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Review of the PhD Thesis

Author:	Mateusz Kwiatkowski
Author's affiliation:	Nicolaus Copernicus University in Toruń, Faculty of Biological and Veterinary Sciences
Title:	"Phosphodiesterases in higher plants - a missing link in cyclic nucleotide signal transduction"
Supervisor:	Prof. UMK dr hab. Krzysztof Jaworski
Foreign supervisor:	Prof. Chris Gehring

The formal basis for the review is the letter by prof. Werner Ulrich, the Dean of the Faculty of Biological and Veterinary Sciences of the Nicolaus Copernicus University in Toruńfrom the 25th of May 2022. I was appointed as a reviewer based on the resolution of the Scientific Council in the Discipline of Biological Sciences of the Nicolaus Copernicus University in Toruń on the 20th of May 2022.

The dissertation consists of:

- two published original articles,
- the self-presentation (containing: List of abbreviations, Introduction, The aim of the study, Discussion, Conclusions, and References),
- abstracts in English and Polish,
- declarations of co-authors about their participation in these works.

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The articles that are considered as a basis of a dissertation for a doctoral degree are:

- 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
- Kwiatkowski, M.; Wong, A.; Kozakiewicz-Piekarz, A.; Gehring, C.; Jaworski, K. In Search of Monocot Phosphodiesterases: Identification of a Calmodulin Stimulated Phosphodiesterase from *Brachypodium distachyon*. *Int. J. Mol. Sci.* 2021, 22, 9654, doi: 10.3390/ijms22179654.

Publications included in the PhD thesis were published in highly-impacted scientific journals. The summarised IF of these two articles is over 13, and the sum of MNiSW points is 240. In both articles, Mr Mateusz Kwiatkowski is the first, and in one of them is also the corresponding author. According to the declaration of all co-authors to the papers, Mr Kwiatkowski contributed 65% to the conceptualisation, methodology, investigation, and writing of both articles. Moreover, Mr Kwiatkowski was supported by the project POWR.03.05.00-00-Z302/17 Universitas Copernicana Thoruniensis in Futuro-IDS "Academia Copernicana".
In the Introduction to the self-presentation and the Introduction to the articles, Mr Kwiatkowski extensively described the current knowledge about cyclic nucleotides, cyclases, and phosphodiesterases (PDEs) in prokaryotes and eukaryotes. It is known that cyclic nucleotides play an important signalling function in plant cells. During the last few decades, more and more data about the role of cyclic nucleotides was gathered. Moreover, computational analysis research allowed for describing plant proteins with nucleotide cyclases activity. Enzymes that degrade cyclic nucleotides are phosphodiesterases, which can play a significant role in damping the signals evoked by these secondary messengers. Our knowledge about phosphodiesterases in plants is modest and, up to 2019, only one PDE cGMP-dependent in *Arabidopsis* was described (Isner et al. 2019, Current Biology, 29: 2580-2585.e4). In addition, 26 proteins were identified as putative cGMP-PDEs in this work.

In connection with the above facts, Mr Kwiatkowski set ambitious goals for analysing the proteome of a model plant *Arabidopsis* and the structural and biochemical properties of candidate PDEs (Publication I). The doctoral student realised a few specific experimental procedures to reach these goals. In a clear way, the author of the dissertation described the methods used to select 32 protein candidates with hypothetical PDE activity. The results of the

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bioinformatics analysis presented in this dissertation (publication I) have shown the first plant PDE motif ([YFW] H x [YFW] R x {20,40} [HRK] [DE]). It is a significant point of study on plant PDEs that will allow researchers to extend knowledge about the metabolism of cAMP, especially as an element turn-off of the signal evoked by this signalling molecule.

Finally, K^+-Uptake Permease 5 (AtKUP5, At4g33530) was chosen for further studies. Interestingly, this protein also contains the adenylate cyclase (AC) domain at N-terminus. The computational evaluation of the AtKUP5 structure confirmed the occurrence of the key functional amino acids from the motif and the characteristic of PDEs pocket protected by a latch. Moreover, analysis of molecular docking indicates that cAMP docks at the PDE domain with good binding affinity and the correct binding position of the adenine head of cAMP and the phosphate tail, and their interactions with the appropriate amino acids. Moreover, studies on PDE motif provided that lysine (K705) plays a significant role in the structure of the catalytic domain. It is also essential data concerning amino acids indispensable in the active domain in plant PDEs. The next step was the subject of the studies biochemical characterisation of recombinant PDEs.

I have a few questions about the obtained results, and I would like to ask the PhD student to explain my doubts.

1) In Figures 2C, and 2D (Publication I), results indicate that phosphodiesterase activity of AtKUP5 depends on calmodulin isoforms (CaM 1, 2 or 9 isoforms), Ca^{2+} ions, and on substrate concentration (panel D). In the Material and methods section, there is a paragraph describing methods used to determine PDE activity that says that samples were incubated for 25 minutes. It was determined V_{max} and K_m for phosphodiesterase activity of GST-AtKUP5$^{573-855}$. Whether during the determination of these parameters were initial velocities also determined? At what points in time?
2) In Figure 2G the authors proposed a model for the dampening role of the PDE in regulating K^+ transport activity. Results indicate that AtKUP5, apart from adenylate cyclase activity, also contains the PDE domain. Is damping the signal evoked by cAMP a consequence of degradation of cAMP (PDE activity), or it can be the effect of inhibition synthesis of cAMP by AMP? Is there any literature data on the inhibition of adenylate cyclase activity by AMP?

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The second publication (Kwiatkowski et al., 2021) contains the results of studies searching for PDEs in monocots. The analysis used a unique and consensus search motif based on key amino acids involved in the catalytic centre of annotated PDEs in different species. These bioinformatics analyses allowed to identify 25 candidate PDEs in *Brachypodium distachyon*. Among them was the selected protein with HD domain – BDPDE1.

Studies on kinetic parameters of BDPDE1 such as V_{max}, K_m, k_{cat}, and catalytic efficiency (k_{cat}/K_m) *in vitro* showed that this protein has a significantly higher affinity to cAMP than to cGMP (Figure 1C). In Figure 2C the authors showed reaction velocity catalysing by BDPDE1 depending on substrate variants such as cAMP or cGMP on their own or in the combination of cAMP and cGMP. Based on these results, the authors indicate a higher substrate specificity for cAMP. In my opinion, parameters such as K_m, k_{cat}, and catalytic efficiency (Figure 1C) are more informative about substrate specificity because data presented in Figure 2C was obtained at only one cAMP and cGMP concentration (0.1 mM) and only at one point in time (after 25 minutes). To determine kinetic parameters like the above, important is initial velocity (v_0) which can be determined at a few points in time and when the points of reaction velocity are on a straight line. Other factors determined that influenced the PDE activity were temperature and divalent cations, such as Mn^{2+} and/or Mg^{2+}. The interesting result is data indicating that phosphodiesterase BDPDE1, similarly to ATKUP5, contains a calmodulin-binding site. Studies on the interaction between calmodulin isoforms (1, 9) with BDPDE1 have shown a higher binding constant (K_b) and lower dissociation constant (K_d) for CML9 than for CaM1. It indicates a higher affinity of tested PDE to CML9 than CaM1 (Figure 3).

Moreover, the activity of BDPDE1 as an enzyme degrading cAMP was induced by the complex CML9/Ca^{2+} (Figure 4). Searching for PDE motifs among proteins in *Brachypodium distachyon* has been started from the alignment of the known PDE centres of ATCN-PDE1, MPCAPE, and ATKUP5 (Figure 6A). It is known from the previous publication (Kwiatkowski et al., 2021) that the protein ATKUP5, and MPCAPE (Kasahara et al. 2016, Sci. Rep. 6(1): 39232), contain the adenylate cyclase domain, apart from the PDE domain. Did the PhD student also analyse the amino acid sequence in the search for the adenylate cyclase domain in BDPDE1?

In summary, studies conducted *in silico* and *in vitro* presented in Mr Kwiatkowski's dissertation shed more light on cyclic nucleotide phosphodiesterases existing in higher plants and their

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participation in cyclic nucleotides signal transduction. Until now, the knowledge about such activity in higher plants has been elusive. Therefore, I state that Mr Kwiatkowski's doctoral dissertation meets the statutory requirements for this type of work and is an original solution to a scientific problem. Moreover, the presented dissertation showed points to the general theoretical knowledge of the doctoral student in cyclic nucleotide metabolism and its functions in plants. In addition, the *in silico* and *in vitro* analyses show that the doctoral student can use appropriate research tools. In my opinion, Mr Kwiatkowski can be confidently considered a person who can conduct scientific work independently. Thus, I submit an application to the Scientific Council in the Discipline of Biological Sciences of the Nicolaus Copernicus University in Toruń for admission of Mateusz Kwiatkowski to further stages of his doctoral dissertation.

Moreover, I would like to apply to the Scientific Council in the Discipline of Biological Sciences of the Nicolaus Copernicus University in Toruń to award Mr Kwiatkowski's doctoral dissertation an appropriate award.

1. Research conducted by Mr Kwiatkowski led to the identification of two proteins AtKUP5 and BDPDE1, from *Arabidopsis thaliana* and *Brachypodium distachyon*, respectively, with cyclic nucleotide phosphodiesterase activity (publications I and II).
2. The above finding was possible thanks to constructing a unique search motif based on key amino acids significant in catalysing the reaction by PDEs and docking simulation. This unique search motif opens the possibility of searching for the following proteins with PDE activity in plants.
3. Studies conducted by Mr Kwiatkowski complement the knowledge, as he titled his doctoral dissertation, about PDEs as the missing link in cyclic nucleotide signal transduction in plants.
4. The high rank of the obtained results is evidenced by the publication impact factor (IF), which for the total of two articles is over 13.

Małgorzata Pietrowska-Borek