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Review

of the PhD thesis, entitled: "N6 Methyladenosine (m6A) and its writer mRNA adenosine methylase (MTA) are required for proper miRNA biogenesis in Arabidopsis thaliana" authored by Susheel Sagar Bhat

Overview

The thesis authored by Susheel Sagar Bhat submitted for the review represents a research study focused on a vital issue related to the RNA metabolism. RNA is regarded as one of the oldest biological molecules, probably constituting the so-called ancient RNA-based progenote world (also referred to as LUCA - the last universal common ancestor). It has been considered that, in the course of evolution from the progenote to the genote, significant transition took place, giving rise to such molecules as DNA and especially proteins, 'marginalizing' RNA as a link between DNA and proteins. As stated by Prof. Harry Noller, "*The invention of protein synthesis, probably instructed and catalyzed by RNA, was not to create a protein world, but to extend the structural, and therefore the functional, capabilities of the RNA world, and initially was the crowning achievement of the RNA world, but also began its demise*". Yet, despite the domination of proteins in a majority of metabolic events, RNAs play an important role in many critical biological processes, being recognized not only as a link between the DNA and protein worlds but also as an active player with such prominent examples as ncRNA. The presented thesis, being focused on RNA, is especially focused on the methyl-adenosine issue, i.e a well-known RNA base modification that influences RNA metabolism. The Author asked the question whether the m6A modification could affect miRNA biogenesis. The analyses were performed with the aid of the *Arabidopsis thaliana* plant experimental model. Susheel Sagar Bhat applied an impressive amount of experimental approaches, including high throughput analyses: RNA sequencing and mass-spectrometry proteomics reinforced by numerous biochemical analyses, including immunoprecipitation, RT-PCR, yeast two-hybrid system together with FLIM-FRET, proximity-ligation, and PIP-seq approaches to evaluate protein-protein interactions, ending up with functional analyses of individual miRNA and its influence on auxin metabolism. Especially, the mRNA adenosine methylase – MTA protein was considered as an experimental object, showing that the MTA might be involved in the methylation of the adenosine base in miRNA, influencing its biogenesis. An important aspect of this work is related to the broad collaboration with several research groups, including the University of Nottingham - UK, Nicolaus Copernicus University – Toruń, and the University of Pennsylvania, USA. This unique fusion of Susheel Sagar Bhat's capabilities with collaborative projects formed a very productive research environment. It shows high research plasticity of the Author, who is able to interact effectively with multidisciplinary research groups.

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Thesis evaluation

The presented thesis falls into canonical presentation broadly accepted by the Polish research system. The thesis has structural organization starting with the abstract, introduction, aim of the study, materials and methods, results, discussion, and references. Additionally, the Author lists published papers along with funding supporting the research and stating collaborators involved in this study, which makes it a well-structured and easy-to-follow thesis.

Initially, the Author provides coherent information about the current knowledge of miRNA, its metabolism, especially discussing the methyl-adenosine issue as a central part of the introduction directly related with the main subject of the thesis. The Author starts with information about the biogenesis of miRNAs in *Arabidopsis*, describing the formation of primary miRNA (pri-miRNA), underscoring the ability of pri-miRNAs to form secondary structures, e.g. a hairpin loop, which can be stabilized by methyladenosine. Additionally, the Author presents a coherent view on the pri-miRNAs processing in plants, with graphical representation shown in Fig. 1. Importantly, Susheel Sagar Bhat describes the biological role of miRNA in plant development, showing that almost all relevant biological process related to plant growth and development are regulated by ncRNA, e.g. early embryo and organ polarity development, flower progression, switch from the juvenile to adult phase in plants, signaling pathways (especially auxin signaling), and regulation of plant response to biotic and abiotic stresses. Subsequently, the methylation and related protein players are characterized with attention focused on the multiprotein methyl transferase complex, underscoring the fact that this is a regulatory process, and m6A is a reversible modification, removed by a group of proteins called m6A 'erasers'. Additionally, the Author mentions the issue of 'readers' belonging to regulatory proteins, which have not been fully explored in the plant system. I agree with the Author that the research field regarding m6A is gaining its momentum and will bring a lot of surprising information, as is the case of this thesis.

The main question asked by the Author is *"could MTA influence miRNA biogenesis as a direct consequence of m6A methylation and/or via interactions with other miRNA biogenesis related proteins"*. All subsequent steps were undertaken to answer this question. The main experimental model that was used by Susheel Sagar Bhat is an *Arabidopsis mta* hypomorphic mutant expressing MTA at a very low level, just to be enough to rescue embryo lethality, and displaying an approximately 90% lower level of m6A than WT plants. The preliminary analyses based on sRNA sequencing showed a decrease in miRNA abundance in the *mta* mutant, with around 51 miRNA that are down regulated, also further verified by RT-PCR. The Author followed this experimental path, applying the well-developed mirEX platform in the lab to test the abundance of almost 300 miRNA from the mutant plant. The analysis showed significant fluctuation in the miRNA expression level. However, in my opinion, there is no clear pattern; some miRNA are upregulated and some of them are down regulated. The Author stated that *These data conclusively show that in the lack of m6A methylation the process of miRNA biogenesis is in-efficient*. Based on the presented data, it is rather perturbed than in-efficient, in my opinion. The next experimental steps were aimed at showing that MTA is involved in miRNA metabolism. The Author performed an elegant analysis with the use of the recently developed

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and broadly used m6A-IP technique followed by NGS, using WT and the *mta* mutant. It showed that 11 miRNA were significantly enriched. Since the enrichment could be connected with MTA, in the next step the Author performed the IP approach and the MTA-GFP hybrid protein was used as a target with the aid of anti-GFP antibodies. In the isolated fraction, 8 out of 10 miRNAs (identified also in the m6A-IP fraction) were significantly enriched in the MTA-GFP sample. - Yes, I do agree that the MTA binds to pri-miRNAs, but the Author also claims that MTA methylates it – was a methylate assay performed to prove this? In the next step, the Author analyzes the structural alterations with RNA, considering the influence of m6A. The work was done in collaboration, using the PIP-seq approach. The analysis showed that pri-miRNAs in *mta* mutants lose secondary structures, thus it might be concluded that the lack of methylation destabilizes the RNA secondary structure. However, in my opinion, this stands in opposition to the accepted notion, which assumes that m6A destabilizes the 2D RNA structure, thus the lack of m6A should contribute to stabilization of the 2D structure – what is the Author's opinion about the role of m6A from the mechanistic point of view in relation to the RNA 2D structure. Thus, there is a possibility that inefficient binding of the microprocessor complex, depicted by the Author as the HYL1:RIP-qPCR experiment, showing that HYL1 cannot bind the pri-miRNAs in the *mta* background, might be related to the lack of m6A rather than to changes in the RNA secondary structure.

Therefore, having established the relationships between MTA and miRNA biogenesis in connection to m6A, the Author focused his attention on possible protein partners of MTA involved in miRNA biogenesis. Initially, Susheel Sagar Bhat used the yeast-two-hybrid system with a selected set of proteins known to be involved in miRNA biogenesis. The analysis showed that TGH and NOT2 might interact with MTA; however, as stated by the Author, the *trans*-activation activity of TGH and NOT2 fused to BD dominated the analysis, giving a possibly false positive effect. Therefore, why did the Author not change the experimental system, fusing TGH or NOT2 to AD? Also, a complementary bacterial two-hybrid system, which is free of such problems, could have been used. Next, the Author validated the MTA-TGH interaction using the elegant FRET-FLIM approach, which may represent *in vivo* analysis. The initial analysis showed that MTA-RFP is located in the nucleus and co-localizes with all analyzed proteins (however, the nucleus has not been visualized; is it possible to stain the nucleus in plants using DAPI to show that this indeed is a nucleus?). In a majority of cases, the fluorescence signal was uniformly distributed within this compartment, except for the co-expression with TGH. The subsequent FRET-FLIM analysis showed that MTA may interact with TGH, but several questions arise in this case. First of all - why is there a discrepancy between the MTA-TGH experimental system and the other analyzed proteins in respect to localization in the nucleus? Is it possible that the behavior of the MTA hybrid protein can be changed completely in the presence of TGH (localization in small loci exclusively)? What does the control experiment where the MTA-RFP protein is expressed alone look like? Finally, what is the distribution of FLIM signals for individual components in relation to GFP? I presume The Author used eGFP, which has been improved in respect to folding, but the eGFP possesses still low folding efficiency (significantly affecting the distribution of FLIM components), thus the distribution of the FLIM signal can be heterogeneous.

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Therefore, it would be interesting to show the distribution of all FLIM components in pseudo-color images across the image and define with the so-called region-of-interest area taken for the analysis. Since the interaction analysis based on FRET-FLIM showed that the interplay between these two proteins is efficient (especially the numerical data are very convincing), I think that this analysis could be supplemented by fluorescence intensity-based FRET, which would additionally validate the FRET-FLIM approach (can be taken at the same time). The next step in the study related to the protein-protein interaction was the proteomics based on the IP analyses with MTA-GFP coupled with MS. The functional complex of MTA was identified, showing that this protein interacts with VIR, MTB, and FIP37, which confirmed its involvement in m6A metabolism. The analysis significantly extends the protein interactom of MTA, indicating that MTA is able to interplay with numerous proteins involved in miRNA biogenesis and mRNA splicing as well; a question arises about the TGH protein partner: is it possible to find this MTA partner in the MS data?

Having established the protein interactom of MTA, Susheel Sagar Bhat continued the protein-protein interaction study, but using an *in vivo* approach based on the proximity ligation assay with RNA polymerase II as a target. The analysis showed clearly that MTA is associated with RNA Pol II, indicating that MTA may act co-transcriptionally in a complex with the whole transcriptional machinery. This is a very original finding, indicating that the methylation of target genes, especially those for miRNA, is coupled with transcription. Therefore, I am just wondering about the regulatory role of methylation; thus, is it possible that the removal of the methyl group may represent a negative regulatory effect for miRNA processing, I mean that miRNAs (or some of them) are constitutively methylated and de-methylation represents a regulatory event. Next, the Author tested the level of methylation using a *tgh-1* mutant to show whether MTA activity is dependent on the TGH protein. It was shown that the level of methylation of pri-miRNAs was not changed, indicating that MTA may work upstream of TGH indeed or TGH is simply not involved in MTA action. Finally, in line with all these experiments, the Author analyzed the microprocessor assembly in the context of RNA Pol II work. Once again, in collaboration with the research group from Toruń, a proximity ligation assay was performed using *mta* and *tgh* mutants and HYL1 and DCL1 proteins were analyzed. The analysis revealed that the intensity of the HYL1 and DCL1 interaction with RNA Pol II was significantly reduced, and the Author concluded that the co-transcriptional formation of the microprocessor is MTA and TGH dependent – very interesting observation. My question is whether it is possible that the microprocessor complex can be assembled independently of RNA Pol II or whether it is stimulated by transcription and what the role of TGH protein is, as the lack of TGH does not affect m6A metabolism.

Last but not least, the Author carried out an analysis to determine the biological significance of the previously reported findings. It has already been reported that there is a link between methylation and auxin metabolism. The Author followed the lead and, using an auxin-responsive reporter genetic system, showed that there is a lower level expression of the GUS reporter gene in the *mta* hypomorphic plant, indicating that a low m6A level may indeed affect negatively auxin metabolism. Subsequently, the Author centered his attention on miR393b, as ncRNA involved in auxin metabolism. As he reported earlier, its level in the *mta* hypomorphic mutant plant

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determined by sRNA sequencing was lower (as a matter of fact, I was not able to find this information in the presented thesis). The Author performed three experiments, namely: RT-PCR coupled with m6A-IP and MTA-GFP-IP and showed that pr-miR393b is methylated and, what is more, is able to interact with MTA in the background of WT vs. the mutant plant. I must admit that this is very informative analysis, which has been confirmed by additional analysis using *Nicotiana* leaves as an experimental system co-transfected with WT or mutated MTA and maturation of exogenous pri-miR393b was followed. It showed that MTA had a positive influence on the analyzed miRNA maturation, once again underscoring the fact that MTA represents a critical element in miRNA metabolism positively influencing its maturation.

Concluding, Susheel Sagar Bhat has provided convincing evidence that the pri-miRNAs in plant species are methylated and the MTA protein is involved in this process via a close physical interaction with a distinct subset of miRNA. Additionally, the lack of methylation perturbs the amount of pri-miRNA and, as shown by the Author, the main reason is the distortion of the RNA 2D structure, which may affect the subsequent steps of miRNA maturation. The provided metabolic picture, in relation to m6A, was enriched by proteomic analysis, which showed that MTA acts in concert with additional protein partners. Especially, the co-transcriptional interplay with RNA Pol II was shown, indicating that methylation and miRNA processing might be coupled events. Finally, in the discussion section, the Author built a comprehensive model showing a web of biologically relevant interplays in miRNA metabolism with the profound example of auxin metabolism.

Final conclusion

The thesis represents high quality research with a clearly defined scientific problem, which has been subsequently resolved. Through numerous frequently sophisticated analyses, the Author provides a set of experimental evidence, which significantly extends our knowledge of the role of methylation of miRNA. The thesis is well written and is highly focused on the problem, showing that the Author has high capability and research skill. Additionally, the information provided in the introduction and especially discussion sections indicates that the Author has profound knowledge and is able to interpret the data and build a general biological picture in a very professional way, taking us beyond the current state of the art, at the same time formulating his own view.

The doctoral dissertation meets the conditions specified in Art. 13 of the Act of March 14, 2003 on academic degrees and titles in science and arts (Journal of Laws 2017, item 1789 as amended). Therefore, I recommend that the Biology Faculty Board of Adam Mickiewicz University in Poznań admit Susheel Sagar Bhat MSc. for the subsequent stages of the doctoral proceedings. Concurrently, I recommend that the research effort made by the doctoral student should be awarded appropriately.

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