Bioscience, Biotechnology, and Biochemistry, 2021, Vol. 85, No. 9, 2003-2010

https:/doi.org/10.1093/bbb/zbab118
Advance access publication date: 30 June 2021
REGULAR PAPER

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Modulation of frequency and height of cytosolic calcium spikes by plasma membrane anion channels in guard cells

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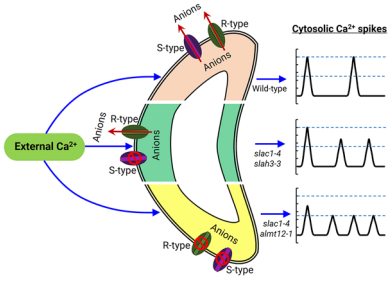
ABSTRACT

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. We 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.

Graphical Abstract



In guard cells, anion channels regulate the height and number of cytosolic calcium spikes elicited by extracellular calcium.

Keywords: ALMT12, cytosolic calcium elevation, membrane depolarization, SLAC1, stomatal closure

Abbreviations: ABA: abscisic acid; $[Ca^{2+}]_{cyt}$: cytosolic calcium concentration; SLAC1: slow anion channel-associated 1; SLAH: slow anion channel-associated homologues; ALMT12: aluminum-activated malate transporter 12; NES-YC3.6:

Received: 12 May 2021; **Accepted:** 21 June 2021

nuclear export signal yellow cameleon 3.6; CFP: cyan fluorescent protein; YFP: yellow fluorescent protein; I_{Ca}: Ca^{2+}-permeable channel

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, Saibo and Van Der Straeten 2005). Stomatal apertures are influenced by several endogenous and exogenous factors (Schroeder *et al.* 2001; Hetherington and Woodward 2003). 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 (Schwartz, Ilan and Grantz 1988; MacRobbie 1992; Fu *et al.* 2011; Hossain *et al.* 2011).

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, Hedrich and Geiger 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 1-4 (SLAH1 to SLAH4), have been identified in Arabidopsis (Negi *et al.* 2008; Dreyer *et al.* 2012). As well as SLAC1, SLAH3 is involved in regulation of stomatal movement (Geiger *et al.* 2011; Deger *et al.* 2015; 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 channel 1/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, Brownlee and Hetherington 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, Hedrich and Roelfsema 2016). The influx of Ca^{2+} is carried by nonselective 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; Siegel *et al.* 2009; Geiger *et al.* 2010; Brandt *et al.* 2015). 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* knockout mutants of *A. thaliana*.

Materials and methods

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 4- to 5-week-old plants were used for all experiments.

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 mMKCl, 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 3 independent experiments were performed.

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

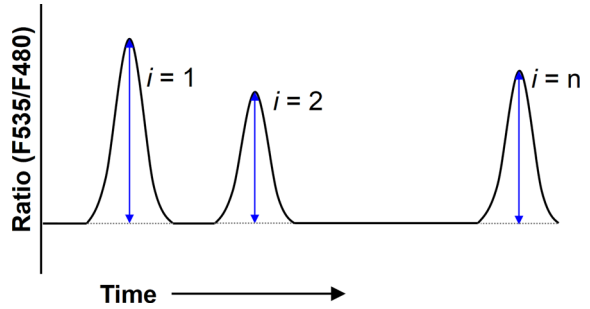


Figure 1. A hypothetical cytosolic calcium ($[Ca^{2+}]_{cyt}$) elevation plot of a guard cell. A $[Ca^{2+}]_{cyt}$ spike was counted when a change in fluorescence ratio (F535/F480) was ≥0.1 unit from the baseline and "peak height of Ca^{2+} spike" for the property analysis is indicated using an arrowed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

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 1 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 2 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 analyzed 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 1). "Height of spike" is defined as the average peak height of Ca^{2+} spikes for each recording by (1). "Height of first spike" is defined as the peak height of first Ca^{2+} spike for each recording by (2). "Number of spikes per unit time" is defined as the number of $[Ca^{2+}]_{cyt}$ spikes between 0 and 40 min for each recording.

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

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

Statistical analysis

The significant differences between mean values of stomatal apertures, height of spike, height of first spike, and number of spikes 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 (χ^2) test.

Results

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 ($P < .01$) but not in the *slac1-4 slah3-3* double mutant ($P = .900$) or in the *slac1-4 almt12-1* double mutant ($P = .760$) (Figure 2a-i). Time course observation showed that the stomatal aperture was decreased from 4.23 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 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.

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 3a) and 92.9% of cells treated with 10 mM $CaCl_2$ ($n = 28$) (Figure 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+} ($P < .0001$) (Figure 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 3c) and 93.8% of cells treated with 10 mM $CaCl_2$ ($n = 32$) (Figure 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 3e) and 100% of cells treated with 10 mM $CaCl_2$ ($n = 32$) (Figure 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+} ($P < .0001$) (Figure 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 ($P = .25$) (Figure 3g) while the percentage of the *slac1-4 almt12-1* guard cells was larger than that of the wild-type guard cells ($P < .05$) (Figure 3g).

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* ($P < .001$) and *slac1-4 almt12-1* ($P < .0001$) mutant guard cells (Figure 4a). The height of the first spike was lower in the *slac1-4 almt12-1* guard cells than that in the wild-type guard cells ($P < .05$) (Figure 4b). The number of Ca^{2+} spike per unit time was larger in *slac1-4 slah3-3* ($P < .05$) and *slac1-4 almt12-1* ($P < .0001$) double mutants guard cells than that in wild-type guard cells (Figure 4c). The *slac1-4 almt12-1* guard cells showed significantly lower height

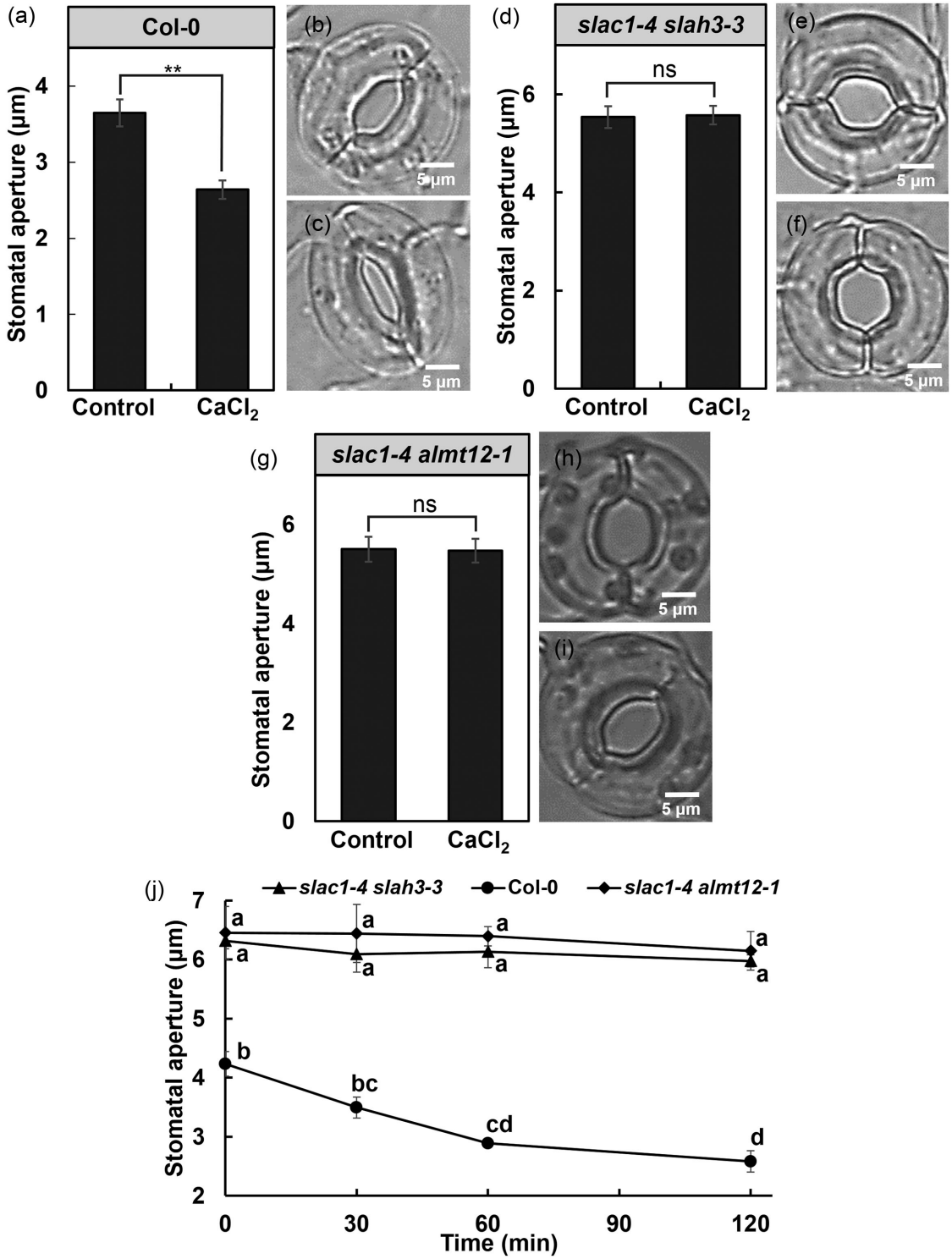


Figure 2. Stomatal response to external Ca^{2+} in Arabidopsis. Effects of external 10 mM $CaCl_2$ on stomatal apertures (width, μm) of (a) wild type (Col-0), (d) *slac1-4 slah3-3*, and (g) *slac1-4 almt12-1* Arabidopsis. Representative images of control and Ca^{2+}-treated stomata of the (b, c) Col-0, (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 preincubated 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 ($n = 3$ independent experiments, >60 stomata for each data point) and statistical difference was determined by Student's *t*-test, $^{**}P < .01$. The "ns" indicates nonsignificant difference where $P > .05$. (j) Statistical differences were determined by Tukey's test based on $P < .05$. Scale bar = 5 μm.

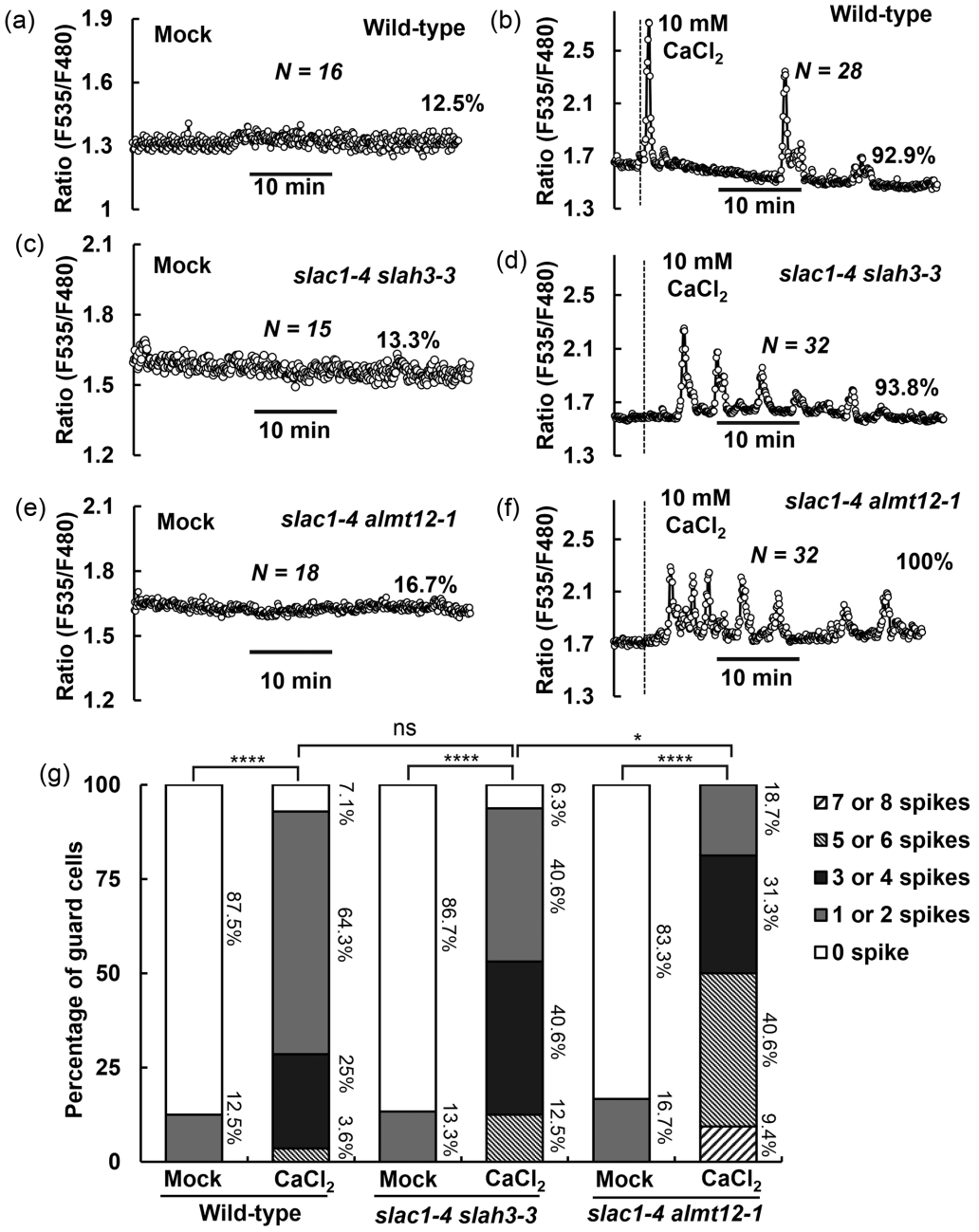


Figure 3. Cytosolic calcium ($[Ca^{2+}]_{cyt}$) elevation in Arabidopsis guard cells induced by external Ca^{2+}. The $[Ca^{2+}]_{cyt}$ elevation in guard cells of wild type, *slac1-4 slah3-3* and *slac1-4 almt12-1* double mutant expressing NES-Yellow Cameleon 3.6 was monitored. (a-f) Representative 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 % 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 (χ^2) test, $^{*}P < .05$, $^{****}P < .0001$. The "ns" indicates nonsignificant difference where $P > .05$.

of spike ($P < .001$) and larger number of spikes per unit time ($P < .001$) than the *slac1-4 slah3-3* guard cells did (Figure 4a and c). The *slac1-4 slah3-3* ($P < .05$) and *slac1-4 almt12-1* ($P < .01$) guard cells had significantly higher baseline $[Ca^{2+}]_{cyt}$ ratios than that of wild-type guard cells (Figure 4d). Fluorescence ratio indicating resting $[Ca^{2+}]_{cyt}$ in guard cells had a negative correlation with height of spike ($R^2 = 0.942$) (Figure 4e) and a positive correlation with number of spikes per unit time ($R^2 = 0.873$) (Figure 4f).

Discussion

In our study, we 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, we found that the external Ca^{2+} induced

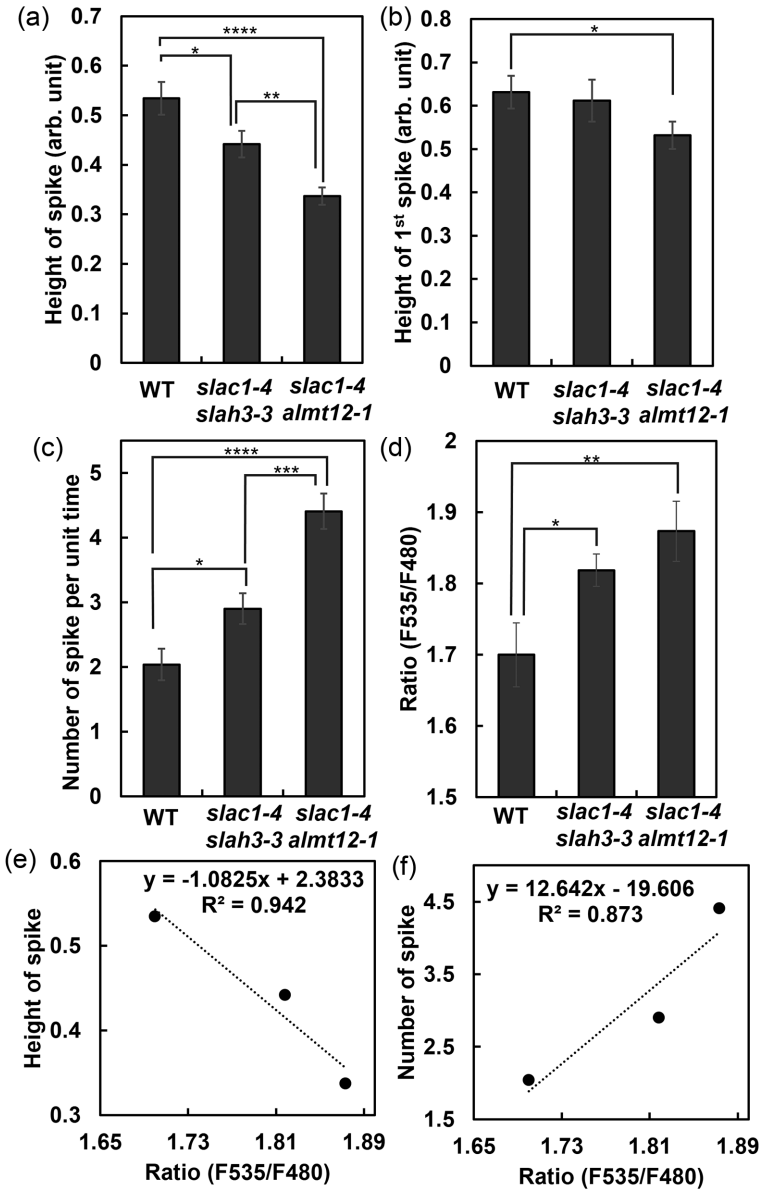


Figure 4. Different properties of cytosolic calcium ($[Ca^{2+}]_{cyt}$) spike. Height of $[Ca^{2+}]_{cyt}$ spike (a), height of first $[Ca^{2+}]_{cyt}$ spike (b), and number of $[Ca^{2+}]_{cyt}$ spike per unit time (c) of wild type ($n = 28$), *slac1-4 slah3-3* ($n = 32$), and *slac1-4 almt12-1* ($n = 32$). (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; $^{*}P < .05$, $^{***}P < .001$, and $^{****}P < .0001$; 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 spikes per unit time (f). R^2, coefficient of determination.

$[Ca^{2+}]_{cyt}$ 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 3b, d, and f). 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 4a and c). 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 first $[Ca^{2+}]_{cyt}$ spike in the guard cells (Figure 4b), indicating that the depolarization by S-type anion channel activation is not involved in the first $[Ca^{2+}]_{cyt}$ spike. On the other hand, the height of the first 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 4b). This result indicates that R-type anion channel activation affect the first $[Ca^{2+}]_{cyt}$ 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 (Schroeder and Hagiwara 1989; Hedrich, Busch and Raschke 1990; Dreyer *et al.* 2012).

The present result obtained using NES-YC3.6 that anion channel mutations increased the resting $[Ca^{2+}]_{cyt}$ in guard cells (Figure 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 4e and f), 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 first spike (Figure 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, Hedrich and Roelfsema 2016).

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

Data availability

The data will be shared on reasonable request to the corresponding author.

Author contribution

M.T.-U.-A., S.M., and Y.M. conceived the research plans; M.T.-U.-A. performed the experiments; T.N. and Y.N. provided suggestions; M.T.-U.-A., S.M., and Y.M. wrote the article with the contribution of all the authors. All authors approved the final manuscript.

Funding

None declared.

Disclosure statement

No potential conflict of interest was reported by the authors.

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