BIOPHYSICS, CHEMISTRY. For the article “Watching proteins fold one molecule at a time,” by Elizabeth Rhoades, Eugene Gussakovskiy, 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) $E_T$ 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 ($\psi^2$) of 2/3. However, the point discussed here (and in the text) does not depend on the exact value of $\psi^2$ or $R_0$. (D–F) Additional $E_T$ trajectories demonstrating slow transitions. These transitions were identified, as already noted, by anticorrelated donor-acceptor intensity changes.

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 Gaynard, 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 “azo-benzenearsonate” 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.
The *Arabidopsis* outward K\(^+\) channel GORK is involved in regulation of stomatal movements and plant transpiration

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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 water 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 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 abscisic acid (ABA). Transpiration measurements on excised rosettes and intact plants (grown in hydroponic conditions or subjected 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 CO\(_2\) toward the inner tissues. Gas exchanges mainly take place through microscopic pores, the stomata. Two highly differentiated epidermal cells surrounding the pore, called guard cells, control stomatal aperture, allowing the plant to cope, under diverse environmental conditions, with the conflicting needs of maintaining a sufficient internal CO\(_2\) 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**

The gork-1 knockout line was obtained by PCR screening of ~40,000 *Arabidopsis thaliana* T-DNA insertion mutants (Was...
silevskija ecotype; library constructed by the Station de Généticue 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 KpnI–SstI 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 hydropsonically 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 ND, 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 osmolarity 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. PC-LAMP 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 expressed the polypeptide encoded by the mutated sequence revealed that the polypeptide was still able to coassemble with wild-type 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 expressed the polypeptide encoded by the mutated sequence revealed that the polypeptide was still able to coassemble with wild-type subunits (obtained by site-directed mutagenesis) able to coassemble with wild-type subunits and to lead to formation of nonfunctional channels (11, 21).

The gork-1 Mutation Prevents Expression of 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 +180 mV in the gork-1 and the gork-dn1 protoplasts are shown at ×10 scale for easier visualization of kinetics in B and C (Right; dotted line, zero current level). Current-voltage relationships at steady state (means ± 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 +180 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) in 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 ∼35% during the first hour after the excision (Fig. 4). The gork-dn1 rosettes displayed an intermediate phe-
by adding 20 mM H9262 (stomatal opening pretreatment) before stomatal closure was induced (Mes-KOH (pH 6.5). (B) Epidermal strips were placed under light for 3 h (stomatal opening pretreatment) before stomatal closure was induced (t = 0) by adding 20 μM azobenzeneasorane in the bath solution (A) or switching the light off (B). (C) Epidermal strips 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 ± SE from four independent experiments, 60 measurements per experiment, are shown).

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...
The whole set of data indicates that transpiration is more developed in our study provides direct support to this hypothesis. The reverse genetics approach could play a major role in stomatal closure and thereby in transpiration control. The 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 ~50% (Fig. 6), depending on the environmental conditions (water availability in these experiments). Thus, whereas disruption of the guard cell inward K$^+$ channel gene KAT1 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, KAT1 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 (azobenzene-carboxate, darkness; Fig. 3 A 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 mV) and rapid (~10-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.

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