

Article Addendum

Plant Cyclic Nucleotide Signalling

Facts and Fiction

Juliana Martinez-Atienza¹

Carl Van Ingelgem²

Luc Roef²

Frans J.M. Maathuis^{1,*}

¹Biology Department; University of York; York, United Kingdom

²Laboratory for Plant Biochemistry and Physiology; University of Antwerp (CGB); Antwerp, Belgium

*Correspondence to: Frans J.M. Maathuis; Biology Department; University of York; York YO10 5DD; UK; Tel.: +44.1904.328652; Fax: +44.1904.328666; Email: fjm3@york.ac.uk

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ABSTRACT

The presence of the cyclic nucleotides 3',5'-cyclic adenylyl monophosphate (cAMP) and 3',5'-cyclic guanylyl monophosphate (cGMP) in plants is now generally accepted. In addition, cAMP and cGMP have been implicated in the regulation of important plant processes such as stomatal functioning, monovalent and divalent cation fluxes, chloroplast development, gibberellic acid signalling, pathogen response and gene transcription. However, very little is known regarding the components of cyclic nucleotide signalling in plants. In this addendum, the evidence for specific mechanisms of plant cyclic nucleotide signalling is evaluated and discussed.

INTRODUCTION

All organisms have to transduce and decode information. Intracellular signalling molecules often function as intermediates in such transductions and these 'second messengers' include Ca^{2+} , lipid based compounds and nucleotides such as 3',5'-cyclic adenylyl monophosphate (cAMP) and 3',5'-cyclic guanylyl monophosphate (cGMP). In animal cells the roles of the cyclic nucleotides cAMP and cGMP are well established and the basic mechanisms of cyclic nucleotide signalling and biochemistry have been well characterized (Fig. 1). In plants, the occurrence and roles of cAMP and cGMP have been more contentious. Nevertheless, sophisticated mass spectrometry-based analytical techniques unequivocally showed the presence of both cAMP and cGMP in several plant species.¹ Several gene products with either adenylylate² or guanylylate³ cyclase activity have been identified as has phosphodiesterase activity but strong functional evidence is still lacking in both cases.

In spite of the seeming lack of clearly identifiable genes and proteins that mediate the basic biochemistry of cyclic nucleotides, many pharmacological and physiological studies implicate cAMP and cGMP in the regulation of important plant processes (Fig. 2). Reports from several laboratories suggest a role for cAMP and/or cGMP in stomatal opening.¹ Studies using plant protoplasts⁴ and intact tissue⁵⁻⁸ have shown direct effects of cyclic nucleotides on cation fluxes sparking interest in these second messengers as potential regulators of the homeostasis of K^+ , Ca^{2+} and Na^+ . In agreement with this notion, recent work showed exposure of intact plants to NaCl generates an increase in cellular cGMP within seconds.⁹ In chloroplast development, cGMP has been demonstrated to be involved in the phytochrome mediated induction of the gene encoding chalcone synthase, a key enzyme in the biosynthesis of anthocyanins.¹⁰ Exposure of barley aleurone layers to gibberellic acid generates a transient increase in cGMP levels and gene transcription, which leads ultimately to α -amylase production for the conversion of starch into sugars.¹¹ Transcription of defence genes such as PAL and PR-1 has been shown in response to nitric oxide (NO) with cGMP as an intermediate.¹² Treatment of plants with membrane permeable cGMP affects the transcription of many genes, particularly of those encoding transporter proteins.⁸

A ROLE FOR CYCLIC NUCLEOTIDE DEPENDENT KINASES?

Clearly, cyclic nucleotide signalling has been shown to occur in plants and to be important for many cellular processes. In some cases, such as the modulation of cation fluxes, the effects can be explained through direct interaction between cyclic nucleotides and membrane transporters but in others, such as the modulation of gene transcription,^{8,11,12} the action of intermediate signalling components is almost inevitable. This raises the

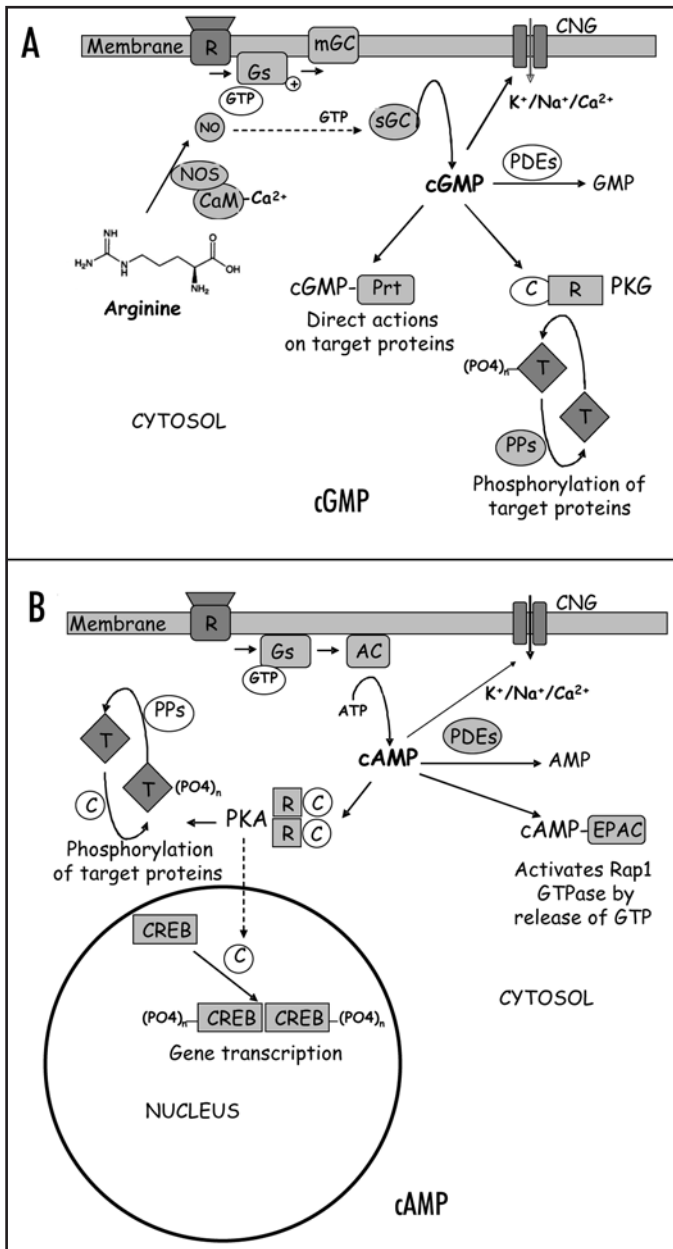


Figure 1. Generic cyclic nucleotide transduction pathways in mammalian cells. (A) Many stimuli activate membrane bound (mGC) or soluble (sGC) guanylate cyclases, often in a nitric oxide (NO) and G-protein dependent manner, which leads to increased levels of cellular cGMP. Cyclic GMP is either broken down by specific phosphodiesterases (PDE) or can directly activates proteins such as cyclic nucleotide gated (CNG) channels. Alternatively, cGMP can activate cGMP-dependent kinase (PKG) which can phosphorylate a large number of target proteins (T). (B) Receptors (R) and G-proteins (Gs) are also involved in stimulation of adenylate cyclases (AC) generating an increase in cellular cAMP. Like cGMP, cAMP can directly modify the activity of proteins such as CNG channels, EPACs which activate Rap-GTPases and the cAMP dependent kinase PKA. Gene transcription is modulated via cAMP responsive element binding proteins (CREBs), transcription factors that dimerise upon phosphorylation by PKA. 'NOS,' NO synthase; 'CaM,' calmodulin; 'PPs,' phosphatases.

question what the components are in these cAMP and cGMP dependent signal transductions. In mammalian cells the activity of some proteins is directly modulated by cyclic nucleotides, (notably that of cyclic nucleotide gated channels or CNGCs), but the majority

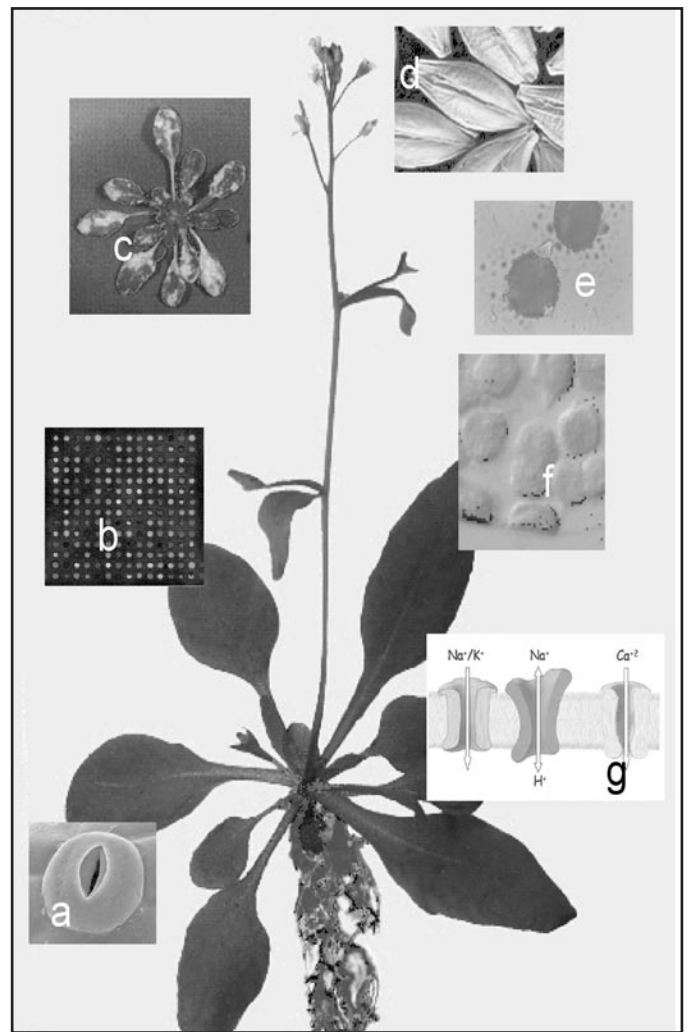


Figure 2. Cyclic nucleotides have been implicated in many important plant processes such as stomatal functioning (a), gene transcription (b), pathogen attack (c), seed germination (d), pollen tube growth (e), chloroplast development (f) and cation fluxes (g).

of cyclic nucleotide signal relays involves phosphorylation as an early down stream step (Fig. 1). The latter occurs through the action of cAMP (PKA) and cGMP (PKG) dependent kinases. Consequently, cyclic nucleotide dependent transcriptional modulation in animals is mediated by phosphorylation of cGMP and cAMP responsive transcription factors such as CREBs which affect transcription of hundreds of genes.¹³ PKA isoforms are also found in many fungal species.

In plants, the broad mechanisms of cyclic nucleotide signalling are still a mystery. A prime concern is the presence (or absence) of kinases that respond to changes in cellular cyclic nucleotide level. Conflicting reports are present whether cyclic nucleotide dependent kinase activity occurs in plants with some studies showing moderate rates of stimulation¹⁴ whereas others could not find any evidence.¹⁵ Bioinformatics analyses show the presence of unique genes in several plant genomes which contain both a cyclic nucleotide binding domain (CNBD) and a kinase domain¹⁶ (Fig. 3). In Arabidopsis, this putative cyclic nucleotide dependent kinase, At2g20040, shows a high degree of homology (E: 2.5×10^{-53}) to mammalian type II PKG with around 48% similarity. While CNBD B of the supposed

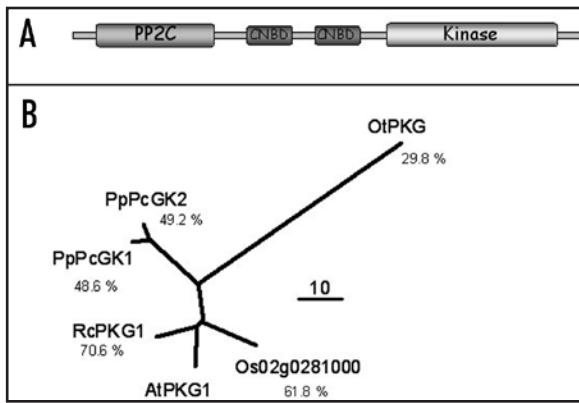


Figure 3. (A) Conserved domain structure of a putative cyclic nucleotide dependent plant kinase. So far, it has been impossible to assign genuine kinase activity to this protein. Interestingly, a PP2C phosphatase moiety is also present in the same open reading frame but its functionality and significance are yet unknown. (B) A single copy gene is present in *Arabidopsis thaliana*, whereas homologous genes are found in the dicot *Ricinus comunis*, in the monocot *Oryza sativa*, in the moss *Physcomitrella patens*, and in the green alga *Ostreococcus tauri*. 'PP2C': phosphatase 2C; 'CNBD': cyclic nucleotide binding domain.

regulatory subunit appears rather degenerate, CNBD A is extremely well conserved, with only one invariant amino acid lacking. Problems are posed however by the absence of the canonical glycine rich loop (G₅₀XG₅₂XXG₅₅) in domain I of the kinase moiety which is considered essential for nucleotide binding and of the catalytic aspartate in the YRD₁₆₄₋₁₆₆ motif of domain VI. Such kinases are often considered 'catalytically dead' and in these cases the kinase domain is often suspected to deliver substrate/interactor binding specificity for other catalytic functions on the protein. Interestingly, the upstream part of what is the same open reading frame also contains a seemingly functional phosphatase domain, an arrangement which is unprecedented so far. The protein has several matching ESTs and promoter-GUS studies indicate it is expressed in vascular tissue and in developing shoot and flower tissues with an expression pattern closely corresponding to the zone of mitotic activity. Transcripts from an ortholog in *Nicotiana tabacum* BY-2 cells accumulate during the cell cycle in a pattern mirroring the fluctuations in cAMP content as described previously (ref. 17) (Roef L, personal communication). Preliminary data also suggest an antagonistic effect between ABA (inducing) and JA (inhibiting) on promoter activity of this gene (Van Ingelgem, Van Onckelen & Roef, personal communication). Unfortunately, expression of several transcript variants in *E. coli* either yields insoluble or inactive proteins. Whether this gene encodes a genuine cyclic nucleotide dependent kinase and/or phosphatase therefore asks for further scrutiny. No obvious phenotypes were observed in mutants with a disrupted kinase moiety in *Arabidopsis* (data not shown) but preliminary results from null mutations in the PP2C and CNBD domains hint at a physiological role during seed germination.

ALTERNATIVE MECHANISMS FOR PLANT CYCLIC NUCLEOTIDE SIGNALLING

The evidence is so far stacked against the presence of either PKA or PKG in plants. However, such enzymes may have very low homology to their fungal and animal counterparts and therefore fail to be identified in bioinformatics analyses. Furthermore, their

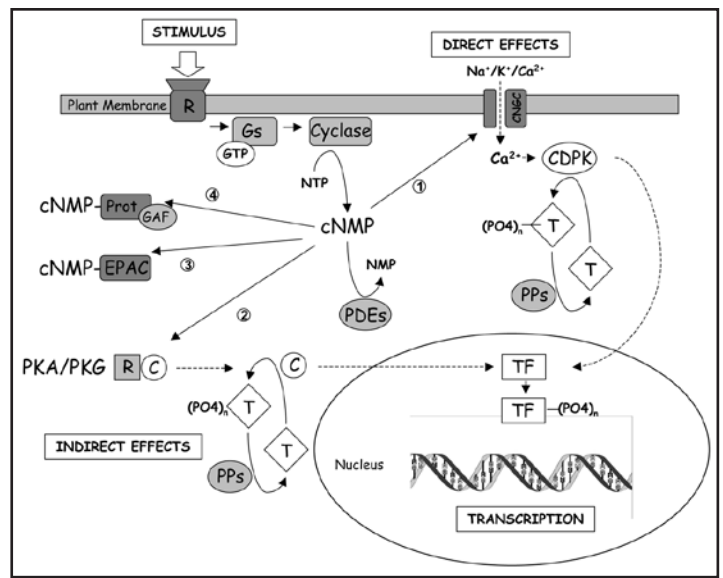


Figure 4. Possible cyclic nucleotide transduction pathways in plants. (1) Cyclic nucleotide gated channels have been characterised in plants and are known to play important roles in pathogen response and cation transport. In addition, they can form a node in the conversion of cyclic nucleotide signals into Ca²⁺ based signalling which can be processed through Ca²⁺ sensors such as Ca²⁺ dependent kinases(CDPKs) and the Ca²⁺ signaling components CIPK-CBL. (2) Although most evidence points to its absence, it cannot be ruled out that, in analogy to mammalian cells, plants contain specific kinases that are activated by cyclic nucleotides. (3) EPACs can activate Rap-GTPases but none has been identified in plants so far. (4) Many plant proteins contain GAF domains but whether these bind and are modulated by cyclic nucleotides remains to be determined.

substrate specificity may also be divergent from that of kinases from other kingdoms, hampering the interpretation of biochemical assays which are typically carried out using 'mammalian' substrates such as glastide and kemptide.¹⁵ Nevertheless, if no such kinases exist in plants, then how do cAMP and cGMP modulate their downstream targets? In plants, the largest group of proteins that contain CNBDs are the CNGCs. Although functional characterisation of plant CNGCs has been fraught with difficulties, there is now good evidence that (a) they are activated by binding cAMP and/or cGMP (b) they have well defined roles in pathogenesis and cation fluxes and (c) they can conduct Ca²⁺. The latter suggests that CNGCs may therefore provide a mechanism to convert cyclic nucleotide signals into Ca²⁺ signals, thus bypassing the need for cyclic nucleotide dependent kinases. The mechanisms of Ca²⁺ signalling in plants are fairly well established and include calcium-dependent protein kinases (CDPKs) and CIPKs (CBL-interacting protein kinases) which form a predominant group of Ca²⁺ sensors and effectors.¹⁸ CDPKs and CIPKs have been identified throughout the plant kingdom but do not occur in animals. Interestingly, CIPK structure shows strong resemblance to that of mammalian PKAs. A scenario where an initial cyclic nucleotide signal is converted into a Ca²⁺ signal could explain the well documented role of plant CNGCs in pathogen responses, the observation that Ca²⁺ fluxes occur during the early phase of plant defence responses, and the fact that both cAMP and cGMP have been implicated in plant defence-related signaling.^{12,19}

In animals, EPACs (Exchange Protein directly Activated by cAMP) carry out diverse functions.^{20,21} EPACs contain GEFs (guanine nucleotide-exchange factors) that activate Rap GTPases

upon binding to cAMP. However, EPAC orthologs have as yet not been identified in any plant genome.

Another family of candidate cyclic nucleotide targets consists of proteins carrying an amino terminal CNBD and a carboxyterminal acyl-CoA thioesterase domain. Several of these can be identified in plant genomes and such proteins would provide a means for cyclic nucleotides to impinge on fatty acid metabolism and possibly protein acylation processes. However, conservation of CNBD signatures is rather low in these proteins and whereas their thioesterase activity has been documented, neither cyclic nucleotide binding nor regulation by cyclic nucleotides has been demonstrated.²²

In bacteria, cAMP is a cofactor of the cAMP-receptor protein (CRP) rather than an activator of protein kinases.²³ CRP or Catabolite gene Activator Protein (CAP) directly binds to DNA upstream of promoters and can either repress or activate gene transcription. CRP/CAP like proteins show some similarity to plant shaker type channels but are otherwise not apparent in plant genomes. The GAF domain (found in cGMP-specific phosphodiesterases, Adenylyl cyclases and Formate hydrogen lyases) is another domain that binds cyclic nucleotides but probably evolved independently of the CNBD.¹⁵ GAFs are particularly prevalent in bacteria²³ where they are believed to allosterically regulate catalytic activities of for example adenylyl cyclases, after binding cGMP. GAF domains are also prevalent in green plants, and are found especially in phytochromes and (putative) ethylene receptors. Such direct modulation of phytochromes via their GAF domain would provide a neat explanation of the role of cGMP in chloroplast development.

CONCLUSIONS AND OUTLOOK

Evidence for the presence and physiological relevance of cAMP and cGMP based signalling in plants is now overwhelming. Yet, some of the key components of these transductions need urgent clarification. Evidence for bona fide PKA and PKG type kinases is scarce but cannot be completely dismissed. Suggestions that the phosphatase function is the key activity of several candidate cyclic nucleotide dependent phosphatase/kinases, may open new perspectives on cyclic nucleotide dependent phosphorylation events in plants. Alternative mechanisms could rely on the conversion of cyclic nucleotide signals into Ca²⁺ signals via CNGCs or on more specific and direct modulation of protein activity via GAF domains on phytochromes. The development of non invasive fluorescent reporters²⁴ for the real time recording of cyclic nucleotides in plant cells (2) would be a great help to further delineate the specific roles of cAMP and cGMP but the main requirement is an unequivocal identification and functional characterisation of (signalling) proteins that bind cAMP and cGMP.

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