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Review of the dissertation
by
Syed Muhammad Muntazir Mehdi, MSc
entitled
„Identification of novel and ABA-regulated miRNAs and characterization of its target gene – *AtBRO1* during growth and abiotic stress response in *Arabidopsis thaliana*"

Abiotic stress triggers intricate molecular reactions in plants, where abscisic acid (ABA) is pivotal in altering gene expression. Despite current knowledge, an understanding of ABA-dependent plant stress responses still needs to be completed, especially considering the regulatory role of microRNA requires further research in this area. Mr. Syed Muhammad Muntazir Mehdi's doctoral thesis, which I reviewed, aligns perfectly with this research trend. The research was supervised by Prof. Agnieszka Ludwików at the Institute of Molecular Biology, Faculty of Biology, Adam Mickiewicz University in Poznań. The research was funded by the SONATA BIS project (2016/22/E/NZ3/00345) led by the supervisor, a doctoral program at the Faculty of Biology, Adam Mickiewicz University (The European Union: Passport to the future - Interdisciplinary doctoral studies at the Faculty of Biology, Adam Mickiewicz University), and the Faculty of Biology 'Dean's grant' (GDWB-09/2019).

The subject of the Thesis and its Scientific Significance

Mr. Syed Muhammad Muntazir Mehdi's doctoral research addresses the regulation of gene expression *via* miRNA molecules in response to exogenous abscisic acid (ABA) in *Arabidopsis thaliana*. Specifically, Ph.D. Candidate identified new miRNAs with altered expression due to exogenous ABA, then identified their target transcripts, and conducted an extensive functional analysis of the role in ABA-dependent stress response of protein encoded by one of the identified target genes.

MicroRNAs play crucial roles in many biological processes, including plants' adaptive responses to abiotic stresses. The abiotic stress response is primarily controlled by ABA, initiating a complex signaling cascade involving in its core pathway three protein classes: ABA receptors (PYR/PYL/RCAR), negative regulators from the PP2C phosphatase family group A, and positive regulators like type 2 protein kinases (SnRK2). Other kinases, like mitogen-dependent kinases (MAP), operate below the primary ABA pathway, e.g., MAPKKK17 and MAPKKK18 are regulated by the main ABA signaling module. These kinase modules, including MAP kinases, likely contribute to generating a persistent signal and maintaining ABA-dependent responses under chronic stress. Earlier studies by Prof. Agnieszka Ludwików's team showed that MAPKKK18 directly interacts with the protein phosphatase ABI1, a negative regulator of the core ABA signaling pathway. ABA induces MAPKKK17 and MAPKKK18 expression, and these kinases seem to have redundant functions in the ABA signaling pathway. Phosphorylation cascades are foundational to ABA signaling, a research area successfully pursued by the team where the Ph.D. Candidate conducted his studies. Advanced sequencing technologies in recent years have identified numerous miRNAs in plant genomes of various species. However, the ABA-dependent regulation of miRNA expression, especially concerning the MAPKKK cascade, remains elusive. **This aspect of the complex molecular network inspired the Ph.D. Candidate's research. I commend the choice of this topic for the doctoral dissertation and find it scientifically intriguing. Understanding the**

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mechanisms governing this process, especially in the context of ABA-dependent responses to abiotic stresses, as undertaken by the Ph.D. Candidate is paramount.

Formal and Substantive Evaluation of the Thesis

Mr. Syed Muhammad Muntazir Mehdi's dissertation is written in English, starting with abstracts in both Polish and English. The abstracts encapsulate the essence of the conducted research. **However, it is surprising that the two versions of the abstracts are not identical ('Streszczenie' vs. 'Abstract')**. The English version provides more detailed information, for instance, about the identified target genes for miRNA. ***I request Ph.D. Candidate to clarify this inconsistency.***

In the subsequent section titled 'Aim of the study', the Author lucidly defines the research hypothesis and overarching objectives, dividing the research plan into two main parts corresponding to the later cited experimental publications. Each general aim is further divided into more detailed goals, guiding the direction of research and experiments to verify the research hypothesis. Such **a clear presentation of research objectives primarily illustrates the Candidate's ability to plan experiments and demonstrates his independence in conducting research.**

The research hypothesis of this dissertation assumed that abscisic acid affects the expression of miRNA, which in turn regulates the effector gene expression from the ABA signaling cascade, including the ABA-dependent group of MAP kinases. The primary objectives of the thesis set by the Ph.D. Candidate were:

1. Identification of novel miRNA molecules in the abscisic acid signaling pathway
2. Characterization of the target gene *AtBro1* for newly identified miRNA (ath-miRn-1) in the previous research phase.

Subsequently, the Author enumerates the funding sources and experimental publications in the dissertation. The following section is a 12-page Thesis Outline, which is concisely written. It commences with a two-page introduction, offering an overview of the current knowledge related to the dissertation's research topic. **The introductory section indicates the Ph. D. Candidate's proficiency in the research theme.** However, parts of this section employ generic phrases and descriptions, more akin to popular science than strict academic discourse (i.e. page 9 "controls plant responses via numerous effector proteins", page 10: "ABA also affects transcription of several microRNAs", page 15 "Bro1 (...) is involved in a number of different processes". Such phrasing, like "and so on," detracts from the rigor expected in a doctoral dissertation. The candidate then delves into the conducted research and the obtained results, dedicating significant space to the methods employed. The research showcases a series of precisely designed experiments, demonstrating the Candidate's mastery over advanced research techniques and their application. Among the methods used were high-throughput small RNA sequencing, qRT-PCR, 5' RLM RACE, molecular techniques for generating transgenic Arabidopsis lines, spatiotemporal gene/protein expression analyses using reporter genes, Illumina transcriptome sequencing, and various phenotyping methods for plants under different abiotic stresses. The research material comprised insertion lines and transgenic overexpression in the genes: *ABI1*, *MPKKK17*, *MPKKK18*, and *BRO1*, as detailed in publications [1] and [2] constituting the dissertation, respectively.

After the Thesis Outline, the Author presents a one-page summary. However, this section needs a deeper interpretation of the results and an outline of prospects based on the findings. Instead, it essentially reiterates statements from the Outline. Comments like, "It will be interesting to investigate the role of the remaining ABA-responsive novel miRNA that we reported here..." come across as somewhat trivial. ***At this juncture, I would expect a well-crafted conclusion based on the research conducted and clear suggestions for future research that would build on the doctoral thesis. Consequently, I request that the Ph. D. Candidate prepare and present such a summary during the doctoral defense.***

Subsequently, the Author provides a bibliography comprising 52 references. While the sources are aptly chosen, it's notable that only 12 of the cited publications are from the past five years.

The following section, the crux of the dissertation, contains copies of the two published original papers constituting the thesis, along with co-authors' declarations. The research outcomes, central to the dissertation, have been published in two original articles in high-impact JCR scientific journals:

[1] Mehdi, S.M.M.; Krishnamoorthy, S.; Szczesniak, M.W.; Ludwików, A. (2021) Identification of Novel miRNAs and Their Target Genes in the Response to Abscisic Acid in Arabidopsis. Int. J. Mol. Sci.: 22, 7153. https://doi.org/10.3390/ijms22137153 [Ministry points (MEiN) - 140, $IF_{2022-2023}$ - 6.208 (IF_5=6.628)]

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[2] Mehdi, S.M.M., Szczesniak, MW and Ludwików, A. (2023) The Bro1-like domain-containing protein, AtBro1, modulates growth and abiotic stress responses in Arabidopsis. Front. Plant Sci. 14:1157435. doi: 10.3389/fpls.2023.1157435 [Ministry points (MEiN) - 100, $IF_{2022-2023}$ - 6.623 (IF_5=6.3)]

The Ph.D. Candidate is the primary author of each publication, with his leading contribution (75% in the first and 80% in the second publication). Mr. Syed Muhammad Muntazir Mehdi designed and conducted experiments, analyzed results, and drafted manuscripts, testifying to his significant involvement and independence in research.

Given that both papers underwent rigorous peer review in international journals, I'll briefly summarize the main findings. The content, naturally, has piqued my scientific curiosity, leading to questions I've listed in the subsequent 'Comments and Questions' section.

Publication [1] addresses the investigation of the role of miRNA in response to ABA in Arabidopsis. The authors utilized the WT ecotype of *A. thaliana* – Columbia-0 and insertion lines in the Col-0 background in the genes: (i) *ABI1* (*ABA-insensitive 1*) - *abi1td*, encoding phosphatase 2C from the core ABA signaling pathway, (ii) *MAPKKK17* (*Mitogen-activated kinase kinase kinase 17*) – *mkkk17*, and (iii) MAPKKK18 (*Mitogen-activated kinase kinase kinase 18*) – *mkkk18*, encoding mitogen kinases activated by ABA, that, operating redundantly, likely facilitate signal transduction during prolonged stress. miRNA identification was carried out by analyzing high-throughput small RNA sequencing data using Illumina technology. The genetic material was extracted from the plants of the mentioned genotypes, previously treated with 100 μM ABA or grown under control conditions, was subjected to analysis. This enabled the identification of miRNAs with differential expression in the wild type (WT) and mutants. Among the differentially expressed miRNAs, ten were newly identified in the WT after ABA treatment. Subsequently, the authors focused on predicting their secondary structure using bioinformatics methods and the RNA Structure tool. Efforts were made to identify target transcripts for the discovered miRNA molecules using the psRNATarget tool. Using the 5' RLM-RACE method, the authors experimentally confirmed interactions between six newly discovered miRNA molecules and seven target mRNAs. Functional annotation analysis of the genes encoding the target transcripts affirmed their involvement in the ABA signaling pathway. Expression analysis using the qRT-PCR method demonstrated their differential expression in response to exogenous ABA. **The Authors emphasize the significance of this discovery, pointing to the undeniable regulatory role of miRNA in the ABA signaling pathway, the most crucial phytohormone in stress response**.

In the **publication [2],** the Authors present a thorough and highly detailed functional characterization of the *AtBRO1* gene (*Bro1-like domain-containing protein*). These studies continue the results described in the publication [1], as they pertain to one of the identified genes (specifically transcripts) targeted by the newly discovered ABA-dependent miRNAs. To understand the function of the protein encoded by *AtBRO1*, the Authors examined the following genotypes: wild-type ecotype Col-0, an insertion line in the *AtBRO1* gene – *bro1-1*, three transgenic lines overexpressing *AtBRO1*, and two complementation lines obtained by the authors by crossing the insertion line with an overexpression line. **This approach allowed for an exceptionally detailed and very elegant genetic study, of the role of *AtBRO1* in stress response and ABA signaling**. Given that this work represents the first analysis of the *AtBRO1* gene, the Authors began their research by examining expression patterns in response to ABA, salinity, and mannitol across various plant organs. It was demonstrated that *AtBRO1* undergoes increased expression in response to ABA, salinity, and mannitol already within an hour of treatment. Based on qRT-PCR, it was determined that the highest expression level pertains to leaves and flowers. These findings were corroborated by analyses using the GUS reporter gene, which provided a more detailed localization of the *AtBRO1* transcript in anthers. A construct with GFP protein was used to determine cellular localization, proving the membranous location of AtBRO1. The Authors also conducted a detailed analysis of the *AtBRO1* promoter region, indicating the presence of elements characteristic of ABA response genes (ABRE-elements), consistent with obtained expression data in response to ABA.

Subsequent analyses examined the phenotype of the genotypes mentioned above in response to ABA, mannitol, salinity, and drought. It was shown that AtBRO1 serves as a positive regulator in response to ABA and mannitol, while its negative role was observed in response to salinity and drought. Using qRT-PCR, the authors conducted expression analyses of selected genes of ABA synthesis, ABA signaling, and stress-

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response marker genes unrelated to ABA. The authors concluded that AtBRO1 operates in an ABA-dependent manner. These findings prompted a global transcriptome profile analysis in response to ABA in the *bro1-1* mutant and WT, achieved through high-throughput RNA-seq. Specific genes exhibiting differential expression uniquely for the mutant and WT were particularly described, supported by functional annotation analysis. **Beyond pointing out several potentially significant regulatory genes in the context of AtBRO1 function, equally important is the result indicating biological processes overrepresented by genes showing differential expression in WT but not in the mutant. These processes relate to membrane transport and organ development, mainly reflecting previously obtained data by the Authors regarding the cellular location of AtBRO1 and expression patterns.**

Key Findings of Doctoral Thesis

Summarizing the pivotal discoveries from Mr. Syed Muhammad Muntazir Mehdi's doctoral research, the highlights include:

1. Identification of 10 novel miRNA molecules in *Arabidopsis thaliana* with ABA-dependent expression.
2. Experimental demonstration of interactions between seven target mRNAs and six miRNAs, previously predicted using bioinformatics tools.
3. The observation that the highest number of differential miRNA expression pertains to the *abi1td* mutant, suggesting that enhanced phosphorylation in plant cells in response to ABA significantly influences miRNAs.
4. Identification of miRNAs with ABA-specific expression in mutants of MAP kinase genes, *mapkkk17*, and *mapkkk18*.
5. Discovery that the previously uncharacterized gene, *AtBRO1*, in the context of ABA response and identified by the Ph.D. Candidate, as a target for ath-miRn-1, encodes the membrane protein playing a crucial role in ABA-dependent stress response in *Arabidopsis thaliana*.

It's worth noting that the dissertation presented for my review bears the hallmarks of a scientific breakthrough. The findings raise new questions and lead to further experimental avenues, and the overall data tells a cohesive story.

The results presented in Mr. Syed Muhammad Muntazir Mehdi's research are invaluable. They provide a deeper understanding of gene expression regulation related to abiotic stress response through ABA-dependent miRNA pathways. The doctoral candidate has demonstrated an ability to conduct scientific research and address an original scientific problem independently.

Comments and Questions

Reading through Mr. Syed Muhammad Muntazir Mehdi's doctoral dissertation, I found it particularly engaging, leading to several queries regarding the conducted research. ***Specifically, during the doctoral defense, I'd like to discuss the following:***

1. I would like the Ph.D. Candidate to elaborate on a statement from the Thesis Outline '*Plants respond to these effects by initiating diverse metabolic and physiological modifications, facilitated by a number of hormones, known as phytohormones, which are often specific to a certain type of stress*', especially in the context of numerous proofs highlighting dependencies and interactions between phytohormone signaling networks, particularly in stress response. The statement seems to be an oversimplification.

2. In the research published as Mehdi et al 2021 [1], a rich set of biological materials was utilized, including mutants of core ABA signaling pathway (*abi1td*) and ABA-activated MAP kinase cascade mutants (*mpkkk17* and *mpkkk18*). However, I found that the analysis regarding these mutants needs deeper explanation. Beyond a short summary indicating the discovery of miRNAs differentially expressed in both mutants (miR167-3p and miR397a), which in fact is very interesting in my opinion,

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a more profound interpretation seems absent. ***Given the redundant roles of both kinases in ABA signaling as per literature, can we hypothesize that these miRNAs are crucial in regulating long-term stress response? Are there any insights on their expression pattern (of miRNAs) during stress based on literature or genomic repositories? I'd appreciate a more comprehensive explanation during the defense.***

3. In Mehdi et al. 2021 [1], validation of miRNA expression identified through high-throughput small RNA sequencing was conducted. For some, opposing results were obtained when qRT-PCR and sRNAseq were compared. The Authors in the publication (but not in the Thesis Outline) offer a potential reason for this discrepancy, pointing to different sample origins. ***Can these miRNAs identified through sequencing but not confirmed by qRT-PCR, still be viewed as ABA-responsive? I am very curious about the Ph.D. Candidate comment.***

4. In Mehdi et al. 2023 [2], a phylogenetic analysis of the *AtBRO1* gene identified a paralog in *A. thaliana* and orthologs in other species. **I'd like the Ph.D. Candidate to clarify if the term 'ortholog' is appropriately used here.**

5. In Mehdi et al 2023 [2], the authors verified transcriptomic analyses from RNA-seq with an independent method, qRT-PCR. While I find this approach convincing (due to the use of overexpression lines in that experiment), ***I'd like the Ph.D. Candidate to comment on the necessity of validating RNA-seq results using an independent method like qRT-PCR, based on literature insights and personal experience.***

I have some editorial remarks on this generally well-written dissertation alongside these substantive questions arising from this intriguing research. I am presenting these below. Note that these remarks are minor and don't diminish the value of the thesis.

1. Throughout the Thesis Outline, there's inconsistency in the notation of the gene/protein/mutant in the context of BRO1 analyses. The insertion mutant should be noted as "*bro1-1*" and not "bro1", and the gene as *AtBro1* or *AtBRO1* (in italics), with the protein as AtBRO1.
2. I noticed a few editorial errors: *Page 10 – pri-miRNA (primordial miRNA) – I believe it should be 'primary'; Page 10 – typo 'mapkinase kinase 3' should be 'map kinase kinase 3'.*
3. Throughout the Thesis Outline, while using abbreviations (e.g., gene names, proteins), the Ph.D. Candidate doesn't expand them. Abbreviations should be clarified as per good scientific writing practices.
4. In Thesis Outline Author wrote: "AtBro1 is positively involved in the response to ABA and mannitol" and, further "AtBro1 is negatively involved in the response to salt stress". I think it is a shortcut in writing, but in my opinion, it should be stated more in a regulation context, so "AtBro1 positively regulates response to ABA and mannitol" would be a more appropriate statement.
5. In Mehdi et al. 2021 publication [1]: Table 3 lists identified target genes for the discovered miRNAs. The text mentions seven target genes for six miRNAs, but the table shows six target genes for five miRNAs (missing ath-miR-9 and its target gene).
6. In Mehdi et al. 2023 publication [2]: Figure 12 shows inconsistency in the notation of genes presented in the model of *AtBro1* action in stress response in Arabidopsis. For some of them, Authors used Gene ID, and for some gene names.

Final Conclusion

In conclusion, I affirm that the doctoral dissertation presented to me by Mr. Syed Muhammad Muntazir Mehdi offers an original solution to a scientific problem. The Ph.D. Candidate demonstrates comprehensive theoretical knowledge in the research area covered in his dissertation. He also unmistakably exhibits the ability to conduct independent scientific research. The comments presented in the review are technical or arise from the reviewer's scientific curiosity.

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I, therefore, assert that Mr. Syed Muhammad Muntazir Mehdi's doctoral dissertation titled, "Identification of a new ABA-regulated miRNA and characterization of its target gene - AtBro1 in growth processes and response to abiotic stress in Arabidopsis thaliana" meets the criteria specified in Art. 187, paragraphs 1-2, of the Act of 20 July 2018 the Law on Higher Education and Science (Journal of Laws of 2023, item 742). Consequently, I appeal to the esteemed Scientific Council of the Institute of Molecular Biology, Faculty of Biology, Adam Mickiewicz University in Poznań, to accept Mr. Syed Muhammad Muntazir Mehdi for further stages of the procedure for conferring the doctoral degree in exact and natural sciences, in the discipline of biological sciences.

W podsumowaniu stwierdzam, że przedstawiona mi do oceny rozprawa doktorska mgr. Syeda Muhammada Muntazira Mehdiego stanowi oryginalne rozwiązanie problemu naukowego. Po wnikliwym zapoznaniu się z Autoreferatem oraz załączonymi publikacjami naukowymi stwierdzam, że Kandydat do stopnia naukowego doktora prezentuje ogólną wiedzę teoretyczną w obszarze badawczym, który stanowił zagadnienie badane w Jego dysertacji, a także niewątpliwie wykazuje umiejętność samodzielnego prowadzenia pracy naukowej.

Stwierdzam zatem, że rozprawa doktorska Pana mgr. Syeda Muhammada Muntazira Mehdiego pt. "Identyfikacja nowego i regulowanego przez ABA miRNA oraz charakterystyka jego genu docelowego - AtBro1 w procesach wzrostu i odpowiedzi na stres abiotyczny u Arabidopsis thaliana" spełnia wszystkie wymogi określone w art. 187 ust. 1-2 Ustawy z dnia 20 lipca 2018 r. Prawo o Szkolnictwie Wyższym i nauce (Dz.U. 2023 poz. 742).

W związku z powyższym zwracam się do Wysokiej Rady Naukowej Instytutu Biologii Molekularnej, Wydziału Biologii, Uniwersytetu Adama Mickiewicza w Poznaniu o dopuszczenie Pana mgr. Syeda Muhammada Muntazira Mehdiego do dalszych etapów postępowania w sprawie nadania stopnia naukowego doktora nauk ścisłych i przyrodniczych, dyscyplinie nauk biologicznych.

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