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Letter to the Editor

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Direct Modulation of the Guard Cell Outward-Rectifying Potassium Channel (GORK) by Abscisic Acid

Dear Editor,

Abscisic acid (ABA) induces turgor loss and hence stomatal closure by promoting rapid net K^+ efflux from guard cells (GCs) through outward-rectifying K^+ (K^+_{out}) channels (Schroeder et al., 1987; Blatt, 1990). The mechanisms of ABA signaling in GCs are detailed elsewhere (see Munemasa et al., 2015; Weiner et al., 2010; Pandey et al., 2007). Briefly, ABA binds to the PYR/PYL/RCARs, a family of soluble steroidogenic acute regulatory-related lipid transfer (START) proteins, and, in turn, inactivates the downstream PP2C (type 2C protein phosphatase), leading to the activation of SnRK2.6 (SNF1 [sucrose non-fermenting-1-related protein kinase]/OST1 [open stomata 1]) protein kinases. These kinases phosphorylate multiple downstream targets, resulting in ionic changes that drive K^+ efflux through voltage-dependent K^+_{out} channels (Ache et al., 2000) to enable ABA-induced stomatal closure (Munemasa et al., 2015). Processes that are indispensable for stomatal closure include the inhibition of plasma membrane (PM) H^+-ATPase activity, cytosolic alkalinization, increase of cytosolic free Ca^{2+}, and activation of both rapid (R-type) ALMT12/QUAC1 and slow (S-type) SLAC1 anion channels, all of which result in rapid and sustained PM depolarization.

Arabidopsis thaliana guard cell outward-rectifying potassium channel (GORK; At5g37500), a member of the *Shaker* family, encodes the major voltage-gated K^+_{out} channel of the GCs, and disruption of its activity results in impairment of stomatal closure in response to either darkness or ABA (Hosy et al., 2003). It has been previously shown that the onset of PM depolarization and activation of K^+_{out} channels in *Vicia faba* GCs following ABA treatment takes about 1 min (Blatt, 1990; Thiel et al., 1992). Intracellular perception of ABA is now well established, and there is also some evidence for extracellular site(s) of ABA perception in *Arabidopsis* (Jeannette et al., 1999; Pandey et al., 2009). Here, we report that ABA can directly modulate the guard cell outward-rectifying potassium (GORK) channel.

ABA activates a Ba^{2+}-sensitive K^+-selective conductance in excised PM patches of *Vicia faba* GC protoplasts (Lemtiri-Chlieh, F. Direct effects of ABA on single outward potassium channels in guard cells. Presented at: 11th International Workshop on Plant Membrane Biology. Cambridge, 9–14th August 1998). The open probability of the native K^+_{out} channel in excised outside-out membrane patches of *Vicia faba* GC protoplasts increases in the presence of (±)-ABA, and this effect is not observed when using the inactive (−)-ABA isomer (Supplemental Figure 1). Such evidence may imply that ABA can act through a membrane-delimited pathway, possibly by acting on the K^+_{out} channel itself. In addition, ABA being permeable in external acidic condition (bath solution used was pH 5.5) may conceivably interact with the channel at both sides of the membrane.

To evaluate such a possibility, we aligned the amino acid sequences of ABA-binding sites of the PYR/PYL/RCARs ABA receptor family, GORK, and the related stellar K^+-outward rectifier SKOR from different plant species (Figure 1A). The alignment shows that the cytosolic domain of GORK/SKOR channels harbors conserved residues that are similar to those in the latch-like region of ABA-binding sites of the PYR/PYL/RCARs (Melcher et al., 2009). These receptors exhibit a conserved gate-latch-lock mechanism underlying ABA signaling pathway as described (Melcher et al., 2009). We therefore hypothesized that the region in *Arabidopsis thaliana* GORK spanning from position D543 to G575, located at the C terminus downstream of the transmembrane segments and containing the ankyrin protein–protein interaction domain, may contribute to ABA interaction (Figure 1A). The large cytoplasmic C-terminal region of the GORK channel also harbors a putative cyclic nucleotide-binding domain (CNBD). At the end of the C terminus lies the conserved K_{HA} domain that is enriched for hydrophobic and acidic residues, which are believed to have a role in protein–protein interactions, such as tetramerization or stabilization of the heteromers. A model of the predicted ABA-interacting domain (D543 to G575) in GORK against the crystal structure of an engineered protein OR265 (PDB: 4HQD) suggests that ABA may dock at the putative site with favorable free energy and ligand pose (Figure 1A, inset).

To assess direct GORK–ABA interaction, we generated a *GORK-EmGFP* construct by cloning the full-length *Arabidopsis thaliana GORK* (At5g37500) into Vivid Colors pcDNA^{TM}6.2/EmGFP-DEST Gateway vector for transient expression in HEK293 cells. Whole-cell current–voltage recordings of the HEK293 cells transfected with *GORK-EmGFP* display a slow-activating outward sigmoidal component with kinetics typical of a native guard cell K^+_{out} (Supplemental Figures 2 and 3). In addition, this current is inhibited by both external Ba^{2+} (Supplemental Figure 3A) and acidic pH (Supplemental Figure 3B). Importantly, we show that the inclusion of the natural (±)-ABA stereoisomer (50 μM) in the patch pipette increases the GORK current (I_{GORK}) amplitude in HEK293 cells, which is not observed with the less active (−)-isomer at the same concentration (Figure 1B and Supplemental Figure 2A). Typically, after 2–4 min from breaking into whole-cell mode, we observed a statistically significant increase of I_{GORK} magnitude (2.55-fold at V = 60 mV, P = 0.003

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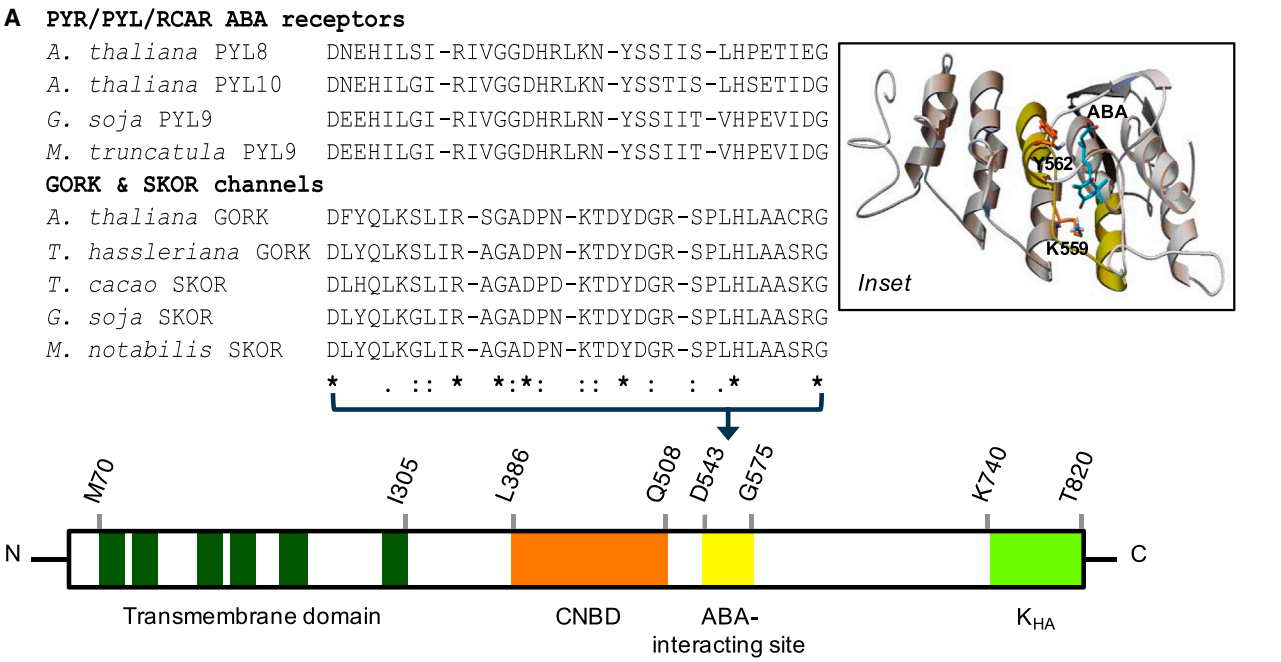

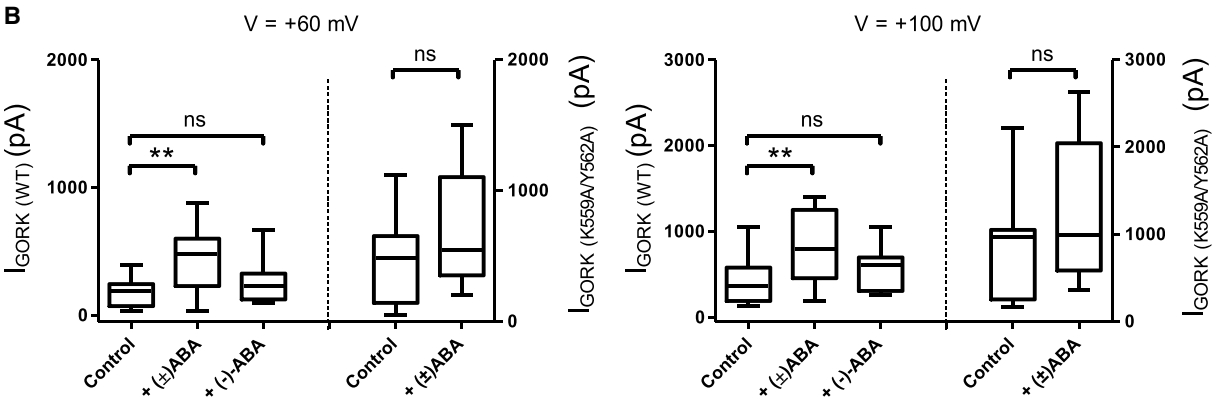

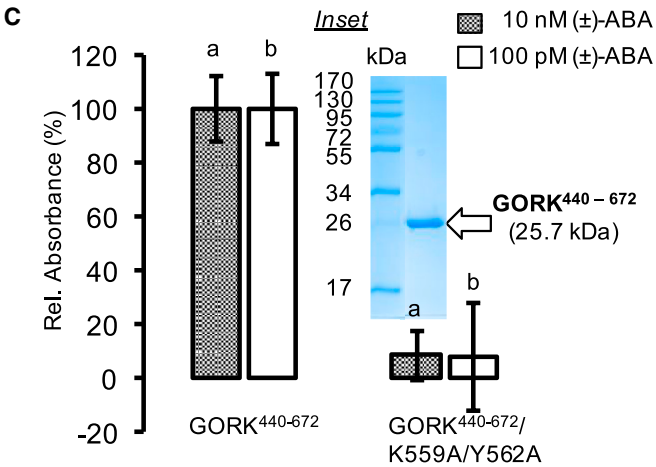

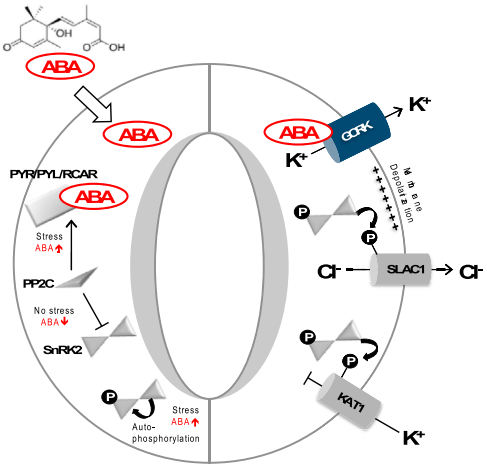

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and 2.03-fold at V = 100 mV, $P = 0.007$) in response to (±)-ABA (Figure 1B and Supplemental Figure 2Aii). The biologically inactive (−)-isomer gave a much reduced and statistically non-significant effect at similar voltages (1.42-fold at V = 60 mV, $P = 0.2$ and 1.37-fold at V = 100 mV, $P = 0.16$; Figure 1B and Supplemental Figure 2Aii, inset). We also observed an enhancement of I_{GORK} (≥65% at V = 100 mV) when the pipette solution was backfilled with 20 μM (±)-ABA (see Supplemental Materials and Methods), although the increase was slightly delayed in time (3 min to onset and 5 min to reach steady state), which is most probably due to the inherent delay introduced by the backfilling technique in order for ABA to reach its target from the cytosolic side (data not shown).

While our data of excised outside-out patches on native IK_{out} channels in *Vicia faba* GC protoplasts (Supplemental Figures 1, 4, and 5) only suggest a membrane-delimited effect of ABA, the experiments performed in HEK293 cells transfected with *GORK-EmGFP* may denote a direct effect on GORK itself. To further examine our hypothesis of predicted ABA-interacting site in the cytosolic domain of GORK, we performed excised inside-out patches of HEK293 cells expressing GORK-EmGFP. In this patch configuration, the cytosolic side of GORK is exposed to the bath solution, therefore allowing the assessment of unitary single-channel recordings to be made before and after ABA application on the same excised membrane patch. Treatment with active (±)-ABA increased the opening probability (Po) of GORK in all four independent patches by an average of 3.6-fold ($V_{holding} = -50$ mV; Supplemental Figure 6A). In contrast, the inactive (−)-ABA isomer was much less effective in all three trials (Supplemental Figure 6B).

To further probe the effect of ABA on GORK, we performed site-directed mutagenesis on the presumptive ABA-interacting site in GORK (Figure 1A). Since the Y562 residue appears to be highly conserved (Figure 1A) and is within 5 Å of the latch residues in the PYL2-ABA complex (Melcher et al., 2009) and the polar K559 that can presumably form a charge interaction with ABA (Figure 1A, inset), we therefore replaced both Y562 and K559 with an alanine residue (K559A and Y562A). In contrast to the wild-type GORK, this double mutation yields a markedly reduced ABA-dependent I_{GORK} activation (1.53-fold increase at V = 60 mV, $P = 0.16$ and 1.38-fold at V = 100 mV, $P = 0.32$), in which the values are almost comparable with the inactive (−)-ABA effect on the wild-type GORK (Figure 1B and Supplemental Figure 2B). This suggests that these two residues, K559 and Y562, may be important for a full activation of the GORK channel by ABA. Interestingly, additional mutations of two other residues, N558A and R565A, in the predicted ABA-interacting site (GORK/N558A/K559A/Y562A/R565A) resulted in a total loss of I_{GORK} (Supplemental Figure 2C). Note that inclusion of (±)-ABA in the patch pipette did not restore any I_{GORK}-like activity nor did it affect the native I_A-like conductance (Supplemental Figure 2C).

Furthermore, we have also cloned and affinity purified the cytosolic domain of $GORK^{440-672}$ and developed a colorimetric-based ELISA (Supplemental Figure 7) to determine whether the predicted ABA-interacting site has affinity for ABA *in vitro*. We noted a signal that developed steadily in a linear manner over time and achieving saturation after 45 min of incubation for recombinant $GORK^{440-672}$ (20 μg/ml) in the presence of 10 nM (±)-ABA (Figure 1C; Supplemental Figure 8A). We also obtained a similar GORK–ABA interaction with a 100-fold lower ABA concentration (Figure 1C; Supplemental Figure 8B). In contrast, this interaction is attenuated at both ABA concentrations in the recombinant $GORK^{440-672}$/K559A/Y562A mutant (Figure 1C). This result is consistent with our electrophysiology data (Figure 1B and Supplemental Figures 2 and 6) and further supports a direct GORK–ABA interaction.

While these data are consistent with the idea of an ABA-interacting site in GORK, it is also conceivable that this site encompasses a larger region than the predicted site (D543 to G575) that closely resembles the "latch" region of the ABA-START receptors (Melcher et al., 2009). Given that the structural feature of GORK is different from that of the canonical ABA receptors, it is conceivable that GORK harbors unique structural scaffolds that may require dimerization with multiple GORK subunits in order to accommodate ABA at this ankyrin-rich region, which has also been previously shown to be involved in GORK gating and regulation (Eisenach et al.,

Figure 1. ABA Rapidly Enhances I_{GORK} in HEK293 Cells By Directly Interacting with its C Terminus Cytosolic Domain (D543-G575).

(A) Alignment of GORK with two *Arabidopsis thaliana* ABA-START receptor family proteins (PYL8 and PYL10). *Glycine soja* and *Medicago truncatula* ABA receptors (PYL9), *Arabidopsis thaliana* GORK (At5g37500) and other GORK and its SKOR (stellar K^+ outward rectifier) homolog across different plant species (*Tarenaya hassleriana*, *Theobroma cacao*, *Glycine soja* and *Morus notabilis*). The asterisk (*) signifies an identical amino acid, the colon (:) stands for a conservative replacement, and a dot (.) for a semi-conservative replacement. Domain organization of *Arabidopsis thaliana* GORK channel, which comprises a short N-terminal sequence, six annotated transmembrane segments (S1–S6; shaded in dark green) with a pore domain formed between S5 and S6, and a large cytoplasmic C-terminal region (approximately two-thirds of the protein) that includes a cyclic nucleotide-binding domain (CNBD; highlighted in orange), the predicted ABA-interacting domain (D543-G575; shaded in yellow), which coincides with the ankyrin-repeat domain and a conserved K_{HA} domain (HA, hydrophobic/acidic; shaded in light green) at the end of the C terminus. Inset: Ribbon model representation of the recombinant $GORK^{440-672}$ and molecular docking of ABA to the predicted ABA-interacting domain. The conserved amino acid residues selected for mutagenesis studies are labeled accordingly (see Supplemental Materials and Methods for homology modeling and docking simulation).

(B) ABA enhances wild-type GORK current amplitude in a heterologous expression system (HEK293 cells) but has no statistically significant effect on the GORK/K559A/Y562A mutant. Boxplots showing the effect of active (±)-ABA versus the less active isomer (−)-ABA (left y axis) on wild-type GORK and the effect of (±)-ABA on the GORK/K559A/Y562A mutant (right y axis) for two different test voltages: +60 mV (left) and +100 mV (right); n > 10, two-tailed paired t test, $^{**}P < 0.01$; ns, non-significant.

(C) Immunoassay characterization of GORK–ABA interaction represented by the relative absorbance (%) for recombinant $GORK^{440-672}$ and the $GORK^{440-672}$/K559A/Y562A mutant in the presence of 10 nM and 100 pM (±)-ABA (n = 3). a, $P < 0.005$; b, $P < 0.0005$. All data are expressed as means ± SEM. Inset: SDS–PAGE showing the band (~25.7 kDa) corresponding to the affinity-purified recombinant $GORK^{440-672}$.

(D) A model of the role of ABA-dependent activation of GORK in stomatal closure, which proposes a fast ABA signaling response in driving stomatal closure by directly activating the GORK channel activity to promote net efflux of K^+ from the guard cells in the event of stress conditions such as drought.

2014; Lefoulon et al., 2016). We therefore speculate that the GORK–ABA mechanism of interaction differs from that of the canonical PYR/PYL/RCARs ABA receptors. A detailed analysis of the physical nature of the GORK–ABA interaction will be the scope of future investigations.

In summary, we propose that ABA can directly enhance K^+-efflux through the GORK channel, enabling a hitherto unknown mechanism to close the stomata that is independent from the currently annotated ABA signaling components (Figure 1D). Such a mechanism may implicate ABA in the direct and rapid stomatal responses to the onset of external stresses.

SUPPLEMENTAL INFORMATION

Supplemental Information is available at *Molecular Plant Online*.

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AUTHOR CONTRIBUTIONS

Conceptualization, F.L.-C. and C.G.; Methodology, A.O., F.L.-C., and A.W.; Investigation, A.O., F.L.-C., and A.W.; Writing – Original Draft, A.O., F.L.-C., A.W., and C.G.; Writing – Review & Editing, A.O., F.L.C., and C.G.; Supervision and Funding Acquisition, C.G.

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