Title: Involvement of OST1 Protein Kinase and PYR/PYL/RCAR Receptors in Methyl Jasmonate-Induced Stomatal Closure in Arabidopsis Guard Cells

Running Title: MeJA signaling in guard cells and ABA perception

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Abbreviations: ABA, abscisic acid; BCECF-AM, 2',7'-bis-(2-carboxyethyl)-5,(6)-carboxyfluorescein acetoxymethyl ester; COI, coronatine-insensitive; Col, Columbia-0; DAF-2DA, 4,5-diaminofluorescein-2 diacetate; GCPs, guard cell protoplasts; H_2DCF-DA, 2',7'-dichlorodihydrofluor -escein diacetate; JA, jasmonic acid; JA-Ile, jasmonoyl-isoleucine; Ler, Landsberg *erecta*; MeJA, methyl jasmonate; NCED, 9-*cis*-epoxycarotenoid dioxygenase; OST, open stomata; pH_{cyt}, cytosolic pH; PYL, pyrabactin resistance-like; PYR, pyrabactin resistance; qRT-PCR,quantitative RT-PCR; RCAR, regulatory component of ABA receptors; ROS, reactive oxygen species; RT-PCR, reverse transcription-PCR; VSP, vegetative storage protein.

Abstract

Methyl jasmonate (MeJA) induces stomatal closure. It has been shown that stomata of many ABA-insensitive mutants are also insensitive to MeJA and a low amount of ABA is a prerequisite for the MeJA response. However, the molecular mechanisms of the interaction between ABA and MeJA signaling remain to be elucidated. Here we studied interplay of the two hormone signaling in guard cells using the quadruple ABA receptor mutant, *pyr1 pyl1 pyl2 pyl4* and ABA-activated protein kinase mutants, *ost1-2* and *srk2e*. In the quadruple mutant, MeJA-induced stomatal closure, H_2O_2 production, NO production, cytosolic alkalization and plasma membrane Ca^{2+}-permeable current (I_{Ca}) activation were not impaired. Meanwhile, the inactivation of inward-rectifying K^+ current was impaired. Contrary to the quadruple mutant, MeJA-induced stomatal closure, H_2O_2 production, NO production and cytosolic alkalization were impaired in *ost1-2* and *srk2e* as well as *aba2-2*, the ABA-deficient mutant. The activation of I_{Ca} was also impaired in *srk2e*. Collectively, these results indicated that OST1 was essential for MeJA-induced stomatal closure, while PYR1, PYL1, PYL2 and PYL4 of ABA receptors were not sufficient factors. MeJA did not apparently activated OST1 kinase activity. This implies that OST1 mediates MeJA signaling through un-detectable level of activity or a non-enzymatic action. MeJA induced the expression of an ABA synthesis gene, *NCED3*, and increased ABA contents only modestly. Taken together with previous reports, this study suggests that MeJA signaling in guard cells is primed by ABA and is brought about not through the pathway mediated by PYR1, PYL1 PYL2 and PYL4.

Keywords: ABA • ABA receptors • *Arabidopsis thaliana* • Guard cells • Methyl jasmonate • OST1 protein kinase.

Introduction

Stomatal pores, which are surrounded by a pair of guard cells, play a crucial role in gas exchange of land plants. Guard cells respond to various environmental stimuli and regulate stomatal aperture to optimize photosynthetic activity and transpiration rate under environmental fluctuation. In this process, environmental signals are integrated in a guard cell and the integrated information is transduced to proton pump and ion channels, which drive stomatal movement (Ward et al. 1995, Assmann and Shimazaki 1999, Murata et al. 2015). In order to securely response to changing stimuli, integration and transfer of information in a cell have to be robust against signal disturbance that a guard cell experiences.

Methyl jasmonate (MeJA) regulates various developmental processes and defense responses (Liechti and Farmer 2002, Turner et al. 2002). Stomatal closure is a response to MeJA (Gehring et al. 1997, Suhita et al. 2003, 2004). Previous studies have demonstrated that MeJA mobilizes a series of second messengers in common with ABA, leading to stomatal closure (Suhita et al. 2004, Munemasa et al. 2007, 2011a, 2011b, Saito et al. 2009, Islam et al. 2010). A line of evidence has been reported that many ABA-insensitive mutants exhibit MeJA insensitivity in stomata, indicating a large body of signaling components is shared by ABA signaling and MeJA signaling in guard cells (for review, see Munemasa et al. 2011b). CORONATINE-INSENSITIVE1 (COI1), the jasmonate receptor, is exceptionally not shared between two hormones; i.e. *coi1* mutant

was insensitive in MeJA-induced stomatal closure, but intact in ABA responses (Munemasa et al. 2007).

9-CIS-EPOXYCAROTENOID DIOXYGENASE3 (*NCED3*) plays a role in the dehydration-inducible biosynthesis of ABA (Urano et al. 2009). It was shown that MeJA increased the expression of *NCED3* (Hossain et al. 2011) that might cause an increase of ABA content. Hossain et al. (2011) reported that a very low concentration of ABA is a prerequisite for MeJA-induced stomatal closure. These results implicate an important role of ABA in MeJA signaling and furthermore are suggestive of a mechanism of robustness of MeJA signaling against environmental and biochemical noises in such a way that an additional triggering cue of ABA is necessary to functionalize it. However, functionalization of MeJA signaling by ABA remains to be further examined.

The ABA receptor family PYR/PYL/RCAR consists of 14 members in the Arabidopsis genome (Ma et al. 2009, Park et al. 2009, Raghavendra et al. 2010). ABA-induced stomatal closure was impaired in loss-of-function mutants of ABA receptors, *pyr1 pyl1 pyl2 pyl4* (Nishimura et al. 2010, Wang et al. 2013, Yin et al. 2013). It was shown that *pyl4* and *pyl5* mutants exhibited hypersensitivity in jasmonic acid (JA) response in root growth and reduced JA-induced anthocyanin accumulation (Lackman et al. 2011). The SNF-related protein kinase, OPEN STOMATA1 (OST1), which is the ABA-activated protein kinase expressed in Arabidopsis guard cells, plays a pivotal role downstream of the ABA receptors through an action of protein phosphatase 2C (PP2C) (Yoshida et al. 2006, Hirayama and Shinozaki 2007, Cutler et al. 2010, Nishimura et al. 2010, Weiner et al. 2010, Umezawa et al. 2010). Potential involvement of this so-called “core

ABA signaling" (Umezawa et al. 2010) in the functionalization of MeJA-induced stomatal closure (Hossain et al. 2011) has not been fully investigated.

In this study, we investigated the involvement of the early ABA signaling components, PYR/PYL/RCAR ABA receptor and OST1 kinase, in MeJA response of guard cells to dissect the interplay of ABA signaling and MeJA signaling in guard cells.

Results

MeJA-induced stomatal closure is impaired in OST1 kinase mutants

To investigate interplay between ABA and MeJA signaling in guard cells, MeJA-induced stomatal closure was examined in two alleles of OST1 kinase loss-of-function mutants, *ost1-2* and *srk2e*, in which ABA-induced stomatal closure was reported to be impaired (Suhita et al. 2004，Yin et al. 2013).

Stomatal closure was induced by exogenous application of MeJA to pre-opened stomata of Columbia-0 ecotype plants (Col, wild-type background of *srk2e* mutant). By contrast, MeJA-induced stomatal closure in the presence of 1 μM and 10 μM MeJA was substantially abolished in *srk2e* (Fig. 1A). Impairment of ABA-induced stomatal closure in *srk2e* has already been reported elsewhere (see Fig. 5 in Yin et al. 2013). The effects of exogenous ABA and MeJA on stomatal aperture of an allelic mutant in another genetic background, *ost1-2,* were also examined. Application of either ABA or MeJA (1 μM and 10 μM) induced stomatal closure in Landsberg *erecta* ecotype (Ler, wild-type background of *ost1-2*) (Fig. 1B and C). However, stomatal closure by ABA and MeJA (1 μM and 10 μM) was significantly impaired in *ost1-2* (Fig. 1B and C).

This was consistent with the previous report (Suhita et al. 2004). Collectively, these results indicate that OST1 kinase is involved in MeJA signaling as well as in ABA signaling in guard cells.

OST1 kinase is involved in MeJA-induced reactive oxygen species production and nitric oxide production, and MeJA-induced cytosolic alkalization in guard cells

To further assess the function of OST1 in MeJA signaling in guard cells, reactive oxygen species (ROS) and nitric oxide (NO) productions, and cytosolic alkalization were examined in *srk2e* and *ost1-2*. In guard cells of wild-type plants, exogenous application of MeJA induced an increase of fluorescence of 2',7'-dichlorofluorescein (DCF), which indicated increase of intracellular H_2O_2 level (Fig 2A and D). This observation was consistent with previous studies (Suhita et al. 2004, Munemasa et al. 2007). Unlike wild types, *srk2e* and *ost1-2* showed no detectable increase of H_2O_2 level in guard cells (Fig. 2A and D). ABA-induced H_2O_2 production was also abolished in *ost1-2* (Fig. 2D) coinciding with the previous result of *srk2e* (Yin et al. 2013).

MeJA-induced NO production in guard cells was examined utilizing 4,5-Diaminofluorescein diacetate (DAF-2DA). In contrast to the increase in wild-type guard cells, no increase of NO level was detected in *srk2e* and *ost1-2* guard cells (Fig. 2B and E).

MeJA-induced cytosolic alkalization of guard cells occurred in wild types as indicated by an increase of 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) fluorescence, but not in *srk2e* and *ost1-2* (Fig. 2C and F). These

results indicates that OST1 plays a crucial role upstream of ROS and NO production, and cytosolic alkalization in MeJA signaling as a positive regulator, as well as in ABA signaling.

Induction of stomatal closure, ROS and NO productions, and cytosolic alkalization in the quadruple mutant of ABA receptors, *pyr1 pyl1 pyl2 pyl4*, and an ABA-deficient mutant

The effect of exogenous MeJA on stomata of the quadruple mutant was examined to gain insight into the function of ABA receptors in the interaction with the MeJA signaling.

Application of 1 μM and 10 μM MeJA induced stomatal closure in the quadruple mutant to a similar degree as in Col (Fig. 3). This indicates that PYR1, PYL1, PYL2, and PYL4 ABA receptors are not sufficient factors for the ABA action in MeJA-induced stomatal closure.

Production of H_2O_2 and NO, and alkalization of cytosol was examined in quadruple mutant guard cells with H_2DCF-DA, DAF-2DA and BCECF-AM, respectively (Fig. 4). The quadruple mutant showed an elevation of H_2O_2 level in guard cells to a similar extent as Col (Fig. 4A). NO production in guard cells was induced in the quadruple mutant's guard cells as well as in Col (Fig. 4B). MeJA induced cytosolic alkalization in both Col and quadruple mutant guard cells (Fig. 4C). These results indicate that mobilization of these second messengers in MeJA signaling is totally functional in quadruple mutant guard cells.

Previously, we showed that stomata of an ABA deficient mutant were insensitive to MeJA. However, the impairment of the mobilization of these

second messengers has not been examined, while the impairment of the initiation of cytosolic Ca^{2+} oscillation was demonstrated (Hossain et al. 2011). Here, we examined the production of H_2O_2 and NO in the ABA-deficient mutant, *aba2-2*. MeJA did not induce production of H_2O_2 nor NO in the mutant likewise MeJA-induced stomatal closure (Fig.5). This demonstrates the necessity of ABA for MeJA signaling in guard cells and a tight association of these second messengers with stomatal movements.

MeJA-induced activation of plasma membrane Ca^{2+} permeable current (I_{Ca}) and inactivation of inward-rectifying K^+ current (I_{Kin}) in the quadruple mutants.

The activation of I_{Ca} is suggested to be involved in Ca^{2+}-dependent ABA and MeJA signaling pathways, which give rise to eventual stomatal closure (Hamilton et al., 2000; Pei et al., 2000; Munemasa et al., 2007). Here, we examined the activation of I_{Ca} in GCPs of *srk2e* and the quadruple mutants. Contrary to the activation of typical hyperpolarization-dependent rapid fluctuating I_{Ca} in Col (Fig. 6A-C), no apparent activation of I_{Ca} was observed in *srk2*e mutant even in the presence of ABA or MeJA (Fig. 6D-F). The lack of this I_{Ca} activation in *srk2e* did not seem to be attributed to impotence of the channels but the impairment of ABA and MeJA signaling, since the application of hydrogen peroxide to *srk2e* GCPs unambiguously evoked I_{Ca} (Supplementary Fig. 2). As shown in Fig. 6G-I, the activation of I_{Ca} in the quadruple mutant GCPs by ABA was substantially diminished, whereas it by MeJA was intact. This responsiveness of mutant's

guard cells to ABA and MeJA was well correlated with the responsiveness of stomata and mobilization of other second messengers (Fig. 1-4).

Inactivation of I_{Kin} is not a critical but a landmark event in guard cell during stomatal closure (Raschke et al., 1988; Hedrich and Schroeder, 1989; MacRobbie, 1998; Kwak et al., 2001). Exogenous application of MeJA inactivated the typical slow-activated hyperpolarization-dependent I_{Kin} in Col, whereas the inactivation was not significant in the quadruple mutant (Fig. 7, the representative raw traces and the current-voltage curve of the corresponding control experiment are shown in Figure 3 of Yin et al., 2013). This indicates that I_{Kin} is regulated by MeJA through the action of PYR1 PYL1 PYL2 and PYL4 ABA receptors, unlike ROS production, NO production, cytosolic alkalization and I_{Ca} activation.

Activation of OST1 kinase by MeJA was not apparently detected

It has been shown that ABA activates OST1 kinase to phosphorylate substrate proteins (Mustilli et al. 2002, Yoshida et al. 2002, Acharya et al. 2013). The previous and this study showed molecular biological evidence that stomata of loss-of-function mutants of *OST1* were MeJA insensitive (Suhita et al. 2004, Figs. 1 and 2 in this study). Here we biochemically tested whether MeJA activates OST1 kinase activity.

Protein extracts of isolated wild-type guard cell protoplasts (GCPs) were subjected to the in-gel phosphorylation assay following a treatment of GCPs with 0 or 10 µM ABA. Two radioactive bands indicating OST1 kinase activity was detected in GCPs that were treated with 10 µM ABA, while no activity band was

detected in GCPs with 0 µM ABA (Fig. 8A and B). The activity band with an apparent molecular mass of 42 kDa should correspond to the activated OST1 due to its molecular mass deduced from the primary structure. The activity band with a smaller apparent molecular mass might be OST1 with an unknown modification that increased mobility in the electrophoresis. ABA did not activate OST1 kinase activity in ABA insensitive mutants: the *quadruple*, *srk2e* and *ost1-2* mutants.

To our surprise, the activation of OST1 was not detected in wild-type GCPs treated with 10 µM MeJA in contrast to ABA (Fig. 8A and B). Nor was the activation of OST1 in *srk2e*, *ost1-2* and quadruple mutant plants. These results indicate that the activation of OST1 protein kinase by MeJA did not occur or was below the detection limit, even in plants of which stomata exhibited apparent MeJA sensitivity.

The effect of the serine/threonine protein kinase inhibitor, staurosporin on MeJA-induced stomatal closure was examined to test the possibility of the involvement of kinase activity. Ten µM MeJA induced stomatal closure in Col. Staurosporin (2 µM) partially but significantly inhibited MeJA-induced tomtal closure (Supplementary Fig. 2). This indicates that some protein kinase activity is involved in MeJA-induced stomatal closure and is sort of supportive to the involvement of OST1 kinase activity. However, we cannot conclude the requirement of OST1 kinase activity, since the specificity of staurosporin is broad and do not exclude the possible involvement of other protein kinases.

Increase of ABA content in response to MeJA

The previous study demonstrated that MeJA induced expression of the gene encoding one of the key ABA synthesis enzymes, *NCED3* (Hossain et al. 2011). Therefore, we hypothesized that MeJA treatment induces an increase of ABA contents. We first carried out a replication study of Hossain et al. (2011). DNA fragment of *NCED3* amplified by reverse transcription (RT)- PCR was detected scarcely at 0 min, and its intensity increased gradually along with incubation time up to 60 min after MeJA spray (Fig. 9A), confirming the previous report (Hossain et al., 2011). A quantitative RT-PCR demonstrated that the transcript level of *NCED3* was transiently increased at 60 min and decreased back to the original level at 120 min (Fig. 9B). The up-regulation of mRNA level of genes encoding hormone-synthesizing enzymes is not necessarily accompanied with an actual increase of hormone contents in theory. We determined the contents of ABA, JA and JA-Ile by liquid chromatography-triple quadrupole mass spectrometry (LC-MS/MS) following a spray with MeJA onto Arabidopsis plants. Contents of JA and JA-Ile dramatically increased by a 30-min incubation after a spray of 10 μM MeJA, reaching to 4000 ng·g FW^{-1} and 150 ng·g FW^{-1} from the resting levels: 83.5 ng·g FW^{-1} and 3.1 ng·g FW^{-1} respectively (Fig. 9C and D). The formation of these hormones from MeJA is most likely through demethylation of MeJA catalyzed by methyl esterases (Findling et al. 2014) and successive formation of the isoleucine conjugate catalyzed by JASMONIC ACID RESISTANT1 (Staswick and Tiryaki 2004). ABA content was 6 ng·g FW^{-1} without MeJA treatment. After an exposure to 10 μM MeJA, ABA contents increased to 10 ng·g FW^{-1} and 12 ng·g FW^{-1} at 30 min and 120 min, respectively (Fig. 9E). We

confirmed that MeJA increased *NCED3* mRNA level and in turn ABA content, although it was only a modest.

Discussion

Role of OST1 protein kinase and PYR/PYL/RCAR ABA receptors in MeJA signaling in guard cells

MeJA signaling in Arabidopsis guard cells has been shown to involve many known ABA signaling components, such as catalytic subunits of NAD(P)H oxidase, type 2C protein phosphatases, the regulatory subunit of protein phosphatase 2A, calcium-dependent protein kinase and myrosinases (Suhita et al. 2004, Munemasa et al. 2007, Saito et al. 2008, Islam et al. 2009, Munemasa et al. 2011a). These genetic evidences imply that a large body of MeJA signaling component in guard cells is overlapped with ABA signaling. Meanwhile, the involvement of PYR/PYL/RCAR ABA receptors and OST1 kinase in signaling of jasmonates has not been well investigated.

It is generally accepted that OST1 kinase is activated by a sequence of biochemical events after perception of ABA by PYR/PYL/RCAR receptors, as follows: inhibition of PP2C by ABA-PYR/PYL/RCAR complexes and disinhibition of OST1 kinase through the suppression of PP2C activity. Then, active OST1 protein kinase phosphorylates target proteins (Geiger et al. 2009, Sato et al. 2009, Sirichandra et al. 2009). Stomata of *OST1* loss-of-function mutants, *ost1-2* and *srk2e,* were impaired in both ABA and MeJA-induced closure (Suhita et al. 2004, Yin et al. 2013, and Fig.1). Due to these molecular evidences for the indispensable role of OST1 in MeJA-induced closure, we hypothesized that

MeJA would activate OST1 activity in a similar way as in ABA signaling. However, the hypothesis seems likely to be false. The activation of OST1 kinase by MeJA was not apparently detected (Fig. 6), while the same concentration of MeJA unambiguously induced stomatal closure (Figs. 1 and 3). Therefore, it seems that two possibilities of OST1 function in MeJA signaling in guard cells remain: (1) the activity of OST1 kinase is not required for MeJA signaling and (2) a very low activity, which is not detectable by the in-gel phosphorylation assay, is critical for MeJA signaling.

Hossain et al. (2011) showed that MeJA-induced stomatal closure was impaired in the ABA-deficient mutant *aba2-2* and MeJA induces the expression of the gene encoding the key enzyme of ABA synthesis, *NCED3*. Alternatively, it was shown that *coi1* stomata were MeJA insensitive, while they were ABA sensitive (Munemasa et al. 2007). Therefore, we suspected that MeJA induced the elevation of ABA contents and in turn induced stomatal closure in a sequential manner. If so, the quadruple mutant of ABA receptors, in which ABA-induced stomatal closure is impaired (Nishimura et al. 2010, Yin et al. 2013), would be MeJA insensitive as well. However, the stomatal movement of the *pyr1 pyl1 pyl2 pyl4* quadruple mutant was comparable to that of wild type (Fig. 3), and mobilization of major second messengers, ROS and NO production, and cytosolic alkalization, in the quadruple mutant guard cells was also comparable to that in wild-type (Fig. 4). Taken together with the fact that MeJA did not apparently activate OST1 kinase activity (Fig. 6), these results demonstrate that MeJA signaling and ABA signaling in guard cells are not simply sequential.

Here we postulate that other ABA perception mechanism(s) besides PYR1, PYL1, PYL2, and PYL4 plays a role in potentiation/priming of MeJA signaling likely through an unidentified ABA signaling pathway in response to a very low concentration of ABA. OST1 protein kinase may be involved in this unidentified ABA signaling pathway with its very low (below a detectable level by the present method) but meaningful activity. The requirement of potential ABA potentiation/priming in MeJA signaling may prevent erroneous firing of MeJA signaling under biochemical fluctuation or noisy environments, and reinforce signal robustness. The postulated ABA-potentiation/priming mechanism of MeJA signaling and mode of action of OST1 in the signaling remain to be unraveled.

There may be a role-sharing among ABA receptors in potentiation/priming of MeJA in guard cells. It was suggested that ROS production, NO production, cytosolic alkalization and I_{Ca} activation were regulated by MeJA without the involvement of PYR1, PYL1, PYL2 and PYL4. On the other hand I_{Kin} was shown to be regulated by MeJA through PYR1, PYL1, PYL2 and/or PYL4 (Fig. 7). This can be explained by an assumption that there are at least two parallel MeJA signaling pathways, which are potentiated/primed with two different sets of ABA receptors (Supplementary Fig. 3). The pathway that regulates ROS, NO, pH and I_{Ca} is co-regulated with ABA through unidentified ABA receptor. One regulating I_{Kin} is co-regulated with either of all of PYR1, PYL1, PYL2 and/or PYL4.

Increase of ABA in response to MeJA and robustness of MeJA signaling in stomatal response

We determined the contents of JA, JA-Ile, and ABA in leaves exposed to MeJA (Fig. 9). In contrast to the remarkable increase of JA and JA-Ile contents, ABA content increased only twice of the resting condition. This low level of increase may not be sufficient to fully up-regulate the core ABA signaling (Weiner et al. 2010). Most likely, it was just satisfactory to functionalize MeJA signaling as discussed by Hossain et al. (2011). Here, we hypothesized that the trace amount of ABA alternatively synthesized after MeJA exposure and/or endogenous level at resting condition binds to unidentified ABA receptors, which might comprised of other PYL genes and primes the COI-dependent JA signaling (Munemasa et al. 2007, Hossain et al. 2011). We should not exclude possible contribution of an unidentified ABA receptor besides the PYR/PYL/RCAR family.

The presence of only a modest but meaningful amount of ABA may reinforce the robustness of MeJA signaling in guard cells against environmental and biochemical noises in a manner such that the logical product (AND operation) of signals cues stomatal closure in response to JA and ABA stimuli, but a single signal does not proceed with the action. At present, biochemical, cytochemical and molecular aspects of the signal conjunction remains unknown.

While a small increase of ABA contents was observed in this study, Lu et al. (2015) reported that the *ABA INSENSITIVE1*-controlled signaling pathway functions without any sign of increase of ABA contents during high carbon-low nitrogen response. They hypothesized that an unknown specific ABA signaling pathway besides the canonical ABA signaling was involved in high carbon-low

nitrogen response. We cannot rule out that the potential involvement of such non-canonical ABA signaling in MeJA signaling in guard cells.

Interaction of ABA signaling and JA signaling

Although the mechanism functionalizing MeJA signaling in guard cells by ABA remains unknown, the role of ABA in potentiation of systemic JA-dependent defense mediated by MYC2 transcription factor has been elucidated (e.g. Vos et al. 2013). Co-application of ABA with larvae feeding of a specialist herbivorous insect to plants potentiates induction of *MYC2* and *VSP1* genes. In this process, MYC2 is thought to be the master component of the interaction of two hormones (Kazan and Manners, 2013). It is tempting to speculate that MYC2 also function in guard cell signaling as the hub of interaction of ABA and MeJA signaling and a subset of ABA receptors functions in this process. Currently it remains unknown that MYC2 transcription factor is involved in the process. The concrete evidence for the involvement of *de novo* gene expression during ABA- and MeJA-induced stomatal closure has not been demonstrated to date, and the contribution of transcription factors in ABA- and MeJA-induced stomatal closure is uncertain.

ABA-induced stomatal closure is an early pre-invasive defense mechanism upon perception of microbes (Ton et al. 2009) as well as drought response. JA is supposed to function at local post-invasive defense and systemic defense. As stomatal closure apparently works as a pre-invasive defense but not post-invasive defense, it can be postulated that ABA primed/potentiated MeJA-indued stomatal closure play a role in systemic defense to prevent further microbe invasion by limiting stomatal opening. Here,

we propose a role of ABA as a mediator of systemic pre-invasive defense by closing stomata in cooperation with jasmonates, through potentiation of the JA signal.

Materials and Methods

Plant materials

Arabidopsis thaliana wild type (Columbia-0 and Landsberg *erecta* ecotypes), *srk2e* mutant (Yoshida et al. 2002), *ost1-2* mutant (Mustilli et al. 2002), *pyr1 pyl1 pyl2 pyl4* quadruple mutant (Park et al. 2009) and *aba2-2* mutant (Hossain et al. 2011) were grown as described previously (Yin et al. 2013).

Measurement of stomatal aperture

Width of stomatal aperture was examined as previously described (Yin et al. 2013). In brief, excised rosette leaves of 4- to 6-week-old plants were floated on the opening buffer containing 5 mM KCl, 50 µM $CaCl_2$ and 10 mM MES-Tris (pH 6.15) for 2 h in the light (80 µmol m^{-2} s^{-1}) to pre-open stomata. Aperture width was measured under a microscope after an additional 2-h incubation in the presence of any given concentrations of MeJA or ABA.

Measurement of H_2O_2 and NO production

The production of H_2O_2 and NO in guard cells was examined utilizing the fluorescence indicators, H_2DCF-DA (2',7'-dichlorodihydrofluorescein diacetate, Sigma, St. Louis, MO, USA) and DAF-2 DA respectively, as previously reported (Yin et al. 2013).

Examination of pH_{cyt} change of guard cells

A pH-sensitive fluorescent dye, BCECF-AM was used to examine relative change in pH_{cyt} of guard cells as previously reported (Yin et al. 2013).

Patch clamp analysis

I_{Ca} was recorded essentially according to Mori et al. (2006). The bath solution contained 100 mM $BaCl_2$, 0.1 mM DTT and 10 mM MES-Tris (pH 5.6), and its osmolarity was adjusted to 485 mmol kg^{-1} with D-sorbitol. The pipette solution contained 10 mM $BaCl_2$, 0.1 mM DTT, 4 mM EGTA, 1 mM NADPH and 10 HEPES-Tris (pH 7.1), and its osmolarity was adjusted to 500 mmol kg^{-1} with D-sorbitol. Voltage ramp speed and sweep repeats were changed to 100 mV s^{-1} and 10 times with a 1-min interval, respectively. In order to examine the effect of ABA, MeJA and H_2O_2, the bath was perfused with the corresponding solution after the establishment of whole cell configuration and recording of the resting condition I_{Ca} was recorded at least up to 30 min after the perfusion (3 time x 10 sweeps). Typically, MeJA-activated I_{Ca} appeared 15-20 min after the perfusion. Patch clamp data were recorded using MultiClamp 700B amplifier, CV-7B headstage, Digidata 1440A digitizer and pClamp 10.3 software, and analyzed using Clampfit 10.3 software (Molecular Devices LLC, California, USA).

I_{Kin} was recorded by the same method as reported (Yin et al., 2013). In brief, the bath solution contained 30 mM KCl, 2 mM $MgCl_2$, 40 mM $CaCl_2$ 10 mM MES-Tris (pH 7.1), and its osmolarity was adjusted to 485 mmol kg^{-1}. The pipette solution contained 30 mM KCl, 70 mM K-glutamate, 2 mM $MgCl_2$, 3.35 mM

$CaCl_2$, 6.7 mM EGTA and 10 mM HEPES-Tris (pH 7.1), and its osmolarity was adjusted to 500 mmol kg^{-1}. In order to examine the effect of MeJA, GCPs were treated with 10 µM MeJA for 2 h before gigaohm seal establishment.

In-gel phosphorylation assay

Guard cell protoplasts (GCPs) were isolated from 5- to 7- week-old Arabidopsis plants according to the methods described elsewhere (Ueno et al. 2005) with slight modifications. Approximately one thousand of fully expanded rosette leaves were blended in ice-cold distilled water with a Waring Commercial blender for 1–2 min. The epidermal fragments were collected on a piece of nylon mesh (opening = 100 µm), rinsed with ice-cold distilled water and incubated in the first digestion buffer containing 0.5% (w/v) cellulase R-10 (Yakult Pharmaceutical Industry Co., Tokyo, Japan), 0.002% (w/v) pectolyase Y-23 (Seishin Pharmaceutical Co., Tokyo, Japan), 0.1% (w/v) polyvinylpyrrolidone K-30, 0.2% (w/v) bovine serum albumin (BSA), 0.25 M mannitol, 1 mM $CaCl_2$ and 10 mM MES-KOH, pH 5.4, at 24°C for 1 h on a reciprocal shaker (70 strokes min^{-1}). The epidermal fragments were collected on a piece of nylon mesh and rinsed with 0.3 M mannitol containing 1 mM $CaCl_2$. To adjust the osmotic pressure of cells, the epidermal peels were incubated in the mannitol solution for 30 min on ice. The epidermal fragments were collected on nylon mesh and incubated in the second digestion buffer containing 1.5% (w/v) cellulase RS, 0.02% (w/v) pectolyase Y-23, 0.2% (w/v) BSA, 0.4 M mannitol and 1 mM $CaCl_2$, pH 5.4, at 27°C for 40–50 min on a reciprocal shaker (50 strokes min^{-1}). When the shape of most guard cell protoplasm became round, the epidermal

fragments in the second digestion buffer were gently pipetted with a glass pipette several times to release GCPs, followed by passing through a piece of nylon mesh. The filtrate was further passed through two layers of nylon mesh (opening = 10 µm), and released GCPs were collected by centrifugation at 400 × *g* for 6 min. The pellet was rinsed three times with 0.4 M mannitol containing 1 mM $CaCl_2$ and 10 mM Mes-KOH, pH 5.4. The GCP suspension was diluted with 0.4 M mannitol containing 1 mM $CaCl_2$ and 10 mM Mes-KOH, pH 5.4. GCPs density was adjusted to 1×10^7 cells mL^{-1}.

GCP suspension was dispensed in 2 mL microcentrifuge tubes and incubated at room temperature for 30 min in the light (80 µmol m^{-2} s^{-1}) for temperature equilibration. Ten µM (final concentration) MeJA, 10 µM ABA or 0.1% ethanol (solvent control) was added to GCP suspension and incubated for 30 min. GCPs were collected by a centrifugation at 400 x *g* for 6 min at 4°C. Then, 50 µL of the extraction buffer (100 mM HEPES, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, NaOH for pH 7.6, 0.5 mM dithiothreitol [DTT], 5 mM Na_3VO_4, 10mM NaF, 5mM beta-glycerophosphate 2Na, 0.5% Triton X-100, 1/200 volume of protease inhibitor [P8465, SIGMA] and 1/200 volume of phosphatase inhibitor [P5726, SIGMA]) was immediately added to the pellet to disrupt GCPs. After a centrifugation at 13000 x *g* for 30 min at 4°C, supernatant was collected in a new tube. Protein concentration was determined using BCA protein assay kit (Thermo Scientific). Ten µg of protein per lane was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis [10% gel, containing 0.25 mg of Histone III-S (Sigma-Aldrich) per mL of the gel as the artificial substrate of kinases]. After a run, the gel was washed three times with the

washing buffer (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} BSA and 0.1% Triton X-100) for 20 min at room temperature, followed by washing with the renaturing buffer (25 mM Tris–HCl, pH 8.0, 1 mM DTT, 0.1 mM Na_3VO_4 and 5 mM NaF) for 30 min at room temperature twice and an additional washing at 4°C overnight. The gel was equilibrated with 100 mL of the reaction buffer (25 mM HEPES, pH 7.5 adjusted with NaOH, 2 mM EGTA, 12 mM $MgCl_2$, 1 mM DTT and 0.1 mM Na_3VO_4) for 30 min at room temperature and then incubated in 20 mL of the reaction buffer containing 1.85 MBq [gamma-^{32}P]ATP (NEG502A, PerkinElmer, specific radioactivity: 111 TBq $mmol^{-1}$) for 90 min at room temperature. The reaction was stopped by immersing the gel in the stop solution containing 5% trichloroacetic acid and 1% pyrophosphoric acid. The gel was successively washed with the stop solution until no radioactivity was virtually detected in the solution. The gel was dried on a sheet of absorbent paper and contacted to a KODAK BioMax Light Film (Scientific Imaging Film, 13x18cm) for 4-7 days at -80°C. Radioactive bands were visualized by development of the film.

RNA extraction, semi-quantitative RT-PCR and quantitative RT-PCR

Arabidopsis plants grown in a pot for 6-weeks were sprayed with 10 µM MeJA or the solvent control (ethyl alcohol) to wet all rosette leaves evenly and incubated for 0, 30, 60 and 120 min in a growth chamber (80 µmol photon m^{-2} s^{-1}, 22 ± 2 °C, 60 ± 10 % relative humidity). Total RNA was extracted and purified using Sepasol-RNA I Super G according to the instruction provided by the manufacture (Nacalai Tesque, Kyoto, Japan). Single strand complementary

DNA was synthesized from 1 mg of RNA using Moloney murine leukemia virus reverse transcriptase (TaKaRa Bio Inc.) using oligo(dT)$_{18}$ as the primer.

Semi-quantitative RT-PCR was performed with BIOTAQ DNA polymerase (Nippon Genetics, Tokyo). PCR conditions were 32 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 1 min for *AtNCED3*; and 32 cycles of 94°C for 30 sec, 65°C for 30 sec and 72°C for 1 min for *ACTIN2*. DNA sequences of primers were 5′-AGCTAACCCACTTCACGAGC-3′ and 5′-CGAATTTGACGGCGTGAACC-3′ for *AtNCED3*, and 5′-TCTTAACCCAAAGGCCAACA-3′ and 5′-CAGAATCCAGCACAATACCG-3′ for *ACTIN2*.

qRT-PCR analysis was performed using SYBR Premix ExTaq II (Takara Bio, Japan) and LightCycler 2.0 (Roche Diagnostics K.K.). DNA sequeces of the primers were 5′-GGTAACATTGTGCTCAGTGGTGG-3′ and 5′-AACGACCTTAATCTTCATGCTGC-3′ for *NCED3*, and 5′-GCTGCGGTTTCTGGGAGAT-3′ and 5′-GTCGGAGCTTTGAGAAGACGAT-3′ for *ACTIN2*. Relative expression level of *NCED3* versus *ACTIN2* was determined by comparative C_T method (Schmittgen and Livak, 2008).

Quantification of JA, JA-Ile and ABA with LC-MS/MS

Rosette leaves were sprayed with 10 µM MeJA or the solvent control (0.1% ethyl alcohol) as mentioned in the previous section. Excised leaves (approximately 0.1 g) were snap frozen in liquid nitrogen and ground into fine powder by vigorously vortexing in a 14-mL round bottom plastic tube with a 10-mm

diameter Zirconia bead for 30 sec 4 times. Crude hormones were extracted in 4 mL of 80% (v/v) acetonitrile containing 1% (v/v) acetic acid at 4°C for 60 min. Deuterium-labeled ABA and JA, and ^{13}C-labeled JA-Ile were added to the extraction solvent at this step as internal standards (d_6-ABA, Icon Isotopes, Summit, NJ, USA; (±)-jasmonic acid 9,10-d_2, Tokyo Kasei, Japan; $^{13}C_6$-JA-isoleucine, kindly provided by Dr. Yusuke Jikumaru, Plant Science Center, Riken [Jikumaru et al. 2004]). Following a centrifugation, supernatant was collected and the pellet was rinsed with 4 mL of 80% acetonitrile containing 1% acetic acid. Rinsing solvent was combined with the first supernatant. Acetonitrile was evaporated with a centrifugal evaporator, and remaining aqueous solution was loaded onto Oasis HLB cartridge (1 cc, Waters, Milford, MA, USA) equilibrated with 1% acetic acid. After washing the cartridge with 1 mL of 1% acetic acid in water, hormones were eluted with 2 mL of 80% acetonitrile containing 1% acetic acid. Eluate was then loaded onto Oasis MCX cartridge (1 cc, Waters) following evaporation of acetonitrile. The cartridge was washed with 1% acetic acid and eluted with 80% acetonitrile containing 1% acetic acid. Eluate containing hormones was evaporated to remove acetonitrile and further applied to Oasis WAX cartridge (1 cc, Waters). After a successive washing with 1 mL of 1% acetic acid and 1 mL of 80% acetonitrile; JA, JA-Ile and ABA were eluted with 2 mL of 80% acetonitrile containing 1% acetic acid. This fraction was evaporated to dryness and dissolved in 50 µL of 1% acetic acid. Contents of JA, JA-Ile and ABA were determined by LC-MS/MS (Agilent 6410B, Agilent Technologies Inc., Santa Clara, CA, USA) equipped with Eclipse XDB-C18 column (2.1 x 50 mm, 1.8 µm particle size, Agilent Technologies Inc.). Area of

peaks in the chromatogram was quantified with MassHunter software (Agilent Technologies Inc.). The detailed condition of LC-MS/MS is shown in Table 1.

Statistical analysis

Significance of difference of means between data sets was assessed by Student's *t*-test in all parts of this article unless otherwise stated. Difference at the level of $p < 0.05$ was regarded as significant.

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Disclosures

The authors have no conflict of interest to declare.

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References

Acharya, B.R., Jeon, B.W., Zhang, W. and Assmann, S.M. (2013) Open stomata 1 (OST1) is limiting in abscisic acid responses of Arabidopsis guard cells. *New Phytol.* 200: 1049-1063.

Assmann, S.M. and Shimazaki, K. (1999) The multisensory guard cell. stomatal responses to blue light and abscisic acid. *Plant Physiol.* 119: 809–815.

Cutler, S.R., Rodriguez, P.L., Finkelstein, R.R. and Abrams, S.R. (2010) Abscisic acid: emergence of a core signaling network. *Annu. Rev. Plant Biol.* 61: 651-679.

Findling, S., Fekete, A., Warzecha, H., Krischke, M., Brandt, H., Blume, E., et al. (2014) Manipulation of methyl jasmonate esterase activity renders tomato more susceptible to *sclerotinia sclerotiorum*. *Funct. Plant Biol.* 41: 133-143

Gehring, C.A., Irving, H.R., McConchie, R. and Parish, R.W. (1997) Jasmonates induce intracellular alkalinization and closure of *Paphiopedilum* guard cells. *Ann. Bot.* 80: 485–489.

Geiger, D., Scherzer, S., Mumm, P., Stange, A., Marten, I., Bauer, H., et al. (2009) Activity of guard cell anion channel SLAC1 is controlled by drought-stress signaling kinase-phosphatase pair. *Proc. Natl. Acad. Sci. USA* 106: 21425-21430.

Hamilton, D.W.A., Hills, A., Köhler, B. and Blatt, M.R. (2000) Ca^{2+} channels at the plasma membrane of stomatal guard cells are activated by hyperpolarization and abscisic acid. *Proc. Natl. Acad. Sci. USA* 97: 4967-4972.

Hedrich, R. and Schroeder, J.I. (1989) The physiology of ion channels and electrogenic pumps in higher plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 40: 539-569.

Hirayama, T. and Shinozaki, K. (2007) Perception and transduction of abscisic acid signals: keys to the function of the versatile plant hormone ABA. *Trends Plant Sci*. 12: 343–351

Hossain, M.A., Munemasa, S., Uraji, M., Nakamura, Y., Mori, I.C. and Murata, Y. (2011) Involvement of endogenous abscisic acid in methyl jasmonate-induced stomatal closure in Arabidopsis. *Plant Physiol.* 156: 430-438.

Islam, M.M., Hossain, M.A., Jannat, R., Munemasa, S., Nakamura, Y., Mori, I.C., et al. (2010) Cytosolic alkalization and cytosolic calcium oscillation in Arabidopsis guard cells response to ABA and MeJA. *Plant Cell Physiol.* 51: 1721–1730.

Islam, M.M., Tani, C., Watanabe-Sugimoto, M., Uraji, M., Jahan, M.S., Masuda, C., et al. (2009) Myrosinases, TGG1 and TGG2, redundantly function in ABA and MeJA signaling in Arabidopsis guard cells. *Plant Cell Physiol.* 50: 1171–1175.

Jikumaru, Y., Asami, T., Seto, H., Yoshida, S., Yokoyama, T., Obara, N., et al. (2004) Preparation and biological activity of molecular probes to identify and analyze jasmonic acid-binding proteins. *Biosci. Biotechnol. Biochem.* 68: 1461–1466.

Kazan, K. and Manners, J.M. (2013) MYC2: the master in action. *Molecular Plant* 6: 686-703.

Kwak, J.M., Murata, Y., Baizabal-Aquirre, V.M., Merrill, J., Wang, M., Kemper, A., et al. (2001) Dominant negative guard cell K^+ channel mutants reduce inward-rectifying K^+ currents and light-induced stomatal opening in Arabidopsis. *Plant Physiol*. 127: 473-485.

Lackman, P., González-Guzmán, M., Tilleman, S., Carqueijeiro, I., Pérez, A.C., Moses, T., et al. (2011) Jasmonate signaling involves the abscisic acid receptor PYL4 to regulate metabolic reprogramming in *Arabidopsis* and tobacco. *Proc. Natl. Acad. Sci. USA* 108: 5891–5896.

Liechti, R. and Farmer, E.E. (2002) The jasmonate pathway. *Science* 296: 1649-1650.

Lu, Y., Sasaki, Y., Li, X., Mori, I.C. Matsuura, T., Hirayama, T., et al. (2015) ABI1 regulates carbon/nitrogen-nutrient signal transduction independent of ABA

biosynthesis and canonical ABA signaling pathways in *Arabiodpsis*. *J. Exp. Bot.* 66: 2763-2771.

Ma, Y., Szostkiewicz, I., Korte, A., Moes, D., Yang, Y., Christmann, A., et al. (2009) Regulators of PP2C phosphatase activity function as abscisic acid sensors. *Science* 324: 1064-1068.

MacRobbie, E.A.C. (1998) Signal transduction and ion channels in guard cells. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 353: 1475–1488

Mori, I.C., Murata, Y., Yang, Y., Munemasa, S., Wang, Y.F., Andreoli, S., et al. (2006) CDPKs CPK6 and CPK3 function in ABA regulation of guard cell S-type anion- and Ca^{2+}-permeable channels stomatal closure. *PLoS Biol.* 4: e327.

Munemasa, S., Hossain, M.A., Nakamura, Y., Mori, I.C. and Murata, Y. (2011a) The Arabidopsis calcium-dependent protein kinase, CPK6, functions as a positive regulator of Methyl Jasmonate signaling in guard cells. *Plant Physiol.* 155: 553–561.

Munemasa, S., Mori, I.C. and Murata, Y. (2011b) Methyl jasmonate signaling and signal crosstalk between methyl jasmonate and abscisic acid in guard cells. *Plant Signal. Behav.* 6: 939-941.

Munemasa, S., Oda, K., Watanabe-Sugimoto, M., Nakamura, Y., Shimoishi, Y. and Murata, Y. (2007) The *coronatine-insensitive 1* mutation reveals the hormonal signaling interaction between abscisic acid and methyl jasmonate in Arabidopsis guard cells. Specific impairment of ion channel activation and second messenger production. *Plant Physiol.* 143: 1398-1407.

Murata, Y., Mori, I.C. and Munemasa, S. (2015) Diverse stomatal signaling and the signal integration mechanism. *Annu. Rev. Plant Biol.* 66: 21.1-21.24.

Mustilli, A., Merlot, S., Vavasseur, A., Fenzi, F. and Giraudat, J. (2002) Arabidopsis OST1 protein kinase mediates the regulation of stomatal aperture by abscisic acid and acts upstream of reactive oxygen species production. *Plant Cell* 14: 3089–3099.

Nishimura, N., Sarkeshik, A., Nito, K., Park, S-Y., Wang, A., Carvalho, P.C., et al. (2010) PYR/PYL/RCAR family members are major *in-vivo* ABI1 protein phosphatase 2C-interacting proteins in Arabidopsis. *Plant J.* 61: 290–299.

Park, S-Y., Fung, P., Nishimura, N., Jensen, D.R., Fujii, H., Zhao, Y., et al. (2009) Abscisic acid inhibits type 2C protein phosphatases via the PYR/PYL family of START proteins. *Science* 324: 1068-1071.

Pei, Z.P., Murata, Y., Benning, G., Thomine, S., Klüsener, B., Allen, G.J., et al. (2000) Calcium channels activated by hydrogen peroxide mediate abscisic acid signalling in guard cells. *Nature* 406: 731–734

Raghavendra, A.S., Gonugunta, V.K., Christmann, A. and Grill, E. (2010) ABA perception and signalling. *Trends Plant Sci.* 15: 395-401.

Raschke, K., Hedrich, R., Reckmann, U. and Schroeder, J.I. (1988) Exploring biophysical and biochemical components of the osmotic motor that drives stomatal movements. *Bot. Acta* 101: 283-294.

Saito, N., Munemasa, S., Nakamura, Y., Shimoishi, Y., Mori, I.C. and Murata, Y. (2008) Roles of RCN1, regulatory a subunit of protein phosphatase 2A, in methyl jasmonate signaling and signal crosstalk between methyl jasmonate and abscisic acid. *Plant Cell Physiol.* 49: 1396–1401.

Saito, N., Nakamura, Y., Mori, I.C. and Murata, Y. (2009) Nitric oxide functions in both methyl jasmonate signaling and abscisic acid signaling in Arabidopsis guard cells. *Plant Signal. Behav.* 4: 119-120.

Sato, A., Sato, Y., Fukao, Y., Fujiwara, M., Umezawa, T., Shinozaki, K., et al. (2009) Threonine at position 306 of the KAT1 potassium channel is essential for channel activity and is a target site for ABA-activated SnRK2/OST1/SnRK2.6 Protein kinase. *Biochem. J.* 424: 439-448.

Schmittgen, T.D. and Livak, K.J. (2008) Analyzing real-time PCR data by the comparative C_T method. *Nat. Protoc*. 3: 1101-1108.

Sirichandra, C., Gu, D., Hu, H.C., Davanture, M., Lee, S., Djaoui, M., et al. (2009) Phosphorylation of the Arabidopsis AtrbohF NADPH oxidase by OST1 protein kinase. *FEBS Lett*. 583: 2982-2986.

Staswick, P.E. and Tiryaki, I. (2004) The oxylipin signal jasmonic acid is activated by an enzyme that conjugates it to isoleucine in Arabidopsis. *Plant Cell* 16: 2117-2127.

Suhita, D., Kolla, V.A., Vavasseur, A. and Raghavendra, A.S. (2003) Different signaling pathways involved during the suppression of stomatal opening by methyl jasmonate or abscisic acid. *Plant Sci.* 164: 481-488.

Suhita, D., Raghavendra, A.S., Kwak, J.M. and Vavasseur, A. (2004) Cytoplasmic alkalization precedes reactive oxygen species production during methyl jasmonate- and abscisic acid-induced stomatal closure. *Plant Physiol.* 134: 1536-1545.

Ton, J., Flors, V. and Mauch-Mani, B. (2009) The multifaceted role of ABA in disease resistance. *Trends Plant Sci.* 14: 310-317.

Turner, J.G., Ellis, C. and Devoto, A. (2002) The jasmonate signal pathway. *Plant Cell* 14: S153-S164.

Ueno, K., Kinoshita, T., Inoue, S., Emi, T. and Shimazaki, K. (2005) Biochemical characterization of plasma membrane H^+-ATPase activation in guard cell protoplasts of Arabidopsis thaliana in response to blue light. *Plant Cell Physiol.* 46: 955–963.

Umezawa, T., Nakashima, K., Miyakawa, T., Kuromori, T., Tanokura, M., Shinozaki, K., et al. (2010) Molecular basis of the core regulatory network in ABA responses: sensing, signaling and transport. *Plant Cell Physiol.* 51: 1821-1839.

Urano, K., Maruyama, K., Ogata, Y., Morishita, Y., Takeda, M., Sakurai, N., et al. (2009) Characterization of the ABA-regulated global responses to dehydration in Arabidopsis by metabolomics. *Plant J*. 57: 1065–1078.

Vos, I.A., Verhage, A., Schuurink, R.C., Watt, L.G., Pieterse, C.M.J. and Van Wees, S.C.M. (2013) Onset of herbivore-induced resistance in systemic tissue primed for jasmonate-dependent defenses is activated by abscisic acid. *Front Plant Sci*. 4: 539.

Wang, Y., Chen, Z., Zhang, B., Hills, A. and Blatt, M.R. (2013) PYR/PYL/RCAR ABA receptors regulate K^+ and Cl^- channels through reactive oxygen species-mediated activation of Ca^{2+} channels at the plasma membrane of intact Arabidopsis guard cells. *Plant Physiol.* 163: 566–577.

Ward, J.M., Pei, Z-M. and Schroeder, J.I. (1995) Roles of ion channels in initiation of signal transduction in higher plants. *Plant Cell* 7: 833-844.

Weiner, J.J., Peterson, F.C., Volkman, B.F. and Cutler, S.R. (2010) Structural and functional insights into core ABA signaling. *Curr. Opin. Plant Biol.* 13: 495–502.

Yin, Y., Adachi, Y., Ye, W., Hayashi, M., Nakamura, Y., Kinoshita, T., et al. (2013) Difference in abscisic acid perception mechanisms between closure induction and opening inhibition of stomata. *Plant Physiol.* 163: 600-610.

Yoshida, R., Hobo, T., Ichimura, K., Mizoguchi, T., Takahashi, F., Aronso, J., et al. (2002) ABA-activated SnRK2 protein kinase is required for dehydration stress signaling in *Arabidopsis*. *Plant Cell Physiol.* 43: 1473–1483.

Yoshida, R., Umezawa, T., Mizoguchi, T., Takahashi, S., Takahashi, F. and Shinozaki, K. (2006) The regulatory domain of SRK2E/OST1/SnRK2.6 interacts with ABI1 and integrates abscisic acid (ABA) and osmotic stress signals controlling stomatal closure in Arabidopsis. *J. Biol. Chem.* 281: 5310–5318.

Table 1. Parameters of LC-MS/MS analysis

Liquid chromatography

Solvent A	Solvent B	Gradient and flow rate
Water containing 0.01% acetic acid	Acetonitrile containing 0.05% acetic acid	3 to 50% in 20 min 0.4 ml min^{-1}

Mass spectrometry

Compounds	Retention time (min)	ESI	Multiple reaction monitoring (m/z)	Collision energy (V)	Fragmentor voltage (V)
ABA	12.6	Negative	263 - 153	5	130
$[^2H_6]$-ABA			269 - 159		
JA	14.4	Negative	209 - 59	15	135
$[^2H_2]$-JA			211 - 59		
JA-Ile	18.0	Negative	322 - 130	14	140
$[^{13}C_6]$-JA-Ile			328 - 136		

ESI, electrospray ionization

Figure legends

Figure 1. Induction of stomatal closure by MeJA and ABA in *ost1* mutants. (A) MeJA-induced stomatal closure in Columbia-0 (Col) and *srk2e* mutant (*srk2e*). Averages of stomatal aperture widths from six independent experiments (n = 6, 120 total stomata) are represented. (B) ABA-induced stomatal closure in Landsberg *erecta* (Ler) and *ost1-2* mutant (*ost1-2*). Averages of stomatal aperture widths from four independent experiments (n = 4, 80 total stomata) are represented. (C) MeJA-induced stomatal closure in Ler and *ost1-2* mutant. Averages of stomatal aperture widths from four independent experiments (n = 4, 80 total stomata) are represented. Error bars represent standard error of the mean. ** and * indicate $P \leq 0.01$ and $P \leq 0.05$, respectively. Representative image of stoma is shown in the upper part of each panel. Scale bar = 10 μm.

Figure 2. MeJA-induced H_2O_2 production, NO production and cytosolic alkalization in guard cells of wild types and *ost1* mutants. H_2O_2 production (A and D), NO production (B and E) and cytosolic alkalization (C and F). The vertical scale represents the percentage of DCF, DAF and BCECF fluorescence levels, respectively. Fluorescent intensities are normalized to the control value

taken as 100% for each experiment. Bars indicate averages of five independent experiments (n = 5, 100 total guard cells per bar). Error bars represent standard error of the mean. ** indicates P ≤ 0.01. Representative image of guard cell fluorescence is shown in the upper part of each panel. Scale bar = 10 μm.

Figure 3. Induction of stomatal closure by MeJA in *pyr1 pyl1 pyl2 pyl4* quadruple mutant. MeJA-induced stomatal closure in Columbia-0 (Col) and the quadruple mutant (*quadruple*) is represented by percentage of 0 μM MeJA treatment. Mean values of stomatal aperture widths were 3.10±0.11, 2.76±0.06 and 2.65±0.03 μm for Col; and 3.53±0.09, 3.19±0.1 and 3.15±0.09 μm for *quadruple* in the presence of 0, 1 and 10 μM MeJA, respectively. Average values from six independent experiments (n = 6, 120 total stomata) are shown. Error bars represent standard error of the mean. n.s. indicates P > 0.05. Representative image of stoma is shown in the upper part of each panel. Scale bar = 10 μm.

Figure 4. MeJA-induced H_2O_2 production, NO production and cytosolic alkalization in Col and *quadruple* mutant guard cells. H_2O_2 production (A), NO production (B) and cytosolic alkalization (C). The vertical scale represents the

percentage of DCF, DAF and BCECF fluorescence intensities in Col and quadruple mutant. Fluorescent intensities are normalized to the control value taken as 100% for each experiment. Bars indicate averages of five independent experiments (n = 5, 100 total guard cells per bar). Error bars represent standard error of the mean. n.s. indicates P > 0.05. Representative image of guard cell fluorescence is shown in the upper part of each panel. Scale bar = 10 μm.

Figure 5. Impairment of MeJA-induced stomatal closure, H_2O_2 production and NO production in *aba2-2* mutant. (A) MeJA- and ABA-induced stomatal closure in *aba2-2* mutant. Averages of stomatal aperture widths from three independent experiments (n = 3, 60 total stomata) are presented. (B) MeJA-and ABA-induced H_2O_2 production in *aba2-2* mutant. (C) MeJA- and ABA-induced NO production in *aba2-2* mutant. The vertical scale represents the percentage of DCF and DAF fluorescence levels. Fluorescent intensities are normalized to the control value taken as 100% for each experiment. Bars indicate averages of three independent experiments (n = 3, 60 total guard cells per bar). Error bars represent standard error of the mean. P values are indicated above the pair of

bars. Representative image of stoma and guard cell fluorescence is shown in the upper part of each panel. Scale bar = 10 µm.

Figure 6. Plasma membrane Ca^{2+}-permeable current (I_{Ca}) activation by ABA and MeJA in *srk2e* and *quadruple* GCPs. (A, D and G) Representative whole cell currents of Col, *srk2e* and *quadruple* GCPs in the absence (0.1% ethylalcohol, open symbols) and the presence of 10 µM ABA (closed circles) and 50 µM MeJA (closed triangles). (B, C, E, F, H and I) Average current-voltage curve. Col, A-C. *srk2e*, D-F. *quadruple,* G-I. E_m, membrane potential. *I*, current. Asterisks indicate $p<0.05$. Error bars indicate standard error.

Figure 7. Inactivation of inward-rectifying K^+ current (I_{Kin}) by MeJA in Columbia-0 (Col) and *pyr1 pyl1 pyl2 pyl4* (*quadruple*) GCPs. Representative trace and step pulse membrane potential protocol (A), and average current-voltage curve (B) of I_{Kin} of Col and *quadruple* in the presence of 10 µM MeJA. (C) Comparison of amplitude of *I* at E_m = -160 mV. Difference among data sets was examined by analysis of variance with Tukey-Kramer *post hoc* test. Different character

indicates significant difference at $\alpha = 0.05$. The data of 0 µM MeJA-treatment is taken from Yin et al. (2013).

Figure 8. In-gel phosphorylation assay of GCP extracts of wild types, *srk2e, ost1-2* and the quadruple mutant. Protein kinase activity bands of GCPs from Columbia-0 (Col), *srk2e* mutant (*srk2e*) and *pyr1 pyl1 pyl2 pyl4* mutant (*quadruple*) (A), and that of Landsberg *erecta* (Ler) and *ost1-2* mutant *(ost1-2*) (B) were visualized by autoradiography after in-gel phosphorylation. GCPs were treated with 10 µM ABA or 10 µM MeJA for 20 min. The ticks on left indicate the position of molecular mass markers.

Figure 9. Induction of ABA synthesis in Arabidopsis leaves by MeJA spray. RT-PCR analysis of *NCED3* transcript in Arabidopsis leaves (A and B). Arabidopsis plants (Columbia-0 ecotype) were treated with 0.1% ethylalcohol (-) or 10 µM MeJA (+). (A) A representative gel image of ethidium bromide-stained RT-PCR amplified DNA fragments of *NCED3* and *ACTIN2* separated in agarose gel electrophoresis (1% agarose in TAE buffer). (B) Quantitative RT-PCR analysis of the relative expression level of *NCED3* normalized to *ACTIN2*. The

expression level was examined by the comparative C_T method (n = 3). Contents of jasmonic acid (C), jasmonoyl-isoleucine (D) and abscisic acid (E) in *Arabidopsis thaliana* (Columbia-0 ecotype) exposed to 0.1% ethanol (white circles) and 10 μM MeJA (black circles). Horizontal axis represents time after spray. Averages from three independent experiments are shown. Error bars represent standard error of the mean. ** indicates $P \leq 0.01$.