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Evaluation of PhD thesis from Mrs. Katarzyna Agata Knop

The following report provides an evaluation of the PhD thesis entitled "Biogenesis of selected abiotic-stress responsive plant microRNAs", which has been submitted by Mrs. Katarzyna Agata Knop to the Faculty of Biology of the Adam Mickiewicz University in Poznan.

The PhD project of Mrs. Knop addresses developmental and stress regulation as well as mechanistic aspects of microRNA (miR) generation in plants. A particular focus of this work is the interplay between miR processing and other post-transcriptional RNA processing events, namely precursor mRNA splicing and 3' end processing. Studies in the past decade have revealed that splicing and 3' end processing are not only essential steps in the maturation of most mRNAs, but actually significantly increase transcriptome diversity by selecting alternative splicing and 3' end processing sites in higher eukaryotes. Furthermore, splicing and 3' end processing can influence each other. The present work from Mrs. Knop considers the interplay between these processes in the maturation of miRs from precursor structures that can be located in exonic and intronic regions of genes. Previous studies in animals and plants have shown functional coupling of these processing steps, however, our current understanding is based on only a few case studies. Given our limited knowledge of the interplay between these post-transcriptional regulatory mechanisms and their importance in plant development and stress responses, the work from Mrs. Knop addresses several fundamental questions of particular relevance.

The introduction of Mrs. Knop thesis provides a comprehensive overview of the relevant topics, starting with a description of miR processing and the major factors involved in it. Furthermore, structures of plant miR genes are presented, including detailed information on all known intronic miRs from Arabidopsis. The introduction then gives an overview of splicing and polyadenylation mechanisms in plants. The last part of the introduction describes post-transcriptional and stress regulation of miR biogenesis, the topics that have also been studied in the course of this thesis. The introduction, as well as the rest of the thesis, is clearly written and nicely illustrated. The thesis is written in English of very good quality, with only few minor grammatical flaws. The materials and methods section contains all information that would be required to repeat the experiments from this thesis.

The experimental strategy of this work can be divided into three major parts. First, levels of the 29 intronic miRs from Arabidopsis are detected in seedlings, rosette leaves, and inflorescence leaves, and 5' ends of pri-miRs are analyzed via RLM-RACE. Second, processing and stress control of miR402 are characterized in detail. Third, the biogenesis of miR319b and miR319b.2, which are derived from the same pre-miR, is analyzed. The experimental strategy is clearly defined and illustrated with many models and cartoons. Furthermore, the figures are of high quality and easy to understand, in particular the inclusion of gene models with primer binding sites is very helpful. Expression analysis showed low steady state levels of most intronic miRs in the stages analyzed here. The RACE analysis identified several novel transcriptional start sites compared to what is annotated for the host pre-mRNA. In order to allow a direct comparison, a RACE analysis for the host mRNAs would have been helpful as the annotation might not be correct. This aspect is included in the discussion, but could have already been mentioned in the result section.

The major part of the result section describes regulation of miR402 production. Mrs. Knop found induction of the pri- and mature miR402 under heat, salt, and drought stress. This induction was accompanied by a decrease of the mRNAs targeted by miR402. Interestingly, stress caused less efficient splicing of the miR402-containing intron and increased use of a newly identified 3' end processing site within this intron. Half-life measurements revealed that stress did not alter the stability of pri-miR402. Further evidence for up-regulation of this miR under stress due to impaired splicing of the hosting intron was provided by treatment with the splicing inhibitor herboxidiene. Using transient expression in *Nicotiana benthamiana*, splicing and miR processing of reporter constructs based on the pri-miR402 sequence and mutated versions thereof were analyzed. In line with the stress data, impaired splicing of the miR-containing intron caused higher accumulation of the pri- and mature miR402. The data are overall of high quality and provide novel and important insight into regulation of miRNA production by its coupling to stress-regulated alternative splicing. For some experiments, I would have suggested additional analyses. Mutation of the splice sites resulted in the activation of cryptic splice sites. In order to assess the full diversity and relative abundance of these splicing variants, co-amplification with primers spanning the region of interest would have been more informative than performing only qPCR of two defined splicing variants. Furthermore, the decay curves from the half-life assays should have been included. The separate analysis of transcripts produced from 3' end processing at the proximal or distal site is a very interesting aspect. However, based on my understanding, the primer pair designated as "proximal" gave amplification of all transcripts, i.e. only "distal" is specific. The last set of experiments analyzing miR402 generation tested the role of SERRATE (SE) by using the mutants *se-1* and *se-2*. The mutant *se-1*, but not *se-2* still interacts with U1 splicing components. In line with this, stronger inhibition of splicing for the miR-containing intron was seen in *se-2* compared to *se-1*, while miR accumulation was lower in *se-1* than *se-2*. This finding is again in line with a competition mechanism of splicing and miR processing. Given that SE is involved in both miR processing and splicing, however, separating these functions in miR402 production is difficult. Analyzing levels of the pri-miR402 in the mutants might have been helpful, as its accumulation would primarily depend on the

splicing defect of SE; production of the mature miR then also depends on the SE defect in miR processing.

The third topic of the thesis was the mechanism of miR319b and miR319b.2 production from the same pre-miR molecule, with a focus on potential coupling of the production of the two mature miRs. To address this question, constructs containing miR319b in its authentic gene context with or without exchanges or deletions in the miR sequences were cloned and transformed into an Arabidopsis mutant deficient in miR319b. Subsequently, levels of the pri-miR, the mature miRs, and the target mRNAs were compared between the wild type and mutant constructs. While some interesting tendencies are visible from this data, e.g. production of miR319b.2 might dependent on the miR319b sequence/processing, the strong variation between the three lines analyzed per construct complicates data interpretation. I guess this variation is at least in part a consequence of copy numbers or insertion sites of the transgenes. Normalizing for expression variation, e.g. by considering the transcript levels of the BASTA resistance gene, might have helped to reduce the variation. Alternatively, levels of mature miRs might have been expressed relative to pri-miRs levels, providing an indicator of processing efficiency. Furthermore, it would have been interesting to determine whether RNA stabilities are altered in the mutant constructs.

The discussion provides an overall balanced assessment of the findings from this thesis in the context of the relevant literature. Limitations of the current data are mentioned, e.g. that it is unknown if the novel transcriptional start sites are unique for the pri-miRs or just are not annotated for the corresponding host mRNAs. Furthermore, key experiments for future studies are mentioned, such as analyzing RNA PolII occupancy and pri-miR stability for the miR319b constructs. The discussion also includes a model on the cross-talk between the microprocessor and spliceosome. I think this model nicely summarizes key findings from this thesis. The major role of SE in this regulation, however, is from my point of view only one option as miR processing and splicing might also compete against each other in an SE-independent manner.

Taken together, this thesis provides novel and interesting insight into the competition of post-transcriptional processing events of mRNAs. Mrs. Knop has clearly demonstrated that she is able to experimentally address fundamental biological questions, present her findings according to scientific standards, and discuss her results in the context of the relevant literature. I therefore fully recommend acceptance of this doctoral dissertation.

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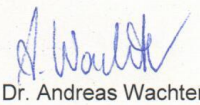

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