A Suppressor Screen of the Chimeric AtCNGC11/12 Reveals Residues Important for Intersubunit Interactions of Cyclic Nucleotide-Gated Ion Channels

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To investigate the structure-function relationship of plant cyclic nucleotide-gated ion channels (CNGCs), we identified a total of 29 mutant alleles of the chimeric AtCNGC11/12 gene that induces multiple defense responses in the Arabidopsis (Arabidopsis thaliana) mutant, constitutive expresser of PR genes (cpr22). Based on computational modeling, two new alleles, S100 (AtCNGC11/12:G459R) and S137 (AtCNGC11/12:R381H), were identified as counterparts of human CNGA3 (a human CNGC) mutants. Both mutants lost all cpr22-mediated phenotypes. Transient expression in Nicotiana benthamiana as well as functional complementation in yeast (Saccharomyces cerevisiae) showed that both AtCNGC11/12:G459R and AtCNGC11/12:R381H have alterations in their channel function. Site-directed mutagenesis coupled with fast-protein liquid chromatography using recombinantly expressed C-terminal peptides indicated that both mutations significantly influence subunit stoichiometry to form multimeric channels. This observation was confirmed by bimolecular fluorescence complementation in planta. Taken together, we have identified two residues that are likely important for subunit interaction for plant CNGCs and likely for animal CNGCs as well.

Cyclic nucleotide-gated ion channels (CNGCs) were first discovered in retinal photoreceptors and olfactory sensory neurons (Zagotta and Siegelbaum, 1996; Kaupp and Seifert, 2002). CNGCs play crucial roles for the signal transduction in these neurons that are excited by photons and odorants, respectively. In mammalian genomes, six CNG genes have been found and named CNGA1 to CNGA4, CNGB1, and CNGB3 (Kaupp and Seifert, 2002). It has been reported that in mammalian cells, CNGCs function as heterotetramers that are composed of A and B subunits with cell-specific stoichiometry (Kaupp and Seifert, 2002; Cukkemane et al., 2011). For example, CNGCs in rod photoreceptors are composed of three A1 subunits and one B1a subunit, whereas in cone photoreceptors, they are believed to be composed of two A3 and two B3 subunits (Zhong et al., 2002; Peng et al., 2004). The structure of each subunit is similar to that of the voltage-gated K+ selective ion channel (Shaker) proteins, including a cytoplasmic N terminus, six membrane-spanning regions (S1–S6), a pore domain located between S5 and S6, and a cytoplasmic C terminus (Zagotta and Siegelbaum, 1996). However, CNGCs are only weakly voltage dependent and are opened by the direct binding of cyclic nucleotides (cAMP and cGMP), which are universally important secondary messengers that control diverse cellular responses (Fesenko et al., 1985). The cytoplasmic C terminus contains a cyclic nucleotide-binding domain (CNBD) and a C-linker region that connects the CNBD to the S6 domain. CNGC activity is also regulated by feedback inhibitory mechanisms involving the Ca2+ sensor protein, calmodulin (CaM). CaM-binding sites in animal CNGCs have been found in various regions of both the C- and N-terminal domains (Ungerer et al., 2011). It has been reported that the subunit composition has significant influence on the mode of CaM-mediated regulation (Kramer and Siegelbaum, 1992; Bradley et al., 2004; Song et al., 2008).

On the other hand, plant CNGCs have only been investigated much more recently. The first plant CNGC, HvCBT1, was identified as a CaM-binding transporter protein in barley (Hordeum vulgare; Schuurink et al., 1995). Subsequently, several CNGCs were identified from Arabidopsis (Arabidopsis thaliana) and tobacco (Nicotiana tabacum; Arai et al., 1999; Köhler and Neuhaus, 1998; Köhler et al., 1999). Interestingly, the Arabidopsis genome sequencing project identified a large family comprising 20 members (AtCNGC1–AtCNGC20), indicating a significant expansion of Arabidopsis CNGCs that suggests a higher level of diversity and diversity.
functional importance in plants (Mäser et al., 2001). To date, possible biological functions of Arabidopsis CNGCs in development, ion homeostasis, thermal sensing, as well as pathogen resistance have been reported (Kaplan et al., 2007; Chin et al., 2009; Dietrich et al., 2010; Moeder et al., 2011; Finka et al., 2012). With respect to structure, plant CNGCs are believed to have a similar architecture to their animal counterparts (Chin et al., 2009). However, only a handful of studies on the structure-function analysis of plant CNGCs have been published so far, and this field is still very much in its infancy (Hua et al., 2003; Bridges et al., 2005; Kaplan et al., 2007; Baxter et al., 2008; Chin et al., 2010).

Previously, we have reported two functionally important residues in plant CNGCs (Baxter et al., 2008; Chin et al., 2010). These residues were discovered using a suppressor screen of the rare gain-of-function Arabidopsis mutant \textit{constitutive expresser of PR genes} \textsuperscript{22} (\textit{cpr22}; Yoshioka et al., 2006). The \textit{cpr22} mutant, which has a deletion between \textit{AtCNGC11} and \textit{AtCNGC12} resulting in a novel but functional chimeric CNGC (\textit{AtCNGC11/12}), exhibits multiple resistance responses without pathogen infection in the hemizygous state and conditional lethality in the homozygous state (Yoshioka et al., 2001, 2006; Moeder et al., 2011). It has been reported that the \textit{cpr22} phenotype is attributable to the expression of \textit{AtCNGC11} and its channel activity (Yoshioka et al., 2006; Baxter et al., 2008), thereby making the suppressor screen an invaluable tool for identifying intragenic mutants to further elucidate the structure-function relationship of plant CNGCs (Baxter et al., 2008; Chin et al., 2010).

In this study, we describe a total of 29 mutant alleles of \textit{AtCNGC11/12}, including the three previously published alleles (Baxter et al., 2008; Chin et al., 2010), and compare their predicted three-dimensional structural positions with equivalent mutations of a human CNGC, CNGA3. In this analysis, two \textit{AtCNGC11/12} mutations emerged as counterparts of human mutations (Wissinger et al., 2001). Both the \textit{AtCNGC11/12} as well as the human CNGA3 mutations were computationally predicted to affect intersubunit interactions. This prediction was experimentally validated by size-exclusion chromatography (FPLC) as well as bimolecular fluorescence complementation (BiFC) in combination with site-direct mutagenesis using recombinant C-terminal peptides.

**RESULTS**

**Chimeric AtCNGC11/12 (cpr22) Suppressor Screening Identified 29 Mutant Alleles of AtCNGC11/12**

The suppressor screen of \textit{cpr22} was reported previously (Baxter et al., 2008). Through this screen, a total of 29 mutant alleles in \textit{AtCNGC11/12} have been discovered. Figure 1 shows a summary of the positions of these new alleles. This includes six premature stop codon mutations in various domains (Fig. 1; Supplemental Table S1), which further supports the previously reported

![Figure 1](https://www.plantphysiol.org/online-only/...)

**Figure 1.** Locations of the 29 mutations with respect to the proposed topological model of AtCNGC11/12, including the six transmembrane domains (S1–S6), the ion pore (P), the C-linker, and the CNBD. For details, see Supplemental Table S1. [See online article for color version of this figure.]
findings that cpr22 (AtCNGC11/12)-mediated phenotypes are attributable to the expression of AtCNGC11/12 (Yoshioka et al., 2006; Baxter et al., 2008). The remaining 23 mutations involve single amino acid substitutions caused by point mutations. In agreement with the mutagenizing effect of the alkylating agent, ethyl methanesulfonate, 22 out of 23 mutations are G/C to A/T conversions (Supplemental Table S1). One exception, suppressor81 (S81), has two single nucleotide mutations within one triplet, including a rare conversion of A to G. The mutations are located across all domains except the cytosolic N terminus. All mutants exhibited no readily discernible phenotypes compared with wild-type plants (data not shown).

### S100 and S137 Are Counterparts of Achromatopsic Human CNGA3 Mutants and Show Complete Suppression of AtCNGC11/12-Induced Phenotypes in Planta and Lost Channel Function in Yeast

CNGA3 is a human CNG gene that encodes one subunit of the cGMP-gated cone photoreceptor CNGC (Kaupp and Seifert, 2002). Wissinger et al. (2001) reported the first comprehensive screen for CNGA3 mutations in families with hereditary cone photoreceptor disorders. They described 46 mutations in CNGA3 that cause autosomal recessive complete achromatopsia linked to chromosome 2q11. Previously, we reported that the AtCNGC11/12 suppressor73 (S73), which has a mutation in the CNBD, is a counterpart of one of these human mutants (Fig. 1; Supplemental Table S1; Baxter et al., 2008). This suggested an inherent level of conservation between plant and animal CNGCs and further indicated that the suppressor screen for cpr22 is a useful tool to discover residues that are functionally important for CNGCs in general. In this study, we further investigate other mutations in AtCNGC11/12 that are in structurally equivalent positions to those in human mutants.

First, three-dimensional computational analysis was conducted for 12 suppressor mutations (S17, S23, S35, S81, S83, S84, S85, S100, S135, S137, S140, and S144) that are located in the cytoplasmic C-terminal region (Fig. 1; Supplemental Table S1), since this region contains important regulatory domains. As a template, the crystal structure of the cytoplasmic C-terminal region of the Sea urchin hyperpolarization-activated cyclic nucleotide-modulated (HCN) channel, SpIH (crystalized with cAMP; Flynn et al., 2007; Protein Data Bank no. 2PTM), that possesses the highest structural similarity to AtCNGC11/12 among the currently available crystal structures of cytosolic C termini, was used. Simultaneously, human CNGA3 mutations from Wissinger et al. (2001) that are located in similar areas to the selected 12 suppressor mutations were also modeled using the SpIH crystal structure. Interestingly, two mutants, S100 and S137, were identified to be equivalent to two CNGA3 mutants. S100 has a single amino acid substitution, Gly-459 to Arg (G459R), that is located at the loop between the first and second β-sheets (β1 and β2) in the β-barrel structure in the CNBD (Supplemental Fig. S1). S137 also has a single amino acid substitution, Arg-381 to His (R381H), that is located in the B’ helix of the C-linker domain (Supplemental Fig. S1). From a structural point of view, AtCNGC11/12:G459R (S100) and AtCNGC11/12:R381H (S137) are located in equivalent positions to the human mutants, CNGA3:G513E and CNGA3:R436W, respectively (Supplemental Fig. S1; Wissinger et al., 2001). These two residues are well conserved in both plants and animals. Both Gly-459 and Arg-381 are conserved in 19 out of 20 Arabidopsis CNGCs. The conservation of Gly-459 and Arg-381 was investigated using various CNGCs from different organisms. As shown in Table I, both residues are remarkably well conserved among a diverse range of organisms except the bovine olfactory epithelium CNGC channel, which has a Lys (K) instead of an Arg (R). However, Lys and Arg are similar in that they are both basic residues. Thus, the high degree of conservation of these two residues among CNGCs of various organisms strongly suggests the importance of these two positions for CNGC channel function.

As shown in Figure 2A (top panels), the Arabidopsis suppressor mutants, S100 and S137, show no detectable morphological differences compared with wild-type plants, indicating an alteration of AtCNGC11/12 function. As mentioned, the original mutant, cpr22, induces multiple pathogen resistance responses, including hypersensitive response-like programmed cell death without pathogen infection (Yoshioka et al., 2001). Thus, spontaneous cell death and pathogen resistance phenotypes were analyzed. As shown in Figure 2A (bottom panels), microscopic analysis revealed the abolishment of spontaneous cell death in both S100 and S137, which correlated with the suppression of morphological phenotypes (i.e. lethality). This was confirmed quantitatively by ion leakage analysis (Fig. 2B). Similarly cpr22-mediated pathogen resistance was also suppressed in both mutants (Fig. 2C). The expression of AtCNGC11/12 (AtCNGC11/12:G459R in S100 and AtCNGC11/12:R381H in S137) was confirmed by semiquantitative reverse transcription (RT)-PCR. No significant difference in the expression levels of AtCNGC11/12 was detected in S100 and S137 compared with cpr22.
indicating that the suppression of the cpr22-related phenotypes in S100 and S137 is attributable to alterations in channel function rather than a loss of expression of AtCNGC11/12.

To confirm the suppression of AtCNGC11/12-induced phenotypes, Agrobacterium tumefaciens-mediated transient expression of AtCNGC11/12:G459R and AtCNGC11/12:R381H was conducted in Nicotiana benthamiana. This system was previously established to analyze hypersensitive response-like cell death development and was recently used to show that AtCNGC11/12 induces cell death in a synchronized manner (Urquhart et al., 2007). As shown in Figure 3A, cell death was induced by the transient expression of AtCNGC11/12 but not by AtCNGC11/12:G459R, AtCNGC11/12:R381H, or the empty vector control. Transcription and translation of AtCNGC11/12, AtCNGC11/12:G459R, and AtCNGC11/12:R381H were monitored by semiquantitative RT-PCR and fluorescence of GFP that was fused to the C-terminal end of each gene. As shown in Figure 3, B and C, there is no significant difference between the expression of the original AtCNGC11/12 and the two mutated genes. Therefore, the suppression of cell death is not due to the expression levels of the mutated genes. Taken together, the two mutations, G459R and R381H, in AtCNGC11/12 suppress the induction of AtCNGC11/12-mediated phenotypes in planta.

Figure 2. Characterization of the suppressor mutants S100 and S137. A, Morphological phenotypes and spontaneous cell death formation of the wild type (Wt), cpr22, and suppressors S100 and S137. A cpr22 homozygous plant is shown in the white square. cpr22 homozygotes are lethal after the cotyledon stage under ambient temperature and humidity (22˚C, 65% relative humidity). Cell death was detected by trypan blue (TB) staining (black arrows indicate dead cells). Approximately 4-week-old plants were used. Bars = 1 cm. B, Quantitative analysis of cell death by electrolyte leakage in cpr22, S100, S137, and the wild type. C, Interaction phenotype with H. arabidopsidis isolate Emwa1. Dark-stained dots indicated by white arrows are oospores. D, RT-PCR analysis of cpr22, S100, and S137 for AtCNGC11/12 expression. β-tubulin served as a loading control (25 cycles). [See online article for color version of this figure.]

Figure 3. Cell death induced by AtCNGC11/12 in N. benthamiana was suppressed by S100 (G459R) and S137 (R381H). A, Induction of cell death in N. benthamiana after infiltration of A. tumefaciens carrying AtCNGC11/12:GFP, AtCNGC11/12:G459R:GFP, AtCNGC11/12:R381H:GFP, or empty vector (EV). B, RT-PCR analysis of leaf discs from N. benthamiana leaves expressing AtCNGC11/12:GFP, AtCNGC11/12:G459R:GFP, or AtCNGC11/12:R381H:GFP 24 h after infiltration. Actin served as a loading control (25 cycles). C, Expression of AtCNGC11/12:GFP, AtCNGC11/12:G459R:GFP, or AtCNGC11/12:R381H:GFP in N. benthamiana leaves at 32 h post infiltration was monitored by confocal microscopy. [See online article for color version of this figure.]
So far, precise characterizations of S73 and S58 have been reported, and it has been suggested that the two mutations affect AtCNGC11/12 function in fundamentally different ways: S73 (AtCNGC11/12:E519K) abolished basic channel function of AtCNGC11/12, whereas S58 (AtCNGC11/12:R557C) retained its channel function but displayed alterations in its regulation (Baxter et al., 2008; Abdel-Hamid et al., 2010; Chin et al., 2010).

To investigate whether AtCNGC11/12:G459R and AtCNGC11/12:R381H have lost basic channel function, functional complementation analysis was conducted in the trk1, trk2 K+ uptake-deficient yeast mutant, RGY516 (Ali et al., 2006). Enhanced growth of mutant yeast has been demonstrated previously upon the expression of various plant CNGCs (Köhler et al., 1999; Leng et al., 1999; Ali et al., 2006; Urquhart et al., 2007; Baxter et al., 2008; Chin et al., 2010). RGY516, transformed with AtCNGC11/12, AtCNGC11/12:G459R, or AtCNGC11/12:R381H, was tested in APG medium (see “Materials and Methods”). As shown in Figure 4A, AtCNGC11/12 was able to complement the trk1, trk2 growth-defect phenotype as expected, whereas AtCNGC11/12:G459R and AtCNGC11/12:R381H could not. Expression of the genes in yeast was confirmed by semiquantitative RT-PCR, and no significant difference between AtCNGC11/12 and mutants was detected (Fig. 4B).

Collectively, these data indicate that both G459R and R381H mutations in AtCNGC11/12 suppress AtCNGC11/12-induced phenotypes by abolishing AtCNGC11/12 channel function.

Computational Analysis Suggests an Effect of G459R and R318H on Intersubunit, But Not Intrasubunit, Interactions of CNGCs

To investigate the molecular mechanisms that cause the suppression of channel function by the G459R or R381H mutation, computational analyses were conducted. A previously published mutation, AtCNGC11/12:E519K (S73), caused alterations in intrasubunit interactions (Baxter et al., 2008). However, our computational modeling did not find any obvious disruption of intrasubunit interactions by G459R or R381H (Supplemental Fig. S2). This was also the case with equivalent mutants in human CNGA3, G513E and R436W (Supplemental Fig. S3), suggesting that these residues do not play significant roles in mediating intrasubunit interactions. We then analyzed the possible alterations of these mutations on intersubunit interactions. As mentioned, it has been reported that native photoreceptor CNGCs are heterotetrameric complexes composed of two structurally related subunit types, A and B subunits (Kaupp and Seifert, 2002). On the other hand, so far there is no experimental evidence describing the multimerization of plant CNGC subunits. However, in analogy to animal CNGCs, it has been speculated that plant CNGCs also form tetramers. Thus, the tetramer of the cytosolic C-terminal region of AtCNGC11/12 (same as wild-type AtCNGC12) was modeled (Fig. 5A). This model was created as a homotetramer, since there is no information about the composition of plant CNGCs, except that both AtCNGC11 and AtCNGC12 subunits can function as homomeric channels when expressed in yeast (Yoshioka et al., 2006; Urquhart et al., 2007; Baxter et al., 2008). As shown in Figure 5B (top left panel), Gly-459 does not have any interactions with a neighboring subunit in this tetramer model, whereas G459R creates a new salt bridge with Asp-433 in a neighboring subunit (Fig. 5B, top right panel). This was also observed when we modeled the mutant of CNGA3 at the equivalent position, CNGA3:G513E. As mentioned, it has been suggested that in CNGCs of native rod cells, two distinct subunits, CNGA3 and CNGB3, form heterotetramers comprising three CNGA3 and one CNGB3 subunits (Kaupp and Seifert, 2002). In this case, two different types of neighboring subunit interactions, CNGA3-CNGA3 and CNGA3-CNGB3, exist (Supplemental Fig. S4A). Figure 5B (bottom panels) shows the area of Gly-513 in a CNGA3-CNGB3...
interaction in this heterotetramer. Gly-513 in CNGA3 does not interact with another subunit, similar to Gly-459 in AtCNGC11/12 (Fig. 5B, bottom left panel), whereas G513E created a repulsive interaction with Glu-457 in a neighboring subunit (Fig. 5B, bottom right panel). A similar prediction was also made in a CNGA3-CNGB3 interaction (Supplemental Fig. S4B).

Collectively, these models suggest that both G459R in AtCNGC11/12 and G513E in CNGA3 created a new interaction with a residue in a neighboring subunit, indicating that this residue is important for channel structure and that any alteration disrupts channel function.

On the other hand, the intersubunit interaction model of AtCNGC11/12 indicates that Arg-381 forms a salt bridge with Glu-412 in a neighboring subunit (Fig. 5C, top left panel). This salt bridge was disrupted by the R381H mutation (Fig. 5C, top right panel). As expected, the counterpart mutation of AtCNGC11/12:R381H in CNGA3, R436W, also disrupts a salt bridge between Arg-436 and Glu-467 of the neighboring subunit in the CNGA3-CNGA3 interaction (Fig. 5C, bottom panels). Additionally, R436W also disrupts an additional salt bridge formed between Arg-436 and Asp-507 in the same subunit (intrasubunit interaction). Again, a similar prediction was made for the CNGA3-CNGB3 interaction (Supplemental Fig. S4C).

Taken together, these computational models strongly suggest that Gly-459 and Arg-381 in AtCNGC11/2 likely influence the interaction between subunits similar to their counterparts in human CNGA3. Furthermore, these models demonstrate that the structural roles of these residues in both plant and animal CNGCs are conserved.

**FPLC Analysis Indicates Alterations of Multimerization of S100 and S137 Mutants**

Electrophysiological studies and biochemical analyses of animal CNGCs have indicated that several distinct subunits form functional channels in vivo (Kaupp and Seifert, 2002). In contrast, the native composition of CNGCs in planta remains to be elucidated, and there is currently no information about the intersubunit interactions in plant CNGCs (Moeder et al., 2011). Thus, to explore the above hypothesis that the two mutations, AtCNGC11/12:G459R and AtCNGC11/12:R381H, affect channel function by altering the tetramerization of the channels, recombinant proteins of the cytosolic C-terminal domain of both mutants as well as AtCNGC11/12 were expressed in Escherichia coli and multimerization was analyzed by FPLC.

Previously, the recombinant cytosolic C-terminal region of AtCNGC11/12 was expressed in E. coli (Baxter et al., 2008). Although it was successful, the expression level was relatively low (J. Baxter and K. Yoshioka, unpublished data). To improve the expression efficiency, a new construct was made in accordance with published data on the crystal structures of the C-terminal regions of HCN2 and SpIH (Zagotta et al., 2003; Flynn et al., 2007). Structural modeling of the C-terminal domain of AtCNGC11/12 was performed using the structure prediction tool Swiss-model (http://swissmodel.expasy.org//SWISS-MODEL.html) with both SpIH and HCN2 as templates to detect the C-terminal region of the AtCNGC11/12 that structurally aligned with the crystallized C-terminal region of these channels. Based on this model, a fragment beginning at Thr-352, just after the end of the sixth transmembrane domain.
(S6), extending through the C-linker and CNBD, and ending at Ala-567, was expressed in *E. coli*. The expressed protein showed the expected size of 26 kD including the poly-His tag, and the yield of the expressed protein was significantly higher compared with that of Baxter et al. (2008; data not shown).

Expressed proteins were then subjected to FPLC analysis using a size-exclusion column (Superdex-200; GE Healthcare). As shown in Figure 6A, the elution of the AtCNGC11/12 C-terminal recombinant protein resulted in two peaks. The second peak is about approximately 26 kD, which is the size of the predicted monomer peptide. The first peak was eluted much earlier (suggesting a mass of more than 150 kD), indicating the multimerization of monomer peptides (Fig. 6A). These peaks were collected separately and subjected to SDS-PAGE. Under denaturing conditions, both peaks showed the monomeric peptide of approximately 26 kD, supporting the idea that the first peak is a multimer of the AtCNGC11/12 C-terminal recombinant protein (Fig. 6B, top panel). The ratio of the protein amounts of the first and second peaks was 0.35:1 (Fig. 6A). To investigate if the two mutations, G459R and R381H, affect this multimerization pattern, both recombinant proteins, the C termini of AtCNGC11/12: G459R (S100) and AtCNGC11/12:R381H (S137), were also subjected to FPLC analysis. As shown in Figure 6C, the same two peaks were observed, but the elution pattern of the AtCNGC11/12:G459R protein was shifted toward the first peak, resulting in a ratio of the first and second peaks of 0.6:1. On the other hand, although the same two peaks were observed again, the elution pattern of AtCNGC11/12:R381H was shifted toward the second peak, resulting in a ratio of the first and second peaks of 0.15:1. The two peaks of AtCNGC11/12:R381H and AtCNGC11/12:G459R were also collected and subjected to SDS-PAGE as well as western blotting using an anti-His antibody (Bioshop) to detect the His-tagged proteins. It was confirmed that the first peak is a multimer of the second peak, similar to AtCNGC11/12 (Fig. 6B, bottom panel; data not shown). This analysis was repeated at least three times using independently extracted recombinant proteins for all three constructs with almost identical results.

**Figure 6.** Analysis of recombinant cytosolic C-terminal peptides. A, Size-exclusion chromatography (Superdex-200 16/60 FPLC column) of the AtCNGC11/12 C terminus revealed two peaks. The ratio of the first (multimer) to second (monomer) peak was 0.35:1. Molecular size markers are indicated by the square (ADH; 150 kD), triangle (bovine serum albumin [BSA]; 66 kD), and star (CA; 29 kD). B, Top panel, SDS-PAGE of first and second peaks of AtCNGC11/12. Both peaks migrated as monomers under denaturing conditions. Bottom panel, western blot of first and second peaks of AtCNGC11/12. Both peaks migrated as monomers under denaturing conditions. Bottom panel, western blot of first and second peaks of AtCNGC11/12:G459R and AtCNGC11/12:R381H using an anti-His antibody. C, FPLC analysis of the AtCNGC11/12: G459R C-terminal peptides showed two peaks. The ratio of the first to second peak was 0.6:1. D, FPLC analysis of the AtCNGC11/12:R381H C terminus detected two peaks. The ratio of the first to second peak was 0.15:1. E, FPLC analysis of the AtCNGC11/12:D433S C terminus detected two peaks. The ratio of the first to second peak was 0.35:1. F, FPLC analysis of the AtCNGC11/12: E412L C terminus detected two peaks. The ratio of the first to second peak was 0.15:1. All experiments were repeated at least two times using independently extracted peptides with almost identical results. [See online article for color version of this figure.]
The FPLC analysis supports the hypothesis generated by the computational prediction. As shown in Figure 5B (top right panel), G459R seems to create a new salt bridge with Asp-433 in the neighboring subunit. This interaction may cause tighter subunit interactions, resulting in the shift toward multimerized peptides shown by the FPLC analysis. Similarly, as explained earlier, R381H seems to disrupt the interaction between Arg-381 and Glu-412 (Fig. 5C, top right panel). This may be the reason for the shift toward the monomer peak seen in the FPLC analysis. However, the possibility of non-specific aggregation of the peptides cannot be excluded.

Thus, we created mutant peptides by site-directed mutagenesis to address the specificity of the effect of the mutations. The expressed peptides were subjected to the same FPLC analysis. For AtCNGC11/12:D433S, the AtCNGC11/12:D433S construct was generated. Asp-433 is the predicted partner of the salt bridge with G459R (Fig. 5B). In D433S, the charge of Asp-433 changes from negative to neutral (Asp to Ser; Supplemental Fig. S5, top panels). Since Gly-459 is also neutral, this D433S mutation should not create a salt bridge with Asp-433 (Supplemental Fig. S5, bottom panels). In AtCNGC11/12:R381H, the partner of the salt bridge of Arg-381 (Glu-412) was mutated to change the charge of this position from negative (Glu) to neutral (Leu). By this mutation, the same disruption of the salt bridge should be recreated from the salt bridge partner side. As shown in Figure 6F, the FPLC elution pattern of AtCNGC11/12:R381H shifted toward the second peak, as seen with AtCNGC11/12:R381H in Figure 6D. The ratio between the first and second peaks was 0.15:1, which is identical to that observed for AtCNGC11/12:R381H.

Taken together, the FPLC analysis indicates that both AtCNGC11/12:D459R and AtCNGC11/12:R381H influence the multimerization of CNGC subunits through the creation/disruption of salt bridges between neighboring subunits, supporting the predictions from our computational modeling.

**BiFC Shows That G459R and R318H Affect Intersubunit Interactions in Planta**

To date, no subunit interaction of plant CNGCs has been demonstrated in planta. However, BiFC, a powerful tool to study in vivo protein-protein interactions, can be used to visualize CNGC subunit interaction in planta. Thus, the interaction of AtCNGC11/12, AtCNGC11/12:G459R, or AtCNGC11/12:R381H as a homomultimer in planta was analyzed by BiFC via A. tumefaciens-mediated transient expression in N. benthamiana. For this analysis, three versions of each gene were made, fused with (1) full-length yellow fluorescent protein (YFP), (2) the N-terminal half of YFP (nYFP), or (3) the C-terminal half of YFP (cYFP). As shown in Figure 7A, YFP fluorescence signals were detected for all three constructs with full-length YFP to similar levels, and they localized to the plasma membrane, as we have observed with our GFP fusion in this study (Fig. 3C) as well as previous work (Urquhart et al., 2007; Baxter et al., 2008). Note that, occasionally, punctuated signals that overlay with chloroplasts were observed. However, these signals were likely autofluorescence of chloroplasts, not YFP signals, since they can also be seen when we infiltrate only A. tumefaciens without BiFC vectors.

When we cotransformed with pairs of split YFP fusions (nYFP and cYFP), relatively weak but steady YFP fluorescence signals were detected with AtCNGC11/12 (11/12:nYFP + 11/12:cYFP in Fig. 7B), indicating that AtCNGC11/12 creates a homomultimer, likely a homotetramer, in planta. However, such YFP signals were not observed with either of the mutant constructs (G459R: nYFP + G459R:cYFP and R381H:nYFP + R381H:cYFP in Fig. 7B). To confirm the specificity of this interaction, we created another set of constructs for AtCNGC2, which is an unlikely partner of AtCNGC11/12 (Moeder et al., 2011), as a negative control. As shown in Supplemental Figure S6, YFP fluorescence signals were detected for AtCNGC2 with full-length YFP. However, when we cotransformed with pairs of split YFP fusions for AtCNGC11/12 and AtCNGC2, only background signals, similar to AtCNGC11/12:G459R or AtCNGC11/12:R381H, were detected (Fig. 7B; Supplemental Fig. S6). Taken together, these data indicate that the signals we observed with AtCNGC11/12 are specific and that both mutations cause a disturbance in subunit interaction in planta.

**DISCUSSION**

The oligomerization (tetramerization) of plant channels has been well studied for plant Shaker-type potassium channels such as AKT1, SKOR, GORK, KDC1, KAT1, and KAT2, which have a similar structure to plant CNGCs (Daram et al., 1997; Dreyer et al., 1997, 2004; Urbach et al., 2000; Zimmermann et al., 2001; Naso et al., 2009). For example, Daram et al. (1997) demonstrated the tetramerization of Arabidopsis AKT1 biochemically. They successfully expressed full-length AKT1 in Sf9 insect cells. Size-exclusion chromatography using recombinant AKT1 revealed monomers, dimers, and tetramers of AKT1. Here, although we have not observed peaks that show the exact size of dimers or tetramers, we have detected monomers and multimers...
by size-exclusion chromatography. The reason for not seeing peaks that show the exact size of the tetramers is currently unknown, but it could be related to the difference in biochemical characteristics between AKT1 and Arabidopsis CNGCs.

Using a yeast two-hybrid system, Daram et al. (1997) also identified two important domains in AKT1 that are involved in subunit interactions. Both domains are located in the cytosolic C-terminal region: one at the beginning of this region including the CNBD and the other at the C-terminal end of this region. Interestingly, the former one, which was revealed to be the essential domain for subunit interactions, is equivalent to the C-linker domain of plant CNBDs, which contains mutation S137 characterized in this study. This indicates the importance of the C-linker domain for subunit interaction of both plant Shaker-type channels and CNGCs in general.

S137 (R381H) is one of the most interesting positions among the 29 mutations discovered in this study. Not only is this mutation equivalent to a human CNGA3 mutation that causes achromatopsia (Wissinger et al., 2001), but this position was previously reported to be important for subunit interaction, affecting channel gating in HCN2 and CNGA1 (Craven and Zagotta, 2004). The C-linker domain comprises six α-helices (A’–F’), and the CNBD comprises three α-helices (A–C) and eight antiparallel β-strands (β1–β8) that form a β-barrel structure between the A and B helices. Through the crystal structure of the tetramer of the cytosolic C-terminal region of HCN2, Zagotta et al. (2003) discovered that all of the intersubunit interactions in the cytosolic C-terminal region occur between the C-linker domains of each subunit. The interacting region was described as “elbow on the shoulder,” where the A’ and B’ helices of one subunit (elbow) rest...
on the C' and D' helices (shoulder) of its neighboring subunit, and their interaction involves many hydrogen bonds, hydrophobic interactions, and salt bridges (Zagotta et al., 2003). Craven and Zagotta (2004) further conducted a precise analysis of one of these C-linker interactions and identified two salt bridges: an important intersubunit interaction between the C-linkers of neighboring subunits and a less important intrasubunit interaction between the C-linker and its CNBD. These two salt bridges are created by three residues, one positively charged residue in the B' helix of the C-linker (Arg/Lys) and two negatively charged residues: one on the D' helix at the C-linker domain (Glu) and another in the loop between B1 and B2 of the CNBD domain (Asp; Fig. 8A). Based on our model, the positions of these three residues are equivalent to Arg-436 (positively charged), Glu-467, and Asp-507 (negatively charged) in CNGA3 (Fig. 5C; bottom left panel).

Craven and Zagotta (2004) created point mutations to disrupt these salt bridges in HCN2 and CNGA1 and discovered that the disruption of these salt bridges favored channel opening. In other words, these interactions are involved in stabilizing the resting configuration (closed form) of the channels. These three residues are very well conserved among HCN and CNGCs (Craven and Zagotta, 2004; Fig. 8A), further indicating their importance. As explained in “Results,” Arg-436 in CNGA3 is the equivalent position to Arg-381 in AtCNGC11/12 (identical to 12; see “Materials and Methods”). Furthermore, our computational modeling revealed a salt bridge between Arg-381 and Glu-412 in the neighboring subunit (Fig. 5C; top left panel), which is almost identical to the interaction between Arg-436 and Glu-467 in CNGA3 (Fig. 5C; bottom left panel) as well as the interaction in HCN2 and CNGA1 described by Craven and Zagotta (2004). Arg-381 and Glu-412 are conserved in 19 out of 20 Arabidopsis CNGCs, further indicating the importance of this salt bridge (data not shown). The less important second salt bridge between Arg-436 and Asp-507 in CNGA3 (Fig. 5C; bottom left panel), which is involved in intrasubunit interaction, does not exist in 18 out of 20 Arabidopsis CNGCs, including AtCNGC12 (11/12). However, this second and likely less important salt bridge was predicted when we modeled homotetramers of AtCNGC2 and AtCNGC4 (Fig. 8B). Both AtCNGC2 and AtCNGC4 have a negatively charged residue, Glu-556 and Glu-521, respectively, instead of Ser-450 at this position in AtCNGC11/12 (12), and this residue creates a salt bridge with Arg-485 and Arg-450 in AtCNGC2 and AtCNGC4, respectively, similar to that formed between Asp-507 and Arg-436 in CNGA3 or the equivalent positions in HCN2 and CNGA1 (Fig. 8A; Craven and Zagotta, 2004). Based on sequence similarity, the 20 members of the Arabidopsis CNGC family are classified into four groups, I, II, III, and IV.
while group IV was further divided into IVA and IVB. AtCNGC2 and AtCNGC4 are the sole members of group IVB and share high sequence similarity as well as physiological function (Mäser et al., 2001; Moeder et al., 2011). Furthermore, recent bioinformatics analyses using moss (Physcomitrella patens) and Arabidopsis CNGCs indicated a diversification of biological function of AtCNGC2 and AtCNGC4 along with AtCNGC19 and AtCNGC20 from the rest of the CNGCs (Finka et al., 2012). It will be of interest to explore whether the structural difference predicted here can discriminate these two members from the others in terms of their channel-gating machinery.

There are four known subunits of HCN channels, HCN1 to HCN4 (Robinson and Siegelbaum, 2003). The subunit composition of native HCN channels is not known. But since HCN2 can form functional homomeric channels in heterologous systems, Zagotta et al. (2003) solved the crystal structure of HCN2 as a homotetramer, and their work has become the foundation for subsequent findings on the gating machinery of HCN as well as CNGCs. Similarly, there are 20 members of Arabidopsis CNGCs, and to date, no information about the subunit composition of native channels is available. However, since the expression of AtCNGC1, AtCNGC2, AtCNGC3, AtCNGC11, AtCNGC12, or AtCNGC11/12 can functionally complement various yeast ion channel mutants by themselves (Köhler et al., 1999; Leng et al., 1999; Yoshioka et al., 2006; Baxter et al., 2008; Chin et al., 2009, 2010), these subunits are believed to form functional homomeric channels in this heterologous system. In addition, electrophysiological studies using AtCNGC1, AtCNGC2, and AtCNGC4 have shown that these subunits also can function as homooligomers (Leng et al., 1999; Balagüé et al., 2003). Therefore, in this study, we generated a model of a homotetramer and identified two mutations that likely affect subunit interactions. This prediction was validated experimentally by FPLC and in planta BiFC analysis. To our best knowledge, this is the first report demonstrating plant CNGC subunit interactions in planta. For future work, elucidating the subunit composition of native channels will be essential. Since the 20 Arabidopsis CNGC subunits can potentially create countless combinations of heterotetramers, BiFC used in this study can be an effective tool to identify the subunit composition in the future.

**MATERIALS AND METHODS**

**Plant Growth Conditions**

Arabidopsis (Arabidopsis thaliana) plants were grown on Pro-Mix soil (Premier Horticulture) in a growth chamber under ambient humidity as described by Silva et al. (1999). Nicotiana benthamiana plants were grown on the same soil in a growth chamber under a 9/15-h light/dark regimen at 22°C (day) and 20°C (night).

**Pathogen Infection**

Infection of Hyaloperonospora arabidopsidis isolate Emw1 was performed as described previously (Yoshioka et al., 2001).

**Suppressor Screening and Identification of the S100 and S137 Mutants**

Suppressors S100 and S137 were identified in the same screen that was described by Baxter et al. (2008).

**Agrobacterium tumefaciens-Mediated Transient Expression**

A. tumefaciens-mediated transient expression in N. benthamiana was performed as described by Urquhart et al. (2007). The expression of these genes was confirmed by semiquantitative RT-PCR (see “RNA Extraction and RT-PCR”) and confocal microscopy (see “Confocal Microscopy”).

**Ion Leakage Analysis and Trypan Blue Staining for Cell Death Detection**

For ion leakage analysis, four leaf discs (0.5 cm) were floated in 5 mL of distilled water. After 15 min, the conductivity was determined using an Oakton Corr® Acorn series conductivity meter (Oakton Instruments). Trypan blue staining was performed as described previously (Yoshioka et al., 2001).

**RNA Extraction and RT-PCR**

Small-scale RNA extraction was carried out using the TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. RT-PCR was performed using complementary DNA (cDNA) generated by SuperScript II Reverse Transcriptase (Invitrogen) according to the manufacturer’s instructions. For the detection of CNGC and Actin gene expression in N. benthamiana, CNGC and Actin gene expression in yeast, and β-tubulin gene expression in Arabidopsis, the same sets of primers described by Baxter et al. (2008) were used.

**Plasmid Construction and Site-Directed Mutagenesis**

The yeast expression vector plasmid pYES2-empty vector (Invitrogen) and pYES2-AcNGC12 were constructed as described previously (Yoshioka et al., 2006; Baxter et al., 2008). For pYES2-AcNGC12:G459R and pYES2-AcNGC12:R381H, total RNA was extracted from S100 and S137 plants, respectively, and cDNA was generated as described above. Cloning into pYES2 was performed as described previously for S88 by Chin et al. (2010). For site-directed mutagenesis, the point mutations D435S and E412L were created in the AcNGC12 cDNA in pllluvector SK+ (Baxter et al., 2008) using the QuickChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. For protein expression, the portions from the beginning of the C-linker to the end of the CNBD (T1048–G1708) of the cDNA of AcNGC11/12, AcNGC11/12:R381H, AcNGC11/12:G459R, AcNGC11/12: D435S, and AcNGC11/12:E412L were subcloned into the BamHI-NdeI sites of the Escherichia coli expression vector pET28a (Novagen; http://www.emdbiosciences.com) under the control of the T7 promoter with a His tag. All constructed plasmids were sequenced for fidelity and transformed into the E.coli strain BL21 (DE3 codon plus). For BiFC analysis, full-length AcNGC11/12, AcNGC11/12:G459R, or AcNGC11/12:R381H cDNA was subcloned into the Gateway entry vector pDONR207 (Invitrogen; http://www.invitrogen.com) using the primers CNGC11-GATEWAY-F (5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCTGC-3') and CNGC11-GATEWAY-R (5'-GGGGACCATTTGTACAAAGAAGCTGGTGCTGC- TTCAGGGCTTGGAAACT-3'). Subsequently, all clones were subcloned into the destination vectors for plant expression, pK2GWYn9 (nYFP), pL2GWYc9 (cYFP; kind gifts from Dr. Joseph Kieber), or pEARLEY101 (full-length YFP; Earley et al., 2006), using LR Clonase according to the manufacturer’s instructions. All constructed plasmids were sequenced for fidelity.

**Functional Complementation in Yeast**

The K+ uptake-deficient yeast strain RGY516 (trk1, trk2) was transformed with the yeast expression vector pYES2 (EV) and pYES2 containing AcNGC11/12, AcNGC11/12:R381H, and AcNGC11/12:G459R. Overnight cultures were grown in selective minimal medium supplemented with 1% KCl. Cultures were then washed five times with sterile water prior to inoculation. Growth assays were performed by inoculating transformed RGY516
yeast in 3 mL of APG medium (10 mM Arg, 8 mM phosphoric acid, 2% Glc, 2 mM MgSO4, 1 mM KCl, 0.2 mM CaCl2, trace minerals/elements, and vitamins, pH 3.2) on sterile 12-well plates supplemented with 15 mM K+. Yeast growth was monitored by measuring optical density at 600 nm at 24, 48, and 72 h at 30°C (Ali et al., 2006).

Confocal Microscopy

*Arabidopsis thaliana* was performed as described by Urquhart et al. (2007) at 22°C. Sections of the infiltrated area were excised and used for confocal microscopy. Confocal fluorescence images were acquired using a Leica TCS SP5 confocal system with an Acousto-Optical Beam Splitter (HCX PL APO CS 40× immersion oil objective; numerical aperture, 1.25), with the Acousto-Optic Tunable Filter for the argon laser set at 20% and the detection window at 500 to 600 nm for GFP and 525 to 600 nm for YFP (Leica Microsystems). Autofluorescence of chloroplasts was detected at 630 to 700 nm.

Computational Modeling and Sequence Alignment

The tertiary structure modeling of AtCNGC11/12 was conducted as described previously (Baxter et al., 2008) using the crystallographic structure of the cytoplasmic C terminus of the invertebrate CNGC, SpIH (Flynn et al., 2007; Protein Data Bank no. 2F7M). The protein fold recognition server (Phyre; Kelley and Sternberg, 2009) was used to model the protein coordinates with an estimated precision of 100%. The tetramer structure modeling of the C terminus of AtCNGC11/12 was conducted using SymmDock (Prediction of Complexes with n-fold cyclic/rotational (Cn) symmetry by Geometry BasedDocking: http://bioinfo3d.cs.tau.ac.il/SymmDock/; Schneidman-Duhovny et al., 2003), where the top 20 solutions were compared with the asymmetric unit of the structure of SpIH (Flynn et al., 2007). The symmetry that resembled the SpIH oligomerization was usually in the first five top solutions. The superimpositions were generated using DaliLite (Holm and Park, 2000). All the remaining images were generated using PyMOL (DeLano, 2002). The sequence alignments of the amino acid sequences of the 20 Arabidopsis CNGCs and other ion channels, starting from the B helix of the C-linker to the end of the second β-strand of the CNBD, were performed with ClustaW (Thompson et al., 1994). Although the C-terminal cytosolic region is identical between AtCNGC11/12 and AtCNGC12, numbers of residues used in the figures are from AtCNGC11/12.

Recombinant Protein Expression and FPLC Analysis

Protein expression in BL21 cells was induced by autoinduction as described by Studier (2005). Proteins were extracted in lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 2 mM MgSO4, 1 mM KCl, 0.2 mM CaCl2, trace minerals/elements, and vitamins, pH 3.2) on sterile 12-well plates supplemented with 15 mM K+. Yeast growth was monitored by measuring optical density at 600 nm at 24, 48, and 72 h at 30°C (Ali et al., 2006).

AtCNGC11/12 and AtCNGC2, numbers of residues used in the figures are from AtCNGC11/12.

Supplemental Data

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

Supplemental Figure S1. Superimposition of structural models of AtCNGC12 and CNGA3.

Supplemental Figure S2. Computational structural modeling of the cytoplasmic C-terminal region of AtCNGC11/12, AtCNGC11/12/G459R and CNGA3/11/12/R381H.

Supplemental Figure S3. Computational structural modeling of the cytoplasmic C-terminal region of CNGA/G513E and CNGA3/R436W.

Supplemental Figure S4. Computational structural modeling of tetramer structure of the cytoplasmic C-terminal region of human rod cell CNGC hetero-tetramer.

Supplemental Figure S5. Predicted interactions of G459 and R381 in A/CNGC11/12 tetramer.

Supplemental Figure S6. Confocal analysis of *N. benthamiana* leaves Agrobacterium-infiltrated with A/CNGC11/12 and A/CNGC2.

Supplemental Table S1. Summary of intragenic mutations in A/CNGC11/12.

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