Distinct Abscisic Acid Signaling Pathways for Modulation of Guard Cell versus Mesophyll Cell Potassium Channels Revealed by Expression Studies in *Xenopus laevis* Oocytes[1]

Fedora Sutton*, Sunil S. Paul[2], Xi-Qing Wang, and Sarah M. Assmann

Plant Science Department, South Dakota State University, Box 2108, Brookings, South Dakota 57007 (F.S., S.S.P.); and Biology Department, Pennsylvania State University, 208 Mueller Laboratory, University Park, Pennsylvania 16802 (X.-Q.W., S.M.A.)

Regulation of guard cell ion transport by abscisic acid (ABA) and in particular ABA inhibition of a guard cell inward K^+ current (I_{Kin}) is well documented. However, little is known concerning ABA effects on ion transport in other plant cell types. Here we applied patch clamp techniques to mesophyll cell protoplasts of fava bean (*Vicia faba* cv Long Pod) plants and demonstrated ABA inhibition of an outward K^+ current (I_{Kout}). When mesophyll cell protoplast mRNA (mesophyll mRNA) was expressed in *Xenopus laevis* oocytes, I_{Kout} was generated that displayed similar properties to I_{Kout} observed from direct analysis of mesophyll cell protoplasts. I_{Kout} expressed by mesophyll mRNA-injected oocytes was inhibited by ABA, indicating that the ABA signal transduction pathway observed in mesophyll cells was preserved in the frog oocytes. Co-injection of oocytes with guard cell protoplast mRNA and cRNA for KAT1, an inward K^+ channel expressed in guard cells, resulted in I_{Kin} that was similarly inhibited by ABA. However, oocytes co-injected with mesophyll mRNA and KAT1 cRNA produced I_{Kin} that was not inhibited by ABA. These results demonstrate that the mesophyll-encoded signaling mechanism could not substitute for the guard cell pathway. These findings indicate that mesophyll cells and guard cells use distinct and different receptor types and/or signal transduction pathways in ABA regulation of K^+ channels.

K^+ plays a significant role in plant cells. This role has been best studied in guard cells where transmembrane K^+ fluxes regulate cell volume, stomatal aperture, and thus gas exchange across the leaf. However, in all cell types, K^+ plays a major role in osmoregulation. Therefore, plasma membrane K^+ channels (for review, see Cherel et al., 1996; Maathuis et al., 1997) and their control are of importance in understanding this fundamental process. Inwardly rectifying plant K^+-channel cDNA clones have been isolated. The first, *AKT1* and *KAT1*, were isolated from Arabidopsis (Anderson et al., 1992; Sentenac et al., 1992) and characterized by functional expression in *Xenopus laevies* oocytes or yeast (Anderson et al., 1992; Sentenac et al., 1992; Bertl et al., 1994; Schachtman et al., 1994). *KAT1* is expressed primarily in guard cells, whereas *AKT1* is expressed in roots (Cao et al., 1995). Other cDNA clones encoding inwardly rectifying K^+ channels have been isolated from Arabidopsis and potato (Cao et al., 1995; Muller-Rober et al., 1995; Ketchum and Slayman, 1996).

Two cDNA clones, *KCO1* (Czempinski et al., 1997) and *SKOR* (Gaymard et al., 1998), encoding voltage activated outwardly rectifying K^+ channels, have been isolated from Arabidopsis. Properties of KCO1 (Czempinski et al., 1997) were determined by expression studies in baculovirus-infected insect cells (Czempinski et al., 1997). SKOR was characterized by expression in *X. laevis* oocytes (Gaymard et al., 1998). In contrast to the limited molecular characterization of plant outwardly rectifying K^+ channels, numerous studies describe the electrophysiological characteristics of outwardly rectifying K^+ currents (I_{Kout}) in isolated plant protoplasts. Studies of ion channels in the plasma membranes of fava bean (*Vicia faba* cv Long Pod) and Arabidopsis guard cells (Blatt, 1988; Schroeder and Hedrich, 1989; Lemtiri-Chlieh, 1996) and mesophyll cells (Li and Assmann, 1993; Romano et al., 1998), tobacco mesophyll cells (Bei and Luan, 1998), Arabidopsis root stelar tissues (Gaymard et al., 1998), and potato leaves (Brandt and Fisahn, 1998) have resulted in the characterization of voltage-activated I_{Kout}. Stretch-activated K^+ channels in the plasma membrane of fava bean guard cells have also been described (Cosgrove and Hedrich, 1991).

Effects of abscisic acid (ABA) on K^+-channel function in guard cells have been studied extensively

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[2] Present address: 4710 Sam Peck Road, #2138, Little Rock, AR 72223.

* Corresponding author; e-mail fedora_sutton@sdstate.edu; fax 605–688–4024.

(Blatt, 1990; MacRobbie, 1993; Assmann et al., 1994; Schwartz et al., 1994). ABA is synthesized and translocated within the plant under stress conditions, and its actions promote physiological responses leading to drought tolerance, osmotic adjustment, and dormancy (Davies and Mansfield, 1983). I_{Kout} in intact stomatal guard cells is rapidly enhanced by ABA, and ABA inhibits an inward K^+ current (I_{Kin}) (Blatt, 1990; Lemtiri-Chlieh, 1996). These results are consistent with the function of that cell type in which ABA is known to inhibit K^+ uptake and promote K^+ loss, thus deflating the guard cells and causing stomatal closure, which improves water conservation under stress conditions. Roberts (1998) described the effect of ABA on maize root K^+ channels. However, in contrast to our knowledge of guard cell mechanisms, the effects of ABA on K^+ channels in other cell types have not been well studied.

In the present study we performed a patch clamp analysis of ABA regulation of I_{Kout} in fava bean mesophyll protoplasts and a two-electrode voltage clamp analysis (TEVC) of *X. laevis* oocytes expressing an I_{Kout} derived from mesophyll mRNA. ABA inhibited the I_{Kout} expressed by the oocytes. These results provide evidence for a mesophyll ABA receptor that modulates a mesophyll outwardly rectifying K^+ channel. We also used the *X. laevis* oocyte system to compare the ABA signaling pathways in mesophyll and guard cells. Co-injection studies with *KAT1* cRNA encoding the guard cell KAT1 channel revealed that the mesophyll ABA signaling pathway that modulates I_{Kout} is not functionally interchangeable with the guard cell ABA signaling pathway that modulates I_{Kin} mediated by KAT1.

RESULTS

Mesophyll Protoplast I_{Kout} Is Modulated by ABA

To determine whether ABA modulates K^+ currents in mesophyll cells, the effect of ABA on I_{Kout} of mesophyll protoplasts was examined by patch clamp analysis. Figure 1A depicts a typical current recording obtained from patch clamp analysis of a mesophyll protoplast. This current was previously characterized and shown to be mediated by a channel with high, although not complete, selectivity for K^+ (Li and Assmann, 1993). I_{Kout} is elicited when the protoplast membrane is depolarized from −47 mV to voltages between −15 and +85 mV. The rundown of mesophyll cell K^+ currents is shown in Figure 1B. There is no significant decay. I_{Kout} at 15 min is 13.0 ± 2.0 picoampere per picofarad (pA pF^{-1}) and the current at 30 min is 12.9 ± 2.4 pA pF^{-1} at the +65 mV step (n = 5) (Fig. 1E). The effect of 25 μM ABA on a typical current recording is shown in Figure 1, D versus C. The average effect of 25 μM ABA was to reduce I_{Kout} by approximately 40% from 11.6 ± 1.8 pA pF^{-1} to 7.0 ± 1.0 pA pF^{-1} at +65 mV (n = 11; Fig. 1E).

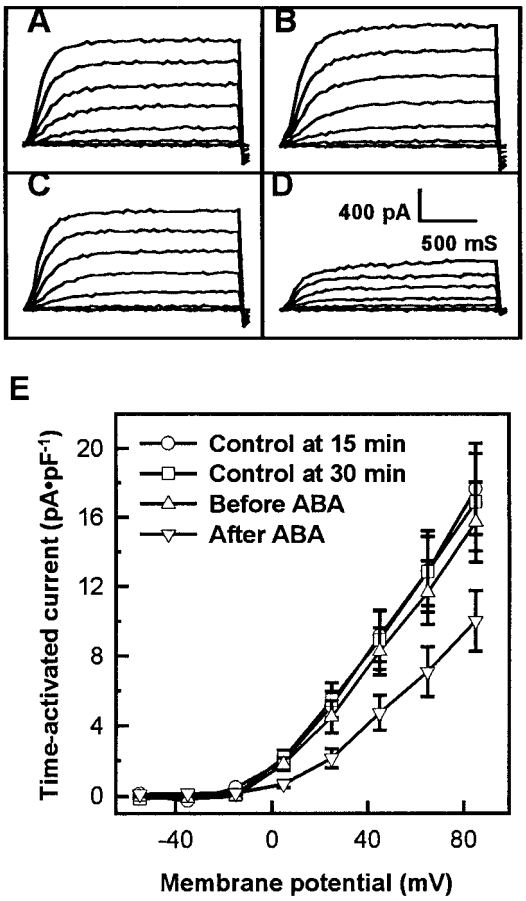

Figure 1. ABA inhibits outward K^+ current (I_{Kout}) from fava bean mesophyll protoplasts. A typical whole-cell recording (current versus time) from mesophyll protoplasts at 15 min (A) or 30 min (B) and before (C) and after ABA application (25 μM) (D) is presented. After the whole-cell configuration was obtained, the membrane potential was clamped at −47 mV. Test pulses were from −55 to +85 mV in 20-mV steps. ABA (± cis/trans, Sigma) (25 μM) was added near the protoplast under study 15 min after achieving the whole-cell configuration and recordings obtained 10 to 15 min later. I_{Kout} obtained from a mesophyll protoplast before ABA treatment (C) was reduced after application of ABA (D). Mean values of time-activated whole-cell currents were normalized by cell capacitance and plotted as a function of voltage. (x ± SE, n = 5 for control, n = 11 for ABA treatment) (E).

Mesophyll mRNA-Injected Oocytes Express Outward Current

Figure 2, A and B, depicts typical whole-cell recordings in 96 mM Na^+ solution from mesophyll mRNA and water-injected oocytes, respectively. Oocytes were voltage clamped at a holding potential of −60 mV; test potentials were from −60 to 180 mV in 20-mV steps. An outward current was observed in oocytes injected with mesophyll mRNA that activates with depolarization and does not appear to saturate. The current per voltage curve from mRNA-injected oocytes versus control oocytes shows an approxi-

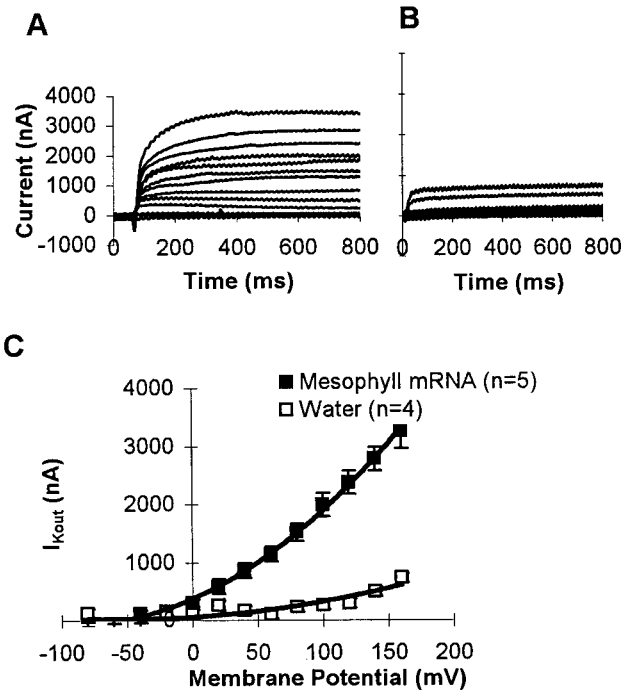

Figure 2. Fava bean mesophyll mRNA expresses an outward current in *X. laevis* oocytes. Typical current recording from *X. laevis* oocytes injected with mRNA (50 ng) (A), water (B). C, Current per voltage relationship of outward current expressed by mRNA-injected oocytes (■) and water-injected oocytes (□). Maximum outward current for each test potential was measured at 800 ms. n, Number of oocytes used for each data point presented. I_{out} was monitored in 96 mM Na^+ solution at a holding potential of −60 mV. Voltage protocol consisted of test potentials stepped from −60 to 180 mV in 20-mV steps.

mately 10-fold greater outward current from mRNA-injected oocytes (Fig. 2C).

The Outward Current Is Carried Mainly by K^+

The reversal potential (E_{rev}) of the outward current observed in oocytes was determined to identify the major ionic species involved. Tail current analysis (Wollmuth and Hille, 1992) of mesophyll mRNA-injected oocytes in 96 mM K^+ solution yielded an E_{rev} of −20 mV. In 2 mM K^+ (i.e. 96 mM Na^+ solution) E_{rev} shifted to −70 mV (Fig. 3A). The voltage protocol consisted of membrane depolarization from −60 to 0 mV for 750 ms followed by −20-mV steps to potentials from 0 to −180 mV .

Analysis of ion channel blockage aids in identification of the ionic species involved. Figure 3B depicts outward currents measured at 180 mV, 800 ms by TEVC analysis of mesophyll mRNA- and water-injected oocytes in the absence and presence of 20 or 40 mM $BaCl_2$. Barium had no effect on outward currents from control oocytes. However, the outward current from mesophyll mRNA-injected oocytes was reduced by approximately 50% in 20 mM $BaCl_2$ and by 75% in 40 mM $BaCl_2$ solution.

I_{Kout} from Mesophyll mRNA-Injected Oocytes Is Modulated by ABA

Typical I_{Kout} recordings were observed from TEVC analysis of oocytes injected with mesophyll mRNA (Fig. 4A) and water (Fig. 4C) in 96 mM Na^+ solution minus ABA. After the initial analysis in the absence of ABA, 50 μM ABA in 96 mM Na^+ solution was bath applied, and 5 min later TEVC analysis was repeated on the same oocyte. ABA treatment resulted in a decrease in I_{Kout} from mesophyll mRNA-injected oocytes (Fig. 4, A and B). Control oocytes showed no significant change of outward current in response to ABA (Fig. 4, C and D).

The effect of different concentrations of ABA on I_{Kout} is represented in Figure 4E. Recovery of I_{Kout} after ABA inhibition varied. Therefore, different oocytes were used for each ABA exposure. Approximately 50% inhibition of I_{Kout} was obtained with 10 μM ABA (Fig. 4E). Outward current from control oocytes was not influenced by application of ABA.

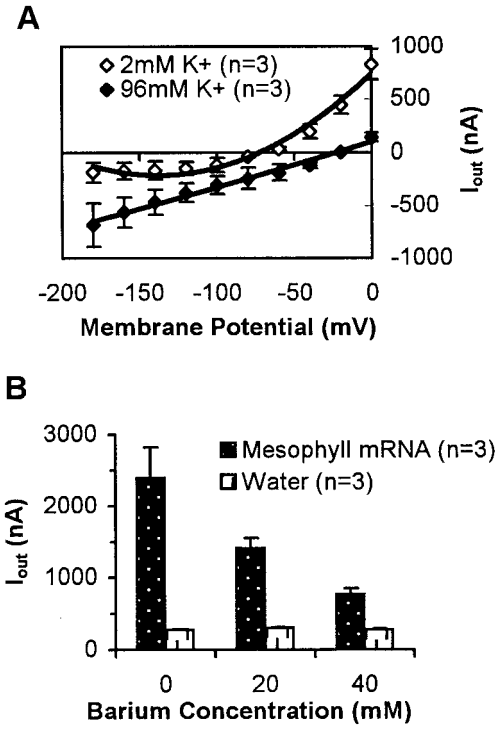

Figure 3. The outward current is specific for K^+. A, Comparison of I_{tail} versus test potential at 800 ms for mesophyll-mRNA-injected oocytes monitored in 2 mM K^+ (96 mM Na^+) solution and 96 mM K^+ solution. I_{Kout} reversed at −70 mV in 2 mM K^+ and shifted to −20 mV when monitored in 96 mM K^+ solution. The membrane potential was held at −60 mV and then depolarized to 0 mV for 750 ms. The membrane potential was then stepped to more negative potentials in −20-mV intervals. B, Effect of Ba^{2+} on outward current from water-injected or mRNA-injected oocytes. Maximum I_{Kout} at 160 mV, 800 ms, versus concentration of Ba^{2+} (0, 20, 40 mM) is depicted.

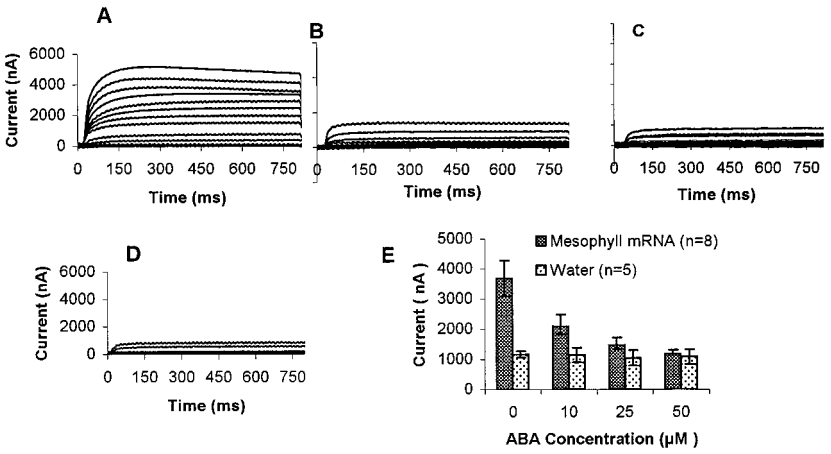

Figure 4. ABA inhibits I_{Kout} in oocytes. I_{Kout} traces in 96 mM Na^+ solution minus ABA from oocytes injected with mesophyll mRNA (A) or water (C). ABA (50 μM) in 96 mM Na^+ solution was applied to the bath and 5 min later I_{Kout} was again monitored from the same oocytes injected with mRNA (B) or water (D). Voltage protocol was as described in Figure 2 legend. E, Effect of 10, 25, and 50 μM ABA concentrations on maximum I_{Kout}. I_{Kout} was measured at 160 mV, 800 ms.

The Mesophyll ABA Signal Transduction Pathway Cannot Functionally Substitute for the Guard Cell ABA Signal Transduction Pathway

The finding that ABA modulates K^+-channel response in mesophyll cells led us to examine whether the ABA signaling pathway encoded by mesophyll mRNA can functionally substitute for the ABA signaling pathway encoded by guard cell mRNA. To ensure that we were examining modulation of the same K^+ channel and also to limit differences in signaling to the receptor and components upstream of the K^+ channel, the inwardly rectifying K^+ channel of guard cells, KAT1, was used for this comparison. Reciprocal experiments with the mesophyll cell outwardly rectifying channel of fava bean were not possible as that channel has not yet been cloned.

I_{Kin} produced by *KAT1* cRNA-injected oocytes in 96 mM K^+ solution is depicted in Figure 5A. ABA (50 μM) had no effect on I_{Kin} (-761 ± 202 nA [mean $\pm$ SE] before ABA, -798 ± 180 nA [mean $\pm$ SE] after ABA, $n = 6$) elicited from oocytes injected with *KAT1* cRNA alone (Fig. 5, C and F [lane 1]). Oocytes injected with only guard cell mRNA or only mesophyll mRNA displayed no significant I_{Kin} (Fig. 5B). Since one would expect guard cell mRNA to include the *KAT1* message, the absence of significant levels of

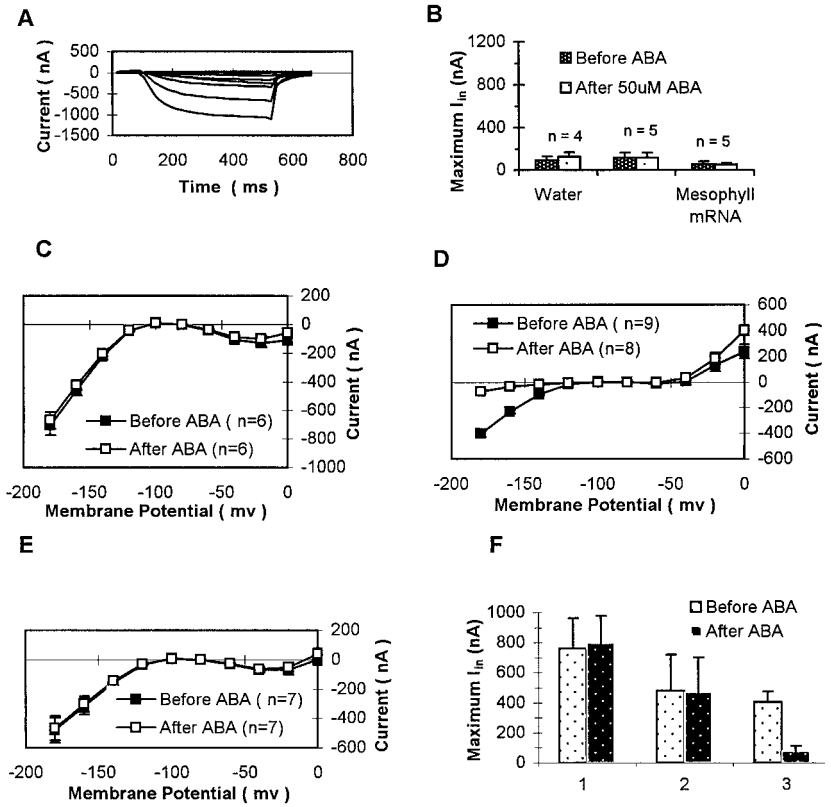

Figure 5. The mesophyll mRNA encoded-ABA signaling pathway is not functionally equivalent to that encoded by guard cell mRNA. I_{Kin} was activated from a holding potential of -60 mV stepped between -180 mV and 0 mV in 20-mV increments. A, I_{Kin} from *KAT1* cRNA-injected oocytes measured in 96 mM K^+ solution. B, Effect of ABA on I^{Kin} (presented as absolute values) obtained from control oocytes (water-injected, guard cell mRNA-injected, and mesophyll mRNA-injected). C, Application of ABA (50 μM) did not reduce I_{Kin} from *KAT1*-cRNA-injected oocytes. D, Effect of ABA on I_{Kin} produced by oocytes co-injected with guard cell mRNA and *KAT1* cRNA. E, Effect of ABA on oocytes co-injected with mesophyll mRNA and *KAT1* cRNA. F, Comparison of the maximum negative current (presented as absolute values) at -180 mV obtained from oocytes injected with: (1) *KAT1* cRNA ($n = 7$); (2) *KAT1* cRNA and mesophyll mRNA ($n = 7$); (3) *KAT1* cRNA and guard cell mRNA ($n = 9$).

I_{Kin} generated by this mRNA suggests that the *KAT1* message may be of low abundance. Oocytes co-injected with *KAT1* cRNA and guard cell mRNA express an I_{Kin} (-406 ± 69 nA [mean $\pm$ SE], $n = 9$) that is inhibited by 50 μM ABA (-76 ± 38 nA [mean $\pm$ SE], $n = 8$) (Fig. 5, D and F [3]). Replacement of the guard cell mRNA-encoded ABA receptor and possible associated signal transduction components by co-injection of *KAT1* cRNA with mesophyll A^+ RNA produced an I_{Kin} (-482 ± 238 nA [mean $\pm$ SE], $n = 7$) which did not, however, respond to ABA (-470 ± 231 nA [mean $\pm$ SE], $n = 7$) (Fig. 5, E and F [2]). Figure 5F depicts average I_{Kin} measured at -180 mV before and after application of ABA in three situations: (a) oocytes injected with *KAT1* cRNA only; (b) oocytes co-injected with *KAT1* cRNA and mesophyll mRNA; (c) oocytes co-injected with *KAT1* cRNA and guard cell mRNA. A 1.8-fold larger I_{Kin} is observed for *KAT1* cRNA-only injected oocytes compared with the co-injected samples. This could be due to competition with other transcripts for translation of the *KAT1* cRNA in co-injected samples or possible inhibition of I_{Kin} by components of the mRNA preparations or proteins encoded by mesophyll or guard cell mRNA. Nevertheless, it is evident that the mesophyll mRNA cannot substitute for the guard cell mRNA in transducing the inhibitory ABA signal.

DISCUSSION

Our patch clamp results on fava bean mesophyll cells demonstrate that the I_{Kout} of these cells is inhibited by ABA. Among other roles, ABA is involved in plant acclimation to drought, osmotic, and salt stress (Davis and Mansfield, 1983). All of these stresses would tend to decrease the water potential of plant tissue, and to the extent that ABA inhibition of K^+ efflux channels counteracts loss of cellular K^+, it may help to preserve the water status of mesophyll tissue. It is also possible that the K^+ channels responsible for I_{Kout} have a limited permeability to Na^+ influx as described for rye root K^+ channels (White and Ridout, 1995). If so, ABA-induced reduction of I_{Kout}-channel activity during salt stress may protect mesophyll cells against harmful Na^+ uptake.

The exact components of the signaling pathway by which ABA inhibits I_{Kout} are not yet known. We previously observed that elevated intracellular Ca^{2+} concentrations and G-protein activators also inhibit this current (Li and Assmann, 1993), suggesting possible elements of an ABA signal transduction pathway that could be studied in future experiments. It is notable that I_{Kin} of guard cells is likewise inhibited by ABA, elevated Ca^{2+}, and G-protein activators (Blatt, 1988; Schroeder and Hagiwara, 1989; Fairley-Grenot and Assmann, 1991; Wu and Assmann, 1994; Lemtiri-Chlieh, 1996). These comparisons led us to question whether there was a universal ABA-signaling pathway that modulated K^+-channel activity in these two cell types. We tested this hypothesis by analyzing ABA regulation of these channels as expressed in *X. laevis* oocytes.

Mesophyll mRNA apparently encodes an outwardly rectifying channel (Fig. 2) that results in production of outward current under TEVC analysis. The dependency of E_{rev} on K^+ concentration and the susceptibility of the outward current to block by Ba^{2+} (Fig. 3), a property also observed for the mesophyll outward K^+ channel when studied in planta (Li and Assmann, 1993), indicates the outward current is due mainly to K^+ flux. The I_{Kout} E_{rev} of -20 mV in 96 mM K^+ solution and -70 mV in 2 mM K^+ solution (96 mM Na^+ solution); (Fig. 3) are very similar to those described by Li and Assmann (1993) who observed E_{rev} of -35 to -25 mV in 100 mM K^+ solution and -75 mV in 1 mM K^+ solution. These values indicate that the channel is K^+ permeable but not completely K^+ selective. We cannot completely exclude the possibility that a translation product of the injected mesophyll mRNA stimulates expression of an oocyte-encoded channel. A delayed rectifier K^+ current endogenous to oocytes has been described (Lu et al., 1990). However, the oocyte channel does not share the same pharmacological characteristics as those described here. The endogenous K^+ currents in oocytes are insensitive to 10 mM barium (Lu et al., 1990). The susceptibility of the K^+ current expressed by mesophyll mRNA-injected oocytes to barium (Fig. 3) suggests that the I_{Kout} is not due to activation of the endogenous K^+ channel. In addition, the close similarity of the oocyte and mesophyll protoplast I_{Kout} currents suggest that the current observed in oocytes indeed results from expression of a plant message.

Analysis of the ABA effect on I_{Kout} in oocytes (Fig. 4) also agreed with that observed in planta (Fig. 1). The dose-dependent inhibition of I_{Kout} by ABA (Fig. 4) suggests the involvement of an ABA receptor. These results indicate that mesophyll mRNA not only encodes an outwardly rectifying K^+ channel but also all or part of an ABA-signaling pathway that modulates the channel. The strong correlation between the results obtained from mesophyll mRNA-injected oocytes with those obtained from direct patch clamp analysis of mesophyll protoplasts provided confidence for use of the oocyte system to address questions regarding modulation of mesophyll K^+ channels by ABA.

KAT1 cRNA expression in oocytes has been characterized (Schachtman et al., 1994; Very et al., 1995), and our results of I_{Kin} mediated by KAT1 are similar to those of other researchers. The lack of ABA inhibition of I_{Kin} from oocytes injected only with *KAT1* cRNA shown in Figure 5 provides evidence for the absence of endogenous ABA receptors or signaling components in oocytes that can couple to the KAT1 channel. ABA inhibition of I_{Kin} obtained from co-expression of guard cell mRNA and *KAT1* cRNA in oocytes (Fig. 5) shows clearly that guard cell mRNA encodes all or

part of an ABA-signaling pathway that functions to inhibit I_{Kin}, just as observed in the in planta experiments on this channel (Blatt, 1990; MacRobbie, 1993; Assmann and Wu, 1994; Schwartz et al., 1994).

These data strongly indicate the presence of an ABA signaling pathway encoded by guard cell mRNA that modulates I_{Kin} and a mesophyll cell mRNA-encoded ABA signaling pathway that modulates I_{Kout}. To determine whether these two ABA pathways are the same or similar enough to function interchangeably, we co-injected *KAT1* cRNA with mesophyll mRNA. The I_{Kin} current obtained from *KAT1* cRNA and mesophyll mRNA co-injected oocytes is due to KAT1 expression, since oocytes injected with only mesophyll mRNA do not express detectable $I_{Kin.}$ The inability of the mesophyll mRNA-encoded ABA signaling pathway to modulate KAT1 leads us to conclude that this ABA signaling pathway cannot function interchangeably with the guard cell mRNA-encoded ABA signaling pathway.

If we assume that the oocytes are providing the second messenger components (membrane-associated and/or cytoplasmic) then we can conclude that two different ABA receptor types are involved in modulation of I_{Kin} and I_{Kout} in guard cells versus mesophyll cells. Alternatively, it is possible that identical ABA receptors are present in the two cell types, but they are coupled to distinct downstream signaling elements in guard cells versus mesophyll cells.

To date there has been one other study using the oocyte system to dissect hormonal signal transduction pathways of plants. Leyman et al. (1999) previously observed an ABA-stimulated Ca^{2+}-dependent Cl^- current in oocytes injected with mRNA from drought-stressed tobacco leaves. A syntaxin-like protein of tobacco, Nt-SYR1 was implicated in ABA signal transduction by these experiments (Leyman et al., 1999). They also showed that application of clostridium botulinum type C, known to disrupt syntaxin function in animal systems, prevented ABA inhibition of I_{Kin} in guard cells as well as altering ABA regulation of I_{Kout} and anion channels in this cell type. Additional studies can now be pursued to further distinguish the two tissue-specific ABA signal transduction pathways described in the present report. Expression cloning strategies (Hollmann et al., 1989) may now be used to identify key components of the unique ABA signal transduction pathway of mesophyll cells.

MATERIALS AND METHODS

Plant Material

Fava bean (*Vicia faba* cv Long Pod) plants were grown for 3 weeks in plant growth chambers with a 10-h light (100 μmol m^{-2} s^{-1} white light, 21°C) and 14-h dark (18°C) regime. Bifoliate leaves with 80% to 90% expansion were used to isolate protoplasts.

Mesophyll Protoplasts

Isolation of mesophyll cell protoplasts was performed as described by Li and Assmann (1993) with some modification. Leaves were cut into slices after removing the abaxial epidermis and midrib, and the slices were put into an enzyme digestion solution containing 0.6 M mannitol, 1 mM $CaCl_2$, 0.4% (w/v) macerozyme (Yakult R-10, Karlan Research Products, Santa Rosa, CA), 1% (w/v) cellulase (Onozuka R-10), 0.1% (w/v) polyvinypyrrolidone 40, and 0.2% (w/v) bovine serum albumin, pH 5.5. The mixture was vacuum infiltrated for 2 min followed by a 5-min digestion at 24°C with fast shaking. The debris and disintegrated cells were discarded by decantation. New enzyme solution was added, and leaf slices were digested for 1 h at 24°C with gentle shaking. The digestion mixture was filtered through 60-μm mesh. The filtrate was centrifuged twice at 200*g* for 3 min, and each time the supernatant was decanted and the pellet containing the protoplasts resuspended in 5 mL of rinsing medium (0.6 M manitol/1 mM $CaCl_2$). Protoplasts for electrophysiological analysis were incubated in darkness on ice for at least 1 h before starting patch clamp experiments. Protoplasts for RNA isolation were quick frozen in liquid N_2.

Guard Cell Protoplasts

Guard cell protoplasts were prepared as described by Miedema and Assmann (1996). Epidermal peels, obtained by blending leaf sections followed by filtration through cotton mesh, were treated with an enzyme solution containing 0.7% Cellulysin (*Trichoderma viride,* Calbiochem, La Jolla, CA). The digest was shaken in the dark at 27°C for 30 min, diluted with basic medium (0.45 M mannitol, 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.5 mM ascorbic acid, 10 μM KH_2PO_4, and 5 mM MES pH 5.5) and shaken for a further 5 min. Peels were collected on 220-μm nylon mesh and transferred to a second enzyme solution containing 1.5% (w/v) Onozuka RS cellulase (Yakult Honsha, Tokyo), 0.02% (w/v) Pectolyase Y-23 (Seishin, Tokyo). This digest was continued with shaking in the dark at 19°C for 20 min. The speed was reduced and the digest continued for an additional 25 min. Resulting protoplasts were filtered through 30-μm nylon mesh and collected by centrifugation at 200g for 4 min. Pellets were rinsed in a medium consisting of 0.35 M mannitol and 1 mM $CaCl_2$, and then quick frozen in liquid N_2.

RNA Isolation

Frozen protoplasts were thawed and homogenized in 4 M guanidinium thiocyanate solution. The homogenate was centrifuged at 8,000*g* for 10 min at 4°C. The resulting supernatant was layered over a 5.7 M CsCl cushion. Ultracentrifugation was performed as per the procedure of Chirgwin et al. (1979). Poly(A^+) RNA was obtained by chromatography on oligo(dT) columns (Qiagen USA, Valencia, CA). The mRNA for injection into oocytes was resuspended in water. All water used for RNA resuspension or oocyte injection was autoclaved twice after treatment with diethyl pyrocarbonate (Sigma, St. Louis).

In Vitro Transcription

KAT1 cDNA was subcloned into the pCITE-4c vector (Novagen, Madison, WI; Li et al., 1998). Since pCITE-4c contains a T7 terminator, uncut plasmid containing *KAT1* cDNA (1 μg) was used in the in vitro transcription reaction. Standard in vitro transcription was performed at 37°C for 1 h with T7 RNA polymerase as per mMessage kit (Ambion, Austin, TX). Resulting transcripts were precipitated with LiCl (3 M), washed with 70% (v/v) ethanol, and resuspended in water.

Oocyte Preparation and Injection

Oocytes were obtained from *Xenopus laevis*. Follicular membranes were removed at room temperature by digestion with collagenase at 2 mg/mL, (type 1A, Sigma) dissolved in Ca^{2+}-free saline solution (82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, and 5 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid]/NaOH, pH 7.4). Oocytes were injected with 50 nL of (1 ng/nL) RNA or 50 nL of water (control). Following injection, oocytes were incubated for 3 to 7 d in "96 mM Na^+ solution" (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, and 5 mM HEPES/NaOH, pH 7.5) supplemented with 25 mM sodium pyruvate, 50 mM gentamycin, 10 μg/mL penicillin/streptomycin, and 0.5 mM theophylline.

Recordings

Patch Clamp Measurements

The pipette solution contained 98 mM K-Glu, 2 mM K_2-EGTA, 2 mM $MgCl_2$, 2 mM KCl, 10 mM HEPES, 500 mM mannitol, and 2 mM MgATP titrated to pH 7.2. The bath (external) solution consisted of 10 mM K-Glu, 1 mM $CaCl_2$, 4 mM $MgCl_2$, 550 mM mannitol, and 10 mM HEPES titrated to pH 6.0. The osmolalities of pipette and bath solution were 640 mmol kg^{-1} and 735 mmol kg^{-1}, respectively. ABA (± cis/trans, Sigma) was added to the bath solution at a final concentration of 25 μM from a stock solution by ejection from a pipette tip placed close to the cell under study. ABA was added 15 min after achieving the whole-cell configuration, and ABA effects were measured 15 min later (i.e. 30 min into the recording).

Whole-cell patch clamp recordings were conducted in the dark at 20°C. Seal resistance was between 1 and 3 GΩ. Cell capacitance was measured for each cell using the capacity compensation device of the amplifier (Bookman et al., 1991). Whole-cell currents were measured using an Axopatch-200A amplifier (Axon Instruments, Foster City, CA), which was connected to a microcomputer via an interface (Digidata 1200 Interface, Axon Instruments). pCLAMP (version 6.0.3, Axon Instruments) software was used to acquire and analyze the whole-cell currents. Voltage pulse protocols were generated using pCLAMP software and applied to the clamped cell during data acquisition. After the whole-cell configuration was obtained, the membrane was clamped to −47 mV (holding potential). Test pulses were from −55 to +85 mV in 20-mV steps. Whole-cell current was filtered at l kHz by the clamp's Bessel filter before storage (1 ms per sample) on computer disc. Whole-cell currents were leak-subtracted before generating whole-cell current-voltage relations. Leak currents for each cell were determined at 20 ms after the membrane potential was stepped from the holding voltage to the test voltages. The mean values of steady-state whole-cell currents were determined as the average of samples obtained between 1.4 and 1.8 s after imposition of the test voltage (when current amplitude had reached its plateau). After subtraction of leak currents, the time-activated whole-cell currents were expressed as the current per unit capacitance (picoampere per picofarad) to account for variations in cell surface area.

TEVC Measurements

Ion channel expression in oocytes was monitored by the TEVC technique as described (Dascal and Cohen, 1987; Sutton et al., 1988) using an Axoclamp 2A amplifier (Axon Instruments). Voltage and current microelectrodes were filled with 3 M KCl; electrodes measuring 0.5- to 2-MΩ resistance in 96 mM Na^+ solution were used. Whole-cell recordings were obtained from oocytes placed in a chamber perfused with various solutions at 0.6 mL/s. Outward currents were recorded in 96 mM Na^+ solution. I_{Kin} was recorded in "96 mM K^+ solution" (96 mM Na^+ solution substituting 96 mM NaCl with 96 mM KCl and 2 mM KCl with 2 mM NaCl). ABA (± cis/trans, Sigma) 10^{-2} M stock was prepared in acetone. Working ABA solutions were prepared by dilution with 96 mM Na^+ or 96 mM K^+ solution for monitoring effects on I_{Kout} and I_{Kin}, respectively. Data acquisition, analysis, and voltage command signals were achieved with pClamp software (version 6.03) (Axon Instruments). Figures were prepared from pClamp data files downloaded to Microsoft EXCEL (Microsoft, Redmond, WA).

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