**Role of Calcium** S**ignaling in Plant Defense Mechanism**

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**INTRODUCTION**

Calcium as Ca2+ ion is an important divalent signalling molecule in plant cells. Plants are constantly facing several biotic stresses like fungi, bacteria and viruses and abiotic stresses like changes in temperature, high salinity, cold and drought. In absence of any stress, Ca2+ concentrations of the cytosol are usually maintained at lower level because of CalciumATPases and Calcium-Hydrogen antiporters in plant cell membranes (Bush, 1995: Sanders’ *et al* 1999). Recognition of biotic and abiotic stresses often leads to increase in cytosolic free Ca2+ level in plant cells to activate signalling responses for analyzing both internal and external signals, converting them into physiological and gene expression responses. Several families of calcium permiable ion channels mediate the effects of cytosolic Ca2+ which include annexins, two-pore channel 1 (TPC1), ionotropic glutamate receptors, cyclic nucleotide-gated channels (CNGCs), and various varieties of mechanosensitive channels, are primarily engaged in cell signaling. Plasma membrane, tonoplast, endoplasmic reticulum, chloroplast and nuclear membranes of plant cells contain these channels that mediate calcium influx into cytosol. The specificity of the calcium signal to produce appropriate defense reaction is believed to be encoded by different amplitude, temporal or spatial changes in cytoplasmic calcium concentration (Trewavas, 1999: Malho, 1998: McAinsh, 1998). Calcium transients in plant cells is an essential early events following the perception of different environmental stimuli. Ca2+ concentration changes are detected by calcium- modulated proteins or calcium sensors including calmodulin, calcium-dependent protein kinases, calmodulin-like proteins (CMLs), calcium and calcineurin B-like (CBLs) proteins, which decode the encoded calcium signals into specific cellular and physiological responses in order to survive environmental challenges. Among calcium sensors kinases represent ‘responders’ and are ability to regulate downstream targets directly through catalytic activity, whereas CaMs/CMLs and CBLs are non-catalytic relay sensors. The interaction of Calcium with Ca2+ sensors either directly stimulate the kinase activity or cause a conformational change that will allow them to engage with downstream effectors. (Harmon et al., 2000). A second level of specificity is made possible by the variety of Ca2+ sensors and their downstream targets, permitting the conversion of varied initial inputs into different biological responses (Hashimoto and Kudla, 2011).

**CHARACTERISTICS OF CALCIUM ION PERMEABLE CHANNELS**

**CYCLIC NUCLEOTIDE-GATED CHANNELS (CNGCS)**

The second messengers cyclic adenosine monophosphate (**cAMP**) and cyclic guanosine monophosphate  (cGMP), are ubiquitous signaling molecules that are essential for controlling a variety of biological functions, gene expression, and signal transduction (Newton RP and Smith CJ, 2004: Trewavas et al., 2002). Cyclic nucleotide gated ion channels (CNGCs) provide a pathway for Ca2+ transport across the plant cell membrane and elevates cytosolic Ca2+ concentration in response to biotic and abiotic stimuli that results in the activation of CNGCs by increasing cyclic nucleotides concentration which leads to generation of important signaling molecules such as nitric oxide (NO) and hydrogen peroxide (H2O2), which play crucial role in the development of the hypersensitive response (HR) in plant cell. The elevated cytosolic calcium ion also compete with cyclic nucleotide for binding to the CNGC, obstructing further Ca2+ conductance by the calcium channel. It is found that during abiotic heat stress signaling in *Arabidopsis* and *Physcomitrella* *patens*, CNGCs participate in heat-induced cytoplasmic calcium (Finka et al., 2012; Gao et al., 2012) which increases cAMP production in Arabidopsis and activates plasma membrane HACC in root cells (Gao et al., 2012). Atcngc6 mutant, lack HACC resulting in decreased thermotolerance and abnormal expression of the heat shock protein. CNGCs are usually expressed at the plant cell membrane and are numerous in the plant root and leaf epidermis where they most likely function to detect and record environmental inputs and in leaf epidermis CNGCs are found in guard cells and the mesophyll, where they play important role in control of stomata closure and photosynthesis (Gobert et al., 2006; Jammes et al., 2011). Additionally, CNGCs facilitate a targeted nuclear release of the ER Ca2+ store, which is responsible for the nuclear Ca2+ alterations in the symbiotic signaling pathway in the roots of legumes. (Charpentier et al., 2016). PTI is also regulated by one of the calcium channel in Rice (OsCNGC9) which is divalent cation-selective inward calcium permeable channel which is activated by OsRLCK185-mediated phosphorylation (Wang et al, 2019).

Thor et al., 2019, identified a plant Ca2+ channel and its associated activation mechanisms that underlie stomatal closure during immunological signaling. Upon being exposed to pathogen-associated molecular patterns (PAMPs), OSCA1.3 is quickly phosphorylated. After being exposed to the peptidic PAMP fg22, produced from bacterial fagellin, for a short period of time, the immune receptor-associated cytosolic kinase BIK1 interacts with and phosphorylates the N-terminal cytosolic loop of OSCA1.3 within minutes. According to genetic and electrophysiological evidence, OSCA1.3 is Ca2+ permeable, and BIK1-mediated phosphorylation of its N terminus enhances its activity. Notably, OSCA1.3 does not control stomatal closure in response to sensing of abscisic acid, a plant hormone linked to abiotic stressors; instead, OSCA1.3 and its phosphorylation by BIK1 are essential for stomatal closure during immunological signaling.

**IONOTROPIC GLUTAMATE RECEPTORS**

The glutamate receptors (GLRs) channels are non-selective that regulates Ca2+ influx in several species of higher plants in both dicotyledons and monocotyledons. In plants the GLRs are encoded by 20 genes and elevate cytosolic Ca2+ level and are variably triggered by Glu and Gly as well as by other amino acids. (Chiu et al., 2002; Qi et al., 2006; Stephens et al., 2008). According to Kim et al. (2001) and Demidchik and Maathuis (2007), GLRs are crucial for plant nutrition as well as for modulating Ca2+ responses upon cold stress. Overexpression of Arabidopsis glutamate calcium channel, At GLR3.1, impaired long-term stomatal closure but the kinetics of Ca2+ alterations forced by extracellular Ca2+ were unaffected (Cho et al., 2009). Price et al. (2013) predicted that GLR proteins are structurally comprised of three transmembrane domain configurations: a pore-forming domain and two potential ligand binding hand motifs that are preferentially expressed in root tissues and some motif subunits are expressed in leaf mesophyll, guard cells and pollen tubes (Weiland et al., 2015). The Glutamate calcium channel, GLR1.2 has been shown to be expressed in pollen tubes by Michard et al. (2011) and is actively involved in the polar Ca2+ influx necessary for pollen tube growth and elongation. GLRs take part in immunity, photosynthesis, pollen incompatibility, and metal-ion homeostasis (Weiland et al., 2015). In Arabidopsis roots, exogenous glutamate leads to a build up of extracellular ATP (Dark et al., 2011) which then activates plasma membrane Ca2+ influx channels by generating ROS by a NADPH oxidase (Demidchik et al., 2009).

**TWO-PORE CHANNEL 1 (TPC1):**

TPC 1 is avoltage-gated organellar cation calcium channel in tonoplast in Charophyte algae and in all terrestrial plants and requires both voltage and cytosolic calcium concentration for activation. This voltage gated TPC1 channel is coded by a single gene in *A. thaliana* (Peiter et al., 2005) with six transmembrane domain (6-TM) structure having several canonical Ca2+-binding sites (motifs) and are activated by cytosolic Ca2+ and its relative expression controls the amount of Ca2+ storage capacity in vacuoles. (Gilliham et al., 2011). Opening of Two Pore Channel 1 is determined by binding of Ca2+ to the cytosolic EF-hand motifs (Kintzer & Stroud, 2016; Guo et al., 2016). These channels play crucial role in long-distance Ca2+ signalling and for rapid Ca2+ signal propagation along the root in response to local NaCl application (Choi et al., 2014). Wang et al., 2017 identified these channels as the oxygen modulators in plants under flooding conditions. TPC1s channel allows Ca2+ entry across the vocuolar membrane in response to cold shock, sucrose,, salicylic acid, as well as elicitors. Changes in the cytosolic Ca2+ levels of plant cells in response to pathogen exposure have been observed and recognized as a vital early event for plant defense responses like salt stress-induced Ca2+ ROS waves. The important signaling molecule ROS activated the calcium channel present in plasma membrane and the increasing cytosolic calcium activate TPC1 proteins for calcium release from vacuole and thus giving rise to a self-propagating ROS/Ca2+ wave. The communication between two cells of plant is mediated by either diffusion of ROS through the apoplast or Ca2+ through the plasmodesmata. The TPC1 mutant Knock out mutant, tpc1, affects the both the ABA-induced germination suppression and the stomatal response to extracellular Ca2+ are affected by functional SV channel activity. TPC1 contributes to cytosolic Ca2+ homeostasis indicated by ABA and CO2-induced stomatal closure and ABA-, K+- and Ca2+dependent root growth phenotypes were no different in tpc1 compared with wild-type plants. Ca2+ signaling activity was found in AtTPC1, rice (OsTPC1) and wheat (TaTPC1).

**Annexins**

Annexins are potential multifunctional proteins actively engaged in regulating trafficking of Ca2+ channel to a membrane, located and expressed in roots and are involved in root cell elongation and is activated by hyperpolarization and extracellular hydroxyl radicals ( Demidchik et al., 2003; Foreman et al., 2003; Laohavisit et al., 2012). Knockout mutant of Annexins in Arabodopsis was found to affect Ca2+ channel in epidermal and root hair apical plasma membrane (Atann1), with mutant root hairs found to be shorter than wild-type root hairs (Laohavisit et al., 2012). The ability of the maize annexins to create a Ca2+ conductance was found in planar lipid bilayers (PLB). But it is unknown how these annexins increased the cytoplasmic calcium concentration of *Arabidopsis* protoplasts, whether by directly establishing a Ca2+ influx pathway or by activating additional channels (Laohavisit et al., 2009). These annexin proteins conduct Ca2+ across planar lipid bilayers (PLB), changing from being voltage independent to hyperpolarization activated when malondialdehyde is incorporated into the PLB to mimic lipid peroxidation (Laohavisit et al., 2009, 2010). Arabidopsis possess eight putative genes that encode these cytoplasmic proteins and 25 and 11 genes were found in wheat and barley, respectively (Xu et al., 2016). Two maize annexin proteins ANN33 and ANN35 are permeable to both ions K+ and Ca2+ and suggested that the annexin protein family could act as Ca2+ channels in calcium signaling processes.

**MECHANOSENSITIVE CHANNELS**

Plants are constantly exposed to extrinsic mechanical stimuli, such as wind, compression, stretch, touch etc can signal a hazard to plants, so mechanosensing and subsequent defense responses are particularly important plants to grow and flourish under mechanically demanding situations. Calcium permeable mechanosensitive channels being important component of mechano-sensing located in plasma membrane, endoplasmic reticulum, apoplast elicits an immediate calcium transient in the cytoplasm upon perception of mechanical stimuli. These calcium permeable channels were first identified in Arabidopsis (Nakagawa et al., 2007) and structurally these channels form homotetramer and have several motifs, such as an EF hand-like motif, coiled-coil motif, and plac8 (DUF614) motif as well as a few predicted putative transmembrane segments present in the cytosol. The putative Ca2+ permeable mechanosensitive channels of *Arabidopsis* MCA1 (At4g35920) and MCA2 (At2g17780) are regulated by the EF hand-like motif, which can sense calcium transient in cytoplasm. In Rice and Tobacco, hypoosmotic stress activate mechanosensitive proteins to increase cytoplasmic calcium (Kurusu et al., 2012a: Kurusu et al., 2012b). Reactive oxygen species (ROS) are produced in the apoplast when calcium binds to EF-hand motifs and then these ROS serve as substrates for peroxidases, which impact cell wall metabolism and cellular responses, such as the activation of genes that are induced by mechanical stimuli (Takeda et al., (2008). Hypo-osmotic stress results in activation of calcium permeable mechanosensitive channels OsMCA1 to form calcium transient and production of ROS in cultured rice cells (Kurusu et al., 2012a: Kurusu et al., 2012b). Mechanosensitive channels play important role in regulation of programmed cell death (Veley et al., 2014), water and ion balance in pollen tubes (Hamilton & Haswell, 2017).

**CALCIUM SENSORS**

Upon perception of external biotic and abiotic stimuli, there is elevation of calcium concentration in cytoplasm, these Ca2+ transients are sensed by Ca2+-binding proteins called calcium sensors or modulators. Calmodulin, calcium-dependent protein kinases, and calcineurin B-like proteins are a few classes of calcium binding sensory proteins found in plants that translate Ca2+ signals into particular cellular and physiological responses in order to grow and flourish in environmental conditions (Aldon et al., 2018). Structurally these proteins have protein kinase and calmodulin-like domains in a single polypeptide showing affinities for calcium ions and their location within the cell, will control their behaviour. When CPKs are taken into consideration, calcium binding to Ca2+ sensors will cause a conformational change that either causes their association to downstream target proteins or stimulate kinase activity directly (Harmon *et al.,* 2000). The Ca sensors and their downstream effects contributes to a second layer of specificity by varying protein phosphorylation and gene expression patterns, allowing the conversion of various external environmental stimuli into distinct biological responses (Hashimoto and Kudla, 2011).

**CALMODULIN (CAM) AND CAM BINDING PROTEINS (CAMBPS)**

The most important calcium modulators are Calmodulin (CaM) and calmodulin-like (CML) proteins which are involved in regulation of gene expression during immune reactions of plant cells. Calmodulin proteins sense the elevated Ca2+ levels and culminates calciumtransients into cellular responses through Ca2+dependent control of subsequent effectors. Calmodulins are a major and prototype class of calcium modulators found in eukaryotic cells. They have two distinct globular domains, two EF-hand motifs, and a helix-loop-helix structure and each CaM is tightly bound to 4 Ca2+ ions. The calmodulin modulator generally lacks catalytic activity, but when it binds to Ca2+ through the EF-hand motif, it changes its structure and exposes hydrophobic areas that create high affinity binding sites for target proteins (Lecourieux *et al*., 2006). Therefore, CaM controls the actions of several downstream CaMBPs by interacting to them which results in additional level of specificity by CaMBPs for Ca2+ signaling and thus activate different physiological reactions (Cheval *et al.,* 2013). Upon the recognition of pathogen by plants, these CaM sensors regulates plant defense by inducing rapid production of nitric oxide (NO) which serves as a modulator of disease resistance by inducing hypersensitive cell death and stimulating the expression of multiple defensive genes (Hong et al., 2008). CBP60g, an important CaM-binding protein, is involved in the expression of SA biosynthesis gene ICS1, providing a path for Ca2+ signal to modulate defense responseslike activation of stress response genes like chaperones, increase resistance of plant to SAR and stimulate production of antioxidants.

**CALCIUM DEPENDENT PROTEIN KINASE (CDPK)**

Plants possess unique and ubiquitous calcium sensor CDPK play role in defense responses to biotic and environmental stresses. The first CDPK known to be engaged in Effector triggered immunity, induced by the fungal elicitor Avr9 is Nicotiana tabaccum NtCDPK2, while CDPK found in Arabidopsis, AtCPK1, phosphorylate phenylalanine ammonialyase (PAL), an alternative pathway to create SA in vitro. This calcium sensor structurally consists of 4 EF-hand motifs that binds to calcium and C-terminus of a Ser/Thr kinase domain with a junction of an autoinhibiotory domain (Harmon *et al.,* 2000) and the binding of Ca2+ to the EF-hand motif induces a configurational change that results in kinase activation. These calcium oscillations result in phosphorylation events by CDPK sensors, which then cause physiological responses in the form of hormone signaling, oxidative burst and gene expression (Wernimont *et al.,* 2010). ROS produced extracellularly is the main aspect of the plants defense mechanism and act as direct toxicants to pathogens, and play role in reinforcement of physical barriers, phytoalexin synthesis, defense gene activation, programmed cell death. These CDPK sensors are involved in the expression of PR genes in both the infected tissue as well as the uninfected tissues exhibiting SA.

**CALCINEURIN B-LIKE PROTEINS (CBLS)**

CBL calcium modulators are the third most significant plant specific small calcium binding proteins. To decode Ca2+ signals, plant-specific Ca2+ sensor proteins have four EF-hand motifs as calcium binding domain in their structure and interact particularly with the CBL-interacting protein kinase (CIPK) family of Ser/Thr protein kinases (Kim et al., 2000). They interact with protein kinases as CBL-CIPK and interaction with CIPK alter the Ca2+binding properties of CBLs. Different Ca2+ responsive complexes are developed by the diversity of CBL/CIPK coupling. The structure of two important sensor proteins of *Arabidopsis* (CBL2 and CBL4) consist of two globular domains, each of which contains one EF hand motif pair, separated by a short linker region between the globular domains. Plant evolution suggests that CBLs-CIPKs evolved simultaneously with the process of plant adaptation and colonization on the land as well as with their increasing ability to tackle the changing environmental conditions. Crystal structural investigation of CBL2 from *Arabidopsis*, the EF hand Ca2+ binding domain has 14 amino acids rather than the 12 amino acids found in classical Ca2+ binding loops (Nagae et al., 2003). In *Arabidopsis*, ion homeostasis is regulated by the calcium sensor CBL10, which mediates salt tolerance, this CBL10 interacts with a family of serine-threonine protein kinases known as CBL-interacting protein kinases (CIPKs). A new Ca2+ regulated salt tolerance pathway, consists of CBL10 and CIPK24, these sensors controls how Na+ is compartmentalized or sequestered into the vacuoles of green tissues. Pea CBL and CIPK were coordinatedly raised in response to various circumstances like cold and salinity but not dehydration stress. During plant-pathogen interactions, the signaling module composed of the proteins calcineurin B-like protein 10 (Cbl10) and calcineurin B-like interacting protein kinase 6 (Cipk6) is thought to be involved in ROS signaling (Torre et al. (2013). Additionally, PTI (PAMP-triggered immunity) has been demonstrated to involve the CIPKs in rice (OsCIPK14) and OsCIPK15. CIPK24 forms a complex with either CBL1/CBL4 or CBL10 to produce a dual functioning kinase, which is how CBL/CIPK function is achieved in salt stress reactions. At the plasma membrane of roots, CBL4-CIPK24 control Na+extrusion through the Na+/H+ exchanger SOS1, whereas CBL10-CIPK24 complexes are localized at the vacuole in shoots where they may control Na+ sequestration into this organelle (Torre et al. (2013).

**Ca2+ EXTRUSION SYSTEMS:**

The cytosolic baseline [Ca2+]cyt increases by a factor of several orders of magnitude when external Ca2+ activity increases (Demidchik et al., 2002). Plant cells typically respond to phytohormones and environmental cues by elevating their [Ca2+]cyt concentrations by 0.5 to 10 lM. Even with these outside stimuli present, the basal [Ca2+]cyt concentration returns within a short period of time. Ca2+ extrusion and sequestration processes, which work against the electrochemical gradient, mediate this recovery, which calls for energy-intensive Ca2+ transporters. Ca2+/H+ exchangers of the CAX family (calcium/cation exchangers), which get their energy from the electrochemical gradient of protons across membranes facing the cytosol, and P-type Ca2+ATPases, which use the energy released during ATP hydrolysis, are the two different types of active Ca2+ transport systems found in plants. (Bonza & De Michelis, 2011; Huda et al., 2013).

**Ca2+ ATPase activity in plants and its regulation**:

Plant Ca2+ pumps are members of the P-type superfamily of ATPases, which is how they get their energy from ATP hydrolysis by attaching the c-phosphate of ATP to the aspartate residue in the P-domain's DKTGT motif (Palmgren & Harper, 1999). The E1 and E2 states are two different conformations for this enzyme. The first binds Ca2+ at the cytosolic side of the membrane with a high affinity. The pump changes conformation to the E2 state following ATP hydrolysis and phosphorylation, which has a significantly reduced affinity for Ca2+ and an ion binding site on the opposite side as a result, Ca2+ dissociates from the protein on the outer side of membrane (Kabala & Klobus, 2005).

**CAX operation and regulation**

The transmembrane helix of CAX proteins has two cation-binding sites known as the a1- and a2-repeat sections. Within these repeat regions, Ca2+ and H+ binding are antagonistic (Nishizawa et al., 2013; Waight et al., 2013), indicating the possibility of an exchange process where one H+ goes in and one Ca2+ comes out (Pittman & Hirschi, 2016). H+ binding reverts the protein structure. The physical interaction between the N-terminus and a nearby N-terminal region is what causes autoinhibition of CAX proteins (Manohar et al., 2011). Modulation of CAX activity may result from phosphorylation, pH variations, and responses to regulatory proteins such the serine/threonine kinase SOS2 with the CAX N-terminal domain (Demidchik & Shabala, 2018).

**CONCLUSION:**

Changes in the cytosolic Ca2+ levels of plant cells after a pathogen exposure have been noted and recognized as an early occurrence essential for plant defense responses. It was discovered through the identification and investigation of CaM-binding proteins, CDPKs, and other calcium sensors that Ca2+ signaling plays a variety of roles in plant defense responses. Mechanosensitive channels have made it possible to elucidate their important roles in a variety of processes, including programmed cell death, responses to salt stress, control of organelle shape, and ROS sensing. These procedures involve ROS sensing as well as the perception of mechanical stimuli. The majority of Ca2+ permeable channels have been electrophysiologically examined and cloned in heterologous expression methods. Plasma layer a ROS-Ca2+ hub is a self-amplifying system formed by the interaction of Ca2+permeable channels and Ca2+ activated NADPH oxidase. This mechanism may be able to convert and amplify the initial Ca2+ or ROS stimulation into a longer-lasting response, which could have effects on cell development, hormone signaling, and stress reactions. Mechanism for Ca2+ removal from the cytosol is formed by the interaction of Ca2+extruding systems, such as Ca2+ATPases and Ca2+/H+ exchangers, with Ca2+permeable channels. This protein has a biphasic activity in Ca2+ extrusion, which increases with [Ca2+]cyt., as shown by the crystal structure of the Ca2+ ATPase autoinhibitory domain, which explains its role in signaling cascades. The function of other alleged calcium exchangers, such as the calcium sodium exchanger (Wang *et al*., 2012). The critical roles of calcium transport systems in intracellular signaling, Ca2+ and Mg2+ nutrition, elongation growth, cytoskeleton regulation, biotic and abiotic stress responses, programmed cell death, gravity sensing, ROS, hormones, temperature changes, mechanical stimuli, control of stomatal closure, and photosynthesis have been revealed in numerous studies using KO mutants and overexpressing lines of Ca2+ transporting systems. Ca2+ transporting machinery is a very appealing target for plants that are being genetically improved for environmental fitness. The practical challenge of reprogramming stress resilience and control over plant development and productivity is incredibly difficult because numerous Ca2+ carrying systems and the complexity of their regulation. Because there are so many Ca2+ carrying systems and their regulation is so intricate, it is extremely challenging to reprogram stress resilience and control over plant growth and production.

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