

Corrections

BIOPHYSICS, CHEMISTRY. For the article “Watching proteins fold one molecule at a time,” by Elizabeth Rhoades, Eugene Gussakovsky, and Gilad Haran, which appeared in issue 6, March 18, 2003, of *Proc. Natl. Acad. Sci. USA* (**100**, 3197–3202; First Published February 28, 2003; 10.1073/pnas.2628068100), the

locants for Fig. 6 on page 3201 were incorrect. Locant *C* should have appeared as *B*, *E* should have appeared as *C*, *B* should have appeared as *D*, and *D* should have appeared as *E*. The corrected figure and its legend appear below.

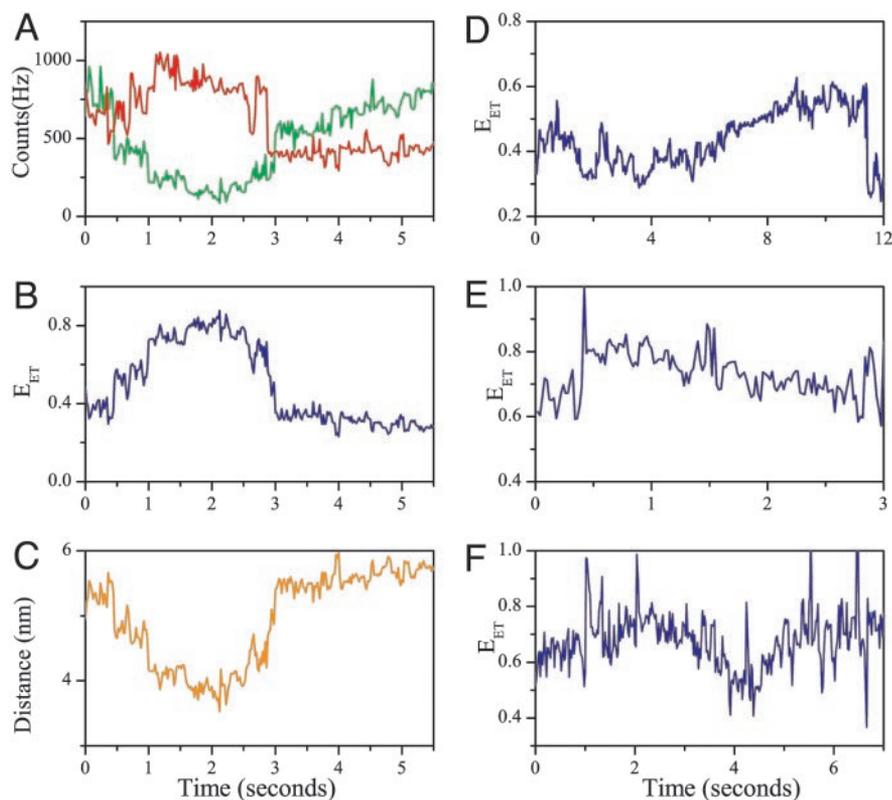


Fig. 6. Time-dependent signals from single molecules showing slow folding or unfolding transitions. (A) Signals showing a slow folding transition starting at ≈ 0.5 sec and ending at ≈ 2 sec. The same signals display a fast unfolding transition as well (at ≈ 3 sec). The acceptor signal is shown in red, and the donor is shown in green. (B) E_{ET} trajectory calculated from the signals in A. (C) The interprobe distance trajectory showing that the slow transition involves a chain compaction by only 20%. The distance was computed from the curve in B (32) by using a Förster distance (R_0) of 49 Å. This Förster distance was calculated by assuming an orientational factor (κ^2) of 2/3. However, the point discussed here (and in the text) does not depend on the exact value of κ^2 or R_0 . (D–F) Additional E_{ET} trajectories demonstrating slow transitions. These transitions were identified, as already noted, by anticorrelated donor-acceptor intensity changes.

www.pnas.org/cgi/doi/10.1073/pnas.1232163100

PLANT BIOLOGY. For the article “The *Arabidopsis* outward K^+ channel *GORK* is involved in regulation of stomatal movements and plant transpiration,” by Eric Hosy, Alain Vavasseur, Karine Mouline, Ingo Dreyer, Frédéric Gaymard, Fabien Porée, Jossia Boucherez, Anne Lebaudy, David Bouchez, Anne-Aliénor Véry, Thierry Simonneau, Jean-Baptiste Thibaud, and Hervé Sentenac, which appeared in issue 9, April 29, 2003, of *Proc. Natl. Acad. Sci. USA* (**100**, 5549–5554; First Published April 1, 2003; 10.1073/pnas.0733970100), the authors note that the word “azobenzene arsonate” should have read “abscisic acid” throughout the article. This error occurred in line 20 of the abstract; on page 5551, left column, second line from the bottom; on line 6 of the Fig. 3 legend; and on page 5553, right column, 11 lines from the bottom. The conclusions presented are unaffected by this change.

www.pnas.org/cgi/doi/10.1073/pnas.1332514100

The *Arabidopsis* outward K⁺ channel *GORK* is involved in regulation of stomatal movements and plant transpiration

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Edited by Enid MacRobbie, University of Cambridge, Cambridge, United Kingdom, and approved February 5, 2003 (received for review July 4, 2002)

Microscopic pores present in the epidermis of plant aerial organs, called stomata, allow gas exchanges between the inner photosynthetic tissue and the atmosphere. Regulation of stomatal aperture, preventing excess transpirational vapor loss, relies on turgor changes of two highly differentiated epidermal cells surrounding the pore, the guard cells. Increased guard cell turgor due to increased solute accumulation results in stomatal opening, whereas decreased guard cell turgor due to decreased solute accumulation results in stomatal closing. Here we provide direct evidence, based on reverse genetics approaches, that the *Arabidopsis* *GORK Shaker* gene encodes the major voltage-gated outwardly rectifying K⁺ channel of the guard cell membrane. Expression of *GORK* dominant negative mutant polypeptides in transgenic *Arabidopsis* was found to strongly reduce outwardly rectifying K⁺ channel activity in the guard cell membrane, and disruption of the *GORK* gene (T-DNA insertion knockout mutant) fully suppressed this activity. Bioassays on epidermal peels revealed that disruption of *GORK* activity resulted in impaired stomatal closure in response to darkness or the stress hormone azobenzearsonate. Transpiration measurements on excised rosettes and intact plants (grown in hydroponic conditions or submitted to water stress) revealed that absence of *GORK* activity resulted in increased water consumption. The whole set of data indicates that *GORK* is likely to play a crucial role in adaptation to drought in fluctuating environments.

The epidermis of the aerial organs of terrestrial plants presents a waxy cuticle that prevents water loss and desiccation but impedes diffusion of atmospheric CO₂ toward the inner tissues. Gas exchanges mainly take place through microscopic pores, the stomata. Two highly differentiated epidermal cells surrounding the pore, named guard cells, control stomatal aperture, allowing the plant to cope, under diverse environmental conditions, with the conflicting needs of maintaining a sufficient internal CO₂ concentration for photosynthesis and of preventing excessive transpirational water loss (1, 2).

Regulation of stomatal aperture relies on turgor changes of the two guard cells; an increase in turgor, caused by increased solute accumulation, promotes pore opening, whereas a decrease in turgor, caused by solute efflux, leads to stomatal closure (1, 2). The available information indicates that the main solutes involved in the osmoregulation process are sucrose, K⁺, and accompanying anions (malate and chloride), depending on the environmental conditions. In the normal diurnal cycle, stomatal opening in the morning would mainly result from K⁺ salt accumulation. In the afternoon, it would mainly rely on sucrose accumulation (3, 4).

The changes in guard cell K⁺ contents contributing to stomatal opening/closure have been shown to involve various channels working in a coordinated way in the plasma membrane and tonoplast. Two types of K⁺-permeable voltage-gated channels, either inwardly or outwardly rectifying, have been extensively characterized in the plasma membrane. The inwardly rectifying K⁺ channels activate on membrane hyperpolarization and are therefore mainly involved in K⁺ entry into the cell. The outwardly rectifying channels activate on membrane depolarization, at membrane potentials more positive than the K⁺-equilibrium potential, and thus allow K⁺ release (5, 6). Molecular and electrophysiological analyses support the hypothesis that these channels are encoded by genes of the *Shaker* family. This family comprises nine members in *Arabidopsis* (7). Three of them, *KAT1* and *KAT2*, which encode inwardly rectifying channels, and *GORK*, which encodes an outwardly rectifying channel, display high expression levels in guard cells as suggested by *GUS* reporter gene approaches (8) and/or quantitative RT-PCR analyses (9, 10). Reverse genetics approaches have been developed to investigate the role of *KAT1* in stomatal opening, using an *Arabidopsis* mutant carrying a knockout mutation in this gene (10) or transgenic *Arabidopsis* lines expressing dominant negative *KAT1* polypeptides (11). The knockout mutation and the expression of dominant negative polypeptides affected inward K⁺ channel activity in the guard cell membrane but did not result in total suppression of the inward K⁺ current, probably because of inwardly rectifying K⁺ channel redundancy. Although the plants expressing dominant negative mutant *KAT1* polypeptides displayed reduced light-induced stomatal opening (11), the relative contribution of the different guard cell inward K⁺ channels in stomatal movements remains unclear.

In the present study, we provide evidence that the *Arabidopsis* *GORK* gene encodes the major voltage-gated outwardly rectifying K⁺ channel of the guard cell membrane. We also demonstrate that *GORK* plays a role in the control of stomatal movements and allows the plant to significantly reduce transpirational water loss.

Materials and Methods

Isolation of the T-DNA-Tagged Mutant *gork-1* Disrupted in the *GORK* Gene. The *gork-1* knockout line was obtained by PCR screening of ≈40,000 *Arabidopsis thaliana* T-DNA insertion mutants (Was-

This paper was submitted directly (Track II) to the PNAS office.

See commentary on page 4976.

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silevskija ecotype; library constructed by the Station de Génétique et Amélioration des Plantes, Versailles, France; ref. 12), with primers corresponding to the *GORK* gene and to the T-DNA left and right borders. Selection on kanamycin 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 of the positive line.

Obtention of the *gork-dn* Dominant Negative Transgenic Lines. The sequence encoding the hallmark GlyTyrGlyAsp motif in the *GORK* pore domain (typical of K⁺-selective channels) was replaced by ArgArgGlyAsp (by site-directed mutagenesis) in the *GORK* gene. The mutated gene, named *gork-dn* (8.78 kb in total, with 2.714 kb upstream from the initiation codon) was cloned into the *Kpn*I–*Sst*I sites of the binary vector pBIB-HYGRO (13). The resulting plasmid was introduced into *Agrobacterium tumefaciens* GV3010 (pMP90) strain (14). *A. thaliana* (Wassilevskija ecotype) was transformed by using the floral dip method (15). Selection on hygromycin allowed us to identify transformed lines displaying a single insertion locus.

Intact Plant Transpiration Measurements. *Arabidopsis* plants were hydroponically grown in a growth chamber (22°C, 65% relative humidity, 8 h/16 h light/dark, 300 μmol·m⁻²·s⁻¹) for 4 weeks before being transferred (a single plant per experiment) to an experimental chamber allowing gas exchange measurements (dew-point hygrometers and infrared gas analyzer) as described (16). The root compartment (500 ml, 21°C) contained an aerated half-strength Hoagland solution. The shoot compartment (23°C, 8 h/16 h light/dark, 400 μmol·m⁻²·s⁻¹; HQI-TS NDL, Osram, Berlin) was attached to an open-flow gas circuit (air flow: 160 liters/h). At the inlet, the water-vapor pressure was held constant (1.5 kPa) and controlled with a dew-point hygrometer (Hygro, General Eastern Instruments, Woburn, MA). Humidity at the outlet was measured with a second dew-point hygrometer. The water-vapor pressure deficit in the leaf chamber was 0.9 ± 0.1 kPa (leaf and air temperatures measured with thermistors). Transpiration and photosynthesis were monitored for at least 8 days, including a 3-day adaptation period (the data corresponding to the adaptation period were not taken into account in the analyses). Photosynthesis was proportional to the leaf fresh weight and leaf area (data not shown).

Stomatal Aperture Measurements. Leaves from 4- to 6-week-old *Arabidopsis* plants were excised at the end of the night period, and epidermal strips were prepared as described (17). After peeling, epidermal strips were placed in Petri dishes containing 5 ml of the incubation solution (usual buffer unless otherwise noted: 30 mM KCl/10 mM Mes-iminodiacetic acid, pH 6.5). To standardize the initial state, epidermal strips were kept in the incubation solution for 30 min in darkness. Then, they were submitted to different treatments at 20°C in darkness or light (300 μmol·m⁻²·s⁻¹). Stomatal apertures were measured (pore width; at least 60 measurements per experiment in <5 min) with an optical microscope (Optiphot, Nikon) fitted with a camera lucida and a digitizing table (Houston Instruments) linked to a personal computer. Each experiment was performed at least in triplicate.

Electrophysiological Recordings. Plants were grown for ≈5 weeks in compost (individual containers) in a growth chamber (21°C, 70% relative humidity, 8 h/16 h light/dark, 300 μmol·m⁻²·s⁻¹). Guard cell protoplasts were isolated by digestion of leaf epidermal peels. The digestion solution contained 1 mM CaCl₂, 2 mM ascorbic acid, Onozuka RS cellulase (1% wt/vol, Yakult Pharmaceutical, Tokyo), Y-23 pectolyase (0.1% wt/vol, Seishin Pharmaceutical, Tokyo), and 1 mM Mes-KOH (pH 5.5). The osmo-

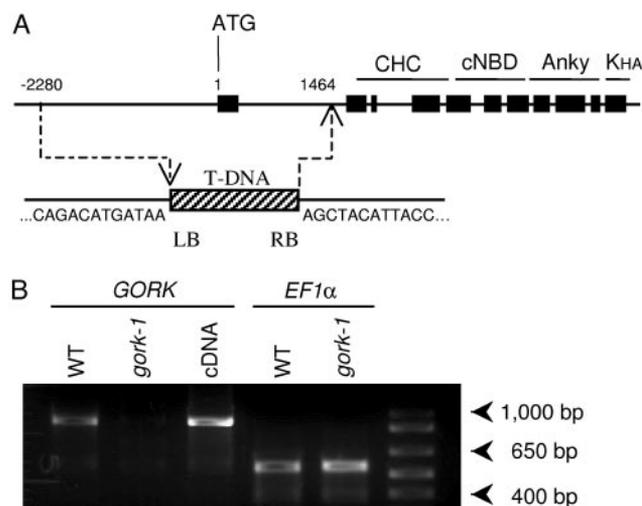


Fig. 1. Identification of a T-DNA insertion mutant. (A) Schematic diagram of the *GORK* gene indicating the site of insertion of the disrupting T-DNA in the *gork-1* mutant line. Black boxes represent exons. Typical domains of plant Shaker channels (30) are indicated as follows: CHC, channel hydrophobic core; cNBD, putative cyclic-nucleotide binding domain; Anky, ankyrin domain; KHA, KHA domain. The hatched box represents T-DNA. The nucleotide sequence flanking the T-DNA left (LB) and right (RB) borders in the disrupted gene are given. The T-DNA insertion resulted in a 3.7-kb deletion in the 5' region of the gene, from nucleotide -2,280 (upstream from the initiation codon) to nucleotide 1,464 (downstream from this codon). (B) Absence of *GORK* transcripts in the *gork-1* mutant. RT-PCR analyses were performed on total RNA extracted from aerial parts of either wild-type or *gork-1* plants (WT and *gork-1* lanes) by using primers (sequences available on request) specific for *GORK* or for the *Arabidopsis EF1α* elongation factor gene (used as a control). The cDNA lane shows PCR-amplified DNA fragment using the same *GORK* primers on the *GORK* cDNA (control).

larity was adjusted to 420 mosM with D-mannitol. The epidermal peels were digested for 40 min at 27°C. Filtration through 50-μm mesh allowed recovery of protoplasts. The filtrate was mixed with three volumes of conservation buffer (10 mM potassium glutamate/1 mM CaCl₂/2 mM MgCl₂/10 mM Mes-HCl, pH 5.5, with osmolarity adjusted to 500 mosM with D-mannitol). The protoplast suspension (≈10% of guard cell protoplasts, based on microscopic observations) was kept on ice. Patch-clamp pipettes were pulled (P97, Sutter Instruments, Novato, CA) from borosilicate capillaries (Kimax-51, Kimble, Toledo, OH) and fire polished (L/M CPZ 101, List Medical, Darmstadt, Germany). The pipette solution contained 1 mM CaCl₂, 5 mM EGTA, 2 mM MgCl₂, 100 mM potassium glutamate, 2 mM MgATP, 10 mM Hepes-NaOH, pH 7.5, with osmolarity adjusted to 520 mosM with D-mannitol. The bath solution contained 20 mM CaCl₂, 2 mM MgCl₂, 100 mM potassium glutamate, 10 mM Mes-HCl, pH 5.5, with osmolarity adjusted to 500 mosM with mannitol. In these conditions, the pipette resistance was ≈10 MΩ. Seals with resistance >5 GΩ were used for electrophysiological analyses. Whole-cell recordings were obtained by using an Axon Instruments Axopatch 200A amplifier. PCLAMP 6.0.3 software (Axon Instruments, Foster City, CA) was used for voltage pulse stimulation, online data acquisition, and data analysis. The voltage protocol consisted in stepping the membrane potential to voltages from -200 to +80 mV in 20-mV increments from a holding potential of -60 mV.

Results

Obtention of *Arabidopsis* Mutants Affected in *GORK* Activity. An *Arabidopsis* mutant line, named *gork-1*, was identified by PCR screening of a collection of T-DNA-transformed plants (Fig. 1A; T-DNA insertion flanking sequences indicated in the figure).

Growth tests on selection medium revealed a single insertion locus (data not shown). The T-DNA insertion was shown to result in a 3.7-kb deletion in the 5' region of the gene (Fig. 1). RT-PCR experiments performed on total RNA extracted from aerial parts of 4-week-old plants grown in a greenhouse indicated that *GORK* transcripts were not accumulated in homozygous *gork-1* plants (Fig. 1B).

Shaker channels are tetrameric proteins (18–20). This structural feature allows us to develop reverse genetics approaches by using dominant negative mutant subunits (obtained by site-directed mutagenesis) able to coassemble with wild-type subunits and to lead to formation of nonfunctional channels (11, 21). Mutations were introduced in the *GORK* coding sequence to replace the hallmark motif GlyTyrGlyAsp, expected to play a crucial role in the formation of the channel-conducting pathway (22), by ArgArgGlyAsp. Experiments in *Xenopus* oocytes revealed that the polypeptide encoded by the mutated sequence was electrically silent (not shown) and indeed endowed with dominant negative behavior; for instance, coinjection of mutant cRNA with wild-type cRNA (*in vitro* transcription) with a mutant/wild-type stoichiometry of 1:1 resulted in $82 \pm 6\%$ ($n = 10$) inhibition of the GORK current. The corresponding mutation was introduced in the *GORK* gene (8.7 kb in total, with a 2.7-kb promoter region), and the mutated gene was introduced into *Arabidopsis* plants. Single locus transformed lines (checked on hygromycin selection medium) were obtained. Patch-clamp experiments were performed on homozygous transgenic plants from the progeny (F_4 generation) of two of them, named *gork-dn1* and *gork-dn2* in this article.

Homozygous *gork-1*, *gork-dn1*, or *gork-dn2* plants were grown in a greenhouse or growth chamber (20°C, 50% relative humidity, 8 h/16 h light/dark, $300 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). Comparison with control wild-type plants grown in parallel in these conditions did not reveal any obvious phenotype. Because previous RT-PCR experiments had shown that *GORK* transcripts are also present in root hairs (23), root systems from plants grown *in vitro* on agar plates were examined under microscope. No obvious effect of the *gork-1* mutation on root hair density or development could be detected (data not shown).

The *gork-1* Mutation Prevents Expression of the Major Voltage-Gated Outwardly Rectifying K^+ Channel of the Guard Cell. Patch-clamp experiments on guard cell protoplasts prepared from wild-type plants revealed both inward and outward currents (Fig. 2A), in agreement with previous analyses (6). The outward current was dominated by a slowly activating sigmoidal component, appearing beyond a threshold potential of ≈ 0 mV (with 100 mM K^+ in both the bath and pipette solutions) and strongly reminiscent of that recorded in *Xenopus* oocytes expressing *GORK* cRNA (10). Similar experiments were performed on guard cell protoplasts prepared from homozygous *gork-1* plants (Fig. 2B) or from *gork-dn1* or *gork-dn2* plants (Fig. 2C). They revealed inward K^+ currents quite similar to those recorded in wild-type protoplasts but strongly reduced outward currents. The time course of the remaining outward K^+ current observed in *gork-dn1* (see the current trace at +80 mV magnified $\times 10$ in Fig. 2C) and *gork-dn2* (data not shown) protoplasts suggested some residual GORK activity. No GORK activity could be detected in *gork-1* guard cells, as shown by the flat kinetics of the residual outward current (current trace at +80 mV magnified $\times 10$ in Fig. 2B). It can therefore be concluded that *gork-1* is actually a knockout mutation.

Bioassays on Epidermal Peels Indicate That Disruption of GORK Activity Affects Stomatal Functioning. The role of *GORK* in stomatal movements was investigated *in vitro* by submitting epidermal peels from wild-type, *gork-1*, or *gork-dn1* plants to treatments inducing either stomatal closure (azobenzeneearsonate or darkness; Fig. 3A and B, respectively) or opening (light; Fig. 3C).

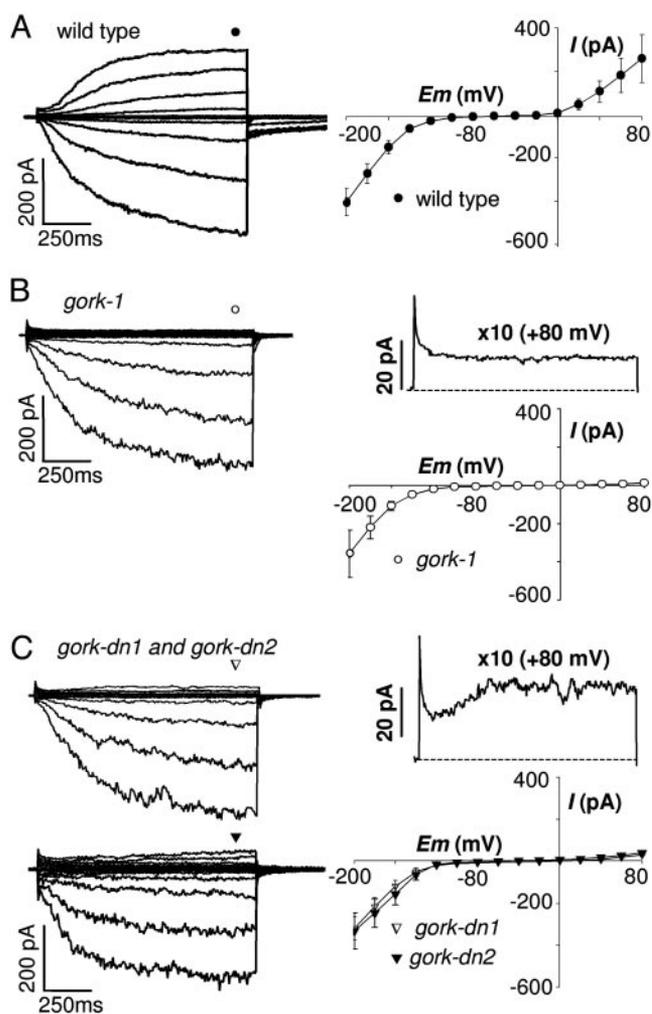


Fig. 2. *GORK* encodes the major voltage-gated outwardly rectifying K^+ channel of the guard cell membrane. Patch-clamp experiments were performed on guard cell protoplasts prepared from wild-type plants (A), homozygous *gork-1* mutant plants (B), or homozygous *gork-dn1* or *gork-dn2* plants, which both express a *GORK* dominant negative mutant polypeptide (C). (A–C Left) Typical examples of inward and outward currents recorded in whole protoplasts. The current traces obtained at +80 mV in the *gork-1* and the *gork-dn1* protoplasts are shown at $\times 10$ scale for easier visualization of kinetics at steady state (means \pm SE; $n = 9$ for wild type, 8 for *gork-1*, 5 for *gork-dn1*, and 5 for *gork-dn2*) are shown (Right). The bath and pipette solutions contained 100 mM potassium glutamate. The voltage steps ranged between -200 and $+80$ mV in 20-mV increments from a holding potential of -60 mV.

Stomatal closure was strongly altered in the *gork-1* and, to a lesser extent, in the *gork-dn1* plants (Fig. 3A and B), indicating that GORK activity was required for efficient stomatal closure. On the other hand, the *gork-1* mutation weakly increased light-induced stomatal opening (Fig. 3C). It is worth noting, however, that *gork-1* plants consistently displayed slightly larger apertures, e.g., by 10–15% at the end of the 3-h light pretreatment in Fig. 3A and B ($t = 0$).

Excised Rosettes from *gork-1* Plants Display Increased Transpirational Water Loss. As a first step in investigating the role of *GORK* in the control of leaf transpiration, we measured water loss (decrease in weight) of rosettes excised from wild-type, *gork-1*, or *gork-dn1* plants. The *gork-1* rosettes displayed greater water loss than the wild-type ones, by $\approx 35\%$ during the first hour after the excision (Fig. 4). The *gork-dn1* rosettes displayed an intermediate phe-

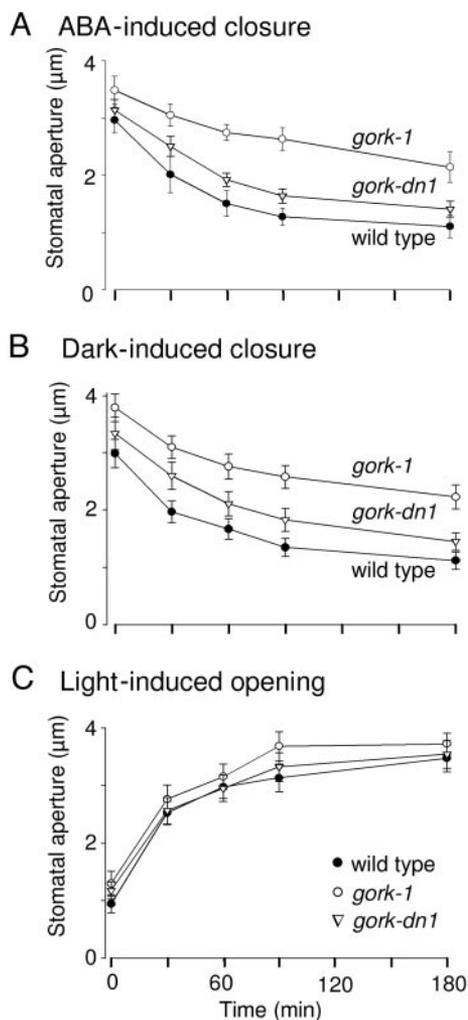


Fig. 3. Disruption of GORK activity affects stomatal movements. Epidermal strips were peeled from wild-type (●), homozygous *gork-1* (○), or *gork-dn1* (▽) plants at the end of dark period and transferred to 30 mM KCl and 10 mM Mes-KOH (pH 6.5). (A and B) Epidermal strips were placed under light for 3 h (stomatal opening pretreatment) before stomatal closure was induced ($t = 0$) by adding 20 μM azobenzene arsonate in the bath solution (A) or switching the light off (B). (C) Epidermal peels were kept in darkness for 3 h. Then, the light was switched on to induce stomatal opening. Measurements of stomatal apertures were performed at various times on the same strips during 3 h. Means \pm SE from four independent experiments, 60 measurements per experiment, are shown).

notype. This provided the first experimental evidence that GORK activity participates in leaf transpiration control.

Intact Plant Transpirational Water Loss Is Increased by the *gork-1* Mutation. In a second set of experiments, the effect of the *gork-1* mutation on intact plant transpiration was investigated by using an experimental setup that allowed us to control the water-vapor pressure deficit (0.9 kPa) and to continuously record the plant transpiration and CO_2 fixation rates (open-flow gas circuit). Switching light on and off induced an increase and decrease, respectively, in transpiration rate, leading to a new steady state (Fig. 5A). Analysis of the steady-state data (Fig. 5B) revealed that the *gork-1* plants displayed higher transpiration rates than the wild-type controls, by $\approx 25\%$ in darkness and $\approx 7\%$ in light (the latter difference, however, being not statistically significant). The kinetics of the transitions between the successive steady states were fitted (least-squares adjustment) with first

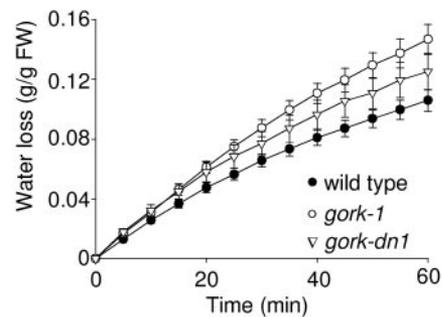


Fig. 4. Disruption of GORK activity results in increased water loss from excised rosettes. Rosettes from wild-type (●), *gork-1* (○), and *gork-dn1* (▽) plants grown on compost in growth chamber (21°C, 70% relative humidity, 8 h/16 h light/dark, 300 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) were excised just before the end of the light period and transferred to darkness. Water loss was determined by monitoring the decrease in fresh weight of the excised rosettes. Means \pm SE of six independent measurements are shown. The difference in transpiration between the wild-type and *gork-1* genotypes is statistically significant (paired values, Student's t test $P \ll 0.01$).

order exponential functions to derive the time constants, τ (Fig. 5C), assumed to reflect the rate of stomatal movements. The *gork-1* mutation was without any significant effect on the rate of stomatal opening but resulted in a strong decrease in the rate of stomatal closure, the mean τ value being shifted from ≈ 12 to 19 min. The former value is consistent with previous analyses of dark-induced stomatal closing in *Arabidopsis* (24) and of dark-induced Rb^+ efflux kinetics in *Commelina* (25).

In another experiment, 6-week-old wild-type and *gork-1* plants grown on compost were submitted to a progressive drought stress over 7 days, leading to loss of leaf turgor. The *gork-1* plants displayed higher transpirational water loss (weight loss) than the wild-type plants, during both the night and the light periods (Fig. 6). The relative difference was clearly more important at the end than at the beginning of the stress treatment (≈ 30 –50% and 5–15%, respectively), suggesting that the relative contribution of GORK to transpiration control is more important in conditions of water shortage.

Discussion

GORK Encodes the Major Outwardly Rectifying K^+ Channel Active in the Guard Cell Membrane. *In planta* (electro)physiological analyses have led to the conclusion that K^+ release from the guard cell, leading to stomatal closure, involves the activity of K^+ -selective slowly activating voltage-gated outwardly rectifying channels (6, 26). The functional features of these channels characterized *in vivo* in guard cells of *Arabidopsis* and of a number of other species are very similar to those displayed by the *Arabidopsis* SKOR and GORK channels expressed in heterologous systems (10, 27). These two channels belong to the so-called Shaker family, which comprises nine members in *Arabidopsis* (7). SKOR is expressed in root stelar tissues, where it plays a role in K^+ secretion into the xylem sap (27). RT-PCR experiments have revealed GORK transcripts in guard cells and in root hairs (9, 23). This information supported the hypothesis that the outward K^+ channel active in guard cells was encoded by the GORK gene (in *Arabidopsis*; ref. 10). Here, we show that expressing a dominant negative mutant allele of the GORK gene in *Arabidopsis* leads to a strong decrease in the outward K^+ conductance of the guard cell membrane and that disruption of the GORK gene results in full suppression of this conductance (Fig. 2). Thus, the present data demonstrate that GORK and SKOR are not redundantly expressed in guard cells and that functional expression of the GORK gene is required for voltage-gated outwardly rectifying K^+ channel activity in the guard cell plasma membrane. The

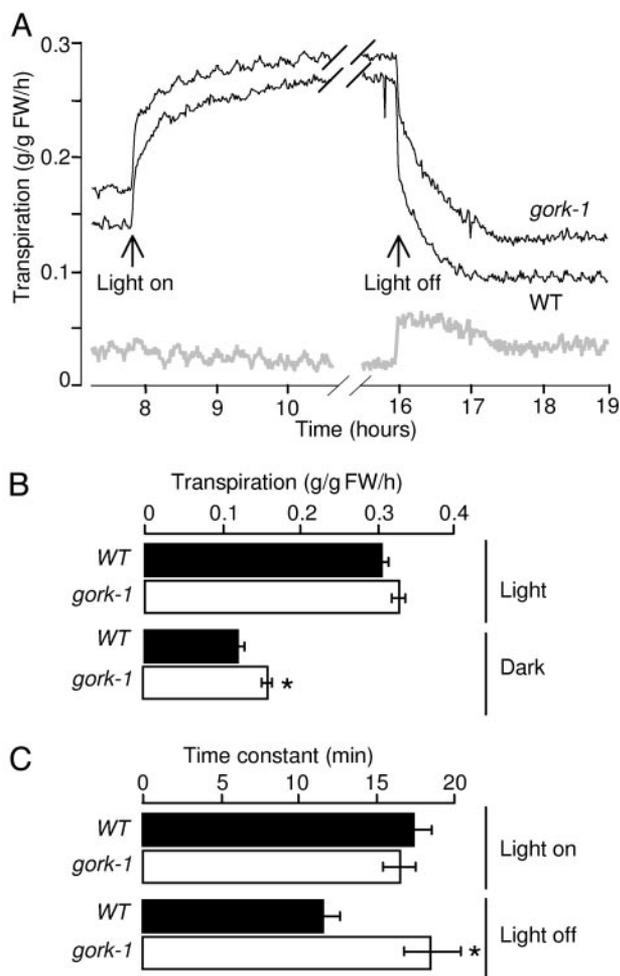


Fig. 5. Effect of the *gork-1* mutation on intact plant transpiration in hydroponic conditions. (A) Typical examples of transpiration recordings. A single hydroponically grown wild-type or *gork-1* plant was present in the experimental chamber. The gray curve shows the difference in transpiration between the two plants. (B) Transpiration rates measured as described in A at the end of the light and dark periods (means \pm SE, four plants per genotype, five successive photoperiods taken into account for each plant). (C) Time constants describing the changes in transpiration rates induced by light or darkness. To derive time constants reflecting the rate of stomatal opening and closure, experimental kinetics (as those shown in A) describing the changes in transpiration rates observed when light was switched on or off were fitted by exponential equations (dashed lines; least-squares fitting, Levenberg-Marquardt algorithm), respectively (means \pm SE, four plants, five photoperiods per plant). Asterisks indicate that the corresponding differences in dark-induced stomatal closure (lower bars in B and C) are statistically significant (Student's *t* test, $P < 0.01$).

simplest hypothesis is that a single gene, *GORK*, encodes the major voltage-gated outwardly rectifying K^+ channel characterized in this membrane (6).

GORK Activity Is Involved in the Control of Stomatal Movements.

Circumstantial evidence supported the hypothesis that, by mediating K^+ release, the outward rectifier of the guard cell membrane could play a major role in stomatal closure and thereby in transpiration control. The reverse genetics approach developed in our study provides direct support to this hypothesis. The whole set of data indicates that transpiration is more important in *gork-1* than in wild-type plants, from 10% (Fig. 5) up to $\approx 50\%$ (Fig. 6), depending on the environmental conditions (water availability in these experiments). Thus, whereas disrup-

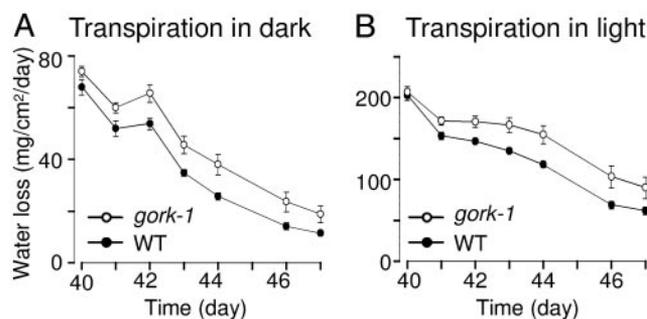


Fig. 6. Increased contribution of GORK to water saving during water stress. Wild-type or *gork-1* plants were grown in parallel for 6 weeks on compost in a growth chamber (20°C, 50% relative humidity, 8 h/16 h light/dark, 300 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). The experimental procedure ensured that water loss (decrease in weight) resulted from leaf transpiration (mean total leaf area of $\approx 90\text{ cm}^2$ per plant at this stage). Periodic weighing and watering allowed us to maintain the water content of the compost at $\approx 70\%$ (wt/wt) during this period. Then, watering of the plants was stopped ($t = 0$; beginning of the water stress period). Only small amounts of water were daily added to the *gork-1* plant containers, after weighing the devices, to strictly compensate for the difference in transpiration rates between the two genotypes. The compost water content of every container decreased in a similar (and almost linear) way, whatever the plant genotype. The devices were weighed twice a day, at the end of the dark and light periods. The transpirational water loss (mean \pm SE; $n = 9$ for the wild-type genotype and 7 for the *gork-1* genotype) during the dark (A) and light (B) periods was expressed on a leaf area basis, the plants being photographed daily (leaf area determined by using OPTIMAS 6.1 software). Plants conspicuously began to suffer from water stress (loss of leaf turgor) 4 days after watering was stopped. The differences between the two genotypes in A and B are statistically significant (paired values, Student's *t* test, $P \ll 0.01$).

tion of the guard cell inward K^+ channel gene *KATI* has been found to be without any effect on the regulation of stomatal aperture (9), that of the outward channel *GORK* results in both impaired stomatal movements and impaired transpiration control. This difference is likely to result from the fact that expression of inward K^+ channel activity in guard cells would involve at least two different genes, *KATI* and *KAT2* (8), whereas the outwardly rectifying K^+ channel activity relies mainly on a single gene, *GORK*.

In vitro analyses using epidermal peels are likely to give a distorted view of *in planta* stomatal control because of the lack of functional interactions with the surrounding epidermal cells. The bioassays shown in Fig. 3 would suggest larger differences in transpiration between wild-type and *gork-1* plants than those observed in Fig. 5. However, this discrepancy could result, at least in part, from the fact that hydroponic conditions (Fig. 5) lessen the importance of stomata, and thus of *GORK* activity, in the control of transpiration. Indeed, larger differences in transpirational water loss between the wild-type and *gork-1* genotypes were observed when the plants were grown in compost and submitted to water shortage (Fig. 6).

Larger steady-state stomatal apertures were found in *gork-1* than in wild-type epidermal peels not only at the end of closure-inducing treatments (azobenzenearsonate, darkness; Fig. 3A and B) but also at the end of 3-h light-induced opening pretreatments/treatments (experimental points corresponding to time 0 in Fig. 3). Consistent with these observations, *in planta* analyses revealed that *gork-1* plants displayed higher transpiration rates than wild-type plants not only in darkness but also in light (Figs. 5 and 6). These results suggest that *GORK* could act as a negative regulator of stomatal opening in light, in addition to playing a role in stomatal closure. It has been shown that the membrane voltage in guard cells can undergo large ($>100\text{ mV}$) and rapid ($\approx 10\text{-s}$ period) oscillations between hyperpolarized

values allowing K⁺ uptake and depolarized values allowing K⁺ release (28). This has led to the hypothesis that control of stomatal movements and steady-state aperture depends on variations in the pattern of these oscillations, enabling the net guard cell K⁺ content to increase or to decrease as a result of changes in the balance of K⁺ uptake through hyperpolarization-activated channels and K⁺ release through depolarization-activated channels (28). The proposal that GORK might act as a negative regulator of stomatal opening stands coherently within the framework of this hypothesis.

Physiological Significance and Role in Natural Conditions. The available information indicates that the dominant solutes involved in guard cell osmoregulation and control of stomatal aperture are K⁺ salts and sugars (mainly sucrose), depending on the environmental conditions and the time of the day (4, 29). Genetic tools allowing the assessment of the relative contribution of these solutes and of the mechanisms responsible for their transport and accumulation are highly needed. The present data provide direct genetic evidence that *GORK* encodes the major voltage-gated outwardly rectifying K⁺ channel expressed in the guard cell plasma membrane, its disruption resulting in a dramatic decrease in the membrane outward K⁺ conductance. However, when grown in standard controlled conditions or even in the greenhouse, *gork-1* plants do not display any obvious phenotype. Furthermore, the *gork-1* stomata can undergo large movements (Fig. 3). This indicates that, in the absence of the GORK channel, different processes, likely to involve activity of other K⁺ efflux systems, can efficiently contribute to the decrease in turgor pressure leading to stomatal closure. Based on the present data, it cannot be said whether these processes/systems significantly contribute to stomatal closure also in the presence of GORK activity or whether they correspond to compensation

mechanisms resulting from the loss of GORK activity. It is worthy to note that the very low level of GORK current remaining in the plants expressing the dominant negative mutant channel resulted in a stomatal phenotype closer to that of the wild-type plants than to that of the *gork-1* plants (Fig. 3). The dominant negative mutant lines could be valuable genetic tools to further investigate why stomatal physiology requires the guard cell membrane to be fitted with a large voltage-gated outwardly rectifying K⁺ conductance.

In hydroponic conditions, the absence of GORK activity resulted in an increase in steady-state transpiration, by ≈7% in light and 25% in darkness (Fig. 5B). During the light-to-dark transition, the increase in water loss reached higher percentages (up to 40–50%; see the peak displayed by the gray curve in Fig. 5A) because of the slower closure kinetics during the transition from the open to the closed steady state once light was turned off. Thus, the contribution of GORK to water saving would be more important in natural conditions with fluctuating environmental conditions requiring rapid adaptation of stomatal opening (e.g., sudden changes in water-vapor pressure deficit and/or light intensity). Also, the data shown in Fig. 6 highlight that drought conditions amplify the relative contribution of GORK to water saving. Reduced water consumption by ≈10–20% can allow the plant to postpone dehydration by several days. Thus, the *GORK* gene is likely to play an important role in drought adaptation and to be under high selection pressure in natural fluctuating environments.

We are grateful to I. A. Lefevre and S. Zimmermann for helpful discussions and comments on the manuscript. This work was partly supported by a Marie Curie Fellowship of the European Union (to I.D.) and an Indo-French Center for the Promotion of Advanced Research grant (to A.V.).

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