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Interaction and Crosstalk Between Calcium and Redox Signaling Events in Plants

Ashish Kumar Srivastava* • **Penna Suprasanna** • **Stanislaus Francis D'Souza**

Nuclear Agriculture and Biotechnology Division, Bhabha Atomic Research Centre, Mumbai 400085, India

Corresponding author: * **ashishbarc@gmail.com; ashish@barc.gov.in**

ABSTRACT

In response to any stimulus, various cellular responses are triggered among, which the most rapid responses include the induction of calcium and reactive oxygen species (ROS) transients. The induction of calcium transient is due to the concerted action of calcium dependent channels, pumps, and carriers situated in the plasma membrane and different sub-cellular compartments. The spatial and temporal nature of the calcium transient is defined as cellular "Ca^{2+} signature" and is responsible for the activation of stimulus-specific calcium sensor and decoder elements. The redox state of the cell under any condition is defined as the integrative ratio of reduced to oxidized form of redox couples present inside the cell. The induction of calcium transient is coherent with the significantly higher level of ROS, which shifts the redox status of the cell to a more oxidized state. This change occurs in a dose dependent manner and is sensed in calcium signaling dependent manner. The complex and coordinated interaction of calcium and redox events is responsible for the generation of stimulus-specific response. The present article deals with the overview of calcium and redox signaling events and their possible crosstalk to regulate different plant functions under normal and stressful environment.

Keywords: ABA, $[Ca^{2+}]_{cyt}$-transients, calcium sensor/effector elements, crosstalk, redox and ROS

CONTENTS

INTRODUCTION

Unlike animals, plants being a sessile organism cannot escape from the surroundings and have to adapt themselves to the changing environments by a series of molecular responses. The physiological basis for these molecular responses is the integration of various signal transduction events into a comprehensive network that finally leads to the activation of stimulus-specific response. The calcium and redox dependent changes are considered as central players in this network. In response to any external stimulus, one of the early responses is the change in calcium signal in the form of transient increase in cytosolic free Ca^{2+} ($[Ca^{2+}]_{cyt}$), which arise because of the flux of Ca^{2+} into the cytosol, either from the external medium or from sub cellular compartments, where the concentration of Ca^{2+} is higher as compared to that of cytosol. The increase in $[Ca^{2+}]_{cyt}$ concentration led Webb *et al.* (1996) to formulate the concept of "Ca^{2+} signatures", which is defined as the repetitive oscillations or spiking of $[Ca^{2+}]_{cyt}$. The frequency (period), amplitude and shape (e.g. sinusoidal, square-wave) of Ca^{2+} signature are determined by the nature and magnitude of the stimulus. It is thought that stimulus specific temporal changes in $[Ca^{2+}]_{cyt}$ enable the Ca^{2+} ion to encode stimulus-specific information within this so-called calcium signature/transient (Dodd *et al.* 2010). An additional level of regulation and specificity is achieved by a set of calcium binding toolkit, which includes the Ca^{2+}-binding proteins functioning as Ca^{2+} signal sensors (CBLs, calcineurin B-like proteins) and effectors (CIPKs, CBL-interacting protein kinases) that together relay the information encoded within calcium signatures.

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Invited Review

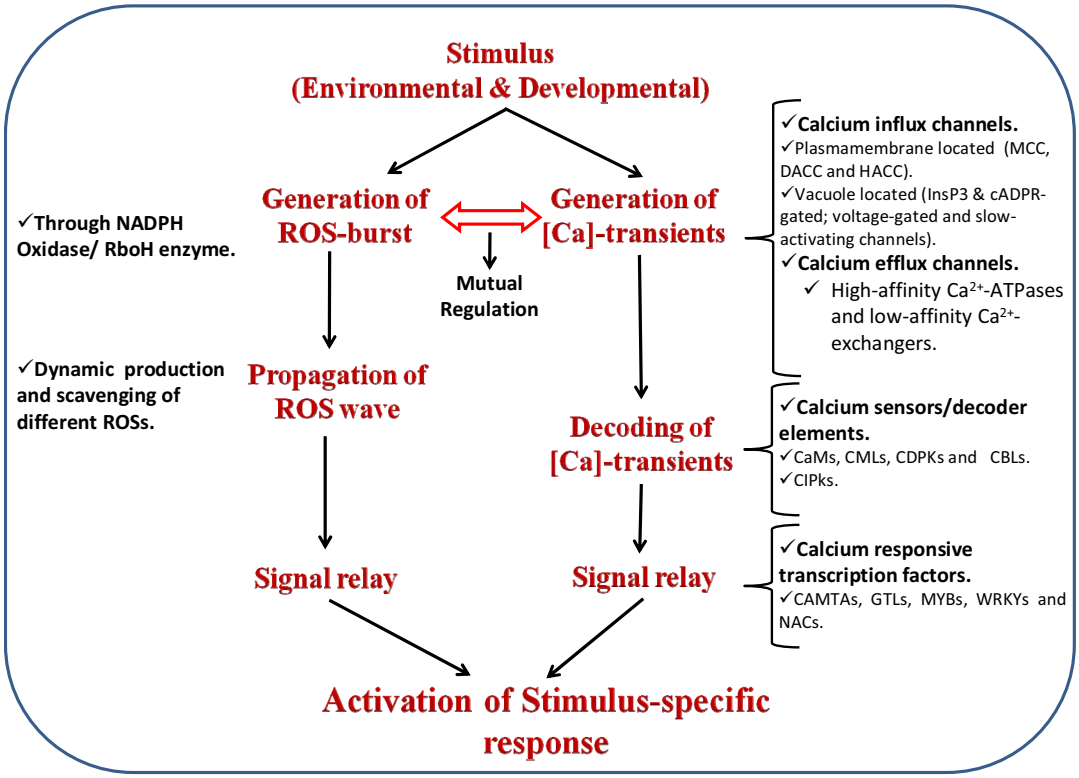

Fig. 1 Overview of calcium and redox signaling response generated in response to various environmental and developmental stimuli. In response to different stimuli, the earliest responses are the induction of calcium- and ROS-transients, which activates the calcium and redox signaling, respectively. Both these signaling mechanisms are under the tight control of each other and their mutual interaction and cross talk is responsible for the induction of stimulus-specific response.

The redox homeostasis is another important process and redox signals play an important role in regulating the plant responses towards different stresses. The redox state of the cell is governed by the level of individual ROS, ROS-producing enzymes, antioxidants, their oxidized forms, and/or oxidation/reduction states (Potters *et al.* 2010). Any external stimulus causing oxidative stress shifts the cellular redox status to a more oxidized state. Such a change in the cellular redox state acts as a cellular messenger that in conjunction with the mediators such as calcium transmits the signal to the nucleus for the activation of the stimulus specific response (Foyer and Noctor 2005). Thus, the coordinated induction of calcium and redox signaling mechanisms is an important step required for the perception and necessary action of plants towards various external stimuli.

In animals, the interaction between the Ca^{2+} and ROS signaling is well documented and it is suggested that any agent, which can cause cellular oxidative stress may results into the abnormal calcium signals that can finally lead to a variety of diseases (Hidalgo and Donoso 2008; Bogeski *et al.* 2011). In plants, although sufficient progress has been made towards understanding of redox (Mittler *et al.* 2011) and calcium signaling (Kudla *et al.* 2010), not much information is available on the their possible interaction and crosstalk. In the present review, we have first briefly described the calcium and redox signaling pathways and then discussed their crosstalk in terms of plant function under normal and stress conditions.

CALCIUM SIGNALING SYSTEM IN PLANTS

All organisms, including plants, use a network of signal transduction pathways to cope with their environment, to control their metabolism and to realize their developmental programs. Calcium has emerged as ubiquitous secondary messenger involved in many of these processes because of its flexibility to exhibit different coordination numbers and complex geometries (Gilroy and Trewavas 2001). The fact that Ca^{2+} serves as a messenger and regulator in so many different processes raises a fundamental question of how specificity in information processing and output determination is achieved (Dodd *et al.* 2010). In this regard, the first clue comes from the stimulus-specific Ca^{2+}-signature, which signifies the spatial and temporal changes in the cytosolic Ca^{2+} level. The second level of specificity comes from the specific set of calcium sensor/effector elements responsible to relay the information into nucleus so that the actual response can be activated. In order to understand the coding system of calcium signature, single-cell systems (guard cells, growing pollen tubes, or root hairs) represent excellent models. However, an entirely different level of complexity may be imposed when a single cell differentiates into mature organs. Since, distinct tissues and organs mainly manifest final response of the plant to external stimuli therefore, research on plant Ca^{2+}-signaling has although taken advantage of single-cell model systems but in parallel moves forward to elucidate Ca^{2+} dynamics in the tissue context and in the whole organism. In the present context, we have reviewed the determinants responsible for shaping calcium signature and about decoding and relay system of calcium signaling (**Fig. 1**).

Major determinants of calcium signature

At the organ level, the induction of the calcium-transient is mainly in the form of single spike. A representative profile of calcium signature in root under NaCl stress is depicted in **Fig. 2A**. The induction of stimulus-specific $[Ca^{2+}]$-signature is dependent on the Ca^{2+} influx channels at the plasma membrane and endomembrane system (both mediate Ca^{2+} release into the cytosol) and Ca^{2+} efflux transporters (responsible for the removal of Ca^{2+} from cytosol). Although, the complete range of Ca^{2+} influx channels and efflux transporters have been extensively reviewed (McAinsh and Pittman 2009; Kudla *et al.* 2010), however, a specific-role of each components of Ca^{2+} regulatory machinery in shaping plant Ca^{2+} signatures is not well defined.

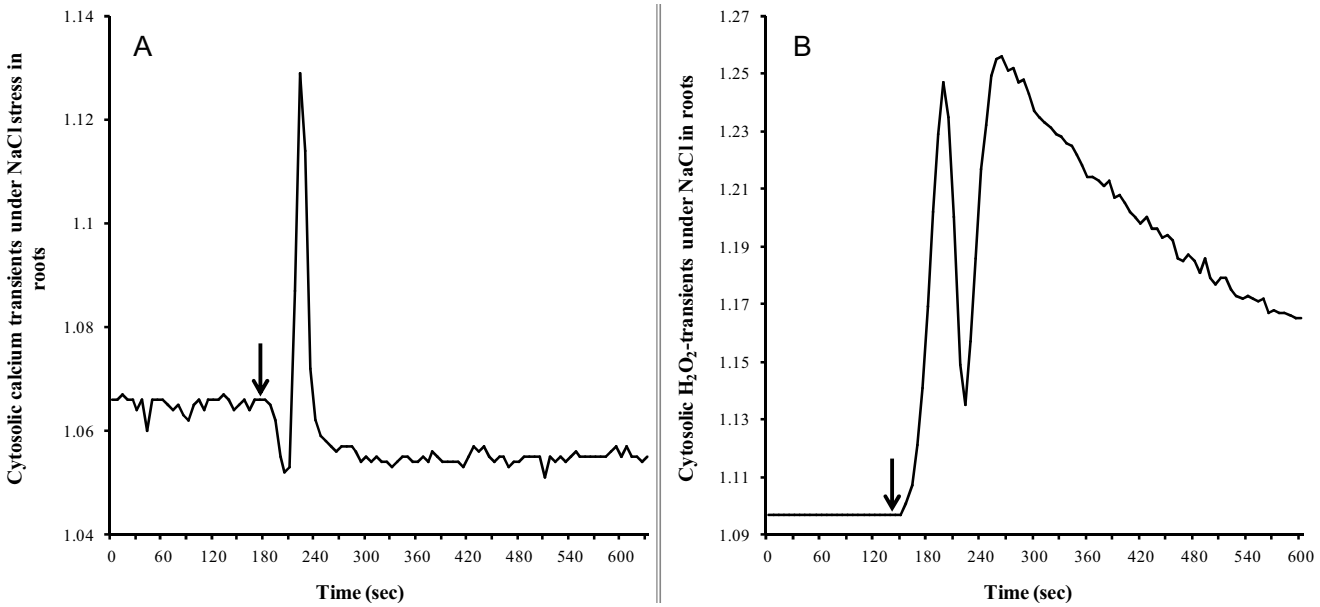

Fig. 2 Overview of NaCl-specific calcium- and ROS-signature in roots of *Arabidopsis* seedlings. The panel A and B represents the typical profile of Ca^{2+}- and ROS transient, respectively in roots subjected to NaCl stress. The down arrow represents the exact time point for the application of NaCl stress. The measurement of Ca^{2+}- and ROS transient was performed using YC 3.6 and Hyper transformed *Arabidopsis* seedlings (unpublished data). Both the signatures can be explained in the terms of their horizontal and vertical components which denote the duration and strength of the response, respectively.

Ca^{2+} influx channels

Different Ca^{2+} channels/transporters can be either classified on the basis of their activation mechanism as voltage-, ligand- and stretch- activated or on the basis of their location as plasma membrane- or endo-membrane localized. By electrophysiological analysis, three distinct groups of Ca^{2+}-permeable channels have been characterized on the plasma membrane of plant cells. These are the mechano-sensitive Ca^{2+} channel (MCC), the depolarization-activated Ca^{2+} channel (DACC) and the hyperpolarization-activated Ca^{2+} channel (HACC). All these three are together termed as nonselective cation channel (NSCC). Both DACC and HACC are termed as voltage-dependent channels (Demidchik and Maathuis 2007). The MCC have been recorded in various cell types and are responsible for shaping mechanically induced $[Ca^{2+}]_{cyt}$-signature. However, despite the identification of 10 MCC-like genes in Arabidopsis genome, limited information is available on the specific functions of this channel type. The DACC is activated in response to stress-induced depolarization and contribute to the short transient influx of Ca^{2+}. In contrast, HACC contributes to a sustained Ca^{2+} influx. In *Arabidopsis*, the HACC activity is localized in the apical region and is down-regulated in sub-apical regions of growing root hairs and at the tips of mature hairs, suggesting its role in generating the root hair apical $[Ca^{2+}]_{cyt}$ gradient (Very and Davies 2000). In contrast to plasma membrane channels, the electrophysiological characterization of Ca^{2+} channels in the endomembrane is not possible in intact cells, imposing additional challenges when assigning the role of channel for the generation of specific Ca^{2+} signatures. Apart from plasmamembrane, at least four Ca^{2+}-permeable channels have been identified at the vacuolar membrane such as inositol 1,4,5-trisphosphate (InsP3)- and cyclic ADP-ribose (cADPR)-gated channels, voltage-gated Ca^{2+} (VVCa) and slow-activating vacuolar (SV) channels (Pottosin and Schonknecht 2007). Whether InsP3- and cADPR-gated Ca^{2+}-permeable channels reside solely in the vacuolar membrane or more widely distributed at the endoplasmic reticulum (ER) remains to be established. In *Arabidopsis*, SV channel is encoded by the AtTPC1 (two-pore channel 1) gene. The demonstration that AtTPC1 encodes the Arabidopsis SV channel has permitted the first functional analysis of endomembrane Ca^{2+}-permeable channel involvement in the generation of plant Ca^{2+} signatures at the molecular level (Peiter *et al.* 2005). In addition, Ca^{2+} release from the vacuole and ER in response to inositol hexakisphosphate (InsP6) and nicotinic acid adenine dinucleotide phosphate (NAADP), respectively also highlights the role of ligand-gated endomembrane Ca^{2+}-permeable channels in shaping the Ca^{2+} signatures.

Ca^{2+} efflux channels

Calcium is an essential nutrient, yet in all organisms Ca^{2+} is extremely toxic when present at high concentrations in the cytosol. This is because it readily forms insoluble complexes with ATP and makes the cell energy deficient. Thus transport mechanisms to rapidly remove Ca^{2+} from the cytosol developed early during evolution (Case *et al.* 2007). In addition, the Ca^{2+} efflux transporters are also important in generating Ca^{2+} signature. Plants have two main pathways for $[Ca^{2+}]_{cyt}$ removal for instance high-affinity Ca^{2+}-ATPases and low-affinity Ca^{2+}-exchangers. The high-affinity Ca^{2+} efflux ATPase is basically a subgroup of P-type ATPase (the P2-ATPase) (Baxter *et al.* 2003). These are further divided into P2A-ATPases, which include sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) in animals, and the ER-type Ca^{2+}-ATPase (ECA) in plants, and P2B-ATPases, including the animal CaM-regulated plasma membrane Ca^{2+}-ATPase (PMCA) and the autoinhibited Ca^{2+}-ATPase (ACA) in plants. The low-affinity Ca^{2+} exchangers are energized by the counter exchange of another ion. These transporters are usually of lower Ca^{2+} affinity than the Ca^{2+} pumps but transport Ca^{2+} from the cytosol rapidly and at high capacity. In animal cells, Ca^{2+} efflux by Na^+/Ca^{2+} exchangers is coupled to Na^+ flux, while plants possess a structurally related family of cation exchanger (CAX) genes that encode H^+/Ca^{2+} exchangers (Shigaki and Hirschi 2006). Arabidopsis has six CAX genes plus five related genes, designated cation/Ca^{2+} exchanger (CCX) that are more similar to an animal Na^+/Ca^{2+} exchanger isoform (Shigaki *et al.* 2006). H^+/Ca^{2+} exchange activity has long been known to be a major route for Ca^{2+} removal from the cytosol into the vacuole (Schumaker and Sze 1985; Blumwald and Poole 1986), although exchange activity has also been detected at the plasma membrane (Kasai and Muto, 1990). CAX genes encoding tonoplast H^+/Ca^{2+} exchangers have been subsequently identified from various plant species and have predominant roles in maintaining low

$[Ca^{2+}]_{cyt}$ (Mei *et al.* 2007).

Decoding and relay of Ca^{2+} Signals

The cytosolic Ca^{2+} changes are perceived by a large number of proteins termed as Ca^{2+}-sensors. A majority of such sensors possess classical helix-loop-helix EF-hand motif responsible for Ca^{2+}-dependent conformational change. Upon binding with Ca^{2+}, these sensor proteins can either act as an activator (sensor relays) or else may bind with another group of proteins to activate the downstream signaling (sensor transducer). The entire group of calcium sensor proteins includes calmodulin (CaMs), calmodulin-like proteins (CMLs), Ca^{2+}-dependent protein kinase (CDPKs) and the calcineurin B-like proteins (CBLs). In CDPKs, the Ca^{2+} sensing (EF-motifs) and a responding function (protein kinase activity) is combined within a single protein and hence termed as sensor responders. In contrast, CaMs/CMLs have no enzymatic function and they alter the downstream target activities via Ca^{2+}-dependent protein-protein interaction. Therefore, they represent the *bonafide* sensor relay proteins. CBLs also belong to sensor relay proteins due to the lack of any enzymatic activity. However, CBLs specifically interact with a family of protein kinases designated as CBL-interacting protein kinases (CIPKs). Consequently, CBL-CIPK complexes could be considered as bimolecular sensor responders. In Arabidopsis, a total 10 CBLs and 26 CIPKs are reported and substantial progress has been made to understand the signaling between SOS3 (CBL4) and SOS2 (CIPK24), which is responsible for mediating the salt-stress response (Qiu *et al.* 2002). In response to salt, the $[Ca^{2+}]_{cyt}$ is increased, which is perceived by SOS3 (CBL-4; a Ca^{2+} sensor). The SOS3 protein interacts with SOS2 (CIPK-24; a Ca^{2+} effector) protein kinase and SOS3-SOS2 complex then activates the SOS1 protein (a plasma membrane Na^+/H^+ antiporter) and thereby re-establishes the Na^+ homeostasis in cells. The activated calcium sensor/effector elements finally manifest their effect through a group of transcription factors such as CaM-binding transcription factors (CAMTAs), GT-element-binding proteins (GTLs), MYBs, WRKYs and NACs.

REDOX OR ROS-DEPENDENT SIGNALING IN PLANTS

Like calcium, it is now widely accepted that redox signals are the central regulators of plant metabolism, morphology and development. The redox state of the cell is governed by the level of individual ROS, ROS-producing enzymes, antioxidants, their oxidized forms, and/or oxidation/reduction states (Potters *et al.* 2010). Any external stimulus, which causes the formation of ROS shifts the cellular redox status to more oxidized state. Such a change in the cellular redox state acts as a cellular messenger that in conjunction with mediators such as calcium transmits the signal to the nucleus for the activation of a stimulus specific response coupling (Foyer and Noctor 2005). Thus, the signaling dependent upon ROS formation is defined as "Redox Signaling" (**Fig. 1**).

The redox signaling offers several possible advantages to plants. The capacity of plants to produce different forms of ROS facilitates them to perceive diverse signals simultaneously. The restricted movement of ROS helps them to perform different functions in various sub-cellular compartments. For example, superoxide cannot passively transfer across the membrane. However, if it gets converted to hydrogen peroxide (H_2O_2) then it can readily transfers across membrane either passively or through water channels. Superoxide and H_2O_2 can also mediate the formation of lipid peroxides that would be membrane soluble. Another possible advantage of ROS signaling is that they could be used as rapid long distance auto-propagating signals transferred throughout the plant. Each individual cell along the path of the signal could activate its own ROS producing mechanism(s) in an autonomous manner carrying a ROS signal over long distances. Thus, ROS have the advantage of being versatile signaling molecules with regard to their properties and mobility within cells (Mittler *et al.* 2011).

ROS formation in plants

A baseline concentration of ROS is always present in plants. It has been estimated that approximately 2% of the O_2 consumed by plants is sidetracked to produce ROS in various subcellular compartments (Bhattachrjee 2005). The O_2 molecule naturally exists as a diradical, as it has two unpaired electrons that have the same spin quantum number (triplet state). This spin restriction makes O_2 a preferred molecule to accept electrons leading to the generation of ROS. The process of generation of ROS as a reduction product of O_2 is depicted in **Fig. 3** (De Gara *et al.* 2010). The single electron reduction of O_2 results in the generation of the $O_2^{\bullet-}$. The dismutation of $O_2^{\bullet-}$ is unavoidable and results into the formation of H_2O_2. Furthermore, $O_2^{\bullet-}$ can also be protonated to form the $HO_2^{\bullet}$ (perhydroxyl radical). Additionally, in the presence of transition metals, such as copper and iron, further reactions take place, e.g. Haber-Weiss or Fenton-type reactions, giving rise to OH^- and $OH^{\bullet}$, which are the most reactive chemical species in the biological world. $^1O_2$ is another form of ROS in which an electron is elevated to a higher energy orbital, thereby freeing O_2 from its spin-restricted state. $^1O_2$ can be formed by photoexcitation of chlorophyll and its reaction with O_2.

The dynamics of ROS signaling

The dynamic production and scavenging of ROS causes rapid alteration in their levels. This generates a signal, which is mainly in the form of two distinguished peaks (Nishimura and Dangl 2010). A representative profile ROS signature in root under NaCl stress is depicted in Fig. 2-B. Recent researches using advanced imaging tools (such as plants expressing HyPer or luciferase system) revealed that the initial burst of ROS production could trigger a cascade of cell-to-cell communication events that result in the formation of a ROS wave, which propagates throughout the plant. Thus, the initial concept of "ROS burst" is now modified into a spatio-temporal concept of "ROS wave". The propagation of ROS wave can be explained by the continuous production/scavenging of ROS along its path. In a plant cell, the production of ROS takes place mainly in chloroplast, mitochondria and peroxisomes. The misdirection of electron from chloroplast PS-I to O_2 generates $O_2^{\bullet-}$. A membrane bound copper/zinc superoxide dismutase (Cu/Zn SOD) in the vicinity of PS-I converts $O_2^{\bullet-}$ to produce H_2O_2. The singlet oxygen ($^1O_2$) is also generated at PS-II by excited triplet-state chlorophyll at the P_{680} reaction center and in the light-harvesting complex when the ETC is over-reduced (Asada 2006). In mitochondria, complex I and complex III in the respiratory ETC are the major sites of ROS production (Moller *et al.* 2007). Ubisemiquinone intermediate formed at complex I and III donates electrons to O_2 and generates $O_2^{\bullet-}$ that is, in turn, reduced to H_2O_2 (Raha and Robinson 2000; Rhoads *et al.* 2006). Peroxisomes produce H_2O_2 and $O_2^{\bullet-}$ at high rates through several metabolic processes. Oxidation of glycolate by glycolate oxidase in peroxisomes accounts for the majority of H_2O_2 production during photorespiration (Noctor *et al.* 2002). The scavenging mechanism of ROS includes low-molecular weight non-enzymatic [ascorbate (ASC) and glutathione (GSH)] and enzymatic [superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), ascorbate peroxidase (APX; EC 1.11.1.11)] antioxidant system (Miller *et al.* 2009). Superoxide dismutase is the major scavenger of superoxide radicals ($O_2^{\bullet-}$) and its enzymatic action results in the formation of hydrogen peroxide (H_2O_2), which is then regulated by CAT and various classes of peroxidases. The detoxification of H_2O_2 by APX in ASC-GSH cycle utilizes ASC as an electron donor, which gets oxidized to dehydroascorbate (DHA) in the process. The regeneration of DHA

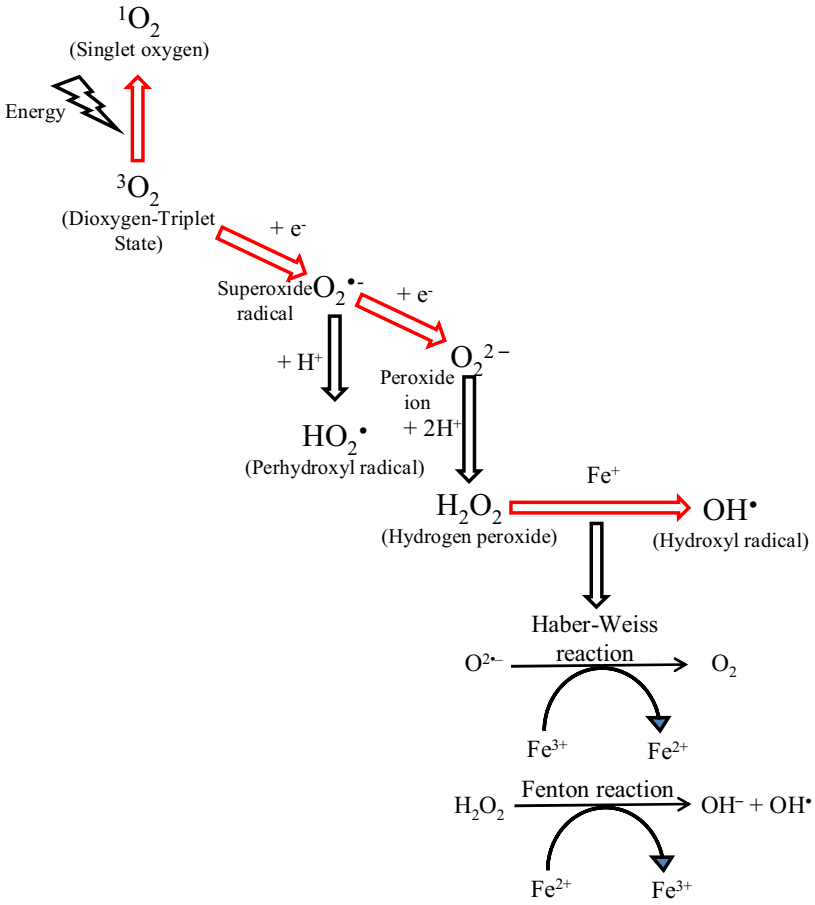

Fig. 3 Different kinds of ROS produced in plants. Different types of ROSs such as triplet oxygen, superoxide radical and peroxides are produced as a reduction product of oxygen.

to ASC is performed by monodehydroascorbate reductase (MDHAR; EC 1.6.5.4) and dehydroascorbate reductase (DHAR; EC 1.8.5.1), utilizing reduced glutathione (GSH) as a reductant, which, in turn, gets converted to oxidized glutathione (GSSG). The final reaction of the cycle is catalyzed by glutathione reductase (GR; EC 1.6.4.2) leading to conversion of GSSG back to GSH. In addition, both ASC and GSH may also directly quench ROS (Noctor 2006).

APPROACHES FOR DEMONSTRATING THE CROSSTALK BETWEEN CALCIUM AND REDOX SIGNALING

There are many ways to explore and demonstrate the possible interaction between calcium and redox signaling events. For instance, various chemical compounds such as Ca^{2+} chelators (EGTA), Ca^{2+} channel blockers (La^{3+} and verapamil) or NADPH oxidase inhibitors (diphenylene iodonium, and pyridine) can be used to specifically block the calcium response and then its effect on ROS machinery can be monitored (Jiang and Zhang 2003). Ozone can be used to alter the cellular redox state and then differential cytosolic calcium signature in *Arabidopsis* seedlings was demonstrated in response to H_2O_2 as compared to that of control (Evans *et al.* 2005). Similar results were also obtained when BSO (L-buthionine-[S,R]-sulfoximine; inhibitor of glutathione biosynthesis) was also used instead of ozone (Rentel and Knight 2004). Both these studies confirm that the nature of H_2O_2-induced calcium signature is dependent upon the redox state. Unlike oxidative agents like ozone and BSO, various reducing agents have also been used to delineate the crosstalk between ROS and calcium. In *Nicotiana tabacum*, Gomez *et al*. (2004) used physiological (glutathione) and non-physiological (DTT and β-ME) thiols to demonstrate that cytosolic calcium level under any particular condition is dependent upon its redox state. The exogenous calcium supplementation (in the form of $CaCl_2$) was also shown to induce the formation of ROS inside the plants and this effect was lost when calcium was supplemented along with the Ca^{2+}-inhibitors and CaM antagonists (Sang *et al.* 2008). The calmodulin antagonists (such as W7) were also used to establish the link between calcium and H_2O_2 mediated signaling in maize plant (Xu 2010). In an earlier study, we have used $LaCl_3$ to block the calcium-signaling pathway and observed that the effect of redox-modulators such as thiourea is partially lost (Srivastava *et al.* 2010). The thiourea can also be used as a chemical probe to delineate the crosstalk between calcium and ROS in *Arabidopsis* roots under salt stress (Srivastava et al., unpublished research).

PLANT PROCESSES DEPENDENT UPON REDOX AND CALCIUM SIGNLAING CROSSTALK IN PLANTS

In recent years, a variety of plant processes are being identified, which involves the crosstalk between ROS and calcium signaling pathways. In most of these pathways, either Ca^{2+} is involved in the activation of NADPH oxidase that results in the formation of ROS or Ca^{2+} serves as a target for ROS produced from NADPH Oxidase (Sagi and Fluhr 2006). The brief description of the plant mechanisms involving ROS-calcium crosstalk is discussed next.

Development of root hairs

In plants, root hairs are extremely important as they facilitate the water and nutrient uptake from soil solution. They are also helpful in anchoring plant into soil. Initially, it was thought that the growth of root hairs is dependent upon the tip-focused cytoplasmic calcium gradient, targeted vesicular traffic and actin cytoskeleton (Carol and Dolan 2002). The

elevated Ca^{2+} concentration in the root hair tip is achieved by increased Ca^{2+}-uptake mediated by plasma membrane hyperpolarization-activated cation channels (Miedema *et al.* 2001). The elongating root hairs maintain high cytoplasmic Ca^{2+} gradient till the cessation of root growth. Such a calcium gradient was not formed in Arabidopsis *rhd2* (root hair defective 2) mutants that have very short root hairs. The RHD2 protein is characterized as an NADPH oxidase that transfers an electron from NADPH to an electron acceptor and causes the formation of ROS, mainly the superoxide radicals. The supplementation of extracellular ROS rescued the phenotype of *rhd2* Arabidopsis mutants (Foreman *et al.* 2003). This becomes the first line of evidence to show that the formation of cytoplasmic Ca^{2+} gradient and the root hair development are dependent upon ROS formation. The patch-clamp studies showed that ROS could activate the opening of calcium channels required for the calcium influx (Foreman *et al.* 2003). The existence of a positive feedback to activate the NADPH oxidase by Ca^{2+} is also being proposed (Takeda *et al*. 2008). This kind of dual mechanism helps to maintain the active growth of root hairs. Apart from ROS, recently it has also been proposed that formation of cytoplasmic Ca^{2+} gradient at the root tip is also under the independent control of root pH (Cardenas 2009). This is supported by the fact that *rhd2* mutant phenotype can be rescued by growing them at high pH. This suggests an additional level of regulation for calcium channel activation and regulation of root hair growth (Monshausen *et al.* 2007).

ABA-dependent guard cell signaling

Plant growth and development are largely affected by the availability of water. Hence adaptation to water deficit stress is a common to all plants. Abscisic acid (ABA) is an endogenous hormone that reduces the water loss through stomatal pores on the leaf surface. Increased biosynthesis and redistribution of the ABA in response to water deficit stress initiates a network of signaling pathways in guard cells leading to stomatal closure (Bray 1997). Perception of ABA requires an enhanced uptake of Ca^{2+} through ROS-activated Ca^{2+}-permeable channels in the plasma membrane of guard cells. The H_2O_2 (Pei *et al.* 2000) and nitric oxide (NO; Desikan *et al.* 2002) are the major ROS responsible for ABA-induced calcium increase. The increased ROS synthesis required for ABA response is achieved by two partially redundant NADPH oxidase catalytic subunit genes (AtrbohD and AtrbohF). Hence, the *atrbohD/F* double mutants of Arabidopsis become insensitive towards ABA-mediated response. The exogenous supplementation of H_2O_2 to this mutant rescued their phenotype both at the level of Ca^{2+} channel activation and stomatal closure (Kwak *et al.* 2003). Apart from ABA, the exogenous calcium (Ca^{2+}_{exo}) supply also increases the intracellular calcium (Ca^{2+}_{int}) level leading to stomata closure (MacRobbie 1992). The Ca^{2+}_{exo}- activates the CAS (calcium-sensing receptor) pathway to enhance the synthesis of ROS (NO and H_2O_2), which triggers Ca^{2+}_{int}-transients and finally the stomatal closure. Like ABA, the *atrbohD/F* mutant was also insensitive towards Ca^{2+}_{exo} (Wang *et al.* 2012). The Ca^{2+}_{exo} and ABA induced synthesis of ROS involves the G-protein mediated signaling (Zhang *et al.* 2010).

Plant-defense against pathogens

Plant defense against a variety of pathogens is initiated through the recognition of conserved microbe- or pathogen-associated molecular patterns (MAMPs/PAMPs) or plant-derived damage-associated molecular patterns (DAMPs). These molecular patterns are pathogen-specific and responsible for the activation of pattern-recognition receptors (PRR) and finally the pathogen-defense response (Boller and Felix 2009). To date, the best-studied PAMP/PRR pairs are flg22 (flagellin-22)/FLS2 (Flagellin sensitive-2; Gomez-Gomez *et al.* 1999) and EF-Tu/EFR (EF-Tu receptor; Zipfel *et al.* 2006).

One of the earliest signaling events after MAMPs/DAMPs perception is the rapid oscillation in cytosolic calcium concentration (Ca^{2+}_{cyt}) (Blume *et al.* 2000). Subsequently, the reactive oxygen species (ROS) are generated that restrict the growth of pathogen either directly through its toxic effects and cell wall strengthening or indirectly by initiating ROS-mediated signaling (Torres *et al.* 2006). The generation of ROS in response to pathogen attack is mediated by Arabidopsis NADPH oxidases (RbohD and RbohF). The superoxide radical (generated by NADPH-oxidase) rapidly converts into the membrane-permeable hydrogen peroxide (H_2O_2), which enters the cytosol and nucleus to activate MAP kinase (MAP3K–MKK4/MKK5–MPK3/MPK6 and MEKK1–MKK1/MKK2–MPK4; Rodriguez *et al.* 2010) and Ca^{2+}-dependent kinase (CPK4/5/6/11; Boudsocq *et al.* 2010) mediated signaling. This finally leads to re-programming of gene expression responsible for the plant-defense response. The pre-treatment of Arabidopsis seedlings with calcium influx inhibitors (such as LaCl or BAPTA) suppressed the ROS burst as well as the MAMP-induced change in gene expression. This confirms that the MAMP-specific response is controlled by the induction of calcium signature followed by the ROS response (Segnozac *et al.* 2011). However, the disturbance in ROS accumulation, either in the *rbohD* mutant or through application of ROS inhibitor, partially reduces the Ca^{2+} response. This suggests the possibility of ROS-mediated feedback control on MAMP-induced Ca^{2+} signaling (Ranf *et al.* 2011).

Besides the pathogen infection, mechanical wounding created by the insect attack also triggers cytosolic Ca^{2+}-transients. Direct crosstalk between calcium, ROS and MAP Kinase signaling has been proposed for mediating the wound response. In Arabidopsis, mitogen-activated protein kinase 8 (MPK8) links the protein phosphorylation, Ca^{2+} and ROS (Takahashi *et al.* 2011). MPK8 is activated through mechanical wounding in a Ca^{2+}-dependent manner. MPK8 can also be phosphorylated and activated by a MAPKK, MKK3 and the complete activation of MPK8 requires the coordinated interaction between the Ca^{2+} and MKK3. The MPK8 pathway negatively regulates ROS accumulation through RbohD. These findings suggest that two major activation modes in eukaryotes, Ca^{2+}/CaMs and the MAP kinase phosphorylation cascade converge at MPK8 to monitor or maintain ROS homeostasis.

Endoplasmic reticulum associated degradation of unfolded proteins

Eukaryotic organisms have quality-control mechanisms that allow the misfolded or proteins to be retained in the endoplasmic reticulum (ER) and subsequently degraded by ER-associated degradation (ERAD). The proper functioning of ERAD pathway is very important under control as well as under stress conditions because misfolded proteins are either toxic to the cells or they may endow with undesirable biological activities. Unlike animals, the details of ERAD pathway in plant are not yet reported. Recently, Liu et al. (2011) demonstrated that in response to an increase in unfolded protein, the Ca^{2+} is released from the ER and it coordinates with reactive oxygen species (ROS) to initiate protein degradation.

Plant response under mechanical stress

Plants are exquisitely sensitive to mechanical stimuli. However, mechanical stresses are not only imposed by the external environment but are also generated endogenously as an inevitable consequence of the expansive growth of pressurized cells. For highly polarized cells, such as root hairs and pollen tubes, control of expansion-related mechanical stresses seems to be a fundamental aspect of growth regulation (Monshausen *et al.* 2008). At the tissue and organ level, mechanical forces may even drive cellular patterning (Hamant *et al.* 2008). The molecular basis for the percep-

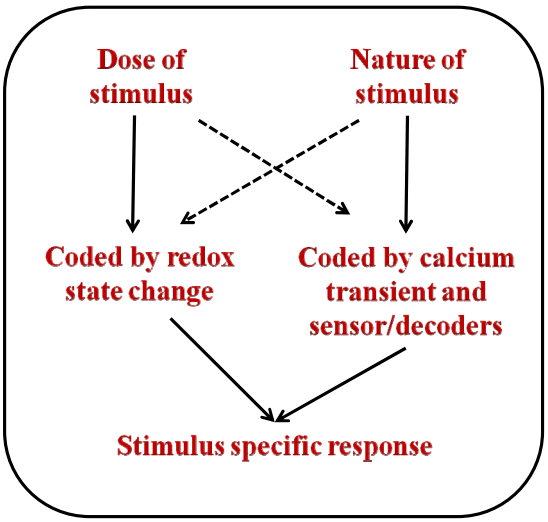

Fig. 4 Hypothesis for the possible interaction of redox and calcium signaling process for the induction of stimulus-specific response. In response to any stimulus, the redox state is changed depending upon its dose which then induces the $[Ca^{2+}]_{cyt}$-transient to activate stimulus-specific calcium sensor/decoder elements that finally initiates the necessary downstream signaling.

tion of mechanical stimulus is the rapid increase in cytosolic Ca^{2+} level (Monshausen and Gilroy 2009). In *Arabidopsis thaliana*, the mechanical stimulation in roots is shown to trigger the rapid and transient increase in $Ca^{2+}_{(cyt)}$ concentration, which is simultaneous with the induction of apoplastic ROS of same kinetics (Monshausen *et al.* 2009). However, the detailed molecular mechanism of ROS and calcium crosstalk is not yet known.

CONCLUSION AND FUTURE DIRECTIONS

The present article deals with an overview of crosstalk between Ca^{2+} and redox signaling in regulating various plant processes. As discussed in this article, ROS or Ca^{2+} channel-dependent generation of Ca^{2+}-signature represents a primary step in the perception of stimulus and generation of a specific response. So far, it has not been established whether the induction of stimulus-specific Ca^{2+}-signatures regulates the ROS burst or it is regulated by upstream ROS signature. However, the data available from the mutants impaired in ROS generation signifies the importance of redox state as a central regulator for the induction of Ca^{2+}-signature that in turn performs the dual function to activate the stimulus-specific effectors and also to enhance the ROS formation required for the feedback regulation of calcium signaling. Further, the concept of redox state can also be integrated into the present understanding of calcium signaling to explain how calcium acts as a ubiquitous messenger to encode the information about the dose and nature of stimulus (**Fig. 4**). In response to any stimulus, the redox state is changed depending upon its dose, which then induces the $[Ca^{2+}]_{cyt}$-transient to activate stimulus-specific calcium sensor/effector elements that finally initiates the necessary downstream signaling. Thus, a complete understanding of the role of Ca^{2+}- and redox-signaling requires a systematic and independent high-throughput analysis of all loss- and gain-of-function genotypes related to components necessary for initiation and progression of the Ca^{2+}- and ROS-signature. This includes the use of all possible 'omics' approaches with the computational tools, combined with high-resolution phenotypic analysis and investigation of spatial and temporal dynamics of sub-cellular Ca^{2+} and ROS-transients and their downstream receptors. This will help to build a complete network of the calcium and redox signaling that functions under normal and stress situation. The next step will be to explore the elements responsible for the calcium and redox crosstalk. For instance, the putative calcium sensor elements that are responsible for the perception of any change in redox state can be identified and characterized. The stimulus-specific nature of these redox-sensitive calcium sensors needs to be explored in detail. In this direction, an additional challenge is to characterize different parameters of calcium- and ROS-signature and to assign them either with the nature or dose of stimulus. The understanding of calcium and ROS crosstalk can be further advanced by evolving the technique for the simultaneous measurement of calcium- and ROS-burst in cytosol and other sub-cellular compartments.

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