Identification of Cyclic GMP-Activated Nonselective Ca^{2+}-Permeable Cation Channels and Associated *CNGC5* and *CNGC6* Genes in Arabidopsis Guard Cells[1][W][OPEN]

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Cytosolic Ca^{2+} in guard cells plays an important role in stomatal movement responses to environmental stimuli. These cytosolic Ca^{2+} increases result from Ca^{2+} influx through Ca^{2+}-permeable channels in the plasma membrane and Ca^{2+} release from intracellular organelles in guard cells. However, the genes encoding defined plasma membrane Ca^{2+}-permeable channel activity remain unknown in guard cells and, with some exceptions, largely unknown in higher plant cells. Here, we report the identification of two Arabidopsis (*Arabidopsis thaliana*) cation channel genes, *CNGC5* and *CNGC6*, that are highly expressed in guard cells. Cytosolic application of cyclic GMP (cGMP) and extracellularly applied membrane-permeable 8-Bromoguanosine 3′,5′-cyclic monophosphate-cGMP both activated hyperpolarization-induced inward-conducting currents in wild-type guard cells using Mg^{2+} as the main charge carrier. The cGMP-activated currents were strongly blocked by lanthanum and gadolinium and also conducted Ba^{2+}, Ca^{2+}, and Na^{+} ions. *cngc5 cngc6* double mutant guard cells exhibited dramatically impaired cGMP-activated currents. In contrast, mutations in *CNGC1*, *CNGC2*, and *CNGC20* did not disrupt these cGMP-activated currents. The yellow fluorescent protein-CNGC5 and yellow fluorescent protein-CNGC6 proteins localize in the cell periphery. Cyclic AMP activated modest inward currents in both wild-type and *cngc5cngc6* mutant guard cells. Moreover, *cngc5 cngc6* double mutant guard cells exhibited functional abscisic acid (ABA)-activated hyperpolarization-dependent Ca^{2+}-permeable cation channel currents, intact ABA-induced stomatal closing responses, and whole-plant stomatal conductance responses to darkness and changes in CO_2 concentration. Furthermore, cGMP-activated currents remained intact in the *growth controlled by abscisic acid2* and *abscisic acid insensitive1* mutants. This research demonstrates that the *CNGC5* and *CNGC6* genes encode unique cGMP-activated nonselective Ca^{2+}-permeable cation channels in the plasma membrane of Arabidopsis guard cells.

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cGMP-activation of channels was found by I.M. and impairment in *cngc5*, *cngc6* and *cngc5 cngc6* was found by Y.-F.W. at UCSD. S.M., N.N., N.R., M.H., and S.L. contributed important data. Most experiments were conducted at UCSD with exception of patch-clamp experiments in Figures 2E and 8 (S.M.), qPCR (Fig. 3C and Supplemental Fig. S3C; H.-M.R., Y.-F.W.) and stomatal conductance analyses (I.P., H.K.). The project was proposed by J.I.S. Y.-F.W., S.M., N.N., and J.I.S. wrote the manuscript.

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Plants lose water via transpiration and take in CO_2 for photosynthesis through stomatal pores. Each stomatal pore is surrounded by two guard cells, and stomatal movements are driven by the change of turgor pressure in guard cells. The intracellular second messenger Ca^{2+} functions in guard cell signal transduction (Schroeder and Hagiwara, 1989; McAinsh et al., 1990; Webb et al., 1996; Grabov and Blatt, 1998; Allen et al., 1999; MacRobbie, 2000; Mori et al., 2006; Young et al., 2006; Siegel et al., 2009; Chen et al., 2010; Hubbard et al., 2012). Plasma membrane ion channel activity and gene expression in guard cells are finely regulated by the intracellular free calcium concentration ($[Ca^{2+}]_{cyt}$; Schroeder and Hagiwara, 1989; Webb et al., 2001; Allen et al., 2002; Siegel et al., 2009; Kim et al., 2010; Stange et al., 2010). Ca^{2+}-dependent protein kinases (CPKs) function as targets of the cytosolic Ca^{2+} signal, and several members of the CPK family have been shown to function in stimulus-induced stomatal closing, including the Arabidopsis (*Arabidopsis thaliana*) CPK3, CPK4, CPK6, CPK10, and CPK11 proteins (Mori et al., 2006; Zhu et al., 2007; Zou et al., 2010; Brandt et al., 2012; Hubbard et al., 2012). Further research found that several CPKs could activate the S-type anion channel SLAC1 in *Xenopus laevis* oocytes, including CPK21, CPK23, and CPK6 (Geiger et al., 2010; Brandt et al., 2012). At the same time, the Ca^{2+}-independent protein kinase Open Stomata1

mediates stomatal closing and activates the S-type anion channel SLAC1 (Mustilli et al., 2002; Yoshida et al., 2002; Geiger et al., 2009; Lee et al., 2009; Xue et al., 2011), indicating that both Ca^{2+}-dependent and Ca^{2+}-independent pathways function in guard cells.

Multiple essential factors of guard cell abscisic acid (ABA) signal transduction function in the regulation of Ca^{2+}-permeable channels and $[Ca^{2+}]_{cyt}$ elevations, including *Abscisic Acid Insensitive1* (ABI1), ABI2, Enhanced Response to Abscisic Acid1 (ERA1), the NADPH oxidases AtrbohD and AtrbohF, the Guard Cell Hydrogen Peroxide-Resistant1 (GHR1) receptor kinase, as well as the Ca^{2+}-activated CPK6 protein kinase (Pei et al., 1998; Allen et al., 1999, 2002; Kwak et al., 2003; Miao et al., 2006; Mori et al., 2006; Hua et al., 2012). $[Ca^{2+}]_{cyt}$ increases result from both Ca^{2+} release from intracellular Ca^{2+} stores (McAinsh et al., 1992) and Ca^{2+} influx across the plasma membrane (Hamilton et al., 2000; Pei et al., 2000; Murata et al., 2001; Kwak et al., 2003; Hua et al., 2012). Electrophysiological analyses have characterized nonselective Ca^{2+}-permeable channel activity in the plasma membrane of guard cells (Schroeder and Hagiwara, 1990; Hamilton et al., 2000; Pei et al., 2000; Murata et al., 2001; Köhler and Blatt, 2002; Miao et al., 2006; Mori et al., 2006; Suh et al., 2007; Vahisalu et al., 2008; Hua et al., 2012). However, the genetic identities of Ca^{2+}-permeable channels in the plasma membrane of guard cells have remained unknown despite over two decades of research on these channel activities.

The Arabidopsis genome includes 20 genes encoding cyclic nucleotide-gated channel (CNGC) homologs and 20 genes encoding homologs to animal Glu receptor channels (Lacombe et al., 2001; Kaplan et al., 2007; Ward et al., 2009), which have been proposed to function in plant cells as cation channels (Schuurink et al., 1998; Arazi et al., 1999; Köhler et al., 1999). Recent research has demonstrated functions of specific Glu receptor channels in mediating Ca^{2+} channel activity (Michard et al., 2011; Vincill et al., 2012). Previous studies have shown cAMP activation of nonselective cation currents in guard cells (Lemtiri-Chlieh and Berkowitz, 2004; Ali et al., 2007). However, only a few studies have shown the disappearance of a defined plasma membrane Ca^{2+} channel activity in plants upon mutation of candidate Ca^{2+} channel genes (Ali et al., 2007; Michard et al., 2011; Laohavisit et al., 2012; Vincill et al., 2012). Some CNGCs have been found to be involved in cation nutrient intake, including monovalent cation intake (Guo et al., 2010; Caballero et al., 2012), salt tolerance (Guo et al., 2008; Kugler et al., 2009), programmed cell death and pathogen responses (Clough et al., 2000; Balagué et al., 2003; Urquhart et al., 2007; Abdel-Hamid et al., 2013), thermal sensing (Finka et al., 2012; Gao et al., 2012), and pollen tube growth (Chang et al., 2007; Frietsch et al., 2007; Tunc-Ozdemir et al., 2013a, 2013b). Direct in vivo disappearance of Ca^{2+} channel activity in *cngc* disruption mutants has been demonstrated in only a few cases thus far (Ali et al., 2007; Gao et al., 2012). In this research, we show that CNGC5 and CNGC6 are required for a cyclic GMP (cGMP)-activated nonselective Ca^{2+}-permeable cation channel activity in the plasma membrane of Arabidopsis guard cells.

RESULTS

8Br-cGMP Activates Inward Nonselective Cation Currents in Arabidopsis Guard Cell Protoplasts

Cyclic nucleotide-activated ion channels function as a type of cyclic nucleotide-gated Ca^{2+}-permeable channel in mammalian cells, and some Arabidopsis CNGCs have been reported to encode functional cyclic nucleotide-gated Ca^{2+}-permeable channels (Leng et al., 2002; Ali et al., 2007; Gao et al., 2012). We performed experiments to test whether cyclic nucleotides could trigger currents in Arabidopsis guard cells. We found that inward currents were activated in Columbia wild-type guard cells upon extracellular application of 500 μM membrane-permeable 8-Bromoguanosine 3′,5′-cyclic monophosphate (8Br)-cGMP (Fig. 1). We used Mg^{2+} as the main divalent cation in the bath solution because it has been established that Ca^{2+}-permeable channels in plants cells are often permeable to multiple divalent cations, including Ca^{2+}, Ba^{2+}, and Mg^{2+}, and Mg^{2+} would be unlikely to interfere with Ca^{2+}-dependent responses (Thuleau et al., 1994; Pei et al., 2000; Véry and Davies, 2000; Wang et al., 2004; Finka et al., 2012).

Since Mg^{2+} and Cl^- were the main ions in the bath and pipette solutions under the imposed conditions, it was possible that the 8Br-cGMP-activated inward currents result from either the influx of Mg^{2+} ions or the efflux of

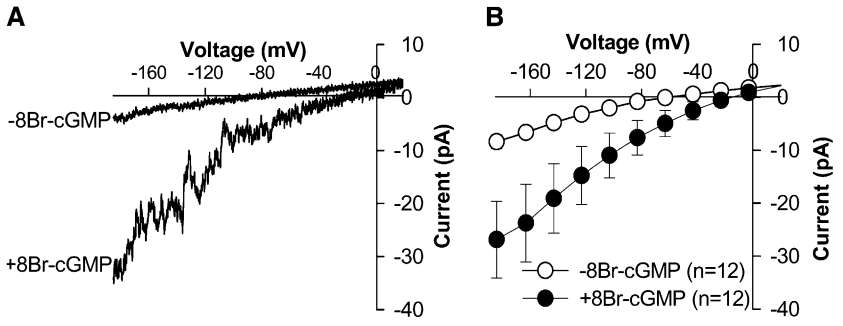

Figure 1. Whole-cell currents recorded in Columbia wild-type guard cell protoplasts of Arabidopsis. A, Typical whole-cell recording in a Columbia wild-type guard cell protoplast before (−) and after (+) application of the membrane-permeable cGMP analog 8Br-cGMP (500 μM) to the bath solution. B, Average current-voltage curves of steady-state whole-cell currents recorded in Columbia wild-type guard cell protoplasts before (−) and after (+) application of 500 μM 8Br-cGMP (n = 12). Values depict means ± SE.

Cl^- across the plasma membrane of Arabidopsis guard cells. Therefore, we analyzed the reversal potential of the 8Br-cGMP-activated currents. Due to the voltage dependence of the 8Br-cGMP-activated currents, the reversal potential could be approximately extrapolated as the voltage where the average 8Br-cGMP-activated currents merged with the average control currents in wild-type Arabidopsis guard cells, which was approximately +21 mV (Fig. 1B) after correction of the liquid junction potential of −4 mV measured as described previously (Ward and Schroeder, 1994). Under the imposed conditions, equilibrium potentials of Mg^{2+} and Cl^- were +23 and −81 mV after correction of ionic activities, respectively. The reversal potential of the 8Br-cGMP-activated currents at +21 mV was close to the equilibrium potential of Mg^{2+} (+23 mV) but far from that of Cl^- (−81 mV). These data show a Mg^{2+} permeability of the 8Br-cGMP-activated currents. We tested the effects of La^{3+} and Gd^{3+}, two well-known plant Ca^{2+} channel blockers (Allen and Sanders, 1994; Grabov and Blatt, 1999; Lemtiri-Chlieh and Berkowitz, 2004; Wang et al., 2004; Gao et al., 2012), on the 8Br-cGMP-activated inward currents in guard cells. We found that the 8Br-cGMP-activated inward currents were strongly inhibited by 100 μM La^{3+} (n = 3) or 100 μM Gd^{3+} (n = 3; Fig. 2, A and B). We further tested the selectivity of 8Br-cGMP-induced currents to cations by replacing Mg^{2+} with the large cation, *N*-methyl-D-glucamine (NMDG), in the bath solution and found that the 8Br-cGMP-activated inward currents were abolished (Fig. 2C). In contrast, similar current amplitudes were observed after $MgCl_2$ was replaced by $BaCl_2$ at the same concentration in the bath solution (Fig. 2D), showing that the 8Br-cGMP-activated inward currents recorded in guard cells were carried mainly by these divalent cations. We also analyzed the 8Br-cGMP-activated currents using simple $BaCl_2$ solutions, as described previously (Pei et al., 2000), and found the 8Br-cGMP-activated Ba^{2+} currents (Fig. 2E). The reversal potential of the recorded 8Br-cGMP-activated currents was +19 mV (Fig. 2E), close to the equilibrium potential of Ba^{2+} (+20 mV) but distant from the equilibrium potential of Cl^- (−53 mV). 8Br-cGMP-activated Ba^{2+} currents were also recorded using step-wise voltage pulses, indicating that the currents are hyperpolarization activated, but the activation is not time dependent (Supplemental Fig. S1). The "spiky" nature of these 8Br-cGMP-activated currents is similar to Ca^{2+}-permeable cation channel currents recorded previously in guard cells (Schroeder and Hagiwara, 1990; Hamilton et al., 2000; Pei et al., 2000). These inward currents were observed after $BaCl_2$ was replaced by $CaCl_2$ in the bath solution with $BaCl_2$ in the pipette solution (n = 9) and the reversal potential was +17 mV, far from the equilibrium potential of Cl^- (−53 mV; Fig. 2E). The permeability ratio for Ba^{2+} relative to Ca^{2+} was 1.46, as determined according to the Goldman-Hodgkin-Katz equation (Hille, 1992). These results show that 8Br-cGMP activates nonselective Ca^{2+}-permeable

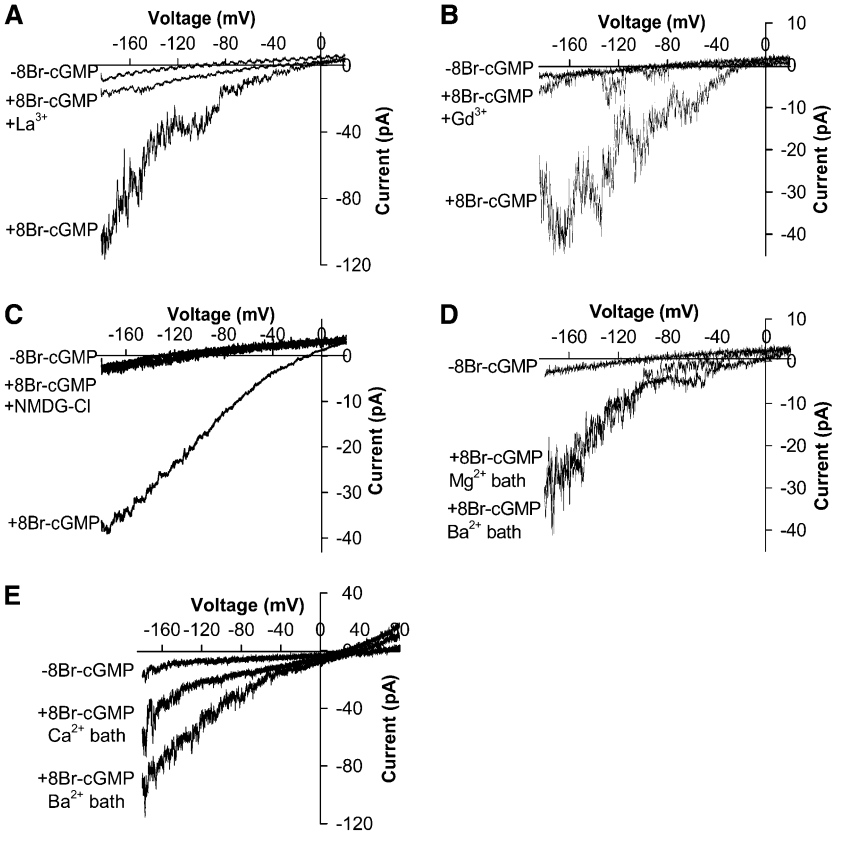

Figure 2. Ion selectivity and blocker sensitivity of 500 μM 8Br-cGMP-activated channels recorded in Arabidopsis Columbia wild-type guard cell protoplasts. A, Whole-cell recording in a Columbia wild-type guard cell protoplast showing the inhibition of 8Br-cGMP (500 μM)-activated currents by the Ca^{2+} channel blocker La^{3+} (100 μM). B, Whole-cell recordings in a Columbia wild-type guard cell protoplast showing 8Br-cGMP-activated channel inhibition by the Ca^{2+} channel blocker Gd^{3+} (100 μM). Two overlapping traces after Gd^{3+} application are depicted. C, Whole-cell recording of a Columbia wild-type guard cell protoplast showing the inhibition of 8Br-cGMP-activated currents by the replacement of Mg^{2+} in the bath solution by 100 mM NMDG-Cl. Two overlapping traces before 8Br-cGMP application and after replacement of Mg^{2+} in the bath solution by 100 mM NMDG-Cl are depicted. D, Whole-cell recording of 8Br-cGMP-activated currents in a Columbia wild-type guard cell protoplast showing similar current amplitudes when MgGlu and $MgCl_2$ in the bath solution were replaced with 100 mM $BaCl_2$. E, 8Br-cGMP-activated currents in a Columbia wild-type protoplast recorded using $BaCl_2$ pipette and bath solutions without NADPH in the pipette solution (see "Materials and Methods"). 8Br-cGMP-activated currents were recorded in a 100 mM $BaCl_2$ bath solution first, and then 100 mM $BaCl_2$ in the bath solution was replaced with 100 mM $CaCl_2$.

cation currents. Further experiments showed that the 8Br-cGMP-activated currents can also be carried by the monovalent cation Na^+ (Supplemental Fig. S2), similar to ABA-activated Ca^{2+}-permeable cation (I_{Ca}) channels in guard cells (Kwak et al., 2003). Taken together, these results showed that cGMP activates nonselective Ca^{2+}-permeable cation channels in the plasma membrane of guard cells. Therefore, we named the 8Br-cGMP-activated nonselective Ca^{2+}-permeable cation currents $I_{cat\text{-}cGMP}$.

Guard Cell-Expressed Putative CNGC Genes

Genes encoding defined plasma membrane Ca^{2+}-permeable channels remain poorly understood in guard cells. To identify putative Ca^{2+}-permeable channel genes underlying $I_{cat\text{-}cGMP}$, we analyzed guard cell-specific microarray sets of Arabidopsis (Yang et al., 2008; Pandey et al., 2010) and found that *CNGC1* (At5g53130), *CNGC2* (At5g15410), *CNGC5* (At5g57940), *CNGC6* (At2g23980), *CNGC15* (At2g28260), and *CNGC20* (At3g17700) genes were highly expressed in guard cells (Supplemental Table S1). We obtained *cngc* transfer DNA (T-DNA) insertion mutant lines for these *CNGC* genes. As *CNGC5* and *CNGC6* are among the two most closely related of the 20 CNGC genes, we generated *cngc5 cngc6* double mutants by crossing the single mutants for further experiments (Fig. 3). Reverse transcription PCR and quantitative real-time (RT)-PCR analyses showed that the mRNA levels of *CNGC5* and *CNGC6* were greatly reduced or abolished in *cngc5-1cngc6-1* (Fig. 3, B and C).

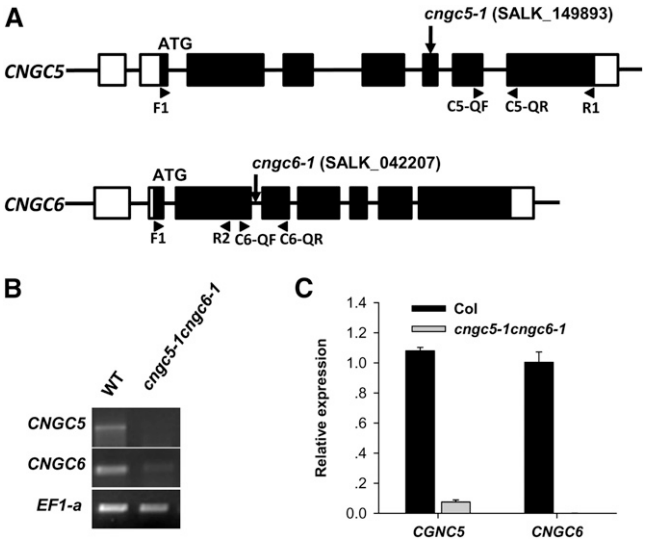

Figure 3. *CNGC5* and *CNGC6* transcript analysis in *cngc5-1* and *cngc6-1* mutants. A, Cartoon showing the genomic structures of *CNGC5* and *CNGC6* and the T-DNA insertion sites of the *cngc5-1 cngc6-1* double mutant. To amplify each CNGC-specific band, RT-PCR was performed with primer sets as indicated by the arrowheads. B, RT-PCR analysis of the *cngc5-1 cngc6-1* double mutant and the Columbia wild type (WT) showing that no clear *CNGC5* transcripts were detected in the *cngc5-1 cngc6-1* mutant, but a possible low level of *CNGC6* transcripts was detected in the *cngc5-1 cngc6-1* mutant after 35 PCR cycles of amplification. C, Quantitative RT-PCR analysis of the *cngc5-1 cngc6-1* double mutant shows that the transcript levels of *CNGC5* and *CNGC6* were greatly reduced.

Mutations in *CNGC5* and *CNGC6* Impaired $I_{cat\text{-}cGMP}$

To pursue the identification of genes encoding ion channels that mediate $I_{cat\text{-}cGMP}$ in Arabidopsis guard cells, we performed patch-clamp experiments on *cngc5-1* and *cngc6-1* single mutant and *cngc5-1 cngc6-1* double mutant guard cells as well as on *cngc2* and *cngc1 cngc20* double mutants. Patch-clamp experiments showed that average current magnitudes of $I_{cat\text{-}cGMP}$ were only partially reduced in the two single mutants, including an insignificant average reduction in the *cngc6-1* single mutant (Fig. 4, B and C; $P \leq 0.005$ for *cngc5-1* versus the wild type, $P = 0.585$ for *cngc6-1* versus the wild type at −184 mV). Note that all data were included in these analyses, and the data from *cngc6-1* guard cells included one guard cell with a large leak-like current after 8Br-cGMP addition, thus adding to a larger degree of error (Fig. 4C; for an independent *cngc6-1* data set, see Fig. 7C below). We analyzed *cngc5-1 cngc6-1* double mutant guard cells, which showed strongly impaired 8Br-cGMP-activated currents (Fig. 4, A and D), compared with the Columbia wild type (Fig. 1; $P < 0.001$ at –184 mV). These data indicate that CNGC5 and CNGC6 contribute to the activity of the $I_{cat\text{-}cGMP}$ channel currents in Arabidopsis guard cells.

To further test the effects of mutations in *CNGC5* and *CNGC6* on $I_{cat\text{-}cGMP}$, we generated a second double mutant allele, *cngc5-2 cngc6-2*, in the Wassilewskija (Ws) accession. *CNGC6* mRNA levels in the *cncg5-2 cngc6-2* mutant were greatly reduced (Supplemental Fig. S3). The *CNGC5* transcript has two splice isoforms in Ws (Supplemental Fig. S3). We thus amplified several independent *CNGC5* transcripts in *cngc5-2*. The 5′ untranslated region insertion in *cngc5-2* showed either greatly reduced transcripts for two different primer pairs, including primers that amplified transcript starting 490 bp 5′ of the *CNGC* start site (Supplemental Fig. S3), or an increased transcript level with primers amplifying from the predicted start (ATG) site of the *CNGC5* transcript (Supplemental Fig. S3, B and C). These data indicate that the *CNGC6* gene was clearly reduced, whereas the *CNGC5* gene was less clearly affected in the *cngc5-2 cngc6-2* double mutant. We confirmed significant activation of $I_{cat\text{-}cGMP}$ by 20 μM cGMP applied in the pipette solution in Ws wild-type guard cells (Supplemental Fig. S4). In contrast, impaired activation was observed in *cngc6-2* mutant guard cells (*cngc5-2 cngc6-2* double mutant allele; Supplemental Fig. S4), providing evidence for an important function of CNGC6 in mediating the cGMP-activated current in the Ws accession. The effect of the T-DNA insertion in *CNGC5* and aberrant *CNGC5* transcripts amplified in *cngc5-2 cngc6-2* cannot, at present, unequivocally define or exclude a contribution of *CNGC5* to this phenotype in the Ws accession.

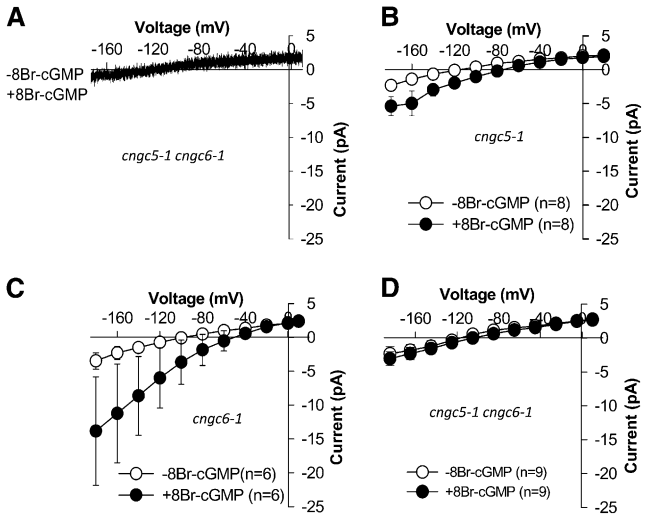

Figure 4. Mutations in *CNGC5* and *CNGC6* impair 500 μM 8Br-cGMP-activated inward currents in Arabidopsis guard cells. A, Typical whole-cell recording in a *cngc5-1 cngc6-1* double mutant guard cell protoplast before (−) and after (+) application of the membrane-permeable cGMP analog 8Br-cGMP (500 μM) to the bath solution. B to D, Average current-voltage curves of steady-state whole-cell currents recorded in *cngc5-1* single mutant (B; $n = 8$), *cngc6-1* single mutant (C; $n = 6$), and *cngc5-1 cngc6-1* double mutant (D; $n = 9$) guard cell protoplasts. Values depict means ± SE.

Microarray data sets suggest that several other *CNGC* genes are expressed in guard cells, including *CNGC1*, *CNGC2*, *CNGC15*, and *CNGC20* (Leonhardt et al., 2004; Yang et al., 2008; Pandey et al., 2010; Bauer et al., 2013; Supplemental Table S1), and it has been reported that *CNGC2* functions as a cAMP-activated Ca^{2+}-permeable channel in Arabidopsis guard cells (Ali et al., 2007). Therefore, we isolated *cngc1*, *cngc2*, and *cngc20* T-DNA insertion mutants and generated a *cngc1 cngc20* double mutant line in the Ws ecotype background. The *cngc15* insertion mutant line (SALK_017995) had a T-DNA insertion in the 5′ untranslated region of *CNGC15* and did not exhibit a reduction in *CNGC15* transcript level; thus, *CNGC15* could not be analyzed in this study. We performed further patch-clamp experiments on *cngc2* and *cngc1 cngc20* mutant guard cells and found that there was no significant difference of $I_{cat\text{-}cGMP}$ in *cngc2* mutant guard cells (Fig. 5B) and its Columbia wild type (Fig. 5A; $P = 0.474$ at −184 mV) as well as between the *cngc1 cngc20* double mutant (Fig. 5D) and its Ws wild type (Fig. 5C; $P = 0.245$ at −184 mV). These results indicate that 8Br-cGMP mainly activated CNGC5 and CNGC6 in the wild type relative to CNGC1, CNGC2, and CNGC20 in Arabidopsis guard cells under the imposed conditions.

cAMP has been shown to activate Ca^{2+}-permeable currents in Arabidopsis guard cells using Ba^{2+} as the main divalent cation in both bath and pipette solutions (Lemtiri-Chlieh and Berkowitz, 2004). As reported above, 8Br-cGMP-activated inward currents could be carried by Ba^{2+} (Fig. 2, D and E). We analyzed cAMP-activated inward currents using Mg^{2+} as the main charge carrier. The results showed that only modest cAMP-activated inward currents were observed in both Columbia wild-type (Supplemental Fig. S5A) and *cngc5-1 cngc6-1* double mutant (Supplemental Fig. S5B) guard cells with 100 μM cAMP applied into the pipette solution. There was no significant difference between Columbia wild-type and *cngc5-1 cngc6-1* double mutant guard cells (Supplemental Fig. S5; $P = 0.151$ at −184 mV). These data and the lack of impairment of cGMP activation in *cngc2* guard cells (Fig. 5B), compared with cAMP response impairment in *cngc2* (Ali et al., 2007), suggest that the cGMP-activated currents in the Mg^{2+} solutions analyzed here differ from the cAMP-activated currents.

YFP-CNGC5 and YFP-CNGC6 Localize to the Periphery of *Nicotiana benthamiana* Protoplasts

To gain insight into the cellular targeting of yellow fluorescent protein (YFP)-tagged CNGC5 and CNGC6 proteins, we transiently expressed *CNGC5* and *CNGC6* fused to enhanced-YFP (*EYFP*) in *N. benthamiana* protoplasts and conducted EYFP fluorescence imaging experiments using confocal microscopy. The results showed localization of YFP-CNGC5 in microdomains in the periphery of *N. benthamiana* protoplasts (Fig. 6). Similar microdomain localizations of plant GFP-CNGC fusions have been observed previously, including for

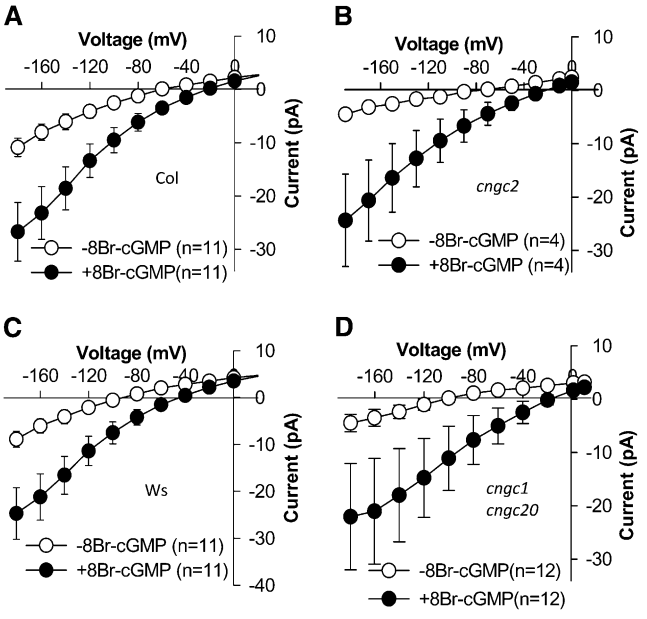

Figure 5. Mutations in *CNGC1*, *CNGC20*, and *CNGC2* did not disrupt the currents activated by 8Br-cGMP added to the bath solution. Average current-voltage curves of whole-cell recordings show 8Br-cGMP (500 μM)-activated inward currents recorded in guard cell protoplasts of the Columbia (Col) wild type (A; $n = 11$), the *cngc2* mutant (B; $n = 4$), the Ws wild type (C; $n = 11$), and the *cngc1 cngc20* double mutant (D; $n = 12$). Values depict means ± SE.

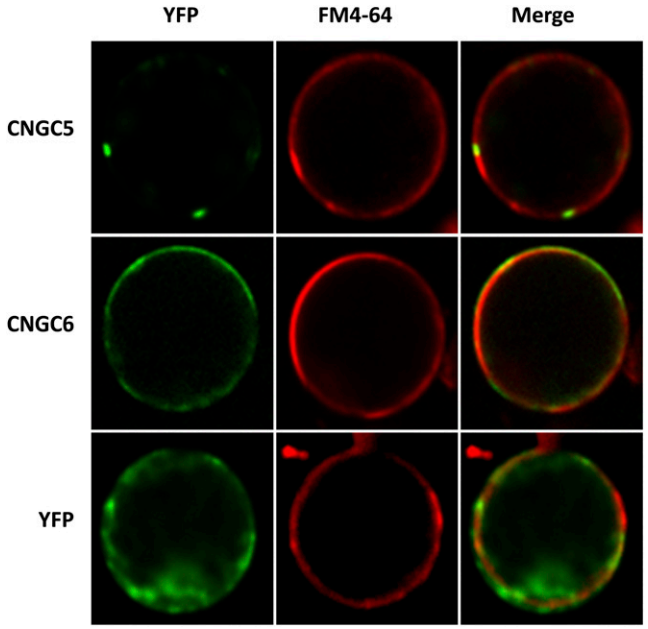

Figure 6. Subcellular localization analysis of YFP-CNGC5 and YFP-CNGC6 in *N. benthamiana* protoplasts. Image columns depict YFP, plasma membrane label FM4-64, and merged images of YFP and FM4-64 fluorescence of *N. benthamiana* protoplasts expressing YFP-CNGC5, YFP-CNGC6, and YFP control.

CNGC1, CNGC11, and CNGC12 (Ali et al., 2006; Urquhart et al., 2007). YFP-CNGC6 also localized to the periphery of *N. benthamiana* protoplasts. YFP-CNGC6 did not show a strong concentration in microdomains (Fig. 6) and overlapped partially with the plasma membrane stain FM4-64 (Bolte et al., 2004; Fig. 6). In contrast, control YFP-expressing protoplasts showed more broadly distributed YFP fluorescence signals, including in the cytoplasm and nuclei (Fig. 6). These results provide initial evidence that CNGC5 and CNGC6 are targeted to the periphery of plant cells at the plasma membrane, which correlates with the strong reductions in $I_{cat\text{-}cGMP}$ activity in the plasma membrane of *cngc5-1 cngc6-1* double mutant guard cells and is consistent with recent YFP fusion localization analysis of CNGC6 (Gao et al., 2012).

Application of Intracellular cGMP Activates Currents in Wild-Type Arabidopsis Guard Cells

We used membrane-permeable 8Br-cGMP to activate large currents in guard cells, allowing facile analyses before and after application (Figs. 1, 2, 4, and 5). To test whether intracellular cGMP can activate $I_{cat\text{-}cGMP}$, we conducted further patch-clamp experiments using cGMP at a lower concentration (20 μM) added to the pipette solution in whole-cell recordings. We observed obvious $I_{cat\text{-}cGMP}$ currents in Columbia wild-type guard cells (Fig. 7A). $I_{cat\text{-}cGMP}$ was reduced in *cngc5-1* (Fig. 7B), *cngc6-1* (Fig. 7C), and *cngc5-1 cngc6-1* (Fig. 7D) mutant guard cells compared with the Columbia wild type (Fig. 7A; $P = 0.013$, 0.044, and 0.032 for *cngc5-1*, *cngc6-1*, and *cngc5-1 cngc6-1* mutants, respectively, compared with the wild type at −184 mV), indicating that intracellular cGMP is capable of activating $I_{cat\text{-}cGMP}$ and CNGC5 and CNGC6 function in the intracellular cGMP response. Note that we cannot exclude that an additional *CNGC* gene(s) may be up-regulated in these *cngc* insertion mutant lines or that additional *CNGC* genes contribute to the small average residual currents in *cncg5 cngc6* double mutant guard cells. However, clearly, both *CNGC5* and *CNGC6* function in the establishment of the cGMP-activated currents. We also tested the effects of 20 μM cAMP added in the pipette solution. We did not observe obvious activation of inward currents in Columbia wild-type guard cells (Supplemental Fig. S6), indicating that cAMP activation may occur through other channels or at higher cAMP concentrations.

CNGC5 and CNGC6 Are Not Solely Essential for ABA, CO_2, and Light/Dark Transition-Induced Stomatal Signaling

ABA triggers cytosolic Ca^{2+} increases and activates nonselective Ca^{2+}-permeable cation channels in Arabidopsis guard cells (Schroeder and Hagiwara, 1990; Hamilton et al., 2000; Pei et al., 2000; Murata et al., 2001; Kwak et al., 2003; Miao et al., 2006; Hua et al., 2012).

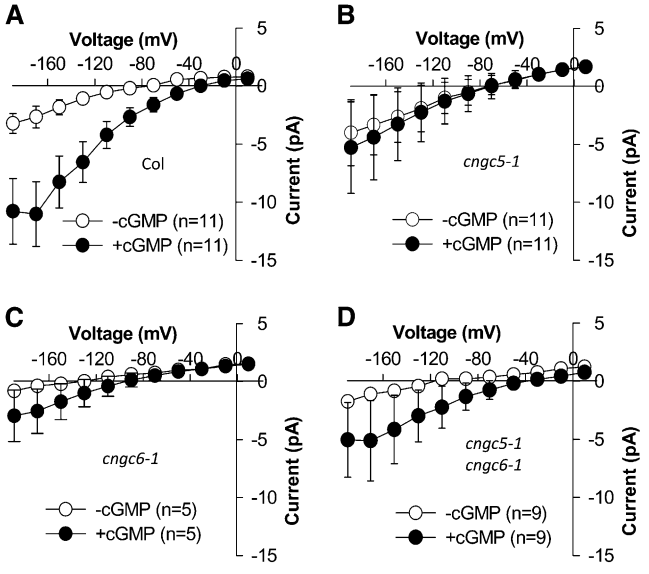

Figure 7. cGMP (20 μM) added to the pipette solution activated obvious currents in Columbia wild-type guard cells, but currents activated by 20 μM cGMP were dramatically impaired in *cngc5-1*, *cngc6-1*, and *cngc5-1 cngc6-1* guard cells. Average current-voltage curves of whole-cell recordings represent the Columbia (Col) wild type (A; $n = 11$), the *cngc5-1* single mutant (B; $n = 11$), the *cngc6-1* single mutant (C; $n = 5$), and the *cngc5-1 cngc6-1* double mutant (D; $n = 9$). Values depict means ± SE.

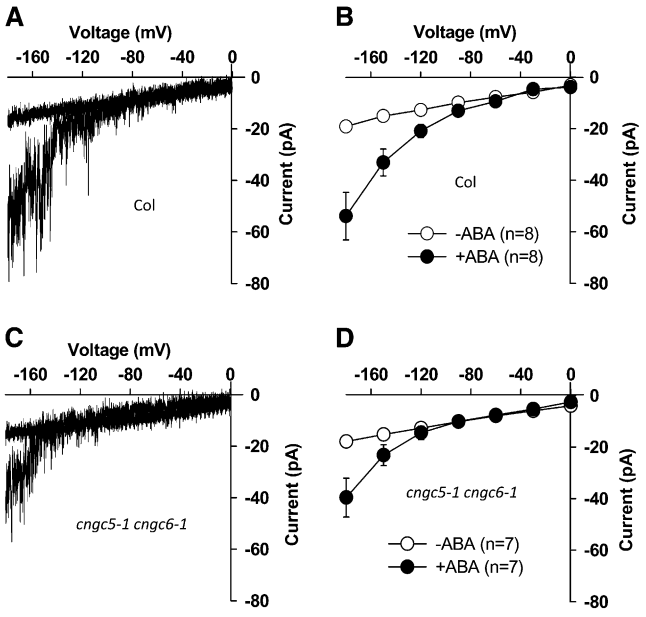

Figure 8. Mutations in *CNGC5* and *CNGC6* did not impair ABA (50 μM) activation of I_{Ca} channels in Arabidopsis guard cells. A, Typical whole-cell recording of ABA-activated I_{Ca} in a Columbia (Col) wild-type guard cell protoplast. B, Average current-voltage curves of whole-cell I_{Ca} recordings in the Columbia wild type (n = 8). C, Typical whole-cell recording of ABA-activated I_{Ca} in a *cngc5-1 cngc6-1* double mutant guard cell protoplast. D, Average current-voltage curves of whole-cell I_{Ca} recordings in the *cngc5-1 cngc6-1* double mutant (n = 7). Values depict means ± SE.

Similar to $I_{cat\text{-}cGMP}$, ABA-activated I_{Ca} channels are permeable to Ba^{2+} (Pei et al., 2000; Murata et al., 2001), Na^{+} (Kwak et al., 2003), and Mg^{2+} (Supplemental Fig. S7). Therefore, we performed patch-clamp experiments to test whether CNGC5 and CNGC6 function as ABA-activated I_{Ca} channels using Ba^{2+} as the main divalent cation in both bath and pipette solutions, as reported previously (Pei et al., 2000; Murata et al., 2001). Genotype-blind patch-clamp experiments showed that measurable ABA activation of I_{Ca} currents was observed in *cngc5-1 cngc6-1* guard cells ($P < 0.037$; Fig. 8, C and D) as well as in Columbia wild-type guard cells ($P < 0.018$; Fig. 8, A and B). A small effect of the *cngc5 cngc6* double mutation on ABA-activated I_{Ca} channel currents could not be excluded, based on slightly smaller average currents and an apparent slight shift in the activation potential. The ABA-insensitive mutants *growth controlled by abscisic acid2* (*gca2*) and *abscisic acid insensitive1* (*abi1-1*) show impaired ABA activation of I_{Ca} channel currents (Pei et al., 2000; Murata et al., 2001). We next analyzed $I_{cat\text{-}cGMP}$ in *gca2* and *abi1-1* mutant guard cells as well as in Landsberg *erecta* wild-type guard cells and found that 8Br-cGMP-activated $I_{cat\text{-}cGMP}$ currents were not disrupted in *gca2* (Fig. 9B) and *abi1-1* (Fig. 9C) guard cells compared with the Landsberg *erecta* wild type (Fig. 9A). These observations together strongly suggest that CNGC5 and CNGC6 alone are not essential for ABA-activated I_{Ca} channels in Arabidopsis guard cells. To further test whether CNGC5 and CNGC6 function in ABA signaling, we pursued ABA-induced stomatal closure analyses and found that both the Columbia wild type and the *cngc5-1 cngc6-1* double mutant showed functional ABA-induced responses (Fig. 10), in line with patch-clamp analyses (Figs. 8 and 9). Moreover, cytosolic Ca^{2+} has also been shown to play a role in CO_2-induced stomatal movements (Schwartz et al., 1988; Webb et al., 1996; Young et al., 2006; Xue et al., 2011). Therefore we also addressed CO_2-induced changes in whole-plant stomatal conductance of CNGC5 and CNGC6 double mutants. The results show that mutation of *CNGC5* and *CNGC6* did not disrupt CO_2-induced stomatal closure and opening (Fig. 11). Moreover, light/dark transitions showed similar intact plant stomatal conductances in wild-type and *cngc5-1 cngc6-1* double mutant plants (Supplemental Fig. S8).

DISCUSSION

Intracellular Ca^{2+} plays an essential role in the regulation of guard cell ion channels and stomatal movements (Schroeder and Hagiwara, 1989; McAinsh et al., 1990; Webb et al., 1996; Grabov and Blatt, 1998;

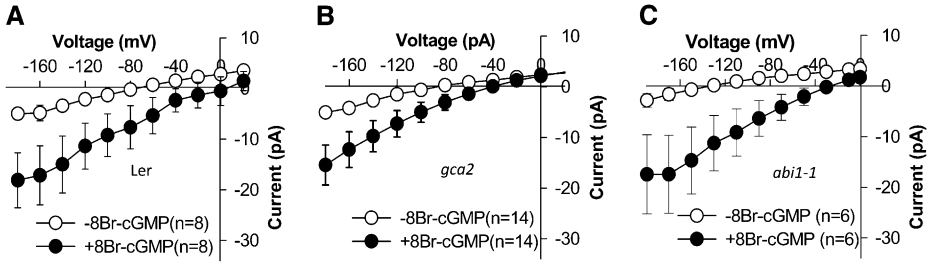

Figure 9. *gca2* and *abi1-1* mutants showed intact cGMP-activated currents in guard cells. Average current-voltage curves of whole-cell recordings showing 8Br-cGMP-activated inward currents were recorded in guard cell protoplasts of the Landsberg *erecta* (Ler) wild type (A; n = 8), the *gca2* mutant (B; n = 14), and the *abi1-1* mutant (C; n = 6). A concentration of 500 μM 8Br-cGMP was added to the bath solution by bath perfusion after establishing whole-cell recordings and analysis of background currents in guard cells. Values depict means ± SE.

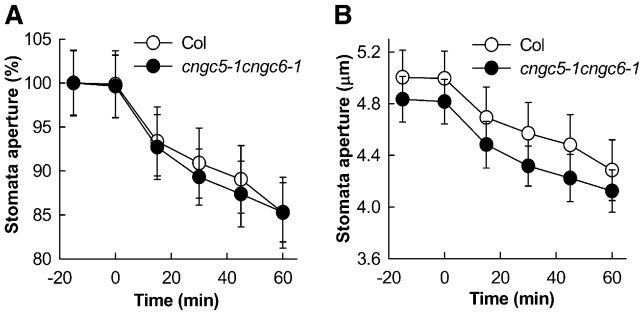

Figure 10. ABA-induced stomatal closure in the Columbia wild type and the *cngc5-1 cngc6-1* double mutant. A, Percentage changes in stomatal apertures relative to the stomatal apertures prior to 1 μM ABA incubation. B, Stomatal apertures from the same experiments shown in micrometers. Time-course experiments were pursued for ABA-induced stomatal closing in the Columbia (Col) wild type and the *cngc5-1 cngc6-1* double mutant (genotype-blind experiments). Stomatal apertures were individually mapped, and images were captured and measured before and after the addition of 1 μM ABA (Siegel et al., 2009). Average stomatal apertures at time −15 min were 5.0 ± 0.2 μm (Columbia wild type) and 4.83 ± 0.18 μm (*cngc5-1 cngc6-1*); $n = 24$ individually mapped stomata each for the Columbia wild type and the *cngc5-1 cngc6-1* double mutant. Values depict means ± SE.

MacRobbie, 2000; Fan et al., 2004; Mori et al., 2006; Young et al., 2006; Marten et al., 2007; Siegel et al., 2009; Chen et al., 2010). Stimulus-triggered Ca^{2+} influx across the plasma membrane of plant cells plays essential roles in many Ca^{2+} signaling responses (Dodd et al., 2010). Two gene families have been proposed to encode Ca^{2+} channels in plants, the Glu receptor-like gene family (Lacombe et al., 2001) and the CNGC gene family (Kaplan et al., 2007). However, there are only a few studies that have shown disruption of a defined Ca^{2+}-permeable channel activity in mutant plants in defined candidate plant Ca^{2+} channel genes (Ali et al., 2007; Gao et al., 2012; Laohavisit et al., 2012).

Activation mechanisms of plasma membrane Ca^{2+}-permeable I_{Ca} currents have been characterized in Arabidopsis guard cells (Hamilton et al., 2000; Pei et al., 2000; Murata et al., 2001; Köhler and Blatt, 2002; Kwak et al., 2003; Miao et al., 2006; Mori et al., 2006; Hua et al., 2012). However, the identities of genes encoding defined plasma membrane Ca^{2+} channels in guard cells remain unknown. CNGCs have been found to be key players as cation channels in mammalian cells, and the Arabidopsis genome includes 20 *CNGC* genes (Kaplan et al., 2007; Ward et al., 2009). Some of these Arabidopsis CNGCs were found to function in pollen tube growth, plant immune responses, and heat responses (Yu et al., 1998; Frietsch et al., 2007; Ma and Berkowitz, 2011; Finka et al., 2012; Mach, 2012; Abdel-Hamid et al., 2013; Fischer et al., 2013; Tunc-Ozdemir et al., 2013a, 2013b). But direct voltage-clamp recordings of a *cngc* mutant in planta has been reported for two *cngc* mutants to date (Ali et al., 2007; Gao et al., 2012). In this research, we identified *CNGC5* and *CNGC6*, which are required for cGMP-activated nonselective Ca^{2+}-permeable cation channel activity in Arabidopsis guard cells (Figs. 2, 4, and 7). T-DNA insertion mutants in these *CNGC* genes show that CNGC5 and CNGC6 encode a new type of nonselective cation channel in the plasma membrane of Arabidopsis guard cells.

A previous study showed cAMP-activated Ca^{2+}-permeable currents in Arabidopsis guard cells using Ba^{2+} in the bath and pipette solutions by adding membrane-permeable 1 mM dibutylyl-cAMP to the bath solution (Lemtiri-Chlieh and Berkowitz, 2004; Ali et al., 2007). In addition, it was recently reported that 50 μM dibutylyl-cAMP together with the animal phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine activated significant inward Ca^{2+} currents in Arabidopsis root protoplasts using Ca^{2+} as the main charge carrier, but effects of cGMP were not analyzed (Gao et al., 2012). Under our experimental conditions, we added cyclic nucleotides in the pipette solution at a lower concentration (20 and 100 μM for cAMP, 20 μM for cGMP) and observed limited cAMP activation of cation currents in guard cells (Supplemental Figs. S5 and S6) but a significant activation of the cation currents by 20 μM cGMP (Fig. 7) using solutions in which Mg^{2+} was the main divalent cation. Our findings do not exclude that higher cAMP concentrations may activate such channels in a Mg^{2+}-containing bath solution. In addition, cAMP might have weaker effects on these CNGCs compared with cGMP in guard cells. Indeed, it has been reported that mammalian *CNGCs* expressed in *X. laevis* oocytes could be activated by cGMP approximately at a 100-fold lower concentration than cAMP (Gordon and Zagotta, 1995). Further research will be needed to determine whether different interacting proteins, including possible different CNGC heteromeric complexes, or conditions affect channel activation properties in these different plant cell types, and a direct comparison of cAMP- or cGMP-activated currents in the same type of cells (root cells or guard cells) would require further experiments.

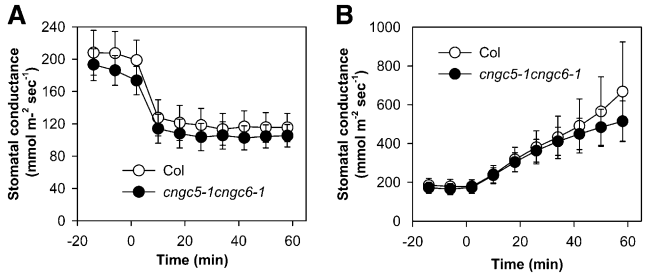

Figure 11. Time-resolved patterns of intact whole-plant rosette stomatal conductances in response to elevated (A) and reduced (B) CO_2 concentration in *cngc5-1 cngc6-1* double mutant and Columbia (Col) wild-type plants. To analyze elevated CO_2-induced stomatal closure, the ambient CO_2 concentration was increased from 400 to 800 μL L^{-1} at time zero (A). To analyze low-CO_2-induced stomatal opening, CO_2 was decreased from 400 to 0 μL L^{-1} at time zero (B). Data represent averages of 12 individual plants ± SE.

Note that, although cGMP and cAMP can activate plant ion channels, it remains unknown whether these small molecules act as second messengers in cells of terrestrial plants. Indeed, whether cAMP and cGMP are produced in response to stimuli in higher plants is controversial and remains a matter of debate. Nevertheless, the ability of cGMP to activate *CNGC5*- and *CNGC6*-dependent ion channel activity in guard cells, with little effect of cAMP, indicates a preference in nucleotide activation mechanisms for CNGC5 and CNGC6. Furthermore, proteins with possible guanylate cyclase activity in plants have been proposed and debated (Ludidi and Gehring, 2003; Qi et al., 2010; Ashton, 2011; Berkowitz et al., 2011). Further research is needed to determine whether this animal paradigm can be applied to plant ion channel regulation. It is conceivable that CNGC5 and CNGC6 are activated in vivo in guard cells by other natural stimuli than cGMP, and more research will be needed to investigate this question. For example, ABA activates Ca^{2+}-permeable channels with properties very similar to these currents, via distinct signaling mechanisms (Murata et al., 2001; Pei et al., 2000; Köhler and Blatt, 2002; Kwak et al., 2003; Miao et al., 2006; Mori et al., 2006; Hua et al., 2012). These data indicate that other or higher order *cngc* mutants might well function as the ABA-activated I_{Ca} channels.

In previous studies of reactive oxygen species and ABA activation of Ca^{2+}-permeable channels, no ATP was added in the pipette solutions for electrophysiological analyses (Pei et al., 2000; Mori et al., 2006). In this study, we used ATP-free solutions containing 0.1 mM dithiothreitol (DTT) for both ABA-activated I_{Ca} and cGMP-activated $I_{cat\text{-}cGMP}$ recordings, indicating that ATP was not strictly required for the activation of these channels. NADPH was included for ABA activation. Similarly, ATP was not required for ligand-activated Ca^{2+}-permeable cation channels in pollen (Wu et al., 2011). Note, however, that these results do not exclude additional regulation of Ca^{2+}-permeable channels by ATP-dependent mechanisms or protein kinases, as demonstrated previously for guard cell I_{Ca} channels (Köhler and Blatt, 2002; Mori et al., 2006), given that conditions prior to whole-cell patch clamping did not preclude ATP-dependent reactions; thus, the phosphorylation state of these channels may be preset prior to patch clamping. For example, ABA activation of I_{Ca} channels is disrupted in the Ca^{2+}-dependent protein kinase *cpk6* and *cpk3* mutant guard cells (Mori et al., 2006).

This study and a recent study (Gao et al., 2012) provide evidence that YFP fusions of CNGC6 are localized in the vicinity of the plasma membrane, consistent with patch-clamp analyses of T-DNA insertion mutants. YFP fusions of the close homolog YFP-CNGC5 showed localization in microdomains in the cell periphery. Similar results were reported previously for other plant membrane proteins, including CNGC1, CNGC11, and CNGC12 channels (Brady et al., 2004; Ali et al., 2006; Sutter et al., 2006; Urquhart et al., 2007; Gutierrez et al., 2010). Further research is needed to determine the relevance of such microdomain accumulation of membrane proteins.

Gene chip data show that several *CNGCs* are expressed in Arabidopsis guard cells, including *CNGC1*, *CNGC2*, *CNGC5*, *CNGC6*, *CNGC15*, and *CNGC20*, and the expression level of *CNGC5* and *CNGC6* is relatively high compared with other homologs (Yang et al., 2008; Supplemental Table S1). Patch-clamp results show that mutations in *CNGC5* and *CNGC6* impaired 8Br-cGMP-activated currents, but T-DNA insertion mutations in *CNGC1*, *CNGC2*, and *CNGC20* did not, indicating that CNGC5 and CNGC6 are important targets for guard cell cGMP-activated channel activity in the plasma membrane of Arabidopsis guard cells under the imposed conditions. Additional CNGCs may be involved in the overlapping, partially redundant formation of regulated ion channels in guard cells. Therefore, our findings do not exclude that other CNGCs in guard cells, in addition to or together with CNGC5 and CNGC6, form regulated ion channels in vivo.

ABA-activated I_{Ca} channel activity was not disrupted by mutations in *CNGC5* and *CNGC6* (Fig. 8). In addition, we found that the ABA-insensitive *gca2* and *abi1-1* mutants, for which ABA activation of I_{Ca} channels is impaired (Pei et al., 2000; Murata et al., 2001; Miao et al., 2006), showed $I_{cat\text{-}cGMP}$ similar to the wild type (Fig. 9). Taken together, we conclude that CNGC5- and CNGC6-mediated ion channels alone are not essential for these ABA responses. It has been suggested that cGMP is required, but not sufficient, for ABA- and nitric oxide-induced stomatal closure (Dubovskaya et al., 2011). Analyses of additional guard cell-expressed cGMP-dependent proteins, in particular CNGCs, in addition to CNGC5 and CNGC6 are needed for understanding a proposed link between cGMP and ABA signaling. This includes analyses of higher order *cngc* mutants, including expansion of the *cngc5-1 cngc6-1* double mutants analyzed here, as higher order (partial) redundancy cannot be excluded at this time.

In this study, we identify two genes, *CNGC5* and *CNGC6*, that are required for the function of a new type of nonselective Ca^{2+}-permeable cation-permeable channel activity in the plasma membrane of plants. *CNGC5* and *CNGC6* are required for the cGMP activation of $I_{cat\text{-}cGMP}$ in the guard cell plasma membrane.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) plants (Columbia, Landsberg *erecta*, and Ws ecotypes) were grown in soil (Sunrise) in a growth chamber (Conviron) under a 16-h-light/8-h-dark cycle at a photon fluence rate of approximately 75 μmol m^{-2} s^{-1} during the day, a humidity of approximately 75%, and a temperature of 21°C ± 0.5°C. The *cngc2* (SALK_129133), *cngc5-1* (SALK_149893), and *cngc6-1* (SALK_042207) mutants were obtained from the Arabidopsis Biological

Resource Center. *cngc5-2* (FLAG_295E04) and *cngc6-2* (FLAG418_D11) were in the Ws background and obtained from the INRA. *cngc1* and *cngc20* were in the Ws background and kindly provided by Dr. Hillel Fromm (INRA) and the Arabidopsis knockout facility at the University of Wisconsin Biotechnology Center (Krysan et al., 1999; Sunkar et al., 2000).

Patch-Clamp Experiments

Guard cell protoplasts of Arabidopsis were isolated enzymatically as described previously (Vahisalu et al., 2008). For patch-clamp experiments of $I_{cat\text{-}cGMP}$, the bath solution contained 92.5 mM MgGlu, 7.5 mM $MgCl_2$, and 10 mM MES-HCl (5 mM HCl), pH 5.6, and osmolarity was adjusted to 485 mmol L^{-1} using D-sorbitol. The pipette solutions contained 9.75 mM MgGlu, 0.25 mM $MgCl_2$, 4 mM EGTA, and 10 mM HEPES-Tris, pH 7.1, and osmolarity was adjusted to 500 mmol L^{-1}. Each day, 0.1 mM DTT was freshly added in the bath and pipette solutions, and no NADPH was added in the pipette solution for all cGMP-dependent $I_{cat\text{-}cGMP}$ recordings. Control experiments performed in the absence of DTT showed that 8Br-cGMP continued to activate $I_{cat\text{-}cGMP}$ ($n = 4$ guard cells). A voltage ramp protocol from −180 to +20 mV (holding potential, 0 mV; ramp speed, 200 mV s^{-1}) was applied for $I_{cat\text{-}cGMP}$ recordings. Whole-cell currents were recorded every minute for 10 min after accessing whole-cell configurations with patch-clamp seal resistances of no less than 10 GΩ. Data prior to 8Br-cGMP exposure were used as preexposure baseline control conditions. Subsequently, cyclic nucleotide-activated currents were recorded 30 times per minute after cyclic nucleotide (8Br-cGMP) was added to the bath solution. For experiments analyzing the effects of cAMP and cGMP added to the pipette solution, the first trace recorded after accessing the whole-cell configuration was used as a baseline control, and the traces recorded subsequently were analyzed for effects of cyclic nucleotides. Liquid junction potential was −4 ± 1 mV, measured as described previously (Ward and Schroeder, 1994) and corrected in Figure 1B. No leak subtraction was applied to the depicted data. A 1 M KCl agar bridge was used as a bath electrode to stabilize bath electrode potentials, which is needed in particular when bath Cl^- concentrations are changed during recordings.

Whole-cell patch-clamp recordings of ABA activation of I_{Ca} were performed as described previously (Munemasa et al., 2007; Vahisalu et al., 2008). The pipette solution contained 10 mM $BaCl_2$, 4 mM EGTA, and 10 mM HEPES-Tris, pH 7.1, osmolarity was adjusted to 500 mmol L^{-1} using D-sorbitol, and 0.1 mM DTT and 5 mM NADPH were freshly added each day. The bath solution contained 100 mM $BaCl_2$ and 10 mM MES-Tris, pH 5.6, osmolarity was adjusted to 485 mmol L^{-1} using D-sorbitol, and 0.1 mM DTT was freshly added each day. A ramp voltage protocol from +20 to −180 mV (holding potential, 0 mV; ramp speed, 200 mV s^{-1}) was used for I_{Ca} recordings. Initial control whole-cell currents were recorded once each minute for 16 times starting 1 to 3 min after accessing the whole-cell configurations. The seal resistance was no less than 10 GΩ. Then, ABA was added to the bath solution by perfusion, and guard cell protoplasts were exposed to ABA in the bath solution for 3 min. Subsequently, I_{Ca} was recorded for another 16 min. Liquid junction potential of −18 mV was not corrected in the figures, and leak currents were not subtracted.

RNA Isolation, RT-PCR, and Quantitative RT-PCR Experiments

Total RNA was isolated with an RNeasy Plant Mini Kit (Qiagen). After treatment with RNase-free DNase I (Qiagen), first-strand complementary DNA (cDNA) was synthesized from 1 μg of total RNA using a first-strand cDNA synthesis kit (GE Healthcare) according to the manufacturer's instructions. DNA fragments for *CNGC5* and *CNGC6* were amplified by 35 PCR cycles using specific primers (Supplemental Table S2). The EF-1α transcript was amplified for 22 PCR cycles.

For real-time quantitative RT-PCR analysis, total RNA was extracted from 3-week-old plants using Trizol reagent (Invitrogen). cDNA was synthesized from RNA using Moloney murine leukemia virus reverse transcriptase (Promega) with oligo$(dT)_{15}$ primers (Promega). Real-time quantitative PCR was performed using TransStart Green qPCR SuperMix on a Bio-Rad CFX Connect Real Time PCR system according to the manufacturer's protocols with specific primers (Supplemental Table S2). Quantification of relative gene expression was achieved by normalization to 18S ribosomal RNA.

YFP Fusion Protein Expression Analyses

The coding regions of the *CNGC5* and *CNGC6* cDNAs were cloned into pENTR/D-TOPO vector using specific primers (Supplemental Table S2), sequenced, and then transferred to the N-terminal fusions to YFP destination vector pH35YG by Gateway LR recombination reaction (Invitrogen). The *Agrobacterium tumefaciens* strain GV3101 carrying the gene of interest was used and infiltrated at an optical density at 600 nm of 0.5 together with the p19 strain in *Nicotiana benthamiana*. Mesophyll protoplasts were isolated from the leaves after 5 d of infiltration according to instructions (Asai et al., 2002; Cheng et al., 2002) and then treated with FM4-64 (Invitrogen). Fluorescence imaging was analyzed by confocal microscopy (Nikon Eclipse TE2000-U) using 488-nm excitation and 500- to 550-nm emission filters for YFP or 568-nm excitation and 580- to 650-nm emission filters for FM4-64.

Stomatal Movement Imaging

All experiments were conducted as genotype-blind experiments. ABA-dependent stomatal apertures were analyzed as described previously (Vahisalu et al., 2008; Siegel et al., 2009).

Whole-Rosette Stomatal Conductance Measurements

To analyze CO_2- and light/dark transition-induced changes in whole-plant stomatal conductance, we used 25- to 28-d-old plants and a custom-made gas-exchange device. The device and plant growth conditions have been described previously (Kollist et al., 2007; Vahisalu et al., 2008).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers *CNGC1* (At5g53130), *CNGC2* (At5g15410), *CNGC5* (At5g57940), *CNGC6* (At2g23980), *CNGC15* (At2g28260), and *CNGC20* (At3g17700).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Barium currents activated by 8Br-cGMP in guard cells.

Supplemental Figure S2. Na+ permeability of the 8Br-cGMP-activated currents in guard cells.

Supplemental Figure S3. *CNGC5* and *CNGC6* transcript analysis in *cngc5-2 cngc6-2* mutant.

Supplemental Figure S4. Inward currents activated by 20 mM cGMP.

Supplemental Figure S5. Modest inward currents activated by 100 μM cAMP.

Supplemental Figure S6. Twenty millimeters of cAMP failed to activate obvious inward currents in Columbia wild-type guard cells.

Supplemental Figure S7. ABA-activated I_{Ca} currents are Mg^{2+} permeable.

Supplemental Figure S8. Whole plant stomatal conductances in response to light/dark transition.

Supplemental Table S1. Transcriptome-derived raw expression data of *CNGC* transcripts in guard cells.

Supplemental Table S2. Oligonucleotides used in this work.

ACKNOWLEDGMENTS

We thank A. Boisson-Dernier for technical advice and T. Demura for the pH35YG plasmid.

AUTHOR CONTRIBUTIONS

The cGMP-activation of ionic currents in guard cells was initially found by I.M. and impairment in these currents in *cngc5*, *cngc6* and *cngc5 cngc6* mutants were found by Y.-F.W. both at UCSD. N.R. and N.N. isolated and genotyped *cngc* mutant alleles. S.M. and Y.-F.W. independently confirmed functional ABA-activation of I_{Ca} channels in mutant guard cells. N.N. analyzed the membrane localizations of YFP-CNGC proteins, and with S.L. and M.H. analyzed ABA-induced stomatal closing in *cngc* double mutant alleles, and H.-M.R. conducted additional stomatal response analyses. Most experiments were

conducted at UCSD with exception of patch clamp experiments in Figures 2E and 8 (S.M.), qPCR experiments in Figures 3C and S3C (H.-M.R., Y.-F.W.) and intact plant stomatal conductance analyses (H.K., I.P.). This project was proposed by J.I.S. Y.-F.W., J.I.S., N.N. and S.M. wrote the manuscript.

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