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The role of cyclic nucleotides in plant signalling

Úloha cyklických nukleotidů v signalizaci rostlin

Bachelor's thesis

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Byl to bolestivej porod, a plod má ještě mnoho chorob

- Prago Union

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Prohlášení

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

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Abstract:

Cyclic nucleotides, specifically cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) are well-established second messengers in most eucaryotic and procaryotic cells. Although they are some of the most ubiquitous second messengers in animal tissues, the significance of cyclic nucleotides in plant signaling has long been underestimated, leading to a period of neglect in plant cyclic nucleotide research. Recently, however, these molecules have been recognized as crucial components of plant signaling pathways, resulting in renewed research interest. Advanced methods tailored to studying cyclic nucleotides are now being employed to identify and characterize novel proteins involved in their signaling and to elucidate the pathways and processes they mediate. Despite the increased interest and rapid advancements in this field, our understanding of cyclic nucleotide signaling in plants remains fragmented. Numerous questions regarding their signaling mechanisms and physiological effects are emerging. This thesis aims to provide a comprehensive review of the current knowledge on cyclic nucleotides in plant signaling, highlighting significant findings and identifying critical research gaps that warrant further investigation.

Keywords: cyclic nucleotides, cAMP, cGMP, adenylate cyclase, guanylate cyclase, phosphodiesterase, cyclic nucleotide-gated channel, phytohormones

Abstrakt:

Cyklické nukleotidy, konkrétně cyklický adenosinmonofosfát (cAMP) a cyklický guanosinmonofosfát (cGMP), patří mezi nejběžnější druhé posly ve většině eukaryotických a prokaryotických buněk. Přestože je signalizace cyklických nukleotidů podrobně popsána v živočišných tkáních, dlouho nebyly považovány za významné pro rostlinou signalizaci a jejich výzkum byl minimální. V posledních letech však začíná vycházet najevo, jak klíčové jsou tyto molekuly jako komponenty signalizačních drah v rostlinách, a v jejich výzkumu nastává období rozkvětu. Pokročilé metody specifické pro studium cyklických nukleotidů umožňují identifikaci a popis nových proteinů zapojených do jejich signalizace a objasnění drah a procesů, se kterými jsou cyklické nukleotidy spojené. Navzdory rychlému pokroku ve výzkumu cykických nukleotidů jsou poznatky o jejich signalizaci v rostlinách roztříštěné. V souvislosti s mechanismy jejich regulace a vlivu na rostlinou fyziologii přibývá mnoho otázek. Tato bakalářská práce si klade za cíl poskytnout celistvou rešerši současného výzkumu cyklických nukleotů v rostlinné signalizaci, a vytyčení výzkumných otázek, které by zasloužily být zodpovězeny.

Klíčová slova: cyclické nukleotidy, cAMP, cGMP, adenylát cykláza, guanylát cykláza, fosfodiesteráza, kationtové kanály řízené cyklickými nukleotidy, rostlinné hormony

Outline

1. Introduction

As complex and dynamic organisms, plants depend on an intricate signaling pathway network to regulate their growth, development, and responses to environmental stimuli. Key players in this signaling network include second messengers, such as calcium ions (Ca^{2+}) and reactive oxygen species (ROS), which amplify and distribute signals within plant cells. Despite their established role as second messengers in other organisms, cyclic nucleotides have been initially considered insignificant in plants. Recent studies indicate that cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) are involved in key signaling pathways in plants, raising new questions about their specific roles and mechanisms of action. (Wheeler *et al.*, 2017; Qi *et al.*, 2022)

To gain more insight into the roles of cyclic nucleotides in plants, it is valuable to understand their functions in other organisms. In bacteria, cyclic nucleotides regulate metabolic pathways, virulence factors, and developmental processes (Gomelsky, 2011; Hall and Lee, 2018). A well-studied example can be found in *E. coli*, where cAMP binds to the catabolite repressor protein (CRP). Depending on its DNA-binding position, the cAMP-CRP complex either activates or represses transcription in operons such as *lac* and *gal*, which control the utilization of alternative carbon sources (Soberón-Chávez *et al.*, 2017).

In animals, cAMP and cGMP regulate neural signaling, muscle contraction, cardiac function, and vision by mediating the downstream effects of first messengers such as hormones, neurotransmitters, and light (Beavo and Brunton, 2002). The cyclic nucleotide-synthesizing proteins - guanylate cyclases (GC) and adenylate cyclases (AC) - in animal cells exist in both membrane-bound and soluble forms, and calcium ions can regulate their activity through calmodulin or protein kinase C (Tian, Yang and Gao, 2020). This regulation illustrates the complex multi-level interactions between calcium and cyclic nucleotides. For instance, calcium ions also regulate phosphodiesterases (PDEs), enzymes that degrade cyclic nucleotides (Yuan *et al.*, 1999). Human PDEs are established drug targets, and many of their inhibitors are clinically used to treat various cardiovascular diseases (Francis, Blount and Corbin, 2011).

Other potential pharmaceutical targets are the downstream effectors of cAMP and cGMP, notably cyclic nucleotide-gated channels (CNGC) and protein kinase A or G (PKA or PKG). (Francis and Corbin, 2000). Further supporting the interplay with calcium, the sensitivity of CNGCs in mammalian photoreceptors and olfactory cells to cyclic nucleotides can be modulated by calmodulin (Molday, 1996). Because PKAs and PKGs can phosphorylate numerous

substrates, various isoforms are localized to specific cellular compartments, like the enzymes metabolizing cyclic nucleotides and the second messengers themselves (Beavo and Brunton, 2002).

Besides their well-established roles in animal and bacterial signaling, cyclic nucleotides are present in many other organisms. Interestingly, cAMP functions as a universal second messenger in all kingdoms of life, while cGMP is notably absent in certain bacteria and fungi. Moreover, other cyclic nucleotides such as cCMP and cUMP have been detected in some mammalian cells, with low levels of cUMP also observed in plant cells, suggesting potential, yet not understood, signaling roles ('cAMP, cGMP, cCMP and cUMP concentrations across the tree of life: High cCMP and cUMP levels in astrocytes', 2014). In bacteria, specific cytidyl and uridyl cyclases have been identified - which leads to the question of whether these cyclases exist in plants as well.

The physiological relevance of cAMP and cGMP in plants has been increasingly evident for some time, with these second messengers mediating responses to phytohormones, light, gravity, and various stresses (Thomas *et al.*, 2013; Shih *et al.*, 2015; Bianchet *et al.*, 2019; Yang *et al.*, 2021). They also significantly contribute to maintaining cellular homeostasis by regulating potassium and calcium ion fluxes(Lu *et al.*, 2016; Al-Younis *et al.*, 2018); and are involved in developmental processes, such as seed germination, stomatal movement, and pollen tube growth (Teng, Xu and Ma, 2010; Dubovskaya *et al.*, 2011; Vaz Dias *et al.*, 2019).

The number of plant ACs, GCs, PDEs, and CNGCs being characterized in plants is quickly growing, and unlike their animal orthologs, they are often integrated into complex multi-domain proteins with different primary functions, which are known as moonlighting proteins (Turek and Irving, 2021). A recent study revealed that the TIR1/AFB auxin receptors, primarily known for their well-established role in regulating transcription, encode a functional adenylate cyclase domain (Qi *et al.*, 2022). This finding represents a breakthrough for both cyclic nucleotide and auxin signaling research and points to a significant role of cAMP in plant hormonal, and other signalling.

Although cyclic nucleotides are now established as crucial components of plant signaling, the precise mechanisms by which cyclic nucleotides operate in plants remain mostly unknown, and further research is needed to integrate them into the broader network of plant signaling.

2. Aims of the thesis

Recent discoveries are shining a new light on cyclic nucleotides in plants, and many questions regarding their signaling and physiological effects are emerging. This thesis aims to provide a comprehensive review of the current knowledge on cyclic nucleotides in plants. The specific objectives include:

- Evaluating the methods used to study cyclic nucleotide signaling in plants, highlighting their strengths and limitations.
- Summarizing the existing research of plant proteins involved in cyclic nucleotide signaling, and exploring how their structural and functional features can be utilized to identify more components of the signaling pathways.
- Assessing the previously proposed functions of cAMP and cGMP in plant physiology, and how they align with recent discoveries, with an emphasis on their roles in hormonal signaling.
- Discussing the significance of understanding cyclic nucleotide signaling in plants and highlighting potential areas for future research.

3. Cyclic nucleotides - second messengers in plants

To comprehend the roles cyclic nucleotides play in plants, it is crucial to understand their structure, metabolism, mechanism of action, and the methods currently used to study them. This chapter serves as an introduction to the study of cyclic nucleotides in plants and reviews the evolving perception of these molecules as second messengers over the past decades.

3.1. Cyclic nucleotides were controversial in the past

Despite the recognition of cyclic nucleotides (further abbreviated as cNs) as ubiquitous second messengers across the Tree of life, their research in plants has faced significant challenges. Initially, the concentrations of cNs in plants appeared low compared to animals. They were often undetectable by the available methods, which raised doubts about their biological relevance (Newton *et al.*, 1999; Gehring, 2010). Thanks to technological advancements, many studies have since demonstrated that cNs are physiologically relevant in plants even at nanomolar concentrations (Alqurashi, Gehring and Marondedze, 2016; Gehring and Turek, 2017), and one study even suggested that cAMP levels detected in plants and animals are similar, contradicting previous assumptions ('cAMP, cGMP, cCMP and cUMP concentrations across the tree of life: High cCMP and cUMP levels in astrocytes', 2014). Due to the limited sensitivity and precision of detection methods used in the 1970s and 1980s (that mainly analyzed whole-tissue extracts), research from these decades is not included in this thesis.

Another challenge to the study of cNs in plants has been the inconsistent functional evidence for components associated with cN signaling, such as nucleotide cyclases or kinases, which have been well-characterized in other organisms (Martinez-Atienza *et al.*, 2007). After the release of the Arabidopsis genome (The Arabidopsis Genome Initiative, 2000), the existence of cN signaling in plants was questioned yet again, because no orthologs of animal, fungal, or bacterial cN-related proteins were identified using homology-based prediction methods. This was possibly due to the complex multi-domain nature of these proteins, and the low sequence conservation of the catalytic domains (Wong *et al.*, 2018). Although several proteins with cN-related functions (mostly cyclases) have been identified in multiple plant species, their roles in plant physiology often remain elusive (Ludidi and Gehring, 2003; Sehlabane *et al.*, 2022). The known plant proteins involved in cN signaling and metabolism, and the methods to study them, are covered in Chapters 5 and 4, respectively.

The concept of cNs as second messengers was associated with controversy even at the very beginning when cAMP was isolated from dog liver by E. W. Sutherland. (Rall and Sutherland, 1958). Sutherland´s hypothesis that hormonal signals are mediated through the formation of cAMP, which then initiates various downstream effects within the cell, initially faced strong criticism. The idea that a single molecule is an intermediary between unrelated hormones and their diverse effects, seemed implausible. It was eventually experimentally confirmed that cAMP is a crucial component of numerous hormonal signaling pathways, and Sutherland received the Nobel prize (Kresge, Simoni and Hill, 2005; Qi and Friml, 2023).

Nonetheless, a question arose: how can a molecule, essentially omnipresent in all subcellular compartments, execute countless specific cellular responses to different inputs? A plausible explanation lies in the concept of compartmentation, which is essentially the creation of subcellular cN pools through protein colocalization. In mammals, for instance, cN-synthetizing cyclases are located in proximity to cN-effector proteins, as well as phosphodiesterases which prevent cN diffusion into the cell (Stangherlin and Zaccolo, 2012; Johnstone *et al.*, 2018; Qi and Friml, 2023). It is presumed that similar compartmentalization occurs in plants, which might partially explain the low observed concentrations of cyclic nucleotides.

3.2. Chemistry of cyclic nucleotides: structure, isoforms and metabolism

Having discussed the initial skepticism surrounding cyclic nucleotides as second messengers in plants, it is now important to review their chemical structure, as well as their synthesis and degradation. Cyclic nucleotide monophosphates (cNMPs) are nucleotides containing a single phosphate group with a cyclic bond linking the sugar and phosphate groups. Based on the different positions of this cyclic bond, there are two naturally occurring isoforms: 3′,5′-cNMP and 2′,3′-cNMP. (Qi and Friml, 2023)

3′,5′-cNMPs are produced by nucleotide cyclases, specifically cAMP-producing adenylate cyclases (AC) and cGMP-producing guanylyl cyclases (Bradham and Cheung, 1982). The reaction involves the removal of two phosphate groups and the formation of a cyclic phosphate bond and it is exergonic. Degradation of cyclic nucleotides is catalyzed by phosphodiesterases (PDEs), which hydrolyze the cyclic phosphate bond, converting 3’,5’-cAMP to 5’-AMP and 3’,5’-cGMP to 5’-GMP. This process is essential for the regulation of intracellular cyclic nucleotide diffusion. 2′,3′-cNMPs are synthesized through DNA/RNA degradation. (Qi and Friml, 2023)

3.3. Methods for studying cyclic nucleotides in plants

As touched upon earlier, the study of cyclic nucleotides in plants has evolved significantly with technological advancements. The rise of high-resolution analytical methods, genomic technologies, and live-cell imaging has enabled scientists to gain deeper insights into the roles of cNs in plant signaling. Measuring the concentrations of cNs offers a means to monitor the effects of upstream modulators on cN levels, their fluctuation during various developmental processes and responses to external factors, to assess the localization of cN signalling in different organs, and to conduct activity assays of nucleotide cyclases. This section reviews the current methodologies used for detecting and quantifying cyclic nucleotides, both *in vitro* and *in vivo*.

Recent studies predominantly used *in vitro* immunoassays and tandem liquid chromatography/mass spectrometry (LC-MS/MS) to quantify cAMP and cGMP levels in plant tissues (Wheeler *et al.*, 2017; Qi *et al.*, 2022), because their high sensitivity allows the detection of cyclic nucleotides at physiological levels.

Among immunoassays, which use antibodies to detect cNs, enzyme immunoassays (EIA), and most notably the enzyme-linked immunosorbent assay (ELISA), are commonly used due to their convenience and accuracy (Qi *et al.*, 2022; Sehlabane *et al.*, 2022; Ruzvidzo and Chatukuta, 2023). Other, previously employed methods include radioimmunoassays (RIA), which utilize ^{125}I-labeled cAMP/cGMP as tracers and are generally more sensitive to lower concentrations, however less convenient with the radioactive tracers being difficult to handle (Sprenger and Nikolaev, 2013; Gehring and Turek, 2017).

To validate the results obtained using immunoassays, high-performance liquid chromatography/mass spectrometry (HPLC-MS/MS) is utilized (Newton, 1996; Qi *et al.*, 2022; Sehlabane *et al.*, 2022), because it offers significantly higher sensitivity and specificity, with the ability to detect as little as 5 fmol/µL of cyclic nucleotide monophosphates (Raji and Gehring, 2017). In addition to these *in vitro* quantification methods, ambient ionization MS is applicable for the analysis of living tissues (Freund *et al.*, 2018).

However, all of these widely used methods only detect total intracellular content of cNs after cell lysis, which prevents monitoring of the rapid dynamics and spatial distribution within the cell.

Therefore, specific genetically encoded biosensors have been developed to detect cN distribution in living cells.

FRET-based (Förster resonance energy transfer) biosensors have been widely used to detect cNs in living animal cells. This method employs biosensors containing a cN-binding domain (BD), positioned between donor and acceptor fluorophores. FRET between the two fluorescent molecules is modulated by cN binding and subsequent change of conformation, which allows for dynamic intensiometric measurement of cN levels in real-time, without the need for tissue homogenization (Calamera *et al.*, 2019). Although FRET can be recorded with standard optical microscopy, the signal can be affected by various conditions, such as pH changes, which may require advanced imaging techniques to ensure accuracy, making it difficult to use.

An alternative non-invasive method that provides high-resolution imaging using standard microscopy employs δ-FlincG, a fluorescent biosensor for endogenous cGMP. This sensor is versatile, applicable for numerous plant species and tissues, and sensitive, detecting cNs in the range of around 20–2000 nM (Isner and Maathuis, 2011). A more recently developed sensor for endogenous cAMP named R-FlincA (Red Fluorescent indicator for cAMP) utilizes the cAMP-binding motif of the PKA regulatory subunit as a sensing domain and can detect fluctuations in cAMP levels at sub-μM concentrations (Ohta *et al.*, 2018).

Other genetically encoded biosensors employed in various organisms include single fluorescent proteins (like GFP) utilizing cN-sensing domains (like Epac in mammals), and BRET (bioluminescent resonance energy transfer). The bioluminescence during BRET occurs between the donor luciferase and an acceptor fluorescent protein. These and other sensors for cAMP and cGMP are more thoroughly reviewed here: (Kim, Shin and Bae, 2021; Ovechkina *et al.*, 2021)

While *in vitro* methods offer high sensitivity and accuracy for quantifying total intracellular cN content, *in vivo* biosensors enable real-time visualization of cN distribution and fluctuations within living cells. Using both approaches is important for a comprehensive understanding of cN signaling. However, there is still room for the development of new cN-specific sensors and other advanced detection technologies.

4. What we know about the effects of cyclic nucleotides in plant physiology

The earliest evidence of cNs in higher plants was obtained from tobacco pith cells, where radioimmunoassay techniques detected both cAMP and cGMP and revealed a positive correlation between cAMP levels and auxin-dependent cell enlargement (Lundeen, Wood and Braun, 1973). More than 20 years later, the first specific response to cAMP was described in *Vicia faba* mesophyll cells: an electrophysiological study (patch-clamp recordings) showed that application of intracellular cAMP increases K^+ efflux, suggesting that this is achieved either directly by a gating mechanism or via a cAMP-regulated protein kinase (Li *et al.*, 1994). A later study established that cGMP also modulates ion homeostasis: according to electrophysiological recordings and amino acid sequence analysis, the voltage-dependent K^+ channel KAT1 is gated by cGMP (Hoshi, 1995).

Later on, thanks to the advancements in quantitative methods establishing the presence of cNs in plants, and the first cN-generating cyclases being identified (Moutinho *et al.*, 2001; Ludidi and Gehring, 2003), the number of studies exploring the roles of cNs in different physiological processes in higher plants started growing. Apart from quantitative analysis and electrophysiological recordings of ion currents, most studies at the time utilized a pharmacological approach commonly used in mammalian research, such as various AC, GC, PDE, PKA, and PKG inhibitors or activators (Durner, Wendehenne and Klessig, 1998; Volotovski *et al.*, 1998; Dubovskaya *et al.*, 2011; Nan *et al.*, 2014). Furthermore, the application of membrane-permeable 8-br-cGMP, dibutyryl-cAMP and other analogs was, and still is employed (Hua *et al.*, 2003; Cousson, 2010; Yang *et al.*, 2021).

A large number of studies proposing the roles of cNs in many physiological processes has been published, but in most cases, they only provide a basis for further research that would clarify the molecular details of how cNs modulate the implied processes. It has been suggested that cNs are involved in an array of developmental processes, among which are seed germination (Uematsu *et al.*, 2007; Uematsu and Fukui, 2008; Teng, Xu and Ma, 2010), pollen tube growth (Moutinho *et al.*, 2001; Prado, Porterfield and Feijó, 2004), root gravitropism (Hu *et al.*, 2005) primary root growth, lateral root formation and adventitious root development (Nan *et al.*, 2014), symbiotic root nodule formation (TERAKADO *et al.*, 1997), photoperiodic flower induction (Szmidt-Jaworska *et al.*, 2004), stomatal movement (Dubovskaya *et al.*, 2011), chloroplast development, cell cycle progression(Ehsan *et al.*, 1999), and phytochrome-mediated

anthocyanin synthesis (Bowler *et al.*, 1994). Furthermore, other preliminary studies suggested that cNs mediate both abiotic and biotic stress responses, likely through the regulation of ion channels (Durner, Wendehenne and Klessig, 1998; Donaldson *et al.*, 2004). They also significantly contribute to maintaining cellular homeostasis by regulating potassium and calcium ion fluxes (Hoshi, 1995; Lemtiri-Chlieh and Berkowitz, 2004).

Some of the aforementioned roles of cNs physiological processes are supported by more recent molecular evidence. For instance, a guanylyl cyclase domain has been discovered in the DGK4 diacylglycerol kinase, which is an important signaling component in pollen tube growth. This supports the previously implied role of cGMP as a second messenger in gametophyte development. (Vaz Dias *et al.*, 2019). Furthermore, the CNGC18 ion channel, an established key component of polarized tip growth of pollen, is positively regulated by cGMP. (Frietsch *et al.*, 2007; Gao *et al.*, 2016)

Another example of a more defined pathway regulated by cNs was demonstrated in a recent study. According to this study, melatonin positively regulates the expression of a novel GC, BcGC1, and causes elevation of cGMP in pak choi. It was proposed that cGMP mediates delayed leaf senescence downstream of melatonin (Liu *et al.*, 2024).

4.1. Cyclic nucleotides and hormonal signaling

Furthermore, cyclic nucleotides have been linked to multiple plant hormones, notably auxin (Nan *et al.*, 2014), abscisic acid (ABA) (Dubovskaya *et al.*, 2011), gibberellic acid (GA) (Uematsu and Fukui, 2008; Bastian *et al.*, 2010), jasmonic acid (JA) (Isner, Nühse and Maathuis, 2012), salicylic acid (SA) (Hao *et al.*, 2010), and brassinosteroids (BL) (Kwezi *et al.*, 2007).

The interaction of cNs and the phytohormone ABA is supported by the discovery of a novel AC in maize, ZmRPP13-like protein 3, which mediates the response to heat stress. Based on assays of the AC and cAMP concentrations in ABA-deficient mutants under heat stress, it was proposed that cAMP at least partially mediates ABA-regulated heat resistance, although the activation mechanism remains unknown (Yang *et al.*, 2021).

In the case of stress-related hormone JA, a model pathway, where JA causes an increase of cAMP, which acts downstream via stimulation of AtCNGC2 Ca2+ conductance, has been proposed (Lu *et al.*, 2016). Although a specific JA-activated AC has not been characterized yet, it has been established that cAMP positively regulates AtCNGC2 Ca2+ conductance (Wang *et al.*, 2017).

An upstream role of cGMP in the regulation of SA has been implied by confirmed co-expression of the GC domain of wall associated kinase WAKL10 with genes involved in biosynthesis of SA and other early pathogen defense signaling molecules (Meier *et al.*, 2010).

The relationship between brassinosteroids and cNs is supported by the GC activity of the BRI1 brassinosteroid receptor. (Kwezi *et al.*, 2007; Wheeler *et al.*, 2017). It was demonstrated that the cGMP production is dependent on the kinase activity stimulated by BL binding, that cGMP activates phosphorylation of the downstream brassinosteroid signaling kinase 1 (BSK1), and that higher cGMP concentrations suppress the kinase activity. This points to the important role of cGMP in BL signaling, which regulates both developmental processes and stress responses (Manghwar *et al.*, 2022)

A breakthrough study identified a functional adenyl cyclase domain in TIR1/AFB, which is part of the SCF-type E3 ubiquitin ligase complex (Qi *et al.*, 2022). This study showed, that auxin stimulates AC activity *in vitro* in the presence of AUX/IAA coreceptors, and *in vivo*. Although the binding of auxin isn´t affected by the inactivation of the AC activity, it negatively impacts the auxin-induced transcription and auxin-mediated root growth inhibition, suggesting that AC is involved in canonical (transcriptional) auxin signaling. AC activity doesn´t seem to mediate rapid auxin responses like apoplastic alkalization. It remains to be elucidated whether cAMP acts as a downstream signal of TIR or if its main function is intramolecular regulation of the TIR1/AFB function.

As for cGMP in auxin signalling - evidence exists that cGMP is involved in auxin-dependent primary root elongation and lateral root formation, through either rapid phosphorylation of target proteins or the initiation of auxin-regulated gene expression and auxin/IAA degradation (Nan *et al.*, 2014). A possible downstream effect of cGMP during root growth and gravitropism might be the regulation of cytosolic Ca2+ transients, for example via CNGC14, which is important for auxin-induced rapid root growth inhibition (Shih *et al.*, 2015). However, the activity of CNGC14 hasn´t been tested in the presence of cNS as of now. Focusing on the regulation of cation currents might be key to how cNs mediate rapid responses to auxin.

4.2. Concluding remarks on cyclic nucleotide effects

Both cAMP and cGMP have been associated with various plant physiological processes, ranging from hormone-dependent growth and development to NO-induced stress responses. However, the knowledge of specific cN-signalling components is still scarce, and as of now, no

cN-mediated signaling pathway in plants has been described in detail. Current knowledge of cN signaling in plants consists of many scattered clues and non-contextual findings. There are two major factors to consider when trying to decipher both the system-level and intracellular effects of cyclic nucleotides. Firstly, the previously touched upon highly localized impact of cNs: functioning in confined cellular pools allows cNs to operate at lower concentrations, and to affect a wide array of processes in the same compartment. Secondly: the prevalence of cross-talking between signaling pathways allows plants to distinctively readjust their responses to various combined inputs. This cross-talk occurs between second messengers, hormones, through “promiscuous” kinases with multiple activators, via different pathways inducing expression of the same genes, and possibly through moonlighting proteins with several functions. (Aerts, Pereira Mendes and Van Wees, 2021). Considering these factors, further study of the biological functions and signaling pathways currently connected with cNs is necessary.

4.3. Effects of non-canonical cyclic nucleotides

Much less is known about the roles of non-canonical cyclic nucleotides 2’,3’-cAMP and 2’,3’-cGMP in plants. Recently, a protein that synthetizes the 2’,3’-isoforms through RNA or DNA hydrolysis has been discovered in the nucleus of *Arabidopsis thaliana*. The cN-synthetic activity is a secondary function of the Toll/interleukin-1 receptor (TIR) domains in nucleotide-binding leucine-rich repeat (NLR) immune receptors, which are established NADases (NAD degrading enzymes) mediating cell death (Yu *et al.*, 2022). Cell death mediated by TIR is dependent on the synthetase activity, which suggests a role of the 2’,3’-isoforms in plant immunity and stress responses and corresponds with previously detected rapid increases in 2’,3’- isoforms levels in response to wounding stress (Van Damme *et al.*, 2014). Additionally, the nudix hydrolase NUDT7 displays 2',3'-cAMP/cGMP phosphodiesterase activity and suppresses TIR-mediated cell death. (Yu *et al.*, 2022). According to Kwiatkowski et al. (2024), 2’,3’-cAMP and 2’,3’-cGMP act as mediators of system-level stress responses at higher concentrations than the locally operating 3’,5’-cNs (Kwiatkowski *et al.*, 2024).

These findings highlight the emerging importance of non-canonical 2',3'-cAMP and 2',3'-cGMP in plant signaling. More research is needed to identify additional proteins capable of synthesizing or hydrolyzing 2',3'-cNs, elucidate the mechanisms by which these 2',3' isoforms mediate cell death and other stress responses and explore their potential involvement in other processes in plants.

5. Downstream signaling of cyclic nucleotides

The downstream targets of cNs consist of cN-dependent protein kinases, transcription factors, ion channels and PDEs. There are two evolutionarily distinct groups of cN binding domains (CNBDs) - CNB domains and GAF domains. CNBDs are found in animal PKAs, PKGs, and Epac proteins, and CNGCs, whereas GAF domains are found in mammal PDEs. The name "GAF" is based on the first three protein families this domain was identified in - specifically cGMP-dependent PDEs, ACs, and bacterial FhlA (Bridges, Fraser and Moorhead, 2005; Heikaus, Pandit and Klevit, 2009). According to Bridges et al. (2005), only two plant protein families containing GAF domains exist - phytochrome proteins and ethylene receptor proteins. It remains to be experimentally confirmed whether these proteins can directly bind cNs.

5.1. Cyclic nucleotide-gated channels

Although bioinformatic searches haven´t identified any plant homologs of animal PKA, PKG, Epac and PDE proteins (Gehring and Turek, 2017), homology-based searches with animal CNB domains led to the discovery of the majority of the known plant CNGCs (Zelman, Dawe and Berkowitz, 2013), making them the only experimentally confirmed plant proteins regulated by cNs for two decades.

Plant CNGCs are the most well-described downstream effectors of cNs in plants. The *A. thaliana* proteome contains 20 homotetrameric CNGCs, and many more CNGCs have been predicted in numerous other higher plants. These channels transport Ca^{2+}, K^{+}, and Na^{+} ions, and are involved in many processes, from development to stress responses. Whether each of the 20 AtCNGCs has been studied in the presence of cNs along with their functions and structure is extensively reviewed here: (Jarratt-Barnham *et al.*, 2021).

In the past, most CNGCs were characterized through electrophysiological assays of cloned genes expressed in heterologous systems such as HEK293T cells (Gao *et al.*, 2016). However, the employment of a genetically encoded buffer for cAMP based on a human PKA regulatory subunit which functions as a "cAMP sponge" (cAS) and causes cAMP depletion in the cell, is now enabling the study of CNGCs (and other cAMP targets) in the absence of cAMP (Lefkimmiatis *et al.*, 2009). Although the reduction of cAMP levels achieved with this buffer might not be completely effective, thus unreliable for studying the consequences of cAMP depletion in some cases. (Sabetta *et al.*, 2019)

The cytosolic C-terminal region of CNGCs contains a CNBD which overlaps with a Calmodulin binding domain (CaMBD). Plant CNGCs can contain multiple phosphorylation sites in the C-terminal region, and some have multiple CaMBDs in both N- and C-terminal regions (Jarratt-Barnham *et al.*, 2021). This complex structure allows for intricate regulation and cross-talk. There are multiple ways in which cNs may regulate CNGCs, and vice versa. Firstly, in the case of co-localization of the respective CNGC and GC or AC at the plasmatic membrane, direct regulation via the cAMP or cGMP binding to the CNBD is possible. CNGCs might also be regulated by specific downstream components of cN signaling, such as various kinases and phosphatases which are implied as regulators of multiple CNGCs (Tian *et al.*, 2019). For instance, CNGC17 and the AtPSKR1 cGMP synthesizing phytosufokine receptor both interact with the AtBAK1 kinase and are components of the same pathway mediating protoplast expansion (Ladwig *et al.*, 2015; Tian *et al.*, 2019). Additionally, cytosolic Ca^{2+} can act as a molecular switch between the activities of moonlighting proteins - in the case of AtPSKR1, Ca2+ inhibits kinase and stimulates GC activity (Muleya et al. 2014). This feedback could also be indirect - for example via calmodulin-dependent kinases.

5.2. Cyclic nucleotide-dependent kinases

Indirect evidence of the existence of PKAs and PKGs in plants was gathered in older studies, but the majority of them used non-specific pharmacological approaches such as inhibitors of cN-dependent kinases from mammals (Nan *et al.*, 2014). Because none of these proteins have been confirmed with molecular and genetic evidence as functional cN-dependent kinases, the only confirmed cN-dependent kinase in plants is a PKG with a role in GA signaling in rice. This PKG displays dual kinase and phosphatase activities and is thus capable of reversible phosphorylation of GAMYB, a GA-related transcription factor. This protein in rice was identified through a database query with a conserved sequence of human PKGI which revealed another plant PKG in *A. thaliana*. An *in silico* analysis of domain architecture showed that plant PKGs contain two cyclic nucleotide-binding domains, CNBD-A and CNBD-B, a Pkinase domain in the C-terminal region and a type 2C protein phosphatase (PP2C) domain in the N-terminal region. The CNBDs contain a conserved phosphate-binding motif (Shen *et al.*, 2019).

Furthermore, cNs have been shown to regulate post-translational modifications of proteins, as supported by studies describing cGMP-dependent phosphorylation and methionine oxidation of *Arabidopsis* proteins (Isner, Nühse and Maathuis, 2012; Marondedze *et al.*, 2013). This coincides with the idea that cGMP-dependent phosphorylation in *A. thaliana* might be executed

by a PKG protein. Recently, quantitative phosphoproteomics combined with cAS-transformed tobacco BY-2 cells identified a large number of proteins whose phosphorylation status was affected by low cAMP levels. Among these proteins were MAP kinases, calcium-dependent protein kinases, receptor-like kinases, and multiple proteins involved in RNA-splicing (Domingo *et al.*, 2023). This evidence points to the existence of plant PKAs, that would execute the phosphorylation of the downstream targets of cAMP signaling.

5.3. Other cyclic nucleotide-dependent proteins

Because the previously used pharmacological approaches haven´t been successful in identifying indirect or direct downstream targets of cN signaling, more recent studies have employed proteomics (mostly using LC/MS) to decipher the effects of cNs on protein levels. A proteomics study in *A. thaliana* revealed changes in the abundance of several proteins in response to cAMP with a role in light- and temperature-dependent responses, for example, photosystem II subunit P-1 (Thomas *et al.*, 2013). Another proteomics study in *Arabidopsis* with cAMP revealed 20 differentially expressed proteins with roles in response to cold and salinity stress, and glycolysis (Alqurashi, Gehring and Marondedze, 2016). Analysis of cAS-overexpressing *A. thaliana* plants attacked by a pathogen revealed that lower endogenous cAMP levels negatively affected the strength of the immune response, and identified numerous cAMP-dependent immune response proteins based on being differentially expressed in the cAS and WT plants. Notably, cAMP buffering delayed spikes in cytosolic Ca^{2+} and H_2O_2 formation after the pathogen attack (Sabetta *et al.*, 2019). In a more recent study that used thermal proteome profiling to find cAMP-binding proteins, 51 proteins which included metabolic enzymes, proteins involved in translation, and most notably, actin, were identified. Although the interaction between actin and cAMP isn´t direct, the influence of cAMP on cytoskeleton dynamics adds another layer of complexity to our understanding of cN signaling (Figueroa *et al.*, 2023). Future studies will hopefully explore whether any of the proteins acting downstream from cNs in plants interact with cNs in a direct manner, or whether they are regulated by other cN effector proteins, such as various transcriptional factors.

In a study from 2016, twelve cCNBP candidates were identified in *A. thaliana* with affinity purification. Eight of these proteins contain domains with similarity to CNB and GAF domains (Donaldson, Meier and Gehring, 2016). As of now, none of these candidate proteins have been experimentally confirmed. It is possible, that the plant proteome contains proteins with unique

CNBDs that remain to be identified in the future. There is clearly a need for more effective screening methods to identify plant proteins that contain cN-binding domains.

5.3. Phosphodiesterases

The first plant protein with PDE activity, mpCAPE (COMBINED AC with PDE), was found in liverwort, a basal plant with motile sperm. As the name suggests, mpCAPE has both AC and PDE activities, and may be involved in the male reproductive process (Kasahara *et al.*, 2016). The putative PDE was further characterized by Hayashida et al. (2022) (Hayashida et al., 2022).

The first PDE discovered in higher plants is AtCN-PDE1. This protein is specific to 3',5'-GMP and inhibits plant growth in response to UVA radiation in *A. thaliana*. Illumination with UVA causes a reduction in cytosolic cGMP levels in guard and mesophyll cells. Cytosolic cGMP was detected with δ-FlincG fluorescence and ELISA. To identify a plant PDE involved in this response, hidden Markov models, large-scale cross-species comparison methods, and gene-model-free searches were employed. This approach identified 26 candidate cGMP-PDEs in *A. thaliana*. AtCN-PDE1-like proteins don't exist in animals, therefore more plant PDE-encoding genes might be unique to plants. (Isner *et al.*, 2019)

A tandem motif-based approach to identify candidate PDEs in plants was described by Kwiatkowski et al. (2021). A consensus search motif was derived from yeast and animal PDE catalytic centers and used to query an A. thanilana database. The consensus amino acid sequence was [YFW]HX[YFW]Rx{20,40}[HRK][DE]. One of the identified PDEs was a potassium uptake permease AtKUP5 with AC activity, which is essential for K+ transport. (Kwiatkowski *et al.*, 2021)

Twin AC-PDE architecture, where enzymes exhibit adenylate cyclase and phosphodiesterase activities, enabling them to both synthesize and degrade cyclic nucleotides seems to be a recurrent theme in plant cyclic nucleotide signaling. The third moonlighting protein with AC-PDE architecture is BnFolD from *Brassica napus,* involved in folate metabolism. AC activity is essential for BnFolDs dehydrogenase activity (Kwiatkowski et al., 2022).

Together, these findings support the role of cNs as molecular tuners and of ACs as meditators of crosstalk between signalling pathways

5.5. Concluding remarks on downstream signaling of cyclic nucleotides

To date, the only well-characterized proteins in plants that are directly regulated through the binding of cNs include CNGCs across many species and one PKG in rice. Another group of plant proteins that contain cN-binding domains are PDEs, although only a little is known about them apart from the aforementioned CN-PDE1 and moonlighting proteins with dual PDE and AC activities. Over the past two decades, studies have identified many proteins indirectly regulated by cNs through post-translational modifications or regulation of gene expression. This indirect evidence suggests the existence of multiple plant cN-dependent kinases and cN-dependent transcription factors. More research employing advanced *in silico* and *in vitro* methods is needed to identify these and other potential CNBPs, as well as novel CNBDs with motifs unique to plants.

6. Nucleotide cyclases

Most of recent cN research has been focused on the identification and characterization of new nucleotide cyclases. The number of known cyclases is quickly growing and new roles of cNs are being discovered through functional characterization of these cN-synthetizing proteins.

Moreover, ACs and GCs are potential targets in crop improvement - research is being conducted not just in arabidopsis but also economically significant plants such as maize, apple, jujube, tomato, and pear (Yuan *et al.*, 2008, 2022, 2023; Rahman *et al.*, 2020; Yang *et al.*, 2021; Liu *et al.*, 2023).

6.1 Identification of nucleotide cyclases

The first plant AC was identified in maize pollen based on a truncated cDNA encoding a protein homologous to fungal adenylyl cyclase that has been isolated from a maize pollen cDNA library (Moutinho and others 2001).

To tackle the previously mentioned challenges (low sequence conservation, low enzymatic activity) in the search for nucleotide cyclases in plants, a consensus 14 amino acid sequence was created using alignments of various prokaryotic and eukaryotic GC domains from different species. The construction of this motif was based on the assumption that only the functionally assigned amino acid residues are conserved in functional centers of complex multi-domain proteins (Ludidi and Gehring, 2003).

The currently implemented GC and AC functional center motifs are [RKS]x[GCTHS]x(10)[KR] and [RKS]x[DE]x(10)[KR], respectively. An AC motif was generated by substituting the amino acid in the 3rd position of the GC motif requisite for substrate specificity to GTP with residue-specific for binding ATP (Bianchet *et al.*, 2019). The residue at position 1 forms a hydrogen bond with the purine base, and the residue at position 14 stabilizes the transition state from NTP to cNMP.

The diagnostic motifs were then used for rapid screening of the *A. thaliana* proteome using pattern matching. The screenings for both GCs and ACs were refined by creating extended motifs with other residues commonly found outside of the catalytic center of plant cyclases, for example, Asp or Glu in position 16 or 17, which bind Mg^{2+} or Mn^{2+} cofactors. (Wheeler *et al.*, 2017; Bianchet *et al.*, 2019). The conserved AC and GC motifs have been identified in multiple plant species, which highlights their versatility (Świeżawska *et al.*, 2017; Yang *et al.*, 2021).

For an additional increase in confidence levels in the candidates retrieved from protein databases using motif-based searches, the GCPred and ACPred web tools can be used (Xu *et al.*, 2018; Schaduangrat *et al.*, 2019). The functionality of the prospective cyclases can be predicted through further *in silico* structure assessment. Homology modeling is used to create 3D models and docking simulations are conducted to predict the binding site (Trott and Olson, 2010; Wong *et al.*, 2018). More tools have been described by Zhou et al. (Pettersen *et al.*, 2021; Zhou *et al.*, 2021). The importance of individual amino acids, or the enzymatic activity they are assigned to in moonlighting proteins, is usually examined in *vitro* through site-directed mutagenesis.

6.2. Known plant adenylate and guanylate cyclases

Apart from the previously described group of proteins with dual AC/PDE activity, plant cyclases can be sorted into multiple other categories based on their domain architecture.

Leucine-rich repeat receptor-like kinases (LRR RLKs) with GC activity represent a significant group of moonlighting plant cyclases. This group includes the brassinosteroid receptor insensitive 1 BRI1 (Wheeler *et al.*, 2017), the phytosulfokine receptor 1 PSKR1 (Kwezi *et al.*, 2011), the pathogen peptide receptor 1 PepR1 (Qi *et al.*, 2010), and the wall-associated kinase-like 10 WAKL10. In AtPSKR1, Ca^{2+} acts as a molecular switch between the GC and kinase activities (Muleya *et al.*, 2014). AtBRI1, AtPSKR1 and AtPepR1 all display intramolecular crosstalk: the activated kinase stimulates cGMP synthesis and cGMP inhibits the kinase through a negative feedback loop (Kwezi *et al.*, 2018).

Furthermore, in the case of the diacylglycerol kinase 4 (AtDGK4) (Vaz Dias *et al.*, 2019) which is a moonlighting kinase with GC activity, cGMP negatively regulates the primary kinase activity. Similar intramolecular interactions were observed in AC moonlighting proteins, for instance the AtTIR1 the functional AC center is essential for its role as part of the SCF-type E3 ubiquitin ligase complex regulating auxin-dependant transcription (Qi *et al.*, 2022) AtPSKR1, AtBRI, AtPepR1, and AtDGK4

The *Arabidopsis* pentatricopeptide repeat protein (AtPPR1) (Dikobe *et al.*, 2024) is the first member of a novel group moonlighting proteins with dual AC and kinase activities. Ca^{2+} and cAMP act as molecular switches between the two activities.

Some plant ACs consist of multiple AC domains, notably ZmRPP13-LK3 (Yang *et al.*, 2021), and AtLRRAC1 (Ruzvidzo, Gehring and Wong, 2019).

Most recently identified cyclases are BcGC1 in pak choi, which mediates leaft senescence delay by melatonin (Liu *et al.*, 2024) and AtSnRK (SNF-1 related protein kinase) which has three active enzymatic centers GC, AC, PDE in the regulatory subunit (Kwiatkowski, Wong, *et al.*, 2024)

A new class of ACs in plants - VII ACs was established in plants based on phylogenetic analysis (Liu *et al.*, 2023) and it consists of jujube proteins ZjAC1, ZjAC2, ZjAC3, apple proteins TTM1, TTM2, pyrus x bretscheinderi PbrTTM1, and hippeastrum hibridum HpAC1. This class encodes the EXEXK motif, whereas other ACs contain the previously mentioned [RKS]X[E]X{9,11}[KR]X{1,3}[DE] motif.

Lastly, plant nucleotide cyclases can be either transmembrane or soluble proteins. The previously mentioned LRR RLKs are examples of transmembrane nucleotide cyclases, and examples of putative soluble nucleotide kinases are the GCs AtGC-1 and PnGC-1(Ludidi and Gehring, 2003; Szmidt-Jaworska *et al.*, 2009)

6.3. Nucleotide cyclase activity assays

Enzyme activity assays of plant nucleotide cyclases should be conducted in reaction mixtures containing Mn^{2+} and/or Mg^{2+} ions as cofactors, to determine if there is a preference. Modulators, such as Ca^{2+}, are also added to the reaction mixture to assess their effect on the cN levels. The absence of modulators can result in lower enzymatic activity *in vitro* compared to *in vivo* conditions. In some proteins with dual activities, Ca^{2+} can act as a molecular switch via effects on the functional centers. For example, Ca^{2+} enhances GC activity and inhibits the kinase activity of PSKR1 (Muleya *et al.*, 2014).

Furthermore, in the case of proteins with dual kinase and GC/AC functions, phosphorylation of certain kinase residues might be necessary for the activation of both of the functional centers (Wheeler *et al.*, 2017; Kwezi *et al.*, 2018). In addition, it might be relevant to test the impact of the respective cN on the primary activity of the moonlighting protein. For example, BRI1, PSKR1 and PePR1 are all inhibited by cGMP, pointing to autonomous regulation of the proteins (Kwezi *et al.*, 2018). Additionally, the production of cAMP by functional AC centers in AtKUP5 and BnFolD is necessary for their proper functioning as a K^+ transporter and a dehydrogenase, respectively (Al-Younis et al. 2018; Kwiatkowski et al. 2022). Therefore, intramolecular allosteric regulation between the functional domains of putative nucleotide cyclases are perhaps quite common and should be considered during activity assays.

7. Conclusion

Cyclic nucleotides, which have been deemed insignificant for a long time, are now recognized as crucial components of plant signaling pathways. This renewed research interest has fueled rapid advancements in the study of the cN interactome, using methods such as bioinformatics screening, RNA sequencing, highly sensitive quantitative assays, gene knockout screens, and *in silico* analysis. Many novel proteins connected to cyclic nucleotide signaling are being identified, and new evidence for both previously implied and novel effects of cyclic nucleotides is emerging. Despite this recent progress, our understanding of cyclic nucleotide signaling in plants remains fragmented. The impact of cNs in plants appears to be both subcellular compartment- and organ-specific, necessitating further research with highly specific live cell-imaging methods to detect cNs under physiological conditions in real-time, while considering their highly localized downstream impacts. Current data suggest that cyclic nucleotides are important signaling components capable of fine-tuning enzymatic activities to achieve higher specificity of impact, and mediating signaling crosstalk through the moonlighting activities of nucleotide cyclases, phosphodiesterases, cyclic-nucleotide gated channels, and other candidate cn-dependent proteins. However, the specific mechanisms of cyclic nucleotide signaling remain to be fully delineated. This thesis summarizes some of the highlights of what seems to be the first chapter of an unfolding story, that is likely to keep surprising us as it develops and reveals more about the main characters and their roles in plant signaling.

List of abbreviations

cGMP	Cyclic guanosine monophosphate
cCMP	Cyclic guanosine monophosphate
GC	Guanylate cyclase
AC	Adenylate cyclase
PDE	Phosphodiesterase
CNGC	Cyclic nucleotide-gated channel
PKA	Protein kinase A
PKG	Protein kinase G
cUMP	Cyclic uridine monophosphate
cCMP	Cyclic cytidine monophosphate
TIR1/AFB	Transport Inhibitor Response 1 and Auxin-Signaling F-box
cN	Cyclic nucleotide
MS	Mass spectrometry
LC/MS	Light chromatography/mass spectrometry
ELISA	enzyme-linked immunosorbent assay
FRET	Förster resonance energy transfer
FlincG	Fluorescent indicators of cGMP
Epac	Exchange protein activated by 3'-5'-cyclic adenosine monophosphate
BRET	Bioluminescent resonance energy transfer
GTP	Guanosine triphosphate
ATP	Adenosine triphosphate
NTP	Nucleoside triphosphate
ABA	abscisic acid
GA	gibberellic acid
JA	jasmonic acid
SA	salicylic acid
BL	Brassinosteroid
RPP13-like	Recognition of Peronospora Parasitica 13-like
WAKL10	WALL ASSOCIATED KINASE-LIKE 10
BRI1	Brassinosteroid insensitive-1
BSK1	brassinosteroid signaling kinase 1

NO	Nitric oxide
TIR	Toll/interleukin-1 receptor
NLR	nucleotide-binding leucine-rich repeat
NUDT7	Nudix Hydrolase 7
CNBD	Cyclic nucleotide binding domain
FhlA	formate hydrogen lyase A
HEK293T	Human embryonic kidney 293
CaMBD	Calmodulin binding domain
PSKR1	Phytosulfokine receptor 1
BAK1	BRI1-associated receptor kinase
GAMYB	gibberellin- and abscisic acid-regulated MYB
MYB	Myeloblastosis
BY-2	Bright Yellow - 2
MAP kinase	Mitogen-activated protein kinase
cAS	cAMP sponge
WT	Wild type
CAPE	(COMBINED AC with PDE).
CN-PDE1	cGMP-activated phosphodiesterase
KUP5	K+ uptake permease 5
LRR RLK	leucine-rich repeat receptor-like kinases
PSiP	PC4 And SRSF1 Interacting Protein
SnRK	SNF-1 related protein kinase
PPR	pentatricopeptide repeat
SCF	Skp1, Cullins, F-box proteins
TTM	triphosphate tunnel metalloenzyme
PNPR1	Pathogenesis-Related Protein
PEPR	Perception of the Arabidopsis Danger Signal Peptide
DGK4	diacylglycerol kinase 4
H-NOX	Heme-Nitric Oxide or Oxygen-binding

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