Plant Cell Physiol. 49(9): 1396–1401 (2008)
doi:10.1093/pcp/pcn106, available online at www.pcp.oxfordjournals.org

Short Communication

Roles of RCN1, Regulatory A Subunit of Protein Phosphatase 2A, in Methyl Jasmonate Signaling and Signal Crosstalk between Methyl Jasmonate and Abscisic Acid

Naoki Saito [1], Shintaro Munemasa [1], Yoshimasa Nakamura [1], Yasuaki Shimoishi [1], Izumi C. Mori [2] and Yoshiyuki Murata [1,*]

[1] *Graduate School of Natural Science and Technology, Okayama University, 3-1-1 Tsushima-naka, Okayama 700-8530, Japan*
[2] *Research Institute for Bioresources, Okayama University, Kurashiki, Okayama 710-0046, Japan*

Methyl jasmonate (MeJA) as well as abscisic acid (ABA) induces stomatal closure with their signal crosstalk. We investigated the function of a regulatory A subunit of protein phosphatase 2A, RCN1, in MeJA signaling. Both MeJA and ABA failed to induce stomatal closure in *Arabidopsis rcn1* knockout mutants unlike in wild-type plants. Neither MeJA nor ABA induced reactive oxygen species (ROS) production and suppressed inward-rectifying potassium channel activities in *rcn1* mutants but not in wild-type plants. These results suggest that RCN1 functions upstream of ROS production and downstream of the branch point of MeJA signaling and ABA signaling in *Arabidopsis* guard cells.

Keywords: Abscisic acid — *Arabidopsis thaliana* — Guard cells — K^+ channel — Methyl jasmonate — Reactive oxygen species.

Abbreviations: $[Ca^{2+}]_{cyt}$, cytosolic calcium concentration; I_{Kin}, inward-rectifying K^+ channel currents; MeJA, methyl jasmonate; OA, okadaic acid; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; ROS, reactive oxygen species.

Stomatal pores that are formed by pairs of guard cells respond to various environmental stimuli, including phytohormones and elicitors. Methyl jasmonate (MeJA), which mediates various plant defense responses (Liechti and Farmer 2002, Turner et al. 2002), has been reported to induce stomatal closure (Gehring et al. 1997, Suhita et al. 2003, Suhita et al. 2004). It has been suggested that reactive oxygen species (ROS) function as a second messenger in abscisic acid (ABA) and MeJA signaling cascade in guard cells (Suhita et al. 2004, Bright et al. 2006, Munemasa et al. 2007).

NAD(P)H oxidase-mediated ROS production is necessary for activation of calcium permeable non-selective cation channels (I_{Ca} channels) by ABA (Pei et al. 2000, Murata et al. 2001, Kwak et al. 2003). Elevation of cytosolic free calcium concentration ($[Ca^{2+}]_{cyt}$) is involved in ABA-induced stomatal closure via calcium-dependent protein kinases (CDPK), CPK3, CPK6, CPK4 and CPK11 (Mori et al. 2006, Zhu et al. 2007). It has also been shown that ROS mediate activation of I_{Ca} channels in MeJA signaling as well as ABA signaling and that both MeJA and ABA elicit S-type anion currents and NO production in guard cells (Munemasa et al. 2007). Previous studies suggest a crosstalk of MeJA and ABA signaling (Suhita et al. 2004, Munemasa et al. 2007).

Arabidopsis rcn1 protein phosphatase 2A (PP2A) A subunit knockout mutant is impaired in ABA-induced stomatal closure, calcium oscillation and activation of S-type anion channels in guard cells, whereas hydrogen peroxide (H_2O_2) successfully induces stomatal closure (Kwak et al. 2002). These results suggest that *rcn1* mutation disrupts upstream of ROS production in the ABA signaling cascade. It is as yet unclear whether *rcn1* mutation impairs ROS production induced by ABA. In contrast to *rcn1* mutant, *pp2ac-2* mutant, which is impaired in PP2A catalytic subunit, is hypersensitive to ABA (Pernas et al. 2007). These results suggest that RCN1 could not regulate PP2AC-2 activity. Furthermore, the function of RCN1 in MeJA signaling remains to be clarified.

It has been demonstrated that activation of inward-rectifying K^+ channel currents (I_{Kin}) in the plasma membrane of guard cells is favorable for stomatal opening (Kwak et al. 2001) and suppression of I_{Kin} is favorable for stomatal closure (Schroeder and Hagiwara 1989, Blatt et al. 1990, McAinsh et al. 1990). Recently, it was demonstrated that MeJA suppressed I_{Kin} and activated outward potassium currents (I_{Kout}) in guard cell protoplasts (GCPs) of *Vicia faba* (Evans 2003). However, no study has elucidated whether MeJA regulates I_{Kin} in *Arabidopsis* guard cells.

*Corresponding author: E-mail, muta@cc.okayama-u.ac.jp; Fax, +81-86-251-8310.

In this paper, we used the ABA-insensitive mutant, *rcn1* (Kwak et al. 2002), in order to elucidate roles of RCN1 in MeJA signaling and to shed light on the signaling crosstalk between MeJA and ABA in guard cells. We examined production of ROS, suppression of I_{Kin} and closure of stomata induced by MeJA. We also investigated effect of okadaic acid (OA), the protein phosphatase 1 (PP1) and PP2A inhibitor, on MeJA-induced stomatal closure and ROS production.

To clarify the involvement of the regulatory subunit of PP2A, RCN1, in MeJA signaling in guard cells, we examined stomatal movements of the known ABA-insensitive mutant, *rcn1* (Fig. 1A and B). ABA-induced stomatal closure was impaired in *rcn1* mutant as previously reported (Kwak et al. 2002). Similarly, MeJA-induced stomatal closure was also impaired in *rcn1* mutant, but not in wild type, suggesting that RCN1 functions in MeJA signal cascade in guard cells.

Kwak et al. (2002) demonstrated that RCN1 functions as a positive transducer of ABA signaling in guard cells. As previously reported by Kwak et al. (2002) did not provide evidence that ABA fails to induce ROS production in guard cells of *rcn1* mutants. Several parallel signaling pathways, including ROS-dependent pathway exists in ABA signaling in guard cells (Pei et al. 2000). It has not been clarified which signaling branch RCN1 functions in.

In order to elucidate role of RCN1 in MeJA and ABA signaling pathways in guard cells, we examined effects of the *rcn1* mutation on ROS production induced by MeJA or ABA using the ROS-detection fluorescence dye, 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) (Fig. 1C and D). MeJA ($P<0.03$) as well as ABA ($P<0.001$) induced ROS production in wild-type guard cells, which is consistent with the previous reports (Pei et al. 2000, Murata et al. 2001, Munemasa et al. 2007). Neither MeJA nor ABA promoted ROS production in *rcn1* guard cells unlike wild-type guard cells. Our results confirmed that RCN1 functions upstream of ROS production.

We examined the involvement of RCN1-associated PP2A in MeJA signaling cascade in guard cells by

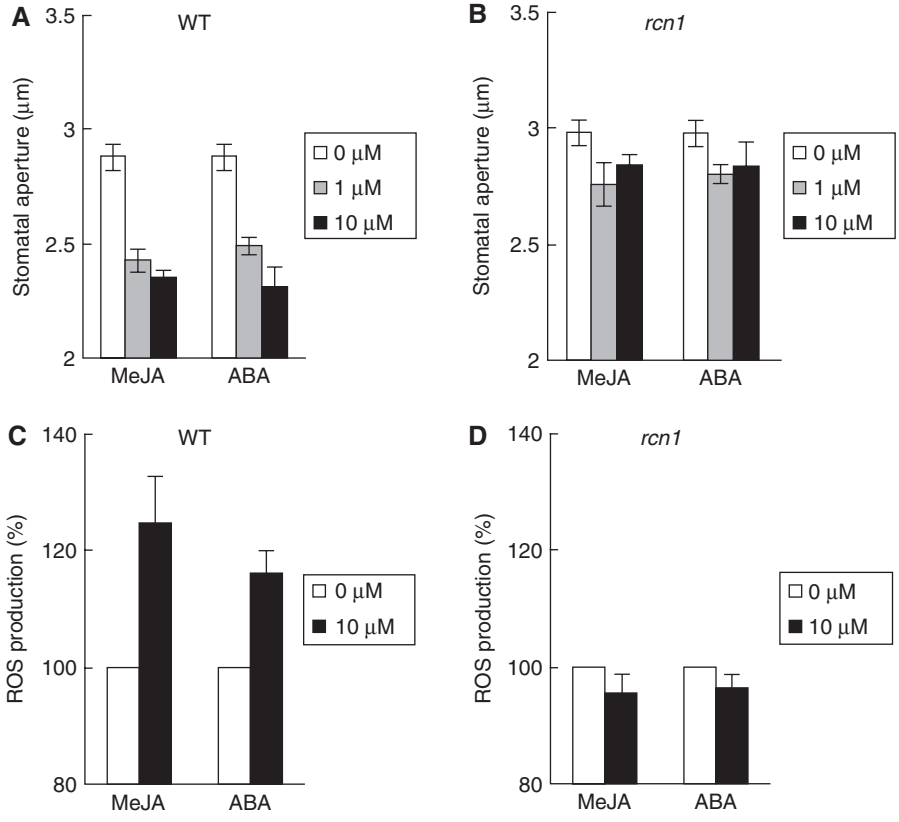

Fig. 1 Impairment of MeJA-induced stomatal closure and ROS production in guard cells of *rcn1* mutant. (A) Effects of MeJA ($n=4$) and ABA ($n=3$) on stomatal closure in wild-type guard cells. (B) Effects of MeJA ($n=4$) and ABA ($n=3$) on stomatal closure in *rcn1* guard cells. (C) Effects of MeJA ($n=4$) and ABA ($n=7$) on ROS production in wild-type guard cells. (D) Effects of MeJA ($n=4$) and ABA ($n=4$) on ROS production in *rcn1* guard cells. In (C) and (D), the vertical scale represents the percentage of H_2DCF-DA fluorescent levels when fluorescent intensities of MeJA- or ABA-treated cells are normalized to control value taken as 100% for each experiment. Data were obtained from at least 60 guard cells. Error bars represent standard errors.

pharmacological analysis, using a PP1 and PP2A inhibitor, OA. First, we investigated the effect of OA on MeJA-induced stomatal closure (Fig. 2A). ABA-induced stomatal closure was inhibited by OA ($P<0.03$), which is consistent with the previous results (Pei et al. 1997). OA also inhibited the MeJA-induced stomatal closure ($P<0.03$). The results suggest that RCN1-associated PP2A is involved in MeJA-induced stomatal closure as well as ABA-induced stomatal closure.

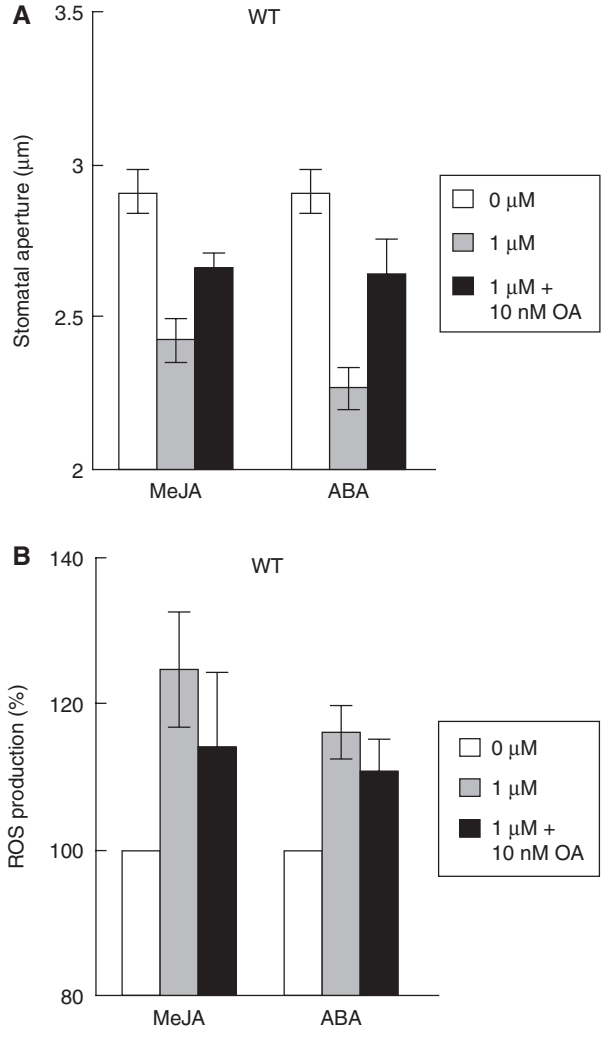

Fig. 2 Inhibition of MeJA-induced stomatal closure and ROS production by OA in guard cells of wild-type plants. (A) Effects of OA on ABA- and MeJA-induced stomatal closure in wild-type guard cells. Rosette leaves of wild-type plants were treated with 10 nM OA for 30 min. Then, rosette leaves pretreated with OA were treated with 1 μM MeJA ($n=6$) or 1 μM ABA ($n=4$). (B) Effects of OA on ABA- and MeJA-induced ROS production in wild-type guard cells. H_2DCF-DA-loaded epidermal tissues of wild-type plants were treated with 10 nM OA for 20 min. Then, epidermal tissues pretreated with OA were treated with 10 μM MeJA ($n=4$) or 10 μM ABA ($n=7$). Data were obtained from at least 60 guard cells. Error bars represent standard errors.

We accessed the effect of OA on MeJA-induced ROS production (Fig. 2B). In the presence of 10 nM OA, neither MeJA nor ABA significantly induced ROS production. These results indicate that RCN1 functions upstream of ROS production as a positive transducer of MeJA signaling in guard cells.

Pharmacological data using OA (Fig. 2) was consistent with the results obtained using *rcn1* mutants (Fig. 1). Both genetic and pharmacological evidence shows that RCN1 positively regulates MeJA as well as ABA signaling cascade in Arabidopsis guard cells.

ABA suppresses I_{Kin}, which is favorable for ABA-induced stomatal closure (Schroeder and Hagiwara 1989, Blatt et al. 1990, McAinsh et al. 1990). We examined the effect of MeJA on I_{Kin} to compare with the effect of ABA (Fig. 3). A whole-cell patch-clamp study demonstrated that ABA ($P<0.01$ at -180 mV) significantly reduced I_{Kin} in *Arabidopsis* guard cell protoplasts (GCPs), as reported in *Vicia faba* (Lemtiri-Chlieh and MacRobbie 1994). MeJA also significantly suppressed I_{Kin} in GCPs ($P<0.03$ at -180 mV). In accordance with stomatal closure and ROS generation results, neither MeJA nor ABA suppressed I_{Kin} in *rcn1* GCPs.

Our previous results show that MeJA activates I_{Ca} channels following ROS production and elicits S-type anion currents in *Arabidopsis* guard cells (Munemasa et al. 2007). A patch-clamp study with Ca^{2+} imaging shows that activation of I_{Ca} channels mediates elevation of $[Ca^{2+}]_{cyt}$ (Pei et al. 2000).

In this study, MeJA suppressed I_{Kin} of guard cell protoplasts in the same way as ABA (Fig. 3). Our present and previous study (Munemasa et al. 2007) demonstrated that MeJA induces ROS production and activates I_{Ca} currents in guard cells as well as ABA, suggesting elevation of $[Ca^{2+}]_{cyt}$ is presumably induced in response to MeJA in guard cells. Elevation of $[Ca^{2+}]_{cyt}$ was reported to suppress I_{Kin} in the plasma membrane of guard cells (Schroeder and Hagiwara 1989). These results suggest that MeJA and ABA could modulate I_{Kin} via elevation of $[Ca^{2+}]_{cyt}$ in both MeJA and ABA signaling in guard cells.

KAT1 mediates plasma membrane I_{Kin} in *Arabidopsis* guard cells (Schachtman et al. 1992, Ichida et al. 1997, Kwak et al. 2001). ABA-activated protein kinase (AAPK) in *Vicia* guard cells is activated by another protein kinase (Furuichi et al. 2005) and in turn phosphorylates KAT1 (Mori et al. 2000). MeJA might also reduce I_{Kin} via AAPK activation. However, it remains to be clarified whether MeJA activates AAPK or OST1 kinase, the AAPK ortholog of *Arabidopsis thaliana* (Mustilli et al. 2002), and inactivates I_{Kin} via phosphorylation of inward-rectifying K^+ channels of guard cells.

We propose a simple model of MeJA and ABA signaling in guard cells (Fig. 4). RCN1, ROS production

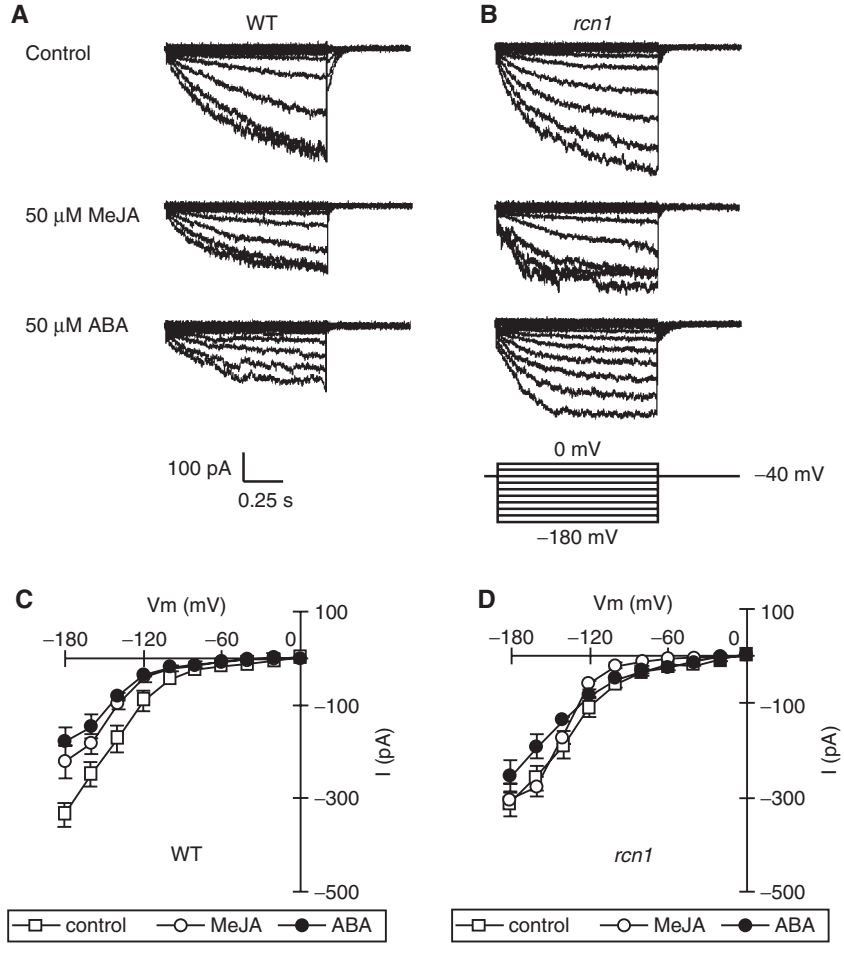

Fig. 3 Suppression of I_{Kin} by MeJA. (A) Whole-cell recordings of I_{Kin} in wild-type GCPs treated with no hormones (top trace), 50 μM MeJA (middle trace), or 50 μM ABA (bottom trace). (B) Whole-cell recordings of I_{Kin} in *rcn1* GCPs treated with no hormones (top trace), 50 μM MeJA (middle trace), or 50 μM ABA (bottom trace). (C) Steady-state current-voltage relationships for MeJA ($n=4$) and ABA ($n=3$) activation of I_{Kin} in wild-type GCPs as recorded in A. (D) Steady-state current-voltage relationships for MeJA ($n=3$) and ABA ($n=5$) activation of I_{Kin} in *rcn1* GCPs as recorded in B. The voltage protocol was stepped up from 0 mV to –180 mV in 20 mV decrements (holding potential, –40 mV). GCPs were treated with 50 μM MeJA or 50 μM ABA for 2 h before recordings. Error bars represent standard errors.

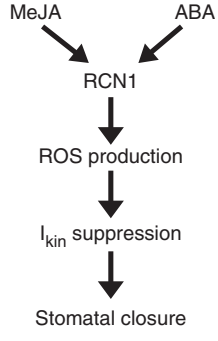

Fig. 4 A model of signal crosstalk in MeJA- and ABA-induced stomatal closure. Neither MeJA nor ABA induces ROS production, I_{Kin} and stomatal closure in *rcn1* mutant. These results suggest that RCN1 functions as a positive transducer upstream of ROS production and downstream of the branch point of MeJA signaling and ABA signaling in *Arabidopsis* guard cells.

and I_{Kin} play roles in both MeJA and ABA hormone signal cascade in the model. RCN1 functions upstream of ROS production and downstream of the blanching point of MeJA and ABA signaling. Our previous results demonstrated that MeJA as well as ABA induced ROS production and NO production, and activated I_{Ca} and S-type anion channels. Taken together, MeJA signal cascade and ABA signal cascade could share signal components downstream of ROS and NO production. In addition, the RCN1-associated PP2A is likely to be different from the PP2A reported by Pernas et al. (2007).

Materials and Methods

Throughout this study we used the *Arabidopsis* ecotype Wassilewskija (WS) as the wild-type plant. WS and the *rcn1*

mutant (WS accession) were grown in growth chambers (22°C, 80 mmol·m^{-2}·s^{-1} under a 12-h light/12-h dark regime).

Stomatal aperture measurements were performed as described previously (Pei et al. 1997, Murata et al. 2001, Munemasa et al. 2007). Excised rosette leaves were floated on medium containing 5 mM KCl, 50 mM $CaCl_2$, and 10 mM MES-Tris (pH 6.15) for 2 h in the light (80 mmol·m^{-2}·s^{-1}) to induce stomatal opening, followed by the addition of MeJA or ABA. After 2 h incubation stomatal apertures were measured. Leaves were blended for 30 s and epidermal peels were collected. Twenty stomatal apertures were measured on each epidermal peel.

ROS production in guard cells was analyzed by using H_2DCF-DA (Lee et al. 1999, Murata et al. 2001, Suhita et al. 2004, Munemasa et al. 2007). Epidermal peels were first incubated for 3 h in the medium containing 5 mM KCl, 50 mM $CaCl_2$ and 10 mM MES-Tris (pH 6.15), and then for 20 min with 50 mM H_2DCF-DA in the medium at room temperature. After the incubation, the peels were washed with distilled water to remove excess dye. The dye-loaded tissues were treated with 10 mM MeJA or 10 mM ABA for 20 min and then DCF fluorescence was imaged and analyzed using AQUA COSMOS software (Hamamatsu Photonics, Hamamatsu, Japan).

For whole-cell patch-clamp recordings of I_{Kin}, *Arabidopsis* guard cell protoplasts were prepared from rosette leaves of 4- to 6-week-old plants with the digestion solution containing 1.0% Cellulase R10, 0.5% Macerozyme R10, 0.5% bovine serum albumin, 0.1% kanamycin, 10 mM ascorbic acid, 0.1 mM KCl, 0.1 mM $CaCl_2$, and 500 mM D-mannitol (pH 5.5 with KOH) (Pei et al. 1997, Kwak et al. 2001). Whole-cell currents were recorded using a CEZ-2400 patch-clamp amplifier (Nihon Kohden, Tokyo, Japan). The resulting values were corrected for liquid junction potential and leak currents were not subtracted. For data analysis, pCLAMP 6 software (Molecular Devices, Sunnyvale, CA) was used. The patch-clamp solutions contained 30 mM KCl, 70 mM K-Glu, 2 mM $MgCl_2$, 6.7 mM EGTA, 3.35 mM $CaCl_2$, 5 mM ATP, and 10 mM HEPES-Tris, (pH 7.1) in the pipette and 30 mM KCl, 40 mM $CaCl_2$, 2 mM $MgCl_2$, and 10 mM MES-Tris, (pH 5.5) in the bath. Osmolarity was adjusted to 500 mmol/kg (pipette solution) and 485 mmol/kg (bath solution) with D-sorbitol. Guard cell protoplasts were treated with 50 mM MeJA or 50 mM ABA for 2 h before recordings.

Significance of differences between data sets was assessed by Student's *t*-test analysis in all parts of this paper. We regarded differences at the level of $P < 0.05$ as significant.

Arabidopsis Genome Initiative number for the gene (*RCN1*) discussed in this article is AT1G25490.

Acknowledgements

This research was supported in part by The Asahi Glass Foundation, NOVARTIS Foundation (Japan) for the Promotion of Science, and Grant-in-Aid for Young Scientists and Grants for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

Blatt, M.R. (1990) Potassium channel currents in intact stomatal guard cells: rapid enhancement by abscisic acid. *Planta* 180: 445–455.

Bright, J., Desikan, R., Hancock, J.T., Weir, I.S. and Neill, S.J. (2006) ABA-induced NO generation and stomatal closure in Arabidopsis are dependent on H_2O_2 synthesis. *Plant J*. 45: 113–122.

Evans, N.H. (2003) Modulation of guard cell plasma membrane potassium currents by methyl jasmonate. *Plant Physiol*. 131: 8–11.

Furuichi, T., Mori, I.C. and Muto, S. (2005) Protein kinase cascade involved in rapid ABA-signaling in guard cells of Vicia faba. *Z. Naturforsch*. 60c: 769–773.

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.

Ichida, A.M., Pei, Z.M., Baizabal-Aguirre, V.M., Turner, K.J. and Schroeder, J.I. (1997) Expression of a Cs^+-resistant guard cell K^+ channel confers Cs^+-resistant, light-induced stomatal opening in transgenic Arabidopsis. *Plant Cell* 9: 1843–1857.

Kwak, J.M., Murata, Y., Baizabal-Aguirre, V.M., Merrill, J., Wang, M., Kemper, A., Hawke, S.D., Tallman, G. and Schroeder, J.I. (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.

Kwak, J.M., Moon, J.H., Murata, Y., Kuchitsu, K., Leonhardt, N., DeLong, A. and Schroeder, J.I. (2002) Disruption of a guard cell-expressed protein phosphatase 2A regulatory subunit, RCN1, confers abscisic acid insensitivity in Arabidopsis. *Plant Cell* 14: 2849–2861.

Kwak, J.M., Mori, I.C., Pei, Z.M., Leonhardt, N., Torres, M.A., Dangl, J.L., Bloom, R.E., Bodde, S., Jones, J.D.G. and Schroeder, J.I. (2003) NADPH oxidase AtrbohD and AtrbohF genes function in ROS-dependent ABA signaling in Arabidopsis. *EMBO J*. 22: 2623–2633.

Lee, S., Choi, H., Suh, S., Doo, I.-S., Oh, K.-Y., Choi, E.J., Taylor, S.A.T., Low, P.S. and Lee, Y. (1999) Oligogalacturonic acid and chitosan reduce stomatal aperture by inducing the evolution of reaction oxygen species from guard cells of tomato and *Commelina communis*. *Plant Physiol*. 121: 147–152.

Lemtiri-Chlieh, F. and MacRobbie, E.A. (1994) Role of calcium in the modulation of *Vicia* guard cell potassium channels by abscisic acid: a patch-clamp study. *J. Membr. Biol*. 137: 99–107.

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

McAinsh, M.R., Brownlee, C. and Hetherington, A.M. (1990) Abscisic acid-induced elevation of guard cell cytosolic Ca^{2+} precedes stomatal closure. *Nature* 343: 186–188.

Mori, I.C., Uozumi, N. and Muto, S. (2000) Phosphorylation of the inward-rectifying potassium channel KAT1 by ABR kinase in Vicia guard cells. *Plant Cell Physiol*. 41: 850–856.

Mori, I.C., Murata, Y., Yang, Y., Munemasa, S., Wang, Y.F. et al. (2006) CDPKs CPK6 and CPK3 function in ABA regulation of guard cell S-type anion- and Ca^{2+}-permeable channels and stomatal closure. *PLoS Biol*. 4: 1749–1762.

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., Pei, Z.M., Mori, I.C. and Schroeder, J.I. (2001) Abscisic acid activation of plasma membrane Ca^{2+} channels in guard cells requires cytosolic NAD(P)H and is differentially disrupted upstream and downstream of reactive oxygen species production in abi1-1 and abi2-1 protein phosphatase 2C mutants. *Plant Cell* 13: 2513–2523.

Mustilli, A.C., 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.

Pernas, M., Garcia-Casado, G., Rojo, E., Solano, R. and Sanchez-Serrano, J.J. (2007) A protein phosphatase 2A catalytic subunit is a negative regulator of abscisic acid signaling. *Plant J*. 51: 763–778.

Pei, Z.M., Kuchitsu, K., Ward, J.M., Schwarz, M. and Schroeder, J.I. (1997) Differential abscisic acid regulation of guard cell slow anion channels in Arabidopsis wild-type and abi1 and abi2 mutants. *Plant Cell* 9: 409–423.

Pei, Z.M., Murata, Y., Benning, G., Thomine, T., Klusener, B., Allen, G.A., Grill, E. and Schroeder, J.I. (2000) Calcium channels activated by hydrogen peroxide mediate abscisic acid signalling in guard cells. *Nature* 406: 731–734.

Schachtman, D.P., Schroeder, J.I., Lucas, W.J., Anderson, J.A. and Gaber, R.F. (1992) Expression of an inward-rectifying potassium channel by the Arabidopsis KAT1 cDNA. *Science* 258: 1654–1658.

Schroeder, J.I. and Hagiwara, S. (1989) Cytosolic calcium regulates ion channels in the plasma membrane of *Vicia faba* guard cells. *Nature* 338: 427–430.

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.

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

Zhu, S.-Y., Yu, X.-C., Wang, X.-J., Zhao, R., Li, Y. et al. (2007) Two calcium-dependent protein kinases, CPK4 and CPK11, regulate abscisic acid signal transduction in Arabidopsis. *Plant Cell* 19: 3019–3036.

(Received June 11, 2008; Accepted July 18, 2008)