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Plant adaptation to fluctuating environment and biomass production are strongly dependent on guard cell potassium channels

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At least four genes encoding plasma membrane inward K⁺ channels (K_{in} channels) are expressed in *Arabidopsis* guard cells. A double mutant plant was engineered by disruption of a major K_{in} channel gene and expression of a dominant negative channel construct. Using the patch-clamp technique revealed that this mutant was totally deprived of guard cell K_{in} channel (GCK_{in}) activity, providing a model to investigate the roles of this activity in the plant. GCK_{in} activity was found to be an essential effector of stomatal opening triggered by membrane hyperpolarization and thereby of blue light-induced stomatal opening at dawn. It improved stomatal reactivity to external or internal signals (light, CO₂ availability, and evaporative demand). It protected stomatal function against detrimental effects of Na⁺ when plants were grown in the presence of physiological concentrations of this cation, probably by enabling guard cells to selectively and rapidly take up K⁺ instead of Na⁺ during stomatal opening, thereby preventing deleterious effects of Na⁺ on stomatal closure. It was also shown to be a key component of the mechanisms that underlie the circadian rhythm of stomatal opening, which is known to gate stomatal responses to extracellular and intracellular signals. Finally, in a meteorological scenario with higher light intensity during the first hours of the photophase, GCK_{in} activity was found to allow a strong increase (35%) in plant biomass production. Thus, a large diversity of approaches indicates that GCK_{in} activity plays pleiotropic roles that crucially contribute to plant adaptation to fluctuating and stressing natural environments.

Arabidopsis | circadian rhythm | inward Shaker | stomata | transpirational water loss

The leaf epidermis is covered with a waxy cuticle that prevents water loss but also impedes diffusion of atmospheric CO₂ toward the inner photosynthetic tissues. Gas exchanges mainly occur through microscopic pores in the epidermis, named stomata. By controlling stomatal aperture, the plant copes with the conflicting needs of allowing CO₂ entry for photosynthesis and of preventing excessive water loss (1). An increase or decrease in turgor of the guard cells lining the pore opens or closes the stoma, respectively. K⁺ and accompanying anions (malate and chloride) are among the major solutes involved in this osmotically driven process (2–6).

Changes in guard cell K⁺ content involve K⁺ channel activity (7, 8) encoded by Shaker K⁺ channel genes (9). A single Shaker gene in *Arabidopsis*, *GORK*, encodes the guard cell membrane outward conductance, and disruption of this gene results in impaired stomatal closure in response to darkness or abscisic acid (ABA) (10). Regarding the inward K⁺ conductance, *Arabidopsis* guard cells express at least four genes coding for Shaker α -subunits involved in formation of inward channels (11). Here we assess the importance of the guard cell membrane inward K⁺ channel (GCK_{in}) activity in stomatal movements and whole-

plant physiology by engineering an *Arabidopsis* mutant totally deprived of this activity.

Results

Genetic Engineering of an *Arabidopsis* Mutant Deprived of GCK_{in} Activity. In a first step, we screened candidate mutant lines in which expression of inward K⁺ channels is affected, by looking for altered transpiration as the most probable phenotype. At least four inward K⁺ channel genes, *KAT1*, *KAT2*, *AKT1*, and *AKT2*, all belonging to the Shaker family, are expressed in guard cells, the first two at higher levels than the others (11, 12). Disruption of *KAT1* was known to barely affect leaf transpiration (11). To assess the role of *KAT2*, we isolated a T-DNA mutant plant in which expression of this gene was totally abolished [supporting information (SI) Fig. S1]. When compared with WT plants, this knockout mutant, named *kat2-1*, displayed neither difference in transpiration rate (Fig. S2) nor any apparent phenotype (data not shown). Alternatively, we developed a dominant negative strategy based on the fact that Shaker channels are homotetrameric or heterotetrameric proteins, formed from subunits encoded by one or several Shaker genes (13–16). WT plants were transformed with a dominant negative (domneg) *kat2* construct (15) under control of the *KAT2* promoter. The resulting transgenic plants, named *domneg-1* (T₃ generation, homozygous for the *domneg* transgene), did not display any defect in transpiration (Fig. S2) or any apparent phenotype (data not shown). Interestingly, a transpiration phenotype appeared when the *kat2 domneg* construct was introduced in the *kat2-1* knockout background. The resulting double mutant (T₃ generation, homozygous for both mutations) displayed decreased transpirational water loss during the light period when compared with WT plants (Fig. S2; see also below). It did not display any phenotype in terms of leaf K⁺ contents or stomatal densities (data not shown). Back-crossing this double mutant with the WT plant indicated that the transpiration phenotype was linked to the simultaneous presence of both mutations and not to the locus of insertion of the *domneg* construct (data not shown).

Patch-clamp experiments were then performed to determine

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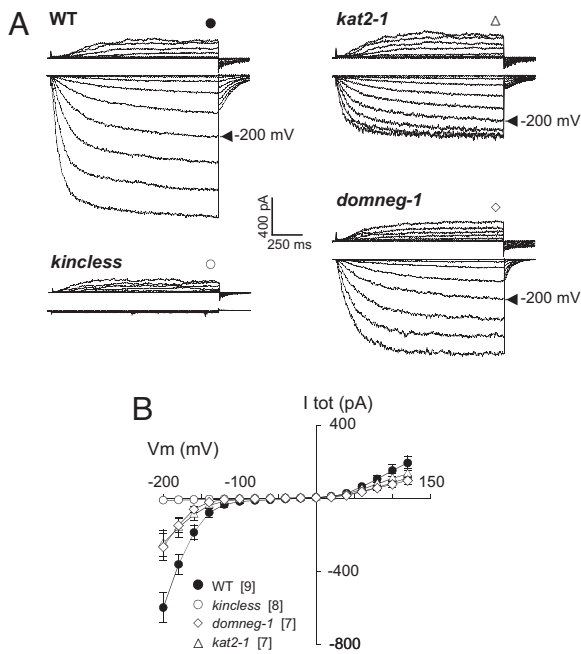


Fig. 1. Absence of inward K^+ channel activity in guard cells of the *kinless* mutant. (A) Representative macroscopic current traces recorded with the patch-clamp technique in guard cell protoplasts from WT (Upper Left), *kat2-1* (knockout mutant disrupted in the *KAT2* gene) (Upper Right), *domneg-1* (expressing a dominant negative *kat2* construct in WT background) (Lower Right), or *kinless* (expressing the same *domneg* construct in the *kat2-1* background) (Lower Left) plants. In all recordings, the holding potential was -100 mV; voltage steps were applied to potentials ranging between -100 and $+120$ mV in 20 -mV increments (top traces for each genotype) or between -100 and -260 mV in increments of -20 mV (bottom traces for each genotype). (B) Comparison of current-voltage relationships from the different genotypes. Total current (I_{tot} , sampled at time marked by symbols over the recordings in A) is plotted against membrane potential. Data are means \pm SE (with the number of repeats in brackets).

the effect of the mutations on GCK_{in} activity. The individual mutations *kat2-1* and *domneg-1* were found to result in partial reduction of GCK_{in} current, by $\approx 60\%$ and 55% , respectively, at -200 mV (Fig. 1). The fact that reduction in GCK_{in} activity was not total in the *domneg-1* mutant suggested that the expression level of the *domneg* construct (under control of the *KAT2* promoter) was not high enough to result in integration of at least one *domneg* polypeptide in every K_{in} channel (see Discussion). Interestingly, the combination of the two mutations *kat2-1* and *domneg-1* totally abolished the current (Fig. 1). The corresponding mutant was therefore named *kinless* (K^+ inward current-less). It was used to investigate the role of GCK_{in} activity in stomatal and whole-plant physiology.

GCK_{in} Activity Plays an Essential Role in Stomatal Opening Triggered by Membrane Hyperpolarization. Stomatal opening is triggered by light at the beginning of the daily cycle. The blue component of white light acts as a specific signal (4, 17, 18). It activates guard cell plasma membrane H^+ -ATPases, leading to membrane hyperpolarization and thereby to increased K^+ uptake through K^+ transport systems. To assess the actual contribution of GCK_{in} activity to this process, we evaluated the effects of fusicoccin (FC), a toxin known to up-regulate plant plasma membrane H^+ -ATPases (17), of Cs^+ , a classical inhibitor of K_{in} channel activity (19) and of blue, red, or white light on stomatal opening in epidermal peels from WT and *kinless* plants. The results (Fig. 2) can be summarized as follows. (i) Cs^+ did not further affect stomatal opening in the *kinless* mutant, providing additional

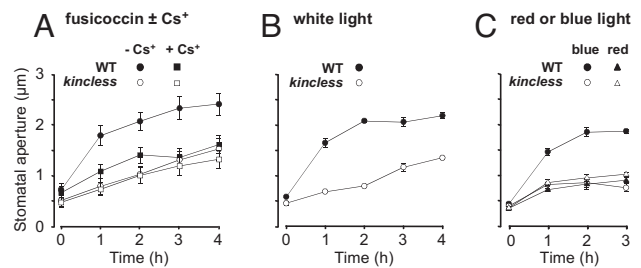


Fig. 2. Disruption of inward K^+ channel activity affects stomatal opening. Epidermal strips were peeled from WT (filled symbols) or *kinless* (open symbols) plants at the end of the dark period. Stomatal opening was induced ($t = 0$) by fusicoccin ($10 \mu\text{M}$) in the presence or absence of Cs^+ (30 mM) (A) or by switching on white light (B) or blue or red light (C). K^+ concentration in the bath solution: 10 mM (A) or 30 mM (B and C). Data are means \pm SE. $n > 100$ in A, $n > 150$ in B, and $n > 200$ in C.

evidence for total absence of GCK_{in} activity. (ii) Stomatal opening was strongly dependent on GCK_{in} activity when it was triggered by fusicoccin or blue light, two treatments that have been extensively characterized as leading to membrane hyperpolarization. (iii) On the contrary, stomatal opening was poorly dependent on GCK_{in} activity when it was triggered by red light. The mechanisms of red light-induced stomatal opening are still unclear, and conflicting data have been reported on the actual contribution of H^+ -ATPase activation and membrane hyperpolarization to the process (18).

GCK_{in} Activity Underlies Stomatal Responsiveness to Changes in Light, Air Humidity, and CO_2 Availability in Intact Plants. Consequences of the absence of GCK_{in} activity on leaf transpiration were then investigated by using a setup that continuously monitors transpirational water loss in an intact plant. Consistent with the above results, absence of GCK_{in} activity affected the increase in transpiration induced by light (Fig. 3A). Fitting the increase in transpiration with monoexponential functions led to time constants approximately six times higher in the mutant (≈ 2 h) than in the WT (≈ 20 min) plants (Fig. 3A). These quantitative analyses demonstrate that stomatal opening induced by blue light in intact plants depends very strongly on GCK_{in} activity.

Besides light conditions, regulation of stomatal aperture integrates plant water status and CO_2 availability. Transpiration recordings revealed that GCK_{in} activity improved stomatal responsiveness to a sudden reduction in leaf to air water vapor pressure difference (VPD) (Fig. 3B). Such sharp changes in micrometeorological conditions are likely to be frequent in natural environments, e.g., upon a decrease in wind velocity. GCK_{in} activity was also found to improve stomatal responsiveness to a reduction in CO_2 availability (Fig. 3C).

Absence of Inward K^+ Channel Activity Results in Impaired Control of Stomatal Aperture in the Presence of Na^+ in the Medium. In far from physiological conditions, when epidermal peels were bathed in solutions containing high Na^+ concentrations (30 mM NaCl) but no K^+ at all, stomata were able to open upon light (Fig. S3), presumably by taking up Na^+ as an osmoticum in place of K^+ (4, 20). However, opening in such conditions subsequently precluded normal stomatal closure (upon ABA addition; Fig. S3), in agreement with previous reports in other plant species, probably because Na^+ cannot be rapidly excreted from the cell (4, 20). Interestingly, introducing K^+ in the bath solution (in the presence of Na^+ , 20 mM NaCl , and 10 mM KCl) before inducing stomatal opening (by switching light on) rescued WT but not *kinless* stomata from the deleterious effects of Na^+ on the subsequent stomatal closure (Fig. 4 A and B). Thus, in the

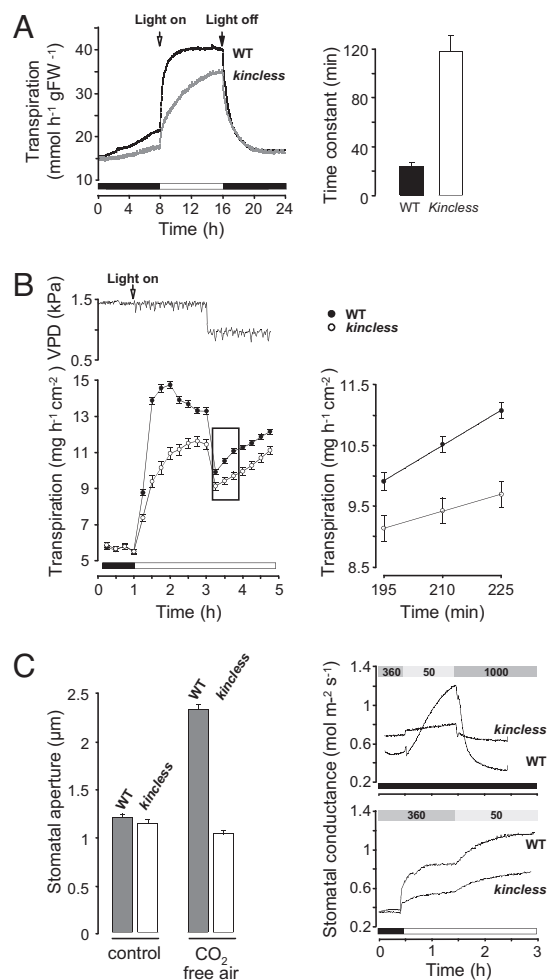


Fig. 3. Inward K⁺ channel activity underlies stomatal responsiveness to changes in light, VPD, and CO₂ conditions. (A) Rate of light-induced stomatal opening. The consequences of the absence of GCK_{in} activity on leaf transpiration were then investigated by using a setup allowing to continuously monitor transpirational water loss in an intact plant (10). (Left) Typical recordings of transpiration in a WT or *kinless* plant during a whole nycthemeral period (8-h/16-h day/night). (Right) Time constants describing the increase in transpiration rate induced by light (derived by fitting the kinetics with monoexponential functions). Data are means ± SE; n = 4. For each of the four plants tested per genotype, the individual time constant integrated values derived from the recording of transpiration rate during at least five successive photoperiods. (B) Responsiveness to a decrease in VPD. An automated growth chamber [Phenopsis robot (28)] was used to impose rapid changes in VPD. (Left) WT or *kinless* plant transpiration (bottom) during a climatic scenario comprising (top) a dark-light transition (open arrow) 2 h before a sudden decrease in VPD. Data are means ± SE; n = 60. (Right) Kinetics of the increase in transpiration rate due to stomatal aperture readjustment during the first half hour (boxed region in Left) after the change in VPD. (C) Responsiveness to CO₂ availability. (Left) Epidermal peel bioassays. Epidermal strips were peeled at the end of the dark period and incubated in the dark in 30 mM K⁺. Stomatal opening was induced by bubbling CO₂-free air for 3 h. Stomatal aperture was measured just before (control values, two bars on the left) and after (two bars on the right) this 3-h treatment. (Right) *In planta* stomatal conductance measurement in WT and *kinless* leaves. Time courses of stomatal conductance in response to changes in CO₂ concentration under dark (Upper) or light (Lower) conditions. Dark and light periods are indicated by black and white boxes under the graphs. The changes in CO₂ concentration in the air flow are indicated (expressed in ppm) in the gray boxes. The decrease in CO₂ concentration in the air flow under dark conditions mimics depletion of internal CO₂ driven by photosynthetic activity (independent of light signal and photosynthesis).

presence of Na⁺, stomatal closure itself (and not only stomatal opening) depends on K⁺-selective GCK_{in} activity.

A second set of experiments was then performed on intact

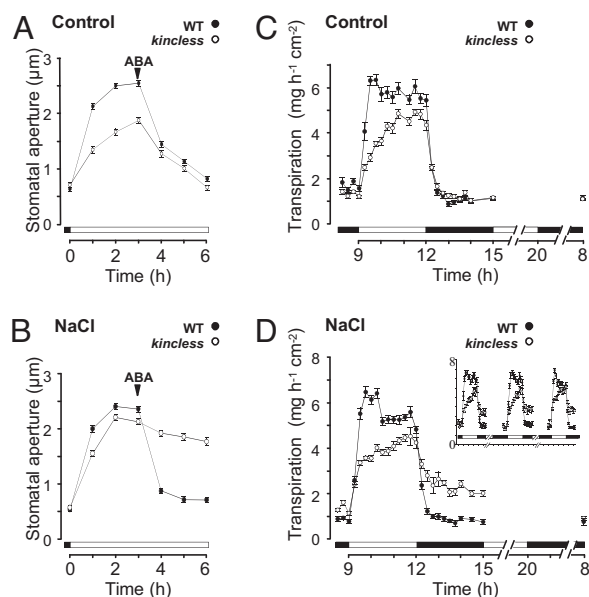


Fig. 4. Disruption of GCK_{in} activity results in impaired stomatal closure upon salt stress. (A and B) Epidermal peel bioassays. Epidermal strips peeled from WT and *kinless* plants were bathed in either 30 mM KCl (A) or 10 mM KCl and 20 mM NaCl (B). Stomatal opening was induced (*t* = 0) by switching light on, and stomatal closure was triggered by adding the plant stress hormone ABA (10 μM). (C and D) Experiments on intact plants. White and black boxes under the curves indicate light and dark periods, respectively. The photoperiod was of 8 h in total: 3 h (9 a.m. to 12 p.m.) and five additional hours (3 p.m. to 8 p.m.). Plant transpiration was recorded between 8 a.m. and 3 p.m. Data are means ± SE; n = 15 plants per genotype. (C) Control treatment: 5-week-old plants continuously grown in the absence of Na⁺. (D) NaCl treatment: same plants as in C, but 12 h after introduction of 70 mM Na⁺ in the nutrient solution. (Inset) Transpiration recordings for 3 successive days after Na⁺ introduction in the nutrient solution.

plants. WT and *kinless* mutant plants were grown for 5 weeks in standard nutrient solution (containing 4.5 mM K⁺). Then, the solution was supplemented with Na⁺ (70 mM). This treatment rapidly (12 h) affected stomatal physiology in the mutant plant: absence of GCK_{in} activity strongly impaired stomatal closure when the light was turned off (Fig. 4 C and D).

Inward K⁺ Channel Activity Renders Stomatal Opening Independent of Previous Illumination Periods. Stomatal opening in *kinless* plants was strongly affected when illumination was reduced to 3 h during the two preceding photoperiods (compare Fig. 5 C and D) and totally inhibited after 48 h in darkness (Fig. 5A), whereas the same treatments had no significant effect in the WT plants (see arrows in Fig. 5A). It is worth noting that stomatal closure itself was affected in the mutant plants when the duration of the preceding photoperiods was reduced (Fig. 5D).

Absence of Inward K⁺ Channel Activity Affects Stomatal Circadian Rhythm. In WT plants, significant stomatal preopening occurred during the last hours of the night period, as shown by recording transpirational water loss (Fig. 5 A and B), attesting circadian rhythm in stomatal movements. Furthermore, such circadian movements persisted under extended dark conditions (Fig. 5A, 48 h dark treatment). These two features were strongly affected in *kinless* mutant plants (Fig. 5 A and B).

Dependence of Plant Growth on Inward K⁺ Channel Activity. Based on the above results, we assessed the importance of GCK_{in} activity in plant growth under challenging light conditions, with higher light intensity during the first hours of the photophase. In

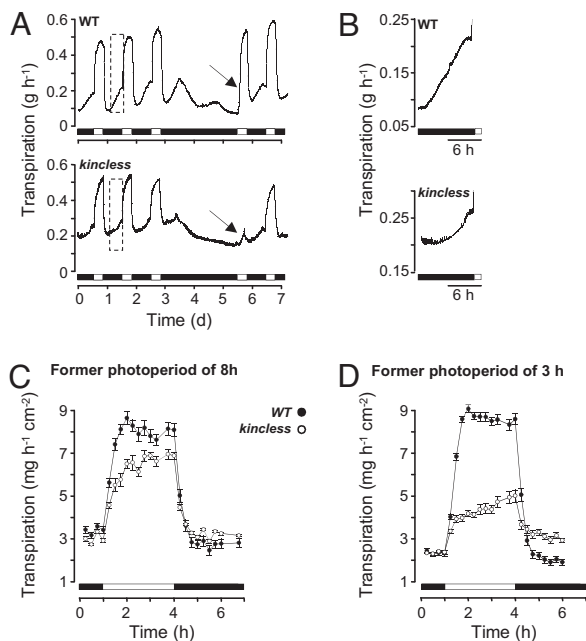


Fig. 5. Disruption of inward K^+ channel activity affects stomatal circadian rhythm and renders stomatal opening sensitive to the duration of the preceding photoperiods. (A and B) Effect on stomatal circadian rhythm. (A) Continuous recording of the transpiration rate in a single WT or *kinless* plant during 7 days. Black and white boxes under the curves indicate dark and light periods, respectively. Two photoperiods were suppressed, on days 4 and 5. (B) Enlargement from A (dotted boxed regions) highlighting stomatal preopening in darkness in the WT plant. (C and D) Sensitivity to the duration of the preceding photoperiods. (C) Transpiration rates recorded in 5-week-old WT or *kinless* plants exposed to an 8-h photoperiod since sowing. (D) Transpiration rates recorded in the same plants after exposure to a 3-h photoperiod during 2 days. Data are means \pm SE; $n = 15$ plants per genotype.

standard growth chambers, with $250 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ light intensity, increasing light intensity to 650 or $1,000 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ (approximately two-thirds of light intensity on a bright sunny day) for the first 2 h of light period (8 h in total) resulted in large differences in growth between WT and *kinless* mutant plants: biomass production (shoot fresh weight of 6-week-old plants) was higher in WT plants than in mutant plants, by $\approx 9.6 \pm 3.1\%$ or $36 \pm 7.5\%$ ($n > 12$) for the $650\text{-}\mu\text{E}$ or $1,000\text{-}\mu\text{E}$ treatments, respectively. No significant difference in biomass production between the two genotypes was observed when light intensity was kept constant, at $250 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, during the whole light period.

Discussion

Large Reductions in GCK_{in} Activity Do Not Affect Plant Transpiration in Standard Conditions. Disruption of the *KAT2* gene and expression of a *kat2 domneg* construct (under control of the *KAT2* promoter region) both resulted in a large decrease in GCK_{in} activity, by $\approx 60\%$, but did not affect plant transpiration, consistent with previous analyses showing that disruption of the *KAT1* gene, which leads to a $>50\%$ reduction in GCK_{in} activity, did not affect stomatal opening (11). Also, when a *kat1 domneg* construct was overexpressed (by means of a tandem repeat of the CaMV 35S promoter) in *Arabidopsis*, impairment in stomatal opening was detectable only in the transgenic plants displaying the highest reduction rates in GCK_{in} activity, by $\approx 70\text{--}80\%$ (21). This suggested that GCK_{in} activity may be much higher than expectedly required for physiological needs, because it could be strongly reduced without any apparent consequence on plant phenotype (8, 21). In this context, it was implicitly assumed that engineering plants with down-regulated GCK_{in} activity could be

a valuable strategy to decrease leaf transpirational water loss and improve plant tolerance to drought. However, based on the present report, it is very likely that such plants would display reduced adaptation to fluctuating and stressing environmental conditions, and ultimately decreased biomass production.

A Mutant Devoid of GCK_{in} Activity. Two-hybrid experiments in yeast and functional analyses after coexpression in *Xenopus* oocytes have provided evidence that *KAT2* can interact and form heteromeric channels with other Shaker subunits, at least *KAT1* and *AKT2* (15, 22). Expression of a *domneg kat2* gene in a *kat2* knockout background is shown to result in total suppression of GCK_{in} activity. Shaker genes expressed in guard cells, besides *KAT2*, are *KAT1* and, probably at a lower level, *AKT1* and *AKT2* (11). We propose that, in a *kat2* knockout background, the *domneg kat2* polypeptides interact with the other WT Shaker subunits expressed in guard cells, preventing formation of functional channels.

The *domneg kat2* construct was expressed under control of *KAT2* promoter, which is mainly active in guard cells and in leaf phloem (22). Based on quantitative real-time PCR, leaf phloem cells express *AKT2* and *KAT1* Shaker genes at much higher levels than *KAT2* (23). Thus, expression of the *kat2 domneg* construct under control of *KAT2* promoter may mainly affect K_{in} channel activity in guard cells, leaving phloem K_{in} channels largely unaffected. Consistent with this hypothesis, K^+ assays revealed similar leaf K^+ contents in *kinless* and control WT plants. It is also worth noting that lack of GCK_{in} activity affected stomatal movements in epidermal peels, i.e., in the absence of control by the leaf and whole plant. Furthermore, all of the data obtained in intact plants are coherent with those obtained in epidermal peels, when comparisons can be made, regarding the kinetics of light-induced stomatal opening or of dark-induced stomatal closure, and the sensitivity to CO_2 and to Na^+ . Thus, the whole set of data indicates that the *kinless* phenotype analyzed in this report, in terms of transpirational water loss by intact plants, reflects defects in stomatal movements rather than in plant K^+ status.

Roles of GCK_{in} Activity in Stomatal Opening upon Membrane Hyperpolarization and Adaptation to Environmental Conditions. Stomata can open in the absence of GCK_{in} activity but with a time constant (≈ 120 min) approximately six times greater than that measured in control WT plants. Fig. 2 clearly shows that signals inducing membrane hyperpolarization (fusicoocin, blue light) are poorly efficient in triggering stomatal opening in the mutant plant devoid of GCK_{in} activity. This means that membrane hyperpolarization of the guard cell membrane due to H^+ -ATPase activation is unable, in the absence of GCK_{in} activity, to trigger rapid accumulation of osmotica from the apoplast, for instance via $\text{H}^+:\text{K}^+$ or $\text{H}^+:\text{sugar}$ symporters. In other words, GCK_{in} activity is an essential effector of rapid stomatal opening by membrane hyperpolarization, a role that was generally assumed but which lacked the support of direct genetic and (electro)physiological tests described here. It is thus crucial for blue light-induced stomatal opening at dawn and control of opening during the first hours of the day period, when K^+ salts are the dominant osmotica responsible for increased guard cell turgor. Accumulation of organic solutes resulting from photosynthesis has been shown to contribute to guard cell turgor and stomatal aperture during the second half of the daily cycle (6, 24).

GCK_{in} activity is also involved in plant responses to a decrease in internal CO_2 availability (Fig. 3C) or in evaporative demand (Fig. 3B). Such roles probably reflect the fact that GCK_{in} activity is the sole transport activity that can enable membrane hyperpolarization to rapidly trigger stomatal opening. Conversely, the fact that GCK_{in} activity plays a role in these responses indicates that they rely on membrane hyperpolarization.

GCK_{in} Activity Protects Stomatal Closure Against Detrimental Effects of Na⁺. Experiments using epidermal peels in K⁺-free solution indicate that stomata can open by accumulating Na⁺ instead of K⁺, but that stomatal closure is thereafter impaired, probably because cells are unable to rapidly release Na⁺ ions. This might be because of lack of a large outwardly directed Na⁺ concentration gradient able to drive rapid efflux upon membrane depolarization. Indeed, Na⁺ is compartmentalized into the vacuole because high concentrations of Na⁺ would be toxic in the cytosol, and thus the concentration gradient of Na⁺ across the plasma membrane cannot build up to become as steep as the K⁺ gradient. The present data show that, in physiological conditions, when K⁺ and Na⁺ are both present in the medium, GCK_{in} activity is required for efficient stomatal functioning (Fig. 4). It allows stomata to open by preferentially using K⁺, setting up the conditions for rapid stomatal closure, the large outward conductance of the membrane to K⁺ (10) and the steep electrochemical gradient of this cation between the cytoplasm and the cell wall, ensuring rapid efflux upon membrane depolarization. In other words, a prerequisite to efficient stomatal closure is selective K⁺ uptake in guard cells during stomatal opening, and this is guaranteed, in the presence of both K⁺ and Na⁺, by the highly K⁺-selective GCK_{in} activity. Noteworthy is the observation that treating plants lacking GCK_{in} activity with Na⁺, resulted in higher transpirational water loss for several hours at night. Greater transpiration in the absence of photosynthesis would decrease plant water use efficiency. Increased transpiration may result in more Na⁺ being moved to the shoot through the xylem to an extent that is potentially toxic.

A Crucial Role of GCK_{in} Activity in Circadian Rhythm. In plants, the circadian clock ensures an optimal phase relation between physiology and daylight cycle, providing the basis for anticipation of recurrent changes in environmental conditions and improving photosynthesis and water use efficiency (25, 26). In natural conditions, stomatal reopening during the last hours of the night promotes CO₂ fixation during the early part of the day, when solar radiation is already high but air temperature and VPD are not yet challenging the plant water status (26). Our results indicate that GCK_{in} activity is central to the mechanisms that underlie the circadian rhythm of stomatal opening, thereby rendering stomatal functioning predictive of, and not merely reactive to, the fluctuating environment.

Conclusion

Rapid and selective K⁺ uptake by inward K⁺ channels from the Shaker family is shown to be the major transport process that converts membrane hyperpolarization into rapid guard cell swelling and stomatal opening. Disruption of this activity in guard cells appears to have more detrimental effects than that of outward K⁺ channel (GCK_{out}) activity. For instance, absence of GCK_{in} activity results in a 6-fold increase in the time constant of stomatal opening, from ≈20 min to 2 h, whereas the time constant of stomatal closure induced by disruption of GCK_{out} activity is increased only 2-fold, from 10 to 20 min (10). Also, at least four Shaker genes code for inward K⁺ channels in guard cells (11), whereas a single one [*GORK* (10)] encodes the outward K⁺ conductance. This might mean that ensuring rapid and selective K⁺ uptake upon membrane hyperpolarization is more essential for stomatal physiology and/or more complex to achieve than wholesale K⁺ secretion upon membrane depolarization.

By enabling membrane hyperpolarization to rapidly trigger stomatal opening, GCK_{in} activity improves stomatal reactivity to changes in internal, environmental, and micrometeorological conditions (CO₂ availability, light, and evaporative demand). GCK_{in} activity also allows stomatal opening to be independent of past illumination period and photosynthesis, maybe because accumulation of K⁺ is likely to be less costly, from an energetic point of view, and/or more straightforward to achieve than accumulation of

organic osmotica. Furthermore, GCK_{in} activity underlies the circadian rhythm of stomatal opening, allowing stomatal opening to anticipate changes in light conditions and not merely to react to illumination. This might explain why GCK_{in} activity strongly impacted biomass production when the plants were exposed to high light intensities only for a short period at the beginning of the day, a meteorological scenario used to mimic conditions that limit stomatal opening later in the day (such as decrease in light intensity from shadowing, high air temperature and VPD, or water shortage). Finally, under salt stress, large and selective GCK_{in} activity prevents detrimental Na⁺ uptake by guard cells upon membrane hyperpolarization, thereby ensuring efficient stomatal function and decreasing transpirational water loss. In conclusion, a robust and K⁺-selective inward channel activity at the guard cell plasma membrane is a major actor in plant adaptation to both atmospheric and soil fluctuating and challenging conditions.

Materials and Methods

Isolation of the T-DNA-Tagged Mutant *kat2-1* Disrupted in the *KAT2* Gene. The *kat2-1* knockout line was obtained by PCR screening of 40,000 *Arabidopsis thaliana* T-DNA insertion mutants [Wassilevskija ecotype; library constructed by Institut National de la Recherche Agronomique, Versailles (27)]. A Southern blot with a probe targeting the T-DNA right border revealed a single insertion locus. The exact position of the T-DNA insertion was determined by sequencing the T-DNA flanking sequences. Plants homozygous for the disruption were selected by PCR in the F₃ progeny.

Generation of the *domneg-1* and *kinless* Mutants. Site-directed mutagenesis was performed on the *KAT2* cDNA to replace the selectivity filter GlyTyrGlyAsp motif (hallmark of K⁺-selective channels) by ArgArgGlyAsp. The mutated cDNA, named *domneg*, was cloned downstream of the *KAT2* promoter region (2.258 kb upstream from the initiation codon) into a binary vector (pBIB-HYGRO), and the resulting plasmid was introduced into *Agrobacterium tumefaciens* GV3010 (pMP90) strain as described (10). WT plants (Wassilevskija ecotype) and *kat2-1* mutant plants were transformed by using the floral dip method (27). Selection on hygromycin allowed us to identify transformed lines and to obtain a fixed transgenic *domneg-1* or *kinless* line, respectively.

Reverse Transcription Experiments. Total RNA was extracted from *Arabidopsis* leaves by using TRIzol reagent (Invitrogen). After conversion to first-strand cDNA, *KAT2* and *EF1α* were amplified by PCR from the same amounts of cDNA by using the following couples of primers: *KAT2-3000* (5'-gcgctttagcagagtttagctgc-3') and *KAT2-3930* (5'-ccgtgaaataggtgagcttct-gaacgattggg-3') or *EF1α-350* (5'-ccaccactgggtgtttagaggctggtac-3') and *EF1α-900* (5'-cattgaaccaactggtgtcactggaag-3').

Intact Plant Transpiration Measurements. Plants were individually grown on compost in plastic containers in a growth chamber (21°C, 70% relative humidity, 8-h/16-h light/dark, 300 μE·m⁻²·s⁻¹). Each container was closed by a screw cap to avoid evaporation from the compost surface. After sowing, each plant grew through a hole pierced in the middle of the cap. This experimental device ensured that water loss (decrease in weight) could be ascribed to leaf transpiration. Periodic addition of water into the container maintained the compost water content close to 75% (wt/wt). The transpirational water loss was expressed on leaf area basis, estimated from daily plant photographs using OPTIMAS 6.1 software.

A controlled automated growth chamber [Phenopsis robot (28)] was used to impose variable micrometeorological conditions (light and VPD; the containers were weighed every 15 min). To study the effect of Na⁺ on transpiration, the plants were hydroponically grown in half-strength Hoagland solution using the same device (plastic containers) as described above for plants grown on compost. Na⁺ was added to the nutrient solution as a chloride salt.

Transpiration and CO₂ Assimilation in Individual Whole Plants. Plants were hydroponically grown in a growth chamber (22°C, 65% relative humidity, 8-h/16-h light/dark, 300 μE·m⁻²·s⁻¹) for 4 weeks before being transferred (a single plant per experiment) to an experimental chamber allowing continuous gas exchange measurements (as described in ref. 10).

Stomatal Conductance. Measurements of stomatal conductance were performed in intact leaves on intact plants using a Li-COR 6400 infrared gas analyzer-based gas exchange system (Li-COR). Leaves were kept at 75% ± 2% relative humidity and 22°C.

Stomatal Aperture Measurements. Epidermal peels were prepared from abaxial epidermis as described (10) and incubated in 30 or 10 mM KCl and 10 mM Mes-iminodiacetic acid (pH 6.5) at 20°C, unless otherwise noted. To standardize the initial state, the epidermal strips were kept in the incubation solution for 30 min in darkness. Then, they were exposed to treatments inducing stomatal aperture: white light ($300 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, 30 mM KCl), blue light ($30 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, 30 mM KCl), red light ($80 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, 30 mM KCl), fusicoccin (10 μM , 10 mM KCl), or CO_2 -free air (30 mM KCl). Effects of Cs^+ or Na^+ on stomatal behavior were investigated either in 30 mM CsNO_3 and 10 mM KCl, using fusicoccin (10 μM) to trigger stomatal opening, or in 20 mM NaCl and 10 mM KCl, using white light ($300 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) to trigger stomatal opening. Stomatal apertures were measured (pore width; at least 40 measurements per epidermis for each experimental point) with an optical microscope (Optiphot; Nikon) fitted with a camera lucida and a digitizing table (Houston Instruments) linked to a personal computer.

Guard Cell Protoplast Isolation and Electrophysiological Recordings. Plants were grown on compost for 5 weeks in a greenhouse. Guard cell protoplasts were

enzymatically isolated, and patch-clamp experiments were carried out as described (10). The pipette solution contained 100 mM K-glutamate, 2 mM MgATP, 5 mM EGTA, 1 mM CaCl_2 (50 nM free Ca^{2+}), 0.5 mM MgCl_2 , 300 mM D-mannitol, and 20 mM HEPES-KOH (pH 7.25). The bath solution contained 20 mM CaCl_2 , 2 mM MgCl_2 , 100 mM K-glutamate, 225 mM D-mannitol, and 10 mM Mes-HCl (pH 5.5). Liquid junction potentials at the pipette–bath interface were measured and corrected.

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1. Hetherington AM (2001) Guard cell signaling. *Cell* 107:711–714.
2. Assmann SM, Wang X-Q (2001) From milliseconds to millions of years: Guard cells and environmental responses. *Curr Opin Plant Biol* 4:421–428.
3. Blatt MR (2000) Cellular signaling and volume control in stomatal movements in plants. *Annu Rev Cell Dev Biol* 16:221–241.
4. Roelfsema MR, Hedrich R (2005) In the light of stomatal opening: New insights into 'the Watergate.' *New Phytol* 167:665–691.
5. Schroeder JI, Allen GJ, Hugouvieux V, Kwak JM, Waner D (2001) Guard cell signal transduction. *Annu Rev Plant Physiol Plant Mol Biol* 52:627–658.
6. Talbot LD, Zeiger E (1996) Central roles for potassium and sucrose in guard-cell osmoregulation. *Plant Physiol* 111:1051–1057.
7. MacRobbie EA (1998) Signal transduction and ion channels in guard cells. *Philos Trans R Soc London B* 353:1475–1488.
8. Schroeder JI, Raschke K, Neher E (1987) Voltage dependence of K channels in guard-cell protoplasts. *Proc Natl Acad Sci USA* 84:4108–4112.
9. Lebaudy A, Véry A-A, Sentenac H (2007) K⁺ channel activity in plants: Genes, regulations and functions. *FEBS Lett* 581:2357–2366.
10. Hossy E, et al. (2003) The Arabidopsis outward K⁺ channel Gork is involved in regulation of stomatal movements and plant transpirations. *Proc Natl Acad Sci USA* 100:5549–5554.
11. Szyroki A, et al. (2001) KAT1 is not essential for stomatal opening. *Proc Natl Acad Sci USA* 98:2917–2921.
12. Véry A-A, Sentenac H (2002) Cation channels in the Arabidopsis plasma membrane. *Trends Plants Sci* 7:168–175.
13. Reintanz B, et al. (2002) AtK1, a silent Arabidopsis potassium channel alpha-subunit modulates root hair K⁺ influx. *Proc Natl Acad Sci USA* 99:4079–4084.
14. Dreyer I, et al. (1997) Plant K⁺ channel alpha-subunits assemble indiscriminately. *Biophys J* 72:2143–2150.
15. Xicluna J, et al. (2007) Increased functional diversity of plant K⁺ channels by preferential heteromerization of the shaker-like subunits AKT2 and KAT2. *J Biol Chem* 282:486–494.
16. Véry A-A, Sentenac H (2003) Molecular mechanisms and regulation of K⁺ transport in higher plants. *Annu Rev Plant Biol* 54:575–603.
17. Kinoshita T (2001) Phot1 and phot2 mediate blue light regulation of stomatal opening. *Nature* 414:656–660.
18. Shimazaki K, Doi M, Assmann SM, Kinoshita T (2007) Light regulation of stomatal movement. *Annu Rev Plant Biol* 58:219–247.
19. Ichida AM, Pei Z-M, Baizabal-Aguirre VM, Turner KJ, Schroeder JI (1997) Expression of a Cs(+)-resistant guard cell K⁺ channel confers Cs(+)-resistant, light-induced stomatal opening in transgenic arabidopsis. *Plant Cell* 9:1843–1857.
20. Robinson MF, Véry A, Sanders D, Mansfield TA (1997) How can stomata contribute to salt tolerance. *Ann Bot* 80:387–393.
21. Kwak JM, et al. (2001) Dominant negative guard cell K⁺ channel mutants reduce inward-rectifying K⁺ currents and light-induced stomatal opening in arabidopsis. *Plant Physiol* 127:473–485.
22. Pilot G, et al. (2001) Guard cell inward K⁺ channel activity in arabidopsis involves expression of the twin channel subunits KAT1 and KAT2. *J Biol Chem* 276:3215–3221.
23. Ivashikina N, et al. (2003) Isolation of AtSUC2 promoter-GFP-marked companion cells for patch-clamp studies and expression profiling. *Plant J* 36:931–945.
24. Vavasseur A, Raghavendra AS (2005) Guard cell metabolism and CO₂ sensing. *New Phytol* 165:665–682.
25. Dodd AN, et al. (2005) Plant circadian clocks increase photosynthesis, growth, survival, and competitive advantage. *Science* 309:630–633.
26. Webb AAR (2003) The physiology of circadian rhythms in plants. *New Phytol* 160:281–303.
27. Bechtold N, Ellis J, Pelletier G (1993) In planta Agrobacterium mediated gene transfer by infiltration of adult Arabidopsis plants. *C R Acad Sci* 316:1194–1199.
28. Granier C, et al. (2006) PHENOPSIS, an automated platform for reproducible phenotyping of plant responses to soil water deficit in Arabidopsis thaliana premitted the identification of an accession with low sensitivity to soil water deficit. *New Phytol* 169:623–635.