Ca^{2+} signalling in plant cells: the big network!

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Significant advances in Ca^{2+} and calmodulin signalling in whole plants and individual cells have been recently reported. Particular relevant contributions have been made to the study of the modification of gene expression by osmotic, light and gravity signals and the growth of root hairs and pollen tubes.

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Abbreviations

ABA	abscisic acid
$[Ca^{2+}]_c$	cytosolic free calcium
cADP-R	cyclic adenosine phosphate ribose
CaM	Ca^{2+}/calmodulin
CBP	calcium binding protein
CDPK	Ca^{2+} dependent protein kinase
cADP-R	cyclic ADP-ribose
ER	endoplasmic reticulum
IP3	inositol (1,4,5) triphosphate

Introduction

Cytosolic free Ca^{2+}, $[Ca^{2+}]_c$, is a convergence point for many disparate signalling pathways. Each signal creates its own Ca^{2+} fingerprint: a spatially unique structure involving specific combinations of several hundred proteins acting in concert. Particular combinations of ion flux change and gene expression underpin the eventual physiological response. $[Ca^{2+}]_c$ transients are known to be induced by a range of signals, from Nod factors [1,2] to those which elicit an oxidative burst [3]. It is, therefore, important to distinguish Ca^{2+} changes which are associated with degenerative processes, such as senescence [4], from adaptive responses which are the essence of signalling. Focus on the signalling aspect is emphasised here, mainly on papers published in 1997 and 1998. Previous significant contributions on this subject have been recently reviewed by us in two parallel manuscripts [5••,6••].

Signalling in whole plants and cell cultures

Incubating tobacco seedlings containing transgenic aequorin, a luminescent Ca^{2+}-sensitive reporter protein, in continuous high temperature induces $[Ca^{2+}]_c$ transients lasting 20–25 minutes. New heat shock $[Ca^{2+}]_c$ transients can only be induced after a recovery period of eight hours at ambient temperatures [7]. Throughout this recovery period, however, normal $[Ca^{2+}]_c$ transients can still be induced by cold and wind signals [7]. Heat, cold and wind thus use different transduction pathways or mobilise spatially-distinct pools of $[Ca^{2+}]_c$ to produce a signal-specific $[Ca^{2+}]_c$ fingerprint. The amplitude of the transients is probably modulated by cytoskeletal organisation as shown for cold shocks [8].

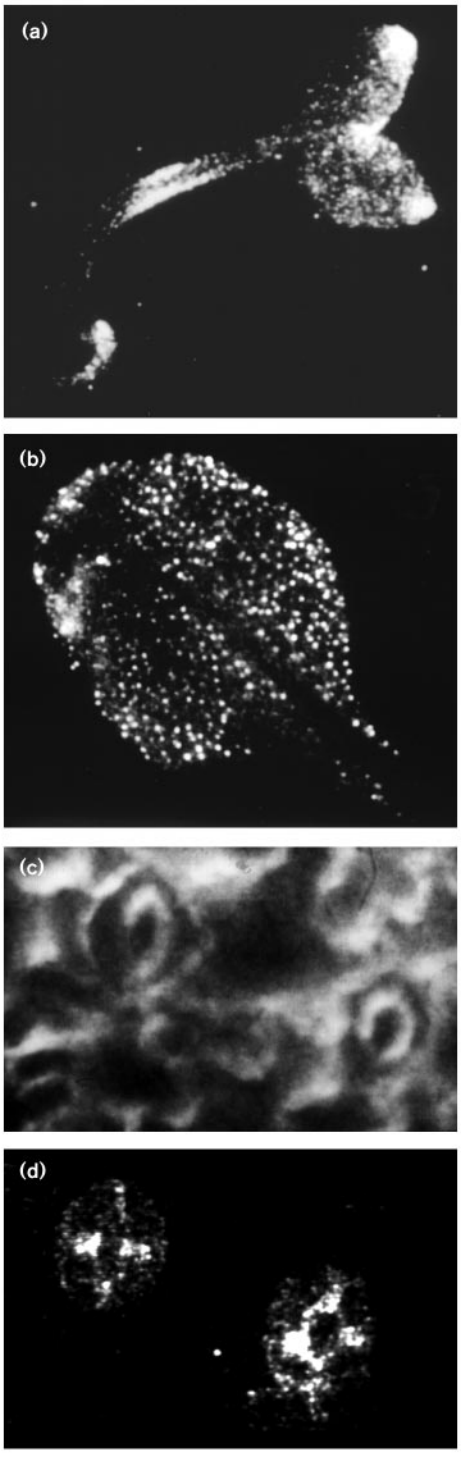
The kinetics of $[Ca^{2+}]_c$ transients induction have been measured in response to some ten signals [6••]. The lag period length before the transient starts, the rise time to the transient peak and the decay time back to the resting level are unique to each signal. Among the possible targets for these transients are plasma membrane anion channels and vacuolar K^+ release [9,10]. Organelle and membrane-targeted aequorins [7,11,12] can help resolve the complexity of $[Ca^{2+}]_c$ signalling and luminescence imaging at the single cell level is now possible (Figure 1).

Hyper-osmotic shock mobilises $[Ca^{2+}]_c$ via Ca^{2+} release from the vacuole and Ca^{2+} entry through the plasma membrane [11]. The mRNA's for Δ^1- pyrrolidone-5-carboxylate synthetase (an enzyme required for proline synthesis), *lti78* and *rab18* accumulate in a Ca^{2+}-dependent manner implicating $[Ca^{2+}]_c$ in cellular drought and salination adaptation. As abscisic acid (ABA) is known to induce *lti78* expression, these data indirectly implicate $[Ca^{2+}]_c$ in the ABA transduction pathway. In contrast, hypo-osmotic shock results in a large $[Ca^{2+}]_c$ transient [13], which can be inhibited by protein kinase inhibitors and is dependent on extracellular Ca^{2+} [14•]. Tobacco cells, shocked in the absence of extracellular Ca^{2+}, could still produce a large $[Ca^{2+}]_c$ transient if Ca^{2+} is added to the cells within a 20 minute period of the initial signal [14•]. These cells are thus able to 'remember' the initial hypo-osmotic signal, perhaps by a similar mechanism to the 30 minute $[Ca^{2+}]_c$ 'memory' induced by cold shock [12]. Hypo-osmotic shock-induced $[Ca^{2+}]_c$ transients are themselves controlled by protein kinases and in turn the transients activate other protein kinase cascades [15].

Ca^{2+} and light regulation

Microinjection of signalling intermediates have implicated cGMP and Ca^{2+}/calmodulin (CaM) in the up regulation of photosystem 1 genes by phytochrome. Down regulation of asparagine synthetase may use the same second messenger pathways [16•]. Whereas phytochrome negatively regulates chalcone synthase (CHS) expression via Ca^{2+}/CaM, the same two signal transduction molecules are used by UV light to positively regulate CHS expression [17]. Results from combined red and UV irradiations suggest that the phytochrome response could be activated first and the UV response subsequently. The apparently antagonistic functions of Ca^{2+} and CaM could, therefore, operate in the same cell because each response is temporally separated from the other.

Figure 1



Luminescence images of tobacco containing the aequorin transgene and subjected to cold shock. **(a)** whole 10 day old seedling **(b)** single cotyledon of whole seedling; note discrete luminescent spots **(c)** bright field image of cotyledon surface on whole seedling containing two guard cells **(d)** luminescence image of the same surface subjected to cold shock; note that some discrimination of internal guard cell structure is possible. Most guard cell cytoplasm is located in the middle and ends of the cell. (Helen Page, Nick Read, Tony Trewavas, unpublished data.)

Powerful though microinjection (and associated caged probe) technologies can be, they are nevertheless invasive techniques. Thus, only a potential role in signalling can be deduced. Demonstration of second messenger involvement requires measurements of signal-enhanced flux through $[Ca^{2+}]_c$ and cGMP as well. Experimentally mimicking the amplitude and kinetics of these $[Ca^{2+}]_c$ and cGMP transients in responsive cells would then enable a significant assessment to be made of the transduction pathways controlling gene expression. Without this information, confusing observations could result as conflicting pathways are artificially activated and indeed natural individual cell variation is eliminated [6••]. For example, red light induces $[Ca^{2+}]_c$ transients in wheat protoplasts but not in *Physcomitrella* [18] or tobacco (G Baum, GI Jenkins and AJ Trewavas, unpublished data). Furthermore, apparent contradictory reports of blue light transduction through $[Ca^{2+}]_c$ between *Arabidopsis* [9] and *Physcomitrella* [18] have been reported.

Ca^{2+} release and uptake

Ca^{2+}-signalling pathways generally involve changes in the concentration of $[Ca^{2+}]_c$. These changes can arise from the activation of membrane-associated transporters — channels and pumps — or through changes in the affinity of Ca^{2+} binding proteins (CBPs). Channels and pumps have now been localised in the plasma membrane, tonoplast and endoplasmic reticulum (ER) and regulate inward Ca^{2+} current (Thuleau *et al.*, this issue pp 424–427). Ion channels located in the tonoplast and plasma membrane are believed to be responsible for most of the Ca^{2+} inward currents. Data collected during an action potential in *Chara*, however, suggests that $[Ca^{2+}]_c$ increases arise mainly from internal stores other than the vacuole [19]. Touch-induced $[Ca^{2+}]_c$ transients also originate from internal stores [20•], although here the nuclear envelope may be a primary source (A van der Luit, MR Knight and AJ Trewavas unpublished data). The second messengers, inositol(1,4,5)triphosphate (IP3) and cyclic adenosine phosphate ribose (cADP-R) mobilise Ca^{2+} release from the internal stores of the vacuole and probably the ER [10,21•]. Phospholipase C is now the proven enzymatic plant source of IP3 and diacylglyceride [22•], whereas cADP-R is synthesised from NAD [23].

CBP's (soluble or attached to the cytoskeleton) still lack critical examination in plant cells. Estimates of the cytosolic buffer capacity for Ca^{2+} (1–20 mM) [24] do suggest that CBP's could play a crucial role in signalling mechanisms. Phosphorylation of CBP's, subsequently modifying Ca^{2+} binding, could act to trigger the initial stimulus or modulate the transduction of the signal or even initiate Ca^{2+} wave propagation [6••,13].

Decoding the Ca^{2+} signalling pathway

Phosphorylation cascades regulated by protein kinases and phosphatases represent primary downstream transduction routes interpreting the $[Ca^{2+}]_c$ signal. Ca^{2+}-dependent

protein kinase (CDPK), Ca^{2+}/Calmodulin-dependent protein kinase (CaM kinase) and a protein kinase C-type enzyme have either been cloned or substantial evidence presented for their presence in plant cells [25,26•,27].

Single $[Ca^{2+}]_c$ transients are sufficient to initiate the accumulation of mRNA of the ABA dependent *lti78* and *kin1/2* genes [12]. The effects of ABA on *lti78* expression can be mimicked either by transformation of maize protoplasts with CDPK cDNA or with simple Ca^{2+} ionophore treatments to increase $[Ca^{2+}]_c$ [28]. *lti78* and *kin2* cDNAs have been loaded into tomato cells as reporter gene constructs. Co-injection of cyclic ADP-ribose (cADP-R) mimics the inducing-effect of ABA and up-regulates *lti78* and *kin2* expression [29••]. From experiments with inhibitors it was confirmed that cADPR-mediated induction of ABA-responsive gene expression is regulated by protein phosphorylation [29••], and the enzyme responsible for this could be CDPK [28]. Heparin, an inhibitor of IP3-dependent Ca^{2+} release failed to block ABA-induced *lti78* and *kin2* cDNA expression suggesting that IP3 may be involved in the secondary rather than primary ABA response. Transduction chains are linked by 'cross talk' [5••], however, and $[Ca^{2+}]_c$ information flow through cADP-R could compensate for inhibition of information flow through IP3. Comparative measurements of $[Ca^{2+}]_c$ flux through cADP-R and IP3 in heparin treated cells are needed. Guard cells can easily switch between Ca^{2+} dependent and Ca^{2+} independent transduction pathways [30].

Mutations in *ABI1* render plants insensitive to ABA and encode proteins with homology to Ca^{2+} regulated ser-thr protein phosphatases. The stomatal guard cells of *abi1* fail to respond to CO_2 and extracellular Ca^{2+} [31]. The signal transduction pathway for all three stimuli must converge on, or be close to, the *ABI1* gene product. CDPK modifies H^+ flux [32] and cytoplasmic pH is thought to be a second messenger mediating ABA action in guard cells [30].

The ubiquity of CDPK in ABA response chains can only be speculated. Specific inhibition of CDPK in aleurone cells using a peptide substrate, syntide-2, indicated its involvement only in a gibberellin-(GA), but not an ABA-dependent $[Ca^{2+}]_c$ transduction pathway [33••]. Furthermore, syntide-2 did not affect the GA-induced increase in $[Ca^{2+}]_c$ suggesting that syntide inhibited GA action downstream of the Ca^{2+} signal. Targets downstream from CDPK are still unknown but may involve a MAP kinase cascade [5••].

Ca^{2+} and calmodulin

CaM is a primary decoder of Ca^{2+} signals in eukaryotic cells. Vos and Hepler [34••] imaged the distribution of fluorescent-tagged bovine CaM during stamen hair cell mitosis. Only a uniform distribution was observed, in conflict with previous data using chemical fixation which suggested strong attachment to the spindle. A fundamental reassessment of much early-acquired data using chemical fixation is thus indicated. A uniform distribution of fluorescent calmodulin in living pollen tubes has also been reported [35•]. When the concentration of exogenous CaM was raised above the endogenous pool, a putative interaction with the actin cytoskeleton could be visualised. Although strict threshold conditions were used in this experiment, it nevertheless suggests the localisation of potential targets for CaM regulation in polarised growth [36]. In contrast with these two studies, microinjection of CaM into developing and polarising *Fucus* zygotes showed an accumulation in the precise cellular region from which the rhizoid emerged several hours later [37]. These observations help support the classical theory of *Fucus* polarity involving $[Ca^{2+}]_c$ which has seen recent difficulties. A putative Ca^{2+} channel identified by dihydropyridine staining was also found to localise in the *Fucus* tip rhizoid [38], but equivalent experiments in pollen tubes failed to reveal tip localisation (L Camacho, AJ Trewavas and R Malhó, unpublished data). Future credible CaM distribution studies urgently require the incorporation of fluorescence polarisation anisotropy technology to image the distribution of free and bound CaM in living plant cells — it is the dynamics of bound CaM which most researchers wish to map.

Tobacco cells transformed with a mutant calmodulin gene, (K115R) exhibit enhanced NAD kinase activity [39••]. When challenged with elicitors or environmental stress signals, these cells also accumulate higher levels of active oxygen species. Transformation with defined CaM mutants is a subtle, but very powerful, future technology which will enable ready dissection of transduction routes through specific CaM binding proteins.

Tip growth in pollen tubes, rhizoids and root hairs

Pollen tubes, root hairs and *Fucus* rhizoids contain a tip-high $[Ca^{2+}]_c$ gradient in the apical 10–40μm [40•,41–44]. Patch clamp data from fungal hyphae, *Fucus* rhizoids and Mn^{2+} quench procedures with higher plant cells [45] suggest a tip-based activity of stretch-activated or mechano sensitive channels [13,46] to be responsible for controlling Ca^{2+} entry to these cells. The extreme tip Ca^{2+} may regulate protein kinases, putative IP3 receptors [47] and Rho GTPases [48•] involved in vesicle fusion/docking and tip growth/orientation.

The $[Ca^{2+}]_c$ gradient oscillates in the Lily pollen tube, the extreme tip varying from 700 nM to 10 μM with a periodicity of less than one minute [40•,41]. Oscillations are in phase with pulsatile growth patterns but out of phase with extracellular Ca^{2+} entry measured by the vibrating probe [40•]. The apparent discrepancy between oscillations in Ca^{2+} entry and $[Ca^{2+}]_c$ was suggested to result from direct movement of extracellular Ca^{2+} into the ER, from where it is subsequently released in pulsatile fashion (so-called capacitative Ca^{2+} entry [5••]) or Ca^{2+} binding to newly secreted wall [40•]. But, the discrepancy may also result

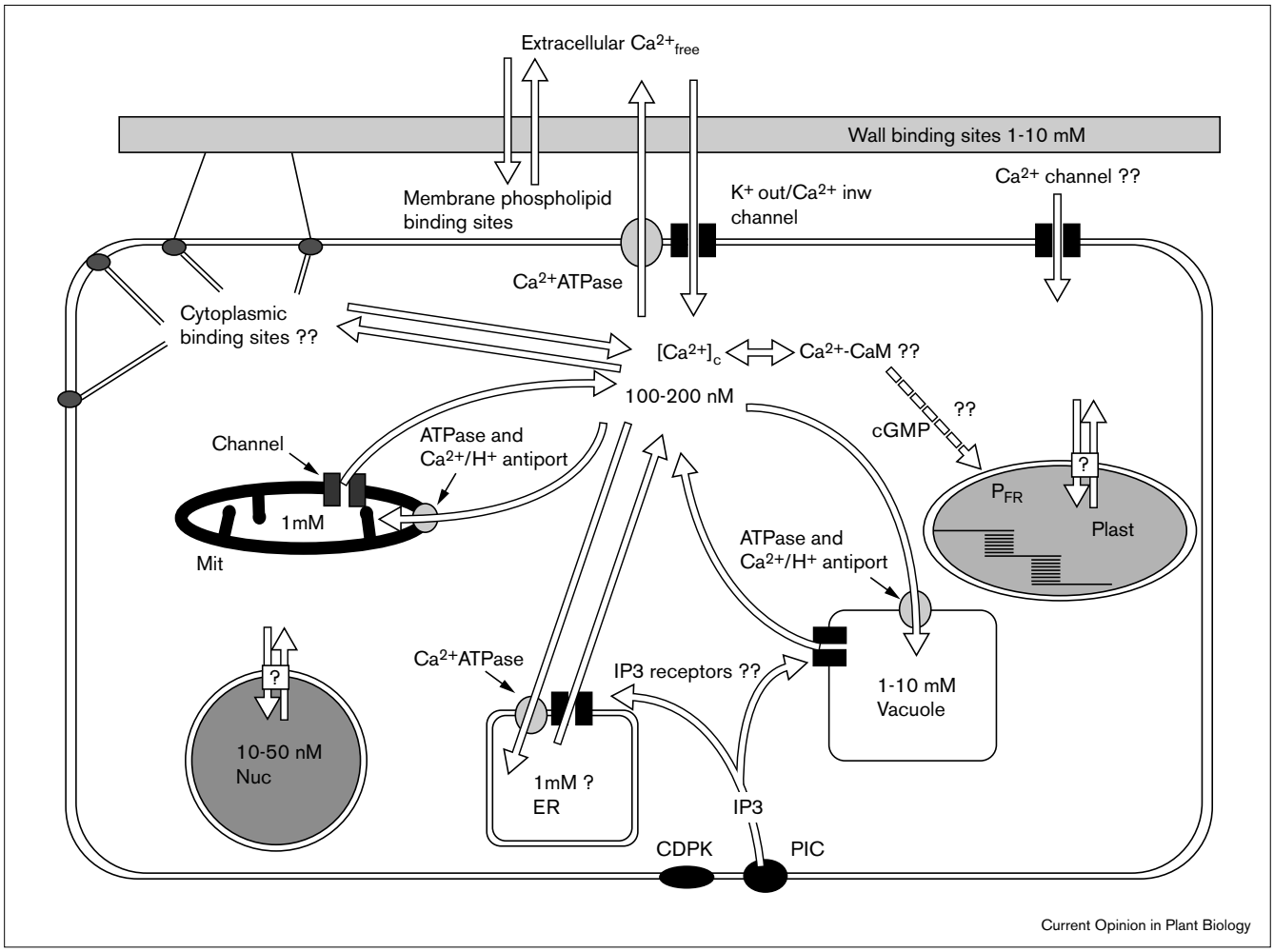
from flawed measurements of the actual peak values. Estimates with aequorin of peak values indicate it is 10–20 μM Ca^{2+} [41], well beyond the reporting range of the fluorescent dye Fura-2 [40•].

Oscillations of $[Ca^{2+}]_c$ could represent a conversion of analogue to digital information, improving environmental sensing by noise reduction [5••]. Alternatively, $[Ca^{2+}]_c$ oscillations may simply originate from a chaotic-like property common to complex systems of interaction but appearing only under discrete conditions. Lily pollen tubes can grow to 1 mm with no detectable $[Ca^{2+}]_c$ oscillations [40•,41]. No difference in overall growth rate accompanies tubes that oscillate from those that do not. $[Ca^{2+}]_c$ oscillations (frequency < 1 min) can be induced in unicellular algae by modifying intracellular mobilisation of Ca^{2+} with caffeine or strontium [49]. The metabolic change from a non-oscillating to an oscillating pollen tube may then be subtle. In root hairs, Nod factors induced $[Ca^{2+}]_c$ oscillations of similar periodicity but these were confined to the nuclear region [1].

Localised photolysis of loaded caged Ca^{2+} in different cytoplasmic regions of pollen tubes and root hairs has shown that $[Ca^{2+}]_c$ modifications in the tip can directly alter growth orientation [50,51••]. But root hairs rapidly recover their initial growth orientation, suggesting an innate fixed polarity. Root hair tips contain spectrin [52] which could permanently anchor a microfilament structure directing a fixed growth orientation. Photolysis of caged IP3 in pollen tube tips did not elicit significant changes in $[Ca^{2+}]_c$ or orientation [47]. IP3 receptors may thus be functionally unique in the tip region.

These studies on single cells have used three different types of $[Ca^{2+}]_c$ measurement. Single wavelength dyes can be excited by visible light. Quantitation of $[Ca^{2+}]_c$ images is precluded because of variations in cell thickness or uneven dye distribution. But single wavelength dyes, the most popular for animal studies, can be used to easily record signal-induced changes in $[Ca^{2+}]_c$ by comparing adjacent time points or adjacent images [50]. Dual wavelength dyes are used for accurate image quantitation but may have to be

Figure 2



Summary of Ca^{2+} signalling knowledge at present in plant cells. Areas of extreme ignorance which most likely deserve future attention are indicated by question marks.

loaded to 20μM to compensate for reduced brightness. Possible participation of the dye in Ca^{2+} signalling can then become a complication and UV excitation with dye breakdown (for Fura-2 or Indo-1) can cause toxicity, impeding the frequency with which images can be collected. Aequorin has weaker binding affinity for Ca^{2+} binding, minimising cell perturbation. But interference from Mg^{2+} and limitations on loading concentrations or expression of transgenes will place constraints on image discrimination. Both single and dual wavelength dyes have been used in pollen tube self-incompatibility $[Ca^{2+}]_c$ measurements [44,53] and $[Ca^{2+}]_c$ oscillations [40•,41]. Root hair and pollen tube $[Ca^{2+}]_c$ gradients and caged Ca^{2+} photolysis have employed both procedures [47,50,51••]. Comparison between these methods has not revealed any fundamental difference. Each method has value in particular circumstances and all are useable, provided the limitations of each are recognised. We recommend use of the simplest technique commensurate with the information desired.

Gravitropism

A rigorous test of the involvement of $[Ca^{2+}]_c$ signalling in gravitropism has been performed in *Arabidopsis* roots [20•]. Variations in $[Ca^{2+}]_c$ on gravi-stimulation were not detected although a mechanical signal was easily imaged. Images were only collected at one minute intervals, however, to avoid cell damage. Short transients could have been missed; those induced by mechanical signals, for example, last less than 10 seconds [6••]. Statocytes, the obvious target for gravisensing, possess a 10 fold higher CaM content than other root cells, which should enable discrimination of much lower, difficult-to-detect, $[Ca^{2+}]_c$ thresholds. Brief, only slight elevations in $[Ca^{2+}]_c$ may modify the activity of Ca^{2+}/CaM-dependent protein kinases [45,54] and could trigger a statocyte response sufficient for gravisensing. Gravi response could instead arise from subtle changes in plasma membrane–cell wall interactions [55].

Conclusions

While knowledge of $[Ca^{2+}]_c$ has gained apace, there are serious areas of ignorance which are illustrated in Figure 2. Characterisation of transduction component interaction and imaging of the spatial movement of transduction components in living cells represents the way forward and is where we predict most spectacular advances will now be made.

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