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 CNGC8 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 cngc5 cngc6 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.
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\(^{2+}\)]\(_{\text{cyt}}\) elevations, including Abscisic Acid Insensitive1 (ABI1), ABI2, Enhanced Response to Abscisic Acid1 (ERA1), the NADPH oxidases AtbodhD and AtbodhF, the Guard Enhanced Response to Abscisic Acid1 (ERA1), the Ca\(^{2+}\) channel genes (Ali et al., 2007; Michard et al., 2011; Hua et al., 2012). Electrophysiological analyses have characterized nonselective Ca\(^{2+}\)-permeable channel activity in the plasma membrane of guard cells (Schuurink et al., 1998; Li et al., 2000; Niu et al., 2000; Hu et al., 2001; Cao et al., 2002; Miao et al., 2006; Hu et al., 2012). [Ca\(^{2+}\)]\(_{\text{cyt}}\) 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; Miao et al., 2006; Mori et al., 2006; Hu 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; Hu 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; Arai 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; Urguhart 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; Friesch 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 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](image-url)
Cl\textsuperscript{−} 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\textsuperscript{2+} and Cl\textsuperscript{−} 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\textsuperscript{2+} (+23 mV) but far from that of Cl\textsuperscript{−} (−81 mV). These data show a Mg\textsuperscript{2+} permeability of the 8Br-cGMP-activated currents. We tested the effects of La\textsuperscript{3+} and Gd\textsuperscript{3+}, two well-known plant Ca\textsuperscript{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\textsuperscript{3+} (n = 3) or 100 μM Gd\textsuperscript{3+} (n = 3; Fig. 2, A and B). We further tested the selectivity of 8Br-cGMP-induced currents to cations by replacing Mg\textsuperscript{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\textsubscript{2} was replaced by BaCl\textsubscript{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\textsubscript{2} solutions, as described previously (Pei et al., 2000), and found the 8Br-cGMP-activated Ba\textsuperscript{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\textsuperscript{2+} (+20 mV) but distant from the equilibrium potential of Cl\textsuperscript{−} (−53 mV). 8Br-cGMP-activated Ba\textsuperscript{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\textsuperscript{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\textsubscript{2} was replaced by CaCl\textsubscript{2} in the bath solution with BaCl\textsubscript{2} in the pipette solution (n = 9) and the reversal potential was +17 mV, far from the equilibrium potential of Cl\textsuperscript{−} (−53 mV; Fig. 2E). The permeability ratio for Ba\textsuperscript{2+} relative to Ca\textsuperscript{2+} was 1.46, as determined according to the Goldman-Hodgkin-Katz equation (Hille, 1992). These results show that 8Br-cGMP activates nonselective Ca\textsuperscript{2+}-permeable channels.
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 Ca²⁺-permeable cation (I₄₋₆) channels in guard cells (Kwak et al., 2003). Taken together, these results showed that cGMP activates non-selective Ca²⁺-permeable cation channels in the plasma membrane of guard cells. Therefore, we named the 8Br-cGMP-activated non-selective Ca²⁺-permeable cation currents I₄₋₆.

Guard Cell-Expressed Putative CNGC Genes

Genes encoding defined plasma membrane Ca²⁺-permeable channels remain poorly understood in guard cells. To identify putative Ca²⁺-permeable channel genes underlying I₄₋₆, 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).

Mutations in CNGC5 and CNGC6 Impaired I₄₋₆

To pursue the identification of genes encoding ion channels that mediate I₄₋₆ 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 current magnitudes of I₄₋₆ 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 ≤ 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 I₄₋₆ channel currents in Arabidopsis guard cells.

To further test the effects of mutations in CNGC5 and CNGC6 on I₄₋₆, we generated a second double mutant allele, cngc5-2 cngc6-2, in the Wassilewskija (Ws) accession. CNGC6 mRNA levels in the cngc5-2 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 CNGC5 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 I₄₋₆ 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.
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-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 \(\mu\)M 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 CAMP-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...
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-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-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-CGMP currents in Columbia wild-type guard cells (Fig. 7A). I_cat-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-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 cngc5 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, CO₂, and Light/Dark Transition-Induced Stomatal Signaling

ABA triggers cytosolic Ca²⁺ increases and activates nonselective Ca²⁺-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).

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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.

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.
Similar to I_{cGMP}, ABA-activated ICa 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 ICa 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 ICa 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 ICa 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 ICa channel currents (Pei et al., 2000; Murata et al., 2001). We next analyzed I_{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_{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 ICa 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 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 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.

Figure 11. Time-resolved patterns of intact whole-plant rosette stomatal conductances in response to elevated (A) and reduced (B) CO2 concentration in cngc5-1 cngc6-1 double mutant and Columbia (Col) wild-type plants. To analyze elevated CO2-induced stomatal closure, the ambient CO2 concentration was increased from 400 to 800 μL L⁻¹ at time zero (A). To analyze low-CO2-induced stomatal opening, CO2 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 Ca2+-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 Ica channels.

In previous studies of reactive oxygen species and ABA activation of Ca2+-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 Ica and cGMP-activated Icat-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 Ca2+-permeable cation channels in pollen (Wu et al., 2011). Note, however, that these results do not exclude additional regulation of Ca2+-permeable channels by ATP-dependent mechanisms or protein kinases, as demonstrated previously for guard cell Ica 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 Ica channels is disrupted in the Ca2+-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 Ica channel activity was not disrupted by mutations in CNGC5 and CNGC6 (Fig. 8). In addition, we found that the ABA-insensitive cca2 and abi1-1 mutants, for which ABA activation of Ica channels is impaired (Pei et al., 2000; Murata et al., 2001; Miao et al., 2006), showed Ica-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 Ca2+-permeable cation-permeable channel activity in the plasma membrane of plants. CNGC5 and CNGC6 are required for the cGMP activation of Icat-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_042237) mutants were obtained from the Arabidopsis Biological
Resource Center. cngc5-2 (FLAG:295E04) and cngc6-2 (FLAG:48H18_D11) were in the Ws background and obtained from the INRA. cngc1 and cngc20 were in the Ws background and kindly provided by Dr. Hielil 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-cGMP}, the bath solution contained 92.5 mM MgGlu, 7.5 mM MgCl₂, and 10 mM MES-HCl (pH 5.6), and osmolarity was adjusted to 485 mMol L⁻¹ using d-sorbitol. The pipette solution contained 9.75 mM MgGlu, 0.25 mM EGTA, and 10 mM HEPES-Tris, pH 7.1, and osmolarity was adjusted to 300 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-cGMP} recordings. Control experiments performed in the absence of DTT showed that 8Br-cGMP continued to activate I_{cat-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-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 Schneider, 1994) and corrected in Figure 1B. No leak subtraction was applied to the depicted data. A 1 kf 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 ICa were performed using a ramp voltage protocol from +20 to 180 mV (holding potential, 0 mV; ramp speed, 200 mV s⁻¹) was applied for I_{cat-cGMP} recordings. Whole-cell currents were recorded every minute for 10 min after 10 mM MES-HCl (pH 5.6), and osmolarity was adjusted to 485 mMol L⁻¹ using d-sorbitol. The pipette solution contained 9.75 mM MgGlu, 0.25 mM EGTA, and 10 mM HEPES-Tris, pH 7.1, and osmolarity was adjusted to 300 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-cGMP} recordings. Control experiments performed in the absence of DTT showed that 8Br-cGMP continued to activate I_{cat-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-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 Schneider, 1994) and corrected in Figure 1B. No leak subtraction was applied to the depicted data. A 1 kf 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.

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

Total RNA was isolated with an RNaseasy 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 CNGC3 and CNGC6 were amplified by 35 PCR cycles using specific primers (Supplemental Table S2). The EF-1α transcript was amplified as a control 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 1 μg of 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 CNGC3s and CNGC6 s 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 FM-64 (Invitrogen). Fluorescence imaging was analyzed by confocal microscopy (Nikon Eclipse TE2000-U) with 488-nm excitation and 500- to 550-nm emission filters for YFP or 568-nm excitation and 580- to 650-nm emission filters for FM-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 CO₂ 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 (At5g31300), 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 GMP.

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_{cat} currents are Mg⁺ 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.

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AUTHOR CONTRIBUTIONS

The CGM-activation of ionic currents in guard cells was initially found by LM. 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_{cat} channels in mutant guard cells. N.N. analyzed the membrane localizations of YFP-CNGC proteins, and with S.L. and M.H. analyzed additional stomatal response analyses. Most experiments were...
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