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The *Arabidopsis* AtPP2CA protein phosphatase inhibits the GORK K⁺ efflux channel and exerts a dominant suppressive effect on phosphomimetic activating mutations

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Running title: *Regulation of GORK K⁺ efflux channel by a PP2C phosphatase*

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ABSTRACT

The regulation of the GORK Shaker channel mediating massive K⁺ efflux in *Arabidopsis* guard cells by the phosphatase AtPP2CA was investigated. Unlike the *gork* mutant, the *atpp2ca* mutants displayed a phenotype of reduced transpiration. We found that AtPP2CA physically interacts with GORK and inhibits GORK activity in *Xenopus* oocytes. Several amino acid substitutions in AtPP2CA active site, including the dominant interfering G145D mutation, disrupted the GORK-AtPP2CA interaction, meaning that the native conformation of the AtPP2CA active site is required for GORK-AtPP2CA interaction. Further, two serines in the GORK ankyrin domain, that mimic phosphorylation (S to E) or dephosphorylation (S to A), were mutated. Mutations mimicking phosphorylation led to a significant increase in GORK activity, whereas mutations mimicking dephosphorylation had no effect on GORK. In *Xenopus* oocytes the interaction of AtPP2CA with “phosphorylated” or “dephosphorylated” GORK systematically led to inhibition of the channel to the same baseline

level. Single-channel recordings indicated that the GORK S722E mutation increases the open probability of the channel in the absence but not in the presence of AtPP2CA. The dephosphorylation-independent inactivation mechanism of GORK by AtPP2CA is discussed in relation with well-known conformational changes in animal Shaker-like channels that lead to the channel opening and closing. In plants, PP2C activity would control stomatal aperture by regulating both GORK and SLAC1, the two main channels required for stomatal closure.

The plant clade A protein phosphatases 2C (PP2Cs) are Mg²⁺ and Mn²⁺-dependent serine/threonine phosphatases which were first identified as components of the abscisic acid (ABA) signal transduction pathway (1,2). The clade A APP2C members in *Arabidopsis* are mostly known as negative regulators of ABA signaling (3). Among this clade, ABI1, ABI2 (4), HAB1 (5) and AtPP2CA (6-8) are well characterized. These proteins are involved in

ABA-regulated germination (7-10). Of these, *ABI1*, *ABI2* and *AtPP2CA* genes are highly induced by ABA in guard cells (5). The *abi1*, *abi2*, *atpp2ca*, and *hab1* mutants exhibit stomatal phenotypes (1,4,5). In particular, the *atpp2ca-1* mutant displays impaired control of stomatal aperture in epidermal strips in response to ABA (7). The role of clade A PP2Cs in stomatal closure in response to ABA has been discovered recently (reviewed by (11,12)). Briefly, the PYR/PYL/RCAR (Pyrabactin resistance/PYR1-like/Regulatory Component of ABA Receptor) soluble ABA receptors undergo a conformational change upon binding ABA, allowing them to bind and inactivate PP2Cs (13-15) and release the SnRK2.6/OST1 kinase. Without ABA PP2Cs can bind to OST1 and inactivate it (16,17). Active OST1 mediates anion efflux through the activation of SLAC1 (18,19). This signaling pathway is important for guard cell plasma membrane depolarization that drives K⁺ efflux and causes stomatal closure. Besides OST1, the Calcineurin B-like Protein Kinase CIPK23 (20) and Calcium-dependent Protein Kinases (CPKs) (21,22) also phosphorylate SLAC1 and its homologue SLAH3. This activation is reversed by dephosphorylation by the PP2Cs. Different mechanisms have been reported for the inhibition of SLAC1 by PP2Cs. For example, *ABI1* inhibits SLAC1 activation by CPK6 in *Xenopus* oocytes, and directly dephosphorylates SLAC1 previously phosphorylated by this kinase (17). Conversely, *ABI1* does not reverse OST1 phosphorylation of SLAC1 but instead physically interacts with OST1, causing an indirect decrease in SLAC1 activity (19). For the SLAC1/*AtPP2CA* pair, a dual mechanism of inhibition by *AtPP2CA* has been proposed: an inhibition of the OST1 kinase that activates the SLAC1 channel, and a direct interaction with the SLAC1 channel (18).

PP2C phosphatases are not only involved in anion channel regulation but can also modulate plant potassium channels. Shaker channels are voltage-gated, K⁺-selective, and are formed by the assembly of four subunits encoded by a family of nine genes in *Arabidopsis thaliana*. A Shaker subunit comprises a short N-terminal sequence, six transmembrane segments (S1-S6), a pore domain between S5 and S6, and a large intracytoplasmic C-terminal region that comprises about two thirds

of the protein, including a C-linker involved in channel targeting and activity (23), a cyclic-nucleotide-binding domain (CNBD), an ankyrin protein-protein interaction domain (absent in KAT1, KAT2 and AtKC1), and a KHA hydrophobic/acidic domain (24). Depending on the subunits used, inwardly- or outwardly-rectifying channels can be formed (25,26). Most genes encoding subunits involved in inward rectifying channels assembly (namely *KAT1*, *KAT2*, *AKT1* and *AKT2*) are expressed in guard cells (5,27,28), whereas GORK is the only outward rectifying channel in this cell type (29). Since GORK is activated by membrane depolarization, it is commonly thought that anion efflux initiates K⁺ efflux through this channel (30). In root peripheral cells, *AKT1*, together with *AtKC1*, is involved in K⁺ uptake from the soil. GORK is also located in these cells although its role remains elusive. GORK may initiate the plasma membrane repolarization after depolarization evoked by environmental changes (26,31).

Two Shaker channels, *AKT1* and *AKT2*, have been reported to be regulated by PP2C phosphatases and a direct interaction has been observed between the phosphatase *AtPP2CA* and *AKT2* (32,33). Another closely related phosphatase, *AIP1/HAI2*, binds and regulates *AKT1* (34,35). The *AKT1* model predicts that different Calcineurin B-like Protein (CIPK) kinases interact with the ankyrin domain to phosphorylate the channel (34,35), while PP2C phosphatases (including *AtPP2CA*) inactivate *AKT1* by interacting specifically with some of these CIPKs. The direct effect of *AIP1* on *AKT1* activity was not addressed.

Inward channel regulation by PP2Cs is now well studied but nothing is known about outward channels/PP2Cs relationships. Previous studies have shown that GORK and its SKOR homologue are sensitive to different signaling factors, such as reactive oxygen species (ROS) (31,36,37), external K⁺ (26,38,39), internal and external pH (38,40), and that clustering of GORK channels is correlated to changes in its gating properties (41). Regarding PP2Cs, early physiological studies in guard cells of *Nicotiana benthamiana* provided evidence that outward K⁺

fluxes were not driven only by depolarization and that PP2C phosphatases could take part in their control. Over-expression in *Nicotiana benthamiana* of the *abi1-1* dominant interfering mutant phosphatase conferring ABA-independent constitutive activity, led to a strong and specific decrease of the amplitude of guard cell outward K^+ currents independent of the cell membrane potential and not observed with K^+ inward and anionic currents (42). This indicated that the K^+ efflux channel, later identified as GORK, was inhibited by ABI1-1 independently of the membrane potential and ABA signaling pathway.

In the present study, we provide evidence for direct GORK inhibition by AtPP2CA. We also show a strong activation of GORK using phosphomimetic mutations and uncover an unexpected suppression of the activating effect of these mutations by co-expression with AtPP2CA.

EXPERIMENTAL PROCEDURES

Plant material - The *atpp2ca-1* (SALK_028132) and *atpp2ca-2* (SAIL_609_G12) mutants were obtained from the SALK institute (43). Homozygous plants were selected by PCR using primers surrounding the insertion site. Both insertions are located in the second exon.

Plant transpiration measurements - Plants were placed under short-day conditions in growth chamber (21°C, 70% relative humidity, 8-h/16-h light/dark, 300 $\mu E \cdot m^{-2} \cdot s^{-1}$), in light-tight pots filled with soil and sealed by a screw lid to prevent evaporation. One hole was drilled in the lid through which a single plant can grow (44). Plants were grown for 5 weeks (late rosette stage) during which they were watered periodically to keep the whole pot weight constant. The rosette surfaces were calculated using ImageJ software, from pictures taken before starting the experiments. Transpiration was measured by weighing the plants at different time points as indicated in the Results part. Plants for excised rosette experiments were grown in the same conditions. The whole rosette was cut from the pot and weighed at different time points to define the water loss.

Two-hybrid system experiments - The cDNA encoding the GORK C-terminal cytosolic

domain (from G313) and *AtPP2CA* full-length ORF were inserted into pGBT9 (45) and pGAD10 (Clontech). Yeast transformation was performed according to (46). After transformation, yeast suspensions were plated on a selective agar medium without leucine and tryptophan but containing histidine (47). Yeast strains used were Y190 for quantitative two-hybrid tests and AH109 for drop tests. Quantitative two-hybrid tests were performed using o-nitrophenol- β -D-galactopyranoside (ONPG) as substrate (45). For drop tests, overnight liquid precultures (without tryptophan and leucine) were diluted 10 times in the same medium, grown for 6 hours, then washed with water. Optical density (600 nm) was adjusted to 0.2 then serial dilutions (1/3, 1/9, 1/27, 1/81) were prepared before pouring drops on the selective agar medium.

Co-purification of AtPP2CA and GORK - AtPP2CA was expressed in *E. coli* as a fusion protein with a N-terminal 6xHis tag. The bacterial extract was loaded onto a mini-column made of nickel-coated beads as described in (33). GORK was expressed in yeast (L40 strain) as a fusion with LexA (in pBTM116 vector). Yeast cells were grown in 500 mL liquid medium supplemented with amino acids but without tryptophan (47). The centrifugation pellet was resuspended in 5 mL CellLytic Y cell lysis solution (Sigma) supplemented with dithiothreitol (5 mM), a protease inhibitor cocktail (Sigma P8215, 1/100° V/V), leupeptin (10 μM), and EDTA (1 mM). Glass beads (3.5 mL, 0.5 mm diameter) were added to the yeast suspension. Yeast cells were broken by 6 cycles of 30 sec vortexing/1 min. chilling on ice. After centrifugation, an aliquot of the pellet was loaded onto the mini-column. Proteins were washed and eluted as previously described (33). Aliquots of the different purification steps (15 μL) were loaded onto SDS-PAGE gels (10 %). Proteins were then transferred onto a nitrocellulose filter and revealed by Western blotting with an anti-LexA antibody (rabbit polyclonal from Invitrogen, catalog n° 46-0710, lot n° 1200262, dilution 1/5000) and goat anti-rabbit IgG/alkaline phosphatase conjugate. The second antibody and chemiluminescent substrate were provided in the Aurora western blotting kit (ICN Biochemicals, catalog n° 821539, antibody lot 6230).

Electrophysiological recordings - GORK, *AtPP2CA*, *ABI2* and *OST1* cDNAs were cloned in a vector derived from pGEMHE (48) between the 5' and 3' untranslated regions of the *Xenopus* β -globin gene. GORK S722A, GORK S722E and *AtPP2CA* G139D point mutations were generated by PCR-mutagenesis using sense and antisense mutated primers (Stratagene, <http://www.genomics.agilent.com>) and *Pfu* ultra polymerase. *In vitro* transcription was performed on linearized plasmid using the mMESSAGE mMACHINE T7 Ultra Kit (Ambion, USA). Stage V-VI oocytes were extracted from mature *Xenopus laevis* then the follicular cell layer was digested with 1 mg.mL⁻¹ collagenase (type 1A; Sigma) for 1 h. Oocytes were injected with a final volume of 50 nL of cRNA preparation, using a micropipette connected to an oocyte injector (Nanoliter 2000, WPI). Injected oocytes were maintained at 19°C during 3 or 4 days in ND-96 solution (2 mM KCl, 96 mM NaCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 5 mM HEPES, 2.5 mM Na-pyruvate, pH 7.4, supplemented with gentamycine 5 μ g/mL). Currents through the oocyte membrane were then recorded using the two-electrode voltage clamp technique as previously described (49). During measurements, oocytes were placed under continuous perfusion in a bath solution containing, unless otherwise stated, 10 mM KCl, 90 mM NaCl, 1 mM CaCl₂, 1.5 mM MgCl₂, and 10 mM HEPES, pH 7.4. The 100 mM KCl solution used for some experiments did not contain NaCl. For oocyte single-channel recordings, oocytes were peeled with tweezers, and analyzed by patch-clamp according to (40). The pipette solution contained 100 mM KCl, 3 mM MgCl₂, 5 mM EGTA, and 10 mM Hepes-NaOH, pH 7.2. The bath solution contained 100 mM KCl, 3 mM MgCl₂, 1 mM CaCl₂, and 10 mM Hepes-NaOH, pH 7.4. All voltage pulse protocol designs, data acquisitions and analyses were done using pCLAMP 9.0 software (Axon instrument). Patch-clamp experiments in COS cells (whole-cell mode) were performed as previously described (50), with 10 mM KCl in the bath solution and 150 mM KCl in the pipette solution.

Isolation of oocyte plasma membranes and western blot - Oocytes (150-200 per assay) were injected with cRNAs and left for 3 days in ND-96 solution for protein expression. They were then

crushed and homogenized at 4°C in 5 mM Na phosphate pH 7.4 buffer containing 5mM MgCl₂, 250 mM sucrose, 0.5 mM Pefablock, 10 μ g leupeptin, 10 μ g aprotinin, 10 μ g pepstatin and 60 μ g chymostatin (homogenization buffer). Homogenates were centrifuged at 500 g for 5 min at 4°C. The supernatants were then overlaid on the top of a discontinuous sucrose gradient (50% and 20% sucrose in homogenization buffer) and centrifuged at 4°C, at 22100 rpm (50000 g) for 4 h in the swinging SW60Ti Beckman rotor. The 20% interface enriched in plasma membrane and the 50% interface enriched in endoplasmic reticulum (51) were collected, resuspended in 5 volumes of homogenization buffer without sucrose and centrifuged at 4°C, 150000 g for 2h. Resulting membrane pellets were resuspended in homogenization buffer before protein determination by Bradford assay.

For Western blot analyses, about 50 μ g of membrane proteins were solubilized with 2x Laemmli solubilization buffer and proteins were separated by SDS-PAGE and further electro-transferred on Millipore Immobilon-P PVDF 0.45 μ m membrane. GORK channels were then detected using a rabbit anti-GORK antibody (1/100) and goat antibodies (1/10000) coupled to horseradish peroxidase. The polyclonal anti-GORK antiserum was raised against a GORK-specific synthetic peptide (amino acids 50-61) and tested as described in (41). The conjugated antibodies came from Agrisera (AS09 602, lot n° 1309).

RESULTS

Atpp2ca mutants display a decreased transpiration phenotype opposite to that of the *gork* mutant - Previous studies indicate that *gork* mutant plants display larger stomatal apertures than wild type plants (29). The difference was slightly more pronounced after the light to dark transition (29). Whole plants were grown in soil to measure water loss in *atpp2ca* mutants using individual sealed pots (44). Under constant light the transpiration rate was slightly reduced (by about 6%) in the *atpp2ca* mutants (Fig. 1A, first diagram pool) compared to wild type. The relative

difference in transpiration rate between the wild type and the *atpp2ca* mutant plants increased after the light was switched off (Fig. 1A, last two diagram pools, respectively 17.3 and 12.6 % reduction compared to the wild type during the first 15 min. after light extinction, 20.9 and 11.9 % between 15 and 45 min.). This shows an increased efficiency of stomatal closing in the mutants when compared to wild type. Similarly, in measurements on excised rosettes, *atpp2ca* mutant plants lost less water than the wild type (Fig. 1B).

Physical interaction between GORK and AtPP2CA - We next tested for a direct interaction between GORK and AtPP2CA by the yeast two-hybrid system. The C-terminal part of GORK downstream from the transmembrane domain (from G313) and the full-length sequence of AtPP2CA were fused to the DNA-binding domain (DBD) or activator domain (ACT) of GAL4. A positive signal was detected using the beta-galactosidase assay (Fig. 2A), showing an interaction between GORK and AtPP2CA. These results were confirmed by growth assay using the *HIS3* reporter gene (Fig. 2B), which proved to be the best assay for detecting interactions with GORK. His-trap purification was then performed to confirm the interaction. A product of the expected size of about 80 kDa (22 kDa for lexA DNA-binding domain and 57 kDa for GORK C-terminal part) was specifically retained on a column bearing AtPP2CA fused to His-tag (Fig. 2C). Thus, the two-hybrid tests provided evidence for physical interactions between AtPP2CA and GORK C-terminal domain, and using His-trap purification gave further evidence to support this results.

Effect of AtPP2CA on GORK activity - In *Xenopus* oocytes, GORK expression resulted in outwardly-rectifying currents displaying a typical sigmoidal activation profile in agreement with previous functional analyses (29,38) (Fig. 3A). The co-expression of AtPP2CA with GORK resulted in an inhibition of GORK-mediated currents (Fig. 3B), with a mean efficiency of approximately 45% (Fig. 3C). The current/voltage curves did not reveal a shift in activation potential upon inhibition. The inhibition of GORK by AtPP2CA was confirmed using COS cells (Fig. 3D). Thus, using two independent heterologous

systems we show evidence for GORK inhibition by AtPP2CA.

To evaluate the specificity of AtPP2CA, we tested another clade A PP2C, ABI2, which is located on the other branch of the phylogenetic tree (3). Like AtPP2CA, ABI2 inhibited GORK currents (Fig. 3E), suggesting that there is a redundancy in the action among the clade A PP2Cs.

AtPP2CA interacts with the OST1 kinase in the ABA signaling pathway (18). We therefore wanted to test this kinase against GORK. No activation of GORK currents was observed (Fig. 3F). This is in agreement with the absence of interaction between GORK and OST1 in previous Bimolecular Fluorescence Complementation tests (19).

Effect of AtPP2CA single mutations on GORK/AtPP2CA interaction - We developed amino acid substitution mutations in AtPP2CA sequence based on mutations previously identified for their negative effect on phosphatase activity in other PP2C proteins. The G139D (G174D in ABI1) mutation of AtPP2CA, close to the DH metal-coordinating residues (DGHG motif), is commonly used (6,18,35). The G139D mutation inactivates AtPP2CA but it does not interfere with its expression in yeast (18,35) or its interaction abilities with kinase partners (6,18,35). We found that this substitution mutation prevents GORK inhibition in oocytes (Fig. 4A). Interestingly, the interaction between AtPP2CA and GORK in the two-hybrid assay was disrupted by this mutation (Fig. 4B lane 3). In order to test whether the phosphatase activity of AtPP2CA is linked to its ability to interact with GORK, we tried other mutations leading to complete or substantial loss of PP2C activity (Fig. 4B and Fig. 5). The dominant interfering G145D mutation in AtPP2CA (G180D or *abil-1* in ABI1, G168D in ABI2, G246D in HAB1) is known to prevent interaction of PP2Cs with PYR/PYL receptors (13,14). In two-hybrid assay, the G145D mutation also disrupted the interaction between AtPP2CA and GORK (Fig. 4B lane 5). We also tested D142N, D327N and D380N mutations in PP2CA, as we know that they target conserved residues in PP2Cs beyond the plant kingdom and correspond to human PP2C α amino acids (D60, D239, D282)

that are essential for metal coordination and catalysis (52). Mutations A294F, P308L, R284C and G287D in AtPP2CA, corresponding to mutations in ABI1 (Fig. 5) that were shown to reverse the *abi1-1* mutation phenotype, were also tested in a two hybrid assay. These four mutations have a strong negative effect on phosphatase specific activity, but R304C and G307D mutant forms in ABI1 retain a significant residual activity (53). Finally, in two-hybrid assays against the C-terminal part of GORK, all the mutations tested completely suppressed the interaction (Fig. 4B and Fig. 5).

Potential phosphorylation sites in GORK C-terminal part act as molecular switches inactivated by AtPP2CA - In order to investigate the mechanism of AtPP2CA action in more detail, we targeted phosphorylation sites in the GORK protein sequence. In GORK, one phosphopeptide has been identified as a target for a plant kinase. The sequence SDFLRLLSSGMNPN contains the LXRXXS motif common to many kinase sites (54) and has been shown to be phosphorylated by protein kinases in the CPK family (55). According to a previously published model of ankyrin repeats (56), the phosphorylatable serine (S649) is at the end of the second helix of the fourth repeat in the GORK ankyrin domain. Another serine located just downstream of the ankyrin domain was also pinpointed as a possible phosphorylation site. This serine (S722) is the only phosphorylatable residue in GORK predicted by the Netphos server that agrees with dephosphorylation sites for HAB1, a close relative of AtPP2CA (57). In the *Arabidopsis* Shaker channel family, the first site is specific to GORK but not predicted as a phosphorylation site in SKOR; whereas the second site is common to the two subunits forming the outwardly-rectifying channels SKOR and GORK, but is not present in other subunits. For each phosphorylation site, two mutations were designed: serine to alanine (mimicking the dephosphorylated state) and serine to glutamate (mimicking constitutive phosphorylation).

We first aimed to determine (i) whether the mutations have an effect on GORK activity, and (ii) whether the dephosphorylation of one of the two sites by AtPP2CA would explain the inhibition of GORK by the phosphatase.

A yeast two-hybrid assay was performed first with AtPP2CA and the S649E and S649A mutant forms of GORK. None of these mutations changed AtPP2CA binding to the GORK C-terminal region (Fig. 6A). We then evaluated the expression of the two mutant forms of GORK in *Xenopus* oocytes. The S649A GORK mimicked a constitutive dephosphorylated state and displayed the same I/V curve as the GORK wild type form; it did not modify GORK activity in this system (Fig. 6B). Strikingly, the constitutively phosphorylated mimic mutant had significantly higher activity (Fig. 6C). This suggests that the constitutive phosphorylation of this residue increases GORK activity. These data support the hypothesis that the GORK channel is not phosphorylated in *Xenopus* oocytes at this site but can be activated by a Ser649 phosphorylation.

AtPP2CA effect on the GORK mutants was also examined in *Xenopus* oocytes (Fig. 6B and 6C). The S649A and S649E GORK mutants were not insensitive to the inhibition by AtPP2CA. This confirms that GORK inhibition by AtPP2CA is not due to the dephosphorylation of the S649 residue.

In this context, for the mutant S649E, we had expected a level of inhibition ("a" in figure 6D) as indicated by the empty arrow. Interestingly, we observed a much higher level of inhibition of GORK activity (Fig. 6C and 6D). The level of GORK S649E + AtPP2CA activity was even significantly lower than the theoretical estimate based on the assumption that the inhibition would be proportional to GORK activity (grey arrow). In fact, the effect of AtPP2CA was dominant over that of the S649E mutation, which was completely suppressed (Fig. 6C and 6D). In summary, our data show that the levels of channel activity are similar for GORK + AtPP2CA and GORK S649E + AtPP2CA.

The effect of similar mimetic mutations in the S722 residue of GORK was also investigated. Our two hybrid results show that neither the GORK S722A nor S772E mutations impaired the GORK/AtPP2CA interaction. Even changing S772 to a Stop codon did not prevent the interaction (Fig. 7A). The effects of S722A and S722E mutations on GORK activity and AtPP2CA interaction were investigated in *Xenopus* oocytes

the same way as for the GORK S649 mutations. Again, we observed no difference between the S722A mutated GORK channel and the native channel concerning both their activity and inhibition by AtPP2CA (Fig. 7B). The S722E mutation also increased the channel activity even more than the corresponding substitution in Ser649 (Fig. 7C). AtPP2CA also strongly reduced the activity of the GORK mutant (mean inhibition of 77% in 7 experiments). Most importantly, the GORK S722E mutation systematically lost its effect in the presence of AtPP2CA (Fig. 7C). Since GORK activity and clustering depend on K^+ external concentration, two different concentrations of 10 and 100 mM were tested in this system. Neither the increase of GORK activity due to the “E” mutation nor the effect of AtPP2CA on native or mutated GORK forms were found to depend on the K^+ concentration of the medium (Fig. 7B and 7C).

We next investigated the increased GORK activity seen in the experiments with the GORK S722E mutant. We tested for an increase of GORK expression or export to the membrane, which could lead to a change of transport efficiency at the oocyte membrane, using a Western blot of oocyte fractionated membranes to verify GORK S722E and S722A expression compared to the native GORK (Fig. 7D). All protein levels were the same under our experimental conditions.

We next asked if these mutations change GORK gating properties. This question was addressed by analyzing GORK activity at the single-channel level (Fig. 8). Our recordings indicated that the mutation did not change GORK unitary conductance, which was close to 20 pS (Fig. 8B). AtPP2CA, which reverses the effect of the mutation, also had no effect on the conductance of the mutant (Fig. 8B). Furthermore, the frequency of opening events was increased by the mutation and this effect was inverted by co-expression with AtPP2CA (Fig. 8A and 8C). In conclusion, the S722E mutation increases GORK opening frequency without changing its unitary conductance, whereas AtPP2CA neutralizes this effect.

DISCUSSION

AtPP2CA, GORK and stomatal closing - Due to the presence of multiple PP2Cs in guard cells, the transpiration phenotype of *atpp2ca* mutants is not predicted to be strong. Conversely, in the triple *abi1 hab1 pp2ca* loss-of-function mutant, only a low residual conductance has been measured (58). In our study, small but significant decreases of transpiration rates in *atpp2ca* plants compared to wild type plants were detected. These differences increased after light/dark transition, which might be explained by a reduced pool of redundant, free active PP2Cs during that period (Fig. 9). The *gork* mutant displays an increase in transpiration rate compared to the wild type. This opposite phenotype compared to *atpp2ca* mutants is consistent with GORK inhibition by AtPP2CA. As GORK activity can be inhibited by other close clade A PP2C members (Fig. 3E), our data are also consistent with published results of the inhibition of K^+ outward currents in tobacco plants overexpressing the ABI1-1 protein, a dominant interfering mutant of ABI1 (42).

SLAC1 and GORK can work together to control stomatal closure. SLAC1 initiates a flux of anions that depolarizes the membrane and this triggers potassium efflux out of the guard cells through GORK. Interestingly, both SLAC1 and GORK are inhibited by AtPP2CA (18) and by other PP2Cs, such as ABI1 and/or ABI2 (19). The transpiration phenotype of the *atpp2ca* mutants is consistent with a co-regulation of GORK and SLAC1 by AtPP2CA. Therefore, PP2Cs plays a central role in the control of stomatal closure by regulating the activities of the three major actors of this process: SnRK2.6/OST1 kinase (16, 17), anion channels (18), and K^+ channels. Due to its regulation by PP2Cs (Fig. 3, Fig. 6, Fig. 7 and Fig. 8), GORK gating is not only the consequence of anion efflux and membrane depolarization. GORK inhibition by PP2Cs also helps slow down stomatal closure rate, and perhaps maintain the depolarization during that process as well.

Relationship between the activity of AtPP2CA and its effect on GORK. - PP2Cs do not always need to dephosphorylate their substrates to inhibit their function. A mechanism of OST1 inhibition by physical interaction with phosphatase has been described for AtPP2CA (34) and the

inhibition of CIPKs by PP2Cs is also thought to involve both physical interaction and dephosphorylation of the kinases (35). The most studied example of a PP2C-substrate interaction is between HAB1 and OST1. Based on the use of an inactive mutant of the HAB1 phosphatase, a two-step mechanism has been proposed for inhibition of OST1 by HAB1. At low concentrations of HAB1, OST1 is inhibited by dephosphorylation of its S175 residue located within its activation loop anchored into the HAB1 catalytic site. If the phosphatase is in excess, a second inhibition mechanism by physical interaction and mutual packing of the HAB1 and OST1 catalytic sites takes place (16). This mechanism involves a conserved tryptophan in the PYL interaction loop of HAB1, which blocks the access of the substrate to the kinase (16). The active site of PP2Cs and the PYL interaction loop are also involved in the interaction with PYR/PYL receptors (16) (Fig. 5).

Mutations that inactivate PP2Cs have been used to address their mechanism of action (16,18,35,52,59). The mutants we designed reproduce amino acid substitutions in critical residues characterized in AtPP2CA or homologous PP2Cs. Mutant proteins could be expressed in protoplasts or heterologous systems for kinetic analyses (6,52,53), and they often retain residual activity (6,52,53,57). The dominant interfering mutant forms in ABI1 and HAB1 (equivalent to G145D in AtPP2CA) retain 2.5 and 4% of the activity against casein as substrate (53,60), but HAB1 G246D only displays a 2-3 times reduction of OST1 inactivation (57). The AtPP2CA G139D G145D double mutant, which has completely lost its PP2C activity (18), retains the ability to bind OST1 (18) and CIPK6 (35) in yeast. It also efficiently inhibits the autophosphorylation of OST1 *in vitro* (18) and the activation of AKT1 by CIPK6 in *Xenopus* oocytes (35). Surprisingly, all nine mutations tested in AtPP2CA disrupted the interaction with the GORK C-terminal domain (Fig. 4B). However, it is unlikely that these results are due to all the AtPP2CA mutants having lost the ability to interact with GORK simply because of a drastic reduction of expression or conformational change. These mutations target sites in conserved domains that are either directly involved in the catalytic activity of the phosphatase (G139D, D142N, G145D, D327N,

D380N), or close to residues forming the catalytic site (R284C, G287D, A294F, P308L in our study) (53) (Fig. 5). R284 and G287 are located in the PYL interaction loop near the conserved tryptophan (Fig. 5). The total absence of interaction between GORK and all AtPP2CA mutants we have tested strongly suggests that the proper conformations of the active site and the PYL interaction loop are required for GORK-AtPP2CA interaction and GORK inhibition. The difference between OST1 and GORK in their ability to bind AtPP2CA mutants is likely due to a second binding site for PP2Cs in the ABA box of OST1 (16). Mutations in several OST1-interacting residues in HAB1 are inefficient at disrupting OST1-HAB1 binding unless they are associated with a mutation in the C-terminal end of HAB1 that is required for interaction with the ABA box of OST1 (16).

Phosphomimetic mutations support a regulation of GORK by phosphorylation - Both S649E and S722E mutations in GORK increase its activity (Fig. 6 and Fig. 7). This strongly suggests that these serine residues can be phosphorylated in plant cells to increase the channel's activity. The previously reported phosphorylation by plant CPKs of serine 649 in a peptide derived from GORK sequence (55) further supports this idea.

Kinases that phosphorylate GORK in plants remain to be identified. Although AtPP2CA physically interacts with OST1 (18), our results indicate that this kinase is not directly involved in GORK activation (Fig. 3F) like it is for SLAC1 (18). Other kinases, such as OST1-related kinases, CIPKs and CPKs, which regulate PP2C phosphatases and/or anion channels SLAC1 and its SLAH3 homologue (21,22,34,35,61) would be good candidates for other regulators of GORK phosphorylation.

Inhibition of GORK by AtPP2CA uncovers a dominant effect - Our results indicate that Ser649 and Ser722 are important sites for GORK activity. We found that GORK inhibition can occur in absence of dephosphorylation of the two serines, which means that S649 or S722 are not good candidates for dephosphorylation by AtPP2CA in *Xenopus* oocytes. Instead, these results reveal an additional mechanism involving the interplay between AtPP2CA and the two serines, as

described below.

Indeed, two routes of inhibition of GORK by AtPP2CA can be distinguished. The first corresponds to the inhibition observed with native GORK in oocytes (“a” in Fig. 6D). Mutations leading to partial or complete loss of AtPP2CA activity did not allow discrimination between inhibition by dephosphorylation or physical interaction involving the active site without dephosphorylation. A phosphorylation-dependent mechanism would suppose that endogenous oocyte kinases are able to phosphorylate GORK. These kinases can phosphorylate a limited number of commercial exogenous animal protein substrates (62) and potassium leak channels (63), and modulate the activity of plant transport systems, such as aquaporins (64,65), sucrose transporters (66), and the AKT2 K⁺ channel (67).

The second level of inhibition (“b” in Fig. 6D), which was studied in more detail, is observed with “E” mutants mimicking phosphorylation states of serines in the C-terminal domain of GORK. This mechanism does not involve dephosphorylation of Ser649 and Ser722. It is interesting to notice that the two mutated serines behaved similarly, suggesting that other phosphorylation sites might also be concerned. Our results suggest that GORK Ser649 and Ser722 are not phosphorylated in oocytes. The extra-activity of GORK due to the “E” mutations was systematically suppressed by AtPP2CA, whatever the percentage of native GORK inhibition in the same experiment (Fig. 6C, 6D and 7C). This suppression of GORK activation is therefore dominant over the effects of the mutations. This might not be restricted to the interaction with GORK. It is worth noting that SLAC1 and its SLAH3 homologue systematically exhibit a dominance of the effect of the phosphatases vs kinases, resulting in a complete or almost complete suppression of SLAC1 and SLAH3 kinase activation in the presence of PP2Cs, even with equal amounts of injected phosphatase and kinase cRNAs (19-22). When the SLAC1 activity can be recorded in *Xenopus* oocytes, the AtPP2CA phosphatase exactly reverts the effect of the OST1 kinase (SLAC1 + OST1 + AtPP2CA = SLAC1 + AtPP2CA).

The activation of GORK by the S722E

mutation and its inactivation by AtPP2CA do not change the channel conductance, but modulate the number and/or duration of opening events (Fig.8). Since the mutation does not change the amount of GORK protein at the plasma membrane, this increase in open states cannot be due to an increase in the number of GORK channels. The conclusion is that GORK opening frequency is increased by the mutation, and reversibly decreased by AtPP2CA. In Shaker channels, voltage gating is driven by the interplay between the voltage sensing module (S1-S4 segments) and the pore-forming module (S5-pore-S6) (24). Some regions of the C-terminal region of Shaker channels (downstream of S6) are also important for KAT-type channel activity and gating, such as the C-linker (23,68) and the cyclic nucleotide-binding domain (CNBD) (68). The increase of channel activity by mutations in or in the vicinity of the ankyrin domain (absent in KAT-type channels) underscores the role of this protein-protein interaction domain, which is located far in the peptide sequence from the transmembrane domains (about 200 amino acid downstream from S6). With respect to GORK gating and regulation, these results suggest a connection between the ankyrin domain and the transmembrane segments. This is in agreement with studies performed with the closest animal homologues of plant Shaker channels (HCN, CNG and KCNH families), demonstrating that the C-terminal cytosolic region is mediating channel gating. The first two domains after the S6 segment, namely the C-linker and the CNBD, play a crucial role. Changes in the conformation of the CNBD, either by a cyclic nucleotide ligand (69), a short intrinsic beta-strand of this domain (70), or mutations in residues of the C-linker and CNBD (71) result in a modulation of channel gating properties (69,70,71), and these changes in conformation of the C-linker are coupled to pore opening (69,72). Similarly, in plant Shaker channels, the first helix in the C-linker was found to be essential for channel activity (23). The ankyrin domain, just downstream from the CNBD, is specific to plant Shaker channels and its function in channel gating is currently unknown. The changes that we observed in GORK gating properties with mutations around the ankyrin domain and their reversibility by AtPP2CA binding suggest the

possibility that conformational constraints of this domain can be transmitted to the other C-terminal domains in the channel to modulate its gating.

In conclusion, the present study reveals (i) that GORK is regulated by PP2C protein phosphatases, (ii) that AtPP2CA active site integrity is likely to be required for the interaction, and (iii) that phosphomimetic mutations in or close to the ankyrin domain lead to an activation of the channel that is suppressed by AtPP2CA, thus suggesting an involvement of the ankyrin domain in channel gating. One of the most surprising observations was the dominant effect of the phosphatase over the activation conferred by phosphomimetic mutations. Such a mechanism of phosphorylation-dependent inactivation without dephosphorylation of these sites would help GORK respond in real time to environmental constraints, since it allows rapid and cost-effective regulation at multiple sites within the channel.

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Author contributions: CL, MB, BM, A-AV and EM performed electrophysiological experiments. EM discovered the inhibition of GORK by AtPP2CA. WS did the western blot in Figure 7. MD provided help in the imaging analyses of Fig. 1B. IG provided helpful discussions, contributed to the writing of the manuscript, and designed Figure 5. IC designed the study, wrote the paper, finalized the figures, and performed interaction experiments. All authors analyzed the results and approved the final version of the manuscript.

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FOOTNOTES

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⁶The abbreviations used are: ABA, abscisic acid; PP2C, protein phosphatase 2C; CIPK, Calcineurin B-Interacting Protein Kinase; CPK, Calcium-dependent Protein Kinase

FIGURE 1. Decreased transpiration in the *atpp2ca* mutants. A, Transpiration rate of wild type (black, $n = 14$), *atpp2ca-1* (light grey, $n = 10$), and *atpp2ca-2* (dark grey, $n = 14$) living plants grown in pots, at different time periods before and after light switch-off (“zero” of time scale). Student tests for the comparisons between the mutants and the wild type: (*) $P < 0.05$ (for *atpp2ca-2*, $P = 0.031$ between 0 and 15 min. and $P = 0.015$ between 15 and 30 min.), (**) $P < 0.01$, (***) $P < 0.001$. Two sets of measurements were performed, and results were reproducible for the two mutants. B, Cumulated water loss in excised rosettes of wild type and *atpp2ca* mutant plants ($n = 10$ to 15, pool of two experiments performed in the same conditions).

FIGURE 2. Physical interaction between AtPP2CA and the C-terminal cytosolic region of GORK. A, Quantitative two-hybrid β -galactosidase activity assays with AtPP2CA as the bait. The reciprocal interactions with GORK as the bait could not be assayed because of the low sensitivity of the test. β -galactosidase activities ($n = 7$, means \pm SD) are expressed as OD420 per min \times 1000/OD600 of the culture medium. B, Two-hybrid tests using *HIS3* as the reporter gene in strain AH109. Drop tests (dilution series, from OD 0,066 at 600 nm (1/3)) showing growth of co-transformed yeast cells on medium without tryptophan and leucine (selection of plasmids, control medium) or also without histidine (interaction tests). GORK was used as positive control (strong GORK-GORK interaction due to subunit assembly *via* the C-terminal intracytoplasmic domains (25)). In this experiment and all subsequent drop test experiments, each series of dilutions was made in duplicate with two different yeast colonies, and tests were repeated with colonies from a different yeast transformation. For (A) and (B): DBD: DNA-binding domain of GAL4, ACT: activator domain. ACT alone indicates transformations with empty pGAD10 vector. C, Co-purification on His-trap column. An *E. coli* extract containing either the 6xHis tag alone (empty vector) or the AtPP2CA-6xHis tag fusion protein was loaded onto Ni-coated beads (control column and AtPP2CA column, respectively). After washing, the *S. cerevisiae* extract containing lexA::CT-GORK protein was loaded on both columns. Unbound proteins were washed again and aliquots (10 μ L) of the last wash fraction (W), the first elution (E1) and the second elution (E2) fractions were loaded on SDS-polyacrylamide gel. The figure (right panel) presents the western blot obtained from the gel, revealed with anti-lexA antibody. Left panel: western blot performed with an extract obtained from yeast expressing the 6xhis tag alone (yeast transformed with empty vector, EV).

FIGURE 3. AtPP2CA inhibits GORK activity in *Xenopus* oocytes. A, Macroscopic current traces recorded in *Xenopus* oocytes. Representative traces obtained with GORK alone and GORK co-expressed with AtPP2CA. B, Current/voltage curves deduced from a pool of recordings. Oocytes were maintained at a holding potential of -60 mV between two voltage pulses. Oocytes were injected with 10 ng of GORK cRNA, either alone or with 20 ng AtPP2CA cRNA ($n \geq 10$). C, Inhibition of GORK (currents at + 50 mV) by AtPP2CA in 8 independent experiments. Currents have been normalized to the level of GORK in each experiment ($n \geq 10$ for each experiment). The dotted line indicates the mean level of inhibition. D, Current/voltage curves obtained from patch-clamp recordings in COS cells expressing GORK or co-expressing GORK and AtPP2CA ($n \geq 10$). Pulses of 1.6 sec were applied, from -100 to + 100 mV with a holding potential of - 40 mV (steps of 20 mV). E and F, Current/voltage curves obtained after expression of GORK alone, or GORK with ABI2 (E), or GORK with OST1 (F) in *Xenopus* oocytes ($n \geq 13$). E and F: Same electrophysiological protocol as in (B). All data are means \pm SEM.

FIGURE 4. Effect of mutations in AtPP2CA. A, GORK inhibition by AtPP2CA or AtPP2CA G139D. The protocol is described in Fig. 3B. Data are means \pm SEM ($n \geq 10$). B, Two-hybrid interaction tests

between GORK C-terminal region and AtPP2CA (or AtPP2CA mutant forms) using *HIS3* as reporter gene. Left, control growth medium, Right, selective medium for detection of interactions. See Fig. 2B for details.

FIGURE 5. Positions of substituted residues in the AtPPCA sequence. Sequence comparison of four Arabidopsis PP2C amino acid sequences ABI1, ABI2, HAB1 and AtPP2CA. Multiple alignment was performed using Clustalw-multialign with Boxshade (<http://mobyli.pasteur.fr/cgi-bin/portal.py#welcome>). Regions of identity are shaded. Mutated amino acids are indicated in red. The locations of invariant metal-coordinating residues (accession : cd00143 NCBI CDD (73) are indicated by blue arrows. Amino acids designated by an asterisk are involved in interaction with PYR/PYL/RCAR receptors. The blue frame indicates the conserved tryptophan (W385) of the PYL interaction loop, which inserts directly into the ligand-binding pocket of the receptor and establishes an ABA contact with the ketone group of ABA (74). This W385 also inserts into the catalytic cleft of kinase thus mimicking receptor-PP2C interactions (16).

FIGURE 6. Effect of mutations on Ser649 on GORK activity and inhibition by AtPP2CA. A, Two-hybrid interaction assay between GORK (or GORK mutated on serine 649) and AtPP2CA. Yeast cells were grown on medium without tryptophan, leucine and histidine. Details of experimental procedures are in the legend of Figure 2B. B, Current/voltage curves with oocytes expressing GORK alone, GORK with AtPP2CA, GORK S649A alone or GORK S649A with AtPP2CA ($n \geq 10$). The holding potential is -60 mV. Applied voltages increase from -90 to +50 mV with an increment of +10 mV. C, Same experiment as in (B) with GORK S649E instead of 649A ($n \geq 12$). In (B) and (C), oocytes were injected with 10 ng of *GORK* cRNA and 20 ng of *AtPP2CA* cRNA. D, Quantitative aspects of experiments presented in (B) and (C) (1 and 2, respectively). Data have been normalized with the level of GORK current in each experiment. (a) is the basic level of inhibition of GORK by AtPP2CA, and (b) the increase of activity due to the mutation S649E. The empty and grey arrows point respectively to the expected levels of GORK S649E + AtPP2CA if (1) the effect of AtPP2CA on GORK were completely independent on the activation of the S649 site, or (2) it were proportional to the channel's activity. The difference between the level of the grey arrow (0.87 ± 0.05) and that of GORK S649E + AtPP2CA (0.62 ± 0.05) is statistically significant (Student test $P = 0.002$). In reality, the increase of inhibition (b') is roughly equivalent to (b), thus highlighting a complete suppression of the positive effect of the mutation. Data are means \pm SEM.

FIGURE 7. Effect of mutations on Ser722 on GORK activity and inhibition by AtPP2CA. A, Two-hybrid interaction tests between GORK (or GORK mutated on serine 722) and AtPP2CA. Interacting partners are listed as bait/prey combinations: fusions with the DNA-binding domain and the activator domain of GAL4, respectively. See Figure 2B for details. Yeast cells were grown on medium without tryptophan, leucine and histidine. B, Steady-state currents recorded at +50 mV (holding potential: -60 mV) from oocytes expressing GORK alone (black bars), GORK + AtPP2CA (light grey bars), GORK S722A (dark grey bars) and GORK S722A + AtPP2CA (white bars) in 10 and 100 mM KCl (two independent experiments, $n \geq 10$ for each bar). C, Steady-state currents recorded at +50 mV (holding potential: -60 mV) from oocytes expressing GORK alone (black bars), GORK + AtPP2CA (light grey bars), GORK S722E (dark grey bars) and GORK S722E + AtPP2CA (white bars) in the experiments including the four oocyte batches in 10 mM and 100 mM KCl ($n \geq 10$ for each bar), three independent experiments, the former and the latter group of plots have been obtained with the same batch of oocytes). Note the similarity with Fig. 4D (GORK + AtPP2CA close to GORK S722E + AtPP2CA). In (B) and (C), oocytes were injected with 10 ng of *GORK* (or *GORK S722E*) cRNA and 20 ng of *AtPP2CA* cRNA. Data are means \pm SEM. D, Western blot with anti-GORK antibody after electrophoresis of oocyte membrane proteins (left), molecular weight markers (middle), and staining of the same protein samples with Coomassie blue R. S: native GORK, A: GORK S722A, E: GORK S722E. The band is at the expected

size (94 kDa for full-length GORK).

FIGURE 8. Single-channel analyses in *Xenopus* oocytes. A, Traces of currents obtained for GORK, GORK S722E and GORK S722E + AtPP2CA (depolarization of +80 mV). B, Current/voltages traces obtained from the current amplitudes of single opening events at different applied depolarizations, and calculations of the corresponding conductances (means of the slopes of the linear regression lines; 3, 6 and 4 repeats). C, Open probabilities calculated from integrated times of channel openings, in two experiments ($n \geq 7$). Comparisons of GORK and GORK + AtPP2CA could not be performed because of low signals, moderate inhibition and variability inherent to single channel measurements. Data are means \pm SEM.

FIGURE 9. Proposed model representing the effect of PP2C phosphatases on GORK. Two subunits are represented for GORK (ovoid transmembrane domain and spherical C-terminal intracytoplasmic region). There is an equilibrium between PP2C phosphatases bound to GORK and bound to PYR/PYL/RCAR receptors. In the *atpp2ca* mutant and during stomatal closure, the equilibrium is shifted to the left (less PP2Cs available for GORK inhibition). When conditions become favorable for stomatal opening, PYL/PYL/RCAR receptors release PP2Cs that inhibit GORK. Phosphorylation sites become inactive.

Figure 1

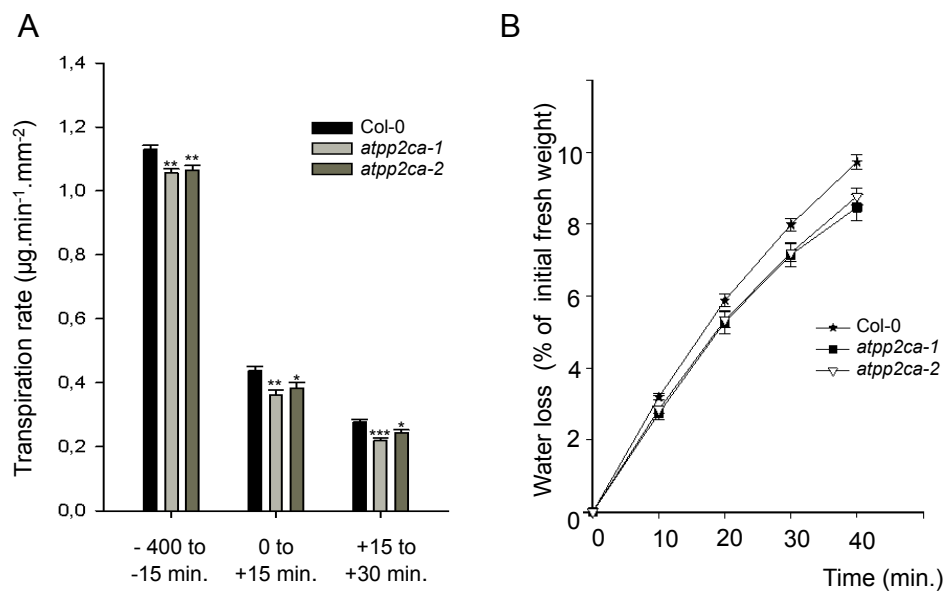


Figure 2

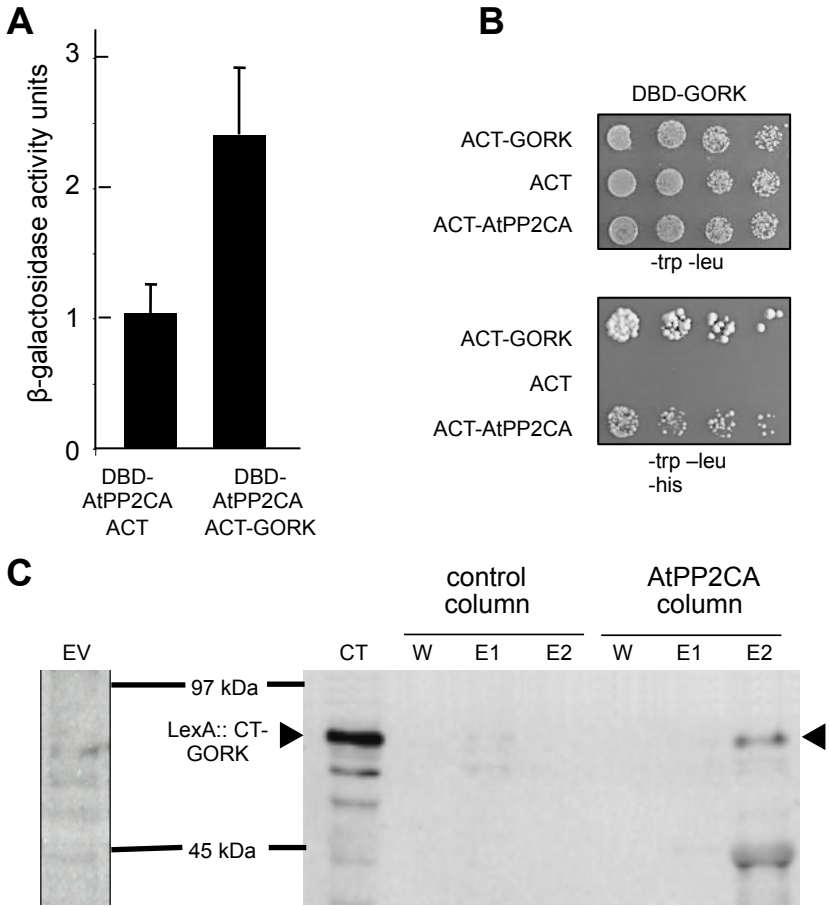
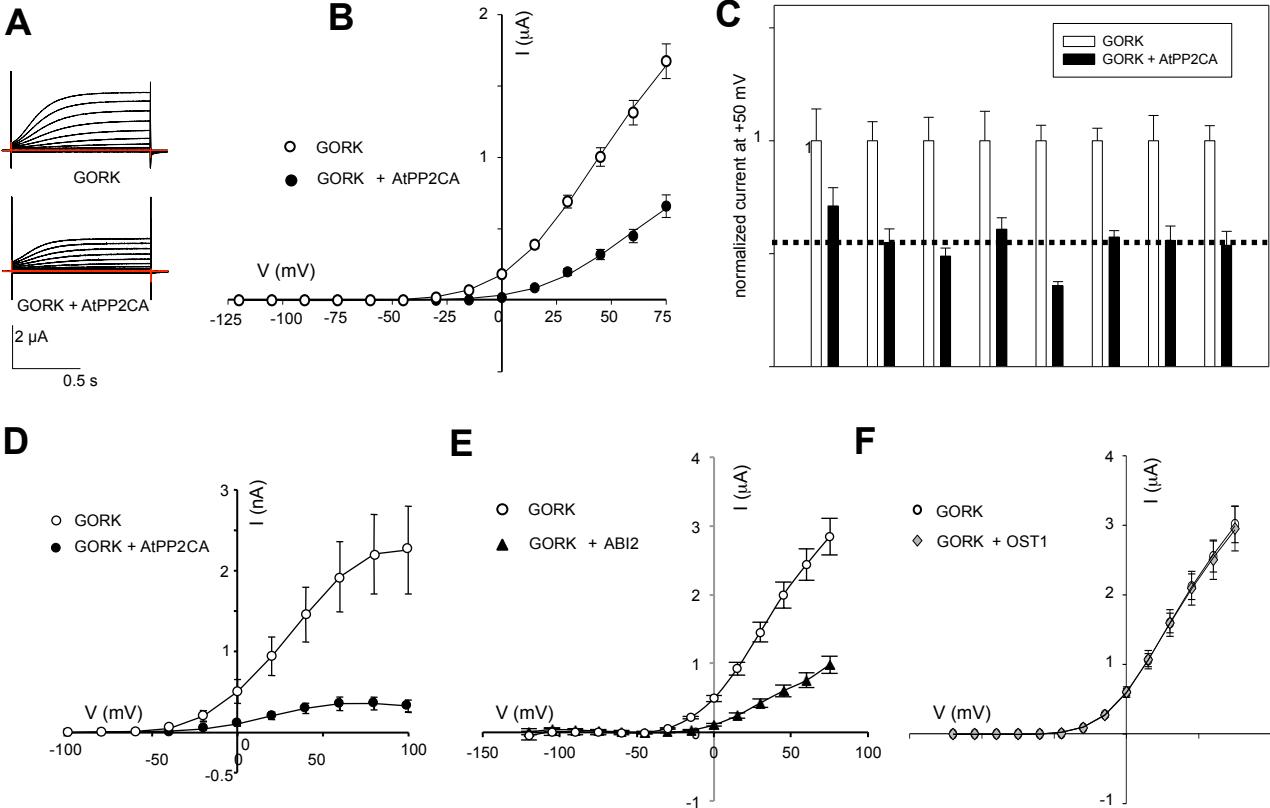
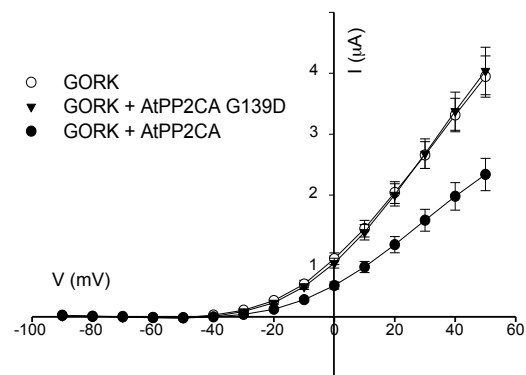


Figure 3



A



B

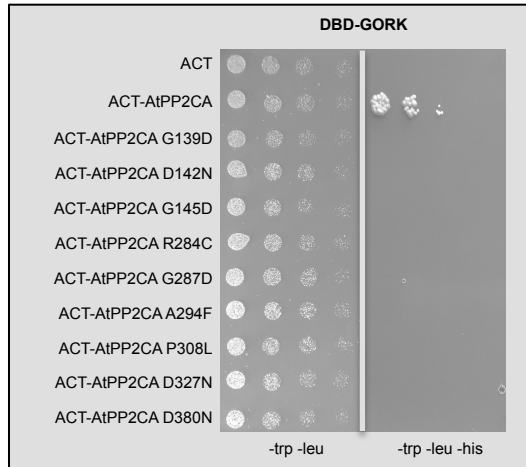


Figure 4

Figure 5

Figure 6

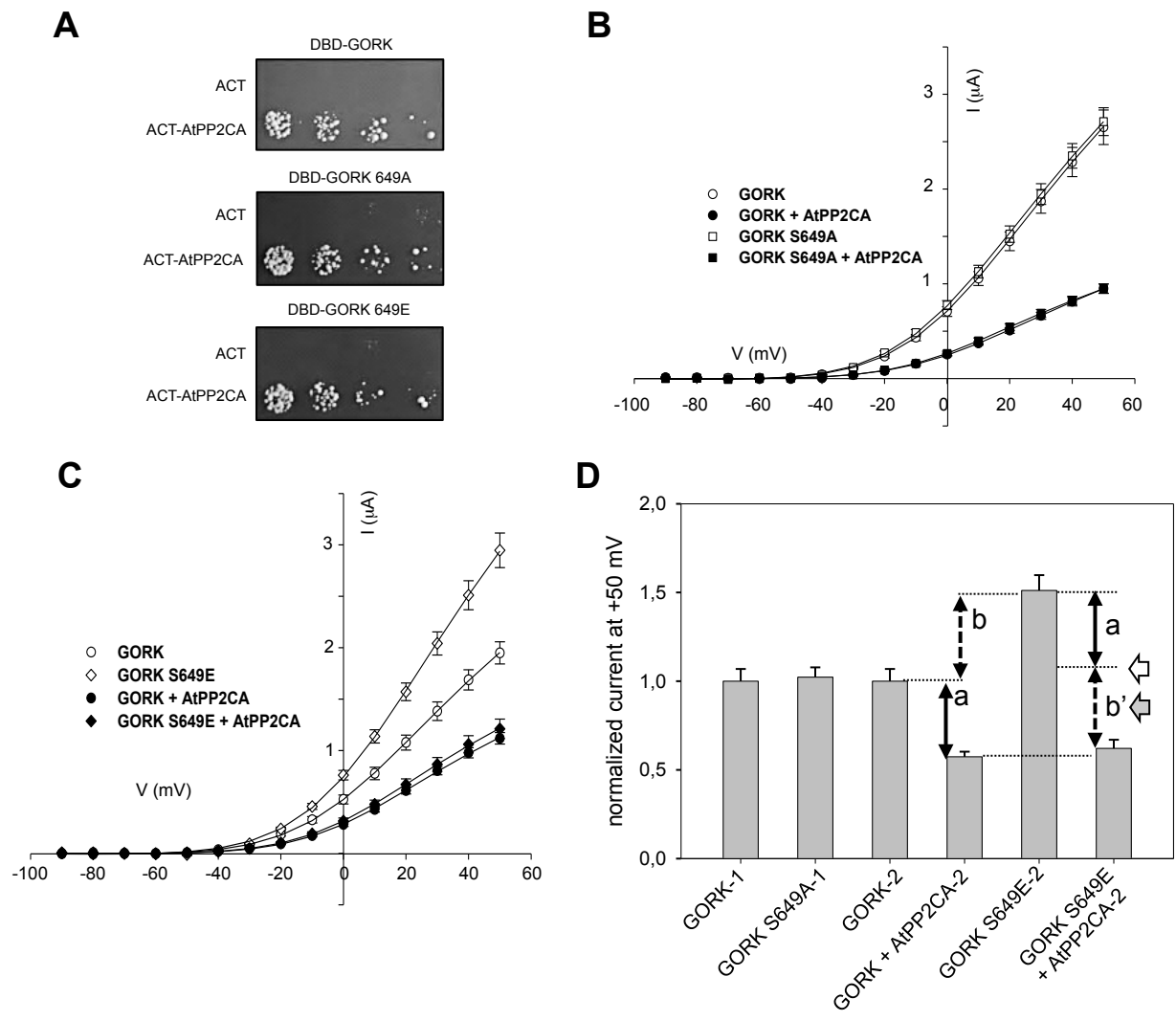


Figure 7

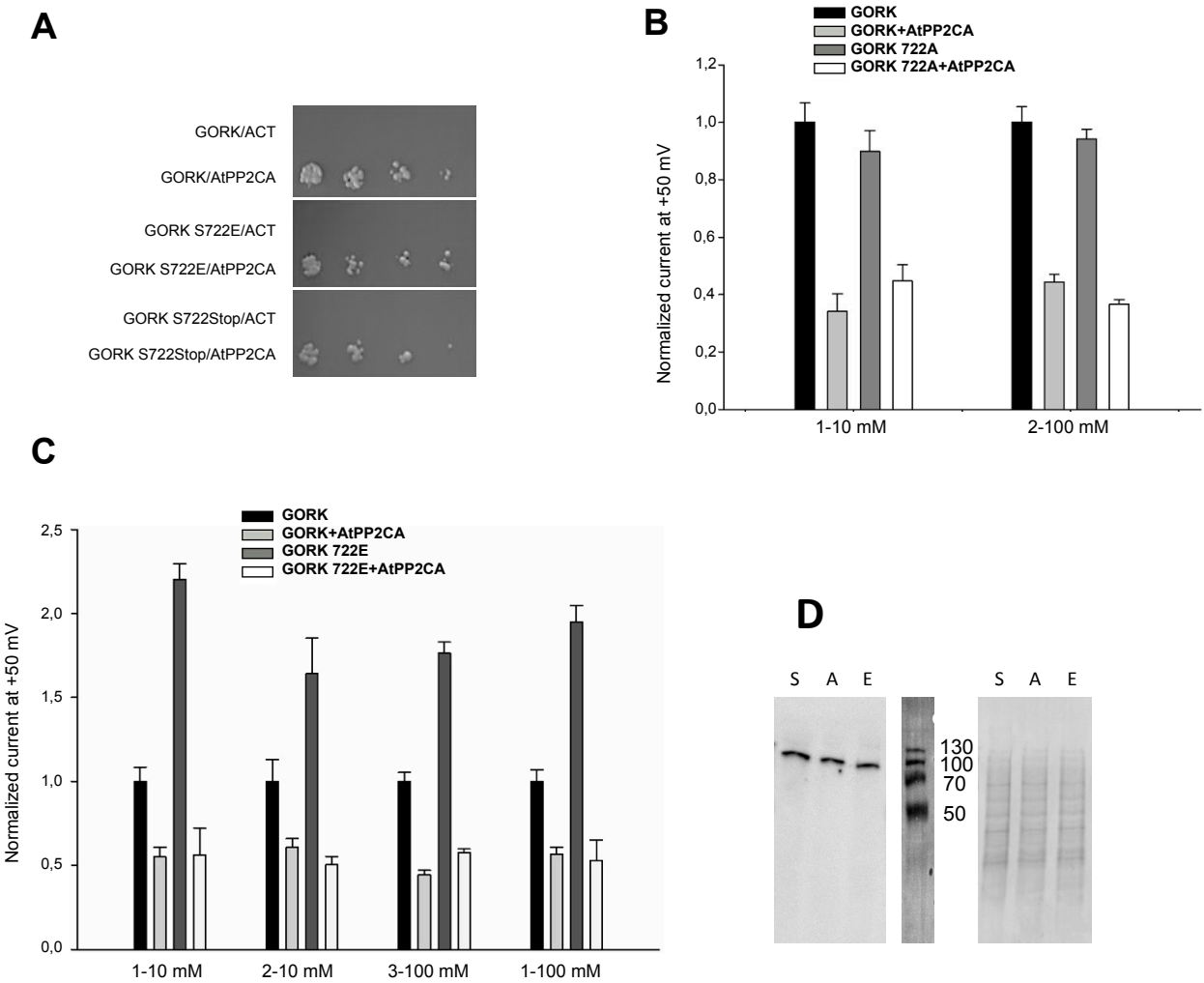


Figure 8

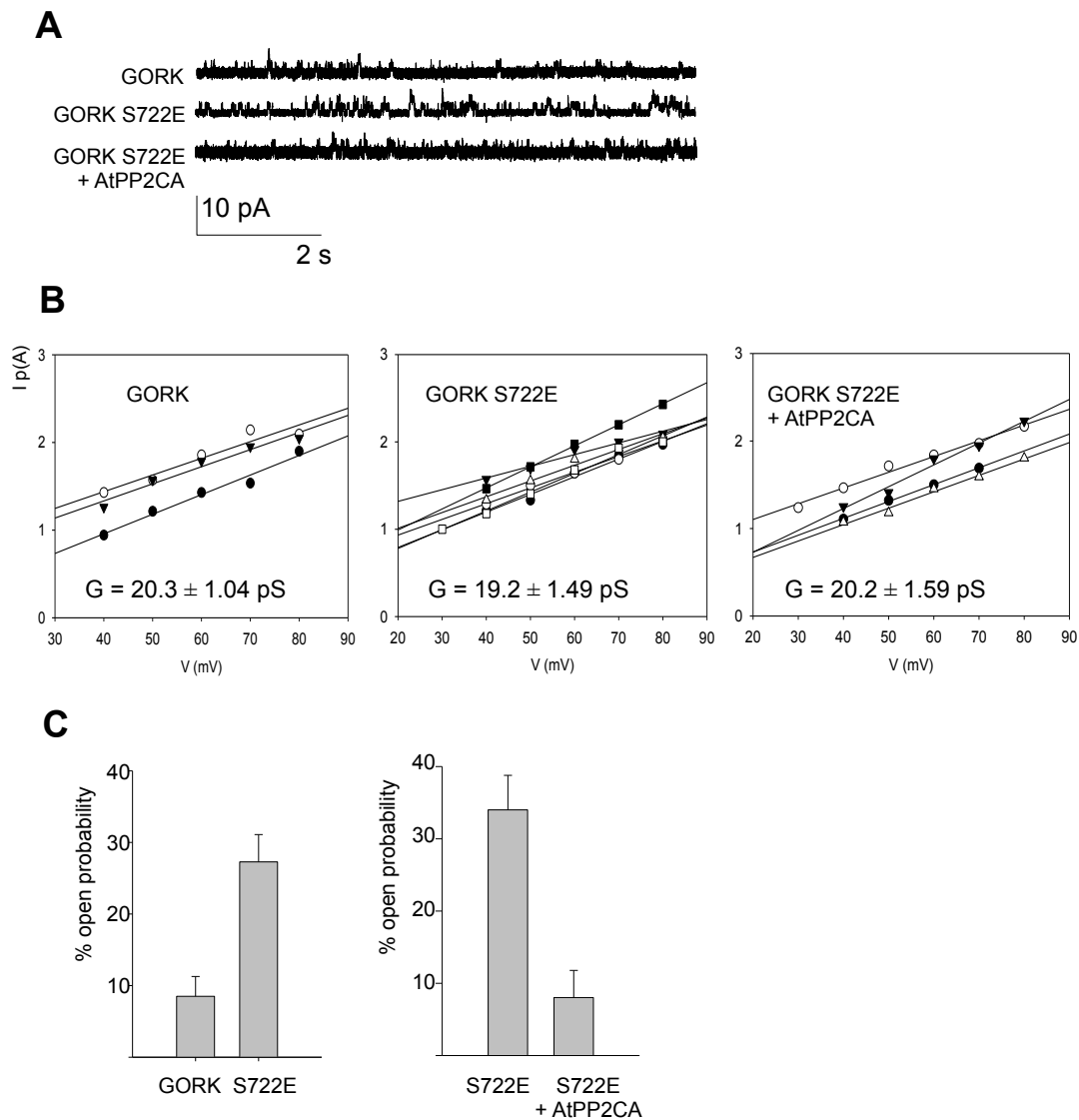
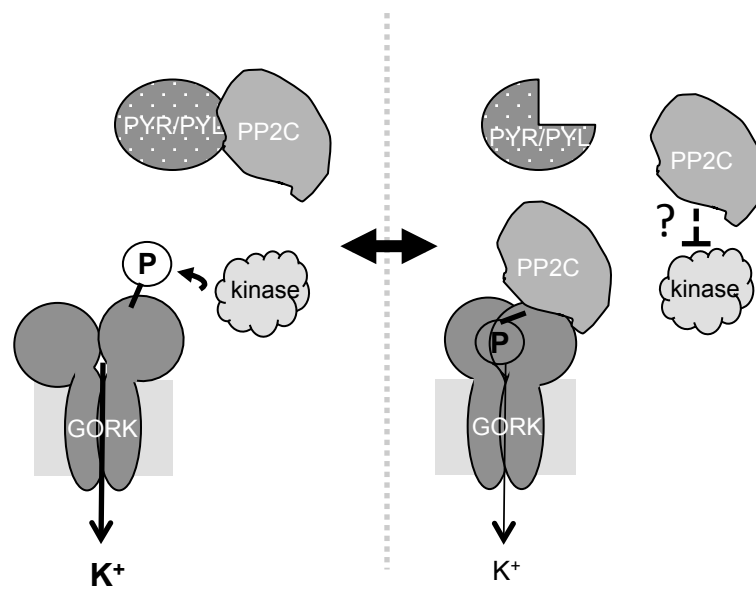


Figure 9



The Arabidopsis AtPP2CA protein phosphatase inhibits the GORK K⁺ efflux channel and exerts a dominant suppressive effect on phosphomimetic activating mutations

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