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Ethylene Inhibits Methyl Jasmonate-Induced Stomatal Closure by Modulating Guard Cell Slow-Type Anion Channel Activity via the OPEN STOMATA 1/SnRK2.6 Kinase-Independent Pathway in Arabidopsis

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Signal crosstalk between jasmonate and ethylene is crucial for a proper maintenance of defense responses and development. Although previous studies reported that both jasmonate and ethylene also function as modulators of stomatal movements, the signal crosstalk mechanism in stomatal guard cells remains unclear. Here, we show that the ethylene signaling inhibits jasmonate signaling as well as abscisic acid (ABA) signaling in guard cells of *Arabidopsis thaliana* and reveal the signaling crosstalk mechanism. Both an ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) and an ethylene-releasing compound ethephon induced transient stomatal closure, and also inhibited methyl jasmonate (MeJA)-induced stomatal closure as well as ABA-induced stomatal closure. The ethylene inhibition of MeJA-induced stomatal closure was abolished in the ethylene-insensitive mutant *etr1–1*, whereas MeJA-induced stomatal closure was impaired in the ethylene-overproducing mutant *eto1–1*. Pretreatment with ACC inhibited MeJA-induced reactive oxygen species (ROS) production as well as ABA-induced ROS production in guard cells but did not suppress ABA activation of OPEN STOMATA 1 (OST1) kinase in guard cell-enriched epidermal peels. The whole-cell patch-clamp analysis revealed that ACC attenuated MeJA and ABA activation of S-type anion channels in guard cell protoplasts. However, MeJA and ABA inhibitions of K_{in} channels were not affected by ACC pretreatment. These results suggest that ethylene signaling inhibits MeJA signaling and ABA signaling by targeting S-type anion channels and ROS but not OST1 kinase and K^+ channels in Arabidopsis guard cells.

Keywords: Abscisic acid • Ethylene • Guard cell • Ion channel • Jasmonate • Stomata.

Introduction

Guard cells, which form stomatal pores mainly in leaf epidermis, can perceive diverse environmental signals and control the stomatal aperture through sophisticated signal integration mechanisms. The plants use the mechanism to optimize photosynthetic activity and transpiration under fluctuating environments (Murata et al. 2015). However, although guard cell signal transduction evoked by individual stimuli such as abscisic acid (ABA) (Munemasa et al. 2015), blue light (Shimazaki et al. 2007, Inoue and Kinoshita 2017) and CO_2 (Engineer et al. 2016) has been extensively studied in the past decade, the signal integration mechanism remains far from clear.

Jasmonic acid and its derivatives are the plant hormones that regulate various developmental processes and defense responses against insects and pathogens (Liechti and Farmer 2002, Turner et al. 2002, Browse, 2009). It has been reported that similar to ABA, jasmonate induces stomatal closure in various plant species (Raghavendra and Reddy 1987, Gehring et al. 1997, Suhita et al. 2003, Suhita et al. 2004, Xin et al. 2005). In guard cells, the jasmonate signal pathway shares several signal components with the ABA signal pathway to induce stomatal closure.

The SNF-related protein kinase, OPEN STOMATA 1 (OST1), which is activated by ABA and plays a crucial role for ABA-induced stomatal closure, is also essential for jasmonates-induced stomatal closure (Mustilli et al. 2002, Yin et al. 2016). It has been shown that the OST1 kinase phosphorylates many downstream targets and that one of the most important targets is SLOW ANION CHANNEL-ASSOCIATED 1 (SLAC1) (Geiger et al. 2009, Lee et al. 2009). The guard cell plasma membrane protein SLAC1 mediates slow, sustained-type (S-type) anion channel activity (Negi et al. 2008, Vahisalu et al. 2008), and activation of the S-type anion channels triggers plasma membrane depolarization, resulting in activation of voltage-dependent outwardly rectifying K^+ channels that mediate K^+ efflux from guard cells (Keller et al. 1989, Schroeder and Hagiwara, 1989, Schroeder and Keller 1992). The efflux of anion and K^+ from guard cells leads to reduction of guard cell turgor pressure, resulting in stomatal closure. It was reported that methyl jasmonate (MeJA) like ABA activates the S-type anion currents (Munemasa et al. 2007, Munemasa et al. 2011, Khokon et al. 2015). In addition, jasmonates inactivate

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inward rectifying K^+ (K^+_{in}) channels in Arabidopsis guard cells (Saito et al. 2008, Yin et al. 2016) and activate outward rectifying K^+ (K^+_{out}) channels in guard cells of *Vicia faba* (Evans 2003).

The gas hormone ethylene inhibits ABA-induced stomatal closure (Tanaka et al. 2005, Wilkinson and Davies 2009, Beguerisse-Díaz et al. 2012, Chen et al. 2013, Watkins et al. 2014). The inhibitory effect of ethylene is likely to be due to flavonol accumulation, which results in reduction of reactive oxygen species (ROS) level in guard cells (Watkins et al. 2014, Watkins et al. 2017). In contrast, it was also reported that ethylene induces stomatal closure via ROS production in guard cells (Desikan et al. 2006, Shi et al. 2015). The reason for these inconsistent results is thought to be the difference in experimental condition (Desikan et al. 2006).

Plant hormone signaling crosstalk is involved in many physiological processes. The signal crosstalk between jasmonate and ethylene in fine-tuned regulation of development, growth and defense responses has been well studied (e.g. Penninckx et al. 1998, Lorenzo et al. 2003, Sun et al. 2006, Pré et al. 2008, Song et al. 2014, Zhang et al. 2014). However, the crosstalk mechanism between jasmonate and ethylene signaling in guard cells is unclear. In this study, we show that ethylene signaling inhibits jasmonate signaling as well as ABA signaling in guard cells via suppression of ROS production and S-type anion channel activity.

Results

Ethylene induces transient stomatal closure and inhibits MeJA-induced stomatal closure

Some studies reported that ethylene inhibits ABA-induced stomatal closure (Tanaka et al. 2005, Wilkinson and Davies 2009, Beguerisse-Díaz et al. 2012, Chen et al. 2013, Watkins et al. 2014) and others reported that ethylene itself induces stomatal closure (Desikan et al. 2006, Shi et al. 2015). Hence, we performed a time-course analysis of stomatal response to ethylene. For ethylene treatment, an ethylene precursor ACC (1-aminocyclopropane-1-carboxylic acid) and an ethylene-releasing compound ethephon were used (Tanaka et al. 2005, Desikan et al. 2006, Shi et al. 2015). Under our experimental condition, ethephon and ACC treatments induced transient stomatal closure, which disappeared 2 h after starting the treatment (**Fig. 1A**). The transient closure was abolished in the ethylene-insensitive mutant *etr1–1* (**Fig. 1B**), which is consistent with previous reports (Desikan et al. 2006, Shi et al. 2015). In addition, both the Ca^{2+} chelator EGTA and the Ca^{2+} channel blocker $LaCl_3$ inhibited the transient closure (**Fig. 1C**), suggesting that the ethylene-induced transient stomatal closure is Ca^{2+}-dependent.

In following experiments, to check the inhibitory effect of ethylene on guard cell MeJA signaling and ABA signaling, rosette leaves were preincubated with ACC or ethephon for >2 h where the transient closure disappeared as shown in **Fig. 1A**. MeJA-induced stomatal closure was inhibited by ACC or ethephon (**Fig. 2A**). As reported previously (Watkins et al. 2014, Tanaka et al. 2005), ethylene signal activation by ACC or ethephon inhibited ABA-induced stomatal closure (**Fig. 2A**). In the ethylene-insensitive mutant *etr1-1*, neither ACC nor ethephon pretreatment significantly inhibited MeJA-induced stomatal closure (**Fig. 2B**). In addition, the ethylene overproducing mutant *eto1-1* displayed an attenuation of MeJA-induced stomatal closure as well as ABA-induced stomatal closure (**Fig. 2C**). These results suggest that ethylene inhibits MeJA signaling as well as ABA signaling in guard cells.

Ethylene inhibits MeJA- and ABA-induced ROS production in guard cells but does not inhibit ABA activation of OST1 kinase

ROS act as a second messenger in guard cell ABA signaling, and plasma membrane NAD(P)H oxidases are responsible for ABA-induced ROS production in guard cells (Pei et al. 2000, Kwak et al. 2003). It was reported that ROS also play a crucial role in guard cell MeJA signaling (Suhita et al. 2004, Munemasa et al. 2007); therefore, next we tested the effect of ethylene signal activation on MeJA-induced ROS production. The guard cell ROS accumulation was visualized using the ROS detection fluorescent dye, 2′,7′-dihydrodichlorofluorescein diacetate (H_2DCF-DA) (Pei et al. 2000, Zhang et al. 2001). Exogenous application of MeJA- or ABA-induced ROS production in guard cells (**Fig 3A;** $P < 0.05$ for Control vs. MeJA and Control vs. ABA), as reported previously (Suhita et al. 2004, Munemasa et al. 2007). We found that ACC pretreatment inhibited MeJA-induced ROS production, as well as ABA-induced ROS production (**Fig 3A;** $P = 0.30$ for ACC vs. MeJA+ACC and $P = 0.47$ for ACC vs. ABA+ACC) as previously reported (Watkins et al. 2014).

To further elucidate the mechanism of how ethylene antagonizes jasmonate signaling in guard cells, we also tested the effect of ACC pretreatment on OST1 kinase activity in guard cells. The OST1 kinase is key for both MeJA signaling and ABA signaling in guard cells (Mustilli et al. 2002, Yoshida et al. 2002, Yin et al. 2016). In-gel protein kinase assay revealed that ABA activation of OST1 kinase was not inhibited by ACC pretreatment in guard cell-enriched epidermal peels (**Fig. 3B**). Although MeJA also requires OST1 to induce stomatal closure, it is reported that MeJA does not activate OST1 kinase (Yin et al. 2016). Consistent with the previous observation, the activation of OST1 in guard cell-enriched epidermal peels treated with 10 μM MeJA was not detectable (**Fig. 3B**).

Ethylene inhibits MeJA and ABA activation of S-type anion channels but not MeJA and ABA regulation of K^+ channels

Activation of the S-type anion channel SLAC1 is the crucial event in ABA- and MeJA-induced stomatal closure. Therefore, next we tested the effects of ethylene on MeJA and ABA activation of S-type anion channels by the whole-cell patch-clamp analysis. Three-hour ACC pretreatment alone did not affect S-type anion channel activity (**Fig. 4**). As previously reported (Pei et al. 1997, Munemasa et al. 2007), S-type anion currents were activated by both MeJA (**Fig. 4A, C**) and ABA (**Fig. 4B, D**), and the MeJA and ABA activation of S-type anion currents was attenuated by pretreatment with ACC.

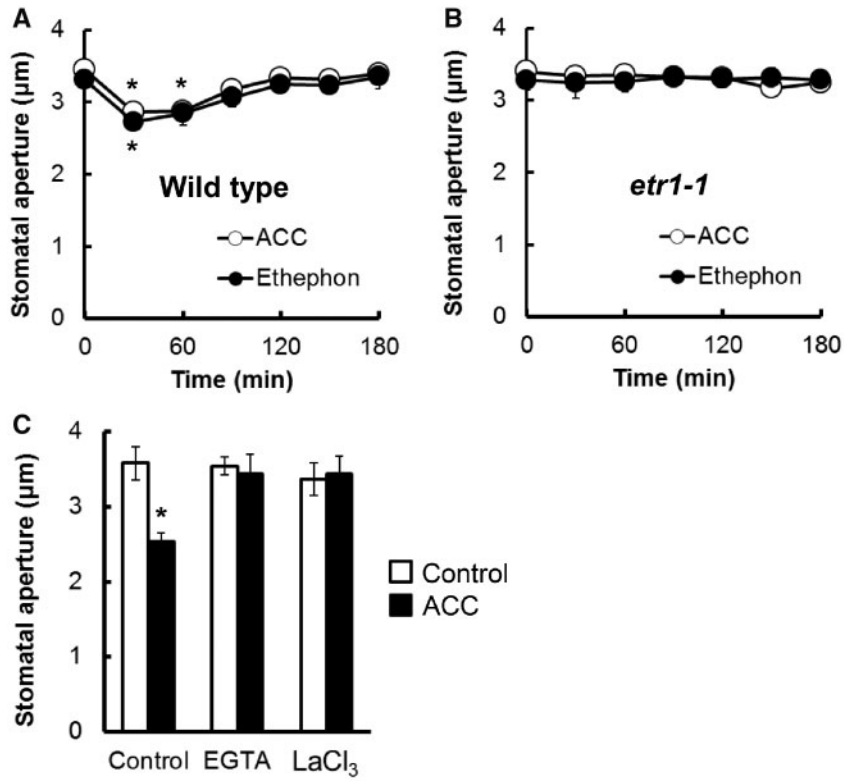

Fig. 1 Ethylene signal activation induces transient stomatal closure. (A) Effects of 10 μM ACC or ethephon on stomatal aperture of wild-type plants. Averages from five independent experiments (100 total stomata per bar) are shown. (B) Effects of 10 μM ACC or ethephon on stomatal aperture of *etr1-1* mutant plants. Averages from three independent experiments (60 total stomata per bar) are shown. (C) Effects of the Ca^{2+} chelator EGTA and the Ca^{2+} channel blocker $LaCl_3$ on ACC-induced transient stomatal closure in wild-type plants. Rosette leaves were preincubated with 10 mM EGTA and 50 μM $LaCl_3$ for 2 h in the light, followed by 30-min incubation with ACC. Averages from three independent experiments (60 total stomata per bar) are shown. Error bars represent standard errors. Asterisk denotes a significant difference vs. corresponding control ($P < 0.05$).

Next, we tested the effect of ethylene on guard cell K^+ channel activity, which also plays an important role for regulation of guard cell turgor pressure. Three-hour ACC pretreatment alone did not affect K^+ channel activity (**Fig. 5**). As previously reported (Saito et al. 2008, Yin et al. 2016), K_{in} channel activities are suppressed by MeJA (**Fig. 5A, C**) as well as by ABA (**Fig. 5B, D**). The MeJA and ABA inhibitions of K_{in}^+ channel were not attenuated by pretreatment with ACC (**Fig. 5**). Under our experimental condition, K_{out}^+ channel activities were not changed by treatment with MeJA as well as ABA, which is consistent with previous studies (Schwartz et al. 1994, Pandey et al. 2002, Becker et al. 2003, Roelfsema et al. 2004, Fan et al. 2008, Acharya et al. 2013).

Discussion

Both ethylene and jasmonate are known to modulate stomatal movement. Although the signal crosstalk has been well studied in the regulation of defense responses and development, the mechanism in guard cells has not been well understood. Here, we found that the activation of ethylene signaling by ACC and ethephon induces transient stomatal closure (**Fig. 1**), and also inhibits MeJA-induced stomatal closure as well as ABA-induced stomatal closure (**Fig. 2A**). These findings support a dual regulatory role of ethylene on stomatal movement (Desikan et al. 2006). The ethylene-induced transient stomatal closure (**Fig. 1A**) is similar to the stomatal response known as 'calcium-reactive closure' (Allen et al. 2001, Cho et al. 2009, Eisenach et al. 2012). Supporting this notion, both the Ca^{2+} chelator EGTA and the Ca^{2+} channel blocker $LaCl_3$ inhibited the ethylene-induced transient closure (**Fig. 1C**). We found that MeJA-induced stomatal closure and ABA-induced stomatal closure were inhibited by ACC pretreatment as well as by ethephon pretreatment (**Fig 2A**). In addition, MeJA-induced stomatal closure and ABA-induced stomatal closure were attenuated in the ethylene-overproducing mutant *eto1-1* (**Fig. 2C**). These results indicate that ethylene signaling inhibits MeJA signaling and ABA signaling downstream of the convergence point of the two signaling (**Fig. 6**). Tanaka et al. (2005) showed that ethylene does not inhibit dark-induced stomatal closure. Together, the ethylene inhibitory effect has some specificity.

It has been shown that NADPH oxidases RBOHs are responsible for ROS production in guard cell ABA, MeJA and ethylene signaling (Murata et al. 2015). We found that the ACC pretreatment inhibits MeJA- and ABA-induced ROS production (**Fig. 3A**), consistent with previous studies (Watkins et al.

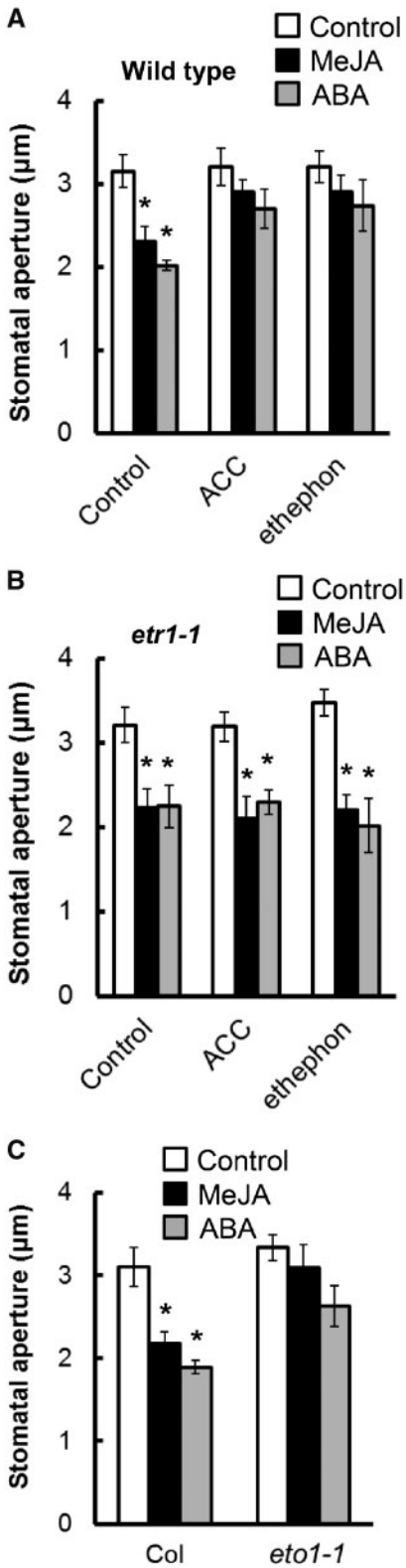

Fig. 2 Ethylene signal activation inhibits MeJA-induced stomatal closure as well as ABA-induced stomatal closure. (A, B) Effects of 10 μM ACC and ethephon on MeJA-induced stomatal closure and ABA-induced stomatal closure in wild-type plants (A) and *etr1-1* mutant plants (B). (C) MeJA- and ABA-induced stomatal closure in the ethylene overproducing *eto1-1* mutant. Averages from three-independent experiments (>60 total stomata per bar) are shown. Error bars represent standard errors. Asterisk denotes a significant difference vs. corresponding control ($P < 0.05$).

2014, Watkins et al. 2017). Thus, ROS function as a signal integrator for ethylene, jasmonate and ABA signaling in guard cells. The SNF-related protein kinase, OST1/SnRK2.6 is the ABA-activated protein kinase and plays a crucial role in guard cell ABA signaling (Mustilli et al. 2002, Yoshida et al. 2002). It was reported that disruption of the ABA-activated protein kinase, OST1/SnRK2.6 impairs MeJA-induced stomatal closure (Mustilli et al. 2002, Yin et al. 2016), indicating the indispensable role of OST1 not only in ABA signaling but also in MeJA signaling in guard cells. Yin et al. (2016) showed that although MeJA requires OST1/SnRK2.6 kinase to induce stomatal closure, OST1/SnRK2.6 kinase is not apparently activated by MeJA. In this study, we also found that MeJA treatment did not activate OST1/SnRK2.6 kinase (**Fig. 3B**). However, the ABA activation of OST1/SnRK2.6 kinase was not inhibited by ACC pretreatment (**Fig. 3B**). The simple interpretation of these results is that ethylene signaling antagonizes ABA signaling and also MeJA signaling downstream of, or in parallel with OST1/SnRK2.6.

The whole-cell patch-clamp experiments revealed that MeJA and ABA activation of S-type anion channels is suppressed by ACC pretreatment (**Fig. 4**), indicating that signal integration of ethylene, jasmonate and ABA is achieved upstream of SLAC1 channel activation. Unlike S-type anion channels, ACC pretreatment does not affect MeJA and ABA regulation of K_{in}^+ channels (**Fig. 5**). Together with ROS and OST1 results (**Fig. 3**), these results imply that ROS mainly contribute to regulate S-type anion channels but not K_{in}^+ channels in guard cell MeJA and ABA signaling.

We found that ABA as well as MeJA does not significantly enhance K_{out}^+ channel activity (**Fig. 5**), which is consistent with the previous studies (Schwartz et al. 1994, Pandey et al. 2002, Becker et al. 2003, Roelfsema et al. 2004, Fan et al. 2008, Acharya et al. 2013). Contrary to these results, several reports show that ABA enhances outward K^+ channel activity (Blatt 1990, Blatt and Armstrong 1993, Lemtiri-Chlieh and MacRobbie 1994, Garcia-Mata et al. 2003), which might be mediated via cytosolic alkalization. One possible explanation for the inconsistency is that in our patch-clamp experiment, the pipette solution might neutralize the cytosolic alkalization-dependent regulation. Differences in plant growth conditions may also need to be considered (Schwartz et al. 1994).

Our data show that the ethylene inhibition of guard cell MeJA and ABA signaling is partial (**Figs. 2–4**). In addition, ACC and ethephon even at higher concentration (30–50 μM) could not completely inhibit MeJA- and ABA-induced stomatal closure (data not shown). These findings are in line with a previous study (Tanaka et al. 2005). The partial inhibition could be explained by ethylene-independent branches of the MeJA and ABA signaling pathway. For example, OST1 directly phosphorylates and activates SLAC1 S-type anion channel (Geiger et al. 2009, Lee et al. 2009), which appears to be independent of ethylene (**Figs. 3B, 6**).

In conclusion, our present data demonstrate that Arabidopsis guard cells integrate jasmonate, ABA and ethylene

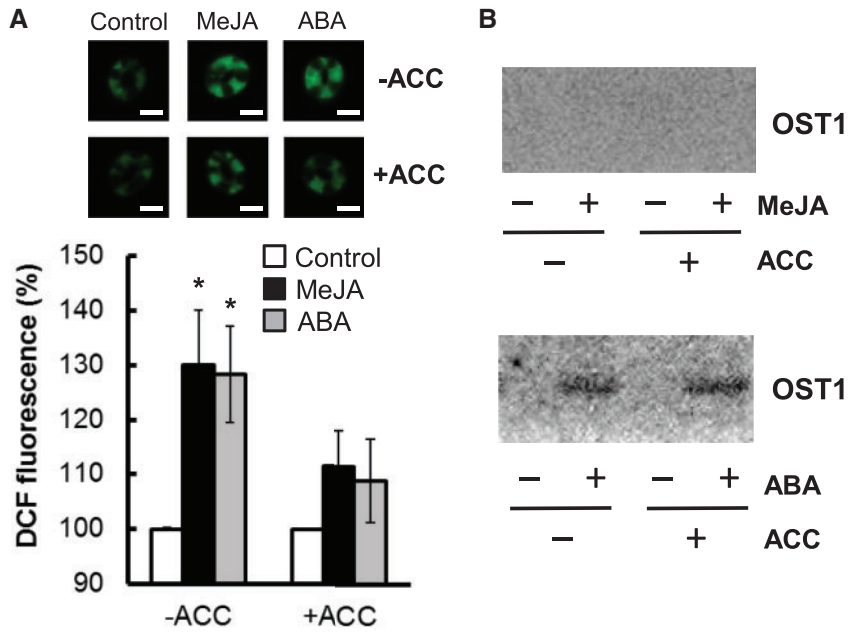

Fig. 3 ACC inhibits ROS production but not OST1 kinase activation in guard cells. (A) Effects of 10 μM ACC on MeJA- and ABA-induced ROS production in guard cells. The ROS-sensitive dye, 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) was used for ROS detection in guard cells (see Materials and Methods section). Scale bar = 5 μm. Averages from five independent experiments (>50 guard cells per bar in total) are shown. Error bars represent standard errors. Asterisk denotes a significant difference vs. corresponding control ($P < 0.05$). (B) Effects of 10 μM ACC on OST1 kinase activity. Protein extracts from guard cell-enriched epidermal tissues were subjected to in-gel protein kinase assay. Histone III-S was used as kinase substrates as previous reports (see Materials and Methods section).

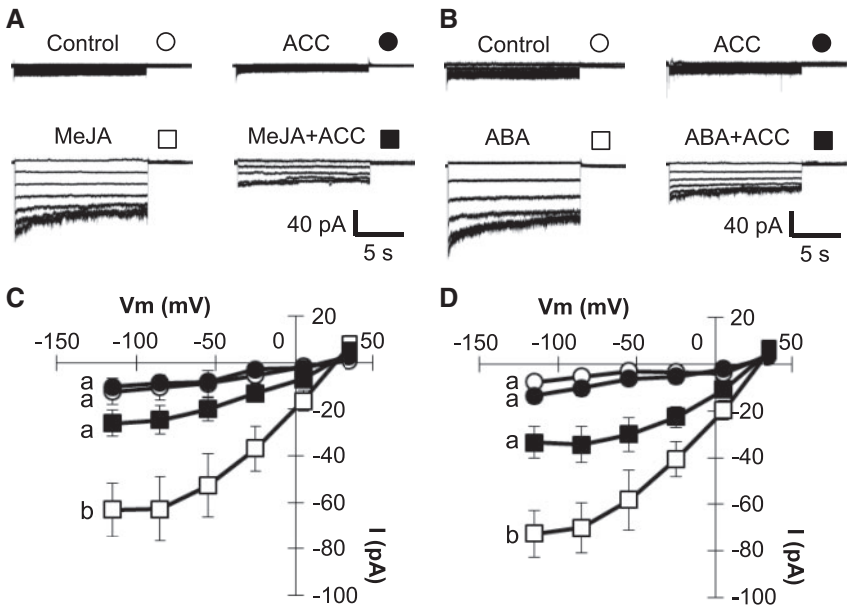

Fig. 4 S-type anion channels are downregulated by ACC pretreatment. Representative current traces (A, B) and average voltage–current relationships (C, D) are shown. MeJA-activation of S-type anion channels and its regulation by ACC are shown in (A, C). ABA activation of S-type anion channels and its regulation by ACC are shown in (B, D). The number of tested guard cell protoplasts was $n = 4$ for Control; $n = 3$ for ACC; $n = 8$ for MeJA; $n = 5$ for MeJA+ACC in (C) and $n = 5$ for Control; $n = 4$ for ACC; $n = 7$ for ABA; $n = 5$ for ABA+ACC in (D). Error bars represent standard errors. Statistical significant differences at −115 mV are denoted with different lowercase letters.

signaling upstream of S-type anion channel regulation in an OST1-independent manner (**Fig. 6**). Guard cell cytosolic ROS are involved in the signal integration process. In addition, our data revealed that K_{in}^+ channels are not targets of guard cell ethylene signaling. Further studies will be required to address the detailed mechanisms underlying the signal integration downstream of ROS that specifically targets regulation of S-type anion channels but not K_{in}^+ channels.

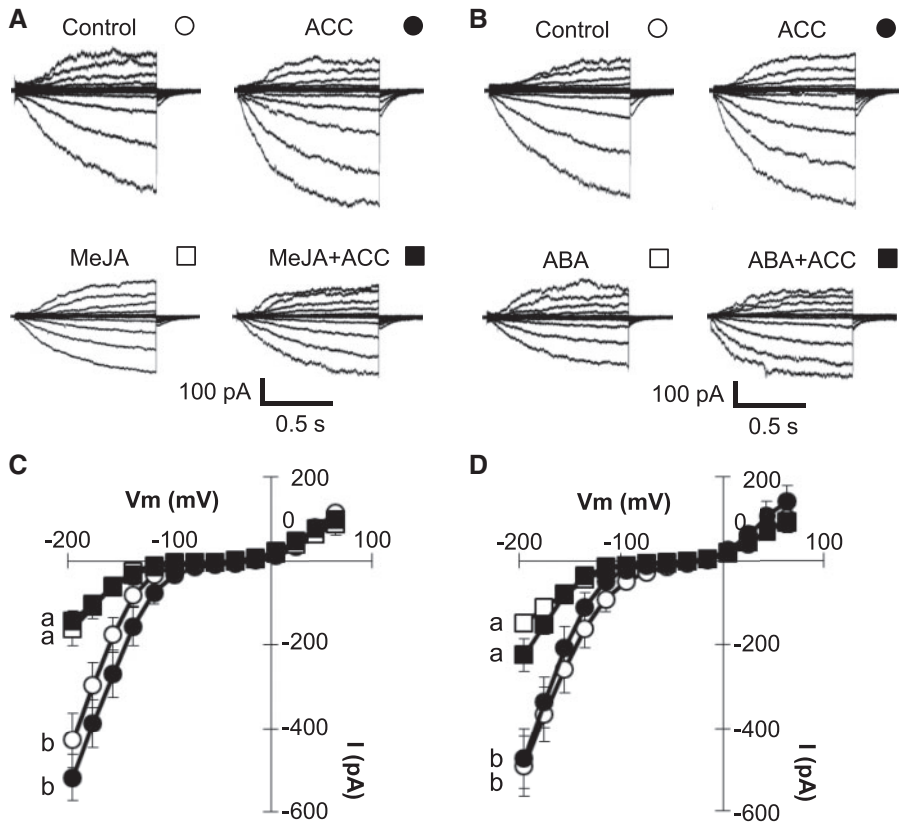

Fig. 5 K^+_{in} and K^+_{out} channel activities are not changed by ACC pretreatment. Representative current traces (A, B) and average voltage–current relationships (C, D) are shown. MeJA-regulation of K^+ channels and its regulation by ACC are shown in (A, C). ABA regulation of K^+ channels and its regulation by ACC are shown in (B, D). The number of tested guard cell protoplasts was $n = 7$ for Control; $n = 7$ for ACC; $n = 7$ for MeJA; $n = 5$ for MeJA+ACC in (C) and $n = 7$ for Control; $n = 6$ for ACC; $n = 6$ for ABA; $n = 7$ for ABA+ACC in (D). Error bars represent standard errors. Statistical significant differences at −195 mV are denoted with different lowercase letters.

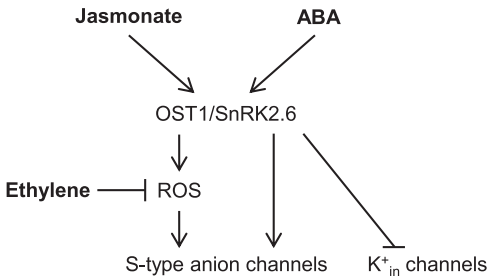

Fig. 6 A schematic model of jasmonate, ABA and ethylene signal crosstalk in Arabidopsis guard cells. Ethylene signaling is integrated into jasmonate and ABA signaling via inhibition of ROS production and S-type anion channel activation. OST1 kinase activity is not inhibited by ethylene (Fig. 3B), suggesting that the signaling branches where OST1 activates S-type anion channel and inactivates K^+_{in} channel via phosphorylation of SLAC1 (Geiger et al. 2009, Lee et al. 2009), KAT1 (Sato et al. 2009), and AKSs (Takahashi et al. 2013) are independent of ethylene signaling. This might explain the partial inhibitory effect of ethylene on jasmonate- and ABA-induced stomatal closure.

Materials and Methods

Plant material and growth

Arabidopsis thaliana plants were grown on a soil mixture [soil:vermiculite (v/v) 1: 1] in a growth chamber at 21°C under 18 h light/6 h dark photoperiod with photon flux density of, 80 μmol·m^{-2}·s^{-1}. Ecotype Columbia (Col-0) was used as wild type. The plants were watered with deionized water containing 0.1% Hyponex (Hyponex Japan, Osaka, Japan) once a week and sprayed with deionized water every day. Five- to 6-week-old plants were used in all experiments.

Stomatal aperture measurements

Stomatal apertures were measured as described previously (Munemasa et al. 2007). To examine effect of ACC and ethephon on MeJA- and ABA-induced stomatal closure, excised rosette leaves were floated on stomatal bioassay buffer containing 5 mM KCl, 50 μM $CaCl_2$ and 10 mM MES-Tris (pH 5.6) with or without ACC or ethephon, and then preincubated for 2.5 h in the light in a growth chamber. After that, MeJA or ABA was added to stomatal bioassay buffer, and leaves were further incubated for 2 h in a growth chamber. After the incubation, leaves were shredded in a warner blender and epidermal tissues were collected using a nylon mesh. ACC, ethephon, MeJA and ABA were used at 10 μM. At least 20 stomatal apertures were measured on each individual experiment using WinRoof Ver 3.0 software (Mitani Corporation, Fukui and Tokyo, Japan). To examine the effect of EGTA and $LaCl_3$ on ACC-induced stomatal closure (Fig. 1C), rosette leaves were preincubated with 10 mM EGTA and 50 μM $LaCl_3$ for 2 h in the light, followed by 30-min incubation with ACC.

ROS measurements

Guard cell ROS production was evaluated using the fluorescent dye 2′,7′-dihydrodichlorofluorescein diacetate (H_2DCF-DA) as described previously (Pei et al. 2000, Murata et al. 2001, Munemasa et al. 2007). Detached rosette leaves were extensively shredded in a warner blender with ice-cold tap water and epidermal tissues are collected using a 100-μm pore nylon mesh. The epidermal tissues

were kept in stomatal bioassay buffer for 2.5 h in a growth chamber with or without 10 μM ACC, and then incubated with 50 μM H_2DCF-DA in the dark for 30 min. After the dye loading, the epidermal tissues were collected using a 100-μm pore nylon mesh and gently rinsed with stomatal bioassay buffer. The tissues were resuspended in stomatal assay buffer and then incubated with or without 10 μM MeJA or ABA. After the 10-min incubation, fluorescent signals were captured using a fluorescence microscope (Biozero BZ-8000, Keyence, Osaka, Japan) and analyzed using ImageJ 1.42q software (NIH, USA).

In-gel protein kinase assay

The in-gel protein kinase assay for OST1 kinase activity evaluation was performed as described previously (Brandt et al. 2015, Waadt et al. 2015) with slight modifications. Detached rosette leaves were extensively shredded in a warner blender with ice-cold tap water and epidermal tissues are collected using a 100-μm pore nylon mesh. The epidermal tissues were kept in stomatal bioassay buffer for 3 h and then incubated in a growth chamber for 2.5 h with or without ACC, followed by an additional 30-min incubation with or without MeJA or ABA. The epidermal tissues were collected using a nylon mesh and then frozen in liquid nitrogen. Proteins were extracted using sea sand, a mortar, and a pestle in extraction buffer containing 100 mM HEPES-NaOH (pH 7.5), 5 mM EDTA, 5 mM EGTA, 0.5% Triton X-100, 150 mM NaCl, 0.5 mM DTT, 10 mM NaF, 5 mM Na_3VO_4, 5 mM beta-glycerophosphate 2Na, 0.5% (v/v) protease inhibitor (P8465, Sigma-Aldrich, MO, USA), and 0.5% (v/v) phosphatase inhibitor 3 (P0044, Sigma-Aldrich). The homogenates were centrifuged at 15,000 × *g* for 30 min at 4°C and supernatants were collected as protein extracts. The protein concentration was measured using the BCA method (Pierce BCA Protein Assay Kit, Thermo Fisher Scientific, MA, USA). Twenty micrograms of protein per lane was subjected to SDS-PAGE on a standard 10.5% polyacrylamide gel polymerized with 0.25–0.5 mg·ml^{-1} of Histone III-S (H5505, Sigma-Aldrich). The gel was washed three times each for 30 min at room temperature using a washing buffer that contains 25 mM Tris-HCl (pH 8.0), 0.5 mM DTT, 0.1 mM Na_3VO_4, 5 mM NaF, 0.5 mg·ml^{-1} bovine serum albumin and 0.1% Triton X-100. The gel was further washed two times each for 30 min at room temperature with renaturation buffer containing 25 mM Tris-HCl (pH 8.0), 1 mM DTT, 0.1 mM Na_3VO_4 and 5 mM NaF, followed by one wash at 4°C overnight. After 30-min equilibration at room temperature with reaction buffer containing 25 mM HEPES-NaOH (pH 7.5), 2 mM EGTA, 12 mM $MgCl_2$, 1 mM DTT and 0.1 mM Na_3VO_4, the gel was incubated in 20 ml of reaction buffer supplemented with 1.85 MBq of [γ-^{32}P]ATP (NEG502A, PerkinElmer, MA, USA) for 90 min at room temperature. The kinase reaction was terminated by washing TCA-PPi solution (5% trichloroacetic acid, 1% sodium pyrophosphate). The wash was performed several times until no radioactivity was detected in the TCA-PPi solution. Then the gel was stained with Coomassie Brilliant Blue and dried on filter paper. The radioactivity of the gel was visualized using Imaging Plate BAS-IP MS 2325 (FUJIFILM, Tokyo, Japan) and BAS imaging analyzer FLA-7000 (FUJIFILM).

Electrophysiology

The whole-cell patch-clamp recording was performed as previously described (Pei et al. 1997, Pandey et al. 2002, Munemasa et al. 2007, Fan et al. 2008) with slight modifications. For S-type anion channel recording, Arabidopsis guard cell protoplasts were prepared from epidermal tissues with a digestion solution containing 1.0% (w/v) Cellulase R10 (Yakult Pharmaceutical Industry Co., Tokyo, Japan), 0.5% (w/v) Macerozyme R10 (Yakult Pharmaceutical Industry Co.), 0.5% (w/v) bovine serum albumin, 0.1% (w/v) kanamycin, 10 mM ascorbic acid, 0.1 mM KCl, 0.1 mM $CaCl_2$ and 500 mM D-mannitol (pH 5.5 with KOH). The pipette solution for S-type anion channels contained 150 mM $CsCl_2$, 2 mM $MgCl_2$, 6.7 mM EGTA, 5.58 mM $CaCl_2$, 5 mM Mg-ATP and 10 mM HEPES-Tris (pH 7.1). The bath solution for S-type anion channels contained 30 mM $CsCl_2$, 2 mM $MgCl_2$, 1 mM $CaCl_2$ and 10 mM MES-Tris (pH 5.6). The pipette and bath solutions were adjusted with D-sorbitol to an osmolality of 500 and 485 mOsmol·kg^{-1}, respectively.

For K^+ channel recording, Arabidopsis guard cell protoplasts were prepared from epidermal tissues using a small-scale protocol described in Pandey et al. (2002) with modifications. Basic solution contained 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 μM KH_2PO_4, 550 mM D-sorbitol, 0.5 mM ascorbic acid and 5 mM MES-Tris (pH5.6). Epidermal tissues obtained from three to four rosette leaves were incubated in a 50-ml Erlenmeyer flask with 10 ml of osmotic solution that contained 55% (v/v) distilled deionized water and 45% (v/v) basic solution at 21°C for 30 min with 140 rpm shaking in the dark. Then 30 ml of basic solution was added into the flask, followed by an additional 5-min incubation. Epidermal tissues were collected using a nylon mesh and transferred into a 50-ml Erlenmeyer flask with 10 ml of an enzyme solution that is composed of 1.5% (w/v) Onozuka RS cellulase (Yakult Pharmaceutical Industry Co.), 0.02% (w/v) Pectolyase Y-23 (Kyowa Chemical Products Co., Osaka, Japan), 0.25% (w/v) bovine serum albumin and 0.5 mM ascorbic acid in the basic solution. The flask was incubated for 30 min with 60 rpm shaking in the dark at 21°C. The suspension was filtered through a nylon mesh and the mesh was washed using the basic solution. The filtrate was centrifuged for 10 min at 200 × *g*. The supernatant was removed and guard cell protoplasts were suspended in the basic solution, followed by an additional 10-min centrifugation. After removing the supernatant, guard cell protoplasts were resuspended in the basic solution and kept on ice until use. The pipette solution for K^+ channels contained 80 mM K-glutamate, 20 mM KCl, 5 mM Mg-ATP, 10 mM HEPES-Tris (pH 7.5). The bath solution for K^+ channels contained 10 mM K-glutamate, 5 mM $MgCl_2$, 1 mM $CaCl_2$ and 10 mM MES-Tris (pH5.6). The pipette and bath solutions were adjusted with D-sorbitol to an osmolality of 560 and 550 mOsmol·kg^{-1}, respectively.

The whole-cell currents were recorded using a CEZ-2200 patch-clamp amplifier (NIHON KOHDEN, Tokyo, Japan) and pCLAMP 8.1 software (Molecular Devices, Inc., CA, USA). Liquid junction potential was not corrected and leak currents were not subtracted. Guard cell protoplasts were pretreated with ACC for 2.5 h and then treated with MeJA or ABA for 30 min before starting patch-clamp experiments.

Statistical analysis

For data analysis, the Dunnett's test was used for **Figs. 1A**, **1B**, **2**, **3**. The Student's *t*-test was used for **Figs. 1C** and **2**. The Tukey–Kramer test was used for **Figs. 4**, **5**. A *P*-value <0.05 was considered as a significant difference.

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Disclosures

The authors have no conflicts of interest to declare.

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