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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<sup>+</sup> channels (Kin channels) are expressed in Arabidopsis guard cells. A double mutant plant was engineered by disruption of a major Kin 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 Kin channel (GCKin) activity, providing a model to investigate the roles of this activity in the plant. GCK<sub>in</sub> 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<sup>+</sup> instead of Na<sup>+</sup> during stomatal opening, thereby preventing deleterious effects of Na<sup>+</sup> 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<sub>in</sub> activity was found to allow a strong increase (35%) in plant biomass production. Thus, a large diversity of approaches indicates that GCK<sub>in</sub> 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  $CO_2$ 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  $CO_2$  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<sup>+</sup> and accompanying anions (malate and chloride) are among the major solutes involved in this osmotically driven process (2–6). plant physiology by engineering an *Arabidopsis* mutant totally deprived of this activity.

#### Results

Genetic Engineering of an Arabidopsis Mutant Deprived of GCKin Activity. In a first step, we screened candidate mutant lines in which expression of inward K<sup>+</sup> channels is affected, by looking for altered transpiration as the most probable phenotype. At least four inward K<sup>+</sup> 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 (T<sub>3</sub> 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 (T<sub>3</sub> 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<sup>+</sup> 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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Changes in guard cell K<sup>+</sup> content involve K<sup>+</sup> channel activity (7, 8) encoded by Shaker K<sup>+</sup> 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<sup>+</sup> conductance, *Arabidopsis* guard cells express at least four genes coding for Shaker  $\alpha$ -subunits involved in formation of inward channels (11). Here we assess the importance of the guard cell membrane inward K<sup>+</sup> channel (GCK<sub>in</sub>) activity in stomatal movements and whole-

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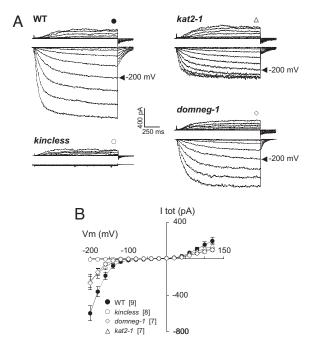
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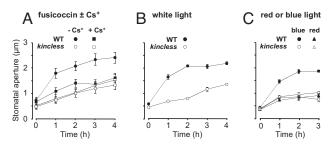
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**Fig. 1.** Absence of inward K<sup>+</sup> channel activity in guard cells of the *kincless* mutant. (A) Representative macroscopic current traces recorded with the patch-clamp technique in guard cell protoplasts from WT (*Upper Left*), *kat2-1* (knockout mutant disrupted in the *KAT2* gene) (*Upper Right*), *domneg-1* (expressing a dominant negative *kat2* construct in WT background) (*Lower Right*), or *kincless* (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 increments of -20 mV (bottom traces for each geno-type). (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  $\pm \text{SE}$  (with the number of repeats in brackets).

the effect of the mutations on GCK<sub>in</sub> activity. The individual mutations *kat2-1* and *domneg-1* were found to result in partial reduction of GCK<sub>in</sub> current, by  $\approx 60\%$  and 55%, respectively, at -200 mV (Fig. 1). The fact that reduction in GCK<sub>in</sub> 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<sub>in</sub> 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 *kincless* (K<sup>+</sup> inward currentless). It was used to investigate the role of GCK<sub>in</sub> activity in stomatal and whole-plant physiology.

GCK<sub>in</sub> 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<sup>+</sup>-ATPases, leading to membrane hyperpolarization and thereby to increased K<sup>+</sup> uptake through K<sup>+</sup> transport systems. To assess the actual contribution of GCK<sub>in</sub> activity to this process, we evaluated the effects of fusicoccin (FC), a toxin known to up-regulate plant plasma membrane H<sup>+</sup>-ATPases (17), of Cs<sup>+</sup>, a classical inhibitor of K<sub>in</sub> channel activity (19) and of blue, red, or white light on stomatal opening in epidermal peels from WT and *kincless* plants. The results (Fig. 2) can be summarized as follows. (*i*) Cs<sup>+</sup> did not further affect stomatal opening in the *kincless* mutant, providing additional



**Fig. 2.** Disruption of inward K<sup>+</sup> channel activity affects stomatal opening. Epidermal strips were peeled from WT (filled symbols) or *kincless* (open symbols) plants at the end of the dark period. Stomatal opening was induced (t = 0) by fusicoccin (10  $\mu$ M) in the presence or absence of Cs<sup>+</sup> (30 mM) (A) or by switching on white light (B) or blue or red light (C). K<sup>+</sup> 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<sup>+</sup>-ATPase activation and membrane hyperpolarization to the process (18).

GCK<sub>in</sub> Activity Underlies Stomatal Responsiveness to Changes in Light, Air Humidity, and CO<sub>2</sub> Availability in Intact Plants. Consequences of the absence of GCK<sub>in</sub> 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<sub>in</sub> activity affected the increase in transpiration induced by light (Fig. 3*A*). Fitting the increase in transpiration with monoexponential functions led to time constants approximately six times higher in the mutant ( $\approx$ 2 h) than in the WT ( $\approx$ 20 min) plants (Fig. 3*A*). These quantitative analyses demonstrate that stomatal opening induced by blue light in intact plants depends very strongly on GCK<sub>in</sub> 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<sup>+</sup> Channel Activity Results in Impaired Control of Stomatal Aperture in the Presence of Na<sup>+</sup> in the Medium. In far from physiological conditions, when epidermal peels were bathed in solutions containing high Na<sup>+</sup> concentrations (30 mM NaCl) but no K<sup>+</sup> at all, stomata were able to open upon light (Fig. S3), presumably by taking up Na<sup>+</sup> as an osmoticum in place of K<sup>+</sup> (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<sup>+</sup> cannot be rapidly excreted from the cell (4, 20). Interestingly, introducing K<sup>+</sup> in the bath solution (in the presence of Na<sup>+</sup>, 20 mM NaCl, and 10 mM KCl) before inducing stomatal opening (by switching light on) rescued WT but not *kincless* stomata from the deleterious effects of Na<sup>+</sup> on the subsequent stomatal closure (Fig. 4 *A* and *B*). Thus, in the

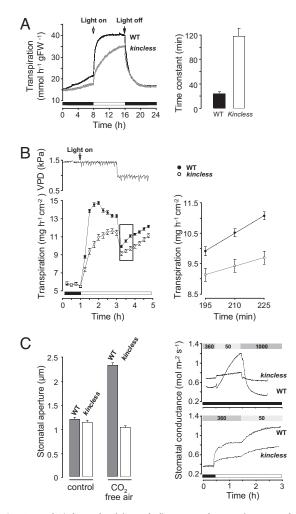
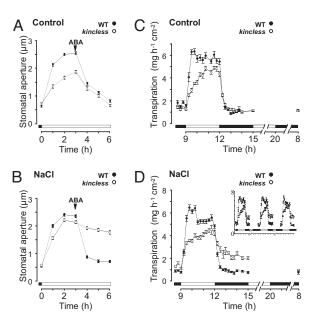


Fig. 3. Inward K<sup>+</sup> channel activity underlies stomatal responsiveness to changes in light, VPD, and CO<sub>2</sub> conditions. (A) Rate of light-induced stomatal opening. The consequences of the absence of  $GCK_{in}$  activity on leaf transpiration were then investigated by using a setup allowing to continuously monitor transpirational water loss in an intact plant (10). (Left) Typical recordings of transpiration in a WT or kincless 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  $\pm$  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 kincless 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  $\pm$  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<sup>+</sup>. 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 kincless leaves. Time courses of stomatal conductance in response to changes in CO<sub>2</sub> 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 CO<sub>2</sub> concentration in the air flow are indicated (expressed in ppm) in the gray boxes. The decrease in  $CO_2$  concentration in the air flow under dark conditions mimics depletion of internal CO<sub>2</sub> driven by photosynthetic activity (independent of light signal and photosynthesis).

presence of Na<sup>+</sup>, stomatal closure itself (and not only stomatal opening) depends on K<sup>+</sup>-selective GCK<sub>in</sub> activity.

A second set of experiments was then performed on intact



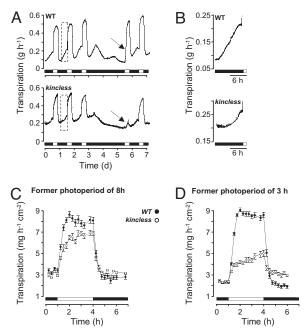
**Fig. 4.** Disruption of GCK<sub>in</sub> activity results in impaired stomatal closure upon salt stress. (*A* and *B*) Epidermal peel bioassays. Epidermal strips peeled from WT and *kincless* plants were bathed in either 30 mM KCl (*A*) or 10 mM KCl and 20 mM NaCl (*B*). Stomatal opening was induced (t = 0) by switching light on, and stomatal closure was triggered by adding the plant stress hormone ABA (10  $\mu$ M). (*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  $\pm$  SE; n = 15 plants per genotype. (C) Control treatment: 5-week-old plants continuously grown in the absence of Na<sup>+</sup>. (*D*) NaCl treatment: same plants as in *C*, but 12 h after introduction of 70 mM Na<sup>+</sup> in the nutrient solution. (*Inset*) Transpiration.

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

Inward K<sup>+</sup> Channel Activity Renders Stomatal Opening Independent of Previous Illumination Periods. Stomatal opening in *kincless* 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<sup>+</sup> 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 *kincless* mutant plants (Fig. 5 A and B).

**Dependence of Plant Growth on Inward K<sup>+</sup> 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<sup>+</sup> 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 *kincless* 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 *kincless* 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  $\mu$ E·m<sup>-2·s<sup>-1</sup></sup> light intensity, increasing light intensity to 650 or 1,000  $\mu$ E·m<sup>-2·s<sup>-1</sup></sup> (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 *kincless* mutant plants: biomass production (shoot fresh weight of 6-week-old plants) was higher in WT plants than in mutant plants, by ≈9.6 ± 3.1% or 36 ± 7.5% (n > 12) for the 650- $\mu$ E or 1,000- $\mu$ E treatments, respectively. No significant difference in biomass production between the two genotypes was observed when light intensity was kept constant, at 250  $\mu$ E·m<sup>-2·s<sup>-1</sup></sup>, during the whole light period.

#### Discussion

Large Reductions in GCK<sub>in</sub> 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<sub>in</sub> 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<sub>in</sub> 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<sub>in</sub> activity, by  $\approx 70-80\%$  (21). This suggested that GCK<sub>in</sub> 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 GCKin 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<sub>in</sub> 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<sub>in</sub> 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 Kin channel activity in guard cells, leaving phloem Kin channels largely unaffected. Consistent with this hypothesis, K<sup>+</sup> assays revealed similar leaf K<sup>+</sup> contents in kincless and control WT plants. It is also worth noting that lack of GCKin 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<sup>+</sup>. Thus, the whole set of data indicates that the kincless phenotype analyzed in this report, in terms of transpirational water loss by intact plants, reflects defects in stomatal movements rather than in plant K<sup>+</sup> status.

Roles of GCK<sub>in</sub> Activity in Stomatal Opening upon Membrane Hyperpolarization and Adaptation to Environmental Conditions. Stomata can open in the absence of GCKin activity but with a time constant ( $\approx$ 120 min) approximately six times greater than that measured in control WT plants. Fig. 2 clearly shows that signals inducing membrane hyperpolarization (fusicoccin, blue light) are poorly efficient in triggering stomatal opening in the mutant plant devoid of GCKin activity. This means that membrane hyperpolarization of the guard cell membrane due to H<sup>+</sup>-ATPase activation is unable, in the absence of GCKin activity, to trigger rapid accumulation of osmotica from the apoplast, for instance via H+:K+ or H+:sugar symporters. In other words, GCKin activity is an essential effector of rapid stomatal opening triggered 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<sup>+</sup> 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. 3*C*) or in evaporative demand (Fig. 3*B*). 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<sup>+</sup>. Experiments using epidermal peels in K<sup>+</sup>-free solution indicate that stomata can open by accumulating Na<sup>+</sup> instead of K<sup>+</sup>, 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<sup>+</sup> concentration gradient able to drive rapid efflux upon membrane depolarization. Indeed, Na<sup>+</sup> is compartmentalized into the vacuole because high concentrations of Na<sup>+</sup> would be toxic in the cytosol, and thus the concentration gradient of Na<sup>+</sup> across the plasma membrane cannot build up to become as steep as the K<sup>+</sup> gradient. The present data show that, in physiological conditions, when  $K^+$  and  $Na^+$  are both present in the medium, GCKin activity is required for efficient stomatal functioning (Fig. 4). It allows stomata to open by preferentially using K<sup>+</sup>, setting up the conditions for rapid stomatal closure, the large outward conductance of the membrane to K<sup>+</sup> (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<sup>+</sup> uptake in guard cells during stomatal opening, and this is guaranteed, in the presence of both K<sup>+</sup> and Na<sup>+</sup>, by the highly K<sup>+</sup>-selective GCK<sub>in</sub> activity. Noteworthy is the observation that treating plants lacking GCKin activity with Na<sup>+</sup>, 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<sup>+</sup> being moved to the shoot through the xylem to an extent that is potentially toxic.

A Crucial Role of GCK<sub>in</sub> 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 preopening during the last hours of the night promotes  $CO_2$  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<sub>in</sub> 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<sup>+</sup> uptake by inward K<sup>+</sup> 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<sup>+</sup> channel (GCK<sub>out</sub>) activity. For instance, absence of GCK<sub>in</sub> activity results in a 6-fold increase in the time constant of stomatal opening, from  $\approx 20$  min to 2 h, whereas the time constant of stomatal closure induced by disruption of GCK<sub>out</sub> 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<sup>+</sup> uptake upon membrane hyperpolarization is more essential for stomatal physiology and/or more complex to achieve than wholesale K<sup>+</sup> secretion upon membrane depolarization.

By enabling membrane hyperpolarization to rapidly trigger stomatal opening, GCK<sub>in</sub> activity improves stomatal reactivity to changes in internal, environmental, and micrometeorological conditions (CO<sub>2</sub> availability, light, and evaporative demand). GCK<sub>in</sub> activity also allows stomatal opening to be independent of past illumination period and photosynthesis, maybe because accumulation of K<sup>+</sup> is likely to be less costly, from an energetic point of view, and/or more straightforward to achieve than accumulation of organic osmotica. Furthermore, GCKin 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 GCKin 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 GCKin activity prevents detrimental Na<sup>+</sup> uptake by guard cells upon membrane hyperpolarization, thereby ensuring efficient stomatal function and decreasing transpirational water loss. In conclusion, a robust and K<sup>+</sup>-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 F<sub>3</sub> progeny.

Generation of the domneg-1 and kincless Mutants. Site-directed mutagenesis was performed on the KAT2 cDNA to replace the selectivity filter GlyTyrGlyAsp motif (hallmark of K<sup>+</sup>-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 kincless line, respectively.

**Reverse Transcription Experiments.** Total RNA was extracted from *Arabidopsis* leaves by using TRIzol reagent (Invitrogen). After conversion to firststrand cDNA, *KAT2* and *EF1* $\alpha$  were amplified by PCR from the same amounts of cDNA by using the following couples of primers: KAT2-3000 (5'gcgtcttagacgagttagctcgc-3') and KAT2-3930 (5'-ccgtgaaataggtagacgttctgaacgattggg-3') or EF1 $\alpha$ -350 (5'-ccaccactggtggttttgaggctggtatc-3') and EF1 $\alpha$ -900 (5'-cattgaacccaacgttgtcacctggaag-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  $\mu$ E·m<sup>-2</sup>s<sup>-1</sup>). 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<sup>+</sup> 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<sup>+</sup> was added to the nutrient solution as a chloride salt.

**Transpiration and CO<sub>2</sub> 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  $\mu$ E·m<sup>-2</sup>·s<sup>-1</sup>) 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%  $\pm$  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  $\mu$ E·m<sup>-2</sup>·s<sup>-1</sup>, 30 mM KCl), blue light (30  $\mu$ E·m<sup>-2</sup>·s<sup>-1</sup>, 30 mM KCl), blue light (30  $\mu$ E·m<sup>-2</sup>·s<sup>-1</sup>, 30 mM KCl), blue light (30  $\mu$ E·m<sup>-2</sup>·s<sup>-1</sup>, 30 mM KCl), this coccin (10  $\mu$ M, 10 mM KCl), or CO<sub>2</sub>-free air (30 mM KCl). Effects of Cs<sup>+</sup> or Na<sup>+</sup> on stomatal behavior were investigated either in 30 mM CsNO<sub>3</sub> and 10 mM KCl, using fusicoccin (10  $\mu$ M) to trigger stomatal opening, or in 20 mM NaCl and 10 mM KCl, using white light (300  $\mu$ E·m<sup>-2</sup>·s<sup>-1</sup>) 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

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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<sub>2</sub> (50 nM free Ca<sup>2+</sup>), 0.5 mM MgCl<sub>2</sub>, 300 mM D-mannitol, and 20 mM Hepes-KOH (pH 7.25). The bath solution contained 20 mM CaCl<sub>2</sub>, 2 mM MgCl<sub>2</sub>, 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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