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Thesis Review Report

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

By Ms. Bharti Aggarwal

Overview of the thesis:

Life on earth began with simplest life forms that faced extreme environmental conditions. Primitive plants, such as *Marchantia polymorpha*, possess sophisticated mechanisms to sustain in harsh environments. These adaptive mechanisms included structural, physiological and reproductive modulations such as cuticle evolution, resilience to prolonged dehydration, alternation of generations, development of extracellular structural components (sporopollenin / lignin / pectic acid), and cell to cell communication networks. These alterations were accompanied with molecular changes in the various regulatory networks involving different transcription factors (TFs) and small non-coding RNAs (miRNAs and siRNAs). Among these, miRNAs are the key regulators which helps to fine tune the gene expression which facilitates the physiological and biochemical changes required to sustain under prevailing environmental challenges. Ms. Bharti selected the one of three bryophyte lineages, *M. polymorpha* to elucidate the molecular mechanisms involving microRNAs. The present thesis aimed to enhance the current understanding of such molecular mechanisms involved in *M. polymorpha* development with special emphasis on sexual organ differentiation, thereby establishing a foundation for understanding sporophytic development in lower plants. Nine miRNA families including miR160, miR166, miR171, miR319, miR390, miR529/miR156, miR408, miR530/1030 and miR536 known to be conserved in all other land plants, were found in *M. polymorpha*. Previous studies used RNA NGS and northern blot analysis which showed that the liverwort-specific PenmiR8163/MpmiR11737a/b and PenmiR8170/MpmiR11865* exhibited differential expression in Marchantia's vegetative and generative organs. In addition, another study identified PenmiR8185/MpmiR11889 and MpmiR11887 and MpmiR11796 which exhibited intriguing expression patterns across Marchantia's organs.

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Research work and important findings

The present thesis performed the detailed studies on four miRNAs including the *MIR* gene structure prediction, pri-miRNA transcript levels, *MIR* gene promoter activities, and the identification of putative targets for these four liverwort-specific miRNAs. Additionally, the PhD scholar also performed the functional characterization of MpmiR11887 and MpmiR11796, thereby elucidating their roles in Marchantia's reproductive development. Previous sRNA NGS data and northern blot hybridization revealed that MpmiR11737a exhibited accumulation in male and female vegetative thalli, while the low level of accumulation was observed for MpmiR11737b. The pre-MpmiR11737a and pre-MpmiR11737b possessed classical stem loop structures. RT-qPCR analysis further validated these results and showed that higher transcript levels of pri-miR11737b were present in reproductive organs. These results suggested that MpmiR11737a and MpmiR11737b exhibited opposite accumulation patterns in *Marchantia*. The full-length transcript of Mp*MIR11737a* gene of 1131 bp was obtained using 5'-RLM and 3'-RACE. The scholar found that MpmiR11737b was located within the 5'-UTR of Mp*8g07030* gene. The attempts to amplify MpmiR11737b were unsuccessful due to low expression levels of pri-MpmiR11737b. The TSS of Mp*MIR11737a* gene was found to be present within the long intron, while the termination site was present in the third exon of its host gene Mp*5g12920*. Based on the degradome sequencing data, Mp*1g15010* gene encoding an uncharacterized protein was identified as a putative mRNA target for MpmiR11737a. The putative miRNA cleavage site was found to be present within the 3'-UTR region. In addition, based on the RT-qPCR, the inverse correlation was observed between the expression level of MpmiR11737a and its putative target Mp*1g15010* transcript. Another liverwort-specific miRNA Pen-miR8170/MpmiR11865* was identified from sRNA NGS data of *M. Polymorpha.* MpmiR11865* exhibited higher expression in the female vegetative thalli and archegoniophores. These results were further confirmed through northern blot analysis. In contrast to NGS data, northern blot analysis showed that MpmiR11865* predominantly accumulated in the antheridiophores. On the contrary, the mature MpmiR11865 exhibited higher accumulation in archegoniophores. These results suggested that both these miRNA species exhibited opposite expression profiles. The

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pri-MpmiR11865 transcript showed higher accumulation in both antheridiophores and archegoniophores. However, the differential accumulation of MpmiR11865* and MpmiR11865 highlighted their different regulatory role in antheridiophores and archegoniophores, respectively. Based on 5'-RLM and 3'-RACE, the full length of MpmiR11865 gene was found to be 1588 bp. Moreover, the TSS and termination site of this gene was not present within any protein-coding genes, representing an intron-less independent transcriptional unit. Degradome sequencing data analysis identified Mp*1g05970* gene encoding a tRNA ligase1 protein as the potential target of MpmiR11865*. This gene produces four alternatively spliced mRNA variants that can all be potentially cleaved with the putative cleavage site present within the 3'-UTR region. In addition, the identified target mRNA exhibited low expression levels, while higher expression was observed for MpmiR11865* in antheridiophores. Likewise, the PhD scholar identified a potential new target (Mp*6g13460* gene) of MpmiR11865 which encodes a nuclear AAA+ATPase (Valosin-containing protein subfamily) protein. This gene produces two mRNA variants. The putative cleavage site was located within the 3'-UTR region. The degradome data was validated by RT-qPCR analysis in which the Mp*6g13460* expression was downregulated in archegoniophores, exhibiting an inverse correlation between expression level of target mRNA and accumulation level of MpmiR11865. The PhD scholar functionally characterized the liverwort-specific miRNAs, MpMIR11887 which is exclusively expressed in antheridiophores and MpMIR11796 which shows dominant expression in archegoniophores. MpmiR11796 is specific two liverwort species: *Lunularia cruciata* and *M. polymorpha,* while MpmiR11887 is specific to one liverwort species, *M. polymorpha.* sNGS data and qRT-PCR showed that both pri-MpmiR11887 and mature MpmiR11887 were present specifically in the antheridiophores. MpmiR11887 sequence was found to overlap with with the 3'-UTR of Mp*6g01830* gene, encoding putative protein of unknown function. In the 5'-RLM and 3'-RACE analysis, the full-length of Mp*MIR11887* gene was determined to be ~1211 bp. It further confirmed that *MpMIR11887* gene was an independent transcriptional unit and the upstream sequence of pre-MpmiR11887 might encode for putative 65 aa long miPeP. The promoter analysis of *MpMIR11887* gene using GUS reporter assay revealed that the GUS expression was induced

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in young antheridia, spermatogenous cells and also in the apical notch of female vegetative thalli and archegonia. The PhD scholar functionally characterized *MpmiR11887* in *M. polymorpha* by generating knock-out (KO) mutants using CRISPR/Cas9 system. Several guide RNAs were designed which targeted 5'-end of the pre-MpmiR11887 or mature MpmiR11887 regions. Five mutants were developed using sgRNA1, while 4 mutants were developed using sgRNA2. Total 9 mutant plants (8 male and 1 female mutant plants) were confirmed for the presence of mutations. Northern blot hybridization confirmed the absence of *MpmiR11887* gene expression in all the mutant plants. Only two mutant plants showed no conformational changes in the structure of pre-MpmiR11887, still no *MpmiR11887* gene expression was observed in these mutants. Three KO (knock out) mutants were selected for functional characterization of *MpmiR11887* gene. The KO plants exhibited differences in the early stages of gemmae development with larger thallus area as compared to WT Tak-1 plants. Another developmental difference was also observed, where 5-week-old KO plants possessed the developed antheridiophores, while, in WT plants, they had only emerged. These results suggested that the MpmiR11887 might be involved in regulating the developmental timing of male gametophyte production. The KO plants exhibited increased diameter of antheridial discs which contained larger antheridia more frequently. This might produce more sperm cells thereby showing higher fertilization efficiency which was further confirmed by achieving successful fertilization. Based on degradome sequencing data, several target mRNAs of MpmiR11887 including *Mp1g20730* (β-tubulin), *Mp7g16780* (*MpATX1*) and *Mp3g14390* (*MpPRM*) were identified. These target mRNAs could not be validated through qRT-PCR and RACE. For the functional characterization of *MpmiR11796*, sRNA NGS and northern hybridization performed previously showed higher accumulation of *MpmiR11796* in the female gametophyte - archegoniophores. However, lower levels of pri-*MpmiR11796* were observed in archegoniophores compared to female vegetative thalli owing to the fact that the pre-MpmiR11796 is more rapidly processed into mature *MpmiR11796*. In the 5'-RLM and 3'-RACE analysis, the full-length of MpmiR11796 gene was determined to be ~505 bp. It was further confirmed that MpmiR11796 gene was an independent transcriptional unit with overlapping sequence with the putative protein encoding gene. The promoter analysis of

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MpmiR11796 gene using GUS reporter assay revealed that the promoter activity was observed in rhizoids present in digitate rays of archegoniophores, stalk of archegoniophore archegonia, ventral scales of female vegetative thallus, antheridiophores and male vegetative thallus. The PhD scholar also generated knock-out (KO) mutants of MpmiR11796 using CRISPR/Cas9 system. Two guide RNAs were designed which targeted miRNA region. One mutant was developed using sgRNA1, while 5 mutants were developed using sgRNA2. Total 6 mutant plants (4 male and 2 female mutant plants) were confirmed for the presence of mutations. Northern blot hybridization confirmed the absence of *MpmiR11796* gene expression in all the mutant plants. All the mutant plants showed conformational changes in the the structure of pre-*MpmiR11796*. The KO plants exhibited limited growth and smaller thallus area as compared to WT. In addition, mutants produced significantly decreased number of gemma cups. In contrast to WT, the structure and layering of ventral scales is disrupted in one of the KO plants. These observations indicated that MpmiR11796 was involved in the development of various structures of female vegetative thallus in *Marchantia*. The KO plants had extremely smaller archegonial receptacles (AR) and often produce an extra small arm on the AR. In addition, in one KO plant, archegoniophores was not able to produce rhizoids and ventral scales as compared to WT. Moreover, the stalks of archegoniophore in same KO plants did not showed any elongation, while WT archegoniophores began to mature and developed elongated brown stalks.The mutant Δ*mpmir11796#29* had significantly lesser area in the archegoniophore stalk which resulted in the reduction in the size of two grooves of rhizoids and simultaneously lesser bundles of pegged rhizoids comparative to WT. Two mutants also exhibited various abnormalities during gemmae development in male. Both KO plants exhibited decreased number of smooth rhizoids during gemmae development. These results suggested that MpmiR11796 is required to mediate archegoniophore growth and development. Δ*mpmir11796#29* KO plants exhibited few aberrations in the archegonial cells, however, round shape of egg cell (EC) was observed in WT archegonia. The ventral canal cell (VCC) in the venter of WT archegonia gradually disintegrates into mucilaginous strands, while Δ*mpmir11796#29* KO plants produced atypical masses of archegonia possibly due to unusual division between the VCC and EC during the development. Various archegonia with

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mucilaginous masses or blobs on the EC were also observed. Altogether, these results suggested that *MpmiR11796* plays an important role in the asymmetrical divisions during egg cell development in *Marchantia*. The PhD scholar also analyzed the fertilization efficiency of Δ*mpmir11796#29* KO by crossing them with WT sperm cells and Δ*mpmir11796#12* male mutant sperm cells. Both of them exhibited low fertilization efficiency, while cross with WT sperm cells producing more adverse effects. Total 9 putative mRNA targets were identified for *MpmiR11796* based on transcriptomic and degradome data. In addition, 10 novel miRNAs were also identified using the sRNA NGS data and accumulation of 3 miRNAs (FAN1, FAN2 and FAN3) were confirmed. sRNA NGS and northern blot hybridization showed that FAN1 was highly accumulated in archegoniophores and less accumulated in antheridiophores. Furthermore, the sequence of pre-miRNA does not overlap with any protein coding gene, however it has a classical stem-loop structure. Based on sRNA NGS data, FAN3 exhibited accumulation in the generative organs of *Marchantia* with significant enrichment observed in archegoniophores. In contrast, northern hybridization showed that FAN3 expression was downregulated in antheridiophores and archegoniophores as compared with male and female vegetative thalli. Its precursor also possessed a classical stem-loop structure. Both sRNA NGS and northern blot hybridization showed that FAN-4 was enriched in archegoniophores. Its precursor also possessed a classical stem-loop structure. It was observed that FAN-4 and previously identified Mpo-miR11886 are sequence isomiR variants derived from the same precursor. In addition, its precursor (44 bp) overlaps with the 5'-UTR of protein-coding gene of unknown function, encoded from the opposite strand.

Chapter 1 introduction provides an overview of *M. polymorpha* with detailed description of its life cycle. It highlights its role as model organism for forward and reverse genetic analysis. The chapter also described the role of miRNA regulation in plant development.

Chapter 2 provides the aim for the research and outlines the key objectives driving the study.

Chapter 3 details the materials utilized in the thesis, including plant material, bacterial strain, culture media and reagents employed in this study. It specifies the composition of buffers used

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in molecular biology and GUS staining protocol. It also provides the information regarding vectors, enzymes and kits used for the present study.

Chapter 4 elaborates on the methodologies used for the present study including bacterial transformation, *Agrobacterium*-mediated transformation, DNA and RNA extraction, PCR and CRISPR/Cas9 based genome editing. It also outlines the procedures for crossing of *Marchantia* plants and construction of promoter reporter plants. Additionally, the chapter describes methodologies used for the transcriptomic study.

Chapter 5 provides a comprehensive account of the results obtained in the thesis. This chapter details the experimental findings, including the characterization study of liverwort specific miRNAs during developmental stages. Data are presented in both tabular and graphical formats to facilitate interpretation and comparison with existing literature. Appropriate controls and statistical analyses are included to support the findings, demonstrating a solid grasp of the study's broader implications.

Chapter6 gives a thorough discussion on the outcomes of the present thesis. Each outcome was discussed in detail concerning the previous reports. Moreover, the results were discussed in relation to the research of others, and the present thesis demonstrates a good understanding of the implications of the work in a broader scientific context.

Chapter 7 provides a detailed conclusion of the thesis, offering a clear summary of the key points and an overview of the main findings from the research. It also provides future perspectives of the present study.

At the end, it lists all the sources and papers used or consulted in writing the entire thesis. Author has written all the references cited in the thesis in a uniform format. Also, it provides supplementary data generated throughout the study, including details of sequences of primers and identified target genes.

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Question to be asked from the Candidate:

1. How will the results of the study be applicable to economically important crops such as wheat, barley, rice etc.
2. It is stated that "MpmiR11796 plays an important role in the overall development of Marchantia, but predominantly in sexual reproductive organ formation and function". Can authors draw/propose a possible interaction network of MpmiR11796 with various genes/TFs at different developmental stages to support their conclusion?
3. Which tissue was used for degradome sequencing, whether it was thalli, antheridia or archegonia or a collective tissue sample? Do you expect differences on the degradome data, if you do an analysis of individual tissues?

Final recommendation:

The present study provides valuable insights into the role of several liverwort specific miRNAs in the development and reproduction of *M. polymorpha*. This study enhances our understanding of how miRNA regulatory networks help these lower land plants to adapt and thrive under harsh environmental conditions. Hence, the present thesis is scientifically and technically sound, presenting a well-crafted piece of research work. I recommend that this thesis be accepted for the award of Ph.D degree after completing all the necessary guidelines laid down by the faculty of biology at AMU, Poznan.

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