Plant K Channel Proteins

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INTRODUCTION

Almost 40 years ago, Epstein *et al.* (1963) developed the seminal concept of passive (i.e. low affinity, occurring at high soil solution [K]) and active (occurring at low soil K) pathways for cation nutrient (specifically K) uptake by crop plants. Since that time, numerous plant K transport proteins have been cloned, and associated with specific transport properties of plant cell membranes. We now have a fairly clear, but still incomplete understanding of the specific proteins that facilitate K uptake into, and transport within the plant. Following the model developed by Epstein, passive K uptake into plants, whereby K movement follows the electrochemical gradient across root cell membranes, has been shown to be facilitated by ion channels (passive transport). Active uptake of K (i.e. against the chemical gradient) is facilitated by proton pumps (H-ATPases) and K/H symporters.

The first protein components of these transport systems to be cloned were the proton pumps (Boutry *et al.*, 1989; Harper *et al.*, 1989). At this time, there are 45 known sequences of plant H-pump ATPases in the genome of the model plant *Arabidopsis thaliana* (Axelsen and Palmgren, 2001); it is likely that there are homologous genes encoded in the chromosomes of all crop plants. The large number of individual members in this gene family (and other families of plant transport proteins mentioned in this chapter) likely corresponds to the requirement for varying expression of proton pumps in different plant organs, cell and intracellular membranes, and at varying developmental stages of plants.

The proton ATPase pumps in the root cell membranes of plants establish the electrochemical gradient which drives both passive and active uptake of K into plants. The action of the proton pumping ATPase dominates the electrical properties of plant cell membranes. Proton pumping out of the cell by this enzyme results in a strongly negative (~ –200 mV) membrane potential

across the plant cell membrane with respect to the cell exterior (Serrano, 1989). This membrane potential drives passive K uptake against a concentration gradient through K-conducting ion channels. The Nernst equation tells us that a membrane potential of this magnitude could allow for passive accumulation of K moving into cells through K channels to a level two orders of magnitude greater than external [K]. In fact, cytosolic K is typically 100-150 mM in plant cells (while K in the soil solution is often < 1 mM). The action of the proton pump facilitates passive K uptake into roots at external K concentrations as low as 10 µM.

There are 55 genes (many already cloned) encoding K transporters in Arabidopsis (homologs of many of these genes have also been cloned from crop plants) which can be divided into co-transporters (Na/K symporters, H/K antiporters, and K/H symporters), and channels (Maser *et al.*, 2001). It is currently envisioned that at very low (~µM) soil solution [K], active K uptake into plants occurs primarily through K/H symporters (Maathius and Sanders, 1994; Schachtman and Schroeder, 1994). In this case, the pH gradient (approximately 2 pH units, which contributes an additional 120 mV to the driving force for K uptake; Maser *et al.*, 2001) generated from the action of the proton pumping ATPase is used by the K/H symporter to couple K uptake against the K electrochemical gradient to passive proton influx (Maathius and Sanders, 1994). It should be noted that current reviews (Schachtman, 2000) speculate that inward rectified K channels in roots may contribute significantly to high affinity K uptake into plants, although it is unclear how this could occur at low soil solution [K].

Since 1992, when the first plant K channels were cloned (Anderson *et al.*, 1992; Sentenac *et al.*, 1992), subsequent work has shown that plant genomes encode five families of K-conducting ion channels . Members of four of these groups of channels are voltage-gated. Voltage gated channels facilitate rectified conductance across membranes. Inward-rectified channels are only open at hyperpolarizing membrane potentials. Outward rectifiers, conversely, are open when the plant cell membrane is depolarized from the typical electrical potential (see above). Inward rectified K channels have been shown to be expressed in the root epidermis, cortex, and endodermis, and contribute to K uptake into plant roots (Hirsch *et al.*, 1998). Inwardly conducting K channels are known to be also expressed in leaves and likely facilitate K uptake from the xylem sap (Cao *et al.*, 1995). Inward (and outward) K channels play important roles in turgor changes of guard cells of the stomatal complex. Outward rectified K channels have been recently shown to be expressed in

the stele of plant roots and to be involved in K loading into the xylem of the vascular system for long distance transport to leaves (Gaymard *et al.*, 1998). Several excellent reviews have been published within the past year which describe the molecular properties, electrical characteristics, and physiological functions of plant voltage gated K channels (Maser *et al.*, 2001; Schachtman, 2000). This chapter will focus, therefore, on a fifth family of plant K-conducting ion channels which have only recently been cloned and functionally characterized.

Members of this fifth class of K-conducting ion channels have been recently cloned from barley, tobacco, and Arabidopsis (Leng *et al.*, 1999). These channels open in the presence of the cyclic nucleotides cAMP and cGMP (Leng *et al.*, 1999), and are therefore referred to as cyclic nucleotide gated channels (cngc's). The cyclic nucleotides cAMP and cGMP are thought to be present in the cytosol of plant cells, and are involved in signal transduction pathways which regulate many aspects of cellular metabolism (Assmann, 1995). The experimental results presented in this chapter will focus on the functional characterization of the electrophysiological properties of these newly cloned plant ion channels. This characterization of cloned members of this plant ion channel family was undertaken by expression of the coding sequence in heterologous systems, and use of voltage clamp methods to characterize the functional properties of the channels encoded by cngc cDNAs. Coding sequences of the cDNAs were expressed in frog (*Xenopus laevis*) oocytes, or human embryonic kidney (HEK293) cells.

MATERIALS AND METHODS

Cloning and DNA manipulation: CDNAs encoding a tobacco (*Nicotiana tobacum*) cngc (NtCBP4), and Arabidopsis cngc's (AtCNGC1 and AtCNGC2) were used in the work reported here. GenBank accession numbers for the cDNAs encoding plant cngc's used in this study are AF079872, (NtCBP4); Y16327, (AtCNGC1); and AF067798, (AtCNGC2). The coding sequences of the channels were subcloned into the pGEM-HE plasmid. The pGEM-HE plasmid contains untranslated regions (UTR's) of the endogenous ß globulin protein of oocytes flanking the insertion site; the cRNA generated from this plasmid will contain the ß globulin UTR's, enhancing expression of the recombinant protein in oocytes (Liman *et al.*, 1992). Methylated, capped, runoff transcripts encoding sense cRNAs were generated from NtCBP4, AtCNGC1 and AtCNGC2 cDNAs using the Epicentre AmpliScribe Transcription Kit

(Epicentre Technologies, Madison, WI). Purified cRNAs were used directly for injection into oocytes (50 nL/oocyte containing 50 ng cRNA). Unless otherwise noted standard molecular biology procedures were performed for all DNA and RNA manipulations (Ausubel *et al.*, 1987). All plasmids were verified by sequencing before utilization in any experimental procedure.

Expression in HEK cells: AtCNGC2 cDNA described above was expressed in the human embryonic kidney cell line HEK 293 (American Type Culture Collection, Rockville, MD) for voltage clamp measurements following methods modified from that of Immke *et al.* (1998). HEK cells were cultured in a Napco (Winchester, VA) CO_2 (5%) incubator at 37°C in maintenance medium (Dulbecco's Modified Eagle Medium (Gibco BRL, Grand Island, NY) with 10% (w/v) fetal bovine serum (Gibco) and 1% (w/v) penicillin/streptomycin added). HEK cells were co-transfected with the pcDNA3.1 plasmid (20 µg/0.2 mL) containing the AtCNGC2 coding sequence, and a plasmid encoding the CD8 antigen (1 µg/0.2 mL) by electroporation (Gene Pulser 2 electroporator; Bio-Rad, Hercules, CA) at 75 µF and 366 V. After electroporation, cells were plated on protamine (1 mg/mL)-coated glass cover slips submerged in maintenance medium and incubated for one to two days prior to use for electrophysiological studies. On the day of recording, cells were washed with maintenance medium and incubated with M450 Dynabeads conjugated with anti-CD8 antibody at 1 µL/2 mL (Dynal, Oslo, Norway). Successful transfection was ascertained by the adherence of Dynabeads to a cell (Jurman *et al.*, 1994). These cells were used for electrophysiological recordings in the whole cell configuration at room temperature. Recordings were made with electrodes made from N51A glass pipettes (Garner Glass Co., Claremont CA) which were pulled on a Sutter P87 instrument (Novato, CA) and fire polished using a Narishige MF83 heater (East Meadow, NY). Perfusion bath and pipette solutions were modified from those Rich *et al.* (2000) used for whole cell voltage clamp analysis of the olfactory cngc upon expression in HEK cells. Bath solution contained 145 mM KCl (or NaCl as noted), 10 mM HEPES-KOH (or NaOH when appropriate) pH 7.4, 10 mM D-glucose, and 0.1 mM $MgCl_2$. Pipettes were filled with 145 mM *N*-methyl-D-glucamine (NMG), 10 mM HEPES-KOH pH 7.4, and 0.5 mM $MgCl_2$. Cells were perfused with bath solution containing 100 µM dibutyryl-cAMP in order to activate channel currents. Voltage stimuli were generated and currents were recorded using pClamp 8.04 software (Axon Instruments, Foster City, CA), an Axopatch 200B amplifier (Axon), and a Digidata 1320 analog/digital interface (Axon). Currents were filtered at 1 kHz and a –60 mV holding potential was used for all recordings. Data were analyzed with Clampfit

component of pClamp; and plotted using Sigma Plot 3.0 software (SPSS Scientific, Chicago, IL). Results are presented as means ± S.E.

Expression in oocytes: Whole cell and patch recordings were made of plant cngc's after expression in *Xenopus laevis* oocytes. Frog culture, oocyte preparation, and channel expression in oocytes was as described previously (Leng *et al.*, 1999). Current recordings in the two-electrode configuration were obtained with a GeneClamp 500 amplifier (Axon) and 1320 analog/digital interface, and filtered at 2 kHz. Pipettes were pulled from KIMAX-51 capillaries (KIMBLE Products, Vineland, NJ) and filled with 3 M KCl for use as electrodes. The bath solution contained 96 mM KCl (or 96 mM chloride salts of other monovalent cations), 1.8 mM $CaCl_2$, 1.8 mM $MgCl_2$, and 10 mM HEPES-KOH (NaOH, LiOH, or CsOH as appropriate) pH 7.5 unless otherwise noted. The bath solution was perfused at 2 mL/min into the 1 mL oocyte chamber. Holding potential for two-electrode recordings was –60 mV in all cases, and currents were evoked by adding 100 µM (or other concentrations as noted) of a lipophilic analog of cAMP (dibutyryl-cAMP) to the perfusion bath solution. Typically, cngc currents were recorded in the two-electrode configuration from whole oocytes just prior to, and then after exposure of the oocyte to (lipophilic) cAMP for approximately 40 min; in some cases (as noted), exposure times were longer. In some experiments, AtCNGC2 currents were recorded from inside-out membrane patches pulled from oocytes. In this case, the bath and pipette solutions contained 130 mM KCl, 0.2 mM EDTA, 2 mM HEPES-KOH pH 7.2. For patch recordings, voltage stimuli were generated and currents were recorded using pClamp 8.04 software, an Axopatch 200B amplifier, and a Digidata 1320 analog/digital interface. Currents were evoked by adding 100 µM cAMP to the bath solution with a gravity-driven, multi-barrel perfusion system.

RESULTS AND DISCUSSION

Many K-conducting ion channels display remarkable selectivity for K; others can be classified as non-selective cation channels. Members of the predominant family of plant K-selective channels whose molecular structures have been delineated share the following characteristics (Schachtman, 2000). They are composed of four pore-forming (α) subunits which traverse the membrane and coalesce to form an ion-conduction pathway. Each of these α subunits has the following primary amino acid structure. They have six membrane-spanning regions (S1-S6), a voltage sensor (the S4 membrane-spanning

domain), a pore region with an ion selectivity filter dipping into the membrane, a cyclic nucleotide binding site, and cytosolic amino- and carboxy-terminal regions (Maser *et al.*, 2001). The selectivity filter within the pore of K-selective channels can be considered a remarkable biological structure. These proteins show selectivity for K over Na by a factor of 10,000 (Doyle *et al.*, *1998*), despite the fact that these two monovalent cations have nearly identical Pauling radii (1.33 Anstroms and 0.95 Anstroms, respectively). Sequence analysis (not shown) of the twenty genes in the Arabidopsis encoding cngc's indicates that these α subunits have a similar primary structure. However, in the case of cngc's, embedded within the cyclic nucleotide binding domain is a calmodulin binding domain. Calmodulin is an important cytosolic signaling protein in plants which, upon binding to Ca, can then bind to many proteins such as ATPases and ion channels and modulate their function (Snedden and Fromm, 2001). Calmodulin is known to bind to animal cngc's and prevent cyclic nucleotide activation of the channel (Zagotta and Siegelbaum, 1996). The primary structure of a plant cngc is shown in **Figure 1**.

In a landmark study, Doyle *et al.* (1998) crystallized a portion of a K-selective ion channel (Kcsa), subjecting the crystals to X-ray diffraction analysis, allowing for the first three dimensional characterization of the structure of an ion channel. This important work generated a 'road map' of

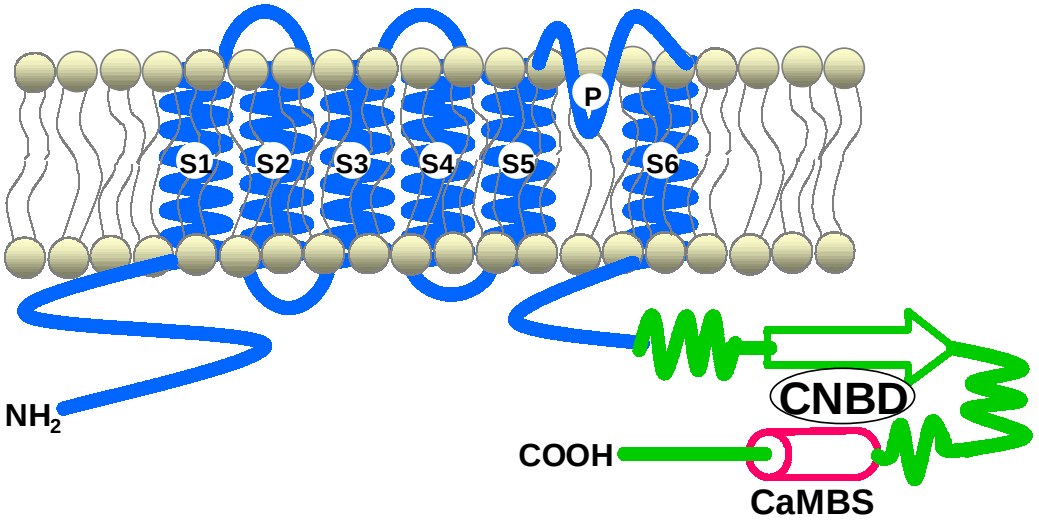

Figure 1. *Transmembrane topological representation of a plant cyclic nucleotide gated channel. Six* α *helices (S1-S6) are shown traversing the membrane, with the pore (P) between S5 and S6 dipping into the membrane from outside the cell. Between the transmembrane domains and the carboxy-terminus, three* α *helices (shown as coils) form the cyclic nucleotide binding domain (CNBD). The calmodulin binding domain (CaMBS) is shown partially overlaying the most carboxy-terminal helix.*

sorts for modeling the three dimensional structure of other ion channel proteins, as is shown in **Figure 2**. Putative three-dimensional structural models were generated for AtCNGC1 and AtCNGC2 in the following manner. In order to identify appropriate modeling templates, query sequences corresponding to the plant cngc's structural domains were run through the Swiss-Model Blast Protein Modeling Server. This utility searches the ExNRL-3D database derived from the protein data base (PDB). Upon identification of a positive structural "hit" (i.e. the PBD record 1BL8, which is the three dimensional structure generated by Doyle *et al.* (1998), the PDB records were downloaded for subsequent analysis. The experimental sequences were sent back through the Swiss-Model Protein Modeling Server using the identified (crystallized) templates. Utilizing the Swiss-Model "First Approach" mode with a lower BLAST P (N) limit of 0.00001, positive structures was rendered

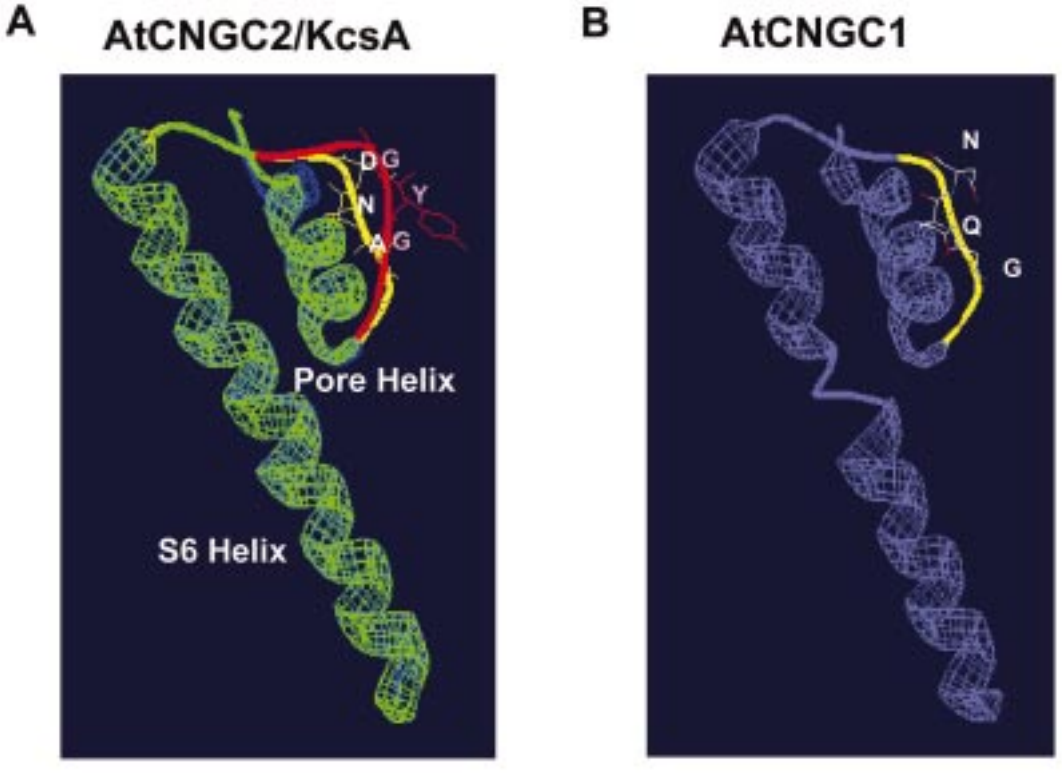

Figure 2. *Three-dimensional renderings of the pore region of K-conducting ion channels. These images are Swiss-Model Protein Data Base Viewer projections. (A) The three dimensional rendering of the transmembrane helices flanking the pore of the K-selective channel Kcsa and the plant cngc AtCNGC2 are nearly superimposeable; the predicted models differ in the selectivity filter region of the pore. Kcsa is modeled to present the amino acids GYG into the pore, while the amino acids 'AND' are projected into the pore in the AtCNGC2 rendering. (B) A similar portion of the plant cngc AtCNGC1 is shown, with the selectivity filter predicted to contain a 'GQN' triplet.*

and analyzed locally through the Swiss-PdbViewer version 3.5 (Glaxo Wellcome Experimental Research). Reproductions of the modeled structures were either rendered by the Persistence of Vision Ray Tracer (POV-Ray) software, or loaded directly into Microsoft PowerPoint as bitmap files and annotated (Guex and Peitsch, 1997).

Figure 2A shows the three dimensional structure of the S6, pore selectivity filter, and pore membrane spanning helix of AtCNGC2 overlaid upon the corresponding domains of Kcsa. The three-dimensional structures of the K-selective channel Kcsa and the plant cngc AtCNGC2 are nearly identical; the exception is the selectivity filter of the two channels. Kcsa, and other (plant and animal) K-selective channels have the amino acid triplet 'GYG' within the selectivity filter (Schachtman 2000; Heginbotham *et al.*, 1994). This amino acid triplet has been shown to be required for the selective conduction of K and exclusion of Na by K-selective channels (Heginbotham *et al.*, 1994). Animal cngc's do not contain this GYG selectivity filter; this molecular aspect of cngc's has been identified as the basis for the fact that this family of animal ion channel proteins do not discriminate between K and Na conductance (Zagotta and Siegelbaum, 1996). Sequence analysis (not shown), and our modeling of plant cngc three-dimensional structure (**Figure 2**) indicates that the 23 plant cngc's which have their deduced amino acid sequences identified to date also do not contain the GYG (glycine-tyrosine-glycine) motif in the selectivity filter within the pore of the channel. Our modeling analysis indicates that AtCNGC2 has the triplet 'AND' (alanine-asparagine-aspartate) within the selectivity filter (**Figure 2A**), and AtCNGC1 has the triplet 'GQN' (glycine-XXX-asparagine) in this region of the pore (**Figure 2B**). The modeling of these plant cngc's suggests a similar structure for the other plant cngc's.

Results shown in **Figures 3** and **4** indicate that the plant cyclic nucleotide gated channel AtCNGC2, with the pore triplet 'AND', does show selectivity for K conductance over Na. In **Figure 3**, the AtCNGC2 coding sequence was expressed in *Xenopus* oocytes. All currents shown are 'leak subtracted' (i.e. the current generated in the absence of (dibutyryl) cAMP was subtracted from those obtained in the presence of cyclic nucleotide, as is the convention with electrophysiological analyses of cngc channels (see refs. in Zagotta and Siegelbaum, 1996). AtCNGC2 is shown to conduct K and a number of other monovalent cations, but discriminate against Na conductance. Similar results were obtained when AtCNGC2 was expressed in HEK cells (**Figure 4**). In **Figure 4**, AtCNGC2 currents are presented without leak subtraction. In the absence of cAMP, K currents were no greater than those obtained from control

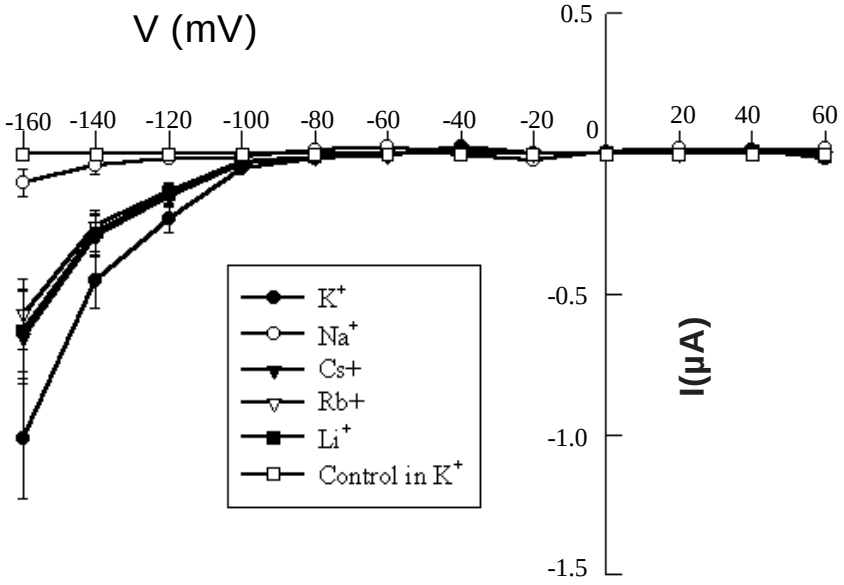

Figure 3. *Current/voltage (I/V) relationship of AtCNGC2 currents recorded from oocytes (two-electrode configuration) with a range of monovalent cations in the perfusion bath. All currents (except for the control) are presented as leak subtracted recordings. Holding potential was –60 mV and command voltages were applied in 20 mV steps between +60 mV and –160 mV. For each monovalent cation shown (all at 96 mM), currents were recorded with a bath solution lacking dibutyryl-cAMP. cAMP-activated currents were recorded after 40 min perfusion with 100 µM dibutyryl-cAMP. In each case, the leak current was subtracted from the cAMP-activated current, yielding the current values shown at each command voltage tested. The control treatment was recorded with KCl and dibutyryl-cAMP in the bath. Current values are presented as means (n=6 (control), 11 (K), 8 (Na), 7 (Cs), 6 (Rb), and 4 (Li)) ± S. E.*

HEK cells (i.e. not transfected with the AtCNGC2 cDNA). Na currents, both in the presence and absence of cAMP, were also no greater than those obtained from control cells. Upon addition of cAMP to the perfusion bath in which the recordings were made, the plant cngc was shown to conduct a significant level of current above both control cells, and cells transfected with AtCNGC2 when cAMP was absent. In these presentations of recordings of ion channel current, negative currents indicate movement of cations (K) into the cells. At the hyperpolarizing voltages (-100 to –160 mV) which can be present across plant cell membranes, then, this ion channel conducts K into the cell. At depolarizing membrane potentials (less negative, and positive voltages), the channel conducts no current; demonstrating that this channel is inward rectified. The insert in **Figure 4** shows representative time-dependent currents recorded from a single cell, in the presence of either K or Na. This presentation indicates that the AtCNGC2 K currents were non-inactivating; i.e. once the

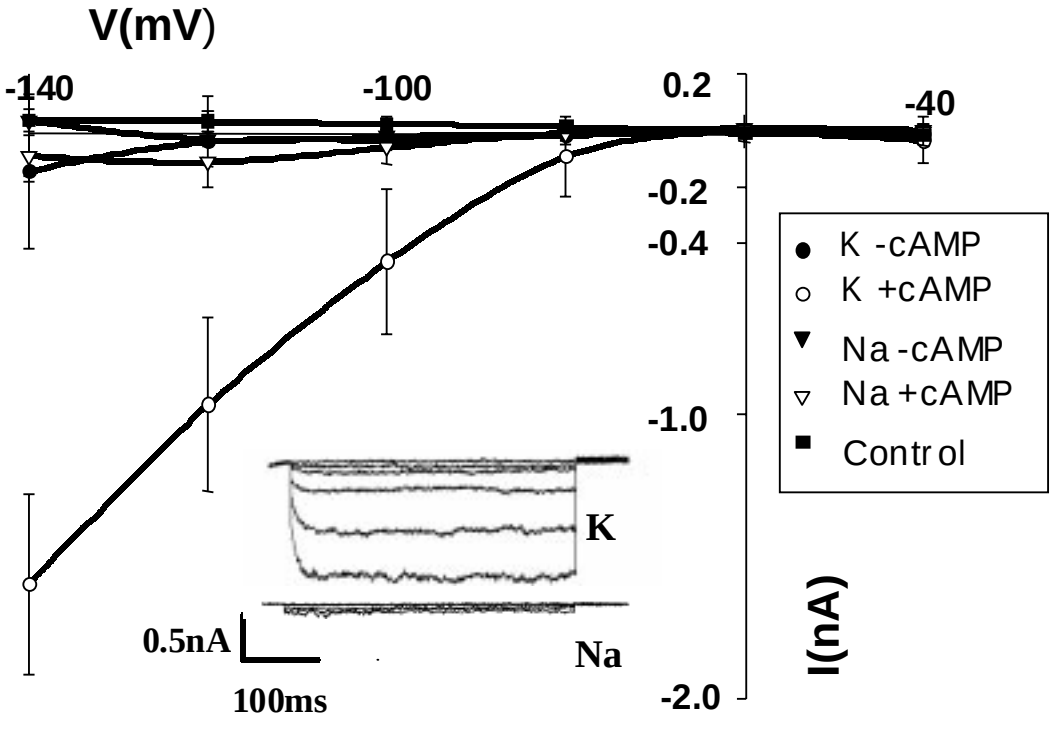

Figure 4. *Current/voltage relationship of AtCNGC2 currents recorded from HEK cells (whole cell configuration) with 145 mM K or Na in the perfusion bath, in the presence and absence of dibutyryl-cAMP. Holding potential was –40 mV and command voltages were applied in 20 mV steps between –40 mV and –140 mV. The currents recorded in the presence of 100 µM dibutyryl-cAMP are presented in this figure without leak subtraction; i.e. separate current values are shown for K or Na in the presence, and absence of cAMP. The control treatment shown in* ***Figure 2A*** *is the current (recorded in the presence of cAMP) measured from HEK cells that were not transfected with AtCNGC2. Results are presented as means (n=6)±S. E. The insert shows representative time-dependent currents for HEK cells expressing AtCNGC2 in the presence of cAMP and either K or Na in the bath solution.*

channel is opened in the presence of cAMP, the channel remains open during the time course of the recording period. Unlike most animal K-conducting ion channels, all K channels cloned to date from plants which have been electrophysiologically characterized are non-inactivating (Schachtman, 2000). It is thought that plant ion channels are non-inactivating because they provide a conduction pathway for nutrient uptake (Schroeder *et al.*, 1994). Animal channels often are involved in action potentials of excitable cells (e.g. nerve and muscle cells) and, therefore, are often gated so that they are quickly inactivated to facilitate action potentials. The data presented in **Figures 3** and **4** indicate that the plant gene AtCNGC2 encodes an ion channel that is inward rectified, non-inactivating, and activated by cAMP. In other experiments (not shown), AtCNGC2 was demonstrated to be activated by cGMP also.

Cyclic nucleotide gated channels are defined functionally as ligand-gated channels which are *activated* by ligand (cAMP or cGMP) binding to the channel protein (Zagotta and Siegelbaum, 1996). Conductance facilitated by some plant and animal voltage-gated K channels is also affected by cyclic nucleotides, but in a different manner (Hoshi, 1995). In this case, the rectified conductance of channels is activated by voltage, but direct binding of cyclic nucleotide to the protein modulates the voltage:current relationship. However, these channels are structurally and functionally distinct from cngc's. Binding of cyclic nucleotide to this class of plant channels results in a reduction of current at a given voltage, but voltage is the primary determinant of conductance (Hoshi, 1995). Cytosolic cyclic nucleotides are also known to modulate the conductance of other classes of K-selective channels, but in an indirect fashion (Zagotta and Siegelbaum, 1996), through cyclic nucleotide-dependent protein kinase phosphorylation of the channel which alters channel conductance (Wang and Giebisch, 1991; Rudy *et al.*, 1991). K currents across some native plant cell membranes are, in fact, modulated by cAMP-dependent protein kinase phosphorylation of the channel (Li *et al.*, 1994).

Further evidence that AtCNGC2 is a plant cngc is presented in **Figure 5**. We have thus far demonstrated (**Figures 3** and **4**) that when a lipophilic analog (dibutyryl-cAMP) of a cyclic nucleotide is added to the perfusion bath, whole cell recordings of HEK cells or two-electrode voltage clamp recordings from intact oocytes expressing the AtCNGC2 coding sequence show increased amplitude of inward K currents. In these experiments, the possibility that cyclic nucleotide activation of the channel is indirect (e.g. via channel phosphorylation mediated by a cyclic nucleotide-dependent kinase) cannot be discounted. The experiments shown in **Figure 5** address this possibility. These recordings were made from (inside-out) patches pulled from oocytes injected with AtCNGC2 cRNA. In this configuration, the lipophilic form of the cyclic nucleotide is not required for activation; cAMP added to the perfusion bath can reach the cyclic nucleotide binding site of the channel without diffusing through the cell membrane. Since these recordings are made from membrane patches, the cytosolic contents of the cell are absent; the effect of cAMP on the channel cannot, therefore, be attributed to cytosolic signaling systems such as kinase-dependent phosphorylation. When a membrane patch is clamped at 0 mV, and then exposed to a 60 mV step potential in the absence of cAMP, no channel currents are evoked (**Figure 5A**). When the same membrane patch is exposed to the 60 mV step potential while cAMP is present in the perfusion bath solution, K^+ currents are evident (**Figure 5B**). A portion of the recording shown in **Figure 5B** is shown in an

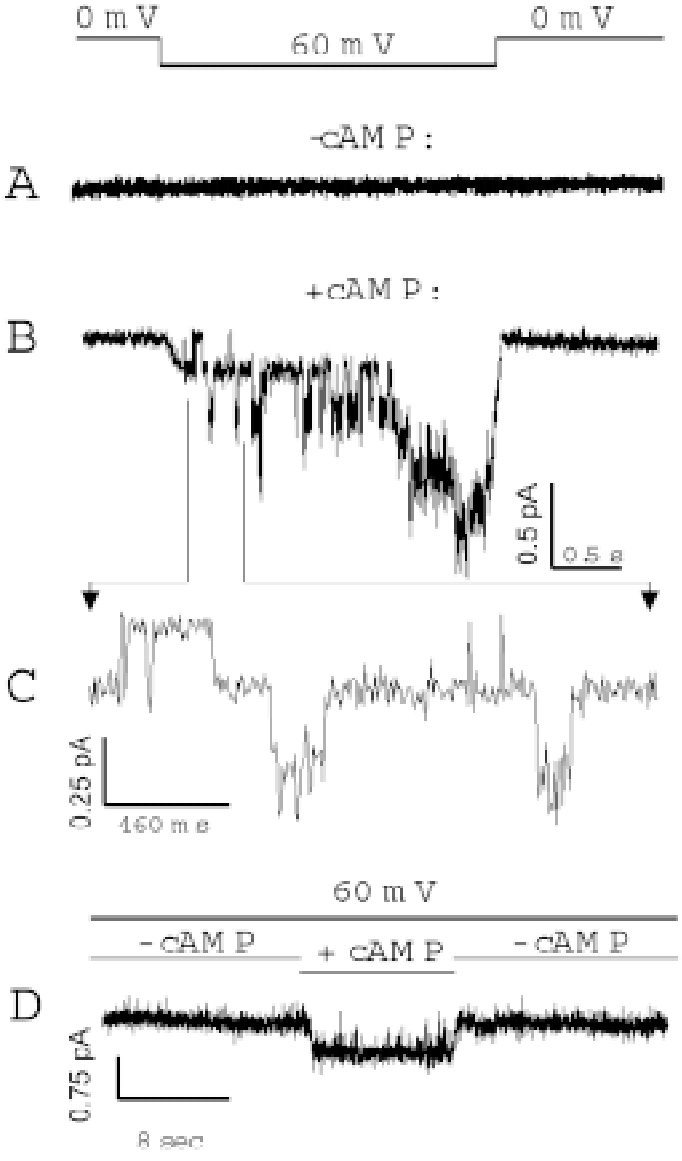

Figure 5. *K currents recorded (inside-out patch configuration) in the presence and absence of 100 µM cAMP from membranes of oocytes expressing AtCNGC2. Currents shown in A and B are from the same patch. The bar at the top indicates the patch was held at 0 mV, and a 60 mV step voltage was applied as indicated for the recordings shown in A and B. Currents shown in A were obtained in the absence of cAMP, and the recording shown in B is from the same patch after perfusion with bath solution containing cAMP. The current tracing in C is a portion of the recording in B presented with an expanded scale. The current tracing in D is from a different patch, which was held at 60 mV command potential during the time course of the recording. The bars above the current trace indicate time periods when cAMP was absent, or applied to the bath solution.*

expanded scale in **Figure 5C**. The expanded scale presentation in **Figure 5C** shows several discreet channel-opening events; the current evoked by application of cAMP to the patch was generated from the opening of four to five individual channels during the time period shown in **Figure 5B**. AtCNGC2 current recordings obtained from another oocyte membrane patch are shown in **Figure 5D**. In this case, a 60 mV potential gradient was imposed across the membrane during the entire time course. During this time course (as indicated) cAMP was applied to the patch, and then the patch was again perfused with bath solution lacking cAMP. In this case, current was generated across the patch only during the application of cAMP. The results shown in **Figure 5**, therefore, provide evidence consistent with the presence of cngc's in the membrane of oocytes injected with AtCNGC2 cRNA. Currents were evoked by the application of cAMP in the absence of the cytosolic factors that could be present within the intact cell (HEK or oocyte). These data support the contention that cAMP activation of AtCNGC2 currents is a direct effect of the ligand binding to the channel.

We have recently attempted to undertake the elctrophysiological characterization of other plant cngc's. We have focused our attention on the Arabidopsis cngc AtCNGC1, and the tobacco channel NtCBP4. Recent studies with these channels have raised the possibility that these (putative) K-conducting channels may play important roles in plant response to soil solutions that negatively impact growth of crop plants. AtCNGC1, and a tobacco cngc, NtCBP4, may be involved in Ca and/or K uptake from the soil. In these roles, they may provide a pathway for deleterious heavy metal (Pb) or Na uptake. Sunkar *et al.* (2000) have recently provided clear evidence that NtCBP4 provides a pathway for Pb uptake into plants; translational arrest of the gene reduced Pb sensitivity of transgenic tobacco in these studies. Maathuis and Sanders (2001) have recently suggested that AtCNGC1 may be expressed in plant roots, and, as a K uptake pathway into plants, possibly provide a mechanism for deleterious Na uptake into plants growing in saline soils. Understanding the role plant cngc's play in plant growth and development could be aided by characterization of their molecular properties.

Our screens of plant cngc expression in oocytes have routinely failed to demonstrate AtCNGC1 and NtCBP4 currents (data not shown). We have observed no inward K currents in an oocyte expressing one (AtCNGC1) of these channels at low (100 μM) [cAMP], while increasing bath cAMP up to the mM range did induce currents in the same oocyte (data not shown). When higher perfusion bath cAMP concentrations are used (as compared to

those used to evaluate AtCNGC2 currents), along with longer incubation times (post application of cyclic nucleotide), we have observed repeatable induction of inward K currents in oocytes injected with AtCNGC1 (**Figure 6**) and NtCBP4 (**Figure 7**) cRNA. In both cases, currents through these plant cngc's are inward rectified and non-inactivating (see inserts in **Figures 6** and **7**), as is the case with AtCNGC2 (**Figure 4**). Currents were evoked from oocytes expressing NtCBP4 and AtCNGC1 after unusually long incubation times with relatively high concentrations of bath lipophilic cAMP. At this time, we are unclear as to why the functional analysis of these plant cngc's requires different experimental conditions that those which we have used to document the ion

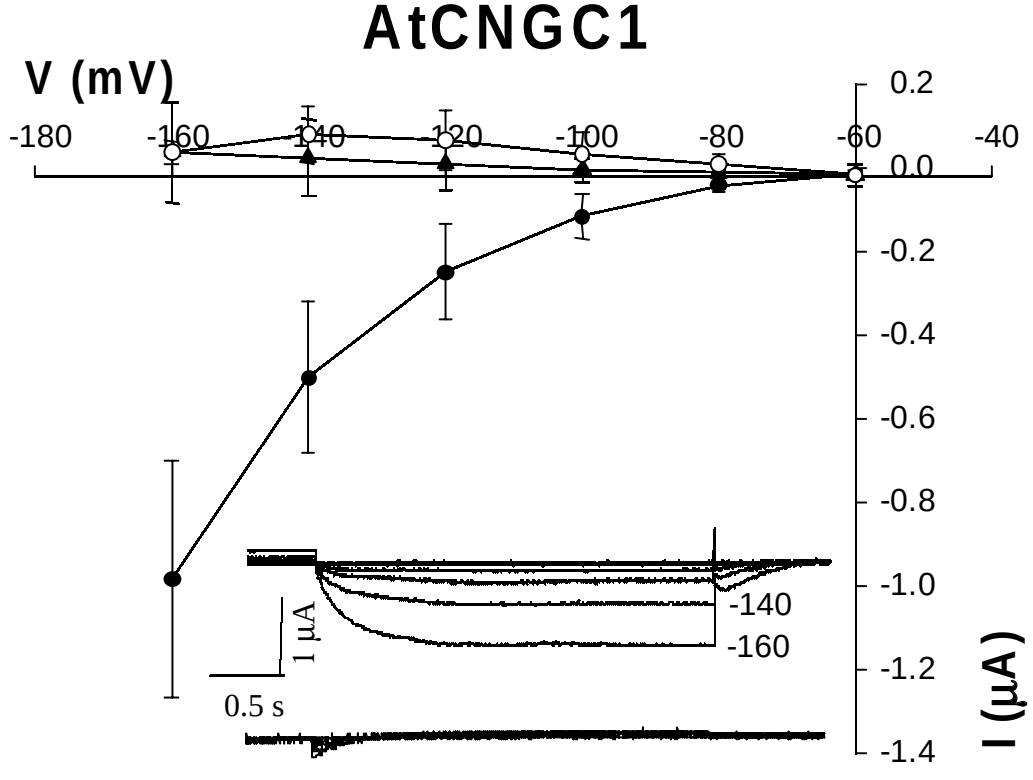

Figure 6. *Current/voltage relationship of AtCNGC1 K (96 mM) currents recorded from oocytes (two-electrode configuration). Recording conditions were as in the experiment shown in* ***Figure 3****, except that dibutyryl-cAMP was at either 0 (triangles) or 5 mM (circles). Command voltages were applied in 20 mV steps between –60 mV and –160 mV. Results are presented without leak subtraction. Recordings were made from oocytes injected with AtCNGC1 cRNA prior to addition of dibutyryl-cAMP to the perfusion bath solution (triangles), and 3 h after addition of dibutyryl-cAMP to the perfusion bath solution (closed circles) in this experiment. Water-injected oocytes were also exposed to dibutyryl-cAMP for 3 h prior to recording currents (open circles). Results are presented as means (n=4)±S. E. The insert shows representative time-dependent currents recorded from oocytes expressing AtCNGC1 prior to (bottom traces) and 3 h after addition of dibutyryl-cAMP (top of insert).*

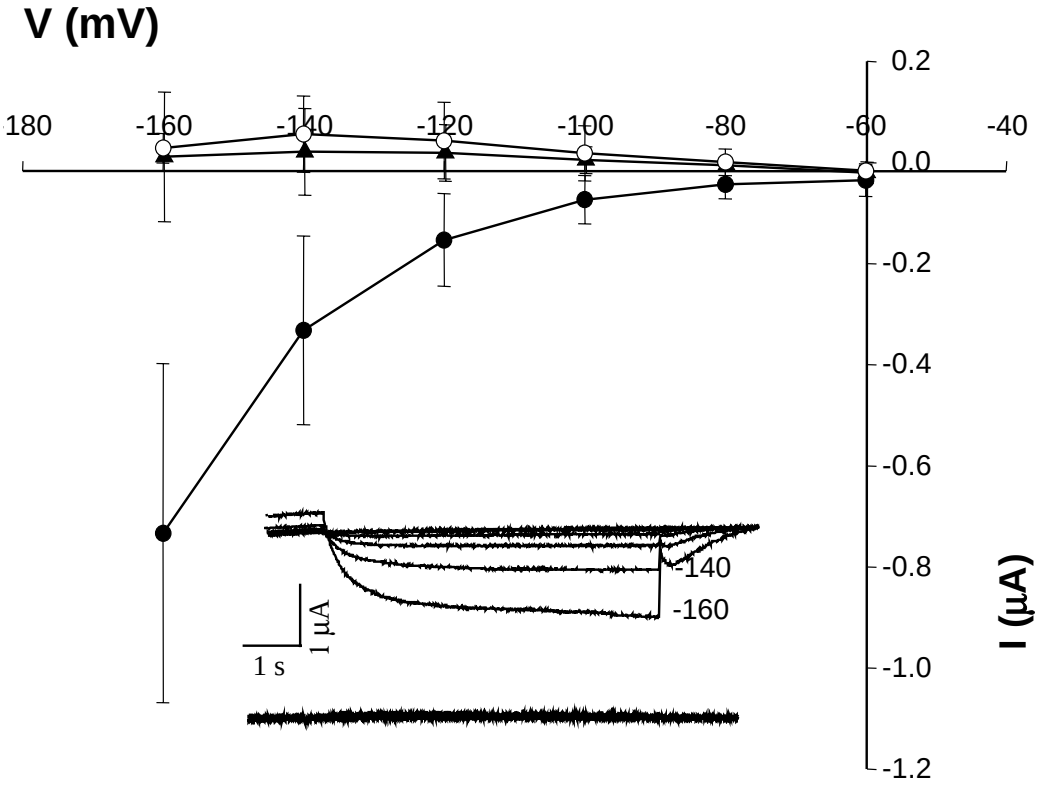

Figure 7. *Current/voltage relationship of NtCBP4 K (96 mM) currents recorded from oocytes (two-electrode configuration). Recording conditions were as in the experiment shown in* ***Figure 3****, except that dibutyryl-cAMP was at either 0 (triangles) or 5 mM (circles). Command voltages were applied in 20 mV steps between –60 mV and –160 mV. Results are presented without leak subtraction. Recordings were made from oocytes injected with NtCBP4 cRNA prior to addition of dibutyryl-cAMP to the perfusion bath solution (triangles), and 1 h after addition of dibutyryl-cAMP to the perfusion bath solution (closed circles) in this experiment. Water-injected oocytes were also exposed to dibutyryl-cAMP for 1 h prior to recording currents (open circles). Results are presented as means (n=4)±S. E. The insert shows representative time-dependent currents recorded from oocytes expressing NtCBP4 prior to (bottom traces) and 1 h after addition of dibutyryl-cAMP (top of insert).*

channel characteristics of AtCNGC2 (**Figures 3-5**). Nonetheless, the data presented in this chapter provides a functional characterization of a newly cloned family of K-conducting ion channels.

Our current work (not shown) has provided preliminary evidence that, in contrast to the selectivity for K over Na conductance of AtCNGC2, AtCNGC1

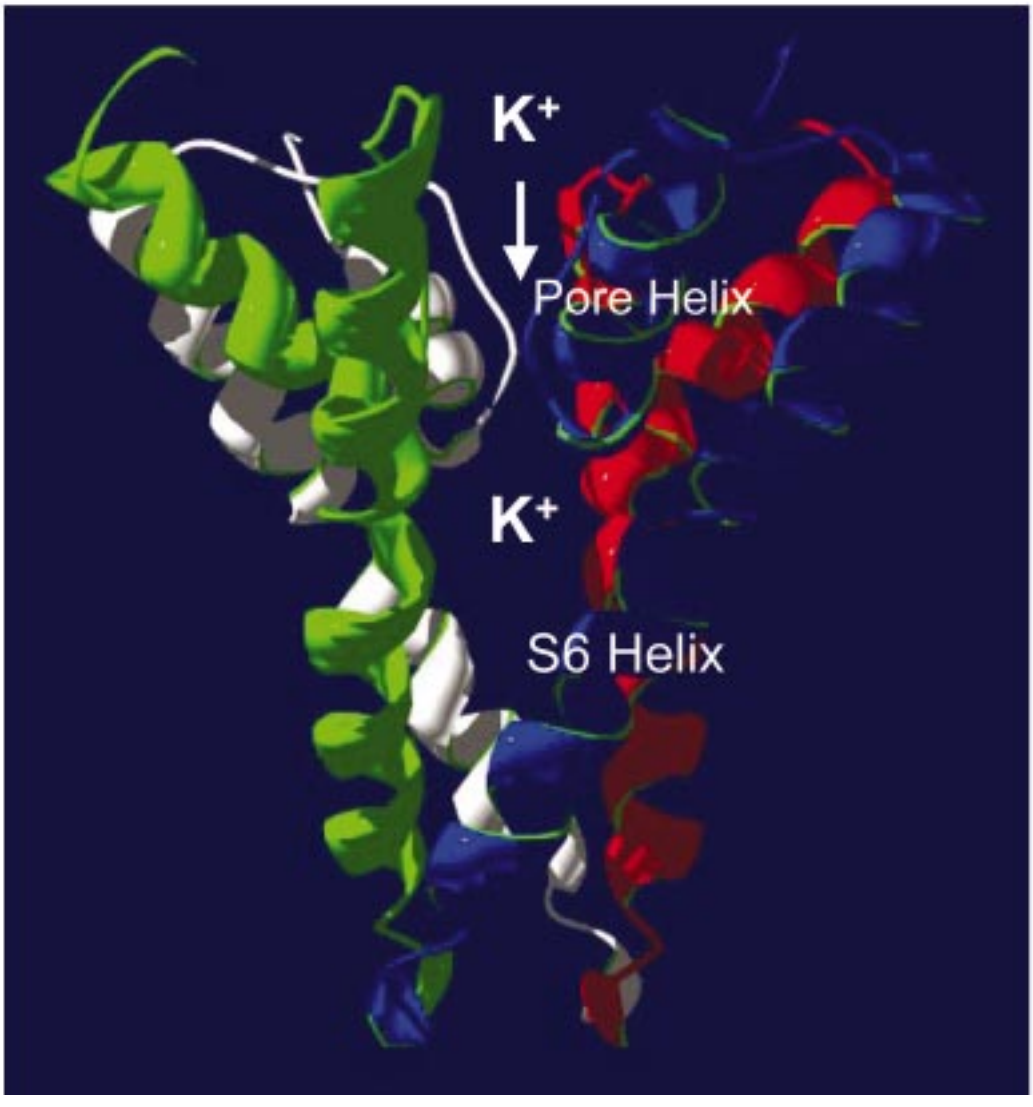

Figure 8. *Persistence of Vision Ray Tracer (POV-Ray) projection of the quaternary protein structure of a portion of the plant cyclic nucleotide gated channel AtCNGC2. The protein projection is a homotetramer of the AtCNGC2* α *subunit portrayed in* ***Figure 2A****. The tetramer forms an 'inverted tee-pee' with the larger portion of the protein (at the top) oriented towards the extracellular side of the membrane. External K moves into the cell through the pore formed across the membrane.*

may not discriminate between K and Na. This finding does not necessarily contradict with the results shown in **Figures 3** and **4**. Our protein modeling studies (**Figure 2**) suggest that, of the 23 known sequences of putative plant cngc's, only AtCNGC2 has the 'AND' motif in the selectivity filter. The unique selectivity for K over Na conductance demonstrated for the plant cngc

AtCNGC2 may not be repeated in other plant cngc's. In concert with current work in a number of laboratories which suggests that plant cngc's are expressed in roots and that expression of this channel may impact cation uptake by plants from the soil solution, the work presented here provides an initial characterization of a new family of plant K-conducting ion channels which may play heretofore unknown roles in a number of important physiological processes in plants.

A model of a representative plant cngc channel protein complex is shown in **Figure 8**. This model is of the K-conducting pathway formed by the oligomerization of four individual AtCNGC2 subunits. The four plant cngc subunits are envisioned to form an ion-conducting pathway across the plant cell membrane that appears as an 'inverted tee-pee' in **Figure 8**. As in the case of other K-conducting ion channel proteins, the amino acids that line the pore form the selectivity filter. At this point, we speculate that the specific amino acids which form this part of the channel protein complex may be different in various members of this plant ion channel family, and provide a molecular basis for uptake of K, along with possibly other cations into plants when these channel genes are expressed in roots.

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