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Review of the PhD dissertation titled

"Charakterystyka funkcjonalna miRNA specyficznych dla wątrobowców w rozwoju narządów płciowych *Marchantia polymorpha*"

Functional characterization of liverwort-specific miRNAs in sexual organ development of *Marchantia polymorpha*

authored by Bharti Aggarwal

Overview This doctoral dissertation, authored by Ms. Bharti Aggarwal under the supervision of Prof. Dr. hab. Zofia Szweykowska-Kulińska and Dr. Halina Pietrykowska has been completed at Adam Mickiewicz University in Poznań, Poland. The research presented in the thesis was supported by the Polish National Science Center NCN (OPUS grant: UMO/2020/39/B/NZ3/00539) and IDUB (The Initiative of Excellence Research University 05/IDUB/2019/94) at Adam Mickiewicz University. Some findings from this dissertation have been published in the research paper *"MicroRNAs differentially expressed in vegetative and reproductive organs of Marchantia polymorpha- insights into their expression pattern, gene structures and function"* in RNA Biology (2024). Additional publications co-authored by the candidate include two review articles in Plant Molecular Biology (2023) and Environmental Pollution (2022), a research article in Journal of Plant Biochemistry and Biotechnology (2021), and several book chapters (2024, 2023, 2021).

The dissertation examines the role of liverwort-specific microRNAs (miRNAs) in the development of the sexual organs in *Marchantia polymorpha*, a model organism representing one of the earliest land plant lineages. Proper sexual organ development and reproductive success require precise developmental control, which relies on complex genetic networks, often fine-tuned by small non-coding RNAs, such as miRNAs. While conserved miRNAs present in all land plants have been studied to some extent in *Marchantia*, the roles of liverwort-specific miRNAs are largely unexplored.

Topic and Novelty of the Dissertation

The dissertation addresses fundamental aspects of plant developmental biology, with a focus on the evolutionarily early mechanisms of gene regulation. The choice of *Marchantia polymorpha* as a research object is well justified due to its phylogenetic position as a representative of one of the oldest land plant lineages. Most importantly, *M. plymorpha* serves as a model organism in molecular biology with a well-developed set of genetic and genomic tools.

The plant has several advantageous characteristics for genomic studies, including a relatively small and well-annotated genome, a dominant haploid phase that simplifies genetic analyses, efficient transformation techniques, and the availability of CRISPR/Cas9 genome editing. Its evolutionary position as a basal lineage of land plants enables the study of traits and regulatory mechanisms that predate the divergence of vascular plants.

Significant achievements in studies of *Marchantia* include the identification of key conserved developmental regulators, elucidation of hormonal signaling pathways (e.g., auxin, gibberellin), discoveries in organogenesis and morphogenesis, and the development of genetic tools and mutant libraries. Studies on *Marchantia* have greatly enriched our knowledge of plant development and evolution by revealing how ancient genetic and epigenetic programs regulate organ formation, stress responses, and reproductive strategies. Research on *Marchantia* has clarified the roles of transcription factors, chromatin remodelers, and small RNAs in developmental plasticity. It has also helped bridge gaps in our understanding of the early innovations that facilitated terrestrial colonization by plants.

The reviewed PhD dissertation presents research on the functional roles of selected non-conserved miRNAs in reproductive organ development. The novelty of the study lies in the pioneering examination of liverwort-specific miRNAs in the context of sexual development. Analysis of the mutants, including CRISPR/Cas9-generated knockouts, allowed the candidate to define specific biological functions of two miRNAs in the development of male and female reproductive structures.

Chapter 1- Introduction of the dissertation provides a well-structured introduction to *Marchantia polymorpha* as a model organism for plant developmental studies. It describes the evolutionary significance of bryophytes and highlights the advantages of *Marchantia* studies for genetic and molecular biology research. The chapter also offers a comprehensive overview of its anatomy, life cycle, reproductive strategies, and the pivotal role of miRNAs in gene regulation. The integration of historical context, technical detail, and current research challenges sets a solid foundation for the thesis and justifies the research focus on liverwort-specific miRNAs.

Chapter 2: Aim of the Thesis: The central hypothesis of the thesis is that liverwort-specific miRNAs are involved in the reproductive development of *Marchantia*. To verify this, the detailed tasks aimed to:

- Identify selected liverwort-specific *MIR* gene structures, pri-miRNA expression patterns, and mRNA target predictions.
- Select promising miRNA-mRNA target modules for in-depth analysis.
- Generate GUS reporter lines for selected *MIR* gene promoters.
- Create CRISPR/Cas9 knock-out (KO) mutants for selected *MIR* genes.
- Perform phenotypic characterization of the KO mutants.
- Define the function of the selected miRNAs in vegetative and reproductive development.

Chapters 3 & 4: Materials and Methodology

Chapters 3 and 4, covering Materials and Methods, provide a detailed and methodical account of the experimental procedures used throughout the dissertation. The author describes the propagation

of *Marchantia polymorpha*, the usage of bacterial strains, the composition of culture media, and provides comprehensive lists of reagents, kits, and software tools. The methodologies include precise protocols for molecular biology techniques such as RNA extraction, RT-qPCR, CRISPR/Cas9 gene editing, GUS reporter assays, RNA-seq, degradome sequencing, northern blotting, 5′ RLM-RACE, mutant line analysis, and confocal microscopy, demonstrating technical rigor. The protocols are articulated and reproducible, indicating careful planning and execution.

Chapter 5: Results

Chapter 5 presents the results in a comprehensive and logically structured manner, demonstrating an understanding of the functional roles of liverwort-specific miRNAs in *Marchantia*. The author skillfully combines sRNA and degradome sequencing, northern blotting, RACE experiments, GUS reporter assays, and CRISPR/Cas9 knockouts to characterize selected miRNAs and their targets. The dissertation focused on six liverwort-specific miRNAs: MpmiR11737a/b, MpmiR11865*/11865, MpmiR11887, and MpmiR11796. The findings show distinct expression patterns and functional effects of miRNAs on reproductive organ development. The data are visualized and convincingly interpreted, providing evidence for the developmental roles of two miRNAs: MpmiR11887 and MpmiR11796. Overall, **the results are novel, validate the thesis hypothesis, and have significant original contributions to knowledge about the structure and function of miRNA in *Marchantia*.**

Key Findings

The key findings from Chapter 5 of the dissertation can be summarized as follows:

- **Liverwort-specific miRNAs show distinct organ-specific expression**:
  Several miRNAs, particularly MpmiR11887 and MpmiR11796, are explicitly expressed in reproductive organs—antheridiophores and archegoniophores, respectively—suggesting specialized roles in sexual development.
- **MpmiR11887 is crucial for male reproductive development**:
  - It accumulates exclusively in antheridiophores.
  - CRISPR/Cas9 knockouts of MpMIR11887 lead to early and enhanced development of male reproductive structures
  - Promoter: GUS activity confirmed specific expression in spermatogonia.
  - It influences the timing and size of antheridiophore/antheridia development.
  - MpmiR11887 likely fine-tunes chromatin-associated gene expression in spermatogenesis; the candidate target MpATX1 encodes a chromatin related protein and is the homolog of histone lysine methyl transferase ATX2 in Arabidopsis.

  However, the direct physical interaction between the miRNA and its candidate target mRNA could not be confirmed by RACE analysis and the targets remain to be validated,

- **MpmiR11796 plays a significant role in female development**:
  - It is highly expressed in archegoniophores, especially in pegged rhizoids and the neck of archegonia.
  - Knockouts result in severe developmental defects: reduced thallus and archegoniophore size, fewer rhizoids, shorter stalks, and abnormal egg cell formation.

- It is crucial for the proper growth and morphology of female vegetative and reproductive structures, including rhizoids, ventral scales, archegonial receptacles, stalk elongation, egg cell development,
- Fertility and gamete development are impaired in subsequent generations, showing their role in reproductive success.

- **Non-coding genes encoding MpmiR11887 and MpmiR11796 are independently transcribed**:
  Detailed RACE analyses revealed that these miRNAs are transcribed from independent, intron-less units, sometimes overlapping with protein-coding genes, but functionally distinct.

Altogether, the results based on classical (mutant) and advanced molecular tools demonstrated the significant roles of MpmiR11887 and MpmiR11796 in male and female reproductive development, respectively. The findings suggest that liverwort-specific miRNAs are not merely lineage-specific but functionally essential regulators of reproductive organ development in *Marchantia polymorpha*.

Chapter 6: Discussion

The Discussion chapter synthesizes the experimental findings and places them in the broader context of plant developmental biology and miRNA evolution. It highlights the significance of liverwort-specific miRNAs—particularly MpmiR11887 and MpmiR11796—as key regulators of male and female reproductive development in *Marchantia polymorpha*. The author thoughtfully interprets the expression patterns, knockout phenotypes, and potential target interactions, emphasizing the evolutionary implications of miRNA specialization in early land plants. Limitations in target validation are acknowledged, and future directions are proposed, including deeper functional analyses and cross-species comparisons.

I appreciate the author's thoroughness in discussing the results; however, I believe that the role of the Discussion section is to provide a selective and coherent interpretation of the most important findings. Excessive reference to detailed results may obscure the main conclusions and make it more difficult to highlight and appreciate the key outcomes of the study.

The identification of targets for MpmiR11887, including the *MpATX1* gene encoding a chromatin-related protein, was unsuccessful. As stated in the Results section (p. 109), this part of the study was inconclusive: changes in *MpATX1* expression in the *mpmir11887* mutant were not statistically significant, and RACE analysis did not reveal a miRNA-guided cleavage site in the candidate mRNA. Despite these negative findings, the Discussion section still proposes further investigation of *MpATX1* as a potential regulatory target of MpmiR11887. I would expect the author to clarify this apparent inconsistency.

Chapter 7: Conclusions and Future Perspectives

Chapter 7 involves the main conclusions of the dissertation, emphasizing that liverwort-specific miRNAs, particularly MpmiR11887 and MpmiR11796, play crucial and non-redundant roles in the reproductive development of *Marchantia polymorpha*. The work demonstrates that these miRNAs are transcriptionally regulated, organ-specific, and influence developmental timing, organ morphology, and gamete formation. The chapter also outlines future perspectives, such as identifying precise miRNA targets, exploring regulatory networks, and expanding studies to other liverwort species.

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Besides summing up the experiments and results, some of Conclusions also highlights the relevance of these findings for understanding the evolution of gene regulation in early land plants. Moreover, the chapter also considers future research which are required to verify the hypothesis and clarify the final function (by target identification) of the studied miRNAs in the reproductive development of *Marchantia*.

General opinion on this chapter: In my opinion, Chapter 7 rather summarizes the results in detail, than presents general, consistent and the most important conclusions. I would appreciate it if the re-formulated conclusions could be included in the presentation during the Ph.D. defense.

Remarks regarding linguistic style and editorial accuracy

From a linguistic and editorial perspective, the dissertation is generally well written and clearly structured. The scientific language is appropriate and accurate, though minor typographical errors and occasional stylistic inconsistencies are present. For example, the spelling and formatting of gene names should be corrected in accordance with standard conventions. In *Marchantia*, as in other plants, gene names are written in *italics*, e.g., MpACT7, to distinguish from proteins or gene products, which are written in roman type (non-italicized) with capitalization, e.g., MpACT7. These shortcomings should be corrected; however, they in no way diminish the overall high quality of the manuscript.

Questions/comments to discuss during PhD defense:

- I would like the candidate answering the following questions during the defense:
    - Results: Mp4g20750 mRNA, which encodes a protein belonging to linker histone H1, was identified as a potential target for MpmiR11796 (published in Aggarwal et al. 2024). However, "*RT-qPCR analysis showed downregulation of Mp4g20750 mRNA in male Δmpmir11796ko plants (graphs not shown in the thesis). This suggests that Mp4g20750 is probably not the true target of MpmiR11796*" –p. 133 of the PhD Thesis. I would appreciate the candidate clarifying these apparently contradictory results.
    - Relevant to my above comment on some evolutionary aspects missed in the Discussion: are there any known functional analogs of MpmiR11887 and MpmiR11796 in higher plants (in particular, angiosperm) despite their apparent lineage-specificity in liverworts?
    - Do you see potential for translational applications of your results beyond basic research?

Final statement

Overall, the dissertation provides valuable new insights into the functional roles of liverwort-specific miRNAs in shaping the unique developmental processes of *Marchantia polymorpha*, highlighting their importance beyond the conserved miRNA repertoire and contributing to the understanding of land plant evolution. The reviewed PhD dissertation demonstrates that the candidate, Ms. Bharti Aggarwal, demonstrates in-depth knowledge in molecular and plant biology, proficiency in applying an impressive array of methods in genetics and genomics, as well as the ability to conduct sound scientific analysis and reasoning.

I state with complete confidence that **PhD thesis of Bharti Aggarwal fully meets the legal requirements for the award of the doctoral degree**, as stipulated in Article 187 sections 1–2 and Article 190 section 3 of the Act of 20 July 2018 – Law on Higher Education and Science (Journal of Laws 2024, item 1571, as amended).

Accordingly, **I hereby submit a request to the Scientific Council for the Discipline of Biological Sciences at Adam Mickiewicz University in Poznań to admit Ms. Bharti Aggarwal, MSc, to the subsequent stages of the doctoral procedure in the field of Natural Sciences, within the discipline of Biological Sciences.**

Sincerely,

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