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Review

Emerging roles in plant biotechnology for the second messenger cGMP - guanosine 3', 5'-cyclic monophosphate

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Second messengers are small transient molecules that transmit and/or modulate environmental or hormonal signals linking them to complex and often systemic physiological responses. Recent reports have renewed interest in the second messenger guanosine 3', 5'-cyclic monophosphate (cGMP) since it has been shown to directly mediate an ever increasing number of plant responses ranging from the modification of cyclic nucleotide gated ion channels to the regulation of transcription of many cGMP responsive genes. Here some of these cGMP dependent responses will be reviewed emphasising their role in plant stress responses. Finally, potential applications of cGMP in plant biotechnology will be discussed.

Key words: Second messenger, 3', 5'-cyclic guanosine monophosphate (cGMP), cyclic nucleotide gated ion channels, stress responses, *Arabidopsis thaliana.*

INTRODUCTION

It is critical for growth and differentiation that plants can rapidly and systemically respond to environmental changes such as light, temperature, gas, water and nutrient availability as well as biotic and abiotic stresses. Many of the responses are, at least in part mediated by either the classical amino acid derived hormones (Kende and Zeevaart, 1997), peptidic hormones (Lindsey et al., 2002), plant steroid hormones (Schaller, 2003) or nitric oxide (NO) (Neill et al., 2003; Shapiro, 2005). The link between environment and hormones and the physiological response are second messengers that form a network of molecular transducers. Second messengers relay information and directly or indirectly affect biological processes such as gating of ion channels (Hoshi, 1995) or the regulation of transcription (Maathuis, 2006). To do this effectively their cellular activities must be transient and specific (Hetherington and Brownlee, 2004; Plieth, 2005).

The most extensively studied second messenger is Ca^{2+}. Rapid cytosolic Ca^{2+} changes are observed in response to e.g. nodulation factors (Hetherington and Brownlee, 2004) and NaCl and drought stress responses (Donaldson et al., 2004). However, over the last decade an increasing number of additional second messengers and signaling mechanisms have been discovered which either act on their own or in conjunction with Ca^{2+}. These include protons in the form of cytosolic pH changes which have been shown to be important in the response to plant hormones including auxin, ABA and cytokinin (Gehring et al., 1990; Irving et al., 1992) and cyclic ADP ribose (Galione et al., 1998; Grill and Ziegler, 1998; McAinsh et al., 2000). Many phospholipids such as phosphatidic acid have also emerged as second messengers (Munnik, 2001) as well as cyclic nucleotides, notably adenosine 3',5'-cyclic monophosphate (cAMP) and guanosine 3',5'-cyclic monophosphate (cGMP) (Newton and Smith, 2004).

Here, we shall be concerned mainly with cGMP (Figure 1), its biological roles as well as guanylyl cyclases, the

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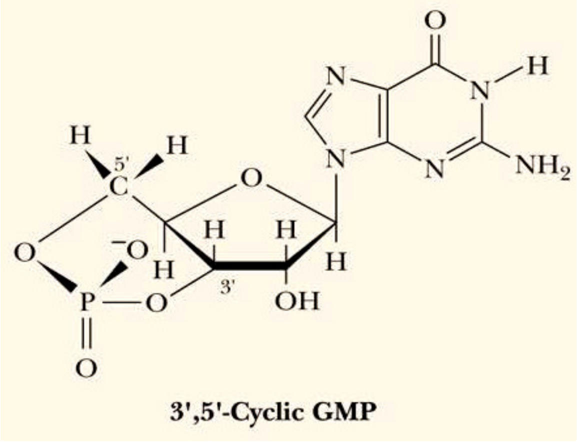

Figure 1. Representation of the chemical structure of the second messenger 3', 5' cyclic guanosine monophosphate – cGMP.

enzymes that catalyse the synthesis of cGMP from guanosine 5'-triphosphate (GTP) and finally with cGMP applications in the area of plant biotechnology.

cGMP AS UNIVERSAL MESSENGER

There are two major classes of guanylyl cyclases that catalyse the synthesis of cGMP, the membrane-bound homodimers, also referred to as particulate guanylyl cyclases (pGCs), and soluble cytosolic guanylyl cyclases (sGCs) which are heterodimers (Pyriochou and Papapetropoulos, 2005). The membrane bound pGCs are stimulated by specific peptide ligands including the natriuretic peptides (NPs) (Kuhn, 2004) while many sGCs can be activated by the gaseous signalling molecule nitric oxide (NO) (Bicker, 2005). The downstream cellular targets or effectors of cGMP include cyclic nucleotide gated ion channels (GNGs), cGMP dependent protein kinases (PKGs) and cGMP regulated phosphodiesterases (G-PDE) which metabolise cGMP to the inactive monophosphate GMP (Potter et al., 2006).

Over the past few decades, a vast body of literature has implicated cGMP as a universal messenger involved in the regulation of an ever increasing number of diverse cellular and physiological processes in vertebrate and invertebrate eukaryotes. In vertebrates, cGMP has been shown to be an important signalling molecule in regulating electrolyte and fluid homeostasis (Kuhn, 2004) as well as a number of other functions including differentiation, growth and apoptosis (Steinbrecher et al., 2002).

In invertebrates, cGMP has also been implicated in diverse roles including controlling chemotaxis of aggregating cells in *Dictyostelium discoideum* (Bosgraaf et al., 2002) and in the modulation of expression of anti-microbial peptides in *Drosophila melanogaster* (Davies, 2006).

Here we are particularly concerned with the role of cGMP in ion homeostasis and the possible lessons for plant biotechnology to be learnt from analogies to animal responses. In vertebrates, cGMP has been documented to be an essential signaling molecule in regulating electrolyte and fluid homeostasis. In both the mammalian intestine and the kidney cGMP mediates the effects of the potent natriuretic peptides guanylin, uroguanylin as well as other natriuretic peptides. These peptides are all synthesized in response to high dietary salt intake or elevated sodium plasma levels and activate membrane bound guanylyl cyclases located in the intestine and renal tubules resulting in elevated intracellular cGMP levels and increased sodium excretion by reducing sodium absorption into the blood. By doing so, they co-regulate blood volume and thus blood pressure. In the intestine guanylin induced elevations of intracellular levels of cGMP inhibit epithelial apical sodium absorption mediated by Na^+/H^+ exchange and additionally activate PKG II and protein kinase A resulting in increased luminal secretion of Cl^-, HCO_3^- and water via activation of the cystic fibrosis transmembrane regulator (CFTR) (Sindic et al., 2005).

In the kidney, natriuretic peptide induced rises in cGMP inhibit sodium and water re-absorption throughout the nephron. In the proximal tubules, cGMP inhibits angiotensin II stimulated sodium and water transport (Harris et al., 1987), apical Na^+/H^+ exchange and K^+ channels which depolarise the proximal tubule cells and lower the electrical driving force for electrogenic Na^+ transport(Sindic et al., 2005).. In the inner medullary collecting duct, cGMP has been shown to reduce apical epithelial sodium re-absorption by inhibiting an amiloride sensitive cation channel. This was shown to occur via a dual mechanism - a direct phosphorylation independent mechanism and by activating cGMP dependent protein kinases (Light et al., 1990). The literature also supports a role for cGMP regulating the fluid balance in invertebrates since NO and cGMP were shown to increase fluid transport and calcium efflux in Type I (principal) cells in Drosophila tubules(Davies, 2006).

DIVERSE ROLES OF cGMP IN PLANT RESPONSES

For decades the presence and more so the possible biological functions of cyclic nucleotides in plants were subject of fierce debate (Newton et al., 1999). In the absence of identified nucleotide cyclases, indirect evidence for cGMP dependent processes was based on either the inhibition or suppression of biological processes with known inhibitors of nucleotide cyclases or stimulation of processes with cell permeant cyclic nucleo-

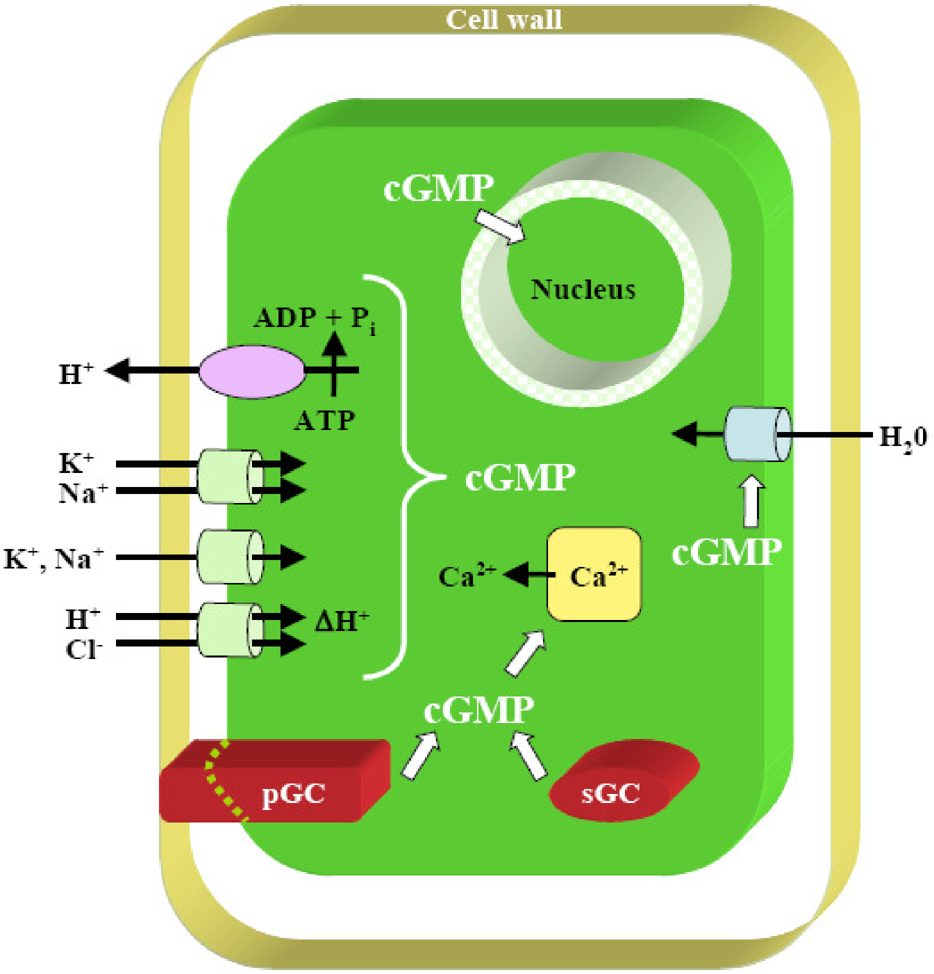

Figure 2. Model of cGMP generation and cGMP dependent cellular effects. Cyclic GMP is generated by particulate guanylyl cyclases (pGCs) that have not yet been characterised *in planta* and soluble guanylyl cyclases (sGCs). The second messenger can than directly or indirectly affect different ion channels and pumps, cause cytosolic Ca^{2+} transients by stimulating release from intracellular stores and affect the transcriptome. It is also conceivable that H_2O uptake via aquorins may also be affected by cGMP.

tides such as 8-Br-cGMP.

In higher plants cGMP-mediated processes have been shown to affect the transcriptome in general (Maathuis, 2006) (Figure 2) and in particular control phytochrome dependent gene expression required for chloroplast development and anthocyanin biosynthesis (Bowler et al., 1994; Chamovitz and Deng, 1996) and gibberellic acid (GA) dependent α-amylase synthesis and secretion (Penson et al., 1996).

In plants as well as in animals, NO is operating as a redox-active signaling molecule (Shapiro, 2005) and NO donors have been shown to induce expression of some defense related genes and tobacco mosaic virus dependent increases of NO synthase activity occurs in resistant plants only (Durner et al., 1998). NO does elevate cGMP levels in plants and NO induced expression of some defense related genes was found to be mediated by the second messengers cGMP and cyclic ADP-ribose both of which also operate in animal responses to NO (Durner et al., 1998). NO dependent and cGMP mediated processes are also involved in *Lilium longiflorum* pollen growth and orientation (Prado et al., 2004) as well as gravitropic responses in soy bean roots (Hu et al., 2005). In addition, specific NO induced Ca^{2+} release from intracellular stores in *Vicia faba* guard cells that can be blocked by antagonists of guanylyl cyclases (Garcia-Mata et al., 2003) - the enzyme that catalyses the formation of cGMP from guanosine 5'-triphosphate (GTP).

Significant and transient increases in intracellular cGMP levels have been reported in response to the plant hormones GA (Penson et al., 1996), cytokinins (Pharmawati et al., 2001), vertebrate atrial natriuretic peptides and plant natriuretic peptides (Pharmawati et al., 1999; Pharmawati et al., 2001; Ludidi et al., 2002) thus linking hormone responses to physiological and transcriptional changes.

cGMP AS KEY MODULATOR IN SALINITY AND DROUGHT RESPONSES

Since the abiotic stresses caused by high salinity and drought are a major reason for crop loss and hence a serious target for biotechnological stress tolerance improvement strategies (Denby and Gehring, 2005) there is also increasing interest in elucidating the role of second messengers and in particular cGMP in the regulation of ion homeostasis. The first indication that cGMP could play a direct role in this process came from electrophysiological experiments (Hoshi, 1995) when it was shown that ion transport is in part dependent on cyclic nucleotides (Hoshi, 1995; Pharmawati et al., 1999). Such regulation can occur in plant voltage gated K^+ channels where binding of cGMP modulates the voltage/current relationship (Hoshi, 1995). Plants also contain cyclic-nucleotide gated low affinity cation channels where binding of cAMP and cGMP to the intracellular portion leads to direct gating (Leng et al., 1999). Recently, voltage independent channels (VICs) without selectivity for particular monovalent cations have been characterised in Arabidopsis. VICs showed no selectivity among monovalent cations and their gating was found to be voltage independent while µM concentrations of cAMP or cGMP at the cytoplasmic side of the plasma membrane caused rapid decreases in channel open probability (Maathuis and Sanders, 2001). It was shown that short-term unidirectional Na^+ influx is reduced in the presence of cyclic nucleotides and importantly that membrane permeable cyclic nucleotide can improve salinity tolerance presumably by reducing net Na^+ uptake.

Further evidence for a role of cGMP in abiotic stress responses came from the demonstration that cGMP levels in Arabidopsis seedlings increase rapidly (< 5 s) and to different degrees after salt and osmotic stress, and that the increases are prevented by treatment with LY, an inhibitor of soluble guanylyl cyclases (Donaldson et al., 2004). In addition, evidence was presented to suggest that salt stress can activate two cGMP signalling pathways – an osmotic, calcium independent pathway and an ionic, calcium dependent pathway (Donaldson et al., 2004).

Cell permeable cGMP and cAMP analogues can also cause swelling due to net water uptake in plant protoplasts (Volotovski et al., 1998) while opening of the stomatal pore, resulting from swelling of two neighbouring guard cells has been observed in response to cell permeable cGMP analogues (Pharmawati et al., 1998; Cousson, 2003). Taken together it appears that cGMP is critically involved in maintaining cell and whole plant water and salt balance through the regulation of ion channels, net water uptake as well as the regulation of stomatal aperture.

DISCOVERING GUANYLYL CYCLASES IN HIGER PLANTS

Considering the functional evidence for the presence of cGMP in higher plants together with the fact that the unicellular green alga *Chlamydomonas reinhardtii* contains well over fifty annotated (based on sequence similarity rather than experimentally proven function) nucleotide cyclases (Schaap, 2005), it is somewhat surprising that until recently there was no report of any guanylyl cyclase in higher plants. Since the complete genome of *Arabidopsis thaliana* could be queried in 2000, it became obvious that guanylyl cyclases in higher plants were either very different or possibly evolutionarily unrelated to known guanylyl cyclases or that the similarities were confined to a small catalytic centre which was below the detection limit of conventional homology search engines like BLAST. To test this hypothesis a search motif based on functionally assigned amino acids in the catalytic centre of annotated guanylyl cyclases (Hurley, 1998; McCue et al., 2000) was designed, tested and used to query the Arabidopsis genome (Ludidi and Gehring, 2003). The motif occurred in seven candidate proteins, one of which was tested experimentally and proven to have guanylyl cyclase activity *in vitro* (Ludidi and Gehring, 2003). This molecule termed AtGC1 (At5G05930) belongs to a new class of guanylyl cyclases with a novel domain organisation, an N-terminal guanylyl cyclase domain and a C-terminal protease domain (Ginalski and Zemojtel, 2004). While all annotated guanylyl cyclases to date are multi-domain molecules - a classical example being the atrial natriuretic peptide (ANP) receptors A and B, containing an extracellular ligand binding domain, a transmembrane domain, a kinase domain and a guanylyl cyclase domain (Garbers and Lowe, 1994) - the combination of guanylyl cyclase and protease is unique. The biological role of AtGC1 is not yet fully elucidated; however microarray expression data (www.genevestigator.ethz.ch) would indicate that transcription is up-regulated in response to *Pseudomonas syringae* infection and interestingly much more so when infected with an avirulent than a virulent strain. This may suggest a very specific role in a biotic stress response while a high level of transcript present in the seeds and early stages of imbibing is indicative for a role in rapid homeostatic adjustments.

Of the other candidate genes two encode kinases, one of which is a wall associated kinase-like (WAKL) protein, two encode F-box proteins and the remaining two encode a PPR-repeat protein and a transferase.

The identification of an increasing number of guanylyl cyclases in higher plants is consistent with the many and complex roles of cGMP in plant responses, the number of annotated guanylyl cyclases in both prokaryotes and eukaryotes (Schaap, 2005) and the green alga Chlamydomonas in particular as well as recent reports of an increasing number of cGMP regulated genes. The number of rapidly up- or down-regulated cGMP responsive Arabidopsis genes invites the use of computational tools to try to identify cGMP co-regulated genes and gene expression networks based on common cis elements (and transcription factor binding sites) in the promoters of these genes.

BIOTECHNOLOGICAL PERSPECTIVES: AN OUTLOOK

It is generally accepted that drought and salinity stress are possibly the most significant contributing factor to crop loss especially in marginal semi-arid agricultural areas (Denby and Gehring, 2005) and it is becoming increasingly clear that to counteract this growing problem, biotechnological solutions might be desirable. This has lead to a significant body of published data showing that the overexpression of some genes can confer increased tolerance to these abiotic stresses, however, the down side is that such an approach may cause a metabolic burden that is unacceptable and unprofitable and this in turn may well be the main reason why no transgenics with increased salinity or drought tolerance are in the field to-date. To overcome the problems of metabolic imbalance a more systemic approach will be required and bioengineering may have to concentrate on regulatory genes and/or genes encoding molecules that activate signalling cascades in a stimulus specific way(Denby and Gehring, 2005).

We argue that guanylyl cyclases may be a promising target group of genes that hold potential for such approaches for the following three reasons. Firstly, it has been shown that cGMP is a specific second messenger in both salinity and drought responses(Donaldson et al., 2004). Secondly, it has been demonstrated that increasing cytosolic cGMP levels caused by external addition of cell permeant cGMP analogues can increase salinity tolerance in *A. thaliana* by directly affecting sodium up-take in roots (Maathuis and Sanders, 2001). Thirdly, cGMP induced transcripts (Maathuis, 2006) include genes encoding proteins that are directly involved in the maintenance of ion and water homeostasis such as monovalent cation transporters including nonselective ion channels and cation: proton antiporters. Furthermore, other expressed cGMP dependent genes encode proteins that regulate homeostasis such as H^+-ATPases or proteins that have a role in cellular protection such as the DNA replication - related element (DRE)-binding protein (S.K. Meier, University of the Western Cape, unpublished data).

However, continuous spatially and developmentally undifferentiated overexpression of guanylyl cyclases is almost certainly not the answer to sustainable increased stress tolerance in transgenics. A more differential approach would be to put guanylyl cyclases or modified functional guanylyl cyclase domains encoding transgenes under the control of tissue specific promoters (e.g. root epidermis) that are rapidly responsive to adverse changes in environmental conditions (Kasuga et al., 1999). Most valuable resources for biotechnological research that have become available recently are the complete plant genomes from Arabidopsis and rice both in the public domain. This enables promoter analyses of stress induced genes and can contribute to building regulatory networks that in turn are a key to understanding and ultimately modulating systemic responses (Mohanty et al., 2005). A further resource of great value is the amount of Arabidopsis gene expression data, and in particular microarray data, available in the public domain. This rapidly growing repository holds significant information on response patterns in both wild-type as well as mutants that can inform research in salt and drought tolerance. In addition, user-friendly tools allow non-specialists in the field of computational biology to extract and analyse data sets and make use of genome wide expression profiling data and this will aid target gene discovery, selection and evaluation via profiling of transgenic plants.

A future system-wide approach to understanding the role of guanylyl cyclases and cGMP in plant function and stress responses in particular will integrate expression data with ecophysiological, physiological, biochemical and metabolic information from both wild-type, mutant and knock-out lines and will significantly contribute to rational bioengineering strategies that will eventually lead to safe and sustainable crops with enhanced stress resistance traits.

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