

# Background ion channel activities in *Arabidopsis* guard cells and review of ion channel regulation by protein phosphorylation events

Zhen-Ming Pei<sup>1</sup>, Julian I. Schroeder and Martin Schwarz<sup>2</sup>

Department of Biology and Center for Molecular Genetics, University of California, San Diego, La Jolla, CA 92093–0116, USA

Received 10 June 1997; Accepted 17 November 1997

## Abstract

In this paper, results on background conductances in *Arabidopsis* guard cells are presented and recent advances pointing to a central role of protein phosphorylation/dephosphorylation events during stomatal signalling are reviewed. Recently, patch clamp studies of *Arabidopsis* guard cells were developed to characterize early abscisic acid signalling. It is reported here that whole-cell recordings from *Arabidopsis* guard cells showed three types of background conductances: two large single channel conductances activated by depolarization and hyperpolarization, respectively, and another instantaneous conductance. The reversal potentials of depolarization-activated single channel currents were in the range of  $-7$  mV and were independent of the CsCl gradient, even at an 86-fold imposed gradient. These data suggest that depolarization-activated single channel currents in *Arabidopsis* guard cells represent non-selective channels. The instantaneous background currents disappeared when the impermeable anions, acetate or glutamate, were loaded into the cytosol of guard cells via the patch electrode. These data and reversal potentials suggest that the small instantaneous background conductance represents a basal activity of anion currents in guard cells. Possible physiological functions of the single channel currents and background anion conductances are discussed. Furthermore, recently reported guard cell ion channel regulation mechanisms are reviewed in the discussion and brought in context of stomatal movement studies.

Key words: Abscisic acid, *abi* mutants, non-selective ion channel, protein phosphorylation, stomatal movements.

## Introduction

Guard cells have become a popular system for characterizing the physiological functions of ion channels in higher plants and for unravelling very early signal transduction events. Several major classes of ion channels were identified by patch clamp studies of the plasma membrane of *Vicia faba* guard cells including outward- and inward-conducting  $K^+$  channels and slow and rapid anion channels (for reviews see Hetherington and Quatrano, 1991; Assmann, 1993; Ward *et al.*, 1995). In addition, non-selective ABA-activated ion channels were found with a permeability to  $Ca^{2+}$  ions (Schroeder and Hagiwara, 1990) and several differing types of single channels were shown to be activated by membrane stretch (Cosgrove and Hedrich, 1991).

Recent comparative studies, suggest that by analysing guard cell ion channels in different species, new regulation mechanisms or differences among species may be revealed (Armstrong *et al.*, 1995; Schmidt *et al.*, 1995; Pei *et al.*, 1997). In the present study whole-cell patch clamp analyses of *Arabidopsis* guard cells demonstrate a low basal activity of non-selective ion channels in the plasma membrane. These data indicate that different species can 'bring out' certain ion channel activities more than others, even if the signalling cascades are similar. The constitutive reproducible background activity of these non-selective ion channels in *Arabidopsis* guard cells has allowed their initial description, as presented here. The large conductance ion channels could be of significance for regulation

<sup>1</sup> To whom correspondence should be addressed. Fax: +1 619 534 7108.

<sup>2</sup> Present address: Center for Molecular Neurobiology Hamburg (ZMNH), Hamburg University, Martinistrasse 52, D-20246 Hamburg, Germany.

of stomatal movements in *Arabidopsis* as well as in other species. In addition, a background anion conductance in *Arabidopsis* is described here. Proposed physiological functions of the background anion conductance in guard cells are discussed in relation to recent studies of stomatal movements in the presence of anion channel modulators, suggesting the presence of a basal anion conductance. In addition, recent studies on regulation of guard cell ion channels by phosphorylation events are reviewed in the discussion. In particular, species-specific differences suggest added complexity to early ABA signal transduction in guard cells.

## Materials and methods

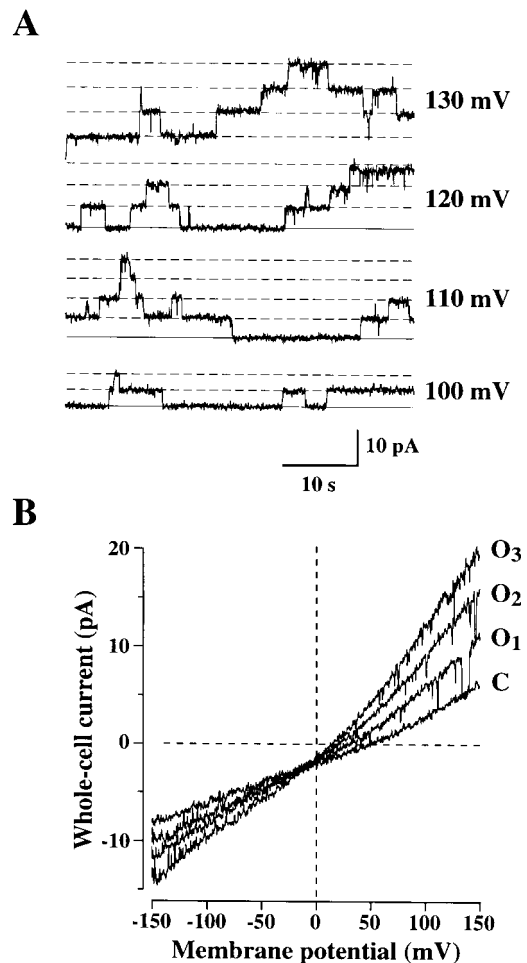
*Arabidopsis thaliana* plants were grown in a controlled environment growth chamber. Four to six-week-old plants were used in experiments. *Arabidopsis* guard cell isolation was accomplished using the same protocol described by Pei *et al.* (1997). Patch clamp techniques were applied to *Arabidopsis* guard cells as described previously (Pei *et al.*, 1997). Voltage-clamp was performed on guard cell protoplasts using an Axopatch 200 amplifier (Axon Instruments, Foster City, CA). Currents from whole-cell recordings were filtered at 1 kHz with an eight-pole Bessel low pass filter and stored with a 486 DX2-based microcomputer. Data were further filtered at 100 Hz and analysed using Axograph software (version 2.0, Axon Instruments). The pipette solution that equilibrates with the cytosol in patch clamp studies contained 150 mM CsCl, 2 mM MgCl<sub>2</sub>, 5 mM Mg-ATP, 6.7 mM EGTA, 3.35 mM CaCl<sub>2</sub>, and 10 mM HEPES-TRIS, pH 7.1. Bath solutions contained 30 mM CsCl, 1 mM CaCl<sub>2</sub>, 2 mM MgCl<sub>2</sub>, and 10 mM MES-TRIS, pH 5.6. For CsCl gradient experiments in Fig. 2, 30 mM CsCl in the bath solution was changed to 1.5, 15 and 150 mM CsCl. Other components were the same as given above. Osmolality of the bath solution was adjusted to 485 mmol kg<sup>-1</sup>, and those of the pipette solutions were adjusted to 500 mmol kg<sup>-1</sup> by addition of D-sorbitol. The bath solution (total volume ≈ 0.1 ml) was exchanged by bath perfusion at 0.25 ml min<sup>-1</sup> for 4 min (1 ml) using a peristaltic pump (Rainin, Woburn, MA). CsCl activities in Fig. 2 have been corrected for ionic activities in solutions. Experiments were performed at room temperature.

## Results

Whole-cell recordings of *Arabidopsis* guard cells under conditions that favour analysis of anion channels by using CsCl solutions (Schroeder and Hagiwara, 1989) showed small background currents in the absence of ABA (Pei *et al.*, 1997). Under these conditions, at least three types of background currents were observed in the *Arabidopsis* guard cells analysed here.

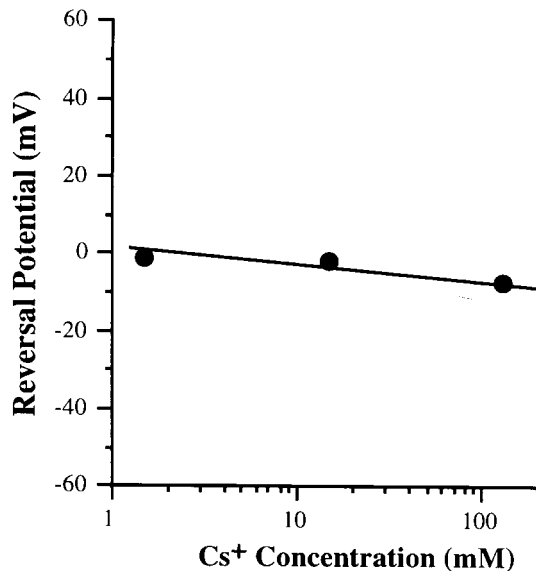
### Depolarization-activated background single channels in *Arabidopsis* guard cells

At strongly depolarized membrane potentials large single channel currents were observed that showed a large open probability (Fig. 1A). Note that in *Vicia faba* guard cells, large single channel currents are also found, when the



**Fig. 1.** Ion channel currents activated at positive membrane potentials during whole-cell recordings in *Arabidopsis* guard cells. (A) Single channel currents at different positive potentials in whole-cell recordings. The membrane potentials are indicated to the right of current traces. (B) Whole-cell currents recorded during voltage ramps from +150 mV to -150 mV at a rate of 230 mV s<sup>-1</sup>. The holding potential between ramps was +150 mV. Activity of three ion channels was resolved. At positive membrane potentials, the bottom current trace shows the closed state of the three channels as indicated by 'C', and the top three current traces show open states of three different channels 'O<sub>1</sub>, O<sub>2</sub> and O<sub>3</sub>'. The CsCl concentrations in the pipette and bath solutions were 150 mM and 30 mM, respectively. Other components of the solutions are described in Materials and methods.

membrane potential is depolarized to very positive membrane voltages (JI Schroeder, unpublished data). The reversal potential of depolarization-activated single channel currents in *Arabidopsis* guard cells shown in Fig. 1B was determined by applying voltage ramps to cells. In the example shown in Fig. 1B, three open-channels were observed with clear closed and open state transitions. Voltage ramps to hyperpolarizing membrane potentials showed that the single channel currents reversed at a value of  $-7 \pm 2.3$  mV (Fig. 2B;  $n = 11$  guard cells). These data show that the single channel currents recorded at strongly depolarized membrane potentials are not solely selective for only Cs<sup>+</sup> or only Cl<sup>-</sup> ions, because the Cs<sup>+</sup>



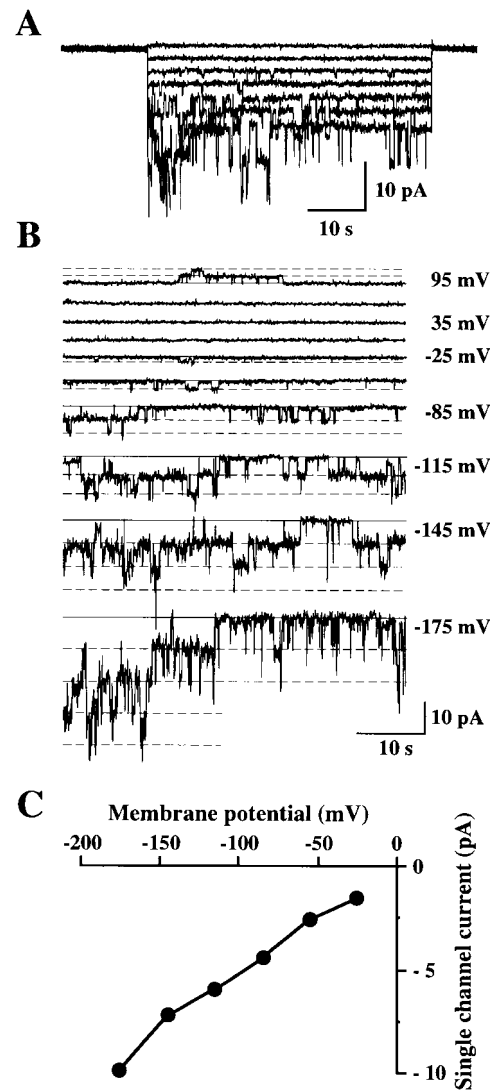
**Fig. 2.** Reversal potentials of the depolarization-activated ion channels were unaffected by changing CsCl activities in the bath solution from 1.5 mM to 14.6 and 129.0 mM, demonstrating that single channel currents recorded during depolarizations are not highly selective for only  $\text{Cl}^-$ ,  $\text{Cs}^+$ ,  $\text{Ca}^{2+}$  or protons (see text). Experiments were performed using voltage ramps as in Fig. 1B to allow analysis of channel currents activated by depolarization. Data shown were averaged from two different cells showing similar results.

equilibrium potential was  $\approx -35$  mV and that for  $\text{Cl}^-$  was  $\approx +30$  mV.

Experiments were performed to test further the selectivity of the single channel currents observed at depolarized potentials. A larger CsCl gradient was applied by lowering the bath concentration to 15 mM CsCl with 150 mM CsCl in the cytosol. The reversal potential of the single channels at this  $\text{Cs}^+$  concentration gradient was similar to the reversal potential recorded with 30 mM CsCl in the bath (Fig. 2). Indeed, applying an 86-fold gradient (calculated after correction for ionic activities) by adding only 1.5 mM CsCl to the bath solution, resulted in a reversal potential of these channels of  $-8$  mV (Fig. 2;  $n=2$  guard cells). These findings confirm that the single channel currents correspond to ion channels that are not specifically selective to only  $\text{Cl}^-$  or only  $\text{Cs}^+$ .

#### Hyperpolarization-activated background single channels

As illustrated in Fig. 3A, in whole-cell recordings large single channel currents were also frequently observed at negative membrane voltages. These single channel currents showed a main conductance state of  $54 \pm 3.5$  pS (Fig. 3B, C), which differed from the conductance of the single channel currents described above at negative potentials (Fig. 1B). During voltage ramps to negative membrane potentials, the single channel current amplitude of the depolarization-activated channels at  $-100$  mV was  $\approx -1.9$  pA (Fig. 1B), while the single channel current amplitude of ion channels shown in Fig. 3B and C was



**Fig. 3.** Small instantaneous currents and single ion channels activated at negative membrane potentials in Arabidopsis guard cells. (A) Whole-cell recordings at potentials from  $-145$  to  $+35$  mV in 30 mV increments, with a holding potential of  $+30$  mV. Note the opening of large conductance single channel currents, which are regularly observed under these conditions. Also note the instantaneous background conductance in Arabidopsis guard cells. (B) Single channel currents recorded at different potentials under the same conditions shown in (A). (C) Average single channel current–voltage relationship from single channels recorded at negative holding potentials. Solutions used were the same as in Fig. 1.

$-5.2$  pA. Because measurable negative single channel currents were recorded at  $-25$  mV (Fig. 3B, C), it can be concluded that the single channels were not selective for monovalent cations, as the equilibrium potential of the major alkali cation present,  $\text{Cs}^+$ , was  $\approx -35$  mV.

Furthermore, the ion channels observed in Fig. 3 at hyperpolarized potentials as well as those described in Fig. 1 and Fig. 2 are not solely permeable to calcium ions because in all experiments an over 1000-fold  $\text{Ca}^{2+}$  gradient was applied across the plasma membrane of Arabidopsis guard cells. Therefore, the  $\text{Ca}^{2+}$  equilibrium

potential was  $> +87$  mV, which differed significantly from the observed reversal potentials of both channel conductances. In addition, the observed single channel currents were not proton-selective, as pH gradients were applied during patch clamp recordings, by using pipette solutions with a pH of 7.1 and bath solutions with a pH of 5.6 (proton equilibrium potential  $\approx +89$  mV).

It could therefore be concluded that (a) these two types of ion channels were not selective for  $\text{Cs}^+$ , (b) the channels were not solely selective for  $\text{Ca}^{2+}$  ions, (c) the channels were not proton-selective, and (d) the depolarization-activated single channel conductances were not selective for anions. Therefore, the depolarization-activated background single channel currents found in *Arabidopsis* guard cells are either non-selective ion channels that may have partial relative permeabilities to the above ions and/or they are divalent cation channels that have a significant  $\text{Mg}^{2+}$  permeability. Magnesium ions were present in the extracellular solution at a concentration of 2 mM and in the cytosolic solution the free  $\text{Mg}^{2+}$  concentration was 2.4 mM ( $\text{Mg}^{2+}$  equilibrium potential  $\approx -2$  mV). Further experiments will be needed to determine whether the underlying ion channels are permeable to  $\text{Mg}^{2+}$ . Furthermore, even if these single channels conduct divalent cations, the question whether they are also partially permeable to monovalent cations and/or anions would require further ion substitution experiments with impermeable ions, to test for effects on reversal potentials.

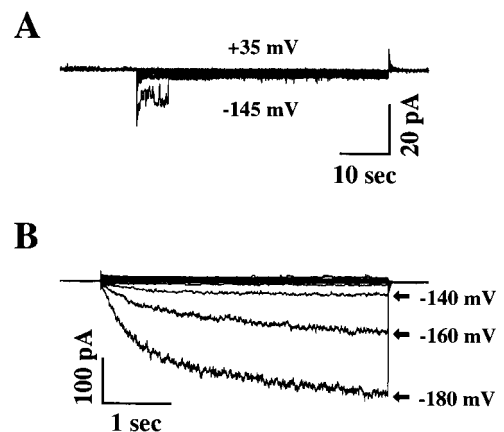
#### Instantaneous background currents in *Arabidopsis* guard cells

In addition to the single channel currents, small instantaneous currents were observed in the majority of whole-cell recordings without addition of ABA (Fig. 3A;  $n=82$  out of 100 guard cells analysed here; see also Pei *et al.*, 1997). The fact that the reversal potential of these small instantaneous whole-cell currents was usually positive with values up to  $+30$  mV, indicated that this instantaneous conductance was not a mere leak conductance related to membrane seals in patch clamp recordings.

One likely source of this small instantaneous conductance in whole-cell recordings is that it includes a low background activity of anion channel currents in *Arabidopsis* guard cells (Pei *et al.*, 1997). A number of stomatal movement assays, using anion channel blockers (Schroeder *et al.*, 1993; Schwartz *et al.*, 1995), protein kinase inhibitors (Schmidt *et al.*, 1995), phosphatase inhibitors (Pei *et al.*, 1997; Esser *et al.*, 1997), extracellular  $\text{K}^+$  (Schwartz *et al.*, 1995), and extracellular malate (Esser *et al.*, 1997) have indicated that guard cell anion channels function as negative regulators during light-induced stomatal opening (see discussion). These light-induced stomatal opening assays suggest that a residual

activity of anion channels exists in guard cells, which by causing depolarization limits the aperture of opening stomatal pores. When anion channel activity is inhibited or blocked, stomatal pores apertures open wider.

Anion channels in guard cells have been previously shown to be largely impermeable to the anions glutamate and acetate (Marten *et al.*, 1992; Schmidt and Schroeder, 1994; Pei *et al.*, 1997). A recent study showed that adding only 30 mM acetate to the cytosol of *Arabidopsis* guard cells led to a reduction of ABA-activated S-type anion current amplitudes (Pei *et al.*, 1997). Figure 4 shows an example of whole-cell currents recorded in *Arabidopsis* guard cells with 120 mM CsCl and 30 mM Cs-acetate in the cytosol (Pei *et al.*, 1997). It is interesting to note that the small instantaneous background current in *Arabidopsis* guard cells was reduced when Cs-acetate was added to the cytosol (compare Figs 3A and 4A). When 150 mM CsCl was replaced by 150 mM Cs-Acetate in the pipette solution, the whole-cell background conductance between  $+30$  and  $-145$  mV was also very small, corresponding to a whole-cell resistance of  $\approx 33.7 \pm 3.8$  G $\Omega$  ( $n=3$ ). The reversal potential and acetate inhibition support the suggestion that the small instantaneous background current corresponds to an anion conductance that is largely impermeable to cytosolic acetate, as are also the large ABA-activated S-type anion currents (Pei *et al.*, 1997). Whether the mechanism underlying acetate reduction of this background current is down-regulation, block or other reasons was not analysed here. Note that the large single channel currents continued to appear when using acetate in the cytosol, showing that they are not



**Fig. 4.** When acetate is added to the cytosol of guard cells, the instantaneous background current is reduced, while the large single channel currents remain (data in Fig. 4 are reproduced with permission of *The Plant Cell*, Pei *et al.*, 1997). (A) When the cytosol of *Arabidopsis* guard cells is dialysed with a solution containing 30 mM Cs-acetate, 120 mM CsCl and 50  $\mu\text{M}$  ABA, the instantaneous background currents typically observed in guard cells are reduced. Large ABA-activated S-type anion currents are substantially reduced (Pei *et al.*, 1997). (B) Extracellular replacement of 30 mM  $\text{Cs}^+$  ions with 30 mM  $\text{K}^+$  ions in the bath solution shows that  $\text{K}^+$  channel currents are not inhibited by cytosolic acetate (Pei *et al.*, 1997).

acetate-inhibited channels (Fig. 4A). As reported previously, replacement of  $\text{Cs}^+$  in the bath solution by  $\text{K}^+$  resulted in large inward-rectifying  $\text{K}^+$  channel currents (Fig. 4B; Pei *et al.*, 1997). These data show that acetate does not inhibit  $\text{K}_{\text{in}}^+$  channels and, furthermore, illustrate, together with capacitance measurements, that whole-cell recordings were intact during acetate experiments (Pei *et al.*, 1997).

## Discussion

In the present study, an initial description of background ion conductances in *Arabidopsis* guard cells is given. First, two frequently occurring large single channel conductance states were shown. The depolarization-activated single channel currents were shown to correspond to ion channels that are not specifically selective to only one of the following ionic species:  $\text{Cs}^+$ ,  $\text{Cl}^-$ ,  $\text{Ca}^{2+}$  or protons. The question whether these ion channels are  $\text{Mg}^{2+}$  permeable and whether partial relative permeabilities to the above listed ions exist, requires further analysis. Such a necessary analysis on the selectivity of the slow vacuolar channels has only recently been completed and is relatively complex for non-selective channels (Ward *et al.*, 1995; Allen and Sanders, 1996). In addition, a small instantaneous background conductance observed in the absence of ABA in *Arabidopsis* guard cells could correspond to an anion conductance that is impermeable to cytosolic glutamate and cytosolic acetate.

Previous studies in *Vicia faba* guard cells have shown the presence of non-selective ion channels. A study of early ABA signal transduction showed that in *Vicia faba* and *Commelina communis* guard cells ABA causes a rapid rise in cytosolic  $\text{Ca}^{2+}$  (McAinsh *et al.*, 1990; Schroeder and Hagiwara, 1990). The most rapid electrophysiological response in guard cells characterized to our knowledge is the rapid activation of non-selective  $\text{Ca}^{2+}$  permeable ion channels in the plasma membrane, which can produce initial transient rises in cytosolic  $\text{Ca}^{2+}$  (Schroeder and Hagiwara, 1990). Interestingly the reversal potential of these non-selective ABA-activated currents was in the range of  $-10$  mV under the imposed conditions (Schroeder and Hagiwara, 1990). The reversal potential of single channel currents observed in the present study and those found in *Vicia faba* guard cells are similar. The question whether the low level of constitutive activity of single channels observed in *Arabidopsis* guard cells here, is related to ABA-activated non-selective  $\text{Ca}^{2+}$  permeable channels will require further analysis. A recent study showed elicitor-activated large conductance non-selective  $\text{Ca}^{2+}$ -permeable channels in parsley protoplasts (Zimmermann *et al.*, 1997), indicating that non-selective channels may play roles in more diverse early signalling events in plants. In parsley cells these channels were reported to show a constitutive (elicitor-independent) background activity.

In another study on *Vicia faba* guard cells the effect of membrane stretching on single channel currents in plasma membrane patches was studied. In this study several single channel conductances were found that were activated by membrane stretching (Cosgrove and Hedrich, 1991). Single channel currents of various permeabilities and conductances were observed including  $\text{Cl}^-$ , and cations including  $\text{Ca}^{2+}$ . Among these stretch-activated channels, non-selective channels of different conductances were found (Cosgrove and Hedrich, 1991). The association of the various stretch-activated single channel conductances to the well-described ion channel types in the plasma membrane of *Vicia faba* guard cells remains unknown. It is possible that the non-selective single channel activities observed here in *Arabidopsis* guard cells are related to one of the stretch-activated non-selective channel conductance states.

A recent study in *Commelina communis* guard cells showed that increasing the extracellular  $\text{Ca}^{2+}$  concentration produces repetitive increases in cytosolic  $\text{Ca}^{2+}$  of guard cells (McAinsh *et al.*, 1995). When  $\text{Ca}^{2+}$  in the bath solution was replaced by  $\text{Mn}^{2+}$  ions, it was found that the initial increases in cytosolic  $\text{Ca}^{2+}$  were quenched. These data show that a  $\text{Ca}^{2+}$  permeable channel is responsible for the initial and rapid phase of cytosolic  $\text{Ca}^{2+}$  increases in response to extracellular  $\text{Ca}^{2+}$  (McAinsh *et al.*, 1995). Early studies on the  $\text{Ca}^{2+}$  sensitivity of ABA signalling in guard cells also indicated an important role for extracellular  $\text{Ca}^{2+}$  in mediating stomatal closing. DeSilva *et al.* (1985) and Schwartz (1985) showed that both buffering extracellular  $\text{Ca}^{2+}$  with  $\text{Ca}^{2+}$  chelators and extracellular application of  $\text{Ca}^{2+}$  channel blockers inhibited ABA-induced stomatal closing. These data correlate to activation of plasma membrane  $\text{Ca}^{2+}$  influx channels during ABA signalling. Note that  $\text{K}_{\text{in}}^+$  channel regulation studies have provided evidence for parallel ABA-induced intracellular  $\text{Ca}^{2+}$  release (Lemtiri-Chlieh and MacRobbie, 1994). ABA-activated  $\text{Ca}^{2+}$  permeable channels in the plasma membrane have been suggested to serve two possible functions in guard cells: (a) to produce an initial rise in cytosolic  $\text{Ca}^{2+}$ ; (b) these non-selective channels can also provide a mechanism for an early pre-depolarization of guard cells, important for inducing stomatal closing (Schroeder and Hagiwara, 1990). The fact that the activity of the single channels observed here is at the level of only 1 to 5 channels per cell, can lead to speculation that stronger activation by signals may occur, as was reported for elicitor-activated non-selective channels in parsley (Zimmermann *et al.*, 1997).

## Review of phosphorylation events regulating guard cell ion channels

Recent studies have shown that differences in the regulation or abundance of different guard cell ion channels

may exist when comparing plant species. The underlying reasons for these differences remain unknown and could encompass emphasis on different elements of identical signalling cascades in different species or perhaps species-specific differences in the signal transduction cascades (Pei *et al.*, 1997). The most striking species-dependent differences in guard cell ion channel regulation found to date are related to phosphorylation events as reviewed here.

A recently characterized example of differences in guard cell signalling is the regulation of S-type anion channels which provide an important regulation mechanism for stomatal closing and negative feedback during stomatal opening (Schroeder and Hagiwara, 1989; Pei *et al.*, 1997). Activation of guard cell anion channels results in anion efflux causing depolarization. This depolarization in turn can activate  $K^+$  release channels and can drive  $K^+$  efflux from guard cells, required for stomatal closing. In *Vicia faba* guard cells, S-type anion channels are strongly activated by phosphorylation events and require hydrolysable ATP for activation (Schmidt *et al.*, 1995). S-type channel activity was inhibited by protein kinase inhibitors and maintained without ATP in the presence of the protein phosphatase inhibitor okadaic acid (OA), providing evidence for the necessity of protein phosphorylation events in S-type channel activation.

On the other hand, in *Arabidopsis* guard cells S-type anion channels are activated by dephosphorylation events and the protein phosphatase inhibitor OA partially inhibits ABA activation of these anion channels (Pei *et al.*, 1997). Further evidence for differences in signalling specificity between *Vicia faba* and *Arabidopsis* guard cells is demonstrated by the fact that ABA regulation of stomatal movements *in vivo* correlates closely to anion channel regulation in the respective species showing opposite regulation by phosphorylation modulators and dephosphorylation modulators in the two species (Schmidt *et al.*, 1995; Esser *et al.*, 1997; Pei *et al.*, 1997). Two possible models to explain these differences have been proposed by Pei *et al.* (1997): (1) the same signalling cascades could emphasize different rate-limiting components in the two species. Or (2) Perhaps distinct differences in the ABA signalling cascades may exist in *Arabidopsis* and *Vicia faba*. The first model would require a more complex ABA signalling cascade, which seems plausible.

Species-dependent differences in S-type anion channel regulation correlate closely to differences in ABA regulation of stomata, suggesting that the observed regulatory mechanisms also determine the physiological response *in vivo* (Schmidt *et al.*, 1995; Pei *et al.*, 1997). Furthermore, this dichotomy extends to two other species, namely to ABA signalling in tobacco and *Commelina communis* stomata: in both *Vicia faba* and *Commelina communis*, studies of ABA-induced stomatal closing and the regulation of S-type anion channels suggest that ABA signals

are transduced by activation of protein kinases and possibly also down-regulation of okadaic acid sensitive protein phosphatases (Schmidt *et al.*, 1995; Esser *et al.*, 1997). On the other hand, in both tobacco and *Arabidopsis*, ABA-induced stomatal movements appear to involve up-regulated protein phosphatases and/or down-regulated protein kinases (Armstrong *et al.*, 1995; Pei *et al.*, 1997). The finding that ABA activates protein phosphorylation events in *Vicia faba* (Schmidt *et al.*, 1995) has been recently directly established in biochemical studies showing ABA activation of a 48 kDa protein kinase activity in *Vicia faba* guard cell extracts (Li and Assmann, 1996; Mori and Muto, 1997).

Another type of anion channel can cause rapid (R-type) and transient depolarizations in guard cells (Hedrich *et al.*, 1990). These rapid anion channels have been reported not to be directly affected by ABA (Marten *et al.*, 1991). Complete block of R-type anion channels by DIDS does not affect ABA-induced stomatal closing (Schroeder *et al.*, 1993). In addition, R-type channels are not regulated by phosphorylation events (Hedrich *et al.*, 1990; Schulz-Lessdorf *et al.*, 1996). Furthermore, R-type activation occurs at acidic cytosolic pH (Schulz-Lessdorf *et al.*, 1996), which contrasts ABA-induced cytosolic alkalization (Irving *et al.*, 1992; Blatt and Armstrong, 1993). In spite of these results, it has been discussed that properties of R-type anion channels still implicate activation during ABA-induced stomatal closing and that these ion channels could provide a contribution to ABA-induced stomatal closing (Hedrich *et al.*, 1990; Schroeder and Keller, 1992; Schroeder *et al.*, 1993). In particular the enhancement of R-type currents by extracellular  $CaCl_2$  and by depolarization (Hedrich *et al.*, 1990) indicate that these anion channels could be active during ABA signalling. However, the question whether ABA activates R-type anion channels remains unknown at present.

During stomatal opening, inward-rectifying  $K_{in}^+$  channels have been characterized as a proposed major pathway for  $K^+$  uptake (Schroeder *et al.*, 1987; for reviews see Assmann, 1993; Ward *et al.*, 1995). These  $K_{in}^+$  channels have been described and characterized in guard cells from several species including *Vicia faba* (Schroeder *et al.*, 1987), maize (Fairley-Grenot and Assmann, 1993), potato (Müller-Röber *et al.*, 1995), tobacco (Armstrong *et al.*, 1995) and *Arabidopsis* (Pei *et al.*, 1997; Roelfsema and Prins, 1997). Comparison of maize and *Vicia faba*  $K_{in}^+$  channels showed that these ion channels in maize show more rapid activation kinetics (Fairley-Grenot and Assmann, 1993).  $K_{in}^+$  channel regulation in *Vicia* guard cells (Li *et al.*, 1994; Thiel and Blatt, 1994) correlates in terms of physiological responses to that of S-type anion channel regulation (Schmidt *et al.*, 1995). For example, in *Vicia faba* okadaic acid enhances ABA-induced stomatal closing and maintains S-type channel activity;

At the same time the inhibition of  $K_{in}^+$  channels by okadaic acid would favour inhibition of stomatal opening, based on the model for  $K_{in}^+$  channel function. Note however that a model (Li and Assmann, 1996) suggesting that the demonstrated down-regulation of outward-rectifying  $K^+$  ( $K_{out}^+$ ) channel activities by phosphorylation events in tobacco (Armstrong *et al.*, 1995) can be explained by the recent evidence for ABA-activated protein kinase activities in *Vicia faba*, would not fit into physiological models of  $K_{out}^+$  channel function. ABA-induced stomatal closing in tobacco would be inhibited if ABA-activated kinases down-regulate  $K_{out}^+$  channels in tobacco.

Regulation of  $K_{in}^+$  and  $K_{out}^+$  channels by various second messengers has been found including cytosolic  $Ca^{2+}$  (Schroeder and Hagiwara, 1989; Lemtiri-Chlieh and MacRobbie, 1994), pH (Blatt, 1992; Miedema and Assmann, 1996) and phosphorylation events. Counteracting effects of distinct protein phosphatases have also been proposed for regulation of guard cell  $K_{in}^+$  channel activity within the same species, implicating a reduction of  $K_{in}^+$  currents both by *activation* of a 'calciurein' protein phosphatase 2B-type (PP2B; Luan *et al.*, 1993) and by *inhibition* of okadaic acid-sensitive protein phosphatases (Thiel and Blatt, 1994; Li *et al.*, 1994). PP2Bs are OA insensitive. Use of two distinct types of phosphatases for distinct targets could explain the proposed counteracting effects of both types of phosphatases.

#### *Background anion conductance in guard cells may function as a negative regulator during stomatal opening*

In addition to the above described non-selective ion channels (Figs 1, 2), instantaneous currents were observed in *Arabidopsis* guard cells that had reversal potentials  $\approx +30$  mV as well as glutamate and acetate impermeability suggesting that they represent a small background activity of anion channels in *Arabidopsis* guard cells. The finding that guard cell anion channels are largely impermeable to acetate, has led to the suggestion the use of  $Cl^-$  in microelectrodes can greatly enhance the resolution of anion channel currents (Pei *et al.*, 1997). This finding should allow future enhanced-resolution microelectrode voltage clamp studies of anion channel regulation. A recent study has provided direct evidence that ABA activates S-type anion channels in guard cells (Pei *et al.*, 1997). Note that in *Arabidopsis* hypocotyls, blue light has also recently been demonstrated to activate anion channels, with properties similar to S-type channels, suggesting a role of these anion channels during hypocotyl movements (Cho and Spalding, 1996). Based on physiological stomatal movement analyses, guard cells of *Vicia faba*, *Commelina communis* and *Arabidopsis* have been predicted to have a background activity of anion channels, even when stomatal pores open in response to light. Evidence

for this suggestion comes from several independent studies showing that stomatal opening in light is wider when anion channels are completely inactivated by five different chemically distinct anion channel blockers (Schroeder *et al.*, 1993; Schwartz *et al.*, 1995) or when using protein kinase inhibitors, which shut down anion channel activity in *Vicia faba* (Schmidt *et al.*, 1995). Furthermore, it is well known that light-induced stomatal opening in *Vicia faba* and *Commelina communis* require extracellular  $K^+$  solutions containing  $\approx 30$  to  $>50$  mM  $K^+$  (Schwartz, 1985). Interestingly it was found that anion channel blockers allowed stomatal opening in response to light in *Commelina communis* at  $K^+$  concentrations of  $\leq 10$  mM (Schwartz *et al.*, 1995).

These data have been interpreted by a model in which a residual activity of anion channels prevails even when stomata are opening in response to light (Schroeder *et al.*, 1993). When this residual activity is down-regulated or blocked, stomata open wider. A further argument in favour of this hypothesis, is the weak voltage dependence of S-type anion channels, which could leave S-type anion channels partially active even when guard cells are strongly hyperpolarized (Schroeder and Hagiwara, 1989; Schroeder and Keller, 1992; Linder and Raschke, 1992). Because active anion channels would allow anion efflux, which produces depolarization, the predicted low background activity of guard cell anion channel currents would represent a negative feedback mechanism during stomatal opening.

Further evidence for this model was recently reported in *Arabidopsis*. In *Arabidopsis* guard cells the protein phosphatase inhibitor okadaic acid inhibits ABA signalling and ABA activation of S-type anion channels (Pei *et al.*, 1997; see above). The *Arabidopsis abil* mutant is ABA insensitive in terms of stomatal closing and anion channel activation (Pei *et al.*, 1997). The *ABII* gene has been shown to encode an okadaic acid-insensitive protein phosphatase type 2C (Meyer *et al.*, 1994; Leung *et al.*, 1994; Bertauche *et al.*, 1996). Interestingly, even in the *abil-1* mutant, guard cells also showed small instantaneous background currents, that were not changed by ABA (Pei *et al.*, 1997). Furthermore, in *Arabidopsis* stomatal movement assays, application of okadaic acid together with ABA causes larger stomatal opening, than with only ABA, which might indicate a background activity of anion channels (even in this ABA-insensitive mutant), which is down-regulated by okadaic acid (see Fig. 5F in Pei *et al.*, 1997). Note that other okadaic acid effects could also account for this effect in *abil*. Nevertheless, patch clamp studies on *abil* guard cells, show a residual instantaneous activity of anion currents, which comprises  $\approx 8\%$  of the ABA-activated levels in wild-type guard cells (Pei *et al.*, 1997).

The strong dynamic range in activity of guard cell anion channels caused by elevation in the cytosolic free

Ca<sup>2+</sup> concentration (Schroeder and Hagiwara, 1989), extracellular Ca<sup>2+</sup> (Hedrich *et al.*, 1990; 1994), extracellular Cl<sup>-</sup> (Lohse and Hedrich, 1995), phosphorylation events in *Vicia faba* (Schmidt *et al.*, 1995) and by ABA (Pei *et al.*, 1997) suggests that the level of anion channel activity in guard cells *in vivo* might depend on the degree of stomatal opening and the applied stimuli. The negative feedback role of slow anion channels during stomatal opening, indicates that during very wide stomatal apertures, S-type anion channels may need to be strongly down-regulated.

Evidence for this hypothesis was obtained in malate regulation experiments. Extracellular malate enhances the activity of R-type anion channels in guard cells (Hedrich *et al.*, 1994). At very high extracellular malate concentrations (40 mM) a partial stomatal closing response can be achieved in *Vicia faba* and *Commelina* guard cells (Esser *et al.*, 1997) when using all conditions as reported elsewhere (Hedrich *et al.*, 1994). Effects of protein phosphatase inhibitors and of protein kinase inhibitors on malate response show a good correlation to anion channel regulation mechanisms (Schmidt *et al.*, 1995; Esser *et al.*, 1997). However, when stomata are opened to very wide apertures in *Commelina* and in *Vicia faba* (e.g. >12 µm in *Vicia faba*) malate was completely ineffective at inducing stomatal closure (Esser *et al.*, 1997). These data support the hypothesis that wide stomatal opening may be accompanied by strong deactivation of guard cell anion channels.

Furthermore, these data suggest that malate can only function in enhancing anion currents when residual activation is present. For this reason and because of other observations of malate effects, the hypothesis that extracellular malate functions as a primary signal for guard cells in response to elevated CO<sub>2</sub> (Hedrich *et al.*, 1994), has been discussed as being unlikely (Esser *et al.*, 1997). An alternative model has been discussed in which extracellular malate is proposed to function as a positive feedback on anion channels during stomatal closing (Ward *et al.*, 1995), because guard cell anion channels are permeable to malate, and therefore malate efflux would occur during stomatal closing (Marten *et al.*, 1992; Schmidt and Schroeder, 1994). These data also provide a functional basis for the finding that ABA causes malate and chloride release from guard cells (Van Kirk and Raschke, 1978; MacRobbie, 1981). To sum up, several studies using different anion channel modulators have indicated that a background activity of anion channels probably prevails under most physiological conditions. The large dynamic range in anion channel activity further indicates that the level of anion channel activity should depend on the physiological status of guard cells during stomatal movements.

Exposure of *Arabidopsis* (*Landsburg erecta*) guard cells to ABA led to a strong activation of S-type anion channels

(Pei *et al.*, 1997). An average 13-fold enhancement of S-type anion channel currents above background was found. Therefore the low instantaneous background conductance reported in the present study represents at most an 8% background anion conductance when compared to the large ABA-activated S-type anion channel currents (Pei *et al.*, 1997). In the present study it has not been determined whether this background conductance is carried by the S-type anion channels that have been previously characterized in *Vicia faba*, *Arabidopsis* and tobacco guard cells (Schroeder and Hagiwara, 1989; Schroeder and Keller, 1992; Linder and Raschke, 1992; Pei *et al.*, 1997; Grabov *et al.*, 1997). It is quite possible that additional types of anion channels exist in guard cells that contribute to small background conductances.

In tobacco guard cells, S-type anion channel with voltage- and time-dependence and okadaic acid stimulation similar to properties in *Vicia faba* were recently described (Grabov *et al.*, 1997). In tobacco guard cells ABA activated S-type anion currents, even in the presence of the dominant *Arabidopsis* *abi1* transgene (Grabov *et al.*, 1997). The observation that transgenic expression of the *Arabidopsis* mutant *abi1-1* cDNA in tobacco does not affect ABA activation of anion channels (Grabov *et al.*, 1997), has been discussed as a possible result of species-specific effects (Pei *et al.*, 1997). Nevertheless, analyses of K<sup>+</sup> channel regulation in tobacco guard cells show interesting effects of the *abi1-1* transgene (Armstrong *et al.*, 1995).

All studies discussed above provide circumstantial evidence for a background activity of anion channels in the absence of ABA in guard cells. This background activity appears to, among other functions, provide a negative feedback mechanism to control the degree of stomatal opening. In the present study the fact that activation of large S-type anion currents in *Arabidopsis* guard cells requires ABA application (Pei *et al.*, 1997) has been used to show that a background anion conductance exists.

In conclusion, patch clamp studies in *Arabidopsis* guard cells have recently been developed in our laboratory (Pei *et al.*, 1997). It is likely that, when patch clamping guard cells from different species, new characteristics and new types of ion channels or regulatory mechanisms become more apparent than in guard cells from other species. In the present study, an initial description of large conductance ion channel types that are reproducibly detected in *Arabidopsis* guard cells has been provided. It has also been demonstrated that there is a background anion conductance in *Arabidopsis* guard cells and the possible roles of these guard cell conductances for stomatal physiology have been discussed. Further studies of these ion conductances as well as other ion channels in *Arabidopsis* guard cells should expand our understanding of early signal transduction mechanisms during stomatal movements.

## Acknowledgements

This research was supported by National Science Foundation grant MCB-9506191 (JIS) and by a Deutsche Forschungs-Gemeinschaft postdoctoral fellowship (MS). Figure 4 in the present manuscript was reproduced from Pei *et al.* (1997) with permission from the American Society of Plant Physiologists.

## References

- Allen GJ, Sanders D. 1996. Control of ionic currents in guard cell vacuoles by cytosolic and luminal calcium. *The Plant Journal* **10**, 1055–69.
- Armstrong F, Leung J, Grabov A, Brearley J, Giraudat J, Blatt MR. 1995. Sensitivity to abscisic acid of guard cell K<sup>+</sup> channels is suppressed by ABI1-1, a mutant *Arabidopsis* gene encoding a putative protein phosphatase. *Proceedings of the National Academy of Sciences, USA* **92**, 9520–4.
- Assmann SM. 1993. Signal transduction in guard cells. *Annual Review of Cell Biology* **9**, 345–75.
- Bertauche N, Leung J, Giraudat J. 1996. Protein phosphatase activity of abscisic acid insensitive 1 (ABI1) protein from *Arabidopsis thaliana*. *European Journal of Biochemistry* **241**, 193–200.
- Blatt MR. 1992. Potassium channels of stomatal guard cells: characteristics of the inward rectifier and its control by pH. *Journal of General Physiology* **99**, 615–44.
- Blatt MR, Armstrong F. 1993. Potassium channels of stomatal guard cells abscisic acid-evoked control of the outward rectifier mediated by cytoplasmic pH. *Planta* **191**, 330–41.
- Cho MH, Spalding EP. 1996. An anion channel in *Arabidopsis* hypocotyls activated by blue light. *Proceedings of the National Academy of Sciences, USA* **93**, 8134–8.
- Cosgrove DJ, Hedrich R. 1991. Stretch-activated chloride, potassium, and calcium channels coexisting in plasma membranes of guard cells of *Vicia faba* L. *Planta* **186**, 143–53.
- DeSilva DLR, Cox RC, Hetherington AM, Mansfield TA. 1985. Synergism between calcium ions and abscisic acid in preventing stomatal opening. *New Phytologist* **101**, 555–63.
- Esser J, Liao Y-J, Schroeder JI. 1997. Characterization of ion channel modulator effects on ABA- and malate-induced stomatal movements: strong regulation by kinase and phosphatase inhibitors, and relative insensitivity to mastoparans. *Journal of Experimental Botany* **48**, 539–50.
- Fairley-Grenot KA, Assmann SM. 1993. Comparison of K<sup>+</sup>-channel activation and deactivation in guard cells from a dicotyledon (*Vicia faba* L.) and a graminaceous monocotyledon (*Zea mays*). *Planta* **189**, 410–19.
- Grabov A, Leung J, Giraudat J, Blatt MR. 1997. Alteration of anion channel kinetics in wild-type and *abi1-1* transgenic *Nicotiana benthamiana* guard cells by abscisic acid. *The Plant Journal* **12**, 203–13.
- Hedrich R, Busch H, Raschke K. 1990. Ca<sup>2+</sup> and nucleotide dependent regulation of voltage dependent anion channels in the plasma membrane of guard cells. *EMBO Journal* **9**, 3889–92.
- Hedrich R, Marten I, Lohse G, Dietrich P, Winter H, Lohaus G, Heldt H-W. 1994. Malate-sensitive anion channels enable guard cells to sense changes in the ambient CO<sub>2</sub> concentration. *The Plant Journal* **6**, 741–8.
- Hetherington AM, Quatrano RS. 1991. Mechanisms of action of abscisic acid at the cellular level. *New Phytologist* **119**, 9–32.
- Irving HR, Gehring CA, Parish RW. 1992. Changes in cytosolic pH and calcium of guard cells precede stomatal movements. *Proceedings of the National Academy of Sciences, USA* **89**, 1790–4.
- Lemtiri-Chlieh F, MacRobbie EAC. 1994. Role of calcium in the modulation of *Vicia* guard cell potassium channels by abscisic acid – a patch-clamp study. *Journal of Membrane Biology* **137**, 99–107.
- Leung J, Bouvier-Durand M, Morris P-C, Guerrier D, Chefdor F, Giraudat J. 1994. *Arabidopsis* ABA response gene *ABI1* – features of a calcium-modulated protein phosphatase. *Science* **264**, 1448–52.
- Li J, Assmann SM. 1996. An abscisic acid-activated and calcium-independent protein kinase from guard cells of fava bean. *The Plant Cell* **8**, 2359–68.
- Li W, Luan S, Schreiber SL, Assmann SM. 1994. Evidence for protein phosphatase 1 and 2A regulation of K<sup>+</sup> channels in two types of leaf cells. *Plant Physiology* **106**, 963–70.
- Linder B, Raschke K. 1992. A slow anion channel in guard cells activating at large hyperpolarization may be principal for stomatal closing. *FEBS Letters* **313**, 27–30.
- Lohse G, Hedrich R. 1995. Anions modify the response of guard-cell anion channels to auxin. *Planta* **197**, 546–52.
- Luan S, Li WW, Rusnak F, Assmann SM, Schreiber SL. 1993. Immunosuppressants implicate protein phosphatase regulation of K<sup>+</sup> channels in guard cells. *Proceedings of the National Academy of Sciences, USA* **90**, 2202–6.
- MacRobbie EAC. 1981. Effects of ABA in 'isolated' guard cells of *Commelina communis* L. *Journal of Experimental Botany* **32**, 563–72.
- Marten I, Lohse G, Hedrich R. 1991. Plant growth hormones control voltage-dependent activity of anion channels in plasma membrane of guard cells. *Nature* **353**, 758–62.
- Marten I, Zeilinger C, Redhead C, Landry DW, Alawqati Q, Hedrich R. 1992. Identification and modulation of a voltage-dependent anion channel in the plasma membrane of guard cells by high-affinity ligands. *EMBO Journal* **11**, 3569–75.
- McAinsh MR, Brownlee C, Hetherington AM. 1990. Abscisic acid-induced elevation of guard cell cytosolic Ca<sup>2+</sup> precedes stomatal closure. *Nature* **343**, 186–8.
- McAinsh MR, Webb AAR, Taylor JE, Hetherington AM. 1995. Stimulus-induced oscillations in guard cell cytosolic free calcium. *The Plant Cell* **7**, 1207–19.
- Meyer K, Leube MP, Grill E. 1994. A protein phosphatase 2C involved in ABA signal transduction in *Arabidopsis thaliana*. *Science* **264**, 1452–5.
- Miedema H, Assmann SM. 1996. A membrane-delimited effect of internal pH on the K<sup>+</sup> outward rectifier of *Vicia faba* guard cells. *Journal of Membrane Biology* **154**, 227–37.
- Mori IC, Muto S. 1997. Abscisic acid activates a 48-kilodalton protein kinase in guard cell protoplasts. *Plant Physiology* **113**, 833–40.
- Müller-Röber B, Ellenberg J, Provart N, Willmitzer L, Busch H, Becker D, Dietrich P, Hoth S, Hedrich R. 1995. Cloning and electrophysiological analysis of KST1, an inward rectifying K<sup>+</sup> channel expressed in potato guard cells. *EMBO Journal* **14**, 2409–16.
- Pei Z-M, Kuchitsu K, Ward JM, Schwarz M, Schroeder JI. 1997. Differential abscisic acid regulation of guard cell slow anion channels in *Arabidopsis* wild-type and *abi1* and *abi2* mutants. *The Plant Cell* **9**, 409–23.
- Roelfsema MRG, Prins HBA. 1997. Ion channels in guard cells of *Arabidopsis thaliana* (L.) Heynh. *Planta* **202**, 8–27.
- Schmidt C, Schelle I, Liao Y-J, Schroeder JI. 1995. Strong regulation of slow anion channels and abscisic acid signalling in guard cells by phosphorylation and dephosphorylation events. *Proceedings of the National Academy of Sciences, USA* **92**, 9535–9.

- Schmidt C, Schroeder JI.** 1994. Anion selectivity of slow anion channels in the plasma membrane of guard cells: large nitrate permeability. *Plant Physiology* **106**, 383–91.
- Schroeder JI, Hagiwara S.** 1989. Cytosolic calcium regulates ion channels in the plasma membrane of *Vicia faba* guard cells. *Nature* **338**, 427–30.
- Schroeder JI, Hagiwara S.** 1990. Repetitive increases in cytosolic  $\text{Ca}^{2+}$  of guard cells by abscisic acid activation of non-selective  $\text{Ca}^{2+}$  permeable channels. *Proceedings of the National Academy of Sciences, USA* **87**, 9305–9.
- Schroeder JI, Keller BU.** 1992. Two types of anion channel currents in guard cells with distinct voltage regulation. *Proceedings of the National Academy of Sciences, USA* **89**, 5025–9.
- Schroeder JI, Raschke K, Neher E.** 1987. Voltage dependence of  $\text{K}^+$  channels in guard cell protoplasts. *Proceedings of the National Academy of Sciences, USA* **84**, 4108–12.
- Schroeder JI, Schmidt C, Sheaffer J.** 1993. Identification of high-affinity slow anion channel blockers and evidence for stomatal regulation by slow anion channels in guard cells. *The Plant Cell* **5**, 1831–41.
- Schulz-Lessdorf B, Lohse G, Hedrich R.** 1996. GCAC1 recognizes the pH gradient across the plasma membrane: a pH-sensitive and ATP-dependent anion channel links guard cell membrane potential to acid and energy metabolism. *The Plant Journal* **10**, 993–1004.
- Schwartz A.** 1985. Role of calcium and EGTA on stomatal movements in *Commelina communis*. *Plant Physiology* **79**, 1003–5.
- Schwartz A, Ilan N, Schwarz M, Scheaffer J, Assmann SM, Schroeder JI.** 1995. Anion-channel blockers inhibit S-type anion channels and abscisic acid responses in guard cells. *Plant Physiology* **109**, 651–8.
- Thiel G, Blatt MR.** 1994. Phosphatase antagonist okadaic acid inhibits steady-state  $\text{K}^+$  currents in guard cells of *Vicia faba*. *The Plant Journal* **5**, 727–33.
- Van Kirk CA, Raschke K.** 1978. Release of malate from epidermal strips during stomatal closure. *Plant Physiology* **61**, 474–5.
- Ward JM, Pei Z-M, Schroeder JI.** 1995. Roles of ion channels in initiation of signal transduction in higher plants. *The Plant Cell* **7**, 833–44.
- Zimmermann S, Nürnberg T, Frachisse J-M, Wirtz W, Guern J, Hedrich R, Scheel D.** 1997. Receptor-mediated activation of a plant  $\text{Ca}^{2+}$ -permeable ion channel involved in pathogen defense. *Proceedings of the National Academy of Sciences, USA* **94**, 2751–5.