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Poznan, Feb 15th, 2017

Review of the PhD thesis
entitled "Biogenesis of selected abiotic stress responsive plant microRNAs"
presented by Katarzyna Agata Knop
to obtain a PhD degree from the
Faculty of Biology, Adam Mickiewicz University in Poznan
Supervisor: Professor Zofia Szweykowska-Kulinska

The research described in the assessed thesis is an important fragment of a broader research program undertaken several years ago at the Department of Gene Expression. The program aims at better understanding of mechanistic links between microRNA biogenesis and other steps of gene expression pathway in normal and abiotic stress conditions, in a model plant *A.thaliana*. Katarzyna Knop focused her efforts on studying biogenesis of several *A.thaliana* microRNAs located in introns of protein coding transcripts.

In the beginning of her work, Ms. Knop characterized the tissue expression of intronic microRNAs and selected miR402 for more detailed investigation. She first studied the effects of heat, salinity and drought stresses on miR402 biogenesis. Then, she extended her analyses to mechanistic interconnections between miRNA biogenesis and splicing, and polyadenylation, paying attention to the role of active 5'splice site and pre-miRNA hairpin localization, along with the role of SERRATE protein in regulation of miRNA maturation. In the second part of her doctoral work, she aimed at functional dissection of two miRNAs: miR319b and miR319b.2, harbored by a single stem-loop pre-miRNA structure.

As for her PhD dissertation itself, it has been written in English, on 197 printed pages, in compliance with traditional structure. It begins with a very detailed *Introduction* section describing the state of knowledge on plant microRNA biogenesis, splicing and polyadenylation mechanisms in plants, including relevant, previous results from the Department of Gene Expression. The briefly presented *Aim* of the work is followed by *Materials and Methods*, and the subsequent, separate sections concerning *Results, Discussion* and *Conclusions*, followed by not a very typical, although appreciated addition, in the form of *Future Perspectives* section.

In this review, I will begin with referring to the importance of the studied research topic, then I will move to commenting on the research strategy and study design, passing further to the most important results obtained, indicating their strong and weaker points, including these of technical nature. I will also ask the author for better explanations concerning some, in my opinion less clearly presented sections.

First, I would like to say, that I agree with Katarzyna Knop that topic of her study is important and needs further, extensive investigation, as existing knowledge regarding mechanistic aspects of intronic miRNA biogenesis in plants is far from satisfactory. This assessment applies in particular to the interplay, in other words crosstalk, that likely occurs between microprocessor complex, spliceosome and polyadenylation machinery.

In this regard, my question to the author is: where, at what localization within the cell nucleus, does this crosstalk take place? In other words, where is the "conversation room" mentioned in the question located? Does it involve dicing bodies (D-bodies) mentioned in the *Introduction* section? Does it involve nuclear speckles, known also as splicing speckles? Or perhaps, are Cajal bodies implicated? Alternatively, does it occur in the nucleoplasm, outside of any known bodies?

Going now further into details, the short RNA northern blots presented in Figure 28 show rather diffuse miRNA bands of moderate intensity. From the presented blots, it is hard to judge whether the miRNAs shown are predominantly single species arising from their highly accurate excision from the respective precursors, or do the diffuse bands result from a suboptimal, northern blotting procedure e.g. small gel etc.?

Going further in this direction: is the northern blotting protocol (Figure 28) the same as the one used to obtain the results presented in Figure 58? What do miRNA deep sequencing results say about the isomiR composition of these microRNAs? I am raising this issue, as miRNA biogenesis, besides its efficiency, has also its specificity characteristics to be considered (the distribution of isomiRs).

As miR402 is a multi-stress responsive miRNA, Katarzyna Knop first studied its biogenesis under temperature stress conditions. The experimental set-up included an analysis of pri-miRNA and mature miRNA accumulation by RT-qPCR and northern blotting, with additional RT-qPCR, respectively, after specific times of stress factor treatment. The representative graphs showing pri-miRNA and miRNA fold-change were presented e.g. in Figure 31. The relevant technical question is: how were the miRNA bands exactly quantified?

The effects of temperature stress were assessed by RT-qPCR for splicing of the miR402 hosting intron, selection of alternative polyadenylation sites (analyzed by 3'RACE PCR), effects of pre-miRNA hairpin position and 5'splice site inactivation. The performed analyses included also the effects of general transcription inhibition by cordycepin, and global splicing impairment by Gex-1A on miR402 biogenesis. In addition, the effects of these factors on miR402 downstream target expression were investigated and demonstrated.

In this regard, my comment relevant to the general strategy for the study is that the author compares the effects of global gene expression perturbations resulting from abiotic stresses, along with global transcription and splicing inhibition, with the effects of very specific molecular alterations affecting a single gene, such as miRNA stem-loop localization within host intron and 5'splice site inactivation. The outcomes of such comparison are either dramatic effects on pri-miRNA-402 levels, as those presented in Figure 41 where Gex-1A treatment resulted in 70-80 fold change (6-7 fold changes of miRNA), or less pronounced effects caused by other factors.

In Figure 46 and Figure 47, the Gex-1A effect is only shown for miRNAs miR837-3p (4-5 fold) and miR1888b (3-4 fold), but not for the respective pri-miRNAs. Were the pri-miRNA accumulations also very high, as in the case of pri-miR402? Could the author comment on the potential causes of these effects?

In spite of the above questions and comments, that are to be answered and clarified during the public defense of the evaluated thesis, I would like to primarily emphasize the main achievements of extensive and comprehensive investigations performed and described by Katarzyna Knop. These achievements include: 1. Demonstration that abiotic stress treatment enhances the intronic miR402 accumulation, which correlates with inhibition of miRNA-hosting intron splicing and alternative polyadenylation site selection. 2. Showing that efficiency of miR402 biogenesis depends on active 5'splice site of the hosting intron, and on the position of pre-miRNA hairpin with regard to this splice site. 3. Formulating a conclusion that competition between the microprocessor and spliceosome, as well as poly-A site selection, are key factors in the regulation of plant intronic miRNA biogenesis. 4. Providing evidence that SERRATE protein is the factor that connects the microRNA biogenesis and splicing processes, by interacting with components of relevant complexes.

The second part of Katarzyna Knop's doctoral work is focused more on the structural aspects of plant miRNA biogenesis, and addresses the following questions: How is the excision of two different miRNAs (miR319b and miR319b.2) residing in a single pre-miRNA hairpin regulated? Is their excision mechanistically coupled or not? Finally, how are these miRNAs, having different targets, functionally linked? By analyzing properties of suitably designed pre-miRNA mutants, she demonstrated that the introduced structural modifications have changed the way in which the miRNA precursor is processed, and showed that there is a possibility of target mRNA cross-regulation by the miR319b and miR319b.2.

To sum up, thanks to enormous efforts undertaken by Katarzyna Knop, numerous valuable insights have been gained, into the way in which biogenesis of plant intronic microRNAs occurs and is affected by the connected steps of gene expression, and various environmental stresses. Concluding, in view of the fact that this highly comprehensive and insightful study is truly outstanding, I have no reservations in awarding Ms. Knop a doctoral degree with distinction.

Włodzimierz Krzyżosiak