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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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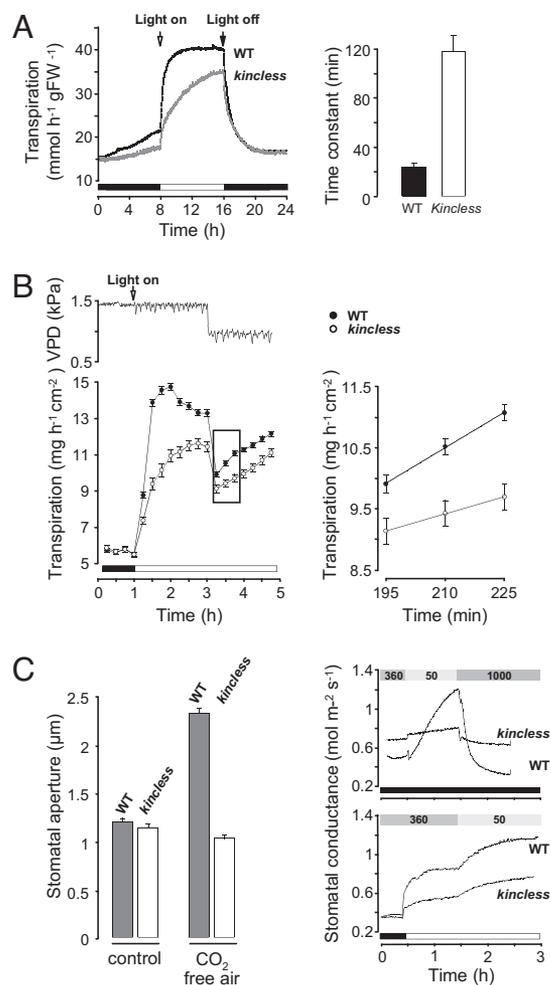


Fig. 3. Inward K⁺ channel activity underlies stomatal responsiveness to changes in light, VPD, and CO₂ conditions. (A) Rate of light-induced stomatal opening. The consequences of the absence of GCK_{in} activity on leaf transpiration were then investigated by using a setup allowing to continuously monitor 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

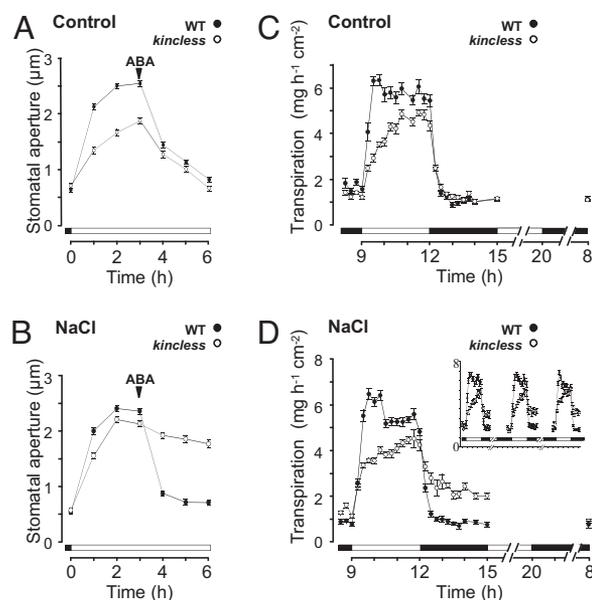


Fig. 4. Disruption of GCK_{in} activity results in impaired stomatal closure upon salt stress. (A and B) Epidermal peel bioassays. Epidermal strips peeled from WT and *kinless* plants were bathed in either 30 mM KCl (A) or 10 mM KCl and 20 mM NaCl (B). Stomatal opening was induced (*t* = 0) by switching 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

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 CO₂-free air (30 mM KCl). Effects of Cs⁺ or Na⁺ on stomatal behavior were investigated either in 30 mM CsNO₃ 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 CaCl₂ (50 nM free Ca²⁺), 0.5 mM MgCl₂, 300 mM D-mannitol, and 20 mM HEPES-KOH (pH 7.25). The bath solution contained 20 mM CaCl₂, 2 mM MgCl₂, 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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