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Minireview

Cyclic nucleotide-gated channels in plants

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Abstract **Until recently the role of cyclic nucleotide monophosphates (cNMPs) in plants had been controversial, with equivocal data about their concentrations, biosynthetic and degrading enzymes, and cellular targets. This review discusses the current knowledge in this field, with focus on the largest class of cNMP targets in plant cells, the cyclic nucleotide-gated channels (CNGCs). Aspects of structure and function are addressed, with reference to studies in heterologous systems and in planta. The picture emerging, albeit still fragmented, is of proteins with diverse functions in the control of ion homeostasis, development, and defense against biotic and abiotic threats.**

Keywords: cAMP; cGMP; Ion channels; Signal transduction; Calmodulin; Stress

1. Signal transduction and cyclic nucleotides

All living cells harbor a network of signal transduction pathways to conduct developmental programs, obtain nutrients, control their metabolism, and cope with their environment. When organisms are challenged by external physical and chemical stimuli, their cytoplasmic compartments receive this information via changes in the concentration of a plethora of second messengers. One group of second messengers that has been discovered and studied in mammalian cells for 50 years is the cyclic nucleotide monophosphates (cNMP; 3′,5′-cAMP and 3′,5′-cGMP) (reviewed by Newton and Smith [1]). These molecules have since been implicated in a wide range of physiological responses including liver metabolism, sensory transduction (visual and olfactory systems), cardiac function, and others. Consequently, cNMPs and their signal transduction intermediates became a major target for drug discovery. In addition, over the years, a role for cNMPs has been established in other eukaryotes including unicellular organisms (e.g. yeast), and in prokaryotes including archaea and eubacteria [1].

In contrast, much less is known about cNMPs in plants. For decades, their occurrence and functions in plants had been fiercely debated [1]. However, progress in the past decade provided solid evidence for physiologically relevant concentrations of both cAMP and cGMP [1,2]. In addition, biochemical and molecular approaches provided evidence for the existence of the enzymes responsible for cNMP synthesis, namely, adenylyl- and guanylyl-cylase [3,4, respectively]. Further biochemical evidence suggested also the existence of plant cNMP phosphodiesterases (reviewed by Newton and Smith [1]).

2. Physiological roles of cNMPs in plants

Increasing evidence for the physiological roles of cNMPs in plants helped convince the skeptics to accept cNMPs as bona-fide and important second messengers. Studies by Chua and colleagues suggested a role for cGMP in photo transduction downstream of phytochrome, in the pathway that leads to activation of *Chalcone Synthase* (*CS*) and anthocyanin biosynthesis (reviewed by Ref. [5]). These researchers also found that the cGMP-dependent signaling pathway antagonizes the phytochrome signaling pathway mediated by calcium/calmodulin (Ca^{2+}/CaM), which is involved in chloroplast development (and *CAB* expression). Similarly, the latter pathway is antagonistic to the cGMP-mediated pathway of *CS* [5]. Further studies using the same system revealed *cis*-elements within the *rbcS* and *CS* genes that are activated by Ca^{2+} and cGMP, respectively, and attenuated by cGMP and Ca^{2+}, respectively [6]. A more recent comprehensive analysis of the effect of cGMP on gene expression in plants, using DNA microarrays, revealed ~1000 genes that responded (>2-fold) to 10 μM of membrane-permeable cGMP within 2 h [7]. Interestingly, cNMPs mediate phytochrome signaling in the cyanobacterium *Anabaena* [8], and also participate in photo transduction in vertebrate retina. Hence, the role of cNMPs in photo transduction may be evolutionarily conserved from prokaryotes through plants to mammals.

Cyclic nucleotides have been suggested to be involved in plant responses to both biotic and abiotic stresses. First, cyclic nucleotides have been found to mediate responses to pathogens, downstream of nitric oxide (NO). NO may activate cGMP pathways for both defense gene induction and potentiation of ROI-induced cell death [9]. Second, cNMPs also help protect plants against salt stress [10]. Furthermore, cNMPs participate in various developmental processes in addition to photomorphogenesis. For example, cAMP acts as a second messenger in pollen

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Abbreviations: cNMP, 3′,5′-cyclic nucleotide monophosphate; CaM, calmodulin; CNGC, cyclic nucleotide-gated channel; CaMBD, CaM-binding domain; CNBD, cNMP-binding domain; HR, hypersensitive response; PR, pathogenesis related; WT, wild-type

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tube growth and reorientation [3], and cGMP is a second messenger in auxin-induced adventitious rooting [11]. This may be related to a previous report on the correlation between cAMP levels and mitotic division in tobacco cells [12,13]. Previous studies established the role of cGMP in the response of cereal aleurone cells to gibberellic acid (GA; [13]).

Ample evidence for the role of cNMPs in controlling ion homeostasis in plants has also accumulated in the past decade and earlier. Kurosaki and Nishi [14] found that cAMP-stimulated carrot cells exhibited enhanced Ca^{2+} influx. A similar enhanced Ca^{2+} influx by cGMP into tobacco protoplasts was also reported [15]. Furthermore, salt and osmotic stress cause rapid increases in cGMP levels in *Arabidopsis thaliana* [2], and Ca^{2+} transients seem to be dependent on cGMP elevation in response to ionic stress [2]. These studies are consistent with findings that cAMP and cGMP improve tolerance to salt stress [10]. Interestingly, improved salt tolerance correlated with cNMP-dependent decrease of channel open probability, and reduced influx of Na^+ [10,16]. Lemtiri-Chlieh and Berkowitz [17] demonstrated by single-channel patch clamp analysis the occurrence of Ca^{2+}-permeable channels triggered by cNMPs in guard-cell plasma membranes.

3. Cellular targets of cNMPs

An important aspect of cNMPs signal transduction has been the identification and characterization of cNMP receptors, such as cNMP-dependent protein kinases [18] and cNMP-gated ion channels (CNGCs) [19] that mediate many of the effects of cyclic nucleotides. Bioinformatics investigations of plant genomes for cNMP target proteins rely on the phylogenetic conservation of cNMP-binding domains in proteins from prokaryotes to mammals [20]. There are two known types of cNMP-binding domains: GAF (e.g., present in phosphodiesterases) and CNBD, present in most known cellular targets of cNMPs. Bioinformatics revealed two types of plant proteins with GAF domains: phytochrome and ethylene receptors [20]. However, there is yet no evidence for the involvement of these domains in cNMP signaling. The more common CNBD is found in the family of CNGCs in plants (20 genes in *Arabidopsis*; Fig. 1) and in the five groups of the shaker-like potassium channels [21]. Other than these proteins, there seem to be either very few additional cNMP-regulated targets in plants (e.g. an Acyl-CoA thioesterase; [20]), or more targets that are not conserved in sequence, which therefore elude bioinformatics investigations. Biochemical studies revealed contradicting evidence regarding cNMP-dependent protein kinase activities in extracts of various plants [1,20] but, to date, genes coding for plant cNMP-dependent protein kinases have not been cloned. Therefore, it seems that the main targets of cNMPs in plants are cation channels (K^+-selective and non-selective). In this review, we focus on the basic structure and function of plant CNGCs. Previous reviews on the subject should be noted [22,23].

4. Heterologous expression for functional characterization

Nine years after the first cloning and characterization of a CaM-binding putative CNGC from barley [24], an elaborate functional characterization of plant CNGC properties is still missing [25]. In this part, we review the functional aspects of plant CNGCs, mainly focusing on its regulation by cNMPs, Ca^{2+}/CaM, and its selectivity for different cations.

Characterization of plant CNGCs in heterologous systems was attempted in *Saccharomyces cerevisiae* mutants deficient in cation uptake. Yeast mutants lacking both of their K^+-uptake transporters, *trk1*, *trk2*, are often used to test complementation of K^+ uptake by channels. The barley CNGC isoform HvCBT1 could not complement the mutant on low K^+ concentration [24]. An attempt to change the pore of HvCBT1 to the GYGD consensus sequence, found in K^+-selective channels (Fig. 2), not only did not improve the growth of the yeast mutant on low K^+, but rather attenuated it [24]. In contrast, the *Arabidopsis* isoforms AtCNGC1 and AtCNGC2 could partially complement the yeast mutant (compared to complementation with the *Arabidopsis* KAT1) even in the absence of permeable cNMPs [26]. Contradicting the latter, Leng et al. [27], showed that AtCNGC2 could partially complement the yeast mutant at low K^+ concentration only in the presence of membrane-permeable cAMP (dibutyryl-cAMP). Another *Arabidopsis* CNGC isoform, AtCNGC3, could also partially complement a yeast mutant lacking the K^+ transporters, again, even in the absence of membrane-permeable cNMPs [28].

Recent studies used a modified complementation protocol for the *trk1*, *trk2* yeast mutant. Hygromycin B was found to inhibit the growth of this mutant even at high K^+ concentrations, because this cationic amino glycoside accumulates in the cell cytosol due to the negative membrane potential in this mutant [29]. Addition of membrane-permeable cAMP enhanced the growth of the yeast mutant expressing AtCNGC1, AtCNGC2, or AtCNGC4 [30]. However, recent results could not verify complementation of the mutant by AtCNGC1 [25]. Instead, a C-terminus truncated AtCNGC1 lacking part of the CaMBD was shown to repress the mutant phenotype. Yoshioka and colleagues [31], using a similar protocol, showed that AtCNGC11 and AtCNGC12, and a chimeric AtCNGC11/12 were all able to enhance yeast mutant growth even in the absence of lipophilic cNMPs. Nonetheless, addition of membrane-permeable cAMP enhanced growth even more, while addition of membrane-permeable cGMP did not. It is important to note that the *trk1,trk2* yeast mutant is not an ideal model for proving K^+ channel permeability. The mutant cells are highly hyperpolarized (negative potential inside; [29]), which is not a "natural" environment. Hence, some membrane proteins fail to function as K^+ transporters at this excessive hyperpolarization state, and, on the other hand, some membrane proteins may gain "unnatural" potassium permeability [29].

Other yeast mutants were used as well to study the function of plant CNGCs. A salt-sensitive yeast strain, carrying deletions in the major Na^+ extruding pumps ENA1-4 was used by Gobert and colleagues [28] to characterize the function of AtCNGC3. The mutant expressing AtCNGC3 was more sensitive to high salt and accumulated significantly more Na^+ than cells with the empty vector control, suggesting that AtCNGC3 forms a functional Na^+ permeable channel in these yeast cells [28]. Another haploid yeast mutant lacking two major Ca^{2+} transporters CCH1 and MID1, involved in signal transduction in response to an alpha mating factor (pheromone), was used to characterize the Ca^{2+} permeability of AtCNGC1 [25]. In this mutant exposure to the mating factor leads to growth arrest. While full-length AtCNGC1 could not complement this yeast mutant in response to the pheromone, a C-terminus-

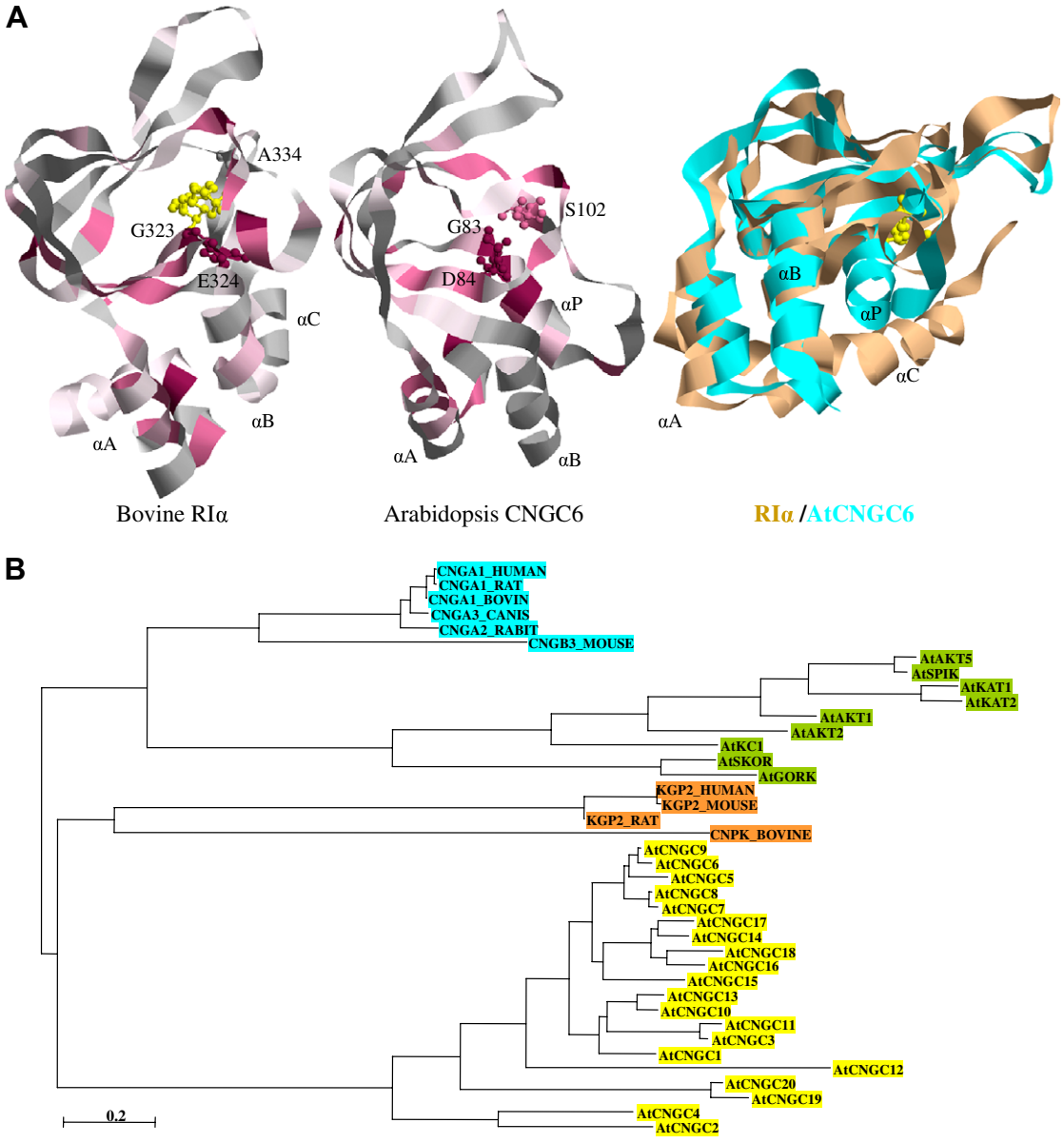
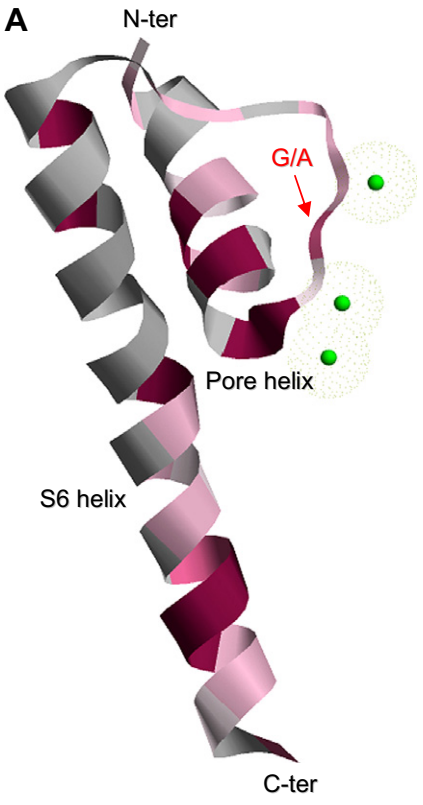


Fig. 1. Structural analysis of plant and non-plant CNBDs. (A) Thirty-nine CNBDs from different protein families and organisms were aligned using ClustalX. The multiple sequence alignment was further subjected to analysis using the ConSurf server (http://consurf.tau.ac.il/; [50]). ConSurf is a program designed to give a general view of amino acid conservation throughout proteins within a given multiple sequence alignment on a known protein structure (PDB file). The program scores the most conserved amino acid as 9, and the most variable as 1. In this figure only amino acids scoring 6–9 are colored (light pink to magenta, respectively). Grey indicates amino acids scoring lower than 6, or amino acids with insufficient data. Left: The B domain of the Bovine cAMP-regulated protein kinase CNBD (PDB 1RGS; X-ray solved structure Y245-V376). The ligand (cAMP) is presented in yellow, and amino acids interacting with the ligand are depicted in ball and stick configuration (G323, E324 and A334). Middle: The *Arabidopsis thaliana* CNGC6 CNBD (PDB 1WGP; NMR-solved structure). Amino acids that may interact with cAMP are in ball and stick configuration (G83, D84 and S102). Right: Superimposition of the CNBD of bovine 1RGS (light brown) on the *Arabidopsis* CNBD of CNGC6 (1WGP, light blue) using the FATCAT server (http://fatcat.burnham.org/). The PDB generated by FATCAT was colored in the RasTop software (1RGS brown and 1WGP cyan). The three helices in CNBDs are marked αA, αB, and αC (see Fig. 6 in Ref. [36]). The *Arabidopsis* protein used for structural analysis (PDB 1WGP) is shorter than that of the bovine CNBD at the C-terminus, and therefore lacks the αC helix, which contains the CaMBD [36]. However, the *Arabidopsis* CNBD structure has an extra unique helix (designated here as αP, for plant-unique α-helix). This helix corresponds to amino acids DELLTWALD of AtCNGC6, which is almost identical to the tobacco NtCBP4 sequence EELLTWALD located in the 'loop' between β-sheets β6 and β7 [36]. This loop is much longer than that of the corresponding bovine CNBD loop. (B) A phylogenetic tree generated from the multiple sequence alignment of the same 39 CNBDs described in A. Phylogentic tree data in Newick format was obtained from the ConSurf results page. Data were then entered into the NJplot tree-drawing tool (http://pbil.univ-lyon1.fr/software/njplot.html), and finalized in MS PowerPoint. Among the sequences are all the *Arabidopsis thaliana* (At) CNGCs (yellow), all the *Arabidopsis thaliana* shaker-like K^+ channels (green), mammalian cNMP-regulated kinases (orange) and mammalian CNGCs (light blue). The AGI genome codes and GenBank Accession numbers for *Arabidopsis* CNGCs are according to [22], and those of the *Arabidopsis* shaker-like K^+ channels are according to [21]. Mammalian cNMP-regulated protein kinase accession numbers: KGP2_MOUSE: AAA02572, KGP2_RAT: Z36276, KGP2_HUMAN: X94612, KGP2_MOUSE: L12460. Mammalian CNGC GenBank accession numbers: CNGB3_MOUSE: AJ243572, CNGA1_RAT: U48803, CNGA1_HUMAN: M84741, CNGA1_BOVINE: X51604, CNGA2_RABBIT: X59668, CNGA3_CANIS: XM_538462. Scale bar represents the number of changes per site.



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AtCNGC2    KILYPIFWGLMTLSTFANDLEPTSNWLEVIFSIVMVLSGLLLFTLLIGNIQVFL
AtCNGC4    KILFPIFWGLMTLSTFGN-LESTTEWSEVVFNIIVLTSGLLLVTMLIGNIKVFL
AtCNGC1    KFFYCFWWGLQNLSSLGQNLKTSTYIWEICFAVFISIAGLVLFSFLIGNMQTYL
NtCBP4     KFFYCFWWGLQNLSSLGQNLQTSTFIWEMCFAVFISIAGLVLFAFLIGNMQTCL
AtCNGC10   KFFYCFWWGLRNLSALGQNLQTSKFVGEIIFAISICISGLVLFALLIGNMQKYL
AtCNGC13   KFFYCFWWGLRNLSALGQNLNTSKFVGEIIFAVSICISGLVLFALLIGNMQKYL
AtCNGC3    KFFYCFWWGLRNLSALGQNLKTSAFEGEIIFAIVICISGLVLFALLIGNMQKYL
AtCNCG11   KFVYCFWWGLRNISALGQNLETSNSAGEIFFAIIICVSGLLLFAVLIGNVQKYL
AtCNGC12   KFVYCFWWGLRNISALGQNLETSNSAGEIFFAIIICVSGLLLFAVLIGNVQKYL
AtCNGC15   KYFYCLWWGLKNLSSLGQNLATSTYAGEILFAIIIATLGLVLFALLIGNMQTYL
AtCNGC16   KYFYCLWWGLRNLSSYGQSLAASTLSSETIFSCFICVAGLVFFSHLIGNVQNYL
AtCNGC18   KYLYCLWWGLRNLSSYGQNITTSVYLGETLFCITICIFGLILFTLLIGNMQSSL
AtCNGC14   KYLYCLWFGLQNLSSYGQNLSTSTSVLETMFAILVAIFGLVLFALLIGNMQTYL
AtCNGC17   RYFYCLWWGLQQLSSYGQNLSTTMFMGETTFAVLIAIFGLVLFAHLIGNMQTYL
AtCNGC6    KYFFCLWWGLQNLSTLGQGLETSTYPGEVIFSITLAIAGLLLFALLIGNMQTYL
AtCNGC9    KYFFCLWWGLQNLSTLGQGLETSTYPGEVIFSIALAIAGLLLFALLIGNMQTYL
AtCNGC5    KYCYCLWWGLQNLSTLGQGLETSTYPMEIIFSISLAISGLILFALLIGNMQTYL
AtCNGC7    KFCYCLWWGLQNLSTLGQGLQTSTFPGEVLFSIAIAIAGLLLFALLIGNMQTYL
AtCNGC8    KFCYCLWWGLQNLSTLGQGLQTSTYPGEVLFSIAIAVAGLLLFALLIGNMQTYL
AtCNGC19   RFSYSLYWGFQQISTLAGNLSPSYSVGEVFFTMGIIGLGLLLFARLIGNMHNFL
AtCNGC20   RYSYSLFWGFQQISTLAGNQVPSYFLGEVFFTMGIIGLGLLLFALLIGNMQNFL
BRET       KYVYSLYWSTLTLTTIGETPPPVRDS-EYFFVVADFLIGVLIFATIVGNIGSMI
BOLF       EYIYCLYWSTLTLTTIGETPPPVKDE-EYLFVIFDFLIGVLIFATIVGNVGSMI
AtKAT1     RYVTALYWSITTLTTTGYGDFHAENPREMLFDIFFMMFNLGLTAYLIGNMTNLV
AtKAT2     RYVTALYWSITTLTTTGYGDLHAENPREMLFFVFFMLFNLGFTSYLIGNMTNLV
AtAKT1     RYVTSMYWSITTLTTVGYGDLHPVNTKEMIFDIFYMLFNLGLTAYLIGNMTNLV
AtAKT2     RYIAAIYWSITTMTTVGYGDLHASNTIEMVFITVYMLFNLGLTAYLIGNMTNLV
AtKC1      GYTYSMYWSIVTLTTVGYGDLHAVNSREKTFNMFYMLFNIGLTSYIIGIMTNLV
AtSPIK     RYNTAMYWSITTFSTTGYGDIHGVNSREMTFILFYMVFNLGLSAYIIGNMTNLV
AtAKT5     RYVTAMYWSITTFSTTGYGDIHGNNAEERAFILFYMIFNLGLLAYIIGNMTNLV
AtSKOR     RYTTSMYFAVVTMATVGYGDIHAVNMREMIFAMVYISFDMILGAYLIGNMTALI
AtGORK     RYTTALYFAIVTMATVGYGDIHAVNLREMIFVMIYVSFDMVLGAYLIGNITALI
KCSA       TYPRALWWSVETATTVGYGDLYPVTLWGRLVAVVVMVAGITSFGLVTAALATWF
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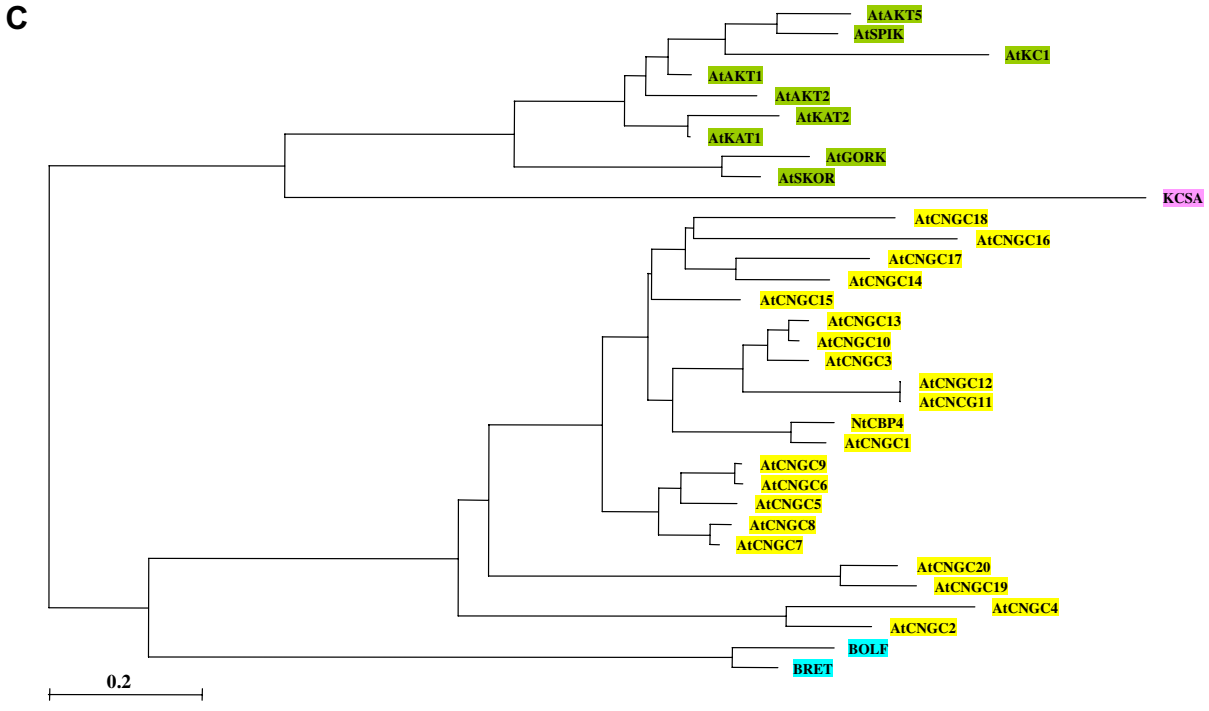

Fig. 2. Pore sequence and structure of plant and non-plant cation channels (A) An image (generated using ConSurf) representing amino acid variance in the pore helix and S6 region of cation channels from different organisms based on the known structure of the *Streptomyces lividans* K^+-selective channel (PDB 1BL8). Colors are as explained in Fig. 1. The G/A (Gly/Ala) amino acid corresponds to the first Gly in the GYGD selectivity filter in all K^+-selective channels and in mammalian CNGCs, which is either Gly or Ala in plant CNGCs (see B). The green spheres represent K^+ ions in contact with the pore loop. (B) Alignment of the amino acid sequences of the S6 and pore region of 20 *Arabidopsis* CNGCs [22] and shaker-like K^+-selective channels [21], tobacco NtCBP4 (similar to *Arabidopsis* CNGC1; [40]), Bovine CNGCs and the *Streptomyces lividans* K^+ channel using ClustalX. The presumed selectivity filter is highlighted in red. Accession numbers are as follows. NtCBP4: AF079872, *Streptomyces lividans* K^+ channel: Z37969, for BRET and BOLF see [33]. (C) Phylogenetic tree derived from the alignment in B. AtCNGC's are in yellow, At K^+ channels in green, Bovine in light blue, and *Streptomyces lividans* in pink. Scale bar represents the number of changes per site.

deleted protein suppressed this mutation, suggesting that AtCNGC1 is permeable to Ca^{2+} ions, and that this permeation is inhibited by CaM (see working model in Fig. 3).

The first functional characterization of a plant CNGC by electrophysiological means was reported by Leng et al. [27]. In this report, expression of AtCNGC2 in *Xenopus laevis* oocytes was undertaken for voltage clamp studies. Addition of cNMPs (cAMP or cGMP) to the bath solution resulted in cNMP-dependent K^+ currents. Moreover, depolarizing voltages resulted in no current in these oocytes, suggesting that AtCNGC2 is an inwardly-rectified cNMP-gated cation channel. Similar results were obtained in human embryonic kidney (HEK) cells expressing AtCNGC2, as cAMP-dependent K^+ currents were recorded and found to be inwardly rectifying and non-inactivating [32]. Moreover, in human HEK cells expressing AtCNGC2, Ca^{2+} permeability was observed only in the presence of membrane-permeable cNMPs [27]. Further experiments with HEK cells or *Xenopus* oocytes expressing AtCNGC2 suggest that AtCNGC2 is also permeable to other monovalent cations, but not to Na^+ [27,32,33]. Relative conductance showed highest values for K^+, which was greater than those for Li^+, Rb^+, Cs^+, in this order [32]. Notably, AtCNGC2 single-channel conductivity was substantially lower than that of animal CNGCs or other potassium selective channels [32]. Animal CNGCs do not contain the GYGD selectivity pore for K^+-selectivity, instead they have a conserved

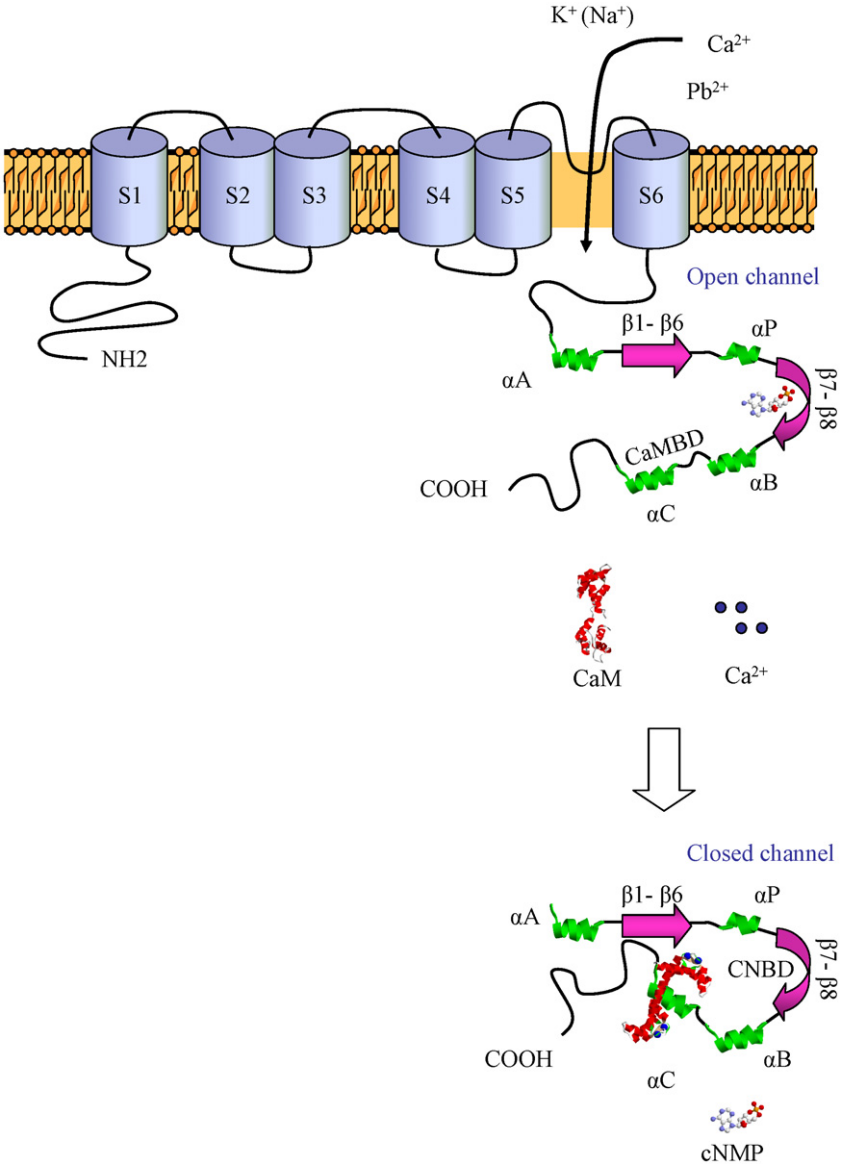

Fig. 3. Working model for plant CNGCs. When cNMP (ball and stick structure) is bound to the CNBD of a CNGC it causes opening of the channel, and cations can pass through the pore subjected to the selectivity barrier (between transmembrane helices 5 and 6). According to Hua et al. [33], AtCNGC2 is not permeable to Na^+, in contrast to some of the other tested plant CNGCs (e.g. AtCNGC1 and AtCNGC3). Other plant CNGCs are permeable to specific divalent cations including Ca^{2+} and Pb^{2+}. Upon elevation of Ca^2 levels in the cytosol, Ca^{2+} binds to CaM, and Ca^{2+}/CaM binds to the CaMBD of CNGCs, thus probably preventing binding of cNMPs to the channel and forcing it to close. In this model the αP helix unique to plant CNGCs (explained in Fig. 1) is included. However, we note that this model is constructed from fragmented data from studies of different plant CNGCs, and therefore may not apply to all CNGCs.

amino acid sequence GETP (Fig. 2), and are found to be non-selective for either divalent or monovalent cations [19]. They do not discriminate between K^+, Na^+ and Ca^{2+}. AtCNGC2 also does not contain the GYGD sequence but rather ANDL (Fig. 2). Nevertheless, it was shown to be K^+ selective [33]. Changing its selectivity filter within the pore sequence to AETL, which is more similar to the animal CNGC pore (Fig. 2), shifted AtCNGC2 from a K^+-selective channel to a cAMP-dependent K^+ and Na^+ non-selective channel in both whole cell configuration in HEK cells and membrane patches of *Xenopus* oocytes [33]. At this point, it is important to note that a channel that is permeable to Ca^{2+} and K^+ but not to Na^+ [27,32] is extraordinary and, to our knowledge, is not present in any other species but plants. Generally, the plant CNGC pore amino acid sequence is different from all known K^+-selective channels and from animals CNGCs (Fig. 2). Also, within the plant CNGC family, 16 of the 20 members have GQNL or similar selectivity filter sequence, which is substantially different from the AtCNGC2 sequence (ANDL; Fig. 2). In addition to the suggestion that AtCNGC2 is Ca^{2+} permeable, it was shown [32] that at high external Ca^{2+} concentrations, total K^+ currents are reduced significantly, similar to the animals counterparts.

Later on, it was shown that HEK cells expressing AtCNGC1 displayed cNMP-activated currents with either K^+ or Na^+ [33]. Importantly, AtCNGC1, which seems to be a non-selective channel, allowing Na^+ permeation through its pore, has a sequence of GQNL in its selectivity filter which is the most common sequence among plant CNGCs (Fig. 2). Thus, CNGCs are among the best candidates to function as non-selective cation channels in plant membranes. Furthermore, ion current recordings obtained from a single successful experiment in *Xenopus* oocytes [34] expressing a different *Arabidopsis* CNGC isoform (AtCNGC4), suggested that this CNGC is also activated by cNMPs. In this experiment direct activation by both cAMP and cGMP, with the latter more efficient, was recorded. The cGMP-gated ion currents showed a weak outward rectification, with permeability to both Na^+ and K^+. However, unlike AtCNGC2, these currents were blocked by Cs^+. Notably, AtCNGC4 has a completely different selectivity filter sequence compared to other AtCNGCs (Fig. 2), with higher similarity to bovine olfactory and retinal CNGCs (Fig. 2). In summary, a detailed analysis of the *Arabidopsis thaliana* isoform AtCNGC2 in yeast, *Xenopus* oocytes, and HEK cells indicate that AtCNGC2 is a cNMP-gated K^+-selective over Na^+ inward-rectifying non-inactivating channel. In addition, it was also suggested to be permeable to Ca^{2+}, and, on the other hand, to be blocked by high external Ca^{2+} concentration. Its presumed selectivity filter sequence suggests a different mechanism of selectivity from all other known channels investigated to date [33].

However, in spite of the remarkable progress in studying CNGCs in heterologous systems, several papers reporting on plant CNGC expression and current recordings in heterologous systems added words of caution. Leng and colleagues [32] described a problem of expression or current recording in *Xenopus* oocytes and HEK cells with all the plant CNGCs they were testing. Similar arguments were provided for AtCNGC4 for which only one experiment out of five was successful [34]. Therefore, conclusions about plant CNGCs based on heterologous systems are not unequivocal. Electrophysiological experiments with other isoforms of plant CNGCs, except for AtCNGC2, were sporadic and insufficient to reach valid conclusions regarding their ion conductance and gating mechanism.

5. CNGC regulation by Ca^{2+}/CaM

Animal CNGCs contain a CaM-binding domain in the N-terminal part of the protein, or, in some instances of heteromeric channels, on a different subunit of the channel, which interacts with the CNBD in the C-terminal half of the protein, a process that leads to channel inactivation [35]. In plants, the CaM-binding domain (CaMBD) coincides with one of the three conserved helices of the CNBD [36]. Therefore, it was suggested that although the mechanisms of interaction of Ca^{2+}/CaM with CNGCs may be different in plant and animal CNGCs, functionally the plant and animal CNGCs may respond similarly to Ca^{2+}/CaM. Namely, in the presence of Ca^{2+}, CaM binds to the plant CNGC at the αC helix of the CNBD and thus blocks channel gating by cNMPs ([36]; working model in Fig. 3). Consistent with this, Hua et al. [37] found (in HEK cells using the whole-cell configuration) that CaM reverses cAMP activation of AtCNGC2 in a Ca^{2+}-dependent manner. Another plant CNGC, AtCNGC10, was also shown to be modulated by cNMPs and CaM in a heterologous system [38]. When transformed with AtCNGC10 cDNA, the *Escherichia coli* LB650 K^+-uptake deficient strain was able to grow on 2 mM K^+, while bacteria transformed with the empty vector failed to do so. Both grew well on 100 mM K^+. To test the effect of CaM and cNMPs on the activity of AtCNGC10 as a K^+ channel, *E. coli* cells were transformed with both CaM and AtCNGC10 cDNAs, or with only the AtCNGC10 cDNA, as a control. A 40% decrease in cell growth rates was observed upon addition of 1 mM Ca^{2+} to the growth medium of the co-transformed bacteria, while the control bacteria showed no significant growth changes. This 40% reduction was abolished upon addition of 1 mM of EGTA or cGMP. Treatment of non-transformed bacteria with cGMP or cAMP had no effect on bacteria growth rates. These results suggest that Ca^{2+}/CaM inhibits, and cyclic nucleotides enhance K^+ conductance through AtCNGC10, consistent with previous predictions [36] and as presented in Fig. 3.

6. Functional analysis of plant CNGCs by reverse genetics: tolerance and sensitivity to various cations

Several plant mutants of CNGCs, and transgenic plants expressing full-length or mutant CNGCs, were characterized and found to exhibit various phenotypes compared to wild-type (WT) plants. One class of phenotypes is related to ion homeostasis, uptake, and transport. A tobacco plasma membrane CNGC isoform designated NtCBP4 [39] was over-expressed in tobacco. Transgenic lines were indistinguishable from WT under normal growth conditions. However, the former exhibited improved tolerance to Ni^{2+}, which was associated with reduced Ni^{2+} accumulation, and hypersensitivity to Pb^{2+}, associated with enhanced Pb^{2+} accumulation [39]. In contrast, seedlings that expressed a truncated version of this protein, from which the C-terminal with the CaMBD and part of the CNBD was removed, showed improved tolerance to

Pb^{2+} with attenuated accumulation of this metal [40]. Furthermore, disruption by T-DNA insertion of the *Arabidopsis CNGC1* gene, which encodes a homologous protein, also conferred Pb^{2+} tolerance [40]. Hence, the tobacco NtCBP4 and *Arabidopsis* AtCNGC1 are likely involved in metal uptake across the plant plasma membrane. Indeed, recent studies indicate that *AtCNGC1* is primarily expressed in roots of *Arabidopsis* seedlings [41], and seedlings lacking this protein contained slightly lower shoot Ca^{2+} than WT plants. In addition, primary roots of *Atcngc1* T-DNA knockout seedlings grew faster than roots of WT plants, and had larger angles and less nitric oxide upon gravistimulation. Hence channels formed by AtCNGC1 may contribute to Ca^{2+} uptake into plants, and affect aspects of growth in the primary root of *Arabidopsis* seedlings.

Similarly, two knockout alleles of *AtCNGC2* were tested for their sensitivity to several cations including Ca^{2+}, Mg^{2+}, Na^+, K^+ and H^+. Both knockout alleles showed exclusive hypersensitivity to Ca^{2+} compared to WT controls [42]. This hypersensitivity was shown in both seedling and mature plants, affecting plant development and fertility. Notably, AtCNGC2 mutants show pleiotropic phenotype defects including dwarfism and low fertility under normal growth conditions [43]. However, preliminary data suggest that Ca^{2+} hypersensitivity is not associated with Ca^{2+} accumulation in these mutants. Rather, Ca^{2+} sensitivity might be due to impaired signaling pathways at high Ca^{2+} concentration [42].

Another CNGC that has an apparent role in ion homeostasis is AtCNGC3 [28]. In comparison to WT, *cngc3* mutants had lower germination rates when grown on 100-140 mM NaCl. Germination of *Atcngc3* on KCl or NH_4Cl did not have the same inhibitory effects. Plants were also germinated on an iso-osmolar medium of sorbitol, which had no significant effect on the mutants when compared to WT. Thus, in comparison to WT, *cngc3* is more susceptible to Na^+ toxicity during germination. Other tested conditions including exposure to pathogens, heavy metals, high or low Ca^{2+} concentrations, and gravitropic stimulation did not cause any significant differences in *cngc3* compared to WT. In order to evaluate the possible role of CNGC3 in ion homeostasis, ion content was measured in WT and mutants that were grown on high levels of KCl or NaCl. Seedlings had no difference in Na^+ content, but *cncg3* had 50% lower K^+ than WT. Na^+ uptake experiments in plants suggested that CNCG3 had a limited role in salinity adaptation. However, as indicated above, AtCNGC3 has the ability to transport Na^+ in yeast. Using a *AtCNGC3 promoter::GUS* construct in transgenic plants, revealed expression throughout plant development, mainly in the embryo, leaves (in vascular tissues) and in roots (epidermal, cortical but not stelar). Combined with K^+ and Na^+ uptake experiments, it was suggested that in mature plants AtCNGC3 might take part in distribution/translocation of ions from the xylem, and if knocked out, excess accumulation of these ions may cause damage by impairing water potential. In seedlings, AtCNGC3 may be part of an uptake pathway and when knocked out, fewer ions accumulate, rendering seedlings less sensitive to high concentrations of Na^+. Another CNGC isoform, AtCNGC10, was studied in the *Arabidopsis akt-1* mutant. The gene was able to partially complement the mutant for K^+ uptake by transformation with a *35S::AtCNGC10* construct, whereas transforming WT plants with an *AtCNGC10* antisense construct led to a 40% lower K^+ levels in comparison to non-transformed WT plants [44]. Therefore, in planta reverse genetic studies demonstrate the role of different CNGCs in cation uptake and homeostasis.

7. CNGCs and plant response to pathogens

Clough et al. [43] first came across *AtCNGC2* (*DND1*) while screening mutants in the gene-for-gene disease resistance pathway. When inoculated with an avirulent strain of *Pseudomonas syringae*, *cngc2* (*dnd1*) mutants managed to maintain lower growth rates of bacteria compared to WT, even though the mutant failed to produce hypersensitive response (HR). This was accomplished by sustaining high levels of salicylic acid, leading to constitutive expression of pathogenesis-related (PR) genes, and other defense responses. These mutants were named *dnd* for their "*Defense, No Death*" phenotype. In a later work, Kohler *et al.* [45] analyzed expression of *CNGC2 promoter::GUS*, and expression of the endogenous *CNGC2* gene, revealing a possible role for this protein in developmentally regulated cell death programs, cotyledon and leaf senescence, and flower and silique dehiscence.

AtCNGC4 (*DND2, HLM1*) is closest in sequence to *AtCNGC2* [22], and it also plays a role in plant defense. The *Atcngc4* mutant exhibits a lesion-mimic phenotype, constitutive PR gene expression, high salicylic acid (SA) levels, and lack HR upon avirulent pathogen inoculation [34,46]. Hence, the lack of CNGC4 also causes a "*Defense, No Death*" phenotype.

Another defense-related mutant is the *Arabidopsis cngc11/12* chimera. Yoshioka et al. [31] discovered that a mutant of the constitutive expressor of PR gene 22 (*cpr22*) harbored a 3 kb genomic deletion that fused AtCNGC11 with AtCNGC12, thus, creating a chimeric CNGC. The *cpr22* mutant shows altered defense responses, stunted growth and curly leaves. Analyzing *cngc11* and *cngc12* lines separately revealed a higher degree of susceptibility to *H. parasitica Emwa1* compared to WT. No differences were observed in morphology, PR gene expression, or lesion formation. When the two mutant lines were crossed, the F1 progeny exhibited a phenotype similar to that of their parents. These results suggest that the *cpr22* phenotype is not caused by the loss of one of these genes or due to a CNGC11/*cngc11*, CNGC12/*cngc12* genotype, but is rather a consequence of the AtCNGC11/12 chimeric gene. Importantly, a few alleles of a lesion-mimic mutant of barley (*nec1*) are also caused by the lack of function of a CNGC, which in sequence is most similar to that of the *Arabidopsis CNGC4* gene [47]. Therefore, CNGCs are involved in plant defense against pathogens in both dicotyledon and monocotyledon plants.

8. CNGCs and plant development

Borsics et al. [44] investigated *AtCNGC10* antisense lines and found high starch accumulation (twice as much as in WT), bigger starch granules, a more frequent appearance of peroxisomes, reduced root growth and elongation, early flowering, and delayed bending in response to gravistimulation. The fact that no homozygous T-DNA insertion lines of *AtCGNC10* could be obtained, and that only a few antisense lines were generated, led Borsics et al. [44] to conclude that *AtCNGC10* is an essential gene with low redundancy. These authors

suggested that AtCNGC10 is part of a light signal transduction pathway, consistent with previous evidence for cNMP involvement in photomorphogenesis [5].

The role of CNGCs in plant development is also apparent from phenotypes of some of the other mutants already discussed above. Both *cngc2* and *cngc4* mutants, but not the *cngc1* mutant, exhibit slow growth and low fertility compared to WT, so it is clear that the role of CNGC2 and CNGC4 is not confined to plant defense. A recent study of transporter genes expressed in *Arabidopsis* pollen at different stages of male gametophyte development [48] revealed that four *CNGCs* (*CNGC7, 8, 16*, and *CNGC18*) are either specifically or preferentially expressed at different stages of pollen development. In addition, a T-DNA knockout of *AtCNGC18* was reported to be male sterile [49]. Other *CNGC*s were also expressed in the developing male gametophyte but not exclusively. These data strongly suggest a role for CNGCs in pollen development, consistent with previous evidence for the involvement of cNMPs and Ca^{2+} in pollen growth and orientation [3]. Therefore, plant CNGCs are involved in different physiological roles throughout plant development.

9. Summary and future perspectives

Although plant CNGCs show similarities in amino acid sequence and overall structure to the family of six trans-membrane-domain K^+-selective shaker family channels and to animal non-selective cation CNGCs, they also differ in several important aspects. First, the pore sequence of plant CNGCs does not contain the GYGD K^+-selectivity filter sequence, nor does it contain the sequence of the animal CNGC selectivity filter (Fig. 2). Hence, the plant CNGC selectivity filter has unique properties in sequence (Fig. 2) and in function [33]. Therefore, plant CNGCs may differ from their animal counterparts in selectivity towards K^+, Na^+, Ca^{2+}, and possibly towards other cations.

In addition, the putative CNBDs of plant CNGCs diverged from all other known CNBDs of eukaryotes or prokaryotes (Fig. 1B); whereas the CNBDs of plant K^+ channels cluster together with those of mammalian CNGCs and protein kinases, the CNBDs of plant CNGCs form a separate group (Fig. 1B). Nevertheless, the CNBD sequences are conserved within the family of plant CNGCs (Fig. 1B). Moreover, based on the NMR solution structure of AtCNGC6 (Fig. 1A; PDB 1WGP), at least some plant CNGCs contain an additional short alpha helix (designated here as αP; Fig. 1), which corresponds to a region previously described as a "loop" between two β-sheets (explained in the legend to Fig. 1).

Importantly, in plant CNGCs a CaMBD was found to partially overlap helix C (αC) of the CNBD (Fig. 1; [36]), a feature that is different from animal CNGCs, which are inactivated by Ca^{2+}/CaM through CaMBDs located far from the CNBD, or even on a different channel subunit [35]. These sequence and structural differences between plant and animal CNGCs suggest that the former may possess unique features in channel gating. Although Ca^{2+}/CaM binding to plant CNGCs was the first identified biochemical feature of this family, Ca^{2+}/CaM modulation of plant CNGCs is yet not well understood. Recent studies identified Ca^{2+}-permeable cNMP-gated channels in plant guard cells [17], which is consistent with the working model for plant CNGCs (Fig. 3). However, there is also solid evidence that plants possess voltage-independent Na^+-permeable cation channels that are inactivated by cNMPs [10,16]. If these channels appear to be members of the CNGC family, it would mean that some plant CNGCs are gated differently from their animal counterparts. Future studies that would take these differences into account would likely shed more light on the function and regulation of plant CNGCs.

Regarding the physiological roles of plant CNGCs, the most obvious is their involvement in response to pathogens. In *Arabidopsis*, mutations in either *CNGC2* or *CNGC4* cause a "*Defense, No Death*" phenotype. It was therefore suggested that these genes are involved in programmed cell death. A similar *CNGC* gene from barley, the *NEC1* gene, was also found to be involved in cell death [47]. In addition, ample evidence suggests a role for CNGCs in pollen development, consistent with independent evidence for the role of cNMPs in pollen development. However, the mechanisms and signal transduction intermediates involved in CNGC-mediated responses and developmental processes are still unknown.

To date, only a few of the plant CNGC expression profiles have been studied at the cell-specific level using promoter::reporter constructs. This approach needs to be expanded to include all CNGCs for more precise mapping of their expression profiles during development and in response to biotic and abiotic stimuli. Immunohistochemistry and in situ hybridizations could also contribute to refine their expression patterns. In addition, although some CNGCs were localized to the plasma membrane, subcellular localization of most plant CNGCs has not been determined.

What is completely missing in studies of plant CNGCs is the biochemical analysis of their structure (except for the NMR-based structure of the CNBD of AtCNGC6; Fig. 1), subunit composition, and interacting proteins. In other organisms CNGCs function as tetramers. Hence, in view of the large number of CNGCs in plants, the possible combinations that could form functional heteromeric channels are huge. Other aspects that need to be further investigated are the signals that regulate CNGCs. While Ca^{2+} and CaM are clearly regulators of plant CNGCs, other signals may be operating either directly or through interacting proteins. However, to date, such interacting proteins have not been reported. Detailed mutation analysis of the various functional domains would also provide more knowledge about ion selectivity, the gating mechanism, and regulatory properties.

In conclusion, the challenge of deciphering the functions of plant CNGCs is immense not only because of the number of CNGCs in plants, but also because of their complex regulatory properties, diverse expression profiles [23], and interactions with other signaling pathways. A multidisciplinary approach to investigate plant CNGCs is hence inescapable.

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