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Cytosolic Alkalization and Cytosolic Calcium Oscillation in Arabidopsis Guard Cells Response to ABA and MeJA

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Abscisic acid (ABA)- and methyl jasmonate (MeJA)-induced stomatal closure are accompanied by cytosolic alkalization in guard cells. However, it remains to be clarified how the alkalization functions in not only ABA signaling but also MeJA. We investigated cytosolic alkalization in guard cells during ABA-, MeJA- and Ca^{2+}-induced stomatal closure of wild type, *abi1-1*, *abi2-1*, *ost1-2* and *coi1* using a pH-sensitive fluorescent dye, BCECF-AM. ABA induced cytosolic alkalization in guard cells of wild-type and *coi1* but not in *ost1-2* guard cells whereas MeJA elicited cytosolic alkalization in wild-type and *ost1-2* guard cells but not in *coi1*. Neither ABA nor MeJA induced cytosolic alkalization in *abi1-1* and *abi2-1* guard cells. Exogenous Ca^{2+} induced stomatal closure accompanied by cytosolic alkalization in guard cells of wild-type, *abi1-1*, *abi2-1*, *ost1-2* and *coi1* plants. An agent to acidify cytosol, butyrate, suppressed Ca^{2+}-induced cytosolic alkalization and ABA-, MeJA- and Ca^{2+}-induced cytosolic Ca^{2+} oscillation in wild-type guard cells to prevent stomatal closure. These results indicate that cytosolic alkalization and cytosolic Ca^{2+} oscillation coordinately function in ABA and MeJA signaling in Arabidopsis guard cells.

Keywords: Abscisic acid • *Arabidopsis thaliana* • Cytosolic calcium • Cytosolic pH • Guard cells • Methyl jasmonate.

Abbreviations: ABA, abscisic acid; BCECF-AM, 2′,7′-bis-(2-carboxyethyl)-5,(6)-carboxyfluorescein acetoxymethyl ester; $[Ca^{2+}]_{cyt}$, cytosolic calcium concentration; MeJA, methyl jasmonate; pH_{cyt}, cytosolic pH; PP2Cs, protein phosphatases 2C; ROS, reactive oxygen species.

Introduction

Guard cells, which form stomatal pores in leaf epidermis of higher plants, respond to numerous biotic and abiotic signaling stimuli (Schroeder et al. 2001, Hetherington and Woodward 2003, Melotto et al. 2006). Many studies have shown that ABA and MeJA stimulate stomatal closure in many plant species (Irving et al. 1992, Blatt and Armstrong 1993, Gehring et al. 1997, Suhita et al. 2004, Saito et al. 2008, Islam et al. 2009, Islam et al. 2010).

Stomatal closure occurs with increment of cytosolic calcium concentration, $[Ca^{2+}]_{cyt}$, and oscillation of $[Ca^{2+}]_{cyt}$ in guard cells in response to ABA, Ca^{2+}, H_2O_2 and CO_2 (Allen et al. 1999a, Allen et al. 2000, Pei et al. 2000, Allen et al. 2001, Mori et al. 2006, Young et al. 2006). Recently, it has been reported that MeJA-induced stomatal closure is accompanied by $[Ca^{2+}]_{cyt}$ oscillation in guard cells (Islam et al. 2010). Biochemical and genetic analyses indicate that ABA and MeJA induce reactive oxygen species (ROS) production mediated by NAD(P)H oxidase (Kwak et al. 2003, Suhita et al. 2004) and that ROS elicits $[Ca^{2+}]_{cyt}$ elevation required by ABA- and MeJA-induced stomatal closure in Arabidopsis (Pei et al. 2000, Murata et al. 2001, Munemasa et al. 2007). Elevation of $[Ca^{2+}]_{cyt}$ activates S-type anion currents and inhibits inward K^+ currents in the plasma membrane of guard cells to accelerate efflux of anion and K^+ from cytosol to apoplast, which leads to stomatal closure (Schroeder and Hagiwara 1989, Allen et al. 1999a, Vahisalu et al. 2008).

Increment of cytosolic pH (pH_{cyt}) (alkalization) in guard cells is a common phenomenon in both ABA- and MeJA-induced stomatal closure in Arabidopsis, *Pisum* and *Paphiopedilum* (Irving et al. 1992, Gehring et al. 1997, Suhita et al. 2004, Gonugunta et al. 2008). It has been reported that ABA elevates $[Ca^{2+}]_{cyt}$ in accordance with increment of pH_{cyt} in *Paphiopedilum* guard cells (Irving et al. 1992), but to our knowledge, there is no report of interaction between MeJA-induced $[Ca^{2+}]_{cyt}$ oscillation and cytosolic alkalization. The $[Ca^{2+}]_{cyt}$ increment inactivates inward K^+ currents, which is favorable to ABA-induced stomatal closure (Schroeder and Hagiwara 1989, McAinsh et al. 1990). Cytosolic alkalization also activates outward K^+ currents and inactivates inward K^+ currents to promote net efflux of K^+ in *Vicia* guard cells (Blatt and Armstrong 1993). Grabov and Blatt (1997) showed that cytosolic acidification activates inward K^+ currents but does not significantly

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change $[Ca^{2+}]_{cyt}$. These results indicate that changes in pH_{cyt} are closely involved in the modulation of ion mobilization to lead stomatal movement.

It has been demonstrated that H_2O_2 induced stomatal closure with cytosolic alkalization in *Vicia* guard cells (Zhang et al. 2001). Suhita et al. (2004) concluded that alkalization is upstream of ROS production based on the time course of alkalization and ROS production in Arabidopsis guard cells. Recently, Gonugunta et al. (2008) suggest without results of $[Ca^{2+}]_{cyt}$ in guard cells that $[Ca^{2+}]_{cyt}$ elevation functions upstream of ABA-induced cytosolic alkalization to induce stomatal closure in *Pisum sativum*. It remains unclear whether cytosolic alkalization functions in not only ABA signal cascade but also MeJA signal cascade in guard cells.

Protein phosphatases type 2C (PP2Cs), ABI1 and ABI2 are known as negative regulators of ABA signaling and *abi1-1* and *abi2-1* mutation disrupt both ABA and MeJA signaling in Arabidopsis (Gosti et al. 1999, Merlot et al. 2001, Murata et al. 2001, Munemasa et al. 2007). OST1 is one of the ABA-activated protein kinases and *ost1* mutation impairs ABA-induced stomatal closure but not MeJA-induced stomatal closure (Mustilli et al. 2002, Suhita et al. 2004). COI1 is one of the F-box proteins and *coi1* mutation disrupts MeJA-induced stomatal closure but not ABA-induced stomatal closure (Munemasa et al. 2007). However, the effects of these mutations on cytosolic alkalization in ABA and MeJA signaling are still unclear.

In this study, we used protein phosphatase mutants *abi1-1* and *abi2-1*, protein kinase mutant *ost1-2* and F-box protein mutant *coi1* in order to elucidate the role of guard cell Ca^{2+}_{cyt} in the modulation of guard cell pH_{cyt} induced by ABA, MeJA and Ca^{2+}. We examined (i) ABA- and MeJA-induced cytosolic alkalization in guard cells of Arabidopsis mutants, (ii) Ca^{2+}-induced stomatal closure and cytosolic alkalization and $[Ca^{2+}]_{cyt}$ oscillation in guard cells and (iii) the effect of butyrate, a cytosol-acidifying agent, on Ca^{2+}-induced stomatal closure, cytosolic alkalization and ABA-, MeJA- and Ca^{2+}-induced $[Ca^{2+}]_{cyt}$ oscillation in guard cells. Based on our findings, we propose an interaction of cytosolic alkalization and $[Ca^{2+}]_{cyt}$ oscillation on ABA signaling and MeJA signaling in Arabidopsis guard cells.

Results

ABA- and MeJA-induced cytosolic alkalization in Arabidopsis guard cells

ABA and MeJA increase pH_{cyt} in guard cells to induce stomatal closure (Irving et al. 1992, Gehring et al. 1997, Suhita et al. 2004, Gonugunta et al. 2008). In this study, we analyzed ABA- or MeJA-induced cytosolic alkalization in guard cells of wild-type, *abi1-1*, *abi2-1*, *ost1-2* and *coi1* plants using a pH-sensitive fluorescent dye, 2′,7′-bis-(2-carboxyethyl)-5,(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) in order to make it clear where cytosolic alkalization functions in ABA and MeJA signaling of guard cells.

As shown in **Fig. 1A**, 10 μM ABA ($P<0.008$) and 10 μM MeJA ($P<0.004$) increased BCECF fluorescence intensity in wild-type [Landsberg (Ler)] guard cells. This result indicates that ABA and MeJA induce cytosolic alkalization, which is similar to a previous report (Suhita et al. 2004). ABA did not induce cytosolic alkalization ($P>0.09$) but MeJA induced cytosolic alkalization ($P<0.007$) in *ost1-2* guard cells. Neither ABA nor MeJA changed pH_{cyt} in *abi1-1* (ABA: $P>0.06$; MeJA: $P>0.53$) and *abi2-1* (ABA: $P>0.92$; MeJA: $P>0.06$) guard cells. These results are consistent with ABA- and MeJA-induced stomatal closure phenotype in these mutants (Murata et al. 2001, Suhita et al. 2004, Munemasa et al. 2007, data not shown).

Like Ler wild-type plants, application of 10 μM ABA ($P<0.005$) and 10 μM MeJA ($P<0.04$) increased pH_{cyt} in wild-type [Columbia-0 (Col-0)] guard cells (**Fig. 1B**), which does not conflict with the results of Suhita et al. (2004). ABA ($P<0.009$) significantly increased pH_{cyt} in *coi1* whereas MeJA ($P>0.19$) failed to increase pH_{cyt} in *coi1* guard cells (**Fig. 1B**). These results are consistent with the result that *coi1* mutation impairs MeJA-induced stomatal closure but not ABA-induced stomatal closure (Munemasa et al. 2007). Time-course experiments show that pH_{cyt} in guard cells was elevated by ABA and MeJA with incubation time and reached its plateau level at 20 min (**Fig. 1C**). **Fig. 1D** shows representative fluorescence images of control epidermal tissue and ABA-treated and MeJA-treated tissues. There is no significant difference between ABA-induced alkalization and MeJA-induced cytosolic alkalization in guard cells ($P>0.22$). These results indicate that MeJA-induced stomatal closure as well as ABA-induced stomata closure requires cytosolic alkalization.

Extracellular Ca^{2+}-induced stomatal closure in ABA- or MeJA-insensitive mutants

Many previous studies illustrated that exogenous Ca^{2+} induced stomatal closure in *Commelina*, *Vicia*, *Pisum* and Arabidopsis (Schwartz et al. 1988, Allen et al. 2000, Li et al. 2004, Gonugunta et al. 2008, Islam et al. 2009, Islam et al. 2010). Exogenous Ca^{2+} elicited $[Ca^{2+}]_{cyt}$ elevation, resulting in stomatal closure in Arabidopsis guard cells (Allen et al. 1999a). The Ca^{2+} chelator EGTA prevents exogenous Ca^{2+}-induced stomatal closure (Schwartz 1985, Gonugunta et al. 2008). We examined exogenous Ca^{2+}-induced stomatal closure in wild-type plants (Ler and Col-0) and Arabidopsis mutant plants.

As shown in **Fig. 2A**, exogenous Ca^{2+} induced stomatal closure in a dose-dependent manner in *abi1-1*, *abi2-1*, *ost1-2* mutants and wild-type (Ler) plant. Exogenous Ca^{2+} at 2.5 mM significantly decreased stomatal apertures in wild type ($P<0.009$) and *abi1-1* ($P<0.006$), *abi2-1* ($P<0.0002$) and *ost1-2* ($P<0.0001$) mutants, which is similar to previous reports (Allen et al. 1999a, Mustilli et al. 2002).

Application of Ca^{2+} induced stomatal closure in wild-type (Col-0) and *coi1* mutant plants (**Fig. 2B**). Exogenous Ca^{2+} at 2.5 mM induced stomatal closure in wild type ($P<0.007$) and *coi1* ($P<0.002$). **Fig. 2A, B** indicates that Ca^{2+} promotes stomatal closure in these mutants as well as in wild type. These results

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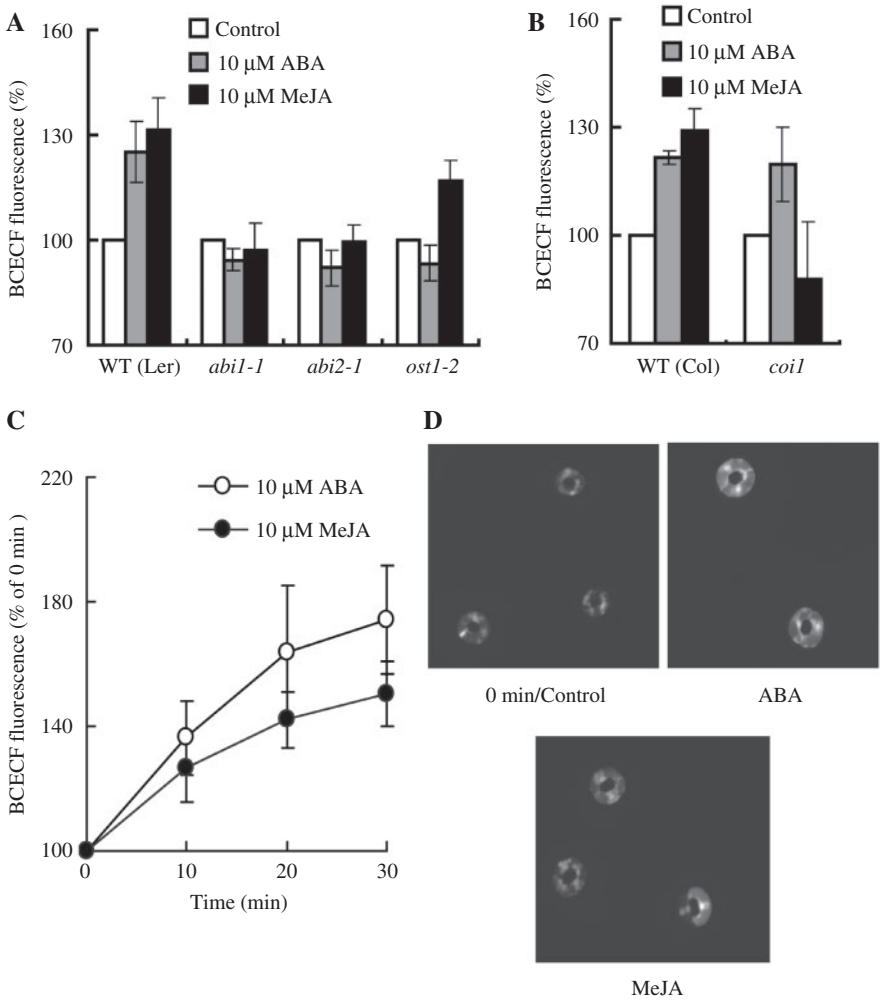

Fig. 1 ABA- and MeJA-induced cytosolic alkalization in Arabidopsis guard cells. (A) Effects of ABA ($n=3$) and MeJA ($n=3$) on BCECF fluorescence in wild-type (Ler), *abi1-1*, *abi2-1* and *ost1-2* mutant guard cells. (B) Effects of ABA ($n=3$) and MeJA ($n=3$) on BCECF fluorescence in wild-type (Col-0) and *coi1* mutant guard cells. (C) Time course of BCECF fluorescence in wild-type guard cells in response to ABA (open circle) and MeJA (closed circle) ($n=3$). The vertical scale represents the percentage of BCECF fluorescence when fluorescence intensities of ABA- or MeJA-treated cells are normalized to the control value taken as 100% for each experiment. Data were obtained from at least 50 guard cells. Error bars represent SD. (D) Representative fluorescence images of epidermal tissues at 0 min for control and 20 min after treatment with ABA and MeJA.

suggest that the ABA and MeJA signaling cascade downstream of [Ca^{2+}]$_{cyt}$ elevation is not impaired in *abi1-1*, *abi2-1*, *ost1-2* and *coi1* mutant plants.

Ca^{2+}-induced cytosolic alkalization in Arabidopsis guard cells

ABA- and MeJA-induced stomatal closure requires not only [Ca^{2+}]$_{cyt}$ elevation but also cytosolic alkalization in guard cells of Arabidopsis, *Paphiopedilum* and *Pisum* (Irving et al. 1992, Allen et al. 1999a, Suhita et al. 2004, Mori et al. 2006, Gonugunta et al. 2008). However, it is unclear whether exogenous Ca^{2+} evokes cytosolic alkalization in Arabidopsis guard cells. Thus, we investigated cytosolic alkalization in guard cells treated with Ca^{2+} using BCECF-AM.

As shown in **Fig. 2C, D**, external 2.5 mM Ca^{2+} increased pH$_{cyt}$ in wild-type (Ler: $P<0.02$; Col-0: $P<0.0003$) guard cells. External Ca^{2+} increased pH$_{cyt}$ in *abi1-1* ($P<0.05$), *abi2-1* ($P<0.01$), *ost1-2* ($P<0.005$) and *coi1* ($P<0.006$) guard cells. In **Fig. 2A, B**, Ca^{2+} induced stomatal closure in both wild type (Ler and Col-0) and these mutants. These results indicate that external Ca^{2+} induces stomatal closure with cytosolic alkalization in Arabidopsis guard cells.

Effects of butyrate on Ca^{2+}-induced stomatal closure and Ca^{2+}-induced cytosolic alkalization

To investigate whether Ca^{2+}-induced stomatal closure requires cytosolic alkalization, we used an intracellular acidifying agent, sodium butyrate, to inhibit cytosolic alkalization in guard cells (Leipziger et al. 2000, Stewart et al. 2001). Exogenous Ca^{2+} at 0.5, 1.0 and 2.5 mM (0.5 mM, $P<0.03$; 1.0 mM, $P<0.05$; 2.5 mM, $P<0.002$) induced stomatal closure in the absence of butyrate but failed to induce stomatal closure in the presence of butyrate

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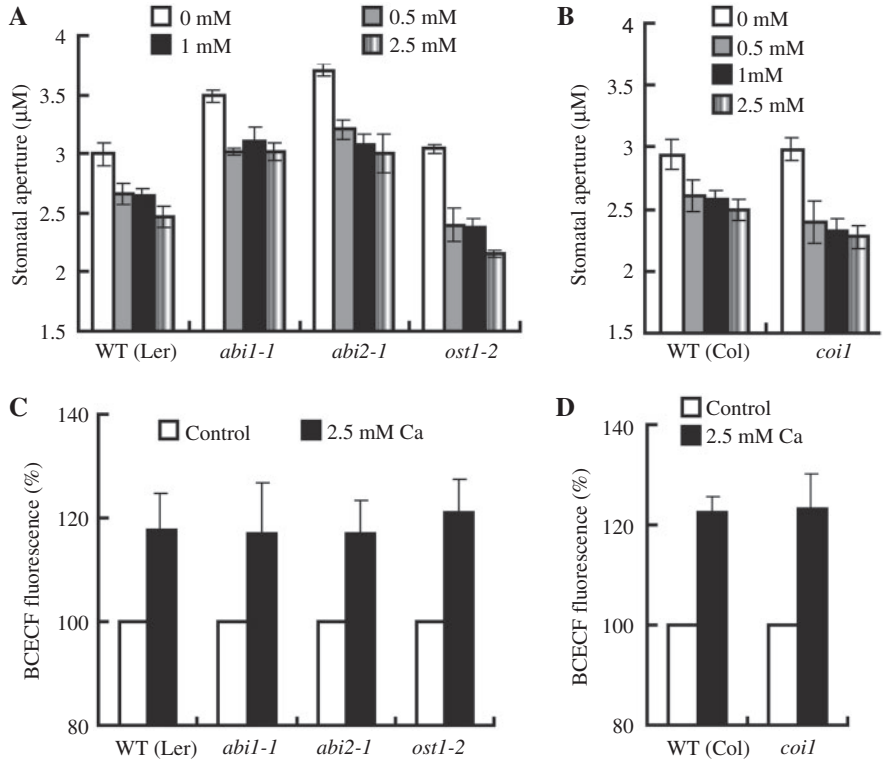

Fig. 2 Exogenous Ca^{2+}-induced stomatal closure and cytosolic alkalization in Arabidopsis guard cells. (A) Exogenous Ca^{2+} ($n=3$) reduces stomatal aperture in wild type (Ler), *abi1-1*, *abi2-1* and *ost1-2* mutants. (B) Exogenous Ca^{2+} ($n=3$) reduces stomatal aperture in wild type (Col-0) and *coi1* mutant. Data were obtained from 60 total stomata per bar. (C) Effects of Ca^{2+} ($n=3$) on BCECF fluorescence in wild-type (Ler), *abi1-1*, *abi2-1* and *ost1-2* mutant guard cells. (D) Effects of Ca^{2+} ($n=3$) on BCECF fluorescence in wild-type (Col-0) and *coi1* mutant guard cells. The vertical scale represents the percentage of BCECF fluorescence when fluorescent intensities of Ca^{2+}-treated cells are normalized to the control value taken as 100% for each experiment. Data were obtained from at least 50 guard cells. Error bars represent SD.

(0.5 mM, $P>0.07$; 1.0 mM, $P>0.13$; 2.5 mM, $P>0.10$) in wild-type plants (**Fig. 3A**), whereas butyrate did not significantly affect the stomatal apertures of Ca^{2+}-untreated wild-type plants ($P>0.09$).

We also assessed the effects of butyrate on Ca^{2+}-induced changes in pH_{cyt} in guard cells. Wild-type guard cells showed cytosolic alkalization in response to exogenous Ca^{2+} ($P<0.01$) whereas butyrate inhibited cytosolic alkalization in wild-type guard cells ($P>0.06$) (**Fig. 3B**). Suhita et al. (2004) demonstrated that butyrate prevents ABA- and MeJA-induced cytosolic alkalization along with stomatal closure in wild-type plants. Taken together with the results in **Fig. 3A**, these results suggested that ABA-, MeJA- and Ca^{2+}-induced stomatal closure is also mediated by cytosolic alkalization in guard cells and that cytosolic alkalization is involved in Ca^{2+}-dependent pathway in ABA and MeJA signaling in Arabidopsis guard cells.

Effect of butyrate on Ca^{2+}-, ABA- and MeJA-induced $[Ca^{2+}]_{cyt}$ oscillation in guard cells

Application of ABA, MeJA and Ca^{2+} induce $[Ca^{2+}]_{cyt}$ oscillations in Arabidopsis guard cells, leading to stomatal closure (Allen et al. 2000, Allen et al. 2001, Allen et al. 2002, Islam et al. 2010). We examined the effects of butyrate on Ca^{2+}-, ABA- and MeJA-induced $[Ca^{2+}]_{cyt}$ oscillations in guard cells using the Yellow Cameleon (YC) technique.

Exogenous Ca^{2+} at 2.5 mM elicited $[Ca^{2+}]_{cyt}$ oscillations (three or more oscillation, 83%; one or two oscillations 17%, $n=12$) in wild-type guard cells (**Fig. 4A, D**). Application of butyrate suppressed Ca^{2+}-induced $[Ca^{2+}]_{cyt}$ oscillations (three or more oscillations, 20%; one or two oscillations, 35%; no oscillation, 45%; $n=20$) in wild-type guard cells (**Fig. 4B, D**), whereas butyrate did not induce $[Ca^{2+}]_{cyt}$ oscillations (**Fig. 4C, D**). There is a significant difference in frequency of oscillation between untreated cells and butyrate-treated cells ($\chi^2=47.25$, $P<0.001$). These results are consistent with the result that butyrate inhibited Ca^{2+}-induced stomatal closure in the wild type (**Fig. 3A**).

Application of 10 μM ABA (**Fig. 5A, E**) and 10 μM MeJA (**Fig. 5C, F**) elicited $[Ca^{2+}]_{cyt}$ oscillations (three or more oscillation 38%; one or two oscillations 41%; no oscillation 21%, $n=34$ for ABA and three or more oscillation, 31%; one or two oscillations, 50%; no oscillation 19%, $n=26$ for MeJA) in wild-type guard cells, which is consistent with ABA- and MeJA-induced stomatal closure (Allen et al. 2000, Islam et al. 2010).

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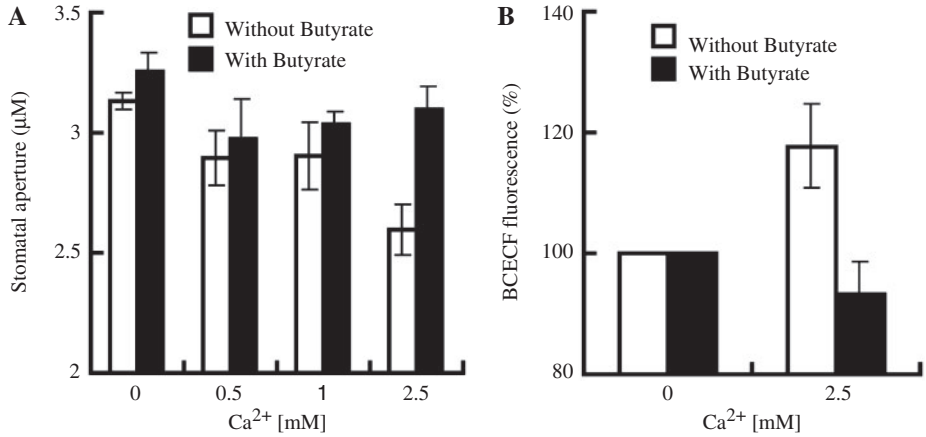

Fig. 3 Inhibition of Ca^{2+}-induced stomatal closure and cytosolic alkalization in guard cells by an acidifying agent, butyrate, in wild type (Ler). (A) Rosette leaves of wild-type plants were incubated for 2 h in light. Light-treated leaves were further treated with sodium butyrate (0.5 mM) under light for 2 h. Then, the leaves were treated with Ca^{2+} under light for 2 h. Data were obtained from 60 total stomata per bar. (B) Leaf epidermal tissues of wild-type plants were incubated for 3 h in light. Light-treated tissues were treated with 20 μM BCECF-AM for 30 min in the dark. Epidermal peels were rinsed with incubation buffer and then 0.5 mM sodium butyrate was added for 20 min. The epidermal tissues were treated for 20 min with 2.5 mM Ca^{2+} or water as control treatment. The vertical scale represents the percentage of BCECF fluorescence when fluorescent intensities of Ca^{2+} treated cells are normalized to the control value taken as 100% for each experiment. Data were obtained from at least 50 guard cells. Error bars represent SD.

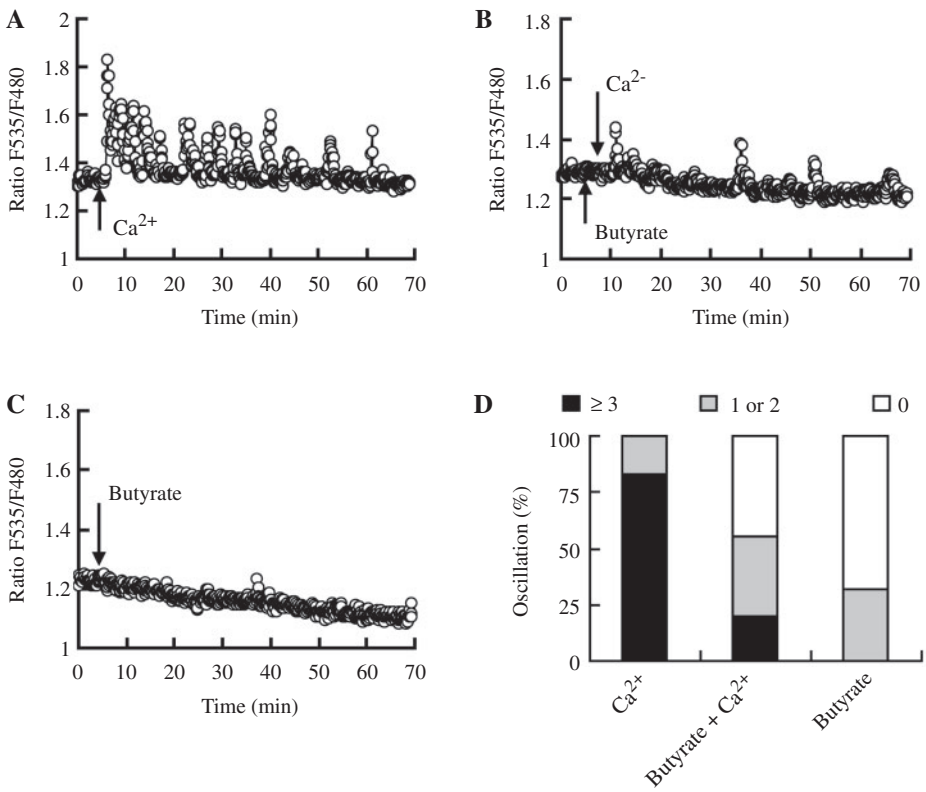

Fig. 4 Effects of 0.5 mM butyrate on Ca^{2+}-induced $[Ca^{2+}]_{cyt}$ oscillations in wild-type guard cells. The fluorescence emission ratio (535/480 nm) was measured in guard cells expressing yellow cameleon 3.6 (YC 3.6). (A) Ca^{2+}-induced $[Ca^{2+}]_{cyt}$ oscillations in wild-type guard cells untreated with butyrate. (B) Ca^{2+}-induced $[Ca^{2+}]_{cyt}$ oscillations in wild-type guard cells treated with butyrate. (C) $[Ca^{2+}]_{cyt}$ oscillations in wild-type guard cells treated with butyrate. (D) Frequencies of $[Ca^{2+}]_{cyt}$ oscillation in guard cells. $[Ca^{2+}]_{cyt}$ transients were counted when changes in $[Ca^{2+}]_{cyt}$ ratios were ≥0.1 units.

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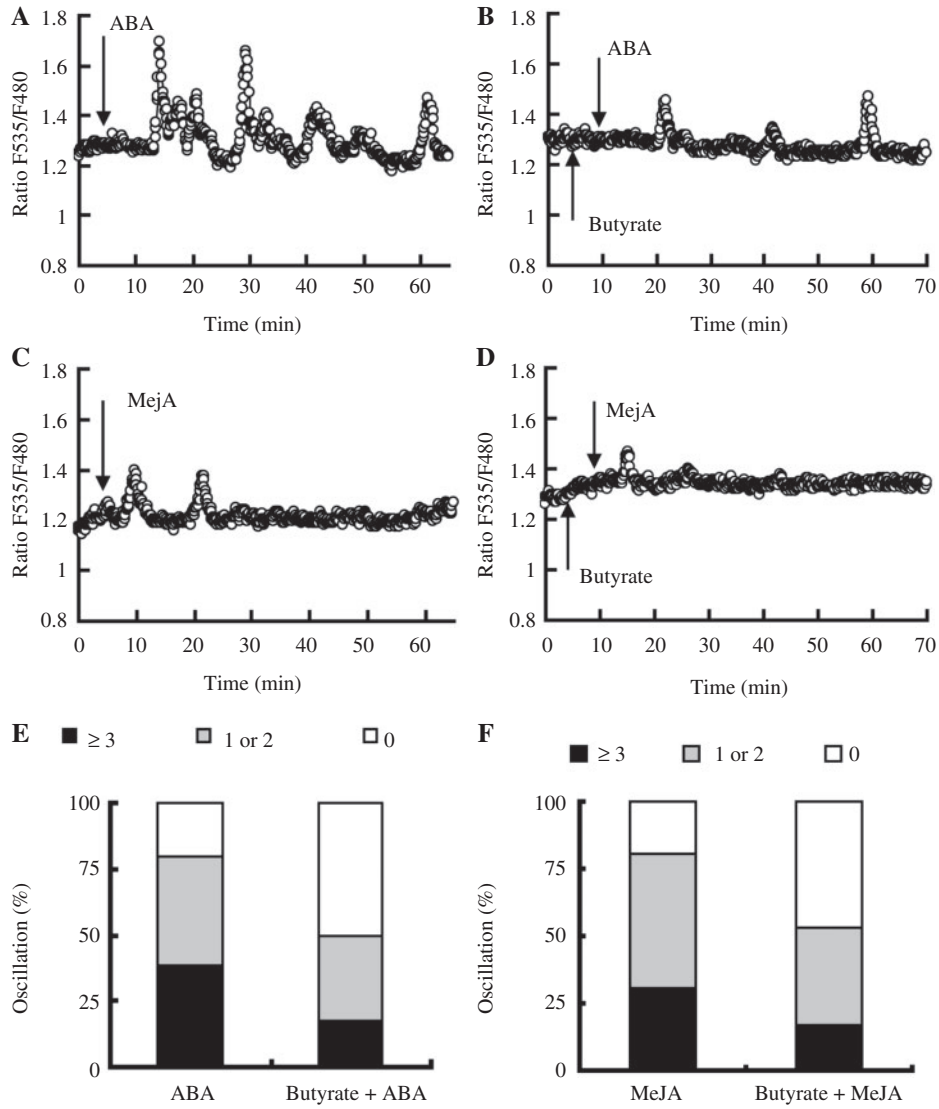

Fig. 5 Effects of 0.5 mM butyrate on ABA- and MeJA-induced $[Ca^{2+}]_{cyt}$ oscillations in wild-type guard cells. Fluorescence emission ratio (535/480 nm) was measured in guard cells expressing yellow cameleon 3.6 (YC 3.6). (A) ABA-induced $[Ca^{2+}]_{cyt}$ oscillations in wild-type guard cells untreated with butyrate. (B) ABA-induced $[Ca^{2+}]_{cyt}$ oscillations in wild-type guard cells treated with butyrate. (C) MeJA-induced $[Ca^{2+}]_{cyt}$ oscillations in wild-type guard cells untreated with butyrate. (D) MeJA-induced $[Ca^{2+}]_{cyt}$ oscillations in wild-type guard cells treated with butyrate. (E) Frequencies of ABA-induced $[Ca^{2+}]_{cyt}$ oscillation in guard cells in the absence or presence of butyrate. (F) Frequencies of MeJA-induced $[Ca^{2+}]_{cyt}$ oscillation in guard cells in the absence or presence of butyrate. $[Ca^{2+}]_{cyt}$ transients were counted when changes in $[Ca^{2+}]_{cyt}$ ratios were ≥0.1 units.

Application of butyrate suppressed ABA- and MeJA-induced $[Ca^{2+}]_{cyt}$ oscillations (three or more oscillations, 18%; one or two oscillations, 50%; no oscillation, 34%; $n=34$ for ABA and three or more oscillations, 16%; one or two oscillations, 37%; no oscillation, 47%; $n=30$ for MeJA) in wild-type guard cells (**Fig. 5B, E and D, F**). There is a significant difference in the frequency of oscillation between butyrate-untreated cells and butyrate-treated cells ($\chi^2=18.36$, $P<0.001$ for ABA and $\chi^2=16.36$, $P<0.001$ for MeJA). These results are consistent with the results that butyrate inhibited ABA- and MeJA-induced stomatal closure in the wild type (Suhita et al. 2004).

Discussion

ABA activates vacuolar H^+-ATPase activity in *Mesembryanthemum*, resulting in cytosolic alkalization (increment of pH_{cyt}) (Barkla et al. 1999). Both ABA and MeJA stimulate ROS production in wild-type guard cells to increase $[Ca^{2+}]_{cyt}$ via activation of plasma membrane non-selective cation channels during stomatal closure in Arabidopsis (Pei et al. 2000, Munemasa et al. 2007). In guard cells, $[Ca^{2+}]_{cyt}$ and pH_{cyt} function as second messengers to activate anion efflux and K^+ to lead stomatal closure (Schroeder and Hagiwara 1989, Blatt and Armstrong 1993).

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In this study, we investigated signal interaction between $[Ca^{2+}]_{cyt}$ elevation and cytosolic alkalization during ABA- and MeJA-induced stomatal closure in Arabidopsis.

Cytosolic alkalization in guard cells during ABA- and MeJA-induced stomatal closure

Previous studies have reported that stomatal closure is triggered by elevating guard cell $[Ca^{2+}]_{cyt}$ and pH_{cyt} in response to ABA or MeJA in Arabidopsis and *Pisum* (Suhita et al. 2004, Gonugunta et al. 2008). Pharmacological and genetic experiments indicate that ROS is one of common signal components for ABA- and MeJA-induced stomatal closure (Munemasa et al. 2007). Protein kinase OST1 is involved in ABA signaling in guard cells and the *ost1* mutation impairs ABA-induced stomatal closure but not MeJA-induced stomatal closure (Suhita et al. 2004). F-box protein COI1 is a jasmonate receptor and the *coi1* mutation disrupts MeJA signaling but not ABA signaling in guard cells (Munemasa et al. 2007, Yan et al. 2009). The PP2C ABI1 interacts with ABA receptors RCARs and PYR/PYL in ABA signaling (Ma et al. 2009, Park et al. 2009) but interestingly the *abi1* mutation impairs both ABA- and MeJA-induced stomatal closure (Murata et al. 2001, Munemasa et al. 2007).

Present results confirm previous work that *abi1-1* mutation impaired both ABA- and MeJA-induced guard cell cytosolic alkalization and *ost1-2* mutation impaired only ABA-induced guard cell cytosolic alkalization (**Fig. 1A**), which is consistent with stomatal movement in response to ABA or MeJA in *abi1-1* and *ost1-2* mutants (Suhita et al. 2004). Unlike ABA, MeJA failed to increase pH_{cyt} in *coi1* mutants (**Fig. 1B**), which is also consistent with the results that *coi1* mutation impairs MeJA-induced stomatal closure but not ABA-induced stomatal closure (Munemasa et al. 2007). These results also indicate that cytosolic alkalization is required by ABA- and MeJA-induced stomatal closure and a common signal component of ABA and MeJA signaling.

It has been shown that the *abi2-1* mutation disrupts downstream of ROS production in the ABA and MeJA signal cascade of guard cells (Murata et al. 2001, Munemasa et al. 2007). Application of exogenous H_2O_2 induces cytosolic alkalization in guard cells to lead stomatal closure in *Vicia faba* (Zhang et al. 2001). In this study, the *abi2-1* mutation also impairs ABA- and MeJA-induced pH_{cyt} elevation in guard cells (**Fig. 1A**). Taken together, these results indicate that increment of pH_{cyt} (cytosolic alkalization) functions downstream of ROS production in ABA signaling and MeJA signaling of guard cells, which is consistent with the recent finding of Gonugunta et al. (2008).

Suhita et al. (2004) have concluded that alkalization is upstream of ROS production based on the time course of alkalization and ROS production, which is in conflict with other results (Zhang et al. 2001, Gonugunta et al. 2008). The time-course experiments probably missed undetectable earlier ROS production in early response to ABA and MeJA because the reaction rate of DCF formation is slower than that of change in BCECF fluorescence, i.e. ROS detection using H_2DCF requires peroxidase mediation before formation of fluorescent DCF and pH measurement using BCECF is based on protonation/deprotonation. The reaction rate of the former could be slower than that of the latter, suggesting that the time-course experiment could not detect earlier ROS production followed by cytosolic alkalization in guard cells.

Exogenous Ca^{2+}-induced stomatal closure requires cytosolic alkalization and $[Ca^{2+}]_{cyt}$ oscillation in guard cells

Exogenous Ca^{2+} as well as ABA and MeJA induce $[Ca^{2+}]_{cyt}$ elevation and elicit $[Ca^{2+}]_{cyt}$ oscillation in guard cells, resulting in stomatal closure (Allen et al. 2000, Allen et al. 2001, Allen et al. 2002, Pei et al. 2000, Munemasa et al. 2007, Islam et al. 2010). In this study, exogenous Ca^{2+} also elicited $[Ca^{2+}]_{cyt}$ oscillation (**Fig. 4A**), which is consistent with stomatal closure (**Fig. 2A, B**). We also investigated change in pH_{cyt} in wild type, *abi1-1*, *abi2-1*, *ost1-2* and *coi1* in response to exogenous Ca^{2+}.

The present study shows that application of Ca^{2+} induces cytosolic alkalization in *abi1-1*, *abi2-1*, *ost1-2* and *coi1* guard cells as well as wild-type guard cells (**Fig. 2C, D**), which is consistent with stomatal phenotype in these mutants in response to exogenous Ca^{2+} (**Fig. 2A, B**). In *Pisum*, changes in $[Ca^{2+}]_{cyt}$ are involved in changes in pH_{cyt} in guard cells during ABA-induced stomatal closure (Gonugunta et al. 2008). These results indicate that $[Ca^{2+}]_{cyt}$ elevation is accompanied by cytosolic alkalization in guard cells, resulting in stomatal closure and suggest that cytosolic alkalization functions downstream of $[Ca^{2+}]_{cyt}$ elevation in Ca^{2+} signaling in guard cells.

Both ABA and MeJA cause stomatal closure following ROS production, activation of plasma membrane I_{Ca} currents by ROS, $[Ca^{2+}]_{cyt}$ oscillation (elevation), which activate anion channels and outward K^+ channels and inactivate inward K^+ channels. Therefore, it is suggested that a Ca^{2+}-dependent pathway contributes to ABA- and MeJA-induced stomatal closure (McAinsh et al. 1990, Pei et al. 2000, Murata et al. 2001, Evans 2003, Mori et al. 2006, Munemasa et al. 2007, Saito et al. 2008, Islam et al. 2010). On the other hand, it is also suggested that the Ca^{2+}-independent pathway mediates ABA-induced stomatal closure (Gilroy et al. 1991, Allan et al. 1994). In addition, exogenous Ca^{2+} activates anion currents and inhibits inward K^+ currents, which are favorable to stomatal closure (Schroeder and Hagiwara 1989, Grabov and Blatt 1999, Mori et al. 2006). This present study indicates that cytosolic alkalization is involved in a Ca^{2+}-dependent pathway leading to stomatal closure.

Interaction between cytosolic alkalization and $[Ca^{2+}]_{cyt}$ oscillation on stomatal closure

We examined effects of an intracellular acidifying agent, sodium butyrate, on Ca^{2+}-induced stomatal closure and Ca^{2+}-induced $[Ca^{2+}]_{cyt}$ oscillation. Butyrate inhibited ABA-, MeJA- and Ca^{2+}-induced stomatal closure and cytosolic alkalization (**Fig. 3A, B**; Suhita et al. 2004, Gonugunta et al. 2008), which also indicates that Ca^{2+}-induced stomatal closure requires cytosolic alkalization in guard cells and that $[Ca^{2+}]_{cyt}$ elevation is an earlier

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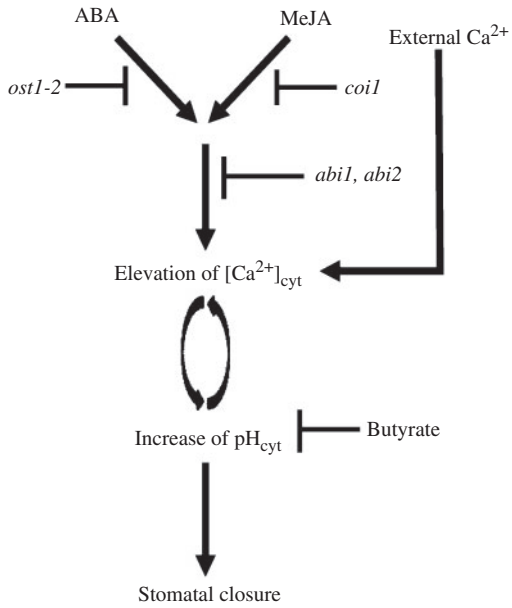

Fig. 6 Simplified model for ABA- and MeJA-induced stomatal closure. Cytosolic alkalization is a downstream signal component of $[Ca^{2+}]_{cyt}$ elevation and feedback-regulates $[Ca^{2+}]_{cyt}$ oscillation in ABA and MeJA signaling of Arabidopsis guard cells.

essential signaling component to increase pH_{cyt} in response to ABA, MeJA and Ca^{2+} in Arabidopsis guard cells. The present study shows that butyrate suppresses Ca^{2+}-, ABA- and MeJA-induced $[Ca^{2+}]_{cyt}$ oscillation (**Figs. 4, 5**), which suggests that cytosolic alkalization feedback-regulates $[Ca^{2+}]_{cyt}$ oscillation.

Intracellular pH is crucial to various cellular functions and intracellular alkalization is associated with an increase in cytosolic calcium in animal cells (Ilino et al. 1994, Alfonso et al. 2000). ABA activates vacuolar H^+-ATPase (V-ATPase) activity in *Mesembryanthemum* (Barkla et al. 1999) and elicits $[Ca^{2+}]_{cyt}$ elevation and $[Ca^{2+}]_{cyt}$ oscillation (Allen et al. 1999b, Allen et al. 2000, Murata et al. 2001, Mori et al. 2006, Islam et al. 2010), suggesting that pH_{cyt} is closely related to $[Ca^{2+}]_{cyt}$ in plant cells.

In conclusion, cytosolic alkalization is elicited following $[Ca^{2+}]_{cyt}$ elevation and feedback-regulates $[Ca^{2+}]_{cyt}$ oscillation in ABA and MeJA signaling of Arabidopsis guard cells (**Fig. 6**).

Materials and Methods

Plant materials and growth conditions

In this study, we used *Arabidopsis thaliana* plants both Landsberg and Columbia-0 ecotypes as the wild type. Arabidopsis *abi1-1*, *abi2-1*, *ost1-2* (Landsberg accession) and *coi1* (Columbia accession) mutants were grown in plastic pots filled with a homogenized mixture of 70% (v/v) vermiculite (Asahi-kogyo, Okayama, Japan) and 30% (v/v) Kureha soil (Kureha Chemical, Tokyo, Japan) in a growth chamber under a 16 h light/8 h dark cycle. The temperature and relative humidity in the chamber was 22±2°C and 60±10%, respectively. Water with Hyponex solution (0.1%) was applied to the plants growing tray twice a week. Homozygous *coi1* seedlings were screened by luciferase activity (Ellis and Turner 2001). The solution containing 3 mM luciferin (Promega) with 0.01% Triton X-100 was used on detached leaves. The plants were treated with 10 µM MeJA for 24 h before using luciferin solution. Homozygous *coi1* seedlings leaves did not show luciferase expression to MeJA.

Analysis of stomatal apertures

Stomatal apertures were measured according to the method of (Munemasa et al. 2007). Excised rosette leaves of 5- to 6-week-old plants were floated on stomata assay solution containing 5 mM KCl, 50 µM $CaCl_2$ and 10 mM Mes–Tris (pH 6.15) under light for 2 h incubation and then ABA, MeJA and $CaCl_2$ were added to the preincubated leaves. After 2 h incubation, stomatal apertures were measured after the leaf was blended for 25 s and the epidermal tissues were collected with nylon mesh. Stomatal apertures were observed under a microscope (Inverted Microscope 1X71–22TPH; Olympus, Japan).

Measurement of pH_{cyt}

A pH-sensitive fluorescent dye, BCECF-AM, used to analyze cytosolic pH (pH_{cyt}) in guard cells treated with ABA, MeJA, or $CaCl_2$ as described previously (Suhita et al. 2004) with a slight modification. Four or five leaf epidermis strips were isolated from leaves of 5- to 6-week-old plants with a commercial blender. The collected epidermal strips were incubated for 3 h in light containing 50 mM KCl and 10 mM MES–KOH (pH 6.5). After this incubation, BCECF-AM was added as indicated to a final concentration of 20 µM from a stock solution in dimethylsulfoxide and then epidermal tissues were incubated for 30 min in the dark at room temperature to load the dye. Then the tissues were rinsed several times with incubation buffer in order to remove excess dye. The dye-loaded epidermal tissues were treated for 20 min with 10 µM ABA, 10 µM MeJA or 2.5 mM $CaCl_2$. Ethanol (0.1%) for ABA and MeJA treatment and distilled water for Ca^{2+} treatment was used as solvent control. Fluorescence was quantified using a fluorescence microscope with the following settings: excitation 480 nm and emission 535 nm. AQUA COSMOS software (Hamamatsu Photonics, Hamamatsu, Japan.) was used for image analyses. Each datum was obtained from at least 50 guard cells.

Measurement of $[Ca^{2+}]_{cyt}$

Rosette leaves of Arabidopsis YC3.6-expressing plants were used to examine $[Ca^{2+}]_{cyt}$ oscillations in guard cells as described (Islam et al. 2010). The abaxial side of the excised leaf was gently mounted on a glass slide by using a medical adhesive, followed by removal of the adaxial epidermis and the mesophyll tissue with a razor blade in order to keep intact the lower epidermis on the slide. The mounted abaxial epidermal peel was kept in an incubation solution containing 5 mM KCl, 50 µM $CaCl_2$ and 10 mM MES–Tris (pH 6.15) under light for 2 h at 22°C. The turgid guard cells were considered for ratiometric $[Ca^{2+}]_{cyt}$ measurement. Then, guard cells were treated with the

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incubation solution supplemented with 2.5 mM Ca^{2+} with a peristatic pump after 5 min from the start of measurement. Observation of YC3.6 was done by dual-emission ratio imaging using 440DF20 excitation filter, 445DRLP dichroic mirror and two emission filters 480DF30 for CFP and 535DF25 for YFP. The CFP and YFP fluorescence intensity of guard cells was imaged and analyzed using AQUA COSMOS software.

Statistical analysis

Significance of differences between mean values was assessed by Student's *t*-test except $[Ca^{2+}]_{cyt}$ oscillation data and significance of differences in $[Ca^{2+}]_{cyt}$ oscillations between with butyrate and without butyrate treated cells induced by Ca^{2+} ABA and MeJA, which were determined by chi-squared test. Differences at $P < 0.05$ were considered significant.

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