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Cyclic Nucleotides and Nucleotide Cyclases in Plant Stress Responses

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1. Introduction

The cyclic nucleotides monophosphates (cNMP) and in particular adenosine 3',5'-cyclic monophosphate (cAMP) and guanosine 3',5'-cyclic monophosphate (cGMP) are cyclic catalytic products of adenosine 5'-triphosphate (ATP) and guanosine 5'-triphosphate (GTP) respectively. These cNMPs are universal second messengers with key roles in many and diverse physiological responses and processes in prokaryotes, and in both higher and lower eukaryotes.

Cyclic AMP is arguably one of the most extensively studied second messengers in animals, lower eukaryotes and bacteria where it has critical roles in signaling the metabolic status. In bacteria, cAMP is involved in the positive regulation of the *lac* operon where in an environment of a low glucose, cAMP accumulates and binds to the allosteric site of the cAMP receptor protein (CRP), a transcription activator protein. Once the CRP is in its active configuration, it binds to a *cis*-element upstream of the *lac* promoter and activates transcription. At high glucose concentrations, cAMP concentration decreases and CRP disengages from the *lac* operon promoter (Meiklejohn & Gralla, 1985). Cyclic AMP signaling is also critical for many aspects of the development of the slime mold *Dictyostelium discoideum* that grows unicellularly, but develops as multicellular organism (Kimmel & Firtel, 2004; McMains et al., 2008). Cyclic AMP has a role in chemotaxis and cAMP oscillations act as intracellular feedback loops in the transcriptional regulation of many regulatory pathways. Post aggregation, cAMP-dependent signals mediate cell sorting, pattern formation, and morphogenetic changes and cAMP receptor affinity can control wave dynamics, geometry and morphogenesis (Dormann et al., 2001). In animals, cAMP can be produced e.g. by G_s-coupled activation of adenylate cyclases (ACs) which triggers a signal cascade that includes the modification of Ca^{2+} channels, the phosphorylation of target proteins including enzymes involved in regulating glycogen metabolism and eventually the cAMP-dependent activation of the transcription factor, cAMP response element-binding (CREB) (Bolwell, 1995; Karin & Smeal, 1992). Cyclic AMP also plays a role in excitation-

contraction where voltage-dependent potentiation of L-type Ca^{2+} channels is due to phosphorylation by cAMP-dependent protein kinase (Sculptoreanu et al., 1993). It has also been shown that bicarbonate can directly stimulate a mammalian soluble adenylyl cyclase (sAC) (Chen, 2000; Kaupp & Weyand, 2000) and that this sAC is most similar to adenylyl cyclases from cyanobacteria, and that bicarbonate regulation of cyclase activity is conserved in these early forms of life making it likely to be an ancient and fundamental regulatory component.

The other cyclic nucleotide under investigation here, cGMP, also has many and diverse roles in bacteria, and lower and higher eukaryotes. In bacteria, the work on cGMP undertaken in the early 1970s stalled mainly because firstly, the levels of cGMP are significantly lower than those of cAMP and secondly, no specific binding target for cGMP could be identified (for review see Linder, 2010). More recently, a specific bacterial guanylyl cyclase (GC; Cya2) from unicellular cyanobacterium *Synechocystis* sp. PCC 6803 has been identified and biochemically and structurally characterized (Ochoa De Alda et al., 2000). It was reported that Cya2 has the catalytic requirements for activity and purine specificity of being a GC and insertional mutagenesis of *cya2* caused a marked reduction in cGMP content without altering the cAMP content. Currently an increasing body of literature is accumulating that reports cGMP dependence of many bacterial processes for instance the control of cyst development in the α-proteobacterium *Rhodospirillum centenum* (Marden et al., 2011). In lower eukaryotes, cGMP is also implicated in signal transduction (Rericha & Parent, 2008; Shpakov & Pertseva, 2008) and *Dictyostelium discoideum* GCs, which interestingly share topological similarities with mammalian ACs, have been reported (Roelofs et al., 2001a; Roelofs et al., 2001b). In vertebrates, cGMP has been implicated in broad range of physiological processes, many of which are linked to specific receptors (Garbers, 1999). The processes include retinal phototransduction (Azadi et al., 2010; Luo et al., 2008) and signaling by the homeostasis regulating Atrial Natriuretic Peptide (Acher, 2002; Chinkers et al., 1989). Apart from the particulate membrane bound receptor GCs, there is a second class, the soluble GCs located in the cytosol (Arnold et al., 1977; Gao, 2010) that are involved in nitric oxide (NO) signaling.

While there is a large body of literature on cyclic nucleotide signaling in lower and higher eukaryotes, both dealing with biological functions and mechanisms of action, the acceptance that cyclic nucleotides play a role in plant signaling was delayed and not without controversy since the levels of cAMP and cGMP in plants appear to be generally lower than in animals or lower eukaryotes (for review see Bolwell, 1995; Gehring, 2010; Newton & Smith, 2004). Another and possibly more important reason for the reluctant acceptance of these signaling molecules in higher plants is that until quite recently, there was no genetic or molecular evidence of nucleotide cyclases (NCs) in higher plants. Here we undertake the review of the literature on biological and molecular aspects of cyclic nucleotide signaling in plants and higher plants in particular. In addition, we will examine the history of cyclic NCs in higher plants and the methods developed that led to their discovery. Finally, we will apply these search methods to generate and evaluate a list of candidate NCs and propose how they might impact on stress signaling in plants.

2. The discovery of adenosine cyclic monophosphate in plants

Perhaps the most convincing data towards directly establishing a specific function for cAMP came from whole-cell patch-clamp current recordings in *Vicia faba* mesophyll protoplasts that revealed that the outward K^+-current increased in a dose-dependent

fashion following intracellular application of cAMP - and not AMP, cGMP or GMP - and indirect evidence indicated that this modulation occurred through a cAMP-regulated protein kinase (Li et al., 1994). Furthermore, cAMP-dependent up-regulation of a calcium-permeable conductance activated by hyperpolarization was also reported in guard cells as well as mesophyll cells of *Arabidopsis thaliana* and *Vicia faba* (Lemtiri-Chlieh & Berkowitz, 2004). Despite this compelling evidence, the history of cAMP function in plants has not been free of controversy and much more is needed before we have a clear picture of the generation and modes of action of cAMP in plant physiology and plant stress responses in particular.

2.1 Cyclic AMP in plants: the long road to acceptance

The question of whether or not cAMP exists and functions in plants was fiercely debated for almost three decades starting in the late 1960s and this during a time when cAMP was accepted as second messenger in animals, lower organisms and prokaryotes. Early published experimental data and various claims from several laboratories suggesting comparable roles for cAMP in higher plants were highly criticized (i) as lacking specificity in the effects elicited by cyclic nucleotides, (ii) uncertainty about cyclic nucleotide-generating enzymes, (iii) ambiguous identification of endogenous putative cyclic nucleotides largely due to inadequate chromatographic identification (Amrhein, 1977; Newton & Smith, 2004). For unequivocal proof of cAMP in plants and its role as a second messenger, one would have had to show for example phosphorylation response of proteins in response to elevated cAMP levels, unambiguously identified cAMP-dependent enzyme function and not least the presence of cAMP itself. Early papers were based on observations of various physiological and metabolical responses after the exogenous application of cAMP, cAMP analogues and phosphodiesterase (PDE) inhibitors known at the time, but these indirect pharmacological approaches were used without sufficient knowledge of the fundamental metabolic mechanisms in plants (Newton et al., 1999).

One of the reports disputing the existence of cAMP in higher plants was a review entitled "Evidence against the occurrence of adenosine 3':5'-cyclic monophosphate in higher plants" (Amrhein, 1974). In it, the author strongly disagreed with the then published literature attempting to demonstrate the occurrence of cAMP in tissues of higher plants including the work by numerous authors (Azhar & Krishna Murti, 1971; Becker & Ziegler, 1973; Brown & Newton, 1973; Janistyn, 1972; Kessler, 1972; Narayanan et al., 1970; Ownby et al., 1973; Pollard & Venere, 1970; Pradet et al., 1972; Raymond et al., 1973; Salomon & Mascarenhas, 1971, 1972; Wellburn et al., 1973). The main reason for disputing the presence of cAMP *in planta* was the low sensitivity of the methods employed to detect cAMP at the picomole level. For example, it was reported that a radiolabeled product derived from the incubation of 8-^{14}C-adenine of barley (*Hordeum vulgare*) seedlings could not be chromatographically separated from cAMP since the resolving power of the chromatographic system used was insufficient to resolve cAMP from the RNA catabolic intermediate, 2',3'-cAMP (Pollar & Venere, 1970). An attempt to demonstrate the chromatographed product as cAMP was carried out by measuring the hydrolysis of cAMP to AMP by cAMP PDE (Narayanan et al., 1970). This method also yielded inconclusive results because PDE had not been confirmed at that time to have specificity for cAMP. Some publications disputing the existence of cAMP in higher plants include explicit and categoric statements such as " … cyclic AMP does not mediate the action of gibberellic acid" (Keates, 1973).

Skepticism about the occurrence of cAMP in plants on the other hand spurred the quest to develop scientific approaches that would eventually generate clear and direct evidence for cAMP and its role in plants. Since the mid-1980s, more reports discussing the presence and potential functions of cyclic nucleotides in plants have been published. The potential roles of cAMP in plants include the regulation of ion channels (Bolwell, 1995) and ion transport in *Arabidopsis thaliana* (Anderson et al., 1992; Trewavas, 1997), activation of phenylalanine ammonia lyase (Bolwell, 1992), cAMP-dependent signal transduction pathways and the cell cycle progression in tobacco BY-2 cells (Ehsan et al., 1998). Cyclic AMP has also been shown to play a role in stimulating protein kinase activity in rice (*Oryza sativa*) leaves (Komatsu & Hirano, 1993), and more recently, to eliciting stress responses and plant defense (Choi & Xu, 2010). For example, increased levels of cAMP coincide with the early stages of the response to phytoalexins and mediate the production of 6-methoxymellein and the activation of calcium uptake into cultured carrot (*Daucus carota*) cells (Kurosaki & Nishi, 1993; Kurosaki et al., 1987).

It is noteworthy that in the early 1970s, the same techniques for tissue extraction, purification and detection techniques employed in animals were applied to plant tissue without taking into account the particular characteristics of the (bio-)chemistry of plants. This led to disputes that were only resolved in the late 1970s when experimental procedures to analyze cAMP were adapted to suit the plant cellular and extracellular environments. Since then a number of research groups reported more reproducible data, for example, the activity of PDE to hydrolyze 3',5'-cAMP through enzyme inhibition reaction by methylxanthines (Brown et al., 1977) and the localization of AC activity in meristems of young pea (*Pisum sativum*) hypocotyl (Hilton & Nesius, 1978). Efforts to identify endogenous cAMP in plant cells coincided with the development of sequential chromatographic and electrophoretic methodologies for the extraction and isolation of cAMP (Brown & Newton, 1973) that led to improved cAMP recovery by co-chromatography. Together, these techniques enabled separation of cAMP from 2′,3′-cAMP and other known naturally occurring adenine nucleotides. Yet, some scientists continued to dispute the claims arguing that the results were due to unidentified adenine compounds in higher plants that may have identical chromatographic properties as cAMP (Amrhein, 1974; Keates, 1973; Lin, 1974).

The elucidation of the structure and molecular conformation (Fig. 1) of cAMP was first established in animals (Sutherland & Rall, 1960) and helped to determine the chemical properties and biological function of this messenger. The molecular conformation and 3D structure of cAMP was established by X-ray crystallography and proton-NMR (Sundralingham, 1975).

2.2 Discovering cAMP in higher plants

The mode of action of cAMP as the second messenger for mammalian hormones had been accepted as early as the 1960s and were done long before any attempts to discover cAMP and cAMP functions in higher plants. Cyclic AMP was originally discovered as the intracellular mediator or activator of many peptides and catecholamine hormones in animals (Robison & Sutherland, 1971; Sutherland & Rall, 1960). Since then, cAMP has been implicated in signaling system that intercede cellular responses to imbalances of the external biosphere.

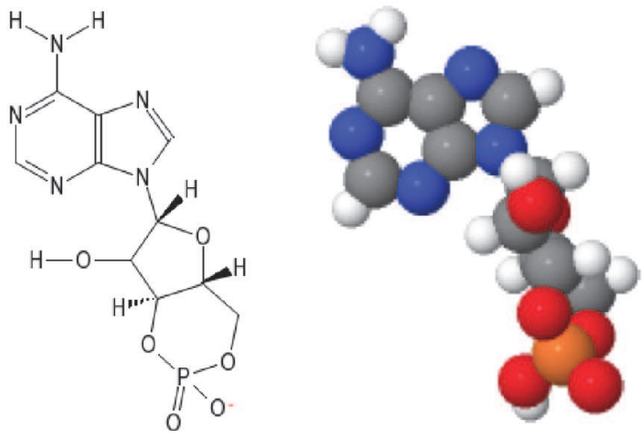


Fig. 1. Chemical and 3D structures of cAMP (Molecular formula: $C_{10}H_{12}N_5O_6P$)

The first functional clue that cAMP may play a role in hormone signaling in higher plants came from findings in barley aleurone layers suggesting that the nucleotide could substitute gibberellic acid (GA) in the induction of α-amylase synthesis (Duffus, 1969). The first evidence of the formation of cAMP in higher plants was demonstrated through the incorporation of ^{14}C-adenosine into cAMP by a method developed by Gilman (Gilman, 1970). The Gilman method is based on the competitive displacement of unlabelled nucleotide present in the complex containing the cAMP binding protein in the sample. However, incorporation of radioactively labeled adenine in plant tissues was observed to give rise to a complex with properties similar to cAMP (Azhar & Krishna Murti, 1971; Pollard, 1970; Salomon & Mascarenhas, 1971). Hence, the use of labeled products was not judged convincing enough to prove the synthesis of cAMP in plant cells, mainly due to the sensitivity limits, the specific activity of labeled precursor applied and the efficiency of conversion to cAMP. At the time, evidence for plant analogs of animal cAMP was limited and as such, endogenous cAMP could not easily be detected. Nevertheless, exogenous application of un-physiologically high levels of cAMP were shown to mimic some biological activities of auxin (Pollard & Venere, 1970) and GA (Kessler, 1972). It was later confirmed that indeed, cAMP is capable of entering plant cells causing an increase of the endogenous level of cAMP by a factor of 30 (Wiedmaier & Kull, 1976). However, these 'pharmacological' studies lacked biological significance and did not prove the existence of cAMP in higher plants.

Based on the specific activity of ATP, the substrate for cAMP formation, the upper limits of cAMP concentrations were estimated to be in the range of pmoles/g fresh weight. For instance, 0.04 pmole/mg protein in barley aleurone layers (Keates, 1973), 7-11 pmoles/g fresh weight in oat (*Avena sativa*) coleoptiles (Ownby et al., 1975) and 0.37 pmoles/g fresh weight in lettuce (*Lactuca sativa*) seedlings (Bressan et al., 1976). In the latter study, incorporation of adenosine-8-^{4}C into compounds, with chemically related properties to cAMP, was measured in germinating lettuce seeds. In addition to tracing radioactively labeled adenine, a modified Gilman binding assay and protein kinase activation assay were used to measure endogenous levels of cAMP in coleoptile leaf segments of oats, tubers of potato (*Solanum tuberosum*), callus of tobacco (*Nicotiana tabacum*), and germinating seeds of lettuce (Bressan et al., 1976). The binding assay proved reliable for mouse and rat liver

analyses, but not sensitive or specific enough for plant tissues as it also responded to various components from lettuce and potato tissues that were chromatographically similar but not identical to cAMP. While the protein kinase activation assay was much more specific, it exhibited positive response to some compounds not chromatographically identical to cAMP. In this study, the concentrations of cAMP found in the plant tissues tested were below the detection limit (Bressan et al., 1976), a problem also encountered in other studies (Amrhein, 1974; Niles & Mount, 1974). Additionally, other authors claimed that cAMP detected in plants was a result of bacterial infection or contamination (Bonnafous et al., 1975), a claim that was refuted by the fact that bacteria contributed to less than 0.1% of the cAMP in plant cells (Ashton & Polya, 1978). By and large, reported cAMP levels were lower than those in animal tissues that typically range from 100 to 500 pmoles/g of fresh weight or 0.5 to 2.6 pmoles/mg protein (Robison et al., 1968). A comprehensive listing of cAMP concentrations in various plants are reported elsewhere (Newton & Brown, 1986).

The ubiquitous presence of cAMP in plant tissues assumes the definite existence of NCs and the likely presence of cyclic nucleotide PDEs. At present, there is hardly any doubt about the presence of adenylate cyclases and cAMP-dependent signaling system in plants. However, the role of these systems in various physiological and biochemical processes is yet to be properly elucidated. Cyclic AMP formation is catalyzed by adenylate cyclases (E.C.4.6.1.1) that convert ATP to cAMP and pyrophosphate (Helmreich et al., 1976), while the enzyme cAMP PDE (E.C.3.1.4.17) hydrolyzes 3′,5′-cAMP to 5′-AMP (Robison & Sutherland, 1971). Endogenously applied cAMP can be used as an indicator of functional activity for adenylate cyclase signaling system (Lomovatskaya et al., 2011). The presence and localization of AC activity was first experimentally demonstrated in meristems of young pea hypocotyls (Hilton & Nesius, 1978) making use of adenylyl-imidodiphosphate as a substrate specific for the enzyme and lead nitrate as a precipitating product that can be visualized with electron microscopy and quantitated *in situ*. Lead precipitate was found to be localized in distinct areas bound to the smooth endoplasmic reticulum in differentiated cells of the pea root cap (Hilton & Nesius, 1978). Later, the use of mass spectrometric techniques explicitly identified the reaction product of AC activity in pea for the first time, thus erasing doubts casted on the methodology (Pacini et al., 1993). In this study, plant extracts from roots of pea seedlings and incubated with ATP and Mg^{2+} produced cAMP as measured by tandem mass spectrometry and hence are the first experimental proof for AC activity in higher plants. Unambiguous cAMP synthesis was later confirmed by mass spectrometric analysis of alfalfa (*Medicago sativa*) cell cultures after exposure to the glycoprotein elicitor of the phytopathogenic fungus *Verticilium alboatrum* (Cooke et al., 1994). Cyclic AMP synthesis by adenylyl cyclases was also reported to occur in apical hook plasma membrane from bean (*Phaseolus vulgaris*) (Roef et al., 1996). This study exploited the use of polyclonal antibodies raised in chicken against an adenosine 3',5' monophosphate-diphtheria toxoid antigen construct in developing an immunoaffinity purification procedure to measure cAMP and hence AC activity.

Technological advances providing an even better reproducibility of quantitative data brought the detection limit of cyclic nucleotides down to 25 fmoles in plant extracts by combining liquid chromatography and electrospray ionization (ESI) mass spectrometry (Witters et al., 1996; Witters et al., 1997; Witters et al., 1999). Due to the high sensitivity of ESI, it has become the technique of choice for the analysis of polar biomolecules. Other methods currently in use to detect cNMP concentrations in plants include the Gilman

method (Gilman, 1970), radio-immune assays (Rosenberg et al., 1982) and the bioluminescent method (Isner & Maathuis, 2011; Nikolaev & Lohse, 2006). The radio-immune assay is based on the primary binding of standard antigen with antibodies followed by unlabeled antigen that competitively replace the radioactively labeled antigen, while the bioluminescent method permits the monitoring of cAMP levels in the living system, however, the use of the latter requires fluorophores with different spectrum properties than chlorophyll and phenols. A recent improved enzyme immunoassay method for determining cAMP concentration in plant tissue was proposed (Lomovatskaya et al., 2011) and allows for the detection of cAMP down to 5 pM, and this is about 10 times more sensitive than previously reported methods.

Enzymes that hydrolyze cAMP - cAMP PDEs – were first reported in pea seedlings (Lieberman & Kunishi, 1969), but apparently, such an activity was not detected in barley (Vandepeute et al., 1973). It has been demonstrated that plant PDEs differ from their animal counterpart in that they have several isoforms, each having its own substrate specificity. One isoform extracted from pea was shown to have an acidic isoelectric point and a substrate preference for 2′,3′-cAMP while its isomer preferred 3′,5′-cAMP (Lin & Varner, 1972). Partially purified PDE isoforms from scarlet runner bean (*Phaseolus coccineus*) showed similar enzymatic properties to those of mammalian PDEs (Brown et al., 1975, 1977). A multitude of PDEs have been reported in species like potato (Ashton & Polya, 1975), purslane (*Portuluca oleracea*) (Endress, 1979) and carrot (Kurosaki & Kaburaki, 1995; for review see Newton & Smith, 2004).

In the early 1980s, three cAMP-responsive protein kinases were identified in duckweed (*Lemna paucicostata*) and they catalyze the phosphorylation of histones (for review see Kato et al., 1983). Later, a cAMP-dependent protein kinase A (PKA) was reported in maize (*Zea mays*) and coconut (*Cocos nucifera*) (Janistyn, 1988, 1989); in addition, PKA activity was also detected in petunia (*Petunia hybrid* var. Old Glory Blue) (Polya et al., 1991) and rice (Komatsu & Hirano, 1993). Plant cAMP was shown to play a physiological role in regulating gene expression through eliciting dissociation of PKA (Inamdar et al., 1991). In this study, an isolated cDNA clone from *Vicia faba* with close resemblance to the animal cAMP response element-binding protein (CREB) was used to provide evidence for the presence of an analogous CREB system. In principle, PKA is a tetramer made up of two isoforms, and once cAMP binds to the regulatory subunits, it elicits dissociation of PKA yielding a regulatory dimer and two free catalytic subunits. Each of the regulatory dimer subunit is capable of binding two molecules of cAMP. In the free state, the catalytic subunits are active and able to phosphorylate other proteins thus altering their activity by increasing the surface charge. The active PKA catalytic subunits have also been shown to migrate to the nucleus allowing phosphorylation of the transcription factor CREB (De Cesare et al., 1999; Inamdar et al., 1991).

In summary, the existence of cAMP in higher plants has now been established using advanced analytical tools. In addition to presence of the cAMP, we also have conclusive evidence for the presence of PDEs and cAMP-binding proteins and a number of cAMP-dependent physiological responses in higher plants. Systematic studies of the function of cAMP are emerging and they include the study of the role of cAMP in ion transport e.g. in *Arabidopsis thaliana* (Anderson et al., 1992), in signal transduction and in cell cycle progression in tobacco BY-2 cells (Ehsan et al., 1998), in plant defence response (Kurosaki et al., 1987), in cAMP activation of phenylalanine ammonia lyase (Bolwell, 1992) and in stress

responses (Choi & Xu, 2010; Meier & Gehring, 2006). Overall, this shows that regardless of the low and seemingly un-physiological levels of cAMP in plants as compared to animals, the perception that plants have also a functional cAMP-dependent signal system remains alive and awaits detailed elucidation.

3. The discovery of guanosine cyclic monophosphate in plants

3.1 Early evidence for cGMP in plants

Not long after the identification and characterization of cAMP, another cyclic nucleotide, cGMP, was synthesized *in vitro* (Smith et al., 1961) and subsequently degraded in an enzymatic reaction similar to the hydrolysis of cAMP (Drummond & Perrotty, 1961). Two years later, endogenous cGMP was detected in rat (*Rattus norvegicus*) urine (Ashman et al., 1963). In 1964, an enzyme capable of breaking the 3′,5′-phosphodiester bond of cGMP was characterized (Kuriyama et al., 1964). The same laboratory confirmed the detection of cGMP in rat urine and proposed that the cGMP synthesis is catalyzed by a cyclase (Price et al., 1967). The chemical and 3D structures of cGMP are shown in Fig. 2. In 1966, a PDE hydrolysing cAMP as well as cGMP was partially purified from dog (*Canis lupus*) heart (Nair, 1966) and by 1969, guanylyl cyclases (GCs) and PDEs were recognized as the enzymes responsible for the synthesis and hydrolysis, respectively, of cGMP. Unlike the ACs that synthesize cAMP, GCs are mostly detected in the soluble fractions of animal tissue homogenates. The activity of GC was barely detected in the presence of Mg^{2+}, whereas it was stimulated by a factor of 10 upon replacement of Mg^{2+} by Mn^{2+} (Hardman & Sutherland, 1969). Seven families of PDEs were reported, of which at least two are cGMP-dependent (Beavo, 1995). In the early seventies, further insights into cGMP-specific PDEs were obtained from different rat and bovine (*Bos taurus*) tissues (Beavo et al., 1970; Cheung, 1971; Kakiuchi et al., 1971; Thompson & Appleman, 1971). That time also saw the isolation of PDEs from various plant sources and tissues including pea seedlings (Lin & Varner, 1972), soybean (*Glycine max*) calluses (Brewin & Northcot, 1973), bean seedlings (Brown et al., 1979) and spinach (*Spinacia oleracea*) chloroplasts (Brown et al., 1980). To date, it is accepted that upon activation, GCs convert cytosolic GTP into cGMP and that hydrolysis of 3′,5′-cGMP to 5′-GMP is catalyzed by the enzyme family of PDEs (Hofmann et al., 2002; Reggiani, 1997). The latter enzymes are valuable as pharmaceutical targets for the development of several commercial drugs such as Sildenafil (Viagra) that keeps cellular cGMP levels elevated (Ghofrani et al., 2006). At least in animals, cellular levels of cGMP are regulated by the rate of synthesis and accumulation of the GCs. It is also regulated by the availability of the GTP substrate and Mg^{2+} co-factor, by the cGMP release from cells (transport and extrusion) and the rate of degradation by PDEs (Murad, 2006).

Although the importance of cGMP as a second messenger was recognized in the early 1970s in animals, appreciation of its significance in plants was initially rather slow. This was in part due to the controversy surrounding the existence of cAMP in plants. The occurrence of cGMP and other cyclic nucleotides including uridine 3′,5′-cyclic monophosphate (cUMP), cytidine 3′,5′-cyclic monophosphate (cCMP), inosine 3′ ,5′-cyclic monophosphate (cIMP) and 2′-deoxythymidine 3′,5′-cyclic monophosphate (c-dTMP) remained a matter of speculation and controversy until unambiguous demonstration by mass spectrometry in pea roots was achieved at the end of the 1980s (Newton et al., 1989).

While the occurrence of cGMP was further confirmed in several plant species and tissues including pea roots (Haddox et al., 1974) and bean seedlings (Newton et al., 1984), it was not

fully accepted as a *bona fide* second messenger in plants before 1994 when three key articles were published. In the first, a significant and rapid increase in cGMP levels in response to nitric oxide (NO) exposure was detected in spruce pine (*Picea abies*) needles, thus suggesting the presence of NO-dependent guanylyl cyclases much like in animals (Pfeiffer et al., 1994). In another series of reports, cGMP was linked to light responses and chloroplast biogenesis (Bowler & Chua, 1994; Neuhaus et al., 1997).

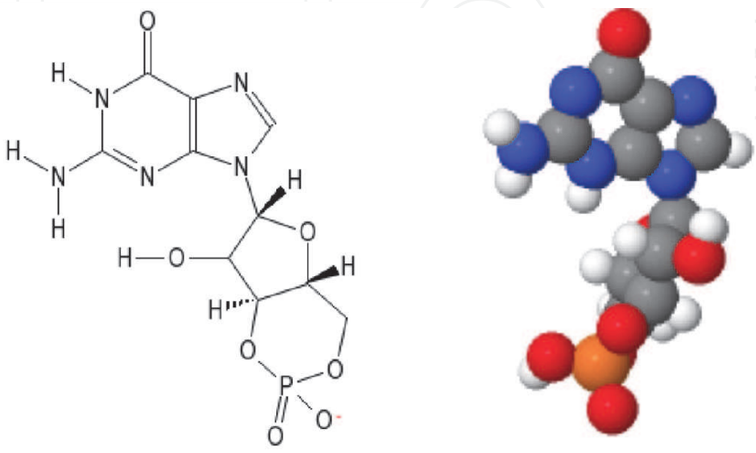


Fig. 2. Chemical and 3D structures cGMP (Molecular formula: $C_{10}H_{12}N_5O_7P$)

3.2 Methods for the detection of cGMP in plants

Cyclic GMP, much like cAMP, occurs at comparatively low concentrations in plant tissues and cells. As stated above, early research was hampered by the lack of sensitive and specific methods for measuring their small amounts and the requirement of complicated purification (and enrichment) methods prior to assaying (Brooker et al., 1968; Goldberg et al., 1969; Hardman et al., 1966). Cyclic GMP was first detected by measuring the radioactive molecule after injection of ^{32}P labeled substrate (Ashman et al., 1963). Three types of methods for measuring cGMP rapidly emerged: enzymatic methods (Kuo & Greengard, 1970), protein binding assay (Gilman, 1970) and immunoassay. The latter is based on immunological detection and ^{125}I-iodinated tracers. Two main techniques, the radioimmunoassay (Steiner et al., 1969) and enzyme immunoassay (Harper & Brooker, 1975) were developed subsequently and allowed determination of concentrations at the femtomole level (10^{-15} mol) with reasonable confidence. Nevertheless, unknown compounds from plant extracts may also cross-react, leading to inconsistencies. Thus, HPLC-mass spectrometric confirmation of the identity of the compound is advised (Brown & Newton, 1992). Other methods are based on the derivatization of cGMP and the successive purification by HPLC combined with UV-detection (Ohba et al., 2001; Soda et al., 2001). Recently, the combination of HPLC with mass spectrometry detection proved superior in terms of selectively and sensitivity (Cordell et al., 2008; Martens-Lobenhoffer et al., 2010; Witters et al., 1996). Additional information on the various assays and techniques developed to date is reviewed elsewhere (Schmidt, 2009) and most recently, an *in vivo* method for the detection of cGMP in real time in a non-invasive manner was described (Isner & Maathuis, 2011).

What are the cGMP concentrations in plants? For example, in pine (*Pinus densiflora*) pollen, cGMP was detected at the level of 60 pmol/g fresh weight (Takahashi et al., 1978). In maize seedlings, cGMP was estimated to be between 35 and 72 pmol/g fresh weight by GC-MS (Janistyn, 1983). Concentrations of cGMP ranging from 0.4 to 20 nmol/g fresh weight were detected in roots of bean (Haddox et al., 1974). In tobacco pith parenchyma, cGMP levels were estimated below 0.1 nmol/g dry weight (Lundeen et al., 1973). However, in the medicinal plants, *Evodia rutaecarpa, E. officinalis* and *Zizyphus jujuba*, levels as high as 10-50 nmol/g dry weight were reported (Cyong & Takahashi, 1982; Cyong et al., 1982). The rather wide range in cGMP concentrations is probably due, at least in part, to the presence of interfering substances and/or secondary metabolites that may have interfered with the assays. By combining gas chromatography and mass spectrometry on maize seedlings, 35-72 pmol/g fresh weight cGMP was measured (Janistyn, 1983). It is however noteworthy that considerable increases in cGMP levels occur in response to environmental cues such as light (Brown et al., 1989), NO (Pfeiffer et al., 1994) or pathogen attack (Meier et al., 2009), and these increases are a pointer to the functional importance of cGMP.

3.3 Biological functions of cGMP *in planta*

Research of the past few decades has connected the second messenger cGMP to a wide range of cellular and physiological processes in vertebrates and invertebrates, as well as bacteria, fungi, and algae. Receptor proteins can function as GCs and signal via cGMP (Chinkers et al., 1989) activating cGMP-dependent protein kinases (PKG, also abbreviated cGK), members of the PDE family and nucleotide-gated ion channels (CNGC) (Biel, 2009; Craven & Zagotta, 2006; Lincoln & Cornwell, 1993; Murad, 2006; Schmidt, 2009; Zimmerman, 2001), as well as transcription (Penson et al., 1996; Maathuis, 2006).

In invertebrates, cGMP controls homeotaxis of aggregating cells in the amoeba *Dictyostelium discoideum* (Bosgraaf et al., 2002) and modulates the expression of antimicrobial peptides in *Drosophila melanogaster* (Davies, 2006). In vertebrates, the signaling molecule is involved in differentiation, growth, apoptosis, regulation of electrolytes and fluid homeostasis (Kuhn, 2004; Steinbrecher et al., 2002) and in mammals, cGMP regulates a number of intracellular processes including neutrophil activation, phototransduction system of the retina (Cobbs & Pugh, 1985; Dhallan et al., 1992; Fesenko et al., 1985; Matthews et al., 1985; Yau & Nakatani, 1985) and natriuresis (Guillemant et al., 1994), and induces the mediation of smooth muscle relaxation in response to NO (Moncada et al., 1992). The role of cGMP on PDEs can also lead to alteration in the rate of hydrolysis of cAMP (Corbin & Francis, 1999).

In plants, growing evidence points to a central role of cGMP in a wide range of cellular and physiological processes including abiotic and biotic stress-response signaling (Donaldson et al., 2004; Durner et al., 1998; Gottig et al., 2008; Ma et al., 2009), the gating of ion channels (Anderson et al., 1992; Hoshi, 1995; Schuurink et al., 1998; Sentenac et al., 1992), light signal transduction (Bowler & Chua, 1994; Neuhaus et al., 1997; Neuhaus et al., 1993) and hormone signal transduction (Meier et al., 2009; Penson et al., 1996; Pharmawati et al., 1998; Pharmawati et al., 2001). Besides, treatment of Arabidopsis roots with the cell permeant cGMP analog (8-Br-cGMP) revealed that cGMP-mediated processes affect the transcriptome in general, and in particular lead to a transcriptional increase in transporter encoding genes. The transcriptional activation of non-selective ion channels and cation:proton antiporter encoding genes suggests a link between cGMP and the long-term modulation of monovalent cation (Na^+ and K^+) fluxes (Maathuis, 2006).

In 1992, two K^+ channels, KAT1 and AKT1, were determined in *Arabidopsis thaliana* (Anderson et al., 1992; Sentenac et al., 1992). While in mammals, the K^+ channels function in controlling the excitability of nerve and muscle cells (Hille, 1992), in plants and fungi, they are believed to be involved in turgor-mediated growth, osmotic adjustment, cell movement, and mineral nutrition (Gustin et al., 1986; Schroeder & Hedrich, 1989). Presence of a cGMP-binding motif in the C-terminal region suggested that cGMP might affect the channels (Schuurink & Jones, 1995), a role later confirmed (Hoshi, 1995), therefore linking the second messenger to the regulation of plant ion and water homeostasis.

In 1994, using the phytochrome A-deficient *aurea* tomato (*Lycopersicon esculentum*) mutant, phytochrome A, the photoreceptor responsible for regulating a wide range of morphogenesis responses such as flowering, seed germination or diurnal rhythms, was shown to utilize three different signal transduction pathways, dependent upon calcium and/or cGMP, to activate genes in the light (Bowler & Chua, 1994). In the first pathway, cGMP stimulates chalcone synthase and ferredoxin $NADP^+$ oxidoreductase, which in turn triggers anthocyanin synthesis (Bowler & Chua, 1994). The second pathway is dependent on calcium and calcium-activated calmodulin, whereas in the third one, both calcium and cGMP are required for the transcriptional activation of genes encoding photosystem I and cytochrome b6f complexes. Overall the combination of these three signaling systems mediates the development of chloroplasts and the biosynthesis of anthocyanin (Neuhaus et al., 1997).

3.4 Cellular signaling with cGMP

Cyclic GMP is becoming increasingly recognized as an important messenger in plant hormone signal transduction. It was linked for the first time to phytohormones while studying barley aleurone layers (Penson et al., 1996; Penson et al., 1997). Gibberellic acid (GA) was also shown to cause an increase in cytosolic cGMP, which in turn is essential for the induction of synthesis and secretion of ∝-amylase, the enzyme that breaks down starch. Auxin and kinetin, both of which cause stomatal opening, also show signaling via cGMP (Cousson & Vavasseur, 1998; Pharmawati et al., 2001), the former possibly through triggering the synthesis of cADP-ribose, which then influences Ca^{2+} fluxes. Abscisic acid (ABA) has been shown to induce stomatal closure through down-stream intermediates that include NO (Garcia-Mata & Lamattina, 2001) and hydrogen peroxide (McAinsh et al., 1996; Pei et al., 2000; Zhang et al., 2001). Recently, it came to light that germination of Arabidopsis seeds is modulated by cGMP levels, which in turn are dependent upon GA (Teng et al., 2010). However, the downstream effectors of cGMP triggering seed germination remain unknown. Taken together, it appears that cGMP is an effector of a number of phytohormones, leading to the downstream signaling mechanisms necessary for plants growth, development and responses to abiotic and biotic stresses.

In animals, NO acts as a highly instable signaling hormone in the cardiovascular system and the brain (Ignarro, 1999) acting locally and signaling via binding to and stimulating GCs (Arnold et al., 1977) giving rise to cGMP transients (Furchgott & Vanhoutte, 1989; Ignarro, 1991). Roles of NO have mainly been explored in animals where NO has been implicated in a number of physiological and pathological responses such as neurotransmission, smooth muscle relaxation, immunity, and apoptosis (Ignarro, 2000; Jaffrey & Snyder, 1995; Lloyd-Jones & Bloch, 1996; Moncada et al., 1991; Wink & Mitchell, 1998). Downstream effectors of NO includes activation of GCs, transcription factors and ion-channels (Melino et al., 1997;

Stamler, 1994). Detection of NO in plants came in 1994, when cGMP production was shown to be stimulated by NO in spruce needles (Pfeiffer et al., 1994). In 1998, it was confirmed that NO acts indirectly through activating the soluble form of GCs in plants and thus similarly to mammals (Martens-Lobenhoffer et al., 2010). Following stimulation by NO, cGMP induces expression of phenylalanine ammonia lyase (Durner et al., 1998), a key enzyme in the phenylpropanoid biosynthetic pathway. This stimulation is also believed to be implicated in the biosynthesis of salicylic acid (Dixon & Paiva, 1995; Mauch-Mani & Slusarenko, 1996; Pallas et al., 1996). In addition, a number of publications have revealed NO as an ubiquitous plant signaling molecule that also plays a role in growth and development including induction of leaf expansion, root growth as well as cell death and defense against pathogens (Durner et al., 1998; Gouvea et al., 1997; Leshem, 1996; Neill et al., 2008). Nitric oxide has also been linked to the induction, stimulation or inhibition of ABA, GA and ethylene (Beligni et al., 2002; Leshem, 1996; Neill et al., 2002). In analogy with the mammalian system where the majority of NO effects are accomplished through elevation in cGMP levels (Bogdan, 2001; Mayer & Hemmens, 1997), cGMP was demonstrated to be required in NO-induced programmed cell death in Arabidopsis (Clarke et al., 2000).

Cyclic GMP is also a second messenger in plant natriuretic peptide (PNP) responses. PNPs are small peptidic molecules with biological activity at nanomolar concentrations (Gehring, 1999; Gehring & Irving, 2003) that induce rapid and transient increases in cellular cGMP levels (Pharmawati et al., 1998; Pharmawati et al., 2001; Wang et al., 2007) and modulate K^+, Na^+ and H^+ net fluxes (Ludidi et al., 2004; Pharmawati et al., 1999) thus linking cGMP effects to the regulation of cellular homeostasis. PNPs have also been implicated in stress defense and possibly play a role in the changes of host homeostasis, which in turn are a part of plant defense against biotrophic bacteria (Gottig et al., 2009).

4. Cyclic nucleotide gated channels – structure and function

At the turn of the 20th century, one could read statements such as "*Corpora non agunt nisi fixata*" (drugs do not act unless they are bound). John N. Langley in England and Paul Ehrlich in Germany are usually credited for developing the concept of "receptive substance"; that could also act as a transducing engine. Fifty years later, Earl W. Sutherland (in the USA), studying the hyperglycemic response in hepatocytes, discovered that addition of epinephrine and/or glucagon in the presence of ATP and Mg^{2+} to the so called "particulate material" (i.e. fraction containing fragments of plasma membrane) obtained from liver slices, but not to "the soluble system", resulted in the generation of a newly synthesized heat-stable factor that was identified soon after to be cAMP. Moreover, cAMP was able to mimic the effects of the hormones on the liver glycogen phosphorylase when added on its own to the soluble fraction (Sutherland et al., 1968). Sutherland and his team paved the way for the fields of secondary messenger systems, G proteins and cell signaling and thus, both cAMP and cGMP became the focus of extensive studies. They were found to be ubiquitous across kingdoms - however, with considerable ambiguity in higher plants (discussed above) - and to regulate a vast array of cellular functions by controlling the activity of numerous proteins downstream of hormone-receptor interaction. Amongst the first proteins to be found regulated by cyclic nucleotides were the protein kinases (PKA and PKG types activated by cAMP and cGMP, respectively). When activated, these kinases phosphorylate an array of other cellular targets including other kinases, phosphatases, gene transcription factors and an ever-growing list of ion channels (Gray et al., 1998; Montminy,

1997; Shipston, 2001; Thevelein & De Winde, 1999; Trautwein & Hescheler, 1990). Note that while the activity of some ion channels can be enhanced by cAMP and/or cAMP-dependent PKA treatment, the activity of others can be down-regulated following the same treatment (Milhaud et al., 1998; Osterrieder et al., 1982; Shuster et al., 1985; Siegelbaum et al., 1982).

By the mid 1980's, Fesenko and co-workers (Fesenko et al., 1985) published a seminal paper where they showed that cationic channels from the retinal outer segments could be directly gated 'open' (no phosphorylation reaction required) by the addition of cGMP. Nowadays, the rod outer segment channel is classically described as part of a special class of channels: the cyclic nucleotide gated channels or CNGCs. These CNGCs form a distinct branch within the superfamily of pore-loop cation channels (Hofmann et al., 2005; Yu et al., 2005). The family of mammalian CNGCs comprises six homologous members, which are classified as A subunits (CNGA1–4) and B subunits (CNGB1 and CNGB3). Based on the detection of their respective mRNAs, CNGCs were found in the heart, brain, muscle, liver, kidney, and testes (Kaupp & Seifert, 2002; Zimmerman, 2001) but that the analysis of CNGC knock-out mice did not provide convincing evidence for relevant physiological function raising questions about CNGC expression in non-sensory system cells (Biel & Michalakis, 2009).

Two cyclic binding domains (CNB), named S1 and S2, were originally shown in the regulatory subunits of both RI and RII monomers of the bovine PKA (Corbin & Francis, 1999). The bacterial protein catabolite activator protein (CAP), also contains this CNB domain, which is linked to a DNA binding domain, and mediates gene transcription in the absence of glucose. The elucidation of many bacterial genomes indicates that this domain is much more diverse. There are literally hundreds of proteins that share this common ancestral structural domain made of about 120 amino acid residues and comprising both β-strands and α-helical elements (Berman et al., 2005; Wu et al., 2004). So, besides the mammalian CNGC, this CNB structure is found in a protein that binds calcium in the cytosol: calmodulins (CaM), in channels like the hyperpolarization-activated cyclic nucleotide-gated channels (HCN), Eag-like K^+-channels (Berman et al., 2005; Biel, 2009; Biel & Michalakis, 2009; Craven & Zagotta, 2006). This feature is also shared by K^+-channels from higher plants; indeed KAT1 and AKT1 were shown to contain the structural domain and/or to be regulated by cGMP (Gaymard et al., 1996; Hoshi, 1995; Sentenac et al., 1992).

In 1998, Schuurink and colleagues demonstrated the existence of CNG-like channels in *Arabidopsis thaliana* plants (Schuurink et al., 1998). They employed a strategy whereby 'CaM conjugated to horseradish peroxidase', was used to screen a complementary DNA expression library for CaM binding proteins in barley aleurones. One of the cDNAs obtained by this screen was shown to be a unique protein of 702 residues; the molecular architectural structure of the deduced translation product resembled that of the superfamily of genes encoding the voltage-gated ion channels. The *Arabidopsis thaliana* genome was discovered to encode 20 putative members of the CNGC family and homologs of these proteins were also found in major crop plants like rice, maize and cotton (*Gossypium spp.*). Plant CNGCs were classified into four main groups: Group I, II, III and IV (group IV is subdivided in two groups: A & B). For more details, the reader is encouraged to consult the following excellent reviews on this subject: (Demidchik et al., 2002; Dietrich et al., 2010; Kaplan et al., 2007; Martinez-Atienza et al., 2007; Mäser et al., 2001; Sherman & Fromm, 2009; Talke et al., 2003; Very & Sentenac, 2002; Ward et al., 2009; White et al., 2002).

Biochemical (Peng et al., 2004; Weitz et al., 2002; Zhong et al., 2002) and biophysical data (Zheng et al., 2002; Zheng & Zagotta, 2004) as well as patch clamp recordings from CNGCs

in animal native sensory neurons and those obtained from heterologous co-expression of distinct subunit combinations (Chen et al., 1993; Kaupp et al., 1989; Korschen et al., 1995; Matthews & Watanabe, 1987; Torre et al., 1992), all strongly suggest that animal CNGC subunits must assemble into tetrameric complexes with stoichiometries that are variable from one type of sensory neuron to another (reviewed in Biel, 2009). For instance, the CNGC expressed in cilia of olfactory sensory neurons consists of three different subunits: CNGA2, CNGA4, and a short isoform of the CNGB1 subunit (CNGB1b) (2:1:1 stoichiometry). The CNG channel of rod photoreceptors consists of the CNGA1 subunit and a long isoform of the CNGB1 subunit (CNGB1a) (3:1 stoichiometry), while the cone photoreceptor channel consists of the CNGA3 and CNGB3 subunits (2:2 stoichiometry). The hydrophobic core of each of the subunits consists of the classic six putative α-helical membrane-spanning segments (named S1 to S6), a re-entrant pore (P) loop between S5 and S6, and hydrophobic N- and C-termini regions, both projecting into the cytosol (Fig. 3). In animals, CNGCs have the CaM binding- and CNB domains (CaMBD and CNBD, respectively) found in the opposite N- and C termini (Fig. 3A). However in plants, both CaM and CNB domains co-exist at the carboxyl terminus (Fig. 3B). Three-dimensional modeling of the CNBD structure of an *Arabidopsis thaliana* CNGC (AtCNGC2) revealed that cAMP binding to CNBD occurs in a pocket formed by a β barrel structure appressed against a shorter (relative to animal CNBD) αC helix (Hua et al., 2003b).

All channels from the voltage-gated channels superfamily including Na_v, K_v and HCN contain a highly positively charged S4 helix: the sensor for the allosteric modulation of gating by membrane voltage (Flynn et al., 2001). S4 is classically described as carrying three to nine regularly spaced arginine or lysine residues at every third position and these residues may confer voltage sensitivity to the channels (Biel, 2009; Jan & Jan, 1992). But in CNGCs it was proposed that glutamate residues in the vicinity of S4 contribute negative charges that may neutralize positively charged arginine and lysine residues leaving the S4 domain with a lower net positive charge with respect to K_v channels and rendering it less sensitive to voltage (Wohlfart et al., 1992). Moreover, it was found that the S4–S5 linker is shorter (11 amino acids instead of 20) and probably forms a loop in CNGCs. This is in contrast to related K_v and HCN channels, where the S4–S5 linker forms an α-helical structure and thus a rigid connection between the voltage sensor domain and the pore lining domain S5 (Ohlenschlager et al., 2002). Consequently, this causes the motion of the S4 domain to be less efficiently coupled to the S6 domain as in K_v and HCN channels (Anselmi et al., 2007). CNG current recordings in whole cell mode show no voltage-dependent inactivation and analysis of their current-voltage (I-V) relations shows only a weak voltage-dependence (especially at saturating or near-saturating ligand concentration and in the absence of divalent cations; Haynes & Yau, 1985; Picones & Korenbrot, 1992b; Zagotta & Siegelbaum, 1996). The weak rectification seen in I-V relations from recordings made in intact animal cells may originate from voltage-dependent channel block by Ca^{2+} and Mg^{2+} (Zimmerman & Baylor, 1992).

Much of our current understanding of the molecular mechanisms of channel ion conduction is in large part due to the elucidation, by MacKinnon and co-workers, of the X-ray crystal structure of KcsA, a K^+-channel from *Streptomyces lividans* (Doyle et al., 1998). The molecular architecture of the putative pore region (P-loop) between S5 and S6 forms the basis of ion permeation and selectivity. In all the P-loop-containing protein family of channels including animal and plant CNGCs, this structure (Fig. 3) dips into the membrane bilayer as an α-helix

(pore helix) and exits back extracellularly as an uncoiled strand (Biel, 2009; Flynn et al., 2001; Hua et al., 2003b). In K^+-channels, a highly conserved amino acid triplet, GYG, seems to be a pre-requisite for K^+ selectivity (Doyle et al., 1998). Sequence alignments of the pore helix shows that both animal and plant CNGCs lack the GYG motif. Animal CNGC1 and CNGC2 have a GXET triplet; where the crucial glutamic acid (E) in place of the tyrosine (Y), confers nonselective cation permeation properties (Doyle et al., 1998; Flynn et al., 2001; Zagotta & Siegelbaum, 1996). CNGCs discriminate poorly among monovalent cations with no significant permeability to anions (example of selectivity sequence in rods: $Li^+ \geq Na^+ \geq K^+ > Rb^+ > Cs^+$), with Ca^{2+} and Mg^{2+} being more permeable than the other cations (Yau & Baylor, 1989; Zagotta & Siegelbaum, 1996; Zimmerman, 2001). In the absence of divalent cations, the single channel conductance of animal CNGCs can be as high as tens of picoSiemens (pS; at least two conductance states have been resolved in rods: ~ 8 - 10 pS and ~ 25 – 30 pS; and in cones and olfactory cells: 45 to 50 pS with apparent sub-conductance states; Haynes et al., 1986; Ildefonse & Bennett, 1991; Matthews, 1987; Ruiz & Karpen, 1997; Taylor & Baylor, 1995; Zimmerman & Baylor, 1992).

To study the biophysical properties of CNGCs (or any other channel for that matter), electrophysiologists took advantage of an elegant and powerful technique that allows heterologous expression of the clones (cDNA and/or mRNA) in a suitable system (HEK cells or oocytes). The end product (ion channel) should in theory 'correctly' fold and get inserted into the membrane to allow further analysis using the patch- or any another voltage clamp technique. Unfortunately, this technique, which by the way yielded important key biophysical information for animal CNGCs (and has also being used successfully to study plant KAT1 and AKT1 ion channels) turned out to be surprisingly unproductive for the electrophysiological study of plant CNGCs. Witness of this statement is the rather scarce number of reports related to this issue (see recent reviews from Dietrich et al., 2010; Ward et al., 2009). Speculations were advanced that maybe the functional analysis of these plant CNGCs upon expression in heterologous systems could be hindered by interaction of the plant protein with regulatory systems/molecules present in the host cell (Ali et al., 2006; Very & Sentenac, 2002). To date, only two groups have produced functional data with some electrophysiological characterization of plant CNGCs (Balague et al., 2003; Hua et al., 2003a; Hua et al., 2003b; Leng et al., 2002; Leng et al., 1999). The *Arabidopsis thaliana* channel AtCNGC2 was the first cloned plant channel shown to be permeable to Ca^{2+} (Leng et al., 1999). A few years later, AtCNGC4 was found to be permeable to both Na^+ and K^+ as is the case for animal CNGCs (Balague et al., 2003). AtCNGC2 was also found to discriminate poorly between all monovalent cations tested (K^+, Li^+, Rb^+ and Cs^+) except for Na^+. Indeed, AtCNGC2, unlike any animal or plant CNGCs cloned to date, is impermeable to Na^+ (Hua et al., 2003a). A molecular analysis of what is presumed to form the selectivity filter in the P-loop indicates that AtCNGC2 has an "AND" triplet that differs from the selectivity filter motifs of all other known CNG channels. The same authors found that specific amino acids within the AtCNGC2 pore selectivity filter, namely Asn-416 and Asp-417, facilitated K^+ permeation over Na^+ (Hua et al., 2003b). They proposed that the "AND" motif can confer selectivity against Na^+, as does the "GYG" motif in K^+-selective channels (Doyle et al., 1998; Jiang & MacKinnon, 2000). Note that most plant CNGCs, for instance AtCNGC1, or their homologs from other plants like NtCBP4 or HvCBT1 have a GQN triplet. It was suggested that glutamine (Q)

could equally compromise the K^+ selectivity in animal cation channels, as does the glutamic acid (E) residue (Kerr & Sansom, 1995; Leng et al., 2002).

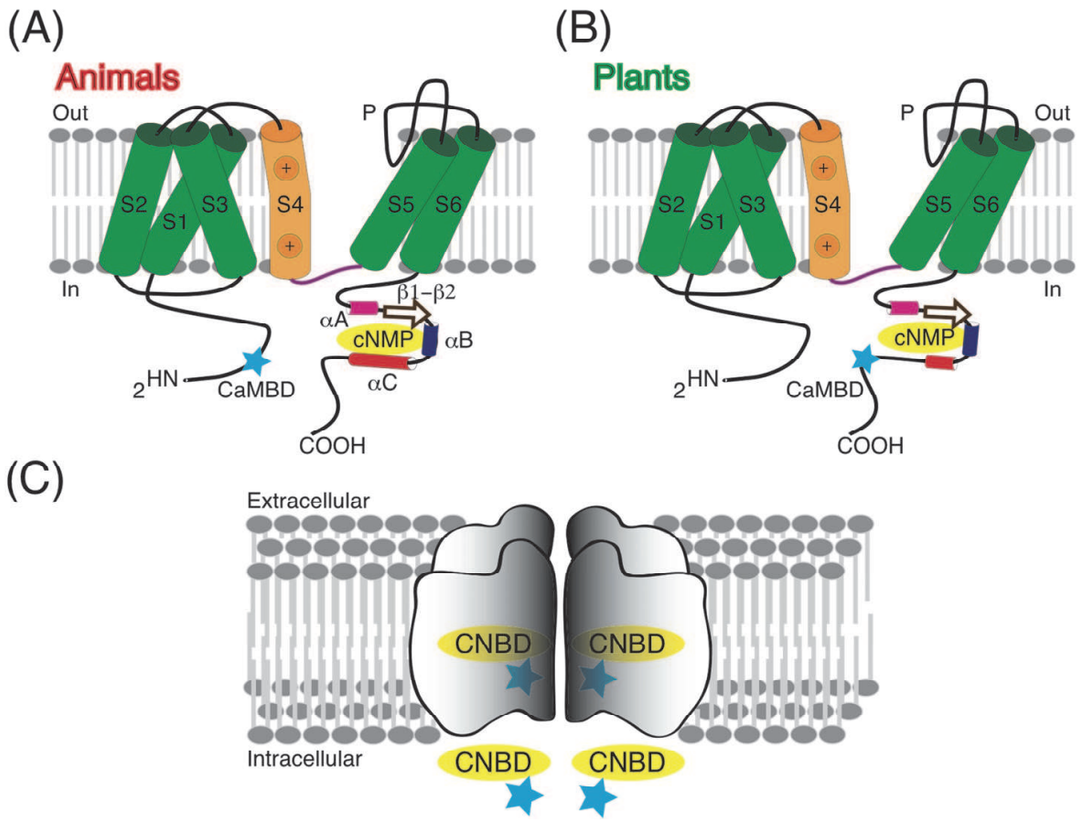


Fig. 3. Animal and plant CNGC membrane topologies and domain structure models. (A) Animal and (B) plant CNGCs share the common six α-helical transmembrane segments (S1-S6) and a pore loop (P) between S5 and S6, which is thought to contain the selectivity filter. The S4 segment is composed of a series of positively charged residues that forms the voltage sensor in all voltage-dependent channels, but in CNGCs, S4 is believed to carry a diminished net positive charge resulting in weaker voltage sensitivity. The N- and C-termini of plant and animal CNGCs extend into the cell cytosol. In the cytosolic C terminus, all CNGCs subunits carry a CNBD that is functionally coupled to the transmembrane channel core via the C-linker domain which is formed by three α-helices (A-C) and two β sheets (β1 and β2) forming a β barrel between the αA and αB helices. In sensory neurons, binding of cNMPs to the CNBD is thought to produce an allosteric conformational change that increases the open probability of the channel pore. The CaMBD is located at the N-terminus in animal CNGCs while in plants, it is located at the C-terminus, beginning at the truncated αC helix. (C) Four subunits assemble to form functional tetramers in animal CNGCs. Plant CNGCs are also predicted to form tetramers with each subunit associated to one CNBD and one CaMBD (figure adapted and modified from Biel, 2009 and Hua et al., 2003b).

In addition to their direct activation by cNMPs, another distinguishing feature of CNGC in native (animal) membranes is their remarkable fast gating kinetics (open-shut transitions)

when recording from toad rods in cell-attached or excised patch modes (Matthews & Watanabe, 1987). This rapid flickery behaviour occurs even in the absence of any added cations like Ca^{2+} and Mg^{2+} that are known to result in flicker block mode (Zimmerman, 2001). However, when the channels were purified and reconstituted, or cloned and heterologously expressed, the channels performed like any other typical channels, i.e. they had slower gating kinetics (Kaupp et al., 1989). It was found that the flickery gating pattern from the native membranes resulted partly from the presence of at least one more type of subunit that was missing in the original reconstitution and expression studies (Chen et al., 1993; Korschen et al., 1995).

To date, there are only few reports in the literature showing any kind of modulation (i.e. direct or indirect) by cNMPs of an ion channel in a native plant membrane. It was suggested (Li et al., 1994) that cAMP, in the presence of 3-isobutyl-1-methylxanthine (IBMX), a cyclic mononucleotide phosphodiesterase inhibitor, modulated an outward K^+-current ($I_{K,out}$) in *Vicia faba* mesophyll cells indirectly through a PKA. Although the amount of cAMP used in the paper was high (1.5 mM), this modulation was specific and could not be reproduced by cGMP or other non-cyclic nucleotides (AMP or GMP). In addition, this modulation was affected with all the known usual suspects of the PKA pathway: PKI and Rp-cAMP, both inhibitors of the PKA, also inhibited $I_{K,out}$ and more importantly, the catalytic subunit of PKA enhanced the magnitude of $I_{K,out}$ in the absence of any added cAMP. In contrast to this, it was also shown that cAMP added alone did not affect ion channels in Vicia guard cells, but if added after ABA and/or internal Ca^{2+} treatments, cAMP was able to antagonize their well-characterized inhibitory effect on the inward K^+-current ($I_{K,in}$) (Jin & Wu, 1999). Other lines of evidence that suggest a role for cNMPs in plants come from studies of the modulation of stomatal aperture and/or internal free cytosolic Ca^{2+} levels (Jin & Wu, 1999; Kurosaki et al., 1994; Moutinho et al., 2001; Pharmawati et al., 1998; Pharmawati et al., 2001; Volotovski et al., 1998). The work of Volotovski and coworkers (1998) clearly and unambiguously demonstrated that physiological responses such as protoplast swelling and change in cell Ca^{2+} homeostasis occurred in response to cAMP and cGMP. Indeed, both cNMP-evoked elevation in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) and other pharmacological compounds such as forskolin (a direct activator of AC), IBMX (a cAMP PDE inhibitor) and zaprinast (a cGMP PDE inhibitor) could all mimic the effect of cNMPs on $[Ca^{2+}]_{cyt}$. The elevation in $[Ca^{2+}]_{cyt}$ was suppressed by verapamil, suggesting to the authors that a significant proportion of the $[Ca^{2+}]_{cyt}$ response occurs via activation of verapamil-sensitive Ca^{2+}-channels. Having said that, the authors could not exclude that internal Ca^{2+} stores could also be involved in the cNMP-elevation of $[Ca^{2+}]_{cyt}$. All these reports point to an indirect effect of cNMPs on plant cells through PKA or other cross talk mechanisms. In addition, support for a role of cNMPs in regulating plant ion channels comes indirectly from work done on the regulatory effects of a PNP hormone that is thought to operate through a cGMP-dependent pathway (Pharmawati et al., 2001; Wang et al., 2007). Using MIFE, a non-invasive microelectrode technique for measurements of net ion fluxes, it has been found that NP and cGMP activates non-selective cation channels (NSCCs) in maize root conductive tissue (Pharmawati et al., 1999). It is important to note that although, these authors referred to a direct effect of cGMP on NSCCs, they used that term to distinguish between NP and cGMP action. Indeed, unlike NP, cGMP was found not to stimulate the plasma membrane H^+-pump. In the same line of indirect evidence, NO which was shown

in plant cells to dramatically and transiently increase cGMP levels (Dubovskaya et al., 2011; Durner et al., 1998), was found to induce stomatal closure and confer increased tolerance to plant water deficits (Garcia-Mata & Lamattina, 2001). Nitric oxide raised $[Ca^{2+}]_{cyt}$ by promoting Ca^{2+} release from intracellular stores. Antagonists of GCs and cyclic ADP ribose-dependent endomembrane Ca^{2+}-channels blocked the increase of free cytosolic Ca^{2+}. It was proposed that a cGMP-dependent cascade might be the origin of this effect (Garcia-Mata et al., 2003). Recently, the evidence of cGMP acting upstream of $[Ca^{2+}]_{cyt}$ increases following either H_2O_2 and/or NO treatment was provided (Dubovskaya et al., 2011). Also, H_2O_2 and NO were found to act downstream of ABA to close the stomata and the changes in NO synthesis were induced by H_2O_2, resulting in increases in [cGMP] and $[Ca^{2+}]_{cyt}$.

A direct effect of cGMP (not cAMP) on the plant K^+ channels KAT1 (Hoshi, 1995) and AKT1 (Gaymard et al., 1996) has been proposed. However, in these studies a cGMP-ATP antagonism was described and indirect effects through possible kinases could not be ruled out (Gaymard et al., 1996). The effect of cGMP on these channels was to shift the activation voltage to more negative potentials, thus cGMP effectively reduced current through these channels over the physiological range of voltages. The first clear-cut evidence for a direct, effect of cNMP on any plant ion channel came from the work of Maathuis and Sanders (2001). They demonstrated cNMP inhibition of a root voltage-insensitive channel (VIC) that showed weak selectivity amongst monovalent cations and which probably provides a pathway for Na^+ uptake in root cells (Maathuis & Sanders, 2001). They suggested that this NSCC could be the target for the direct effect of cNMP seen in excised inside-out patches, and hence, be considered as candidate for the presence of functional CNGCs *in planta*. This effect was somehow unexpected since most animal and plant CNGCs, native and/or heterologously expressed, are rather activated by addition of cNMPs not deactivated. It was argued that cNMPs might exert their attenuating effect on Na^+ toxicity (Essah et al., 2003; Maathuis & Sanders, 2001; Rubio et al., 2003) not only by decreasing root cell NSCC activity and thus Na^+ uptake but also by activating CNGCs, like *At*CNGC2 involved in Ca^{2+} signaling (Leng et al., 1999), thus providing a possible role for CNGCs in plant salinity tolerance (Talke et al., 2003). There is also strong evidence to support a direct effect of cAMP in the shoot. Indeed, cAMP (or its membrane permeable derivative, dibutyryl cAMP) regulated a calcium conductance in both whole-cell mode and excised patches from *Vicia faba* guard and mesophyll cells (Lemtiri-Chlieh & Berkowitz, 2004). In this work, the authors showed that addition of cAMP consistently activated a channel with a 13 pS chord conductance. It was concluded that cAMP-activated calcium current seen in the whole protoplast configuration could be explained by a direct effect of cAMP binding on the Ca^{2+}-channel itself or a close entity to it. Like animal native CNGCs (Matthews & Watanabe, 1987), cAMP induced a rapid flickery gating behavior of the plant channel, but unlike them, cAMP binding was not the exclusive activator. Voltage (hyperpolarization) also activated the channel even in the absence of any added cAMP, resembling a feature of the mammalian HCN channels (Biel & Michalakis, 2009; Craven & Zagotta, 2006). However, CNGCs in the photoreceptor outer segments from striped bass, exhibit a very low level of activity even in the absence of added CNs, and the kinetics of this activity were the same as those measured in the presence of cGMP (Picones & Korenbrot, 1992a).

5. Evidence of nucleotide cyclases in higher plants

Given that the role of cNMP is recognized in many biological processes as both essential and sufficient to transduce signals and/or elicit physiological responses ranging from protein phosphorylation to transcriptional activation of specific genes, it is somewhat surprising how little we still know about NCs, the enzymes that catalyze the conversion of nucleotide triphosphate to cNMP and pyrophosphate. This gap in our understanding is particularly noticeable in higher plants.

In late 1997, a potentially highly influential paper was published in "Nature" linking an AC to the signaling of the plant hormone auxin in tobacco. The AC gene (*axi141*) was claimed to have AC activity and protoplasts transformed with it and treated with the AC stimulating compound forskolin could grow in the absence of cAMP, thus establishing the messenger as necessary and sufficient for auxin-dependent growth. However, the paper has been retracted since *axi141* does not confer auxin-independent growth and we do not know if indeed the reported data on AC activity are factual. To-date, the only annotated and experimentally confirmed cAMP generating molecule in plants is a maize pollen AC required for polarized pollen tube growth, which in turn depends on cAMP (Moutinho et al., 2001). The Arabidopsis orthologue of this protein (At3g14460) is annotated as a disease resistance protein belonging to the nucleotide-binding site-leucine-rich repeat (NBS-LRR) family used for pathogen sensing with a role in defense responses and apoptosis (DeYoung & Innes, 2006). NBS-LRR proteins directly bind pathogen proteins and associate with either a modified host protein or a pathogen protein, leading to conformational changes in the amino terminal and LRR domains of NBS-LRR proteins that are thought to promote the exchange of ADP for ATP by the NBS domain. It is thus conceivable that NBS-LRR downstream signaling is enabled by cAMP.

The discovery of the first GC (AtGC1; At5g05930) in higher plants is even more recent (Ludidi & Gehring, 2003) and was made possible by the deduction of a 14 amino acid (AA) long search term (GC core motif, see Fig. 5) based on an alignment of conserved and functionally assigned amino acids in the catalytic centre of annotated type III GCs from lower and higher eukaryotes (Liu et al., 1997; McCue et al., 2000). It has since been shown that the Arabidopsis brassinosteroid receptor (AtBRI) (Kwezi et al., 2007), a stress responsive wall associated kinase-like molecule (AtWAKL10; At1g79680) (Meier et al., 2010) and the Arabidopsis peptide signaling molecule (Pep1) receptor (AtPepR1; At1g73080) (Qi et al., 2010) also contain functional GC domains and this is commensurate with the presence of the amino acid residues essential for catalysis. The discovery of increasing complexities in the molecular architecture of higher plant NCs and in particular GCs (Fig. 4) is entirely compatible with findings in the single celled green alga *Chlamydomonas reinhardtii*, where catalytic NC domains appear in >20 different domain combinations including H-NOX, periplasmic binding protein, GAF-like, protein kinase-like domain, ATPase domain of HSP90, ribonuclease-H domain, G protein-coupled receptors and cysteine proteinase (Meier et al., 2007).

If we assume that many of these multiple domain proteins operate as multifunctional units, they may well have highly diverse and complex roles in cellular signaling (Meier et al., 2007). There are potentially several signaling modes that could be in operation in these complex multi-domain NCs, one of which is intramolecular cross-talk where the GCs

activated in a stimulus or ligand specific way are generating a cytosolic cGMP signature which in turn modifies the activity of a second domain (e.g. kinase) and thereby enabling intra-molecular cross-talk.

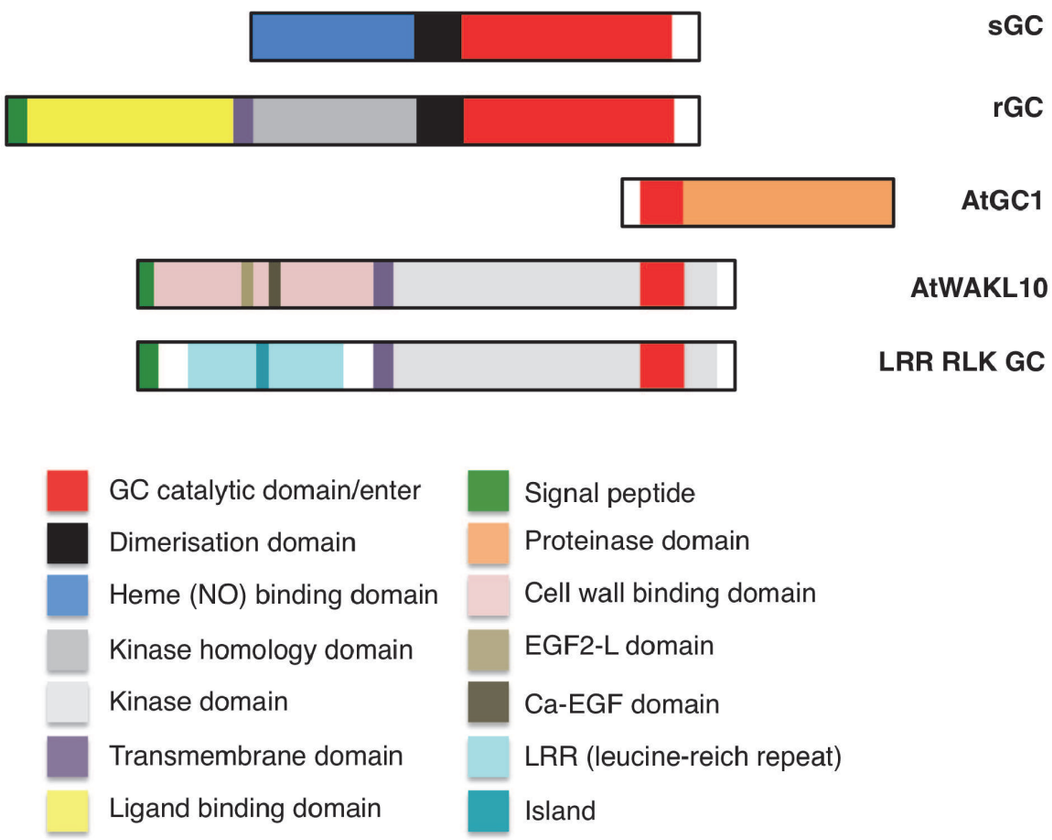


Fig. 4. Guanylyl cyclase domain organisations. Soluble GCs typically contain heme-binding domains allowing for NO dependent activation while reticulate GCs (rGC) contain transmembrane domains and extracellular ligand domains. The signal peptides direct the peptide to the cell membrane. AtWAKL10 contains an extracellular region that is tightly associated with the cell wall and contains several epidermal growth factor (EGF) repeats that may act as ligand binding domains (Meier et al., 2010). The AtWAKL10 protein has previously been predicted to contain an extracellular calcium binding EGF-like domain and a degenerate EGF2-like domain (He et al., 1999).

6. In search of novel nucleotide cyclases in higher plants

It has long been evident that BLAST searches using annotated or experimentally proven prokaryote or lower and higher eukaryote AC and GC sequences do not identify plausible plant candidate NC (Ludidi & Gehring, 2003) with low e-values since the degree of conservation in the NC catalytic center of these multi-domain enzymes is too low. Instead, we have argued that the 14 AA catalytic centre (not including the metal binding residues) may be sufficiently conserved to identify NCs in higher plants. For this reason, we have

deduced a rational query motif based on specific functional residues in the GC catalytic center, which has yielded seven candidates (Fig. 5) (Ludidi & Gehring, 2003), one of which (AtGC1) was functionally tested and proved to be the first GC in higher plants. Recently, a homologue of this gene was identified in Morning Glory (*Pharbitus nil*) and shown to have catalytic activity *in vitro* as well as being a potential element of the light signalling responses *in vivo* (Szmidt-Jaworska et al., 2009).

The original GC motif:

```
--[RKS][YFW][CTGH][VIL][FV]X[DNA]X[VIL]X(4)[KR]--
    1    2    3     4   5  6  7  8  9 10-13 14
```

The relaxed GC motif:

```
--[RKS][YFW][CTGH][VIL][FV]X(3)[VIL]X(4)[KR]X(1,2)[D]--
    1    2    3     4   5         9 10-13 14
```

The relaxed AC motif:

```
--[RKS]X[DE]X(9,11)[KR]X(1,3)[DE]--
    1    3         12/14
```

The extended AC motif:

```
--[RK][YFW][DE][VIL][FV]X(8)[KR]X(1,3)[DE]--
   1    2   3    4   5        14
```

Fig. 5. Guanylyl cyclase and adenylyl cyclase motifs employed in the identification of nucleotide cyclases in higher plants. In the original GC motif the residue (red) in position 1 does the hydrogen bonding with the guanine, the amino acid in position 3 confers substrate specificity and the residue in position 14 stabilises the transition (GTP/cGMP). The Mg^{2+}/Mn^{2+}-binding site is in the C-terminal (green). In the derived AC motifs position 3 (magenta) has been substituted to [DE] to allow for ATP binding.

In a further step, we used site directed mutagenesis to reduce stringency of the 14 AA GC core motif but added additional AAs to the search term to include the C-terminal Mg^{2+}/Mn^{2+}-binding site and the N-terminal PPi-binding site and this has yielded a further 27 candidate GCs in Arabidopsis, two of which (AtBRI1 and AtPepR1) have already been shown to be catalytically active *in vitro*. We have also undertaken a gene ontology analysis (Al-Shahrour et al., 2004) of these genes with a view to determine if this group is enriched for specific functions and found a significant over-representation ($p < 1e^{-5}$) in the Fatigo+ (level 4) categories of phosphorus metabolic processes, protein metabolic process, cellular macromolecular metabolic process and biopolymer metabolic process. It is noteworthy that several are annotated as leucine rich repeat receptor like kinases (LRR RLKs) and that the GC domain identified lies within the cytosolic kinase domain. Furthermore, three genes of this group (CLAVATA1 receptor (At4g20270) and ERECTA (At5g07180 and At2g26330)) have roles in plant development and meristem development in particular while one candidate (At3g46400) has similarity to a light repressible protein kinase. The key challenge

is to elucidate GC activity of the untested GCs *in vitro* and *in vivo* to establish the role(s) of cGMP in downstream signaling which will yield important new insights into this family of plant proteins.

As mentioned above, currently the only annotated and experimentally confirmed AC in plants is a maize pollen protein, which can generate cAMP that in turn acts as second messenger in polarized pollen tube growth (Moutinho et al., 2001). The *Arabidopsis thaliana* orthologue of this protein (At3g14460) is annotated as disease resistance protein and belongs to the NBS-LRR family that is used for pathogen sensing and also has a role in defense responses and apoptosis (DeYoung & Innes, 2006). NBS-LRR proteins directly bind pathogen proteins and associate with either a modified host protein or a pathogen protein leading to conformational changes in the amino-terminal and LRR domains of NBS-LRR proteins that are thought to promote the exchange of ADP for ATP by the NBS domain. It is thus conceivable that NBS-LRR downstream signaling (DeYoung & Innes, 2006) is enabled by cAMP.

Given that cyclic nucleotides have important and diverse roles in plant signaling via cyclic nucleotide-responsive protein kinases, -binding proteins and -gated ion channels (Newton & Smith, 2004), it is unlikely that a single AC or GC can account for all cAMP and cGMP dependent processes in higher plants. In line with this hypothesis is the fact that a number of Arabidopsis molecules with highly variable domain combinations and experimentally confirmed GC activity have recently been reported (Kwezi et al., 2007; Ludidi & Gehring, 2003; Meier et al., 2010). It is likely that what is true for GCs will also hold true for ACs. This then leaves us with the task to identify them, a task made complicated by the fact that BLAST searches with known and/or experimentally confirmed NCs from lower or higher eukaryotes do not return plausible candidate molecules. We also note that Prosite signatures for class one and class two ACs (EYFG[SA]X(2)LWXLYK and YRNXW[NS]E[LIVM]RTLHFXG, respectively) are not present in the *Arabidopsis thaliana* proteome even if we allow two mismatches.

Since in Arabidopsis, GCs have been identified with a catalytic motif search (Fig. 5), it was reasoned that a similar approach might lead to the discovery of novel ACs. In the modified AC search motifs the amino acid residues that confer substrate specificity (position 3 in Fig. 5) are substituted to [DE]. Consequently, the AC core motif within the catalytic centre consists of the functionally assigned residue that does the hydrogen bonding with the adenine (position 1), the amino acid that confers substrate specificity for ATP (position 3) and the amino acid that stabilizes the transition state from ATP to cAMP ([K,R], position 12-14). Additional diagnostic residues are the Mg^{2+}/Mn^{2+} binding amino acid [D,E], usually 1 – 3 amino acids removed from C-terminal of the transition state stabilizing residue. Such a relaxed AC motif ([RKS]X[DE]X(9,11)[KR]X(1,3)[DE]) (Fig. 5) is indeed present in a maize AC (AJ307886.1) which is the only experimentally tested AC in plants. It is also present in the *Sorghum bicolor* ortholog (gb|EER90437.1) and the related ($2e^{-70}$) Arabidopsis NBS-LRR class protein (At3g14460).

In many annotated GCs and all experimentally confirmed GCs in Arabidopsis (Kwezi et al., 2007; Ludidi & Gehring, 2003; Meier et al., 2010; Szmidt-Jaworska et al., 2009), the position between the assigned residue that does the hydrogen bonding (position 1) and the amino acid that confers substrate specificity (position 3) of the core motif is [YFW] and this is also the case in the confirmed maize AC which incidentally shares some similarity ($5e^{-05}$) with an annotated *Neurospora crassa* AC (XP_965280.1).

In Arabidopsis (TAIR: www.arabidopsis.org) there are currently only three annotated but functionally unconfirmed ACs (At1g26190, At1g73980 and At2g11890) and they all do contain a relaxed AC motif ([RKS]X[DE]X(9,11)[KR]X(1.3)[DE]) (Fig. 5). The first, a phosphoribulokinase/uridine kinase family protein shows similarity ($3e^{-108}$) to an AC domain-containing protein from the cellular slime mold *Polysphondylium pallidum* PN500. This protein contains a conserved CYTH-like domain typical for the superfamily of enzymes that hydrolyze triphosphate-containing substrates and requires metal cations as cofactors. The term CYTH derives from bacterial class IV adenylyl cyclases (CyaB) and thiamine triphosphatase and the domain occur in RNA triphosphatases, membrane-associated polyphosphate polymerases, tripolyphosphatases, nucleoside triphosphatases, nucleoside tetraphosphatases and other proteins with unknown functions. Furthermore, searches initiated from the C-terminal region to the uridine kinase from *Oryza sativa* identified archaeal CyaB homologs (Iyer & Aravind, 2002). The second candidate AC (At1g73980) has a similar domain organization and high homology ($6e^{-109}$) to a *Dictyostelium discoideum* AX4 AC domain containing protein. The third has only some similarity to non-plant proteins, one being a *Trichomonas vaginalis* G3 AC family protein ($2e^{-04}$). In addition, there is one putative Arabidopsis AC (At3g21465) annotated at NCBI (http://www.ncbi.nlm.nih.gov/protein/51968402). It does contain the core motif, but has no annotated domains or known functions and does not share any similarity with annotated and/or experimentally confirmed ACs but appears to be transcriptionally up-regulated in response to biotic stress.

While the presence of the relaxed AC motif may prove useful as supporting criterion for the identification of candidate ACs, it is not stringent enough to identify candidate ACs *ab initio* with any reasonable degree of confidence. In order to achieve this, we have proposed to use an extended AC motif that accounts for the specificity for ATP rather than GTP binding and with the C-terminal metal binding residue ([RK][YFW][DE][VIL][FV]X(8)[KR]X(1,3)[DE]) (Fig. 5). This extended motif retrieves nine putative Arabidopsis ACs (see Table 1).

The candidate ACs include two F-box proteins (At3g28223 and At4g39756) and a toll interleukin receptor (TIR) NBS-LRR (At3g04220). In the former, the F-box domains (cyclin like; IPR001810) have a role in protein-protein interactions and have also been associated with cellular functions, such as signal transduction and the regulation of the cell cycle that in turn is linked to both auxin responses and changes in cellular cAMP content (Ehsan et al., 1998; Leyser, 1998; Mohanty et al., 2001). Given this association one might be tempted to speculate that *axi*, an auxin independence conferring gene could encode an AC, particularly since the Arabidopsis axi1 protein does contain the AC core motif as well as the C-terminal metal binding residue.

With regards to the latter, At3g04220, we note that LRR proteins with AC domains or AC-like domains have been reported (Suzuki et al., 1990). Further, the maize AC (Moutinho et al., 2001) is structurally similar to plant TIR-NBS-LRR type disease resistance proteins (e.g. ADB66335.1, *Populus trichocarpa*, $4e^{-76}$) and At3g04220 also contains a P-loop NTase signature which also occurs in signal transduction ATPases with numerous domains (STAND) that in turn include ACs (Leipe et al., 2004). If one queries "Panther" (A search tool for a library of protein families and subfamilies indexed by function (http://www.pantherdb.org; Thomas et al., 2003) and look for ACs in *Arabidopsis thaliana*, one retrieves 83 entries with the GO (gene ontology) biological process categories: immune system process, cell surface receptor linked signal transduction, intracellular signaling

cascade, nucleotide and nucleic acid metabolic process and signal transduction. Many of the retrieved sequences are in fact TIR-NBS-LLRs and Coiled-Coil (CC)-NBS-LLRs that also have a role in plant resistance.

Arabidopsis thaliana proteins containing the AC search motif:		
[RK][YFW][DE][VIL][FV]X(8)[KR]X(1,3)[DE]		
At1g25240	KWEIFEDDFCFTCKDIKE	Epsin N-terminal homology
At1g62590	KFDVVISLGEKMQR—-LE	Pentatricopeptide (PPR) protein
At1g68110	KWEIFEDDYRCFDR-—KD	Epsin N-terminal homology
At2g34780	KFEIVRARNEELKK-EME	MATERNAL EFFECT EMBRYO ARREST 22
At3g02930	KFEVVEAGIEAVQR—-KE	Chloroplast protein
At3g04220	KYDVFPSFRGEDVR--KD	TIR-NBS-LRR class protein
At3g18035	KFDIFQEKVKEIVKVLKD	Linker histone-like prot. – HNO4
At3g28223	KWEIVSEISPACIKSGLD	F-box protein
At4g39756	KWDVVASSFMIERK--CE	F-box protein

Table 1. Candidate adenylyl cyclase molecules.

If we were to allow an [S] in position one, 10 more sequences are identified and they include a Type IIA (sarco/endoplasmic reticulum Ca, SERCA-type) Ca^{2+}-ATPase (At4G00900) that catalyzes the efflux of calcium from the cytoplasm and is transcriptionally up-regulated in response to syringolin and flagellin 22 (For expression analyses see https://www.genevestigator.com/gv/index.jsp; Zimmermann et al., 2005). Syringolin A is a molecular determinant secreted by *Pseudomonas syringae* pv *syringae* that is perceived by non-host plant species e.g. rice. It is recognized by wheat and does induce the accumulation of gene transcripts and increases protection against powdery mildew when applied before inoculation. By doing so, it essentially eradicates powdery mildew from infected wheat if applied after inoculation and therefore has a curative effect. Syringolin A has no fungicidal activity against a variety of fungi and its action on wheat cannot be mimicked by the fungicide cyprodinil and has therefore been proposed as counteracting the suppression of host defense reactions (Wäspi et al., 2001). Flagellin 22 (flg22) designates the conserved N-terminal part of flagellin that activates plant defense mechanisms in *Arabidopsis thaliana* and functions via the receptor-like-kinase, flagellin-sensitive-2 (FLS2). Mitogen-activated-protein-kinases (MAPK) are key signaling compounds that mediate the transcriptional regulation of > 900 flg22 responsive genes. While Ca^{2+} has long been recognized as an essential signal in plant defense responses, the mechanisms by which Ca^{2+} signals are sensed and translated into early microbe-associated molecular patterns (MAMPs) signals remain little understood. However, recently, four calcium-dependent protein kinases (CDPKs) were shown to be Ca^{2+}-sensor protein kinases and to be critical for transcriptional reprogramming in plant innate immune signaling (Boudsocq et al., 2010). It was reported that CDPK and MAPK cascades act differentially in MAMP-mediated regulatory programs that control early genes involved in the synthesis of defense peptides and metabolites, cell wall modifications and redox signaling. Double, triple and quadruple cAMP-dependent protein kinase mutants (*cpk* mutant) display progressively diminished oxidative burst and

gene activation induced by flg22, as well as compromised pathogen defense (Boudsocq et al., 2010).
Finally, we have also applied our AC/GC search strategies to the Rice proteome and identified 40 candidate GCs and 25 ACs and many of the putative GC genes are in families with either publications documenting roles in pathogen signalling, or annotated as such. In addition, there are also families of development-related LRR-RLKs, similar to BRI1 and CLAVATA1 and STRUBBELIG-receptor LRR-RLKs involved in immune and self/non-self signaling (Alcazar et al., 2010) and/or signaling during development (Eyuboglu et al., 2007).

7. Cyclic nucleotide cyclases and cyclic nucleotides in plant stress responses

Recently, cyclic nucleotides have been shown to play an important role in pathogen defense (Ma et al., 2009; Meier et al., 2009). Cyclic AMP was shown to be elevated at the initial site of infection initiating pathogen-related cytosolic Ca^{2+} signaling (Ma et al., 2009). Further, recent research (Qi et al., 2010) demonstrated cyclic nucleotides such as cAMP and cGMP are activating ligands for plant CNGCs, including CNGC2 and are thus linked to Ca^{2+} transport and hence Ca^{2+} signalling. Activation of channels by cGMP within a cell may be confined to microdomains near the plasma membrane and so provide spatial resolution of the signal. In addition, the role AtPeps, danger-associated molecular pattern (DAMPs) molecules play in pathogen-defense signaling cascades, and the guanylyl cyclase activity of the AtPep receptor AtPepR1 (Qi et al., 2010) provided a model for linking pathogen perception at the cell surface to intracellular Ca^{2+} signaling and immune responses in plants (Ma et al., 2009; Ryan et al., 2006). This implies that both cAMP and cGMP have specific roles in plant-defense signal transduction cascades, suggesting that specific cyclic nucleotide signatures generated in response to biotic (Ma et al., 2009; Meier et al., 2009) and abiotic (Donaldson et al., 2004) stresses act as second messengers in signaling cascades that critically depend on CNGCs (Kaplan et al., 2007; Talke et al., 2003). This is a concept that is in accord with the suggestion that the largest group of cNMP targets in plant cells are the CNGCs (Kaplan et al., 2007) that have a key role in the control of ion homeostasis and defense against biotic and abiotic stress (Kaplan et al., 2007; Ma et al., 2010).
Further evidence for the role of cGMP in pathogen response has been provided by a direct pathogen dependent increase in tissue cGMP levels in *planta* (Meier et al., 2009). Here, cGMP accumulation in *Arabidopsis thaliana* leaves was measured after inoculation with virulent (DC3000) and avirulent (AvirB) *Pseudomonas syringae* strains and caused marked and sustained increases in cGMP levels in response to the avirulent strain only, while the virulent strain caused smaller increases with a delayed onset. The earlier induction of cGMP by the avirulent strain is compatible with the instant recognition of specific pathogen avirulent (*avr*) gene encoded molecules that together with their corresponding *R* genes in plants triggers activation of plant defense responses. In the delayed response case for virulent DC3000 strain, pathogens are able to grow and spread due to the absence of specific pathogen recognition by plant *R* gene products. In Arabidopsis cell cultures, cGMP has been implicated as required for NO-induced cell death in response to challenge by avirulent bacterial pathogens (Clarke et al., 2000). In tobacco, cGMP has also been implicated in NO-dependent defense responses and required for induction of expression of defense-related genes, pathogenesis-related 1 gene (*PR1*) and the phenylalanine ammonia lyase gene (*PAL*) (Durner et al., 1998; Klessig et al., 2000).

Moreover, specific and transient increases in intracellular cGMP levels have also been reported in response to NaCl, drought stress (Donaldson et al., 2004) and ozone (Pasqualini et al., 2008). Cyclic GMP levels in *Arabidopsis thaliana* have been demonstrated to be time dependent and differing in response to salt and osmotic stress (Donaldson et al., 2004). Salt stress triggers an osmotic conduit that is independent of calcium concentration and the ionic response pathway triggered by high NaCl is calcium-dependent. These results suggest that cGMP also plays a complex role in abiotic stress responses (Donaldson et al., 2004). Remarkably, cGMP increases were observed two hours following ozone treatment in tobacco leaf, suggesting that cGMP is essential for the induction of some late ozone-dependent pathways but not critical in the early signal responses (Pasqualini et al., 2008). In this study, it was shown that early response to ozone and NO caused transcriptional activation of the scavenger coding for proteins *AOX1*, *GPX* and *ACS2* and were cGMP independent, but the early response of *PAL*a and late response of *PR1a* showed critical dependence on cGMP. In this context, it is noteworthy that the early response of *PALa* was observed two hours after fumigation coinciding with increase in cGMP transients.

Cyclic AMP may also have an important role in abiotic stress responses and particularly to salt stress since VICs in *Arabidopsis thaliana* roots have been reported to have open probabilities that are sensitive to micromolar concentrations of cAMP or cGMP at the cytoplasmic side of the plasma membrane. Here, presence of permeable cyclic nucleotides during growth improved plant tolerance to salinity that corresponded to a reduction in sodium accumulation (Maathuis & Sanders, 2001). This may suggest that plants contain a cyclic nucleotide-based signaling response that directly affects sodium transport across VICs in response to salinity stress.

Nucleotide cyclases have also been implicated in playing a critical role in biotic stress. An experimentally confirmed putative Arabidopsis AC (At3g21465) has been shown to be transcriptionally up-regulated in response to biotic stress (Gehring, 2010). The AC F-box domains (cyclin like; IPR001810) have a role in protein-protein interactions and are also associated with signal transduction and cell cycle regulation that are linked to both auxin responses and changes in cellular cAMP content (Ehsan et al., 1998; Leyser, 1998; Mohanty et al., 2001). In addition, microarray analysis of the AtWAKL10 (At1g79680) (Zimmermann et al., 2005) which is also a GC (Ludidi & Gehring, 2003), supports a role for GCs and hence cGMP in plant pathogen signaling. In summary, AC and GC are increasingly understood to have a role in deciphering pathogen perception, response signalling and downstream pathogen defense responses in the plant immune signal transduction cascade (Fig. 6), a view that is supported by older studies where the application of fungal extracts was shown to induce elevation in the endogenous levels of cAMP in cultured French bean cells, alfalfa and carrot (Bolwell, 1992; Cooke et al., 1994; Kurosaki et al., 1987).

Finally, it is noteworthy that cNMPs have a role in both abiotic and biotic stress responses and this is perhaps not surprising given that in biotic interactions pathogens can, directly or indirectly, cause homeostatic disturbances that in turn lead to conditions that amount to abiotic stress. Interestingly, some of the host homeostatic modifications are part of the defense response to pathogens (Garavaglia et al., 2010; Gottig et al., 2008, 2009) where the aim of the host is either to starve the biotroph pathogen by restricting access to water and nutrients or to dehydrate the pathogen by sharply increasing the apoplastic osmotic pressure.

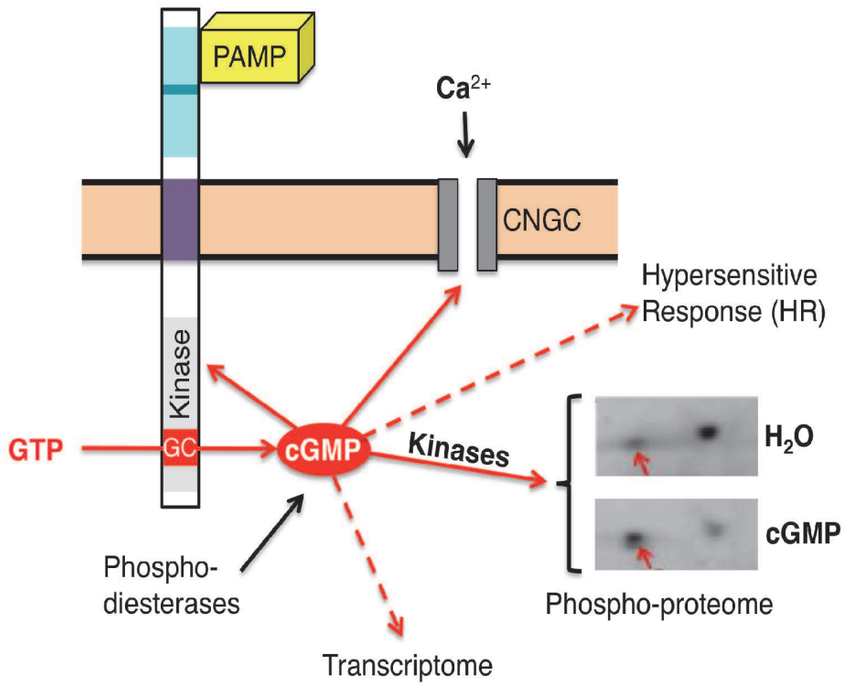


Fig. 6. Guanylyl cyclase mediated signal transduction. A receptor type GC (the domain organisation is annotated in the legend to Fig. 4) is activated by a ligand, here PAMP (Pathogen-associated molecular pattern) and this in turn activates the cytosolic GC domain that generates cGMP that causes Ca^{2+} influx through the regulation of CNGCs (cyclic nucleotide gated channels). Cyclic GMP, possibly together with Ca^{2+} and calmodulin, activates protein kinases which result in a rapid change of the phospho-proteome; cGMP also plays a critical role in the transcriptional regulation of cGMP-dependent gene transcription (Bastian et al., 2010) and the complex processes leading to the "hypersensitive response" (HR). Solid lines represent direct interactions and dashed lines indicate indirect actions that are only partly understood. Additional soluble GCs may also contribute to raises in cGMP levels (not show in this model).

8. Out-look

Given that our understanding of the structural features that enable NCs activity in higher plants is growing, we can expect significant progress in the discovery and experimental confirmation of novel NCs in higher plants in the foreseeable future. This will afford a better understanding of the role of both cAMP and cGMP as second messengers in plant development, responses to environmental stimuli and/or hormones and in particular stress responses. In addition, we will be likely to see cAMP- and cGMP-dependent transcriptomes, (phospho-)proteomes and metabolomes that, together with studies in mutants (e.g. affected in their ability to respond to stress), will afford new and fundamental insights into plant stress signaling. Finally, given the central role of the cNMPs and hence NCs in homeostasis regulation and stress responses, it is conceivable that some of the NCs will be targets for rational bioengineering strategies that will eventually deliver crop plants with improved abiotic stress resistance.

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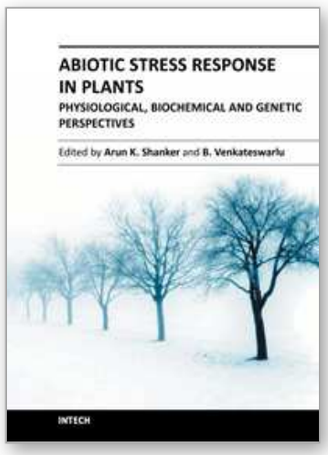
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Abiotic Stress Response in Plants - Physiological, Biochemical and Genetic Perspectives

Edited by Prof. Arun Shanker

ISBN 978-953-307-672-0
Hard cover, 346 pages
Publisher InTech
Published online 29, August, 2011
Published in print edition August, 2011

Plants, unlike animals, are sessile. This demands that adverse changes in their environment are quickly recognized, distinguished and responded to with suitable reactions. Drought, heat, cold and salinity are among the major abiotic stresses that adversely affect plant growth and productivity. In general, abiotic stress often causes a series of morphological, physiological, biochemical and molecular changes that unfavorably affect plant growth, development and productivity. Drought, salinity, extreme temperatures (cold and heat) and oxidative stress are often interrelated; these conditions singularly or in combination induce cellular damage. To cope with abiotic stresses, of paramount significance is to understand plant responses to abiotic stresses that disturb the homeostatic equilibrium at cellular and molecular level in order to identify a common mechanism for multiple stress tolerance. This multi authored edited compilation attempts to put forth an all-inclusive biochemical and molecular picture in a systems approach wherein mechanism and adaptation aspects of abiotic stress are dealt with. The chief objective of the book hence is to deliver state of the art information for comprehending the effects of abiotic stress in plants at the cellular level.

How to reference

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Fouad Lemtiri-Chlieh, Ludivine Thomas, Claudius Marondedze, Helen Irving and Chris Gehring (2011). Cyclic Nucleotides and Nucleotide Cyclases in Plant Stress Responses, Abiotic Stress Response in Plants - Physiological, Biochemical and Genetic Perspectives, Prof. Arun Shanker (Ed.), ISBN: 978-953-307-672-0, InTech, Available from: http://www.intechopen.com/books/abiotic-stress-response-in-plants-physiological-biochemical-and-genetic-perspectives/cyclic-nucleotides-and-nucleotide-cyclases-in-plant-stress-responses

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