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Review

of the Doctoral Dissertation by **Serhii Kozin** entitled "**Enginering of tryptophan specialized metabolism in *Arabidopsis thaliana* for plant immunity**."

Carried out in
The Department of Plant Functional Metabolomics,
Institute of Bioorganic Chemistry Polish Academy of Sciences, Poznan, Poland,
under supervision of prof. dr. hab. Paweł Bednarek.

The thesis is prepared in the form of a manuscript and is written in English. It includes a table of contents, a list of abbreviations, an introduction, a clearly defined aim of the study, as well as sections presenting the results and their discussion and literature list

The Bednarek research group has a long-standing expertise in glucosinolate biology and other sulfur containing phytoalexins and plant resistance to pathogens; consequently, the thesis is well situated within a strong and highly appropriate scientific framework.

This study focuses on the role of tryptophan-derived defense metabolites in plant immunity, specifically in *Arabidopsis thaliana*. The work investigates whether their protective function depends mainly on their chemical properties or on where and when they are produced in the plant. To explore this, both native (camalexin) and foreign (brassinin from *Brassica rapa*)

phytoalexin pathways were engineered in a susceptible mutant line. The results showed that even when these compounds were produced in non-native tissues, they still effectively limited the growth of the fungal pathogen *Plectosphaerella cucumerina*.

The introduction of the dissertation is well written and provides a clear and comprehensive entry into the subject matter. Spanning 28 pages, it effectively presents the background, context, and significance of the research topic. The author successfully guides the reader through the relevant literature and key concepts, creating a solid foundation for the subsequent chapters. Overall, the introduction is coherent, informative, and appropriately structured for a doctoral thesis. One small detail- I would like the more detailed description and characteristics of double mutant used for subsequent transformations as this would ease interpretation of the results.

The Materials and Methods section generally provides a clear description of the conducted experiments and is, for the most part, well presented. However, I have some reservations regarding the figure illustrating the experimental workflow. While the overall experimental design becomes clear after reading the thesis, the figure itself is somewhat confusing and may initially mislead the reader rather than facilitate understanding.

The literature is appropriately selected and well-curated, comprising nearly 200 publications. A substantial portion of the references is recent, reflecting up-to-date research in the field. In my opinion, the cited works are well chosen and relevant to the subject of the dissertation.

The main part of the thesis consists of the Results section, which spans over 40 pages and presents the outcomes of the performed experiments. This section is supported by numerous figures and tables(over 40). The data are generally well described, statistically analyzed, and presented in the form of graphs.

A major achievement of the work was the successful reconstitution of camalexin biosynthesis in transgenic plants to nearly wild-type levels through the expression of the two introduced genes. This not only confirms their essential role in the pathway but also highlights the importance of enzyme co-localization and the possible formation of multi-enzyme complexes in efficient metabolite production.

It is also particularly valuable that the study did not focus solely on the final product, camalexin, but provided a detailed characterization of the entire biosynthetic route by

identifying intermediate metabolites. In my view, this represents a significant strength of the work, especially given the challenges associated with detecting and quantifying intermediates in the absence of commercially available standards. Here I have a question -where do the data regarding the predicted fragmentation patterns of all precursors originate from?

For better understanding of the result, a more detailed characterization of the initial mutant background would have been beneficial. It remains unclear to me whether the mutant is completely deficient in camalexin production under all circumstances. Although the screening included Col-0 and the transgenic lines generated by the PhD candidate, the original mutant line used for transformation was not clearly represented as a reference negative control. In my opinion, including such plants in the analysis would have provided a more robust experimental framework.

It would also be important to clarify whether alternative isoforms of the targeted gene exist or whether other potential bypass pathways for camalexin biosynthesis might be active in the mutant background. Additionally, it is worth considering whether the mutation affected the biosynthesis of other glucosinolates or related secondary metabolites.

Finally, it would be relevant to discuss whether the transgenic approach could have indirectly influenced the expression of other genes, similarly to the downregulation observed for the native PAD3 gene, potentially leading to broader metabolic or regulatory effects. This would be important as pathogen resistance was not fully restored despite the re-establishment of camalexin biosynthesis. What other factors could have been altered in these mutants? Could changes in the metabolic background also have played a role?

For me it was also very interesting that in the double transgenic plants, camalexin-derived compounds such as HCX-Hex are present at low levels even under control conditions and can be induced not only by pathogen infection but also by flg22 treatment, which is not observed in wild-type plants. However, this raises an important question: how can this be reconciled with the lack of a corresponding increase in camalexin accumulation upon flg22 treatment? This is discussed some in the thesis but I would like a PhD candidate to comment more on that.

The section on brassinin demonstrated that it is possible to activate the synthesis of this compound in a plant that does not naturally produce it by introducing single genes, and it also

allowed the determination of which of the approaches is the most effective. The section on cyclobrassinin and spirobrassinin appears incomplete, as no further experiments or analysis are presented. The result section seems to end rather abruptly at this point. Additionally, the Discussion (p. 118) mentions that individual T1 lines producing brassinin derivatives were tested for resistance; however, these data are not included in the manuscript. Could the author clarify this issue? Despite this, the study provides valuable tools for further investigation and I'm sure the work using those generated plants will be continued.

Summarized key achievements of the study:

- Demonstrated that camalexin biosynthesis can be successfully reconstituted in epidermal cells through the expression of CYP71A13 and PAD3, with additional pathway activities being activated upon pathogen infection.
- Revealed that in obtained transgenic plants camalexin undergoes enhanced detoxification in epidermal tissue compared to its native biosynthetic site, indicating strong tissue-specific differences in its metabolic fate.
- Showed that camalexin produced in the epidermis remains biologically active and effectively suppresses pathogen growth (*PcBMM*).
- Identified PEN2-dependent metabolites, I3M-DTC and 4MI3M-DTC, formed in *Arabidopsis thaliana* during pathogen perception.
- Confirmed that the core steps of brassinin biosynthesis from I3G to I3M-DTC are evolutionarily conserved between *A. thaliana* and *Brassica rapa*, and that expression of DTC-MT alone is sufficient to enable brassinin production in *A. thaliana*.
- Demonstrated that the enzyme BABG exhibits higher efficiency in brassinin formation than PEN2,
- Established that DTC-MT lacks activity toward 4MI3M-DTC, highlighting its strict substrate specificity.
- Showed that brassinin is functional in a heterologous *A. thaliana* system, contributes to pathogen resistance, and is subject to detoxification by endogenous plant enzymes via hydroxylation and glycosylation.

- Demonstrated that the formation of spirobrassinin and cyclobrassinin requires *Brassica rapa*-specific enzymes (CYP71CR1 and CYP71CR2) and does not occur with native *A. thaliana* enzymatic machinery.

The 18-page Discussion section is generally well written and effectively interprets the obtained results in the context of the available literature. However, I am not a strong advocate of dividing the Discussion into subsections that mirror the Results section. With such an approach, it might be more appropriate to combine the Results and Discussion into a single chapter. I do, however, appreciate the inclusion of a final summary at the end of the thesis.

I have a few editorial comments; however, I do not intend to list all of them, but rather to highlight the most common issues identified in the thesis in order to support improved scientific writing in the future. There are a few minor instances of awkwardly phrased sentences, for example the statement that "Scientific interest in this species dates back to the 1940s, when Friedrich Laibach first proposed its use due to its small genome and ease of cultivation (Koornneef and Meinke, 2010)." This wording is potentially misleading, as the small genome size of *Arabidopsis thaliana* was not known at that time; instead, the species was selected based on its practical experimental advantages.

There are also several editorial issues that make the manuscript more difficult to follow. For example, some figures or schemes need to be read from right to left, which is not intuitive. References to figures that appear several pages later (e.g., section 4.1.10 on p. 65 referring to a figure on p. 69) disrupt the reading flow, and in some cases it is unclear whether the reference is correct. Figure captions are sometimes split across pages, further reducing readability. The placement of tables can also be problematic, requiring the reader to frequently jump back and forth in the text. In addition, it is unclear what the different shades of gray represent in Figure 4.2.1.

Despite these minor comments, it is clear that a great deal of effort has been invested in this work, both in terms of the experimental design and the analysis of the results. I am convinced that the generated transgenic plants may serve as valuable material for future research. I would also like to ask the author a forward-looking question: if the study were to be continued, which experiments would be prioritized first?

To sum up, the doctoral dissertation submitted for review meets the requirements specified in the Act of 20 July 2018 – Law on Higher Education and Science (Journal of Laws of 2018, item 1668, as amended), as well as in the Procedure for Awarding the Doctoral Degree at the Institute of Bioorganic Chemistry PAS No. 40/2025/Internet/RN_140 of 18 March 2025). I therefore request that the Scientific Council of the Institute of Bioorganic Chemistry PAS admit M. Sc. Serhii Kozin to the further stages of the proceedings for the award of the doctoral degree.

I would like also to recommend awarding a distinction to the doctoral dissertation due to its high scientific quality, originality, and significant contribution to the advancement of the field. The research presented is both theoretically valuable and practically relevant. The thesis not only provides valuable insights into studied biosynthetic pathways but also opens the way for future applied applications.

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