ELEVATION OF CYTOSOLIC CALCIUM IN GUARD CELLS

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Calcium ion (Ca^{2+}) is a ubiquitous intracellular second messenger that regulates many cellular processes in plants as well as in animals. Guard cells respond to environmental and internal stimuli, resulting in a change in a stomatal aperture. This signaling pathway employs Ca^{2+} as a second messenger in the guard cells. Hence, elevation of cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_{\text{cyt}}$) can be observed in guard cells treated with phytohormones such as abscisic acid and methyl jasmonate. Several other second messengers including cyclic ADP-ribose, inositol 1,4,5-trisphosphate, and myo-inositol hexakisphosphate are involved in the $[\text{Ca}^{2+}]_{\text{cyt}}$ elevation in the guard cells. Moreover, several methods have been developed to observe the $[\text{Ca}^{2+}]_{\text{cyt}}$ elevation in the guard cells. In this mini-review, we briefly introduce the roles and regulation of the transient $[\text{Ca}^{2+}]_{\text{cyt}}$ elevation in the guard cells and the methods to monitor the $[\text{Ca}^{2+}]_{\text{cyt}}$.

Key Words: *abscisic acid, cytosolic calcium elevation, guard cell, stomatal closure, Yellow Cameleon*

1. INTRODUCTION

Elevation of the cytosolic calcium concentration ($[\text{Ca}^{2+}]_{\text{cyt}}$) was first observed in guard cells treated with abscisic acid (ABA)¹ and subsequently in guard cells treated with external Ca^{2+} , hydrogen peroxide, and cold^{2,3}. The transient elevation of Ca^{2+} usually appears at intervals of 10 min or more and each elevation persists for 1 min to 4 min⁴.

The roles of $[\text{Ca}^{2+}]_{\text{cyt}}$ elevation in ABA signalling are not yet fully understood. Gilroy *et al.* have demonstrated that the $[\text{Ca}^{2+}]_{\text{cyt}}$ elevation is essential for stomatal closure⁵. Siegel *et al.* have shown slow stomatal closure in Arabidopsis under conditions that prevent $[\text{Ca}^{2+}]_{\text{cyt}}$ elevation in the guard cells⁶. Romano *et al.*, however, have shown by revealing uninterrupted downstream events that the $[\text{Ca}^{2+}]_{\text{cyt}}$ elevation is not needed for stomatal closure⁷. Furthermore, Allan *et al.* have proposed that there may be two ABA signal transduction pathways involved in the ABA-induced stomatal closure; one

is Ca^{2+} -dependent and the other is Ca^{2+} -independent⁸. More recently, Allen *et al.* hypothesized that certain threshold level of the $[\text{Ca}^{2+}]_{\text{cyt}}$ elevation regulates the stomatal closure and that a defined pattern of the $[\text{Ca}^{2+}]_{\text{cyt}}$ elevation is needed to inhibit stomatal reopening or to maintain closure⁹.

In this mini-review, we will briefly describe the roles of the $[\text{Ca}^{2+}]_{\text{cyt}}$ elevation in ABA-induced stomatal closure and the regulation of the $[\text{Ca}^{2+}]_{\text{cyt}}$ elevation by second messengers in guard cells. We will also provide a brief description of the measurement of the $[\text{Ca}^{2+}]_{\text{cyt}}$ elevation in guard cells using a genetically encoded calcium indicator.

2. ROLES OF $[\text{Ca}^{2+}]_{\text{cyt}}$ OSCILLATION IN GUARD CELL ABA SIGNALING

Abscisic acid induces the production of reactive oxygen species (ROS) that can trigger Ca^{2+} entry into

the cytosol by stimulating the hyperpolarization-activated Ca^{2+} -permeable channels in the guard cells (Fig.1)¹⁰. Plasma membrane Ca^{2+} -permeable channels are also involved in the generation of external Ca^{2+} -induced $[\text{Ca}^{2+}]_{\text{cyt}}$ elevation in the Arabidopsis guard cells². The elevation of $[\text{Ca}^{2+}]_{\text{cyt}}$ in guard cells not only depends on the Ca^{2+} entry across the plasma membrane, but also on the release of Ca^{2+} from intracellular stores such as the vacuoles, endoplasmic reticulum, and trans-Golgi network. It has been demonstrated that the inositol 1,4,5-triphosphate (InsP_3)-gated Ca^{2+} -release channels in the endomembranes and cyclic ADP-ribose (cADPR)-sensitive Ca^{2+} -permeable channels in vacuoles participate in the $[\text{Ca}^{2+}]_{\text{cyt}}$ elevation in the guard cells^{11,12}. Estimates show that Ca^{2+} release from intracellular stores accounts for more than 95% of the Ca^{2+} entering the cytosol to raise the $[\text{Ca}^{2+}]_{\text{cyt}}$ ⁴.

The elevated $[\text{Ca}^{2+}]_{\text{cyt}}$ in guard cells inhibits inward-rectifying K^+ channels to prevent K^+ uptake and activates both the slow-activating sustained

Fig.1 A simplified model showing the roles of the $[\text{Ca}^{2+}]_{\text{cyt}}$ elevation in the guard cells. Elevated $[\text{Ca}^{2+}]_{\text{cyt}}$ can activate anion channels and can depolarize the plasma membrane. Depolarization activates the outward-rectifying K^+ channels. Elevated $[\text{Ca}^{2+}]_{\text{cyt}}$ also inhibits inward-rectifying K^+ channels and modulates the activity of H^+ -ATPase in the guard cells.

(S-type) anion channels and rapid transient (R-type) anion channels at the plasma membrane to depolarize the membrane¹⁰. These changes are favorable to K^+

efflux through the outward-rectifying K^+ channels, which lead to stomatal closure (Fig.1)¹⁰. In addition to inhibiting the inward-rectifying K^+ channels and activating anion channels, the $[\text{Ca}^{2+}]_{\text{cyt}}$ elevation can also modulate the activity of the H^+ -ATPase (Fig.1). The elevation in $[\text{Ca}^{2+}]_{\text{cyt}}$ from $0.3 \mu\text{M}$ to $1.0 \mu\text{M}$ inhibits the plasma membrane H^+ -ATPase in the guard cells of *Vicia faba*¹³. This inhibition of H^+ -ATPase is thought to occur via recruitment of Ca^{2+} from intracellular Ca^{2+} stores rather than Ca^{2+} influx from an apoplastic space¹³. The H^+ -ATPase, which can be inhibited by ABA, has been shown to be important in H^+ pumping required for the membrane hyperpolarization that activates the inward-rectifying K^+ channels. In addition, the inhibition of H^+ pumping by ABA-induced $[\text{Ca}^{2+}]_{\text{cyt}}$ elevation prevents membrane hyperpolarization thereby indirectly contributing to membrane depolarization caused by the Ca^{2+} influx through the Ca^{2+} -permeable channels¹⁴.

ABA induces activation of the Ca^{2+} -independent protein kinase Open Stomata 1 (OST1) that activates S-type anion channels, which leads to the release of anions from guard cells and triggers stomatal closure¹⁵. The $[\text{Ca}^{2+}]_{\text{cyt}}$ elevation that occurs before or during stomatal closure augments the activity of the S-type anion channels¹⁶. As a result of a further stimulation of the S-type anion channels in guard cells, the $[\text{Ca}^{2+}]_{\text{cyt}}$ elevation seems to accelerate stomatal closure¹⁶.

The Ca^{2+} -dependent response is likely to be mediated by the two types of Ca^{2+} -sensor proteins, i.e., Ca^{2+} -dependent protein kinases (CPKs) and calcineurin B-like (CBL)-interacting protein kinases (CIPKs) that bind to the CBL proteins^{16,17}. In the *cpk3 cpk6* double and *cpk5 cpk6 cpk11 cpk23* quadruple mutants, ABA-induced stomatal closure was impaired^{18,19}, indicating a Ca^{2+} -dependent stomatal signaling pathway. In addition, a recent study found that CIPK23 in combination with either the CBL1 or CBL9 phosphorylates and activates the guard cell S-type anion channels¹⁷, suggesting the positive role of the CBL1/9-CIPK23 complexes in the stomatal closure in response to ABA.

3. THE OTHER SECOND MESSENGERS INVOLVED IN $[\text{Ca}^{2+}]_{\text{cyt}}$ ELEVATION

Several second messengers have been identified that regulate the $[\text{Ca}^{2+}]_{\text{cyt}}$ elevation in the guard cells. In vacuoles, the nanomolar concentrations of cADPR can stimulate the Ca^{2+} permeable channels¹². In guard cells, cADPR initiates transient $[\text{Ca}^{2+}]_{\text{cyt}}$ elevation and provokes stomatal closure whereas neither an inactive cADPR analog 8-NH₂-cADPR

nor noncyclic ADPR induces $[Ca^{2+}]_{cyt}$ elevation and stomatal closure²⁰⁾. Moreover, treatment with an inhibitor of cADPR production, nicotinamide, inhibited the ABA-induced $[Ca^{2+}]_{cyt}$ elevation in guard cells as well as partly restrained ABA-induced stomatal closure²⁰⁾²¹⁾, suggesting that the pathway involving cADPR functions parallel to other Ca^{2+} -dependent pathways in the ABA signal transduction.

The production of $InsP_3$ from phosphatidylinositol 4,5-bisphosphate by hydrolysis mediated by phospholipase C triggers the $[Ca^{2+}]_{cyt}$ elevation in guard cells, resulting in stomatal closure²²⁾. The $InsP_3$ content slightly increases in the ABA-treated guard cell protoplasts and ABA-induced stomatal closure is delayed by a phospholipase C inhibitor U-73122¹¹⁾. In addition, production of myo-inositol hexakisphosphate ($InsP_6$) induced by ABA inhibits the inward K^+ channels in a Ca^{2+} -dependent manner in the guard cells²³⁾, suggesting that $InsP_6$ is one of the regulators of the $[Ca^{2+}]_{cyt}$ elevation.

4. OBSERVATION OF $[Ca^{2+}]_{cyt}$ CHANGE IN THE GUARD CELLS

Generally, there are two available techniques to observe the $[Ca^{2+}]_{cyt}$ change in the guard cells; one is to use calcium-sensitive fluorescence dyes, such as fluo-3 AM and fura-2, and another is to use calcium sensor proteins. For the $[Ca^{2+}]$ measurement in living cells, the sensor proteins have several advantages versus the fluorescence dyes because the sensor proteins allow us to monitor $[Ca^{2+}]$ in a desired cellular or subcellular location.

One of the sensor proteins, Yellow Cameleon, is composed of a donor chromophore (Cyan Fluorescent Protein, CFP), calmodulin (CaM), a glycylglycine linker, the CaM-binding peptide of myosin light-chain kinase (M13), and an acceptor chromophore (Yellow Fluorescent Protein, YFP) (**Fig.2**)²⁴⁾. The binding of Ca^{2+} to CaM intensifies the intramolecular interaction between CaM and M13, which brings CFP and YFP close to each other, resulting in an increased efficiency of the Förster Resonance Energy Transfer (FRET) between CFP and YFP²⁴⁾.

Fig.2 Simplified structure of Yellow Cameleon used to detect cellular Ca^{2+} changes. Upon an increase in the Ca^{2+} concentration, calmodulin (CaM) binds Ca^{2+} and interacts with M13, which induces a conformational change in the protein. This change leads to an increase in the efficiency of FRET from CFP to YFP.

In other words, CFP and YFP stand apart from each other in the Cameleon at a lower Ca^{2+} concentration, causing a higher intensity of the CFP fluorescence at 480 nm due to the lower FRET efficiency, whereas CFP and YFP stand close to each other at a higher Ca^{2+} concentration, leading to a lower intensity of the YFP fluorescence at 535 nm due to the higher efficiency (**Fig.2**). Consequently, an increase in $[Ca^{2+}]$ elevates the ratio of the YFP fluorescence intensity to the CFP fluorescence intensity. The continuous monitoring of the fluorescence ratio allows the real-time imaging of $[Ca^{2+}]$.

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