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Forisome dispersion in *Vicia faba* is triggered by Ca^{2+} hotspots created by concerted action of diverse Ca^{2+} channels in sieve elements

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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.

Key words: distant injury, electropotential wave, remote sieve tube occlusion, activity of sieve element Ca^{2+} channels, signal cascades, Ca^{2+} hotspots

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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.[1] It is suspected that both occlusion mechanisms are triggered by influx of Ca^{2+} ions during passage of EPWs. Our recent study[2] 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.

Actions Potentials and Variation Potentials as EPW Components

The term EPW includes two modes of propagation namely rapid transient "action potentials" (APs) and slower "variation potentials" (VPs). APs are thought to propagate mainly along sieve tubes[3-5] and are mediated by voltage-sensitive Ca^{2+} channels, VPs are interpreted to result from propagation of pressure waves following tension relaxation in the xylem vessels. These turgor pressure changes lead to local activation of mechano-sensitive Ca^{2+} channels which may directly effects Ca^{2+} concentrations or trigger release of other, as yet uncharacterised, signalling molecules.[6,7]

In *Vicia faba*, EPWs may represent the superposition of both AP- and VP-profiles as different stimuli elicit just AP-responses or AP + VP responses. Furthermore, the two phases of the EPW show differential sensitivity to inhibitors[2] and repeated stimuli. For example, during repeated burning, the AP-phase is visible in the first stimulation followed by the VP-phase (**Fig. 1**). However, the AP-phase is absent from response to the second stimulation, probably due to the refractory period observed for APs, whereas the long VP-phase remains unaffected.

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.[2] Ca^{2+}-resting levels of around 100 nM (**Fig. 2**) were measured in the cytoplasm

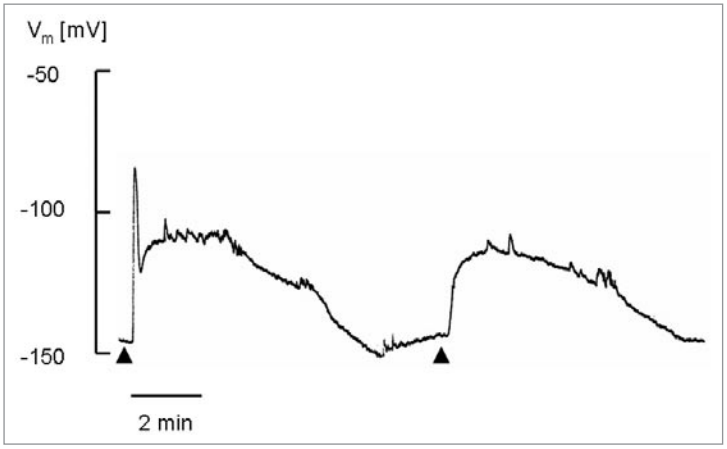

Figure 1. EPWs recorded from SE/CCs of main veins of *Vicia faba* triggered by remote burning consist of an action potential and a variation potential. A first burning stimulus (▲) at the leaf tip triggers an EPW in the main vein (measured at 3 cm distance) composed of a rapid, sharp depolarization during the AP-phase and a long-lasting depolarization in the VP-phase. A second burning stimulus, applied directly after recovery of the membrane potential, induces the VP-like slow kinetic phase, whereas the fast AP-like transient is absent. It appears that EPWs consist of superimposed APs and VPs under these experimental conditions.

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).[2] 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 considerable lower than the 50 µM Ca^{2+} threshold measured for forisome dispersion in vitro[8] and in vivo.[2] 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.[11] Furthermore, whilst the mobility of Ca^{2+} in the cytosol is normally restricted[11,12] as dictated by cytosolic Ca^{2+}-binding capacity,[13] 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.[2] 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,[2] 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+} microgradients 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.[2]

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

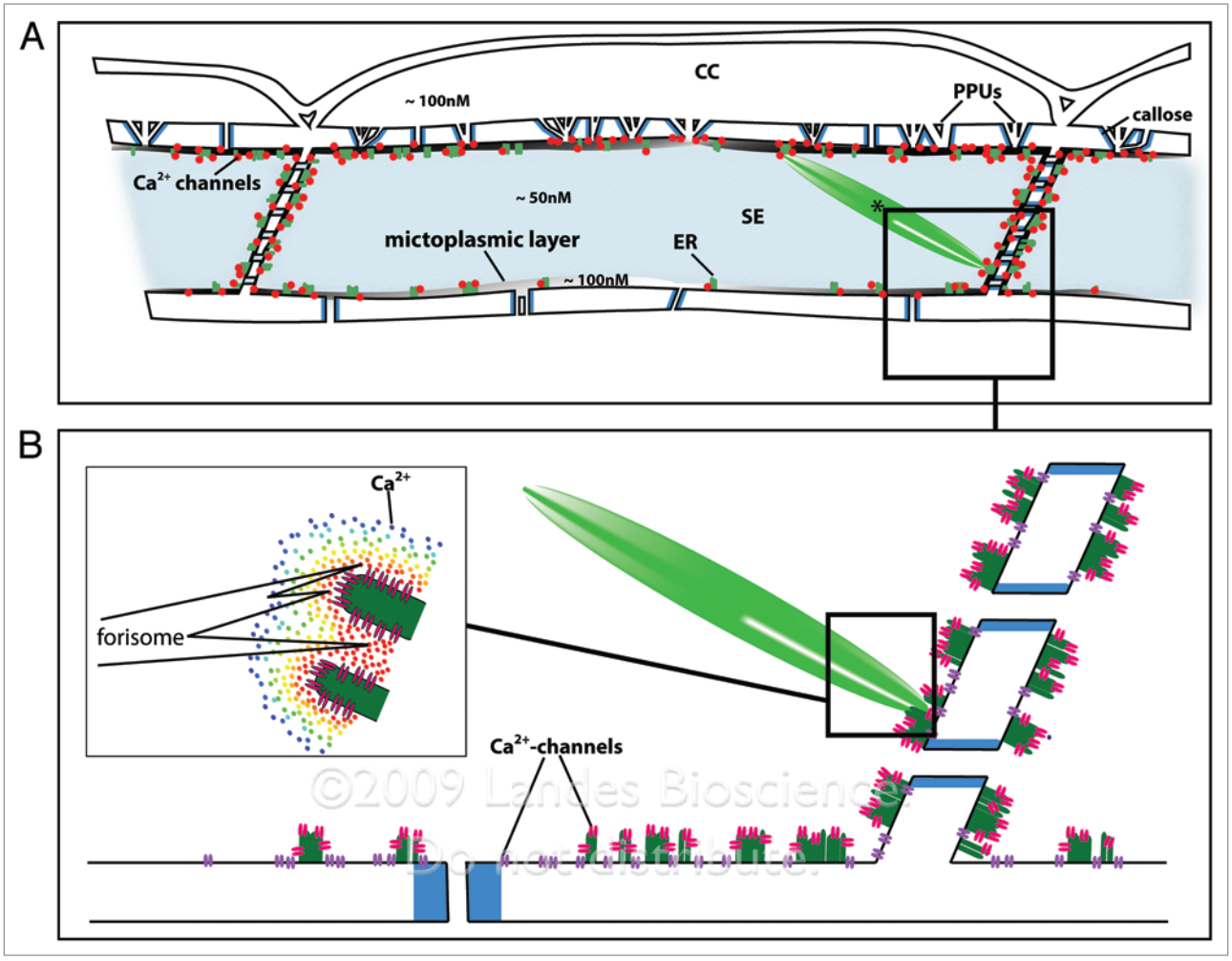

Figure 2. Localization of voltage-sensitive Ca^{2+} channels and Ca^{2+} distribution in SEs of *Vicia faba*. (A) Ca^{2+} channels in sieve elements (SEs) abound in the sieve-element reticulum (SER) which aggregates in the sieve-plate region and around PPU pores.[2] Ca^{2+} channels bound to the sieve-element plasma membrane (SE-PM) are more evenly distributed. However, their numbers tend to be higher near sieve plates and in the SE-PM at the CC-side.[2] (B) Magnification of the sieve-plate region. Voltage-sensitive Ca^{2+} channels are localized at both membrane systems[2] and depicted as purple (SE-PM) and magenta rods (SER membrane). Tight connections between SE-PM and SER by macromolecular anchors[19] may enable electrical or mechanical coupling between both membrane systems. Forisome tips are often associated with SER stacks[2] where steep Ca^{2+} gradients up to 100 µM occur in the vicinity of Ca^{2+}-channel pores,[11] thus exceeding the Ca^{2+} thresholds of around 50 µM required for forisome dispersion, particularly in and around SER interstices.[2] (B) Magnification of a forisome tip, which is occasionally forked (reviewed in ref. 17), loosely attached to the SER and inserted into ER-generated Ca^{2+} microgradients radiating from the non-stirred ER interstices in the mictoplasm. Hypothetical Ca^{2+} distribution is presented by a gradient from red to blue (high to low concentrations). Ca^{2+} influx in response to a remote stimulus is presumed to be proportional to the local density and co-operative activity of Ca^{2+}-channels.[2]

Ca^{2+} influx and forisome dispersion (**Fig. 3C**). Distant burning represents the most aggressive stimulus and triggers a sharp, strong depolarization peaks and a long-lasting depolarization tail, suggestive of massive Ca^{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,[14,15] but is not sufficient to trigger substantial Ca^{2+} release. During the more prolonged VPs, mechano-sensitive[16,17] or putative ligand-activated Ca^{2+} channels may operate, that enable massive Ca^{2+} influx and synergistic activation of Ca^{2+} release from the SER via CICR channels (**Fig. 3**). The evidence for the involvement of various Ca^{2+} channels is given in the legend of the model (**Fig. 3**). Cumulative Ca^{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^{2+} probes and spatially explicit mathematical models of Ca^{2+} dynamics.

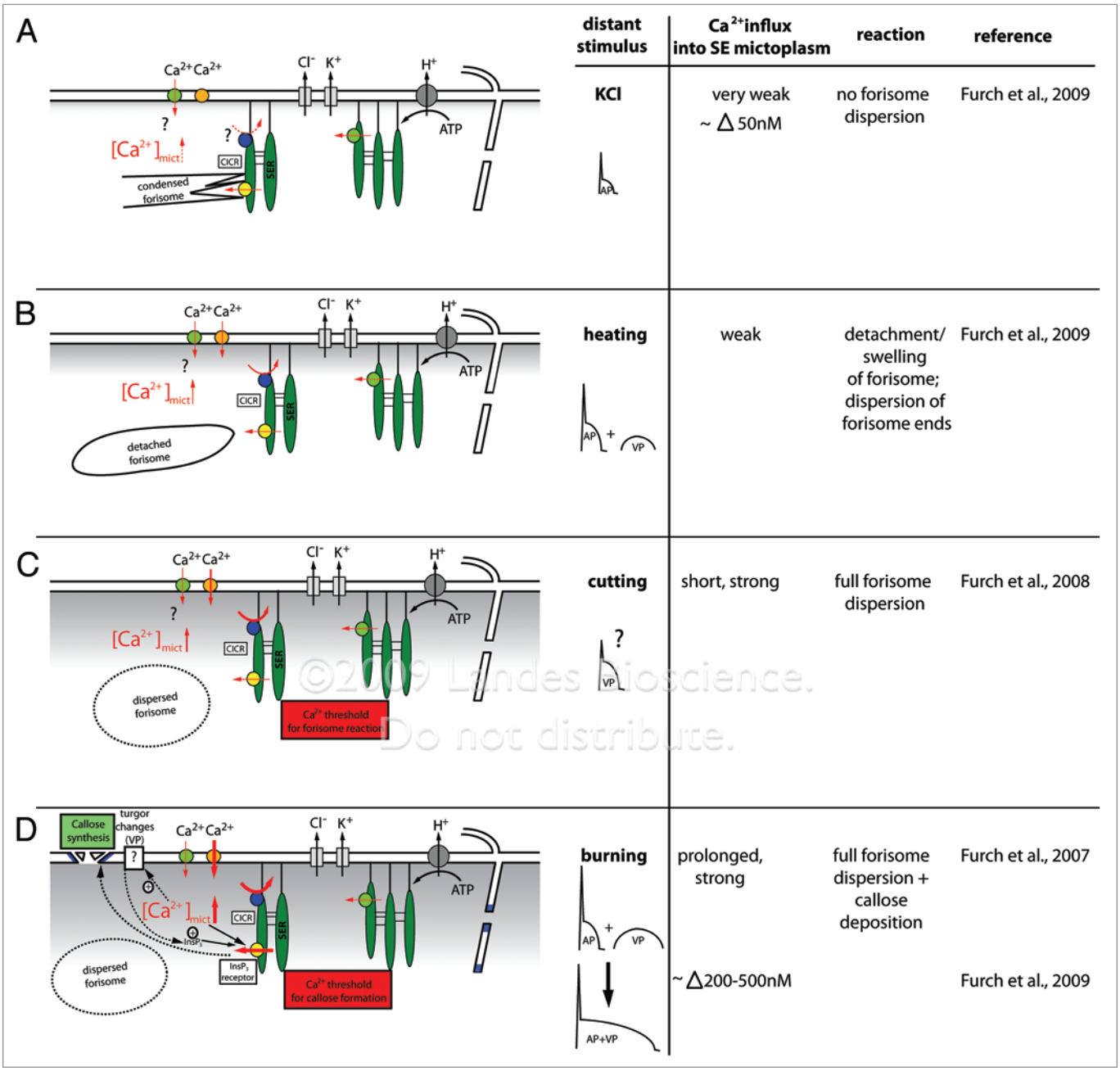

Figure 3. For figure legend, see page 972.

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]

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Figure 3. Hypothetical deployment of Ca^{2+} channels localized at SE-PM and SER as relay stations between remote stimuli and occlusion responses. This model assumes that a graded stimulus-response correlation depends on the increasing involvement of various Ca^{2+} channels with increasing stimulus strength. The latter results in a cumulative Ca^{2+} release into the non-stirred mictoplasm. The impact of stimulus increasing from (A–D) is depicted by symbols representing a putative strength and relative contribution of action (AP) and variation (VP) potential to the EPW. The grey tones visualize Ca^{2+} concentrations effected by various stimuli in the vicinity of SE PM-SER conglomerates, which are connected by nano-anchors (stripes between SER stacks and SE-PM[19]). The thickness of the red arrows represents the magnitude of the Ca^{2+} fluxes. (A) Small AP-like depolarizations induce minute release of Ca^{2+} ($[Ca^{2+}]_{mict}$ stippled arrow) via voltage-gated channels (green symbols) (reviewed in refs. 20 and 21) into the mictoplasm. These may affect forisome attachment, but are insufficient to effect conformational changes of the forisome. (B) Larger AP- respectively VP-associated depolarizations enhance the mictoplasmic Ca^{2+} content ($[Ca^{2+}]_{mict}$ 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 SER[19] to the SER membrane where voltage-dependent Ca^{2+} channels are activated.[14,15] Most likely, voltage-sensitive Ca^{2+} channels (green symbols) in the SER are needed to sustain the rise in Ca^{2+}. Alternatively, or in addition, Ca^{2+}-dependent Ca^{2+}-channels may be activated to initiate Ca^{2+} release (CICR channels; blue symbols) from the SER as reported for storage vacuoles (reviewed in refs. 22 and 23). This implies that Ca^{2+} ions cannot be released from ER cisternae without a preceding PM trigger. This is compatible with the observation that, La^{3+} in the external medium inhibits forisome dispersion.[2] (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[24] induces the release of Ca^{2+} to an extent that brings about full dispersion. (D) A steep AP followed by a massive VP not only induces forisome dispersion, but also callose deposition.[1] It is unclear if the VP is initiated by suppression of SE-PM proton-pump activity leading to activation of voltage-gated Ca^{2+} channels,[20,21,25,26] or by direct Ca^{2+} influx via mechano-sensitive Ca^{2+} channels (orange symbols) or Ca^{2+}-activated Ca^{2+}-channels (blue symbols). To elevate Ca^{2+} concentrations ($[Ca^{2+}]_{mict}$ thick arrow) to the level required for callose formation,[27] further Ca^{2+}-signal amplification may be achieved by involvement of the signal molecule $InsP_3$. High-affinity $InsP_3$-binding sites on the ER suggest the presence of $InsP_3$-gated Ca^{2+}-release channels (yellow symbols).[28] Increase in cellular levels of $InsP_3$ has been reported in response to osmotic shocks[29] as well as burning stimulus.[30]

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