MUNI
FACULTY
OF SCIENCE

Oddělení experimentální biologie rostlin

Review of the Ph.D. Thesis submitted in the Doctoral study program in Experimental Plant Biology at the Faculty of Natural Sciences of Charles University

Student name: George A. Caldarescu

Thesis title: MLOs and EXO70H4 interplay in trichomes cell wall development

Name and affiliation of reviewer: Assoc. Prof. Marketa Samalova, PhD
Department of Experimental Biology of Plants, Faculty of Science, Masaryk University

The doctoral thesis of PhD candidate George A. Caldarescu makes a significant contribution to the field of plant cell biology by elucidating the specific interactions and molecular mechanisms underlying trichome cell wall (CW) biogenesis in *Arabidopsis thaliana*. The research provides novel insights into the interplay between EXO70H4, a subunit of the exocyst complex, and MLO proteins (MLO2, MLO6, and MLO12) in regulating vesicle tethering during secondary CW synthesis in trichomes. Mutant analyses revealed disrupted callose deposition, accompanied by defects in reactive oxygen species (ROS) localization and heavy metal distribution. Notably, CW analysis demonstrated the absence of calcium carbonate, highlighting the role of these proteins in biomineralization and CW hardening. The candidate provided compelling evidence that MLO6 plays a central role in callose distribution in *Arabidopsis* trichomes and that the physical interaction between EXO70H4 and MLO6 is critical for determining vesicle fusion sites.

Using AlphaFold predictions and molecular dynamics simulations, the study unveiled exciting findings, showing that MLO proteins must form trimers to function as calcium transport channels. Furthermore, this trimeric structure is stabilized when incorporated into the plasma membrane, offering new insights into the molecular basis of MLO function. In addition to these discoveries, the candidate successfully established proximity labelling using the TurboID system in the laboratory. This methodological innovation uncovered a previously unrecognized link between exocytosis and autophagy and represents a significant advancement for future research.

The thesis is supported by a manuscript published in the prestigious journal *The Plant Cell* (Impact Factor 10), where the candidate is credited as a first shared co-author (*"Interplay of EXO70 and MLO proteins modulates trichome cell wall composition and susceptibility to powdery mildew"*). The specific contributions of the candidate and other co-authors are clearly stated.

The theoretical part of the thesis examines the structure and function of trichomes, with a particular focus on the roles of callose, calcium, and ROS in plant development and stress responses. The candidate then addresses the vesicle-tethering exocyst complex, emphasizing its critical role in guiding secretory vesicles to specific plasma membrane sites. Mildew Resistance Locus O (MLO) proteins, characterised as seven-transmembrane domain proteins, are discussed in the context of their role in reducing susceptibility to barley pathogens, their

involvement in polar secretion, and recent discoveries identifying them as calcium channels. These findings served as the foundation for the research hypotheses. Advances in machine learning, including tools such as AlphaFold, molecular dynamics simulations, and proximity labelling, are also highlighted for their transformative applications in plant research, reflecting the candidate's broad engagement with contemporary scientific methodologies.

The aims and research objectives are clearly defined, encompassing the phenotypic analysis of *exo70H4* and *mlo* mutants, molecular-mechanistic investigations of EXO70H4-MLO protein interactions, bioinformatic modelling and prediction of functional MLO complexes, and the establishment of the TurboID methodology. The candidate successfully achieves these goals through well-defined research questions and a logical, stepwise approach.

The experimental design is thorough and well-aligned with the thesis objectives. The candidate used T-DNA-based *mlo* mutant lines together with wild-type (WT) controls, and prepared an additional *mlo6* mutant generated via CRISPR-Cas9 gene editing to corroborate the dominant role of the MLO6 isoform. However, the thesis lacks critical details, such as primer sequences for preparing the lines. Similarly, the preparation of transgenic lines expressing fluorescent protein-tagged constructs for the localization of EXO70H4 or MLO proteins is inadequately detailed, particularly regarding cloning strategies and number of plant generations or their selection. The candidate employed histochemical methods to visualize ROS and heavy metals in trichomes, alongside a range of techniques to characterize CW composition, which demonstrates the candidate's technical proficiency. These included quantification of neutral sugars and cellulose, Fourier-transform infrared (FTIR) spectroscopy for global CW composition (performed by collaborators), energy-dispersive X-ray spectroscopy (EDS) analysis and non-destructive Raman microscopy, that confirmed the absence of calcium carbonate in the triple *mlo* mutant. Further, he analysed the fluorescent lines using confocal laser scanning microscopy (CLSM), and validated protein-protein interactions through FRET-FLIM analysis and yeast two-hybrid assays. Also, a plant luciferase complementation imaging assay is mentioned in the results but not detailed in the Materials and Methods section. Finally, several advanced computational approaches, including AlphaFold and molecular dynamics simulations, were performed by collaborators. The methods are described in sufficient detail to ensure reproducibility and are presented in a logical sequence. Furthermore, the image analysis, graphical data presentation, and statistical analyses were conducted to a high standard.

The result section provides a clear explanation of the purpose of each experiment. The findings are both significant and original, providing compelling evidence for the collaborative roles of EXO70H4 and MLO proteins in trichome CW biogenesis, notably, the disrupted callose deposition and lack of calcium in mutant CWs. The localization of EXO70H4 and MLO proteins tagged with fluorescent markers in trichomes, as well as the effects of loss-of-function mutations on partner localization and the callose synthase PMR4 (Powdery Milddew Resistance 4) in triple mutant trichomes, provides valuable insights. The interaction between MLO proteins and EXO70H4 is confirmed through multiple methods, including FRET-FLIM in transient tobacco assay, the plant luciferase assay, and yeast two-hybrid analysis, indicating a strong interaction of EXO70H4 with the C-terminus of MLO6. The integration of

experimental findings with bioinformatic modelling enhances the interpretation of the results. Proteome analysis of *Arabidopsis* using TurboID proximity labelling was also conducted. However, the rationale for selecting the *exo84b-1* mutant background and the focus on ATG18a protein among the 135 labelled proteins identified are insufficiently explained.

The discussion is thorough and insightful, effectively comparing the results with existing literature and showcasing the candidate's critical thinking and deeper understanding of the scientific field. The interpretations are well-reasoned, and the proposed solutions to unresolved questions highlight the candidate's ability to address complex issues and contribute to advancing the field. The thesis references a wide range of relevant, up-to-date, and high-quality sources, with all citations correctly formatted.

Overall, the thesis is well-written, logically structured, and professionally presented. Minor inconsistencies in gene and protein nomenclature, as well as formatting of plant species names, should be addressed to align with international conventions. Figures and graphs are of high quality, clearly labelled, and effectively support the text. However, not all abbreviations are listed at the beginning of the thesis. On the other hand, “callose” is not an abbreviation:) The formatting and language are of high quality, with only minimal typographical errors noted.

The reviewed PhD thesis represents a significant contribution to the field of plant cell biology, advancing our understanding of vesicle-mediated processes and CW dynamics in *Arabidopsis* trichomes. It is of high scientific quality, demonstrating the candidate’s mastery of the discipline, originality in research, and ability to conduct independent scientific work. The research is innovative, impactful, and rigorously executed.

I strongly recommend this dissertation for defence.

Questions for the PhD candidate:

1. For generating the plant expression constructs and transgenic lines (p. 60), you used cDNAs for *MLO2* and *MLO6* genes but genomic sequences for *MLO12* and *EXO70H4*. Was there a specific reason for this choice? For example, did you observe differences in expression levels between constructs? Additionally, which generation of transgenic plants did you work with, and were they homozygous?

2. In your thesis, you describe Raman spectroscopy as a non-destructive method (p. 27) with high spatial resolution, which allowed you to identify the missing1080 cm^{-1} peak in the *mlo* triple mutant. Could you tell us more details about this novel technique, particularly in the context of its application to plant CWs?

3. In your CLSM localization experiments, you claim that the mCherry-EXO70H4 and MLO-GFP co-localized at the plasma membrane (Figure 9) and that, after plasmolysis, a portion of these proteins was trapped in the CW (Figure 10). In Figure 11, you state that “the

left panels show single planes focused on the CW, and the right panels show single planes focused on the PM". Given the resolution limits of CLSM, how did you achieve this distinction? Do you have images of WT trichomes under the same imaging setting (for mCherry and GFP) that would serve as negative controls? Do similar speckles appear there too?

4. Similarly, in Figure 12, you discuss the localization of GFP-PMR4. Can you definitively determine its localization based on the provided images or videos? On p. 38, you mention "…the typical CW localization pattern in WT…" but later suggest that "…the delivery of the callose synthase to the plasma membrane is perturbed…" Could you clarify these observations?

5. Using the FLIM-FRET method, you confirmed the interaction between MLO6-GFP and mCherry-EXO70H4. On p. 41, you note, "This reduction in the donor fluorescence lifetime strongly suggests an interaction between EXO70H4 and MLO6 in the plant cell cytoplasm (Fig. 13E)." Given that both proteins are localized to the plasma membrane or cell wall, how do you reconcile this observation? Could you provide additional details about the constructs or share images to clarify?

6. In your TurboID proximity labelling experiment you focused on ATG18a. What was the rationale for selecting this protein? What are the potential functional implications of the newly identified EXO84B-ATG18a interaction in coordinating autophagy and exocytosis? In the description of Figure 15, you mentioned that you used MS media with the addition of 100 μM … of what?

7. How do you envision the findings of your thesis contributing to applied research, such as improving crop traits or enhancing plant stress tolerance?

In Brno, 17 January 2025

doc. Mgr. Markéta Šámalová, Ph.D.

Masarykova univerzita | Přírodovědecká fakulta
Oddělení experimentální biologie rostlin
A: Kamenice 735/5 | 625 00 Brno, CZ
T: +420 549 498 476 | W: www.sci.muni.cz/ebr
E: marketa.samalova@sci.muni.cz