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Phosphodiesterases in higher plants - a missing link in cyclic nucleotide signal transduction

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List of abbreviations:

A – alanine

AC – adenylate cyclase

AMP - 5′-adenosine monophosphate

AtKUP5 - *Arabidopsis thaliana* K^+-Uptake Permease 5

ATP - adenosine 5′-triphosphate

BLAST - Basic Local Alignment Search Tool

CaM – calmodulin

cAMP - 3′-5′-cyclic adenosine monophosphate

cCMP - 3′-5′-cyclic cytidine monophosphate

cGMP - 3′-5′-cyclic guanosine monophosphate

CNBD - cyclic nucleotide-binding domain

CNGC - cyclic nucleotide-gated channel

cNMP – cyclic mononucleotide

cUMP - 3′-5′-cyclic uridine monophosphate

D – aspartic acid

E – glutamate

F – phenylalanine

GAF - cGMP-activated PDEs, adenylyl cyclase, and Fh1A domain

GC – guanylate cyclase

GMP - 5′-guanosine monophosphate

GTP - guanosine 5′-triphosphate

H – histidine

I - isoleucine

K – lysine

L – leucine

LC-MS/MS - liquid chromatography tandem mass spectrometry

NO – nitric oxide

PAS - Per-ARNT-Sim domain

PDE – phosphodiesterase

PSK – phytosulfokine

PSKR1 - Phytosulfokine receptor 1

R - arginine

TAIR - The Arabidopsis Information Resource

W – tryptophan

Y - tyrosine

Introduction

Signal transduction is the process in which the information carried by extracellular and intracellular signals are received by the cell and then transmitted through it by inducing a chain of intracellular biochemical reactions that convert input signals into an appropriate response such as cell growth, division, differentiation or cell death [1]. This is possible thanks to the presence of the cell's regulatory mechanisms consisting of signal receiving receptor, elements responsible for signal transmission and effector systems that forms the response. The initiation of intracellular signaling mainly occurs through the specific recognition of cytosolic or cell surface transmembrane receptor proteins activated by physical or chemical agents like hormones, cytokines, steroids, light or neurotransmitters, called first messengers. Activation of the receptor triggers transduction of signal, which involves modulating the behavior of series of information components in the cascade, turning them on or off like a switch [2]. The reversible mechanism of protein phosphorylation is one such fundamental mechanism. It thus modifies the shape or/and behavior of many proteins. In the process of phosphorylation, protein kinases attach phosphate groups (PO_4) to the polar groups of various amino acids. This allows the targeted protein to change activity or conformation when interacting with other molecules [3]. Ligand activated receptors can also stimulate the cytoplasmatic appearance of second messengers - a group of small intracellular molecules like cyclic mononucleotides (cNMP), nitric oxide (NO) or Ca^{2+} ions, that amplify or relay extracellular signals, which in turn activate channels or enzymes constituting a biochemical cascade [4]. In the resting state, secondary messengers are stored in parts of the cell or their synthesis is inhibited, hence they occur in low concentrations in the cytoplasm. Following receptor activation, their levels elevate rapidly and then return quickly to baseline. The levels of second messengers in the cytosol are precisely tuned by a number of homeostatic mechanisms to ensure the precision of cell signaling. Thus, changes in transmitters concentration and their duration constitute a signal that initiates a cascade of events leading to a specific physiological response [5]. Cyclic NMPs, together with calcium, are such main universal regulators of cell functions. Cyclic NMPs can be divided into four types: 3′-5′-cyclic adenosine monophosphate – cAMP; cyclic guanosine monophosphate – cGMP; 3′-5′-cyclic cytidine monophosphate – cCMP; 3′-5′-cyclic uridine monophosphate - cUMP, which of cAMP and cGMP are key regulators of cell functions.

Cyclic AMP was first isolated from the liver of a dog during studies on the effects of epinephrine and glucagon on the activity of glycogen phosphorylase [6]. This discovery was a foundation of the second messenger concept and for his work, regarding the mechanics of

hormonal action and the isolation of cAMP, Earl Wilbur Sutherland was awarded the Nobel Prize in 1971. Shortly thereafter, cGMP was discovered [7]. Cyclic AMP is a molecule consisting of an adenine ribonucleotide with a phosphate group bound to the oxygen molecules at the 3′ and 5′ positions of the sugar moiety. The structure of cyclic GMP is similar except that the nucleotide is a guanine [8]. Cyclic AMP and cGMP, due to their broad regulatory functions in several hormone-dependent signal transduction pathways as well as activation of specific protein kinases, are present in almost all living organisms, ranging from simple procaryotes to complex *Homo sapiens*. By contrast, the existence of the cyclic pyrimidine nucleotides cCMP and cUMP has been controversial for decades. Currently, their presence is documented in eukaryotes, although their biological role remains to be elucidated [9]. Bacteria have evolved their virulence strategies to interfere with host cNMP levels [10]. Cholera and pertussis toxin use cAMP as the crucial pathogenic signal, whereas the heat-stable enterotoxins modulate cGMP levels in host intestinal cell [11,12]. Apart from being involved in virulence, cAMP signaling participates in the metabolic processes of the cell during exponential growth by coordinating the expression of catabolic proteins with biosynthetic and ribosomal proteins [13]. Protozoan signaling processes, including cNMP metabolism, are not as well characterized as in animals due to the poorly described downstream cNMP effector proteins however, cAMP may participate in cell cycle regulation [14] and a cGMP-activated ion channel in the protist *Stentor coeruleus* may be involved as an effector in the photosensory transduction pathway leading to the motile photophobic response [15]. The role of cNMP in fungi and animals is best understood because information on the occurrence and role of cNMP in morphological and metabolic processes in fungi, like control of morphogenesis or fruiting body formation, began to appear in the early 1970s [16,17]. In animals, cAMP transduces the stimuli of a wide range of hormones and neurotransmitters and is capable of regulating at least one enzyme in every known mammalian metabolic pathway. Cyclic GMP is established as a metabolic regulator and appears to have a much more restricted role, participating in response to light activation of retinal pigments, mediating the contraction/relaxation cycle in smooth muscle in response to NO or in the movement of sodium ions and water across membranes [18].

Cyclic NMPs are formed from nucleoside triphosphate and this process is catalyzed by nucleotide cyclases. Adenylate cyclase (AC) convert ATP (adenosine 5′-triphosphate) into cAMP and pyrophosphate. Cyclic GMP and pyrophosphate are produced from the GTP (guanosine 5′-triphosphate) substrate by guanylate cyclase (GC). The AC enzyme family can be divided into six classes on the basis of sequence homologies in their catalytic domains.

Eukaryotic ACs and all known GCs belong to the largest class III, and enzymes from this group are found in almost every organism [19]. In mammals, the ACs group consists of nine transmembrane enzymes (AC1–AC9) and one soluble AC10. Transmembrane ACs are glycoproteins with a molecular weight of 120 kDa, contain variable N-terminal regions, followed by a six transmembrane helices domain, the first catalytic domain C1, a second six transmembrane helices domain, and the second catalytic domain C2 [20]. Soluble AC10 consist of two heterologous catalytic domains C1 and C2 forming the 50 kDa amino terminus of the protein [21]. The other part of the C-terminus of the enzyme can consist of putative regulatory domains. Soluble ACs have been identified in organelles such as the nucleus or mitochondria and are directly regulated by Ca^{2+}, pH, bicarbonate and ATP concentration [19]. In mammals, four soluble GC subunits and seven single membrane-spanning forms were discovered. The soluble GCs exist as heterodimers containing a N-terminal heme-binding, dimerization and C-terminal catalytic domains. The transmembrane cyclase members are homodimers containing an extracellular ligand-binding, transmembrane, kinase homology, dimerization and C-terminal catalytic domains [22].

Cyclic nucleotides enable complex signaling critical to interactions with the environment. These signals in addition to signal strength also have a temporal and spatial dimension. At some point, cNMP stimulation of the effector has to be terminated. A diverse superfamily of cyclic nucleotide phosphodiesterases (PDEs) selectively catalyze the hydrolysis of the 3′ cyclic phosphate bonds in cAMP and cGMP to generate 5′-AMP (5′-adenosine monophosphate) and 5′-GMP (5′-guanosine monophosphate), which are inactive in the cNMP signaling pathways [23]. The hydrolysis of cNMP is catalyzed by three classes of catalytic domains. Metazoans, including mammals, express mostly PDEs belonging to class I. In the genome of *H. sapiens* there are 21 annotated genes encoding PDEs containing a class I catalytic domain. Due to the substrate specificity, the PDE family can be divided into three groups that hydrolyze cAMP, cGMP or both substrates. Three of the eleven PDE families selectively hydrolyze cAMP (PDEs 4, 7, 8), three are selective for cGMP (PDEs 5, 6, 9) and five hydrolyze both cNMPs with varying degree of efficiency (PDEs 1, 2, 3, 10, 11) [24]. The catalytic domain of class I PDE contain three subdomains consisting largely of 16 helices. These structures create a deep pocket where the substrate or inhibitors can bind. The active site is formed at the junction of the helices by residues that are highly conserved among all the PDEs. At one end of the catalytic cleft are two divalent metal binding sites. A tightly bound zinc is coordinated by conserved histidine (H) and aspartic acid (D), while magnesium binds less tightly to the

catalytic pocket. These conserved residues are subset of the larger HD domain structure that is found in an enzyme superfamily possessing phosphohydrolase activity [24,25]. Additionally, in each structure of the mammalian PDE there seems to be a conserved glutamine that forms one or two hydrogen bonds (depending on the PDE) with the purine in the binding pocket, potentially stabilizing the hydrolytic process [26]. Phosphodiesterases, depending on the group, have a regulatory domain often at their N-terminus, that impacts the catalytic process. These regulators are Ca^{2+}/calmodulin-binding domains which allow for cross-talk between the Ca^{2+} and cNMP signaling pathways [27] and GAF domains (cGMP-activated PDEs, adenylyl cyclase, and Fh1A) which function as regulatory elements that bind nucleotides or other small molecules. GAF domain participate in PDE2 dimerization and cGMP binding [28]. Many PDEs have in their structure phosphorylation and autoinhibitory domains, but PDE8 can also be regulated by PAS (Per-ARNT-Sim) domains that participate in ligand binding and protein-protein interactions. [23,24].

The discovery of cyclic NMPs over half a century ago in animals, bacteria and fungi suggested that they are universal signaling molecules throughout the tree of life and having key major roles in a wide spectrum of processes. However, the identification of these molecules in the plant kingdom has been controversial, mainly due to the apparently low concentrations in which cNMPs are present in plant cells. The first convincing work on the presence of cNMPs in higher plants comes from a report published in 1973 that showed changes in cNMPs levels during cell expansion and division in tobacco [29]. However, one year after the publication of this report, the detection of cAMP in plants has been severely criticizing the lack of sufficiently sensitive and quantitative methods for detecting cNMPs at the femtomolar level [30]. The skeptical approach to the presence of cNMPs in plants was overcome when analytical techniques based on mass spectrometry unequivocally showed the presence of both cAMP and cGMP in plant tissues [18]. Over time, more and more publications began to appear in which the concentrations of cAMP and cGMP were measured and accurately given, ranging from a femtomolar to even nanomolar concentrations depending on plant species and tissues [31].

After years of controversy in the plant science community, the roles of cNMPs in plants have finally been established and the discovery of plant physiological processes that are critically dependent on changing cNMPs levels continues. It has been shown that cAMP is involved in the regulation of cellular Ca^{2+} and K^{+} flux, indicating a potential role as a regulator of ion and water homeostasis [32,33]. Cyclic AMP participates in pollen tube growth and reorientation [34], mediates in biotic and abiotic stresses through the regulation of stomatal

opening and defense-related genes [35,36], promotes seed germination [37] or cell cycle progression [38]. In chloroplast development, cGMP contributes to the biosynthesis of anthocyanins [39]. Fluxes of K^+ and Na^+ modulated by cGMP via the activation of non-selective ion channels were observed in the roots of *Zea mays* [40]. In addition, cGMP has been strongly implicated in NO signaling in plants, which is a gaseous regulator of development and signaling present at each step of the plant life cycle [41]. In *Pharbitis nil* cGMP was involved in the regulation of the flowering [42]. In plant cNMP signaling it is worth referring to another secondary messenger with which cNMPs can closely cooperate and this is Ca^{2+}. Signaling pathways involving these two messengers can form complex networks with specific checkpoints and the modulation of physiological processes are mediated by cyclic nucleotide-gated channels (CNGC) which can be considered an intersection of cNMP and Ca^{2+} signaling [43]. CNGCs are cationic channels with varying ion selectivity. CNGCs are specifically located in the plasma membrane and allow diffusion of divalent and monovalent cations, including Ca^{2+} and K^+ [44]. All eukaryote CNGCs have six membrane-spanning regions (S1–S6), a positively charged transmembrane domain (S4), and a P-loop between the 5th and 6th domain. In their structure there is cyclic nucleotide-binding domain (CNBD) and a calmodulin binding domain (CaMBD). This type of architecture allows calmodulin (CaM) to compete with cNMP as a ligand in allosteric gating of channel conductance, thereby regulating cellular responses [44,45]. Interactions between CNGC, cNMPs and Ca^{2+} were observed in the protoplasts of *Nicotiana plumbaginifolia* [46], pollen tubes of *Pyrus pyrifolia* [47] or guard cells of *Arabidopsis thaliana* [48]. Crosstalk between Ca^{2+} and cNMP can also occur via other enzymes like cyclases, as seen in phytosulfokine (PSK) signaling, where the GC activity of the hormone receptor (PSKR1) is significantly enhanced by Ca^{2+} [49].

Although the presence of cNMPs in plants has been established and the processes they participate in have been well described, for a long-time it was difficult to find the cyclases which are responsible for their synthesis. One of the first reports of the alleged presence of GC in plants in 1984 came from investigations of guanylate cyclase-like activity in bean chloroplasts [50]. With the passage of time and the availability of complete plant genomes, many GCs and ACs have now been discovered and more putative cyclases remain to be tested experimentally.

Using the basic local alignment search tool (BLAST) to search the *A. thaliana* genome, matching ACs and GCs from lower and higher eukaryotes, do not return any plausible candidates and only a few GC candidates were found in cyanobacteria. This result implied a

separate evolution of plant ACs and GCs, where catalytic domains of "classic cyclases" may have been lost [51,52]. When the standard homology approach was unsuccessful, a change to the method was proposed in which an alignment of annotated prokaryotic and eukaryotic cyclases was created and the conserved amino acids necessary for catalysis were identified. Thus, a 14 amino acid motif was developed and the available *A. thaliana* proteome was scanned, returning seven GC candidates. Using the motif-based approach, it was finally possible to identify and describe *in vitro* activity of the first GC in higher plants (AtGC1, At5g05930) [51]. Over time, the GC motif was modified by relaxing it or adding new amino acids whose participation in catalysis was confirmed in other species by mutagenesis, what gradually increased the database of experimentally tested GCs [53–55]. Despite the fact that cAMP was discovered earlier than cGMP, for a long time, the only experimentally confirmed ACs in plants was the *Z. mays* pollen protein, which plays a role in the growth of pollen tube [34] and HpAC1 involved in stress signaling in *Hippeastrum x hybridum* [56]. Given that cAMPs play an important role in plant signaling and have been found in many pathways, it is unlikely that there will be only one AC responsible for all these processes. The search was also complicated by the fact that, Prosite signatures for ACs from class I and II were not present in the *A. thaliana* proteome, even when 2 mismatches were allowed [57]. Much like the discovery of the first GCs, a motif-based approach was used. In this case, no new motif was isolated from an alignment of annotated eukaryotic ACs but the already established GC motif was modified. In the modified AC search terms, the amino acid residues that form the hydrogen bonding with the adenine (position 1) and stabilizes the transition state from ATP to cAMP (position 12-14) were substituted [57]. The key step to the success was substitution of amino acids in position 3 to [DE], that confer substrate specificity. The resulting motif ([RKS]X[DE]X(9,11)[KR]X(1,3)[ED]) was also present in the AC from *Z. mays* discovered earlier. The implementation of an amino acid search motif and its further modifications has enabled the discovery of many ACs, most of which are hidden as a small domain in a complex, multi-functional proteins [58–61]. Such proteins can be referred to as moonlight protein and are classified as single chain polypeptides that are capable of performing two or more biochemically distinct functions [62]. The term moonlighting protein can be applied to the cNMP domain containing proteins in plants, since most of the discovered AC and GCs are part of multidomain proteins. While there is no simple explanation why plants have evolved cyclases embedded in complex multifunctional proteins, it can be speculated that it may have proven advantageous in intramolecular tuning in particular when localized and changing cNMP levels may themselves activate/inhibit neighboring domains [49,59].

Until recently, insights into cNMP homeostasis in plants was incompletes since their degradation mechanism has remained elusive. Despite identifying plant cyclases and describing them relatively well, for a long time no PDEs have been identified, isolated and characterized *in vitro*. The presence of cNMP in plant cells indicated that signaling with its participation must be terminated at some point which necessitates PDE activity. Therefore, the search for evidence of the presence of these enzymes in plants began, and the first records of the detection of PDE activity in a protein extract appeared in the 1970s [63,64]. Decades later, in 2016, reports appeared on the isolation and characterization *in vitro* of a PDE in the liverwort *Marchantia polymorpha* [65]. The work focused on the search for genes encoding AC in the transcriptome database of *M. polymorpha*, using the amino acid sequence of cyanobacteria AC, CyaC. In addition to identifying the domain significantly similar to the CyaC catalytic AC domain on the C-terminus, the deduced amino acid sequence also contained the sequence of the PDE catalytic domain located in the N-terminus. Before 2020, only one PDE which is a cGMP-dependent AtCN-PDE1 (At1g17330) was reported in higher plants. This PDE belongs to the HD-domain/PDEase-like protein superfamily, responds to UV and is implicated in stomatal movements [66]. This PDE was detected using hidden Markov models, large-scale cross-species comparison methods, and gene-model-free searches. Such approach resulted in the identification of 26 putative cGMP-PDEs in *A. thaliana*. However, PDEs responsible for cAMP hydrolysis in higher plants have still not been identified.

The aim of the study

The central argument of my doctoral thesis is that the newly discovered plant PDEs – which degrade cAMP and/or cGMP – are central to cNMP signaling in that they constitute the "off" signal. It is therefore reasonable to ask whether, how many and which PDEs does the proteome of the model species *Arabidopsis thaliana* harbor and what are the structural and biochemical properties of these candidate PDEs.

The specific aims of experimental procedure are:

- Designing a rational method to identify novel candidate PDEs
- Structural description of proteins with predicted PDE activity
- Comparison of orthologous candidate PDEs from different species
- Biochemical characterization of recombinant PDEs
- Inferring a biological role for the novel PDEs with a view to build a model of cNMP homeostasis

The outcomes of the investigation framed above are presented in two original articles [67,68] appended.

Discussion

Sequence similarity searches to identify homologous sequences is an effective and reliable step in identifying orthologues of proteins that are well established in one system but not yet discovered in another, particularly in proteins of ancient origin and a conserved domain architecture. For this purpose, homology search programs such as BLAST, PSI-BLAST, SSEARCH, FASTA or HMMER3 can be used [69]. In some cases, the proteins among all prokaryotic and eukaryotic organisms will have quite a different amino acid sequence. In the course of evolution, multidomain proteins, which make up the majority of proteins encoded in the genomes of higher organisms, frequently changed the arrangement and composition of the domains [70]. Despite this shuffling of domains, the overall structure of the protein can remain conserved, so that searching for homologous sequences in other organisms gives positive results [71]. However, sometimes the reorganization of the domains could be so substantial that some proteins cannot be found, even by searching for localized regions between sequences, such as active sites. One such example is plant phosphodiesterase, enzyme responsible for degradation of cNMP by breaking the phosphodiester bond. When searching the *A. thaliana* proteome with the sequences of the annotated animal or yeast PDEs, the BLAST program does not return plausible plant orthologs. In addition, despite belonging to the plant kingdom, no orthologs were found in higher plants during screening of PDEs from green algae *Chlamydomonas reinhardtii*, which is closely related to class I mammalian PDEs 1A and 3A [72]. This does not indicate an absence of PDEs in plants, because their activity has been experimentally confirmed in protein extracts [63,73]. Since the PDE orthologs are not present in the proteomes of higher plants, it can be concluded that plant PDEs may not be homologous to the currently annotated PDEs but that they may contain a significant degree of similarity only in the catalytic center. This may be related to the complex structure of proteins that can contain several functional domains [74]. Within this complex architecture there may be functional centers that contain only the key amino acids required for catalysis and these being few, are likely to remain beyond the detection limit of BLAST homology searches [75]. Thus, I formulated hypothesis that such functional centers should be conserved among all known PDEs, because the catalytic mechanism for degradation of cNMPs, based on hydrolysis of the 3′ cyclic phosphate bond of cAMP and cGMP is the same [24]. Moreover, plant cyclase domains discovered to-date exhibit embedded functional centers [52]. Considering the above assumptions regarding the conservation of functional centers, it is possible to construct consensus sequences - also termed

motifs - with which it is possible to query databases and identify candidate PDE catalytic sites some of which may indeed be hidden in multidomain plant proteins.

In order to construct the motif, it was necessary to determine the PDE catalytic center from various organisms, to isolate the key amino acids involved in the recognition and hydrolysis of cNMP. The catalytic center of all known PDEs consists of a hydrophobic pocket that can accommodate cAMP or cGMP substrate. Usually, the substrate is recognized and bound by an aromatic and hydrophobic amino acid residues like tyrosine (Y), phenylalanine (F), leucine (L) or isoleucine (I). At the bottom of the pocket are hydrolysis centers of PDEs that contain two divalent metal cations like Zn^{2+}, Mg^{2+} or Mn^{2+}. In all PDE groups, the first metal is coordinated by 4 basic and charged amino acids, two histidine residues, and two aspartate residues, and the second metal is coordinated by only one D residue [76–78]. Based on this knowledge, in my first research article entitled "*A tandem motif-based and structural approach can identify hidden functional phosphodiesterases"* [67] I identified the sequence of PDE catalytic core in the moss *Physcomitrella patens*, which is a close orthologue of the previously discovered PDE in the liverwort *M. polymorpha* [65]. The sequence in *P. patens* (pfam00233) appeared as annotated PDE in the Pfam database, which is a large collection of protein families. The combination of amino acid residues participating in the nucleotide binding and hydrolyzing 3′ cyclic phosphate bond is present at the beginning of catalytic center. Subsequently, a comparative sequence analysis of the two tested PDEs from *M. polymorpha* and from *A. thaliana* was performed. Each sequence contained the same amino acid combination at the catalytic site consisting of substrate recognition residues followed by a gap and then by residues involved in hydrolysis process. After aligning the plant sequences, highly conserved amino acids were isolated and chemically related amino acids were added at the key positions in the motif, like aromatic tryptophan (W) for nucleotide base binding, charged arginine (R), lysine (K) and glutamate (E) as a substitution for the H or D. The alignment was then extended with the sequence of annotated PDEs from fungi and animals. Thus, the first plant PDE motif ([YFW] H x [YFW] R x {20,40} [HRK] [DE]) was created (Figure 1).

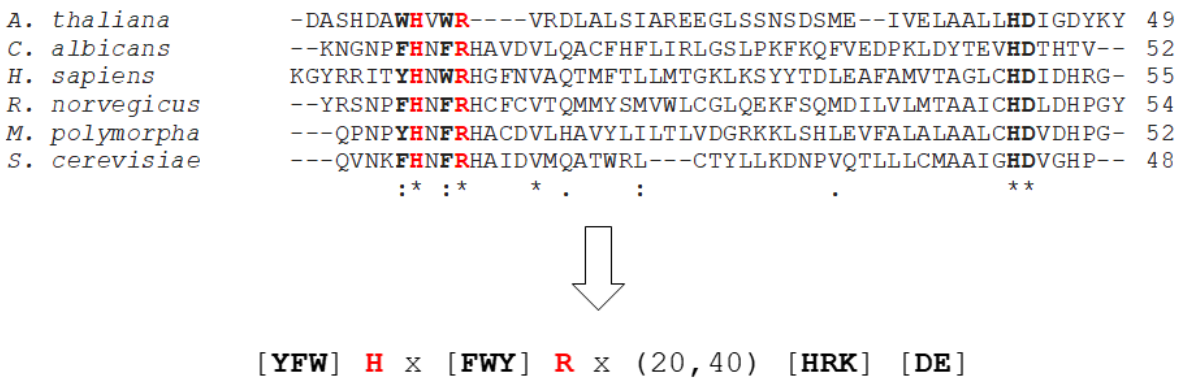

Figure 1. Alignment of plant PDE sequences from *A. thaliana* (At1g17330), *M. polymorpha* (PTQ35772.1) and annotated fungi and animal PDEs from *Candida albicans* (Q8NJP9), *Saccharomyces cerevisiae* (C7GNQ8), *Homo sapiens* (P35913, PDE6B) and *Rattus norvegicus* (Q8QZV1, PDE9A).

The deduced plant PDE motif was used to query the *A. thaliana* proteome using pattern matching tool (PatMatch) feature, designed to find short sequence matches [79] in The Arabidopsis Information Resource (TAIR) website. It retrieved a list of 32 candidates, most of which already had an annotated functions in the cell. Moreover, this motif occurs in 829 sequences in Prosite database which scan sequences from various organisms. Among these hits, 97 were annotated PDEs. This result supports the hypothesis of the existence of PDE functional centers masked by larger protein domains.

Of the 32 candidates, the *Arabidopsis thaliana* K^+-Uptake Permease 5 (AtKUP5, At4g33530) was selected for further analysis, due to the earlier work in which the AC domain at N-terminus was identified in this protein [59]. The KUP proteins are described as an electrochemical potential-driven group of transporters and are likely K^+-H^+ symporters [80]. Despite having an AC domain at the N-terminus, this protein appeared to have a PDE domain at the C-terminus, after the 12 transmembrane domains. The presence of both an AC and a PDE domain on the cytosolic side suggested the possibility of channel regulation mediated by cAMP and hence the control of K^+ by these domains. If so, searching for sequences using motifs may prove helpful in analyzing complex intramolecular regulatory mechanisms. To strengthen the evidence for specificity in the motif-based approach we used structural assessments in the form of homology modeling and ligand docking simulations of selected candidates. This approach allows to visualize the three-dimensional structure of the protein and may confirm the participation of the designated amino acids in the catalysis process before conducting *in vitro* experiments. The computational evaluation of the AtKUP5 structure confirmed the presence of the key functional amino acids from the motif and the characteristic for PDEs pocket protected

by a latch [76,77]. In addition, cAMP docks at the PDE domain with good binding affinity and the correct binding position has been established, where the adenine head of cAMP points towards [YFW] H x [FWY] R and the phosphate tail points towards [HRK] of the motif.

To confirm the validity of the results obtained by the tandem motif based and structural modeling approach, expression of the recombinant AtKUP5 protein fragment was performed and the *in vitro* activity was determined. The AtKUP5$^{573-855}$ fragment generated AMP from the substrate cAMP, thus becoming the first active cAMP-specific PDE discovered in higher plants. The obtained PDE domain was unable to catalyze hydrolysis of cGMP substrate as detected using sensitive liquid chromatography tandem mass spectrometry (LC-MS/MS) method. Moreover, as with cyclases, the activity of this plant PDE is much lower than that of animal PDEs [81,82], which is likely to be related to the generally lower concentrations of cNMP in plant tissues. In order to experimentally confirm the PDE motif, the mutagenesis of the key amino acid K705, towards which cAMP phosphate is pointed, was performed. Mutations of the K705 to A705 or D705 in the wild-type AtKUP5 PDE domain significantly reduced enzyme activity thus indicating a disturbance in the structure of the catalytic domain.

PDEs provide a mechanism for controlling intracellular cAMP and cGMP concentrations and enable termination of cNMP dependent signaling *in vivo*. In animals, both cyclases and PDEs are not evenly distributed, which may result in the formation of cNMP gradients in the cell [83,84]. Due to the nature of plant cyclases and PDEs as small domains within complex proteins, it appears that cNMP signaling can be transiently and dynamically regulated by controlling the localized concentration of cNMP by many proteins with such masked activities. In the context of AtKUP5, a model of functional protein regulation by changes in intracellular cAMP levels was propose, where the PDE has a dampening role on AtKUP5 in K^+ homeostasis. Cyclic AMP produced by the AtKUP5 AC domain can cause a cascade of cell signaling pathways either by activating cAMP-dependent protein kinases that will phosphorylate and thus activate other K^+ channels, or by direct activation of CNGC to directly increasing K^+ or Ca^{2+} transport. The PDE domain, which is activated by the CaM/Ca^{2+} complex serves as a checkpoint to regulate K^+ homeostasis through the hydrolyzation of cAMP to abolish the signal. The discovery of multiple plant PDEs as small domains of larger proteins may indicate that PDEs are not simply a means of terminating a cNMP signal in a linear fashion but may offer an intramolecular regulation of entire protein complexes.

The previously identified cGMP-activated AtCN-PDE1 was discovered using the profile hidden Markov model approach [66]. However, the PDE motif is also present in AtCN-

PDE1. Since plant PDE domains are masked in larger multi-domain proteins, the possibility that such a structure is a common feature had to be considered. To do so, I refined the motif-based approach for plant PDEs domains searches. In my second article entitled "*In search of monocot phosphodiesterases: identification of a calmodulin stimulated phosphodiesterase from Brachypodium distachyon"* [68] the *A. thaliana* PDE ortholog was used to search for PDE center in plants from the monocotyledonous family. To this end, the AtCN-PDE1 sequence was used to query the *Brachypodium distachyon* proteome in the BLAST program. After initial analysis, the BdPDE1 ortholog was identified with 66% identical amino acids. Comparative analysis of the BdPDE1 sequence with its orthologue showed that both proteins belong to the family of YpgQ-like proteins that are members of the highly conserved HD superfamily, acting as 2'-nucleotidase, pyrophosphohydrolase, phosphatase or 2 ', 3'-cyclic phosphodiesterase [85,86]. YpgQ proteins type contain an HD motif that is shared with the family of metal hydrolases and class I PDEs. Therefore, proteins referred to as YpgQ hydrolases may act as putative phosphodiesterases, and this has been confirmed with the AtCNC-PDE protein [66]. BdPDE1 also shows hydrolytic activity towards cGMP, however, a higher affinity was noted for cAMP. Considering the results of the previous publication it can be noted that, as with animals, plant PDEs have a preference for substrates and like different groups of animal PDEs, are able to hydrolyze one or both of the cNMPs. Despite the detected PDE activity in the BdPDE1 protein, the PDE motif was not found in its structure. Of the key amino acids in the original PDE motif [YFW] **H** x [YFW] **R** x {20,40} [**H**RK] [**D**E] reported in publication I, only H122, R125, H155 and D156 are present in the PDE center. Instead of an aromatic amino acid (YFW), alanine (A) and leucine occupy position 1 and 4 of the motif, forming a distinct cavity that dock cAMP or cGMP. The presence of leucin in the formation of the hydrophobic pocket has previously been described for human PDE9 [78]. Moreover, these two amino acids are also conserved in other monocot orthologs. To accommodate the broader identification of PDE active sites in plants, conserved amino acids A and L were incorporated into an expanded, created earlier, PDE motif.

In order to ascertain the correct orientation of the amino acids from the motif in the PDE active site, the BdPDE1 structure was built on the metal-dependent HD domain-containing hydrolase from *Bacillus halodurans* template, and docking was performed with the cAMP substrate. Molecular docking confirmed the correct configuration of amino acids from the PDE motif and their participation in catalysis. Moreover, additional amino acids R233 and Y237 that could also interact with cAMP were found downstream of D156. These amino acids are not

only spatially close to the substrate, but they also orientate toward cAMP at the PDE center, which may imply their key to catalytic functions. The mutagenesis of the amino acid Y237 confirmed the structural assessment, reducing the enzyme activity fivefold. Significantly, it was found that R and Y amino acids also appear downstream in bacterial orthologs, which further justifies their inclusion into an expanded PDE motif. Thus, considering the sequence, biochemical and structural analyses in this study, the second PDE motif ([AYFW] H x [LEYFW] R x {20,40} [HRK] [DE] x {60,90} R x {3} [YFW]) was created. It identified additional 25 putative PDEs in the *B. distachyon* proteome and additional 42 candidates in *A. thaliana*.

Conclusions

The presented doctoral dissertation set out to identify enzymes with cyclic nucleotide phosphodiesterase activity, which have remained elusive in higher plants. The results obtained during the implementation of the above-mentioned studies made it possible to formulate the following final conclusions:

- By construction of a unique consensus search motif based on key amino acids directly involved in the molecular function of ligand binding sites that are conserved in various species it is possible to identify novel plant PDE candidates but also PDEs from other groups of organisms such as bacteria and animals including *H. sapiens*
- Motif-based proteome searches combined with structure visualization and docking simulation is a promising approach to find and establish suitable PDE candidates, prior to taking any experimental steps
- In plants, PDE domains are part of multifunctional complex proteins and could coexists with cyclase domain, acting as an on/off-switch for cyclic nucleotide dependent signaling

The findings of the doctoral thesis have important consequences for signaling mediated by cyclic nucleotides in plants since until recently the mechanism of their degradation remained unknown. Currently, there is an increasing emphasis in plants research on discovering the pathways in which cNMP are involved. With the tools discovered and applied here we are now in a position to detect PDEs even at low levels. Moreover, the discovery of PDE domains existing together with cyclase domains in one protein suggests a novel but highly efficient way to elucidate cNMP dependent cellular signaling both at the molecular and systems level.

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Abstract of PhD thesis entitled: “Phosphodiesterases in higher plants - a missing link in cyclic nucleotide signal transduction”

Signal transduction is a process in which chemical or physical stimuli reaching the cell are registered by specialized sensors and transferred through the cell membrane to the cytoplasmic elements of the effector system. Binding of an extracellular ligand to a receptor triggers a cascade of events of a biochemical and genetic nature leading to a specific physiological response. The process of transmitting information contained in a stimulus activates a series of signal particles. Cyclic nucleotides (cNMP), classified as secondary messengers, are one of such particles. Appearing in the response to receptor activation, they play key roles in many physiological and developmental processes in both prokaryotes and eukaryotes. For many years, signal transduction with the participation of cNMP in plants was controversial, mainly due to the technical impossibility of their determination in plant cells. With the discovery of techniques such as LC-MS/MS, allowing the determination of concentrations of substances at the trace amounts, the position of cNMP as a secondary messenger in plants was stabilized, and the enzymes responsible for their synthesis began to be discovered. However, the enzymes responsible for the degradation of cNMP, phosphodiesterases (PDEs), remained elusive in higher plants. Taking into account the fact that the termination of cNMP signaling is possible only by the action of PDE, I have hypothesized that PDEs are present in plants, and the difficulty in their discovery is related to a structure different from that known for animal PDEs.

Based on the analysis of the sequence and structural properties of the catalytic sites of phosphodiesterases from various organisms, I have developed a unique, universal amino acid sequence that allows the identification of plant PDEs, based on the key amino acids involved in ligand binding and hydrolysis. This led to the discovery of over 30 PDEs in the proteome of the model plant *Arabidopsis thaliana*. The implementation of protein structure visualization and ligand docking simulation techniques allowed to state that plant PDEs are not stand-alone enzymes, but their domains are embedded in larger, multifunctional proteins. By analyzing the structure of PDE orthologs of various plant species in which the original PDE motif was absent and by using a mutagenesis tool, I modified the motif by adding new, unique amino acids involved in PDE catalytic activity. The resulting tool significantly increased the amount of PDEs found in higher plants. The *in vitro* biochemical studies carried out with the use of the LC-MS/MS detection system confirmed the correctness of the PDE motifs, because both

proteins that were tested showed hydrolytic activity against the cyclic nucleotides cAMP and cGMP.

In summary, implementation of the tandem motif based - molecular docking approach, led to a significant expansion of our knowledge about plant PDEs. The analysis of individual candidates shows that plant PDEs, unlike their animal orthologs, are not individual proteins, but small domains embedded in the structure of larger proteins. This distribution of enzymes responsible for cNMP metabolism sheds new light on the different evolution of cNMP metabolism in plants, which in the future will directly contribute to a better understanding of the processes in which these secondary messengers are involved.

Streszczenie rozprawy doktorskiej pt.: „Fosfodiesterazy u roślin wyższych – brakujące ogniwo w transdukcji sygnału cyklicznych nukleotydów”

Transdukcja sygnału jest procesem, w którym bodźce chemiczne bądź fizyczne docierające do komórki są rejestrowane przez wyspecjalizowane sensory i przenoszone przez błonę komórkową na cytoplazmatyczne elementy układu efektorowego. Wiązanie zewnątrzkomórkowego liganda z receptorem wywołuje kaskadę zdarzeń o naturze biochemicznej i genetycznej, prowadzące do swoistej odpowiedzi fizjologicznej. Proces przekazywania informacji zawartej w bodźcu uruchamia szereg cząstek sygnalnych. Cykliczne nukleotydy (cNMP) zaliczane do wtórnych przekaźników informacji, są jednymi z nich. Pojawiając się w odpowiedzi na aktywację receptora, pełnią kluczowe role w wielu procesach fizjologicznych i rozwojowych zarówno u prokariontów i eukariontów. Przez wiele lat transdukcja sygnału z udziałem cNMP u roślin budziła kontrowersje, głównie przez techniczny brak możliwości ich oznaczenia w komórkach roślinnych. Wraz z odkryciem technik takich jak LC-MS/MS, pozwalających oznaczać stężenia substancji na poziomie śladowym, pozycja cNMP, jako wtórnego przekaźnika u roślin została ustabilizowana, a enzymy odpowiedzialne za ich syntezę zaczęły być odkrywane. Jednakże wciąż niewiele było wiadomo o enzymach odpowiedzialnych za degradację cNMP u roślin wyższych - fosfodiesterazach (PDE). Biorąc pod uwagę fakt, że zakończenie sygnalizacji z udziałem cNMP możliwe jest tylko przez działanie PDE, postawiłem hipotezę, że u roślin są obecne PDE, a trudność w ich odkryciu związana jest z odmienną budową niż ta poznana dla PDE zwierzęcych.

W oparciu o analizę sekwencji i właściwości strukturalnych centrów katalitycznych fosfodiesteraz pochodzących z różnych organizmów, opracowałem unikalną, uniwersalną, sekwencje aminokwasową, pozwalającą identyfikować roślinne PDE, opartą o kluczowe aminokwasy zaangażowane w wiązanie oraz hydrolizę ligandu, co pozwoliło na odkrycie ponad 30 PDE w proteomie rośliny modelowej *Arabidopsis thaliana.* Zastosowanie technik wizualizacji struktury białek i symulacji dokowania ligandu pozwoliło na stwierdzenie, że roślinne PDE nie są pojedynczymi enzymami, a domenami wchodzącymi w skład większych, wielofunkcyjnych białek. Poprzez analizę struktury ortologów PDE różnych gatunków roślin, w których nie występował pierwotny motyw PDE oraz zastosowanie narzędzia mutagenezy dokonałem modyfikacji motywu, dodając nowe, unikalne, aminokwasy zaangażowane w aktywność katalityczną PDE. Powstałe w ten sposób narzędzie znacząco zwiększyło ilość odkrytych PDE w roślinach wyższych. Przeprowadzone badania biochemiczne *in vitro* z wykorzystaniem systemu detekcji LC-MS/MS potwierdziły prawdziwość motywów PDE,

ponieważ obydwa białka, które zostały poddane testom wykazywały aktywność hydrolityczną względem cyklicznych nukleotydów cAMP oraz cGMP.

Podsumowując, otrzymane wyniki, w których zastosowano tandemowe podejście motyw-dokowanie molekularne doprowadziły do znacznego poszerzenia naszej wiedzy o roślinnych PDE. Analiza poszczególnych kandydatów pokazuje, że roślinne PDE w odróżnieniu do zwierzęcych ortologów nie są pojedynczymi białkami, lecz są małymi domenami osadzonymi w strukturze większych białek. Takie rozmieszczenie enzymów odpowiedzialnych za metabolizm cNMP rzuca nowe światło na odmienną ewolucję metabolizmu cNMP u roślin, co w przyszłości bezpośrednio przyczyni się do lepszego poznania procesów, w których zaangażowane są te wtórne przekaźniki.

Publication list

1.	**Kwiatkowski, M**.; Wong, A.; Kozakiewicz, A.; Gehring, C.; Jaworski, K. A tandem motif-based and structural approach can identify hidden functional phosphodiesterases. *Comput. Struct. Biotechnol. J*. 2021, 19, 970–975. Doi: 10.1016/j.csbj.2021.01.036 Contribution: 65 %	IF – 7.271 $IF_{5\text{-year}}$ – 7.914 MNiSW – 100
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Communications

A tandem motif-based and structural approach can identify hidden functional phosphodiesterases

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ABSTRACT

Cyclic nucleotide monophosphates (cNMPs) are increasingly recognized as essential signaling molecules governing many physiological and developmental processes in prokaryotes and eukaryotes. Degradation of cNMPs is as important as their generation because it offers the capability for transient and dynamic cellular level regulation but unlike their generating enzymes, the degrading enzymes, cyclic nucleotide phosphodiesterases (PDEs) are somewhat elusive in higher plants. Based on sequence analysis and structural properties of canonical PDE catalytic centers, we have developed a consensus sequence search motif and used it to identify candidate PDEs. One of these is an *Arabidopsis thaliana* K^+-Uptake Permease (AtKUP5). Structural and molecular docking analysis revealed that the identified PDE domain occupies the C-terminal of this protein forming a solvent-exposed distinctive pocket that can spatially accommodate the cyclic adenosine monophosphate (cAMP) substrate and importantly, cAMP assumes a binding pose that is favorable for interactions with the key amino acids in the consensus motif. PDE activity was confirmed by the sensitive liquid chromatography tandem mass spectrometry (LC-MS/MS) method. Notably, this activity was stimulated by the Ca^{2+}/CaM complex, the binding of which to the PDE center was confirmed by surface plasmon resonance (SPR). Since AtKUP5 also has adenylate cyclase (AC) activity that is essential for K^+ transport, we propose that this dual moonlighting AC-PDE architecture, offers modulatory roles that afford intricate intramolecular regulation of cAMP levels thereby enabling fine-tuning of cAMP signaling in K^+ homeostasis.

1. Introduction

In plants, much like in animals, cyclic nucleotide monophosphates (cNMP) and in particular 3′,5′-cyclic adenosine monophosphate (cAMP) and 3′,5′-cyclic guanosine monophosphate (cGMP) are key signaling molecules that trigger many physiological and developmental responses [1,2] and molecular mechanisms notably cyclic nucleotide monophosphate-dependent phosphorylation [3–5]. Despite the growing evidence for the role of cAMP in many plant physiological processes, the molecular mechanism of cAMP metabolism remains poorly understood.

In the animal cell, cNMP homeostasis is achieved by the coordinated action of cyclases responsible for the synthesis and cyclic nucleotide phosphodiesterases (cNMP PDEs), enzymes capable of degrading 3′,5′-cNMPs to inactive 5′- and 3′-NMPs [6]. Cyclic NMPs regulate processes by binding to and activating cNMP effectors, such as cAMP- and cGMP-dependent protein kinases (PKA and PKG) [7] or bind to cyclic nucleotide-gated channels (CNGCs) which are responsible for conducting Ca^{2+} ions in signal transduction [8].

In higher plants, while the cAMP generating enzymes (adenylate cyclases (ACs)) have been identified in recent years, the degrading enzymes (the PDEs) have remained somewhat elusive. To-date, only one PDE which is a cGMP-dependent AtCN-PDE1 (At1g17330), was reported in higher plants. This PDE, which belongs to the HD-domain/PDEase-like protein superfamily with orthologs in bacteria and archaea, responds to UV and is implicated in stomatal movement [9,10].

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Here we propose that there are more PDEs in the *A. thaliana* proteome that remain to be discovered and through a combination of complementary computational and experimental approaches, we present an amino acid consensus search motif that can, in tandem with structural and molecular docking assessments, identify seemingly hidden catalytically active PDE centers in complex proteins.

2. Results and discussion

2.1. Identification of candidate PDEs in A. thaliana

We hypothesized that catalytically active PDE centers in *A. thaliana* exist in complex multifunctional proteins masked by much larger primary domains and are thus undetected by homology searches. In order to detect them, we aligned PDE catalytic centers from annotated PDEs from yeast and animals and extracted a consensus search term (motif) (Fig. 1A). The motif [YFW]Hx[YFW]Rx{20,40}[HRK][DE] was then used to query the *A. thaliana* proteome and retrieved 32 candidates not including splice variants (Supplementary Table S1). This type of motif-based search method has previously identified functional centers of diverse molecular functions in complex proteins [11–13]. Additional details and specific steps (and alignments) for the identification of candidate PDEs are provided in Supplementary Methods. Within these candidates we noted two K^+ transporters which also have AC activities and chose AtKUP5 [14] for further investigation.

2.2. Computational evaluation of a candidate PDE

AtKUP5 contains an AC domain located in the N-terminal cytosolic domain with the catalytic core spanning amino acids from 81 to 96 (Fig. 1A) while the PDE catalytic center is located in the C-terminus like calmodulin binding (amino acids 814 – 834) domain. To probe the PDE center, we used the full length AtKUP5 model for structural assessment and cAMP substrate docking simulations. The motif [YFW]Hx[YFW]Rx{20,40}[HRK][DE] appears at the C-terminal (Y669 – E706) in the cytosolic region, and assumes a "helix-loop" secondary fold (Fig. 1). At the tertiary level, the PDE domain contains a cavity protected by a 'latch' (Fig. 1B). Importantly, cAMP docks at the domain with mean binding affinity of −6.02 ± 0.02 kcal/mol and can assume a binding pose that is spatially favorable for interactions with key amino acids in the motif. We consider a "correct binding pose" an orientation where the adenine head of cAMP points towards [YFW]Hx[FWY]R and the phosphate tail points towards [HRK] of the PDE motif. This rationale was based on the functional roles of corresponding amino acids in annotated plant PDEs i.e., *Physcomitrella patens*, *Marchantia polymorpha* and *Arabidopsis thaliana*, from which the PDE motif was constructed (see Supplementary Methods). In AtKUP5, the phosphate of cAMP points towards K705 and the adenine points towards H670 (Fig. 1C and D) and notably, this binding pose has a 72.2% docking frequency in AtKUP5 (Supplementary Figure S2). Key amino acids in the motif, Y669, H670, F672, R673, K705 and E706 are bolded and, other amino acids in the vicinity deemed capable for interactions with cAMP are labeled accordingly (Fig. 1C and D).

We also assess a close homolog of AtKUP5, AtKUP7, that also harbors a PDE center. AtKUP5 shares 73.8% identity with AtKUP7 and assumes a similar structural fold with AtKUP5 at the PDE center. The PDE center is located at the C-terminal (Y668 – E705) in the cytosolic region and assumes a "helix-loop" secondary fold, while at the tertiary level, the PDE domain contains a cavity protected by a 'latch' much like in AtKUP5. Importantly, cAMP docks at the domain with a mean binding affinity of −6.72 ± 0.01 kcal/mol and assumes a positive binding pose frequency of 94.4%. The key amino acids in the motif, Y668, H669, F671, R672 and E705 also appear in AtKUP7 with the exception that K705 in AtKUP5 is replaced by R704 in AtKUP7 (Supplementary Figure S2).

Further, we examined two Arabidopsis proteins AtLARP1 (At5g21160) and AtKING1 (At3g48530) which were among seven PDE candidates identified by the PDE motif [YFW]Hx[YFW]Rx{20, 40}[HRK][DE] (Supplementary Methods). Docking data and representative structures of AtLARP1A and AtKING1 PDE centers docked with cAMP are presented in Supplementary Figure S3. Unlike AtKUP5 and AtKUP7, these two PDE candidates do not contain a "cleft-latch" like structure but the PDE motif occupies clear cavities that spatially accommodate cAMP with mean binding affinities of −4.60 ± 0.06 kcal/mol for AtLARP1A and −5.14 ± 0.34 kcal/mol for AtKING1, respectively. Structural assessments also indicate that the gap x{20,40} between [YFW]Hx[YFW]R and [HRK] in the PDE motif, might be narrower in AtLARP1A and AtKING1. For instance, in AtLARP1A, the phosphate end of cAMP appears to be interacting with K802 which is only 17 amino acids apart while in AtKING1, this gap is only 12 (Supplementary Figure S3). Future experimental validations of candidates with different structural folds than AtKUP5 will guide refinement of the existing PDE motif.

Computational analysis suggests that the PDE motif appears in structurally diverse proteins with varying primary functions e.g., K^+ transport, RNA-binding and kinase; however, they could accommodate cAMP substrate with good binding affinities (mean free energies ranging from −4.60 kcal/mol for AtLARP1A to −6.72 ± 0.01 kcal/mol for AtKUP7) within clear cavities. We propose that the PDE motif identifies catalytic centers that operate as modulators at moonlighting sites in their respective proteins of varying primary functions [15] and it is therefore conceivable that they assume diverse structural folds which are different than the existing classes of PDEs. We note that since the strength of this motif-based approach is to enable the identification of PDE centers hidden within complex proteins which would otherwise be undiscovered through standard homology approaches, experimental validations will be required to ascertain the functionality, substrate selectivity and catalytic rates of candidates identified through this approach.

2.3. Biochemical evaluation of PDE activity in AtKUP5

PDE catalytic activity was assessed by enzymatic assays with cAMP as the substrate and the AMP product was detected and quantified using the sensitive liquid chromatography tandem mass spectrometry (LC-MS/MS) method (Fig. 2A). AtKUP5$^{573\text{-}855}$ generates AMP from the substrate cAMP with a V_{max} of 1.17 pmole AMP min^{-1} μg $protein^{-1}$ (0.25 mmol AMP s^{-1} mol $protein^{-1}$), and a K_m of 5.35 μM. Cyclic GMP is not a substrate for AtKUP5$^{573\text{-}855}$ (Supplementary Figure S1). Comparing the obtained results with other plant PDEs, we noted that AtKUP5 has the lowest rate e.g., the PDE of MpCAPE has 100x higher activity [16] while the V_{max} of AtCN-PDE1 is 50x greater [9].

We also assessed the effect of Ca^{2+} and CaM isoforms derived from *A. thaliana* and bovine brain on the PDE activity. All CaM isoforms stimulate cAMP hydrolysis, after the formation of the active CaM/Ca^{2+} complex (Fig. 2C) where four calmodulin EF-hand motifs bind single Ca^{2+} ion [17]. The addition of the active CaM/Ca^{2+} complex causes increases in the V_{max} (1.78 pmole min^{-1} μg $protein^{-1}$) and the affinity of the enzyme for the substrate (K_m 4.24 μM) (Fig. 2D). The formation of the active CaM/Ca^{2+} complex is not possible when using EGTA, which chelates Ca^{2+} ions. This results in no noticeable increase in PDE activity despite the addition of CaM/Ca^{2+}.We then used surface plasmon resonance (SPR) to assess the binding affinity of CaM to PDE. Fig. 2E shows the sensogram for interactions between immobilized GST-AtKUP5$^{573\text{-}855}$ and CaM9 without, and in the presence of, Ca^{2+} ions. In both cases, an increase

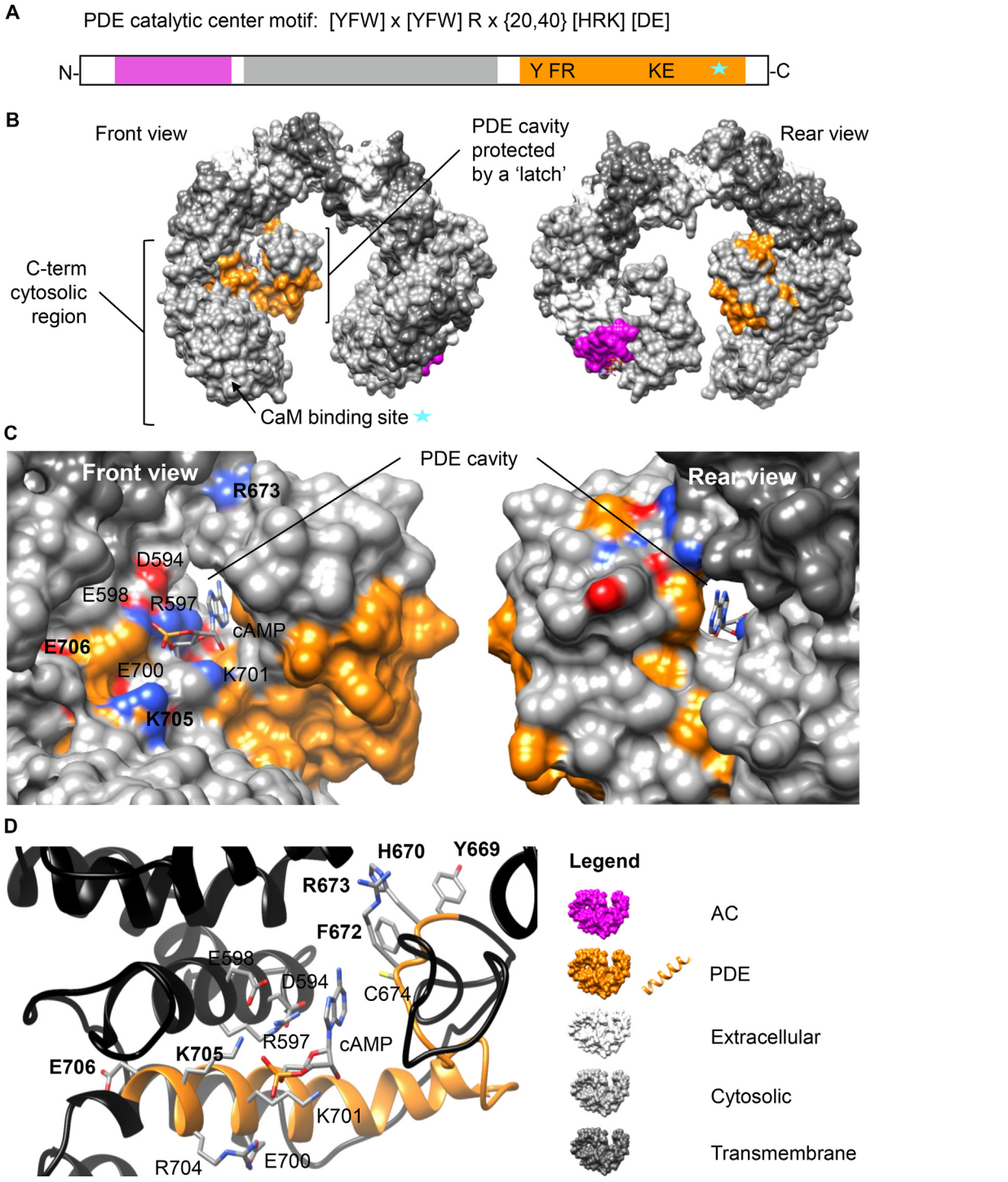

Fig. 1. Computational assessment of the PDE center in AtKUP5. (A) The amino acid motif of annotated and experimentally tested PDE catalytic centers. The following box diagram presents the amino acid sequence of the AtKUP5 K^+ transporter encoding a PDE and AC. The PDE domain is located in the cytosolic C terminal region and is shown in orange, with critical residues of the motif and calmodulin binding domain (blue star). The AC catalytic center is located in the cytosolic N terminal region and is shown in purple. The 12 transmembrane domains are shown in gray. (B) The full-length AtKUP5 model showing the location of the PDE domain at the solvent-exposed cytosolic C-terminal region. Dockings and interactions of cAMP with key residues at the PDE domain of AtKUP5 are shown as (C) surface and as (D) ribbon models respectively. The PDE domain is highlighted in orange and the amino acid residues implicated in interactions with cAMP are colored according to their charges in the surface models and shown as individual atoms in the ribbon model. Key amino acids in the PDE motif [YFW]Hx[YFW]Rx{20,40}[HRK][DE] are bolded and other amino acids in the vicinity capable for interaction with cAMP are labeled accordingly. The full-length AtKUP5 structure was modeled against the AtKUP7 template [28] using the MODELLER (ver. 9.25) software [29]. cAMP docking simulations were performed using AutoDock Vina (ver. 1.1.2) [30]. Molecular graphics and analyses were performed with the UCSF Chimera package [31]. Orientations and substrate binding poses were analyzed with the UCSF Chimera package [31]. Chimera was developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIGMS P41-GM103311). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

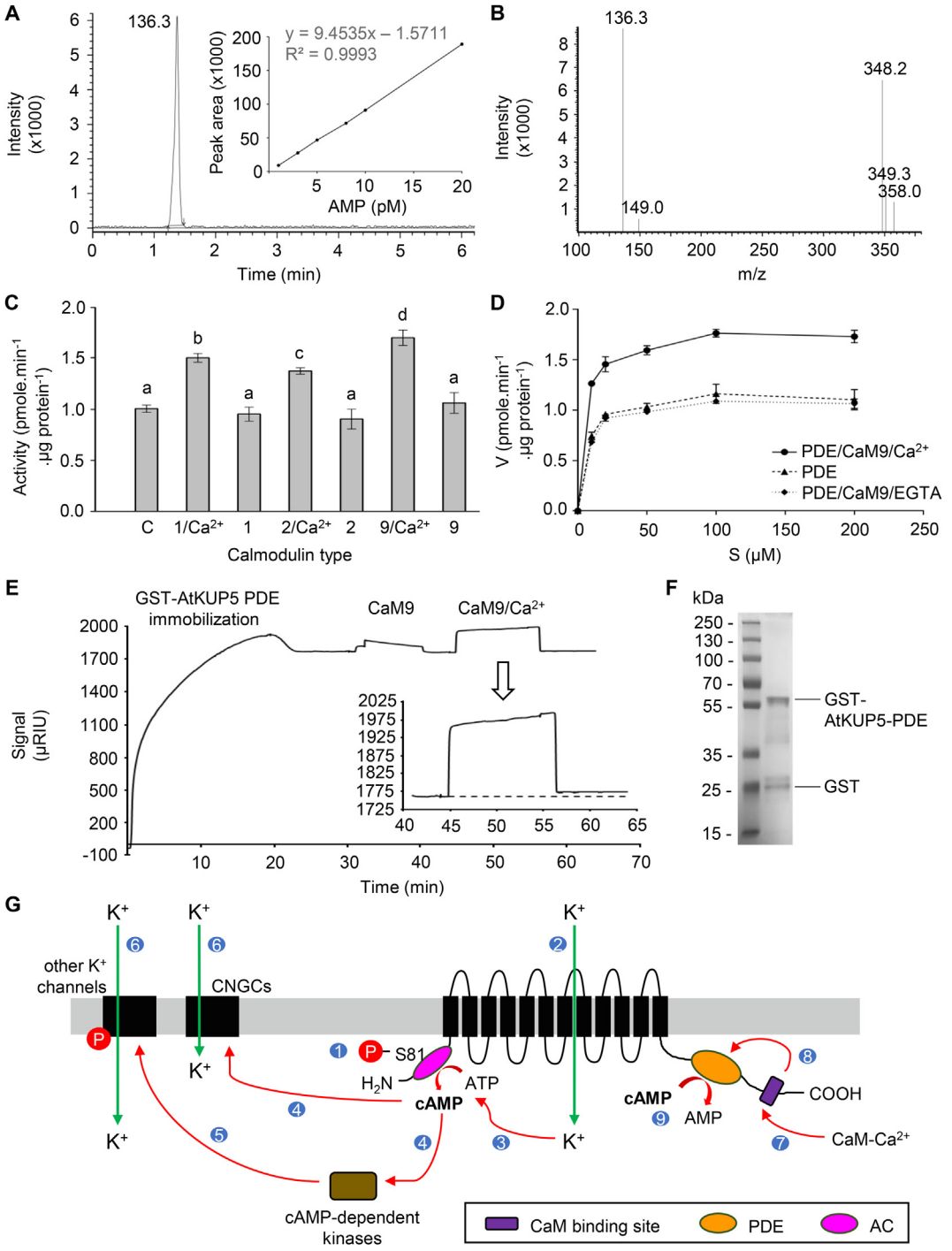

Fig. 2. PDE activity of AtKUP5 and a model for the dampening role of PDE in K^+ homeostasis. (A) Ion chromatogram of AMP together with calibration curve. (B) Inset showing the parent AMP ion at *m/z* 348.20 $[M + H]^+$ and the corresponding fragmented daughter ion at *m/z* 136.30 $[M + H]^+$. The fragmented product ion was used for quantitation. (C) GST-AtKUP5$^{573\text{-}855}$ activity in the presence of various CaM isoforms, with or without addition of Ca^{2+} ions. Data are mean values (n = 3) and error bars show standard error of the mean. Statistical analysis was performed by one-way ANOVA followed by a Tukey–Kramer multiple comparison test. Different letters indicate significantly different data. (D) Michaelis-Menten plot of the phosphodiesterase activity of GST-AtKUP5$^{573\text{-}855}$, in the presence of CaM9/Ca^{2+} and CaM9/Ca^{2+} with addition of an ion chelating compound EGTA, that is intended to prevent the formation of the CaM active complex. Values indicate means ± SD (n = 3). (E) Binding analysis of CaM9 to GST-AtKUP5$^{573\text{-}855}$ on the surface of gold discs covered with glutathione. The increasing µRIUs reflect the mass change on the surface of modified gold disc, i.e., the binding of the GST-AtKUP5$^{573\text{-}855}$, CaM9, and CaM9/Ca^{2+} complex, respectively. The signal rapidly increases and then plateaus as the system reaches equilibrium. When the injection ends, the CaM9 and CaM9/Ca^{2+} complex dissociates, resulting in a decrease in the signal. (F) The purified GST-AtKUP5$^{573\text{-}855}$ protein (3 µg) was analyzed by SDS-PAGE. (G) A model for the dampening role of PDE in the regulation of K^+ transport. It was previously speculated that phosphorylation of S81 (1), Y92, T75 and/or S76 located within or close to the AC center of AtKUP5, activates its K + transport activity (2). K^+ uptake will in turn stimulate the AC activity of AtKUP5, converting ATP into the second messenger cAMP [14] (3). cAMP will orchestrate a cascade of cellular signaling pathways through the activation of cAMP-dependent protein kinases (4) which can phosphorylate and thus activating, other K^+ channels (5), thereby further increasing cytosolic K^+ (6). Alternatively, cAMP can also activate cyclic nucleotide gated channels (CNGCs) (4) to directly increase the transport of K^+ into the cell (6). Ca^{2+} activated calmodulin (CaM-Ca^{2+}) can bind to the C-terminal of AtKUP5 (7) and activates the activity of the PDE (8) (shown in this study), converting cAMP into AMP. Degradation of cAMP by PDE dampens the cAMP-mediated signaling processes (9) that include K^+ transport.

in signal was observed, but for the binding of CaM/Ca^{2+} complex the increasing μRIUs was higher and signal amplitude did not decline to the baseline level after sample washing (11 μRIU difference). This implies that the CaM/Ca^{2+} complex binds to AtKUP5$^{573-855}$.

2.4. Conclusion and outlook

Considering our current findings in the context of the whole protein and its broader cellular signaling functions, we propose a model (Fig. 2G) where the PDE has a dampening role on AtKUP5 in K^+ homeostasis. It was previously speculated that phosphorylation of S81, Y92, T75 and/or S76 located within or close to the AC center of AtKUP5, activates its K^+ transport activity [18–21]. K^+ uptake will in turn stimulate the AC activity of AtKUP5, converting ATP into the second messenger cAMP [14]. Cyclic AMP will orchestrate a cascade of cellular signaling pathways either, 1) through the activation of cAMP-dependent protein kinases which can phosphorylate and thus activate other K^+ channels thereby further increasing cytosolic K^+ [22] or, 2) by activating cyclic nucleotide gated channels (CNGCs) to directly increase the transport of K^+ into the cell [23–25]. Ca^{2+} activated calmodulin (CaM-Ca^{2+}) is predicted to bind at the C-terminal of AtKUP5 which in turn activates the PDE (Fig. 2C-E), thus affecting K^+ net flux and homeostasis (Fig. 2G).

In conclusion, we have constructed a consensus search motif for the identification of candidate PDEs based on key amino acids directly involved in the molecular function of ligand binding sites that are conserved in various species. This method has identified novel PDE candidates in *A. thaliana*. It appears that many PDEs, much like ACs, are part of multifunctional multi-domain proteins. One of the candidates is AtKUP5 which has been previously shown to have AC activity essential for its role as a K^+ transporter. Incidentally, a similar domain combination, AC-PDE, was recently reported in the liverwort *Marchantia polymorpha* [16]. It is thus conceivable that this domain architecture represents an ancient signaling module where one protein can transiently and dynamically regulate localized cAMP concentration. Taken together, our results have assigned the PDE center in AtKUP5 to a dampening role of the cAMP signal and we propose that this dual AC-PDE architecture, affords intricate control and concomitantly also the fine-tuning of cellular cAMP signaling and in the broader K^+ homeostasis.

3. Material and methods

3.1. Construction of a PDE consensus sequence motif

For the identification of *A. thaliana* candidate PDEs we used a method based on motif searches as described previously [26,27]. The motif was built from an alignment of key residues in the catalytic center of annotated PDEs from different species. The motif ([YFW]Hx[YFW]Rx{20,40}[HRK][DE]) was used to query Swiss-Prot and PatMatch function in The Arabidopsis Information Resource (TAIR). Amino acids between square brackets are those that are allowed in a position, "x" stands for any amino acid and curly brackets delineate the number of undetermined amino acids. Detailed description and steps for the construction of the PDE motif are provided in Supplementary Methods. The calmodulin binding site was predicted using Calmodulin Target Database at Cellular Calcium Information Server (http://calcium.uhnres.utoronto.ca/).

3.2. Structural analysis of the PDE center in AtKUP5

The full-length AtKUP5 structure was modeled against the AtKUP7 template as described in [28] using the MODELLER (ver. 9.25) software [14,28,29]. Docking simulations of cAMP to the PDE domain of AtKUP5 was performed using AutoDock Vina (ver. 1.1.2) [30]. In docking simulations, all bonds in the cAMP ligand were allowed to move freely but the protein was set rigid. Docking orientations of cAMP were evaluated based on a pre-determined "correct binding pose" where the orientation of cAMP deemed favorable for catalysis is presumably to be as follows: adenine head pointing towards [YFW]Hx[FWY]R and the phosphate tail pointing towards [HRK] of the PDE motif. This rationale was based on the functional roles of corresponding amino acids in annotated plant PDEs i.e., *Physcomitrella patens*, *Marchantia polymorpha* and *Arabidopsis thaliana*, from which the PDE motif was constructed (Supplementary Methods). Docking simulations consider both spatial and charge at the vicinity of the catalytic center based on predetermined grids that cover the entire PDE center and can afford free rotation of cAMP substrate which we have been set prior to docking experiments. The cAMP docking poses were analyzed, and all images created by UCSF Chimera (ver. 1.10.1) [31]. Chimera was developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIGMS P41-GM103311). All 3D structures and the PDE domains of AtKUP7, AtLARP1A and AtKING1 were generated in the same manner by homology modeling using the MODELLER (ver. 9.25) software [29] and docked to cAMP using AutoDock Vina (ver. 1.1.2) [30]. All structures, binding poses, and images were analyzed and created using UCSF Chimera (ver. 1.10.1) [31].

3.3. PDE biochemical assay and LC-MS/MS detection

PDE *in vitro* activity was determined by using LC-MS/MS to determine the rate of AMP formation. The reaction mixture contained: 3 mM Tris-HCl (pH 8.0), 0,1 mM cAMP, 0.1% (v/v) 2-mercaptoethanol, 5 μg of GST-AtKUP5$^{573-855}$, 0.5 mM $MgCl_2$ and $MnCl_2$. To check if calmodulin regulates the PDE activity, three different CaM isoforms (*A. thaliana* CaM1, CaM9 and bovine CaM2) were added to the reaction in the concentration of 2 μM and the GST-AtKUP5$^{573-855}$ protein concentration was 0,625 μM. Samples were incubated at 37 °C for 25 min. The enzyme reaction was terminated by incubation at 100 °C for 10 min and the samples were centrifuged at 13,200 × g for 10 min.

LC-MS/MS experiments were performed using the Nexera UHPLC and LCMS-8045 integrated system (Shimadzu Corporation). The ionization source parameters were optimized in positive ESI mode using pure AMP dissolved in HPLC-grade water (Sigma). Samples were separated using a ReproSil Star 100 ZIK HiliC column (150 × 2 mm, 3 μm, Dr. Maisch GmbH). A gradient of solvent A (0.1% (v/v) formic acid with 25 mM ammonium formate) and solvent B (100% (v/v) acetonitryle) was applied over 6 min: B: 95% − 35% with a flow rate of 0.6 mL/min. The interface voltage was set at 4.0 kV for positive (ES +) electrospray. Data acquisition and analysis were done with LabSolutions workstation for LCMS-8045.

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CRediT authorship contribution statement

Mateusz Kwiatkowski: Conceptualization, Methodology, Investigation, Writing - original draft, Funding acquisition.

Aloysius Wong: Conceptualization, Formal analysis, Visualization, Funding acquisition, Writing - original draft, Writing - review & editing. **Anna Kozakiewicz:** Methodology, Investigation. **Christoph Gehring:** Conceptualization, Writing - original draft, Writing - review & editing, Supervision. **Krzysztof Jaworski:** Conceptualization, Writing - original draft, Writing - review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.csbj.2021.01.036.

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Publication II

In Search of Monocot Phosphodiesterases: Identification of a Calmodulin Stimulated Phosphodiesterase from *Brachypodium distachyon*

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In Search of Monocot Phosphodiesterases: Identification of a Calmodulin Stimulated Phosphodiesterase from *Brachypodium distachyon*

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Abstract: In plants, rapid and reversible biological responses to environmental cues may require complex cellular reprograming. This is enabled by signaling molecules such as the cyclic nucleotide monophosphates (cNMPs) cAMP and cGMP, as well as Ca^{2+}. While the roles and synthesis of cAMP and cGMP in plants are increasingly well-characterized, the "off signal" afforded by cNMP-degrading enzymes, the phosphodiesterases (PDEs), is, however, poorly understood, particularly so in monocots. Here, we identified a candidate PDE from the monocot *Brachypodium distachyon* (BDPDE1) and showed that it can hydrolyze cNMPs to 5′NMPs but with a preference for cAMP over cGMP in vitro. Notably, the PDE activity was significantly enhanced by Ca^{2+} only in the presence of calmodulin (CaM), which interacts with BDPDE1, most likely at a predicted CaM-binding site. Finally, based on our biochemical, mutagenesis and structural analyses, we constructed a comprehensive amino acid consensus sequence extracted from the catalytic centers of annotated and/or experimentally validated PDEs across species to enable a broad application of this search motif for the identification of similar active sites in eukaryotes and prokaryotes.

Keywords: phosphodiesterase (PDE); cAMP; cGMP; calmodulin (CaM); calcium ions; protein–protein interactions; *Brachypodium distachyon*

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1. Introduction

Cyclic nucleotide monophosphates (cNMPs), such as 3′,5′-cyclic adenosine monophosphate (cAMP) and 3′,5′-cyclic guanosine monophosphate (cGMP), are well-established as essential signaling and effector molecules in both prokaryotes and eukaryotes [1,2]. The presence and physiological relevance of cyclic nucleotides in plants was controversial for a long time because of their low concentrations as compared to animals (for review, see Reference [3]). However, recent evidence has established cNMP-dependent processes in plants ranging from signaling to the control of transcription, translation and metabolism [4–8] The cyclic NMP levels are dependent on the activities of two key enzymes, the cyclic mononucleotide cyclases and cyclic mononucleotide phosphodiesterases (PDEs). Adenylyl (AC) and guanylyl (GC) cyclases catalyze the conversions of ATP and GTP to the respective products cAMP and cGMP. Cyclic AMP and cGMP, in turn, serve as "on signals" for cNMP-dependent cellular processes. Consequently, the "off signal", the hydrolysis of cAMP or

cGMP to AMP or GMP, is enabled by PDEs that convert cNMP into 5′NMP by hydrolyzing the 3′-phosphodiester bonds [9]. Although cNMPs and plant nucleotide cyclases are increasingly recognized as essential components of many plant functions [10–15], our understanding of plant PDEs is still scant. To date, only two proteins in dicotyledonous [16,17] and one in liverwort [18] have been reported to have PDE activity, and notably, no PDE has yet been found in monocotyledonous plants. Our knowledge of the physiological processes in which cNMP participate is still expanding, and the discovery and characterization of novel PDEs will yield insights into the complex functions of cNMP-dependent processes at the molecular and systems levels.

In plants, cAMP and cGMP signaling may be tuned by Ca^{2+}, ROS (reactive oxygen species) or NO (nitric oxide) [1], and interactions between Ca^{2+} and cNMP have been reported to modulate physiological processes, e.g., via cyclic nucleotide-gated channels (CNGC) that can be considered intersections of cNMP and Ca^{2+} signaling [19]. Crosstalk between Ca^{2+} and cNMP can also occur via nucleotide cyclases, as seen in phytosulfokine (PSK) signaling, where the GC activity of the hormone receptor (PSKR1) is significantly enhanced by Ca^{2+} [20]. In the case of plant ACs, there is, as yet, no experimental evidence of interactions with Ca^{2+}, but it is well-documented that animal ACs are directly and indirectly regulated by Ca^{2+} and/or calmodulin [21]. Considering the various regulatory regions of animal PDEs, especially in group 1 phosphodiesterases (PDE1), which are allosterically regulated by calmodulin [22], it is likely that Ca^{2+} may also modulate the plant PDE activity and, hence, contribute to cNMP homeostasis. In view of recent reports on plant PDEs, it may turn out that they act as intramolecular regulators moonlighting in complex multifunctional proteins [17,18].

In this study, we identified and characterized the activity of a candidate PDE (BDPDE1) from the monocot *Brachypodium distachyon* and then derived a comprehensive and inclusive search term (amino acid motif) based on the catalytic centers of annotated PDEs across species to enable the discovery of similar PDE centers that might be hidden in complex multidomain proteins, particularly in plants, where investigations on the mechanisms that govern cNMP metabolism are still in their infancy.

2. Results and Discussion

2.1. Identification and Characterization of PDE Activity in BDPDE1

Here, we set out to discover the as yet elusive monocot phosphodiesterases that are key to the regulation of the cAMP and cGMP levels in the cell. First, we explored if the *Arabidopsis thaliana* PDE ATCN-PDE1 (At1g17330) [16] had any orthologs in the monocot *B. distachyon*. When the sequence of ATCN-PDE1 was used to query the *B. distachyon* proteome, we found an ortholog, BDPDE1 (NCBI: XP_003574089.2), with 66% identical amino acids covering 69% of the protein. A comparative analysis of the BDPDE1 sequence with its ortholog showed that both proteins belong to the family of YpgQ-like proteins, which are members of the highly conserved HD superfamily (Pfam 01966). This group of enzymes exhibits broad substrate specificity, acting as a 2′-nucleotidase, pyrophosphohydrolase, phosphatase or 2′,3′-cyclic phosphodiesterase [23–25]. YpgQ-like proteins contain an HD motif, which is shared with the family of metal hydrolases and class I PDEs; therefore, proteins referred to as YpgQ hydrolases can function as putative phosphodiesterases. This is consistent with the fact that ATCN-PDE1, which participates in the opening of stomata in response to UVA, reduces the pool of cGMP [16].

The functional evaluation of the catalytic activity of the candidate PDE was done in vitro by enzymatic assays using cAMP and/or cGMP as a substrate. Reaction products AMP and GMP were detected and quantified using the sensitive liquid chromatography tandem mass spectrometry (LC-MS/MS) method (Figure 1A,B). BDPDE1 generates both AMP and GMP as a result of the reaction; however, BDPDE1 shows a higher affinity towards cAMP (Figure 1C). The V_{max} for cAMP as a substrate was 2.45-nmol AMP min^{-1} mg $protein^{-1}$ and a K_M of 0.0115 mM, whereas in the case of cGMP as a substrate, the V_{max} was 1.01-nmol GMP min^{-1} mg $protein^{-1}$, and the affinity for cGMP was almost 10-

fold lower (K_M of 0.1133 mM). The activities obtained by BDPDE1 were somewhat lower compared with those of the *A. thaliana* ortholog (V_{max} 58.22-nmol min^{-1} mg $protein^{-1}$ and K_M 0.0258 mM) [16] and comparable to the *A. thaliana* K^+ transporter ATKUP5 (V_{max} 1.17-nmol AMP min^{-1} mg $protein^{-1}$ and K_M 0.0053 mM) [17], which also contained a moonlighting PDE domain.

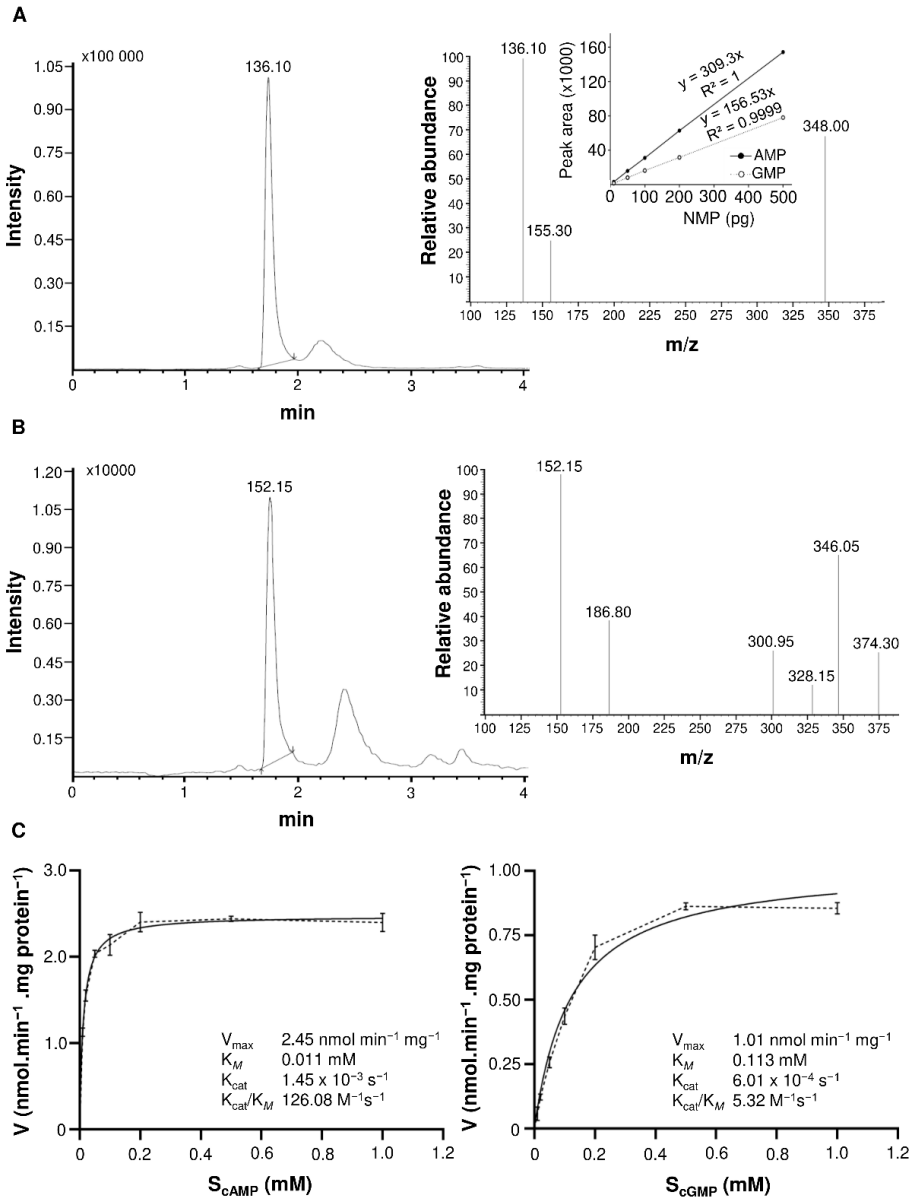

Figure 1. Kinetic parameters of BDPDE1. (**A**) Ion chromatogram of AMP with the inset showing the daughter AMP ion at *m/z* 136.10 $[M + H]^+$. The fragmented product ion was used for quantitation of the phosphodiesterase activity. Inside is a calibration curve for AMP and GMP. (**B**) Ion chromatogram of GMP with the inset showing the daughter GMP ion at *m/z* 152.15 $[M + H]^+$. The fragmented product ion was used for quantitation of the phosphodiesterase activity. (**C**) Michaelis–Menten plots for the cyclic nucleotide phosphodiesterase activity of BDPDE1. The V_{max} for a cAMP substrate was 2.45-nmol AMP min^{-1} mg $protein^{-1}$ and a K_M of 0.011 mM, respectively. The V_{max} for a cGMP substrate was 1.01-nmol GMP min^{-1} mg $protein^{-1}$ and a K_M of 0.113 mM, respectively. The reaction time was 25 min, and the standard reaction mixture contained: 3-mM Tris-HCl (pH 8.0), cAMP or cGMP in a concentration ranging from 0.01 mM to 1 mM, 0.1 % (*v/v*) 2-mercaptoethanol, 5 µg of GST-BDPDE1 and 0.5-mM $MgCl_2$ and $MnCl_2$ in a final volume of 100 µL.

Next, we characterized the influence of temperature, ion cofactors and the PDE inhibitor IBMX (3-Isobutyl-1-methylxanthine) on the catalytic properties of the enzyme. The enzymatic activity was tested in the temperature range from 10 °C to 45 °C. As the temperature increased, a significant increase in activity was noticeable, tailing off at 37 °C (Figure 2A). These results are comparable with the experiments carried out on the cGMP-stimulated PDE from calf livers [26], where the highest catalytic efficiency was achieved at 37 °C. Subsequently, enzymatic reactions were carried out in the presence or absence of the cofactors 0.5-mM Mg^{2+} and 0.5-mM Mn^{2+}. Divalent cations stimulated the enzymatic activity (Figure 2B), and the addition of both the Mg^{2+} and Mn^{2+} ions resulted in the PDE reaching the highest activity. In the case of animal PDEs, there are structural reasons for the dependence on two metal ions: the coordinated conserved histidine and aspartate residues that interact with Zn^{2+} and the weaker binding of Mg^{2+} in the catalytic pocket [27,28]. It appears that, in BDPDE1, the role of the metal ion is taken over by Mn^{2+}, since, in its presence, the enzyme has >3-fold higher activity than in the presence of Mg^{2+}. A similar effect was observed previously in MPCAPE-PDE (*Marchantia polymorpha*), where among the tested ions, the enzymatic activity was higher in the presence of Mn^{2+} [18]. Furthermore, the use of a nonselective PDE inhibitor IBMX at a concentration of 50-µM reduced the enzymatic activity two-fold.

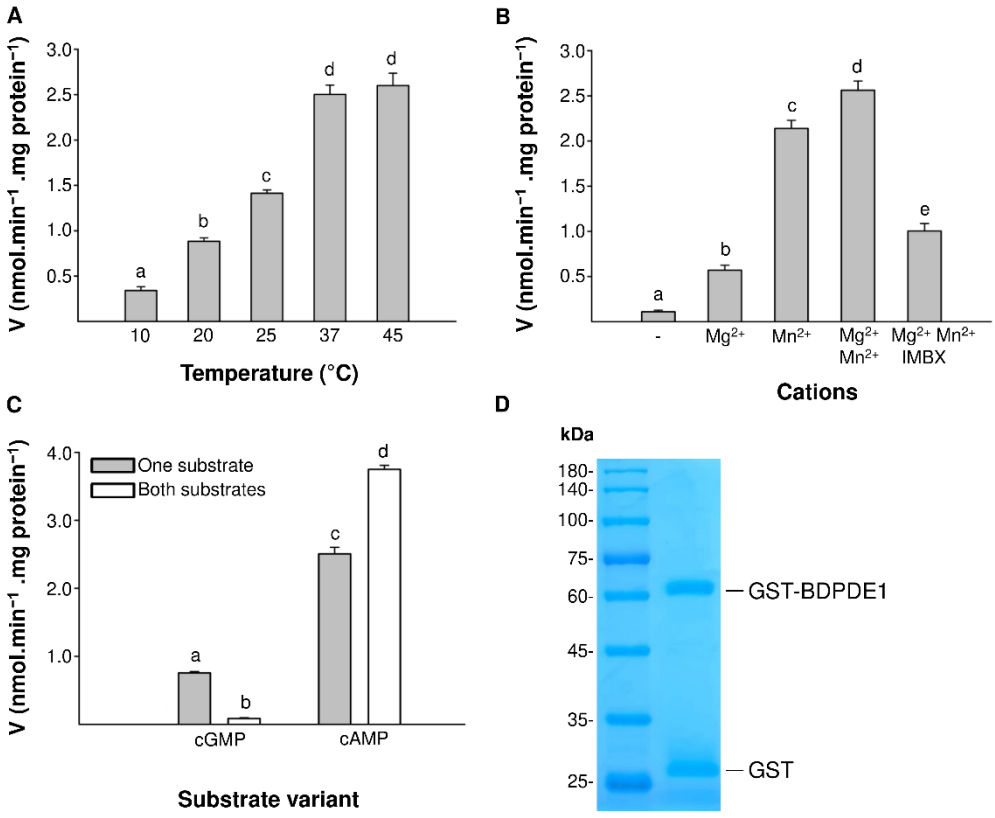

Figure 2. Characterization of the biochemical properties of BDPDE1. (**A**) Influence of temperature on the PDE activity. The enzymatic activity was tested in the temperature range from 10 °C to 45 °C. The reaction time was 25 min in the standard reaction mixture containing 0.1-mM cAMP and 5 µg of GST-BDPDE1. (**B**) Effect of divalent cations and inhibitor on the PDE activity of GST-BDPDE1. The reaction was carried out in the standard reaction mixture containing 0.1-mM cAMP, 5 µg of GST-BDPDE1 and, depending on the reaction variant, 0.5-mM $MgCl_2$ and/or $MnCl_2$, and 50-µM IMBX was added. (**C**) Substrate specificity of GST-BDPDE1. The reaction was performed in the presence of 5 µg of GST-BDPDE1, and depending on the substrate variant, 0.1-mM cAMP and/or 0.1-mM cGMP was added. In all the error bars, different letters indicate significant differences at $p < 0.05$. (**D**) The purified GST-BDPDE1 protein (4 µg) was analyzed by SDS-PAGE.

The kinetics of BDPDE1 showed that the enzyme is capable of hydrolyzing both cAMP and cGMP. To check if there is a competition of cAMP and cGMP at the catalytic core of the PDE domain, assays measuring the BDPDE1 activity in the presence of two substrates simultaneously were performed, both in the same 0.1-mM concentrations. BDPDE1 favored the cAMP substrate, having a greater V_{max} and affinity for it. Interestingly, when both substrates are present in the reaction mixture, the activity of this enzyme towards cAMP increases >1.5-fold (Figure 2C). At the same time, the cGMP hydrolysis is negligible, decreasing nine-fold compared to the base value. The activity of cAMP hydrolysis was activated by the addition of cGMP, which is similar to a rat PDE2, where cGMP binds to an allosteric site regulating the enzymatic activity [29,30]. These results indicate that, while acting primarily as a plant cAMP PDE, BDPDE1 may also function as a cAMP-inhibited cGMP PDE. It is therefore conceivable that cAMP-mediated signal transduction can also cross-regulate the signaling strength of cGMP.

2.2. The Calmodulin/Ca^{2+} Complex Stimulates BDPDE1 Activity

Calcium-dependent cellular processes are regulated through intracellular Ca^{2+}-binding proteins, of which the best-studied are calmodulin (CaM), calmodulin-like proteins (CMLs) and calcium-dependent protein kinases (CDPKs). These proteins bind Ca^{2+} ions through the EF hand motif, a conserved helix–loop–helix structure that binds a single Ca^{2+} ion, thereby causing a change in the conformation that, in turn, can activate target proteins or cause self-activation [31–34]. Since it was reported that CaM/Ca^{2+} affects the activity of group I animal PDEs and the PDE domain of the ATKUP5 [17,35] and that a protein sequence analysis revealed that BDPDE1 also contains a predicted CaM-binding site between amino acids 50 and 70, we investigated the possible interactions of CaM and CaM-like isoforms with plant PDE using fluorescence spectroscopy. Due to the high structural similarities of CaM isoforms, we chose one representative of the CaM, using CaM1 and CaM-like isoform 9 (CML9). The BDPDE1 fluorescence spectra in the presence of CaM1 and CML9 at 37 °C are shown in Figure 3. The control emission spectra of BDPDE1, the buffer (TRIS), CaM1 and CML9 are shown in Supplementary Figure S1.

The results demonstrated that, at an excitation with 280 nm, BDPDE1 has a distinct peak of fluorescent emission at 333 nm deriving from tryptophan residues. CaM1 and CML9 lack tryptophan residues and instead contain tyrosine residues, which makes it possible to study their interaction with BDPDE1. We found that the plant PDE fluorescence intensity increases with the increasing concentration of CaM1 and CML9, while the maximum emission wavelength does not change. The results are indicative of an interaction between investigated CaM1 and CML9 with BDPDE1. The fluorescence data of the formation of the BDPDE1-CaM1 and BDPDE1-CML9 complexes were analyzed using the following equation assuming a 1:1 stoichiometry [36]:

$$\frac{PL}{[P]_t} = \frac{[P]_t + [L]_a + K_d - \sqrt{([P]_t + [L]_a + K_d)^2 - 4[P]_t[L]_a}}{2[P]_t} \quad (1)$$

where K_d is the dissociation constant, $[P]_t$ is the concentration of the protein, $[L]_a$ is the total concentration of the ligand and $[PL]$ is the concentration of the protein–ligand complex. The obtained fluorescence data were fitted to the one-site binding model with an applied nonlinear least-squares regression using OriginPro software (Figure 3).

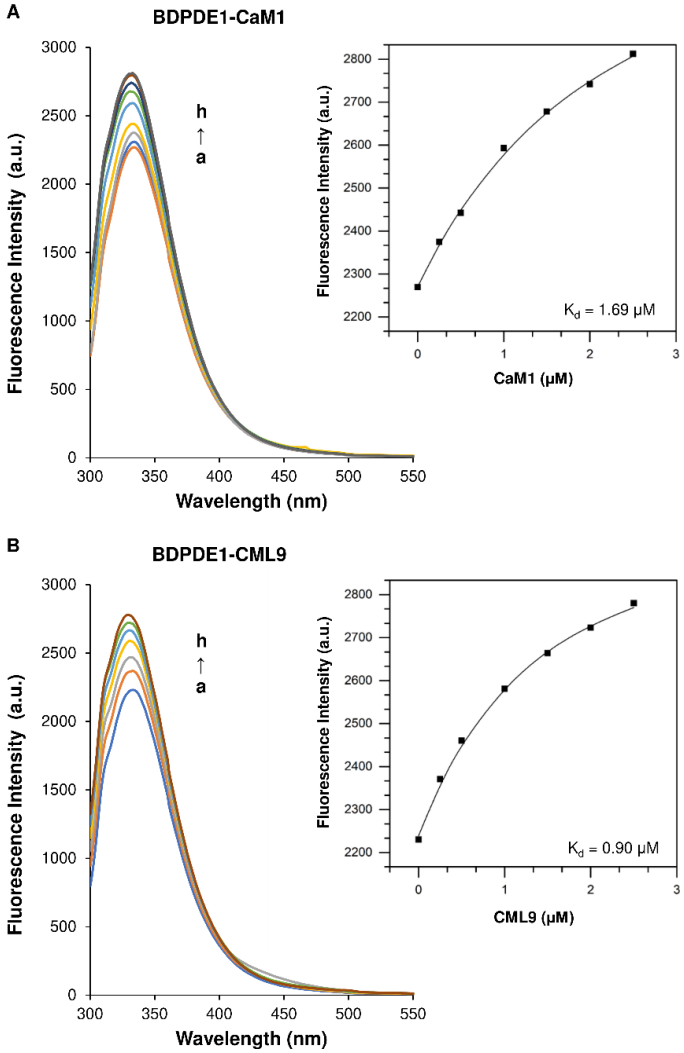

Figure 3. The fluorescence data of BDPDE1 in the presence of (**A**) CaM1 and (**B**) CML9 at 37 °C. (left). Emission spectra of BDPDE1 in the presence of CaM1 and CML9 (right) and the dependence of the fluorescence intensity on the CaM1 and CML9 concentrations. The concentration of BDPDE1 was 0.5-μM. The concentrations of CaM1 and CML9 from "a" to "h" were 0, 0.25, 0.5, 1.0, 1.5, 2.0 and 2.5 μM, respectively.

Our studies revealed that CaM1 and CML9 strongly bind to BDPDE1, as evidenced by the binding constant (K_b) value in the order of 10^6 M^{-1}. We observed that CaM1 has a lower affinity (0.59 ± 0.09 L/mol), while CML9 binds to BDPDE1 in a higher binding constant of 1.11 ± 0.19 L/mol. We also determined that the dissociation constants (K_d) value for CaM1 and CML9 were 1.69 ± 0.28 μM and 0.90 ± 0.16 μM, respectively.

Further, we examined the influence of the four CaM isoforms (1, 3, 7 and CML9) on the PDE activity. Since calmodulins are highly conserved among plant species and show no significant differences in their structures (Supplementary Figure S2), CaM isoforms derived from *A. thaliana* were used in the experiment. All CaM isoforms stimulated both cAMP and cGMP hydrolysis after the formation of the active CaM/Ca^{2+} complex in the presence of 10-μM Ca^{2+} ions (Figure 4A,B). The highest increase in activity for both cAMP and cGMP hydrolysis were noted with the addition of CML9. For individual CaM isoforms, the increase in PDE activity was 3.5-fold for cGMP (Figure 4B) and 1.5-fold for cAMP (Figure 4A), and for CML9, it was 4.5-fold for cGMP and three-fold for cAMP. After the addition of 1-mM EGTA, which chelates Ca^{2+} ions, the active CaM/Ca^{2+} complex was not formed, and the presence of CaM isoforms did not affect the reaction.

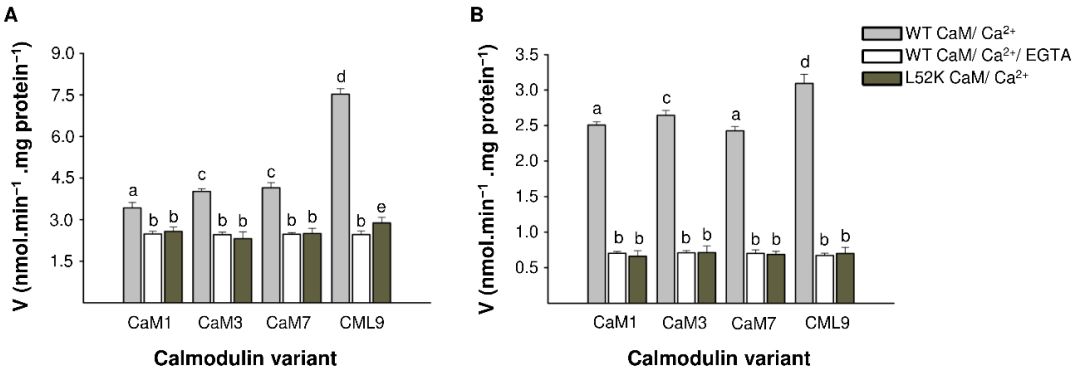

Figure 4. Regulation of the enzyme activity by CaM and CaM-like isoforms. (**A**) BDPDE1 and BDPDE1^{L52K} activity in the presence of various CaM isoforms, which were in active or inactive complexes (addition of 1-mM EGTA chelate). The reaction was carried out for 25 min in the standard reaction mixture containing 0.1-mM cAMP, 0.625-µM GST-BDPDE1, 2-µM of each CaM isoform, 12.5-µM $CaCl_2$ and 1-mM EGTA, depending on the variant. Different letters indicate significantly different data between the groups at $p < 0.05$. (**B**) The BDPDE1 and BDPDE1^{L52K} activity in the presence of various CaM isoforms, where 0.1-mM cGMP was used as a substrate.

We further investigated the effect of the CaM-binding on BDPDE1 activity with site-directed mutagenesis. In the determined CaM-binding site, leucine at position 52 was mutated to lysine, which disrupts CaM-binding [37]. This substitution resulted in no increase in BDPDE1 activity in the presence of an active CaM/Ca^{2+} complex. No CaM isoform had an effect on the cAMP and cGMP hydrolysis reactions; however, a slight increase in BDPDE1 activity was noted for CML9. This may be due to the fact that CMLs show significant structural differences from CaMs, and their binding may also alter the conformation of plant proteins in other ways.

Given that, in plants, many proteins have been reported as calcium-binding (e.g., CDPKs (calcium-dependent protein kinases), SnRKs (SNF-related serine/threonine-protein kinases) or SOSs (salt overly sensitive) and responsive to calcium [32,38], we wanted to determine if we could rule out the possibility of a nonspecific increase in activity due to the presence of Ca^{2+}. Therefore, we tested the effect of different concentrations of Ca^{2+} ions (0–10 µM) on the BDPDE1 enzymatic activity and noted no significant differences in the enzyme activities (Figure 5). This is consistent with no unspecific binding of Ca^{2+} to BDPDE1.

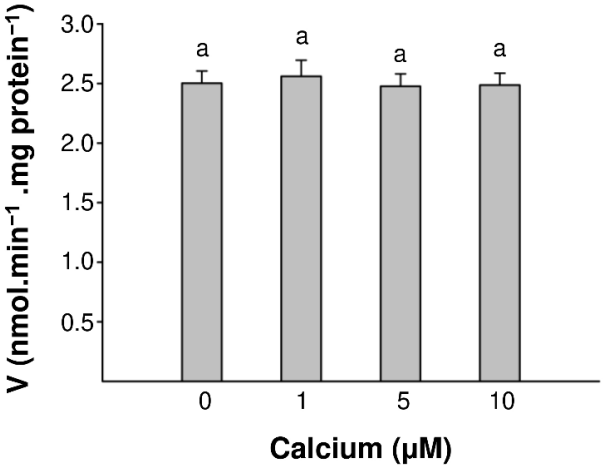

Figure 5. Impact of Ca^{2+} ions on the GST-BDPDE1 activity. AMP generated in vitro after 25 min by 5 µg of GST-BDPDE1 in the presence of 0.1-mM cAMP, 0.1 % (*v*/*v*) 2-mercaptoethanol, 0.5-mM $MgCl_2$ and $MnCl_2$ and $CaCl_2$ in the concentration range from 0 to 12.5 µM. The same letters indicate no statistically significant differences at $p < 0.05$.

2.3. Probing the Catalytic Center of BDPDE1 and Construction of an Expanded PDE Search Motif

Previous studies using a search motif approach to discover novel plant PDEs have shown that it might identify functional PDEs that moonlight in complex, multidomain proteins [17]. The motif [YFW]Hx[YFW]Rx{20,40}[HRK][DE] reported previously [17] is present in the catalytic centers of all the previously experimentally confirmed plant PDEs (Figure 6A). However, the PDE motif that was deliberately made stringent to identify candidate PDEs with high probability may be insufficiently broad, as no monocot orthologs harbor the full motif. Since BDPDE1 is functionally active in vitro, we probed the PDE catalytic center through mutagenesis studies, which are guided by a combination of sequence and structural analyses, to reveal other functionally important amino acids, especially in monocots. Through a sequence analysis, we found that, in BDPDE1, which is the monocot ortholog of ATCN-PDE1, alanine (A) and leucine (L) rather than an aromatic amino acid (YFW), occupy position 1 and 4 of the motif. Notably, these two amino acids are also conserved in other monocot orthologs, such as *Oryza sativa* (NCBI: EEC67204.1) and *Zea mays* (NCBI: NP_001148226.1) (Figure 6B). To account for a broader identification of PDE active sites in monocots and dicots, we therefore decided to include the conserved A and L amino acids in an expanded PDE motif.

We then probed the structure at the catalytic center of BDPDE1 to visualize how catalysis at the predicted PDE center might occur. Since the BDPDE1 crystal structure is unknown, we employed a homology modeling strategy to build a 3D model for BDPDE1 using the crystal structure of a metal-dependent HD domain-containing hydrolase from *Bacillus halodurans* (PDB ID: 3DTO) as the template. This has a 37.04% identity to BDPDE1 at the region between R80 to A315 and covers 91% of the queried amino acids. There is, however, no suitable template structure to model the N-terminal region of BDPDE1 that contains the predicted CaM-binding site at N50–F70 (Figure 6C) and was thus omitted from the structural analysis.

Based on the BDPDE1 model, the PDE center is solvent-exposed and occupies a distinct cavity that could dock cAMP with a binding affinity of −4.9 kcal/mol, as predicted by molecular docking simulations (Figure 7A). Of the key amino acids in the original PDE motif [YFW]**H**x[YFW]**R**x{20,40}[**H**RK][**D**E] reported in Reference [17], only H122, R125, H155 and D156 are present in the PDE center (labeled black), while the additional amino acids R233 and Y237 (labeled green) that could also interact with cAMP are found in positions 77 and 81 downstream of D156, respectively (Figure 6B). These amino acids are not only spatially close to the substrate, but they also orientate toward cAMP at the PDE center (Figure 7A), thus implying that they could participate in key catalytic functions. The structural analysis not only provides confidence that the predicted PDE center in BDPDE1 could bind cAMP, which is the first step for catalysis and, presumably, also the subsequent hydrolysis of cAMP to 5′AMP, but also guides mutagenesis experiments to probe the role of the key amino acids in the PDE motif.

Based on our sequence and structural analyses, we identified L124, H155, D155 and Y237 as possible loss-of-function mutations to glycine (G) or glutamine (E), since these residues are spatially close to, and orientate towards, the substrate and are present in monocots, as well as in dicots and/or bacterial orthologs. The mutagenesis of the key amino acids H155 and D156, which appeared in the original PDE motif reported in Reference [17], reduced the enzymatic activity by four-fold, generating only 0.604 and 0.572-nmol AMP min^{-1} mg $protein^{-1}$, respectively. While L124E mutagenesis did not significantly affect the catalytic efficiency, the $BDPDE1^{Y237E}$ mutant, however, generated only 0.5-nmol AMP min^{-1} mg $protein^{-1}$, which is approximately five-fold lower than the wild-type BDPDE1 (Figure 7B). This is consistent with our structural evaluations, which also revealed the possibility of R233 and Y237 being located downstream of the original PDE motif to interact with cAMP. Significantly, we found that these amino acids also appear downstream in bacterial orthologs, and this further justifies their inclusion into an expanded PDE motif (Figure 6B).

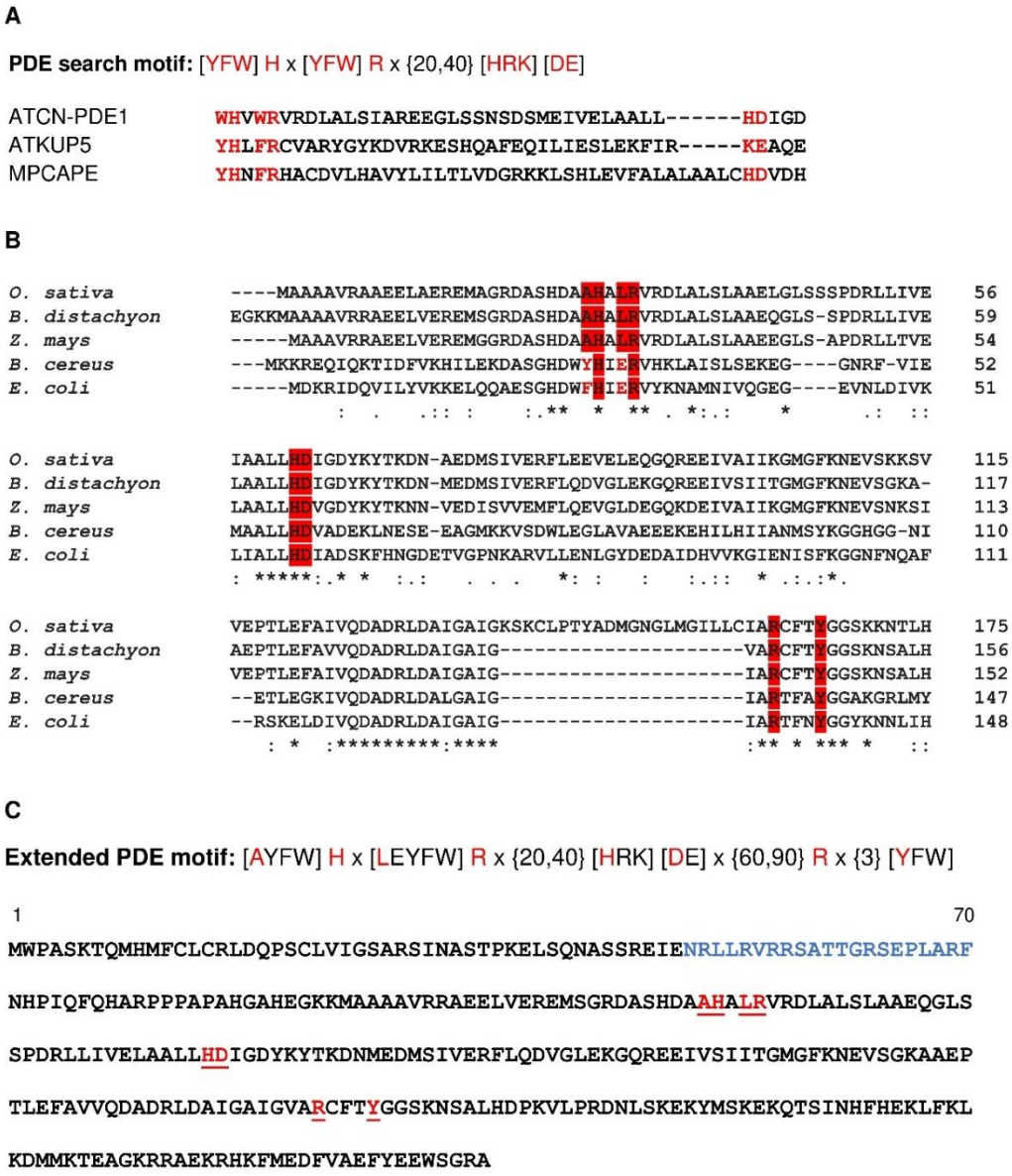

Figure 6. (**A**) Alignment of the known PDE centers of ATCN-PDE1 (At1g17330), ATKUP5 (At4g33530) and MPCAPE (Mapoly0068s0004). An * (asterisk) indicates positions that have a single, fully conserved residue. A: (colon) indicates conservation between groups of strongly similar properties. A. (period) indicates conservation between groups of weakly similar properties. (**B**) Alignment of the BDPDE1 orthologs in monocots *O. sativa* (NCBI: EEC67204.1) and *Z. mays* (NCBI: NP_001148226.1) and prokaryotes *B. cereus* (NCBI: CUB11224.1) and *E. coli* (NCBI: OJR83528.1). (**C**) Sequence of the extended PDE motif and amino acid sequence of the BDPDE1 protein (NCBI: XP_003574089.2). The key amino acids of the PDE motif are marked as red letters, and the calmodulin binding site is marked as blue letters.

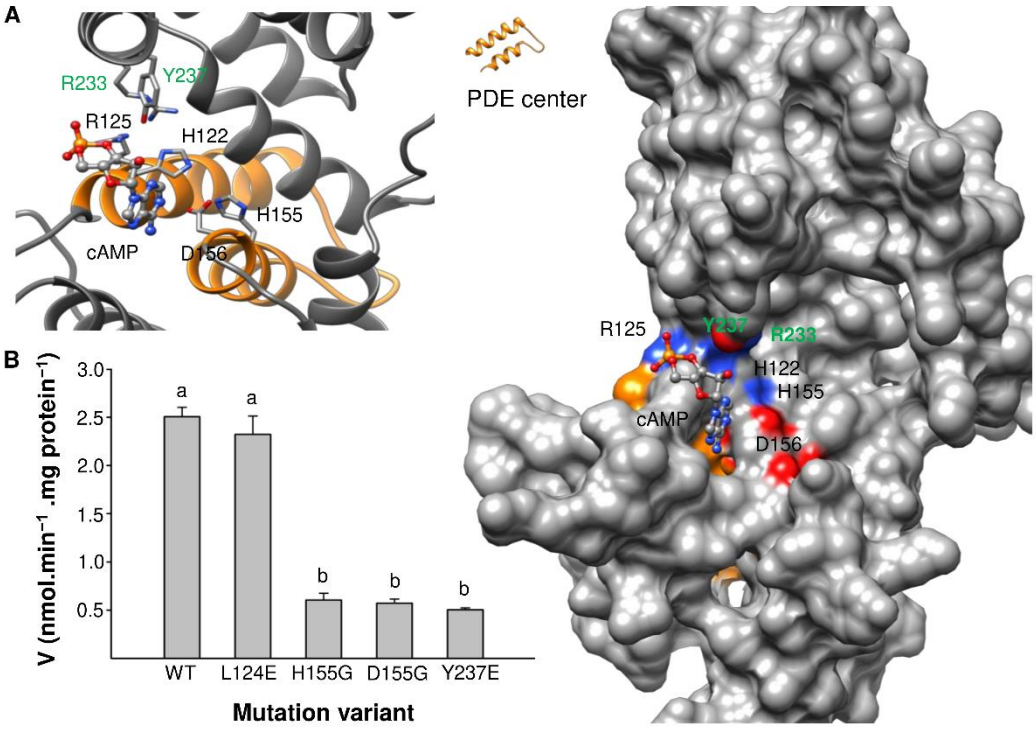

Figure 7. Computational assessment of the PDE catalytic center in BDPDE1. (**A**) The PDE center of BDPDE1 is shown as orange in the surface model (right) and as individual atoms in the ribbon model (left). The PDE center is solvent-exposed and occupies a distinct cavity that docks cAMP with a binding affinity of −4.9 kcal/mol. Key amino acids in the original PDE motif [YFW]**H**x[YFW]**R**x{20,40}[**H**RK][**D**E] reported in Reference [17] and in the expanded PDE motif [AYFW]Hx[LEYFW]Rx{20,40}[HRK][DE]x{60,90}Rx{3}[YFW] constructed in this study are labeled black and green, respectively. These amino acids, which could interact with cAMP at the PDE center, are colored according to their charges in the surface model and as individual atoms in the ribbon model. The BDPDE1$^{R80\text{-}A315}$ 3D structure modeled against the crystal structure of a metal-dependent HD domain-containing hydrolase from *B. halodurans* (PDB ID: 3DTO) using MODELLER (ver. 9.25) [39] and cAMP docking simulations were performed using AutoDock Vina (ver. 1.1.2) [40]. Molecular graphics and analyses were performed with the UCSF Chimera package [41]. Chimera was developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIGMS P41-GM103311). (**B**) The effect of site-directed mutagenesis on PDE activity. Mutations of H155G, D156G and Y237E in the wild-type (WT) BDPDE1 domain significantly reduced the enzyme activity, while L124E did not affect the activity. The reaction time was 25 min, and the standard reaction mixture contained 0.1-mM cAMP and 5 µg of the mutated protein. Different letters indicate significantly different values as compared to the control sample ($p < 0.05$).

In the bacterial orthologous of BDPDE1, A is present in position 1 of the PDE motif, much like in monocot PDE candidates, but in position 4, E is present in place of L (Figure 6B). Thus, considering the sequence, biochemical and structural analyses in our study, we included the A and L/E amino acids, which are conserved in monocot and bacterial PDE candidates in positions 1 and 4 of the PDE motif, as well as the R and Y amino acids located downstream of the HD domain, to yield a more comprehensive and inclusive PDE motif [**A**YFW]**H**x[**L**EYFW]**R**x{20,40}[**H**RK][**D**E]x{60,90}**R**x{3}[**Y**FW] (Figure 6C). This motif can be broadly applied to discover PDEs not just in monocots but, also, in other organisms from prokaryotes and eukaryotes and identifies 25 putative PDEs in the *B. distachyon* proteome (Supplementary Table S1). Further bioinformatics and experimental characterizations of these candidates will afford a more complete understanding of the cyclic nucleotide signaling in plants and beyond.

3. Materials and Methods

3.1. Expression Vector Construct

The total RNA was isolated from leaves and stalk of *B. distachyon* using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The first-strand cDNA for RT-PCR was synthesized using the GoScript™ Reverse Transcription System (Promega, Madison, WI, USA) following the manufacturer's instructions. In order to construct the expression plasmid for the GST-BDPDE1, the cDNA fragment was amplified by RT-PCR using specific primers:

-*BDPDE1* (forward)
5′-GGATCCCCAGGAATTCCCATGTGGCCAGCATCCAAAACAC-3′
-*BDPDE1* (reverse)
5′-GATGCGGCCGCTCGAGAATCAAGCCCTGCCACTCCAC-3′

The PCR reactions were performed with cDNA as the template, forward and reverse primers and CloneAmp HiFi PCR Premix (Takara Bio USA, Mountain View, CA, USA). The amplified DNA fragments were purified using an Agarose-Out DNA Purification Kit (Eurx, Gdańsk, Poland). The amplified PCR products were cloned into the pGEX-6P-2 vector (Cytiva, Uppsala, Sweden) in the EcoRI–XhoI restriction sites using an In-fusion Cloning kit (Takara Bio USA, Mountain View, CA, USA).

3.2. Site Directed Mutagenesis of BDPDE1

GST-BDPDE1(L124E), GST-BDPDE1(H155G), GST-BDPDE1(D156G), GST-BDPDE1(Y237E) and GST-BDPDE1(L52K) mutants were constructed by site-directed mutagenesis using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent, Cedar Creek, TX, USA). Specific primers used in the reaction are in the Supplementary Materials.

3.3. Expression and Purification of the Recombinant Protein

The resulting plasmids were introduced into *E. coli* BL21(DE3) pLysS-competent cells (Promega, Madison, WI, USA) in order to produce the fusion proteins with a glutathione-S-transferase (GST) affinity tag. The transformants were grown in LB medium (500 mL) containing ampicillin (100 µg/mL) and 2% glucose at 37 °C. Fusion protein expression was induced by adding isopropyl-ß-D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM at OD_{600} = 0.6 and incubating the culture at 20 °C for 3.5 h. The bacteria were harvested by centrifugation, and the pellet was suspended in lysis buffer (50-mM Tris-HCl, pH 8.0, 150-mM NaCl, 5-mM EDTA, 5-mM EGTA, 1 % (*v/v*) Triton X-100, 1-mM PMSF and 0.2-mg/mL lysozyme) and disrupted by sonication. The cell extract was centrifuged at 18,000× *g* for 35 min, and the supernatant was loaded onto glutathione-Sepharose 4B beads (Cytiva, Uppsala, Sweden). Afterward, the column was washed multiple times with a buffer containing 50-mM Tris-HCl (pH 8.0) and 150-mM NaCl, and the GST fusion protein was eluted with 10-mM glutathione in 50-mM Tris-HCl (pH 9.0). The recombinant calmodulins (CaM1, 3, 7 and CML9) were purified according to Reference [32]. The homogeneity and purity of the eluted protein fraction was analyzed by SDS–PAGE electrophoresis (10% gel) with the Coomassie Blue gel staining.

3.4. Structural Analysis of the PDE Center and CaM-Binding Site in BDPDE1

The $BDPDE1^{R80\text{-}A315}$ 3D structure was modeled against the crystal structure of a metal-dependent HD domain-containing hydrolase from *B. halodurans* (PDB ID: 3DTO) using MODELLER (ver. 9.25) [39], and cAMP docking simulations were performed using AutoDock Vina (ver. 1.1.2) [40]. In the docking simulations, all bonds in the cAMP were allowed to move freely, but $BDPDE1^{R80\text{-}A315}$ was set as rigid. Docking simulations consider both spatial and charge at the vicinity of the PDE center based on predetermined grids that cover the entire catalytic center. Molecular graphics and analyses were performed with the UCSF Chimera package [41]. Chimera was developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIGMS P41-GM103311).

3.5. PDE Biochemical Assay and LC-MS/MS Analysis

PDE in vitro activity was determined by using LC-MS/MS to determine the rate of AMP or GMP formation. The reaction time was 25 min, and the standard reaction mixture contained: 3-mM Tris-HCl (pH 8.0), 0.1-mM cAMP or cGMP, 0.1% (*v/v*) 2-mercaptoethanol, 5 µg of GST-BDPDE1 and 0.5-mM $MgCl_2$ and $MnCl_2$ in a final volume of 100 µL. To investigate if calmodulin regulates the activity of PDE, four different CaM isoforms were added to the reaction in the concentration of 2-µM, together with 12.5-µM $CaCl_2$, and the GST-BDPDE1 protein concentration was 0.625-µM. The concentration of free Ca^{2+} ions was calculated using the Maxchelator program available online at: maxchelator.stanford.edu [42]. The samples were incubated at 37 °C for 25 min. The enzyme reaction was terminated by incubation at 100 °C for 10 min, and the samples were centrifuged at 13,200× *g* for 10 min.

LC-MS/MS experiments were performed using the Nexera UHPLC and LCMS-8045 integrated systems (Shimadzu Corporation, Kyoto, Japan). The ionization source parameters were optimized in positive ESI mode using pure AMP and GMP dissolved in HPLC grade water (Sigma, St. Louis, MO, USA). The samples were separated using a XSelect CSH Phenyl-Hexyl column (100 × 2.1 mm, 3.5 µm, Waters, Dublin, Ireland). A gradient of solvent A (0.05 % (*v/v*) formic acid with 5-mM ammonium formate) and solvent B (100 % (*v/v*) acetonitrile) was applied over 3 min: B: 0–5%, followed by washing and conditioning of the column with a flow rate of 0.4 mL/min. The interface voltage was set at 4.0 kV for positive (ES+) electrospray. Data acquisition and analysis were made with the LabSolutions workstation for LCMS-8045.

3.6. Fluorescence Studies

The fluorescence spectra of plant phosphodiesterase (BDPDE1) in the absence and presence of CaM1 and CML9 in 10-mM glutathione and 50-mM Tris-HCl (pH 9.0) were performed on a JASCO FP-8300 spectrofluorometer with 10-mm quartz cells (Hellma Analytics, Müllheim, Germany). The measurements were recorded in the range of 300–600 nm after excitation at λ = 280 nm at 37 °C. The samples were prepared in 2-mL Eppendorf tubes and contained BDPDE1 at a concentration of 0.5-µM alone or with CaM1 and CML9 at the following concentrations: 0.25, 0.5, 1.0, 1.5, 2.0 and 2.5 µM, and 10-mM glutathione in 50-mM Tris-HCl (pH 9.0) was added to each tube, up to 2 mL. Then, the spectrum was recorded, and the emission spectra were measured three times. The fluorescence data were fitted by applying nonlinear least-squares regression using OriginPro software Version 2016 (OriginLab Corporation, Northampton, MA, USA).

3.7. Statistical Analyses

All experiments were performed in at least triplicate. Values are expressed as the mean ± SE. Differences between the groups were calculated by one-way ANOVA, followed by a Tukey–Kramer multiple comparison test using SigmaPlot 11.0 software. In all cases, the confidence coefficient was set at $p < 0.05$.

4. Conclusions

Degradation of cNMPs by PDEs is an integral component of cyclic nucleotide-dependent signaling in organisms across the tree of life; yet, plant PDEs have remained largely elusive. Given the recent discovery of PDEs in dicots [16,17], we set out to identify novel PDEs in monocots. Our results showed that a *B. distachyon* ortholog of the *A. thaliana* PDE ATCNPDE1, BDPDE1, can hydrolyze cNMPs to 5′NMPs with a preference for cAMP over cGMP in vitro, and importantly, the PDE activity was significantly enhanced by the CaM/Ca^{2+} complex. Through bioinformatics-guided mutagenesis studies, we also ascertained the key residues involved in both PDE catalytic activity and in the interaction of CaM. Our results imply that plant PDE domains may be embedded within complex multidomain proteins where they are likely to modulate intra- and intermolecular domains, thereby acting as tuners of downstream signals. Finally, based on our biochemical, mutagenesis and structural analyses, we constructed a comprehensive amino acid consensus sequence

that is a diagnostic for annotated and/or experimentally validated PDEs across kingdoms, thus affording broad applications of this search motif for the identification of PDE active sites in eukaryotes and prokaryotes.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/ijms22179654/s1.

Author Contributions: Conceptualization, M.K., C.G. and K.J.; methodology, M.K. and A.K.-P.; formal analysis, M.K. and A.W.; investigation, M.K.; writing—original draft preparation, M.K., A.W., A.K.-P., C.G. and K.J.; writing—review and editing, A.W., K.J. and C.G.; visualization, K.J.; supervision, C.G. and K.J. and funding acquisition, M.K. and A.W. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data is contained within the article and the Supplementary Materials.

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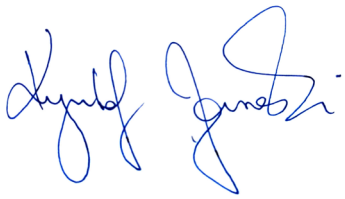
Declaration

I declare that my contribution to the following articles is as follows,

1. Kwiatkowski Mateusz, Wong Aloysius, Kozakiewicz Anna, Gehring Christoph A., Jaworski Krzysztof, (2021) A tandem motif-based and structural approach can identify hidden functional phosphodiesterases, Computational and Structural Biotechnology Journal 19, 970-975, DOI: 10.1016/j.csbj.2021.01.036
   concepition, writing - original draft preparation, review and editing, supervision – search in the article 10%

2. Kwiatkowski Mateusz, Wong Aloysius, Kozakiewicz-Piekarz Anna, Gehring Christoph, Jaworski Krzysztof (2021) In search of monocot phosphodiesterases : identification of a calmodulin stimulated phosphodiesterase from *Brachypodium distachyon*. International Journal of Molecular Sciences, 22 (17) , 1-15, DOI: 10.3390/ijms22179654 concepition, writing - original draft preparation, review and editing, supervision – search in the article 10%



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To who it may concern:

I, Chris Gehring herewith declare that my contribution to the joint publications on which Mateusz Kwiatkowski is the first author, was 10 %. I also affirm that he has conducted all experiments described, interpreted them, and significantly contributed to the development of a novel conceptual framework for cyclic nucleotide signaling.
In short, his contribution to the to the publications,

Kwiatkowski, M. *et al*.
A tandem motif-based and structural approach can identify hidden functional phosphodiesterases.
Comput. Struct. Biotech. J. (2021) **19**, 970-975; doi: 10.1016/j.csbj.2021.01.036.

Kwiatkowski, M. *et al*.
In search of monocot phosphodiesterases: identification of a calmodulin stimulated phosphodiesterase from *Brachypodium distachyon*.
Intl. J. Mol. Sci. (2021) **22**, 9654; doi: 10.3390/ijms22179654.

was in my view significantly above 55%.

With kind regards,

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Wenzhou, Zhejiang May 9, 2022

To whom it may concern,

I hereby declare that my contribution to the article “In search of monocot phosphodiesterases: Identification of a calmodulin stimulated phosphodiesterase from Brachypodium distachyon. Kwiatkowski et al., *Int. J. Mol. Sci.* 2021 22(17), 9654” was 10% (writing-review and editing), and to the article “A tandem motif-based and structural approach can identify hidden functional phosphodiesterases. Kwiatkowski et al., *Comput. Struct. Biotechnol. J.* 2021, 19, 970-975” was 10% (writing-review and editing).

Sincerely,

Aloysius Wong, PhD

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RESEARCH UNIVERSITY
EXCELLENCE INITIATIVE

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To whom it may concern,

I hereby declare my contribution to the articles:

- In search of monocot phosphodiesterases: Identification of a calmodulin stimulated phosphodiesterase from Brachypodium distachyon. Kwiatkowski et al., *Int. J. Mol. Sci.* 2021 22 (17), 9654 was **5%** (studies of protein-protein interactions by fluorescence spectroscopy),
- A tandem motif-based and structural approach can identify hidden functional phosphodiesterases. Kwiatkowski et al., *Comput. Struct. Biotechnol. J.* 2021, 19, 970-975 was **5%** (studies of molecular interactions by surface plasmon resonance).

Sincerely,

Anna Kozakiewicz-Piekarz