2+}\)-channel pores could explain forisome dispersion in vivo. Forisome dispersion will occur preferentially at sites with the highest Ca\(^{2+}\)-channel frequency. This is consistent with observations that the probability of forisome dispersion is highest close to the sieve plate and the lowest in the SE centre.

To get a complete picture of wound responses, we also have to account for the observation that forisome reactivity and stimulus strength are quantitatively related. The impact of EPWs on forisome conformation probably results from cumulative events including variable contributions of diverse Ca\(^{2+}\) channels. Here, a tentative model is presented that causally correlates distant stimulus strength, EPW profiles, location of Ca\(^{2+}\) channels, Ca\(^{2+}\) influx, forisome position and responsiveness (Fig. 3A–D).

In response to passage of weak EPWs forisomes may detach and are seen to move (Fig. 3A), while with somewhat stronger EPWs forisome tips disperse (Fig. 3B). Forisomes fully disperse (Fig. 3C) in reaction to EPWs with an extended depolarization plateau phase. When the depolarization period lasts even longer, not only do forisomes disperse, but callose deposition is also triggered (Fig. 3D). The rationale of the model (Fig. 3) is that EPWs gradually recruit more Ca\(^{2+}\) channels and induce commensurate increases in Ca\(^{2+}\) influx with increasing stimulus strength.

Accordingly, short-lasting, small EPWs evokes by remote KCl depolarisation induce minute transient changes in Ca\(^{2+}\) and minor movements of forisomes (Fig. 3A). Warming the leaf tip by heating the surrounding air provokes sharp EPWs with a larger amplitude and is accompanied by a stronger Ca\(^{2+}\) influx (Fig. 3B). Distant cutting causes sharp EPWs with a large depolarization amplitude but short depolarization tail that initiates prolonged
Ca\textsuperscript{2+} influx and forisome dispersion (Fig. 3C). Distant burning represents the most aggressive stimulus and triggers a sharp, strong depolarization peak and a long-lasting depolarization tail, suggestive of massive Ca\textsuperscript{2+} influx leading to forisome dispersion and callose deposition (Fig. 3D).

According to the model (Fig. 3A), only voltage-sensitive channels in the SE-PM are involved in propagation of APs. Plasmamembrane depolarization may also be relayed to ER-located voltage-gated channels\textsuperscript{14,15} but is not sufficient to trigger substantial Ca\textsuperscript{2+} release. During the more prolonged VPs, mechanosensitive\textsuperscript{16,17} or putative ligand-activated Ca\textsuperscript{2+} channels may operate, that enable massive Ca\textsuperscript{2+} influx and synergistic activation of Ca\textsuperscript{2+} release from the SER via CICR channels (Fig. 3). The evidence for the involvement of various Ca\textsuperscript{2+} channels is given in the legend of the model (Fig. 3). Cumulative Ca\textsuperscript{2+} influx immediately adjacent to the forisomes as a result of the aggregated impact of APs and VPs is presumed to be crucial to give graded coupling between stimulus and response.

Further experimental support for this model is technically challenging as the system needs to be intact to function properly and is recalcitrant to both imaging and electrophysiological approaches. Nevertheless, there are clear improvements that might aid future investigations, such as the use of transgenic ratiometric Ca\textsuperscript{2+} probes and spatially explicit mathematical models of Ca\textsuperscript{2+} dynamics.
Final Remarks

Our work shows that electrical signals of different strength trigger a commensurate release of Ca\(^{2+}\) ions into SEs which exemplifies step-wise graded control of signal cascades in distant organs. It demonstrates that, unlike action potentials in animals, stimulus and response are correlated quantitatively. Why electrical propagation in plants and animals is organized in a different fashion may be explained by differences in physiological intentions. While in animals, the leading requirement of electrical propagation is signal transfer over long distances, the main goal in plants is massive exchange and release of ions along the pathway to initiate responses over a range of length scales.\(^{18}\)

References


11. Trewavas A. Calcium, c’est la vie: calcium makes the observation that, La3+ in the external medium inhibits forisome dispersion. (C) Since cutting close by the observation side causes a massive loss in turgor, most likely a variation potential overlayed with an AP-like transient induces the release of Ca2+ to an extent that brings about full dispersion. (D) A steep AP followed by a massive Ca2+ NP only induces forisome dispersion, but also callose deposition. It is unclear if the VP is initiated by suppression of SE-Pm proton-pump activity leading to activation of voltage-gated Ca2+ channels, 22-25, or by direct Ca2+ influx via mechanically sensitive Ca2+ channels (orange symbols) or Ca2+-activated Ca2+-channels (blue symbols). To elevate Ca2+ concentrations ([Ca2+]i, thick arrow) to the level required for callose formation, 24 further Ca2+-signal amplification may be achieved by involvement of the signal molecule InsP3. High-affinity InsP3-binding sites on the ER suggest the presence of InsP3-gated Ca2+-release channels (yellow symbols). 26 Increase in cellular levels of InsP3 has been reported in response to osmotic shocks as well as burning stimulus. 26


13. Malho R, Moutinho A, Van der Luit A, Trewavas AJ. Spatial characteristics of calcium signalling: the voltage drop along the SE-Pm may be propagated via the anchors between SE-Pm and Ser19 to the Ser membrane where voltage-dependent Ca2+ channels are activated. 15 Most likely, voltage-sensitive Ca2+ channels (green symbols) in the SER are needed to sustain the rise in Ca2+. Alternatively, or in addition, Ca2+-dependent Ca2+-channels may be activated to initiate Ca2+ release (CICR channels; blue symbols) from the SER as reported for storage vacuoles (reviewed in refs. 22 and 23). This implies that Ca2+ ions cannot be released from ER cisternae without a preceding PM trigger. This is compatible with the observation that, La3+ in the external medium inhibits forisome dispersion. (B) Larger AP-, respectively VP-associated depolarizations enhance the microplastic Ca2+ content ([Ca2+]i, thin arrow) to such an extent that forisomes move and/or swell. The voltage drop along the SE-Pm may be propagated via the anchors between SE-PM and SER19 to the SER membrane where voltage-dependent Ca2+ channels are activated. 21, 25, 28 Increase in cellular levels of InsP3 has been reported in response to osmotic shocks as well as burning stimulus. 26


