Cytoplasmic Alkalization Precedes Reactive Oxygen Species Production during Methyl Jasmonate- and Abscisic Acid-Induced Stomatal Closure[1]

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Signaling events during abscisic acid (ABA) or methyl jasmonate (MJ)-induced stomatal closure were examined in Arabidopsis wild type, ABA-insensitive (*ost1*-2), and MJ-insensitive mutants (*jar1*-1) in order to examine a crosstalk between ABA and MJ signal transduction. Some of the experiments were performed on epidermal strips of *Pisum sativum*. Stomata of *jar1*-1 mutant plants are insensitive to MJ but are able to close in response to ABA. However, their sensitivity to ABA is less than that of wild-type plants. Reciprocally, the stomata of *ost1*-2 are insensitive to ABA but are able to close in response to MJ to a lesser extent compared to wild-type plants. Both MJ and ABA promote H_2O_2 production in wild-type guard cells, while exogenous application of diphenylene iodonium (DPI) chloride, an inhibitor of NAD(P)H oxidases, results in the suppression of ABA- and MJ-induced stomatal closure. ABA elevates H_2O_2 production in wild-type and *jar1*-1 guard cells but not in *ost1*-2, whereas MJ induces H_2O_2 production in both wild-type and *ost1*-2 guard cells, but not in *jar1*-1. MJ-induced stomatal closing is suppressed in the NAD(P)H oxidase double mutant *atrbohD/F* and in the outward potassium channel mutant *gork1*. Furthermore, MJ induces alkalization in guard cell cytosol, and MJ-induced stomatal closing is inhibited by butyrate. Analyses of the kinetics of cytosolic pH changes and reactive oxygen species (ROS) production show that the alkalization of cytoplasm precedes ROS production during the stomatal response to both ABA and MJ. Our results further indicate that JAR1, as OST1, functions upstream of ROS produced by NAD(P)H oxidases and that the cytoplasmic alkalization precedes ROS production during MJ or ABA signal transduction in guard cells.

Methyl jasmonate (MJ), a linolenic acid derivative, is involved in plant development and defense and is overproduced during wounding, fruit ripening, and drought stress (Creelman and Mullet, 1997). MJ affects plant transpiration (Lee et al., 1996; Wang, 1999) by promoting stomatal closure (Raghavendra and Reddy, 1987; Gehring et al., 1997; Suhita et al., 2003). MJ-induced stomatal closure is accompanied by an alkalization of the guard cell cytoplasm in *Paphiopedilum* spp. (Gehring et al., 1997). A recent study has shown that this response to MJ requires external calcium and involves a calmodulin-like domain, obviously of a protein kinase (Suhita et al., 2003). Interestingly, Evans (2003) demonstrated that MJ activates the outward potassium channel from guard cell protoplast of *Vicia faba*, the main conductance allowing K^+ efflux and loss of turgor. These steps of cytoplasmic pH modification (Irving et al., 1992) and modulation of potassium channels at the guard cell plasma membrane (Armstrong et al., 1995) are also involved in abscisic acid (ABA)-induced stomatal closure.

In addition, cytoplasmic calcium waves (Allen et al., 2000), protein (de)phosphorylation (Leung et al., 1994, 1997; Meyer et al., 1994; Li et al., 2000; Merlot et al., 2001; Kwak et al., 2002; Mustilli et al., 2002) and reactive oxygen species (ROS) have all been identified to participate in ABA signaling (Gomez-Cadenas et al., 1999; Guan et al., 2000; Pei et al., 2000; Zhang et al., 2001a, 2001b; Klüsener et al., 2002). In guard cells, ABA induces ROS production, which in turn activates Ca^{2+} channels at the plasma membrane (Pei et al., 2000; Murata et al., 2001). Further, ABA-induced elevation in cytosolic Ca^{2+} leads to activation of slow anion channels and inactivation of inward rectifying K^+ channels. The consequences are K^+ efflux, guard cell turgor reduction, and stomatal closure. Interestingly, MJ together with various elicitors also induces an accumulation of H_2O_2 in leaves (Orozco-Cardenas and Ryan, 1999). Thus, it is likely that ABA and MJ transduction pathways leading to stomatal closure involve overlapping signaling elements. Such interaction has already been suggested by Herde et al. (1997) who

[1] This work was supported by grants from the Indo-French Centre for the Promotion of Advanced Research (grant no. 2203–1 to A.S.R. and A.V.) and the Council of Scientific and Industrial Research [grant no. 38(0949)/99/EMR–II to A.S.R.], both from New Delhi. J.M.K. was supported by a fellowship from the Human Frontier Science Program.

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Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.103.032250.

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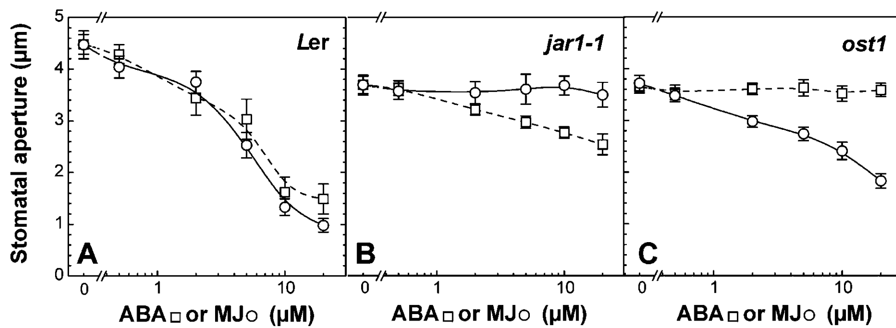

Figure 1. Dose-response curves of ABA- (squares) and MJ- (circles) induced stomatal closure in Landsberg *erecta* (A), *jar1*-1 mutant (B), and *ost1*-2 (C) mutant plants of Arabidopsis, respectively. The stomata of abaxial epidermis from leaves were allowed to open in light for 2 h, then ABA or MJ was applied for 2 h. Results are the averages ± SE (n = 60) from at least 3 independent experiments.

observed that ABA deficient mutants were insensitive to jasmonic acid in reducing the transpiration stream. However, the jasmonate-insensitive mutant *jar1*-1 showed increased sensitivity to ABA inhibition of germination (Staswick et al., 1992). Although these observations suggest that the relationships between MJ and ABA signals affecting stomatal regulation and germination are different, they also indicate the existence of crosstalk between MJ and ABA signaling cascades. It remains largely unknown which molecular components are shared by ABA and MJ signal transduction.

In the study presented here, cytoplasmic pH changes and ROS production in response to ABA or MJ were studied in guard cells of Arabidopsis. Additionally, mutant plants affected in ABA signaling (*ost1*-2; Mustilli et al., 2002), MJ signaling (*jar1*-1; Staswick et al., 1992, 2002), plasma membrane catalytic subunits of the plasma membrane NAD(P)H oxidases (*atrbohD/F*; Kwak et al., 2003) or guard cell outward K^+ channel (*gork1*; Hosy et al., 2003) were used to assess the respective roles of these genes in ABA or MJ signaling pathways leading to stomatal closure.

RESULTS

jar1-1 Mutants Are Insensitive to MJ But Not to ABA While ost1-2 Mutants Are Insensitive to ABA But Not to MJ

The *jar1*-1 MJ-insensitive mutant has been isolated on the basis of a diminished sensitivity of root growth to MJ (Staswick et al., 1992). The *jar1*-1 mutation affects the biochemical capability of JAR1 in the adenylation of jasmonic acid (Staswick et al., 2002). The *ost1*-2 mutant has been isolated using infrared thermography and characterized as ABA-insensitive at the stomatal level (Mustilli et al., 2002). OST1 is an ABA-activated protein kinase, an ortholog of the *V. faba* Ca^{2+}-independent ABA-activated protein kinase (Li et al., 2000). The *ost1*-2 mutation (Gly-33 to Arg) affects an invariant residue required for ATP-binding and is thus predicted to abolish OST1 kinase activity (Mustilli et al., 2002).

Figure 1 presents the stomatal sensitivity to ABA and MJ in wild-type plants, *jar1*-1, and *ost1*-2 mutant plants. Dose-response curves for MJ and ABA were quite similar in wild-type plants (Fig. 1A), with a 50% effect observed at around 5 μM. In the case of the *jar1*-1 mutant (Fig. 1B), stomata did not respond to MJ while a residual response to ABA was still observed, 28% of stomatal closure observed in wild-type plant at 20 μM ABA. As previously described (Mustilli et al., 2002), stomata from *ost1*-2 mutant plants were insensitive to ABA (Fig. 1C). However, they were still able to close in response to MJ, with a diminished sensitivity compared to wild-type plants, 60% of stomatal closure observed in wild-type plant at 20 μM MJ. These results demonstrate that JAR1 and OST1 are not absolutely required in a common ABA and MJ signaling pathway leading to stomatal closure. However the diminished response of *ost1*-2 to MJ and *jar1*-1 to ABA suggests a crosstalk between two signaling pathways through interacting elements.

Protein Kinases Are Essential Elements in Stomatal Closure by ABA and MJ

Protein (de)phosphorylation events play important roles in ABA signaling in guard cells (Li et al., 2000; Merlot et al., 2001; Kwak et al., 2002; Mustilli et al.,

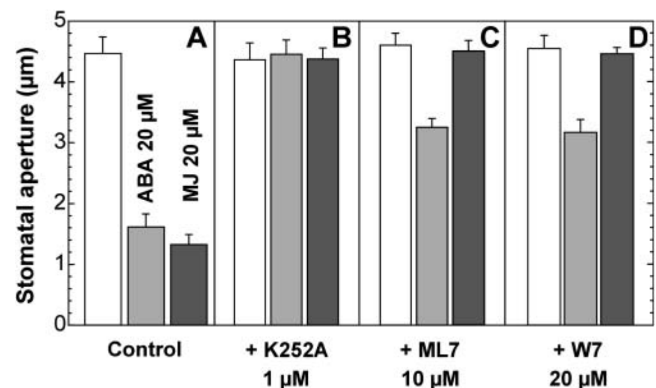

Figure 2. ABA- or MJ-induced stomatal closing (A) in the presence of the protein kinase inhibitors K252a (B), ML7 (C), and the calmodulin antagonist W7 (D). Stomata of leaf epidermis were allowed to open in light for 2 h, and then incubated for 2 h in ABA or MJ. K252a, ML7, and W7 were added 30 min before the addition of ABA or MJ. Results are the averages ± SE (n = 60) from at least 3 independent experiments.

Table I. *ABA- and MJ-induced H_2O_2 production in guard cells and stomatal closure*

	Ler		*jar1*-1		*ost1*-2		*gork1*	
	H_2O_2	*SA*	*H_2O_2*	*SA*	*H_2O_2*	*SA*	*H_2O_2*	*SA*
	%	μm	%	μm	%	μm	%	μm
Control	100.0 ± 3.1	4.47 ± 0.27	94.4 ± 3.8	3.69 ± 0.17	98.6 ± 2.7	3.65 ± 0.12	101.1 ± 3.5	4.51 ± 0.21
MJ	128.4 ± 2.5	1.33 ± 0.16	97.5 ± 3.0	3.50 ± 0.26	112.8 ± 3.5	2.41 ± 0.17	127.4 ± 3.5	4.22 ± 0.15
ABA	121.5 ± 3.1	1.62 ± 0.29	116.0 ± 2.8	2.54 ± 0.20	99.3 ± 3.7	3.59 ± 0.13	120.5 ± 3.7	2.67 ± 0.18

Influence of *jar1*-1, *ost1*-2, and *gork1* mutations on H_2O_2 production and stomatal closure in response to 20 μM MJ or ABA. Changes in ROS levels were analyzed by measuring H_2DCF-DA fluorescence levels in guard cells in response to a 30-min treatment with ABA, MJ, or solvent control addition. To determine the consequence of mutations on stomatal closure, leaf epidermis were allowed to open in light for 2 h, then ABA or MJ was applied for 2 h. Results are the averages ± SE ($n = 60$) of 3 to 4 independent experiments. The extents of H_2O_2 production in the guard cells of wild-type plants, without MJ or ABA, are taken as 100%. SA, stomatal apertures.

2002). Three compounds, K252a (broad range protein kinase inhibitor; Kase et al., 1987), ML7 (Ca^{2+}-calmodulin protein kinase inhibitor; Saitoh et al., 1987; Hidaka and Kobayashi, 1999), and W7 (calmodulin inhibitor; Yorio et al., 1985), were compared in their abilities to inhibit ABA- or MJ-induced stomatal closure (Fig. 2). K252a was found to abolish the ABA- and MJ-induced stomatal closure (Fig. 2B). In contrast, ML7 and W7 were able to suppress the response to MJ but were only partly effective against ABA (Fig. 2).

These results strongly suggest that at least one protein kinase, regulated by calcium and a calmodulin-like domain, is involved in stomatal response to MJ. The results also suggest that the ABA signaling cascade involved Ca^{2+}-dependent and Ca^{2+}-independent protein kinases as previously suggested (Allan et al., 1994; MacRobbie, 1998).

JAR1 and OST1 Are Located Upstream of ABA- and MJ-Induced H_2O_2 Production through an NAD(P)H Oxidase

ROS production in guard cells is induced by not only ABA (Pei et al., 2000; Murata et al., 2001) but also by chitosan, an elicitor of defense reactions (Lee et al., 1999), or in leaves by an exposure to MJ vapors (Orozco-Cardenas and Ryan, 1999). Analysis of ROS levels in guard cells using the fluorescent dye, 2′,7′-dichlorofluorescein diacetate (H_2DCF-DA), confirmed that ROS production was significantly stimulated after a treatment with ABA and unraveled an even greater stimulation by MJ in wild-type plants (Table I). Interestingly, MJ did not increase ROS levels in *jar1*-1 guard cells, while ABA had no effect on ROS level in *ost1*-2 guard cells (Table I). Additionally, ROS production and stomatal closure triggered by ABA were slightly impaired in *jar1*-1, and identical results were obtained when *ost1*-2 mutants were submitted to MJ. However, externally applied H_2O_2 elicited a similar degree of stomatal closure in *jar1*-1 (55% decrease over control, without H_2O_2) and *ost1*-2 (60% decrease over control) as in wild-type plants (53% over control), suggesting that OST1 and JAR1 are placed upstream of ROS production in ABA or MJ signaling.

Pei et al. (2000) reported that diphenylene iodonium (DPI) partially prevented ABA-induced stomatal closure and proposed that DPI could limit H_2O_2 production in guard cells. To test that the fluorescence observed in guard cells was the result of the product of an NAD(P)H oxidase, DPI was supplied to the incubation medium for 30 min before treating the epidermal strips with ABA or MJ. In the presence of 12.5 μM DPI, ROS production was indeed restricted even after a treatment with 20 μM ABA or MJ (Table II). In parallel, stomatal closure by ABA and MJ was also prevented. These results indicate that ROS production by an NAD(P)H oxidase (Auh and Murphy, 1995) or another flavoenzyme (O'Donnell et al., 1993) is a key element in the MJ signaling pathway as already shown for ABA (Pei et al., 2000). A recent work has shown that a double mutant of two isoforms of catalytic subunits of plasma membrane NAD(P)H oxidases

Table II. *The effect of DPI on the patterns of ROS production in response to ABA or MJ in guard cells of Arabidopsis wild-type and mutant plants*

Plant	Hormone (20 μM)					
	None		ABA		MJ	
	No DPI	DPI	No DPI	DPI	No DPI	DPI
Wild type	100 ± 1.9	103 ± 2.5	121 ± 2.2	104 ± 2.1	126 ± 3.5	102 ± 3.2
jar1-1	96 ± 2.9	105 ± 3.2	112 ± 3.5	106 ± 3.2	97 ± 3.4	103 ± 3.1
ost1-2	99 ± 2.8	101 ± 3.0	99 ± 3.1	98 ± 3.0	113 ± 2.8	97 ± 3.6
gork1	101 ± 3.1	102 ± 2.9	120 ± 2.7	103 ± 3.3	127 ± 3.7	106 ± 2.9

The ROS production was monitored by using fluorescence dye, H_2DCF-DA, as described in "Materials and Methods". The extent of ROS production in guard cells, without 20 μM MJ or 20 μM ABA or 12.5 μM DPI in wild type, was taken as control and 100%. Results are averages (±SE) from at least 3 experiments.

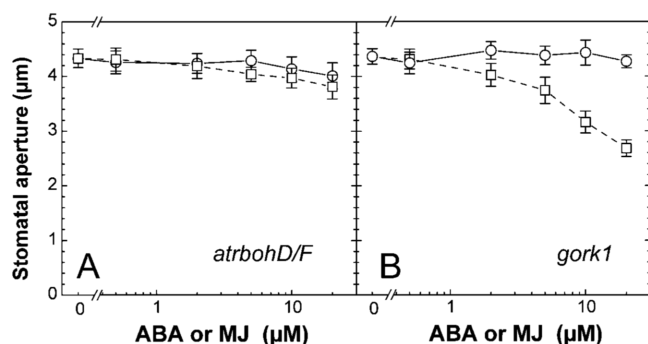

Figure 3. Dose-response curves of ABA-induced (squares) and MJ-induced (circles) stomatal closure in *atrbohD/F* double mutant plants and *gork1* mutant plants. Stomata of leaf epidermis were allowed to open for 2 h under light, then ABA or MJ was applied for 2 h. Results are the average ± SE ($n = 60$) of 3 to 4 independent experiments.

(*atrbohD/F*) led to a diminished ABA sensitivity at the stomatal level (Kwak et al., 2003). Interestingly, stomatal response to MJ was almost abolished in this double mutant (Fig. 3A), underlining the importance of NAD(P)H oxidases during not only ABA- but also MJ-induced stomatal closure.

Alkalization of pH_{Cyt} Is Necessary and Precedes ROS Production in Response to ABA and MJ

A modification of guard cell cytoplasmic pH is essential in ABA-induced stomatal closure (Irving et al., 1992; Blatt and Armstrong, 1993). We have therefore studied the response of the cytoplasmic pH to ABA and MJ. The null point method, first developed to measure cytoplasmic pH ($[pH]_{Cyt}$) in animal cells, has been shown to be valuable in measuring $[pH]_{Cyt}$ changes induced by MJ in barley aleurone protoplast (Van der Veen et al., 1992). The basic principle of this method is that when extracellular solution pH ($[pH]_{Ext}$) is equal to $[pH]_{Cyt}$ and buffering capacity is low, a selective disruption of the plasma membrane by digitonin will not affect $[pH]_{Ext}$. We used this method to evaluate the $[pH]_{Cyt}$ change of guard cell protoplasts (GCPs) in response to MJ or ABA. In preliminary experiments, very little change of $[pH]_{Ext}$ was observed when GCPs were treated with digitonin at $[pH]_{Ext}$ 7.35 ± 0.03 (Fig. 4A). Then, two types of experiments were performed. In a first set $[pH]_{Ext}$ was fixed to 7.35 ± 0.03 using KOH, then changes in pH of external solution, after addition of digitonin to MJ- or ABA-treated GCPs, were recorded (Fig. 4).

In a second batch of experiments, the precise values of $[pH]_{Cyt}$ in presence of ABA or MJ were estimated by changing the external pH by 0.1 unit to determine the null-point value. Based on triplicate measurements, the estimated $[pH]_{Cyt}$ for untreated guard cell protoplasts was 7.33 ± 0.04 ($[pH]_{Cyt}$ ± SE), and 7.47 ± 0.02 or 7.68 ± 0.02 after a 30 min treatment with 20 μM ABA or MJ, respectively. In the presence of MJ or ABA an alteration of the cytoplasmic pH took place in 15 min and then slowly decreased (Fig. 5). During the first 30 min of treatment ABA or MJ induced a ROS production increase in guard cells which then plateaued for at least 30 min (Fig. 5).

Technically it is difficult to use the null-point method for observing pH changes within 15 min. The pattern was therefore cross-checked with another species, *Pisum sativum*, using short durations of incubations and monitoring pH with a fluorescent dye, BCECF-AM. These results again indicated that the rise in pH of guard cells started within 5 min and peaked by 15 min, while the ROS production started only after 10 min and reaches maximum by 30 min (Fig. 6). Interestingly MJ triggered a stronger response than ABA despite kinetics that were similar. These results are in accordance with the results obtained by Gehring et al. (1997) using *Paphiopedilum* spp. guard cells and the fluorescent dye BCECF. The controls without ABA or MJ showed no significant change in pH (Fig. 6A).

Addition of the weak acid butyrate (0.5 mM), which causes an acidification of cytoplasm (Blatt and Thiel, 1994), limited stomatal closure caused by ABA or MJ in wild-type plants as well as in *jar-1* and *ost-1* mutants of Arabidopsis (Table III). Butyrate also diminished ABA- and MJ-induction of ROS production (Table IV; Fig. 7). Butyrate at 0.5 mM did not significantly affect the rates of either photosynthesis (122 μmol mg chl^{-1} h^{-1} in the absence and 120 μmol mg chl^{-1} h^{-1} in the presence of butyrate) or respiration (9 μmol mg chl^{-1} h^{-1} and 10

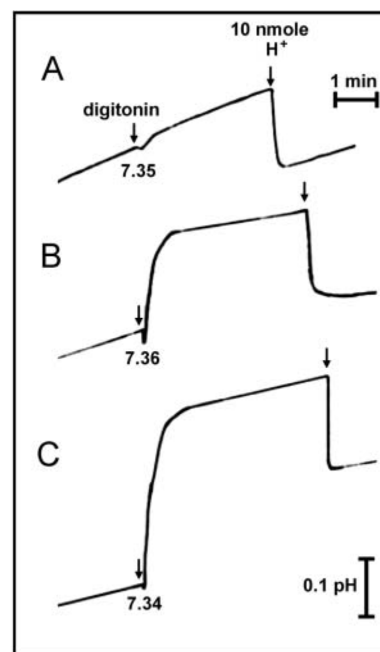

Figure 4. Change in the pH of the external solution after permeation of the guard cell plasma membrane by digitonin in control protoplasts (A), ABA-treated protoplasts (B), or MJ-treated protoplasts (C). Guard cell protoplasts were incubated in the presence of 20 μM ABA or MJ for 30 min before application of digitonin. Values indicate the external solution pH at time of application of digitonin; 10 nmol of HCl were injected into the medium at the end of each experiment for calibration.

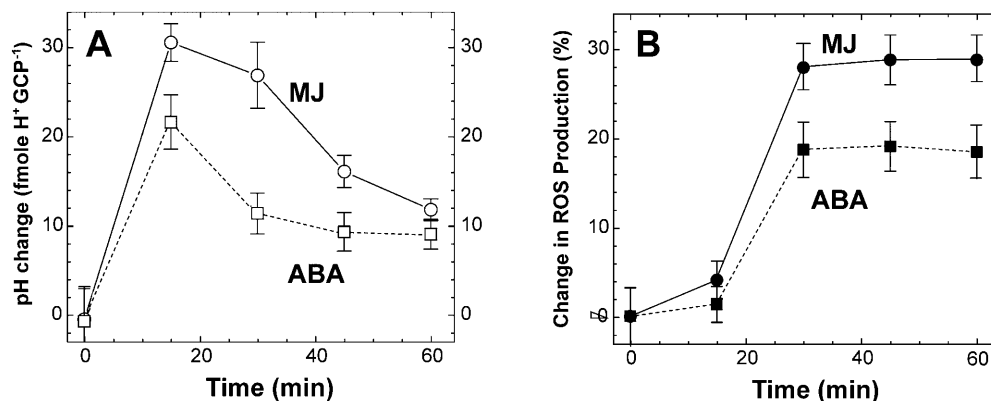

Figure 5. Kinetics of pH change (A) and ROS production (B) in guard cells in response to 20 μM ABA or 20 μM MJ; pH changes (A) were determined using the null-point method and H_2O_2 production (B) using the fluorescent dye H_2DCF-DA as described in "Materials and Methods". Each data point is the mean ± SE from at least 3 independent experiments.

μmol mg chl^{-1} h^{-1}, ±butyrate, respectively). Thus, butyrate at the concentration used in our experiments did not influence the metabolism of guard cells. These results together with the kinetics of ROS production and pH changes (Figs. 5 and 6), gave strong indications that pH changes preceded ROS production during stomatal closure induced by MJ and ABA. Moreover, preincubation of GCPs with 1 μM K252a also led to the suppression of the pH change (Table IV), suggesting that a protein kinase is involved upstream of pH change in ABA and MJ signaling cascades.

Role of Calcium or Calmodulin in pH or ROS Production

Although changes in calcium, pH, and ROS are observed in response to hormonal signals, their interrelationship and the exact sequence of these events have not been clear. It may be argued that changes in pH or ROS production in guard cells are brought out by external/internal calcium. The pattern of pH change and ROS production were therefore assessed after modulating calcium, by the addition of either external calcium or EGTA. Added external calcium or EGTA did not affect ROS production (Table V), confirming the involvement of calcium downstream of ROS production. We noticed that ML-7, a Ca^{2+}-calmodulin (CaM) protein kinase inhibitor, was quite effective in reversing the stomatal closure caused by MJ but not that of ABA. Therefore, the effect of W7 (CaM antagonist) was checked. Again, W7 was effective in reversing the effect of MJ but not of ABA. Thus, a CaM-like domain appears to play a more active role in the case of MJ than that of ABA (Fig. 2, C and D).

Gork1 Mutant Is Insensitive to MJ

The guard cell outward K^+ channel GORK was for a long time suspected to be the main K^+ conductance supporting ion efflux during stomatal closure. The

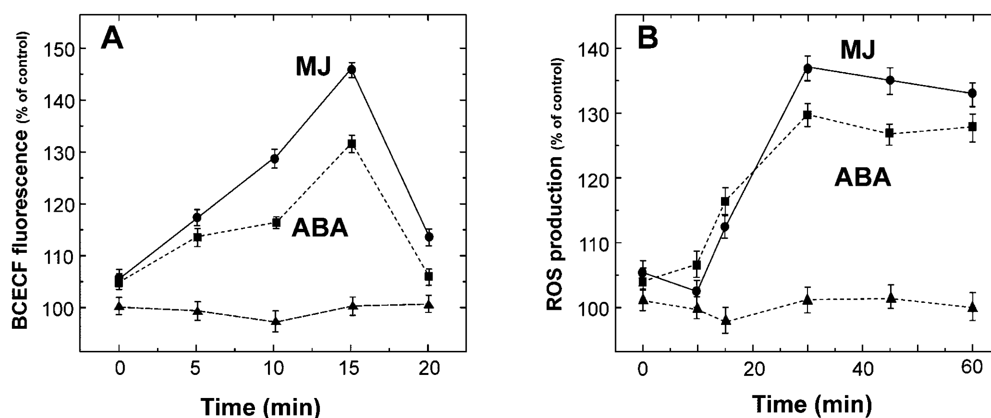

Figure 6. The pH changes and ROS production in guard cells of *P. sativum*. Changes in pH (A) or ROS (B) were monitored by using BCECF-AM or H_2DCF-DA after the addition of ABA (squares) and MJ (circles). The pixel intensities of fluorescence at each given point were determined and the relative changes in pH or ROS production were expressed by considering solvent control at zero time as the standard (100%). Each data point is the mean ± SE from at least 3 independent experiments. Note the different time scales in A and B.

Table III. *Reversal by butyrate of stomatal closure by ABA or MJ in guard cells of Arabidopsis wild-type and mutant plants*

Plant	Control		ABA (20 μM)		MJ (20 μM)	
	No Butyrate	0.5 mM Butyrate	No butyrate	0.5 mM Butyrate	No butyrate	0.5 mM Butyrate
	Stomatal aperture (μm)					
Wild type	4.12 ± 0.2	4.03 ± 0.5	1.09 ± 0.3	2.72 ± 0.2	0.98 ± 0.1	2.96 ± 0.2
jar1-1	3.41 ± 0.2	3.24 ± 0.7	2.54 ± 0.2	2.75 ± 0.2	3.5 ± 0.3	3.29 ± 0.1
ost1-2	3.87 ± 0.2	3.56 ± 0.4	3.59 ± 0.5	3.61 ± 0.1	1.83 ± 0.1	2.62 ± 0.3
gork1	4.19 ± 0.3	4.07 ± 0.2	2.76 ± 0.2	2.89 ± 0.3	4.22 ± 0.2	4.01 ± 0.4

Modulation of pH change in the presence of butyrate on wild type, *jar1*-1, *ost1*-2, and *gork1* with respect to stomatal closure in response to 20 μM ABA or MJ. The leaf epidermis were allowed to open in light for 2 h, then ABA or MJ or 0.5 mM butyrate was applied for 2 h. Results are the averages ±SE ($n = 60$) of 3 to 4 independent experiments.

molecular nature of this ion channel has been recently identified (Ache et al., 2000) and a GORK knockout mutant, *gork1*, characterized at the stomatal level (Hosy et al., 2003). In this mutant, the outward K^+ currents, generally observed upon membrane depolarization, are absent in guard cell protoplasts, and the mutant displays a limited stomatal closure in response to the stress hormone ABA compared to wild-type plants.

We have therefore examined stomatal responses to ABA and MJ in *gork1*. As previously observed, the *gork1* mutation led to a diminished response to ABA (Fig. 3B). Interestingly, stomatal closure in response to MJ was completely suppressed in the *gork1* mutant. A recent work from Evans (2003) has already described a MJ dose-dependent modulation of inward and outward rectifier K^+ channels at the guard cell plasma membrane. The results from our study suggest that GORK contributes to one of the conductances involved in K^+ efflux during ABA-induced stomatal closure while it is an essential element in MJ-induced ion efflux and stomatal closure. MJ did not induce stomatal closure but caused significant ROS production in guard cells of *gork1* (Table II).

DISCUSSION

ABA and MJ play a crucial role in plant adaptation to stress conditions. These two phytohormones inhibit root growth, limit transpiration, interfere with seed germination and cell cycle, and induce stomatal closure (Raghavendra and Reddy, 1987; Staswick et al., 1992; Wang, 1999; Swiatek et al., 2002). Considerable efforts have been devoted to identify signaling elements in the guard cell response to ABA, a fundamental process in drought resistance (Schroeder et al., 2001). In comparison, very few studies have focused on MJ signaling cascade leading to stomatal closure. It has been shown that MJ is produced during water stress (Creelman and Mullet, 1997) and that stomatal closure contributes to diminish the entry of certain pathogens in the leaf tissues (Agrios, 1997). Additionally, stomatal closure could limit plant growth and help redirect plant metabolism toward defense reactions. Previous studies have shown that some events in MJ- and ABA-signaling are similar, e.g. calcium requirement and protein (de)phosphorylation (Suhita et al., 2003), alkalization of the guard cell cytoplasm (Gehring et al., 1997), ROS production (Lee et al., 1999), and modulation of K^+ channels at the guard cell plasma membrane (Evans, 2003).

In the study presented here, we observed that the response to MJ was more sensitive to Ca^{2+}-calmodulin (CaM) protein kinase inhibitors than the stomatal response to ABA (Fig. 2). These inhibitors were able to reverse the response to MJ, while the response to ABA was only partially affected. These findings suggest that at least one protein kinase with a Ca^{2+}-CaM like regulatory domain plays an essential role in MJ response, while such activity appears to participate to a limited extent in the ABA cascade. In contrast, a broad range inhibitor of protein kinase (K252a) was able to suppress both responses, suggesting that Ca^{2+}-

Table IV. *The effect of butyrate and K252a on alkalization and ROS production by guard cells of Arabidopsis in response to ABA, MJ*

Hormone	Rise in pH (fmol H^+ GCP^{-1})			ROS Production (% of Control)		
	Control	+ 0.5 mM Butyrate	+ 1 μM K252a	Control	+ 0.5 mM Butyrate	+ 1 μM K252a
20 μM						
None	0.34 ± 0.01	0.26 ± 0.01	0.87 ± 1.2	100 ± 3.1	101 ± 3.5	101 ± 2.7
ABA	20.8 ± 1.21	8.6 ± 1.3	0.82 ± 2.1	122 ± 3.1	110 ± 3.5	105 ± 3.1
MJ	26.5 ± 1.15	10.1 ± 1.0	0.74 ± 1.4	128 ± 2.5	114 ± 3.7	103 ± 2.9

Changes in pH of external solution were recorded after permeation of guard cell plasma membrane by digitonin in control or hormone-treated protoplasts. The ROS production was monitored by using fluorescence dye, H_2DCF-DA, as described in "Materials and Methods". The extent of ROS production in guard cells, without MJ or ABA or butyrate or K252a (control), is taken as 100%. Results are averages (±SE) from at least 3 experiments.

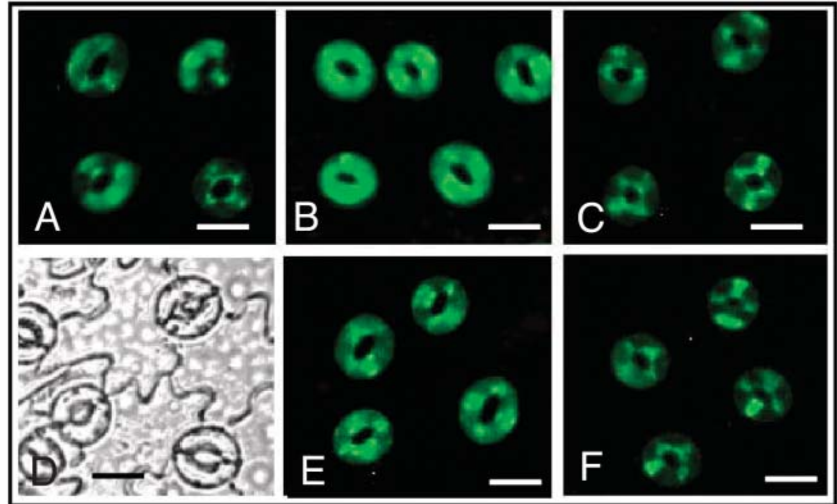

Figure 7. MJ- or ABA-induced H_2O_2 production in guard cells is inhibited by 0.5 mM butyrate. Photographs were taken from a representative lot of guard cells from epidermal strips loaded with H_2DCF-DA, untreated (A) or submitted to a 30-min pretreatment with 20 μM MJ (B), or 20 μM ABA (E). C and F, Effects of 0.5 mM butyrate on MJ- or ABA-induced H_2O_2 production, respectively. Photographs were taken using fluorescence (A–C, E, F) or light microscopy (D); bars represent 10 μm.

dependent and Ca^{2+}-independent protein kinases are involved parallely during the ABA signaling. Interestingly, K252a was able to suppress MJ- or ABA-induced pH changes and ROS production (Table IV), suggesting that a protein phosphorylation event is essential and located upstream of these responses. While the kinetics of ROS production and pH change in response to MJ or ABA were almost similar, the amplitude of responses was always higher with MJ than that with ABA. These results confirm the previous observations from Gehring et al. (1997), who used different techniques and species. Additionally, the limited responses of *jar1*-1 and *ost1*-2 allow placing JAR1 and OST1 upstream of cytoplasmic alkalization in the MJ- and ABA-signaling pathways, respectively.

The outward-rectifying K^+ channels appear to play an important role in stomatal closure. However, the reports on the regulation of these outward-rectifying K^+ channels are ambiguous. These channels were found to be down-regulated by H_2O_2 (Köhler et al.,

Table V. *The effect of calcium or EGTA on stomatal closure, of alkalization, and ROS production in response to ABA or MJ, in guard cells of P. sativum*

Phenomenon and Calcium Modulator	Hormone (20 μM)		
	None	ABA	MJ
Stomatal opening (μm)			
None	4.0 ± 0.5	1.9 ± 1.0	1.8 ± 0.7
10 μM $Ca(NO_3)_2$	2.8 ± 0.5	2.5 ± 1.1	2.0 ± 0.9
2 mM EGTA	4.8 ± 0.8	3.4 ± 1.2	4.4 ± 0.8
Change in pH (% Control)			
None	100 ± 2.7	120 ± 2.1	125 ± 3.1
10 μM $Ca(NO_3)_2$	99 ± 3.2	121 ± 2.8	124 ± 2.9
2 mM EGTA	100 ± 2.9	120 ± 3.2	124 ± 3.7
Change in ROS (% Control)			
None	100 ± 3.3	122 ± 2.8	130 ± 3.2
10 μM $Ca(NO_3)_2$	105 ± 2.8	126 ± 3.1	135 ± 3.0
2 mM EGTA	96 ± 3.4	118 ± 3.4	123 ± 3.7

Change in pH or ROS levels were analyzed by measuring BCECF-AM or H_2DCF-DA fluorescence in guard cells in response to ABA or MJ or solvent control. Stomata were allowed to open in light for 2 h, then ABA or MJ was applied. The cellular pH and ROS production were examined after 15 and 30 min, respectively. The fluorescence intensity of the dye in the control sets (without ABA or MJ or calcium or EGTA) is taken as 100 and other values were expressed in relation to control. Results are the averages ±SE of 3 to 4 independent experiments.

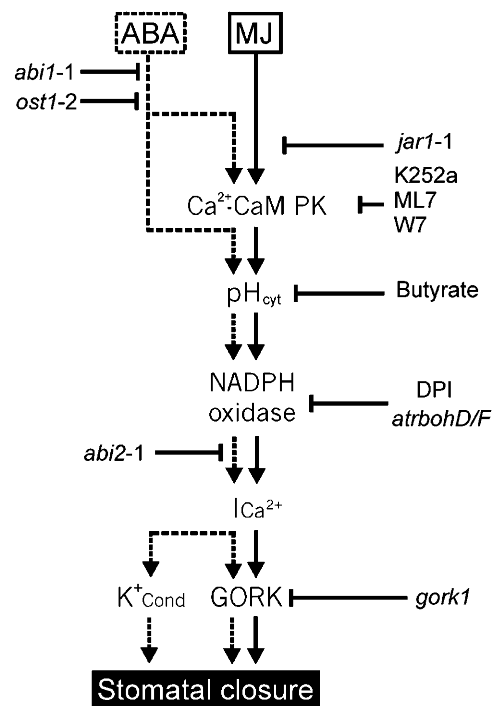

Figure 8. Model for the sequence of events in the MJ signaling cascade leading to stomatal closure. This linear model integrates our results from the different mutants and the use of inhibitors. Ca^{2+}-CaM PK, calcium-calmodulin activated protein kinase; Ica, calcium influx at the plasma membrane. *abi1* and *abi2* have been placed according to Murata et al. (2001) and Mustilli et al. (2002).

Table VI. *Responses of the three ecotypes of Arabidopsis to ABA or MJ*

Ecotype	Control	Hormone (20 μM)	
		ABA	MJ
Stomatal aperture (μm)			
Landsberg	3.9 ± 0.24	1.1 ± 0.21	1.2 ± 0.23
Columbia	3.8 ± 0.17	0.8 ± 0.25	1.0 ± 0.19
Wassilewskija	4.3 ± 0.21	1.2 ± 0.29	0.9 ± 0.13

The stomatal opening was measured as described in "Materials and Methods", in the absence or presence of 20 μM ABA or MJ. Results are averages ±SE of 3 to 4 independent experiments.

2003). However, a rise in cytoplasmic pH (which is expected to raise H_2O_2 levels) led to the up-regulation of these outward K^+ channels (Miedema and Assmann, 1996). In a recent study, Evans (2003) found that MJ down-regulated the outward K^+ channels. As per our observations, the *gork1* mutant, whose outward K^+ channels are impaired, was insensitive to MJ (Fig. 3B). Further, external addition of H_2O_2 caused marked stomatal closure in all the three mutants, including GORK. We suggest that GORK is one of the limiting elements during stomatal response to MJ and possible mechanisms could also be involved, for example modulation of influx of Suc or K^+.

An important point from our study is that the sequence of events signaling stomatal closure can be traced, which appears broadly similar for MJ and ABA. At least one protein phosphorylation event is necessary for the cytoplasmic alkalization, which leads to ROS production by the NAD(P)H oxidase. In turn, ROS would activate hyperpolarization-activated Ca^{2+} channels (Pei et al., 2000; Murata et al., 2001) and the resulting elevation of free cytoplasmic Ca^{2+} triggers plasma membrane anion channels leading to cell depolarization. Hedrich et al. (1990) showed R type anion channel activation by extracellular $CaCl_2$. Schroeder and Hagiwara (1989) showed S type anion channel activation by cytoplasmic Ca^{2+} elevation. ROS would also inhibit inward-rectifying K^+ channels (Köhler et al., 2003; Torsethaugen et al., 1999). Among the last steps in the cascade leading to stomatal closure is the activation of the outward K^+ rectifier from guard cells (Armstrong et al., 1995), allowing potassium efflux and loss of turgor. Thus, GORK appears to be an essential component in the MJ signaling cascade, leading to stomatal closure (Fig. 8).

CONCLUSION

The sequence of events occurring during stomatal closure is often debated. From the work presented here, using different mutants, a general scheme is proposed for stomatal response to MJ (Fig. 8). Comparative analysis of responses to MJ and ABA points out some specificity in the ABA cascade, which appears to involve parallel Ca^{2+}-dependent and Ca^{2+}-independent pathways and K^+-conductance(s) other than GORK. There are still many open questions, e.g. the nature of the component leading to cytoplasmic alkalization, the elements linking pH change and H_2O_2 production in guard cells. The role of calcium also appears complex. From our study, a Ca^{2+}-CaM protein kinase seems to be involved in a very early MJ signaling, whereas H_2O_2 production occurs downstream of phosphorylation events that activate Ca^{2+}-channels at the plasma membrane leading to Ca^{2+} elevation in the cytoplasm. Thus, change in cytoplasmic free calcium could be an important signaling event involved in multiple steps of the signaling pathway leading to complex kinetics (Allen et al., 2000). Future studies will have to focus on these crucial points of stomatal physiology.

MATERIALS AND METHODS

Plant Material and Culture Conditions

The Arabidopsis Landsberg *erecta*, *ost1-2* mutant plants (accession Landsberg, Mustilli et al., 2002), *jar1-1* mutant plants (accession Columbia, Staswick et al., 1992), *atrbohD/F* mutant plants (accession Columbia, Torres et al., 2002), and *gork1* mutant plants (accession Wassilewskija, Hosy et al., 2003) were grown on sand in hydroponic conditions in a growth chamber (8 h light, 300 μmol m^{-2} s^{-1}, 70% relative humidity, 22°C; 16 h darkness, 75% relative humidity, 20°C) for 4 to 5 weeks. Plants were watered four times a day with a half-strength Hoagland solution. Most of the experiments were performed with Arabidopsis at CEA de Cadarache, DEVM/LEMS France.

Additional experiments were conducted with another species, *Pisum sativum* cv Arkel at Department of Plant Sciences, University of Hyderabad, India. Seedlings were grown in plastic trays filled with soil and farmyard manure (3:1, v/v). Plants were grown outdoors under natural photoperiod of approximately 12 h and average daily temperature of 30°C day/20°C night. The first and second fully expanded leaves were picked from 8- to 10-d-old plants.

Stomatal Aperture

Leaves from 4- to 5-week-old plants were harvested at the end of the night. Paradermal sections of abaxial epidermis were incubated in 30 mM KCl, 10 mM MES-KOH, pH 6.5, at 22°C. As indicated, ABA, MJ, K252a, ML7, W7, and other effectors were added to the solution. Stomatal apertures were measured with an optical microscope (Optiphot-2, Nikon, Tokyo) fitted with a camera lucida and a digitizing table (Houston Instrument TG 1017, Austin, TX) linked to a personal computer. For each treatment, at least 60 stomatal apertures were measured; each experiment was at least repeated thrice. It was ascertained that the three ecotypes of Arabidopsis used in this study have similar responses to ABA and MJ at the stomatal level (Table VI).

Guard Cell Protoplasts

GCPs were prepared essentially as reported by Pandey et al. (2002). They were then washed twice in 550 mM mannitol, 10 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM MES-NaOH, pH 7. For kinetic studies, GCPs were incubated in the same solution in the presence of ABA, MJ, or methanol solvent control for the indicated time at room temperature (around 22°C).

Fluorescent Dyes to Monitor ROS and pH

Hydrogen peroxide production in guard cells of Arabidopsis or *P. sativum* was monitored by using H_2DCF-DA, as previously described (Murata et al., 2001). Epidermal leaf peels were mounted on a microscope slide with medical adhesive (Hollister, Libertyville, IL). Epidermal tissues were incubated for 3 h

in 30 mM KCl and 10 mM MES-KOH, pH 6.5. The dye H_2DCF-DA (30 μM) was added to the incubation medium. After 20 min, the excess of dye was removed by three washes with distilled water. When used, DPI (12.5 μM) was added 30 min before the dye to the epidermal strips. Epidermal tissues were then incubated for the indicated time with 20 μM ABA or MJ with an equal volume of methanol added to the control.

Changes in pH were examined in epidermis of *P. sativum* by incubation with 2′,7′-bis(2-carboxy-ethyl)-5(6)-carboxyfluorescein-acetoxymethylester (BCECF-AM) as described earlier by Irving et al. (1992). The epidermal tissues were incubated for 3 h with 50 mM KCl and 10 mM MES-KOH, pH 6.5 in light (350–450 μmol m^{-2} s^{-1}). The strips were then treated with 20 μM BCECF-AM for 30 min in darkness. The strips were rinsed several times in incubation buffer so as to remove the excess dye. The epidermal tissues were then treated with 20 μM ABA or MJ (or methanol in the control) and examined under the fluorescent microscope.

Guard cells were then observed either with an epifluorescence microscope (Optiphot-2) fitted with a CCD camera (AxioCam, Zeiss, Gottingen, Germany) for the ROS fluorescence or with a fluorescence microscope fitted with camera (Eclipse TE 200, Niokon, Tokyo; Coolsnap CF, Photometrics, Roper Scientific, Tucson, AZ) for studies of pH change with BCECF-AM (20 μM) fluorescence. Images were captured and the relative fluorescence emission of guard cells was analyzed using the NIH software, as previously described in Murata et al. (2001).

Measurements of Cytoplasmic pH by the Null-Point Method

The null-point method used for barley aleurone protoplasts, as described in Van der Veen et al. (1992), was adapted for Arabidopsis guard cell protoplasts. Briefly, 10^6 GCPs were placed in a weakly buffered medium (0.5 mM MES, 10 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 500 mM mannitol, pH 7). The suspension was continuously stirred with a magnetic flea at low-speed magnetic stirring to avoid protoplast damage. GCPs were incubated with MJ or ABA 20 μM at room temperature. The $[pH]_{Ext}$ was adjusted to the required value with diluted KOH. Subsequently, digitonin (0.01%, w/v) was added to permeate the protoplast plasma membrane. The resulting pH changes in the external solution were recorded with a combined pH electrode (Ingold 104023522, Wilmington, MA) coupled to a pH-meter (pHM85, Radiometer Copenhagen, Copenhagen). Buffering capacity of the solution was determined by adding 10 nmol of HCl at the end of each experiment.

Chemicals

Chemicals were purchased from Sigma (St. Louis); Cellulase R10, Cellulase RS, and Pectolyase Y-23 from Sheishin Corporation (Tokyo); W7 and protein kinase inhibitors from BIOMOL (Plymouth, PA); and BCECF-AM from Molecular Probes (Juro, Switzerland).

ACKNOWLEDGMENTS

We thank Drs. Elena Marin and Nathalie Leonhardt for their help in guard cell protoplast preparations and critical reading of the manuscript. The Arabidopsis mutants were generous gifts from Dr. Hervé Sentenac (*gork1*, ENSAM, Montpellier, France) and Dr. Jérôme Giraudat (*ost1*-2, ISV, Gif sur Yvette, France). We thank Julian Schroeder for communicating findings on *AtrbohD* and *AtrbohF* gene functions in ABA signaling prior to publication (supported by NIH grant no. GM60396 to J.S.) and for providing the *atrbohD/F* double mutant seeds.

Received August 26, 2003; returned for revision January 7, 2004; accepted January 8, 2004.

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