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Expression of a Cs^+-Resistant Guard Cell K^+ Channel Confers Cs^+-Resistant, Light-Induced Stomatal Opening in Transgenic Arabidopsis

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Inward-rectifying K^+ (K^+_{in}) channels in the guard cell plasma membrane have been suggested to function as a major pathway for K^+ influx into guard cells during stomatal opening. When K^+_{in} channels were blocked with external Cs^+ in wild-type Arabidopsis guard cells, light-induced stomatal opening was reduced. Transgenic Arabidopsis plants were generated that expressed a mutant of the guard cell K^+_{in} channel, KAT1, which shows enhanced resistance to the Cs^+ block. Stomata in these transgenic lines opened in the presence of external Cs^+. Patch–clamp experiments with transgenic guard cells showed that inward K^+_{in} currents were blocked less by Cs^+ than were K^+ currents in controls. These data provide direct evidence that KAT1 functions as a plasma membrane K^+ channel in vivo and that K^+_{in} channels constitute an important mechanism for light-induced stomatal opening. In addition, biophysical properties of K^+_{in} channels in guard cells indicate that components in addition to KAT1 may contribute to the formation of K^+_{in} channels in vivo.

INTRODUCTION

Control of stomatal pore apertures in plant leaves is important for regulating CO_2 flow into leaves for photosynthesis and for controlling water loss through transpiration (Raschke, 1979; Zeiger et al., 1987). The turgor and volume of the pairs of guard cells that surround stomatal pores determine the stomatal aperture. An increase in the turgor and volume of guard cells, which is mediated by an increase in the guard cell K^+ salt and water content, results in the opening of stomatal pores (Imamura, 1943; Humble and Hsiao, 1969; Humble and Raschke, 1971; MacRobbie, 1983).

The plasma membrane of guard cells exhibits inward-rectifying, hyperpolarization-activated K^+ (K^+_{in}) channels that have been proposed to provide a molecular pathway for proton pump–driven K^+ uptake (Schroeder et al., 1984, 1987; Thiel et al., 1992; Fairley-Grenot and Assmann, 1993). Environmental signals, such as light, cause activation of plasma membrane H^+-ATPases, resulting in hyperpolarization (Assmann et al., 1985; Shimazaki et al., 1986) and K^+ uptake (Raschke, 1979; MacRobbie, 1983; Schroeder et al., 1987; Thiel et al., 1992). In addition, extracellular acidification by proton pumps further enhances the activity of guard cell K^+_{in} channels (Blatt, 1992; Ilan et al., 1996).

The model that K^+_{in} channels provide a major pathway for K^+ uptake during stomatal opening has been supported by biophysical, second messenger regulation, and physiological studies (reviewed in Assmann, 1993; Schroeder et al., 1994). However, stomatal movement studies with K^+_{in} channel blockers have failed to show inhibition of stomatal opening by such blockers (Kelly et al., 1995). In addition, molecular physiological studies using cloned K^+_{in} channel cDNAs to study the proposed role of K^+_{in} channels for stomatal opening have, to our knowledge, not been reported.

Several plant K^+ channel cDNAs have been isolated by complementation of yeast mutants and homology screening and include *KAT1*, *AKT1*, *AKT2*, and *KST1* (Anderson et al., 1992; Sentenac et al., 1992; Cao et al., 1995; Müller-Rober et al., 1995). Heterologous expression studies with Xenopus oocytes and yeast showed that the expressed proteins encode the electrical characteristics typical of plant K^+_{in} channel currents (Schachtman et al., 1992; Bertl et al., 1994; Müller-Rober et al., 1995; Ketchum and Slayman, 1996). Based on RNA hybridization analysis and promoter–β-glucuronidase (*GUS*) fusions, *AKT1* is expressed mainly in roots and *AKT2* is highly expressed in shoots and leaves (Cao et al.,1995; Lagarde et al., 1996). The Arabidopsis K^+_{in} channel, *KAT1*, was shown to be expressed mainly in guard cells (Nakamura et al., 1995).

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K^+_{in} channels have been described for a variety of plant species and cell types, such as suspension culture cells of Arabidopsis, maize, and tobacco (Ketchum et al., 1989; Colombo and Cerana, 1991; VanDuijn et al., 1993), guard cells of fava bean, maize, and potato (Schroeder et al., 1987; Fairley-Grenot and Assmann, 1993; Müller-Rober et al., 1995), mesophyll cells (Kourie and Goldsmith, 1992; Spalding et al., 1992), pulvinus cells (Moran and Satter, 1989; Kim et al., 1993), aleurone cells (Bush et al., 1988), coleoptiles (Thiel et al., 1996), and different types of root cells (White and Tester, 1992; Findlay et al., 1994; Gassmann and Schroeder, 1994; Wegner et al., 1994; Maathuis and Sanders, 1995; Roberts and Tester, 1995). The wide distribution and activity levels of plant K^+_{in} channels support theories that these K^+ channels perform fundamental tasks in plant biology (reviewed in Schroeder et al., 1994; Maathuis et al., 1997). In this study, we have pursued the analysis of the guard cell K^+ channel KAT1 (Nakamura et al., 1995) in transgenic Arabidopsis plants to test its proposed role in light-induced stomatal opening at the molecular physiological level.

A *KAT1* mutant with point mutations in the 3′ flanking region of the pore domain (H267T/E269V) of the channel showed increased resistance to an extracellular tetraethylammonium (TEA^+) block, suggesting that this region faces the extracellular milieu (Ichida and Schroeder, 1996). In addition to TEA^+, the H267T/E269V mutant also showed increased resistance to voltage-dependent block by external Cs^+ (Ichida and Schroeder, 1996). Cesium is a relatively effective blocker compared with other known plant K^+_{in} channel blockers. For example, 10 mM Ba^{2+}, which blocks 90% of K^+_{in} channel currents in fava bean guard cells, does not inhibit stomatal opening but rather slows it (Kelly et al., 1995). A clear inhibition of stomatal opening by K^+_{in} channel blockers has to our knowledge not yet been reported, reflecting the need for a molecular physiological analysis of their proposed role in guard cells.

Many K^+_{in} channel blockers may show only small effects because the block of K^+_{in} currents should result in more negative membrane potentials, as a result of the continuing activity of proton pumps. Hyperpolarization in turn enhances K^+_{in} channel activity, lessening the blocker effect (see Kelly et al., 1995). However, in the case of Cs^+ block, hyperpolarization enhances Cs^+ inhibition of K^+_{in} currents, because Cs^+ enters K^+_{in} channels to induce block in a voltage-dependent manner (Kourie and Goldsmith, 1992; Very et al., 1995; Ichida and Schroeder, 1996). The effectiveness and mechanism of the Cs^+ block of K^+_{in} channel currents suggested to us that this blocker could be used for physiological analyses of K^+_{in} channel function in guard cells in vivo. Expression in transgenic Arabidopsis plants of the H267T/E269V mutant *KAT1* channel displaying increased resistance to Cs^+ block was used here to study the molecular physiology of K^+_{in} channels in guard cells. Stomatal opening assays and functional analysis of Arabidopsis guard cell K^+_{in} channel activities by patch–clamp analysis reported here support the model that K^+_{in} channels play an important role in mediating stomatal opening by light.

RESULTS

Arabidopsis Stomatal Opening Is Inhibited by External CsCl

Wild-type Arabidopsis stomata were closed by dark and elevated CO_2 treatment and subsequently opened by 2 hr of exposure to white light in KCl solutions, as shown in Figures 1A and 1B. When 30 mM CsCl was included in the stomatal opening solution, wild-type stomatal opening was significantly inhibited (Figures 1A and 1C). The addition of 10 mM CsCl to the bathing medium resulted in only an intermediate inhibition of stomatal opening (data not shown), which correlates to findings suggesting that small K^+_{in} channel cur-

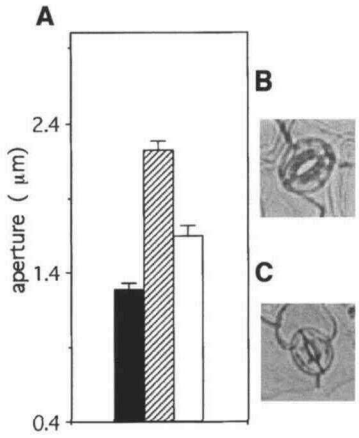

Figure 1. Cs^+ Inhibition of Light-Induced Stomatal Opening in Wild-Type Arabidopsis.

(A) Average stomatal aperture before treatment with stomatal opening conditions (solid bar). Data from wild-type plants show that stomata open after 2 hr of incubation in light with 10 mM KCl (diagonally striped bar). Stomatal pores that are incubated for 2 hr in 10 mM KCl and 30 mM CsCl show a significant inhibition of stomatal opening (open bar). Error bars indicate standard error of the mean (n = 200 stomata measured for each bar in 10 independent experiments).

(B) An example of an Arabidopsis stomatal complex is shown from wild-type Arabidopsis (Columbia ecotype) after 2 hr of exposure to light in 10 mM KCl solution, which produces stomatal opening.

(C) Stomata from wild-type Arabidopsis Columbia ecotype are prevented from opening after 2 hr of exposure to light in 10 mM KCl plus 30 mM CsCl.

Stomatal opening solutions in all experiments in the present study include 10 mM KCl, 10 mM Mes, and 300 μM anthrocene-9-carboxylate, pH 6.15 (Schwartz et al., 1995).

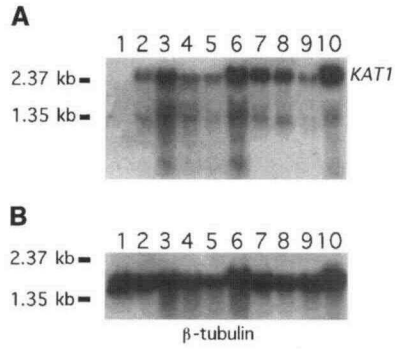

Figure 2. RNA Gel Blot with the *KAT1* Probe Showing mRNA in Transgenic Arabidopsis.

(A) Lane 1 contains wild-type Columbia plant RNA with the guard cell–specific endogenous *KAT1* message. Wild-type plants do not exhibit a detectable signal on whole-plant RNA gel blots (Cao et al., 1995). Lane 2 contains the *KAT1* transgene expressed in Arabidopsis line 1.11; lanes 3 to 6 contain the *KAT1* transgene expressed in four individuals from line 3.14; lanes 7 and 8 contain the H267T/E269V transgene expressed in two individual plants from line 22.12; and lanes 9 and 10 contain the H267T/E269V transgene expressed in two individual plants from line 23.23.
(B) Arabidopsis β-tubulin mRNA shows relative quantities of sample loading on the same blot as **(A)**.
Size markers are indicated at left.

rents seem to be sufficient to allow stomatal opening (Kelly et al., 1995). Increasing the extracellular Cs^+ concentration to 30 mM consistently showed a stronger inhibition of stomatal opening (Figure 1A; $n = 600$ stomata, $P < 2 \times 10^{-9}$).

KAT1 Expression Levels in Transgenic Arabidopsis

To analyze the molecular physiological properties of the guard cell K^+_{in} channel, KAT1, independent transgenic Arabidopsis lines were generated in which either the Cs^+-resistant H267T/E269V *KAT1* mutant or the wild-type *KAT1* gene as a control was stably expressed. To test for *KAT1* expression in transgenic Arabidopsis plants, RNA gel blot hybridization was performed. Figure 2 shows *KAT1* mRNA levels in 10 representative individuals. In wild-type Arabidopsis, *KAT1* mRNA could not be detected by RNA gel blot analysis of whole plants (Figure 2A, lane 1; Cao et al., 1995). This can be attributed to the finding that *KAT1* expression, as determined by a promoter–*GUS* fusion, is limited mainly to guard cells (Nakamura et al., 1995).

RNA gel blot analysis of homozygous transgenic *KAT1*- and H267T/E269V-expressing lines showed high levels of transgene mRNA in kanamycin-resistant lines (Figure 2A, lanes 2 to 10). Lane 2 shows *KAT1* hybridization with total RNA from transgenic Arabidopsis plants expressing the wild-type *KAT1* cDNA. Lanes 3 to 6 show *KAT1* hybridization with total RNA from individuals from another line of transgenic Arabidopsis expressing the wild-type *KAT1* cDNA. Lanes 7 and 8 show *KAT1* hybridization with total RNA from two individuals from one line of transgenic Arabidopsis expressing the Cs^+-resistant mutant H267T/E269V cDNA. Lanes 9 and 10 show *KAT1* hybridization with total RNA from two individuals from another line of transgenic Arabidopsis expressing the H267T/E269V cDNA. Relative quantitation of sample loading was assayed by hybridization to the β-tubulin message (Figure 2B) and by ethidium bromide staining of total RNA (data not shown). These data show successful overexpression of *KAT1* transcripts in transgenic Arabidopsis lines. Because guard cell tissue is a small fraction ($<<0.1\%$) of the total mass of a whole plant, evidence of overall expression of *KAT1* mRNA is not a direct indicator of transgene mRNA expression level in guard cell tissue.

Expression of Mutant K^+_{in} Channel H267T/E269V Confers Cs^+-Resistant Stomatal Opening in Arabidopsis

Transgenic Arabidopsis plants ectopically expressing *KAT1* and the H267T/E269V mutant showed normal whole-plant growth behavior comparable with wild-type plants under standard growth chamber conditions in soil (see Methods; $n > 300$ transgenic plants). This result can be explained by the findings that K^+_{in} channels are expressed in many plant cell types. Ectopic expression of these channels is not expected to give rise to visible phenotypes under nonstress conditions, based on proposed models of K^+_{in} channel function in which K^+_{in} channel activity is necessary but not disruptive for nonstress, nonextreme physiological responses (Schroeder et al., 1994). Because *KAT1* is expressed in Arabidopsis guard cells in vivo (Nakamura et al., 1995), subsequent experiments were pursued to determine the effect of the different *KAT1* transgenes on stomatal movements. Note that all stomatal assays performed for this study were blind assays in which the backgrounds of the plants (transgenic *KAT1*, transgenic H267T/E269V, or the wild type) were not known to the researcher (see Methods).

For control experiments, stomatal opening was assayed in transgenic Arabidopsis lines overexpressing the wild-type *KAT1* cDNA. The average stomatal responses for two independent transgenic lines are shown in Figure 3A (line 3.14, single-insertion homozygous, and line 1.11, estimated at two to four insertions). In *KAT1*-expressing lines, light caused stomatal opening (Figures 3A and 3B). Stomatal opening was partially inhibited by 30 mM CsCl in stomata from transgenic Arabidopsis plants expressing the wild-type *KAT1* cDNA (Figures 3A and 3C; $n = 900$ total stomata from 15 plants). Stomatal opening in wild-type Arabidopsis control lines was inhibited by Cs^+ by an average of 61% (Figure 1; $P < 2 \times 10^{-9}$). Likewise, stomatal opening in *KAT1*-overexpressing lines was inhibited by Cs^+ by an average of 46%

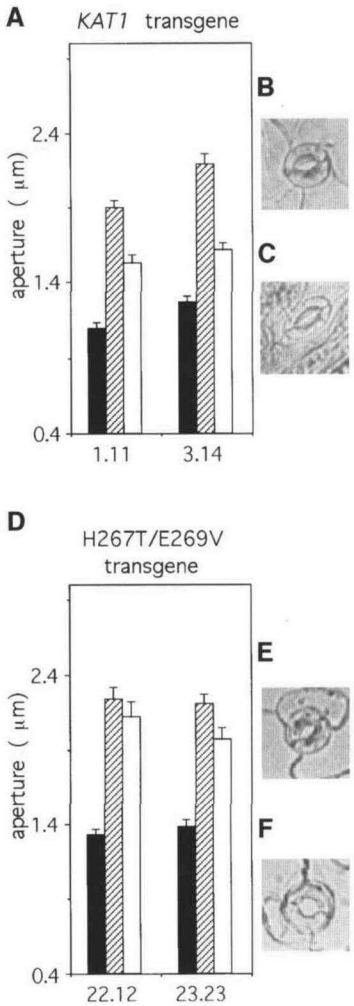

Figure 3. Expression of the H267T/E269V *KAT1* Mutant Transgene Leads to Stomatal Opening That Is Less Sensitive to Cs^+.

(A) Average stomatal aperture before treatment with stomatal opening conditions (solid bars; line 1.11, $n = 200$; line 3.14, $n = 100$). Data from *KAT1*-expressing plants show that stomata open after 2 hr of incubation in 10 mM KCl (diagonally striped bars; line 1.11, $n = 200$; line 3.14, $n = 100$). Stomata that were incubated in 10 mM KCl and 30 mM CsCl show inhibition of stomatal opening (open bars; line 1.11, $n = 200$; line 3.14, $n = 100$). Error bars indicate standard error.

(B) A stomatal complex from an Arabidopsis leaf expressing the *KAT1* transgene opens after 2 hr of exposure to light in a 10 mM KCl solution.

(C) Stomatal pores from Arabidopsis leaf expressing the *KAT1* transgene are prevented from opening after 2 hr of exposure to light in 10 mM KCl plus 30 mM CsCl.

(D) Average stomatal aperture before treatment with stomatal opening conditions (solid bars; line 22.12, $n = 120$; line 23.23, $n = 100$). Data from H267T/E269V-expressing plants show that stomata open after 2 hr of incubation in 10 mM KCl (diagonally striped bars; line 22.12, $n = 120$; line 23.23, $n = 100$). Stomata that were incubated in 10 mM KCl and 30 mM CsCl also open (open bars; line 22.12, $n = 120$; line 23.23, $n = 100$). Error bars indicate standard error.

in line 1.11 ($P < 1.5 \times 10^{-6}$) and by an average of 63% in line 3.14 ($P < 2 \times 10^{-10}$). Relative changes in percentage in stomatal apertures were determined after subtraction of background stomatal apertures at time zero. P values compare data at a t of 2 hr with or without CsCl.

Stomatal opening also was assayed in transgenic Arabidopsis lines overexpressing the Cs^+-resistant *KAT1* mutant H267T/E269V. Stomatal opening was not strongly inhibited by 30 mM CsCl in stomata from H267T/E269V-expressing transgenic Arabidopsis, as shown in Figure 3D ($n = 660$ total stomata measured from 11 plants). Data from two separate single-insertion homozygous transgenic lines are illustrated in Figure 3D. Examples of light-induced stomatal opening in Cs^+-free solution (Figure 3E) and in 30 mM Cs^+-containing solution (Figure 3F) are illustrated. Stomatal opening in H267T/E269V-expressing lines was on average only inhibited by 13% in line 22.12 (Figure 3D; $P < 0.34$) and by 29% in line 23.23 ($P < 0.014$). These data demonstrate the ability of the H267T/E269V-expressing lines to show an enhanced Cs^+ resistance of light-induced stomatal opening.

The height of Arabidopsis stomata can vary within one leaf (Roelfsema and Prins, 1995). Therefore, the height of all stomatal pores was measured and recorded in all experiments (for experimental definitions of stomatal height and aperture, see Methods). Analysis of stomatal pore heights showed that this parameter did not vary significantly among the wild-type and transgenic lines analyzed here. For experiments illustrated in Figure 1, wild-type Columbia stomatal height averaged 21.04 ± 1.14 μm ($n = 600$ stomata, from 10 different plants). In plants expressing H267T/E269V (line 22.12), stomatal height was 22.1 ± 0.63 μm ($n = 360$ from six plants); for H267T/E269V (line 23.23), stomatal height was 23.5 ± 1.8 μm ($n = 300$ from five plants). In plants expressing the wild-type *KAT1* (line 1.11), stomatal height averaged 20.3 ± 0.96 μm ($n = 600$ from 10 plants); for another *KAT1*-expressing line (line 3.14), stomatal height averaged 20.2 ± 0.19 μm ($n = 300$ from five plants). In addition, analysis of light-induced stomatal opening by plotting ratios of stomatal aperture to height (Roelfsema and Prins, 1995) showed results similar to those for stomatal apertures, as illustrated in Figure 4A. Therefore, we concluded that expression of K^+_{in} channels in transgenic plants did not significantly influence stomatal height and that the observed

(E) A stomatal complex from Arabidopsis expressing the H267T/E269V transgene opens after 2 hr of exposure to light in a 10 mM KCl solution.

(F) A stomatal complex from Arabidopsis expressing the H267T/E269V transgene opens after 2 hr of exposure to light in a 10 mM KCl plus 30 mM CsCl solution. An above average wide-open stoma is shown.

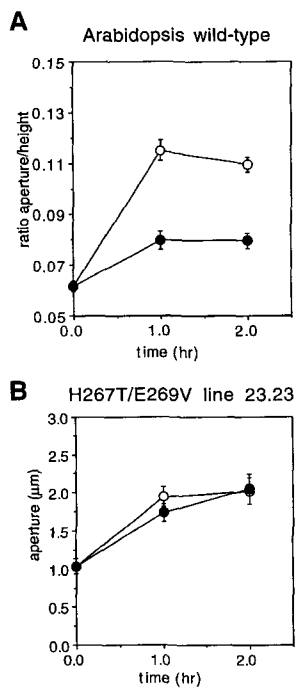

Figure 4. Time Dependence of Light-Induced Stomatal Opening.

(A) Wild-type stomata open when exposed to light and incubated in 10 mM KCl plus 300 μM anthrocene-9-carboxylate (open circles). Opening is blocked by 30 mM external CsCl (filled circles). The ratio of stomatal pore height to pore aperture is shown on the y-axis (n = 200 stomata measured for each graph symbol). Error bars indicate the standard error.

(B) H267T/E269V-expressing plants (line 23.23) have stomata that show time-dependent opening (open circles) and opening in the presence of CsCl (filled circles). Representative data from one plant are illustrated (n = 20 stomata per symbol). Error bars indicate the standard error.

stomatal opening phenotypes were not a result of differences in stomatal pore heights.

The time course of stomatal opening in response to light showed a variability that is typical of stomatal responses in other systems (Gorton et al., 1988). Nevertheless, the 2-hr time period for which average results in Figures 1 and 3 are shown was sufficient to detect differences in stomatal opening, as illustrated in Figure 4. The clear Cs^+ sensitivity of the wild-type line and the Cs^+ resistance of an H269T/E269V-expressing line are apparent (Figures 4A and 4B).

Guard Cells from Arabidopsis Expressing H267T/E269V Have Inward K^+ Currents with an Increased Resistance to Block by Cs^+

Physiological differences in stomatal response to Cs^+, determined by measuring stomatal aperture, indicated that properties of K^+_{in} channel guard cells from Columbia wild-type and the transgenic lines may be different. To establish a high-resolution assay for K^+_{in} channel function in transgenic Arabidopsis lines, patch–clamp studies with Arabidopsis guard cells were pursued. Patch–clamp solutions were used that allowed the resolution of guard cell K^+_{in} channels while reducing contributions of other ion channel activities (Schroeder et al., 1987). Figure 5 illustrates patch–clamp analysis for four representative guard cells. Upon hyperpolarization of Arabidopsis guard cells, K^+_{in} channels were activated, resulting in inward K^+ currents (Figure 5A), similar to those described for fava bean (Schroeder et al., 1987), maize (Fairley-Grenot and Assmann, 1993), tobacco (Armstrong et al., 1995), and potato (Müller-Rober et al., 1995). Wild-type (Columbia) Arabidopsis guard cell K^+_{in} channels were blocked to a large degree by 5 mM external CsCl in the presence of 30 mM external KCl (Figures 5B and 5C). Even in the presence of 5 mM Cs^+, small residual time-dependent K^+ currents were observed (Figure 5B). The average residual time-dependent inward current with 5 mM CsCl block was -8.5 ± 1.3 pA (at -200 mV), which is estimated to be sufficient to allow stomatal opening (see Discussion).

Guard cells from transgenic Arabidopsis plants expressing the wild-type *KAT1* transgene were also analyzed. Guard cells from these transgenic lines also showed K^+_{in} currents (Figures 5D and 5G). Measurable levels of *KAT1* ectopic expression (Figure 2, lanes 2 to 6) did not greatly alter the guard cell K^+ channel response to Cs^+ block (Figures 5D to 5I). K^+_{in} channel currents were smaller in the 1.11 *KAT1*-expressing transgenic line under the imposed conditions when compared with the 3.14 *KAT1*-expressing transgenic line (e.g., compare Figures 5D and 5G).

Guard cells from transgenic plants showing enhanced overall expression of the Cs^+-resistant H267T/E269V transcript were analyzed by patch clamping. K^+_{in} channel currents in guard cells from H267T/E269V-expressing Arabidopsis were dramatically less sensitive to Cs^+ block (Figures 5J to 5L). The difference in guard cell response to Cs^+ block between the *KAT1*- and the H267T/E269V-expressing plants can be seen in representative current–voltage plots (Figures 5C, 5F, 5I, and 5L). The Cs^+ resistance of the H267T/E269V-expressing guard cells correlates well with the enhanced Cs^+ resistance of this mutant analyzed in Xenopus oocytes (Ichida and Schroeder, 1996). These data provide direct evidence that KAT1 is a plasma membrane K^+_{in} channel in vivo, as was predicted from functional characterization in the heterologous yeast and Xenopus oocyte systems (Anderson et al., 1992; Schachtman et al., 1992).

The time course of activation differed among the illustrated recordings (Figures 5A, 5D, 5G, and 5J). Overexpression of H267T/E269V led to larger K^+_{in} currents that also showed a more rapid activation in response to hyperpolarization (Figure 5J). On the other hand, overexpression of the wild-type *KAT1* cDNA produced large K^+_{in} currents that did not show markedly different activation times when compared

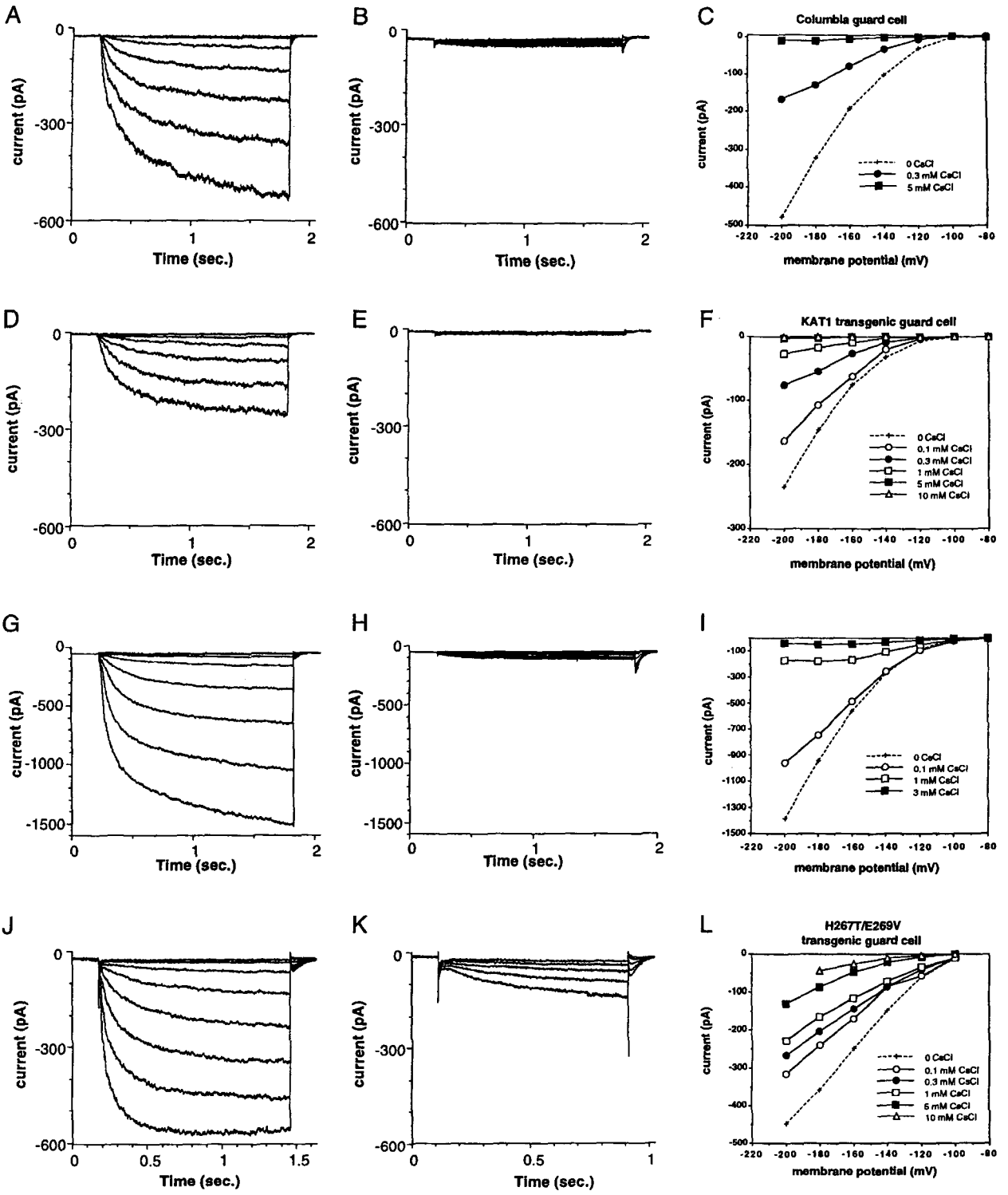

Figure 5. K^+_{in} Channel Currents in Transgenic Arabidopsis Guard Cells.

(A) Wild-type Arabidopsis guard cell inward K^+ currents are shown in the presence of 30 mM KCl and no CsCl.
(B) Wild-type Arabidopsis guard cell inward K^+ currents are blocked by 5 mM CsCl.

with those of the wild type (Figure 5). Enhanced expression of KAT1 in Xenopus oocytes was recently shown to accelerate activation times because of shifts in voltage dependence (Very et al., 1994, 1995; Cao et al., 1995). Whether the rapid activation in H267T/E269V-expressing guard cells is related to similar observations with highly expressed KAT1 (Cao et al., 1995; Very et al., 1995) requires further experimentation and analysis. Variations in activation time courses have been observed for guard cell K^+ channel currents and have been shown to depend on several experimental parameters, such as cytosolic K^+ concentrations (Lemtiri-Chlieh, 1996). In addition, significantly more rapid activation of K^+_{in} channel currents was observed in maize compared with fava bean guard cells (Fairley-Grenot and Assmann, 1993). Because K^+_{in} current activation occurs within half times of 40 to 140 msec (maize and fava bean) (Fairley-Grenot and Assmann, 1993), and because K^+ uptake during stomatal opening occurs on the time scale of hours (Imamura, 1943; Humble and Raschke, 1971; Raschke, 1979), it has been proposed that these differences in activation times are not significant for long-term K^+ uptake during stomatal opening (Fairley-Grenot and Assmann, 1993).

Additional interesting differences were observed when comparing K^+_{in} channel properties in the wild-type Arabidopsis guard cell plasma membrane (Figures 5A to 5C) with those recorded for KAT1 in Xenopus oocytes. When KAT1 is expressed in Xenopus oocytes, with the same 30 mM KCl composition of the bath solution, Cs^+ shows a clear voltage-dependent block of K^+_{in} currents at approximately −150 to −190 mV (Very et al., 1995; Ichida and Schroeder, 1996). Interestingly, wild-type Arabidopsis guard cells (Figure 5C) and the transgenic lines showed no or little voltage-dependent Cs^+ block at the same external K^+ and Cs^+ concentrations (Figures 5F and 5L). However, Figure 6 shows that in Arabidopsis guard cells, the voltage-dependent block of K^+_{in} channels by Cs^+ became more apparent when the membrane was hyperpolarized negative of −200 mV. These data suggest slight structural differences of the channel pore for KAT1 expressed in Xenopus oocytes and native K^+_{in} channels in Arabidopsis guard cells.

The degree to which the H267T/E269V transgene reduced the Cs^+ block of K^+_{in} channels was further quantified. Guard cell K^+_{in} currents were recorded in 30 mM KCl solution without CsCl to establish a 100% current level. Figure 7 shows the percentage of remaining K^+_{in} currents at −200 mV after the addition of different concentrations of CsCl for wild-type Columbia and transgenic lines. Fifty percent of the guard cell K^+_{in} current at −200 mV was blocked by 0.13 ± 0.03 mM CsCl for wild-type Columbia guard cells (n = 8) and 0.21 ± 0.02 mM CsCl and 0.29 ± 0.06 mM CsCl for *KAT1*-expressing plants (line 1.11, n = 4, and line 3.14, n = 4, respectively) (Figures 7A and 7B). In contrast to these control experiments, the H267T/E269V-expressing lines showed a dramatic shift in the Cs^+ sensitivity, with 50% block at 2.16 ± 0.54 mM CsCl for H267T/E269V-expressing plants (n = 3) (Figures 7A and 7B). These data show that expression of the wild-type *KAT1* transgene did not greatly affect Cs^+ block when compared with the Columbia background line (Figure 7A). These results correlate with the finding that *KAT1* is expressed natively in Arabidopsis guard cells (Nakamura et al., 1995).

During stomatal opening, the magnitude of K^+_{in} channel currents that suffice to mediate physiological K^+ uptake rates is significantly smaller than that of the large K^+ currents recorded at −200 mV (Outlaw, 1983; Schroeder et al., 1987; Kelly et al., 1995). Therefore, K^+_{in} channel current

Figure 5. (continued).

(C) Current–voltage curves plotted from recordings from guard cell shown in **(A)** and **(B)** show a reduction in inward K^+ currents as Cs^+ concentrations increase. Instantaneous currents have been subtracted.

(D) K^+_{in} currents in an Arabidopsis guard cell expressing the control *KAT1* transgene (line 1.11) are shown in the presence of 30 mM KCl.

(E) K^+_{in} currents in an Arabidopsis guard cell expressing the control *KAT1* transgene (line 1.11) are blocked in the presence of 30 mM KCl and 5 mM CsCl.

(F) Current–voltage curves show K^+ currents are reduced by the addition of extracellular Cs^+ to the *KAT1*-expressing guard cell shown in **(D)** and **(E)**.

(G) K^+_{in} currents in an Arabidopsis guard cell expressing the control *KAT1* transgene (line 3.14) are shown in the presence of 30 mM KCl.

(H) K^+_{in} currents in an Arabidopsis guard cell expressing the control *KAT1* transgene (line 3.14) are blocked in the presence of 30 mM KCl and 3 mM CsCl.

(I) Current–voltage curves show K^+ currents are reduced by the addition of extracellular Cs^+ to the *KAT1*-expressing guard cell shown in **(G)** and **(H)**.

(J) K^+_{in} currents in an Arabidopsis guard cell expressing the Cs^+-resistant *KAT1* mutant H267T/E269V (line 22.12) are shown in the presence of 30 mM KCl and no CsCl.

(K) K^+_{in} currents in an H267T/E269V-expressing Arabidopsis guard cell show enhanced Cs^+ resistance in the presence of 5 mM CsCl and 30 mM CsCl.

(L) Current–voltage curves for the H267T/E269V-expressing guard cell shown in **(J)** and **(K)** show that inward K^+ currents are more resistant to inhibition by Cs^+.

Membrane potentials in all experiments were stepped in −20 mV increments from −40 to −200 mV. **(B)**, **(E)**, and **(K)** are with 5 mM CsCl and **(H)** is with 3 mM CsCl. Also, the y-axis scales for **(G)** and **(H)** are different from scales for **(A)**, **(B)**, **(D)**, **(E)**, **(J)**, and **(K)**.

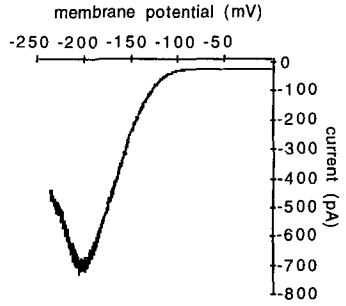

Figure 6. Voltage-Dependent Cs^+ Block in Guard Cells.

The voltage-dependent block with 10 mM KCl and 0.1 mM CsCl becomes evident at membrane hyperpolarizations more negative than −200 mV. A voltage ramp from 0 to −240 mV over 3 min is shown. Data from a guard cell from line 3.14 are shown.

magnitudes were also analyzed at membrane potentials that were less negative. Guard cells from wild-type plants had inward K^+ currents of −50 ± 13 pA (n = 8) at −140 mV. When 1 mM CsCl was applied, K^+ currents were small but detectable (<3 pA). *KAT1*-expressing guard cells (1.11 line) had inward K^+ currents of −14 ± 6 pA (n = 4) at −140 mV. With 1 mM CsCl added, absolute inward K^+ currents were also <3 pA at −140 mV. We note that despite this strong block of K^+_{in} currents by Cs^+, physiologically significant residual inward-rectifying currents of <3 pA prevailed.

In contrast, H267T/E269V-expressing guard cells had inward K^+ currents of −61 ± 23 pA (n = 5) at −140 mV. Even after the addition of 1 mM CsCl to the bathing medium, H267T/E269V-expressing guard cells had inward K^+ currents of −52 ± 12 pA (n = 4) at −140 mV. These data illustrate that at physiological potentials, H267T/E269V-expressing guard cells have inward K^+ currents that are more resistant to Cs^+ block than do wild-type guard cell K^+ currents in the presence of extracellular Cs^+ (Figures 5 and 7). The direct functional analysis of transgenic K^+_{in} channel properties by patch clamping correlates well with results from differential Cs^+ inhibition of light-induced stomatal opening (Figures 1 and 3).

DISCUSSION

K^+_{in} channels have been described in a wide variety of plant species and plant cell types (see Introduction; reviewed in Schroeder et al., 1994; Maathuis et al., 1997). This abundance of K^+_{in} channels in plants supports the theory that K^+ channels are important for fundamental functions of plant cells. The classical function described for K^+ channels in animal cells is to regulate membrane potential, in particular to repolarize the membrane potential during nerve action potentials (Hodgkin and Huxley, 1952). Detailed physiological studies indicate that whereas K^+_{in} channels in plants are clearly important for membrane potential control (e.g., Colombo and Cerana, 1991; Schroeder and Fang, 1991; Maathuis and Sanders, 1995), they have also been proposed to play a major role in long-term K^+ transport.

Although archetypal animal K^+ channels display rapid inactivation that would reduce long-term transport activity (Jan and Jan, 1992), plant K^+ channels described in most

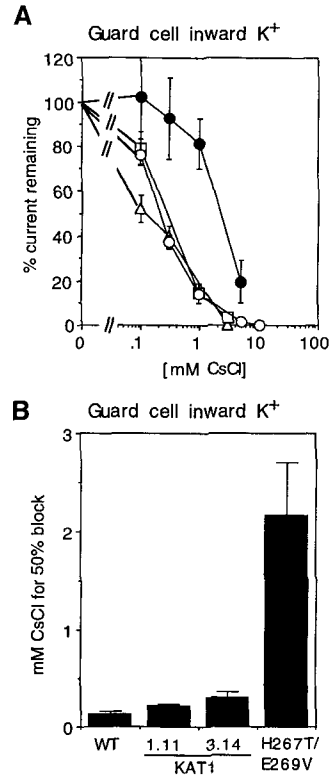

Figure 7. H267T/E269V-Expressing Lines Have Guard Cells with K^+ Currents That Are Blocked Less by CsCl.

(A) The Cs^+ block dose–response curves show that transgenic expression of the Cs^+-resistant H267T/E269V *KAT1* mutant in guard cells produces K^+_{in} channel currents that are more resistant to block by Cs^+ when compared with wild-type Columbia and *KAT1*-expressing guard cell currents. Averaged data for wild-type Columbia are indicated by open triangles (n = 8 cells). Averaged data for control *KAT1*-expressing cells, line 1.11, are indicated by open circles (n = 4), and averaged data for line 3.14 are indicated by open squares (n = 4 cells). Averaged data for H267T/E269V-expressing cells, line 22.12, are indicated by filled circles (n = 3). Error bars indicate the standard error. Data recorded at −200 mV are shown.

(B) A 10-fold higher CsCl concentration is required to block 50% of inward K^+ currents at −200 mV in guard cells expressing the Cs^+-resistant K^+_{in} channel H267T/E269V (line 22.12) than for control wild-type (WT) guard cells or guard cells overexpressing *KAT1*. Error bars show the standard error.

cases show little or no inactivation and therefore are capable of sustaining prolonged K^+ transport, lasting on the order of minutes or hours (Schroeder, 1988). Classic studies with guard cells have shown that K^+ ion movements across the plasma membrane are an essential part of stomatal movements (Imamura, 1943; Humble and Hsiao, 1969; Humble and Raschke, 1971; MacRobbie, 1983). Patch–clamp studies with guard cells have demonstrated that K^+-selective channel currents remain activated for long durations and that K^+ current magnitudes in guard cells are sufficiently large for carrying the physiological rates of K^+ uptake and release required during stomatal movements (Schroeder et al., 1987). Many biophysical and regulation studies support the model that K^+_{in} channels provide a low-affinity K^+ uptake pathway in guard cells (reviewed in Assmann, 1993; Schroeder et al., 1994).

Despite these analyses, it remains to be demonstrated, at the molecular level, that K^+_{in} channels function as a significant physiological K^+ uptake pathway during stomatal opening. The importance of other possible uptake mechanisms and pathways has not been determined or excluded in previous studies with guard cells. It appears that high-affinity K^+ transport mechanisms are unlikely to be significant in guard cells because stomatal opening in various species requires high (millimolar) K^+ concentrations.

Large K^+_{in} Channel Activity in Guard Cells

Light-induced stomatal opening in fava bean and Commelina usually requires high extracellular K^+ concentrations in the range of 50 mM (Schwartz et al., 1995). K^+_{in} channel current magnitudes in guard cells are, under most conditions, $\geq$10-fold larger than physiological K^+ influx rates during stomatal opening (Outlaw, 1983; Kelly et al., 1995). High millimolar K^+ concentrations can reduce the effectiveness of K^+_{in} channel blockers, because K^+_{in} currents are larger at high extracellular K^+ concentrations.

In addition, recent studies have indicated the presence of additional K^+ influx channel types in guard cells (Wu, 1995; Henriksen et al., 1996). The likely complexity of K^+ uptake mechanisms in plants is further highlighted by the recent finding that a T-DNA insertion into the Arabidopsis root K^+_{in} channel, *AKT1*, disrupts plant growth in the micromolar K^+ concentration, illustrating the importance of detailed molecular physiological studies with individual cDNAs (R. Hirsh and M.R. Sussman, personal communication).

The importance of guard cells for gas exchange regulation, the wealth of previous characterizations of mechanisms underlying regulation of K^+ channels and stomatal physiology, and the ability to correlate biophysical properties to stomatal movements render guard cells a suitable model system to study the molecular physiology of plant K^+ channels. We emphasize, however, that a clear inhibition of stomatal opening by guard cell K^+_{in} channel blockers had not been shown previously, for reasons discussed in further detail below. An inhibition in coleoptile elongation by Ca^{2+}, which among other effects can function as a K^+_{in} channel blocker, was reported recently (Thiel et al., 1996).

It has been suggested that in wild-type stomata, K^+_{in} channels play a necessary but not always a rate-limiting role during stomatal opening (Kelly et al., 1995). In Kelly et al. (1995), stomatal opening was slightly slowed but not inhibited by blocking 90% of inward K^+ currents in guard cells by using Ba^{2+}. In fava bean guard cells, the average K^+ uptake rate during stomatal opening corresponds to an absolute K^+ channel current of 8 pA (Outlaw, 1983; Schroeder et al., 1987), whereas absolute K^+_{in} channel current activities in single Arabidopsis guard cells range from $\sim$200 to $>$500 pA (Figures 5A and 5B). On the other hand, blue light–stimulated H^+-ATPase current activities reported in fava bean guard cells were in the range of $\sim$2 to 20 pA, supporting the suggestion that H^+-ATPases are one of the rate-limiting activities in this process (Assmann et al., 1985; Schroeder, 1988). In an early characterization of guard cell K^+_{in} channel currents, it was noted that "residual K^+ currents measured after reduction by 10 mM Ba^{2+} were estimated still to be sufficient to allow stomatal movements in the physiologically observed periods" (Schroeder et al., 1987). The above-mentioned findings and hypotheses imply that a great excess in K^+_{in} channel activity is available, which could support stomatal opening under various conditions compared with the more rate-limiting H^+ pump currents. For the same reason, antisense transformation studies with *KAT1* can be expected to give rise to limited effects on stomatal opening.

The K^+ channel blocker Cs^+ is more effective than Ba^{2+} and can block K^+ influx into guard cells, and as shown here, it is effective in inhibiting stomatal opening when used at high concentrations. In agreement with Ba^{2+} block studies, when low Cs^+ concentrations were used that blocked only 90% of K^+_{in} channel currents, stomatal opening was also not noticeably inhibited in Arabidopsis (Y.J. Liao and A.M. Ichida, data not shown). Also, overexpression of the *KAT1* cDNA caused large K^+_{in} currents but did not increase stomatal opening (Figures 3A and 5G). This provides additional support for the hypothesis that there is a large K^+_{in} channel activity in guard cells.

We show the ability to suppress Cs^+ inhibition of stomatal opening by expression of a Cs^+-resistant K^+_{in} channel mutant. We note that the function of additional Cs^+-blocked K^+ uptake transporters in guard cells (i.e., a redundancy in available K^+ uptake pathways; Wu, 1995; Henriksen et al., 1996) was not excluded here. Indeed, the finding that partial stomatal opening was still observed in the presence of 30 mM CsCl (Figure 1A) indirectly supports the activity of additional accumulation mechanisms for K^+ or other osmolytes. Data presented here, however, do provide molecular physiological evidence that K^+_{in} channels contribute to K^+ uptake during stomatal opening, because the Cs^+ block effect on stomatal opening was suppressed by the H267T/E269V mutant (Figure 3D).

Cs^+ Toxicity Is Related to K^+ Concentration

Cs^+ toxicity has been characterized for Arabidopsis seedling growth and was shown to be alleviated by increasing levels of K^+ in the external media (Sheahan, 1991; Sheahan et al., 1993). Cesium toxicity is most likely mediated by several mechanisms and is affected by several genetic loci (Sheahan et al., 1993). Interestingly, a Cs^+-resistant Arabidopsis mutant, *csi52*, recently has been shown to affect K^+ channel activities and K^+ transport in roots (Maathuis and Sanders, 1996). Although it is clear that Cs^+ affects several enzymes in whole-plant studies (Sheahan et al., 1993), it is still interesting that the block of KAT1 by Cs^+ is also competitively decreased by raising the level of K^+ in the external solution (Ichida and Schroeder, 1996). We found here that the effectiveness of Cs^+ in blocking stomatal opening in Arabidopsis was also dependent on the external K^+ concentration such that Cs^+ was ineffective if external K^+ concentrations were raised (data not shown). We found that stomatal opening is only effectively blocked by Cs^+ when low extracellular K^+ concentrations are used (Figure 1). This correlates with the competitive block of K^+_{in} channels by Cs^+ when KAT1 is expressed in Xenopus oocytes (Ichida and Schroeder, 1996). This correlation provides further pharmacological support for the model that K^+_{in} channels provide a mechanism for K^+ uptake during stomatal opening.

Interactions of K^+_{in} Channels and Anion Channels

Stomatal opening versus closing is achieved by a balance of guard cell solute influx and efflux and parallel control of K^+, anion, and Ca^{2+} channels. In the Arabidopsis stomatal opening experiments described here, stomata were closed initially. Subsequently, light and external KCl were used to induce stomatal opening. Anion efflux would depolarize the cell, working against the hyperpolarization required to activate K^+_{in} channels for K^+ influx. Usually 50 to 100 mM external KCl was required to stimulate stomatal opening by light in Arabidopsis (data not shown; Roelfsema and Prins, 1995), as is well known for fava bean and Commelina stomata (Raschke, 1979; Schwartz et al., 1995). Recent studies have shown that in fava bean and Commelina, use of anion channel blockers results in wider light-induced stomatal opening and enables stomatal opening to occur at lower extracellular K^+ concentrations (1 to 10 mM KCl; Schwartz et al., 1995). In Arabidopsis, when the anion channel blocker anthracene-9-carboxylic acid was added to the external solution, only 10 mM KCl was required to stimulate stomatal opening by light (Figure 1). Opening with 10 mM KCl was not measurable in the absence of this anion channel blocker (data not shown). In effect, blocking of anion channels with anthracene-9-carboxylic acid has been shown to render the stomata more sensitive to opening stimuli and external K^+ (Schwartz et al., 1995).

Because the physiological effects of Cs^+ are dose dependent on the K^+ concentration, it is advantageous that Arabidopsis stomata can be induced to open by light with only 10 mM KCl via downregulation of anion currents (Schwartz et al., 1995). This relatively low level of KCl allowed us to use effective concentrations of Cs^+, which were well below the range where osmotic effects take place (Raschke, 1979), and permitted us to show here that K^+_{in} channel block can reduce stomatal opening. These data provide further evidence that the regulation of stomatal movements depends on the integrated function of many types of ion channels and transporters. We emphasize that our findings do not contradict the contribution of K^+_{in} channel regulation by second messengers, because modulation of K^+_{in} activity in wild-type plants will affect the integrated response if H^+ pumps and anion channels are regulated in parallel (see Schroeder et al., 1994; Kelly et al., 1995).

Stomata from Transgenic Arabidopsis Expressing H267T/E269V Are Resistant to Cs^+ Block of Stomatal Opening

Stomata from transgenic Arabidopsis expressing H267T/E269V, a Cs^+-resistant K^+_{in} channel (Ichida and Schroeder, 1996), are resistant to Cs^+ inhibition of stomatal opening (Figures 3D and 3F). Both the stomatal opening assays in Arabidopsis epidermal fragments and the patch–clamp studies reported here allowed us to study specific effects of transgenes on Arabidopsis guard cells. Because experimental parameters were identical in all assays, with the exception of the specific transgene being expressed, our data provide unequivocal support that the H267T/E269V transgene enables stomatal opening in the presence of Cs^+. Controls in which the nonmutant *KAT1* cDNA was transgenically expressed also support the specific Cs^+ resistance of the H267T/E269V lines (Figure 3).

Cs^+ Resistance of K^+ Currents from Guard Cells Expressing H267T/E269V

To analyze the underlying mechanisms by which transgenic plants produce phenotypic responses, functional or biochemical analyses of the transgenic protein are desirable. For this study, we used patch–clamp analysis of Arabidopsis guard cells as a high-resolution functional assay for directly quantifying effects of transgenes on K^+ channel properties in guard cell plasma membranes. K^+_{in} channel analyses demonstrated that transgenic expression of the H267T/E269V mutant gave rise to K^+_{in} channel currents that were more resistant to block by extracellular Cs^+ (Figures 5 and 7). The shift in Cs^+ sensitivity of K^+_{in} currents in H267T/E269V-expressing lines was similar to the shift in Cs^+ sensitivity of this mutant expressed in Xenopus oocytes (Ichida and Schroeder, 1996). Control experiments with guard cells from the wild-type background Columbia line showed a

markedly larger sensitivity to Cs^+ than did the H267T/E269V lines (Figures 5 and 7).

Interestingly, the quantitative comparison of K^+_{in} channel current properties found here in Arabidopsis guard cells and those of KAT1 expressed in Xenopus oocytes showed differences. Expression of KAT1 alone in Xenopus oocytes produces K^+_{in} channel currents that show a strong voltage-dependent Cs^+ block at approximately −150 to −190 mV (Very et al., 1995; Ichida and Schroeder, 1996). However, K^+_{in} currents in Arabidopsis guard cells under the same K^+ and Cs^+ conditions showed limited or no clear voltage-dependent Cs^+ block at membrane potentials more positive than −200 mV (Figure 5C), suggesting structural differences in the K^+ channel pore in vivo. In guard cells, voltage-dependent block of K^+_{in} channels only became more apparent at more negative membrane potentials (Figure 6). These data directly demonstrate quantifiable functional differences in native guard cell K^+_{in} channel properties compared with cloned single subunits expressed in oocytes and support recent evidence for additional structural components that could make up K^+_{in} channels. A recent study showed that an Arabidopsis K^+ channel β subunit homolog binds to a protein in guard cell membranes that is recognized by an antibody to K^+ channel α subunits (Tang et al., 1996). In addition, it is possible that other K^+_{in} channel α subunits, in addition to KAT1, form heteromultimeric K^+ channels (Isacoff et al., 1990) with KAT1 in vivo. Heteromultimeric channels could change pore properties, such as voltage-dependent Cs^+ block. It is interesting that in spite of these differences, transgenic expression of a Cs^+ block–resistant *KAT1* mutant produced Cs^+-resistant K^+_{in} channels in Arabidopsis guard cells.

At least two differences may exist in guard cells of the wild-type control plants with respect to H267T/E269V-expressing plants. First, in the H267T/E269V-expressing plants, the transgene has mutations at amino acid positions that lie in the proposed pore region, causing structural changes that result in the resistance to Cs^+ block (Ichida and Schroeder, 1996). Second, plants expressing H267T/E269V also have higher overall levels of K^+_{in} channel mRNA, which could result in higher levels of functional K^+_{in} channel proteins in guard cells, leading to larger residual currents after Cs^+ block (Figure 2, lanes 7 to 10). To distinguish whether the observed Cs^+-dependent stomatal movement differences were due to structural changes in the transgene protein that reduce Cs^+ block or to transgene protein levels, plants with ectopic expression of wild-type *KAT1* were analyzed.

The 3.14 line ectopically expressing *KAT1* showed greatly enhanced K^+_{in} channel currents (Figure 5G), which showed a similar Cs^+ sensitivity to that of wild-type guard cells. The 3.14 line also showed a similar Cs^+ sensitivity of stomatal opening to wild-type lines (Figures 1 and 3A). The 1.11 line ectopically expressing *KAT1* showed slightly reduced K^+_{in} current magnitudes under the imposed conditions (Figures 5D and 5F). Possibly, tissue-specific gene silencing ("cosuppression") partially reduced K^+_{in} current activities in the 1.11 line, because cosuppression is expected to occur only in tissues that natively express *KAT1* and not in each transgenic line (Napoli et al., 1990; Van der Krol et al., 1990; Ramachandran et al., 1994; Brusslan and Tobin, 1995). In both the 3.14 and 1.11 lines, transgenic *KAT1*-expressing guard cells showed a Cs^+ sensitivity of K^+_{in} channel currents similar to that of the wild-type Columbia background lines (Figures 5 and 7). Cs^+ sensitivity was independent of the absolute magnitude of K^+_{in} channel currents (Figures 5 and 7), showing that resistance to Cs^+ block of stomatal opening was a specific property of the H267T/E269V mutant.

Conclusions

Transgenic expression of mutant K^+_{in} channels in Arabidopsis together with physiological assays and quantitative patch–clamp analysis of transgene function are useful approaches for studying stomatal physiology and the role of K^+_{in} channels in plant cell physiology. The combined use of guard cell K^+_{in} channel expression, light-induced stomatal opening bioassays, and K^+_{in} channel recordings in Arabidopsis guard cells allowed an initial molecular physiological analysis of the role of K^+_{in} channels during stomatal opening. The enhanced Cs^+ resistance of both K^+_{in} channel block and light-induced stomatal opening in the H267T/E269V lines show that functional transgenic expression of Cs^+-resistant K^+_{in} channels allows light-induced stomatal opening to proceed at Cs^+ concentrations that are toxic to wild-type stomatal opening. These data provide molecular physiological evidence supporting the model that K^+_{in} channels provide a pathway for K^+ uptake during stomatal opening. In addition, patch–clamp analysis of Arabidopsis guard cells indicates that the structure and properties of guard cell K^+_{in} channels in vivo differ slightly from those of the single KAT1 subunit. Continued molecular physiological studies of plant K^+ channels as pursued here should allow the analysis of the various functions of members and subunits of K^+ channel families in plants.

METHODS

Transformation of *Arabidopsis thaliana*

The cDNAs for *KAT1* and the Cs^+-resistant *KAT1* mutant H267T/E269V were subcloned into the *Agrobacterium tumefaciens* plasmid vector pMON530 (Monsanto, St. Louis, MO) by using the XhoI cloning site. Agrobacterium strain C58 was transformed with the pMON530 cDNA constructs. Three-week-old Arabidopsis (Columbia ecotype) plants were infected with the transformed Agrobacterium, using a vacuum infiltration protocol modified from Bechtold et al. (1993). Seeds harvested (T_1) from ~150 vacuum-infiltrated plants were screened for kanamycin resistance on kanamycin growth media plates. Two independent kanamycin-resistant lines were studied in detail for each construct. Kanamycin-resistant T_1 seedlings were transferred to soil. Fifty to 80 self-pollinated T_2 seedlings were tested

for the kanamycin-resistant phenotype to determine the T_1 genotype. T_3 seedlings from individual self-pollinated T_2 plants were further tested for kanamycin-resistant segregation to identify homozygous kanamycin-resistant T_2 plants. Light-induced stomatal opening assays and patch–clamp analyses were performed with tissue from homozygous T_2 plants.

The presence of the *KAT1* and H267T/E269V transgenes was detected on RNA gel blots. Total RNA from tissue from whole plants grown in liquid culture was isolated using a guanidinium isothiocyanate protocol (Ausubel et al., 1987).

Arabidopsis Stomatal Opening Assays

Three- or 4-week-old Arabidopsis plants (Columbia ecotype) grown at 25°C under constant bright light in well-watered soil were placed in the dark for 2 days, and CO_2 levels were elevated by placing a beaker with a saturated solution of $NaHCO_3$ in a tank with the plants to induce stomatal closing (stomata from non-dark-grown and CO_2-pretreated light-grown plants were consistently open). Three to five rosette leaves ~1.5 cm in length were used from each plant assayed. One leaf representing the zero time point was blended in cold water using a Waring blender. To isolate epidermal segments containing stomata, blender contents were filtered through 297-μm polypropylene Spectra/Mesh (Fisher, Hampton, NH). Other leaves from dark-treated plants were submerged in solution A (10 mM KCl, 10 mM Mes, and 300 μM anthracene-9-carboxylate, pH 6.15) and incubated under white light (with a fluence rate of 150 $\mu mol\ m^{-2}\ sec^{-1}$). Other leaves from the same plant were placed in solution B (10 mM KCl, 10 mM Mes, 300 μM anthrocene-9-carboxylate, and 30 mM CsCl). After 2 hr of incubation, one leaf was removed from each solution and blended. Both stomatal aperture and height of 20 stomata were measured for each time point with a Nikon Diaphot 300 microscope (Melville, NY) and NIH Image public domain software (http://rsb.info.nih.gov/nih-image/). The height of stomatal apertures was defined as the dimension perpendicular to the aperture width, stretching from the outer borders of the guard cells. Stomatal responses were continuously analyzed for independent wild-type and transgenic lines over a period of 10 months. P values were calculated by using Excel (*t* test: two-sample assuming unequal variances). Variable 1 is aperture data for a given plant line at $t = 2$ hr with 0 mM CsCl. Variable 2 is aperture data for a given plant line at $t = 2$ hr with 30 mM CsCl. All stomatal aperture measurements for a given condition for each line were pooled to determine P values. All illustrated results were performed as blind experiments during which the identities of the plant lines (wild type or transgenic) under investigation were not known to the researcher.

Stomatal apertures were recorded using a method that gives consistent results with small apertures. Stomatal apertures were small for the following reasons. (1) Not fully expanded leaves were used, because younger leaves show stronger signal-dependent responses. These leaves have smaller stomatal pores in Arabidopsis. (2) A small population of large stomata that can be found in Arabidopsis leaves was not sampled. (3) Light-induced stomatal opening was triggered in leaves with intact neighboring epidermal cells. It appears that wider apertures may be achieved by light exposure of floating isolated epidermal peels in which the neighboring cells are usually disrupted. (4) Using differential interference contrast optics, we found that even within a given sample, two different stomatal apertures can be defined. When the microscope is focused on the optical plane outlining the guard cells, stomatal aperture values are larger (focus on outer edges of guard cells; method A). By focusing away from this optical plane outlining the guard cells, lip structures come into focus that show significantly smaller stomatal apertures (method B; see, e.g., Figures 1B and 1C). These smaller apertures were measured in all experiments because they reflect the gas exchange pore. As an example from typical pilot studies in which the identical stomatal apertures were measured with both methods, light-induced open stomata had average apertures when focusing on guard cell outlines of 3.66 ± 0.25 μm (method A), whereas the aperture lips of the identical stomata had average open apertures of 1.21 ± 0.07 μm (method B) (±SE; $n = 15$). In another experiment, open apertures seen by using method A were 3.88 ± 0.13 μm, and the identical stomatal apertures seen by using method B were 1.20 ± 0.06 μm ($n = 15$). We determined in pilot experiments that regardless of the focal plane used for stomatal measurements, the relative response to a signal is comparable. When the outline of guard cells is in the focal plane, measured aperture values are larger. Use of one consistent method in all experiments is crucial, as was done here and previously (Roelfsema and Prins, 1995; Pei et al., 1997).

Patch–Clamp Analysis of Arabidopsis Guard Cells

Arabidopsis rosette leaves were blended in cold water to isolate epidermal peels, as described previously (Kruse et al., 1989; Kelly et al., 1995). Guard cell protoplasts were isolated from 3- to 5-week-old plants by enzymatic digestion of leaf epidermal strips, as previously described (Pei et al., 1997). Guard cell protoplasts with diameters between 4 and 5 μm were used in patch–clamp experiments. Patch–clamp pipettes were prepared from soft glass capillaries (Kimax 51; Kimble) and pulled on a multistage programmable puller (model p-87; Sutter Instrument Co., Navato, CA). Giga-ohm seals between electrode and plasma membrane (>15 GΩ) were obtained by suction and usually appeared within 2 to 3 min (Hamill et al., 1981). Cells were pulled up to the bath solution surface to reduce stray capacitance. Whole-cell configurations were established by applying increased continuous suction to the interior of the pipette. Protoplasts in the whole-cell configuration were stable and could last for >1 hr during measurements and bath perfusions.

Protoplasts were voltage-clamped using an Axopatch 200 amplifier (Axon Instruments, Foster City, CA). Data were stored and analyzed as described previously (Schroeder and Fang, 1991). The pipette solution, which dialyzes the cytosol for K^+ current measurements, was composed of 30 mM KCl, 70 mM K-glutamate, 2 mM $MgCl_2$, 6.7 mM EGTA, 3.35 mM $CaCl_2$, 5 mM ATP, and 10 mM Hepes-Tris, pH 7.1, and the bath contained 30 mM KCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, and 10 mM Mes-Tris, pH 5.5. To analyze Cs^+ block of K^+ channels, 0.1 to 10 mM CsCl was added to the 30 mM KCl bath solution by perfusion. Osmolalities of all solutions were adjusted to 485 mmol kg^{-1} for bath solutions and 500 mmol kg^{-1} for pipette solutions by the addition of D-sorbitol. Guard cells were held at a membrane potential of −40 mV and stepped to hyperpolarizing potentials by voltage pulses lasting 1 to 2 sec. For current amplitude analysis, instantaneous currents were subtracted for each pulse.

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