Research Article

The group IV-A cyclic nucleotide-gated channels, CNGC19 and CNGC20, localize to the vacuole membrane in Arabidopsis thaliana

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Abstract. Plant cyclic nucleotide-gated channels (CNGCs) are implicated in the uptake of both essential and toxic cations, Ca\(^{2+}\) signalling, and responses to biotic and abiotic stress. The 20 CNGC paralogues of Arabidopsis are divided into five evolutionary groups. Group IV-A is highly isolated and consists only of two closely spaced genes, CNGC19 and CNGC20. Prior studies have shown that both genes are induced by salinity and biotic stress. A unique feature of CNGC19 and CNGC20 is their long hydrophilic N-termini. To determine the subcellular locations of CNGC19 and CNGC20, partial and full-length fusions to GFP\(\text{S65T}\) were generated. Translational fusions of the N-termini of CNGC19 (residues 1–171) and CNGC20 (residues 1–200) to GFP\(\text{S65T}\) were targeted to punctate structures when transiently expressed in leaf protoplasts. In the case of CNGC20, but not CNGC19, the punctate structures were co-labelled with a marker for the Golgi. The full-length CNGC19-GFP fusion co-localized with markers for the vacuole membrane (\(\alpha\)TIP- and \(\gamma\)TIP-mCherry). Vacuole membrane labelling by the full-length CNGC20-GFP fusion was also observed, but the signal was weak and accompanied by numerous punctate signals that did not co-localize with \(\alpha\)TIP- or \(\gamma\)TIP-mCherry. These punctate structures diminished, and localization of full-length CNGC20-GFP to the vacuole increased, when it was co-expressed with the full-length CNGC19-mCherry. Vacuole membrane labelling was also detected \textit{in planta} via immunoelectron microscopy using a CNGC20-antiserum on cryopreserved ultrathin sections of roots. We hypothesize that the role of group IV-A CNGCs is to mediate the movement of cations between the central vacuole and the cytosol in response to certain types of abiotic and biotic stress.

Keywords: Calcium signalling; cation channels; cyclic nucleotide; secretory pathway; vacuole.

Introduction

Plants have evolved several distinct classes of transporters and channels to facilitate the movement of cations across cellular membranes (Mäser et al. 2001). The members of the plant cyclic nucleotide-gated channel (CNGC) family are evolutionarily and structurally related to Shaker-type K\(^{+}\) channels, but are typically permeable to a range of monovalent cations (Leng et al. 2002; Balagué et al. 2003; Hua et al. 2003; Gobert et al. 2006; Christopher et al. 2007). Several CNGCs have also been shown to translocate divalent cations such as Ca\(^{2+}\) and Mg\(^{2+}\) (Leng et al. 1999; Ali et al. 2006; Frietsch et al. 2007; Urquhart et al. 2007; Guo et al. 2010), and there is a growing body of evidence that the molecular function of some CNGCs is to mediate Ca\(^{2+}\) signalling (Talke et al. 2003; Ma et al. 2009). Cyclic nucleotide-gated channels are oppositely regulated by two distinct second messengers, cyclic nucleotides and Ca\(^{2+}\)/calmodulin,
through partially overlapping ligand-binding domains located near the C-terminus (Leng et al. 1999, 2002; Arai et al. 2000; Köhler and Neuhaus 2000; Balagué et al. 2003; Li et al. 2005; Yoshioka et al. 2006). In general, cyclic nucleotides activate CNGCs, whereas Ca²⁺/calmodulin inhibit them. The phenotypic characterization of cngc mutants and antisense lines implicates members of this channel family in the uptake and distribution of monovalent and divalent cations (Sunkar et al. 2000; Li et al. 2005; Gobert et al. 2006; Ma et al. 2006; Christopher et al. 2007; Guo et al. 2008, 2010; Yuen and Christopher 2010), plant defence responses (Yu et al. 1998; Clough et al. 2000; Balaguè et al. 2003; Yoshioka et al. 2006; Moeder et al. 2011), gravitropism (Ma et al. 2006; Borsics et al. 2007) and pollen tube elongation (Chang et al. 2007; Frietsch et al. 2007).

Plant CNGCs are divided into five phylogenetic subfamilies, designated as groups I, II, III, IV-A and IV-B (Mäser et al. 2001). Of the 20 CNGCs in Arabidopsis, only two genes, CNGC19 and CNGC20, constitute group IV-A. These tandem genes are closely spaced on chromosome 3. Whereas CNGC19 is expressed within the vasculature of roots and leaves, CNGC20 is expressed in the root cortex, the carpel and sepal cells of flowers, guard cells and in leaf mesophyll cells proximal to veins (Kugler et al. 2009). Although salt stress influences the expression of both genes (Maathuis 2006; Kugler et al. 2009; Yuen and Christopher 2010), knockout mutations in either gene do not result in hypersensitivity to Na⁺, or altered accumulation of Na⁺ within plant tissues (Maathuis 2006; Kugler et al. 2009). Null mutants of CNGC19 and CNGC20 exhibit reduced resistance to avirulent pathogen infection, indicating a role for these genes in plant defence responses (Moeder et al. 2011). Furthermore, CNGC19 is induced by bacterial infection and the bacterial elicitor, flg22, and T-DNA insertion mutants of CNGC19 display increased susceptibility to the fungal pathogen, Botrytis cinerea (Moeder et al. 2011).

To understand how individual CNGCs contribute to cation fluxes in plants, it is crucial to define their distribution patterns within cells. In plants, CNGCs are present at both the plasma membrane (PM) and the membrane of vacuoles. The subcellular localization of CNGCs has primarily been accomplished through the use of chimeric fluorescent reporter proteins. Fluorescent protein fusions indicate that at least four of the six CNGCs belonging to group I are targeted to the PM: CNGC3 (Gobert et al. 2006), CNGC10 (Christopher et al. 2007) and CNGC11 and CNGC12 (Baxter et al. 2008). CNGC18, a pollen-specific member of group III, was shown to localize to a region of the PM near the tip of expanding pollen tubes (Chang et al. 2007; Frietsch et al. 2007). The group II members, CNGC7 and CNGC8, are also expressed exclusively in pollen, but localize to the tonoplast (Chang et al. 2007). The subcellular distribution of CNGCs has been studied in a few cases by immunolocalization. Using paralogue-specific antibodies, CNGC5 (group II) was detected at the PM of leaf protoplasts by immunofluorescence microscopy, and CNGC10 was shown by high-resolution immunoelectron microscopy to pass through the secretory pathway and localize to the PM (Christopher et al. 2007). In contrast, no experimental information is available on the subcellular localizations of CNGC19 or CNGC20. Protein sorting algorithms predict that CNGC20 resides in the chloroplast (Sherman and Fromm 2009). However, no CNGC-related sequences were identified in two comprehensive, experimentally derived datasets of the Arabidopsis chloroplast proteome, AT_CHLORO (Ferro et al. 2010) and the Plastid Proteome DataBase (van Wijk and Baginsky 2011).

In this report, we utilized fluorescent reporter protein fusions and immunoelectron microscopy to determine the subcellular destinations of CNGC19 and CNGC20. We demonstrated that both channels are targeted to vacuolar membranes. Our findings suggest that group IV-A CNGCs mediate plant responses to salinity and pathogen infection by facilitating the movement of cations between the central vacuole and the cytosol.

**Methods**

**Protein sequence analysis**

Subcellular localization predictions for Arabidopsis CNGCs were performed using TargetP v1.1 (Nielsen et al. 1997). Transmembrane domain predictions were performed using TMHMM v1.0 (Krogh et al. 2001). Genes encoding putative group IV-A CNGCs were identified from the sequenced genomes of Oryza sativa, Populus trichocarpa, and Physcomitrella patens by BLASTP searches, with CNGC19 and CNGC20 serving as the query sequences. The poplar homologues, designated here as PtCNGC-Iva1, PtCNGC-Iva2 and PtCNGC-Iva3 (GenBank: EEE95941.1, EEF07751.1 and EEF07752.1, respectively), and the rice homologue OsCNGC-Iva1 (GenBank: BAD16877.1) were obtained using the NCBI BLAST server, while the moss homologue PpCNGC-Iva1 (COSMOS v1.6: Pp1s204_120V6) was identified by a Phytozome BLAST search. Phylogenetic analysis with all 20 CNGCs from Arabidopsis confirmed that these CNGCs belong to group IV-A (data not shown).

**Generation of translational fusion constructs**

All constructs used for protoplast transfection in this study were generated in cloning vector pBluescript KS(+) (data not shown). The generation of pBL(35S:GFP(S65T)) was...
described previously (Cho et al. 2011). The constructs pBL(35S:CNGC19FL-GFP) and pBL(35S:CNGC20FL-GFP) were created by replacing the EGFP coding sequence of plasmid pBL(35S:CNGC10-EGFP) (Christopher et al. 2007) with GFP (S65T), and the CNGC10 genomic DNA sequence with the full-length genomic DNA sequences of CNGC19 (At3g17690) or CNGC20 (At3g17700), respectively. The GFP (S65T) fragment was amplified from plasmid HBT95: sGFP(S65T)-NOS with primers GFP/NdeI (5′-CACCAGACTAGTATGAGTGAATGGG-3′) and NdeI/BstEII. It was necessary to ligate the CNGC10 and CNGC20 fragments between NcoI and BstEII restriction sites of pBL(35S:CaMV35S-GFP). To amplify CNGC19 was amplified using primers CNGC19/NcoI.F (5′-TAACCATAGTACCTTTACATCCTATC-3′) and CNGC19(N2) using primers CNGC19/NcoI.F and CNGC19(D86)/NdeI.R, while CNGC20 was amplified using primers CNGC20/NcoI.F (5′-TGTCACACCTTTTACATCCTATC-3′) and CNGC20/NdeI.R (5′-AATCATGAATGCTATCCTATCCTATC-3′). The GFP (S65T) fragment was cloned between the NdeI and BstEII restriction sites of pBL(35S:CNGC10-EGFP), and the CNGC19 and CNGC20 fragments between NcoI and NdeI. It was necessary to ligate the CNGC20 ampli- con sequentially as two separate fragments due to the presence of an internal NdeI site. Green fluorescent protein fusions to the N-terminal regions of CNGC19 and CNGC20 were developed as described above, except that the CNGC19(N2) fragment was obtained using the primer pair CNGC19/NcoI.F (5′-GTGACATGATCAGCAGCTCA-3′) and CNGC19(N1) with primers CNGC19/NcoI.F and CNGC19(V166)/NdeI.R (5′-GTCCACATGATCAGCAGCTCA-3′) and CNGC20(N1) using primers CNGC20/NcoI.F and CNGC20(E117)/NdeI.R (5′-GTCCACATGATCAGCAGCTCA-3′) and CNGC20(N2) using primers CNGC20/NcoI.F and CNGC20(V200)/NdeI.R (5′-GTCCACATGATCAGCAGCTCA-3′). The fusion construct pBL(35S:CNGC19FL-mCherry) was generated by ligation of a PCR fragment containing both the coding sequence for mCherry and the nopalin synthase (nos) 3′ untranslated region (UTR) between the NdeI and SacI sites of pBL(35S:CNGC19FL-GFP). This fragment was amplified from plasmid pt-rk (Nelson et al. 2007), using the primers GFP/NdeI.F and Nos/SacI.R (5′-TGGTACCTGAGGATCCATC)-3′). The constructs used for the transient expression of fluorescent markers for the Golgi [pBL(35S:Man49- mCherry)], peroxisomes [pBL(35S:mCherry-5KL1)] and tonoplast [pBL(35S:αTIP-mCherry)] are derivatives of the organelle markers developed by Nelson et al. (2007). The new plasmids were generated by digesting the binary plasmids G-rk, PX-rk and vac-rk (respectively) with SacI and HindIII, and mobilizing the reporter gene cassettes between the corresponding restriction sites of pBluescript KS(+). The tonoplast marker construct, pBL(35S:αTIP-mCherry), was generated by amplification of the full-length genomic DNA sequence of AtαTIP (At1g73190; also referred to as AtTIP3;1) with primers αTIP/XbaIF (5′-AAGTGCTGATCATCTCATG-3′) and αTIP/BamHRI (5′-GTTCGGAGTATCATCTCGTCCAGG-3′), and subsequent ligation of the PCR product between the SpeI and BamHI sites of pBL(35S-γTIP-mCherry). The plastid envelope marker pBL(35S:NTT2-mCherry) was created in a similar manner, using primers NTT2/SpeI.F (5′-GTCTTACTAGTAGTATGAGGAGATG-3′) and NTT2/BamHI.R (5′-ATCATGGATCCATGAGAATGAGTGAAGT-3′) to amplify the genomic DNA sequence of AtNTT2 (At1g15500). pBL(35S:NTT2-GFP) was created by digesting a PCR fragment of pBluescript KS(+) with KpnI and NcoI, and cloning the 3.1-kb fragment containing the cauliflower mosaic virus (CaMV) 35S promoter and AtNTT2 genomic DNA sequence between the KpnI and NcoI sites of pBL(35S:GFP(S65T)). The construct pBL(35S:BiP1- mCherry-HDEL) contains, in order, the CaMV 35S promoter, the AtBiP1 (At5g28540) genomic DNA sequence, the coding sequence of mCherry-HDEL and the nos terminator, inserted between the KpnI and SacI sites of pBluescript KS(+). The CaMV 35S promoter region was amplified with primers 35S/KpnI.F (5′-AACCGGGATCCATGAGGAGAAGTTG-3′) and 35S/SacI.R (5′-ACTGTTACCAATGAGAATGAGTGAAGT-3′), the AtBiP1 gene was amplified from genomic DNA with primers BiP1/XbaI.F (5′-GACTGGCGCGAGAAGTTGCTGCGTACC-3′) and BiP1/XbaI.R (5′-AGACCCGGGATCCATGAGAATGAGTGAAGT-3′), and the mCherry-HDEL coding sequence and nos 3′ UTR were amplified from plasmid ER-rk with primers GFP/XmaI.F (5′-AAGGGTTCGCATCAGTACATCTATCAGTACATCTA-3′) and Nos/XcmI.R.

**Transient expression in leaf protoplasts**

Protoplasts were isolated from 3- to 4-week-old Arabidop- sis leaves and transfected with plasmid DNA using the protocol described by He et al. (2006). For standard transfections involving only one reporter construct, 20 μL of ~1 μg·μL−1 plasmid DNA were added per 200 μL of protoplasts suspended in MMg solution (0.4 M mannitol, 15 mM MgCl₂, 4 mM MES, pH 5.7). In cases where protoplasts were simultaneously co-transfected with two reporter constructs, a 20-μL mixture containing ~10 μg of each plasmid was utilized. After transfection, the protoplasts were incubated in W5 solution (154 mM NaCl, 125 mM CaCl₂, 5 mM KCl, 2 mM MES, pH 5.7). Samples were analysed 18+ hours post-transfection with an Olympus FV-1000 laser scanning confocal microscope. For mitochondria staining, the protoplasts were incubated in W5 solution containing 0.5 μM MitoTracker Orange CMTMRos (Life Technologies, Grand Island, NY, USA) 30 min prior to
analysis. The excitation/emission filters utilized for fluorescence detection were 488/505–525 nm for GFP(S65T), 543/585–615 nm for mCherry and MitoTracker Orange, and 633/650 nm for chlorophyll autofluorescence. Confocal microscopy was performed at the Biological Electron Microscope Facility (University of Hawaii, Manoa, HI, USA).

Immunoelectron microscopy

An affinity-purified rabbit polyclonal antibody was raised against a unique region of CNGC20 (residues 664–678, sequence Ac-CLERSSVNPDGTRIR-amide) by New England Peptide (Gardner, MA, USA). For immunogold labelling, developing roots and leaves were preserved by high-pressure freezing/freeze-substitution techniques as described previously (Andème Ondzighi et al. 2008). The resin-embedded sections were placed on formvar-coated nickel slot grids and blocked for 30 min with 3 % (w/v) non-fat dried milk solution in 0.01 M phosphate-buffered saline pH 7.2 containing 0.1 % Tween-20 (PBST). The sections were washed in PBST and then incubated with a 10-fold dilution of the CNGC20 primary antiserum or the rabbit pre-immune serum (negative control). After washing, a 25-fold dilution of secondary antibody, goat anti-rabbit IgG conjugated to 15 nm gold particles (Ted Pella, Inc.), was added for 2 h at room temperature. Sections were washed and stained with uranyl acetate and lead citrate. All observations were performed using a Philips CM10 microscope (Philips, Hillsboro, OR, USA). The CNGC20 antiserum was tested by immunoblot analysis [as shown in Supporting Information] by the method described in Neuteboom et al. (2009).

Results

The hydrophilic N-terminus of group IV-A CNGCs is novel and conserved

CNGC19 and CNGC20 polypeptides possess the major structural features common to members of the plant CNGC family. These include a central hydrophobic core region consisting of six membrane-spanning α-helices (S1–S6), and partially overlapping cyclic nucleotide-binding and calmodulin-binding domains situated near their C-termini (Fig. 1). Interestingly, the N-terminal hydrophilic ends of CNGC19 and CNGC20 [171 and 205 amino acids (aa) in length, respectively] are substantially longer than the N-termini of other Arabidopsis CNGC paralogues (30–126 aa; Table 1). The hydrophilic N-termini of CNGC19 and CNGC20 share 59.6 % protein sequence identity, but lack significant sequence similarity to the N-termini of other Arabidopsis CNGCs. According to the neural network subcellular prediction program, TargetP, both CNGC19 and CNGC20 potentially harbour chloroplast localization signals (Table 1), with hypothetical transit peptide cleavage sites after positions L48 of CNGC19 and R25 of CNGC20.

We compared the N-terminal segments of CNGC19 and CNGC20 with those belonging to group IV-A CNGCs encoded by the sequenced genomes of poplar (Populus trichocarpa), rice (Oryza sativa L. ssp. japonica) and moss (Physcomitrella patens ssp. patens). The rice and poplar orthologues have N-terminal hydrophilic regions that are comparable in length to CNGC19 and CNGC20 (183–209 aa), while the N-terminus of the P. patens orthologue is substantially shorter (135 aa; Table 1).

Figure 1. Domain arrangement of CNGC19 and CNGC20. Shaded boxes indicate the positions of the N-terminal 19-aa motif, the six transmembrane segments (S1–S6) of the hydrophobic core region and the C-terminal overlapping cyclic nucleotide-binding (CNBD) and calmodulin-binding domains (CaMBD). FL corresponds to the full-length versions of CNGC19 and CNGC20, and N1 and N2 represent portions of the N-terminal region utilized in GFP fusion constructs.
Protein sequence alignment revealed several strictly conserved residues within the hydrophilic N-terminal regions of group IV-A CNGCs, including a 19-aa interval with the conserved sequence LxxSGxLGxCxDPxCxxCP (Fig. 2). Unlike CNGC19 and CNGC20, the group IV-A CNGCs of rice, poplar and moss were not predicted to localize to chloroplasts (Table 1).

**Table 1. N-terminal length and predicted subcellular localization of Arabidopsis CNGCs.** The 20 CNGCs of Arabidopsis are arranged by subfamily, with group IV-A orthologues from other plants included for comparison. Deduced N-terminal lengths are based on the predicted positions of the S1 transmembrane segments by TMHMM v1.0. Here, %19Nterm and %20Nterm indicate percentage identity with the N-terminal regions of CNGC19 and CNGC20; the absence of similarity is denoted by ‘not applicable’ (n.a.). Four predictive scores are obtained using TargetP: cTP, chloroplast transit peptide; mTP, mitochondrial targeting peptide; SP, secretory pathway signal peptide; and others. The highest score among the four categories is indicated in italics.

| Name    | N-terminal region | TargetP          |
|---------|-------------------|------------------|
|         | Length| %19Nterm | %20Nterm | cTP   | mTP   | SP   | Others |
| I       | AtCNGC1 | 97     | n/a      | n/a    | 0.09  | 0.23  | 0.03  | 0.80   |
|         | AtCNGC3 | 85     | n/a      | n/a    | 0.24  | 0.21  | 0.03  | 0.35   |
|         | AtCNGC10 | 81     | n/a     | n/a    | 0.11  | 0.28  | 0.02  | 0.83   |
|         | AtCNGC11 | 42     | n/a     | n/a    | 0.01  | 0.53  | 0.04  | 0.87   |
|         | AtCNGC12 | 42     | n/a     | n/a    | 0.00  | 0.28  | 0.04  | 0.96   |
|         | AtCNGC13 | 81     | n/a     | n/a    | 0.10  | 0.74  | 0.00  | 0.33   |
| II      | AtCNGC5 | 101    | n/a     | n/a    | 0.22  | 0.15  | 0.05  | 0.56   |
|         | AtCNGC6 | 116    | n/a     | n/a    | 0.15  | 0.21  | 0.01  | 0.71   |
|         | AtCNGC7 | 68     | n/a     | n/a    | 0.10  | 0.40  | 0.01  | 0.70   |
|         | AtCNGC8 | 104    | n/a     | n/a    | 0.21  | 0.33  | 0.01  | 0.47   |
|         | AtCNGC9 | 109    | n/a     | n/a    | 0.06  | 0.35  | 0.01  | 0.66   |
| III     | AtCNGC14 | 85     | n/a     | n/a    | 0.07  | 0.10  | 0.19  | 0.93   |
|         | AtCNGC15 | 81     | n/a     | n/a    | 0.02  | 0.31  | 0.08  | 0.89   |
|         | AtCNGC16 | 57     | n/a     | n/a    | 0.19  | 0.75  | 0.01  | 0.09   |
|         | AtCNGC17 | 84     | n/a     | n/a    | 0.12  | 0.09  | 0.04  | 0.88   |
|         | AtCNGC18 | 53     | n/a     | n/a    | 0.03  | 0.51  | 0.10  | 0.30   |
| IV-A    | AtCNGC19 | 171    | 100.0 % | 59.6 % | 0.68  | 0.20  | 0.01  | 0.12   |
|         | AtCNGC20 | 205    | 59.6 %  | 100.0 %| 0.98  | 0.02  | 0.03  | 0.15   |
|         | PlCNGC-Iva1 | 209   | 39.0 %  | 50.2 %  | 0.41  | 0.07  | 0.06  | 0.82   |
|         | PlCNGC-Iva2 | 209   | 39.5 %  | 51.2 %  | 0.29  | 0.07  | 0.06  | 0.85   |
|         | PlCNGC-Iva3 | 196   | 39.4 %  | 46.4 %  | 0.19  | 0.08  | 0.05  | 0.91   |
|         | OsCNGC-Iva1 | 183   | 25.9 %  | 30.9 %  | 0.12  | 0.17  | 0.04  | 0.84   |
|         | PpCNGC-Iva1 | 135   | 22.0 %  | 24.9 %  | 0.07  | 0.23  | 0.07  | 0.86   |
| IV-B    | AtCNGC2 | 126    | n/a     | n/a    | 0.15  | 0.20  | 0.01  | 0.52   |
|         | AtCNGC4 | 92     | n/a     | n/a    | 0.10  | 0.33  | 0.09  | 0.79   |

GFP fusions with CNGC19 or CNGC20 are not targeted to chloroplasts

To determine whether CNGC19 and CNGC20 truly possess N-terminal chloroplast sorting signals, constructs were developed for the expression of the first 88 aa of CNGC19 or the first 117 aa of CNGC20, translationally fused to the N-terminus of the green fluorescent protein variant, GFP(S65T). The corresponding chimeric proteins, designated as CNGC19N1-GFP and CNGC20N1-GFP, were transiently expressed in Arabidopsis leaf protoplasts under the CaMV 35S promoter. Despite harbouring the predicted chloroplast transit peptide sequences of their respective CNGC paralogues (Fig. 1), expression of CNGC19N1-GFP and CNGC20N1-GFP in protoplasts resulted in diffuse cytoplasmic fluorescence (Fig. 3B and C), similar to non-chimeric GFP(S65T) controls performed in parallel.
The CNGC19N1 and CNGC20N1 protein segments span from the initiator Met residue to just prior to the conserved N-terminal 19-aa motif of group IV-A CNGCs. To address the question of whether a longer interval of the N-terminus was required for chloroplast targeting, a second set of GFP fusions was generated using nearly the entire N-terminal region (CNGC19N2: aa 1–166; CNGC20N2: aa 1–200). Unlike their shorter counterparts, the longer CNGC19N2-GFP and CNGC20N2-GFP fusions displayed punctate GFP fluorescence patterns. However, these punctate signals did not overlap with chlorophyll autofluorescence (Fig. 3D and E), indicating that the CNGC19N2 and CNGC20N2 sequences directed the chimeric proteins to intracellular structures which were distinct from chloroplasts.

In addition to the truncated CNGC-GFP fusions, the localization patterns of fusions containing the full-length sequences of CNGC19 (CNGC19FL-GFP) or CNGC20 (CNGC20FL-GFP) were examined. Several distinct localization patterns were observed in protoplasts transfected with the full-length CNGC19 and CNGC20 fusion constructs. The CNGC19FL-GFP fusion labelled elliptical structures (Fig. 4A), short non-spherical endomembranes (Fig. 4B), as well as intracellular structures possessing an extended endomembrane morphology (Fig. 4C). The CNGC20FL-GFP fusion typically exhibited punctate labelling (Fig. 4D), with occasional weak labelling of extended endomembranes (Fig. 4E). The endomembrane localization patterns of CNGC19FL-GFP and CNGC20FL-GFP were not consistent with labelling of the chloroplast envelope, as contiguous endomembranes labelled by the full-length CNGC fusions were sometimes observed wrapping around two or more chloroplasts (arrows in Fig. 4C and E). A marker for the chloroplast envelope inner membrane was generated by fusing *A. thaliana* plastidic nucleotide transporter 2 (NTT2) to the N-terminus of the monomeric red fluorescent protein (RFP) variant, mCherry. Protoplasts simultaneously expressing NTT2-mCherry in combination with either CNGC19FL-GFP or CNGC20FL-GFP did not display significant co-localizing GFP and RFP fluorescence (Fig. 5). The NTT2-mCherry marker consistently labelled the perimeter of individual chloroplasts, indicating that the trafficking of proteins targeted to the chloroplast envelope was not impaired in the leaf protoplast transient expression system.

![Figure 2. Sequence alignment of group IV-A CNGCs from *Arabidopsis*, poplar, rice and moss. Three putative members of group IV-A are present in poplar (GenBank: EEE95941.1, EEF07751.1 and EEF07752.1), one in rice (GenBank: BAD16877.1) and one in *P. patens* (COSMOSS v1.6: Ppi204_120V6). Residues matching the consensus (five or more sequences) are shaded in black, and similar residues are shaded in grey. Amino acids strictly conserved among all sequences are denoted by an asterisk below the alignment. The 19-aa conserved interval (LxxSGxLxPxPxAxxCP) and first transmembrane domain (S1) are boxed in red. The indicated position of S1 is based on TMHMM 1.0 predictions for both CNGC19 and CNGC20.](https://academic.oup.com/aobpla/article-abstract/doi/10.1093/aobpla/plt012/160699)
CNGC19FL-GFP and CNGC20FL-GFP co-localize with vacuole membrane markers αTIP- and γTIP-mCherry

To address the possibility that CNGC19 and CNGC20 are targeted to vacuolar membranes, co-transfection experiments were performed with the vacuole membrane markers, α-tonoplast intrinsic protein (αTIP)-mCherry and γTIP-mCherry. Elliptical structures labelled by CNGC19FL-GFP were co-labelled by αTIP-mCherry (Fig. 6A) and γTIP-mCherry (Fig. 6D). The CNGC19FL-GFP fusion also co-localized with αTIP- and γTIP-mCherry within non-elliptical membranes (Fig. 6B, C and E). Although these membranes occasionally resembled the limiting membrane of the central vacuole (Fig. 6E), in many instances CNGC19FL-GFP co-localized with short regions of intense vacuole marker fluorescence (Fig. 6B and C).

Comparison of the signal pattern of CNGC20N2-GFP with that of αTIP- or γTIP-mCherry revealed that CNGC20N2-GFP co-localized with the vacuole markers in both extended endomembranes (Fig. 7A–C) and ring-like structures (Fig. 7A and B). However, neither vacuole marker co-localized with the punctate CNGC20N2-GFP signals (Fig. 7A–C), implying that a substantial proportion of CNGC20N2-GFP fusion protein was not incorporated into vacuolar membranes.

A CNGC19FL-mCherry fusion was generated to directly compare the subcellular localization patterns of full-length CNGC19 and CNGC20 within the same cell. Protoplasts co-transfected with CNGC19FL-mCherry and CNGC20FL-GFP displayed completely co-localizing patterns of GFP and RFP fluorescence (Fig. 8). In addition, the presence of the frequent punctate structures derived from CNGC20FL-GFP was greatly diminished when it was co-expressed with CNGC19FL-mCherry.

Punctate CNGC20N2-GFP and CNGC20FL-GFP signals co-localize with the Golgi marker Man49-mCherry

Co-transfection experiments with additional organelle markers were performed to investigate the punctate labelling pattern of CNGC20FL-GFP. The CNGC20FL-GFP punctate signals did not overlap with BiP1-mCherry-HDEL [Supporting Information], an endoplasmic reticulum (ER) marker generated by fusing Arabidopsis thaliana ER luminal binding protein 1 (BiP1) to a modified version
of mCherry harbouring the C-terminal ER retention motif, HDEL. We also did not observe co-localization between CNGC20\textsubscript{FL}-GFP and markers for the mitochondria [Supporting Information] or peroxisomes [Supporting Information]. However, co-expression with the Golgi marker Man49-mCherry (Nelson et al. 2007) revealed that some (but not all) of the punctate CNGC20\textsubscript{FL}-GFP signals overlapped with Man49-mCherry fluorescence (Fig. 9A).

The punctate distribution patterns of partial fusion constructs, CNGC19\textsubscript{N2}-GFP and CNGC20\textsubscript{N2}-GFP, were also compared with the Golgi marker. Interestingly, whereas
CNGC19N2-GFP did not co-localize with Man49-mCherry (Fig. 9B), CNGC20N2-GFP fluorescence overlapped with the Golgi marker (Fig. 9C). Not all of the punctate structures labelled by the Golgi marker were co-labelled by CNGC20N2-GFP (Fig. 9C), possibly indicating that CNGC20N2-GFP is only targeted to, or accumulates within, a subset of Golgi.

Localization of native CNGC20 at the membrane of vacuoles by immunoelectron microscopy
To verify that CNGC20 is targeted to vacuolar membranes, immunolocalization experiments were performed using an affinity-purified rabbit polyclonal antiserum raised against a peptide unique to CNGC20. *Arabidopsis* root sections immunolabelled with the CNGC20 antibody were examined by transmission electron microscopy (TEM). Anti-CNGC20 labelling was observed at the membranes of vacuoles (Fig. 10A, C and D). Labelling was also detected at the ER (Fig. 10A and B), and on darker-stained areas within the cytoplasm (Fig. 10A and D), which may represent CNGC20 at various stages along the secretory pathway. We did not detect labelling in control samples challenged with the rabbit pre-immune serum in place of the anti-CNGC20 antibody [Supporting Information] or in leaf sections containing chloroplasts incubated with the anti-CNGC20 antibody [Supporting Information]. Unlike the over-expression that occurs when CNGC20FL-GFP is transiently expressed in protoplasts under the CaMV 35S promoter, immunolocalization allows for the detection of CNGC20 under its normal expression pattern in plants. Thus, immunoelectron microscopy confirmed the presence of native CNGC20 in planta at the membrane of vacuoles.
Discussion

In Arabidopsis, the CNGC phylogenetic tree consists of 20 members, of which CNGC19 and CNGC20 are the sole members of subgroup IV-A. They are distinguished among CNGCs by their long and novel conserved N-termini, yet retain key features common to all CNGCs such as the overlapping cyclic nucleotide- and calmodulin-binding domains of the C-terminus, six predicted membrane spanning domains and a close resemblance to Shaker-type K⁺ channels. To better understand the roles that CNGC19 and CNGC20 play in regulating cation fluxes in plants, we investigated their subcellular locations using the leaf mesophyll protoplast transient expression system. Although the conserved hydrophilic N-termini of CNGC19 and CNGC20 are predicted to contain chloroplast transit peptides, translational fusions of GFP with CNGC19 or CNGC20 did not localize to chloroplasts. We instead found that CNGC19FL-GFP and CNGC20FL-GFP co-localized with markers for the vacuole membrane (αTIP-mCherry, γTIP-mCherry) when simultaneously expressed in protoplasts.

Under our experimental conditions, αTIP- and γTIP-mCherry typically did not appear to label the limiting membrane of the large central vacuole. This is consistent with previous studies showing that epitope-tagged or fluorescent protein-tagged versions of αTIP, δTIP and γTIP strongly label relatively small elliptical structures when transiently expressed in protoplasts or cultured cells (Park et al. 2004; Kim et al. 2006; Saito et al. 2011). These structures may represent either small vacuoles or ring-like extensions of the central vacuole membrane that are referred to as vacuole ‘bulbs’. Vacuolar bulbs were first identified in Arabidopsis transgenic plants stably expressing γTIP-GFP under the CaMV 35S promoter (Saito et al. 2002). The intensity of γTIP-GFP fluorescence in bulbs is ~3-fold higher than that in the limiting membrane of the central vacuole (Saito et al. 2002), and their formation is correlated with the fusion of small vacuoles (Saito et al. 2011). It is speculated that vacuolar bulbs may serve as a reservoir of membranes to facilitate the rapid expansion or transformation of vacuoles or plant response to salt stress (Saito et al. 2002; Boursiac et al. 2005), or as specialized subregions of the vacuole where hydrolitic activities are localized (Saito et al. 2002; Zheng and Staehelin 2011). In addition to labelling bulb-like structures, CNGC19FL-GFP and CNGC20FL-GFP occasionally co-localized with short, non-elliptical membranes of intense vacuole marker fluorescence. We hypothesize that these co-labelled membranes correspond to regions of the tonoplast where vacuole bulbs were unfolded to expand the limiting membrane of the central vacuole.

Protoplasts expressing CNGC20FL-GFP frequently exhibited punctate punctate GFP fluorescence that partially co-localized with the Golgi marker Man49-mCherry. Thus, CNGC20FL-GFP may have a lower efficiency in trafficking to the vacuole than CNGC19FL-GFP under our experimental conditions, resulting in the accumulation of CNGC20FL-GFP in Golgi and possibly other undefined punctate subcellular structures. We observed a decrease in punctate labelling when CNGC20N2-GFP was co-expressed with CNGC19FL-mCherry, with 70–80 % more of the cells exhibiting some vacuolar labelling, possibly indicating an increased efficiency in CNGC20FL-GFP trafficking to the vacuolar

![Figure 9. Comparison of CNGC19N2-GFP and CNGC20N2-GFP with a marker for Golgi. Confocal laser scanning microscope images of leaf protoplasts co-transfected with the Golgi marker Man49-mCherry and (A) CNGC20FL-GFP, (B) CNGC19N2-GFP or (C) CNGC20N2-GFP. Column 1, GFP signal (green); column 2, RFP signal (red); column 3, merged GFP and RFP signals; column 4, merged GFP and RFP signals with chlorophyll autofluorescence (blue). Scale bars represent 5 μm.](https://academic.oup.com/aobpla/article-abstract/doi/10.1093/aobpla/plt012/160699/11)
membrane in the presence of co-expressed CNGC19FL-mCherry. Since plant CNGC polypeptides most likely assemble as tetramers to form functional cation channels (Hua et al. 2003), we speculate that the simultaneous expression of CNGC19FL-mCherry and CNGC20FL-GFP causes the chimeric proteins interacting with each other to form CNGC19/CNGC20 hetero-multimers, which are transported together to the vacuole membrane.

Protoplasts expressing GFP fused to the entire hydrophilic N-terminal region of CNGC19 or CNGC20 (CNGC19N2-GFP; CNGC20N2-GFP) do not display diffuse cytosolic labelling, but instead exhibit punctate labelling. This suggests that the N-terminal regions of CNGC19 and CNGC20 contain information that influences protein sorting, but are not sufficient to direct proteins to the vacuole. Interestingly, the N-terminal regions of group IV-A CNGCs contain a novel 19-aa conserved motif (LxxSGxLgxCxxDPxCxxCP). Since GFP fusions harbouring shortened versions of the N-terminal region truncated just prior to this motif (CNGC19N1-GFP; CNGC20N1-GFP) exhibit diffuse labelling of the cytosol, we speculate that the 19-aa interval may play a role in protein sorting. Interestingly, in protoplast co-transfection experiments, the Golgi marker Man49-mCherry co-localized with CNGC20N2-GFP, but not with CNGC19N2-GFP. This suggests that differences exist between the protein sorting information contained within the N-terminal hydrophilic regions of CNGC19 and CNGC20. These differences may contribute, at least in part, to the dissimilar localization patterns of our full-length CNGC19FL-GFP and CNGC20FL-GFP fusions with regard to the absence or presence of punctate labelling (respectively).

The presence of CNGC19 and CNGC20 at vacuolar membranes suggests that these channels serve as pathways for the passive transport of cations between the vacuole and cytosol. Since the genes encoding CNGC19 and CNGC20 are upregulated by salt stress (Maathuis, 2006; Kugler et al. 2009; Yuen and Christopher, 2010), one possible function of these channel proteins is to ameliorate the effects of deleterious levels of Na\(^+\) in the cytosol by facilitating Na\(^+\) redistribution between the cytosol and vacuole. It is unlikely, however, that CNGC19 and CNGC20 play a direct role in the sequestration of Na\(^+\) to the central vacuole since Na\(^+\) must be actively transported against its electrochemical gradient, a function performed by vacuolar Na\(^+\)/H\(^+\) (NHX) antiporters (Apse et al., 1999, 2003). An alternative possibility is that CNGC19 and CNGC20 facilitate the plant’s response to salinity by mediating Ca\(^{2+}\) signalling. Salt stress induces a rapid rise in cGMP levels, and a transient increase in free cytosolic [Ca\(^{2+}\)]; at moderate salt concentrations (50 mM), suppressing cGMP accumulation with an inhibitor of guanylyl cyclase also diminishes the Ca\(^{2+}\) spike (Donaldson et al., 2004). Cyclic nucleotide-gated channels, which are activated by cyclic nucleotides (Li et al., 2005), could function as a link between cGMP accumulation and the influx of Ca\(^{2+}\) into the cytosol (Donaldson et al., 2004). The potential involvement of vacuolar CNGCs in Ca\(^{2+}\) signalling is circumstantially supported by experiments in tobacco protoplasts that have demonstrated that cAMP and cGMP can trigger the influx of Ca\(^{2+}\) to the cytosol from both intracellular and extracellular Ca\(^{2+}\) stores (Volotovski et al., 1998). In addition to being upregulated by salt stress,
CNGC19 and CNGC20 have been implicated in the Arabidopsis response to infection by bacterial and fungal pathogens (Moeder et al. 2011). If CNGC19 and CNGC20 are indeed Ca\(^{2+}\) channels, they may serve a similar molecular function in the response to both abiotic and biotic stress by mediating calcium signalling through the release of vacuolar Ca\(^{2+}\) into the cytosol.

Conclusions

CNGC19 and CNGC20 are components of vacuole membranes. Under the protoplast transient expression system, CNGC20 is weakly trafficked to the vacuole. However, co-expression of CNGC19 and CNGC20 results in efficient transport of CNGC20 to the vacuolar membrane, possibly due to the formation of heteromultimeric channels. How CNGC19 and CNGC20 influence cation fluxes within plant cells in response to salt stress and biotic stress remains unclear. Future experiments defining the permeability of these channels to various cations will clarify whether CNGC19 and CNGC20 are directly involved in the subcellular redistribution of Na\(^{+}\) or function as mediators of Ca\(^{2+}\) signalling.

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Contributions by the Authors

D.A.C. conceived the project, and D.A.C. and C.C.Y.Y. designed the protoplast transient expression experiments and developed all the new fusion constructs utilized in this study. D.A.C. performed all the immuneelectron microscopy experiments. C.C.Y.Y. conducted protoplast transfections and analysed the samples through confocal microscopy. D.A.C. and C.C.Y.Y. wrote the manuscript, and read and approved the final version for submission.

Conflict of Interest Statement

None declared.

Supporting Information

The following Supporting Information is available in the online version of this article.

**File 1:** Figure. Comparison of CNGC19\(_{FL}\) and CNGC20\(_{FL}\) localization with a marker for the ER. Confocal laser scanning microscope images of leaf protoplasts co-transfected with the ER marker BiP1-mCherry-HDEL and (A) CNGC19\(_{FL}\)-GFP or (B) CNGC20\(_{FL}\)-GFP. Column 1, GFP signal (green); column 2, RFP signal (red); column 3, merged GFP and RFP signals; column 4, merged GFP and RFP signals with chlorophyll autofluorescence (blue). Scale bars represent 5 \(\mu\)m.

**File 2:** Figure. Comparison of CNGC19\(_{FL}\) and CNGC20\(_{FL}\) localization with a marker for mitochondria. Confocal microscopy images of leaf protoplasts transiently expressing (A) CNGC19\(_{FL}\)-GFP or (B) CNGC20\(_{FL}\)-GFP, and stained with MitoTracker Orange. Column 1, GFP signal (green); column 2, MitoTracker Orange signal (red); column 3, merged GFP and MitoTracker Orange signals; column 4, merged GFP and MitoTracker Orange signals with chlorophyll autofluorescence (blue). Scale bars represent 5 \(\mu\)m.

**File 3:** Figure. Comparison of CNGC19\(_{N2}\) and CNGC20\(_{N2}\) localization with a marker for peroxisomes. Confocal laser scanning microscope images of leaf protoplasts co-transfected with the peroxisome marker mCherry-SKL and (A) CNGC19\(_{N2}\)-GFP or (B) CNGC20\(_{N2}\)-GFP. Column 1, GFP signal (green); column 2, RFP signal (red); column 3, merged GFP and RFP signals; column 4, merged GFP and RFP signals with chlorophyll autofluorescence (blue). Scale bars represent 5 \(\mu\)m.

**File 4:** Figure. Pre-immune serum staining of roots. Immunoblotting of Arabidopsis cryo-fixed thin root tissue sections with rabbit pre-immune serum and anti-rabbit 15 nm gold-conjugated secondary antiserum as a negative control. Samples were viewed via TEM as described in the Methods. No labelling was observed. G, Golgi apparatus; Cy, cytoplasm; Vc, vacuole; ER, rough endoplasmic reticulum; Pm, plasma membrane; M, mitochondrion.

**File 5:** Figure. Immunolabelling of leaves. Immunolabelling of Arabidopsis cryo-fixed thin leaf tissue sections with anti-CNGC20 antiserum and anti-rabbit 15 nm gold-conjugated secondary antiserum. Samples were viewed via TEM as described in the Methods. No labelling was observed in the chloroplasts (CT); some labelling was observed in the edges of vacuoles (V).

**File 6:** Figure. Immunoblot analysis of CNGC20 in Arabidopsis total cellular proteins. Immunoblot analysis was performed using the anti-CNGC20-specific peptide antiserum on 40 \(\mu\)g of total seedling proteins (14-day-old) from wild type (WT) and the CNGC20 T-DNA mutant (MUT). The homozygous T-DNA insert (SALK_129133.22.05) is in the fourth exon of the CNGC20 locus (At3g17700). The antiserum detects a single band of \(\sim 84\) kDa in the wild-type protein sample, which is the predicted size of CNGC20, whereas no CNGC20 protein is detected in the mutant. This indicates that the antiserum is specific to CNGC20. Coomassie-stained proteins (COOM) are shown from a duplicate gel.
Literature Cited

Ali R, Zielinski RE, Berkowitz GA. 2006. Expression of plant cyclic nucleotide-gated cation channels in yeast. Journal of Experimental Botany 57:125–138.

Andéme Ondzighi C, Christopher DA, Cho EJ, Chang SC, Staehelin LA. 2008. Arabidopsis protein disulfide isomerase-5 inhibits cysteine proteases during trafficking to vacuoles before programmed cell death of the endothelium in developing seeds. The Plant Cell 20:2205–2220.

Apse MP, Aharon GS, Snedden WA, Blumwald E. 1999. Salt tolerance conferred by overexpression of a vacuolar Na+/H+ antipporter in Arabidopsis. Science 285:1256–1258.

Apse MP, Sottosanto JB, Blumwald E. 2003. Vacular cation/H+ exchange, ion homeostasis, and leaf development are altered in a T-DNA insertional mutant of ANKH1, the Arabidopsis vacuolar Na+/H+ antipporter. The Plant Journal 36:229–239.

Arazi T, Kaplan B, Fromm H. 2000. A high-affinity calmodulin-binding site in a tobacco plasma-membrane channel protein coincides with a characteristic element of cyclic nucleotide-binding domains. Plant Molecular Biology 42:591–601.

Balogué C, Lin B, Alcon C, Flottes G, Malmström S, Köhler C, Neuhaus G, Pelletier G, Gaymard F, Roby D. 2003. HML1, an essential signaling component in the hypersensitive response, is a member of the cyclic nucleotide-gated channel ion channel family. The Plant Cell 15:365–379.

Baxter J, Moeder W, Ullmer W, Andeme-Ondzighi C, Staehelin LA, Christopher DA. 2011. Protein disulfide isomerase-2 of Arabidopsis thaliana catalyzes disulfide bond formation during ER-to-Golgi transport. The Plant Journal 67:485–495.

Borsics T, Webb D, Andeme-Ondzighi C, Staehelin LA, Christopher DA. 2007. The cyclic nucleotide-gated calmodulin-binding channel ATCNGC10 localizes to the plasma membrane and influences numerous growth responses and starch accumulation in Arabidopsis thaliana. Planta 225:563–573.

Bouriasc Y, Chen S, Luu DT, Sorieu M, van den Dries N, Maurel C. 2005. Early effects of salinity on water transport in Arabidopsis roots. Molecular and cellular features of aquaporin expression. Physiologia Plantarum 128:253–265.

Cho EJ, Yuen CY, Kang BH, Ondzighi CA, Staehelin LA, Christopher DA. 2011. Protein disulfide isomerase-2 of Arabidopsis mediates protein folding and localizes to both the secretory pathway and nucleus, where it interacts with maternal effect embryo arrest factor. Molecules and Cells 32:459–475.

Christopher DA, Borsics T, Yuen CY, Ullmer W, Andeme-Ondzighi C, Andres MA, Kang BH, Staehelin LA. 2007. The cyclic nucleotide gated cation channel ATCNGC10 traffics from the ER via Golgi vesicles to the plasma membrane of Arabidopsis root and leaf cells. BMC Plant Biology 7:48.

Clough SJ, Fengler KA, Yu IC, Lippok B, Smith RK Jr, Bent AF. 2000. The Arabidopsis ndd1 ‘defense, no death’ gene encodes a mutated cyclic nucleotide-gated ion channel. Proceedings of the National Academy of Sciences of the USA 97:9323–9328.

Donaldson L, Ludidi N, Knight MR, Gehring C, Denby K. 2004. Salt and osmotic stress cause rapid increases in Arabidopsis thaliana cGMP levels. FEBS Letters 569:317–320.

Ferro M, Brugière S, Salvi D, Seigneurin-Berny D, Court M, Moyet L, Romus C, Miras S, Mellal M, Le Gall S, Kieffer-Jaquinod S, Bruley C, Garin J, Joyard J, Masselon C, Rolland N. 2010. AT_CHLORO, a comprehensive chloroplast proteome database with subplastidial localization and curated information on envelope proteins. Molecular & Cellular Proteomics 9:1063–1084.

Friedtsch S, Wang YF, Sladek C, Poulsen LR, Romanowsky SM, Schroeder JI, Harper JF. 2007. A cyclic nucleotide-gated channel is essential for polarized tip growth of pollen. Proceedings of the National Academy of Sciences of the USA 104:14531–14536.

Gobert A, Park G, Amtmann A, Sanders D, Maathuis FJM. 2006. Arabidopsis thaliana cyclic nucleotide gated channel 3 forms a non-selective ion transporter involved in germination and cation transport. Journal of Experimental Botany 57:791–800.

Guo KM, Babourina O, Christopher DA, Borsics T, Rengel Z. 2008. The cyclic nucleotide-gated channel, ATCNGC10, influences salt tolerance in Arabidopsis. Physiologia Plantarum 134:499–507.

Guo KM, Babourina O, Christopher DA, Borsics T, Rengel Z. 2010. The cyclic nucleotide-gated channel ATCNGC10 transports Ca2+ and Mg2+ in Arabidopsis. Physiologia Plantarum 139:303–312.

He P, Shan L, Sheen J. 2006. The use of protoplasts to study innate immune responses. Plant-Pathogen Interactions 35:1–9.

Hua BG, Mercier RW, Leng Q, Berkowitz GA. 2003. Plants do it differently. A new basis for potassium/sodium selectivity in the pore of an ion channel. Plant Physiology 132:1353–1361.

Kim MJ, Kim HR, Paek K-H. 2006. Arabidopsis tonoplast proteins TIP1 and TIP2 interact with the cucumber mosaic virus 1a replication protein. Journal of General Virology 87:3425–3431.

Köhler C, Neuhaus G. 2000. Characterisation of calmodulin binding to cyclic nucleotide-gated ion channels from Arabidopsis thaliana. FEBS Letters 471:133–136.

Krogh A, Larsson B, von Heijne G, Sonnhammer EL. 2001. Predicting transmembrane membrane topology with a hidden Markov model: application to complete genomes. Journal of Molecular Biology 305:567–580.

Kugler A, Köhler B, Palme K, Wolff P, Dietrich P. 2009. Salt-dependent regulation of a CNG channel subfamily in Arabidopsis. BMC Plant Biology 9:140.

Leng Q, Mercier RW, Yao W, Berkowitz GA. 1999. Cloning and first functional characterization of a plant cyclic nucleotide-gated cation channel. Plant Physiology 121:753–761.

Leng Q, Mercier RW, Hua BG, Fromm H, Berkowitz GA. 2002. Electrophysiological analysis of cloned cyclic nucleotide-gated ion channels. Plant Physiology 128:400–410.

Li XL, Borsics T, Harrington HM, Christopher DA. 2005. Arabidopsis ATCNGC10 rescues potassium channel mutants of E. coli, yeast and Arabidopsis and is regulated by calcium/calmodulin and cyclic GMP in E. coli. Functional Plant Biology 32:643–653.

Ma W, Ali R, Berkowitz GA. 2006. Characterization of plant phenotypes associated with loss-of-function of ATCNGC1, a plant cyclic nucleotide gated cation channel. Plant Physiology and Biochemistry 44:494–505.

Ma W, Qi Z, Smigel A, Walker RK, Verma R, Berkowitz GA. 2009. Ca2+ and cAMP, and transduction of non-self perception during plant immune responses. Proceedings of the National Academy of Sciences of the USA 106:20995–21000.
Maathuis FJ. 2006. The role of monovalent cation transporters in plant responses to salinity. Journal of Experimental Botany 57: 1137–1147.

Mäser P, Thorne S, Schroeder JI, Ward JM, Hirschi K, Sze H, Talke IN, Amtmann A, Maathuis FJM, Sanders D, Harper JH, Tchieu J, Gribskov M, Persans MW, Salt DE, Kim SA, Guerinot ML. 2001. Phylogenetic relationships within cation transporter families of Arabidopsis. Plant Physiology 126: 1646–1667.

Moeder W, Urquhart W, Ung H, Yoshioka K. 2011. The role of cyclic nucleotide-gated ion channels in plant immunity. Molecular Plant 4: 442–452.

Nelson BK, Cai X, Nebenführ A. 2007. A multicolored set of in vivo organelle markers for co-localization studies in Arabidopsis and other plants. The Plant Journal 51: 1126–1136.

Neuteboom LW, Matsumoto KO, Christopher DA. 2009. An extended AE-rich N-terminal trunk in secreted pineapple cystatin enhances inhibition of fruit bromelain and is post-translationally removed during ripening. Plant Physiology 151: 515–527.

Nielsen H, Engelbrecht J, Brunak S, von Heijne G. 1997. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Engineering 10: 1–6.

Park M, Kim SJ, Vitale A, Hwang I. 2004. Identification of the protein storage vacuole and protein targeting to the vacuole in leaf cells of three plant species. Plant Physiology 134: 625–639.

Saito C, Ueda T, Abe H, Wada Y, Kuroiwa T, Hisada A, Furuya M, Nakano A. 2002. A complex and mobile structure forms a distinct subregion within the continuous vacuolar membrane in young cotyledons of Arabidopsis. The Plant Journal 29: 245–255.

Saito C, Uemura T, Arai C, Tominaga M, Ebine K, Ito J, Ueda T, Abe H, Morita MT, Tasaka M, Nakano A. 2011. The occurrence of ‘bulbs’, a complex configuration of the vacuolar membrane, is affected by mutations of vacuolar SNARE and phospholipase in Arabidopsis. The Plant Journal 68: 64–73.

Sherman T, Fromm H. 2009. Physiological roles of cyclic nucleotide gated channels in plants. In: Baluska F, Mancuso S, eds. Signaling in plants. Berlin: Springer, 91–106.

Sunkar R, Kaplan B, Bouche N, Arazi T, Dolev D, Talke IN, Maathuis FJM, Sanders D, Bouche D, Fromm H. 2000. Expression of a truncated tobacco NCBP4 channel in transgenic plants and disruption of the homologous Arabidopsis CNGC1 gene confer Pb2+ tolerance. The Plant Journal 24: 533–542.

Talke IN, Blaudez D, Maathuis FJM, Sanders D. 2003. CNGCs: prime targets of plant cyclic nucleotide signalling? Trends in Plant Science 8: 286–293.

Urquhart W, Gunawardena AH, Moeder W, Ali R, Berkowitz GA, Yoshioka K. 2007. The chimeric cyclic nucleotide-gated ion channel ATCNGC11/12 constitutively induces programmed cell death in a Ca2+ dependent manner. Plant Molecular Biology 65: 747–761.

van Wijk KJ, Baginsky S. 2011. Plastid proteomics in higher plants: current state and future goals. Plant Physiology 155: 1578–1588.

Volotovski ID, Sokolovsky SG, Molchan OV, Knight MR. 1998. Second messengers mediate increases in cytosolic calcium in tobacco protoplasts. Plant Physiology 117: 1023–1030.

Yoshioka K, Moeder W, Kang HG, Kachroo P, Masmoudi K, Berkowitz G, Klessig DF. 2006. The chimeric Arabidopsis cyclic nucleotide-gated ion channel 11/12 activates multiple pathogen resistance responses. The Plant Cell 18: 747–763.

Yu IC, Parker J, Bent AF. 1998. Gene-for-gene disease resistance without the hypersensitive response in Arabidopsis dnd1 mutant. Proceedings of the National Academy of Sciences of the USA 95: 7819–7824.

Yuen CYL, Christopher DA. 2010. The role of cyclic nucleotide-gated channels in cation nutrition and abiotic stress. In: Demidchik V, Maathuis F, eds. Ion channels and plant stress responses. Berlin: Springer, 137–157.

Zheng H, Staehelin LA. 2011. Protein storage vacuoles are transformed into lytic vacuoles in root meristematic cells of germinating seedlings by multiple, cell type-specific mechanisms. Plant Physiology 155: 2023–2035.