

The Shaker Type Potassium Channel, GORK, Regulates Abscisic Acid Signaling in Arabidopsis

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Evolution of adaptive mechanisms to abiotic stress is essential for plant growth and development. Plants adapt to stress conditions by activating the abscisic acid (ABA) signaling pathway. It has been suggested that the ABA receptor, clade A protein phosphatase, SnRK2 type kinase, and SLAC1 anion channel are important components of the ABA signaling pathway. In this study, we report that the shaker type potassium (K⁺) channel, GORK, modulates plant responses to ABA and abiotic stresses. Our results indicate that the full length of PP2CA is needed to interact with the GORK C-terminal region. We identified a loss of function allele in *gork* that displayed ABA-hyposensitive phenotype. *gork* and *pp2ca* mutants showed opposite responses to ABA in seed germination and seedling growth. Additionally, *gork* mutant was tolerant to the NaCl and mannitol treatments, whereas *pp2ca* mutant was sensitive to the NaCl and mannitol treatments. Thus, our results indicate that GORK enhances the sensitivity to ABA and

negatively regulates the mechanisms involved in high salinity and osmotic stresses via PP2CA-mediated signals.

Keywords : abscisic acid, high salinity, osmotic stress, PP2CA

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Global warming and extreme temperatures caused by climate change lead to stress conditions that interfere with plant growth and crop productivity. Plants have been constantly evolving defense mechanisms to overcome these stress conditions, such as high temperatures, drought, high salinity, and cold. Osmotic stress, caused by water deficits, also affects plant growth and causes reduced crop yields. Among osmotic stresses, drought stress is closely related to stomatal adjustment and is one of the major abiotic stresses (Golldack et al., 2014; Lee and Luan, 2012). When plants are exposed to drought conditions, closure of stomata in response to abscisic acid (ABA) occurs to minimize water loss (Golldack et al., 2014; Lee and Luan, 2012).

ABA is a key hormone in plant responses to abiotic stress, especially drought stress (Osakabe et al., 2014), as it modulates several stress-related genes, including protein kinases, phosphatases, transcription factors, and ion channels involved in osmotic adjustment (Sirichandra et al., 2009). However, although previous studies have elucidated aspects of the ABA signaling pathway from the perspective of plant cells, the exact mechanism of the ABA signaling pathway remains unclear (Lee et al., 2009; Lim et al., 2015a; Ma et al., 2009; Park et al., 2009). The three major components involved in ABA-induced stomatal closure are

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the pyrabactin resistance/pyrabactin resistance-like/regulatory component of ABA receptor (PYR/PYL/RCAR) proteins, sucrose non-fermenting 1-related subfamily 2 (SnRK2) protein kinases, and type 2C protein phosphatases (PP2Cs) (Lee and Luan, 2012; Ma et al., 2009; Park et al., 2009). PYR/PYL/RCAR ABA receptors initiate the ABA signaling pathway in cells and transfer the signal to clade A PP2Cs, which negatively modulate the ABA signaling pathway, and to SnRK2s protein kinases, which positively modulate the ABA signaling pathway (Lee and Luan, 2012). PYR/PYL/RCAR ABA receptors interact with clade A PP2Cs (Szostkiewicz et al., 2010), interrupting the interaction between clade A PP2Cs and SnRK2s. Released SnRK2 protein kinases enhance specific gene expression and activate ion channels and transcription factors via phosphorylation of target proteins (Lim et al., 2015a; Lee and Luan, 2012; Lee et al., 2009).

Additionally, ABA decreases the turgor pressure in guard cells by modifying the activities of several ion channels (Schroeder et al., 2001). SLAC1 and shaker type K⁺ channels are ABA-modulated ion channels that regulate anion and cation fluxes across the plasma membrane and tonoplast, which adjusts stomatal apertures (Lebaudy et al., 2010; Lemtiri-Chlieh and MacRobbie, 1994; Negi et al., 2008; Schroeder and Hagiwara, 1989; Schroeder et al., 1987; Vahisalu et al., 2008). In Arabidopsis, the shaker type K⁺ channel is a voltage-gated channel composed of nine genes (Hedrich, 2012). The structure of the shaker type K⁺ channel consists of six transmembrane domains and N-terminal/C-terminal regions in the cytoplasm. The C-terminal region is involved in protein-protein interactions and channel activity and targeting (Dreyer and Blatt, 2009; Nieves-Cordones et al., 2014; Pilot et al., 2003). Among the nine channels, KAT1, KAT2, AKT1, AKT2, and GORK are expressed in guard cells (Dreyer and Blatt, 2009; Lefoulon et al., 2016). It has been widely recognized that the anion efflux of SLAC1 activates GORK channels in guard cells, thus causing stomatal closure. Moreover, PP2C activity modulates stomatal aperture as it controls SLAC1 and GORK activity (Lefoulon et al., 2016).

Moreover, studies have reported that the RCAR2 ABA receptor positively regulates the ABA signaling pathway, whereas the clade A protein phosphatase PP2CA negatively regulates the ABA signaling pathway by modulating the SLAC1 anion channel activity (Lee et al., 2009, 2013). Additionally, these studies have also demonstrated that RCAR2 is associated with drought tolerance. In this study, we isolated the Arabidopsis GORK channel that interacts with PP2CA; we also isolated two T-DNA insertion mutants of *GORK1* that are hyposensitive to ABA, high salin-

ity, and osmotic stress in seeds and seedlings. Our findings indicate that GORK channels modulate ABA-induced stress responses.

Materials and Methods

Plant growth conditions. Seeds of Arabidopsis (*Arabidopsis thaliana*, ecotype Col-0) were grown in soil composed of equal parts of sterilized sand, loam soil, and a compost mixture of peat moss, perlite, and vermiculite (5:3:2, v/v/v). Seeds were grown under white fluorescent light (130 μmol photons/m²/s) at 24 ± 1°C. For the germination and seedling growth experiment, Arabidopsis seeds were grown on Murashige and Skoog (MS) media (Duchefa Biochemie, Haarlem, Netherlands) supplemented with 1% sucrose in a growth chamber at 24 ± 1°C and under a 16-h light/8-h dark period. Seeds were vernalized at 4°C for 2 days to synchronize germination.

Germination and seedling growth. To examine germination, primary root growth, and cotyledon greening, 200 seeds of wild-type plants, *gork* and *pp2ca* mutant plants were plated on MS agar medium containing ABA, NaCl, and mannitol. Germination was determined by radicle emergence. Primary root growth was measured 7 days after plating, and green cotyledons were measured 10 days after plating. Plates were placed in a growth chamber at 24°C for a 16-h light/8-h dark cycle.

RNA isolation and reverse transcription PCR (RT-PCR) analysis. Total RNA isolation and RT-PCR assays were conducted as described by Lim et al. (2015b), using leaf tissues of wild-type and *gork* mutant plants. To synthesize cDNA, we used 1 μg of the total RNA and a Transcript First Strand cDNA Synthesis kit (Roche, Indianapolis, IN, USA), according to the manufacturer's protocol. For the RT-PCR assay, the cDNA was amplified using the iQSYBR Green Supermix (Bio-Rad, Hercules, CA, USA) with GORK specific primers (F: CAGGAACTTCCTTT-GCACGAC; R: TCAAATGAATCATCGGAAAAATC). The *AtACT8* gene was used for internal control.

Subcellular localization of GORK and PP2CA. For the subcellular localization analysis, we used full-length coding regions of GORK and PP2CA without the stop codon and p326GFP vector. To express each transient construct, *Agrobacterium tumefaciens* strain GV3101 was infiltrated to epidermal cells of *Nicotiana benthamiana* p19 strain (1:1 ratio; OD₆₀₀ = 0.5). Four days after such transient expression, tobacco leaves were cut and epidermal cells were

observed with a confocal microscope (510 UV/Vis Meta, Zeiss, Oberkochen, Germany).

Yeast two-hybrid analysis. To verify the interaction between GORK and PP2CA, we used a yeast two-hybrid (Y2H) analysis (Lee et al., 2007). The C-terminal region of GORK and the full-length cDNA of PP2CA were inserted into pGBKT7 and pGADT7 vectors, respectively, and transformed into the AH109 yeast strain using the lithium acetate-mediated method (Ito et al., 1983). To evaluate the interaction between GORK and PP2CA, yeast cells containing each construct were spotted on selection media (SC-adenine-histidine-leucine-tryptophan) and grown for 4 days.

Bimolecular fluorescence complementation (BiFC) analysis. BiFC analysis was conducted as described previously (Lim and Lee, 2016). To generate BiFC constructs, open reading frames without a stop codon for GORK and PP2CA were inserted into Pro35S:SPYNE and Pro35S:SPYCE vectors, respectively (Waadt et al., 2008). To express each transient construct, *Agrobacterium tumefaciens* strain GV3101 was infiltrated into epidermal cells of *N. benthamiana* ($OD_{600} = 0.5$). Four days after agroinfil-

tration, tobacco leaves were cut and epidermal cells were observed with a confocal microscope (510 UV/Vis Meta, Zeiss).

Results and Discussion

Reduced sensitivity of *gork* mutants in response to ABA.

Previous studies have shown that GORK is involved in stomatal closure and water loss by transpiration (Hosy et al., 2003; Lefoulon et al., 2016). Even though it is widely recognized that clade A PP2Cs negatively modulates GORK channel activity and that CPK3 positively modulates GORK channel activity (Corratgé-Faillie et al., 2017; Lefoulon et al., 2016), the biological role of GORK in early stages of a plant's life cycle remains unclear. To investigate the biological functions of GORK in seeds and seedlings, we identified T-DNA insertion mutants *gork-1* (SALK_082258) and *gork-2* (SALK_144737) as knockout alleles (Fig. 1A). First, we performed RT-PCR analysis to verify the knockout of *gork* mutants (Fig. 1B). *GORK* transcripts were detected in wild-type plants but not in *gork* mutants. The identified *gork* mutants showed no obvious phenotype under normal growth conditions (Fig. 1C). However, growth of wild-type and *gork* mutant plants was

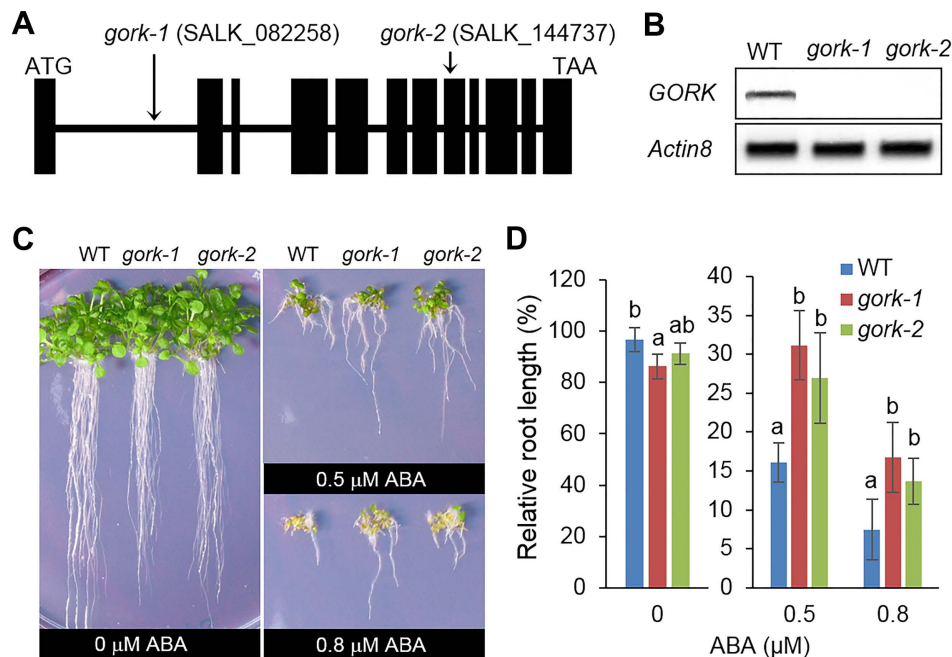


Fig. 1. Decreased sensitivity of *gork* mutants to abscisic acid (ABA). (A) Schematic diagram of the T-DNA insertion sites within the genomic DNA sequence of the *GORK* gene. Boxes refer to exons and bars to introns. (B) Relative expression of the *GORK* gene in wild-type (WT) and *gork* mutant plants. (C, D) Inhibition of primary root growth of WT and *gork* mutant plants in the presence of ABA. Seedlings were grown vertically in 0.5 \times Murashige and Skoog containing 0.5 and 0.8 μ M ABA. After 12 days, images were taken (C) and root lengths of each plant were measured (D). Data represent the mean \pm SE of three independent experiments. Different letters indicate significant difference between WT and mutant plants (ANOVA; $P < 0.05$).

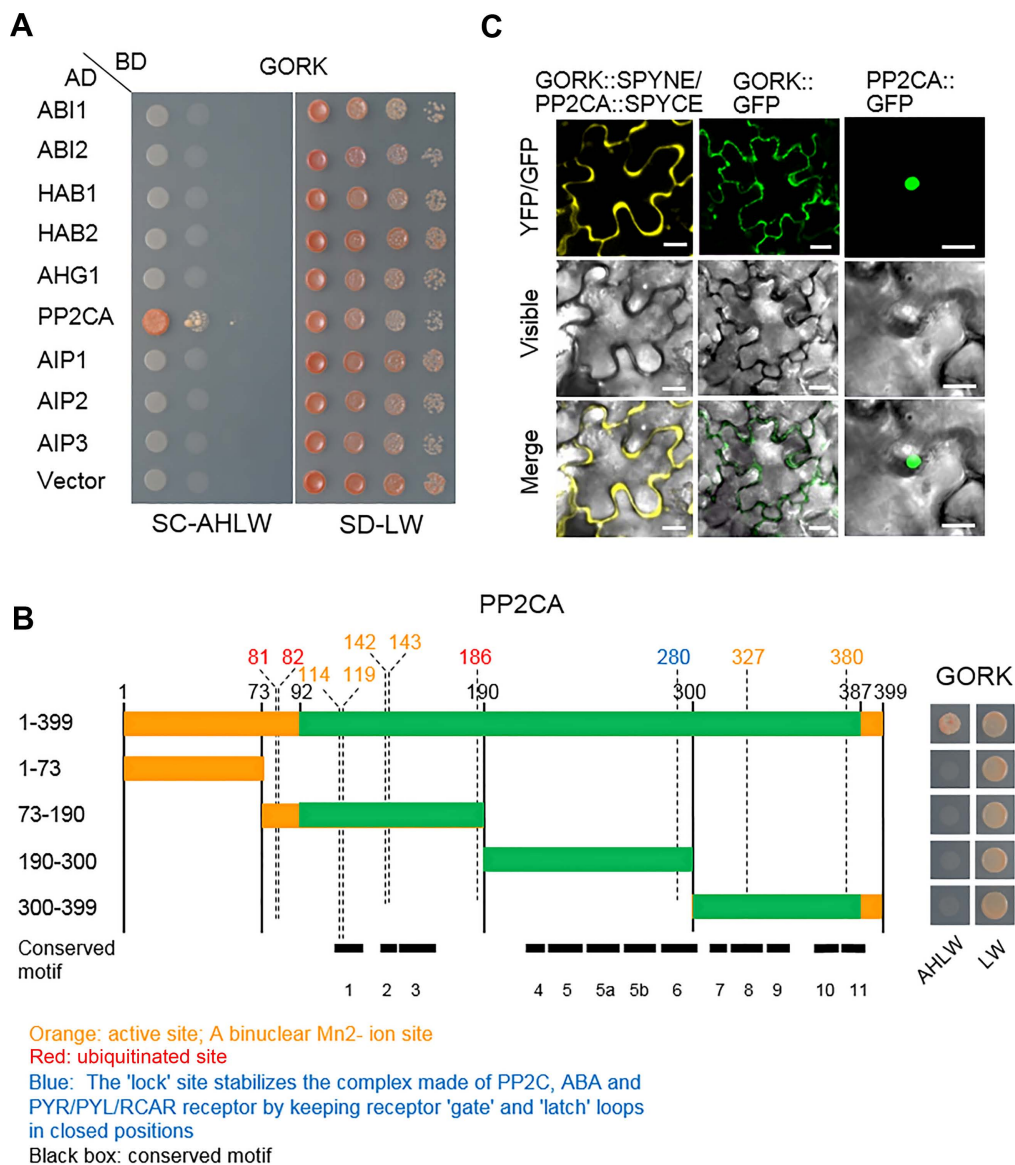


Fig. 2. Physical interactions between GORK and PP2CA. (A) Yeast-2 hybrid (Y2H) assay of the interactions between GORK and PP2Cs. Growth on SC-ALTW was used as an indicator of the interaction (left row). Growth on SC-LW was used as the control (right row). (B) Y2H assay of the interactions between GORK and PP2CA fragment domains. (C) Bimolecular fluorescence complementation assay of the interaction between GORK and PP2CA and their subcellular location in leaves of *Nicotiana benthamiana*. The yellow fluorescence indicates an interaction between the indicated partner proteins. The images were obtained from the YFP channel, bright field, or a merged picture of the two. Scale bars = 20 μ m.

inhibited in the presence of 0.5 and 0.8 μ M of ABA. Additionally, primary root lengths of *gork* mutants were significantly longer than that of wild-type plants (Fig. 1C and D).

Interaction between GORK and PP2CA. Earlier, we demonstrated that PP2CA inhibits the activity of SnRK2.6 kinase, which consequently inhibits the SLAC1 anion channel (Lee et al., 2009, 2013). We conducted a Y2H assay to isolate the clade A PP2C interacting with GORK.

We used the GORK C-terminal region as bait and clade A PP2Cs as prey. We performed a one-by-one Y2H assay with clade A PP2Cs (Fig. 2A); specifically, the yeast with GORK and PP2CA grew on the selective media, suggesting that GORK interacts with PP2CA. It has been demonstrated that the domain of clade A PP2Cs, including specific amino acids, plays an important role in the interaction with RCARs and SnRK2 kinases (Fuchs et al., 2013); however, little is known about the interaction with GORK.

To elucidate which domain is important in the interaction between GORK and PP2CA, we generated truncated forms of PP2CA and performed Y2H. Growth of yeast with GORK was only detected with full-length PP2CA (Fig. 2B), indicating that full-length PP2CA is necessary for the interaction to occur. We used BiFC analysis to determine where the interaction complex was located inside the cell and green fluorescent protein (GFP) analysis to determine where each protein was located inside the cell (Fig. 2C). Co-expression of *GORK*-SPYNE with *PP2CA*-SPYCE was detected as yellow fluorescence predominantly in plasma membrane. The GFP signals of GORK-GFP and PP2CA-GFP were generated in the plasma membrane and nucleus, respectively. Such results agree with the Y2H analysis and indicate a physical interaction between GORK and PP2CA. In addition, it also suggests that GORK and PP2CA are both targeted at the plasma membrane, where they regulate K⁺ efflux thus causing stomatal closure (Corratgé-Faillie et al., 2017; Lefoulon et al., 2016).

ABA sensitivity of GORK and PP2CA mutants. PP2CA has been reported as one of the core ABA signaling component, where it acts as a negative ABA regulator (Baek et al., 2018; Lee et al., 2013; Lim et al., 2015a). To investigate the *in vivo* function of GORK in response to ABA, we compared the germination and seedling growth of wild-type plants, *gork* and *pp2ca* mutant plants (Fig. 3). In the absence of exogenous ABA, we did not find differences between the phenotypes in relation to seed germination and seedling development. However, when grown on media containing ABA, *gork* mutants showed significantly higher germination in comparison to wild-type plants, whereas *pp2ca* mutants showed significantly lower germination rate than wild-type plants (Fig. 3A). We also compared primary root growth and seedling establishment of wild-type, *gork*, and *pp2ca* mutant plants (Fig. 3B-D). ABA-induced inhibition of primary root growth in *gork* mutants was significantly lower in comparison to wild-type and *pp2ca* plants (Fig. 3B). Additionally, the number of green cotyledons in *gork* mutant plants was significantly higher in comparison to wild-type plants; whereas in *pp2ca* mutants, the number of green cotyledons was significantly lower in comparison to wild-type plants (Fig. 3C and D). These results suggest that knockout of GORK decreases sensitivity to ABA during germination, which leads to reduced primary root growth and hinders seedling establishment.

Abiotic stress responses of *gork* and *pp2ca* mutants. ABA signal and the abiotic stress response share common signaling components (Lim et al., 2015a; Sah et al., 2016).

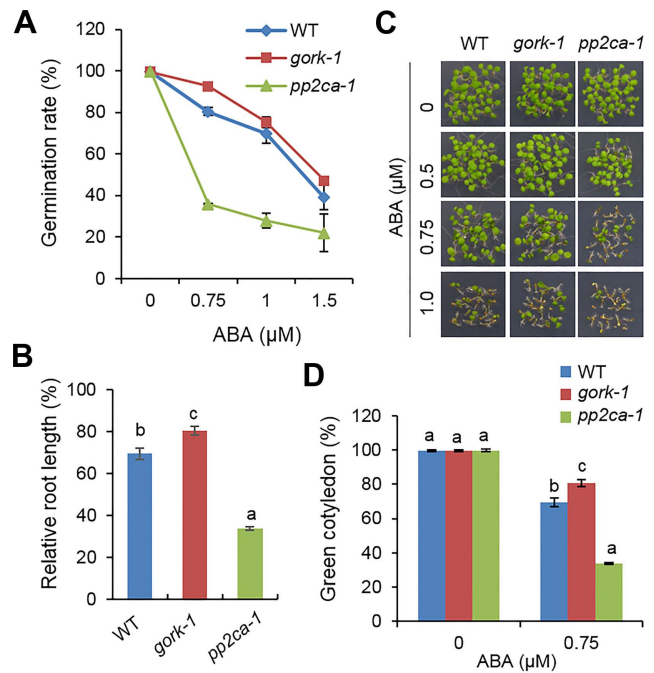


Fig. 3. Abscisic acid (ABA) sensitivity of *gork* and *pp2ca* mutants. (A) Germination of wild-type (WT), *gork* and *pp2ca* mutant plants on 0.5× Murashige and Skoog (MS) media containing different concentrations of ABA. Germination was determined by radicle emergence and was counted 3 days after plating. (B) Inhibition of primary root growth by ABA in WT, *gork*, and *pp2ca* mutant plants. Seedlings were grown vertically in 0.5× MS containing 0.75 μM of ABA for 7 days. (C, D) Number of green cotyledons in WT, *gork*, and *pp2ca* mutant plants in response to ABA. Representative photographs were taken at 10 days after plating (C), and the number of seedlings with expanded cotyledons was counted (D). Values are the mean ± SE from three independent experiments. Different letters indicate significant difference between WT and mutant plants (ANOVA; $P < 0.05$).

We therefore investigated the involvement of GORK and PP2CA in plant responses to abiotic stress, including high salinity and osmotic stress (Figs. 4 and 5). First, we evaluated the plants response to high salinity by comparing different phenotypes (wild-type, *gork* and *pp2ca* mutant plants) treated with NaCl (Fig. 4). Under regular growth conditions, there was no significant difference between wild-type and mutant plants. However, in the presence of NaCl, *pp2ca* mutants showed significantly lower germination rate compared to wild-type and *gork* plants (Fig. 4A). Moreover, the number of green cotyledons was significantly higher in *gork* mutant plants and significantly lower in *pp2ca* mutant plants in comparison to wild-type plants (Fig. 4B and C).

To further examine whether *gork* and *pp2ca* mutants showed different responses to osmotic stress, we measured

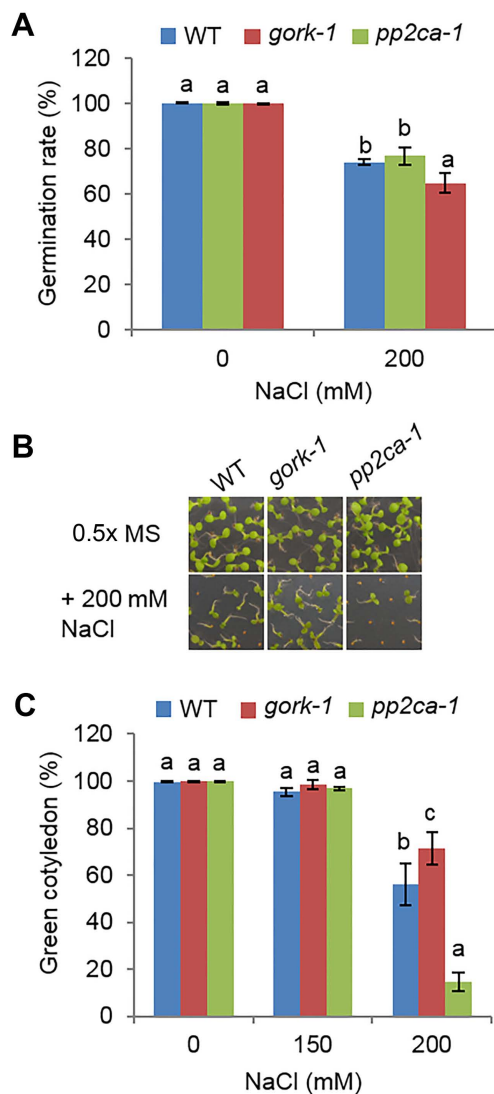


Fig. 4. Response of *gork* and *pp2ca* mutant plants to NaCl. (A) Germination rates of wild-type (WT), *gork*, and *pp2ca* mutant plants on 0.5× Murashige and Skoog (MS) media containing 200 mM of NaCl. Germination was determined by radicle emergence and was evaluated 9 days after plating. (B, C) Number of green cotyledon in WT, *gork*, and *pp2ca* mutant plants in response to NaCl. Representative photographs were taken at 10 days after plating (B), and the number of seedlings with expanded cotyledons was counted (C). Values are the mean ± SE from three independent experiments. Different letters indicate significant difference between WT and mutant plants (ANOVA, $P < 0.05$).

germination and the number of green cotyledons in seeds treated with mannitol (Fig. 5). In contrast to the ABA and NaCl treatments, seed germination of *gork* and *pp2ca* mutants did not differ from that of wild-type plants (Fig. 5A). However, the number of green cotyledons was significantly higher in *gork* mutants and significantly lower in *pp2ca*

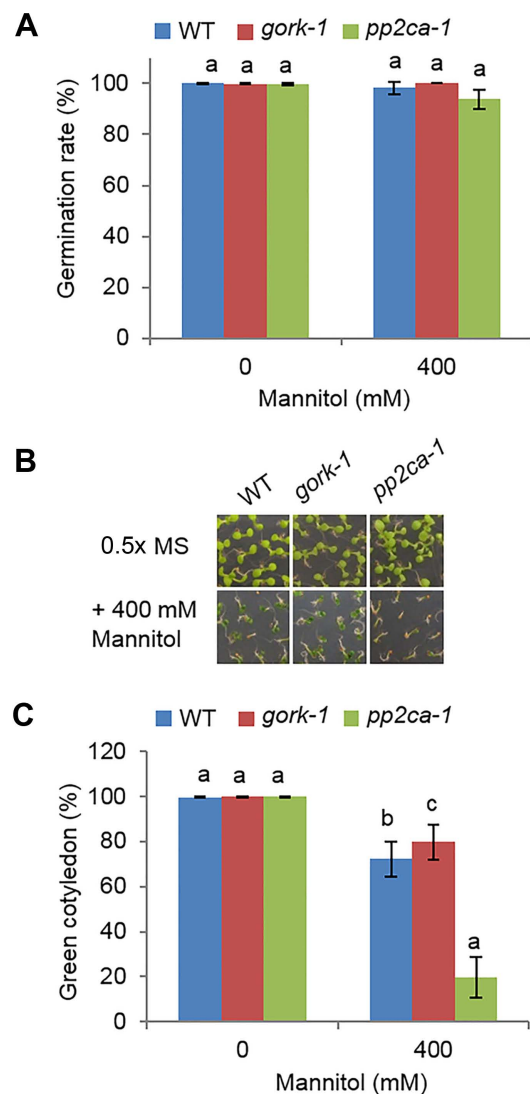


Fig. 5. Response of *gork* and *pp2ca* mutant plants to mannitol. (A) Germination rates of wild-type (WT), *gork*, and *pp2ca* mutant plants on 0.5× Murashige and Skoog media containing 400 mM of mannitol. Germination was determined by radicle emergence and was evaluated 9 days after plating. (B, C) Number of green cotyledons in WT, *gork*, and *pp2ca* mutant plants in response to mannitol. Representative photographs were taken at 10 days after plating (B), and the number of seedlings with expanded cotyledons was counted (C). Values are the mean ± SE from three independent experiments. Different letters indicate significant difference between WT and mutant plants (ANOVA; $P < 0.05$).

mutants in comparison to the number of green cotyledons found on wild-type plants (Fig. 5B and C). These results indicate that both GORK and PP2CA affect the plant responses to high salinity and osmotic stress. Together, these results are consistent with earlier studies and with the genetic analyses performed. In conclusion, the shaker type

K⁺ channel GORK is related to the ABA response as it interacts with clade A PP2C, affecting the plant's response to high salinity and osmotic stress. However, *gork* mutants do not appear to be related in regulating turgor pressure in guard cells in germination and seedling growth. Identification of additional regulators of GORK, such as other kinases or phosphatases, will help clarify the exact functions of GORK in the ABA signaling pathway in seeds and seedlings.

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