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Calcium efflux systems in stress signaling and adaptation in plants

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Transient cytosolic calcium ($[Ca^{2+}]_{cyt}$) elevation is an ubiquitous denominator of the signaling network when plants are exposed to literally every known abiotic and biotic stress. These stress-induced $[Ca^{2+}]_{cyt}$ elevations vary in magnitude, frequency, and shape, depending on the severity of the stress as well the type of stress experienced. This creates a unique stress-specific calcium "signature" that is then decoded by signal transduction networks. While most published papers have been focused predominantly on the role of Ca^{2+} influx mechanisms to shaping $[Ca^{2+}]_{cyt}$ signatures, restoration of the basal $[Ca^{2+}]_{cyt}$ levels is impossible without both cytosolic Ca^{2+} buffering and efficient Ca^{2+} efflux mechanisms removing excess Ca^{2+} from cytosol, to reload Ca^{2+} stores and to terminate Ca^{2+} signaling. This is the topic of the current review. The molecular identity of two major types of Ca^{2+} efflux systems, Ca^{2+}-ATPase pumps and Ca^{2+}/H^+ exchangers, is described, and their regulatory modes are analyzed in detail. The spatial and temporal organization of calcium signaling networks is described, and the importance of existence of intracellular calcium microdomains is discussed. Experimental evidence for the role of Ca^{2+} efflux systems in plant responses to a range of abiotic and biotic factors is summarized. Contribution of Ca^{2+}-ATPase pumps and Ca^{2+}/H^+ exchangers in shaping $[Ca^{2+}]_{cyt}$ signatures is then modeled by using a four-component model (plasma- and endo-membrane-based Ca^{2+}-permeable channels and efflux systems) taking into account the cytosolic Ca^{2+} buffering. It is concluded that physiologically relevant variations in the activity of Ca^{2+}-ATPase pumps and Ca^{2+}/H^+ exchangers are sufficient to fully describe all the reported experimental evidence and determine the shape of $[Ca^{2+}]_{cyt}$ signatures in response to environmental stimuli, emphasizing the crucial role these active efflux systems play in plant adaptive responses to environment.

Keywords: cytosolic calcium, signatures, oscillations, Ca^{2+}-ATPase, calcium exchanger

INTRODUCTION

CYTOSOLIC CALCIUM HOMEOSTASIS AND SIGNALING IN PLANT–ENVIRONMENTAL INTERACTION

Calcium is an essential plant nutrient which plays a crucial structural role in cell walls and maintains membrane integrity. Calcium can easily interact with proteins, membranes, and organic acids through its ability to form different coordination bonds (from six to nine) which results in a high-affinity for carboxylate oxygen, rapid binding kinetics, and complex geometries (Medvedev, 2005; Case et al., 2007). Because of this, calcium can be a toxic cellular compound at higher concentrations as it would trigger aggregation of proteins and nucleic acids, precipitation of phosphates (present in ATP), and affect the integrity of lipid membranes (Case et al., 2007). As a result, plants have evolved efficient Ca^{2+} efflux mechanisms that can keep cytosolic free calcium, $[Ca^{2+}]_{cyt}$, at a constant and very low (submicromolar) level by exporting Ca^{2+} out of the cell or into the intracellular organelles (Dodd et al., 2010). In a typical plant cell, free Ca^{2+} concentrations are in the range of 1–10 mM in the apoplasm, 100–200 nM in the cytoplasm, 0.2–10 mM in the vacuole, ∼1 mM in the endoplasmic reticulum (reviewed in Medvedev, 2005), and 2–6 μM in chloroplast stroma (cf. Ettinger et al., 1999). Such extremely low $[Ca^{2+}]_{cyt}$ creates a unique cellular environment in which $[Ca^{2+}]_{cyt}$ concentration can be elevated by a factor of 10 or 20 rapidly (within seconds) upon sensing stress by using large electrochemical potentials either at the plasma or organelle membranes (Sanders et al., 1999). Indeed, $[Ca^{2+}]_{cyt}$ elevation is an ubiquitous denominator of the signaling network when plants are exposed to soil acidity, salinity, anoxia, ozone, drought, osmotic, oxidative, heat and cold stresses, gaseous pollutants, mechanical cues, light, plant hormones, pathogens, bacterial, and fungal signals (reviewed in Sanders et al., 1999; McAinsh and Pittman, 2009). Resulting $[Ca^{2+}]_{cyt}$ elevations are decoded by Ca^{2+} sensor proteins (e.g., CaM, CMLs, CDPKs, CBL/CIPKs) which regulate downstream targets leading to a stress-specific physiological response (DeFalco et al., 2010).

STRESS-SPECIFIC CALCIUM "SIGNATURES"

Stress or stimuli induced $[Ca^{2+}]_{cyt}$ elevations vary in magnitude, frequency, and shape. These depend on the severity of the stress as well the type of stress experienced, thus creating a unique stress-specific calcium "signature" that is then decoded by signal transduction networks. An example of such stress-specific "signatures" is shown in **Figure 1**, which depicts $[Ca^{2+}]_{cyt}$ elevation in response to abscisic acid (ABA), anoxia, and salt stresses. Very often, not one but a series of repetitive spikes (oscillations) is observed in response to environmental stimuli (e.g., for 10 nM ABA in guard cells **Figure 1A**). In the case of anoxia, $[Ca^{2+}]_{cyt}$ elevation showed two distinctive peaks; one rapid (within a minute), and another one that lasted for hours (**Figure 1B**), whereas $[Ca^{2+}]_{cyt}$ responses to salt stress did not have a second peak (**Figure 1C**).

The magnitude of $[Ca^{2+}]_{cyt}$ elevations shows a clear dose-dependency of external stimulus as was shown for salinity (Tracy et al., 2008), ozone (Clayton et al., 1999), hypo-osmotic shock (Goddard et al., 2000), H_2O_2 (Price et al., 1994), high temperatures (Gong et al., 1998), and apoplastic Ca^{2+} treatments (McAinsh et al., 1995). In addition to the shape and magnitude of $[Ca^{2+}]_{cyt}$ elevation, repetition of $[Ca^{2+}]_{cyt}$ elevations (spikes) could encode stimuli specific information. A classical example comes from Miwa et al. (2006) where *Early Nodulation 11* (*ENOD11*) inductions were observed only when the $[Ca^{2+}]_{cyt}$ spikes lasted for at least 60 min. Jasmonic acid treatment lengthened the period between spikes but did not alter the number of spikes required for *ENOD11* expression, which suggests that indeed the number of spikes carries information required for *ENOD11* expression (Miwa et al., 2006). More specific aspects of temporal encoding mechanisms and the role of cytosolic calcium oscillations in plant–environmental information are discussed in Section "Temporal Encoding Mechanisms: Cytosolic Calcium Oscillations." Altogether, these results demonstrate that information concerning the type and strength of stress are encoded within the magnitude, shape, and frequency of $[Ca^{2+}]_{cyt}$ elevations. While most published papers have focused predominantly on the role of Ca^{2+} influx mechanisms in shaping $[Ca^{2+}]_{cyt}$ signatures, the latter is impossible without involvement of Ca^{2+} efflux mechanisms, removing excess of Ca^{2+} from the cytosol. The purpose of the current review is to emphasize the role of Ca^{2+} efflux systems in the cytosolic Ca^{2+} signaling and shaping $[Ca^{2+}]_{cyt}$ signatures.

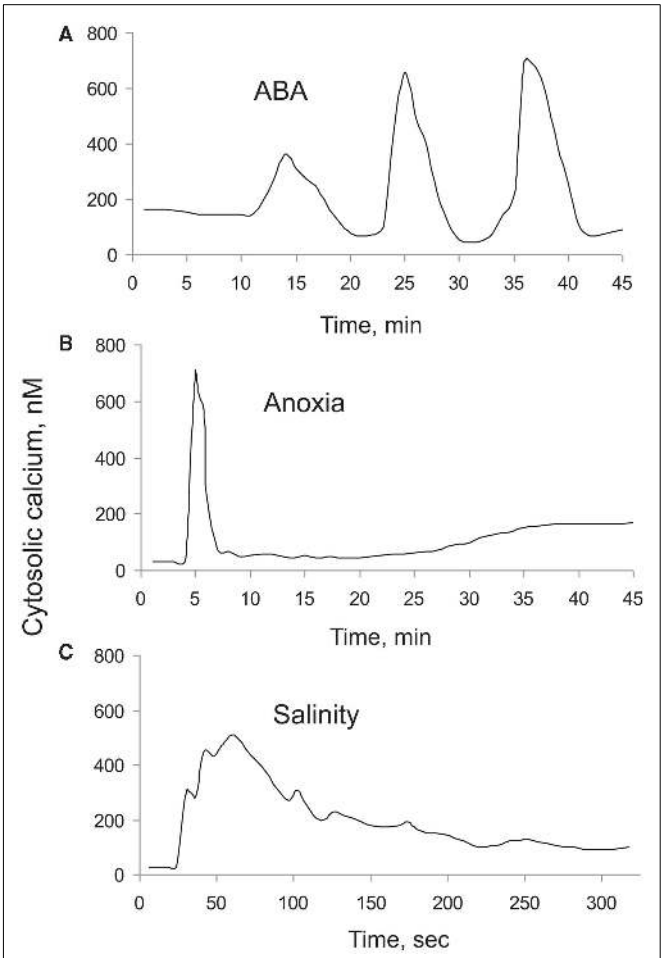

FIGURE 1 | Typical $[Ca^{2+}]_{cyt}$ "signatures" measured in plant cells in response to a range of external stimuli. (A) Response to 10 nM ABA treatment measured in guard cells of *Commelina communis* (Staxén et al., 1999); **(B)** response to anoxia (Sedbrook et al., 1996); and **(C)** response to salt (Tracy et al., 2008) treatments in *Arabidopsis thaliana* seedlings. For **(B)**, luminescence ratio values were converted to $[Ca^{2+}]_{cyt}$ values by considering peak $[Ca^{2+}]_{cyt}$ value as 700 nM.

ENCODING ENVIRONMENTAL INFORMATION BY CALCIUM

SPATIAL ENCODING: INTRACELLULAR CALCIUM MICRODOMAINS

Pleiotropic Ca^{2+} effects on cellular functions are due to a spatio-temporal organization of Ca^{2+} signal, and it would be a gross oversimplification to assume that $[Ca^{2+}]_{cyt}$ is uniform across the cell. Instead, *localized* $[Ca^{2+}]_{cyt}$ increases take place. Several factors make such localization possible. First, due to a presence of different buffer components in the cytosol Ca^{2+} mobility is very low (e.g., diffusion coefficient value of 1 to 5×10^{-11} m^2/s as compared to 8×10^{-10} m^2/s for pure water; Allbritton et al., 1992; Hille, 2001). The second contributing factor is a very specific configuration of different intracellular Ca^{2+} stores and their non-random distribution within a cell. Third, as a result of heterogeneous distribution and clustering of various Ca^{2+} transporters, the contact zones may exist between different organelles and a plasma membrane (PM), creating so-called "Ca^{2+} microdomains." Such microdomains may be only a few nanometers in size, as ones which occur at the immediate vicinity of an open Ca^{2+}-permeable channel (Rizzuto and Pozzan, 2006).

There are numerous examples of a specific subcellular localization of a Ca^{2+} signal for a variety of plant responses, with a preferential involvement of the PM, vacuole, nucleus, mitochondria, chloroplasts, or some combinations of the aforementioned stores (Sanders et al., 1999). The shaping of Ca^{2+} response is very likely dependent on the non-random distribution of Ca^{2+} pumps, with the expression of *ACA 2* in the ER, *ACA 4* and *11* in the tonoplast, and *ACA 8–10* in the PM (Sze et al., 2000; Harper, 2001; Boursiac and Harper, 2007; Kudla et al., 2010).

During polar growth, e.g., of root hairs or pollen tubes, Ca^{2+} increases occur exclusively in the apical zone, which can be explained by a localized expression of some Ca^{2+} influx components. *CNGC18* encoding a putative Ca^{2+}-permeable channel,

whose expression is essential for tube growth, is predominantly expressed in the tip of a pollen tube (Frietsch et al., 2007), whereas *ACA 9* Ca^{2+} pump is expressed uniformly over the tube (Schiøtt et al., 2004). Despite this uniform distribution, *ACA 9* is believed to be active mainly in the high Ca^{2+} region in the tip, due to Ca^{2+}–CaM binding; on the contrary, *CNGC18* is presumed to be inactivated by Ca^{2+}–CaM (Hepler and Winship, 2010). This pair could obviously form a Ca^{2+} oscillator, where *ACA 9* Ca^{2+} pump may prevent long-lasting Ca^{2+} overloads. In addition, in the pollen tube the stimulation of a hyperpolarization-activated Ca^{2+}-influx channel by *external* apoplastic CaM was demonstrated (Shang et al., 2005). This may add an additional feed-forward loop via Ca^{2+}-activated exocytosis of CaM-containing vesicles (Hepler and Winship, 2010). Another Ca^{2+} influx channel, a stretch-activated one, was functionally characterized in patches derived from a tip of pollen tube (Dutta and Robinson, 2004), whose activity may be modulated by a periodic growth-related cell wall loosening.

Growth-related local Ca^{2+} rises are also mediated by PM ROS-activated channels (Foreman et al., 2003), and the activation of ROS-generating enzyme NADPH-oxidase by incoming Ca^{2+} tends to act as a positive feedback (Takeda et al., 2008). It is not surprising then that local Ca^{2+} and ROS changes are often coupled (Foreman et al., 2003; Terada, 2006; Cárdenas et al., 2008). Moreover, integration of NADPH-oxidase in sterol-rich lipid rafts in the tip of a pollen tube is very essential for generation of a tip-focused cytosolic Ca^{2+} gradient, underlying the polarized growth (Liu et al., 2009).

It is widely reported in animal literature that opening of a single Ca^{2+} permeable channel is capable of generating very high (up to 300 μM) local Ca^{2+} increases (Llinas et al., 1992; Naraghi and Neher, 1997; Heidelberger, 1998). The peak amplitude and spatial diffusion of the Ca^{2+} microdomain formed at the mouth of a Ca^{2+} channel and its immediate neighborhood depends on the conductance and Ca^{2+} selectivity of the channel, electrochemical gradient for Ca^{2+} across the membrane, and local intracellular Ca^{2+} buffering. To the best of our knowledge, no such work has been performed on plant systems.

Multiple roles have been attributed to such Ca^{2+} microdomains. Presynaptic termini Ca^{2+} microdomains are believed to control vesicles fusion and exocytosis (Llinas et al., 1992; Heidelberger et al., 1994). In non-excitable cells subplasma membrane Ca^{2+} microdomains control Ca^{2+}-sensitive adenylate cyclases (Mons et al., 1998) and NO synthase (Lin et al., 2000). Strikingly, a very local Ca^{2+} increase at the mouth of an open PM channel may initiate the signaling sequence, leading eventually to gene activation far away (for a review see Rizzuto and Pozzan, 2006). Finally, Ca^{2+} channels themselves are targets for ultra-local Ca^{2+} signaling. Several types of Ca^{2+} channels such as L-type Ca^{2+}-selective channels and CRAC have been shown to be inactivated by incoming Ca^{2+} (Zweifach and Lewis, 1995; Soldatov, 2003). Conversely, opening of several clustered RyR channels required a localized Ca^{2+} spark to be generated (Franzini-Armstrong and Protasi, 1997). Tight contact zones existing between the PM, ER, and mitochondria are not only important for creating local Ca^{2+} microdomains, but may result in direct transport of Ca^{2+} from a translocator in one membrane to its counterpart in another membrane, so-called "linked Ca^{2+} transport" (Poburko et al., 2004). As mitochondrial Ca^{2+} uptake may eventually lead to Ca^{2+} overload followed by the activation of the transition pore and release of apoptotic factors, location of mitochondria in relation of a high Ca^{2+} microdomain may be crucial for determining a cell's fate (Spät et al., 2009).

The physical basis for a non-random distribution of membrane elements is due to the existence of lipid rafts, well established for animal cells and becoming more evident also for plants (Zappel and Panstruga, 2008). In animal cells, recruitment of Ca^{2+} channels, pumps, exchangers into lipids rafts, and their functional consequences are known (Balijepalli and Kamp, 2008). In plants such evidence so far exists only for KAT1 channels (Sutter et al., 2006; Homann et al., 2007), but there is no reason to exclude such possibility also for plant Ca^{2+} permeable channels. Recently, we obtained some indirect evidence that double-pore Ca^{2+} (TPC) channels tend to cluster and communicate via local Ca^{2+} changes, as closed–open transitions of individual channels demonstrated interdependency (Pottosin, unpublished). Such an arrangement seems logical in light of a very high threshold for the TPC channel activation by intracellular Ca^{2+} (several tens of micromolar for the physiological voltage range (Schulz-Lessdorf and Hedrich, 1995; Pottosin et al., 1997; Pérez et al., 2008). Such high free Ca^{2+} concentrations occur only in close proximity of the mouth of an open Ca^{2+}-permeable channel. A high threshold protects the cell from a global Ca^{2+} release from the non-exhaustible store, the central vacuole, which would be fatal. This consideration was obviously overlooked the when original hypothesis for the Ca^{2+}-induced Ca^{2+} release based on the Ca^{2+} activation and Ca^{2+} permeability of vacuolar TPC was formulated (Ward and Schroeder, 1994). Recent studies with *tpc*-mutants show that TPC channels do not significantly contribute to any type of global Ca^{2+} response in plants (Ranf et al., 2008). On the other hand, clustering of the tonoplast TPC channels and/or their contacts with PM Ca^{2+}-permeable channels of other organelles and PM would tend to split the large vacuole into multiple local Ca^{2+} circuits, where local feed-forward-looped Ca^{2+} rises could take place (Pérez et al., 2008). The fact that the tonoplast Ca^{2+}/H^{+} (CAX) exchanger also has a relatively low (K_d ~10–15 μM) affinity for Ca^{2+} (Hirschi, 2001) indirectly indicates that it may encounter very high local Ca^{2+} rises.

TEMPORAL ENCODING MECHANISMS: CYTOSOLIC CALCIUM OSCILLATIONS

Advantages of oscillatory strategy

most other biological systems, cellular metabolism in general, and membrane transporters in particular, are governed by non-liner mechanisms and include a large number of positive and negative feedback loops (Hansen, 1978; Feijo et al., 2001; Shabala et al., 2006). It is not surprising, therefore, that such systems exhibit oscillatory behavior. Moreover, transient oscillatory responses are the most typical response of every feedback-controlled system to step-wise changes in external parameters. It is hardly surprising, therefore, that $[Ca^{2+}]_{cyt}$ oscillations are widely reported in a range of plant systems (see below). Such a periodic behavior confers several functional advantages for the organism (Rapp, 1987), with precision of control and discrimination of true signals from environmental noise being the most important. Theoretical findings by

Rapp et al. (1981) suggest that many biological oscillations reflect the biochemical implementation of analog-digital-analog control strategy; a strategy that provides significant functional advantages for living cells.

Oscillations may also facilitate synchronization of events widely separated in space between subcellular compartments (Lloyd and Stupfel, 1991), and it has been suggested that Ca^{2+} oscillations can act as cellular timekeepers to coordinate related biochemical reactions and enhance their overall efficiency (Izu and Spangler, 1993). Another advantage of oscillatory strategy is that oscillations may enhance signal efficiency specifically at low levels of stimulation. Experiments carried out on lymphocytes T cells revealed that oscillations in cytosolic free Ca^{2+} increase the efficiency and specificity of gene expression (Dolmetsch et al., 1998). The authors showed that this effect arises from the highly non-linear dependence of transcription on $[Ca^{2+}]_{cyt}$, so that oscillations periodically exceed the threshold for activation whereas a small constant $[Ca^{2+}]_{cyt}$ rise of the same average magnitude does not. In other words, oscillatory control optimized sensitivity to weak external stimuli.

Theoretical studies also show that such systems will possess complex dynamics leading to "strange" behavior such as bifurcation and chaos (May, 1989), and both theoretical (Rand et al., 1981) and experimental (Shabala et al., 1997) evidence for the chaotic stomatal behavior were presented. Given the fact that the stomata aperture is controlled by $[Ca^{2+}]_{cyt}$ modulation, evidence for deterministic chaos in $[Ca^{2+}]_{cyt}$ kinetics is waiting to be revealed.

Ca^{2+} oscillations in plant cells

Two major types of $[Ca^{2+}]_{cyt}$ oscillations are known: (i) a baseline spiking, in which the magnitude of the spike remains the same but the frequency of the spiking is affected by environmental stimulus, and (ii) sinusoidal $[Ca^{2+}]_{cyt}$ oscillations, in which agonist dose regulates the amplitude but has no effect on oscillation frequency. In technical terms this is equivalent to the frequency and amplitude modulation (Berridge, 1997).

Over the last decade, calcium oscillations have been a popular subject of numerous reviews (e.g., McAinsh and Hetherington, 1998; Ng and McAinsh, 2003; McAinsh and Pittman, 2009; Roelfsema and Hedrich, 2010) and, thus, are only briefly covered here. Such oscillations have been found in various plant systems such as guard cells (McAinsh et al., 1995, 1997), pollen tubes (Holdaway-Clarke et al., 1997; Feijo et al., 2001), roots (Kiegle et al., 2000), root hairs (Ehrhardt et al., 1996; Monshausen et al., 2008), and some other systems (Bauer et al., 1998; Schonknecht et al., 1998). Oscillations in $[Ca^{2+}]_{cyt}$ result from a dynamic balance of fluxes of Ca^{2+} into and out of the cytosol and include release and uptake from both intracellular stores and external media (Ng and McAinsh, 2003). The frequency of $[Ca^{2+}]_{cyt}$ oscillations ranges typically from ca 20 s (Bauer et al., 1998) to 10–15 min (McAinsh et al., 1995) and shows a clear dependence on the magnitude of external stimulus (discussed in the next section). The latter findings lead to the concept of the existence of a frequency encoding mechanism, which conveys information about the severity of environmental fluctuation (e.g., temperature; heavy metal toxicity; hormonal level) by means of $[Ca^{2+}]_{cyt}$ spikes. The functional role of $[Ca^{2+}]_{cyt}$ oscillations was shown in nodulation experiments with alfalfa. The pronounced $[Ca^{2+}]_{cyt}$ spikes were observed only in nodulating wild type plants, but not in non-nodulating alfalfa mutant (Ehrhardt et al., 1996); they were also absent in roots of tomato plants which are not capable of nodulating.

Both plasma and endomembrane Ca^{2+} channels are considered to be essential for generation of stimulus-induced $[Ca^{2+}]_{cyt}$ oscillations (McAinsh et al., 1995, 1997). Involvement of hyperpolarization-dependent PM Ca^{2+} channels (HACC) has been shown (Pei et al., 2000), and oscillatory coupling between MP and $[Ca^{2+}]_{cyt}$ has been suggested (Grabov and Blatt, 1998; Blatt, 1999). Indirect control via ABA-induced H_2O_2 production and a subsequent activation of Ca^{2+} influx through HACC has also been demonstrated (Pei et al., 2000).

Repetitive transient $[Ca^{2+}]_{cyt}$ spikes were observed in unicellular green alga *Eremosphaera viridis* upon stimulation with Sr^{2+} (Bauer et al., 1998). These spikes were inhibited in cells pretreated with either ruthenium red or ryanodine, two known agents affecting activity of ryanodine/cyclic ADP-ribose type of Ca^{2+} channel, indicating the involvement of ER channels in generation of $[Ca^{2+}]_{cyt}$ oscillations. $InsP_3$-gated Ca^{2+}-release channels have also been implicated (McAinsh and Hetherington, 1998). It was suggested that these channels may form a part of the signal transduction pathway of different stimuli based on difference in distribution, gating properties, and sensitivity.

Ca^{2+} as a component of encoding/decoding mechanism

Increases in $[Ca^{2+}]_{cyt}$ have been observed in several cell types in response to a number of stimuli (see Stress-Specific Calcium "Signatures"). Most of these treatments resulted in long-lasting $[Ca^{2+}]_{cyt}$ oscillations and showed all signs of frequency encoding. As such, a strong correlation between mechanical signal strength and an amplitude of the resulting $[Ca^{2+}]_{cyt}$ spike has been shown in *Nicotiana* cotyledons (Knight et al., 1991, 1992). Sr^{2+}-induced $[Ca^{2+}]_{cyt}$ oscillations in *Eremosphaera* showed an increase in frequency and decrease in amplitude at increasing agonist concentrations (Bauer et al., 1998). In stomata guard cells, the period of $[Ca^{2+}]_{cyt}$ oscillations increased from $T = 8.3$–13.6 min as external Ca^{2+} rose from 0.1 to 1.0 mM (McAinsh et al., 1995). This is consistent with animal models (Tang and Othmer, 1995) and points out that the signal specificity may be encoded by the amplitude and frequency of oscillations.

At the other end of the equation is a decoding mechanism. It was suggested that the Ca^{2+} signature may be decoded by the differential effects of Ca^{2+} on various downstream Ca^{2+}-regulated proteins, such as calmodulin, phosphoinositide-specific phospholipase C, and Ca^{2+}-dependent phosphatases and kinases (Tang and Othmer, 1995; Leckie et al., 1998; McAinsh and Hetherington, 1998). Direct evidence that calmodulin-dependent protein kinase II can decode the frequency of $[Ca^{2+}]_{cyt}$ spikes into distinct amounts of kinase activity was given by De Koninck and Schulman (1998).

Dolmetsch et al. (1997) demonstrated that differential gene transcription in B lymphocytes is achieved through amplitude modulation of the $[Ca^{2+}]_{cyt}$ signaling system. Low $[Ca^{2+}]_{cyt}$ concentrations activate the nuclear factor of activated T cells (NF-AT), whereas much larger elevations stimulate a different set of transcriptional regulators. In their model, mechanisms of AM-modulation are based on the recruitment of a variable number of elementary events, resulting from the opening of either

individual or small group of channels located in the internal stores (organized as a hierarchy). The same group has also shown that $[Ca^{2+}]_{cyt}$ oscillation frequency can discriminate among different transcriptional pathways (Dolmetsch et al., 1998). While low frequency spikes recruited NF-kB alone, higher frequency oscillations ($T < 6$ min) activated NF-AT, Oct/OAP, and NF-kB, resulting in a differential gene expression. Another work used caged $InsP_3$ to induce forced $[Ca^{2+}]_{cyt}$ oscillations in *T* lymphocytes (Li et al., 1998). It was shown that $[Ca^{2+}]_{cyt}$ oscillations were more effective in activation of the NF-AT gene expression than a single, prolonged $[Ca^{2+}]_{cyt}$ increase, provided that the period was roughly 1 min; slower ($T = 2$ min) and faster ($T = 0.5$ min) oscillations were less efficient (Li et al., 1998).

It was also shown in animal systems that $[Ca^{2+}]_{cyt}$ oscillations were more effective in Ca^{2+}-sensitive mitochondrial dehydrogenase (CSMDH) activation than a sustained $[Ca^{2+}]_{cyt}$ increase of similar amplitude (Hajnoczky et al., 1995). In this work, sustained NADPH elevation was achieved when the $[Ca^{2+}]_{cyt}$ oscillation frequency was 0.5–1/min, while sustained increase in $[Ca^{2+}]_{cyt}$ caused only a transient elevation of NADPH. These results suggest that the pulsating organization and frequency modulation of $[Ca^{2+}]_{cyt}$ signaling are superior to amplitude modulation of $[Ca^{2+}]_{cyt}$ responses in controlling mitochondrial metabolism. Thus, it appears that mitochondria are tuned to the oscillating $[Ca^{2+}]_{cyt}$ signal.

SHAPING CYTOSOLIC CALCIUM SIGNALS

As prolonged $[Ca^{2+}]_{cyt}$ elevation is detrimental to normal cell metabolism, the basal $[Ca^{2+}]_{cyt}$ levels must be restored after the signaling process has been completed. This may be achieved by orchestrated action of the cytosolic buffering system, and by the action of Ca^{2+} efflux mechanisms present in the PM and endomembranes. As argued below, the buffering capacity of Ca^{2+}-binding proteins inside the cytosol is rather limited, making the Ca^{2+} efflux system absolutely essential in the above process of restoration of basal $[Ca^{2+}]_{cyt}$ levels.

CYTOSOLIC BUFFERING

Intracellular Ca^{2+} buffering is an important determinant of the Ca^{2+} signal specificity, as both the magnitude and kinetics of Ca^{2+} signatures are critically dependent on Ca^{2+} buffering (Lew et al., 1984; Koopman et al., 2001). At the same time, there appears to be no correlation between cytosol buffering capacity and steady-state free Ca^{2+} level (Rizzuto and Pozzan, 2006). In animal cells cytosolic Ca^{2+} buffering may be described by a simple Michaelis–Menten formalism, with apparent dissociation constant (K_{app}) ranging from 0.4 to 0.7 μM and maximal capacity (B_{max}) of 0.15–0.3 mM. Assuming for simplicity one (high-affinity)-site binding, the total-to-free Ca^{2+} ratio in cytosol could be expressed as

$$Ca_{(tot)}/Ca_{free} = 1 + B_{max}/(Ca_{free} + K_{app})$$

Assuming basal $[Ca^{2+}]_{cyt}$ levels = 0.1 μM, one may calculate that only one of 200–600 Ca^{2+} ions in cytosol is free while all others are bound.

In plants, a very high (15–45 mM) cytosolic buffering capacity for Ca^{2+} was estimated by some authors (Plieth et al., 1997). This estimate, however, included vacuolar Ca^{2+} sequestration, and, more importantly, was made under the assumption that Ca^{2+} and H^+ always bind to the same sites; the assumption was later challenged by other authors (Schönknecht and Bethmann, 1998). Thus, it is generally accepted that the cytosolic buffering capacity in plants is not different from their animal counterparts, and is in a range of $B_{max} = 0.2$–0.5 mM (Trewavas, 1999).

Cytosolic Ca^{2+}-buffering is achieved mainly by Ca^{2+} binding proteins. Most of these proteins also act as Ca^{2+}-sensors (Schwaller, 2009). *Arabidopsis* genome alone harbors 7 calmodulin (CaM) and 50 calmodulin-like genes (McCormack et al., 2005). The estimated CaM concentration in the cytosol is between 5 and 40 μM (Zielinski, 1998). These and some other proteins contain at least one (and up to six) specific helix–loop–helix structural motifs termed as EF-hand, which can interact each with other and bind Ca^{2+} in a co-operative manner, resulting in a protein activation due to a relatively small Ca^{2+} change. Another large group of Ca^{2+} sensor proteins are calcineurin B-like proteins, bearing three EF hands (10 CBL genes in *Arabidopsis*, Luan et al., 2002) and Ca^{2+}-dependents protein kinases (CDPKs) with four EF hands (34 genes for CDPK in *Arabidopsis*, Cheng et al., 2002b). In Ca^{2+} sensors Ca^{2+} binding within EF-hand results in a relatively large scale conformational change, more pronounced than in a few "pure" Ca^{2+} buffering proteins such as calbindin and parvalbumin in animal cells.

The affinity and co-operativity of Ca^{2+} binding could vary greatly in different EF-proteins, and there are at least two classes of binding sites: one highly selective for Ca^{2+} against Mg^{2+}, and another with a lower and comparable affinity for Ca^{2+} and Mg^{2+} (Gifford et al., 2007). In plants, besides EF-hand proteins, there are several other cytosolic Ca^{2+} binding (and normally, also Ca^{2+}-regulated) proteins like phospholipase D and annexins (White and Broadley, 2003; Tuteja and Mahajan, 2007). Other Ca^{2+}-binding proteins have been recently discovered (Ide et al., 2007).

CALCIUM EFFLUX SYSTEMS

The most potent factor in shaping $[Ca^{2+}]_{cyt}$ signatures is the activity of Ca^{2+} efflux systems. These are of utmost importance in both keeping $[Ca^{2+}]_{cyt}$ at submicromolar level and in replenishing Ca^{2+} stores after $[Ca^{2+}]_{cyt}$ signaling is completed. There are two groups of Ca^{2+} efflux mechanisms, Ca^{2+}-ATPases and Ca^{2+} exchangers (CAX), both of which operate at the PM and endomembranes (**Figure 2**). Ca^{2+}-ATPases are high-affinity ($K_m = 0.1$–2 μM) but low-capacity transporters whereas Ca^{2+} exchangers are low-affinity ($K_m = 10$–15 μM) but high-capacity transporters. This suggest that (i) Ca^{2+}-ATPases may be primarily involved in termination of $[Ca^{2+}]_{cyt}$ signaling, whereas (ii) Ca^{2+} exchangers may be primarily involved in removal of $[Ca^{2+}]_{cyt}$ when $[Ca^{2+}]_{cyt}$ elevations are higher than normal (Sze et al., 2000). As a result, both Ca^{2+}-ATPases and CAX transporters contribute to shaping the $[Ca^{2+}]_{cyt}$ signal. Ca^{2+}-efflux transport mechanisms originated early on in biological evolution, and there is significant sequence conservation of these transporters in all forms of life (McAinsh and Pittman, 2009). This has facilitated the work to reveal the molecular identity of these systems. Significant knowledge exists about Ca^{2+}-ATPases and CAX transporters in terms of activation kinetics and regulation, expression pattern, cellular locations, and physiological functions (Sze et al., 2000; Pittman

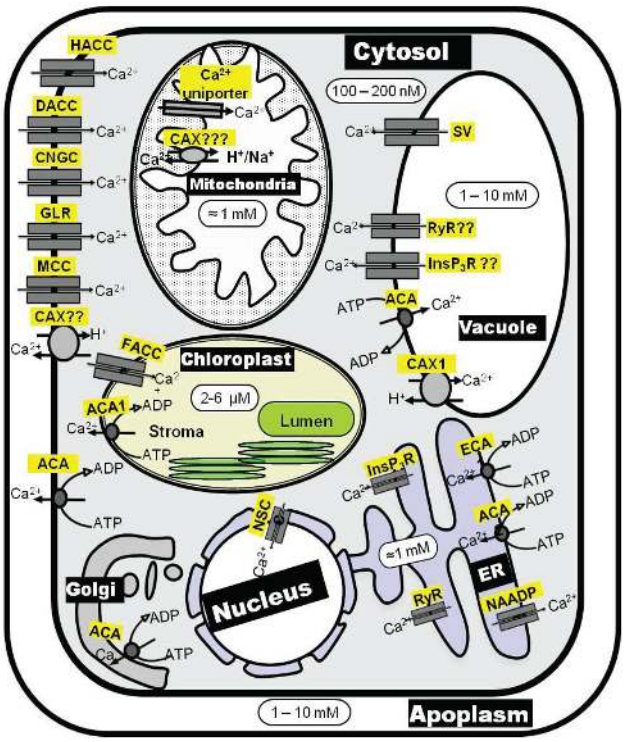

FIGURE 2 | Schematic diagram of Ca^{2+} transporters involved in Ca^{2+} homeostasis maintenance inside the plant cell. ACA, autoinhibited calcium ATPase; CAX, calcium exchanger; CNGC, cyclic nucleotide gated channel; DACC, depolarization activated cation channel; ECA, ER-type calcium ATPase; FACC, fast-activating cation channel; GLR, glutamate receptor-like channel; ACC, hyperpolarization activated cation channel; InsP3R, inositol 1,4,5-trisphosphate receptor-like channel; MCC, mechanosensitive cation channel; NSC, non-selective cation channel; RyR, cyclic ADP-ribose (cADPR)-activator ryanodine receptor-like channel; SV, slow-activating vacuolar channel.

and Hirschi, 2003; Shigaki and Hirschi, 2006; Boursiac and Harper, 2007). Despite this fact, the role of specific Ca^{2+} efflux systems has never been included in any $[Ca^{2+}]_{cyt}$ signaling model.

MOLECULAR IDENTITY OF CALCIUM EFFLUX SYSTEMS

CA^{2+}-ATPASES

Ca^{2+}-ATPases are energized directly by ATP and belong the super family of P-type ATPases, ion pumps that are ubiquitous in all life forms. A hallmark of P-type ATPases is that they form a phosphorylated reaction cycle intermediate during catalysis. Two types of Ca^{2+}-ATPases are known in plants: P_{2A}-ATPase [or ER-type Ca^{2+}-ATPase (ECA)] and P_{2B}-ATPase [or autoinhibited Ca^{2+}-ATPase (ACA); Geisler et al., 2000; Sze et al., 2000]. The structurally most distinctive difference between plant P2A- and P2B-ATPases is the extended N-terminus of P2B-ATPases, which serves a role as an autoinhibitor of pump activity and binds calmodulin. Four members of ECA (ECA 1–4) and 10 members of ACA (ACA1–10) have been identified in *Arabidopsis* (Sze et al., 2000).

ACAs can be present in the PM as well as in endomembranes whereas ECAs are exclusively localized to endomembranes. The cellular locations of ACAs and ECAs in *Arabidopsis* are depicted in **Figure 2**. (i) ACA8 (Bonza et al., 2000), ACA9 (Schiøtt et al., 2004) and ACA10 (George et al., 2008) reside at the PM, (ii) ACA4 (Geisler et al., 2000) and ACA11(Lee et al., 2007) at the tonoplast, (iii) ECA 1 (Liang et al., 1997) and ACA2 (Harper et al., 1998) at the ER, (iv) ECA 3 at the Golgi (Mills et al., 2008) and endosomes (Li et al., 2008), and (v) ACA1 at the plastid envelope (Huang et al., 1993). Apart from ACAs and ECAs, a P_1-ATPase (HMA1) has been implicated in acting as a Ca^{2+}/heavy metal pump at the chloroplast envelope (Moreno et al., 2008).

CA^{2+} EXCHANGERS

Ca^{2+} exchangers are energized by the counter transport of another cation, usually H^+ or Na^+. In *Arabidopsis*, six CAX genes (AtCAX1 to AtCAX6) that encode H^+/Ca^{2+} exchangers plus five CCX (cation/Ca^{2+} exchangers, previously described as AtCAX7 to AtCAX11) that encode K^+-dependent Na^+/Ca^{2+} exchangers, have been identified to date (Mäser et al., 2001; Shigaki et al., 2006). The function of CAX (CAX1 to CAX4) in the tonoplast is widely studied (Hirschi, 1999; Hirschi et al., 2000; Cheng et al., 2002a, 2003, 2005); CAX activity at the PM is also reported (Kasai and Muto, 1990; Luo et al., 2005).

REGULATION OF CALCIUM EFFLUX SYSTEMS ACTIVITY

CA^{2+}-ATPASE REGULATION

Ca^{2+}-ATPases are activated by submicromolar concentrations of Ca^{2+}. For this reason they are defined as high-affinity pumps (Møller et al., 2010). Due to tight coupling between ion binding and ATP hydrolysis, ATP hydrolysis will never take place if Ca^{2+} has not been bound in the membranous region of the Ca^{2+}-ATPase (Morth et al., 2011). Likewise, hydrolysis of ATP is always associated with transport of Ca^{2+}.

As $[Ca^{2+}]_{cyt}$ increases in response to environmental stress, Ca^{2+}-ATPases are immediately activated as a result of Ca^{2+} binding to their transport sites. P2A-ATPases have two Ca^{2+} binding sites in their membrane domain both of which have to be occupied before ATP hydrolysis can occur (Møller et al., 2010). In contrast, P2B-ATPases have a single membranous Ca^{2+} binding site (Brini and Carafoli, 2009) and therefore can proceed to ATP hydrolysis directly after Ca^{2+} binding. Further, in contrast to P2A Ca^{2+}-ATPases, P2B-ATPases are equipped with a Ca^{2+} sensor that allows the pump to change its activation state depending on $[Ca^{2+}]_{cyt}$ (see below). These combined features make P2B-ATPases optimal for responding to increased $[Ca^{2+}]_{cyt}$.

The sensor function of P2B Ca^{2+}-ATPases is achieved by the ability of a terminal autoinhibitory domain to bind calmodulin. A calmodulin binding site was first identified in the C-terminal domain of an animal P2B Ca^{2+}-ATPase (James et al., 1988) and later found in the N-terminal domain of a plant P2B Ca^{2+}-ATPase (Malmström et al., 1997). We now know that the N-terminal localization of an autoinhibitory calmodulin binding domain is a distinctive feature of plant P2B Ca^{2+}-ATPases (Geisler et al., 2000; Boursiac and Harper, 2007). The calmodulin protein binds four Ca^{2+} ions cooperatively and as a result changes from a loose to a compact conformation when binding to the Ca^{2+} pump (Ishida and Vogel, 2010). The calmodulin binding site of the N-terminus of P2B Ca^{2+}-ATPases is thought to interact with a cytoplasmic domain of the pump in this way restricting domain

movements and pump function (Luoni et al., 2004). The sequence of amino acid residues that serves as a calmodulin binding site also functions as a pump autoinhibitor (Baekgaard et al., 2006). This suggests that, as calmodulin binds Ca^{2+}, its affinity for the N-terminal calmodulin binding site increases and, when fully loaded with Ca^{2+}, calmodulin competes favorably with the intramolecular binding site for the autoinhibitor and, as the autoinhibitory sequence becomes neutralized by calmodulin with bound Ca^{2+}, the N-terminal autoinhibition is relieved. In addition to calmodulin, other cellular components such as acidic phospholipids might influence the autoinhibitory effect of the N-terminal domain (Bonza and De Michelis, 2011).

The presence of a regulatory terminal domain in P2B Ca^{2+}-ATPases is not a unique feature of these pumps. The plant PM H^+-ATPase has an extended C-terminal domain with two autoinhibitory sequences (Axelsen et al., 1999) and the PM heavy metal pump HMA4 has an extended C-terminal domain that serves as a Zn^{2+} and Cd^{2+} sensor (Baekgaard et al., 2010). Both these pumps belong to the P-type ATPase superfamily. How are P2A Ca^{2+}-ATPases with very short terminal domains then regulated? At least in animal cells, they interact with inhibitory subunits that are small membrane proteins (Palmgren and Nissen, 2011). Whether in a similar way plant P2A-ATPases are post-translationally regulated by associated subunits remains to be shown.

CAX REGULATION

Plant cation/H^+ exchangers, like plant Ca^{2+}-ATPases, appear to be primarily regulated at the post-translational level, although some form of transcriptional regulation may occur (Shigaki et al., 2010; Manohar et al., 2011). CAX1 may be regulated via an N-terminal autoinhibitory domain, which binds to an adjacent region within the N-terminus (Pittman et al., 2002; Mei et al., 2007). Other forms of regulation via (i) formation of a "hetero-CAX" complex through interaction between CAX1 and CAX3 (Zhao et al., 2009); (ii) phosphorylation (Pittman et al., 2002), or (iii) various CAX interacting proteins (CXIP) including CXIP4 and the Ser/Thr Kinase SOS2 (Cheng and Hirschi, 2003; Cheng et al., 2004a,b) and/or (iv) pH homeostasis (Zhao et al., 2008) have also been observed.

CONTROL OF CA^{2+} ATPASES BY POLYAMINES AND ROS

Our recent findings also suggest that PM Ca^{2+}-efflux systems may be regulated by synergistic effects of polyamines (PAs) and hydroxyl-radicals ($OH^•$). The levels of both PA and $OH^•$ are known to increase dramatically under stress conditions. Also, PAs block a variety of K^+ and non-selective cation channels in plants (Dobrovinskaya et al., 1999; Liu et al., 2000; Shabala et al., 2007; Zhao et al., 2007), whereas $OH^•$ and H_2O_2 activate different PM Ca^{2+} influx channels (Pei et al., 2000; Demidchik et al., 2003, 2007), thus affecting cytosolic ionic homeostasis. There is also a cross-talk between ROS and PAs, as several types of plant responses to environmental clues such as salt or drought involve PAs export to apoplast and further oxidation by available diamine- or polyamine-oxidase, resulting in H_2O_2 and $OH^•$ formation and activation of the Ca^{2+} influx across the PM (An et al., 2008; Moschou et al., 2008).

In our work, $OH^•$ (1 mM Cu/ascorbate) treatment evoked a long-lasting Ca^{2+} influx into pea roots due to $OH^•$ -induced non-selective passive conductance. However, at shorter times, transient Ca^{2+} efflux was measured to be sensitive to eosine yellow, a specific Ca^{2+} pump inhibitor (data not shown). Lowering the amount of $OH^•$ (0.1 mM Cu/ascorbate) shifted the balance between Ca^{2+} uptake and efflux toward net efflux (**Figure 3**), implying a lower threshold for the $OH^•$ -inducible Ca^{2+} efflux system as compared to the Ca^{2+} influx one. Addition of either 1 mM Spm^{4+} or Put^{2+} provoked a massive net Ca^{2+} efflux with very similar kinetics and magnitude (**Figure 3**). Integrating this flux over the period of 30 min and taking into account the root geometry and dimensions, we estimated that the total intracellular Ca^{2+} loss was equivalent to 0.2 mM for $OH^•$ treatment, and ∼0.6 mM for PAs. The latter value may be even in excess of the total cytosolic Ca^{2+}, implying the mobilization of the vacuolar pool.

To the best of our knowledge, PAs effects on Ca^{2+} pumps have never been reported. However, PAs are known to stimulate another P-type pump, H^+-ATPase, presumably via interaction of autoinhibitory domain protein with 14-3-3 proteins (Garufi et al., 2007). PAs may also activate the H^+-pump via an NO-dependent pathway (Tun et al., 2006; Arasimowicz-Jelonek et al., 2009; Zandonadi et al., 2010). Another interesting possibility is that this comes about due to formation of a complex of Mg^{2+}-ATP-spermine, which seems to present an increased rate for catalysis by ATPases as compared to Mg^{2+}-ATP (Meksuriyen et al., 1998). It may also be possible that PAs stimulate the PM H^+ pump, which indirectly affects/stimulates the Ca^{2+} pump in an H^+-coupled mechanism.

There is a large body of data on animal PM Ca^{2+}-pumps (PMCA), showing their *inhibition* by ROS resulting from protein cross-linking, lipid peroxidation, and concurrent inhibition by an oxidized form of calmodulin (Waring, 2005). However, these effects develop slowly (time scale of hours) and, thus, could not be responsible for the rapid induction of net Ca^{2+} efflux in our

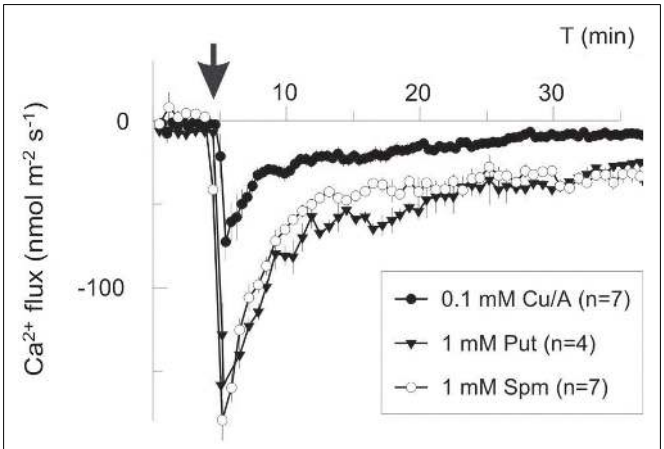

FIGURE 3 | Polyamines and ROS induce Ca^{2+} efflux in the mature zone of pea roots. Polyamines (1 mM of putrescine, Put, or spermine, Spm) and copper-ascorbate mixture to generate $OH^•$ (Cu/A, 0.1 mM) were added externally at the moment indicated by the arrow. Negative flux corresponds to Ca^{2+} efflux from the root to the bath. Data are mean ± SE, with a number of individual roots used as specified (*n*).

experiments. On the contrary, for plants a rapid activation of Ca^{2+}-ATPase by ROS via the CaM–Ca^{2+} binding complex, has been described (Romani et al., 2004). However, the observation of a net Ca^{2+} efflux at lower ROS generated levels (0.1 mM Cu/A; **Figure 3**) implies that the activation of Ca^{2+} pumping in this case is an early event, independent on the Ca^{2+}-pump activation via Ca^{2+}–CaM.

It should be also mentioned that PAs are readily catabolized in the apoplast, releasing H_2O_2, which could be further converted to $OH^{\bullet}$ by apoplastic peroxidases iron and/or diamine oxidases copper (Liszkay et al., 2004; Kukavica et al., 2009). A possible contribution of this mechanism should be validated in direct experiments.

INCORPORATING CALCIUM EFFLUX SYSTEMS INTO EXISTING SIGNALING MODELS

EXPERIMENTAL EVIDENCE

Although transient increases in $[Ca^{2+}]_{cyt}$ are essential for plant responses to a variety of environmental stimuli, long-lasting elevations in $[Ca^{2+}]_{cyt}$ are harmful for cells. Hence, the basal conditions must be restored back to resting level after the signal is completed, enabling cells to react to further signals (Sanders et al., 1999; Beffagna et al., 2005). In animal systems, active Ca^{2+} efflux systems have been widely implicated in a wide range of stress responses (e.g., Jornot et al., 1999; Zaidi and Michaelis, 1999). Much less is known about the involvement of Ca^{2+} efflux systems in stress responses in plant cells. We believe this is wrong, and argue that active Ca^{2+} efflux systems must be incorporated in all stress signaling models.

Biotic stresses

Intracellular calcium signaling is universally accepted as a key component of plant biotic stress defense mechanisms (Grant et al., 2000; Lam et al., 2001, Pike et al., 2005). Elicitor-induced elevations in $[Ca^{2+}]_{cyt}$ were reported during hypersensitive (HR) plant–pathogen interactions (Nurnberger et al., 1994; Blume et al., 2000; Lecourieux et al., 2002); these are believed to be essential for the development of the oxidative burst needed to trigger the activation of several plant defense reactions (Blumwald et al., 1998; Lecourieux et al., 2002). It was shown that Ca^{2+} channel blockers inhibit HR in many species (Atkinson et al., 1990; Levine et al., 1996), suggesting that PM-mediated calcium influx is required for HR initiation. As a result, most preceding reports were focused on the role of calcium cannels in HR induction (Atkinson et al., 1990; Levine et al., 1996, Jabs et al., 1997; Grant et al., 2000, Lam et al., 2001; Balagué et al., 2003, Pike et al., 2005; Hann and Rathjen, 2007). However, recent experiments in our laboratory have suggested that pathogen-induced Ca^{2+} influx occurs only at the first stages of pathogen–host interaction, during 0–7 h after the challenge (Nemchinov et al., 2008). Using *P. syringae*-inoculated tobacco plants we have shown that the initial calcium uptake is subsequently followed by the net calcium efflux initiated between 10 and 12 h and continued up to 48 h after the pathogen challenge. This efflux was inhibited by cyclopiazonic acid, a known inhibitor of Ca^{2+}-ATPase, suggesting that active Ca^{2+} efflux systems play an important role in HR. A new model of a multi-step HR process has been put forward (Nemchinov et al., 2008). According to this model, prolonged Ca^{2+} uptake, which continues to occur 1–7 h after the challenge, reflects the pathogen's successful overcoming the initial PAMP-triggered defense reaction, and sustained increases in $[Ca^{2+}]_{cyt}$ at this stage are necessary for generation of ROS, oxidative burst, and induction of HR (Atkinson et al., 1990; Grant et al., 2000). After HR transduction pathway has been initiated with the help of Ca^{2+} uptake, $[Ca^{2+}]_{cyt}$ levels sharply decline to mediate the last HR phase – an expanded cell death.

We have also recently demonstrated that Ca^{2+} efflux systems play a crucial role in the phenomenon of acquired cross-tolerance to oxidative stress in plants. *Nicotiana benthamiana* plants were infected with *Potato virus X* (PVX) and exposed to oxidative (either UV-C or H_2O_2) stress. It was shown that virus-infected plants had a better ability to control UV-induced elevations in $[Ca^{2+}]_{cyt}$ free Ca^{2+} and prevent structural and functional damage of chloroplasts (Shabala et al., 2011a), and that PM Ca^{2+} efflux systems play a critical role in this process. Several major lines of evidence support this conclusion: (1) significant net Ca^{2+} efflux was measured from UV-treated leaves 2 h after stress exposure. As passive Ca^{2+} leak from the cytosol is thermodynamically impossible, such efflux cannot be attributed to the general change in PM permeability and may be explained *only* by the activation of some Ca^{2+} efflux (active) system at the PM (e.g., either Ca^{2+}-ATPases or Ca^{2+}/H^+ exchanger); (2) PVX-inoculated leaves were actively pumping Ca^{2+} out (net efflux) while mock controls were still taking up Ca^{2+}; (3) PVX-inoculated cells have a much better capacity to activate PM Ca^{2+} efflux systems to deal with UV-induced elevation in $[Ca^{2+}]_{cyt}$ thereby preventing damage to chloroplast structure (Shabala et al., 2011a).

To separate the different types of active Ca^{2+} transport systems, a series of pharmacological experiments was conducted. Neither erythrosine B nor eosin yellow (EY), two known inhibitors of P_{2B}-type Ca^{2+}-ATPases, had a significant impact on the magnitude of net Ca^{2+} fluxes from tobacco mesophyll segments (Shabala et al., 2011a). Also unaffected was Ca^{2+}-ATPase activity in purified PM vesicles from tobacco leaves. This suggested that the PM Ca^{2+}-ATPases play a limited (if any) role in mediating Ca^{2+} efflux under oxidative stress conditions and suggested that the latter is mediated by PM Ca^{2+}/H^+ exchangers.

Not only plasma but also endomembrane Ca^{2+} efflux systems mediate the phenomenon of cross-protection in plants. Using biochemical and electrophysiological approaches, it was revealed that both endomembrane P_{2A} and P_{2B} Ca^{2+}-ATPases play significant roles in adaptive responses to oxidative stress by removing excessive Ca^{2+} from the cytosol, and that their functional expression is significantly altered in PVX-inoculated plants (Shabala et al., 2011b). Taken together, these findings highlight the crucial role of Ca^{2+} efflux systems in acquired tolerance to oxidative stress and open up prospects for practical applications in agriculture.

Abiotic stresses

The evidence for the importance of Ca^{2+}-ATPase in shaping $[Ca^{2+}]_{cyt}$ signatures came from two independent salinity studies. First, knocking out both *AtACA4* and *AtACA2* in *Saccharomyces cerevisiae* can increase NaCl sensitivity, but expression of *AtACA4* or *AtACA2* can provide tolerance to NaCl. Moreover, $[Ca^{2+}]_{cyt}$

elevations upon salinity stress are brought back to resting levels quickly, by expression of *AtACA2* in this yeast mutant (Anil et al., 2008). Secondly, in moss (*Physcomitrella patens*), salinity stress to a loss-of-function mutant of ACA type ATPase (PCA1), resulted in sustained $[Ca^{2+}]_{cyt}$ elevation and never returned to resting level (Qudeimat et al., 2008). ACAs may also e involved in $[Ca^{2+}]_{cyt}$ signal shaping in response to other stresses. For example, *AtACA8* was found to be unregulated, whereas *AtACA10* was found be down regulated in response to cold stress (Schiøtt and Palmgren, 2005).

Earlier Romani et al. (2004) showed that submicromolar concentrations of EY (a P_{2B}-type Ca^{2+}-ATPase inhibitor) prevented both the increase in Ca^{2+} efflux and the transient ROS accumulation in *Egeria densa* in response to ABA treatment. This result was explained by assuming an important role of PM Ca^{2+}-ATPase in switching off the signal triggering ROS production. Another report from the same group implicated PM Ca^{2+}-ATPase activation in plant adaptation to osmotic stress (Beffagna et al., 2005). Interestingly, knocking out *cax1* resulted in increased freezing tolerance (Catalá et al., 2003) but knocking out *cax3* resulted in an increased sensitivity to salinity (Zhao et al., 2008), suggesting that each stress targets a particular CAX transporter within the CAX family. Stress-induced $[Ca^{2+}]_{cyt}$ measurements involving *cax* knock out mutants may provide more insight into the specific role of each CAX transporter in shaping $[Ca^{2+}]_{cyt}$ signals.

THEORETICAL CONSIDERATIONS AND MODELING

The importance of Ca^{2+} efflux systems in shaping Ca^{2+} signatures is further investigated by modeling stimulus-induced changes in $[Ca^{2+}]_{cyt}$. As a starting point, we use the model of Stucki and Somogyi (1994), as adopted by Bauer et al. (1998). This model includes four major components: two Ca^{2+}-permeable channels (*A* and *C*), located respectively at the plasma – and endomembranes, and two active Ca^{2+} efflux systems: PM-based Ca^{2+}-ATPase pump (*B*) and endomembrane-based Ca^{2+}/H^+ exchanger (*D*; **Figure 4**). Unlike the Bauer et al. (1998) model, we assume no leakage from the cytosol. We also assume that activity of endomembrane Ca^{2+} channel is dependent on ROS accumulation in the cytosol; this is parametrized by factor *R*.

The amount *y* of Ca^{2+} in the cytosol, and *x* in the intracellular store is then given by (cf. Stucki and Somogyi, 1994):

$$\frac{dy_{\text{total}}}{dt} = A - (B+D)\,y_{\text{free}} + \left(C\frac{(y_{\text{free}}+R)^n}{K+(y_{\text{free}}+R)^n}\right)(x-y_{\text{free}})$$

$$\frac{dx}{dt} = Dy_{\text{free}} - \left(C\frac{(y_{\text{free}}+R)^n}{K+(y_{\text{free}}+R)^n}\right)(x-y_{\text{free}}) \qquad (1)$$

Here y_{total} denotes the total Ca^{2+} in the cytosol. This includes both bound and free calcium.

The mechanistic (biological) meaning of these equations is as follows. In Eq.(1), the rate of change of Ca^{2+} in the cytosol dy_{total}/dt is affected by both channels (*A* and *C*) and both active Ca^{2+}-efflux systems (*B* and *D*). The latter drive an efflux of Ca^{2+} from the cytosol (hence the minus sign), and this efflux is proportional to the concentration y_{free} of free cytosolic Ca^{2+}. Influx

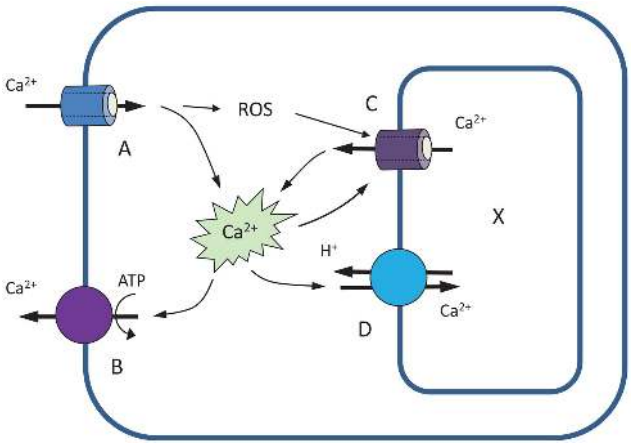

FIGURE 4 | A four-component model illustrating the role of Ca^{2+} efflux systems in shaping up Ca^{2+} signatures (based on Stucki and Somogyi, 1994; Bauer et al., 1998). See text for explanations.

of Ca^{2+} into the cytosol is driven by a concentration gradient between external calcium and the cytosol (assumed to be a constant *A*, since the external calcium is typically plentiful); and an efflux from the intracellular store, driven by the concentration gradient $(x-y_{\text{free}})$. The functional form for the multiplicative factor for *C* is given by the Hill equation, modified to include ROS accumulation (parametrized by *R*). Physically it corresponds to buffering-type kinetics.

In Eq.(2), the rate of change of intracellular store Ca^{2+} concentration, *dx/dt*, is then governed by an efflux due to channel *C* that is equal in magnitude but opposite in sign to the influx into the cytosol as described in Eq.(1); and an influx proportional to the free cytosolic Ca^{2+} concentration due to pump *D*.

The buffering capacity of the cytosol is described by Michaelis–Menten kinetics as

$$\frac{y_{\text{total}}}{y_{\text{free}}} = 1 + \left(\frac{B_{\max}}{y_{\text{free}}+K_{\text{d}}}\right) \qquad (2)$$

The left-hand side of Eq.(1) can then be expressed in terms of y_{free},

$$\frac{dy_{\text{free}}}{dt}\left(1+\frac{B_{\max}K_d}{(y_{\text{free}}+K_d)^2}\right) = A-(B+D)\,y_{\text{free}} + \left(C\frac{(y_{\text{free}}+R)^n}{K+(y_{\text{free}}+R)^n}\right)(x-y_{\text{free}}) \qquad (3)$$

and the expression for amount *x* in the intracellular store remains unchanged.

Model parameters

The equations above are scalable. *A* has dimensions of (amount/time); *B*, *C*, and *D* have dimension (1/time); *K* is in $[\text{amount}^{(1/n)}]$; $B_{\max}$, K_{d} and *R* are in (amount), just as *x* and *y*. This means that the scaled quantities can be related to physical

ones via the initial conditions, $x(t=0)$ and $y(t=0)$ which are given in moles.

The scaling comes from available experimental data for Ca^{2+} flux into the cell. Assuming net Ca^{2+} influx (through A) into the cell being ∼60 nmol/m^2/s (e.g., as in response to ROS treatment; Demidchik et al., 2002, 2007) and cell diameter of 30 μm, then the total amount of Ca^{2+} influx will be 10^{-6} mol/L, giving a physical flux $A_{phys} = 2$ μM/s. Hence, measuring concentrations in micrometer and time in seconds, we set for our default model $A = 2$. Assuming $[Ca^{2+}]_{cyt}$ equal 100 nM and Ca^{2+} concentration inside the internal organelles within 100–1000 μM range (Medvedev, 2005), we adopt $y(t=0) = 0.1$ and $x(t=0) = 160$.

As in Bauer et al. (1998) we assume $n = 4$, $K = 1$. To set the buffering parameters we use the physical values of $B_{max,phys} = 0.2$–0.5 mM (Trewavas, 1999) and $K_{d,phys} = 0.15$–0.6 μM (Martinez-Serrano et al., 1992; Kuratomi et al., 2003). This translates to adopted scaled quantities of $B_{max} = 200$–500 and $K_d = 0.15$–0.6. For the default model, we adopt $B_{max} = 250$ and $K_d = 0.6$.

The time scaling relates to constants A, B, C, and D. A is simply the uptake rate of Ca^{2+} from external media, and has dimensions of (amount/time); and units of [μM/s]. B, C, and D are essentially the inverse of decay-like constants, with dimensions of (1/time). This is easy to see from a simplified version of Equation 1, where each of these variables is described by a term like $(dy/dt) \propto By$, etc. The solution to such a differential equation is an exponential, $y \propto e^{Bt}$, and so $1/B$ is the time it takes for the amount of "stuff" to drop/increase by a factor of e (=2.718).

As discussed above, the time scaling of A is set by the physical parameter describing the influx into the cell (provided no other mechanisms were operating). In our default model $A = 2$, which corresponds to a flux of 2 μM/s. Setting A in this way means the values of B, C, and D are fixed by what "decay" constants are biologically meaningful.

Qualitative behavior

We explore the qualitative behavior of the model and contribution of activities of Ca^{2+}-ATPase pump and Ca^{2+}/H^+ exchanger to the kinetics of $[Ca^{2+}]_{cyt}$. This is done using larger timescales to reflect the oscillatory behavior of $[Ca^{2+}]_{cyt}$ changes. In doing this, we loosely follow the ratios between values adopted by Bauer et al. (1998) and assume the following set parameters: $A = 2$; $B = 20$; $C = 60$; $D = 36$; $R = 0.05$; $B_{max} = 250$; $K_d = 0.6$. We also give the initial conditions $y(t=0) = 0.1$; $x(t=0) = 160$. As explained below, this set of parameters is motivated by experimental results. We then vary each of the parameters B and D in turn, typically through a dynamic range of a few, around the "default" value. **Table 1** summarizes the various parameters and their typical values. Results are shown in **Figure 5** and described below.

Looking at timescales of a few minutes, increase in the activity of either the PM Ca^{2+}-ATPase pump B or the Ca^{2+}/H^+ exchanger D, results in a longer oscillation timescale, and sharper peaks in cytosol concentration (**Figures 5A,C**). Importantly, as one can see, the physiologically relevant set of parameters in our model reproduces oscillation timescales of a few minutes as reported by many experimental studies (reviewed in McAinsh and Hetherington, 1998; Ng and McAinsh, 2003; McAinsh and Pittman, 2009; Roelfsema and Hedrich, 2010).

Shorter-term behavior allows us to decouple the pump/exchanger contributions. The exchanger D only shifts the location of the first peak (**Figure 5D**), while the pump B both shifts the peak and changes the speed with which the cytosol concentration drops (**Figure 5B**) – the "decay constant" discussed in the previous section. Thus, pumps and exchangers appear to have a different role in shaping $[Ca^{2+}]_{cyt}$ signatures.

Table 1 | Model parameters.

Parameter	Description	Units	Default	Min	Max	Touch	Anoxia	Osmotic	Cold
x	Amount of Ca^{2+} in cytosol	μM							
y	Amount of Ca^{2+} in intracellular store	μM							
B	Plasma membrane-based Ca^{2+} pump	μM/s	20	10	30	20	2.5	8	13
D	Endomembrane-based Ca^{2+}/H^+ exchanger	μM/s	36	18	54	36	34	58	50
σ_B	SD in B for cell population	μM/s	0	0	5	0.1	1.7	1	3
σ_D	SD in D for cell population	μM/s	0	0	5	0.1	5	4	3
A	Ca^{2+} permeable channel (external → cytosol)	μM/s	2	Fixed					
C	Ca^{2+} permeable channel (intracellular store → cytosol)	μM/s	60	Fixed					
$x(0)$	Initial amount of Ca^{2+} in cytosol	μM	160	Fixed					
$y(0)$	Initial amount of Ca^{2+} in intracellular store	μM	0.1	Fixed					
R	ROS concentration	μM	0.05	Fixed					
K_d	Buffering parameter	μM	0.6	Fixed					
B_{max}	Buffering parameter	μM	250	Fixed					
K	Half maximal saturating Ca^{2+} concentration	$\mu M^{1/n}$	1	Fixed					
n	Hill coefficient	–	4	Fixed					

*The "default" model is the reference model. Parameters B, D, σ_B, and σ_D are changed in the range minimum – maximum and plotted in **Figures 5** and **6**. Best fit parameters for touch, cold, anoxia, and osmotic stress correspond to curves in **Figure 7**. All other model parameters are held constant in this work.*

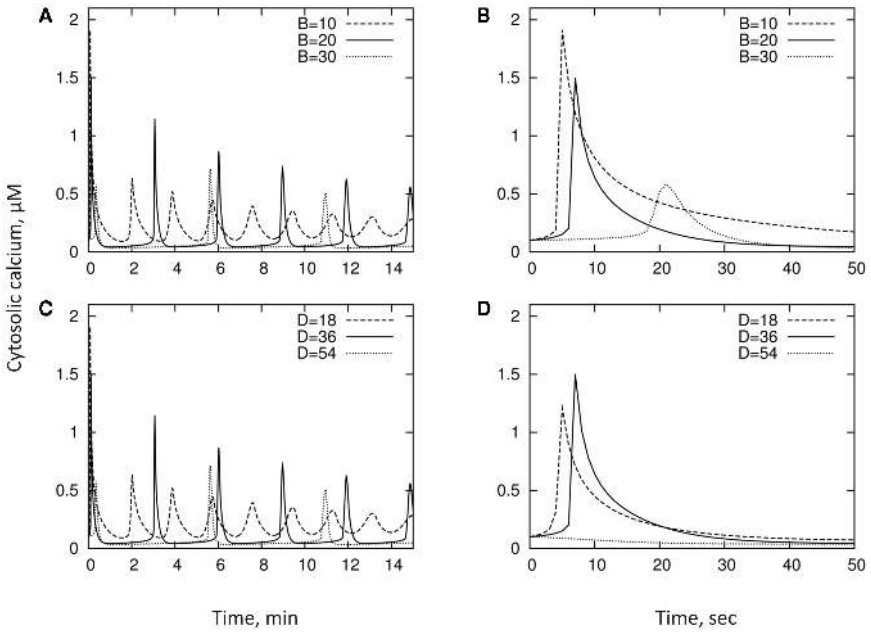

FIGURE 5 | Qualitative behavior of the model and contribution of activities of Ca^{2+}-ATPase pump and Ca^{2+}/H^+ exchanger to the kinetics of $[Ca^{2+}]_{cyt}$. The exchanger *D* [shown in panel **(C)** and zoomed in panel **(D)**] shifts the location of the first peak, while the pump *B* [shown in panel **(A)** and zoomed in panel **(B)**] alters both the peak location and the speed with which $[Ca^{2+}]_{cyt}$ drops.

Accounting for spatial heterogeneity

Stimulus-induced elevations in $[Ca^{2+}]_{cyt}$ usually show marked spatial heterogeneities, displaying both "hot-spots" and Ca^{2+}-quiescent regions (Gilroy et al., 1991; McAinsh et al., 1995; Ng and McAinsh, 2003). It was suggested that such spatial heterogeneity could result from either different accessibility of the primary stimulus to only a subset of the signaling machinery, or the non-uniform distribution of the intracellular signaling machinery (Ng and McAinsh, 2003). Regardless of the reason, the kinetics of $[Ca^{2+}]_{cyt}$ reported in the literature reflect a result of integration of these non-uniform $[Ca^{2+}]_{cyt}$ domains within the cell. Moreover, quite often the measured signal reflects the integrated response of many cells and, thus, may combine responses from several populations of cell types (e.g., epidermal and cortical cells in plant roots). It is logical to expect that these cells may have rather different activities of Ca^{2+} pumps and exchangers and, as a result, display different $[Ca^{2+}]_{cyt}$ kinetics in response to the same stimulus. As a result, the overall measured $[Ca^{2+}]_{cyt}$ signal may be quite different from the response of each individual cell. This is further illustrated in **Figure 6**, which depicts $[Ca^{2+}]_{cyt}$ kinetics from a population of $n = 100$ cells having a normal (Gaussian) distribution of parameters *B* and *D*.

Unsurprisingly, both lowering of the amplitude and broadening of the oscillations are observed. Moreover, the phase coherence between cells degrades with time, resulting in oscillations becoming more and more "smeared out." This is qualitatively consistent with results from the literature.

Fitting experimental data

As a final illustration of our model, we consider experimental data reporting changes in $[Ca^{2+}]_{cyt}$ in response to cold, osmotic stress, touch, and H_2O_2 in *Arabidopsis* seedlings, as per Logan and Knight (2003). In brief, changes in $[Ca^{2+}]_{cyt}$ were observed *in planta* using recombinant aequorin *Arabidopsis* plants. Cold, mannitol, or H_2O_2 treatments were effected by slowly (to prevent a touch response) injecting 0.5 mL of ice-cold water, 0.7 M mannitol, or 20 mM H_2O_2, respectively, into a cuvette containing an *Arabidopsis* seedling floating in 0.5 mL of water at room temperature. Touch treatment was effected by the rapid injection of 0.5 mL of room temperature water into the cuvette. The original data reported in that paper was digitized and is shown in respective panels in **Figure 7**.

As one can see, experimental data (unconnected symbols) can be adequately approximated by the model fits (continuous lines; **Figure 7**; also summarized in **Table 1**). Importantly, this is achieved by using a realistic (i.e., physiologically relevant) set of initial characteristics (see above), validating the adequacy of the model. Even more importantly, it appears that specific $[Ca^{2+}]_{cyt}$ signatures observed in response to four different types of stress (cold, osmotic stress, touch, and H_2O_2 treatments) can be adequately achieved by modifying just the distributions of parameters *B* and *D*, i.e., properties of PM-based Ca^{2+}-ATPase pump and endomembrane-based Ca^{2+}/H^+ exchanger, respectively.

As such, we use the best fit to touch stress as the reference model. The difference between plant responses to cold and touch may be explained by a 1.5-fold increase in parameter *D*, a similar decrease in *B*, and a broadening of their distributions (compared to single-cell responses) by 23 and 6% of the mean, respectively. Much slower rise in $[Ca^{2+}]_{cyt}$ in response to osmotic stress may be explained by still lower values of *B* (with only a minimal change in *D*), while both sustained elevation and reduced peak

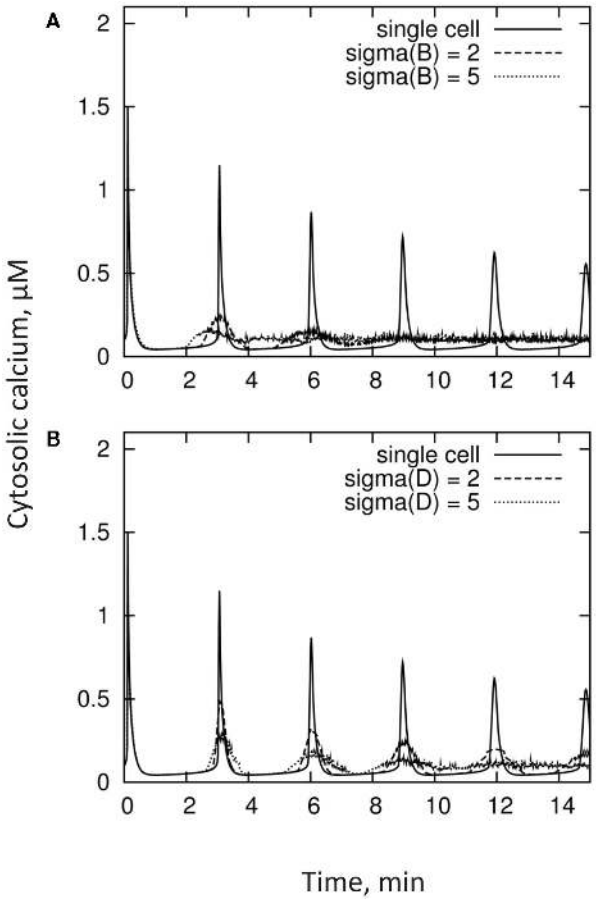

FIGURE 6 | Kinetics of $[Ca^{2+}]_{cyt}$ measured from a population of $n = 100$ cells having a Gaussian (normal) distribution of parameters *B* panel (A) and *D* panel (B), as compared with individual cell responses. Distributions in these parameters broaden the first $[Ca^{2+}]_{cyt}$ peak, and result in substantial decorrelation at subsequent maxima.

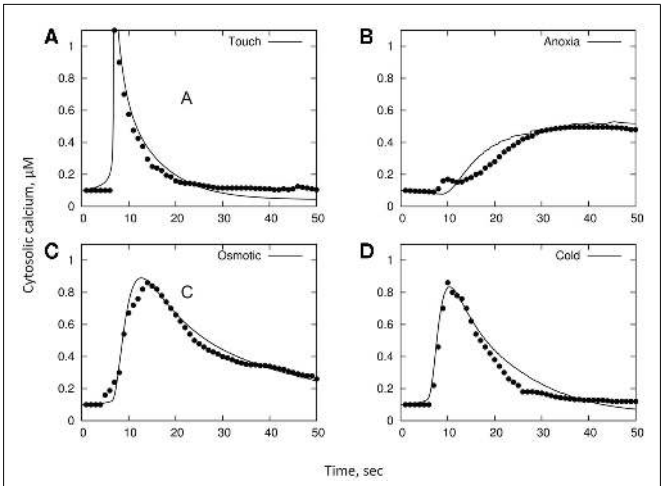

FIGURE 7 | A qualitative comparison between stress-induced $[Ca^{2+}]_{cyt}$ signatures and model simulation. Unconnected symbols are experimental observations (as per Logan and Knight, 2003); lines are model fits. The basic parameters in all models are: $A = 2$; $C = 60$; $R = 0.05$; $B_{max} = 250$; $K_d = 0.6$; $y(t = 0) = 0.1$; $x(t = 0) = 160$. Only distributions in *B* and *D* are varied between treatments. The remaining parameters are: for cold panel **(D)**, $B = 13$, $\sigma_B = 3$, $D = 50$, $\sigma_D = 3$; for osmotic panel **(C)**, $B = 8$, $\sigma_B = 1$, $D = 58$, $\sigma_D = 4$; for touch panel **(A)**, $B = 20$, $\sigma_B = 0.1$, $D = 36$, $\sigma_D = 0.1$; for H_2O_2 panel **(B)**, $B = 2.5$, $\sigma_B = 1.7$, $D = 74$, $\sigma_D = 5$.

$[Ca^{2+}]_{cyt}$ values in response to H_2O_2 treatment are given by a further decrease in *B* and (slightly) higher *D* values.

The dynamic range spanned by *D* in these models is only 0.3 dex (i.e., a factor of 2); and the width of the normal distribution relative to the mean is $\sigma_D/D = 0$–0.07. The dynamic range for *B* is 0.9 dex (factor of 8), and $\sigma_D/D = 0$–0.65. Both these changes are within the physiological range of changes expected under stress conditions. Indeed, the efficiency of ATP production drops 19-fold (from 38 to only two ATP molecules; Gibbs and Greenway, 2003) under anoxic conditions; this is twice as wide as the dynamic range for *B* in the models. Importantly, oxygen profiles in the root differ dramatically between epidermal, cortical, and stellar tissues (Armstrong et al., 1994), even under normoxic conditions. Thus, differential Ca^{2+}-ATPase activity is *expected* between these tissues. This special heterogeneity will confer a broad dynamic range for *B* and may explain the presence of the second peak in $[Ca^{2+}]_{cyt}$ observed 30–40 min after anoxia onset (**Figure 1C**).

CONCLUSION AND PROSPECTS

Restoration of the basal $[Ca^{2+}]_{cyt}$ levels is essential for removing excess Ca^{2+} from the cytosol, to reload Ca^{2+} stores and to terminate Ca^{2+} signaling. (As shown in this paper, it is impossible to achieve this without having efficient Ca^{2+} efflux mechanisms in place). It also appears that physiologically relevant variations in the activity of Ca^{2+}-ATPase pumps and Ca^{2+}/H^+ exchangers are sufficient to fully describe all the reported experimental evidence and determine the shape of $[Ca^{2+}]_{cyt}$ signatures in response to environmental stimuli. This emphases the crucial role these active efflux systems play in plant adaptive responses to environment and suggests that more attention has to be given to elucidation of the spatio-temporal properties and control modes of Ca^{2+}-ATPase pumps and Ca^{2+}/H^+ exchangers in plants.

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