REVIEW

by Assoc. Prof. Samir Izetov Naimov
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On PhD thesis for the award of the educational and scientific degree
“Philosophy Doctor” in:
Field of higher education: 4 Natural Sciences, Mathematics and Informatics.
Professional field: 4.3 Biological sciences.
Doctoral Program: "Molecular Biology"

Author: Nikol Hadzieva
Title: “Identification of specific interactions between Potato Spindle Tuber Viroid and two Bulgarian pepper cultivars"
Supervisor Assoc. prof. Dr. Mariana Gozmanova

1. General description of the submitted materials

By Order No. RD 21-456 from 02. 03. 2023. of the Rector of Plovdiv University "Paisii Hilendarski" I have been appointed as a member of the scientific jury for the procedure for the defense of the dissertation thesis " Identification of specific interactions between Potato Spindle Tuber Viroid and two Bulgarian pepper cultivars " for the obtaining of the educational and scientific degree "Doctor" in the field of higher education: 4. Natural Sciences, professional field: 4.3. Biology, Mathematics and Informatics, Doctoral Programme Molecular Biology. The author of the dissertation is Nikol Slaveva Hadzieva - PhD student at the Department of Plant Physiology and Molecular Biology under supervisor of Assoc. Prof. Dr. Mariana Gozmanova from Paisii Hilendarski Univversity of Plovdiv

The set of documents submitted by Nikol Slaveva Hadzieva is in accordance with Article 36 (1) of the Regulations for the Development of the Academic Staff of PU and includes the following documents:

- a request to the Rector of PU for disclosure of the dissertation defense procedure;
- CV in European format;
- Minutes of the Departmental Council relating to the reporting of readiness for the opening of the procedure and to the preliminary discussion of the thesis;
- Dissertation;

- Author's abstract;
- List of scientific publications on the topic of the dissertation;
- Scientific publications reprints;
- List of citations;
- A declaration of originality and authenticity of the attached documents;
- A reference for compliance with the specific requirements of the faculty concerned;
- The PhD student has applied a total of three scientific publications, two of them with impact factor in quartile 1 journals.

2. Brief biographical data about the PhD student

Nicole Hadzieva was born on the 26th of January in Lovech. In the period 2009-2013 she graduated from secondary education at the High School for Foreign Languages "Exarch Joseph I" with intensive study of English. After that he continued his education in Bachelor's program "Biology and Chemistry" and obtained a Master's Degree in Molecular Biology. Since 2019 Nicole Hadzieva is a PhD student at the Department of Plant Physiology and Molecular Biology under the scientific supervision of Assoc. Prof. Dr. Mariana Gosmanova.

3. Relevance of the subject matter and appropriateness of the set goals and objectives

Viroids are small covalently closed RNA molecules without protein-coding capacity that replicate using the replicative machinery of plant cells. Due to their restricted host range to the plant kingdom, viroids are commonly accepted as a strictly plant pathogens. Several mechanisms of transmission of viroids have been described in the scientific literature, some of which are less efficient, but once in the plant, viroids lead to a number of changes in vegetative organs. This, in turn, leads to losses ranging from reduced productivity and quality to complete crop lost as is the case with CTiVd and CCCVd in coconut palms, for example. While macroscopic changes in the vegetative organs of plants are well documented, changes at the molecular level remain insouciantly studied. This is the major scientific topic of the dissertation submitted for review. In this context, the topic of the thesis is topical and the work itself implies obtaining significant results of fundamental and applied nature. A keyword search of public databases retrieves over 1800 records, 248 of which are for PSTVd. Longitudinally, in 2000, 24 scientific papers were published on the topic while in 2022 their number increased to 173, a trend indicative of the relevance of the topic.

4. Knowledge of the problem

In the Literature Review, the PhD student discusses three main aspects of the topic under development: 1) Vidoids as pathogens; 2) Small interfering RNA molecules; and 3) Pepper as an important agricultural crop. The applicant has analyzed 240 literature sources and has managed to present in an accessible way the main concepts related to the research problem. In a similar way, the PhD student also critically reviewed her own results in the light of currently relevant work by other teams. In the discussion of her own results, the applicant has demonstrates a significant knowledge capacity for and a professional interpretation of the data obtained. In the presentation of the two chapters mentioned above, the PhD student demonstrates an excellent knowledge on the research topic and fluent handling of the literature sources. In this context, I believe that Nikol Hadzieva has acquired a sufficient knowledge of the specific issues and has acquired the necessary skills to work with and analyze literary sources.

5. Research methodology

For the purpose of the research presented in the dissertation, the Nikol Hadzieva has used a number of molecular biological methods, which can be subdivided, into 1) methos for accumulation of scientific data and 2) method for subsequent data analysis and validation. The first group of methods includes the isolation and characterization of RNA and high-throughput sequencing techniques. Through these, the following have been accumulated huge amount of data, some of which is discussed in the thesis, but it should also be noted that the dataset provides an excellent opportunity for further studies and meta-analyses. The results obtained are available for use in the NCBI database, and the part of them that is relevant to the main concept is presented as supplementary files. The overall scientific findings presented are obtained using reliably prepared plant material and adequately conducted controlled infection experiments on the pepper varieties tested. Last, but not least, I would like to draw attention to the adequately selected bioinformatic and statistical set of methods that allow for a correct interpretation of the results. The experimental set-up is in line with the set aim and objectives. During the PhD study, Nikol Hadzieva has been able to master many methods that would allow her to successfully realize herself as a young scientist.

6. Characteristics and evaluation of the thesis

The PhD thesis is presented on 121 pages and includes 7 tables and 33 figures. The text is organized and structured in a standard manner, beginning with a brief introduction of one page, a literature review, presented in 25 standard pages, and aim and objectives-1 page, followed by

materials and methods-7 pages, results-31 pages, discussion in 8 pages, conclusions-1 page, and references-23 pages. The PhD thesis ends with contributions-1 page, list of scientific publications, and participation in scientific conferences. The literature review begins with brief information on viroids as plant pathogens, their classification and replication models. Gradually, the thesis turns the exposition to the specifics of PSTVd, discussing in detail the biology of the viroid itself, the pattern of replication, modes of transmission, and the symptomatology of PSTVd infection at both the macroscopic and microscopic levels. Particular attention is given to what has been described to date in the literature about the molecular interactions associated with infection and host cell responses, with particular emphasis on the involvement of small RNA molecules in the plant defense response. The last few pages of the chapter are devoted to pepper as an agricultural crop, focusing on miRNA-related research. Based on the literature review, the applicant sets the aim of the thesis as "To identify specific interactions between Potato Spindle Tuber Viroid and two Bulgarian pepper varieties." The research tasks are set in a logical sequence, the first one specifying the pepper varieties to be investigated. The tasks are specific, realistically set, and correspond to the technical resources of the training unit. The Materials and Methods chapter offers a description of the methodologies and more specific materials used. The procedures for culturing pepper plants, inoculating them with PSTVd, preparing RNA material for the infection, isolating total RNA, sequencing RNA and the bioinformatic model applied to analyze the results are described in detail. Procedures for validation of the differential gene expression analysis results are described. In analyzing the section, I found the following omissions and inaccuracies: 1) The data presented in Figures 8 and 9 are more appropriate for the Results chapter 2) In section 2.1.1 "Surface sterilization of pepper seeds" should be revised - the reagent used for sterilization could not have been KH_2PO_4, 3) In the description of pepper varieties (par. 1.1 "Description of the plant material") used in the study should provide data for the paper cultivars pedigree, which would have been more relevant to the subsequent interpretation of the results obtained, 4) The method of RNA isolation with the "RNeasy Plant Mini Kit" is not mentioned anywhere else in the thesis. The Results section discusses in chronological order the activities of the presented research. Using *in vitro* transcription, the applicant has obtained the required amounts of viroid RNA, which were subsequently used to infect pepper plants within a bioassay. Although it could be assumed that the aim of the bioassay was to identify PSTVd-susceptible pepper varieties, this was not explicitly mentioned. On my opinion, the bioassays are extremely important procedures to develop and labor intensive, and therefore should be formulated as a individual task. Total RNA was isolated from pooled samples of uninfected

and PSTVd-infected plants. Qiazol reagent was used for this purpose and the methodology for RNA isolation is not describer in the Materials and Methods section or elsewhere. By means of conventional PCR, the Nikol Hadzieva confirmed PSTVd infection and performed a semi-quantitative assessment of viroid load. The latter was found to be higher in the cultivar Julunska rosehip. After grading, the same was sent for sequencing, and the results were subjected to post-filtering, and further analysis as follows: 1) mapping on a PSTVd reference genome, thus demonstrating replication of the viroid in both pepper cultivars, and 2) analyzed for differentially expressed transcripts in response to viroid infection was conducted. Although NGS data indicated the presence of SNPs on positions 288, 298, 301, and 333, these were not demonstrated by cloning and subsequent sequencing. Differential expression of more than 200 miRNAs belonging to more than 60 families has been detected. In most cases, the gene expression regulation profile in the two cultivars shows a specific profile. On this basis, the PhD student concludes that the response to PSTVd is cultivar specific. Similarly, the differential expression of protein-coding genes was assessed and validated and annotated. Overall, the results are presented with sufficient information and appropriate bioinformatic and statistical interpretation. The Discussion section is devoted to a discussion of the results obtained in the context of the scientific work of other teams. The discussion is thorough and adequate to the results obtained. As a result, ten conclusions and five scientific and applied contributions are formulated, which reflect the main achievements of the work. The conclusions are carefully formulated and correspond in scope and content to the set aim and objectives of the thesis. The contributions are mainly of a fundamental nature but with potential for practical applicability. The overall content of the dissertation characterizes it as a serious, complete, and well- structured study. No evidence for plagiarism was found.

7. Contributions and significance of the development for science and practice

The results of the research are summarized in five scientific and applied contributions as follows:

- For the first time, molecular interactions between PSTVd and two Bulgarian pepper varieties were investigated by large-scale expression analyses of small RNAs and iRNAs.
- For the first time, differentially expressed miRNAs and differentially expressed protein- coding genes have been identified in the pepper cultivars KK and DS that collectively determine the incorporation of a cultivar-specific response to PSTVd.

- The described molecular interactions between PSTVd and pepper suggest their involvement in modulating viroid spread, replication, and the development of viroid specific symptomatology, thereby potentially contributing to the development of strategies to improve the control of PSTVd infection among agricultural crops.
- The results of the large-scale sequencing of iRNAs obtained from infected and control pepper plants are annotated in the NCBI biobase
- The results of the large-scale sequencing of small RNAs in this thesis have been annotated in the NCBI SRA bio-database. The contributions are carefully and precisely formulated, meet the aims and objectives and are relevant to the results presented.

8. Assessment of the publications on the dissertation

In the present procedure, the PhD student has submitted three scientific publications directly related to the results of the dissertation. Two of these have an impact factor of 3.4 and 3.9 in quartile 1 journals, and one is in Scientific Proceedings of the UBS. In one of the publications with impact factor, the PhD student is the first author and in the other two-second. At the time of preparing the review, seven citations of the PhD student's scientific articles were found in public databases, four of the citations were of articles reflecting the results of the dissertation. The PhD student has indicated participation in six scientific conferences related to the dissemination of the data from the reviewed work.

9. Personal participation of the PhD student

Taking into account the participation of the PhD student in the writing of three scientific publications related to the dissertation and her participation in six scientific forums, as well as the overall presentation of the dissertation, I believe that the personal participation of the PhD student is in full compliance with the requirements set forth in the Law for the Development of Academic Staff in the Republic of Bulgaria (Law on the Development of Academic Staff in the Republic of Bulgaria), the Regulations for the Implementation of the Law on the Development of Academic Staff in the Republic of Bulgaria (Law on the Development of Academic Staff in the Republic of Bulgaria) and the relevant Regulations of Paisii Hilendarski University.

10. Author's abstract

Two Author's abstract are submitted in Bulgarian and English, respectively. The abstracts fully reflect the main scientific achievements of the applicant The two versions are identical and are 33 and 31 typewritten pages respectively. The abstracts are prepared according to the generally accepted model and reflect the results obtained fully and accurately.

11. Critical comments and recommendations

In addition to the above comments on the thesis, the following omissions should also be noted: the formatting of the tables could be improved and unified; the term "potato tubers" is used incorrectly and should be replaced by "potato tubers"; the nomenclature of the spelling of the names of the restricts is not followed in some places; some figures lack labels (e.g. Fig. 12); In Fig. 17, a band on the agarose gel is labeled as 25S rRNA I assume it is 28S rRNA; The structure of PSTVd is shown in Figs. 4 and 5, and in Fig. 4 the same information is presented in three different ways; Some of the figures and graphs are in English; The resolution of some of the figures makes it impossible to interpret the results presented; In some places scientific jargon is translated incorrectly into English, e.g. PCR instead of PCR.

Overall, these omissions affect the presentation of the dissertation, but do not diminish the value of the results obtained.

12. Personal impressions

My personal impressions of the applicant work are cursory and could not contribute to overall evaluation of her work.

13. Recommendations for future use of the dissertation contributions and results

I don't have any.

CONCLUSION

The dissertation *contains scientific and applied results that represent an original contribution to science* and meet the requirements of the Law for the Development of Academic Staff in the Republic of Bulgaria, the Regulations for the Implementation of the LADAPB and the relevant Regulations of University of Plovdiv “ Paisii Hilendarski”. The dissertation work shows that the PhD student Nikol Hadzieva possesses the necessary theoretical knowledge and professional skills in the scientific specialty of Molecular Biology, demonstrating qualities and skills for independent scientific research. Due to the aforementioned, I confidently give my positive evaluation for the conducted research, presented by the above-reviewed dissertation, abstract, achieved results and contributions, and I propose to the esteemed scientific jury to award the educational and scientific degree "Doctor" to Nikol Hadzieva in the following field of higher education: field of higher education: 4 Natural Sciences, Mathematics and Informatics; professional field: 4.3 Biological Sciences; doctoral program "Molecular Biology".

29.03 2023 г. Reviewer: .....

Assoc. Prof. Samir Naimov