The *Arabidopsis* AtPP2CA Protein Phosphatase Inhibits the GORK K\(^+\) Efflux Channel and Exerts a Dominant Suppressve Effect on Phosphomimetic-activating Mutations*

Cécile Lefoulon\(^{1,2}\), Martin Boeglin\(^{1}\), Bertrand Moreau\(^{1}\), Anne-Aléïnor Very\(^{1}\), Wojciech Szponarski\(^{4}\), Myriam Dauzat\(^{5}\), Erwan Michard\(^{1,3}\), Isabelle Gaillard\(^{1}\), and Isabelle Chérel\(^{1,4}\)

**From the \(^{4}\)Laboratoire de Biochimie et Physiologie Moléculaire des Plantes, CNRS/INRA/SupAgro/UM2, Unité Mixte de Recherche (UMR) 5004, 2 Place Viala, 34060 Montpellier Cedex, France and the \(^{5}\)Laboratoire d’Ecophysiologie des Plantes sous Stress Environnementaux, INRA/SupAgro, UMR 759, 2 Place Viala, 34060 Montpellier Cedex, France**

The regulation of the GORK (Guard Cell Outward Rectifying) Shaker channel mediating a 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 interacts physically with GORK and inhibits GORK activity in *Xenopus* oocytes. Several amino acid substitutions in the 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 the GORK-AtPP2CA interaction. Furthermore, two serines in the GORK ankyrin domain that mimic phosphorylation (Ser to Glu) or dephosphorylation (Ser to Ala) 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 channel opening and closing. In plants, PP2C activity would control the stomatal aperture by regulating both GORK and SLAC1, the two main channels required for stomatal closure.

The plant clade A protein phosphatases 2C (PP2Cs)\(^{5}\) are Mg\(^{2+}\)- and Mn\(^{2+}\)-dependent serine/threonine phosphatases that 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, the 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 the 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 in Refs. 11, 12). Briefly, the Pyrabactin resistance (PYR)/PYR1-like (PYL)/regulatory component of the ABA receptor (RCAR)-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, which 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 homolog SLAH3. This activation is reversed 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 phosphorylated previously by this kinase (17). Conversely, ABI1 does not reverse OST1 phosphorylation of SLAC1 but, instead, interacts physically 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, formed by the component of the abscisic acid receptor; CPK, calcium-dependent protein kinase; CNBD, cyclic nucleotide-binding domain; CIPK, calcineurin B-like interacting protein kinase; GORK, guard cell outward rectifying channel; KAT, K\(^+\) channel in *Arabidopsis thaliana*. 

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\(^{1}\) Both authors contributed equally to this work.

\(^{2}\) Present address: Institute of Molecular, Cellular, and Systems Biology, College of Medical, Veterinary, and Life Sciences, Bower Bldg., University of Glasgow, Glasgow G12 8QQ, UK.

\(^{3}\) Present address: Instituto Gulbenkian de Ciência, Rua da Quinta Grande, 6 p.-2780-156 Oeiras, Portugal.

\(^{4}\) To whom correspondence should be addressed: Biochimie et Physiologie Moléculaire des Plantes, CNRS/INRA/SupAgro/Université de Montpellier, 2 Place Viala, 34060 Montpellier Cedex. E-mail: cherel@supagro.inra.fr.

\(^{5}\) The abbreviations used are: PP2C, protein phosphatase 2C; ABA, abscisic acid; PYR, pyrabactin resistance; PYL, PYR1-like; RCAR, regulatory component of the ABA receptor (RCAR)-soluble ABA receptors; Shaker channel mediating a 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 interacts physically with GORK and inhibits GORK activity in *Xenopus* oocytes. Several amino acid substitutions in the 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 the GORK-AtPP2CA interaction. Furthermore, two serines in the GORK ankyrin domain that mimic phosphorylation (Ser to Glu) or dephosphorylation (Ser to Ala) 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 channel opening and closing. In plants, PP2C activity would control the stomatal aperture by regulating both GORK and SLAC1, the two main channels required for stomatal closure.
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Experimental Procedures

Plant Material—The atpp2ca-1 (SALK_028132) and atpp2ca-2 (SAIL_609_G12) mutants were obtained from the Salk Institute for Biological Studies (La Jolla, CA) (43). Homozygous plants were selected by PCR using primers surrounding the insertion site. Both insertions were located in the second exon.

Plant Transpiration Measurements—Plants were placed under short-day conditions in a growth chamber (21 °C, 70% relative humidity, 8–16-h light/dark, 300 μE.m⁻².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 could grow (44). The 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 under “Results.” Plants for excised rosette experiments were grown under 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 Gly-313) and the AtPP2CA full-length ORF were inserted into pGBT9 (45) and pGAD10 (Clontech). Yeast transformation was performed according to Ref. 46. After transformation, yeast suspensions were plated on selective agar medium without leucine and tryptophan but containing histidine (47). The 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 as a substrate (45). For drop tests, overnight liquid precultures (without tryptophan and leucine) were diluted 10 times in the same medium, grown for 6 h, and then washed with water. The optical density (600 nm) was adjusted to 0.2, and 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 Escherichia coli as a fusion protein with an N-terminal His₆ tag. The bacterial extract was loaded onto a mini-column made of nickel-coated beads as described in Ref. 33. GORK was expressed in yeast (L40 strain) as a fusion with LexA (in the pBTM116 vector). Yeast cells were grown in 500 ml of liquid medium supplemented with amino acids but without tryptophan (47). The centrifugation pellet was resuspended in 5 ml of Celllytic Y cell lysis solution (Sigma) supplemented with dithiothreitol (5 mM), a protease inhibitor mixture (Sigma, catalog no. P8215, 1/100 v/v), leupeptin (10 μg/ml), and EDTA (1 mM). Glass beads (3.5 ml, 0.5 mm in diameter) were added to the yeast suspension. Yeast cells were broken by six cycles of 30 s of vortexing/1 min of chilling on ice. After centrifugation, an aliquot of the pellet was loaded onto the mini-column. Proteins were washed and eluted as described previously (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 antibody (Invit-
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FIGURE 1. Decreased transpiration in the atpp2ca mutants. A, transpiration rate of wild-type (black, $n = 14$), atpp2ca-1 (light gray, $n = 10$), and atpp2ca-2 (dark gray, $n = 14$) living plants grown in pots for different time periods before and after light switch-off (0 in the time scale). Student’s $t$ tests for comparisons between the mutants and the wild type were used. $*, p < 0.05$ (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 ($n = 10–15$, a pool of two experiments was performed under the same conditions).

### Table 1. Antibodies Used for Western Blotting

| Antibody          | Catalog Number | Lot Number |
|-------------------|----------------|------------|
| Polyclonal anti-GORK antiserum | 1/100 | 1/10,000 |
| Goat antibodies (1/10,000) coupled to horseradish peroxidase | | |
| Polyclonal anti-GORK antiserum (1/100) and goat antibodies (1/10,000) coupled to horseradish peroxidase | | |

### Table 2. Water Loss in Excised Rosettes

| Time (min.) | Water Loss (% of initial fresh weight) |
|-------------|---------------------------------------|
| 0           | 0                                     |
| 10          | 2                                     |
| 20          | 4                                     |
| 30          | 6                                     |
| 40          | 8                                     |

### Notes
- Transpiration rate was calculated as the rate of water loss from the excised rosettes.
- Student’s $t$ tests were used to compare the transpiration rates between the mutants and the wild type.

### References
- For further details, please refer to the original article and the cited literature.
Results

Atpp2ca Mutants Display a Decreased Transpiration Phenotype Opposite to That of the Gork Mutant—Previous studies have indicated 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 reduced slightly (by about 6%) in the atpp2ca mutants (Fig. 1A, left set of columns) compared with the 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, center and right sets of columns; 17.3% and 12.6% reduction compared with the wild type during the first 15 min after light extinction and 20.9% and 11.9% between 15 and 45 min). This shows an increased efficiency of stomatal closing in the mutants compared with the 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 with the yeast two-hybrid system. The C-terminal part of GORK downstream of the transmembrane domain (from Gly-313) and the full-length sequence of AtPP2CA were fused to the DNA-binding domain or activator domain of GAL4. A positive signal was detected using a β-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 the lexA DNA-binding domain and 57 kDa for the GORK C-terminal part) was retained specifically on a column bearing AtPP2CA fused to the His tag (Fig. 2C). Therefore, the two-hybrid tests provided evidence for physical interactions between AtPP2CA and the GORK C-terminal domain, and using His trap purification gave further evidence to support these results.

The 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). Co-expression of AtPP2CA with GORK resulted in inhibition of GORK-mediated currents (Fig. 3B) with a mean efficiency of ∼45% (Fig. 3C). The current/voltage curves did not reveal a shift in activation potential upon inhibition. Inhibition of GORK by AtPP2CA was confirmed using COS cells (Fig. 3D). Therefore, using two independent heterologous systems, we showed evidence for GORK inhibition by AtPP2CA.

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 an interaction between GORK and OST1 in previous bimolecular fluorescence complementation tests (19).

The Effect of AtPP2CA Single Mutations on the GORK/AtPP2CA Interaction—We developed amino acid substitution mutations in the AtPP2CA sequence on the basis of mutations identified previously for their negative effect on phosphatase activity in other PP2C proteins. The G139D (G174D in ABI1) mutation of AtPP2CA, close to the DG (aspartate-glycine) 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, third lane). 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 in ABI1, G168D in ABI2, and G246D in HAB1) is known to prevent interaction of PP2Cs with PYR/PYL receptors (13, 14). In a two-hybrid assay, the G145D mutation also disrupted the interaction between AtPP2CA and GORK (Fig. 4B, fifth lane). We also tested the D142N, D327N, and D380N mutations in PP2CA because we knew that they target conserved residues in PP2Cs beyond the plant kingdom and correspond to human PP2C/H9251 amino acids (Asp-60, Asp-239, and Asp-282) that are essential for metal coordination and catalysis (52). The 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 the R304C and G307D mutant forms in ABI1 retain significant residual activity (53). Finally, in two-hybrid assays against the C-terminal part of GORK, all mutations tested completely suppressed the interaction (Figs. 4B and Fig. 5).

Potential Phosphorylation Sites in the GORK C-terminal Part Act as Molecular Switches Inactivated by AtPP2CA—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 SDFLKRLLS
SGMNPN contains the LXXRXS 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 model of ankyrin repeats published previously (56), the phosphorylatable serine (Ser-649) 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 (Ser-722) 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 (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
whether the mutations have an effect on GORK activity and whether 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 current/voltage 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) than the GORK wild-type form; it did not modify GORK activity in this system (Fig. 6C). The levels of channel activity are similar for GORK + AtPP2CA and GORK S649E + AtPP2CA.

The effect of similar mimetic mutations in the Ser-722 residue of GORK was also investigated. Our two-hybrid results show that neither the GORK S722A nor the S722E mutations impaired the GORK/AtPP2CA interaction. Even changing Ser-722 to a stop codon did not prevent the interaction (Fig. 7A). The effects of the S722A and S722E mutations on GORK activity and AtPP2CA interaction were investigated in Xenopus oocytes in 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 channel activity even more than the corresponding substitution in Ser-649 (Fig. 7C). AtPP2CA also strongly reduced the activity of the GORK mutant (mean inhibition of 77% in seven experiments). Most importantly, the GORK S722E mutation systematically lost its effect in the presence of AtPP2CA (Fig. 7C). Because 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 because of 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. 7, B and C).

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 Western blotting of oocyte fractioned membranes to verify GORK S722E and S722A expression compared with the native GORK (Fig. 7D). All protein levels were the same under our experimental conditions.

We next asked whether 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. 8, A and C). In conclusion, the S722E mutation increases the
GORK opening frequency without changing its unitary conductance, whereas AtPP2CA neutralizes this effect.

Discussion

AtPP2CA, GORK, and Stomatal Closing—Because of 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 this study, small but significant decreases of transpiration rates in a tpp2ca plants compared with 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 with the wild type. This opposite phenotype, compared with atpp2ca mutants, is consistent with GORK inhibition by AtPP2CA. Because 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+/H11001 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. Because of its regulation by PP2Cs (Figs. 3 and 6–8), GORK gating is not only the consequence of anion efflux and membrane depolarization. GORK inhibition by PP2Cs also helps to slow down the stomatal closure rate and, perhaps, maintain depolarization during that process as well.

The 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. On the basis of 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 Ser-175 residue located within its activation loop anchored into the HAB1 catalytic site. If the phosphatase is in excess, then 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, which inserts directly into the ligand-binding pocket of the receptor and establishes an ABA contact with the ketone group of ABA (74). This Trp-385 also inserts into the catalytic cleft of kinase, mimicking receptor/PP2C interactions (16).
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 a substrate (53, 60), but HAB1 G246D only displays a 2- to 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. 4). However, it is unlikely that these results are due to all AtPP2CA mutants having lost the ability to interact with GORK simply because of a drastic reduction in expression or conformational change. These mutations target sites in conserved domains that are either involved directly in the catalytic activity of the phosphatase (G139D, D142N, G145D, D327N, and D380N) or close to residues forming the catalytic site.
Arg-284 and Gly-287 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 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).

Phosphomimetic Mutations Support a Regulation of GORK by Phosphorylation—The S649E and S722E mutations in GORK increase its activity (Figs. 6 and 7). This strongly suggests that these serine residues can be phosphorylated in plant cells to increase the activity of the channel. The phosphorylation by plant CPKs of serine 649 in a peptide derived from HAB1 is 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).

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FIGURE 7. Effect of mutations on Ser-722 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 (ACT), respectively. See Fig. 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 columns), GORK + AtPP2CA (light gray columns), GORK S722A (dark gray columns), and GORK S722A + AtPP2CA (white columns) in 10 and 100 mM KCl (two independent experiments, n = 10 for each column). C, steady-state currents recorded at +50 mV (holding potential, −60 mV) from oocytes expressing GORK alone (black columns), GORK + AtPP2CA (light gray columns), GORK S722A (dark gray columns), and GORK S722E + AtPP2CA (white columns) in the experiments, including the four oocyte batches in 10 mM and 100 mM KCl (n = 10 for each column); (three independent experiments, the former and the latter group of plots were obtained with the same batch of oocytes). Note the similarity with Fig. 6D (GORK + AtPP2CA close to GORK S722E + AtPP2CA). B and C, oocytes were injected with 10 ng of GORK (or GORK S722E) cRNA and 20 ng of AtPP2CA cRNA. Data are mean ± S.E. D, Western blotting with anti-GORK antibody after electrophoresis of oocyte plasma membrane proteins (left panel), molecular weight markers (center panel), and staining of the same protein samples with Coomassie Blue R (right panel). S, native GORK; A, GORK S722A; E, GORK S722E. The band is at the expected size (94 kDa for full-length GORK).
from GORK sequence, reported previously (55), further supports this idea.

Kinases that phosphorylate GORK in plants remain to be identified. Although AtPP2CA interacts physically with OST1 (18), our results indicate that this kinase is not involved directly 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 the anion channel SLAC1 and its SLAH3 homolog (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 Ser-649 and Ser-722 are important sites for GORK activity. We found that GORK inhibition can occur in the absence of dephosphorylation of the two serines, which means that Ser-649 and Ser-722 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 (Fig. 6D, a). 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 (Fig. 6D, b), 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 Ser-649 and Ser-722. It is interesting to note that the two mutated serines behaved similarly, suggesting that other phosphorylation sites might also be concerned. Our results suggest that GORK Ser-649 and Ser-722 are not phosphorylated in oocytes. The extra activity of GORK because of the E mutations was suppressed systematically by AtPP2CA, whatever the percent-
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FIGURE 9. Proposed model representing the effect of PP2C phosphatases on GORK. Two subunits are represented for GORK (void 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, PYR/PYL/RCAR receptors release PP2Cs that inhibit GORK. Phosphorylation sites become inactive.

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). Because 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 the GORK opening frequency is increased by the mutation and decreased reversibly 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 KAT1 and KAT2 Shaker channels (downstream of S6) are also important for channel activity and gating, such as the C-linker (23, 68) and the CNBD (68). The increase in channel activity by mutations in or in the vicinity of the ankyrin domain (absent in KAT1, KAT2, and AtKC1) 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 of 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 homologs of plant Shaker channels (HCN (hyperpolarization-activated cyclic nucleotide-gated), CNG (cyclic nucleotide-gated), and KCNH (K⁺ voltage-gated channel, subfamily H) families), demonstrating that the C-terminal cytosolic region is mediating channel gating. The first two domains after the S6 segment, 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 β 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 has been 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 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, this study reveals that GORK is regulated by PP2C protein phosphatases, that AtPP2CA active site integrity is likely to be required for the interaction, and that phosphomimetic mutations in or close to the ankyrin domain lead to an activation of the channel that is suppressed by AtPP2CA, 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 because it allows rapid and cost-effective regulation at multiple sites within the channel.

Author Contributions—C. L., M. B., B. M., A. A. V., and E. M. performed the electrophysiological experiments. E. M. discovered the inhibition of GORK by AtPP2CA. W. S. performed the Western blotting analysis shown in Fig. 7. M. D. helped with the imaging analyses of Fig. 1A. I. G. provided helpful discussions, contributed to the writing of the manuscript, and designed Fig. 5. I. C. designed the study, wrote the paper, finalized the figures, and performed the interaction experiments. All authors analyzed the results and approved the final version of the manuscript.

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