Cyclic nucleotide-gated channel 18 is an essential Ca^{2+} channel in pollen tube tips for pollen tube guidance to ovules in *Arabidopsis*

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In flowering plants, pollen tubes are guided into ovules by multiple attractants from female gametophytes to release paired sperm cells for double fertilization. It has been well-established that Ca^{2+} gradients in the pollen tube tips are essential for pollen tube guidance and that plasma membrane Ca^{2+} channels in pollen tube tips are core components that regulate Ca^{2+} gradients by mediating and regulating external Ca^{2+} influx. Therefore, Ca^{2+} channels are the core components for pollen tube guidance. However, there is still no genetic evidence for the identification of the putative Ca^{2+} channels essential for pollen tube guidance. Here, we report that the point mutations R491Q or R578K in cyclic nucleotide-gated channel 18 (CNGC18) resulted in abnormal Ca^{2+} gradients and strong pollen tube guidance defects by impairing the activation of CNGC18 in *Arabidopsis*. The pollen tube guidance defects of *cngc18-17* (R491Q) and of the transfer DNA (T-DNA) insertion mutant *cngc18-1* (+/−) were completely rescued by CNGC18. Furthermore, domain-swapping experiments showed that CNGC18's transmembrane domains are indispensable for pollen tube guidance. Additionally, we found that, among eight Ca^{2+} channels (including six CNGCs and two glutamate receptor-like channels), CNGC18 was the only one essential for pollen tube guidance. Thus, CNGC18 is the long-sought essential Ca^{2+} channel for pollen tube guidance in *Arabidopsis*.

CNGC18 | pollen tube guidance | Ca^{2+} gradient | Ca^{2+} channel | *Arabidopsis*

Pollen tubes deliver paired sperm cells into ovules for double fertilization, and signaling communication between pollen tubes and female reproductive tissues is required to ensure the delivery of sperm cells into the ovules (1). Pollen tube guidance is governed by both female sporophytic and gametophytic tissues (2, 3) and can be separated into two categories: preovular guidance and ovular guidance (1). For preovular guidance, diverse signaling molecules from female sporophytic tissues have been identified, including the transmitting tissue-specific (TTS) glycoprotein in tobacco (4), γ-amino butyric acid (GABA) in *Arabidopsis* (5), and chemocyanin and the lipid transfer protein SCA in *Lilium longiflorum* (6, 7). For ovular pollen tube guidance, female gametophytes secrete small peptides as attractants, including LUREs in *Torenia fournieri* (8) and *Arabidopsis* (9) and ZmEA1 in maize (10, 11). Synergid cells, central cells, egg cells, and egg apparatus are all involved in pollen tube guidance, probably by secreting different attractants (9–15). Additionally, nitric oxide (NO) and phytosulfokine peptides have also been implicated in both preovular and ovular pollen tube guidance (16–18). Thus, pollen tubes could be guided by diverse attractants in a single plant species.

Ca^{2+} gradients at pollen tube tips are essential for both tip growth and pollen tube guidance (19–27). Spatial modification of the Ca^{2+} gradients leads to the reorientation of pollen tube growth in vitro (28, 29). The Ca^{2+} gradients were significantly increased in pollen tubes attracted to the micropyles by synergid cells in vivo, compared with those not attracted by ovules (30). Therefore, the Ca^{2+} gradients in pollen tube tips are essential for pollen tube guidance. The Ca^{2+} gradients result from external Ca^{2+} influx, which is mainly mediated by plasma membrane Ca^{2+} channels in pollen tube tips. Thus, the Ca^{2+} channels are the key components for regulating the Ca^{2+} gradients and are consequently essential for pollen tube guidance. Using electrophysiological techniques, inward Ca^{2+} currents were observed in both pollen grain and pollen tube protoplasts (31–36), supporting the presence of plasma membrane Ca^{2+} channels in pollen tube tips. Recently, a number of candidate Ca^{2+} channels were identified in pollen tubes, including six cyclic nucleotide-gated channels (CNGCs) and two glutamate receptor-like channels (GLRs) in *Arabidopsis* (37–40). Three of these eight channels, namely CNGC18, GLR1.2, and GLR3.7, were characterized as Ca^{2+}-permeable channels (40, 41) whereas the ion selectivity of the other five CNGCs has not been characterized. We hypothesized that the Ca^{2+} channel essential for pollen tube guidance could be among these eight channels.

In this research, we first characterized the remaining five CNGCs as Ca^{2+} channels. We further found that CNGC18, out of the eight Ca^{2+} channels, was the only one essential for pollen tube guidance in *Arabidopsis* and that its transmembrane domains were indispensable for pollen tube guidance.

Results

CNGC7, -8, -9, -10, and -16 Are Ca^{2+} Channels. We characterized CNGC7, -8, -9, -10, and -16 using a patch-clamping technique by

Significance

In flowering plants, pollen tubes deliver paired sperm cells into ovules for double fertilization by perceiving and responding to multiple attractants secreted from the ovules. Ca^{2+} gradients in pollen tube tips are essential for pollen tube guidance, and plasma membrane Ca^{2+} channels in the pollen tube tips are the core components for the regulation of the Ca^{2+} gradients by mediating and regulating external Ca^{2+} influx. Therefore, the Ca^{2+} channels are essential for pollen tube guidance. However, the molecular identities of the Ca^{2+} channels remain to be addressed. In this research, we found that cyclic nucleotide-gated channel 18, out of eight Ca^{2+} channels present in pollen tubes, is the main one essential for pollen tube guidance in *Arabidopsis*.

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transiently expressing the *CNGCs* in HEK293T cells. Large inward currents were observed in HEK293T cells expressing each of the five CNGCs (Fig. S1). The CNGC-mediated inward currents were strongly decreased upon removal of external Ca^{2+} or after application of either Gd^{3+} (100 μM) or La^{3+} (100 μM) (Fig. S1). The reversal potentials shifted to a more negative potential direction upon removal of external 10 mM Ca^{2+} (Fig. S1 *B*, *D*, *F*, *H*, and *J*). We also observed large inward currents using a 10-mM Ba^{2+} bath solution, and the current amplitudes were similar to that with 10-mM Ca^{2+} bath conditions (Fig. S1). These data demonstrate that these five CNGCs are all Ca^{2+}-permeable channels. We also tested their permeability to monovalent cation K^+. Large inward K^+ currents were observed in HEK293T cells expressing the typical inward K^+ channel KAT1 (Fig. S2), as reported (41, 42), but only background conductance was observed in the mock control or in HEK293T cells expressing any of the five CNGCs (Fig. S2). Thus, the five CNGCs have no obvious permeability to K^+. CNGC18, GLR1.2, and GLR3.7 have been characterized as Ca^{2+} channels (40, 41). Thus, the eight channels present in pollen tubes are all Ca^{2+}-permeable channels.

The CNGC18 Point Mutations R491Q and R578K Lead to Male Sterility in *Arabidopsis*. To investigate the functions of the Ca^{2+} channels in pollen tube guidance, we collected homozygous transfer DNA (T-DNA) insertion mutants of all eight Ca^{2+} channels, except CNGC18 (Fig. S3 and Table S1). Homozygous knockout mutant *cngc18-1* is not available because of its complete male sterility (37). As an alternative, we screened a pool of 23 point mutants and identified two point mutants, *cngc18-17* (R491Q) and *cngc18-22* (R578K) (Fig. 1*A*), that had shorter siliques, fewer seeds per silique, and slightly lower in vitro pollen germination rates than in WT (Fig. 1 *B*–*F*). However, the pollen tubes of the two point mutants looked normal and were comparable with WT with respect to their shape and length (Fig. 1 *E* and *G*). We generated two complementation lines, complemented line 1 (COM1) and complemented line 2 (COM2), by expressing WT *CNGC18* driven by its native promoter in the *cngc18-17* mutant background (Fig. S4 and Table S1). The phenotypes of *cngc18-17* were rescued in COM1 and COM2 (Fig. 1 *B*–*F*).

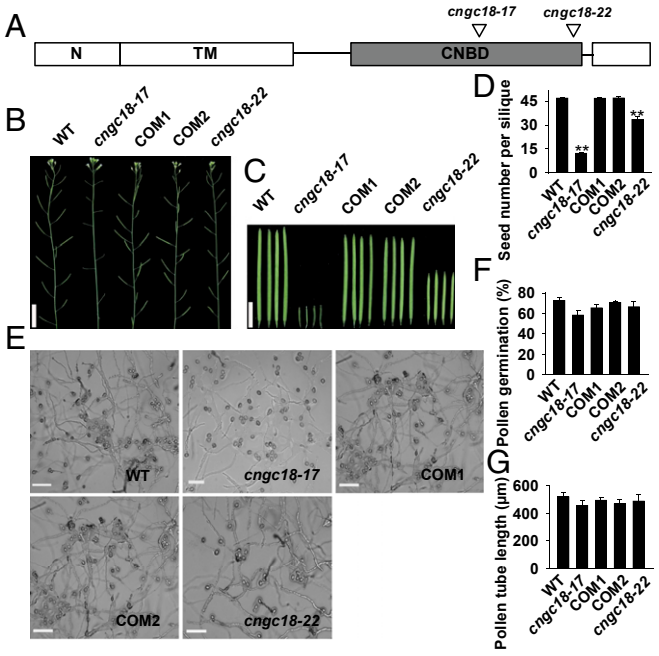

Fig. 1. *cngc18-17* and *cngc18-22* show severe male sterility. (*A*) Schematic model showing the *cngc18-17* (R491Q) and *cngc18-22* (R578K) point mutations. N, TM, and CNBD denote the N-terminal domain, transmembrane domains, and cyclic nucleotide binding domain, respectively. (*B*, *C*, and *E*) Images of plants (*B*), siliques (*C*), and in vitro-grown pollen tubes (*E*). (Scale bars: *B*, 2 cm; *C*, 0.5 cm; and *E*, 100 μm.) (*D*, *F*, and *G*) Average seed number per silique (*D*), pollen germination rate (*F*), and average pollen tube length (*G*). Error bars depict means ± SEM. **Significant differences from WT ($P < 0.01$).

Among the Eight Ca^{2+} Channels Present in Pollen Tubes, CNGC18 Is the only One Critical for Pollen Tube Guidance. We conducted limited pollination experiments using *male sterility 1* (*ms1*) plants (43) as the female parent and the *cngc* and *glr* mutants as male parents. We found that ∼96% of the pollen tubes of seven mutants (*cngc7*, *cngc8*, *cngc9*, *cngc10*, *cngc16*, *glr1.2*, and *glr3.7*) and WT successfully entered into *ms1* micropyles (hereafter termed on-target pollen tubes) (Fig. S5*A*) whereas less than 5% of pollen tubes failed to do so (hereafter termed off-target pollen tubes) (Fig. S5*C*). Interestingly, there were 41.9% and 24.1% off-target pollen tubes for *cngc18-17* and *cngc18-22*, respectively, significantly more than in WT and in the other seven mutants (Fig. S5*C*). Further observation revealed that the off-target pollen tubes fell into two groups. The first group was termed passing-by pollen tubes, which either wandered around a micropyle or passed very close to a micropyle (Fig. 2*A*). The second group was termed branched pollen tubes, which branched and stopped near micropyles (Fig. 2*A*). There were 27.5% and 24.1% passing-by pollen tubes for *cngc18-17* and *cngc18-22*, respectively, much more than the 2.8% in WT (Fig. 2*B*). There were 14.4% branched pollen tubes for *cngc18-17*, but no branched pollen tubes in *cngc18-22* or WT (0%) (Fig. 2*B*). All other *cngc* and *glr* mutants showed no branched pollen tubes, except for *cngc8* (a single branched pollen tube out of 155) (Fig. 2*B*). These data demonstrate that CNGC18 is the main Ca^{2+} channel essential for pollen tube guidance in *Arabidopsis*. CNGCs are downstream targets of cyclic nucleotides (cAMP and cGMP), and cAMP has been reported to function as a second messenger in pollen tube orientation by regulating Ca^{2+} channel activity in pollen tubes (34, 44, 45). Then, we focused on the six CNGCs, especially CNGC18, in further experiments.

We generated transgenic *Arabidopsis* lines by expressing enhanced green fluorescent protein (*eGFP*)-tagged versions of *CNGC7*, *CNGC8*, *CNGC9*, *CNGC10*, and *CNGC16* in *cngc18-17*, under the *CNGC18* promoter, to ensure that these genes were expressed at a level similar to *CNGC18*. We failed to observe an eGFP signal in the transgenic plants because of a weak *CNGC18* promoter (38). However, qRT-PCR (quantitative real-time PCR) data verified the expression of eGFP fused to the C terminus of the CNGCs (Fig. S4 and Table S1). We then performed further limited pollination experiments with these transgenic lines. The percentages of off-target pollen tubes (passing-by pollen tubes plus branched pollen tubes) were 36.2% (25.7% + 10.5%), 38.5% (25.5% + 13%), 39.1% (23.9% + 15.2%), 36.8% (24.3% + 12.5%), and 37.3% (23.3% + 14%) for *cngc18-17* mutants expressing *CNGC7*, *-8*, *-9*, *-10*, and *-16*, respectively (Fig. 2*B* and Fig. S5 *B* and *C*), similar to the *cngc18-17* mutant. However, the percentages of off-target pollen tubes were 16.9% for COM1 (12.7% passing-by pollen tubes and 4.2% branched pollen tubes) and 3.4% for COM2 (including only passing-by pollen tubes and no branched pollen tubes), in comparison with 2.8% for WT and 41.9% for the *cngc18-17* mutant (Fig. 2 *A* and *B* and Fig. S5*C*). These data demonstrate that the pollen tube guidance defects of *cngc18-17* were rescued by CNGC18, but not by the other five CNGCs.

Among the Six CNGCs Present in Pollen Tubes, only CNGC18 Is Able to Completely Rescue the Male Transmission Defect of *cngc18-1* (+/–). To further verify the role of CNGC18 in pollen tube guidance, we expressed *eGFP*-tagged versions of *CNGC7*, *-8*, *-9*, *-10*, *-16*, and *-18* and two point-mutated *CNGC18*, *CNGC18-R491Q* and *CNGC18-R578K*, in T-DNA insertion mutant *cngc18-1* (+/–) under

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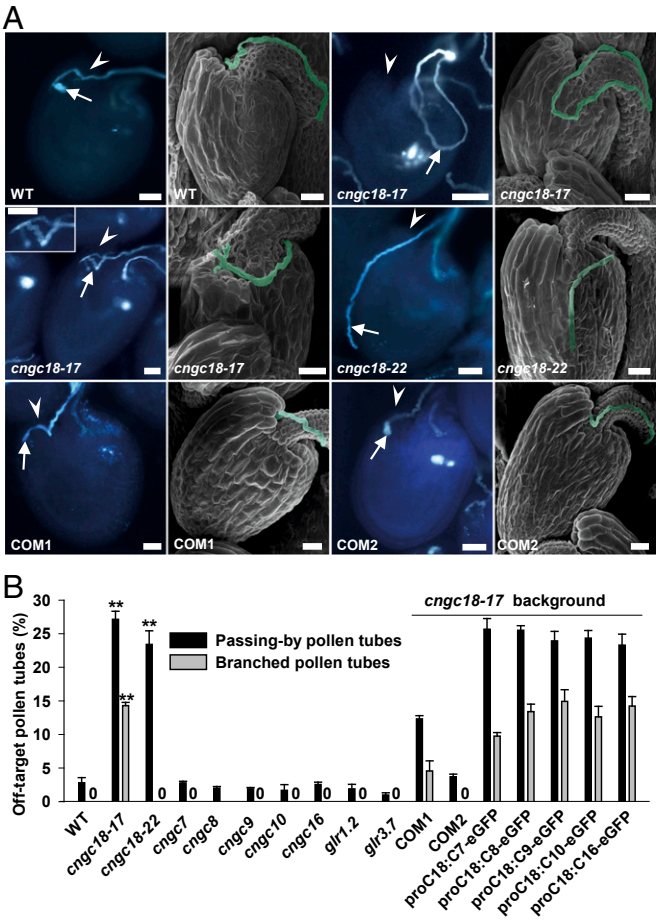

Fig. 2. The R491Q and R578K point mutations in CNGC18 resulted in severe pollen tube growth defects whereas the T-DNA insertion mutations in the other seven Ca^{2+} channels had no pollen tube growth defects. (*A*) In vivo aniline blue staining and scanning electron microscopy images, showing typical on-target pollen tubes of WT, COM1, and COM2, examples of passing-by pollen tubes of *cngc18-17* and *cngc18-22*, and an example of branched pollen tubes of *cngc18-17*. Pollen tubes and micropyles are indicated by white arrows and arrowheads, respectively. (Scale bars: 20 μm.) (*B*) Percentages of passing-by and branched pollen tubes. A "0" indicates that no branched pollen tubes were observed. **Significant difference from WT ($P < 0.01$). Error bars depict means ± SEM.

the *CNGC18* promoter (Fig. S4 and Table S1) and then analyzed whether the male transmission defect of heterozygous *cngc18-1* (+/−) could be rescued by any of the CNGCs. The male transmission defect of *cngc18-1* (+/−) (0%) was completely rescued by CNGC18-eGFP (53%); strongly rescued by CNGC18-R578K-eGFP (22.0%); poorly rescued by CNGC18-R491Q-eGFP (0.83%), CNGC7-eGFP (3.58%), CNGC10-eGFP (0.86%), and CNGC16-eGFP (0.63%); and not rescued by CNGC8-eGFP (0%) and CNGC9-eGFP (0%) (Table 1). The poor rescue of the male transmission defect of *cngc18-1* (+/−) by CNGC18-R491Q-eGFP is consistent with the strong male transmission and pollen tube guidance defects of *cngc18-17* whereas the strong rescue of the male transmission defect of *cngc18-1* (+/−) by CNGC18-R578K-eGFP is consistent with the much weaker male transmission and pollen tube guidance defects of the *cngc18-22* mutant (Figs. 1 and 2 and Table 1). Similarly, the rescue of the transmission defect of *cngc18-1* (+/−) by the other five CNGCs is in agreement with the normal pollen tube guidance in their T-DNA insertion knockout mutants (Fig. 2 and Fig. S5). This genetic evidence clearly demonstrates that CNGC18 is an essential Ca^{2+} channel for pollen tube guidance in *Arabidopsis* whereas the other five CNGCs and two GLRs play no role or negligible roles in this process.

The Transmembrane Domains of CNGC18 Are Essential for Pollen Tube Guidance. CNGC16 is a close homolog of CNGC18, but with no role or a negligible role in pollen tube guidance (Fig. 2, Fig. S5, and Table 1). To investigate which domain of CNGC18 is essential for pollen tube guidance, we exchanged domains between CNGC18 and CNGC16 (Fig. S6). We tested the Ca^{2+} channel activity of the chimeric channels using a patch-clamping technique after expressing the chimeric channels in HEK293T cells. We observed similar Ca^{2+} channel currents and cGMP-induced channel currents in HEK293T cells expressing the chimeric channels (Fig. S7) as for WT CNGC16 (Fig. S1 *I* and *J*) or WT CNGC18, as previously reported (41). These data demonstrate that the chimeric channels have normal Ca^{2+} channel activity and can be activated normally by cyclic nucleotides. We then generated transgenic *Arabidopsis* lines by expressing the chimeric channels driven by the *CNGC18* promoter in the *cngc18-1* (+/−) background. The male transmission defect of *cngc18-1* (+/−) was poorly rescued by CNGC16-18N (0.781%), CNGC16-18C (1.46%), and CNGC18-16TM (0.862%), but was rescued by CNGC16-18TM nearly completely (44.8%), as for WT CNGC18 (53%) (Table 1). We carried out limited pollination experiments and found that the homozygous transgenic plants *CNGC16-18TM/cngc18-1* (−/−) showed no obvious defect in pollen tube guidance compared with the control *qrt* plants (Fig. S8). These data demonstrate that the transmembrane domains of CNGC18 are indispensable domains for its essential role in pollen tube guidance in *Arabidopsis*.

The Point Mutations R491Q and R578K Impaired the Activation of CNGC18 by Cyclic Nucleotides Both in Vitro and in Vivo. To investigate how the two point mutations affected the channel activity of CNGC18, we carried out patch-clamping experiments in HEK293T cells via transient expression. We observed obvious inward Ca^{2+} channel currents in HEK293T cells expressing either *CNGC18-R491Q*, *CNGC18-R578K*, or WT *CNGC18* (Fig. 3 *A* and *B*), showing no significant impairment of the channel activity by the two point mutations in CNGC18. Interestingly, we found that both CNGC18-R491Q and CNGC18-R578K failed to be further activated by the application of 100 μM 8Br-cGMP whereas WT CNGC18 was significantly activated (Fig. 3 *A* and *B*), as previously reported (41). To test whether the point mutations R491Q and R578K impaired the activation of CNGC18 in vivo, we isolated pollen tube protoplasts (46) and measured the whole-cell inward Ca^{2+} currents using a patch-clamping technique (32). In comparison with background currents in a cGMP-free condition, large currents were triggered by the intracellular application of 100 μM cGMP in WT (Fig. 3 *C* and *D*). These cGMP-activated currents were abolished by removal of extracellular Ca^{2+} or by application of the Ca^{2+} channel blockers La^{3+} (100 μM) or Gd^{3+} (100 μM) (Fig. 3 *C* and *D*). The reversal potential shifted from +21.5 mV to +2.9 mV upon removing extracellular Ca^{2+} (Fig. 3*D*). These results demonstrate that the cGMP-activated inward currents in *Arabidopsis* pollen tube protoplasts are Ca^{2+} channel currents. Further patch-clamping results showed that the cGMP-activated Ca^{2+} currents were impaired in *cngc18-17* and *cngc18-22* relative to WT (Fig. 3 *C* and *D*). Together, the patch-clamping data in both HEK293T cells and pollen tube protoplasts demonstrate that the two point mutations R491Q and R578K impaired the cGMP activation of the Ca^{2+} channel CNGC18.

***cngc18-17* and *cngc18-22* Mutants Showed Abnormal Cytosolic Ca^{2+} Oscillation in Pollen Tube Tips.** We tested whether the guidance defect of pollen tubes in *cngc18-17* and *cngc18-22* resulted from an abnormal Ca^{2+} gradient at the pollen tube tips. We monitored the dynamic fluorescence of the Ca^{2+} sensor yellow cameleon version 3.6 (YC3.6) and observed regular and normal $[Ca^{2+}]_{cyt}$ (cytosolic Ca^{2+} concentration) oscillation at different frequencies around a relatively stable $[Ca^{2+}]_{cyt}$ level in growing pollen

Table 1. Transmission analysis of WT, mutants, and transgenic *Arabidopsis* lines

Parent, female × male	Segregation of resistance genes (resistant/sensitive)		
	Expected	Observed	Transmission, %
ms1 × *cngc18-1* (+/–)	298: 298	0:596	0**
ms1 × *proC18:CNGC7-eGFP/cngc18-1* (+/–)	223: 223	16:431	3.58**
ms1 × *proC18:CNGC8-eGFP/cngc18-1* (+/–)	711: 711	0:1,423	0**
ms1 × *proC18:CNGC9-eGFP/cngc18-1* (+/–)	183: 183	0:366	0**
ms1 × *proC18:CNGC10-eGFP/cngc18-1* (+/–)	347: 347	6:688	0.86**
ms1 × *proC18:CNGC16-eGFP/cngc18-1* (+/–)	79: 79	1:157	0.63**
ms1 × *proC18:CNGC18-eGFP/cngc18-1* (+/–)	231: 231	245: 217	53.0
ms1 × *proC18:CNGC18-R491Q-eGFP/cngc18-1* (+/–)	60: 60	1:120	0.83**
ms1 × *proC18:CNGC18-R578K-eGFP/cngc18-1* (+/–)	250:250	110:390	22.0**
ms1 × *proC18:eGFP/cngc18-1* (+/–)	62:62	0:124	0 **
ms1 × *proC18:CNGC16-18NT/cngc18-1* (+/–)	64:64	1:127	0.781**
ms1 × *proC18:CNGC16-18TM/cngc18-1* (+/–)	77:77	69:85	44.8
ms1 × *proC18:CNGC16-18CT/cngc18-1* (+/–)	68:68	2:135	1.46**
ms1 × *proC18:CNGC18-16TM/cngc18-1* (+/–)	58:58	1:115	0.862**

proC18, the promoter of *CNGC18*. **Significant difference from the expected Mendelian segregation ratio (1:1, e.g., 50%) (χ^2 test, χ^2, $P < 0.01$).

tube tips of WT (Fig. 4*A*), similar to previous reports (25, 47). However, most of the growing pollen tube tips of *cngc18-17* (Fig. 4*A*) and *cngc18-22* (Fig. 4*A*) manifested abnormal Ca^{2+} oscillation patterns, especially irregular drifts of basal $[Ca^{2+}]_{cyt}$ levels, whereas, in WT, there were relatively stable $[Ca^{2+}]_{cyt}$ levels (Fig. 4*A*). There were 80.6% and 64.1% pollen tubes showing abnormal drifts of basal $[Ca^{2+}]_{cyt}$ for *cngc18-17* and *cngc18-22*, respectively, compared with 8% for WT (Fig. 4*B*). It has been reported that the $[Ca^{2+}]_{cyt}$ gradients, rather than high frequent $[Ca^{2+}]_{cyt}$ oscillation, are required for pollen tube growth and guidance (24, 30). Therefore, the irregular drift of the basal $[Ca^{2+}]_{cyt}$ levels (i.e., the disturbance of the Ca^{2+} gradients in pollen tube tips) might be the main cause of pollen tube guidance defects in *cngc18-17* and *cngc18-22*.

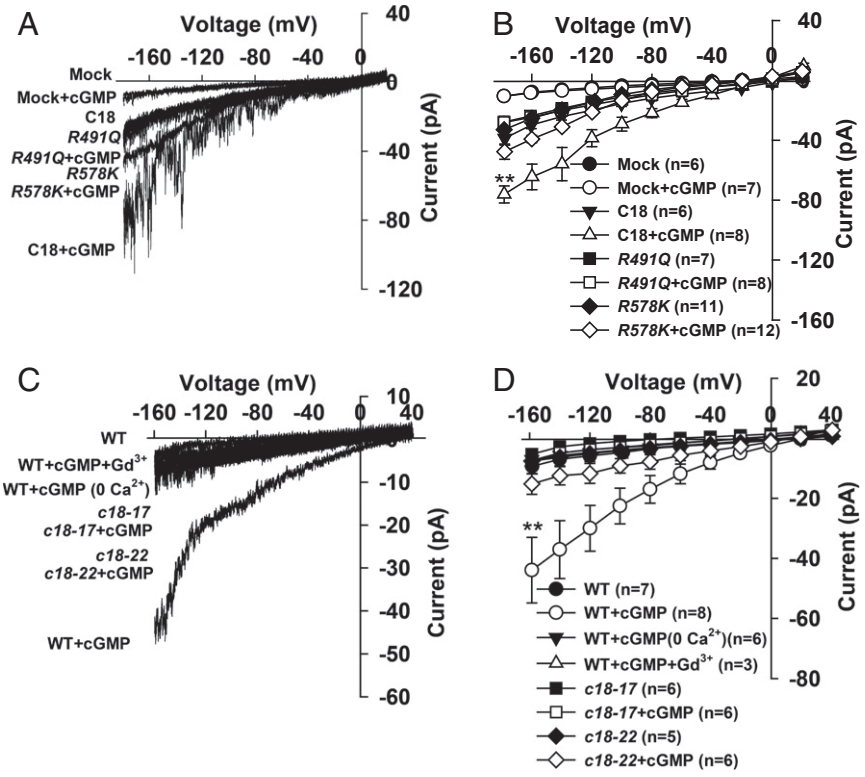

Fig. 3. The CNGC18 point mutations R491Q and R578K impair activation of CNGC18 by cGMP. (*A* and *B*) Typical whole-cell recordings (*A*) and the average current-voltage curves (*B*) of whole-cell currents recorded in HEK293T cells. (*C* and *D*) Typical whole-cell recordings (*C*) and the average current-voltage curves (*D*) of whole-cell currents recorded in *Arabidopsis* pollen tube protoplasts. Extracellular 8Br-cGMP and intracellular cGMP treatments were used to activate channel currents in HEK293T cells and pollen tube protoplasts, respectively. C18, R491Q, R578K, *c18-17*, and *c18-22* denote WT CNGC18, CNGC18-R491Q, CNGC18-R578K, *cngc18-17*, and *cngc18-22*, respectively. Error bars depict means ± SEM. **Significant difference from WT ($P < 0.01$).

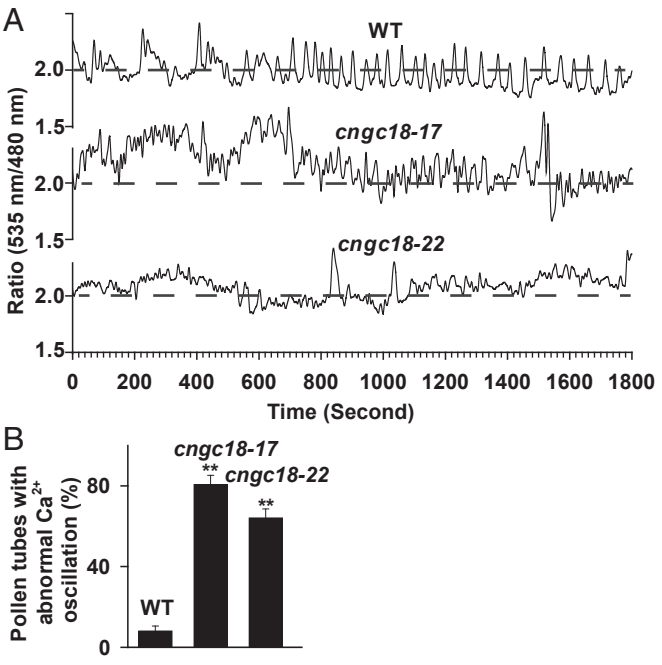

Fig. 4. CNGC18 point mutations R491Q and R578K lead to irregular drifts of basal $[Ca^{2+}]_{cyt}$ levels in *Arabidopsis* pollen tube tips. (*A*) Time course of typical $[Ca^{2+}]_{cyt}$ oscillation in the pollen tube tips of WT, *cngc18-17*, and *cngc18-22*. (*B*) Fractions of pollen tubes showing irregular drifts of basal $[Ca^{2+}]_{cyt}$ levels. Thirty-five pollen tubes were tested for WT, 33 for *cngc18-17*, and 39 for *cngc18-22*. Dashed lines indicate the expected relatively stable basal $[Ca^{2+}]_{cyt}$ levels. Error bars depict means ± SEM. **Significant difference from WT ($P < 0.01$).

Discussion

A number of candidate channel families for Ca^{2+} channels were identified in *Arabidopsis*, including ligand-gated channels, such as CNGC and GLR, and stretch-activated Ca^{2+} channels OSCA (reduced hyperosmolality-induced $[Ca^{2+}]_i$ increase 1) and MCA (MID1-complementing activity 1) families (20, 48, 49). Although eight are present in pollen tubes, only CNGC18 is essential for pollen tube guidance. Therefore, CNGC18 could be the main Ca^{2+} channel for pollen tube guidance in *Arabidopsis*.

It is striking that CNGC18 is essential for pollen tube guidance whereas the other five CNGCs tested apparently have a negligible role or no such role. CNGC18 is mainly localized in the tips of pollen tubes (37) whereas CNGC7 and CNGC8 are mainly localized at the pollen tube flanks (38). A T-DNA insertion in CNGC16 showed no phenotype in normal plant growth conditions (39), and CNGC9 and CNGC10 had no function in pollen tube guidance (Fig. 2 and Fig. S5). Thus, it is plausible that the subcellular localization of CNGC18 might explain its essential function in pollen tube growth and guidance.

CNGC18 may play dual roles in pollen tube tips. First, CNGC18 mediates external Ca^{2+} influx to establish and maintain the Ca^{2+} gradient, which allows the formation and tip growth of pollen tubes. This role of CNGC18 may account for the lethal male sterile phenotype of T-DNA insertion mutant *cngc18-1* (37) because the absence of the Ca^{2+} channel activity and Ca^{2+} gradient is lethal for pollen tubes. Second, CNGC18 is critical for pollen tube guidance. Both R491Q and R578K did not abolish the Ca^{2+} channel activity of CNGC18 but impaired its further activation by cyclic nucleotides (Fig. 3). The Ca^{2+} channel activity of the two point-mutated CNGC18 allows external Ca^{2+} influx to establish the Ca^{2+} gradient and further allows the formation and tip growth of pollen tubes. But the two point mutations in CNGC18 could reduce the sensitivity of CNGC18 to upstream regulators, by impairing its further activation, and consequently lead to abnormal Ca^{2+} gradients and pollen tube guidance defects. The upstream regulators of CNGC18 could be cyclic nucleotides and/or protein kinases (50).

The transmembrane domains of CNGC18 are indispensable for pollen tube guidance (Table 1 and Fig. S8). However, the importance of CNGC18's transmembrane domains does not exclude the importance of its N terminus and especially the C terminus. The free C terminus of CNGC18 might be the main regulatory domain for its Ca^{2+} channel activity because both the cyclic nucleotide binding domain (CNBD) and calmodulin-binding domain are in the C terminus. Furthermore, both the point mutations R491Q and R578K are in the CNBD domain (Fig. 1). These point mutations did not abolish the Ca^{2+} channel activity of CNGC18 (Fig. 3) but impaired its further activation by cyclic nucleotides (Fig. 3) and led to sterility and a pollen tube guidance defect (Figs. 1 and 2 and Fig. S5), supporting the importance of the C terminus for pollen tube guidance. The rescue of the male transmission and pollen tube guidance defects of *cngc18-1* (+/−) by CNGC16-18TM (Table 1 and Fig. S8) might be explained by two possible scenarios. First, the transmembrane domains of CNGC18 might have caused a localization of the chimeric channel at pollen tube tips. Secondly, the C terminus of CNGC16 is similar to that of CNGC18 and thus might have allowed the chimeric channel to be regulated by upstream regulators of CNGC18.

We cannot exclude the involvement of GLRs in pollen tube guidance, but these two GLRs could play only minor roles, if any, considering the normal pollen tube guidance in *glr1.2* and *glr3.7* mutants (Fig. 2 and Fig. S5). GLR and CNGC are quite different Ca^{2+} channels, including their amino acid residue sequences and activating ligands (40, 41). Therefore, it is reasonable to assume that GLRs respond to different upstream attractants than CNGC18.

Taken together, CNGC18 might be the main Ca^{2+} channel for pollen tube guidance and tip growth in *Arabidopsis*.

Materials and Methods

In Vitro Pollen Germination and Tube Growth Assay. The medium for in vitro pollen germination and tube culturing was modified from a previous report (51) and contained 18% (wt/vol) sucrose, 1.6 mM boric acid, 10 mM $CaCl_2$, 1 mM $Ca(NO_3)_2$, 1 mM $MgSO_4$, and 0.5% (wt/vol) agarose, adjusted to pH 6.4 with KOH. In vitro pollen germination and tube culturing experiments were conducted at 22 °C under a 100% saturated relative humidity for 6 h (52) and then photographed using an inverted microscope (model Axio observer D1; Carl Zeiss) equipped with a neo CMOS CCD camera (Andor Technology). In each replicate, about 300 pollen grains were counted for pollen germination analysis, and about 100 pollen tubes were used for pollen tube length analysis. Pollen tube length was measured using Image J (imagej.nih.gov/ij/, 1997–2012). Three replicates were conducted.

In Vivo Pollen Tube Growth Observations. Pollen tube growth was analyzed using limited pollination methods. About 10 pollen grains were manually placed on each *ms1* pistil. After 16 h to allow pollen grains to germinate and pollen tubes to grow, the pistils were cut off and fixed in 3:1 ethanol:acetic acid for 24 h as described (53).

For decolorized aniline blue staining, the fixed pistils were rehydrated in a series of ethanol solutions (70%, 50%, 30%) and then with distilled water, softened in 8 M NaOH for 12 h, and washed five times with distilled water. The pistils were stained overnight in 0.1% decolorized aniline blue (pH 11 in 50 mM K_3PO_4), and pictures were taken under a fluorescence microscope (Model DM6000B; Leica). Three replicates were performed, and no fewer than 40 pollen tubes were analyzed for each assay in each replicate.

For scanning electron microscopy observations, the pistils were fixed in FAA solution (50% ethanol, 5% acetic acid, 3.7% formaldehyde) for 24 h and dehydrated with a series of ethanol solutions (30%, 50%, 70%, 85%, 95%, and 100%) (54). The pistils were subsequently CO_2 critical point-dried, coated with sputtering gold, and then observed using a scanning electron microscope (JSM-6360LV; JEOL).

Whole Cell Patch-Clamping Experiments. CNGC-mediated Ca^{2+} and K^+ current recordings in HEK293T cells were performed as described (41). For whole-cell Ca^{2+} current recordings in *Arabidopsis* pollen tube protoplasts, protoplast isolation was carried out as described (46). cGMP was freshly added each day. A ramp voltage protocol of 2 s duration from −180 mV to +20 mV was applied 1 min after accessing to whole-cell configuration (32). Whole-cell Ca^{2+} currents were recorded every 30 s for 10 min. See *SI Materials and*

Methods for bath and pipette solutions for patch-clamping experiments in HEK293T cells and *Arabidopsis* pollen tube protoplasts.

Cytosolic Ca^{2+} Imaging in Pollen Tube Tips. Ca^{2+} imaging in pollen tube tips was performed by monitoring the ratio (535 nm/480 nm) of YC3.6 using an inverted microscope (Model IX71; Olympus). The interval of image acquisition was 5 s, and the exposure time for excitation light was 200 ms for both 535 nm and 480 nm. Software MAG Biosystems 7.5 (MetaMorph) was used for data acquisition and analysis.

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