Regulation of the ABA-sensitive *Arabidopsis* potassium channel gene *GORK* in response to water stress

D. Becker[a,1,*], S. Hoth[b,1,2], P. Ache[a], S. Wenkel[a,3], M.R.G. Roelfsema[a], O. Meyerhoff[a], W. Hartung[a], R. Hedrich[a]

[a] *Institute for Molecular Plant Physiology and Biophysics, Biocenter, University of Würzburg, Julius-von-Sachs-Platz 2, 97082 Würzburg, Germany*
[b] *Laboratory of Plant Molecular Biology, The Rockefeller University, 1230 York Avenue, New York, NY 10021, USA*

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Abstract **The phytohormone abscisic acid (ABA) regulates many stress-related processes in plants. In this context ABA mediates the responsiveness of plants to environmental stresses such as drought, cold or salt. In response to water stress, ABA induces stomatal closure by activating Ca^{2+}, K^+ and anion channels in guard cells. To understand the signalling pathways that regulate these turgor control elements, we studied the transcriptional control of the K^+ release channel gene *GORK* that is expressed in guard cells, roots and vascular tissue. *GORK* transcription was up-regulated upon onset of drought, salt stress and cold. The wilting hormone ABA that integrates responses to these stimuli induced *GORK* expression in seedlings in a time- and concentration-dependent manner and this induction was dependent on extracellular Ca^{2+}. ABA-responsive expression of *GORK* was impaired in the ABA-insensitive mutants *abi1-1* and *abi2-1*, indicating that these protein phosphatases are regulators of *GORK* expression. Application of ABA to suspension-cultured cells for 2 min followed by a 4 h chase was sufficient to manifest transcriptional activation of the K^+ channel gene. As predicted for a process involved in drought adaptation, only 12–24 h after the release of the stress hormone, *GORK* mRNA slowly decreased. In contrast to other tissues, *GORK* expression as well as K^+_{out} channel activity in guard cells is ABA insensitive, allowing the plant to adjust stomatal movement and water status control separately.**

Key words: Guard cell; *GORK*; K^+ channel; Abscisic acid; Water stress

1. Introduction

The difference between water uptake in the root and shoot transpiration depends on the respective diffusion resistances and reflects the plant water status. During drought or salt stress rising abscisic acid (ABA) levels regulate guard-cell ion transport to promote stomatal closure and thereby reduce transpiration [1–4]. Likewise, root ion transport adapts to limitations in soil water availability [5,6]. In the desert, the *Cactaceae* survive by minimising the boundary water permeability of the root and the shoot while shuttling water, ions and metabolites between the tissues of this 'closed system'. Besides these extremophiles, all land plants operate a network capable of solute, water, and information transport. The long-distance transport system consisting of xylem and phloem is characterised by two bottlenecks: (i) loading of ions and water into the root xylem for transport towards the leaves and (ii) loading of photoassimilates into the phloem of mature leaves to provide, e.g., growing roots with carbon skeletons and energy. Furthermore transport of xylem-derived K^+ in mature (source) leaves has to be loaded into the phloem and addressed to the growing sink organs. To maintain the osmotic and hydrostatic pressure gradients between xylem and phloem, solutes and water have to be exchanged between the vasculature and adjacent cells. Aquaporins are highly expressed at the sites of pronounced solute- and water exchange and thus seem to determine the hydraulic conductivity of, e.g., root hairs, xylem parenchyma cells, hydathodes and guard cells [7,8]. The water movement requires osmotic and/or pressure gradients [9]. The regulation of the plant water status and drought adaptation therefore depends on the activity and number of ion channels and osmolite transporters. Volume regulation of guard cells in particular and turgor control of plant cells in general involves the uptake and release of K^+ and anions [10].

Here, we have focused on the ABA regulation of *GORK*, a gene encoding a K^+-release channel, in the context of plant adaptation to drought, salinity and cold.

2. Materials and methods

2.1. Plant material and growth conditions

Seeds of *Arabidopsis thaliana* ecotype Col-0 were surface sterilised in 70% ethanol and 5% NaOCl+0.01% Triton X-100, and germinated on filter paper moistured with Murashige and Skoog (MS) medium, supplemented with 2% sucrose. Seedlings were grown under sterile conditions in a growth chamber at 8/16 h day/night regime, 21/16°C day/night temperature, 80% relative humidity and photon flux density 100 μmol m^{-2} s^{-1}. Growth conditions for water loss experiments were as described in [11]. Electrophysiological recordings were performed on guard cells from leaves of 6–8-week-old greenhouse-grown *A. thaliana* Col 0 plants. *Arabidopsis* suspension-cultured cells were grown at 26°C on a rotary shaker in medium containing 1× MS+MES (Duchefa, The Netherlands), 58.5 mM sucrose, 0.56 mM myo-inositol, 2.26

*Corresponding author. Fax: (49)-931-888 6157.
E-mail address: dbecker@botanik.uni-wuerzburg.de (D. Becker).

[1] These authors contributed equally to this study.
[2] Present address: Molecular Plant Physiology, FAU Erlangen, Staudtstrasse 5, 91058 Erlangen, Germany.
[3] Present address: Department of Plant Developmental Biology, Max-Planck-Institute for Plant Breeding Research, Carl-von-Linne-Weg 10, 50829 Cologne, Germany.

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μM 2,4 dichlorophenoxyacetic acid, 0.1 mM $FeSO_4 \cdot 7H_2O$, 0.13 mM EDTA, 4.06 μM nicotinic acid, 2.5 μM pyridoxal hydrochloride, and 300 nM thiamine hydrochloride. Cells were subcultured weekly by transferring 20 ml of cells into 50 ml of fresh medium.

2.2. ABA induction and dehydration studies

ABA was dissolved in methanol as a 20 mM stock solution. For cell culture and protoplast experiments 20 μM ABA was directly added to the incubation solution, while control cells were incubated in the corresponding volume of methanol for the times indicated in the figures. Filter-grown *Arabidopsis* seedlings were studied for *GORK* induction by placing filters in fresh Petri dishes containing MS plus 10 μM ABA or methanol. Plant material was harvested after a 4 h treatment and used for RNA isolation, if not stated otherwise. For analysis of ABA mutants, seeds of wild-type (WT) plants and mutants were grown on agar plates and transferred to a hydroponic system containing 50% MS salt base (pH 5.7) and 1% sucrose (cf. [11]). Plants were grown in the hydroponic system for 2 days under long-day conditions. Fresh medium containing 10 μM ABA dissolved in methanol was added. The corresponding volume of methanol was added to the medium of control plants. Dehydration experiments were started at 8.00–9.00 a.m. As a measure for water loss, the weight of eight to 10 leaves cut from greenhouse plants was determined over time. Cold treatment was performed by exposing suspension-cultured cells or plants to 4°C for 10 min followed by a recovery time of 4 h before RNA extraction.

2.3. RNA preparation and quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA of *Arabidopsis* seedlings or cell culture was isolated using the Plant RNeasy Extraction kit (Qiagen, Hilden, Germany). DNA digestion and phenol/chloroform extraction was performed using RNase free DNase I (Amersham Biosciences, Freiburg, Germany) in a standard procedure. Guard-cell protoplasts were isolated as described below, mRNA of protoplasts and epidermal peels was purified twice with the Dynabeads mRNA Direct kit (Dynal, Oslo, Norway) to minimise DNA contaminations. First-strand cDNA was prepared using M-MLV (H^-) reverse transcriptase (Promega) and diluted 20-fold in water for PCR following RT. PCR was performed in a LightCycler (Roche Diagnostics, Mannheim, Germany) with the LightCycler-FastStart DNA Master SYBR Green I kit (Roche Molecular Biochemicals). The following gene-specific primers were used: AKT2/3fwd (5′-AAA ATG GCG AAA ACA C-3′), AKT2/3rev (5′-CGC TGC TTC ACA TAG AA-3′), *GORK*fwd (5′-CCT CCT TTA ATT TAG AAG-3′), *GORK*rev (5′-GCT CCA TCC GAT AG-3′), PP2CAfwd (5′-AAT TGT TGC TGA CTC C-3′), PP2CArev (5′-AAC TCT TAA CCA TCG T-3′), ABI1fwd (5′-CTG CAA TAA CCA ATA CTC-3′), ABI1rev (5′-TCT TCT TCT CGC TAG TAA-3′), KIN2fwd (5′-TCA GAG ACC AAC AAG AAT-3′), KIN2rev (5′-CGA TAT ACT CTT TCC CGC-3′). cDNA quantities were calculated by using LightCycler 3.5.3 (Roche Diagnostics, Mannheim, Germany). All quantifications were normalised to 10 000 molecules of the actin 2/8 gene fragments of 435 bp amplified by ACT2/8fwd (5′-GGT GAT GGT GTG TCT-3′) and ACT2/8rev (5′-ACT GAG CAC AAT GTT AC-3′). To enable detection of contaminating genomic DNA, the primers for AKT2/3, GORK, PP2CA and KIN2 were selected to flank up to three introns. All kits were used according to the manufacturer's protocols. The GenBank accession numbers are as follows: AKT2/3 (U40154/U44745), GORK (AJ279009), ABI1 (AAN13081), PP2CA (P49598), KIN2 (P31169) and *Arabidopsis* actins (cf. [12]).

2.4. Protoplast isolation

Arabidopsis guard-cell protoplasts were essentially isolated as described previously [13]. The enzyme solution contained 0.8% (w/v) cellulase (Onozuka R-10), 0.1% pectolyase, 0.5% bovine serum albumin (BSA), 0.5% polyvinylpyrrolidone, 1 mM $CaCl_2$, 8 mM Mes/Tris pH 5.6. The osmolarity of the enzyme solution was adjusted to 540 mosmol kg^{-1} using D-sorbitol.

2.5. Promoter-GUS assays

The GORK promoter was cloned into the pCRII-TOPO-TA vector (Invitrogen) as an 1525 bp PCR fragment as described in [14]. The GORK promoter was cloned as an *Xho*I/*Bam*HI fragment into the pVKH vector containing the uidA gene (GUS), which was finally used to generate transgenic *A. thaliana* plants. GUS analyses were carried out as described previously [15]. Semi-thin cross-sections (10 μm) were prepared from various samples using a RM 2165 microtome (Leica, Bensheim, Germany) after complete dehydration, and by embedding in Technovit8100 (Heraeus-Kulzer, Wehrheim, Germany).

2.6. Electrophysiological recordings

Leaves of 6–8-week-old *A. thaliana* var. Col 0 plants were pre-incubated for 4 h with or without 20 μM ABA. Following this treatment the leaf tissue was kept in the absence or presence of 20 μM of the phytohormone. Leaf discs of 5 mm diameter were cut and attached with their adaxial side to microscope slides, using silicone adhesive (medical adhesive B liquid, Aromando, Duesseldorf, Germany). Because only guard cells in open stomata of *A. thaliana* can be impaled [16], opening of stomata was induced for at least 2 h, with white light (150 μmol m^{-2} s^{-1}), in the following solution; 50 mM KCl, 0.1 mM $CaCl_2$ and 5 mM Mes/Bis Tris propane pH 6. Stomata in this experimental solution open in the presence of ABA [17]. Guard cells were impaled with double-barrelled electrodes, using the following bath solution: 50 mM KCl and 1 mM Ca/Mes pH 6. The double-barrelled electrodes were pulled from borosilicate glass capillaries (GC 100F-10, Harvard Apparatus, Edenbridge, Kent, UK). Two capillaries were aligned, heated and twisted 360° on a customised horizontal electrode puller (List-Medical-Electronic, Darmstadt, Germany). A first pull was executed on the horizontal puller, while the final pull was carried out on a horizontal laser puller (P2000, Sutter Instrument Co., Novato, CA, USA). The electrodes were filled with 300 mM KAc pH 7.5 and had a tip resistance ranging from 80 to 180 mΩ. The electrodes were connected via Ag/AgCl half cells, filled with 300 mM KCl, to a double microelectrode amplifier (VF-102, Bio-Logic, Claix, France) equipped with headstages of 10^{11} Ω input impedance. Voltage step protocols were applied via an ITC-16 interface (Instrutec Corp., Elmot, NY, USA) under control of Pulse software (Heka, Lambrecht, Germany) fed into a differential amplifier (CA-100, Bio-Logic, Claix, France) that was connected to the microelectrode amplifier.

2.7. ABA analysis

Freeze-dried tissue samples were homogenised and extracted in 80% methanol. Extracts were passed through a Sep-Pak C_{18}-cartridge. Methanol was removed under reduced pressure and the aqueous residue partitioned three times against ethyl acetate at pH 3.0. The ethyl acetate of the combined organic fractions was removed under reduced pressure. The residue was taken up in TBS buffer (Tris-buffered saline; 150 mM NaCl, 1 mM $MgCl_2$, and 50 mM Tris pH 7.8) and subjected to an immunological ABA assay (ELISA). Recoveries of ABA during purification procedures were checked routinely using radioactive ABA and found to be more than 95%. The immunochemicals were generously supplied by Professor Weiler, Ruhr Universität Bochum (Germany).

3. Results

Previous studies have shown that GORK is localised in guard cells as well as root hairs [14,18] and a *gork1-1* mutant lacks the respective outward K^+ currents [19]. To study the expression profile of *GORK* in more detail we performed quantitative, real-time RT-PCR on various *Arabidopsis* tissues using *GORK*-specific primers. These studies revealed that *GORK* is expressed in roots and leaves as well as in *Arabidopsis* suspension-cultured cells (not shown). To confirm the RT-PCR results, the *GORK* promoter was fused to the β-glucuronidase gene (*GUS*) and introduced into WT *Arabidopsis* plants. In several independent transgenic lines we detected GUS staining in guard cells and root cells of *Arabidopsis* seedlings, well in agreement with our previous studies [14]. In addition we observed GUS staining in vascular cells of the root and shoot (Fig. 1A,B).

In intact plants, stomata close within minutes when an ABA pulse reaches the guard cells [20]. Thus, all components for a rapid change in stomatal aperture are expressed in guard

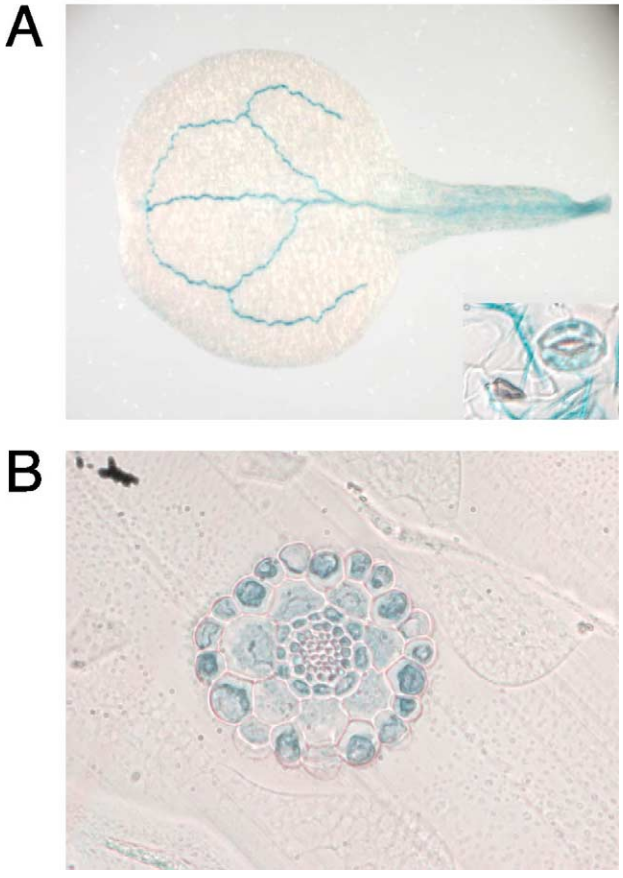

Fig. 1. Localisation of *GORK* expression by promoter-GUS studies. Promoter-GUS-transformed 2-week-old *Arabidopsis* seedlings show *GORK* expression in guard cells and the vasculature (A). Semi-thin section of a Technovit-embedded root (B) reveals ubiquitous *GORK* promoter activity.

cells [21]. The ABA response of the whole plant is much slower and less well understood. Here, we tested, whether adaptation to drought and salinity requires the transcriptional control of K^+ channels by monitoring the stress-induced and ABA-dependent expression of the *GORK* gene.

3.1. *Drought and salt stress induction of* GORK *expression requires ABA signalling*

When leaves were excised from *Arabidopsis* WT plants, the transpirational loss of water reduced the fresh weight by about 60% within 100 min (Fig. 2A). Leaves from the ABA-insensitive mutant *abi1-1*, however, lost more than 90% of their fresh weight over this period. Following mRNA extraction from the respective leaves, *GORK* transcript abundance was determined by quantitative, real-time RT-PCR. In response to dehydration, a six- to seven-fold increase in WT but only a 1.5- to two-fold increase in the *abi1-1* mutant was measured as compared to the K^+ channel message in well-watered controls (Fig. 2B). Similar results were obtained when 20 μM ABA was fed via the petiole of excised leaves (not shown), a treatment that caused stomata to close within 10–30 min [13]. ABA induction of *GORK* in WT but not in the ABA-insensitive mutant *abi1-1* indicates that drought triggers an ABA-signalling pathway leading to increased K^+ channel expression. Besides drought, osmotic stress and salt stress result in ABA biosynthesis and activate ABA response genes [22]. Correspondingly, increasing sorbitol concentrations in the range of 200–650 mOsm mediated a gradual increase in *GORK* expression in suspension-cultured cells in the absence of exogenous ABA (Fig. 2C). Expression levels reached similar values as seen with ABA (compare with Fig. 3B). It should be noted that the ABA concentration in sorbitol-stressed (600 mOsm) suspension-cultured cells raised to 2038 pmol g^{-1} fresh mass as compared to 102 pmol g^{-1} fresh mass in control cells. This value is well in the order of 2533 pmol g^{-1} fresh mass in suspension-cultured cells treated with 20 μM ABA for 4 h. To provide further evidence for the hypothesis that water stress activates *GORK* through ABA biosynthesis, we stressed seedlings with increasing NaCl concentrations (200–1000 mOsm) in the absence of ABA. Thereby we found sodium-chloride-induced water stress as effective as sorbitol with respect to *GORK* induction (not shown). The addition of ABA to salt- or sorbitol-treated seedlings did, however, not further enhance channel transcription (not shown), suggesting that osmolite-induced ABA synthesis provides for the transcriptional control.

To test whether the transcriptional activation of the *GORK*

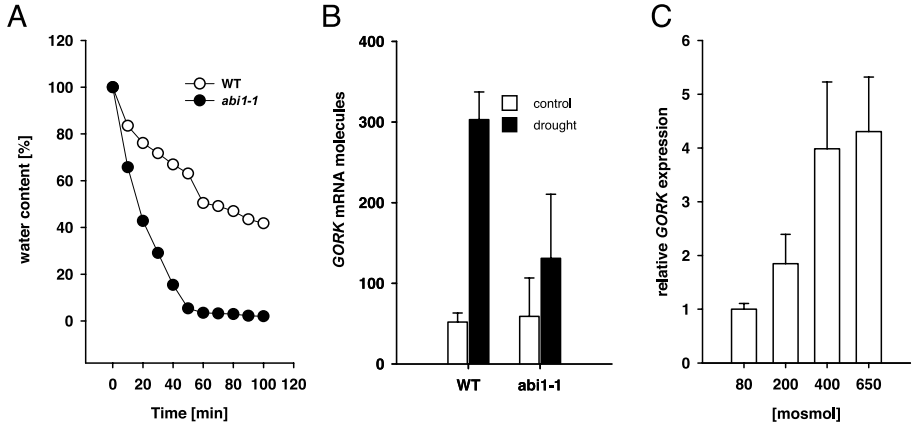

Fig. 2. *GORK* expression is up-regulated by water stress. A: Water loss experiment of *Arabidopsis* WT (○) and *abi1-1* mutant leaves (●). Data shown represent a typical experiment out of three independent measurements. B: Quantitative, real-time RT-PCR of *GORK* transcription in leaves of *Arabidopsis* WT and *abi1-1* mutant plants from (A) after 100 min. The *y*-axis gives the number of *GORK* molecules per 10 000 actin molecules in leaves of control plants (white bars) and those derived from (A) (black bars). C: Induction of *GORK* expression in suspension-cultured cells upon a 4 h sorbitol treatment. Values are given relative to *GORK* mRNA levels under control conditions (cell culture medium, 80 mOsm, no sorbitol) and represent the means of three different experiments ($n = 3$, mean ± S.D.).

gene could be related to any of the so far known ABA-signalling pathways, *GORK* expression was analysed in ABA-signalling mutants by quantitative, real-time RT-PCR. A 4 h treatment of 4-week-old seedlings with 10 μM ABA resulted in a five- to six-fold increase in *GORK* transcript abundance in WT (Fig. 3A). The ABA-insensitive mutants *abi3*, *abi4*, and *abi5* as well as the ABA-hypersensitive mutant *era1* showed a WT-like behaviour. This indicates that neither the two transcription factors ABI3 and ABI4 nor the fatty acid elongase ERA1 represents a component of the ABA-signalling pathway leading to increased *GORK* expression. *GORK* induction, however, was reduced to 1.5- and 2.5-fold in *abi1-1* and *abi2-1*, respectively (cf. [11]), suggesting that *GORK* induction might require protein phosphorylation/de-phosphorylation events. Extending our studies from excised leaves and entire seedlings to root hairs and *Arabidopsis* cell cultures, we found the ABA induction of *GORK* in these cell types, too (Fig. 3B).

3.2. *ABA activates* GORK *in a time- and concentration-dependent manner*

To study the ABA sensitivity of *GORK* transcription in more detail, we followed the increase in *GORK* transcripts with incubation time and ABA concentration. The high resolution of quantitative, real-time RT-PCR, enabled us to detect a two-fold increase in the *GORK* message of WT seedlings after 30 min (not shown). Increased incubation times resulted in a progressive rise in K^+ channel expression (Fig. 4A). While peak induction of *GORK* in the presence of 20 μM ABA was recorded after 4–8 h (up to 500%), the *GORK* message dropped to 70–75% of peak expression after 12 h (Fig. 4A). In contrast, the up-regulation of the ABA-sensitive *Arabidopsis* phloem K^+ channel AKT2/3 mRNA [15,23–25] appeared to be delayed and much less pronounced when compared to the *GORK* message (Fig. 4A). To follow the concentration dependence of the ABA-triggered up-regulation, *GORK* transcripts were determined in response to increasing ABA concentrations (Fig. 4B). Following a 4 h incubation with 0.2, 2, 10, 20, 30, 40, and 50 μM ABA, *GORK* transcript abundance increased in a dose-dependent manner with half-maximal *GORK* activation at 7.5 μM (Fig. 4B).

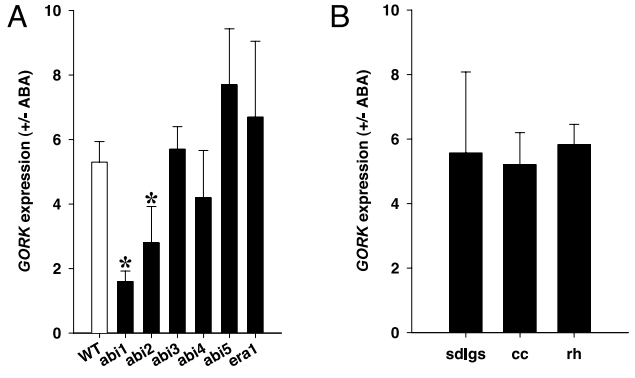

Fig. 3. *GORK* expression is up-regulated by ABA. A: Three-week-old seedlings from *Arabidopsis* WT plants and various ABA mutants were probed for ABA-induced activation of *GORK* expression in response to 20 μM ABA. Differences for ABA induction of GORK transcripts were statistically significant for *abi1-1* and *abi1-2* only, when compared to WT (Student's *t*-test; $P \ll 0.05$). B: ABA-dependent expression of *GORK* in seedlings (sdlgs), suspension-cultured cells (cc), and root hair protoplasts (rh) from *Arabidopsis* WT. Values given represent the ratio of *GORK* mRNA levels in ABA-treated and non-treated plants or cells, respectively ($n = 3$, mean ± S.D.).

Transmission of the ABA signal leading to *GORK* activation was studied with *Arabidopsis* suspension-cultured cells using ABA pulses of 2, 10, and 240 min duration followed by extensive washing and a 4 h chase period. Under these conditions an ABA pulse of 2 min was sufficient to induce the activation of the channel gene (Fig. 4C). Thus, manifestation of the *GORK*-specific ABA signal was fast, while transcripts peaked about 4 h later. In line with a long-term effect, even after a 24 h chase following a 4 h ABA treatment, still 50–60% of peak *GORK* mRNA concentration was detected (not shown).

3.3. *ABA up-regulation of* GORK *is pH independent*

ABA is a weak acid and accumulates in living plant cells due to 'acid trapping'. Previous studies have shown that the protonated form rather than the free acid rapidly enters the cell [26]. To test whether external or cytosolic ABA induces *GORK* expression, we incubated *Arabidopsis* suspension-cultured cells for 4 h with 20 μM ABA buffered to either pH 5, 6, 7, or 8. Following extensive washing, cells were harvested to monitor for the *GORK* transcript level and ABA content. Although ELISA studies confirmed highest tissue concentrations of ABA at pH 5 (not shown), *GORK* up-regulation was not affected by pH changes (pH 5–8) (Fig. 5). This apparent discrepancy, together with the finding that the membrane-impermeable ABA–BSA conjugate induces the expression of *RAB18* [27], points to the existence of an extracellular site for ABA perception. In good agreement, the impermeable ABA–glucose ester up-regulated *GORK* as efficiently as free ABA (not shown).

3.4. GORK *expression in seedlings involves* Ca^{2+} *signalling*

In guard cells of isolated epidermal peels, ABA has been shown to induce changes in cytosolic calcium [28]. Furthermore imposed calcium oscillations in the absence of ABA caused stomata to close [29]. Thus, calcium oscillations seem to be essential and sufficient to trigger short-term turgor loss in guard cells. To test whether calcium is required for *GORK* activation, we analysed *GORK* transcripts in *Arabidopsis* seedlings in response to 20 μM ABA in the presence or absence of the calcium scavenger EGTA or the calcium channel blocker La^{3+} (cf. [27]). Both treatments suppressed the ABA activation of *GORK* indicating that this process is calcium dependent (Fig. 6).

Because low temperatures alike ABA elicit cytoplasmic calcium transients [30], we followed *GORK* transcripts in response to cold. Fig. 7 shows that cold evokes the up-regulation of *GORK*. Transcript abundance following 10 min of cold stress increased four-fold already after 1 h of recovery at room temperature. The ABA- and cold-sensitive protein phosphatase AtPP2CA exhibited a similar expression profile, while the AKT2/3 potassium channel expression did not respond to cold. Thus, the activation of *GORK* in *Arabidopsis* by stress signals like drought, salt or cold seems to involve ABA synthesis and/or cytoplasmic calcium changes.

3.5. *Regulation of* GORK *in guard cells is ABA insensitive*

When guard cells were challenged with ABA, to our surprise, *GORK* expression remained unaffected (Fig. 8A,B, cf. Fig. 3). ABA-induced activation of *GORK* was observed nei-

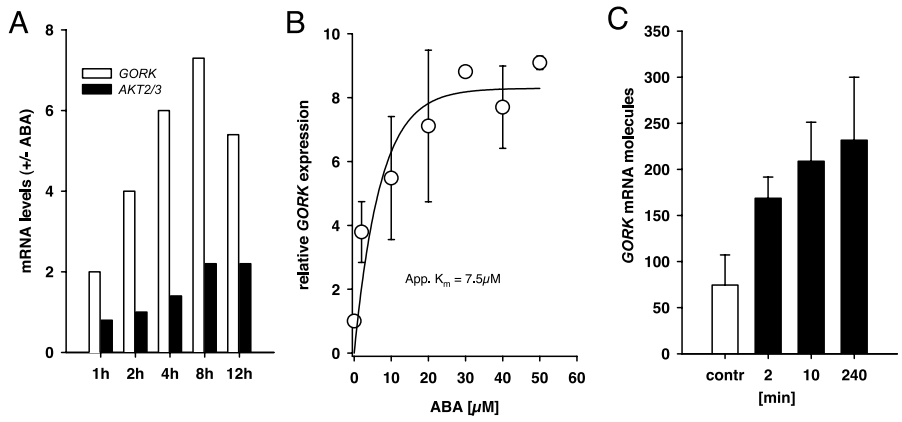

Fig. 4. Kinetics of ABA-dependent activation of *GORK* expression. A: Time dependence of *GORK* and *AKT2/3* expression in 2–3-week-old *Arabidopsis* seedlings following treatment with 20 μM ABA. Representative experiment showing the ratio of channel mRNA levels in ABA-treated and non-treated plants ($n = 2$). B: Dose–response curve of ABA-dependent *GORK* expression. Values represent relative *GORK* mRNA levels in ABA-treated and non-treated plants, the latter was set to 1.0 ($n = 3$, mean ± S.D.). C: ABA-induced *GORK* mRNA expression by application of 20 μM ABA pulses to *Arabidopsis* suspension-cultured cells for the durations indicated followed by a 4 h chase. The *y*-axis gives the number of *GORK* mRNA molecules per 10 000 actin molecules ($n = 3$, mean ± S.D.).

ther in epidermal fragments nor in guard-cell protoplasts. The activation of other ABA-dependent genes expressed in guard cells, such as *ABI1* and *KIN2* [31], was also severely reduced in guard-cell-enriched epidermal fragments when compared to ABA-treated leaves. This de-regulation of either positive or negative regulators of *GORK* expression may therefore underpin guard-cell-specific regulation of *GORK*. Interestingly, the basic transcript level of *GORK* was up to 10-fold higher in guard cells compared to other cell types (Fig. 8A). This strong expression correlates with the existence of seven 'TAAAG motifs' in the *GORK* promoter. These elements have been shown to drive guard-cell-specific expression of KST1, another guard-cell K^+ channel, by interaction with zinc finger transcription factors of the Dof family [32].

To test, whether the lack of ABA induction of *GORK* transcripts in guard cells is manifested at the protein level, too, we followed K^+ channel activity in response to ABA treatment. When applying voltage pulses in the range of −100 to +60 mV on intact guard cells of leaf discs, impaled with double-barrelled microelectrodes, outward K^+ currents were elicited positive of the equilibrium potential for K^+ (Fig. 9A). A 4 h

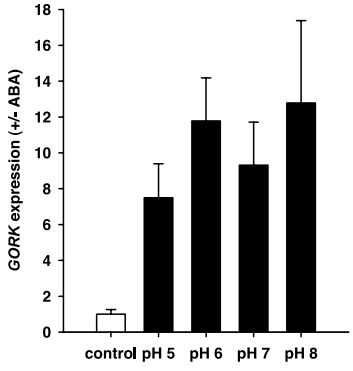

Fig. 5. pH dependence of ABA-induced *GORK* expression. ABA-induced expression of *GORK* mRNA in *Arabidopsis* suspension-cultured cells upon treatment with 20 μM ABA at the indicated external pH values. Values represent the ratio of *GORK* mRNA levels in ABA-treated and non-treated plants ($n = 3$, mean ± S.D.). Control denotes non-treated plants and was set at 1.0.

ABA treatment of intact leaves, sufficient to induce *GORK* transcripts by a factor of at least four in leaf tissue and suspension-cultured cells, had effect on neither the kinetics nor the amplitude of outward K^+ currents (Fig. 9A,B), well in line with the observed ABA insensitivity at the transcriptional level. The ABA treatment did not alter ion currents recorded over the whole voltage range of −200 to +60 mV. An incubation of leaf discs with ABA also had no effect on the conductance properties of the guard-cell plasma membrane (Fig. 9C). In contrast, the outward rectifier in suspension-cultured cells has been shown to increase upon ABA treatment up to five-fold [33].

4. Discussion

ABA-signal transduction not only acts at different stages of plant development, including dormancy, embryo development, seed germination, or growth, but is also involved in stomatal closure as well as the adaptation of plants to various stress factors (see [34–36] for review). Genetic screens have identified mutants impaired in ABA biosynthesis or exhibiting either insensitivity or hypersensitivity to ABA [36,37]. The corresponding genes that were affected in the respective mutants include genes encoding phosphatases, transcription factors, RNA-binding proteins, a G-protein alpha subunit, a protein kinase, and a Ca^{2+}-binding protein (for overview see [38–41]. These gene products represent elements of a complex ABA regulatory network. In addition to genetic screens, the expression of ABA-sensitive genes like *KIN2* [42], the dehydrin *RAB18* [33,43] or many of the late abundant embryogenesis (lea) genes (see [44,45] and references therein) has been employed to decompose the ABA-signalling chain. Recently, the first genome-wide gene expression profiling in plants revealed new targets of ABA [11]. In contrast to many ABA-responsive genes, the expression pattern of the *GORK* gene in the plant is well known and the protein function and regulation have been characterised in vivo and in vitro. Thus, *GORK* is a suitable target to elucidate signalling steps in the ABA pathway(s) and to link them to the physiological response of the plant to the stress hormone.

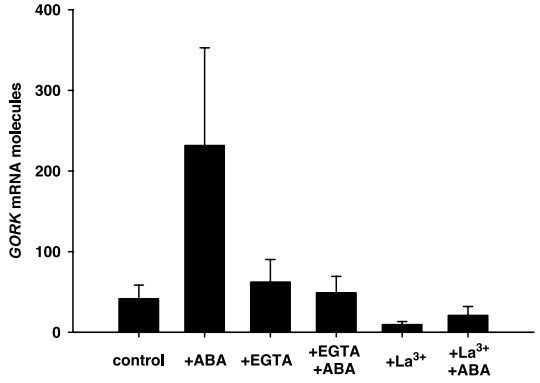

Fig. 6. Ca^{2+} dependence of ABA-induced *GORK* expression. ABA-induced expression of *GORK* mRNA in 2–3-week-old *Arabidopsis* seedlings upon treatment with 20 μM ABA in the presence or absence of 10 mM EGTA or 5 mM $LaCl_3$, respectively. The *y*-axis gives the number of *GORK* mRNA molecules per 10 000 actin molecules ($n = 3$, mean ± S.D.).

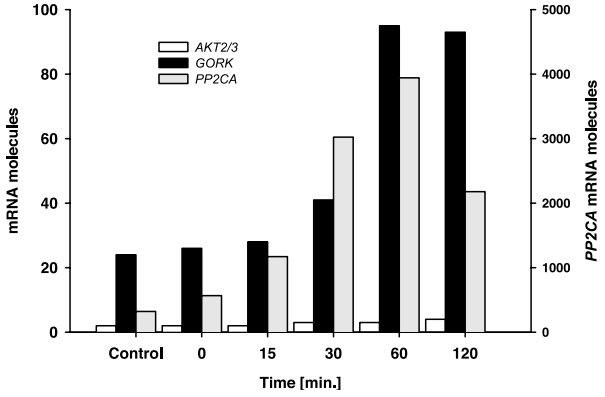

Fig. 7. Cold activates *GORK* expression. Representative cold-treatment experiment. Cold-triggered gene expression was monitored for *GORK* (black bars), *AKT2/3* (white bars) and *PP2CA* (grey bars) after treating *Arabidopsis* suspension-cultured cells for 10 min at 4°C followed by a recovery period of 4 h at room temperature. Expression is given as the relative number of mRNA molecules per 10 000 molecules of actin ($n = 2$).

Our studies show that the K^+-release channel gene *GORK* is widely expressed in *Arabidopsis*, exhibiting strongest signals in roots, shoot vasculature and in guard cells. In contrast the *shaker*-like K^+-release channel SKOR in *A.thaliana* is exclusively expressed in the root stele [46] and *AKT2* in the phloem [23,24,47]. Challenging *Arabidopsis* seedlings with stimuli that are known to elevate endogenous ABA concentrations, including drought, osmotic stress or cold [48], led to the up-regulation of *GORK* transcripts. Likewise, the exogenous application of ABA to excised leaves, seedlings or suspension-cultured cells induced *GORK* expression in a time- and concentration-dependent manner. In line with the potential role of this K^+ channel in long-term stress adaptation, up-regulation of *GORK* was manifested within 2 min of ABA application and persisted for 24–48 h following ABA removal. This indicates that even a very short pulse of ABA is sufficient to trigger ABA-signal transduction and optimise the stress response (Fig. 4C). Several lines of evidence suggest the co-existence of a calcium-independent and -dependent pathway in ABA signalling [49]. *GORK* expression is regulated by the latter, because its ABA-dependent up-regulation in *Arabidopsis* seedlings depended on extracellular calcium (Fig. 6). In this context it has been shown that cold evokes cytosolic calcium transients in plants (for review see [30]). The fact, that *GORK* activation is sensitive to low temperatures provides additional evidence for a Ca^{2+}-dependent step. The *abi1-1* mutation reduced the ABA-induced elevation in cytosolic $[Ca^{2+}]$ in *Arabidopsis* guard cells [50] and resulted in an impaired regulation of *GORK* by ABA or drought (see Figs. 2B and 3A). Genome-wide gene expression studies have revealed a dramatic impairment of ABA-responsive gene expression in the *abi1-1* mutant [11]. A subset of ABA-dependent genes that continued to be appropriately regulated in *abi1-1* suggested the presence of at least two ABA-signalling pathways, only one of which is blocked in *abi1-1* (see [11] and references therein). The ABI1-dependent pathway regulates *GORK* expression in response to ABA. Both protein phosphatases, ABI1 and ABI2, are transcriptionally up-regulated by ABA [11,51]. We therefore assume that these two 2C-type phosphatases represent positive regulators of *GORK* expression. ABA-dependent *GORK* activation did not require the activity of the transcription factors ABI3, ABI4 or ABI5 (Fig. 3A).

In contrast to other cell types, ABA failed to promote up-

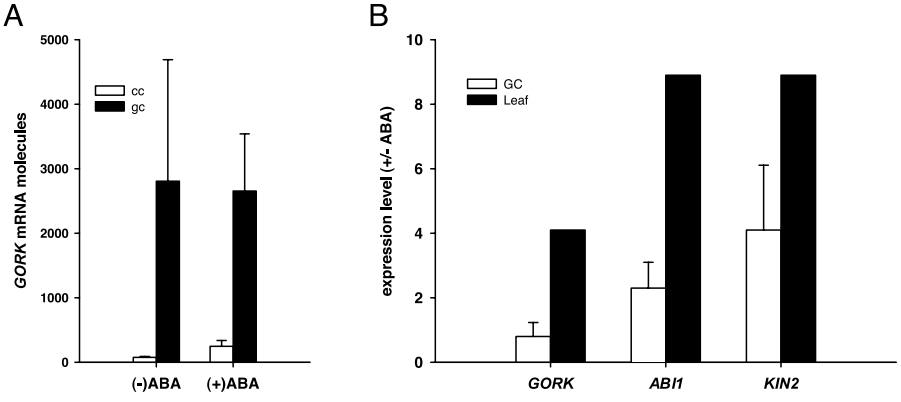

Fig. 8. *GORK* expression in guard cells is ABA insensitive. A: Relative number of *GORK* molecules (given as per 10 000 molecules of actin) in guard-cell-enriched epidermal fragments (black bars) and suspension-cultured cells (white bars). Values represent the means of three different experiments and the error is given as S.E.M. B: Representative data on the effect of ABA on gene expression of *GORK*, *ABI1*, and *KIN2* in guard-cell-enriched epidermal fragments (white bars) and entire leaves (black bars) of *Arabidopsis*. Values represent the ratio of mRNA levels in ABA-treated and non-treated plants or cells, respectively ($n = 3$, mean ± S.D. for guard cells, $n = 2$, mean, for leaf).

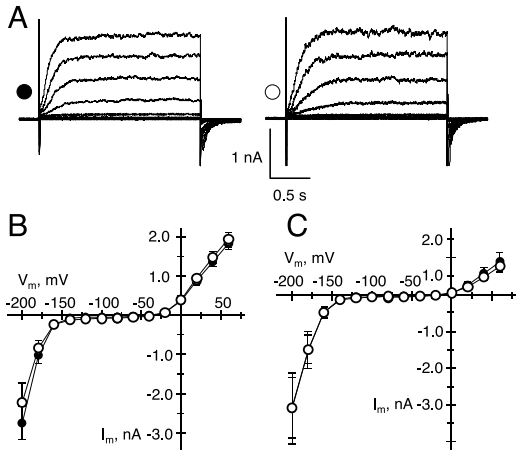

Fig. 9. Guard-cell K^+ channels are unaffected by ABA treatment. Electrophysiological recordings of impaled intact guard cells in leaf discs using the double-barrelled-electrode voltage-clamp technique. A: Plasma membrane conductance of guard cells incubated with (○) or without (●) 20 μM ABA. *A. thaliana* guard cells were clamped from a holding potential of −100 mV to test potentials ranging from −100 to +60 mV. Time-dependent outward currents are carried by outward rectifying K^+-selective channels encoded by GORK. Leaves were incubated for 4 h with or without ABA, afterwards leaf discs were kept with or without 20 μM ABA and stomatal opening was induced using the following solution: 50 mM KCl, 0.1 mM $CaCl_2$, and 5 mM Mes/Bis Tris propane pH 6. B: Average current–voltage relation of guard cells incubated with (○) or without (●) 20 μM ABA as in A. Error bars represent S.E.M., $n=5$ (−ABA) or $n=6$ (+ABA). Note that currents recorded at potentials more positive than −20 mV are predominantly carried by outward rectifying K^+ channels, while currents recorded at potential more negative than −160 mV are predominantly carried by inward rectifying K^+ channels. C: Average current–voltage relation of guard cells incubated with (○) or without (●) 20 μM ABA. In contrast to B, leaves were not pre-incubated but, instead, leaf discs were directly exposed to ABA. Guard cells were recorded after an incubation period of 4 h or more. Error bars represent S.E.M., $n=4$ (−ABA) or $n=6$ (+ABA).

regulation of GORK transcripts in guard cells. Similarly, ABA was ineffective to activate GORK-mediated K^+ efflux [19] in this cell type, while previous studies have documented the up-regulation of GORK-like channels in other cell types by ABA [33]. Guard cells are symplasmically isolated from neighbouring cells. The lack of plasmodesmata enables them to regulate their volume independently of the surrounding tissue. The regulation of guard-cell ion channels during stomatal closure in response to drought stress has been extensively studied and was attributed to fast ABA signalling [21,52]. ABA is known to induce pH and Ca^{2+} changes in the cytoplasm of guard cells and depolarisation of the plasma membrane by activating Ca^{2+}, K^+ and anion channels [14,53–56]. An ABA signal originated from the root is accompanied by an alkalinisation of the xylem sap [57,58] and guard-cell cytoplasm [53] and will thus directly activate the proton-sensitive GORK in guard cells. The finding that the overexpression of *abi1-1* in guard cells of tobacco resulted in both a significant reduction of K^+_{out} currents and insensitivity of the outward rectifier towards ABA [59] indicates the involvement of ABI1 in post-translational modification, too, and thus activation of the guard-cell K^+-efflux channel. Very recently the activity of the guard-cell K^+_{out} channel was clearly attributed to GORK function [19]. Neither a *gork* 'knock-out' nor the corresponding dominant-negative mutants exhibited obvious phenotypes and fast stomatal movement was significantly impaired in *gork1-1* [19]. Due to the high number of *GORK* transcripts in guard cells, ABA-induced stomatal closure in the dominant-negative *GORK* mutant (*gork-dn*1) was comparable to WT. A similar finding exists for the K^+ channel blocker Ba^{2+} [60]. Under long-term drought conditions, however, *gork* mutants exhibited significantly higher water loss when compared to control plants [19]. Thus, potassium released from guard cells in response to the ABA activation of anion channels probably reflects voltage-dependent rather than ABA-dependent activation of the K^+-efflux channel. Studies on the *gork-1* mutant by Hosy et al. [19] show that in guard cells other K^+ transporters are involved in potassium release as well.

Up to now, however, little is known about long-term stress responses in guard cells. Here we show that fast as well as slow ABA signalling of channel activation and transcription in the plant body and in *Arabidopsis* guard cells is regulated differentially. Plants optimise their water use efficiency with respect to CO_2 assimilation by adjusting stomatal conductance. This process is accomplished by rapid changes in the activity of ion channels in guard cells. The high but ABA-insensitive expression of *GORK* in guard cells may thus provide for fast stomatal closure upon water stress onset. Recently ABA-activated protein kinases (AAPKs) have been shown to be involved in fast and slow ABA signalling of stomatal closure in *Vicia faba* [61,62] and *Arabidopsis* [63]. In line with our finding that ABA does not control *GORK* expression in guard cells, the role of AAPKs seems to point to guard-cell-specific ABA signalling. Guard-cell autonomy is in line with the fact that drought stress management requires the hydraulic uncoupling of guard cells from the surrounding mesophyll cells [64]. Thus, the transcriptional up-regulation of the GORK K^+-efflux channel in response to ABA (cf. [33]) in the root and shoot might poise these cell types for stress adaptation. Future studies on plants de-regulated with respect to *GORK* expression will identify the role of this K^+-release valve in stomatal action and whole-plant stress tolerance.

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