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Reviewer Report on the dissertation of George A. Caldarescu "MLOs and EXO70H4 interplay in trichomes cell wall development"

To Prof. Dr. Jana Albrechtova, Chairperson of the Board of Examiners for the PhD defense of George A. Caldarescu PhD student at Charles University, Prague, Czech Republic,

Please, find enclosed my reviewer report on the PhD thesis of George A. Caldarescu submitted to the Faculty of Science at the Charles University, Prague. The practical work towards this thesis was performed in the laboratory of and under supervision of Prof. Dr. Viktor Žárský.

(1) Short overall assessment of the work. This is highly relevant, groundbreaking, novel work on the role of the plant MILDEW RESISTANCE LOCUS O (MLO) proteins, particularly MLO6 and its interaction with the exocyst subunit EXO70H4.

(2) Main hypothesis
The main hypothesis of the work that needed to be tested was that *MILDEW RESISTANCE LOCUS O* (*MLO*) genes and their protein products may interact with the exocyst subunit *EXO70H4* gene and the encoded protein in *Arabidopsis thaliana* (Arabidopsis) leaf trichomes and that both may have a role in trichome cell wall formation.

(3) Specific assessment of the work based on its goals

Along the above hypothesis the doctoral work of George Caldarescu aimed at:

Goal 1) an understanding of the role of the *MILDEW RESISTANCE LOCUS O* (*MLO*) genes *2*, *6* and *12* in comparison to the exocyst subunit *EXO70H4* gene in *Arabidopsis thaliana* (Arabidopsis) trichomes. To address this, George Caldarescu employed microscopic analyses of callose deposition, H_2O_2 reactive oxygen species (ROS) formation, heavy metal accumulation and calcium carbonate accumulation in *exo70H4* as well as several *mlo* mutants. These goals were met by generation of a knew *mlo6-6* CRISPR/Cas9 mutant allele and thorough quantitative analyses of a single *exo70H4* mutant as well as single, double and triple and mutant analysis of the above-mentioned *MLO* genes, employing histological stains for

callose, ROS, and HM accumulation in the wild-type and mutant backgrounds. The levels of all those analyzed components proved to be reduced in *exo70H4* mutant *mlo2 mlo6 mlo12* and triple mutant trichomes. George Caldarescu further employed energy-dispersive X-ray spectroscopy (EDS) analysis to observe calcium carbonate and cell wall polysaccharide accumulation. This revealed a strong reduction of calcium carbonate but not of pectins, cellulose and lignins in the *exo70H4* single and the *mlo2 mlo6 mlo12* triple mutant trichomes. Hence, the thesis provides a thorough, comprehensive, methodologically advanced and sound analysis of the mutant trichomes, revealing a contribution mostly by MLO6, to a lesser extent of MLO2 and to a low (if any) extent by MLO12 to the above-described processes.

Goal 2) aimed at a molecular-mechanistic analysis and understanding of the functional interaction between the EXO70H4 and MLO proteins. This was addressed by localization of fluorescently tagged proteins of MLO2, 6 and 12 in trichomes as well as the co-localization analyses of those proteins with fluorescently tagged EXO70H4, which all proved to co-localize. Localization analyses in the respective other mutant background revealed interdependence of MLO6 and EXO70H4 localization, when tested in the *exo70H4* and *mlo2 mlo6 mlo12* mutant backgrounds, respectively. Moreover, consistent with reduced callose levels in *mlo2 mlo6 mlo12* trichomes, the GFP-tagged PMR4 callose synthase was affected in its localization in the *mlo2 mlo6 mlo12* mutant background. Protein-protein interactions (not only but) preferentially between EXO70H4 and MLO6 were observed with the MLO6 C-terminus in a heterologous yeast two-hybrid system, confirmed by split-luciferase assays in the heterologous *Nicotiana benthamiana* plant system and by FRET/FLIM analysis of mCherry-EXO70H4 and MLO6-GFP overexpressed in *N. benthamiana.* Taken together, the goal of analyzing molecular interactions was well met by employing three independent *in vivo* or *in planta* methods, although testing the interaction in Arabidopsis trichomes could not yet be achieved. A deeper mechanistic understanding of the interaction will require much more than one PhD thesis, but it can already be said that the interaction in yeast occurs via the MLO6 cytosolic C-terminus.

Goal 3) aimed at a bioinformatic modeling and prediction of a functional MLO protein membrane complex. The goal was fulfilled, in part, by providing an AlphaFold 2 model for an MLO6 trimer and an all-atom molecular dynamics simulation of its Ca^{2+} transport activities. I do not have the competence to review the molecular dynamics simulation, but the description of both in the thesis is rather short. Evaluating the AlphaFold model would have benefitted from including a comparison between the probabilities of the overall fold formation and the subunit interface interaction/alignment within the proposed structure for mono-, di- tri and tetramers, so one could judge which model is actually the most likely one. This, however, can be something for a future thesis and the newer AlphaFold 3 program now also provides more possibilities for locating possible calcium binding sites within trimers and for testing the likelihoods of interactions with other ions.

Goal 4) aimed at introducing the TurboID methodology in the laboratory and on studying the exocyst proteome under biotic stress. This was achieved and among 135 proteins identified from plants treated with *Pseudomonas syringae* one highly interesting candidate, ATG18a was already confirmed to co-localize and interact with EXO84B by FRET/FLIM analysis in the heterologous *N. benthamiana* system.

(4) General comments/evaluation

This is a high-quality PhD thesis presenting an impressive amount of thoroughly performed experiments that uncover novel roles of MLO proteins particularly MLO6, in trichome cell

wall differentiation. The thesis is well structured, the introduction contains all necessary information to follow the work. The results are well presented, the figures and graphs are of high quality and contain appropriate statistical analyses. The Materials and Methods sufficiently outline the experimental procedures to allow for reproduction.

The discussion is sufficient and of good quality, integrating and discussing major findings within the field. The thesis has also generated ample results based on which future studies can be performed, such as further modeling and functional analysis of MLO6 function in calcium transport and its mechanistic interaction with EXO70H4 as well as the follow up of the *in planta* function of ATG18a interaction with EXO84B.

The Literature sources used are adequate, relevant and up to date and they are correctly cited in the work. Only, the citation style is not consistent throughout the thesis text. Sometimes only the last author name is used, sometimes the last author name and the initial or the first name.

This PhD thesis contains an impressively large amount of thorough work, employing diverse technologies and, certainly, is far beyond an average PhD thesis. Unfortunately, the formal side is - although still acceptable – not that impressive, as the work includes a larger number of language errors and typos as well as inconsistencies in mutant and species names (variably sometimes in italics, sometimes not) and a couple of sentences that are incomplete (see questions).

Nonetheless, given the overall amount of excellent work, the novelty of the findings, the overall very good structure of the thesis, the statistical analyses performed and especially the groundbreaking nature of the work with respect to the completely novel role of the MLO6 and other proteins in trichome cell wall differentiation, I consider this, despite some formal shortcomings, a very good PhD thesis that has led to a number of groundbreaking findings and a well-deserved first co-authorship in "The Plant Cell" - one of the top journals in plant research.

Thus, this dissertation certainly meets the conditions for holding a defense and I highly recommend the dissertation for holding the defense.

With kind regards,

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(5) Questions to the defendant

1. Page 7, first paragraph, introduction: **Q**: Was there a specific reason not to include GLABRA2 here?

2. Page 8, "All cell wall constituents, including pectins and hemicellulose, are synthesised de novo in the Golgi apparatus and secreted into the apoplast (Mohnen, 2008; Scheller et al., 2010)." **Q**: This sentence is prone to be misunderstood. Why?

3. Page 10 "Furthermore, the pmr4 LOF mutant exhibited enhanced resistance to pathogens, attributed to a higher concentration of salicylic acid. This suggested that PMR4 is a negative regulator of this hormone (Nishimura et al., 2003). **Q**: Can one solidly claim after analysis of a loss-of-function mutant that PMR4 is a regulator of a process? (*A. thaliana* mutant names always in italics, consistently throughout a thesis, please)

4. Page 13: "For instance, EXO84B regulates the polar localisation of PEN3 and NIP5;1 (Mao et al., 2016)." **Q:** Mao et al. did not write that EXO84B regulates PEN3 and NIP5;1. Why?

5. Page 21, figure 1: **Q**: How would you explain the differences in cell wall pixel intensity between the two different *mlo2 mlo6 mlo12* mutant allele combinations in Figs. 1d,e?

6. Page 21, figure 1: **Comment**: Fig. 1B You should give the number "n" that is underlying your statistical analysis does n = 150 or does n = 600 i.e. is one representative experiment displayed or did you pool the numbers of all four experiment? Both may be legitimate, but one cannot find out from the description in the legend and that holds for several figure legends in this thesis. The same issue with Fig. 1D, E is the data in the graph based on 20 or 40 trichomes?

7. Page 22, figure 2b: **Comment**: This should read "µm" not "µ" on the y-axis of the graphs in Fig. 2B. **Q**: Is the average cell wall thickness of 2 µm estimated by fluorescence microscopy for the Columbia-0 trichome a slight overestimate or in good agreement with published values from transmission electron microscopy analyses?

8. Page 26, figure 4b, legend: "Figure 4: The exo70h4-1 mutant and all the MLOs mutants investigated share similar aberrant ROS and HM localisation patterns in rosette leaf trichomes." **Q**: Does your data really support that specific statement? While all mutants similarly differ from the wild type in ROS deposition, there is not a significant difference for heavy metal distribution between wild type and all the mutants and, thus, the heavy metal distribution shows three groups of different significances. Why do you think this is the case?

9. Page 28: "The intensity of the cellulose peak can also help us to distinguish their orientation (Gierlinger, 2018)." **Q:** I guess by "their" you refer to the "cellulose microfibrils" here.

10. Page 39: Figure 12 **Q:** Why was the EXO70H4 rather than the PMR4 promoter used to analyze GFP-PMR4 expression?

11. Page 40: **Q:** Did you consider generating an *exo70h4 mlo6* double or an *mlo2 mlo6 mlo12 exo70h4* quadruple mutant to test for genetic interaction?

12. Page 43: **Q**: Could one perform FRET-FLIM analysis on trichomes expressing both mCherry-EXO70H4 and MLO6-GFP under control of their own promoters, as these transgenes are principally available - or are there technical challenges towards this?

13. Page 44: **Q**: So, what had the highest probability score for overall fold formation and for correct orientation/interface interaction of the subunits - the MLO6 homo di-, tri, or tetramer?

14. Page 44: **Q:** Is there really a high probability for the formation of a heterotrimer in the AlphaFold model and how likely is this relative to an MLO6 homotrimer?

15. Page 44: **Q:** Did you also try this prediction on AlphaFold3 as it came out prior to submission of this thesis and allows for improved modelling of protein-protein interactions as well as protein-ion interactions such as divalent Calcium. Magnesium, Copper, Zinc cations? (if you did not, we can skip the next question)

16. Page 44: **Q:** Did you test as to whether adding calcium cations improves or decreases the probability of the correct structure prediction in AlphaFold and how many calcium ions can be bound to the homomeric subunits without decreasing the structure prediction?

17. Page 47, figure 15, legend: "with the addition of 100 µM on shaker" It lacks what has been added – biotin.

18. Page 49: "we show that MLO must form a trimer to be able to transport calcium". Comment: I did not find experimental evidence towards this, not even towards that it does form a trimer. You provide an *in silico* model that suggests this - which is a good start. **Q**: Is there (/are) there are there way(s) to biochemically address and corroborate this, in the future, as to whether MLO6 forms homotrimers and whether this is a necessity for its function?

19. Page 50: "Analysing and quantifying the parameters that determine the trichome phenotype, such as callose deposition, which, along with the cell wall's autofluorescence, are considered the primary discriminators between Col-0 WT and the exo70h4-1 mutant, and cell wall thickness, which is influenced by the proper delivery to the plasma membrane of the various components used to build up the cell wall." **Q:** This is a very long but, unfortunately, incomplete sentence. Could you explain what you wanted to say here?

20. Page 54, last sentence: **Q**: Is there a possibility that MLO6 (2 and/or 12) could transport heavy metal ions themselves?

21. Page 60: "**Agrobacterium tumefaciens**" should not be bold but in italics here and throughout the thesis the form considering the right spelling and formatting of mutant names, species names etc. is quite inconsistent.

22. **Q**:What is your favourite hypothesis for the function of the EXO84B and ATG18a interaction *in planta* and how would you test forn this?