SZKOŁA GŁÓWNA
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Review of the PhD thesis of Mateusz Kwiatkowski entitled "Phosphodiesterases in higher plants - a missing link in cyclic nucleotide signal transduction"

I am writing to provide my review for the PhD thesis of Mateusz Kwiatkowski. In general, it was a pleasure to read this disertation as the subject of the thesis is of the highest scientific importance and the highest experimental quality.

Mgr Mateusz Kwiatkowski PhD dissertation consists of two peer-reviewed experimental papers, in both of which he is the first author. In one of them mgr Mateusz Kwiatkowski is also the corresponding author. The experimental work was carried out at Nicolaus Copernicus University in Toruń, Faculty of Biological and Veterinary Sciences. The thesis was supervised by dr hab. Krzysztof Jaworski from Nicolaus Copernicus University in Toruń and prof. Chris Gehring from University of Perugia. Dr hab. Krzysztof Jaworski is investigating various aspects of plant growth and development in the context of cellular signalization. His main interest is metabolism of cyclic nucleotide monophosphates (cNMP) with the special impact on purine nucleotide cyclases and cyclic nucleotide phosphodiesterases. He is the recognized expert in this field of study which is of great interest and importance for plant physiologist. Professor Chris Gehring is a world-class specialist in plant stress response and cellular signalization in plants.

Cyclic nucleotides (cNMP) are classified as secondary messengers in cellular signal transduction pathways in all living organisms. For many years, signal transduction *via* cNMP in plants was

Szkoła Główna Gospodarstwa Wiejskiego w Warszawie
Instytut Biologii
Katedra Fizjologii Roślin

prof. dr hab. Agnieszka Gniazdowska-Piekarska

ul. Nowoursynowska 159
02–776 Warszawa
+48 22 59 325 10
+48 22 59 325 30
agnieszka_gniazdowska@sggw.edu.pl
www.sggw.pl

controversial, mainly due to the technical problems in their determination in *planta*. The development of the techniques such as LC-MS/MS allows the determination of cNMP and later on confirmed their role of secondary messengers in plant cells. Nucleotide cyclases: adenylate cyclase (AC) and guanylate cyclase (GC) - the enzymes responsible for cNMP synthesis are well characterized, but phosphodiesterases (PDEs) - the enzymes responsible for the degradation of cNMP till now remained elusive (mysterious) in higher plants. Thus, the main goal of the Mateusz Kwiatkowski PhD thesis - demonstration of the presence of PDE in plants and its characterization is of great scientific impact.

In the dissertation of Mateusz Kwiatkowski the publications are preceded by the brief introduction to the subject of his PhD study. It contains basic information about the background of the research. The PhD candidate formulated the hypothesis and aims of the study. The last part of the introduction is a discussion of the results, containing also some elements of the data presented in both articles. At the end of the Introduction chapter Mateusz Kwiatkowski included the Reference list consisting of 86 citations. The dissertation is accompanied by abstracts written in Polish and English. Authors statements to both papers are attached to the thesis. The authors' statements confirm the PhD candidate's leading participation in the laboratory research and in the preparation of both manuscripts.

Since the experimental data was published in respectful scientific journals (*Computational and Structural Biotechnology Journal;* IF = 7.271 and *International Journal of Molecular Sciences;* IF = 5.924) where they were subjected to peer-review by experts in the field, I do not fill entitled to criticize the study design or obtained results. Just opposite, I have found both papers very well structured with conclusions fully supported by presented experimental data. Moreover, it needs to be underlined that both papers (even published in 2021) were cited by other researchers: Publication I was cited five times and Publication II three times (according to Web of Science, July, 15th 2022), which confirms the visibility of the work in the scientific society, and points at importance of the results.

However, I must confess here I'm somewhat disappointed with the Introduction part of the thesis. Although it would be the redundancy with the attached publications but I was looking for more "round" research background with some graphics to highlight the position of cyclic nucleotides in plant cell signalization. I have also expected a description of the results with some future conclusions. I would like to know if the PhD candidate sees more gaps in our knowledge on

PDEs and their regulatory role in cyclic nucleotides metabolism. Does he plan any other experiments to study in more details the existence of two domains (PDE and cyclase) in one protein?

My criticism concerns also formatting of the References part. The formatting of the References was done carelessly, with no attention to details (punctuation marks, upper and lower case letters, fonts). It is a pity, because it affects the general perception of the Introduction chapter. But this is only an editorial problem, that does not lower scientific value.

A brief description of the most important achievements of the PhD dissertation of Mateusz Kwiatkowski.

Publication I (**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).

Based on sequence analysis and structural properties of canonical PDE catalytic centers, the PhD student developed a consensus sequence search motif and used it to identify PDEs candidate. One of these was 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. Activity of PDE was confirmed by the sensitive liquid chromatography tandem mass spectrometry (LC-MS/MS) method. PDE 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, Mateusz Kwiatkowski proposed 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.

Publication II (**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).

Research conducted in the frame of the paper published in IJMS led to identification of a candidate PDE from the monocot plant Brachypodium distachyon (BDPDE1). PhD candidate proved that it can hydrolyze cNMPs to 5'NMPs but with a preference for cAMP over cGMP *in vitro*. Moreover the PDE activity was significantly enhanced by Ca^{2+} only in the presence of calmodulin (CaM). Based on biochemical, mutagenesis and structural analyses, Mateusz Kwiatkowski 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.

At the end I have few questions steaming from my curiosity. I would like the PhD candidate to speculate on the mechanism of PDE action as regulators of cyclic NMPs concentration in plant response to biotic stresses in which nitric oxide (NO) is known to be involved. Furthermore, being fascinating with the presented data, and taking to account the link of cGMP to NO signal transduction pathways I wonder if there are any information on the control of PDE activity by NO or ROS/RNS. I would like also to know if the PhD candidate has any information on the cyclic NMPs signature - similar to that observed for Ca^{2+} or ROS? If so, in regulation of which physiological processes in plants it could work?

Final decision

The doctoral thesis entitled " Phosphodiesterases in higher plants - a missing link in cyclic nucleotide signal transduction" presented by Mateusz Kwiatkowski **fulfils all requirements needed to obtain the doctoral degree in the biological sciences** in accordance with the relevant legal regulation and customary standards. The reviewed thesis fulfils the requirements necessary to obtain the PhD degree set out in the Act on Academic Degrees and Title (Rozprawa doktorska spełnia warunki określone w art. 187 Ustawy z dnia 20 lipca 2018 r. Prawo o szkolnictwie wyższym i nauce, tekst jednolity: Dz.U. z 2021 r. poz. 478). **Given the excellent results of Mateusz Kwiatkowski thesis presented in two papers published in respectful scientific journals I obviously fully support awarding him a PhD and request Biological Sciences Discipline Board at Faculty of Biological and Veterinary Sciences to admit Mateusz Kwiatkowski to further stages of the doctoral dissertation procedure.**

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Considering the very good publications, on which the thesis is based, innovative findings, achieved by sophisticated, modern methodology, I strongly encourage awarding Mateusz Kwiatkowski an appropriate distinction.

Sincerely yours

Katedra Fizjologii Roślin
Instytutu Biologii
/ Prof. dr hab. Agnieszka Gniazdowska-Piekarska /