Involvement of Endogenous Abscisic Acid in Methyl Jasmonate-Induced Stomatal Closure in Arabidopsis[1][W][OA]

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In this study, we examined the involvement of endogenous abscisic acid (ABA) in methyl jasmonate (MeJA)-induced stomatal closure using an inhibitor of ABA biosynthesis, fluridon (FLU), and an ABA-deficient Arabidopsis (*Arabidopsis thaliana*) mutant, *aba2-2*. We found that pretreatment with FLU inhibited MeJA-induced stomatal closure but not ABA-induced stomatal closure in wild-type plants. The *aba2-2* mutation impaired MeJA-induced stomatal closure but not ABA-induced stomatal closure. We also investigated the effects of FLU and the *aba2-2* mutation on cytosolic free calcium concentration ($[Ca^{2+}]_{cyt}$) in guard cells using a Ca^{2+}-reporter fluorescent protein, Yellow Cameleon 3.6. In wild-type guard cells, FLU inhibited MeJA-induced $[Ca^{2+}]_{cyt}$ elevation but not ABA-induced $[Ca^{2+}]_{cyt}$ elevation. The *aba2-2* mutation did not affect ABA-elicited $[Ca^{2+}]_{cyt}$ elevation but suppressed MeJA-induced $[Ca^{2+}]_{cyt}$ elevation. We also tested the effects of the *aba2-2* mutation and FLU on the expression of MeJA-inducible *VEGETATIVE STORAGE PROTEIN1* (*VSP1*). In the *aba2-2* mutant, MeJA did not induce *VSP1* expression. In wild-type leaves, FLU inhibited MeJA-induced *VSP1* expression. Pretreatment with ABA at 0.1 μM, which is not enough concentration to evoke ABA responses in the wild type, rescued the observed phenotypes of the *aba2-2* mutant. Finally, we found that in wild-type leaves, MeJA stimulates the expression of *9-CIS-EPOXYCAROTENOID DIOXYGENASE3*, which encodes a crucial enzyme in ABA biosynthesis. These results suggest that endogenous ABA could be involved in MeJA signal transduction and lead to stomatal closure in Arabidopsis guard cells.

Stomatal pores are surrounded by pairs of guard cells in the leaf epidermis of higher plants. Guard cells respond to a variety of external and internal stimuli such as light, drought, external Ca^{2+}, pathogen attack, and the phytohormones abscisic acid (ABA) and methyl jasmonate (MeJA) and regulate CO_2 uptake into leaves for photosynthesis, control of transpirational water loss, and innate immunity (Schroeder et al., 2001; Hetherington and Woodward, 2003; Suhita et al., 2004; Israelsson et al., 2006; Melotto et al., 2006; Munemasa et al., 2007; Shimazaki et al., 2007; Islam et al., 2009, 2010b).

MeJA, a linolenic acid derivative, regulates many processes of plant growth and development and mediates various plant defense responses (Liechti and Farmer, 2002; Turner et al., 2002). MeJA as well as ABA stimulate stomatal closure in several plant species (Irving et al., 1992; Gehring et al., 1997; Liu et al., 2002; Suhita et al., 2004; Munemasa et al., 2007; Saito et al., 2008; Islam et al., 2009, 2010b).

MeJA recruits many ABA signaling components to induce stomatal closure, including the NAD(P)H oxidases AtrbohD/F (Suhita et al., 2004), the Snf-related protein kinase *OST1* (Suhita et al., 2004), the protein phosphatases 2C *ABI1* and *ABI2* (Munemasa et al., 2007), a regulatory subunit of protein phosphatase 2A, RCN1 (Saito et al., 2008), and the myrosinase TGG (Islam et al., 2009), suggesting that MeJA signaling is overlapped with ABA signaling in guard cells. Interestingly, it has been demonstrated that MeJA stimulates ABA biosynthesis in plants (Kim et al., 2009; Yoon et al., 2009). Herde et al. (1997) showed that ABA-deficient mutants are insensitive to jasmonic acid in reducing the transpirational stream. From these findings, we hypothesized that MeJA-induced stomatal closure requires endogenous ABA.

Calcium functions as an important second messenger in ABA signaling and MeJA signaling of guard cells. ABA promotes the influx of Ca^{2+} across the plasma membrane and Ca^{2+} release from internal stores, resulting in the elevation and oscillation of cytosolic free calcium concentration ($[Ca^{2+}]_{cyt}$) in

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guard cells (Leckie et al., 1998; Grabov and Blatt, 1999; Allen et al., 2000; Hamilton et al., 2000; MacRobbie, 2000; Pei et al., 2000; Kwak et al., 2003; Marten et al., 2007). It has been demonstrated that MeJA also activates nonselective Ca^{2+}-permeable cation (I_{Ca}) channels of guard cell plasma membrane and elicits $[Ca^{2+}]_{cyt}$ oscillation in Arabidopsis (*Arabidopsis thaliana*) guard cells (Munemasa et al., 2007; Islam et al., 2010a, 2010b). Stomatal closure is accompanied by an increment of $[Ca^{2+}]_{cyt}$ and oscillation of $[Ca^{2+}]_{cyt}$ in guard cells in response to ABA and MeJA (Grabov and Blatt, 1998; Allen et al., 1999a, 2000, 2001; Pei et al., 2000; Klüsener et al., 2002; Mori et al., 2006; Munemasa et al., 2007; Islam et al., 2010a, 2010b). The increment of $[Ca^{2+}]_{cyt}$ activates S-type anion currents and inhibits inward K^+ currents in the plasma membrane of guard cells to accelerate the 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).

Denekamp and Smeekens (2003) have demonstrated that MeJA enhanced osmotic stress and wounding-responsive gene *AtMYB102* expression synergistically in combination with ABA, but MeJA alone had a limited effect on *AtMYB102* expression, suggesting the involvement of ABA in MeJA-inducible gene expression. It has been reported that *VEGETATIVE STORAGE PROTEIN1* (*VSP1*) is a MeJA-inducible gene (Berger et al., 1995; Ellis and Turner, 2001; Liu et al., 2005) and that exogenous ABA has no visible effect on *VSP1* expression (Montiel et al., 2011). It has been demonstrated that Arabidopsis *9-CIS-EPOXYCAROTENOID DIOXYGENASE3* (*AtNCED3*) plays a pivotal role in drought stress-inducible ABA biosynthesis (Iuchi et al., 2001; Urano et al., 2009). Overexpression of *AtNCED3* in Arabidopsis and *PvNCED1* in *Nicotiana plumbaginifolia* enhances ABA accumulation and resistance to water stress (Iuchi et al., 2001; Qin and Zeevaart, 2002). However, there is no report of whether MeJA induces *VSP1* gene expression in the ABA-deficient *aba2-2* mutant and *AtNCED3* expression in wild-type plants.

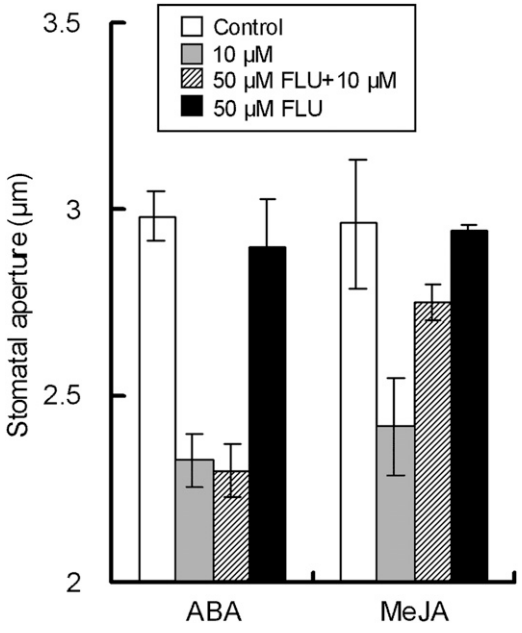

Figure 1. Effect of FLU on ABA-induced and MeJA-induced stomatal closure in wild-type guard cells. Rosette leaves of wild-type plants were treated with 50 μM FLU for 30 min. Then, rosette leaves pretreated with FLU were incubated with 10 μM ABA or 10 μM MeJA. Twenty averages from three independent experiments (60 total stomata per bar) are shown. Error bars represent SE.

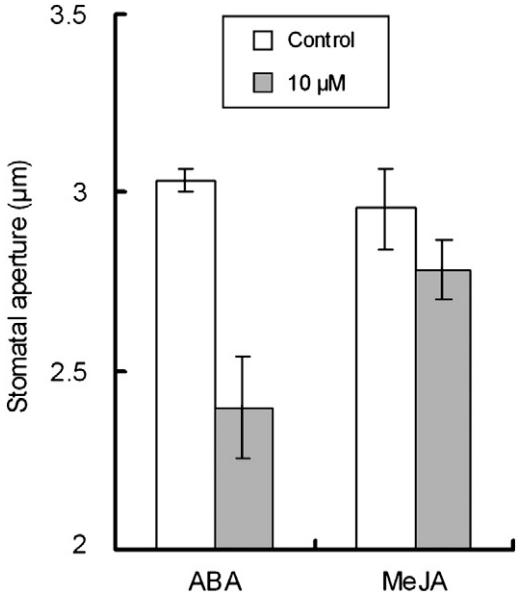

Figure 2. ABA-induced and MeJA-induced stomatal closure in *aba2-2* mutants. Twenty averages from three independent experiments (60 total stomata per bar) are shown. Error bars represent SE.

In this study, we used an inhibitor of ABA biosynthesis, fluridon {FLU; 1-methyl-3-phenyl-5-[3-triflurometh yl (phenyl)]-4-(^{1}H)-pyridinone}, and an ABA-deficient Arabidopsis mutant, *aba2-2*, to clarify the involvement of endogenous ABA in MeJA signaling in Arabidopsis guard cells. FLU is an herbicide that is used as an inhibitor of ABA biosynthesis (Gamble and Mullet, 1986; Kowalczyk-Schroder and Sandmann, 1992), and FLU suppresses the accumulation of endogenous ABA (Moore and Smith, 1984; Moore et al., 1985; Gamble and Mullet, 1986; Cammue et al., 1989; Popova, 1995). Endogenous ABA levels in fresh and dehydrated tissues of the *aba2-2* mutant were about 23% and 2.4%, respectively, of those of wild-type plants (Nambara et al., 1998). We examined (1) the effect of FLU on ABA- or MeJA-induced stomatal closure and $[Ca^{2+}]_{cyt}$ oscillation in wild-type plants, (2) the impairment of MeJA-induced stomatal closure and $[Ca^{2+}]_{cyt}$ oscillation in the *aba2-2* mutant, (3) the effect of FLU and the *aba2-2* mutation on MeJA-inducible *VSP1* expression, (4) *AtNCED3* expression in MeJA-treated leaves of wild-type plants, and (5) the effect of 0.1 μM ABA on MeJA-induced stomatal closure, $[Ca^{2+}]_{cyt}$ oscillation,

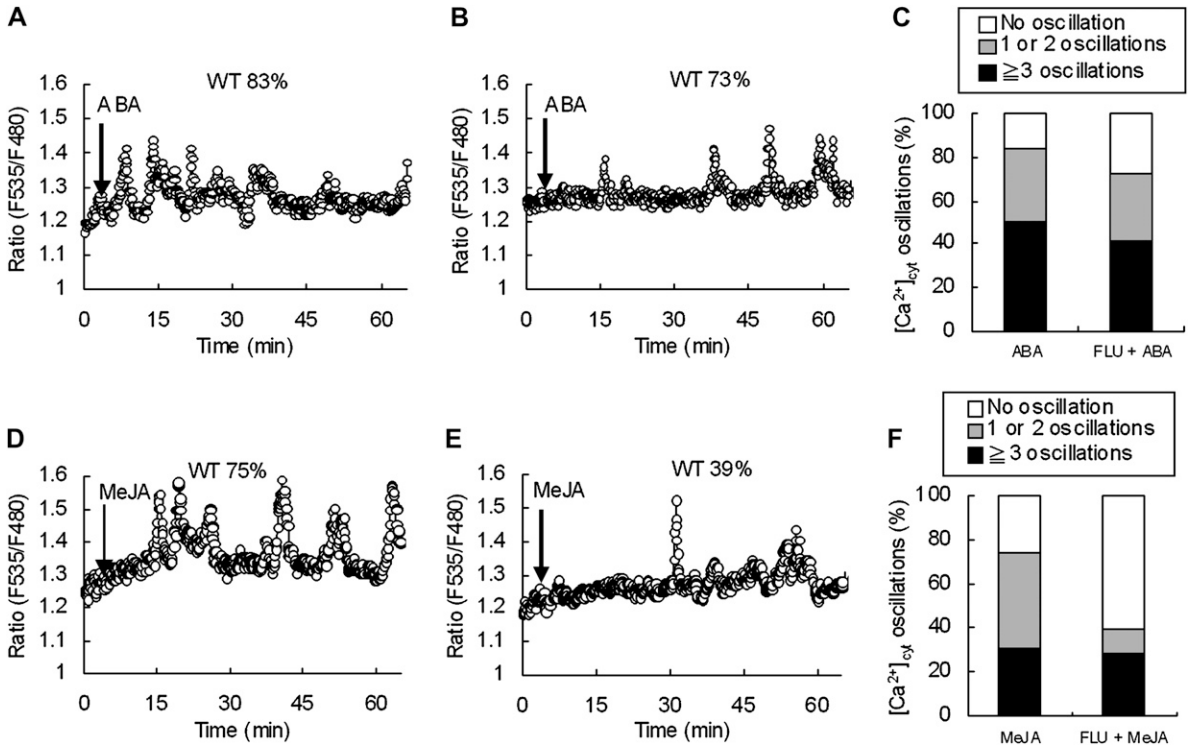

Figure 3. Effect of FLU (50 μM) on ABA-elicited and MeJA-elicited $[Ca^{2+}]_{cyt}$ oscillations (changes in fluorescence emission ratio, 535/480 nm) in wild-type (WT) guard cells. A, When wild-type guard cells were treated with 10 μM ABA, 20 of 24 guard cells (83%) showed $[Ca^{2+}]_{cyt}$ oscillations. B, In the presence of FLU, 16 of 22 guard cells (73%) showed ABA-elicited $[Ca^{2+}]_{cyt}$ oscillations in the wild type. C, Stack column representation of ABA-elicited $[Ca^{2+}]_{cyt}$ oscillations (%) in the absence (ABA; $n = 24$) or presence (FLU + ABA; $n = 22$) of FLU. D, Twenty-four of 32 (75%) wild-type guard cells showed MeJA-elicited $[Ca^{2+}]_{cyt}$ oscillations. E, In the presence of FLU, 11 of 28 guard cells (39%) showed MeJA-elicited $[Ca^{2+}]_{cyt}$ oscillations in the wild type. F, Stack column representation of MeJA-elicited $[Ca^{2+}]_{cyt}$ oscillations (%) in the absence (MeJA; $n = 32$) or presence (FLU + MeJA; $n = 28$) of FLU.

and *VSP1* expression in *aba2-2* mutants. Our results suggest that endogenous ABA could be involved in MeJA signal transduction and lead to stomatal closure in Arabidopsis guard cells.

RESULTS

Effect of FLU on ABA- or MeJA-Induced Stomatal Closure in the Wild Type

To clarify the involvement of endogenous ABA in MeJA signaling in guard cells, the effect of FLU (an inhibitor of ABA biosynthesis) on ABA- or MeJA-induced stomatal closure was examined in wild-type plants. Exogenous application of 10 μM ABA and 10 μM MeJA induced stomatal closure in wild-type plants (Fig. 1). FLU at 50 μM did not inhibit ABA-induced stomatal closure. On the other hand, MeJA-induced stomatal closure was inhibited by FLU, while FLU alone showed no significant effect on stomatal closure (Fig. 1).

Impairment of MeJA-Induced Stomatal Closure by the *aba2-2* Mutation

To obtain more solid evidence for the involvement of endogenous ABA in MeJA signaling in guard cells, we examined whether MeJA induces stomatal closure in the ABA-deficient mutant *aba2-2* (Nambara et al., 1998). Application of 10 μM ABA significantly induced stomatal closure in *aba2-2* mutants, but 10 μM MeJA did not induce stomatal closure in *aba2-2* mutants (Fig. 2). These results indicate that the *aba2-2* mutation is involved in MeJA-induced stomatal closure but not in ABA-induced stomatal closure.

Effect of FLU on ABA- or MeJA-Elicited $[Ca^{2+}]_{cyt}$ Oscillations in the Wild Type

ABA-induced $[Ca^{2+}]_{cyt}$ increases are one of the earliest measurable ABA signaling events in guard cells (McAinsh et al., 1990, 1997; Schroeder and Hagiwara, 1990). Recently, we reported that MeJA elicited $[Ca^{2+}]_{cyt}$ oscillations to lead to stomatal closure like ABA (Islam et al., 2010a, 2010b). In order to determine the involvement of endogenous ABA in MeJA signaling, the effect of FLU on ABA- or MeJA-elicited $[Ca^{2+}]_{cyt}$ oscillation was investigated using wild-type plants expressing the Ca^{2+} reporter Yellow Cameleon 3.6 (YC3.6; Nagai et al., 2004; Mori et al., 2006; Islam et al., 2010a, 2010b).

In the absence of FLU, 83% of wild-type guard cells treated with 10 μM ABA showed $[Ca^{2+}]_{cyt}$ oscillations ($n = 20$ of 24 cells; three or more oscillations, 50%; one

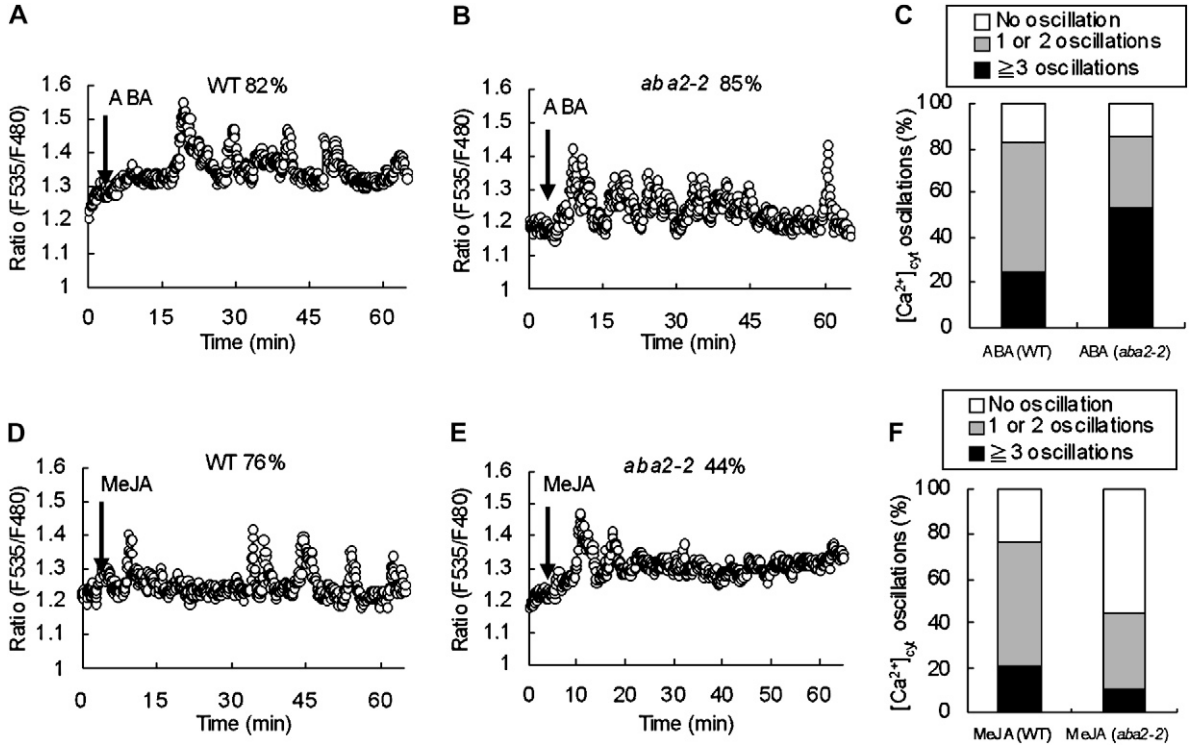

Figure 4. ABA- elicited and MeJA-elicited $[Ca^{2+}]_{cyt}$ oscillations in wild-type (WT) and *aba2-2* guard cells. A, Representative data of fluorescence emission ratio (535/480 nm) showing $[Ca^{2+}]_{cyt}$ oscillations in 10 μM ABA-treated wild-type guard cells (n = 23 of 28 cells; 82%). B, Representative data of fluorescence emission ratio (535/480 nm) showing $[Ca^{2+}]_{cyt}$ oscillations in 10 μM ABA-treated *aba2-2* guard cells (n = 29 of 34 cells; 85%). C, Stack column representation of ABA-induced $[Ca^{2+}]_{cyt}$ oscillations (%) in wild-type guard cells (n = 28) and *aba2-2* guard cells (n = 34). D, Representative data of fluorescence emission ratio (535/480 nm) showing $[Ca^{2+}]_{cyt}$ oscillations in 10 μM MeJA-treated wild-type guard cells (n = 22 of 29 cells; 76%). E, Representative data of fluorescence emission ratio (535/480 nm) showing $[Ca^{2+}]_{cyt}$ oscillations in 10 μM MeJA-treated *aba2-2* guard cells (n = 16 of 36 cells; 44%). F, Stack column representation of MeJA-induced $[Ca^{2+}]_{cyt}$ oscillations (%) in wild-type guard cells (n = 29) and *aba2-2* guard cells (n = 36).

or two oscillations, 33%; Fig. 3, A and C, ABA). In the presence of FLU, 73% of wild-type guard cells showed ABA-elicited $[Ca^{2+}]_{cyt}$ oscillations (n = 16 of 22 cells; three or more oscillations, 41%; one or two oscillations, 32%; Fig. 3, B and C, FLU + ABA). No $[Ca^{2+}]_{cyt}$ oscillation was observed in 17% of wild-type guard cells treated with 10 μM ABA (n = 4 of 24 cells; Fig. 3C, ABA), while 27% of wild-type guard cells showed no response to ABA in the presence of FLU (n = 6 of 22 cells; Fig. 3C, FLU + ABA). These results suggest that FLU did not significantly reduce the number of guard cells displaying ABA-elicited $[Ca^{2+}]_{cyt}$ oscillations in wild-type plants.

MeJA-elicited $[Ca^{2+}]_{cyt}$ oscillations were 75% in wild-type guard cells (n = 24 of 32 cells; three or more oscillations, 31%; one or two oscillations, 44%; Fig. 3, D and F, MeJA). In the presence of FLU, only 39% of wild-type guard cells treated with 10 μM MeJA showed $[Ca^{2+}]_{cyt}$ oscillations (n = 11 of 28 cells; three or more oscillations, 29%; one or two oscillations, 10%; Fig. 3, E and F, FLU + MeJA). Furthermore, 25% of wild-type guard cells showed no response to 10 μM MeJA (n = 8 of 32 cells; Fig. 3F, MeJA), whereas 61% of wild-type guard cells showed no response to 10 μM MeJA in the presence of FLU (n = 17 of 28 cells; Fig. 3F, FLU + MeJA). These results indicate that FLU reduced the number of cells showing MeJA-elicited $[Ca^{2+}]_{cyt}$ oscillations in wild-type guard cells.

ABA-Elicited $[Ca^{2+}]_{cyt}$ Oscillations in Wild-Type and *aba2-2* Plants

To determine whether the *aba2-2* mutation affects mechanisms upstream of $[Ca^{2+}]_{cyt}$ increases in guard cells, we analyzed $[Ca^{2+}]_{cyt}$ oscillations using wild-type and *aba2-2* plants expressing YC3.6. ABA at 10 μM induced $[Ca^{2+}]_{cyt}$ oscillations in 82% of wild-type guard cells (n = 23 of 28 cells; three or more oscillations, 25%; one or two oscillations, 57%; Fig. 4, A and C). By contrast, 85% of *aba2-2* guard cells treated with 10 μM ABA showed $[Ca^{2+}]_{cyt}$ oscillations (n = 29 of 34 cells; three or more oscillations, 53%; one or two oscillations, 32%; Fig. 4, B and C). About 18% of wild-type guard cells showed no $[Ca^{2+}]_{cyt}$ oscillations in response to 10 μM ABA (n = 5 of 28 cells; Fig. 4C), whereas 15% of *aba2-2* guard cells showed no

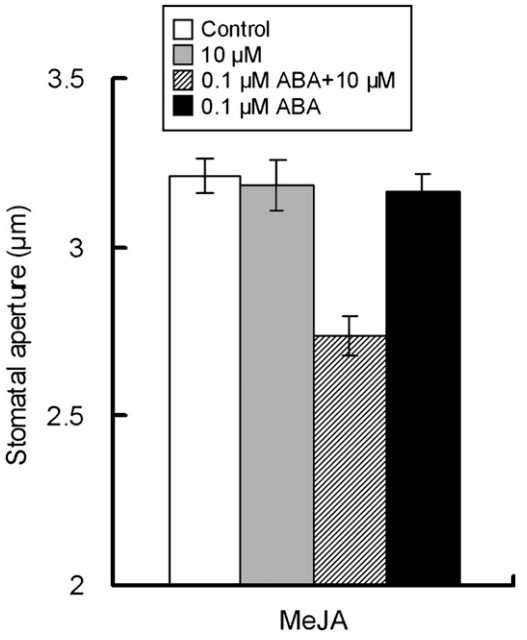

Figure 5. Effect of 0.1 μM ABA on MeJA-induced stomatal closure in *aba2-2* guard cells. Rosette leaves of *aba2-2* plants were treated with 0.1 μM ABA for 30 min. Then, rosette leaves pretreated with 0.1 μM ABA were incubated with 10 μM MeJA. Twenty averages from three independent experiments (60 total stomata per bar) are shown. Error bars represent SE.

response to 10 μM ABA (n = 5 of 34 cells; Fig. 4C). These results suggest that the *aba2-2* mutation does not affect ABA-elicited $[Ca^{2+}]_{cyt}$ oscillations.

MeJA-Elicited $[Ca^{2+}]_{cyt}$ Oscillations in Wild-Type and *aba2-2* Plants

In wild-type plants, 76% of guard cells treated with 10 μM MeJA showed $[Ca^{2+}]_{cyt}$ oscillations (n = 22 of 29 cells; three or more oscillations, 21%; one or two oscillations, 55%; Fig. 4, D and F). On the contrary, only 44% of *aba2-2* guard cells treated with 10 μM MeJA showed $[Ca^{2+}]_{cyt}$ oscillations (n = 16 of 36 cells; three or more oscillations, 11%; one or two oscillations, 33%; Fig. 4, E and F). About 24% of wild-type guard cells showed no $[Ca^{2+}]_{cyt}$ oscillations in response to 10 μM MeJA (n = 7 of 29 cells; Fig. 4F). Compared with the wild-type, a higher percentage of cells with no $[Ca^{2+}]_{cyt}$ oscillation was observed in *aba2-2* guard cells treated with 10 μM MeJA (56%; n = 20 of 36 cells; Fig. 4F). These results suggest that the *aba2-2* mutation suppressed MeJA-elicited $[Ca^{2+}]_{cyt}$ oscillations.

Effect of ABA on MeJA-Induced Stomatal Closure and $[Ca^{2+}]_{cyt}$ Oscillations in *aba2-2* Mutants

To clarify the involvement of increased levels of endogenous ABA in MeJA signaling in guard cells, we examined the effect of 0.1 μM ABA on MeJA-induced stomatal closure in *aba2-2* guard cells (Fig. 5). MeJA did not induce stomatal closure in *aba2-2* mutants, whereas in the presence of 0.1 μM ABA, MeJA induced stomatal closure in *aba2-2* guard cells, where 0.1 μM ABA did not significantly induce either stomatal closure or $[Ca^{2+}]_{cyt}$ oscillations in wild-type plants (data not shown). In wild-type plants, 77% of guard cells treated with 10 μM MeJA elicited $[Ca^{2+}]_{cyt}$ oscillations (n = 17 of 22 cells; three or more oscillations, 54%; one or two oscillations, 23%; Fig. 6, A and C). In the presence of 0.1 μM ABA, 79% of *aba2-2* guard cells showed MeJA-elicited $[Ca^{2+}]_{cyt}$ oscillations (n = 19 of 24 cells; three or more oscillations, 58%; one or two oscillations, 21%; Fig. 6, B and C). No $[Ca^{2+}]_{cyt}$ oscillation was observed in 23% of wild-type guard cells treated with 10 μM MeJA (n = 5 of 22 cells; Fig. 6C), whereas 21% of *aba2-2* guard cells showed no response to 10 μM MeJA in the presence of 0.1 μM ABA (n = 5 of 24 cells; Fig. 6C). These results suggest that the *aba2-2* mutation is

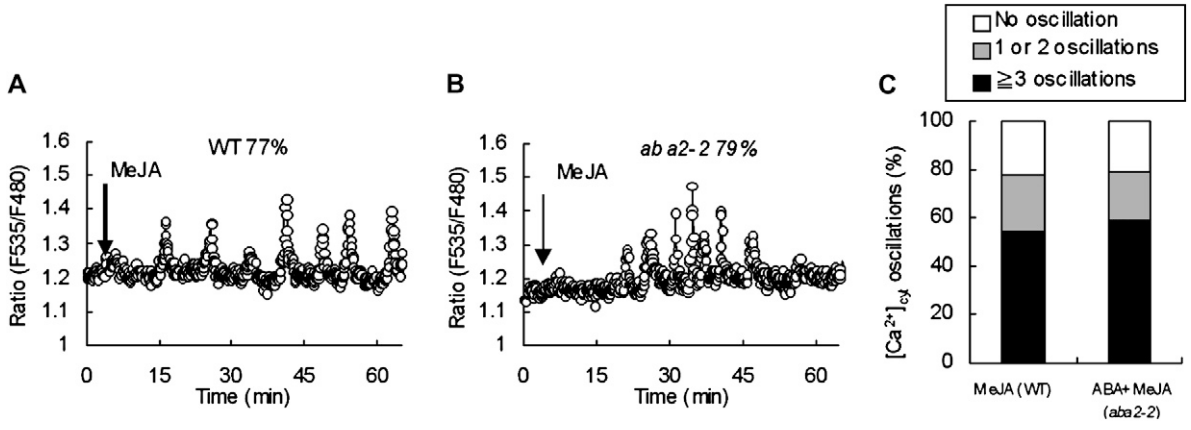

Figure 6. Effect of 0.1 μM ABA on MeJA-induced $[Ca^{2+}]_{cyt}$ oscillations in *aba2-2* guard cells. A, Representative data of fluorescence emission ratio (535/480 nm) showing $[Ca^{2+}]_{cyt}$ oscillations in 10 μM MeJA-treated wild-type (WT) guard cells (n = 17 of 22 cells; 77%). B, Representative data of fluorescence emission ratio (535/480 nm) showing MeJA-elicited $[Ca^{2+}]_{cyt}$ oscillations in *aba2-2* guard cells treated with 0.1 μM ABA (n = 19 of 24 cells; 79%). C, Stack column representation of MeJA-induced $[Ca^{2+}]_{cyt}$ oscillations (%) in wild-type and *aba2-2* guard cells in the absence (WT; n = 22) or presence (*aba2-2*; n = 24) of 0.1 μM ABA.

involved in MeJA-induced stomatal closure as well as MeJA-elicited $[Ca^{2+}]_{cyt}$ oscillations.

MeJA-Inducible *VSP1* Expression in Wild-Type and *aba2-2* Plants

In our experimental conditions, MeJA induced *VSP1* expression in wild-type plants (Fig. 7A), but MeJA did not induce *VSP1* expression in the *aba2-2* mutant (Fig. 7B). In the presence of 0.1 μM ABA, MeJA induced *VSP1* expression in the *aba2-2* mutant, whereas 0.1 μM ABA had no effect on *VSP1* expression. ABA at 10 μM also had no effect on *VSP1* expression in wild-type plants without MeJA (Supplemental Fig. S1). These results suggest the involvement of endogenous ABA in MeJA-inducible gene expression.

Effect of FLU on *VSP1* and *AtNCED3* Expression in the Wild Type

In the presence of FLU, MeJA did not induce *VSP1* expression in the wild type (Fig. 8). FLU alone had a small effect on *VSP1* expression. These results suggest that FLU reduced MeJA-inducible *VSP1* expression in the wild type. In wild-type plants, MeJA induced *AtNCED3* expression (Fig. 8), but in the presence of FLU, MeJA reduced *AtNCED3* expression. FLU alone had limited effect on *AtNCED3* expression.

DISCUSSION

Impairment of MeJA-Induced Stomatal Closure, $[Ca^{2+}]_{cyt}$ Oscillations, and *VSP1* Expression by FLU

Guard cell $[Ca^{2+}]_{cyt}$ change acts as an early second messenger in signal transduction pathways that control stomatal aperture (Allen et al., 1999a, 2000; Murata et al., 2001; Kwak et al., 2002; Islam et al., 2010a, 2010b). GFP-based Ca^{2+} indicator YC3.6 technique is used as a novel tool for direct monitoring of guard cell $[Ca^{2+}]_{cyt}$ oscillations (Allen et al., 1999b, 2000). ABA and MeJAactivate I_{Ca} channels of the guard cell plasma membrane (Kwak et al., 2003; Munemasa et al., 2007), and then activation of I_{Ca} channels contributes to the elevation of $[Ca^{2+}]_{cyt}$ in guard cells, causing stomatal closure (Hamilton et al., 2000; Pei et al., 2000). During drought stress, endogenous ABA levels increased due to the activation of de novo biosynthesis of ABA (Ikegami et al., 2009). FLU inhibited the accumulation of endogenous ABA (Moore and Smith, 1984; Moore et al., 1985; Gamble and Mullet, 1986; Cammue et al., 1989; Popova, 1995). MeJA induced *VSP1* expression in Arabidopsis (Berger et al., 1995; Ellis and Turner, 2001; Liu et al., 2005). In this study, FLU did not affect ABA-induced stomatal closure and ABA-elicited $[Ca^{2+}]_{cyt}$ oscillation (Figs. 1 and 3, B and C) but inhibited MeJA-induced stomatal closure, MeJA-elicited $[Ca^{2+}]_{cyt}$ oscillation, and *VSP1* expression (Figs. 1, 3, E and F, and 8), suggesting that the accumulation of endogenous ABA is required for MeJA-induced stomatal closure as well as MeJA-elicited $[Ca^{2+}]_{cyt}$ oscillation and *VSP1* expression.

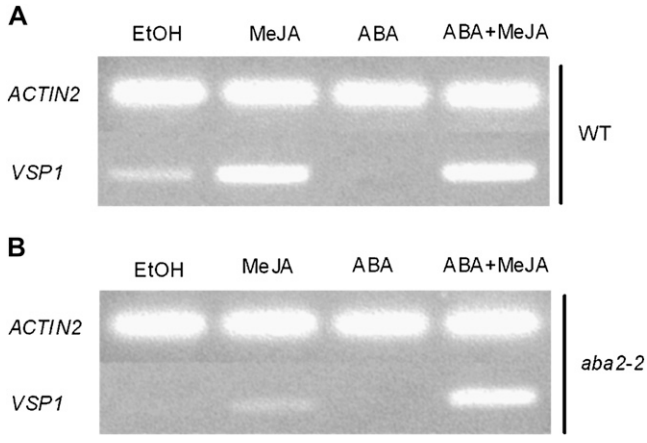

Figure 7. MeJA-inducible *VSP1* expression in wild-type (WT) and *aba2-2* leaves. A, MeJA induced *VSP1* expression in wild-type leaves. B, In the presence of 0.1 μM ABA, MeJA induced *VSP1* expression in *aba2-2* leaves.

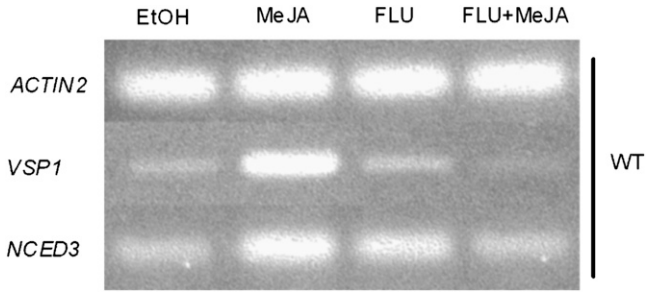

Figure 8. Effect of FLU on MeJA-inducible *VSP1* expression and *AtNCED3* expression in MeJA-treated wild-type (WT) leaves.

The *aba2-2* Mutant Did Not Show MeJA-Induced Stomatal Closure and *VSP1* Expression

The Arabidopsis *ost1* mutation impaired both ABA-induced stomatal closure and MeJA-induced stomatal closure (Suhita et al., 2004). Munemasa et al. (2007) demonstrated that *abi2-1* and *abi1-1* mutations impaired both ABA-induced stomatal closure and MeJA-induced stomatal closure.

The levels of endogenous ABA in fresh and dehydrated tissues of the *aba2-2* mutant were highly reduced compared with those of wild-type plants. As a result, *aba2-2* plants wilt and produce seeds with reduced dormancy (Nambara et al., 1998). In this study, we investigated the involvement of endogenous ABA in MeJA signaling using the *aba2-2* mutant. The *aba2-2* mutation impaired MeJA-induced stomatal closure and *VSP1* expression but did not impair ABA-induced stomatal closure (Figs. 2 and 7B). These

results indicate that endogenous ABA could be involved in MeJA-induced stomatal closure.

In addition to the regulation of stomatal movements, we also checked the MeJA inhibition of root growth (Staswick et al., 1992; Feys et al., 1994; Grunewald et al., 2009; Munemasa et al., 2011) in wild-type and *aba2-2* plants (Supplemental Fig. S2, A and B). MeJA inhibited root growth in wild-type and *aba2-2* plants. ABA at 0.1 μM increased root length in *aba2-2* plants. In the presence of 0.1 μM ABA, MeJA slightly increased the root length in *aba2-2* plants. These results suggest that endogenous ABA is not involved in the MeJA inhibition of root growth.

Impairment of MeJA-Elicited $[Ca^{2+}]_{cyt}$ Oscillations in *aba2-2* Mutants

Like the ABA response, application of MeJA elicits $[Ca^{2+}]_{cyt}$ oscillations, leading to stomatal closure in Arabidopsis (Allen et al., 1999b, 2000, 2001; Islam et al., 2010a, 2010b). Ca^{2+}-permeable cation channels, which are activated by ABA and MeJA, contribute to the elevation of cytosolic free Ca^{2+} in guard cells (Hamilton et al., 2000; Pei et al., 2000; Murata et al., 2001; Munemasa et al., 2007). In this study, we investigated whether the *aba2-2* mutation impairs ABA- or MeJA-induced $[Ca^{2+}]_{cyt}$ oscillations in order to assess the contribution of *aba2-2* to Ca^{2+} signaling in ABA- and MeJA-induced stomatal closure. ABA elicits $[Ca^{2+}]_{cyt}$ oscillations in the *aba2-2* mutants as well as in wild-type guard cells (Fig. 4, A and B). There is no significance difference in ABA-elicited $[Ca^{2+}]_{cyt}$ oscillations between *aba2-2* and wild-type guard cells. MeJA elicits $[Ca^{2+}]_{cyt}$ oscillations in *aba2-2* mutants (Fig. 4E). Compared with wild-type guard cells, MeJA-induced $[Ca^{2+}]_{cyt}$ oscillations were significantly reduced in *aba2-2* guard cells (Fig. 4, D and E). Taken together, it is suggested that the *aba2-2* mutation is involved in MeJA-induced $[Ca^{2+}]_{cyt}$ oscillations in guard cells.

Involvement of Endogenous ABA in MeJA Signaling in Guard Cells

Defective stomatal closure with a wilty phenotype of ABA-deficient mutants is overcome by exogenous application of ABA (Ikegami et al., 2009). ABA-deficient mutants are insensitive to jasmonic acid in reducing the transpirational stream (Herde et al., 1997). MeJA stimulates the production of ABA in plants (Kim et al., 2009; Yoon et al., 2009). In Arabidopsis, overexpression of *AtNCED3* enhances ABA accumulation (Iuchi et al., 2001). In our experimental conditions, MeJA induced *AtNCED3* expression in wild-type plants (Fig. 8). Exogenous application of 0.1 μM ABA is unable to generate $[Ca^{2+}]_{cyt}$ oscillations or stomatal closure in wild-type guard cells (Allen et al., 2002). In this study, MeJA did not induce stomatal closure in *aba2-2* mutants (Fig. 2). In the presence of 0.1 μM ABA, MeJA elicited stomatal closure (Fig. 5) as well as $[Ca^{2+}]_{cyt}$ oscillations and *VSP1* expression in *aba2-2* mutants as in wild-type guard cells (Figs. 6B and 7B). These results suggest that a small amount of ABA could be involved in MeJA-induced stomatal closure, $[Ca^{2+}]_{cyt}$ oscillations, and *VSP1* expression in *aba2-2* mutants like wild-type guard cells. MeJA-dependent biosynthesis of ABA could be suppressed by the *aba2-2* mutation. In guard cell ABA signaling, NAD(P)H oxidases, AtrbohD/F, and the calcium-dependent protein kinase CPK6 function upstream of I_{Ca} channel activation, which contributes to $[Ca^{2+}]_{cyt}$ elevation (Kwak et al., 2003; Mori et al., 2006). It has been shown that AtrbohD/F (Suhita et al., 2004) and CPK6 (Munemasa et al., 2011) are also involved in MeJA-induced stomatal closure. These findings and our data here suggest that MeJA signaling in guard cells requires endogenous ABA to prime/activate such common $[Ca^{2+}]_{cyt}$-regulating signaling components. Taken together, this investigation suggests that endogenous ABA is required by MeJA signaling in Arabidopsis guard cells.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

In this study, we used the Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia as the wild-type plant and *aba2-2* as the ABA-deficient mutant. Wild-type, *aba2-2* mutant, and wild-type (Col) expressing Ca^{2+} reporter YC3.6 plants were grown on soil containing a homogenized mixture of 70% (v/v) vermiculite (Asahi-kogyo) and 30% (v/v) Kureha Soil (Kuhera Chemical) in a growth chamber (photon flux density of 80 μmol m^{-2} s^{-1} under a 16-h-light/8-h-dark regime). The temperature and relative humidity in the growth chamber were 22°C ± 2°C and 60% ± 10%, respectively. Water was applied two to three times per week with Hyponex solution (0.1%) on the plant growth tray.

Measurement of Stomatal Aperture

Stomatal aperture measurements were performed as described previously (Murata et al., 2001; Munemasa et al., 2007; Saito et al., 2008). Excised rosette leaves were floated on medium 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 induce stomatal opening followed by the addition of 10 μM ABA or 10 μM MeJA. Then, stomatal apertures were measured after 2 h of incubation. FLU was added 30 min prior to ABA or MeJA application. Leaves were blended for 30 s, and epidermal peels were collected. Twenty stomatal apertures were measured on each epidermal peel.

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

The *aba2-2* plants that express YC3.6 were generated by crossing *aba2-2* and the YC3.6-transformed Columbia plant. Four- to 6-week-old wild-type plants expressing YC3.6 and *aba2-2* plants expressing YC3.6 were used for the measurement of guard cell $[Ca^{2+}]_{cyt}$ oscillations, as described previously (Islam et al., 2010a, 2010b). The abaxial side of the excised leaf was softly attached to a glass slide using medical adhesive (stock no. 7730; Hollister), and then adaxial epidermis and mesophyll tissues were removed carefully with a razor blade to keep the intact lower epidermis on the slide. Isolated abaxial epidermal peels were incubated in the 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 to promote stomatal opening. The turgid guard cells were considered for the measurement of guard cell $[Ca^{2+}]_{cyt}$ oscillations. Guard cells were treated with different solutions by using a peristatic pump after 5 min from the start of the measurement. For dual-emission ratio imaging of YC3.6, a 440AF21 excitation filter, a 445DRLP dichroic mirror, and two emission filters, 480DF30 for cyan fluorescent protein (CFP) and 535DF25 for yellow fluorescent protein (YFP),

were used. The CFP and YFP fluorescence intensities of guard cells were imaged and analyzed using AQUA COSMOS software (Hamamatsu Photonics). Note that we used the same exposure time for both CFP and YFP.

Analysis of Gene Expression by Reverse Transcription-PCR

RNA extraction and reverse transcription-PCR were performed according to the manufacturer's instructions. Plants were sprayed with 0.1% ethanol or 10 μM MeJA and kept in growth chambers for 2 h. FLU (50 μM) or 0.1 μM ABA was sprayed 30 min prior to MeJA application. Then, RNA was extracted using Trizol reagent (Invitrogen). cDNA was synthesized from 1 μg of RNA using Moloney murine leukemia virus reverse transcriptase (TaKaRa Bio). PCR was performed with 1 μL of reverse transcription reaction mixture using BIOTAQ DNA polymerase (Bioline). Primers used in PCR amplification are as follows: for *VSP1*, 5′-CTCTCTAGTATTCCCTACTACGC-3′ (VSP1F) and 5′-GATTCTCGACAGTGACTTCTGAC-3′ (VSP1R); for *AtNCED3*, 5′-AGCTAACCCACTTCACGAGC-3′ (AtNCED3FW) and 5′-CGAATTTGACGGCGTGAACC-3′ (AtNCED3RV); and for *Actin2*, 5′-TCTTAACCCAAAGGCCAACA-3′ (ACT2F) and 5′-CAGAATCCAGCACAATACCG-3′ (ACT2R).

Assay of Root Growth

Seeds were sterilized by washing with 70% ethanol and subsequent immersion in 5% (v/v) sodium hypochlorite solution for 5 min, followed by washing five times with sterile distilled water. Seeds were sown on Murashige and Skoog (MS) plates (Murashige and Skoog, 1962) containing MS salt mixture (Nihon Pharmaceutical), 2% (w/v) Suc, 3 mg L^{-1} thiamine hydrochloride, 0.5 mg L^{-1} pyridoxine hydrochloride, 5 mg L^{-1} nicotinic acid, and 0.8% (w/v) agar powder (Nacalai Tesque). Plates were kept at 4°C for 3 d and then transferred to growth chambers in a vertical orientation. Four-day-old seedlings were transferred to MS plates containing 10 μM MeJA, 0.1 μM ABA, or 0.1 μM ABA plus 10 μM MeJA. After 5 d, root length was measured.

Statistical Analysis

The significance of differences between mean values of stomatal apertures and root growth were assessed by Student's *t* test, and the frequency of $[Ca^{2+}]_{cyt}$ oscillations was assessed by χ^2 test. Differences at $P < 0.05$ were considered significant.

Arabidopsis Genome Initiative numbers for the genes discussed in this article are as follows: *ABA2* (At1g52340), *VSP1* (At5g24780), *AtNCED3* (At3g14440), and *Actin2* (At3g18780).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Effect of ABA on MeJA-inducible *VSP1* expression in the wild type.

Supplemental Figure S2. MeJA inhibition of root growth in wild-type and *aba2-2* plants.

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