EXPRESSION AND SUB-CELLULAR LOCALIZATION OF CYCLIC NUCLEOTIDE-GATED ION CHANNELS IN *ARABIDOPSIS THALIANA*

A THESIS SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAI'I IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

IN

MOLECULAR BIOSCIENCES AND BIOENGINEERING

MAY 2007

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ACKNOWLEDGEMENTS

I would like to express much gratitude to my advisor, Dr. David Christopher, for his valuable insight and support during the course of my research, and for giving me the opportunity to work in plant molecular biology. I would also like to thank my committee members, Drs. Dulal Borthakur and David Webb for their advice and guidance. Dr. Tamas Borsics made many, many constructs for me in a valiant attempt to express a CNGC-GFP fusion construct in protoplasts. As the resident CNGC expert, he also provided a wealth of useful suggestions, techniques, and support for accomplishing this work. Many thanks to Tina Carvahlo at the microscopy facility for training me on the confocal laser-scanning microscope and providing expertise for my immunolocalization experiments. All of my lab mates have made this experience very enjoyable and one I will always cherish. They have all passed knowledge on to me that has not only helped my current project, but will continue to strengthen me as a scientist for years to come.

Finally, I would like to thank Jared for his love and support and keeping me entertained throughout the process.
ABSTRACT

Cyclic nucleotide gated ion channels (CNGCs) are a 20-member gene family in the model plant, *Arabidopsis thaliana*, and play a role in the transport of both monovalent (ie. Na\(^+\), K\(^-\)) and divalent (ie. Ca\(^{2+}\), Mg\(^{2+}\)) cations across cellular membranes. CNGCs play vital roles in numerous processes required by living organisms, such as sight and smell in animals, and cell division, growth, and development in plants. Structurally, CNGCs contain six transmembrane domains, a pore region, and two regulatory domains, a calmodulin binding domain, and a cyclic nucleotide-binding domain (CNBD).

Previous work on Arabidopsis CNGCs has shown that mutations in several of the genes can cause aberrant regulation of cell death, loss of pathogen defense mechanisms, and altered sensitivity to cation concentration in growth media. Very little research has been done to determine the location and function of these channels at the cellular level. Two previously unstudied CNGCs, CNGC5 and CNGC11, were chosen for sub-cellular localization using immunolocalization and CNGC-GFP fusion protein expression. Both CNGC5 and 11 were found to immunolocalize to the plasma membrane of isolated plant cells. Focusing on CNGC5, T-DNA insertional mutant plants were also identified and found to have a knockdown in CNGC5 expression resulting in a slight growth deficiency under normal conditions. Sub-cellular location, along with T-DNA mutant phenotypes, will provide critical insight into determining the role of these CNGCs at the cellular level.
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<table>
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<tr>
<td>35S</td>
<td>Cauliflower mosaic virus 35S promoter</td>
</tr>
<tr>
<td>CaM</td>
<td>Calmodulin</td>
</tr>
<tr>
<td>CaMBD</td>
<td>Calmodulin binding domain</td>
</tr>
<tr>
<td>cAMP</td>
<td>adenosine 3',5'-cyclic monophosphate</td>
</tr>
<tr>
<td>CaMV35S</td>
<td>Cauliflower mosaic virus 35S promoter</td>
</tr>
<tr>
<td>cGMP</td>
<td>guanosine 3',5'-cyclic monophosphate</td>
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<tr>
<td>CNBD</td>
<td>Cyclic nucleotide binding domain</td>
</tr>
<tr>
<td>CNGC</td>
<td>Cyclic nucleotide gated ion channel</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>LBb1</td>
<td>T-DNA left border primer b1</td>
</tr>
<tr>
<td>LP</td>
<td>Left primer</td>
</tr>
<tr>
<td>mGFP5</td>
<td>Modified green fluorescent protein 5</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PM</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>RP</td>
<td>Right primer</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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<td>TMD</td>
<td>Transmembrane domain</td>
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CHAPTER 1

INTRODUCTION

In plants, cations are both essential macro- and micronutrients, as well as play critical roles in many cellular processes, such as signal transduction, growth, and development. While cations are an important component for normal cellular function, their over-abundance can also cause severe deleterious effects to the plant. Cation translocation across cellular membranes is a process that is tightly regulated by families of cation transporters expressed within the cell. In the model plant, Arabidopsis thaliana, one family of cation transporters is that of the cyclic nucleotide-gated ion channels (CNGCs). The Arabidopsis CNGC family is composed of 20 genes that encode putative non-selective monovalent (ie. K\(^+\), Na\(^+\)) and divalent (ie. Ca\(^{2+}\), Mg\(^{2+}\)) cation channels similar in structure to Shaker-type potassium channels (Maser, et al. 2001). CNGC structure is composed of six membrane-spanning domains (S1-6), a pore region for cation transport, and two regulatory domains, a cyclic nucleotide binding domain (CNBD) and a calmodulin-binding domain (CaMBD). Cation transport through CNGCs is activated by cyclic nucleotides (cAMP or cGMP) and inhibited by calmodulin (CaM).

CNGCs are found in both plants and animals and play critical roles in several physiological processes. In animals, CNGCs are known to function in neurons involved in sight and smell (Zagotta, et al. 1996), while in plants are shown to function in nutrient uptake, pathogen defense, and growth and development. In contrast to animal CNGCs, the two regulatory domains in plant CNGCs overlap at the C-terminus of the protein as
opposed to separate in the N- and C-terminus (Fig. 1). This implies a divergence in regulation between organisms, by means of a different mechanism that cyclic nucleotides and calmodulin physically activate or inhibit the channel, respectively.

**Figure 1.** Structural representation of a plant and animal CNGC.

Although originally discovered in barley in 1998 and subsequently found in many species, CNGCs in plants have been studied most extensively in the model plant *Arabidopsis thaliana*, though research has focused on only a few of the CNCGs encoded
in its genome. Alignment of the amino acid sequences of the 20 predicted Arabidopsis CNGCs results in a phylogenetic tree composed of 4 major groups, with Group IV being the most distantly related to any of the other CNGCs and to the subgroups within itself (Fig. 2, from Maser, et al. 2001). Of these CNGCs, research on CNGC1, 2, 3, 4, 10, 11, and 12 has been published, showing that these channels have significant roles in various physiological functions and have varying conductivity for cations such as K\(^+\), Na\(^+\) and Ca\(^{2+}\).

![Phylogenetic tree of Arabidopsis CNGCs](image)

Figure 2. Phylogenetic relationship of Arabidopsis CNGCs (from Maser, et al. 2001).

Research on Arabidopsis CNGCs has focused on their role in various physiological functions in the plant, as well as their function in heterologous expression.
systems. CNGC1, 2, and 4 were originally identified by groups studying pathogen defense responses in plants. Mutations in these genes were found to alter both the hypersensitive response (a process by which the plant limits the spread of infection by inducing a type of programmed cell death around the point of pathogen entry), as well as the expression of broad-spectrum defense genes (Yu, et al. 1998, Clough, et al. 2000, and Balague, et al. 2003). Later, CNGC11 and 12 were also found to effect pathogen resistance in plants (Yoshioka, et al. 2006). Loss of expression of either CNGC1 or 10 cause altered growth and gravitropic response in the root, phenotypes caused by a reduced uptake of Ca$^{2+}$ or K$^+$, respectively (Ma, et al. 2006 and Borsics, et al. 2006). Mutations in the CNGC3 gene cause a decrease in seed germination and tolerance to toxic levels of both Na$^+$ and K$^+$, suggesting that functional CNGC3 may non-selectively import these cations into the plant (Gobert, et al. 2006).

Despite advances in our knowledge of plant CNGCs, little is known of the cellular location and function of these channels. Localization of these channels to a specific subcellular membrane is a critical step to determining their specific role within the cell. Recently, CNGC10 was localized to the plasma membrane of leaf cells using antibody targeting visualized by electron microscopy (Borsics, et al. 2006). CNGC3 has also been localized to the plasma membrane, as determined by expression of a CNGC3-GFP fusion protein in isolated leaf cells (protoplasts) (Gobert, et al. 2006). GFP, or green fluorescent protein, is a gene identified in jellyfish that can be fused to another gene, and when expressed in cells, acts as a visual reporter for localization of the fused protein. Both of
these techniques, immunolocalization and GFP fusion protein expression, will be used to localize two CNGCs, CNGC5 and 11, to a sub-cellular membrane.

CNGC5 and 11 were selected for sub-cellular localization due to elevated mRNA expression levels relative to other unstudied CNGCs, both are strong candidates for antibody production in rabbits (the antibodies are used for immunolocalization), and there are T-DNA insertional mutant plant lines available for each gene to study the phenotype of a loss-of-function of each CNGC. Both immunolocalization and CNGC-GFP fusion expression were performed using leaf protoplasts, or cells isolated from leaf tissue (Fig. 3). A significant part of the localization process was spent optimizing protoplast isolation technique, the transient transfection procedure required to express the CNGC-GFP fusion in cells, and the fixation and immunolabeling process.

CNGC5 was immunolocalized to the plasma membrane of protoplasts, and CNGC11 was tentatively immunolocalized to the plasma membrane. Neither CNGC could be expressed as a CNGC-GFP fusion to confirm the plasma membrane localization. Expression of the fusion protein may be toxic to the cell, or proper protein folding and cellular targeting could not occur. CNGC5 knockdown mutants were also identified and show growth deficiencies under normal conditions. Localization of the channels to a cellular membrane, as well as the phenotype of plants with an insertional mutation in the gene, will help to illuminate the function of CNGC5 and 11 at the cellular level.
Figure 3. Leaf cell morphology as seen in a leaf section and as protoplasts. (A) Cells in a leaf section as seen by electron microscopy (image by DA Christopher), and as (B) protoplasts, released from the leaf with cell wall-digesting enzymes and visualized using bright light microscopy (40X magnification).
CHAPTER 2
LITERATURE REVIEW

2.1 Cation transport in Arabidopsis

The uptake of cations, or positively charged ions, into plants is not only an essential part of the plant’s growth and development, but also critical to the nutritional value of a plant to its animal consumers. Transport of cations within the plant is critical for plant turgor and osmotic regulation, maintenance of the membrane potential and ionic concentrations that favor enzyme activity and metabolic processes, all essential for normal physiological function in the plant (Gambale, et al. 2006 for review). Two macronutrient cations, potassium (K⁺) and calcium (Ca²⁺), are the most abundant cations found in plants, composing up to 10 and 2% of plant dry weight, respectively (Marschner, 1995). There are also several important micronutrient cations, such as copper, iron, manganese, and zinc that are required in lower concentrations, but also essential for the plant. The translocation of these cations from soil or water into the plant is the responsibility of a large number of cation transport proteins, embedded within cellular membranes. The most extensively studied plant is the model plant Arabidopsis thaliana, from whose genome over 150 different cation transporters have been identified (Maser, et al. 2001 for review). Although these genes have been identified, few of the gene products have been functionally characterized or identified for physiological significance. The best-characterized cation transport proteins are those existing for the movement of K⁺, which will be the main focus in this section of the review.
Cation transport across membranes is driven by the electrochemical gradient established by proton pumps, or H⁺-ATPases (Michelet, et. al. 1995). The movement of positively charged protons out of the cell allows for the exchange of other cations down the electrochemical gradient into the cell, a movement that is facilitated by cation transport proteins embedded in the membrane. Ion channels are pore-forming proteins that allow passive passage of ions down the electrochemical gradient, while ion pumps or transporters are also pore-forming proteins, but have the ability to move ions against the electrochemical gradient using either chemical energy (from the hydrolysis of ATP by ATPases) or from the energy obtained by the movement of other ions down their electrochemical gradient (symporters or antiporters) (Brownlee, 2002 for review). There can exist multiple families of cation transport proteins for movement of a single cation, presumably to transport the cation under varying environmental conditions and in response to specific stimuli.

2.1a Potassium and calcium transport in Arabidopsis

There are at least five families of K⁺ transporters in Arabidopsis: the KUP/HAK/KT transporters, the Trk/HKT transporters, KCO channels, Shaker-type channels, and K⁺/H⁺ antiporter homologs (Fox, et al. 1998 and Maser, et al. 2001). The information gathered on K⁺ transport proteins provides interesting insight to the possible structure and mechanism of other cation channels. There are high affinity K⁺ transporters of the KUP/HAK/KT gene family that import K⁺ when there is low external K⁺ present, (Schachtman, et al. 1994 and Rubio, et al. 1995) and low affinity K⁺ transporters that
passively transport $K^+$ down the electrochemical gradient when $K^+$ concentration is high on one side of the membrane. The majority of $K^+$ transporters are low affinity $K^+$ channels, and are either inward or outward rectifying (Fox, et al. 1998).

The protein structure of the $K^+$ transport proteins in Arabidopsis is composed of variable numbers of transmembrane domains (TMDs) that anchor the protein in a cellular membrane and at least one pore domain (P) that facilitates movement of cations through the channel (Schroeder, et al. 1994, Maathuis, et al. 1997, Doyle, et al. 1998). Many of the genes encode proteins composed of 6TM/1P or 4TM/2P structure, while others are composed of 8TM/4P or even 12TMDs (Maser, et al. 2001). Each gene encodes one subunit of a functional channel. Tetramers of 6TM/1P subunits have been shown to form a functional channel, with the four pore domains forming the cation pore embedded inside the transmembrane domains (Doyle, et al. 1998). A dimer channel has also been shown to form with two 4TM/2P subunits (Goldstein, et al. 1998).

Potassium channels have also been shown to possess other features that may confer pore selectivity, ligand gating, and structural arrangement. Shaker-type $K^+$ channels dominate $K^+$ transport within the cell and have been shown to possess a G-Y-G amino acid sequence in the pore region that selects for $K^+$ permeability at a significantly greater rate than any other cation (Anderson, et al. 1995, Kukuljan, et al. 1995 and Nakamura, et al. 1997). These channels also possess a C-terminal cyclic nucleotide-binding domain (Sentenec, et al. 1992 and Daram, et al. 1997), which may regulate the channel in the presence of cyclic nucleotides, as well as an ankyrin-binding domain that anchors the channel to the cytoskeleton (Daram, et al. 1997). The plant Shaker-type $K^+$
channels can also be broken into subgroups based on the conditions in which they transport K⁺; channels that move K⁺ inward, channels that move K⁺ outward, and those that can move in either direction, dependent on the electrochemical gradient. These channels are also differentially expressed in plant tissues, and show varying K⁺ transport ability dependent on the subunit composition of the channel (Gambale, et al. 2006 for review).

Calcium is a cation that is critical for many cell-signaling events. The maintenance of its concentration in organelles, the cytoplasm, and apoplasm is the responsibility of Ca²⁺ channels, Ca²⁺-ATPases and Ca²⁺/H⁺ antiporters. Calcium is transported passively along the electrochemical gradient by several channels that have been identified in Arabidopsis (White, et al. 2002 for review). The concentration of Ca²⁺ against the electrochemical gradient is important for signaling processes, and is established by Ca²⁺ transporters that use energy in the form of ATP or the energy stored in another ion’s gradient. Concentrated stores of calcium are released in the response to many stimuli, such as light, touch, pathogen infection, gravity, cold, and hormones that stimulate specific pathways in the cell to respond (Fox, et al. 1998).

There are potentially several hundred cation transport proteins in Arabidopsis responsible for the transport of many macronutrient and micronutrient cations essential for basic cellular and organismic functions. Disruption of some of these cation transport genes has shown that cation transport proteins impact a range of physiological responses in the plant, such as nutrient uptake, leaf and stomatal movement, pathogen defense response, phloem loading, pollen tube development, and root development, amongst
others (Fox, et al. 1998, White, et al. 2002, and Gambale, et al. 2006). Although large numbers of cation transport proteins may be relatively specific to one cation, the channels transport that cation under varying conditions and in response to different stimuli, making each transport protein critical to cellular function.

2.2 Cyclic nucleotide-gated ion channels in Arabidopsis

Cyclic nucleotide-gated ion channels (CNGCs) compose a 20-member gene family of non-selective cation channels in Arabidopsis thaliana, with a predicted structure similar to Shaker-type potassium channels. Analysis of the predicted amino acid structure of CNGCs show that the channels are composed of six transmembrane domains (S1-S6), a pore domain, and a cyclic nucleotide-binding domain (CNBD) that is overlapped by a calmodulin-binding domain (CaMBD), both of which act as regulatory domains (Kohler, et al. 1999 and Maser, et al. 2001). Several members of the Arabidopsis CNGC family have been studied and have been shown to transport K\(^+\), Na\(^+\), and Ca\(^{2+}\) to varying degrees as well as confer differing phenotypes in loss-of-function plants (Talke, et al. 2003 for review). Most Arabidopsis CNGCs remain unstudied, with the only CNGCs having published data are CNGC1, 2, 3, 4, 10, 11, and 12, and the exact function of these channels at the cellular level remains to be determined.

2.2a Discovery of CNGCs in plants

CNGCs in plants were originally discovered in 1998 in barley, Hordeum vulgare, after a barley cDNA expression library was probed for calmodulin (CaM) binding proteins. The gene was named HvCBT1, and was found to localize to the plasma
membrane of barley protoplasts when expressed as a HvCBT1-GFP fusion protein (Schuurink, et al. 1998). Nine more CNGCs have recently been identified in barley, two of which alter the expression of defense genes in the plant (Rostoks, et al. 2006). In a similar screen for CaM binding proteins, a CNGC, NtCBP4, was discovered in tobacco, *Nicotiana tabacum*. The channel was found to localize to the plasma membrane as determined by Western blot of cellular membrane fractions. When over-expressed in the plant, the channel conferred tolerance to elevated nickel (Ni\(^{2+}\)) and sensitivity to increased lead (Pb\(^{2+}\)) in the growth media, indicating that NtCBP4 has some selectivity for the toxic metal Pb\(^{2+}\) and not Ni\(^{2+}\) (Arazi, et al. 1999). When NtCBP4 was mutated, plants showed resistance to Pb\(^{2+}\), confirming that the channel can transport that metal specifically (Sunkar, et al. 2000). CNGCs have also been identified in the genomes of rice and bean (Talke, et al. 2003).

### 2.2b Functional characterization of Arabidopsis CNGCs

Despite early advances on CNGCs in barley and tobacco, the majority of research has been conducted on CNGCs expressed in Arabidopsis. CNGCs have been difficult to express in heterologous, or non-host systems, so few have been functionally characterized. Several complementation assays involving expression of CNGC1, 2, 3, 4, 10, 11, and 12 in yeast with cation uptake deficiencies have been reported. All of the CNGCs mentioned previously complement K\(^+\) uptake-deficient yeast, suggesting that they function as K\(^+\) channels (Kohler, et al. 1999, Leng, et al. 1999, Mercier, et al. 2004, Li, et al. 2005, Ali, et al. 2006, Gobert, et al. 2006, and Yoshioka, et al. 2006). The potassium transport ability of CNGC1 and 10 was confirmed by their ability to rescue K\(^+\)
uptake-deficient *E. coli* (Li, et al. 2005 and Ali, et al. 2006). Interestingly, CNGC1 was also able to complement Ca\(^{2+}\) uptake deficient yeast, while CNGC3 was not, but CNGC3 was able complement Na\(^+\) uptake deficient yeast (Ali, et al. 2006 and Gobert, et al. 2006).

Functional characterization of CNGC2 and 4 has also included expression in frog, *Xenopus laevis*, oocytes and the human embryonic kidney cell line HEK293. Voltage clamp analysis of *X. laevis* oocyte membranes was performed after expression of either CNGC2 or 4 in the cells. Voltage clamping works by isolation of a small patch of membrane and application of ions on either side of the membrane. Movement of ions is measured by a change in current across the membrane. CNGC1 and CNGC2 were shown to import K\(^+\) and other monovalent cations such as Cs\(^+\), Rb\(^+\), and Li\(^+\) when activated by the cyclic nucleotides cAMP and cGMP, but not Na\(^+\) (Leng, et al. 1999 and Leng, et al. 2002). CNGC4 was shown to transport K\(^+\) and Na\(^+\), also after activation using cAMP and cGMP (Leng, et al. 1999 and Balague, et al. 2003). When expressed in HEK293 cells, CNGC2 was found to be permeable to Ca\(^{2+}\), only in the presence of cAMP or cGMP (Leng, et al. 1999). Together, the functional characterization of these members of the Arabidopsis CNGC family show that the channels have the ability to transport K\(^+\), Na\(^{2+}\), and Ca\(^{2+}\) to varying extent and only after activation by cyclic nucleotides in some systems.

**2.2c Regulation of CNGCs in Arabidopsis**

Arabidopsis CNGCs possess an overlapping CNBD and CaMBD at the C-terminus, which is in contrast to animal CNGCs that possess a CaMBD separate from the CNBD at the N-terminus of the protein (Liu, et al. 1994, Zagotta, et al. 1996, and
Grunwald, et al. 1998). Cation transport in Arabidopsis CNGCs has been shown to be activated by the cyclic nucleotides cAMP and cGMP (Leng, et al. 1999, Leng et al. 2002, Balague, et al. 2003, Li, et al. 2005, and Yoshioka, et al. 2006), presumably by binding the CNBD and opening the channel for cation transport. Calmodulin has also been shown to bind CNGCs at the CaMBD (Kohler, et al. 1999 and Kohler et al. 2000), and in the case of CNGC10, CaM has been shown to inhibit the CNGC10-mediated complementation of K⁺ uptake-deficient E. coli (Li, et al. 2005). The inhibition of CNGC10 by CaM could be reversed by cGMP, indicating a competition of the two molecules for regulation of the channel.

Cyclic nucleotides (CNs) are secondary messenger molecules produced by nucleotidyl cyclases, and have been implicated in several cellular processes in plants, such as guard cell closure, cell death, root development, gene expression and protein synthesis, phytochrome signaling, root nodulation, and pollen tube growth (Molchan, et al. 2000, Moutinho, et al. 2001, Terakado, et al. 2001, Trewavas, et al. 2002, Pagnussat, et al. 2003, Maksyutova, et al. 2003, Desikan, et al. 2004, Newton, et al. 2004, and Maathuis 2006). Although they are implicated in many cellular processes, the specific downstream targets of CNs in plant cells remains largely unknown. A number of proteins have been identified from the Arabidopsis genome that contain CN binding domains, but the functionality of the domain for most proteins has yet to be determined. CNGCs have been proposed to be one of the major downstream targets for CNs (cAMP, cGMP) in plants (Talke, et al. 2003), thereby activating cation transport in the cell. In plant CNGCs, activation of the channel by CNs is thought to be in competition with
calmodulin (CaM) inhibition. The overlap of these two binding domains suggests that once one site is bound by either a CN or CaM, the other site becomes inaccessible or modified such that it can not be bound. Exactly how the binding of either CNs or CaM modifies the channel to activate or inhibit it is unknown.

Calmodulins are Ca$^{2+}$ sensitive regulatory proteins, and there are at least 7 different CaMs expressed in Arabidopsis (CaM1-7), along with two divergent isoforms, CaM8 and 9, that may not function as typical CaMs (Kohler et al. 2000 and McCormack, et al. 2005 for review). The Ca$^{2+}$ binding motif of the CaM protein acts as a Ca$^{2+}$ sensor, and when Ca$^{2+}$ is bound, a conformational change occurs that allows the protein to interact with CaM binding domains in other proteins, such as CNGCs (Chin, et al. 2000 and Snedden et al. 2001). A study showed that CNGC2 was able to bind CaM2 and 4 in the presence of Ca$^{2+}$, while CNGC1 could only weakly bind those CaM isoforms. Both CNGCs were not able to bind CaM8 and 9 (Kohler, et al. 2000). Arabidopsis CNGCs may be able to bind specific CaM isoforms, indicating differentiation regulation, dependent on the CaM expression and localization. The regulation of CNGC activity may occur not only by differential binding affinity of CaMs and Ca$^{2+}$ fluxes in the cell that “activate” those CaMs, but by expression of various cellular pathways that produce competing CNs as secondary messengers.

2.2d Physiological function of CNGCs in Arabidopsis

Several studies have been published illuminating some of the physiological processes in which CNGCs are involved in Arabidopsis. CNGC2, 4, 11, and 12 have been implicated in plant pathogen defense responses by studying knockout mutant plants
for each gene. CNGC2 mutant plants show an absence of hypersensitive response (HR) to pathogen infection, while maintaining high levels of constitutively expressed defense genes that make the mutants resistant to a broad spectrum of pathogens, including bacteria, fungi, and viruses, despite the loss of HR (Yu, et al. 1998, Clough, et al. 2000, and White, et al. 2001 for review). In contrast, CNGC4 mutant plants display a lesion-mimic phenotype, where spontaneous lesions form on the leaf surface, yet the plants do not have the HR response to pathogens. These mutants had some increased disease resistance to specific pathogens, but did not show resistance to a broad spectrum of pathogens like the CNGC2 mutants (Balague, et al. 2003). CNGC11 and 12 mutant plants show decreased pathogen resistance and normal HR, while plants with an imbalance in both genes show constitutive expression of defense genes and the lesion-mimic phenotype (Yoshioka, et al. 2006). These studies show that CNGCs play a role in the pathogen defense response, and each channel appears to affect different pathways in that response, from programmed cell death to defense gene activation.

Other Arabidopsis CNGCs have been studied for their affect on growth and development of the plant. CNGC1 is expressed mainly in the root and a loss-of-function in the channel resulted in a loss of Ca\(^{2+}\) accumulation in shoot tissue, as well as altered root growth. The roots of CNGC1 mutant plants showed increased elongation relative to WT plants and a partial loss of gravitropic response (root growth in the direction of gravitational pull); both are signs of Ca\(^{2+}\) deficiency in roots (Ma, et al. 2006). A knockdown in CNGC10 expression has shown an array of phenotypes, including early flowering, a reduction in leaf tissue mass, and loss of gravitropic response in roots, starch
accumulation in chloroplasts, and a 50% reduction in plant K⁺ level. (Li, et al. 2005 and Borsics, et al. 2006). These results implicate CNGC10 in K⁺ transport that is required for signaling pathways induced by light, as seen by the affects on leaf development, and gravity stimulation.

CNGC mutant plants have also been tested for their sensitivity to altered cation concentration in growth media. CNGC2 mutant plants were found to be sensitive to elevated Ca²⁺ as measured by average weight and rosette size, but not sensitive to altered levels of Na⁺, Mg²⁺, and H⁺ (Chan, et al. 2003). CNGC3 mutant plants show a decrease in germination in media containing excess Na⁺, but not K⁺, and showed a slight increase in tolerance to excess K⁺, determined by weight, after 10 days of growth (Gobert, et al. 2006). Knockdown CNGC10 plants show reduced growth in low K⁺ levels, confirming its role in K⁺ uptake in plants (Borsics, et al. 2006). A CNGC1 mutant plant was tested for sensitivity to heavy metal cations and found to be sensitive to lead, Pb²⁺, but not nickel, Ni²⁺ (Sunkar, et al. 2000). These data show that the loss of CNGC expression in Arabidopsis can lead to altered cation uptake and salinity tolerance, as well as tolerance to toxic metal cations.

2.2e Localization of Arabidopsis CNGCs

Little information is known on the expression patterns and cellular localization of these channels. CNGC2-GUS fusion expressed in plants show that CNGC2 is expressed in cotyledons, flowers undergoing senescence, in the dehiscence zone of siliques, and transiently in senescing leaf and cell culture (Kohler, et al. 2001). Taken with the CNGC2 mutant plant phenotype (loss of HR), these results support the role of CNGC2 in
programmed cell death. A CNGC3::GUS fusion showed expression of CNGC3 largely in root tissue, but also in focalized spots in shoot tissue (Gobert, et al. 2006). This data also confirms the significance of CNGC3 in the root. Only two Arabidopsis CNGCs have been localized at the cellular level; both CNGC3 and CNGC10 localize to the plasma membrane of leaf cells (Gobert, et al. 2006 and Borsics, et al. 2006). Sub-cellular location is critical to illuminating the function of these channels at the cellular level, and will serve determine the mechanisms by which the channels mediate the previously mentioned physiological processes.
CHAPTER 3
MATERIALS AND METHODS

3.1. Plant material and growth conditions

Experiments were performed using wild-type *Arabidopsis thaliana*, Columbia ecotype, and the CNGC5 T-DNA insertional mutant plant lines, SALK_014491 and SALK_131191. Heterozygous T-DNA mutant plant lines were identified using the T-DNA Express website (http://signal.salk.edu/cgi-bin/tdnaexpress) and were obtained from the Arabidopsis Biological Resource Center.

Seeds were surfaced sterilized by washing with 70% ethanol, rinsing with water, washing with 50% household bleach for 5 minutes, followed by several rinses with water. Seeds were plated on 0.5X MS agar plates and stored at 4°C in the dark for 1-3 days for even germination, then transferred to a growth chamber where they were exposed to a 16/8 hour light/dark cycle at 23°C for 10-14 days before being transferred to soil (Fafard Superfine Germinating Mix).

3.2. RNA isolation and RT-PCR

Total RNA was isolated from WT and insertional mutant plants using phenol-chloroform extraction (as described by Hoffer and Christopher, 1997). Tissues were ground in a mortar and pestle in liquid nitrogen to a fine powder and RNA was extracted using 1:1 acid phenol:chloroform (v/v) and RNA extraction buffer (100mM Tris-HCl pH 8.5, 200mM NaCl, 10mM EDTA, and 0.5% SDS), then precipitated using ethanol, followed by 2M NH₄OAc and isopropanol. The resultant RNA pellet was resuspended in double distilled H₂O and concentration was determined using a spectrophotometer.
Reverse transcription was performed on 3-5μg of total RNA using M-MLV reverse transcriptase (Promega) and oligo d(T) primer to obtain first-strand cDNA. Primers were designed using the Primer3 website (http://frodo.wi.mitedu/cgi-bin/primer3/primer3_www.cgi) to specifically amplify several CNGC coding regions based on DNA sequence obtained from The Arabidopsis Information Resource (http://www.arabidopsis.org/). Primers for each gene are as follows: CNGC5 forward 5'-ATCAAGCGGCATCTCTGTCT-3' and reverse 5'-CTTTGTGTTCGGTTCTGTG-3'; CNGC12 forward 5'-ACAAATGGCGAGAAACCAAG-3' and reverse 5'-GGAGAGCTGGAACAAATCTGC-3'; CNGC13 forward 5'-TGCTTCAGTGATTGTGGTGG-3' and reverse 5'-TAGTTTTGCTCCGGCAATGTC-3'; CNGC14 forward 5'-TCTCGATCTTGTTCGTCGTG-3' and reverse 5'-TTGAGCTTATGAGCCGTCGT-3'; CNGC15 forward 5'-TGCTTGCAACATCGGGAAC-3' and reverse 5'-GTTCTGCTCCAATGGTTGT-3'; and CNGC17 forward 5'-ATTGGACAGTACCACCCTCC-3' and reverse 5'-ATATCGCCTACACCGTTTCC-3'. The constitutively expressed gene Actin was used as positive controls and for normalization of RT-PCR band density. Specific primers for the coding region of each gene are as follows, Actin forward 5'-CTTCCGCTCTTTCTTTCCAAGCTC-3' and Actin reverse 5'-ATCATCTCCTGCAAATCCAGCCTTC-3'. Relative expression levels were obtained by analyzing gel band density using Kodak 1D imaging software.
3.3. DNA isolation and insertional mutant genotyping

Genomic DNA was extracted from 4 week-old leaf tissue for genotyping the T-DNA insertional mutant plant lines. Approximately half of a mid-sized leaf was cut and transferred into a 1.5ml centrifuge tube, flash frozen in liquid nitrogen, and ground to a fine powder. DNA was extracted into solution by adding 10µl of 1M NaOH, heating the sample to 100°C for 1 minute, then suspending the mixture in 100µl Tris, pH 8. For PCR, 1µl of a 1:5 (DNA:ddH2O) dilution was used for a 15µl reaction.

Primers for genotyping the T-DNA insertions were designed using the SIGnAL toolbox (http://signal.salk.edu/tdnaprimers.2.html) and T-DNA Express website (http://signal.salk.edu/cgi-bin/tdnaexpress). A right and left primer (RP and LP) were designed to amplify a wild-type copy of the CNGC5 gene, while the T-DNA insert was identified using the T-DNA left border primer LBb1 (5'-GCGTGGACCGCTTGCTGCAACT-3') in combination with the RP mentioned above. Primers used for genotyping are as follows: SALK_014491 RP 5'-GTGTCCACTGAAATTGGAGG-3' and LP 5'-AAAATCCTCCATTCCACCTTG-3'; SALK_131191 RP 5'-GTGTCCACTGAAATTGGAGG-3' and LP 5'-TCCTTCGAGTGTGAAAGAAGC-3'; SALK_149893 RP 5'-CACGCTCCCTAAGATCTTGTG-3' and LP 5'-GAGCTTTCTGGTTAAGCCGTC-3'; and SALK_026568 RP 5'-GGTGAAACCAGTGGAGGTG-3' and LP 5'-TTTACAGGTGGGAGGTGGT-3'.
3.4. GFP fusion constructs

Truncated coding sequences of CNGC5 and CNGCII were cloned from first-strand cDNA obtained from wild-type *A. thaliana* (Colombia ecotype) into the binary vector pCAMBIA 1302. Primers were designed to PCR-amplify a template of each gene from first-strand cDNA, which was then used to amplify a specific fragment of the gene using primers containing overhanging restriction enzymes sites specific for cloning into the pCAMBIA 1302 vector. Specific PCR amplification of the template CNGC5 was performed using the forward primer 5'-GTCTTAAAGCTTTGCTCTTTC-3' and reverse primer 5'-AGTAAAACCTGAAATGTGTGC-3', and for CNGCII using the forward primer 5'-ATGGAAAAATTGAAAAGTGTTAGAGGACG-3' and reverse primer 5'-ACTGGTTCACCTTCACGCACA-3'. Primers were designed with overhanging Ncol and Spel restriction enzyme sites to amplify a specific fragment off of the above template for insertion between the cauliflower mosaic virus 35S promoter and mGFP5. The CNGC5 fragment was amplified using the forward primer 5'-CATGCCATGGAAACACTCG-3' and reverse primer 5'-CATAAATACGTGGTGCATGGATCTGACTAGTCC-3'; CNGCII using the forward primer 5'-CATGCCATGGAAAAATTGAAAAGTGTTAGAGG-3' and reverse primer 5'-GTCTCGAGTCTGAGGTCTTTTGG-3'. These fragments were then digested with Ncol and Spel and ligated into the pCAMBIA 1302 vector using T4 ligase (Promega), resulting in an insertion of 1623 nucleotides for CNGC5 and 1254 nucleotides for CNGCII. The clones were sequence verified to ensure that the CNGC coding sequence was fused in-frame to mGFP5.

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The 35S-CNGC-mGFP5 fragment of the pCAMBIA clones was sub-cloned into the small vector pBluescript SK+ to increase transfection efficiency in protoplasts. The blunt-end producing enzymes FspI and ScaI (both Fermentas) were used to digest out a fragment of 3438 and 3057 nucleotides for CNGC5 and 11, respectively. The fragments were gel purified using the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare) and ligated into pBluescript SK+, which was digested with EcoRV (Promega) to produce blunt ends for ligation into the vector. All ligation reactions were transformed into E. coli XL-1 blue cells and plated on LB agar plates with antibiotic selection for transformants.

3.5. Protoplast isolation

Protoplasts were isolated from 3-5 week-old wild-type plants (Abel, et al. 1994 and Sheen, 2002). Approximately 0.2-1g leaf tissue was harvested and sliced into thin strips using a fresh razor. The tissue was incubated in 0.5M mannitol in a 10cm Petri dish for 1 hour, after which the mannitol was aspirated and 20mls of enzyme solution (0.5M mannitol, 8mM CaCl2, pH adjusted to 5.5) containing 1% cellulase (MP Biomedicals) and 0.25% pectinase (Calbiochem). The tissue was incubated in enzyme solution overnight in the dark, after which the protoplast were harvested by swirling the plate for several minutes to free cells from the digested leaf tissue, then purified by centrifugation (200rcf, 5 minutes) and washed twice in W5 wash solution (0.5M mannitol, 154mM NaCl, 125mM CaCl2, 5mM KCl, 5mM glucose, pH adjusted to 5.8)). After two washes in W5 wash solution, the cells were resuspended in 2ml Mg/Mannitol solution (0.4M mannitol, 15mM MgCl2, 4mM MES) and purified using a sucrose cushion
(cell suspension added on top of 5ml of 21% sucrose and centrifuged for 10 minutes at 300rcf). Purified cells were removed from the upper layer and counted using a hemocytometer.

3.6. Transient transfection of protoplasts

PEG-mediated transfection was carried out in 12- or 24-well tissue culture plates (Becton Dickinson). 1-2x10^5 protoplasts in 100μl Mg/Mannitol solution were used for each transfection. 10μg of DNA (in 10μl ddH₂O) were added to the cells, followed by 110μl of 40% PEG 4000 (Fluka, dissolved in 0.2M mannitol, 0.1M CaCl₂). The cells were incubated for 30 minutes, followed by addition of 440μl of W5 wash buffer and the cells were allowed to settle by gravity for several minutes and the supernatant was removed. The cells were resuspended in W5 wash buffer and incubated in the dark for 16-48 hours before being visualized using epiflourescent (Olympus BX51) or laser-scanning confocal microscopy (Olympus Fluoview FV-1000 on IX81 inverted microscope).

3.7. Specificity of anti-peptide CNGC5 and 11/12 antibodies

Polyclonal anti-peptide antibodies were generated and purified by New England Peptide, Inc. (Boston, MA) to specific antigenic peptide regions of CNGC5 and 11 (the peptide for CNGC11 is homologous with CNGC12 and will be referred to as anti-CNGC11/12). The specific peptide sequences were selected using predictive software by New England Peptide and are located in between the membrane-spanning helices S3 and S4. The peptides for CNGC5 (Ac-NRAKESVLKSCK-amide) and CNGC11/12 (Ac-CKRAGSDNIRF-amide) were synthesized and injected into rabbits to generate
polyclonal antibodies that were affinity purified. The purified antibodies were tested for specificity to their peptide by dotting varying concentrations of peptides from CNGC5, 10, 11/12, 19 and 20 on nictrocellulose membrane (Protran, Schleicher and Schuell Bioscience) and probing with 1:3000 dilution of primary antibody, followed by a 1:2000 dilution of goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Pierce) for detection using chemiluminescence.

3.8. Immunolocalization of CNGC5 and 11/12 in fixed leaf protoplasts

Protoplasts were isolated as described above and fixed for immunolabeling. After sucrose cushion purification, the cells were spun down again in a 15mL conical tube (Falcon) at 200rcf for 5 minutes at 4°C. The supernatant was discarded and the cells were resuspended in 5mls of fixative (3.5% formaldehyde and 1% glutaraldehyde in 1X PBS). The cells were incubated for 2 hours on an orbital shaker set at 30-40rpm, spun down gently (200rcf for 5 minutes), and resuspended in 2mLs of PBS. Approximately 1x10^5 fixed cells were transferred to one well of a 24-well plate (Falcon) for immunolabeling. The volume of each well was increased to 300μl using 1X PBS and primary antibody was added to a dilution of 1:200. Cells were incubated with primary antibody for 2 hours on an orbital shaker (30-40rpm). After two hours, the plate was removed from the shaker and the cells were allowed to settle by gravity for approximately 2 minutes. The supernatant was carefully removed and the cells were washed in 500μl 1X PBS for 5 minutes with gentle shaking. The supernatant was removed as before and the remnant volume increased to 300μl with 1X PBS. Secondary antibody (goat anti-rabbit Alexafluor 488, Invitrogen) was added to a final dilution of
and incubated with gentle shaking for 1 hour. The cells were washed gently and viewed used confocal laser scanning microscopy.

3.9. CNGC5 cloning into the PET15b vector and recombinant protein expression

A truncated coding sequence of CNGC5 was cloned into the bacterial expression vector pET15b (Novagen) for recombinant protein expression. Primers were designed for amplification of a specific CNGC5 fragment from first-strand cDNA to be digested with NdeI (from an overhanging NdeI site in the forward primer) and BamHI (located within the CNGC5 cDNA) for insertion into the multiple cloning site of pET15b. Primer sequences for amplification of the insert are forward 5'

GCGATGCATATGGCAGGGAAAAG-3' and reverse 5'

CAGGATCCCCTTCCCGCAAG-3'.

Truncated CNGC5 was amplified with the high fidelity DNA polymerase Immolase (Bioline), digested with NdeI (Promega) and BamHI (Promega), and ligated into pET15b using T4 DNA ligase (Promega). The ligation reaction was transformed into *E. coli* XL-1 blue cells for amplification and purification of the plasmid using QIAgene Miniprep Kit (Qiagen). A 1588 nucleotide insertion was confirmed by restriction enzyme digestion and sequencing. For recombinant protein expression, the pET15b/CNGC5 construct and empty pET15b vector were transformed into the expression host BL21(DE3) *E. coli* cells. Expression of the recombinant was induced in 5ml cultures (optical density 0.6) using 0.1mM IPTG, which induces transcription of the T7 promoter upstream of the CNGC5 insert. After 1, 2, and 4 hours of induction, 1ml of culture was removed and used for Western blot analysis.
Westerns were performed by preparing whole cell lysis of the bacterial cultures. To concentrate the protein sample, 40μl of bacterial culture were spun down at 12,000rpm (4°C) for 1 minute, the supernatant was removed, and the cell pellet resuspended in 10μl of 1X PBS and 10μl of 2X protein loading buffer (0.1M Tris-HCl pH6.8, 4% SDS, 0.2M DTT, 20% glycerol, 0.1% Bromophenol Blue). Mechanical lysis of the cells was achieved by passing the sample through a 28 ½ gauge insulin syringe (Becton-Dickinson) several times until a dense foam formed. The samples were heated at 95°C for two minutes to denature before loading into a 10% SDS denaturing polyacrylamide gel. Upon transfer to a nitrocellulose membrane (Protran), the blot was probed with a 1:3000 dilution of anti-CNGC5 primary antibody, followed by a 1:2000 dilution of anti-rabbit horseradish peroxidase-conjugated secondary antibody (Pierce) for chemiluminescent detection of antibody binding.

3.10. Immunoprecipitation using anti-CNGC5 and Western blot

Proteins were extracted from whole plants by grinding in liquid nitrogen to a fine powder, then adding 4 volumes (w/v) of 1X lysis buffer (5% IGEPA, 0.5% PMSF, 0.5% Vanadate, and 0.125M sodium pyrophosphate) and shaking well. Samples were kept on ice for 20 minutes with occasional shaking, then centrifuged at 4,000rpm for 20 minutes at 4°C. The supernatant was removed and 500μl were used for immunoprecipitation.

In a 1.5ml centrifuge tube, 2μg of anti-CNGC5 antibody was added to 500μl of protein extract and the mixture was rotated on an orbital shaker at 4°C for 2 hours. After 2 hours, 15μl of Protein-A-Agarose beads (Invitrogen) were added and the mixture was
rotated for at least 45 minutes at 4°C. After incubation, the agarose beads were pelleted out of solution by centrifuging at 12,000rpm for 30 seconds at 4°C, followed by 3 washes of 1ml 1X lysis buffer using similar centrifugation. After removing the supernatant from the third wash, the pellet was dried using a Hamilton syringe and the beads were resuspended in 25μl of protein loading buffer, heated to 95°C for 8 minutes, and loaded onto a 10% SDS denaturing polyacrylamide gel, being careful to avoid the beads. The proteins were transferred to a nitrocellulose membrane and probed with a 1:3000 dilution of anti-CNGC5 primary antibody, followed by a 1:2000 dilution of anti-rabbit horseradish peroxidase-conjugated secondary antibody (Pierce) for chemiluminescent detection.
CHAPTER 4
RESULTS

4.1 Selection of CNGC5 and 11 for sub-cellular localization

Several criteria were involved in selecting two Arabidopsis CNGCs, CNGC5 and 11, for sub-cellular localization. Initially, several of the 20 Arabidopsis CNGCs were selected based on a lack of previous reports or publications. At the time, there existed publications on CNGC1, 2, 4, and 10, with the remaining CNGCs being unstudied. From this group of 16, CNGC5, 12, 13, 14, 15, and 17 were selected for determination of mRNA expression using reverse transcriptase polymerase chain reaction (RT-PCR). Presumably, the CNGCs with higher mRNA levels would translate to higher protein content within the cell, which would facilitate easier antibody detection for the purpose of immunolocalization. RT-PCR was performed for each of CNGC5, 12, 13, 14, 15, and 17 from both root and leaf RNA of 14 and 35 day-old WT Arabidopsis thaliana (Columbia ecotype). The PCR reactions were run out on a gel and the band density for each gene normalized to that of Actin, a constitutively expressed gene throughout the plant (Fig 4A). From this data, it was seen that each of the CNGCs had varying mRNA expression patterns in the leaf and root tissue, and also in young and mature plants. CNGC13 mRNA was expressed so minimally that it could only be detected by a high-fidelity DNA polymerase in either tissue at both stages of development (data not shown).

CNGC5 and 17 were selected for further analysis due to their overall high mRNA expression level; CNGC5 being expressed highly in root and leaf tissues and CNGC17
highly expressed in leaf and moderately in root. CNGC14 was also selected because of its moderate expression in young root, and very little expression in leaf. This data indicated that it could have an interesting role in root growth and development. Along with CNGC5, 14, and 17, CNGC11 was also selected for further analysis due to its very high expression level in leaf tissue relative to Actin, as determined by quantitative RT-PCR (qRT-PCR) performed by other lab members also studying CNGCs (T. Borsics and K. Gushiken, data not shown). CNGC11 was expressed at about 1.5 times that of Actin, which is significantly higher than the other CNGCs tested.

After selecting CNGC5, 11, 14, and 17 based on mRNA expression, several other criteria were involved in selecting two CNGCs for sub-cellular localization (Fig. 4B). The predicted amino acid sequence (obtained from The Arabidopsis Information Resource, http://www.arabidopsis.org/) for each gene was sent to New England Peptide, Inc. (Boston, MA) to determine the best antigenic peptide within the protein to use to generate anti-peptide antibodies in rabbit. The company’s predictive software located antigenic peptides in the cytosolic region of the protein so that the antibody has an accessible epitope. For CNGC5, 11, and 14, the peptide is located between the S5 transmembrane helix and the pore, and for CNGC17, the peptide is located between the S3 and S4 transmembrane helices.

The candidate peptides for CNGC11, 14, and 17 all had issues that were taken into consideration when selecting genes for localization. The peptide for CNGC11 is 100% homologous to a peptide in the same location of CNGC12, which would make the antibody specific for both genes, and the peptide for
CNGC14 is 45% homologous with a peptide in CNGC16, which could cause some cross-reactivity. The peptide for CNGC17 contained an internal cystein residue, which alters the immune response of the rabbit to the peptide and antibody specificity could be lost. CNGC5 had no peptide issues and became a strong candidate for selection.

Other considerations for selecting CNGCs for localization had to do with their phylogenetic uniqueness, predicted sub-cellular location using computer software, and the availability of T-DNA insertional mutant plant lines to study the knockout mutant phenotype for each gene. It was desired that the two CNGCs reside in different groups on the phylogenetic tree (Fig. 2), in the hope that the CNGCs could have varying function and cation selectivity due to their divergent relationship, resulting in unique phenotypes of the knockout mutant plant lines. The amino acid sequence for each of the CNGCs was also run through several sub-cellular location predictive software programs (work done by K. Gushiken, not shown) and only CNGC5 and 11 produced weak sub-cellular predictions. CNGC5 contains a weak chloroplast-targeting signal, while CNGC11 contains a weak plasma membrane signal (predictions from ChloroP and PsortI software, respectively). Three of the CNGCs, CNGC5, 11, and 17, had at least one SALK T-DNA knockout mutant line available to study the phenotype of a loss-of-function plant.

Based on the information obtained for these CNGCs, CNGC5 and 11 were selected for sub-cellular localization. Both of the genes have high mRNA expression in at least one tissue type, possess a weak sub-cellular targeting signal, have at least one
Figure 4. Transcript levels for several CNGCs and criteria for selection of CNGC5 and 11 for sub-cellular localization. (A) Histogram of mRNA abundance of various unstudied CNGCs, as determined by RT-PCR of total RNA from leaf and root tissues of 14 and 35 day-old wild-type WT *Arabidopsis thaliana* (Col). Band density was normalized to actin mRNA levels, which are constitutively expressed in all tissues. (B) Chart represents the criteria used to select CNGC5 and 11 from four candidate genes for generation of anti-peptide antibodies to use for immunolocalization. The genes were selected based on phylogenetic uniqueness, mRNA abundance, location and specificity of antigenic peptides within each protein, and the availability of T-DNA insertional mutant plants lines to characterize knockout phenotypes.
T-DNA mutant plant line available, and are located on separate branches of the phylogenetic tree. The antigenic peptide selected for antibody production against CNGC11 is 100% homologous to CNGC12, which makes it specific for both proteins and will therefore be referred to as an anti-CNGC11/12 antibody. In order to resolve this issue, a T-DNA mutant for CNGC12 was identified in the SALK T-DNA mutant database and protoplasts from these plants could be used for immunolocalization of CNGC11 alone. However, several attempts were made to genotype both a CNGC11 and 12 mutant plant and neither could be identified. As a result, more attention was paid to localization of CNGC5 and characterization of the CNGC5 mutant plant lines.

4.2 Specificity of anti-CNGC5 and 11/12 antibodies

Anti-peptide antibodies against CNGC5 and 11/12 were generated in rabbits and used for immunolocalization in protoplasts isolated from leaf tissue of 3-4 week-old WT Arabidopsis, Columbia ecotype. The antibodies were generated from small peptides specific to each gene that were chemically synthesized and injected into rabbits to generate an immune response. The peptides selected for each gene reside in the hydrophilic region that links the S5 transmembrane helix to the pore region of the channel (Fig 5A and B). Presumably, the location of the peptide would create an epitope that was accessible to the antibodies, as opposed to a peptide located within the hydrophobic, membrane-bound region of the protein.

The antibodies were tested for specificity both by checking for cross-reactivity to peptides from other CNGCs, and by checking the affinity of each for their specific
CNGC from WT plant protein extracts. Peptides from CNGC5, 10, 11/12, 19, and 20 were dotted onto nitrocellulose membranes in varying concentrations and probed with either the anti-CNGC5 or 11/12 antibody (Fig. 5C). Antibody binding was detected by chemiluminescence using anti-rabbit horseradish peroxidase-conjugated secondary antibody. Both antibodies were specific to their respective peptides and do not cross-react with peptides from other CNGCs. The antibodies were also used for immunoprecipitation (Fig. 5D). Each antibody was mixed with total protein extract from WT plants for two hours and removed from solution using Protein-A-agarose beads. The precipitated proteins were run out on an SDS-PAGE gel for Western blot analysis.

The anti-CNGC5 antibody pulled out a protein of the correct size for the predicted CNGC5 protein produced in vivo (~83kD), as well as three distinct bands over 105kD in size. The size of the larger bands indicates that they may be dimers, trimers, and tetramers of CNGC5, or CNGC5 bound to other proteins (such as other CNGCs, possibly forming a heteromeric channel). As expected, the anti-CNGC11/12 antibody also pulled out proteins of the correct size for CNGC11 (~71.5kD) and CNGC12 (~73kD), but the bands are faint, and there are two stronger bands formed between the 105 and 160kD markers that do not match the correct size for either CNGC or a dimer of each. However, larger bands starting around the 160kD marker may represent dimers, trimers, and tetramers of the gene products.
Figure 5. Epitope location and specificity of anti-peptide antibodies for CNGC5 and CNGC11/12. (A) Amino acid sequences for CNGC5, 11, and 12 with the peptide sequence (red) used to generate polyclonal antibodies in rabbit located between the S5 transmembrane domain and the pore region (blue). The six transmembrane domains are highlighted in gray and the CNBD in purple. (B) Representation of the protein structure of a CNGC with the location of the antigenic peptides indicated by the arrow. (C) Antibodies show specificity to their respective peptides and do not cross-react with peptides from other CNGCs. Peptides were titrated and bound on nitrocellulose membranes in varying concentrations and incubated with the primary antibody (anti-CNGC5 or 11/12), followed by incubation with a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody for chemiluminescent detection. (D) Western blots of anti-CNGC5 and 11/12 immunoprecipitated proteins. Arrow indicates a band of the corresponding size for CNGC5 (82kD) and faint bands for CNGC11 (71.5kD) and 12 (73kD) in the immunoprecipitated proteins. The open arrowheads denote bands of putative CNGC dimers, trimers, and tetramers, and the bracket denotes secondary putative CNGC11 and 12. The bright band at the 50kD marker represents the antibody heavy chain (HC) and un-precipitated protein extract (no IP) was used as a control.
4.3 Immunolocalization of CNGC5 and 11/12 in WT Arabidopsis leaf protoplasts

Immunolocalization experiments were performed on fixed WT leaf protoplasts. Protoplasts were isolated from 3-4 week-old plants by incubating leaf slices in the cell wall-digesting enzymes, cellulase and pectinase. Once cells were freed from the cell wall and cellular debris removed, several different fixation and immunolabeling techniques were attempted to optimize localization using the anti-CNGC5 and 11/12 antibodies. Formaldehyde, glutaraldehyde, and methanol were used as fixatives, and immunolabeling was attempted using varying antibody concentrations and with or without detergents and blocking agents.

After attempting several different fixation techniques, a 2 hour incubation of 3.5% formaldehyde and 1% glutaraldehyde solution in phosphate buffered saline (PBS) was used to fix the cells previous to immunolabeling. The addition of glutaraldehyde as a fixative does cause some increase in background autofluorescence, but it could be filtered efficiently using the appropriate settings on the microscope. It was found that adding detergents (0.1-0.5% Triton-X) to the antibody solution appeared to lyse the cells and cause a precipitate to form, making visualization of unlysed cells impossible. Also, the addition of bovine serum albumin (BSA) as a blocking agent made no difference in reduction of background immunofluorescence. After several rounds of optimization, it was determined that the best technique for immunolabeling the protoplasts was to incubate in a 1:200 dilution of the anti-CNGC primary antibodies in PBS, followed by a five minute wash in PBS, then incubation in a 1:100 dilution of the Alexafluor 488-
conjugated anti-rabbit secondary antibody, another wash in PBS, and visualization using confocal laser-scanning microscopy to detect immunofluorescence.

The anti-CNGC5 and 11/12 antibodies both immunolocalize to the plasma membrane (PM) of fixed WT protoplasts (Fig. 6A-H). As a positive control, an anti-peptide antibody against CNGC10 was used and also showed immunolocalization to the plasma membrane (Fig 6I-L). Previous experiments from this lab have shown that CNGC10 localizes to the plasma membrane using immunogold labeling visualized by electron microscopy (unpublished data). To show that PM immunofluorescence is not an artifact of incubation with a primary antibody or a rabbit polyclonal antibody, protoplasts were immunolabeled with an anti-Pdi5 antibody (Fig. 6M-P). Pdi5 is a protein disulfide isomerase expressed in Arabidopsis and has been shown to localize inside the vacuole and endoplasmic reticulum using electron microscopy (unpublished data). The anti-Pdi5 antibody does not localize to the plasma membrane and does not produce an immunofluorescent signal. Incubation of fixed protoplasts with the Alexaflour 488 secondary antibody alone also produced no signal, indicating that plasma membrane immunolocalization of anti-CNGC5 and 11/12 was a result of primary antibody localization and not background binding of the secondary antibody (Fig 6Q-T).
Figure 6. Immunolocalization of CNGCs 5, 11/12, and 10 to the plasma membrane (PM) of Arabidopsis leaf protoplasts using confocal laser scanning microscopy. (A-D) Localization of anti-CNGC5 antibody to the PM of leaf protoplasts. (E-H) Localization of anti-CNGC11/12 antibody to the PM. (I-L) Positive control shows anti-CNGC10 localization to the PM. (M-P) Negative control shows that an anti-peptide antibody for Pdi5, a protein disulfide isomerase in Arabidopsis, does not localize to the PM. (Q-T) Negative control showing protoplasts incubated with the secondary antibody alone. (A, E, I, M, and Q) The anti-rabbit AlexaFluor 488-conjugated secondary antibody used to visualize primary antibody binding is detected using a 505-525 nm emission filter. (B, F, J, N, and R) Chlorophyll autofluorescence is detected using a 650 nm emission filter. (C, G, K, O, and S) Merge of immunofluorescence and chlorophyll autofluorescence. (D, H, L, P, and T) Merge the previous images with a bright light image of the cell.
4.4 Transient expression of CNGC-GFP fusion proteins in WT Arabidopsis leaf protoplasts

In order to confirm immunolocalization, CNGC5 and 11 were fused to green fluorescent protein (GFP) and transiently expressed in live protoplasts as a means to visualize localization resulting from cellular trafficking of the fusion protein. Both CNGC5 and 11 were truncated within the CNBD, resulting in a fusion that replaces the CaMBD and most of the CNBD with mGFP5 (Fig. 7A). The truncated copy of CNGC5 and 11 was amplified from cDNA, restriction enzyme-digested, and ligated into the binary vector pCAMBIA 1302, which resulted in a CNGC fusion to the modified GFP gene, mGFP5, under the control of the cauliflower mosaic virus 35S promoter (referred to as CaMV35S or 35S) (Fig. 7B). The pCAMBIA 1302 vector is a large plasmid used for Agrobacterium-mediated transformation, and due to its large size (~13kB), PEG-mediated transfection efficiency of live protoplasts is low. To increase transfection efficiency the 35S-CNGC-mGFP5 cassette of pCAMBIA 1302 was removed by restriction enzyme digestion and ligated into a small plasmid, pBluescript SK+ (Fig. 7C).

Despite repeated attempts, transient expression of the CNGC5- and 11-GFP fusions were unsuccessful. Several other constructs containing both full length and truncated CNGC10-GFP fusions also did not express in protoplasts (constructs generated by T. Borsics). Transfection was attempted using varying concentrations of DNA, plasmid purification kits, and length of incubation post-transfection and all were unsuccessful. However, the positive control, 35S-mGFP5, was expressed throughout the
Figure 7. Constructs used for CNGC-GFP fusion protein expression in Arabidopsis leaf protoplasts. (A) Schematic of a CNGC showing the location within the cyclic nucleotide binding domain (CNBD) that the CNGC cDNA was truncated and fused to mGFP5; transmembrane domains S1-6 (TMD); and the calmodulin binding domain (CaMBD). (B) The binary vector pCAMBIA 1302 was used to generate a C-terminal mGFP5 fusion with truncated CNGC5 and 11 cDNA. Both CNGC templates were amplified from cDNA using primers containing specific restriction enzyme sites to generate an mGFP5 fusion in place of the CNBD and CaMBD. Expression of the fusion in plant cells is driven by the cauliflower mosaic virus 35S promoter. (C) In order to increase transfection efficiency, the 35S-CNGC-mGFP5 cassette was blunt-end digested with FspI and Scal and cloned into the EcoRV site of the small vector, pBluescript SK+. 
Figure 8. Transient expression of 35S-mGFP5 in Arabidopsis leaf protoplasts, visualized using confocal laser scanning microscopy. mGFP5 is expressed 16 hours (A-D) and 48 hours (E-H) post-transfection in leaf protoplasts. (I-L) The negative control shows a lack of GFP expression after transfection with the empty vector. (A, E, and I) mGFP5 expression viewed through a 505-525nm emission filter. (B, F, and J) Chlorophyll autofluorescence viewed through a 650nm emission filter. (C, G, and K) merge mGFP5 expression with chlorophyll autofluorescence, and (D, H, and L) merge both with a bright light image of the cell.
cytosol of protoplasts at 16 to 48 hours post-transfection (Fig. 8A-H), showing that the protoplasts were being transfected, but that the CNGC-GFP fusion was unable to be expressed. Protoplasts transfected with the empty vector, pBluescript SK+, do not express GFP (Fig. 8I-L).

4.5 Identification and phenotype of two T-DNA insertional mutant lines for CNGC5

The T-DNA Express website of the Salk Institute Genome Analysis Laboratory (SIGnAL) was searched and three T-DNA insertional mutant lines for CNGC5, and one for CNGC11 were identified. For CNGC5, two T-DNA insertion lines, SALK_014491 and SALK_131191 are located in the 5' untranslated region (UTR) of the gene, while one insertion line, SALK_149893, is located in exon 7 (Fig. 9A). The CNGC11 T-DNA insertion line is located in the last intron, intron 7, of the gene. Only two homozygous T-DNA lines, SALK_014491 and SALK_131191, both for CNGC5, could be identified through PCR-based genotyping. No plants containing a T-DNA insert could be identified for the third CNGC5 T-DNA line, SALK_149893, and for the CNGC11 mutant SALK_026568.

PCR primers were designed to amplify both a WT CNGC5 and the T-DNA insert inside the gene (Fig. 9B). To identify the WT copy of CNGC5, a left and right primer (LP and RP) specific to CNGC5 were used in combination to amplify an approximately 1kb region of the gene that surrounds the putative T-DNA insert. The T-DNA insert was identified using a primer specific to the T-DNA (LBbl) and the RP used for WT CNGC5. Homozygous T-DNA mutant plants were identified by the presence of a PCR band for
the T-DNA insert and lack of a WT band (Fig. 9C). Six homozygous T-DNA insert plants were identified for the SALK_014491 line and one for the SALK_131191 line. Seeds from these plants were obtained and propagated for characterization of CNGC5 expression and phenotype analysis.

RT-PCR was performed on the WT and T-DNA insertional mutant plants to assess CNGC5 mRNA expression levels (Fig. 10A). The T-DNA mutant lines showed an approximately 50-70% reduction in CNGC5 transcript level when normalized to the constitutively expressed gene, CBP20, as opposed to a knockout in expression, which could be expected from the presence of a T-DNA insert in the gene. The SALK_014491 line has a slightly reduced CNGC5 mRNA expression level compared to the SALK_131191 line. The anti-CNGC5 antibody was also used to immunoprecipitate CNGC5 from protein extracts from WT and mutant plants. A Western blot of the immunoprecipitated proteins also shows a reduction in CNGC5 proteins levels in the two mutant plant lines compared to WT (Fig. 10B).

Under normal growth conditions, the two CNGC5 knockdown lines show a slight decrease in growth as determined by measuring average total weight and the weight of root and aerial tissues separately (Fig. 10C). Ten plants were grown for 14 days in two separate experiments and the aerial and root tissues were separated to analyze differences in tissue growth as measured by weight. Both CNGC5 knockdown lines showed a slight decrease in the average total weight and average weight of aerial tissue. The SALK_014491 mutant line showed more growth deficiency in both categories as compared to the SALK_131191 line. A significant reduction in root weight was seen in
Figure 9. Identification of two T-DNA insertional mutant plant lines for CNGC5. (A) Shows the position of three different SALK T-DNA insertion lines for the CNGC5 gene. Open boxes represent exons and dotted boxes represent untranslated regions. (B) Schematic diagram representing the method for PCR-based identification of T-DNA insertions within a gene. A left and right primer (LP and RP) are used in combination to amplify a wild-type CNGC5 band, while a primer specific to the left border region of the T-DNA insert (LBb1) and the right primer are used to amplify the T-DNA insert in the gene. Homozygous T-DNA mutant lines are identified by the inability of a WT band to form as a result of the T-DNA insertion in both alleles. (C) PCR identification of homozygous T-DNA mutants for the SALK_014491 and SALK_131191 lines. Five homozygous lines were identified for SALK_014491 (#3, 8, 16, 18, and 19) and 1 for SALK_131191 (#6).
Figure 10. The CNGC5 T-DNA insertional mutant lines SALK_014491 and SALK_131191 show a reduction in CNGC5 mRNA, protein expression, and average weight. (A) RT-PCR shows that expression of CNGC5 mRNA is reduced in the SALK_014491 and SALK_131191 mutant plants. PCR was performed on first-strand cDNA of WT and mutant plants and limited to 24, 25, and 26 cycles for comparison to the constitutively expressed gene, CBP20. CNGC5 band density for each cycle was normalized to CBP20 for comparison of expression levels. (B) Western blot of immunoprecipitated proteins from WT, SALK_011491 and SALK_131191 plant lines. The two SALK lines show slightly reduced CNGC5 protein expression (arrow) relative to WT. The antibody heavy chain (HC) is indicated as a loading control. (C) Fourteen day-old SALK_014491 and 133191 mutant plants show slightly reduced total and aerial weight compared to WT plants, and SALK_014491 plants show a significant reduction in root weight. The SALK_131191 line does not show this root phenotype.
the SALK_014491 mutant line, which averaged about half the root weight of WT plants. By observation, the difference in root weight was not a result of reduction in length, but of thickness. The roots appeared generally thin and wispy. Despite having a similar knockdown in CNGC5 expression, the SALK_131191 mutant line did not show a significant difference in average root weight compared to WT, with some plants showing diminished root growth like the SALK_014491 plants and some showing root growth similar to WT.

WT and CNGC5 knockdown plants were also grown in media containing elevated levels of KCl, NaCl, CaCl₂, and MgCl₂ to determine whether a decrease in CNGC5 expression effects growth and development in the presence of excess monovalent and divalent cations. No significant different between the WT and mutant plants was seen when the change in total weight, aerial weight, and root weight relative to the control was compared (data not shown).
The physiological significance of several plant CNGCs has been characterized to some extent, but very little information is known of the location and function of these channels at the cellular level. This study attempted to localize two Arabidopsis CNGCs, CNGC5 and 11, to a sub-cellular membrane in leaf protoplasts. Two techniques were used for localization, immunolocalization and expression of CNGC-GFP fusion proteins. Using specific antibodies, CNGC5 was localized to the plasma membrane of fixed leaf protoplasts using immunofluorescence. CNGC11 may also be located in the plasma membrane, but the antibody used for immunolocalization experiments was not as specific as the CNGC5 antibody. Neither CNGC could be expressed as a GFP fusion protein, potentially due to biological issues that prevented its expression. Two T-DNA insertional mutant plant lines were identified for CNGC5 and showed a knockdown in CNGC5 expression as determined by RT-PCR and Western blot of immunoprecipitated proteins. Both knockdown lines showed a slight growth deficiency, as measured by average total weight, when grown in normal conditions, but showed no significant phenotype when grown in salt stress.

At the time of their selection for sub-cellular localization experiments, there were no published reports on either CNGC5 or 11. A subsequent publication during the course of this research showed that CNGC11 is a positive regulator of resistance to an avirulent pathogen in Arabidopsis (Yoshioka, et al. 2006), but no sub-cellular localization of the
channel was reported. The amino acid sequence for each protein was analyzed and an antigenic peptide 11 amino acids long was identified from each protein for use in generating anti-peptide antibodies in rabbits. The peptide used to generate the antibody for CNGC11 was also 100% homologous to a peptide in CNGC12, thereby making the antibody specific for both channels (referred to as anti-CNGC11/12). The obvious problem that this creates for immunolocalization of CNGC11 specifically was intended to be resolved by identification of a T-DNA insertional mutant lines (from the Salk Institute Genome Analysis Laboratory) having a knockout in expression of either CNGC11 or 12, which would eliminate the cross-reactivity of the antibody. However, after several rounds of genotyping, no CNGC11 or 12 mutant plants could be identified and so the focus turned to CNGC5, which did have identifiable T-DNA mutant plant lines.

Immunolocalization experiments showed that the anti-CNGC5 and 11/12 antibodies localized to the plasma membrane of fixed leaf protoplasts. It could not be determined whether the anti-CNGC11/12 antibody was binding CNGC11, CNGC12, or both, so localization of CNGC11 (and CNGC12) to the plasma membrane is only tentative. To show that the plasma membrane signal generated by both the anti-CNGC5 and 11/12 antibodies was not a product of fixation or non-specific binding, an anti-CNGC10 antibody was also used as a positive control and showed localization to the plasma membrane. CNGC10 has already been shown to be located to the plasma membrane of leaf sections using the same antibody (Li, et al. 2005). The antibodies differ in that the epitope for CNGC10 (the peptide used to generate the antibody) is located in the C-terminus of the protein, which is predicted to reside in the cytosol of the
cell. In contrast, the epitopes of both the CNGC5 and 11/12 antibodies are located in a presumably extracellular region of the protein. Thus, the signal generated by the anti-CNGC10 antibody shows that the antibodies can penetrate fixed protoplasts, and yet no internal signal was seen from either anti-CNGC5 or 11/12. However, internal regions of the cell were not completely permeable to the antibody, because no immunofluorescent signal was seen from an anti-Pdi5 antibody used as a control. Pdi5 is a protein disulfide isomerase that has been shown to be located inside the vacuole and endoplasmic reticulum (unpublished data). Therefore, if CNGC5 or 11/12 is located inside an organelle, or the epitope of the antibody is directed inside an organelle, it is likely that this was inaccessible to the antibody and so could not be seen in the immunolocalization experiments.

In order to confirm immunolocalization, a truncated CNGC5 and 11 cDNA was fused to mGFP5 for expression in live leaf protoplasts. The mGFP5 gene produces a green fluorescent protein with a few amino acid modifications for enhanced expression in plants. This is a useful tool for localization in that visualization of the protein is a result of direct fusion to GFP and not an indirect localization that relies on antibody binding. In both cases the CNGCs were truncated in the CNBD, before the CaMBD, and fused to mGFP5 in the hope that the channel would be non-functional and would not disrupt cation transport in the cell. Expression of the CNGC-GFP fusion was put under the control of the cauliflower mosaic virus 35S promoter for constitutive, high expression for easy visualization. It was thought that the CNGC-GFP fusion protein would enter the same biological trafficking pathway as the native CNGC due to an unknown sub-cellular
localization signal encoded in the protein. Despite repeated attempts, the fusion protein for either CNGC5 or 11 was not detected after transient transfection of a plasmid containing the fusion construct in leaf protoplasts. Several variations of a CNGC10-mGFP5 fusion were also attempted and none were detectable (constructs obtained from T. Borsics). These constructs included both full-length and truncated CNGC10 cDNA fusions to mGFP5, as well as a construct under the control of the native CNGC10 promoter. The lack of CNGC-GFP fusion expression was not a result of the transfection procedure. The positive control, 35S-mGFP5, was consistently expressed in protoplasts with between 10-40% transfection efficiency. It is possible that the slightly larger CNGC-GFP fusion construct could not be transfected into the cell, but more likely, the lack of expression is a result of some biological hindrance in the cell. It is possible that due to the GFP fusion, the protein stalled in processing and sub-cellular targeting or was expressed and targeted, but due to the CNGC fusion, the GFP molecule was unable to properly fold and therefore undetectable using microscopy. Another possibility is that expression of the CNGC-GFP fusion was toxic to the cells. The fusion protein could have acted as a functional channel and generated a toxic change in cation concentrations within the cell, or could have replaced native CNGC5 subunits in a functional channel, also causing a disruption in cation transport that killed the cell. While CNGC5, 10, and 11 could not be expressed as GFP fusion proteins, previous work has shown that this can be done with other CNGCs in plants. A CNGC3-GFP fusion protein has been expressed in Arabidopsis leaf protoplasts (Gobert, et al. 2006) as well as a barley CNGC-GFP
(HvCBT1-GFP) in barley aleurone protoplasts (Schuurink, et al. 1998). Perhaps the three CNGCs expressed here are toxic as GFP fusions or are unable to be properly targeted or folded unlike the CNGCs reported.

The two loss-of-function mutants studied for CNGC5 show that this channel plays a role in plant development, as both plant lines showed a decrease in weight when grown in normal conditions as compared to WT plants. However, no clear phenotype was seen when the plants were grown under salt stress (KCl, NaCl, CaCl₂, and MgCl₂), indicating that either CNGC5 does not transport these cations or the partial loss of CNGC5 expression does not affect the plant in these conditions. Another possibility is that there is some functional redundancy between channels that compensates for a partial loss of CNGC5. More extensive studies on these plant lines would need to be conducted to get a clearer picture of the physiological significance of CNGC5.

The work presented here was intended to initiate the study of two little known cation channels in Arabidopsis, CNGC5 and 11, at the cellular level. The sub-cellular location of these channels is a critical component to determining how they function at the cellular level, and how their cellular function affects the plant as a whole. CNGC5 was localized to the plasma membrane of leaf cells using antibody targeting and CNGC11 (and 12) were tentatively localized to plasma membrane as well. In the case of CNGC5, this channel protein likely plays a role in the translocation of cations across the plasma membrane in response to cytosolic signals and effects growth and development at the whole-plant level. More work is needed to confirm localization of CNGC11 and its impact on plant physiology.
References


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