Cyclic Nucleotide Monophosphates in Plants and Plant Signaling

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Abstract

Cyclic nucleotide monophosphates (cNMPs) and the enzymes that can generate them are of increasing interest in the plant sciences. Arguably, the major recent advance came with the release of the complete *Arabidopsis thaliana* genome that has enabled the systematic search for adenylate (ACs) or guanylate cyclases (GCs) and did eventually lead to the discovery of a number of GCs in higher

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plants. Many of these proteins have complex domain architectures with AC or GC centers moonlighting within cytosolic kinase domains. Recent reports indicated the presence of not just the canonical cNMPs (i.e., cAMP and cGMP), but also the noncanonical cCMP, cUMP, cIMP, and cdTMP in plant tissues, and this raises several questions. Firstly, what are the functions of these cNMPs, and, secondly, which enzymes can convert the substrate triphosphates into the respective noncanonical cNMPs? The first question is addressed here by comparing the reactive oxygen species (ROS) response of cAMP and cGMP to that elicited by the noncanonical cCMP or cIMP. The results show that particularly cIMP can induce significant ROS production. To answer, at least in part, the second question, we have evaluated homology models of experimentally confirmed plant GCs probing the substrate specificity by molecular docking simulations to determine if they can conceivably catalytically convert substrates other than ATP or GTP. In summary, molecular modeling and substrate docking simulations can contribute to the evaluation of cyclases for noncanonical cyclic mononucleotides and thereby further our understanding of the molecular mechanism that underlie cNMP-dependent signaling in planta.

Keywords
Adenylate cyclase · *Arabidopsis thaliana* · cAMP · cGMP · Cyclic nucleotide monophosphates · Guanylate cyclase · Plant · Second messenger

1 A Brief History of Cyclic Mononucleotide Signaling in Plants

While there is a large body of literature on cNMP-dependent signaling in lower and higher eukaryotes, both addressing the question of biological functions and mechanisms of action, the acceptance that cNMPs play a role in plant signaling was delayed and not without controversy (Gehring 2010; Irving and Gehring 2013; Lemtiri-Chlieh et al. 2011; Newton et al. 1999). One reason for this is that the levels of cAMP and cGMP reported in plants appear to be generally lower than in animals or lower eukaryotes (for review, see Bolwell 1995; Gehring 2010; Newton and Smith 2004; Schaap 2005). 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 nucleotidyl cyclases (NCs) in higher plants. Additionally, homology searches with annotated adenylate cyclases (ACs) or guanylate cyclases (GCs) from lower or higher eukaryotes do not identify candidate cyclases in higher plants (Ludidi and Gehring 2003; Roelofs et al. 2001). The breakthrough came when a motif search of the *A. thaliana* genome based on conserved and functionally assigned amino acids in the catalytic center of annotated GCs (Liu et al. 1997; McCue et al. 2000; Zhang et al. 1997) from lower and higher eukaryotes returned a candidate that also contains the adjacent glycine-rich domain typical for GCs. In this molecule, termed AtGC1, the catalytic domain

is in the N-terminal part. AtGC1 contains the arginine or lysine that participates in hydrogen bonding with guanine and the cysteine that confers substrate specificity for GTP (Zhang et al. 1997). Since the discovery of the first GC in higher plants, and based on rational modification of the search motif together with site-directed mutagenesis, many more candidate GCs have been predicted (Wong and Gehring 2013a, b) and indeed tested for enzymatic activity. The recently experimentally confirmed GCs include a wall-associated kinase-like GC (AtWAKL10) (Meier et al. 2010), the brassinosteroid receptor (AtBRI1) (Kwezi et al. 2007), the Pep R1 receptor (AtPepR1) (Qi et al. 2010), and the phytosulfokine receptor (AtPSKR1) (Kwezi et al. 2011), which to date is the best studied. Phytosulfokines (PSKs) are sulfated pentapeptides that modulate plant growth and differentiation, as well as responses to biotic stresses (Igarashi et al. 2012; Mosher et al. 2013; Shen and Diener 2013). AtPSKR1 is a leucine-rich repeat receptor kinase with a functional GC catalytic center embedded within its cytoplasmic kinase domain (Irving et al. 2012; Kwezi et al. 2011). Overexpression of AtPSKR1 in Arabidopsis protoplasts causes an over 20-fold increase in endogenous cGMP levels, and this, together with ligand-specific cGMP transients (Kwezi et al. 2011), indicates that the receptor has GC activity in vivo and implies that cGMP has a role in PSK-dependent responses.

The first *bona fide* (Moutinho et al. 2001) AC was reported in the African blue lily (*Agapanthus umbellatus*) and shown to act on pollen tube growth and reorientation that in turn is a prerequisite for fertilization and subsequent seed formation (Moutinho et al. 2001). The authors also showed that growing tubes display a uniform distribution of cAMP with a resting concentration of approximately 100–150 nM and that forskolin and dideoxyadenosine, both modulators of ACs (Cochaux et al. 1982; Florio and Ross 1983), can alter cAMP resting levels. Since this initial report, the search for ACs has continued, and given that several candidate ACs have been predicted (Gehring 2010), we can expect to see in vitro and in planta test results from some of the candidates in the near future.

Interestingly, in the single-cellular green alga *Chlamydomonas reinhardtii*, there are more than 90 annotated ACs and GCs, and they come in over 20 different domain combinations where NCs combine with, for example, leucine-rich receptor kinases or proteases (Meier et al. 2007). Although these NCs have been annotated based only on sequence homology with NCs in animals or lower eukaryotes and not experimentally tested, it is likely that many of them function as cyclases.

There is increasing evidence that cNMPs have complex physiological roles in plants, both as modulators of single reactions and at the systems level. Given that in plants GCs are best established, we present here an overview of cGMP-dependent processes (Fig. 1) and foresee that noncanonical cNMPs share some of the targets or can substitute cGMP. GCs can be activated by ligands such as PSK (Kwezi et al. 2011) or nitric oxide (NO) (Domingos et al. 2015; Mulaudzi et al. 2011) and cause cGMP transients that in turn directly or indirectly affect downstream processes in different cellular compartments such as the cytosol, the chloroplast, the mitochondria, and the nucleus. In the cytosol, cGMP can modify plasma membrane H^+-ATPases (Suwastika and Gehring 1999) and net cation transport (Pharmawati

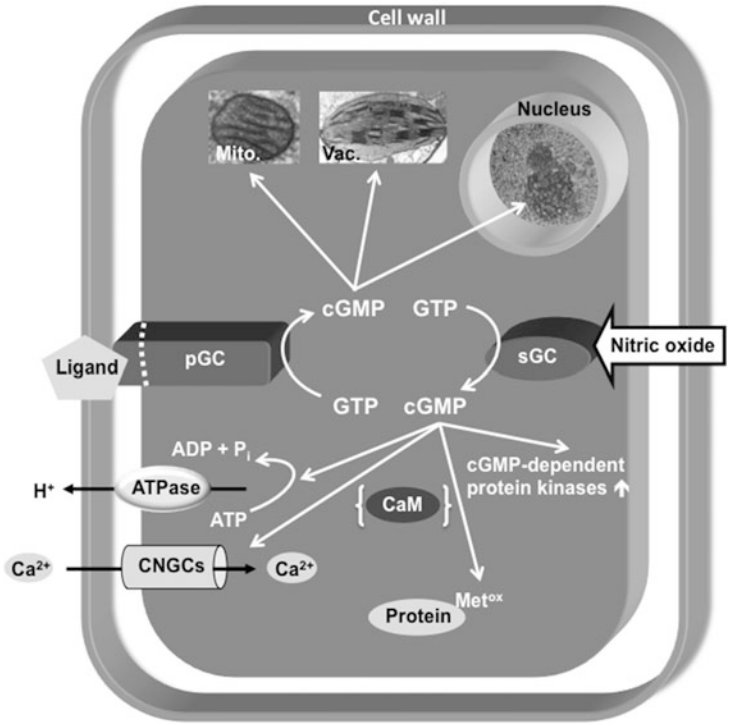

Fig. 1 Overview of cGMP-dependent processes in plant cells. Particulate (pGCs) or soluble GCs (sGCs) catalyze the reaction from GTP to cGMP that in turn modulates processes in the cell membrane, the cytosol, the mitochondria (Mito.), the chloroplast (Chlo.), or the nucleus. Proton transport is modulated by ATPases, while cation transport, including Ca^{2+} uptake, is enabled by cyclic nucleotide-gated channels (CNGCs) and calmodulin (CaM)

et al. 1999) including Ca^{2+} uptake (Ordoñez et al. 2014) conceivably through cyclic nucleotide-gated channels (CNGCs) (Talke et al. 2003; Zelman et al. 2012). Consistent with these responses is a cGMP-dependent accumulation of transcripts encoding monovalent cation transporters such as nonselective ion channels and cation/proton antiporters (Maathuis 2006). It has also been reported that cGMP is necessary and sufficient to transcriptionally induce a set of hormone (Bastian et al. 2010; Penson et al. 1996)-, ozone-, and nitric oxide-dependent (Pasqualini et al. 2009) genes. Furthermore, cGMP also causes distinct changes in the proteome signature (Ordoñez et al. 2014), in particular affecting proteins with a role in ion transport and stress responses and also resulting in specific posttranslational modifications such as phosphorylation (Facette et al. 2013; Yoshida et al. 2015) and methionine oxidation (Marondedze et al. 2013).

2 Evidence for Noncanonical Cyclic Nucleotide Monophosphates in Plants

The first systematic attempt to quantify the complement of cNMPs in plants was undertaken in the late 1980s (Newton et al. 1989). The authors report extraction of endogenous (3′,5′-) cAMP, cGMP, cCMP, cUMP, cIMP, and cyclic deoxyTMP (cdTMP) from meristematic and non-meristematic root tissue of *Pisum sativum* (Newton et al. 1989). Plant extracts were sequentially purified including an adsorption chromatography step on alumina, ion-exchange chromatography, and preparative electrophoresis. The purified samples were then compared to cyclic mononucleotide standards by HPLC and UV absorbance spectrophotometry, and further analysis was carried out by fast atom bombardment mass spectrometry and mass-analyzed ion kinetic energy spectrometry. The results indicated the presence of cAMP, cGMP, cCMP, and cUMP in meristematic tissue and cAMP, cGMP, cCMP, cIMP, and cdTMP in the non-meristematic tissues. The authors also report that there was a significantly higher concentration of cCMP in meristematic tissue as compared to non-meristematic tissues (Newton et al. 1989). The authors speculated that the elevated cCMP levels in the meristem might reflect a role in the rapidly dividing cells, analogous to that proposed for cCMP in mammalian cells, while the higher levels of cUMP in non-meristematic cells might be a reflection of the different rates of cell proliferation in the two types of tissue. However, the authors did not report the concentration of the respective nucleotides within the tissues, and the comparatively low sensitivity of the methods used at the time does not completely eliminate the possibility of artifacts.

In a recent survey of cAMP, cGMP, cCMP, and cUMP concentrations, it was found that *Arabidopsis thaliana* green leaf tissue does contain about 15 pmol mg^{-1} protein of cAMP and about 10 x less cGMP, while no cCMP was detected (Hartwig et al. 2014). Perhaps surprisingly, Arabidopsis leaves do contain 5.21 ± 2.11 pmol mg^{-1} protein of cUMP, which in effect makes the cUMP levels higher than those of cGMP. It has also been speculated that ancestral soluble ACs may be able to catalytically convert CTP and UTP to cCMP and cUMP, respectively (Hartwig et al. 2014; Seifert 2015).

3 Cyclic Mononucleotides and Their Role in Plant Reactive Oxygen Species (ROS) Signaling

In tobacco, defense gene induction has been shown to be critically dependent on ROS, notably nitric oxide (NO), as well as the messengers cGMP and cyclic ADP-ribose that function downstream of NO (Durner et al. 1998; Klessig et al. 2000). There is increasing evidence that cyclic nucleotides also have a role in the responses to ROS in Arabidopsis (Ordoñez et al. 2014). Biologically active ROS include superoxide and hydrogen peroxide (H_2O_2), and the superoxide and hydroxyl radicals are capable of inducing cellular oxidative damage. In plants, these compounds are produced in excess as a result of environmental stresses such

as drought, salinity, and low temperatures as well as biotic stress such as pathogens (Apel and Hirt 2004; Sharma et al. 2012). Production of ROS is confined to cellular compartments that have strong electron flow such as the mitochondria, chloroplasts, and peroxisomes (Choudhury et al. 2013). ROS-mediated oxidative stress has been shown as the main symptom of toxicity; however, plants have a coordinated antioxidant defense or ROS scavenging mechanisms to counteract both enzymatic and nonenzymatic effects of ROS in order to achieve redox homeostasis (Munne-Bosch et al. 2013). In plants, a hypersensitive response leads to apoptosis or programmed cell death where ROS can be involved. It has also been shown that in systemic acquired resistance (a type of plant immune response), ROS interacts with salicylic acid (SA) in SA signaling (O'Brien et al. 2012). Besides oxidative toxicity, ROS can impact positively at low cellular concentrations by acting as a second messenger (Mittler et al. 2004).

In order to assess whether noncanonical cNMPs could play a role in ROS-mediated signaling and oxidation-reduction-related processes, an *Arabidopsis thaliana* cell suspension culture was treated with 100 μM of either cAMP, 8-bromo-cAMP, cCMP, or cIMP in addition to a ROS inducer (pyocyanin), a ROS inhibitor (*N*-acetyl-L-cysteine), or H_2O_2 as controls. The results showed that ROS production was significantly induced within 30 minutes in cell suspension cultures treated with cIMP. This was comparable to the treatment with pyocyanin and consistently observed in four biological replicates of two independent experiments. In the first experiment, ROS production was measured by the total ROS detection kit (Enzo Life Sciences, Lausen, Switzerland), which directly monitors real-time reactive oxygen and/or nitrogen species production in living cells and detects superoxide (Fig. 2a). In the second experiment, an OxiSelect intracellular ROS assay was used to measure H_2O_2 production enabling the monitoring of hydroxyl and other ROS activities within cells (Fig. 2b). Neither cAMP nor the cell-permeant cAMP analog, 8-bromo-cAMP, significantly induced ROS production. This observation has some similarity to mammalian cells, where, for instance, cAMP stimulated a low ROS response in granulocytes from normal patients compared to samples from type 1 and type 2 diabetic patients (Isoni et al. 2009; Nogueira-Machado et al. 2006). We further examined whether the ROS production observed from the live cells follows the same trend when cells are lysed and observed a different trend in the cytosolic fraction. After cell lysis, cAMP induces rapid and significant ROS production in response to treatment. In addition, cIMP, cCMP, and 8-bromo-cAMP as well as cAMP induced ROS production within 30 minutes. The induction of ROS can be interpreted as a type of cellular signaling mechanism that is both cNMP dependent and specific. Similar to cGMP, cIMP is not an oxidizing agent, but it can directly or indirectly induce ROS production. Cyclic GMP has been shown to cause protein oxidation (Marondedze et al. 2013), a role cIMP could conceivably share. We therefore speculate that cNMPs, and in particular cGMP and the noncanonical cIMP, also play key signaling roles in plant cell oxidation-reduction processes. The roles of noncanonical cyclic nucleotides as second messengers in animal systems and their pharmacological implications have recently been reviewed (Beste and Seifert 2013; Seifert et al. 2015), and it appears that the roles and

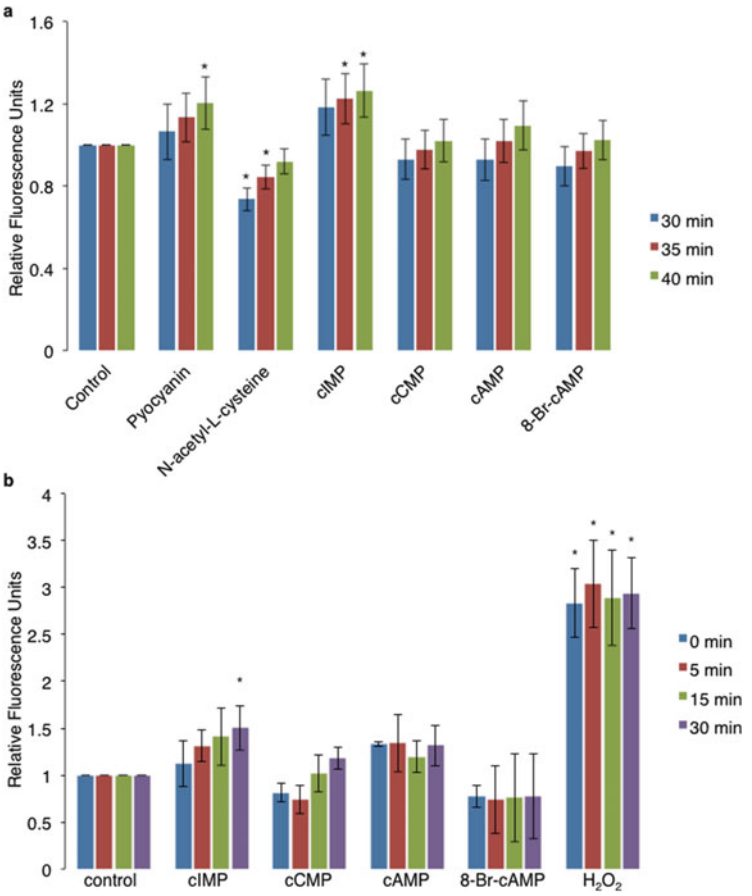

Fig. 2 *Arabidopsis thaliana* cell suspension culture oxidation assay. (**a**) Enzo ROS detection kit (Enzo Life Sciences, Farmingdale, NY) was used to assess in vivo levels of intracellular ROS generation following the assay protocol provided by the manufacturer. Cultured *Arabidopsis thaliana* (Col-0) cells were placed in a black bottom 96-well cell culture plate and a baseline reading was taken. Cells were loaded simultaneously with the treatment solution and the ROS detection solution. The treatment solution contains either positive control (ROS inducer (pyocyanin)), negative control (ROS inhibitor (*N*-acetyl-L-cysteine)), media solution (control untreated samples), or experimental samples containing a 10 μM of cIMP, cCMP, cAMP, or 8-Br-cAMP. Cells were stained for 30 min at 37°C in the dark, and fluorescence in the cells was measured at 30, 35, and 40 min posttreatment at 480/530 nm using a PHERAstar *FS* microplate reader (BMG Labtech GmbH, Germany) and the values plotted. *Each bar* represents data from five biological replicates ($n = 5$), and the *lines* are the standard errors. Treatment with cIMP at the final concentration of 10 μM induces statistically significant differences of the means at $p = 0.05$ using a two-sample *t*-test. (**b**) OxiSelect™ intracellular ROS assay kit (Cell Biolabs, Inc., San Diego, CA) was used in the in vivo oxidation experiments according to the assay protocol provided by the manufacturer. Cultured *Arabidopsis thaliana* (Col-0) cells were placed in a black bottom 96-well cell culture plate for 2 h in a shaking incubator. The 2′,7′-dichlorofluorescein diacetate/media solution was added to the cells prior to incubation for 1 h at 37°C. The dye-loaded cells were then treated with 10 μM of cIMP, cCMP, cAMP, 8-Br-cAMP, or H_2O_2. Fluorescence in the cells was measured at 0, 5, 15, and 30 min posttreatment at 480/530 nm using a PHERAstar *FS* microplate reader (BMG Labtech GmbH, Germany) and the values plotted. *Each bar* represents data from five

mechanisms of action of cIMP remain a source of debate (Seifert 2014). Recently and based on indirect evidence, it was proposed cIMP can be synthesized by soluble GCs and that this reaction mediates hypoxic contraction of coronary arteries (Chen et al. 2014).

4 Can Plant Mononucleotide Cyclases Conceivably Catalyze Noncanonical Nucleotide Triphosphates and Generate Their Corresponding Cyclic Nucleotide Monophosphates?

Given the reports of noncanonical cyclic mononucleotides in plants, we reviewed the catalytic center motif search and modeling approaches that have successfully identified functional GCs and predicted ACs in plants (Ludidi and Gehring 2003; Wong and Gehring 2013a, b) and discuss if the currently identified plant GCs can plausibly accommodate other nucleotide triphosphates (NTPs) and generate their corresponding cNMPs.

We have previously established that many plant GC centers share residence with larger primary domains at sites termed moonlighting centers (Freihat et al. 2014; Irving et al. 2012; Wong et al. 2015) and that these sites have important modulatory and/or signaling roles. They differ from, for example, the animal soluble and particulate GCs in that they are more likely to operate in localized spatial and temporal signaling events by acting as branch points (Freihat et al. 2014; Igarashi et al. 2012; Mosher et al. 2013; Zhou et al. 2013) that divert one pathway to another. In the phytosulfokine receptor (PSKR), the molecular switch is Ca^{2+} that can selectively shift the activation from the primary kinase to its embedded moonlighting GC (Muleya et al. 2014) that then generates cGMP to further inhibit the kinase (Kwezi et al. 2011). This intramolecular micro-regulation would therefore require transient levels of much lower amounts of cytosolic cGMP as compared to animal systems, and this is consistent with activities observed in plant GCs (Freihat et al. 2014). Several groups have detected cyclic mononucleotides in plant tissues (e.g., Donaldson et al. 2004; Hartwig et al. 2014; Isner et al. 2012; Maathuis 2006; Newton et al. 1999) and from in vitro enzymatic reaction of recombinant purified plant proteins (Kwezi et al. 2007, 2011; Ludidi and Gehring 2003; Meier et al. 2010; Mulaudzi et al. 2011; Qi et al. 2010), however at levels lower than those typically measured in animal GCs. Others failed to detect low levels of cyclic mononucleotides (Bojar et al. 2014), and this may be attributed to the lack of sensitive high-performance methods such as the liquid chromatography-mass spectrometry tandem (Beste et al. 2012; Hartwig et al. 2014). Detection by UV absorption of HPLC-separated fractions (Bojar et al. 2014) is not sensitive enough to

Fig. 2 (continued) biological replicates ($n = 5$), and the *lines* are the standard errors. Treatment with cIMP at the final concentration of 10 μM induces statistically significant differences of the means at $p = 0.05$ using a two-sample *t*-test

contrast cGMP from the background signal contributed by salts and cofactors that are present in the reaction mixture. Since a number of plant GC centers require specific conditions such as cofactors and dimerization for optimum catalysis, absence of these requirements, especially in in vitro assays, is one likely reason for low or lack of detection (Berkowitz et al. 2011; Freihat et al. 2014; Wheeler et al. 2013; Wong and Gehring 2013a). The PSKR1, when bound to its natural ligand alpha-phytosulfokine in mesophyll protoplast cells, has elevated GC activity (Kwezi et al. 2011), thus showing further evidence for an intricate transduction role that links ligand perception to the downstream cGMP-mediated cellular responses. Interestingly, the human interleukin 1 receptor-associated kinase 3 (IRAK3), uncovered using sequence homology-guided bioinformatic data-mining tools, was recently shown to generate cGMP at levels typical of plant GCs when expressed as a recombinant protein in both *E. coli* and human embryonic kidney (HEK)-293T cells (Freihat et al. 2014), thus implying that similar modulatory modes also exist in the animal system. This may now help to expand our current understanding of the signaling cascade downstream of the different GCs including the NO-dependent GCs (Domingos et al. 2015; Mulaudzi et al. 2011).

For the assessment of currently known plant GC centers, we used the well-characterized AtPSKR1 as a representative model since accurate homology models can be made based on the availability of highly similar template structures and docking simulations can be performed with confidence. The AtPSKR1 kinase homology model (Fig. 3a) accommodates a GC center that forms a distinct cavity at a separate region that does not overlap with the ATP binding site of the kinase domain. This GC center has an alpha helix fold that is followed immediately by a loop and accommodates the functional residues implicated in catalysis (Wong et al. 2015). Docking simulations implicate that GTP docked with the following binding pose: the hydrophobic nucleobase guanosine sits deep at the catalytic center and at distance close enough for establishing interactions important for catalysis with the experimentally determined functional residues at positions 1 and 3 (serine and glycine) at the GC center, while the negatively charged hydrophilic triphosphates point outward toward the solvent-exposed amino acid residue at position 14 (arginine or lysine) that has a positive net charge. In addition, this orientation also places the triphosphate end in the direction of the cofactor (Mg^{2+} or Mn^{2+}) that interacts with the amino acid (aspartic acid or glutamic acid) located two residues downstream of the GC center (Wong and Gehring 2013a; Wong et al. 2015). We define this substrate orientation as "suitable for catalysis." Further probing of the interacting residues at the catalytic center by site-directed mutagenesis and computational methods has been described previously (Wong and Gehring 2013a) and shown that the PSKR1 GC center, in the presence of docked GTP, displays structural and biochemical properties that are also present in other plant GCs identified to date.

Since NTPs all share very similar structural properties, it is conceivable that plant GCs can recognize them as substrates and are able to generate their corresponding cyclic mononucleotides. This proposition is supported by the fact that GCs and ACs in general poorly discriminate the different NTPs (Beste

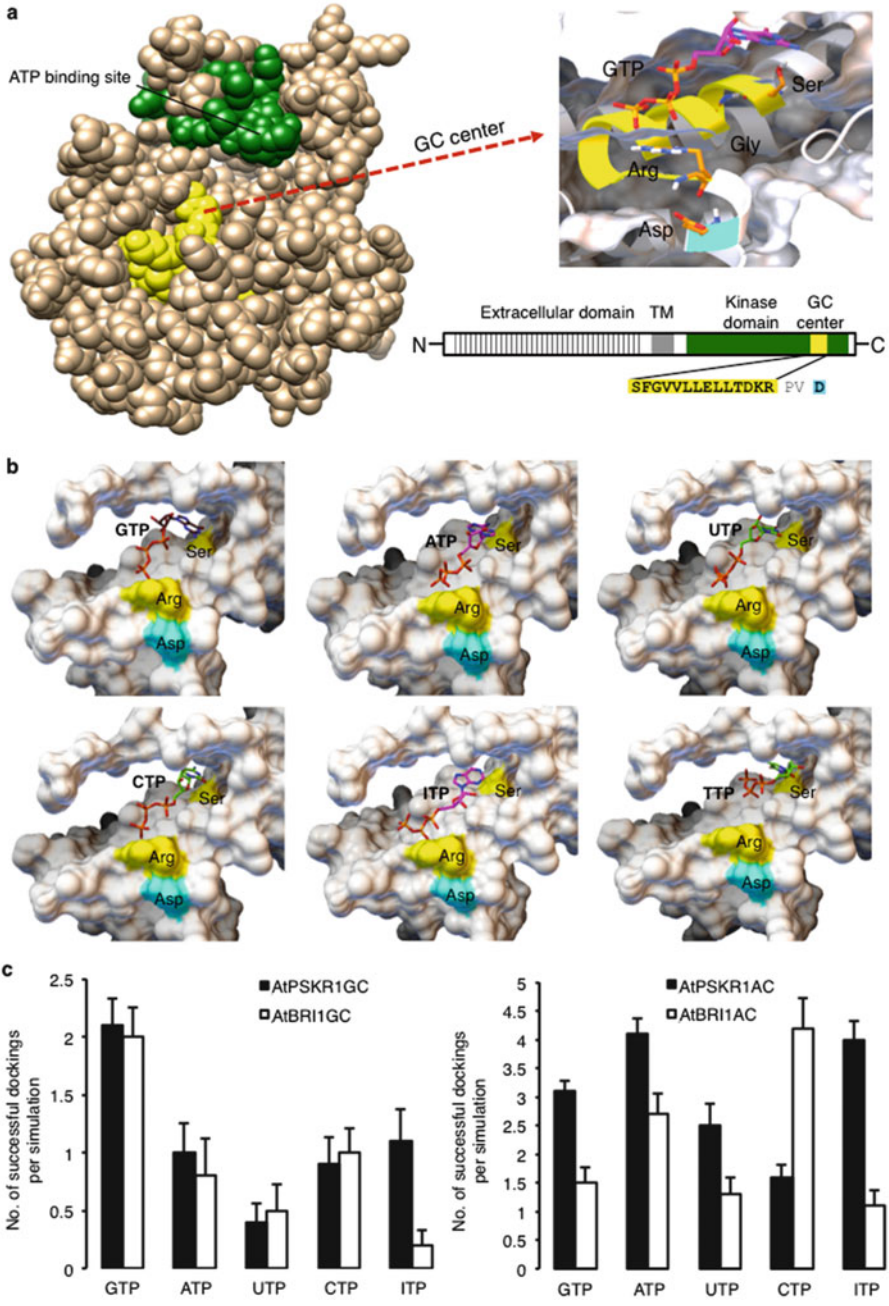

Fig. 3 Homology models of AtPSKR1GC and molecular docking of NTPs. (**a**) The homology model of AtPSKR1 kinase (Phe^{734}–Val^{1008}) and the domain organization of AtPSKR1 (Accession number: NP_178330.1) illustrate the ATP binding site (*green*) at the kinase domain and the GC center (*yellow*), and molecular docking of GTP to the GC center (*inset*) reveals the substrate pose and interactions with key residues at the GC center. Ribbons highlighted in *yellow* and *cyan* indicate the GC catalytic center and the metal-binding residue. (**b**) A representative model of

et al. 2012; Ruiz-Stewart et al. 2004; Sunahara et al. 1998; Surmeli et al. 2015). Perhaps remarkably, the retinal guanylyl cyclase (RetGC-1) can be converted into an AC by substitution of just two amino acid residues at the catalytic center (Tucker et al. 1998), while the *Dictyostelium discoideum* homolog of a mammalian soluble AC encodes a GC (Roelofs et al. 2001). Importantly, the recombinant purified rat soluble GC catalyzes GTP, ITP, XTP, and ATP and generates the corresponding cyclic mononucleotides in the presence of Mg^{2+}, but catalyzes only UTP and CTP when Mn^{2+} was provided in place of Mg^{2+} (Beste et al. 2012). Here, we performed in silico docking simulations using the NTPs: CTP, UTP, ITP, and TTP as substrates and the GC catalytic center of the model AtPSKR1 as the receiving molecule. When GTP was used as the ligand, successful docking that has favorable free binding energy and a binding pose deemed "suitable for catalysis" as previously defined occurred on average of over two times per simulation (Fig. 3c). As expected, the frequency of this binding pose was reduced to approximately one when ITP, ATP, CTP, and UTP were tested in place of GTP (Fig. 3c). A similar docking trend was also observed with the GC center of the AtBRI1, which has structure, domain architecture, and biological functions that are similar to that of PSKR1 (Fig. 3c). The binding poses of NTPs, in particular the noncanonical CTP, UTP, and ITP, satisfy the conditions for catalysis (Fig. 3b), although these solutions occurred less frequently compared to GTP (Fig. 3c). We note that binding *per se* is not diagnostic for catalysis; however, these simulations are useful for conducting initial screens to select plausible candidates and uncover structural insights as substrate binding is usually the defining rate-limiting step of enzymatic reactions (Wong and Gehring 2013a; Wong et al. 2015). Notably, only TTP did not yield any binding pose that can be deemed "suitable for catalysis" (Fig. 3b).

We further tested the substrate preference (or lack thereof) of AtPSKR1GC by replacing the residue at positions 1 and 3 of the catalytic center to arginine and glutamic acid, respectively, that is predicted to convert the GC into an AC. These mutations fit the derived AC motif that has been previously curated based on catalytic centers of ACs from animals and lower eukaryotes (Gehring 2010;

Fig. 3 (continued) AtPSKR1GC catalytic center (Asn^{871}–Glu^{980}) docked with the different NTPs. Amino acid residues that have catalytic functions are indicated in *yellow*, and the residue that is involved in metal binding is highlighted in *cyan*. All structures and images were prepared and analyzed with the UCSF Chimera – a visualization system (Pettersen et al. 2004). (**c**) Docking simulations of NTPs on the GC and the putative AC catalytic centers of AtPSKR1 and AtBRI1 (Accession numbers: NP_178330.1 and NP_195650.1). A total of ten docking simulations were performed, each generating nine solutions. The positive binding modes in each run were determined by analysis using PyMOL (ver 1.7.4) (The PyMOL Molecular Graphics System, Schrödinger, LLC), and the number of successful dockings per simulation were averaged. Homology models of AtPSKR1 (Phe^{734}–Val^{1008}), AtPSKR1GC (Asn^{871}–Glu^{980}), and AtBRI1GC (Leu^{1021}–Arg^{1134}) kinase were based on the AvrPtoB-BAK1 complex (PDB entry: 3TL8) which has a sequence similarity of 43% covering 99% of the queried amino acid sequence, and models were built and assessed using Modeller (ver. 9.10) (Sali and Blundell 1993). NTP docking simulations were performed using AutoDock Vina (ver. 1.1.2) (Trott and Olson 2010)

Wong et al. 2015) and did not cause structural changes at the catalytic center although the surface charge and the hydrophobic environment of the cavity were affected (Wong et al. 2015). Docking simulations were repeated using the putative AtPSKR1AC as the receiving molecule. Interestingly, docking of ATP with binding pose deemed "suitable for catalysis" occurred on average of over four times per simulation, while GTP was only three times (Fig. 3c). Both UTP and CTP have about 2.5 and 1.5 successful dockings (Fig. 3c), while ITP averaged four times. TTP did not yield binding poses that suggest suitability for catalysis (Fig. 3b). When the putative AtBRI1AC was used as the receiving molecule, GTP, UTP, and ITP have significantly lower docking frequency than that of ATP with the exception that CTP averaged a surprisingly high frequency of docking events (Fig. 3c). These mutations have resulted in a clear preference for ATP for the GC-derived AtPSKR1AC and AtBRI1AC over GTP (Fig. 3c), thus demonstrating that plant GCs like their animal counterparts have poor substrate discrimination (Beste et al. 2012).

The fact that in silico docking simulations afforded UTP, CTP, and ITP binding poses that are suitable for catalysis much like those generated from GTP and ATP dockings suggests that the identified plant GCs can rationally use these noncanonical NTPs as substrates and convert them into their respective cyclic forms. Therefore, highly sensitive experimental approaches should be used to investigate both in vitro and in vivo the capability of these known plant GCs to generate noncanonical cMNPs. If proven so, the molecular mechanism that assigns substrate specificity and affords substrate discrimination to these plant GC centers should then be elucidated. Some parallels can be however drawn from the animal systems, for example, the mammalian soluble GC has both allosteric and pseudosymmetric sites that accommodate other NTPs such as ATP, the binding of which modulates the GC activity (Surmeli et al. 2015). Alternatively, ions such as Mg^{2+} or Mn^{2+} can also dictate substrate specificities and affinities as evident in the catalytic activity of the rat soluble GC (Beste et al. 2012). It is also plausible that the sheer amounts of one NTP in relation to another can transiently, spatially, and/or temporally govern catalysis. Such regulations are vital when plant NCs, such as the modeled putative AC forms of AtPSKR1 and AtBRI1 that favors ITP and CTP to a degree similar if not greater than ATP (Fig. 3c), cannot discriminate these signaling molecules and are therefore consistent with the earlier described modulatory role of cyclic nucleotides (Freihat et al. 2014).

The detection of noncanonical cMNPs, in particular cUMP in the leaf of *Arabidopsis thaliana* (Hartwig et al. 2014), implies that cyclases capable of generating these molecules do exist in plants. Although we showed hints of cIMP being involved in ROS signaling in *Arabidopsis thaliana* suspension cultures and ITP can be rationally accommodated as a substrate for plant NCs, however, we note that cIMP has yet to be detected in plant tissues. Perhaps, the more relevant question is whether unique and designated proteins that exclusively generate cNMP molecules are present, and if so, do they exist as catalytic centers that are embedded within allosteric or moonlighting sites of larger multi-domain proteins much like in the currently characterized plant GCs? This will likely be answered as their

physiological significance, cellular signaling roles, and roles in hormone, biotic, and abiotic responses unfold in the near future.

5 Concluding Remarks

With the presence of noncanonical cNMPs established in plants, the quest for both their generation and biological function can be undertaken. A number of specific questions await answers. Firstly, can currently described and experimentally tested plant ACs and GCs catalyze substrates other than cAMP and cGMP, and, secondly, are there dedicated NCs with substrate specificity or preference for, e.g., UTP given that the cUMP levels in plants appear to be higher than those of cGMP. Furthermore, the biological roles of noncanonical cNMPs await elucidation, in particular the following questions: (1) Can noncanonical cNMPs modulate the gating of CNGCs (Zelman et al. 2012), and if so, how does their efficiency compare to cAMP or cGMP? (2) What do noncanonical cNMPs do to cAMP- and cGMP-dependent kinases? (3) Given that cGMP is necessary and sufficient to transcriptionally induce a set of hormone (Bastian et al. 2010; Penson et al. 1996)-, ozone-, and nitric oxide-dependent (Pasqualini et al. 2009) genes, will this also be observed in response to noncanonical cNMPs? (4) Can noncanonical cNMPs induce specific posttranslational modifications such as phosphorylation (Facette et al. 2013; Yoshida et al. 2015) and methionine oxidation (Marondedze et al. 2013)?

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