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


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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 $t=0.5$ sec and ending at $t=2$ sec. The same signals display a fast unfolding transition as well (at $t=3$ sec). The acceptor signal is shown in red, and the donor is shown in green. (B) 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 ($m^2$) of 2/3. However, the point discussed here (and in the text) does not depend on the exact value of $m^2$ or $R_0$. (D–F) Additional ET 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 Moulène, 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.

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**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 vapor loss, relies on guard cell movements and allows the plant to cope, under diverse environmental conditions, with water stress. 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. The plant 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. 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 azobenzenearsonate. Transpiration measurements on excised roots and intact plants (grown in hydroponic conditions or subterranean) revealed that absence of GORK activity resulted in impaired stomatal closure and water stress (8). Importantly, reporter gene approaches (9, 10) and 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, 8). 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. The plant 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.

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. The plant 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.

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**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...
silevskija ecotype; library constructed by the Station de Géné-
tique 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–SrfI sites of the binary vector pBIB-HYGRO (13).
The resulting plasmid was introduced into Agrobacterium tumefaciens
GV3010 (pMP90) strain (14). A. thaliana (Wassilevskija
ectype) 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
hydropotronically 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; HOI-TS NDL, Osrarn,
Berlin) was attached to an open-flow gas circuit (air flow: 160
liters/h). At the inlet, the water-vapor pressure was held con-
stant (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 0.7 ±
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 correspond-
ing 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 epider-
mal peels. The digestion solution contained 1 mM CaCl₂, 2 mM
ascorbic acid, Onozuka RS cellulase (1% wt/vol, Yakult Phar-
maceutical, Tokyo), Y-23 pectolyase (0.1% wt/vol, Seishin Phar-
maceutical, Tokyo), and 1 mM Mes-KOH (pH 5.5). The osmo-

mality 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 boro-
silicate 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, 10 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 Instru-
m ents Axopatch 200A amplifier. pclAMP 6.0.3 software (Axon
Instrument, Foster City, CA) was used for voltage pulse stim-
ulation, online data acquisition, and data analysis. The voltage
protocol consisted in stepping the membrane potential to volt-
age 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. 4A;
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 expressing 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 ± 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 (F2 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 μmol·m−2·s−1). Comparison with control wild-type plants grown in parallel under 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 ×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 ×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 (azobenzenearsonate or darkness; Fig. 3A and B, respectively) or opening (light; Fig. 3C).

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 peels 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 ~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). (Fig. 5A 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 azobenzenearsenate 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 ± SE from four independent experiments, 60 measurements per experiment, are shown).

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 azobenzenearsenate 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 ± 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
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 developed in our study provides direct support to this hypothesis. The reverse genetics approach membrane could play a major role in stomatal closure and thereby in transpiration control. The reverse genetics approach

**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 ± 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 ± 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).

The experimental procedure ensured that water loss (decrease in weight) resulted from leaf transpiration (mean total leaf area of ~90 cm² per plant at this stage). Periodic weighing and watering allowed us to maintain the water content of the compost at ~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 ± 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 < 0.01).

**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 μmol m⁻² s⁻¹). The experimental procedure ensured that water loss (decrease in weight) resulted from leaf transpiration (mean total leaf area of ~90 cm² per plant at this stage). Periodic weighing and watering allowed us to maintain the water content of the compost at ~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 ± 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 < 0.01).

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 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-I plants do not display any obvious phenotype. Furthermore, the gork-I 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-I 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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