# Regulation of Cytosolic Alkalization and Cytosolic Calcium Elevation in Arabidopsis Guard Cells

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**Md. Tahjib-Ul-Arif**

**Graduate School of Environmental and Life Science**

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**OKAYAMA UNIVERSITY, JAPAN**

# Regulation of Cytosolic Alkalization and Cytosolic Calcium Elevation in Arabidopsis Guard Cells

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**Submitted by**

**Md. Tahjib-Ul-Arif**

**Department of Biofunctional Chemistry**

**Graduate School of Environmental and Life Science**

**Okayama University, Japan**

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# Chapter 1

# General Introduction

## 1.1. Stomata

Stomata are microscopic pores that can be found in the epidermis of leaves, young stems, and other plant organs. The stomata are most common on green aerial parts of plants, particularly the leaves. These minute pores are involved in the exchange of carbon dioxide and water between plants and the atmosphere. A pair of specialized parenchyma cells known as guard cell surround the pore and are responsible for controlling the size of the stomatal opening. Plants modify the volume of guard cells to regulate the degree of stomatal openings in order to grow and adapt to changing environmental conditions. Guard cells sense multiple external and internal signals, such as light, drought, $CO_2$, relative humidity, ozone, microbial invasion, and phytohormones, resulting in turgor-driven volume change (Melotto et al., 2008; Murata et al., 2015; Schroeder et al., 2001; Shimazaki et al., 2007). Because of tiny size, fast generation period, huge number of offspring, fully sequenced genome, and availability of mutations, *Arabidopsis thaliana* is frequently used in studies about the mechanisms driving stomatal movement (The Arabidopsis Genome Initiative, 2000).

## 1.2. Abscisic acid and methyl jasmonate

A 15-carbon terpenoid molecule named as abscission II was discovered in cotton fruit and then in sycamore leaves named as dormin. Following the purification, the chemical structures of abscission II and dormin were determined in 1965 and it was found that the both compounds were chemically identical and renamed as abscisic

acid (ABA) (Addicott, 1983). Later, it has been discovered that the levels of ABA increased considerably under drought condition and that ABA induced stomatal closure (Kwak et al., 2002; Murata et al., 2001; Schroeder et al., 2001). ABA plays an important role as signalling molecules in the physiological responses of plants to several environmental stresses (Finkelstein, 2013).

Methyl jasmonate (MeJA) is a methyl ester widely distributed in the plant kingdom. It was first detected as a fragrant compound present in *Jasminum* essential oil and other plant species. MeJA is synthesized via the lipoxygenase pathway and is known to regulate plant growth and response to environmental stresses (González-Aguilar et al., 2006). This natural compound, either as a vapor or as an emulsion, has been shown to control microbial proliferation in fresh and processed fruits and vegetables (Ayala-Zavala et al., 2005). It has been reported that similar to ABA, MeJA induces stomatal closure in various plant species (Gehring et al., 1997; Liu et al., 2005; Raghavendra and Reddy, 1987; Suhita et al., 2003). In guard cells, the MeJA signal pathway shares several signal components with the ABA signal pathway to induce stomatal closure.

### 1.3. Second messengers in stomatal signaling

Several second messengers, such as reactive oxygen species (ROS), nitric oxide (NO), calcium ($Ca^{2+}$), cytosolic pH ($pH_{cyt}$), hydrogen sulfide ($H_2S$), phosphatidic acid, cyclic guanosine monophosphate (cGMP), and cyclic adenosine diphosphate ribose (cADPR), involved in stomatal signal transduction have been identified. $H_2O_2$ and $Ca^{2+}$ are two of them that have been extensively researched and are involved in a variety of different guard cell signaling pathways (Hossain et al., 2014; Khokon et al., 2011; Sobahan et al., 2015; Ye et al., 2013). The roles of ROS, $pH_{cyt}$, and cytosolic

$Ca^{2+}$ in guard cell signaling are summarized in the following sections.

### *1.3.1. Reactive oxygen species*

Reactive oxygen species are chemical species that are formed due to the partial reduction of oxygen, which includes $H_2O_2$, superoxide anion ($O_2^{\bullet-}$), singlet oxygen, and hydroxyl radical. Environmental and endogenous stresses elicit ROS production that is related to guard cell signaling. Mainly, ROS production in guard cells is mediated by two mechanisms such as plasma membrane-localized NAD(P)H oxidases and cell wall class III peroxidases. In ABA and MeJA signaling, ROS synthesis in the guard cell is catalyzed by two NAD(P)H oxidases, AtrbohD and AtrbohF (Jannat et al., 2012, 2011; Kwak et al., 2003), whereas, in salicylic acid and isothiocyanate signaling, ROS production in guard cell is catalyzed by SHAM-sensitive apoplastic class III peroxidases (Hossain et al., 2013; Khokon et al., 2011). Usually, the $H_2O_2$ is produced via dismutation by unknown superoxide dismutases, that $H_2O_2$ can accumulate in guard cell via diffusion and water channels (Bienert et al., 2007; Henzler and Steudle, 2000). $H_2O_2$ plays a vital role in the activation of non-selective $Ca^{2+}$-permeable channels which lead $Ca^{2+}$ influx from guard cells (Pei et al., 2000). In turn, cytosolic $Ca^{2+}$ can both directly and indirectly activate NAD(P)H oxidases, resulting in more $H_2O_2$ production (Kobayashi et al., 2007; Ogasawara et al., 2008). However, it is still unknown, whether this control is necessary for guard cell signaling that leads to stomatal closure.

### *1.3.2. Cytosolic pH*

Stomatal closure is triggered by osmotically driven changes in cell volume caused by $K^+$ and anion efflux through plasma membrane localized channels. ABA and MeJA

cause guard cell shrinkage via cytosolic free calcium concentration ($[Ca^{2+}]_{cyt}$) elevations (Munemasa et al., 2007) and $pH_{cyt}$ changes (Suhita et al., 2004). Changes in $[Ca^{2+}]_{cyt}$ and $pH_{cyt}$ caused by ABA influence inward- and outward-rectifying $K^+$ channels, as well as $Cl^-$ channels, biasing the membrane for KCl efflux and loss of cell turgor (Blatt, 2000). Blatt and Armstrong (1993) demonstrated that external sodium butyrate-mediated acidification of the cytoplasm inhibited the outward $K^+$ channel, prevented ABA-induced cytoplasm alkalization, and blocked ABA-induced activation of the outward $K^+$ channel, arguing for cytoplasmic pH regulation and positioning pH in the signaling chain leading from ABA to this channel (Schroeder and Hagiwara, 1989; Vahisalu et al., 2008). MeJA also reduces inward-rectifying $K^+$ current in Arabidopsis (Saito et al., 2008) and simultaneously stimulates outward-rectifying $K^+$ current in *Vicia* (Evans, 2003). These results indicate that elevation of $[Ca^{2+}]_{cyt}$ and $pH_{cyt}$ function in ion mobilization. It has been reported that cytosolic alkalization is required by ABA- and MeJA-induced stomatal closure and a common signal component of ABA and MeJA guard cell signaling (Islam et al., 2010).

### *1.3.3. Cytosolic calcium elevation*

Cytosolic free $Ca^{2+}$ elevation was first observed in guard cells treated with ABA (McAinsh et al., 1990) and subsequently in guard cells treated with external $Ca^{2+}$, $H_2O_2$, and cold (Allen et al., 2000; McAinsh et al., 1995). The role of $[Ca^{2+}]_{cyt}$ elevation in ABA signaling is not yet fully discovered. One group of scientists has demonstrated that $[Ca^{2+}]_{cyt}$ elevations are essential for stomatal closure (Gilroy et al., 1990). Other groups of researchers have shown slow stomatal closure in Arabidopsis under conditions that prevented $[Ca^{2+}]_{cyt}$ elevations in guard cells (Siegel et al., 2009). Some other groups, however, have shown by revealing uninterrupted downstream

events that $[Ca^{2+}]_{cyt}$ elevations are not needed for stomatal closure (Romano et al., 2000). Furthermore, another group has proposed that there may be two ABA signal transduction pathways involved in ABA-induced stomatal closure: one is $Ca^{2+}$-dependent and another is $Ca^{2+}$-independent (Allan et al., 1994). More recently another group of scientists hypothesized that certain threshold level of $[Ca^{2+}]_{cyt}$ elevations regulates stomatal closure and that defined pattern of $[Ca^{2+}]_{cyt}$ elevations is needed to inhibit stomatal reopening or to maintain closure (Allen et al., 2001).

Live guard cell imaging based on a $Ca^{2+}$-sensing fluorescent protein, yellow cameleon, has greatly advanced our understanding of $Ca^{2+}$ signaling in guard cells (Nagai et al., 2004). From findings based on this technique, we know that stomata respond to a specific pattern of elevation of $[Ca^{2+}]_{cyt}$ to close (Allen et al., 2001). $Ca^{2+}$ influx is essential for elevation of $[Ca^{2+}]_{cyt}$ induced by ABA in guard cells (Klusener et al., 2002; Siegel et al., 2009). $Ca^{2+}$ release from internal stores also contribute to the elevation of $[Ca^{2+}]_{cyt}$ (García-Mata et al., 2003; Klusener et al., 2002). In Arabidopsis, there are a maximum of 250 proteins possibly having the $Ca^{2+}$-binding EF-hand motif (Day et al., 2002), which transduce the signal of $Ca^{2+}$ to downstream events. Two families of proteins are well-studied $Ca^{2+}$ sensors, the $Ca^{2+}$-dependent protein kinases (CDPKs) and the $Ca^{2+}$-sensor protein calcineurin B-like (CBL). CBLs form complexes with CBL-interacting protein kinases (CIPKs).

## 1.4. Proton pumps in guard cells

### *1.4.1. V-type ATPase*

The V-type proton ATPase (V-ATPase, VHA) is a multi-subunit enzyme complex present at the endomembranes of all eukaryotes that shares a common ancestor with the F-type ATP synthase (F-ATPase) and archaebacterial A-ATPases (Nishi and Forgac,

2002). Plants' tonoplast V-ATPase is abundant, accounting for 6.5 to 35% of tonoplast-localized protein (Ratajczak, 2000). V-ATPases are formed by two sub-complexes: a membrane peripheral complex ($V_1$) and a membrane integral complex ($V_o$), both of which are made up of 13 subunits encoded by 27 VHA-genes (Sze et al., 2002). The V1-subcomplex, which is comprised of VHA-A, VHA-B, VHA-C, VHA-D, VHA-E, VHA-F, VHA-G, and VHA-H, is responsible for ATP hydrolysis, whereas the Vo-subcomplex, which is composed of VHA-a, VHA-c, VHA-c", VHA-d, and VHA-e, translocates protons across the membrane (Schumacher, 2014). Phosphorylation of V-ATPase subunits (Hong-Hermesdorf et al., 2006), oxidation and reduction of specific cysteine residues (Dschida and Bowman, 1995), and activation of the V-ATPase by cytoplasmic $Cl^-$ (Ward and Sze, 1992) regulate the activity of V-ATPase. Furthermore, during the light/dark transition, cytoplasmic ATP/ADP ratios influence V-ATPase activity (Dietz et al., 1998).

Pharmacological treatments and mutation analyses can be used to understand more about the V-ATPase function. Concanamycin A (ConcA) and Bafilomycin A (BafA), membrane-permeable macrolide antibiotics that bind to the subunits a and c and thereby hinder proton translocation into the lumen of different compartments, are effective inhibitors of the V-ATPase (Bowman and Bowman, 2005; Huss et al., 2002; Wang et al., 2005).

In Arabidopsis, the VHA-a subunit appears in three distinct isoforms, which regulate where individual V-ATPase complexes are located inside the cell. V-ATPase complexes with the VHA-a1 subunit are found in the trans-Golgi network/early endosome (TGN/EE), whereas VHA-a2 or VHA-a3 isoforms are found in the tonoplast (Dettmer et al., 2006). The vacuolar lumen is alkalized to a pH of 6.4 in the double mutant, *vha-a2 vha-a3*, lacking both tonoplast-localized VHA-a isoforms, compared

to 5.8 in wild-type vacuoles (Krebs et al., 2010). Moreover, the de-etiolated 3 (*det3*) mutant that has a reduced mRNA level of VHA-C resulting in only 40% of the wild-type V-ATPase activity (Schumacher et al., 1999), inhibited $Ca^{2+}$ and oxidative stress induced stomatal closure (Allen et al., 2000). The aforementioned examples demonstrate that a V-ATPase can be involved in the regulation of cytosolic alkalization.

### *1.4.2. P-type proton ATPase*

P-type proton ATPases ($H^+$-ATPases, AHA) are another form of proton pump found in plant plasma membranes (PM). They are in responsible of establishing and maintaining the electrochemical gradient at the plasma membrane, which is employed by secondary transporters to move ions and organic molecules. PM-$H^+$-ATPases are involved in salt tolerance, stomata opening, intracellular pH regulation, and cell elongation, in addition to nutrient intake from the soil and mineral distribution throughout the plant (Morsomme and Boutry, 2000; Sondergaard et al., 2004). Arabidopsis contains 11 members of the PM-$H^+$-ATPase family (*AHA1-11*) (Gaxiola et al., 2007). The highest expression levels are shown by *AHA1* and *AHA2,* with more *AHA1* transcript found in shoots whereas *AHA2* is higher in roots (Haruta et al., 2010).

## 1.5. Anion channels in guard cells

### *1.5.1. S-type anion channel*

S-type anion channels are encoded by a small family of genes that are homologous to transporters found in various kingdoms (Chen et al., 2010; Negi et al., 2008). The founding member of this family is Slow Anion Channel 1 (SLAC1), the name given to an S-type channel by Klaus Raschke (Linder and Raschke, 1992). Loss of SLAC1 results in stomata that are unable close in response to high $CO_2$ levels and low relative

humidity, whereas they close very slowly in darkness. SLAC1 preferentially conducts $Cl^-$ and $NO_3^-$, whereas conductivity for malate is negligible (Chen et al., 2010; Geiger et al., 2009). The SLAC1 Homolog 3 (SLAH3) channel has the highest permeability for $NO_3^-$; in contrast to SLAC1, SLAH3 also requires extracellular $NO_3^-$ to induce its activity (Geiger et al., 2011). Both SLAC1 and SLAH3 are located in guard cells, suggesting that they together contribute to the release of $Cl^-$ and $NO_3^-$ during stomatal closure.

### *1.5.2. R-type anion channel*

A recent study showed that R-type channels are related to the Aluminum-activated Malate Transporters (ALMTs). Loss of an *ALMT* gene leads to a reduction in R-type anion currents in guard cells (Meyer et al., 2010) and slows down stimulus-induced stomatal closure (Meyer et al., 2010; Sasaki et al., 2010). This suggests that the gene product is part of a channel complex that releases anions from guard cells. It is also known as Quick Activating Anion Channel 1 (QUAC1). Among the ALMT family, the root-expressed ALMT1 was identified by comparison of aluminum-tolerant and $Al^{3+}$-sensitive wheat cultivars and was subsequently shown to mediate voltage-independent malate currents. In contrast, ALMT12/QUAC1 is expressed in guard cells transporting malate in an $Al^{3+}$-insensitive and highly voltage-dependent manner (Mumm et al., 2013).

## 1.6. Purpose of the study

The objectives of this study are to answer the follows questions:

- Do ABA- and MeJA-induced cytosolic alkalization in guard cells require V-ATPase or PM-$H^+$-ATPase?
- Do extracellular $Ca^{2+}$- induced cytosolic alkalization and stomatal closure require V-ATPase or PM-$H^+$-ATPase?
- Is tonoplast V-ATPase or TGN/EE V-ATPase required for ABA and MeJA-induced cytosolic alkalization in guard cells?
- Is there a relationship between cytosolic alkalization and $[Ca^{2+}]_{cyt}$ elevation in guard cells?
- Does cytosolic alkalization regulate the $[Ca^{2+}]_{cyt}$ elevations in guard cells?
- Are anion channels in guard cells involved in the regulation of properties of $[Ca^{2+}]_{cyt}$ elevation induced by extracellular $Ca^{2+}$?

# CHAPTER 2

# Regulation of cytosolic alkalization and interaction of cytosolic alkalization with cytosolic calcium elevation in Arabidopsis guard cells

## 2.1. Abstract

Abscisic acid (ABA)- and methyl jasmonate (MeJA)-induced stomatal closure are accompanied by cytosolic alkalization and cytosolic free calcium ($[Ca^{2+}]_{cyt}$) elevation in guard cells. In this study, I investigated the roles of $H^+$-ATPase and V-ATPase on ABA-, MeJA-, and external $Ca^{2+}$-induced cytosolic alkalization in *Arabidopsis thaliana* guard cells using *aha1-9* (plasma membrane $H^+$-ATPase loss-of-function), *det3-1* [(having 60% reduced V-ATPase activity both in tonoplast and trans-Golgi network/ early endosome (TGN/EE)], and *vha-a2 vha-a3* (having no V-ATPase activity only in tonoplast) Arabidopsis mutants. I also investigated the regulatory mechanism of $[Ca^{2+}]_{cyt}$ elevation by cytosolic alkalization. ABA and MeJA induced cytosolic alkalization and stomatal closure in wild-type plant, *aha1-9*, and *det3-1* mutants but delayed in *vha-a2 vha-a3* mutant, whereas external $Ca^{2+}$ elicited cytosolic alkalization and stomatal closure in wild-type plant and *aha1-9* mutant but not in *det3-1* and *vha-a2 vha-a3* mutants. The frequency of ABA-induced $[Ca^{2+}]_{cyt}$ elevation in *aha1-9* and *det3-1*, and *vha-a2 vha-a3* guard cells is comparable to that of wild-type guard cells. In response to external $Ca^{2+}$, *det3-1* and *vha-a2 vha-a3* showed long-lasting $[Ca^{2+}]_{cyt}$ elevation in guard cells, with a frequency that differed significantly from wild-type. Methylamine, an cytosolic alkalizing agent, induced cytosolic alkalization, $[Ca^{2+}]_{cyt}$ elevation, and stomatal closure both in *det3-1* and *vha-a2 vha-a3* mutants. These

results suggest that tonoplast-localized V-ATPase is one the regulators of ABA- and MeJA-induced cytosolic alkalization in guard cells. However, $Ca^{2+}$-dependent cytosolic alkalization solely regulated by tonoplast-localized V-ATPase in guard cells. Plasma membrane $H^+$-ATPase is not involved in cytosolic alkalization in response to ABA, MeJA, and external $Ca^{2+}$. Moreover, our results suggests that cytosolic alkalization regulates transient elevations of $[Ca^{2+}]_{cyt}$ in Arabidopsis guard cells.

## 2.2. Introduction

Pairs of guard cells form stomatal pores on the surfaces of leaves, which regulate water loss and carbon dioxide ($CO_2$) availability for photosynthesis. To acclimatize and thrive under a variety of environmental conditions, plants must optimally regulate stomatal opening and closing. Phytohormones such as abscisic acid (ABA), methyl jasmonate (MeJA), and salicylic acid, higher concentrations of extracellular $Ca^{2+}$, and increased concentration of $CO_2$ cause stomatal closure (Hossain et al., 2011; MacRobbie, 1992; Young et al., 2006), which is accompanied by reactive oxygen species (ROS) production, cytosolic alkalization, and cytosolic free calcium concentration ($[Ca^{2+}]_{cyt}$) elevation (Allen et al., 2001, 2000; Mori et al., 2006; Pei et al., 2000; Young et al., 2006; Kwak et al., 2003; Suhita et al., 2004). In Arabidopsis guard cell, ROS activates $Ca^{2+}$ permeable channels and elicits $[Ca^{2+}]_{cyt}$ elevation (Munemasa et al., 2007; Murata et al., 2001; Pei et al., 2000). The elevated $[Ca^{2+}]_{cyt}$ activates S-type anion channels and inhibits inward $K^+$ channels in the plasma membrane of guard cells to accelerate efflux of anion and $K^+$ from cytosol to apoplast, which causes stomatal closure (Allen et al., 1999; Schroeder and Hagiwara, 1989; Vahisalu et al., 2008).

The cytoplasm of guard cells become alkaline during ABA and MeJA-induced stomatal closure, whereas it become acidic prior to stomatal opening in response to kinetin, indoleacetic acid, and fusicoccin (Gehring et al., 1990; Gonugunta et al., 2009). Cytosolic alkalization activates outward $K^+$ channels and inactivates inward $K^+$ channels to promote net efflux of $K^+$ from guard cells (Blatt and Armstrong, 1993). In contrast, cytosolic acidification activates inward $K^+$ channels (Grabov and Blatt, 1997) and increases $Ca^{2+}$ currents in a wide variety of cells and tissues (Li et al., 2012). These results indicate that changes in cytosolic pH are closely involved in the modulation of ion mobilization in guard cells. Therefore, changes of cytosolic pH is considered as a key factor in the regulation of stomatal movement (Gonugunta et al., 2009). However, the mechanism of ABA- and MeJA-induced cytosolic alkalization in guard cells is still unclear. The underlying mechanisms of cytosolic alkalization-induced changes of $[Ca^{2+}]$ elevation in guard cells are also still not well-understood. Allen et al. (2000) showed that extracellular $Ca^{2+}$ elicited prolonged $[Ca^{2+}]_{cyt}$ elevation and stomatal closure was abolished in de-etiolated 3 (*det3*) mutant (having reduced V-type ATPase activity). Conversely, ABA elicited transient elevations of $[Ca^{2+}]_{cyt}$ in *det3* and stomatal closure occurred normally. However, cytosolic alkalization in this mutant in response to ABA, MeJA, and exogenous $Ca^{2+}$ has not been studied yet.

The changes of cytosolic pH are dependent on the coordinated action of several proton ($H^+$) pumps or transporters at the plasma membrane (PM) and endomembrane. The P-type ATPases ($H^+$-ATPase, AHA) pump $H^+$ to the apoplast, and the V-type ATPases (V-ATPase, VHA) pump $H^+$ to intracellular compartments (Sze and Chanroj, 2018). Plasma membrane $H^+$-ATPases are in responsible for establishing and maintaining the electrochemical gradient at the plasma membrane (Morsomme and Boutry, 2000; Sondergaard et al., 2004). Arabidopsis contains 11 members of

the PM-$H^+$-ATPases (AHA1-11) (Gaxiola et al., 2007). The highest expression levels are shown by AHA1 and AHA2, with more AHA1 transcript found in shoots whereas AHA2 is higher in roots (Haruta et al., 2010). V-ATPase is a highly conserved multi-subunit endomembrane proton pump (Kluge et al., 2003; Ratajczak, 2000) consist of a cytosolic ATP-hydrolyzing $V_1$ domain (subunits A to H) and a membrane-bound proton-translocating $V_0$ domain (subunits a, c, c′, c″, d, and e) (Nishi and Forgac, 2002; Schumacher and Krebs, 2010). Vacuolar pH in the *vha-a2 vha-a3* double mutant that lacks both tonoplast-localized isoforms of VHA-a is increased to 6.4 compared to 5.9 in the wild-type leading to a strong reduction in growth and nutrient storage capacity (Krebs et al., 2010). Previous study also reported that inhibition of V-ATPase activity delayed ABA-induced cytosolic alkalization in guard cells (Bak et al., 2013). However, it remains unclear whether MeJA and extracellular $Ca^{2+}$-induced cytosolic alkalization require V-ATPase.

In the present study, I examined: (i) ABA- and MeJA-induced cytosolic alkalization, $[Ca^{2+}]_{cyt}$ elevation, and stomatal movement, (ii) extracellular $Ca^{2+}$-induced cytosolic alkalization, $[Ca^{2+}]_{cyt}$ elevation, and stomatal closure in Arabidopsis mutants; and (iii) the effect of methylamine, a cytosol-alkalizing agent, on stomatal movement, cytosolic alkalization, and $[Ca^{2+}]_{cyt}$ elevation in Arabidopsis.

## 2.3. Materials and Methods

### *2.3.1. Plant materials and growth conditions*

In this study, *Arabidopsis thaliana* wild-type Columbia-0 (Col-0) and loss-of-function mutant *aha1-9*, *det3-1*, *vha-a2*, *vha-a3*, and *vha-a2 vha-a3* were used. Plants were grown on a soil mixture [vermiculite (Asahi-kogyo, Okayama, Japan): Kureha soil (Kureha Chemical, Tokyo, Japan), 1:1 (v/v)] in the growth chamber at 21±2°C and

80 μmol $m^{-2}$ $s^{-1}$ light intensity under a 16 h light/8 h dark condition. The plants were watered with deionized water containing 0.1% (v/v) Hyponex (Hyponex Japan, Osaka, Japan) once a week. Rosette leaves from four to five week-old plants were used for all experiments.

### *2.3.2. Measurement of stomatal aperture*

Stomatal apertures were measured as described previously (Hossain et al., 2011). The excised rosette leaves were floated on stomatal bioassay solution containing 5 mM KCl, 50 μM $CaCl_2$, and 10 mM MES-Tris (pH 6.15) under light condition (80 μmol $m^{-2}s^{-1}$) for 2 h to open stomata. After that, ABA, MeJA, $CaCl_2$, or methylamine were added to the stomatal bioassay solution and incubated for another 2 h. The final concentrations of ABA and MeJA on stomatal bioassay solution were 10 μM, and of $CaCl_2$ and methylamine were 10 mM and 2 mM, respectively. The incubated leaves were blended by a commercial blender (700BUJ, Waring Commercial, Torrington, Connecticut) and epidermal tissues were collected using a nylon mesh. At least 20 stomatal apertures were measured on each distinct experiment using WinRoof 3.0 software (Mitani Corporation, Fukui and Tokyo, Japan). Following the similar procedure three independent experiments were performed.

### *2.3.3. Detection of ROS in guard cells*

Production of ROS in guard cells was examined using the fluorescent dye 2′,7′-dichlorodihydrofluorescein diacetate ($H_2$DCF-DA; Sigma, St Louis, MO), as described previously (Afrin et al., 2020; Khokon et al., 2011). Briefly, the detached leaves were shredded in the commercial blender and the resulting fragments were collected by a 100 μm pore nylon mesh. The epidermal fragments were soaked in the stomatal

bioassay solution under light for 2 h and then incubated in the bioassay solution supplemented with 50 μM $H_2$DCF-DA at 25±1°C under dark conditions for 30 min. Following this, the epidermal fragments were collected again using 100 μm pore nylon mesh and washed with the stomatal bioassay solution. The samples were resuspended in the bioassay solution and treated with 10 μM ABA, 10 μM MeJA, 10 mM $CaCl_2$, or 2 mM methylamine at 25±1 °C, under dark conditions for another 30 min. Subsequently, fluorescence of guard cells was observed under a fluorescence microscope (Biozero BZ-8000, KEYENCE, Osaka, Japan) with an OP-66 835 BZ filter (excitation wavelength 480/30 nm, absorption wavelength 510 nm, and dichroic mirror wavelength 505 nm). The captured fluorescence images were analyzed using ImageJ 1.52a software (National Institutes of Health, Bethesda, MD, USA). Three independent experiments were performed. In each experiment, four or five leaves were collected from four or five independent plants and the fluorescence images from at least 20 guard cells were analyzed.

### *2.3.4. Detection of cytosolic alkalization in guard cells*

The cytosolic pH of guard cells was monitored using a fluorescent dye BCECF-AM (3′-*O*-acetyl-2′,7′-bis(carboxyethyl)-4 or 5-carboxyfluorescein, diacetoxymethyl ester; Dojindo, Kumamoto, Japan), as described previously (Islam et al., 2010). Epidermal tissues were isolated from four or five leaves with the commercial blender. The collected tissues were incubated for 2 h in the stomatal bioassay solution under light conditions. After this incubation, the tissues were incubated in the bioassay solution supplemented with 20 μM BCECF-AM for 30 min in the dark at 25±1°C to load the dye. The tissues were then washed several times with bioassay buffer in order to remove excess dye. The dye-loaded epidermal fragments were treated with 10 μM

ABA, 10 μM MeJA, 10 mM $CaCl_2$, or 2 mM methylamine for 30 min. Fluorescence images of guard cells were captured using a fluorescence microscope (Biozero BZ-8000, KEYENCE, Osaka, Japan) with the following settings: excitation 480 nm and emission 535 nm. The captured fluorescence images were analyzed using ImageJ 1.52a. Three independent experiments were performed. In each experiment, four or five leaves were collected from four or five independent plants, and the fluorescence images from at least 20 guard cells were analyzed.

### *2.3.5. Imaging of $[Ca^{2+}]_{cyt}$ in guard cells*

Fluctuations in $[Ca^{2+}]_{cyt}$ in guard cells were detected using transgenic Arabidopsis plants expressing Nuclear Export Signal (NES) Yellow Cameleon 2.6 (NES-YC2.6). Yellow Cameleons have been widely-used for plant $[Ca^{2+}]_{cyt}$ imaging but it is localized not only in the cytosol but also in the nucleus. In this study we used NES-tagged YC, which shows no detectable fluorescence signal in the nucleus (Krebs et al., 2012). The experimental procedure described previously by Afrin *et al.* (2020) was followed. The abaxial side of excised leaves were gently mounted on a glass cover slip using a medical adhesive. The adaxial epidermis and the mesophyll tissue were removed, the remaining abaxial epidermal peels were incubated in the stomatal bioassay solution consisted of 5 mM KCl, 50 μM $CaCl_2$, and 10 mM MES-Tris (pH 6.15) in a growth chamber for 2 h to promote stomatal opening. The turgid guard cells were treated with 10 μM ABA, 10 μM MeJA, 10 mM $CaCl_2$, or 2 mM methylamine at 5 min after the start of measurement. Two to three guard cells from one sample leaf for each experiment were selected. This experiment was repeated using different sample leaves on different days. The cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) fluorescence intensities (F535 and F480) of guard cells were measured

under a fluorescence microscope (IX71, Olympus, Tokyo, Japan) equipped with a dual-emission imaging system (W-View system; 440AF21 excitation filter, 445DRLP dichroic mirror and two emission filters, 480DF30 for CFP and 535DF25 for YFP; Hamamatsu Photonics, Hamamatsu, Japan) and a CCD camera (Hamamatsu ORCA-ER digital camera; Hamamatsu Photonics). The fluorescence intensities were ratiometrically analysed using AQUA COSMOS software (Hamamatsu Photonics). Same exposure time was used for both CFP and YFP. For the analysis of $[Ca^{2+}]_{cyt}$, $[Ca^{2+}]_{cyt}$ elevation was counted when changes in fluorescence ratios (F535/F480) were ≥0.1 unit from the baseline and the elevation of ratio observed only because of the increment and reduction of CFP and YFP fluorescence intensities, respectively.

### *2.3.6. Statistical analysis*

The significant differences between mean values of stomatal apertures, ROS accumulation, and cytosolic pH, were assessed by Student's t-test, and the significant differences between number of $[Ca^{2+}]_{cyt}$ elevation were assessed by a chi-squared ($\chi^2$) test.

## 2.4. Results

### *2.4.1. Effects of ABA, MeJA, and external $Ca^{2+}$ on stomatal aperture*

Previous studies showed that ABA, MeJA, and external $Ca^{2+}$ induced stomatal closure in Arabidopsis (Islam et al., 2010). I examined ABA-, MeJA-, and external $Ca^{2+}$-induced stomatal closure in wild-type plants as well as in PM $H^+$-ATPase and V-ATPase Arabidopsis loss-of-function mutants.

ABA and MeJA at 10 μM concentration induced stomatal closure in *aha1-9*, *det3-1*, *vha-a2*, *vha-a3*, and *vha-a2 vha-a3* mutants and in wild-type plant (Figure

2.1a, 2.1b). Stomatal closure elicited by ABA and MeJA was delayed in the *vha-a2 vha-a3* double mutant (ABA, $P<0.021$; MeJA, $P<0.048$) compared to the wild-type plant (ABA, $P<0.001$; MeJA, $P<0.015$) (Figure 2.1a, 2.1b).

External $Ca^{2+}$ at 10 mM also decreased stomatal apertures in wild-type and in *aha1-9*, *vha-a2*, and *vha-a3* mutants, but failed to decrease stomatal apertures in *det3-1* and *vha-a2 vha-a3* mutants (Figure 2.1c). External $Ca^{2+}$-induced stomatal closure occurred slightly later in *vha-a2* ($P<0.047$) and *vha-a3* ($P<0.043$) single mutants than in wild-type plant (Figure 2.1c).

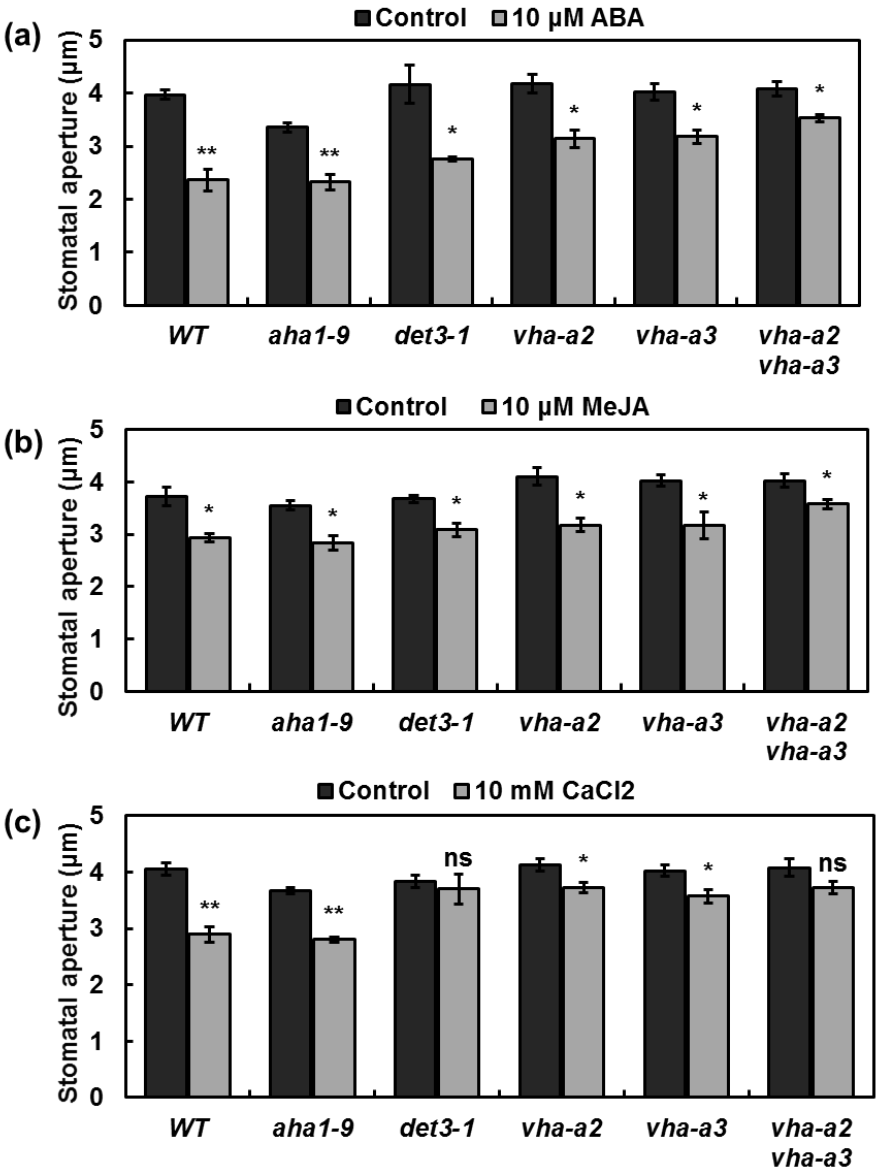


Figure 2.1. Stomatal response to ABA, MeJA, and external $Ca^{2+}$ in Arabidopsis.

Effects of ABA **(a)**, MeJA **(b)**, and external $Ca^{2+}$ **(c)** on stomatal apertures (width, μm) in wild-type, *aha1-9*, *det3-1*, *vha-a2*, *vha-a3*, and *vha-a2 vha-a3* Arabidopsis plants. The abaxial side of rosette leaves were pre-incubated on a stomatal bioassay solution for 2 h under light, followed by 2 h incubation with 10 μM ABA, 10 μM MeJA, or 10 mM $CaCl_2$ containing stomatal bioassay solution under light condition. Data are the mean ± SE (of three independent experiments, >60 stomata for each data point) and statistical difference was determined by Student's t-test, $*P<0.05$ and $**P<0.01$. The 'ns' indicates non-significant difference where $P>0.05$.

### *2.4.2. Effects of ABA, MeJA, and external $Ca^{2+}$ on cytosolic alkalization in guard cells*

ABA, MeJA, and external $Ca^{2+}$ increased $pH_{cyt}$ in wild-type guard cells (Gehring et al., 1997; Gonugunta et al., 2009; Irving et al., 1992; Suhita et al., 2004). In this study, I examined ABA-, MeJA-, and external $Ca^{2+}$-induced cytosolic alkalization in wild-type, *aha1-9*, *det3-1*, *vha-a2*, *vha-a3*, and *vha-a2 vha-a3* guard cells in order to investigate the roles of PM $H^+$-ATPase and V-ATPase in ABA, MeJA, and $Ca^{2+}$ signaling of guard cells.

ABA and MeJA significantly increased BCECF fluorescence intensity in wild-type guard cells (Figure 2.2a). This result indicated that ABA and MeJA induced cytosolic alkalization, which is similar to a previous report (Suhita et al. 2004; Islam et al. 2010). ABA and MeJA also increased BCECF fluorescence intensity in *aha1-9*, *det3-1*, *vha-a2*, *vha-a3*, and *vha-a2 vha-a3* guard cells (Figure 2.2a, 2.2b). However, ABA ($P<0.035$) and MeJA ($P<0.013$) induced cytosolic alkalization were slightly reduced in *vha-a2 vha-a3* mutant (ABA, $P<0.035$; MeJA, $P<0.013$) guard cells than that of wild-type (ABA, $P<0.0006$; MeJA, $P<0.002$) guard cells (Figure 2.2a, 2.2b).

External $Ca^{2+}$ increased BCECF fluorescence intensity in wild-type plants and in *aha1-9*, *vha-a2*, and *vha-a3* mutant but did not in *det3-1* and *vha-a2 vha-a3* mutant guard cells (Figure 2.2c). External $Ca^{2+}$-induced cytosolic alkalization slightly impaired in the guard cells of *vha-a2* ($P$<0.015) and *vha-a3* ($P$<0.029) single mutants than that of wild-type ($P$<0.0001) plant (Figure 2.1c).

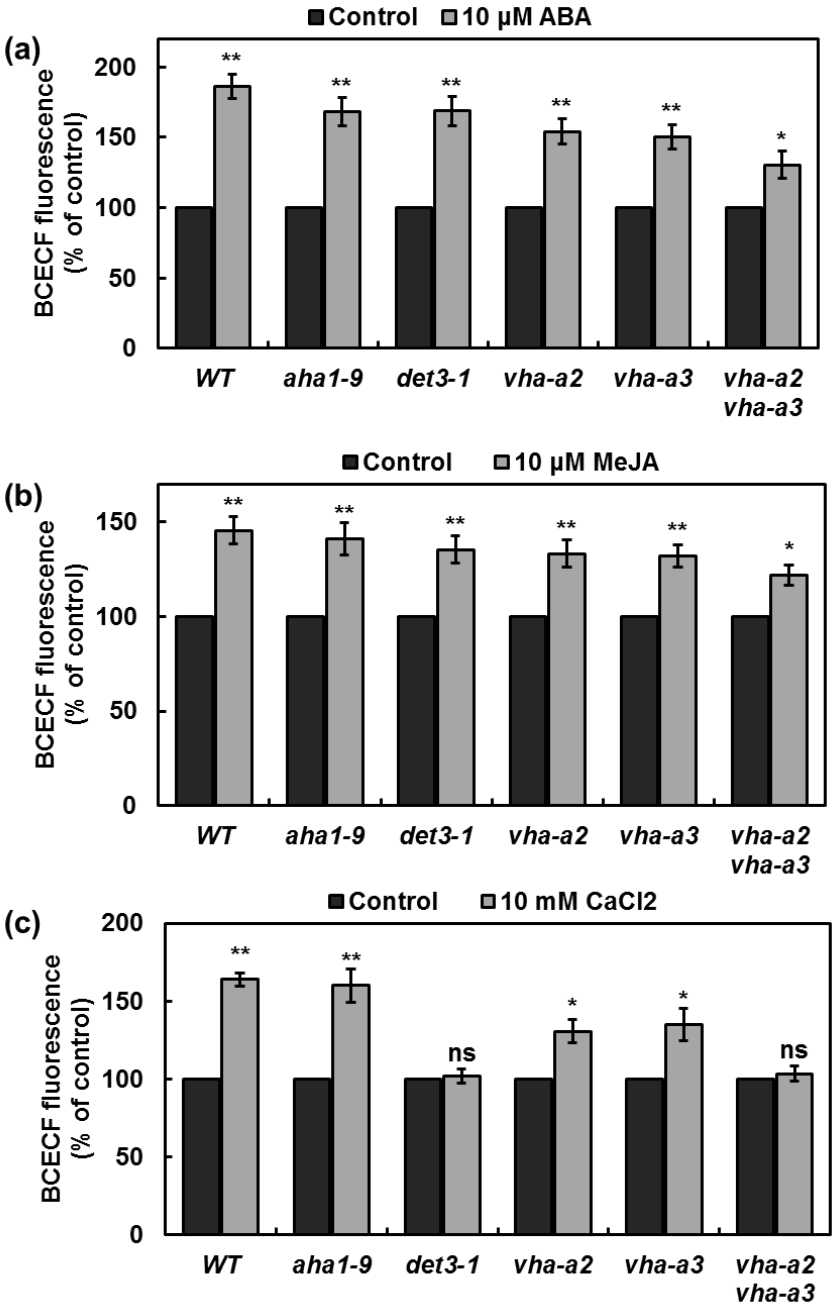


Figure 2.2. Cytosolic alkalization in response to ABA, MeJA, and external $Ca^{2+}$ in Arabidopsis guard cells.

Effects of ABA **(a)**, MeJA **(b)**, and external $Ca^{2+}$ **(c)** on cytosolic alkalization (BCECF fluorescence intensity) in wild-type plant and in *aha1-9*, *det3-1*, *vha-a2*, *vha-a3*, and *vha-a2 vha-a3* mutants. The leaf epidermal tissues were treated with 10 μM of ABA or MeJA, or with 10 mM of $CaCl_2$ for 30 minutes after treatment of BCECF-AM. Data are the mean ±SE (of three independent experiments, >60 stomata for each data point). Statistical difference was determined by Student's t-test, $*P<0.05$ and $**P<0.01$. The 'ns' indicates non-significant difference where $P>0.05$.

### *2.4.3. Effects of ABA, MeJA, and external $Ca^{2+}$ on ROS production in guard cells*

In current study, ABA-, MeJA-, and external $Ca^{2+}$-induced ROS production in the guard cells were examined. Treatment with 10 μM of ABA induced ROS production in the guard cells of *aha1-9*, *det3-1*, and *vha-a2 vha-a3* (Figure 2.3a-2.3c). Similarly, treatment with 10 μM of MeJA induced ROS production in the guard cells of *aha1-9*, *det3-1*, and *vha-a2 vha-a3* (Figure 2.3a-2.3c). External $Ca^{2+}$ induced ROS production in guard cells of *aha1-9* but failed to induce ROS production in the guard cells of *det3-1* and *vha-a2 vha-a3* mutants (Figure 2.3a-2.3c).

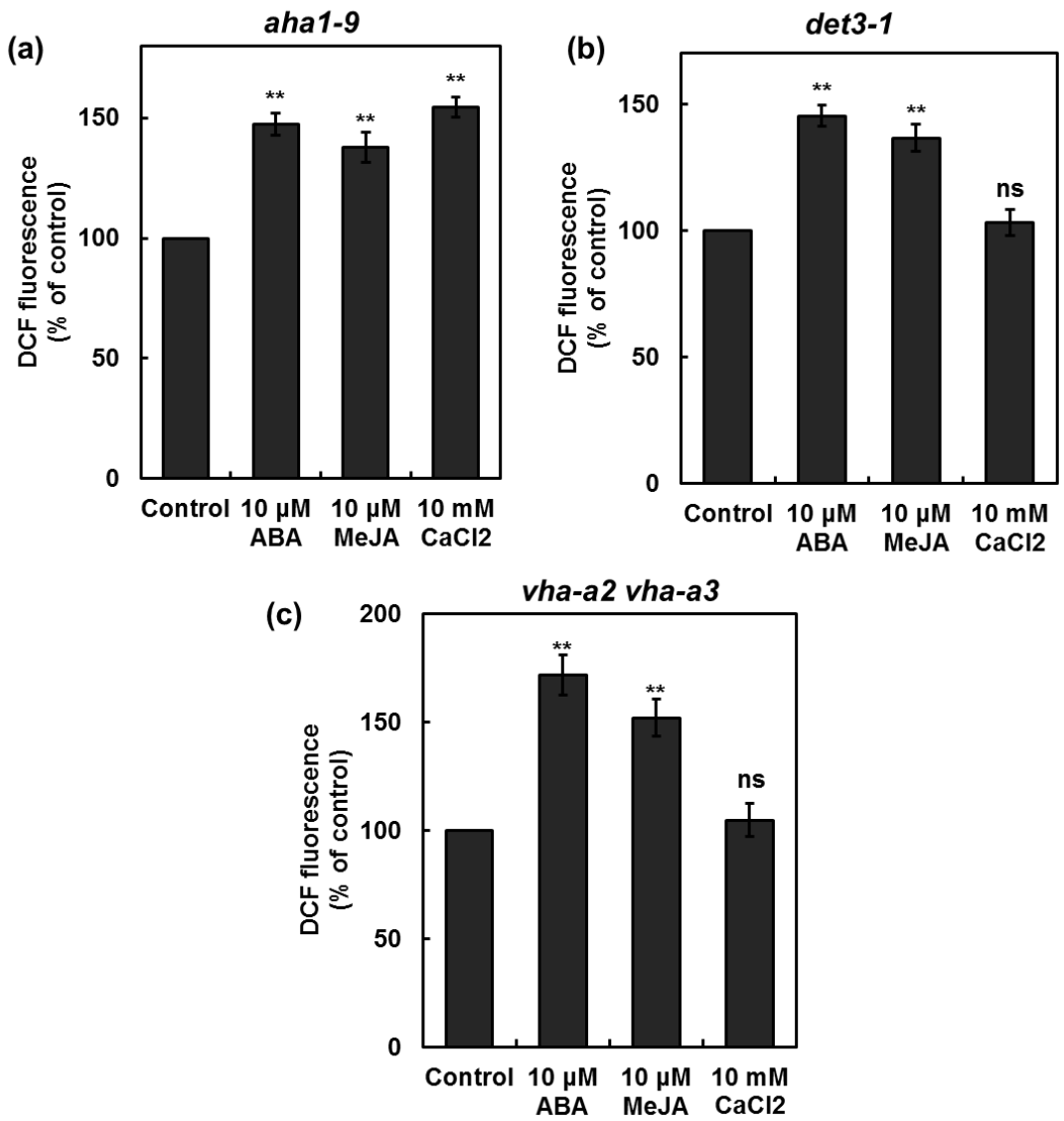


Figure 2.3. ROS production in response to ABA, MeJA, and external $Ca^{2+}$ in Arabidopsis guard cells.

Effects of ABA, MeJA, and external $Ca^{2+}$ on ROS production (DCF fluorescence intensity) in *aha1-9* **(a)**, *det3-1* **(b)**, and *vha-a2 vha-a3* **(a)** Arabidopsis mutants. The leaf epidermal tissues were treated with 10 μM of ABA or MeJA, or with 10 mM of $CaCl_2$ for 30 minutes after treatment of $H_2$DCF-AM. Data are the mean ±SE (of three independent experiments, >60 stomata for each data point). Statistical difference was determined by Student's t-test, $*P<0.05$ and $**P<0.01$. The 'ns' indicates non-significant difference where $P>0.05$.

### *2.4.4. Effects of ABA and external $Ca^{2+}$ on $[Ca^{2+}]_{cyt}$ elevation in guard cells*

Application of ABA and external $Ca^{2+}$ induce $[Ca^{2+}]_{cyt}$ elevation in Arabidopsis guard cells, leading to stomatal closure (Allen et al., 2001, 2000; Islam et al., 2010). In the present study, I examined the effects of ABA and external $Ca^{2+}$ on $[Ca^{2+}]_{cyt}$ elevation in guard cells of wild-type plant and of *det3-1* and *vha-a2 vha-a3* mutant using the YC technique.

In the presence of 10 μM ABA, 69.23% of wild-type guard cells showed $[Ca^{2+}]_{cyt}$ elevation (n = 13 cells; three or more spikes, 15.38%; one or two spikes, 53.85%) (Figure 2.4a, 2.4e). ABA elicited $[Ca^{2+}]_{cyt}$ elevation in 64.28% guard cells of *aha1-9* (n = 20 cells; three or more spikes, 14.28%; one or two spikes, 50%) (Figure 2.4b, 2.4e). ABA also elicited $[Ca^{2+}]_{cyt}$ elevation in 81.25% guard cells of *det3-1* (n = 18 cells; three or more spikes, 18.75%; one or two spikes, 62.50%) (Figure 2.4c, 2.4e) and in 42.85% guard cells of *vha-a2 vha-a3* (n = 13 cells; three or more spikes, 7.14%; one or two spikes, 35.71%) (Figure 2.4d, 2.4e). There is no significant difference in frequency of elevation between ABA-treated guard cells of wild-type and ABA-treated guard cells of *aha1-9*, *det3-1* (Figure 2.4e). However, the frequency of elevation of *vha-a2 vha-a3* was significantly lower than that of wild-type in response to ABA (Figure 2.4e).

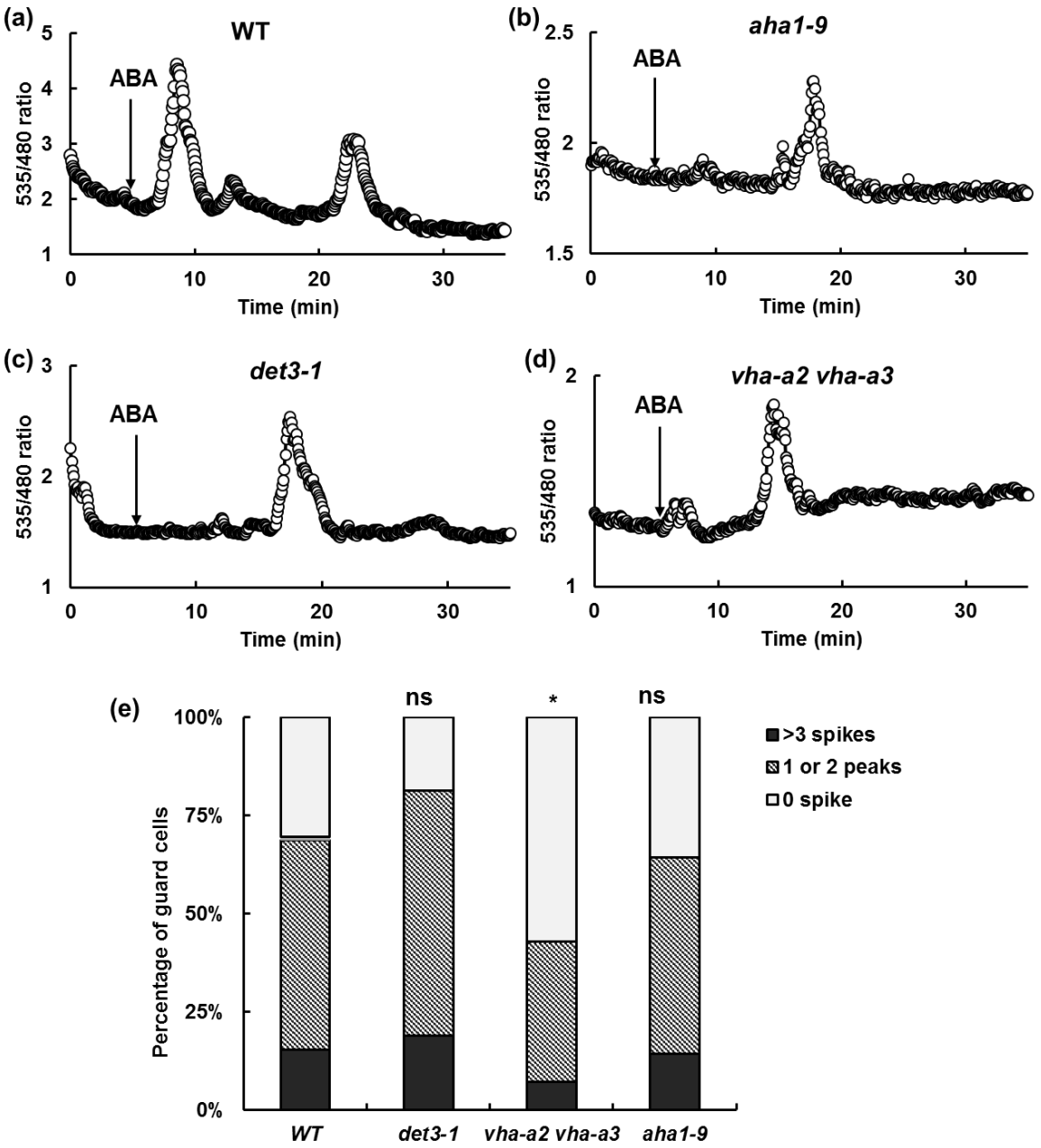


Figure 2.4. Cytosolic calcium ($[Ca^{2+}]_{cyt}$) elevation in Arabidopsis guard cells induced by ABA.

The $[Ca^{2+}]_{cyt}$ elevation in guard cells of wild-type plant and *aha1-9*, *det3-1*, and *vha-a2 vha-a3* mutants expressing NES-Yellow Cameleon 2.6 (NES-YC 2.6) was monitored. Representative traces of fluorescence emission ratios (F535/F480) showing $[Ca^{2+}]_{cyt}$ spikes in guard cells of wild-type plant **(a)** *aha1-9* **(b)**, *det3-1* **(c)**, and *vha-a2 vha-a3* **(d)** mutants. 10 μM of ABA was applied into stomatal bioassay solution containing

epidermal peel treated under light for 2 h. Percentage bar chart **(e)** showing the percent of guard cells showing $[Ca^{2+}]_{cyt}$ elevation of wild-type plant and *aha1-9*, *det3-1*, and *vha-a2 vha-a3* mutants under 10 μM ABA treatment. $[Ca^{2+}]_{cyt}$ elevations were counted when changes in fluorescence ratios (F535/F480) were ≥0.1 unit from the baseline. The significance of differences between different treatments were determined by chi-squared ($\chi^2$) test where 'ns' indicates non-significant difference at $P>0.05$ and '*' indicates significant difference at $P<0.05$.

External $Ca^{2+}$ at 10 mM elicited $[Ca^{2+}]_{cyt}$ elevation in 90% of wild-type guard cells (n = 10 cells; three or more spikes, 60%; one or two spikes, 30%) (Figure 2.5a, 2.4e). External $Ca^{2+}$ elicited $[Ca^{2+}]_{cyt}$ elevation in 88.89% guard cells of *aha1-9* (n = 18 cells; three or more spikes, 33.33%; one or two spikes, 55.56%) (Figure 2.5b, 2.4e). External $Ca^{2+}$ also elicited $[Ca^{2+}]_{cyt}$ elevation in 95.45% guard cells of *det3-1* (n = 25 cells; three or more spikes, 13.36%; one or two spikes, 81.81%) (Figure 2.5c, 2.5e) and in 95% guard cells of *vha-a2 vha-a3* (n = 21 cells; three or more spikes, 20%; one or two spikes, 75%) (Figure 2.5d, 2.4e). There is a significant difference in frequency of elevation between ABA-treated guard cells of wild-type and ABA-treated guard cells of *det3-1* and *vha-a2 vha-a3* (Figure 2.4e).

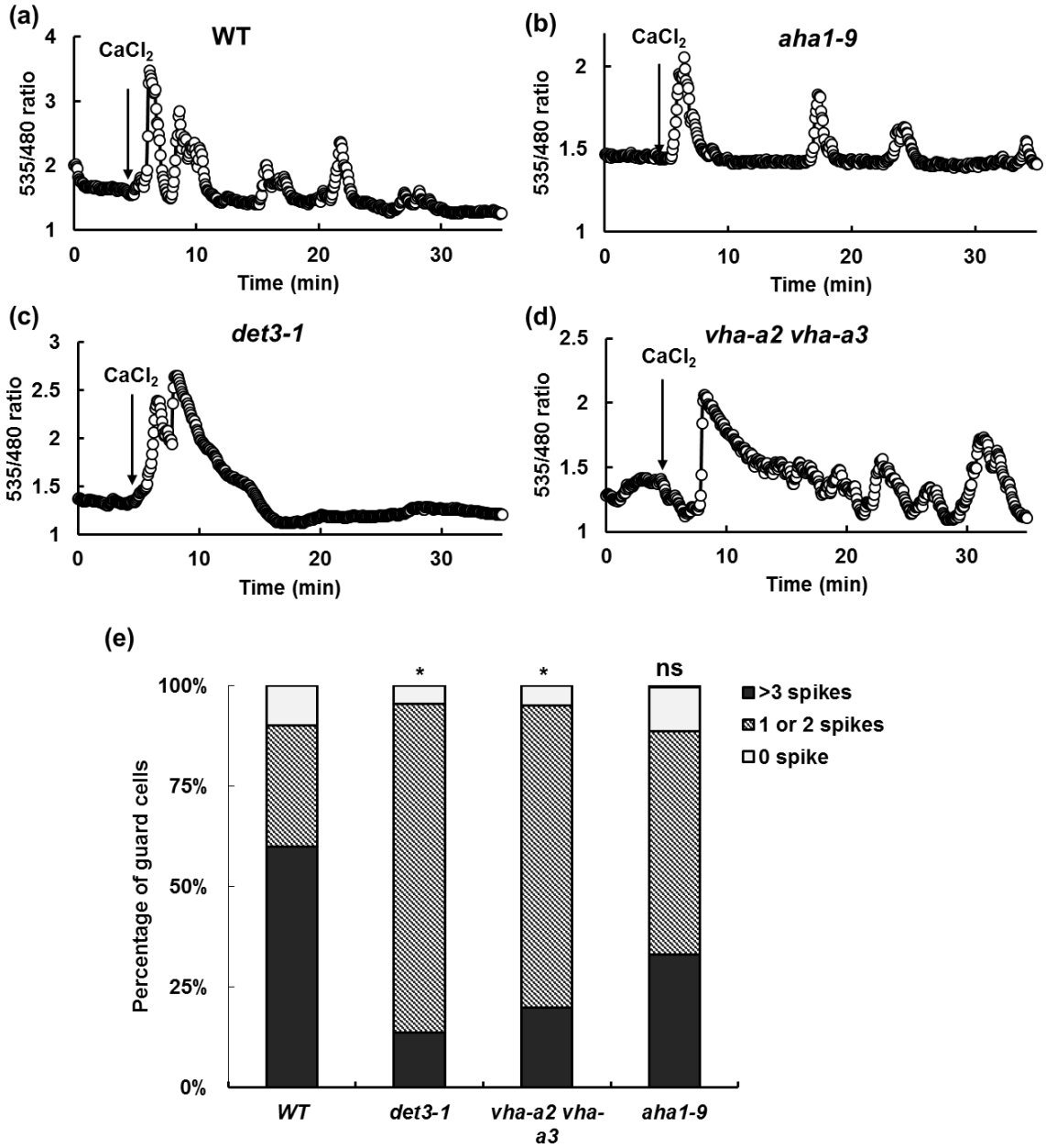


Figure 2.5. Cytosolic calcium ($[Ca^{2+}]_{cyt}$) elevation in Arabidopsis guard cells induced by external $Ca^{2+}$.

The $[Ca^{2+}]_{cyt}$ elevation in guard cells of wild-type plant and *aha1-9*, *det3-1*, and *vha-a2 vha-a3* mutants expressing NES-Yellow Cameleon 2.6 (NES-YC 2.6) was monitored. Representative traces of fluorescence emission ratios (F535/F480) showing $[Ca^{2+}]_{cyt}$ spikes in guard cells of wild-type plant **(a)** *aha1-9* **(b)**, *det3-1* **(c)**, and *vha-a2 vha-a3* **(d)** mutants. 10 mM $CaCl_2$ was applied into stomatal bioassay solution containing

epidermal peel treated under light for 2 h. Percentage bar chart **(e)** showing the percent of guard cells showing $[Ca^{2+}]_{cyt}$ elevation of wild-type plant and *aha1-9*, *det3-1*, and *vha-a2 vha-a3* mutants under 10 mM $CaCl_2$ treatment. $[Ca^{2+}]_{cyt}$ elevations were counted when changes in fluorescence ratios (F535/F480) were ≥0.1 unit from the baseline. The significance of differences between different treatments were determined by chi-squared ($\chi^2$) test where '*' indicates significant difference at $P<0.05$. The 'ns' indicates non-significant difference where $P>0.05$.

### *2.4.5. Effects of methylamine on stomatal aperture, cytosolic alkalization, and $[Ca^{2+}]_{cyt}$ elevation in guard cells*

Methylamine is a cytosolic alkalizing agent and previous studies showed that it induced stomatal closure in Arabidopsis (Zhu et al., 2014). In present study, methylamine at 2 mM also induced stomatal closure in wild-type plants, as well as in *aha1-9*, *det3-1*, and *vha-a2 vha-a3* mutants (Figure 2.6a). Moreover, methylamine treatment caused cytosolic alkalization in the guard cells of wild-type plant and *aha1-9*, *det3-1*, and *vha a2 vha a3* mutants (Figure 2.6b).

Methylamine elicited $[Ca^{2+}]_{cyt}$ elevation in 76.47% guard cells of *det3-1* (n = 17 cells; three or more spikes, 41.17%; one or two spikes, 35.29%) (Figure 2.6c, 2.6e). Methylamine also elicited $[Ca^{2+}]_{cyt}$ elevation in 81.82% guard cells of *vha-a2 vha-a3* (n = 11 cells; three or more spikes, 45.45%; one or two spikes, 36.36%) (Figure 2.6d, 2.6e).

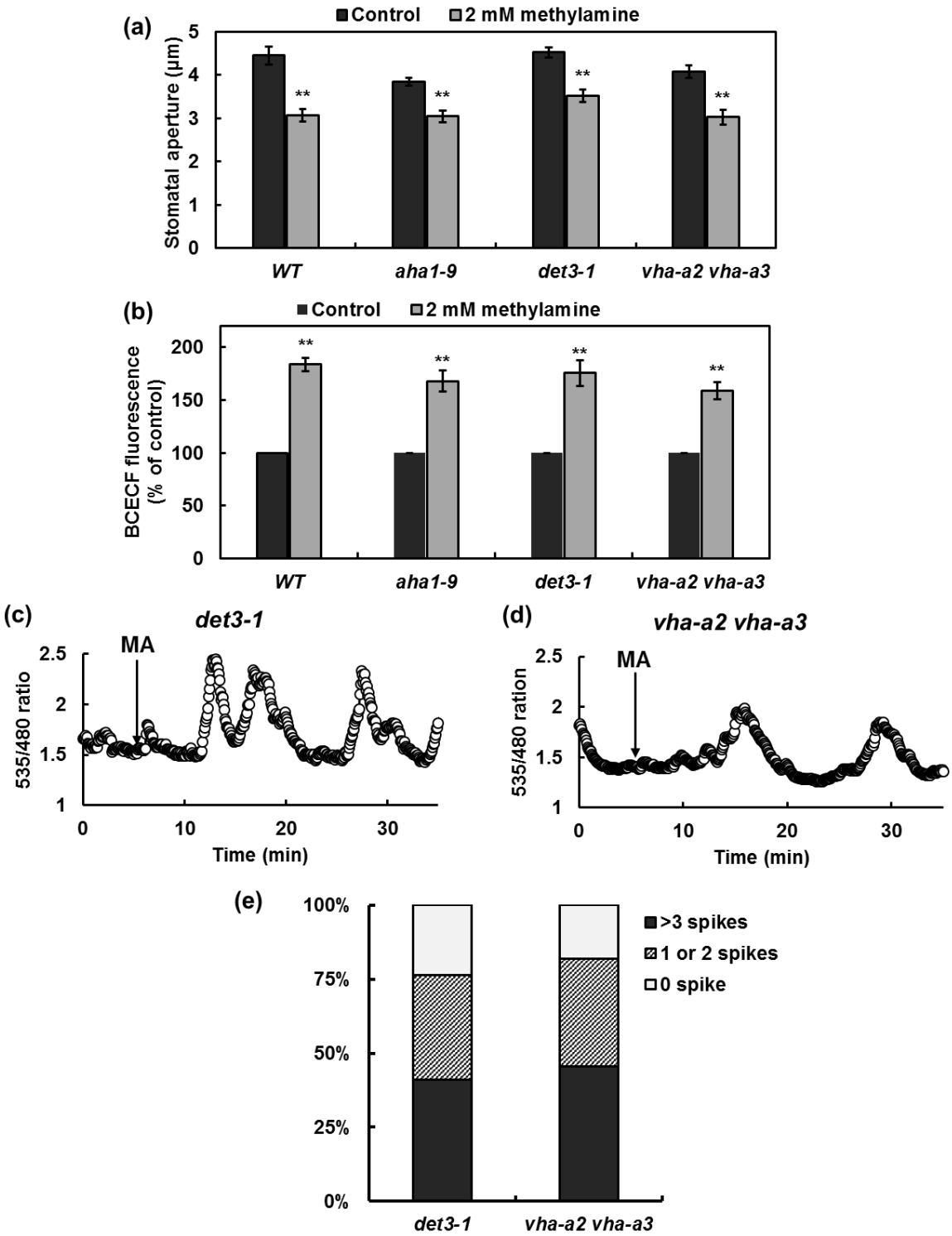


Figure 2.6. Stomatal response, cytosolic alkalization, and cytosolic calcium ($[Ca^{2+}]_{cyt}$) elevation in Arabidopsis in response to methylamine.

Effects of 10 mM methylamine on stomatal apertures (width, μm) **(a)** and cytosolic alkalization **(b)** in wild-type plant and *aha1-9*, *det3-1*, and *vha-a2 vha-a3* mutants.

The $[Ca^{2+}]_{cyt}$ elevation in guard cells of *det3-1* and *vha-a2 vha-a3* mutant expressing NES-Yellow Cameleon 2.6 (NES_YC 2.6) was monitored. Representative traces of fluorescence emission ratios (F535/F480) showing $[Ca^{2+}]_{cyt}$ spikes in guard cells of det3-1 **(c)** and vha-a2 vha-a3 **(d)** mutants. Percentage bar chart **(e)** showing the percent of guard cells showing $[Ca^{2+}]_{cyt}$ elevation of *det3-* and *vha-a2 vha-a3* mutants under 10 mM methylamine treatment. $[Ca^{2+}]_{cyt}$ elevations were counted when changes in fluorescence ratios (F535/F480) were ≥0.1 unit from the baseline. The significance of differences between different treatments were determined by chi-squared ($\chi^2$) test. The 'ns' indicates non-significant difference where $P>0.05$.

## 2.5. Discussion

Efflux of $H^+$ from cytosol to apoplast or intracellular spaces causes cytosolic alkalization. Plasma membrane $H^+$-ATPases pump $H^+$ to the apoplastic space, on the other hand, V-ATPases pump $H^+$ to the tonoplast or other intracellular stores. The key objective of this study was to figure out whether ABA-, MeJA-, and extracellular $Ca^{2+}$-induced cytosolic alkalization in guard cells require PM $H^+$-ATPase or V-ATPase. Previous reports showed that ABA inhibits blue light-dependent $H^+$ pumping via PM $H^+$-ATPase in guard cell protoplasts (Goh et al., 1996; Zhang et al., 2004), which indicates that during stomatal closing ABA inhibits PM $H^+$-ATPase and ABA-induced cytosolic alkalization was not employed by PM $H^+$-ATPase. However, in our study, ABA induced cytosolic alkalization in *aha1-9* mutant guard cells (Figure 2.2a), suggesting that PM $H^+$-ATPase is not required for ABA-induced cytosolic alkalization in guard cells. Moreover, MeJA and extracellular $Ca^{2+}$ caused cytosolic alkalization in *aha1-9* mutant guard cells (Figure 2.2b, 2.1c). This findings indicate that as like as ABA-induced cytosolic alkalization, PM $H^+$-ATPase is not involved in MeJA- and extracellular

$Ca^{2+}$-induced cytosolic alkalization in Arabidopsis guard cells. The downstream responses of cytosolic alkalization, such as $[Ca^{2+}]_{cyt}$ elevations and stomatal closure, in *aha1-9* mutant is identical to that of wild-type plants (Figure 2.1a-2.1c, 2.4b, 2.4e, 2.5b, 2.5e).

To address the specific role of tonoplast-localized V-ATPase in cytosolic alkalization, a double mutant, *vha-a2 vha-a3*, has been used in this study. VHA-a2 and VHA-a3 are isoforms of a large integral subunit localized to the tonoplast. The *vha-a2 vha-a3* mutant lacks tonoplast-localized V-ATPase activity but has a functional V-ATPase in the trans-Golgi network/early endosomes (TGN/EE) (Krebs et al., 2010). A weak V-ATPase mutant, *det3*, has a 60% reduced V-ATPase activity both in tonoplast and TGN/EE (Allen et al., 2000), which has been also used in this study. In current study, I found that both ABA- and MeJA-induced cytosolic alkalization were delayed in *vha-a2 vha-a3* guard cells more than that of *det3-1* or wild-type guard cells (Figure 2.2a, 2.2b). This result indicates that the tonoplast V-ATPase is involved in ABA- and MeJA-induced $pH_{cyt}$ change in Arabidopsis guard cells. Previously it has been reported that ABA activates V-ATPase in *Mesembryanthemum* (Barkla et al., 1999) and ABA induces vacuolar acidification in faba bean (Bak et al., 2013). The vacuolar acidification is necessary to maintain cytosolic pH alkaline during stomatal closing in response to ABA (Bak et al., 2013). However, our results suggest that not only ABA but also MeJA activates V-ATPase to facilitate vacuolar acidification that results in cytosolic alkalization. The *vha-2*, *vha-a3*, and *det3-1* mutants showed almost similar levels of cytosolic alkalization in response to ABA and MeJA (Figure 2.2a, 2.2b), which suggests that TGN/EE V-ATPase might not responsible for ABA- and MeJA-induced cytosolic alkalization in Arabidopsis guard cells.

External $Ca^{2+}$ as well as ABA and MeJA induce cytosolic alkalization and

$[Ca^{2+}]_{cyt}$ elevation in guard cells, resulting in stomatal closure (Allen et al., 2001, 2000; Islam et al., 2010; Munemasa et al., 2007; Pei et al., 2000). In this study, external $Ca^{2+}$ also elicited cytosolic alkalization and $[Ca^{2+}]_{cyt}$ elevation in wild-type plants (Figure 2.2c), which is consistent with stomatal closure (Figure 2.1c). I also studied $pH_{cyt}$ change and $[Ca^{2+}]_{cyt}$ elevation in *det3-1* and *vha-a2 vha-a3* Arabidopsis mutants in response to extracellular $Ca^{2+}$ to investigate the role of V-ATPase in $Ca^{2+}$-induced stomatal signaling. Application of $Ca^{2+}$ did not induce cytosolic alkalization in *det3-1* and *vha-a2 vha-a3* guard cells (Figure 2.2c), as well as did not induce stomatal closure (Figure 2.1c), suggesting the involvement of V-ATPase in $Ca^{2+}$-induced stomatal signalling. Our results indicate that tonoplast V-ATPase regulate cytosolic alkalization in response to extracellular $Ca^{2+}$.

In *det3*, extracellular $Ca^{2+}$ elicited prolonged $[Ca^{2+}]_{cyt}$ elevations rather than a transient elevations and stomatal closure was abolished (Allen et al., 2000). I also found a similar result where extracellular $Ca^{2+}$ induced a single and long duration $[Ca^{2+}]_{cyt}$ elevation and failed to induce stomatal closure in *det3-1* mutant (Figure 2.5c, 2.5d). I also observed an impaired cytosolic alkalization in *det3-1* mutant in response to extracellular $Ca^{2+}$ (Figure 2.2c). Allen et al. (2001) reported that a certain threshold level of $[Ca^{2+}]_{cyt}$ elevations regulates stomatal closure and that defined pattern of $[Ca^{2+}]_{cyt}$ elevations is needed to inhibit stomatal reopening or to maintain closure. Together it implies that cytosolic alkalization is need for a defined pattern of $[Ca^{2+}]_{cyt}$ elevations in guard cells but not need for a prolonged $[Ca^{2+}]_{cyt}$ elevations. More particularly, it can be assumed that cytosolic alkalization regulates the $Ca^{2+}$-induced transient elevations of $[Ca^{2+}]_{cyt}$ in Arabidopsis guard cells.

Calcium-induced stomatal closure requires cytosolic alkalization in guard cells and that $[Ca^{2+}]_{cyt}$ elevation is an earlier essential signaling component to increase

$pH_{cyt}$ in response to ABA, MeJA and extracellular $Ca^{2+}$ in Arabidopsis guard cells (Gonugunta et al., 2008; Suhita et al., 2004). I also observed a similar result where extracellular $Ca^{2+}$ induce cytosolic alkalization (Figure 2.2c). Islam et al. (2010) showed that butyrate suppresses $Ca^{2+}$-, ABA-, and MeJA-induced $[Ca^{2+}]_{cyt}$ elevation, suggesting that cytosolic alkalization feedback-regulates $[Ca^{2+}]_{cyt}$ elevation in Arabidopsis guard cells.

In this study, I examined effects of an intracellular alkalizing agent, methylamine, on cytosolic alkalization, $[Ca^{2+}]_{cyt}$ elevation, and stomatal closure. Delayed cytosolic alkalization and altered frequency of $[Ca^{2+}]_{cyt}$ spikes were observed in response to extracellular $Ca^{2+}$ in *det3-1* and in *vha-a2 vha-a3* mutant guard cells (Figure 2.2c, 2.5c-2.5e), suggesting an interrelation between $pH_{cyt}$ and $[Ca^{2+}]_{cyt}$ in $Ca^{2+}$-mediated guard cell signaling. However, methylamine treatment induced cytosolic alkalization in these mutants and transient elevations of $[Ca^{2+}]_{cyt}$ spikes similar to wild-type and eventually induced stomatal closure both in *det3-1* and in *vha-a2 vha-a3* mutants (Figure 2.6). Together with previous reports, our results suggest that the cytosolic alkalization is a upstream event than $[Ca^{2+}]_{cyt}$ elevation in guard cell signaling and $[Ca^{2+}]_{cyt}$ elevation feedback-regulates cytosolic alkalization.

# CHAPTER 3

# Regulation of cytosolic calcium elevation by plasma membrane anion channels in guard cells

## 3.1. Abstract

Cytosolic calcium ($[Ca^{2+}]_{cyt}$) elevation activates plasma membrane anion channels in guard cells, which is required for stomatal closure. However, involvement of the anion channels in the $[Ca^{2+}]_{cyt}$ elevation remains unclear. I investigated the involvement using *Arabidopsis thaliana* anion channel mutants, *slac1-4 slah3-3* and *slac1-4 almt12-1*. Extracellular calcium induced stomatal closure in the wild-type plants but not in the anion channel mutant plants whereas extracellular calcium induced $[Ca^{2+}]_{cyt}$ elevation both in the wild-type guard cells and in the mutant guard cells. The peak height and the number of the $[Ca^{2+}]_{cyt}$ spike were lower and larger in the *slac1-4 slah3-3* than in the wild-type and the height and the number in the *slac1-4 almt12-1* were much lower and much larger than in the wild-type. These results suggest that the anion channels are involved in the regulation of $[Ca^{2+}]_{cyt}$ elevation in guard cells.

## 3.2. Introduction

The epidermis of the aerial parts of flowering plants contains numerous stomata, which consist of a pair of guard cells. Stomatal opening facilitates carbon dioxide ($CO_2$) uptake into leaves for photosynthesis while stomatal closure prevents excessive water loss by transpiration (Chaerle et al., 2005). Stomatal apertures are influenced by several endogenous and exogenous factors (Hetherington and Woodward, 2003;

Schroeder et al., 2001). Several stimuli such as abscisic acid (ABA), methyl jasmonate, $CO_2$, microbe-derived molecules, and higher extracellular calcium ($Ca^{2+}$) causes rapid stomatal closure in plants (Fu et al., 2011; Hossain et al., 2011; MacRobbie, 1992; Schwartz et al., 1988).

Anion efflux from guard cells mediated by slow (S-type) and rapid (R-type) anion channels triggers membrane depolarization, which is a key step in induction of stomatal closure (Roelfsema et al., 2012). The membrane depolarization activates voltage-dependent $K^+_{out}$ channels, leading to a decrease in guard cell turgor pressure and stomatal closure (Munemasa et al., 2015). SLOW ANION CHANNEL-ASSOCIATED 1 (SLAC1) is an S-type anion channel protein isolated from Arabidopsis (*Arabidopsis thaliana*) (Negi et al., 2008; Vahisalu et al., 2008). Four homologues of SLAC1, the SLOW ANION CHANNEL-ASSOCIATED HOMOLOGUES 1 to 4 (SLAH1 to SLAH4), have been identified in Arabidopsis (Dreyer et al., 2012; Negi et al., 2008). As well as SLAC1, SLAH3 is involved in regulation of stomatal movement (Deger et al., 2015; Geiger et al., 2011; Liu et al., 2019). Guard cells of the *slac1 slah3* double mutant had much lower S-type anion currents than the guard cells of the wild-type or their single mutant plants (Zheng et al., 2018). Arabidopsis QUICK-ACTIVATING ANION CHANNEL1/ALUMINUM-ACTIVATED MALATE TRANSPORTER 12 (QUAC1/ALMT12) represents an R-type anion channel, which is a voltage-dependent channel and regulates the release of several anions such as nitrate and chloride from guard cells (Meyer et al., 2010; Sasaki et al., 2010). Mutation of ALMT12 causes impaired ABA-, and extracellular $Ca^{2+}$-induced stomatal closure in Arabidopsis (Meyer et al., 2010; Sasaki et al., 2010).

Calcium ($Ca^{2+}$) is one of the most important ubiquitous intracellular second messengers in plants (Tuteja and Mahajan, 2007). Stomatal closure occurs with the

transient elevation of cytosolic calcium concentration ($[Ca^{2+}]_{cyt}$) in guard cells in response to numerous stimuli, including ABA (McAinsh et al., 1990), methyl jasmonate (Hossain et al., 2011), extracellular $Ca^{2+}$ (McAinsh et al., 1995), and $H_2O_2$ (Pei et al., 2000). The influx of $Ca^{2+}$ from the apoplast and release of $Ca^{2+}$ from intracellular stores to cytosol cause elevation of $[Ca^{2+}]_{cyt}$ in guard cells (Pei et al., 2000; Voss et al., 2016). The influx of $Ca^{2+}$ is carried by non-selective $Ca^{2+}$-permeable channels that are activated by plasma membrane hyperpolarization (Hamilton et al., 2000; Pei et al., 2000). Pattern of $[Ca^{2+}]_{cyt}$ spike (transient elevation) known as $Ca^{2+}$ signature is involved in regulation of stomatal closure and reopening (Allen et al. 2001).

Electrophysiological studies showed that S-type anion channel activities were increased by $[Ca^{2+}]_{cyt}$ elevation in guard cells (Schroeder and Hagiwara, 1989; Siegel et al., 2009). Impairment of the $[Ca^{2+}]_{cyt}$-dependent signaling by genetic or pharmacological approaches inhibits both the anion channel activation and stomatal closure that are induced by stimuli such as ABA (Allen et al., 2001; Brandt et al., 2015; Geiger et al., 2010; Siegel et al., 2009). These results indicate that the $[Ca^{2+}]_{cyt}$ elevation is a key event upstream of activation of the S-type and R-type anion channels in guard-cell signaling. However, it remains unknown whether these anion channels regulate the $[Ca^{2+}]_{cyt}$ elevation. This study investigated the properties of $[Ca^{2+}]_{cyt}$ elevation induced by extracellular $Ca^{2+}$ in the *slac1-4 slah3-3* and *slac1-4 almt12-1* knock-out mutants of *A. thaliana*.

## 3.3. Materials and methods

### *3.3.1. Plant materials and growth conditions*

In this study, *A. thaliana* wild-type Columbia-0 (Col-0) and loss-of-function mutant

*slac1-4 slah3-3* and *slac1-4 almt12-1* were used. The homozygous *AtSLAC1* and *AtSLAH3* loss-of-function mutant *slac1-4 slah3-3* was prepared by crossing T-DNA insertion mutant *slac1-4* (SALK_137265) and *slah3-3* (SALK_106054). The homozygous *AtSLAC1* and *AtALMT12* loss-of-function mutant *slac1-4 almt12-1* was prepared by crossing *slac1-4* and *almt12-1* (WiscDsLox329D04). Plants were grown on a soil mixture [vermiculite (Asahi-kogyo, Okayama, Japan): Kureha soil (Kureha Chemical, Tokyo, Japan), 1:1 (v/v)] in the growth chamber at 21±2°C and 80 μmol $m^{-2}$ $s^{-1}$ light intensity under a 16 h light/8 h dark condition. The plants were watered with deionized water containing 0.1% (v/v) Hyponex (Hyponex Japan, Osaka, Japan) once a week. Rosette leaves from four to five week-old plants were used for all experiments.

### *3.3.2.* Measurement of stomatal aperture

Stomatal apertures were measured as described previously (Hossain et al., 2011). The excised rosette leaves were floated on stomatal bioassay solution containing 5 mM KCl, 50 μM $CaCl_2$, and 10 mM MES-Tris (pH 6.15) under light condition (80 μmol $m^{-2}s^{-1}$) for 2 h to open stomata. After that, $CaCl_2$ were added to the stomatal bioassay solution and incubated for another 2 h. The final concentrations of $CaCl_2$ on stomatal bioassay solution was 10 mM. The incubated leaves were blended by a commercial blender (700BUJ, Waring Commercial, Torrington, Connecticut) and epidermal tissues were collected using a nylon mesh. At least 20 stomatal apertures were measured on each distinct experiment using WinRoof 3.0 software (Mitani Corporation, Fukui and Tokyo, Japan). Following the similar procedure three independent experiments were performed.

### *3.3.3.* Measurement of guard cell $[Ca^{2+}]_{cyt}$

Fluctuations in $[Ca^{2+}]_{cyt}$ in guard cells were detected using transgenic Arabidopsis plants expressing Nuclear Export Signal (NES) Yellow Cameleon 3.6 (NES-YC3.6). Yellow cameleon 3.6 has been widely-used for plant $[Ca^{2+}]_{cyt}$ imaging but it is localized not only in the cytosol but also in the nucleus. In this study we used NES-tagged YC3.6, which shows no detectable fluorescence signal in the nucleus (Krebs et al., 2012). The experimental procedure described previously by Afrin *et al.* (2020) was followed. The abaxial side of excised leaves were gently mounted on a glass cover slip using a medical adhesive. The adaxial epidermis and the mesophyll tissue were removed, the remaining abaxial epidermal peels were incubated in the stomatal bioassay solution consisted of 5 mM KCl, 50 μM $CaCl_2$ and 10 mM MES-Tris (pH 6.15) in a growth chamber for 2 h to promote stomatal opening. The turgid guard cells were treated with 10 mM $CaCl_2$ at 5 min after the start of measurement. Two to three guard cells from one sample leaf for each experiment were selected. This experiment was repeated using different sample leaves on different days. The cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) fluorescence intensities (F535 and F480) of guard cells were measured under a fluorescence microscope (IX71, Olympus, Tokyo, Japan) equipped with a dual-emission imaging system (W-View system; 440AF21 excitation filter, 445DRLP dichroic mirror and two emission filters, 480DF30 for CFP and 535DF25 for YFP; Hamamatsu Photonics, Hamamatsu, Japan) and a CCD camera (Hamamatsu ORCA-ER digital camera; Hamamatsu Photonics). The fluorescence intensities were ratiometrically analysed using AQUA COSMOS software (Hamamatsu Photonics). Same exposure time was used for both CFP and YFP. For the analysis of $[Ca^{2+}]_{cyt}$, $[Ca^{2+}]_{cyt}$ elevation was counted when changes in fluorescence ratios (F535/F480) were ≥0.1 unit from the baseline and the elevation

of ratio observed only because of the increment and reduction of CFP and YFP fluorescence intensities, respectively.

The ratio data are analyzed with ImageJ 1.52a software (National Institutes of Health, Bethesda, MD, USA) for measuring the height of each $[Ca^{2+}]_{cyt}$ spike (transient elevation of cytosolic $Ca^{2+}$) (Figure 3.1). "Height of spike" is defined as the average peak height of $Ca^{2+}$ spikes for each recording by (1). "Height of 1st spike" is defined as the peak height of 1st $Ca^{2+}$ spike for each recording by (2). "Number of spike per unit time" is defined as the number of $[Ca^{2+}]_{cyt}$ spikes between 0 min and 40 min for each recording.

$$\text{Height of spike} = \frac{1}{n}\sum_{i=1}^{n}(\text{Height of } Ca^{2+} \text{ spike})_i \qquad (1)$$

$$\text{Number of spike per unit time} = \frac{1}{n}\sum_{i=1}^{n}(\text{Number of } Ca^{2+} \text{ spike for 40 min})_i \qquad (2)$$

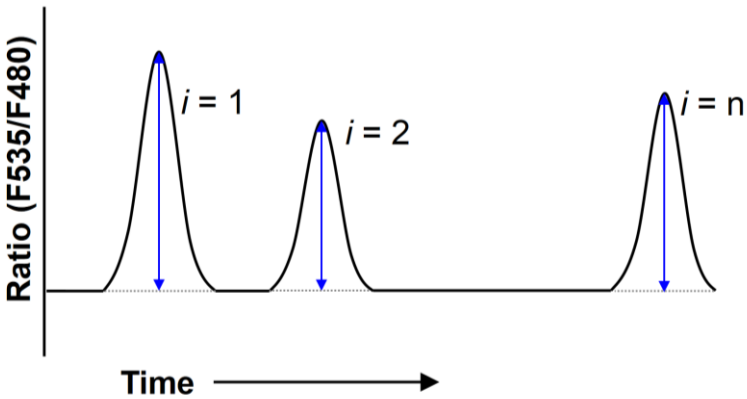


Figure 3.1. A hypothetical cytosolic calcium ($[Ca^{2+}]_{cyt}$) elevation plot of a guard cell. A $[Ca^{2+}]_{cyt}$ spike was counted when a change in fluorescence ratio (F535/F480) was ≥0.1 unit from the baseline and "peak height of $Ca^{2+}$ spike" for the property analysis is indicated using an arrowed blue line. 1, 2, ..., n indicate the number of spikes.

### *3.3.4.* Statistical analysis

The significant differences between mean values of stomatal apertures, height of spike, height of 1st spike, and number of spike per unit time were assessed by Student's t-test, and the significant differences between number of $[Ca^{2+}]_{cyt}$ elevation were assessed by a chi-squared ($\chi^2$) test.

## 3.4. Results

### *3.4.1.* Effects of extracellular $Ca^{2+}$ on stomatal aperture in *slac1-4 slah3-3* double mutant and in *slac1-4 almt12-1* double mutant

Calcium chloride at 10 mM significantly induced stomatal closure in the Col-0 ($P$<0.01) but not in the *slac1-4 slah3-3* double mutant ($P$=0.900) or in the *slac1-4 almt12-1* double mutant ($P$=0.760) (Figure 3.2a-3.2i). Time course observation showed that the stomatal aperture was decreased from 4.23 μm to 2.58 μm in Col-0 in the presence of 10 mM $CaCl_2$ but was not significantly changed in the *slac1-4 slah3-3* double mutant or in the *slac1-4 almt12-1* double mutant (Figure 3.2j). These results indicate that extracellular $Ca^{2+}$ does not induce stomata closure either in the *slac1-4 slah3-3* double mutant or in the *slac1-4 almt12-1* double mutant.

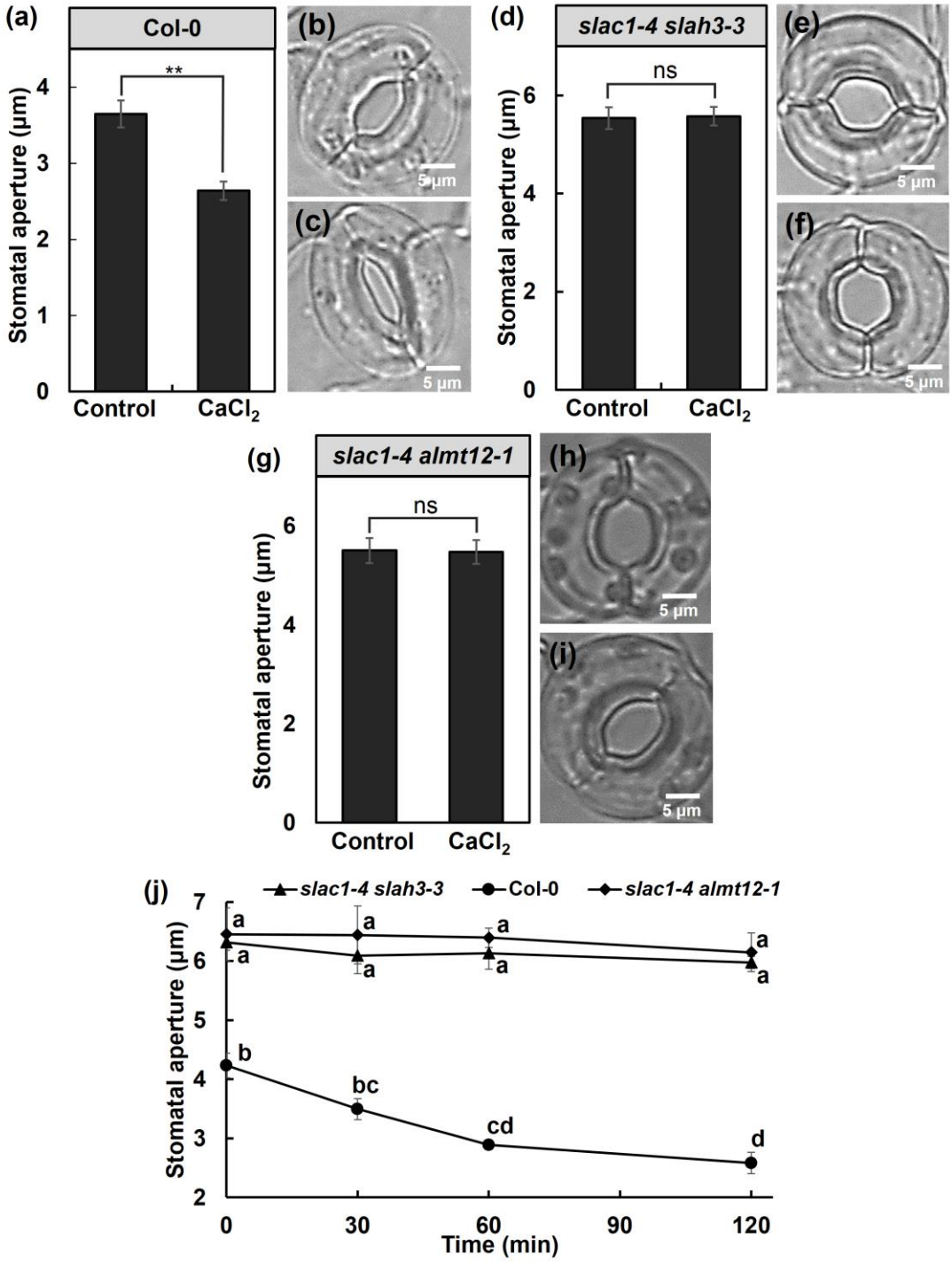


Figure 3.2. Stomatal response to external $Ca^{2+}$ in Arabidopsis.

Effects of external 10 mM $CaCl_2$ on stomatal apertures (width, µm) of **(a)** wild-type (Col-0), **(d)** *slac1-4 slah3-3*, and **(g)** *slac1-4 almt12-1* Arabidopsis. Representative images of control and $Ca^{2+}$-treated stomata of the **(b, c)** Col-0, **(e, f)** *slac1-4 slah3-3*, and **(h, i)** *slac1-4 almt12-1*. **(j)** Time-course changes in stomatal apertures (width,

μm) in Col-0, *slac1-4 slah3-3*, and *slac1-4 almt12-1*. The abaxial side of rosette leaves were pre-incubated on a stomatal bioassay solution for 2 h under light, followed by 2 h incubation with 10 mM $CaCl_2$ containing stomatal bioassay solution. **(a, d, g)** Data are the mean ± SE (*n*=three independent experiments, >60 stomata for each data point) and statistical difference was determined by Student's t-test, ***P*<0.01. The 'ns' indicates non-significant difference where *P*>0.05. **(j)** Statistical differences were determined by Tukey's test based on *P*<0.05. Scale bar=5 μm.

### *3.4.2.* Effects of extracellular $Ca^{2+}$ on $[Ca^{2+}]_{cyt}$ spike in *slac1-4 slah3-3* and in *slac1-4 almt12-1* double mutant guard cells

The $Ca^{2+}$ spikes (transient $Ca^{2+}$ elevation) were observed in 12.5% of guard cells treated with 50 μM extracellular $CaCl_2$ (*n*=16) (Figure 3.3a) and 92.9% of cells treated with 10 mM $CaCl_2$ (*n*=28) (Figure 3.3b). The number of $Ca^{2+}$ spikes in the wild-type guard cells treated with 10 mM $Ca^{2+}$ was larger than that in the wild-type guard cells treated with 50 μM $Ca^{2+}$ (*P*<0.0001) (Figure 3.3g), which is consistent with the previous results (Allen et al., 2001; Siegel et al., 2009). In the *slac1-4 slah3-3* mutant guard cells, $Ca^{2+}$ spikes were observed in 13.3% of guard cells treated with 50 μM extracellular $CaCl_2$ (*n*=15) (Figure 3.3c) and 93.8% of cells treated with 10 mM $CaCl_2$ (*n*=32) (Figure 3.3d). In the *slac1-4 almt12-1* mutant guard cells, $Ca^{2+}$ spikes were observed in 16.7% of guard cells treated with 50 μM extracellular $CaCl_2$ (*n*=18) (Figure 3.3e) and 100% of cells treated with 10 mM $CaCl_2$ (*n*=32) (Figure 3.3f). The numbers of $Ca^{2+}$ spikes in the guard cells of *slac1-4 slah3-3* and *slac1-4 almt12-1* guard cells treated with 10 mM $Ca^{2+}$ were larger than those in the guard cells of both mutants treated with 50 μM $Ca^{2+}$ (*P*<0.0001) (Figure 3.3g). The percentage of guard cells that displayed $Ca^{2+}$ spike in the *slac1-4 slah3-3* guard cells

was not significantly different from that in the wild-type guard cells ($P$=0.25) (Figure 3g) while the percentage of the *slac1-4 almt12-1* guard cells was larger than that of the wild-type guard cells ($P$<0.05) (Figure 3.3g).

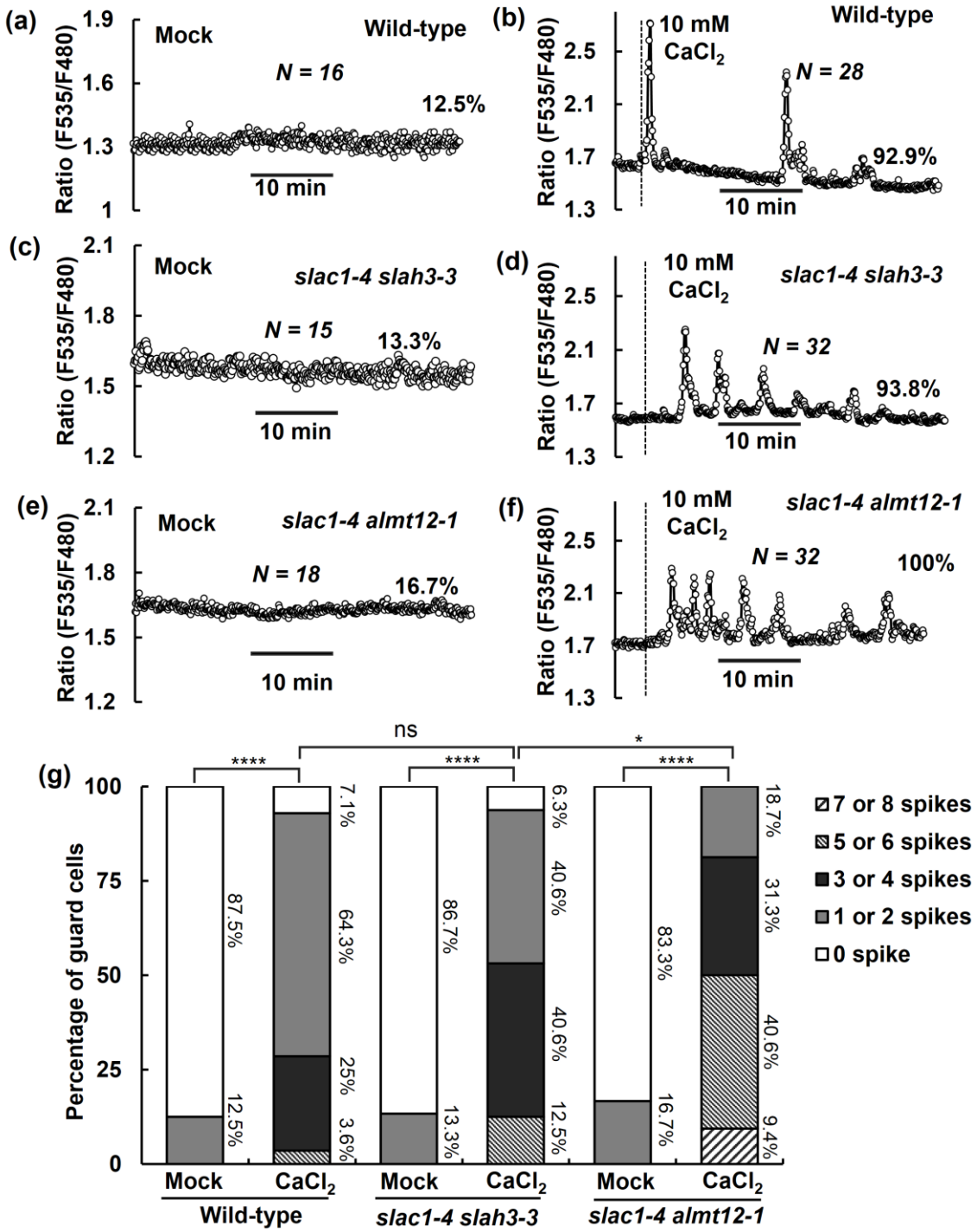


Figure 3.3. Cytosolic calcium ($[Ca^{2+}]_{cyt}$) elevation in Arabidopsis guard cells induced by external $Ca^{2+}$.

The $[Ca^{2+}]_{cyt}$ elevation in guard cells of wild-type, *slac1-4 slah3-3* and *slac1-4 almt12-1* double mutant expressing NES-Yellow Cameleon 3.6 was monitored. **(a-f)** Representative traces of fluorescence emission ratios (F535/F480) showing $[Ca^{2+}]_{cyt}$ spikes in guard cells. In mock treatments, epidermal peel was bathed into stomatal bioassay solution **(a, c, e)**, and in $Ca^{2+}$ treatments, 10 mM $CaCl_2$ was applied into stomatal bioassay solution **(b, d, f)**. Percentage bar chart **(g)** showing the percent of guard cells showing $[Ca^{2+}]_{cyt}$ elevation of wild-type, *slac1-4 slah3-3*, and *slac1-4 almt12-1* double mutant under mock and 10 mM $CaCl_2$ treatment. $[Ca^{2+}]_{cyt}$ elevations were counted when changes in fluorescence ratios (F535/F480) were ≥0.1 unit from the baseline. The significance of differences between different treatments were determined by chi-squared ($\chi^2$) test, **P*<0.05, *****P*<0.0001. The 'ns' indicates non-significant difference where *P*>0.05.

### *3.4.3.* Characterization of extracellular $Ca^{2+}$-induced $[Ca^{2+}]_{cyt}$ elevation in *slac1-4 slah3-3* and *slac1-4 almt12-1* mutant guard cells

Analysis of $[Ca^{2+}]_{cyt}$ spike properties revealed that in response to external $Ca^{2+}$, wild-type guard cells had the highest spike height, which was significantly decreased in *slac1-4 slah3-3* (*P*<0.001) and *slac1-4 almt12-1* (*P*<0.0001) mutant guard cells (Figure 3.4a). The height of the 1st spike was lower in the *slac1-4 almt12-1* guard cells than that in the wild-type guard cells (*P*<0.05) (Figure 3.4b). The number of $Ca^{2+}$ spike per unit time was larger in *slac1-4 slah3-3* (*P*<0.05) and *slac1-4 almt12-1* (*P*<0.0001) double mutants guard cells than that in wild-type guard cells (Figure 3.4c). The *slac1-4 almt12-1* guard cells showed significantly lower height of spike (*P*<0.001) and larger number of spike per unit time (*P*<0.001) than the *slac1-4 slah3-3* guard cells did (Figure 3.4a, 3.4c). The *slac1-4 slah3-3* (*P*<0.05) and *slac1-4*

*almt12-1* ($P$<0.01) guard cells had significantly higher baseline $[Ca^{2+}]_{cyt}$ ratios than that of wild-type guard cells (Figure 3.4d). Fluorescence ratio indicating resting $[Ca^{2+}]_{cyt}$ in guard cells had a negative correlation with height of spike ($R^2$=0.942) (Figure 3.4e) and a positive correlation with number of spike per unit time ($R^2$=0.873) (Figure 3.4f).

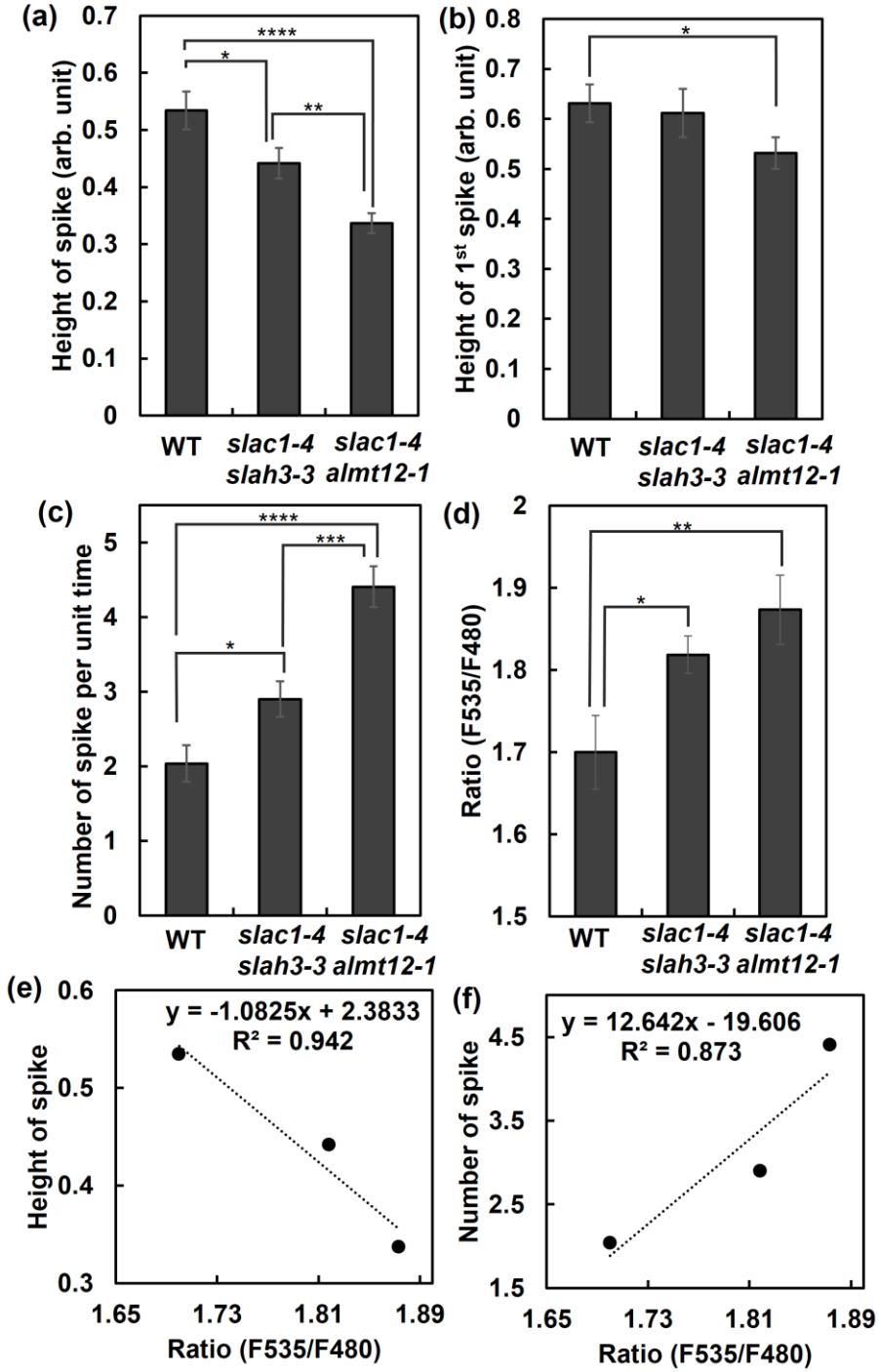

Figure 3.4. Different properties of cytosolic calcium ($[Ca^{2+}]_{cyt}$) spike.

Height of $[Ca^{2+}]_{cyt}$ spike **(a)**, height of 1st $[Ca^{2+}]_{cyt}$ spike **(b)**, and number of $[Ca^{2+}]_{cyt}$ spike per unit time **(c)** of wild-type (*n*=28), *slac1-4 slah3-3* (*n*=32), and *slac1-4 almt12-1* (*n*=32). **(d)** Baseline cytosolic $Ca^{2+}$-dependent ratiometric fluorescence levels in wild-type (*n*=15), *slac1-4 slah3-3* (*n*=17), and *slac1-4 almt12-1* (*n*=16) guard cells transformed with NES-YC 3.6. Data are the mean ± SE and statistical differences were determined using Student's t-test; **P*<0.05, ****P*<0.001, and *****P*<0.0001. arb. unit, arbitrary unit. Plots showing the correlation: fluorescence ratio (F535/F480) versus height of spike **(e)** and fluorescence ratio (F535/F480) versus number of spike per unit time **(f)**. $R^2$, coefficient of determination.

## 3.5. Discussion

In our study, I examined the roles of the guard cell plasma membrane anion channels on regulation of $[Ca^{2+}]_{cyt}$ elevation. The elevated $[Ca^{2+}]_{cyt}$ activates S-type channels (Schroeder and Hagiwara, 1989; Siegel et al., 2009). Hence, the $[Ca^{2+}]_{cyt}$ elevation is recognized as the upstream event of activation of the anion channels in the guard-cell signaling but involvement of the anion channels in the regulation of $[Ca^{2+}]_{cyt}$ elevation remains to be clarified. In this study, I found that the external $Ca^{2+}$ induced $[Ca^{2+}]_{cyt}$ elevation in the *slac1-4 slah3-3* double mutant and in the *slac1-4 almt12-1* double mutant, as well as in the wild-type guard cells (Figure 3.3b, 3.3d, 3.3f). However, the number of $[Ca^{2+}]_{cyt}$ spike was larger and the height of $Ca^{2+}$ spike was lower in the *slac1-4 slah3-3* and *slac1-4 almt12-1* than in the wild-type (Figure 3.4a, 3.4c). These results suggest that the S-type anion channels and the R-type anion channels also regulate the properties of $[Ca^{2+}]_{cyt}$ elevation that functions upstream of the anion channel activation in the guard-cell signaling.

The S-type anion channel mutant *slac1-4 slah3-3* and the wild-type showed similar height of the 1st $[Ca^{2+}]_{cyt}$ spike in the guard cells (Figure 3.4b), indicating that the depolarization by S-type anion channel activation is not involved in the 1st $[Ca^{2+}]_{cyt}$ spike. On the other hand, the height of the 1st spike is lower in the S-type and R-type anion channel mutant *slac1-4 almt12-1* than in the S-type anion channel mutant *slac1-4 slah3-3* mutant and the wild-type (Figure 3.4b). This result indicates that R-type anion channel activation affect the 1st $[Ca^{2+}]_{cyt}$ spike, which might be accounted for both by the inactivation of R-type anion channel and by the activation of S-type anion channel at hyperpolarized membrane potentials (Dreyer et al., 2012; Hedrich et al., 1990; Schroeder and Hagiwara, 1989).

The present result obtained using NES-YC3.6 that anion channel mutations increased the resting $[Ca^{2+}]_{cyt}$ in guard cells (Figure 3.4d), which is consistent with the previous result obtained using YC3.6 that the *slac1* mutation raised the resting $[Ca^{2+}]_{cyt}$ (Laanemets et al., 2013). These results suggest that the resting $[Ca^{2+}]_{cyt}$ is attributed to the change in plasma membrane anion channel activity. Furthermore, resting $[Ca^{2+}]_{cyt}$ in guard cells was correlated positively with height of spike and negatively with number of spike (Figure 3.4e, 3.4f), which might be accounted for by the result that raising $[Ca^{2+}]_{cyt}$ reduced the open probability of plasma membrane $Ca^{2+}$ channels in *Vicia faba* guard cells (Hamilton *et al.* 2000). Taken together, these results suggest that the properties of $[Ca^{2+}]_{cyt}$ spike are closely related to the kinetics of anion channel activity.

The plasma membrane hyperpolarization-activated $Ca^{2+}$-permeable ($I_{Ca}$) channels mediate the early $[Ca^{2+}]_{cyt}$ elevation in guard cells (Hamilton et al., 2000; Pei et al., 2000). The $[Ca^{2+}]_{cyt}$ elevation leads to S-type anion channel activation (Schroeder and Hagiwara, 1989; Siegel et al., 2009), which is consistent with the

present result that the *slac1 slah3* mutation did not affect the height of 1st spike (Figure 3.4b). Since $I_{Ca}$ channels are activated by hyperpolarization, the depolarization by S-type anion channel activation is unfavorable for the activation of $I_{Ca}$ and the late and repetitive $[Ca^{2+}]_{cyt}$ spikes. In addition, the lower steady-state level of cytosolic $Ca^{2+}$ is also partially attributed to the anion channel activation. Hence, The late spikes might be accounted for by $Ca^{2+}$ release from intracellular stores such as $Ca^{2+}$-induced $Ca^{2+}$ release (CICR) (Grabov and Blatt, 1999; Voss et al., 2016).

Taken together, it can be concluded that both the early $[Ca^{2+}]_{cyt}$ response as 1st spike and the later response including spike numbers are regulated by two different types of anion channels, S-type and R-type.

# Summary

Stomata, formed by pairs of guard cells in the epidermis of terrestrial plants, regulate gas exchange including transpiration and $CO_2$ entry for photosynthesis, thus playing a critical role in plant growth. Stomatal apertures are influenced by several endogenous and exogenous factors. Several stimuli such as ABA, MeJA, $CO_2$, microbe-derived molecules, and higher extracellular calcium ($Ca^{2+}$) cause rapid stomatal closure, which is accompanied by reactive oxygen species (ROS) production, cytosolic alkalization, and cytosolic free calcium ($[Ca^{2+}]_{cyt}$) elevation. Elevation of $[Ca^{2+}]_{cyt}$ activates S-type anion channels and inhibits inward $K^+$ channels in the plasma membrane of guard cells. In Chapter 2, I investigated the mechanism of ABA-, MeJA-, and external $Ca^{2+}$-induced cytosolic alkalization and regulation of $[Ca^{2+}]_{cyt}$ elevation by cytosolic alkalization in *Arabidopsis thaliana* guard cells using *aha1-9* (plasma membrane $H^+$-ATPase loss-of-function), *det3-1* (having 60% reduced V-ATPase activity both in tonoplast and TGN/EE), and *vha-a2 vha-a3* (having no V-ATPase activity in tonoplast). During ABA-, MeJA-, and $Ca^{2+}$-induced stomatal closure in wild-type, *aha1-9*, *det3-1*, and *vha-a2 vha-a3*, I detected cytosolic alkalization using a pH-sensitive fluorescent dye, BCECF-AM and $[Ca^{2+}]_{cyt}$ elevation using NES-Yellow Cameleon 2.6 calcium sensor. ABA and MeJA induced cytosolic alkalization and stomatal closure in wild-type, *aha1-9*, and *det3-1* but delayed in *vha-a2 vha-a3*, whereas external $Ca^{2+}$ elicited cytosolic alkalization and stomatal closure in wild-type and *aha1-9* guard cells but not in *det3-1* and *vha-a2 vha-a3* guard cells. The frequency of ABA-induced $[Ca^{2+}]_{cyt}$ elevation in *aha1-9* and *det3-1* guard cells is comparable to wild-type but in *vha-a2 vha-a3*, the $[Ca^{2+}]_{cyt}$ elevation is significantly different than wild-type. In response to extracellular $Ca^{2+}$, *det3-1* and *vha-a2 vha-a3*

showed long-lasting $[Ca^{2+}]_{cyt}$ elevation in guard cells, with a frequency that differed significantly from wild-type. Methylamine, an cytosolic alkalizing agent, induced cytosolic alkalization, $[Ca^{2+}]_{cyt}$ elevation and stomatal closure both in *det3-1* and *vha-a2 vha-a3*. These results suggest that tonoplast-localized V-ATPase is one the regulators of ABA- and MeJA-induced cytosolic alkalization in guard cells. However, $Ca^{2+}$-dependent cytosolic alkalization solely regulated by tonoplast-localized V-ATPase in guard cells. Plasma membrane $H^{+}$-ATPase is not involved in cytosolic alkalization in response to ABA, MeJA, and external $Ca^{2+}$. Moreover, our results suggests that cytosolic alkalization regulates transient elevations of $[Ca^{2+}]_{cyt}$ in guard cells. In Chapter 3, I investigated the involvement of the S- and R-type anion channels in the $[Ca^{2+}]_{cyt}$ elevation using *A. thaliana* anion channel mutants, *slac1-4 slah3-3* and *slac1-4 almt12-1*. Extracellular $Ca^{2+}$ induced stomatal closure in the wild-type plants but not in the anion channel mutant plants whereas extracellular $Ca^{2+}$ induced $[Ca^{2+}]_{cyt}$ elevation both in the wild-type guard cells and in the mutant guard cells. The peak height and the number of the $[Ca^{2+}]_{cyt}$ spike were lower and larger in the *slac1-4 slah3-3* than in the wild-type and the height and the number in the *slac1-4 almt12-1* were much lower and much larger than in the wild-type. These results suggest that the anion channels are involved in the regulation of $[Ca^{2+}]_{cyt}$ elevation in guard cells.

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