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HR EXCELLENCE IN RESEARCH

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REVIEW OF A DOCTORAL DISSERTATION

RECENZJA ROZPRAWY DOKTORSKIEJ

Preeti Shakya, M.Sc.

"Proteomic approach to understand the mechanisms underlying interactions between *Hypericum perforatum* L. and *Agrobacterium tumefaciens*"

„Podejście proteomiczne do zrozumienia mechanizmów interakcji pomiędzy *Hypericum perforatum* L. i *Agrobacterium tumefaciens*"

Conducted at the Department of Integrative Plant Biology
Institute of Plant Genetics – Polish Academy of Sciences
under the supervision of Franklin Gregory, PhD, DSc, Professor at IGR PAS
with Assistant Supervisor – Robert Malinowski, PhD, DSc, Professor

Legal basis for the Review

This Review was prepared in response to the resolution of the Scientific Council of the Institute of Plant Genetics, Polish Academy of Sciences in Poznań, dated December 16, 2025 (No. 8/IX/2025), appointing reviewers for the aforementioned Dissertation, and pursuant to the provisions of the Act of July 20, 2018 – Law on Higher Education and Science (Journal of Laws of 2018, item 1669, as amended), hereinafter referred to as the "Act." The Review was prepared based on the doctoral Dissertation of the above candidate for the academic degree of Doctor (hereinafter, the "PhD candidate"), received on January 13, 2026.

Overview and relevance of the topic

Hypericum perforatum L. (St. John's wort) is of significant importance in the pharmaceutical market as a herbal raw material, used to produce preparations with antidepressant, anti-inflammatory, and wound-healing properties. Consequently, in the horticultural and agricultural sectors, it is considered a medicinal plant of high economic value. There is an urge to improve *H. perforatum* cultivars to increase and stabilize the production of secondary metabolites, such as hypericin and hyperforin. This action would allow the production of raw materials of higher, more consistent pharmaceutical quality.

Genetic transformation is a useful tool for increasing the biosynthesis efficiency of active compounds and improving plant functional characteristics. It is most commonly performed using the vector method with the bacterium *Agrobacterium tumefaciens* (also known as *Rhizobium radiobacter*). There are many examples of agricultural and horticultural plant species that have been successfully transformed using this approach. However, in the case of *Hypericum perforatum*, transformation using *Agrobacterium* can be difficult and has low efficiency. The reasons for these difficulties are not fully understood (they are likely related to the plant's specific defense mechanisms and secondary metabolism) and require further research.

In her doctoral Dissertation, Preeti Shakyav, M.Sc., undertook the ambitious task of comprehensively explaining the molecular and physiological mechanisms underlying *Hypericum perforatum* resistance to *Agrobacterium tumefaciens*-mediated transformation, thereby expanding knowledge of plant-bacteria interactions.

This research problem is of significant scientific and practical importance. Many studies have examined the influence of various factors on the efficiency of plant transformation, but the genetic factor (i.e., species-dependent plant responses) makes this issue particularly complex. To date, few studies have explored the causes of the low efficiency of genetic transformation in *Hypericum perforatum*, focusing primarily on the transcriptome and metabolome levels. However, proteome studies are lacking. Exploring this topic could lead to improved plant transformation efficiency toward higher secondary metabolite content, with applications in the phytopharmaceutical and cosmetic industries, as well as in the production of plants with increased utility value. Furthermore, this research provides new insights into the physiological and molecular mechanisms of plants of the *Hypericum* genus.

Formal evaluation of the Dissertation

The subject of the following Review is a doctoral Dissertation prepared based on a series of three thematically coherent scientific publications: two review articles and one original research paper. The articles constituting the scientific achievement were printed and submitted (also in electronic format) along with declarations regarding the contribution of the PhD candidate and co-authors to these papers. These include:

1. Hou W., **Shakya P.**, Franklin* G. (2016). A perspective on *Hypericum perforatum* genetic transformation. *Frontiers in Plant Science* 7: 879. DOI:10.3389/fpls.2016.00879 (IF_{2016}: 4.291, $MNiSW_{2016}$: 40)
2. **Shakya P.**, Marslin G., Siram K., Beerhues L., Franklin* G. (2019). Elicitation as a tool to improve the profiles of high-value secondary metabolites and pharmacological properties of *Hypericum perforatum*. *Journal of Pharmacy and Pharmacology*, 71: 70-82. DOI:10.1111/jphp.12743 (IF_{2019}: 2.571, $MNiSW_{2019}$: 70)
3. **Shakya P.**, Selvakesavan R.K., Perlikowski D., Antonydhason V., Kiirika L., Franklin* G. (2025). *Agrobacterium tumefaciens*-induced proteomic remodelling and physiological adaptations in *Hypericum perforatum* L. *Plant Stress*, 18. DOI:10.1016/j.stress.2025.100986 (IF_{2025}: 6.9, $MNiSWIF_{2025}$: 20)

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The above papers were published in recognized, peer-reviewed international scientific journals (Frontiers in Plant Science, Journal of Pharmacy and Pharmacology, Plant Stress) and are indexed in the Journal Citation Reports (JCR) database. All of these journals publish exclusively in English and are assigned to the discipline of agriculture and horticulture (according to the Polish Ministry of Science and Higher Education). According to the data presented in the Dissertation, the summary IF of these publications was 14.9, and the total number of points from the Polish Ministry of Science and Higher Education was 190. However, it appears the PhD candidate used the current parameters (from 2025) when providing scientometric data. Instead, she should have provided data consistent with the year of publication. In this situation, the summary IF of the publications is 13.762, and the total Polish Ministry of Science and Higher Education points are 130.

Nevertheless, I consider the bibliometric indicators of the attached papers to be high in the field of agricultural sciences. The papers published in Front. Plant Sci. and J. Pharm. Pharmacol. were cited 33 and 97 times, respectively; a total of 130 (both total and without self-citation; according to Web of Science). Considering that they were published relatively recently, i.e., within the last 10 years, this is a very good score. The third article has not yet received any citations, which may be due to

its recent publication (2025). Conceptual illustrations provided by Ms. Shakya (pp. 37, 61, and 80) help understand the papers' concepts.

All three articles are multi-authored (with three to six authors), and in two, the PhD candidate is the first author and in one the second author, underscoring Ms. Shakya's significant contribution to the creation of these works. The PhD candidate's contribution included the literature review, information collection, preparation and revision of the original draft, as well as the design of figures and tables (for the review and research articles) and the conduct of experiments, investigations, and formal analysis (for the original research article). It is a pity that the PhD candidate did not participate in developing the study concept and did not serve as the corresponding author on any of the papers, which would have helped learn about the editorial process. On the plus side, these works were created in international collaboration with scientists from Australia, Germany, Portugal, India, and the USA. According to the PhD candidate, the research presented in the Dissertation was also conducted in cooperation with the Proteomics Core Facility, Masaryk University, CEITEC (Central European Institute of Technology), Brno, Czech Republic. However, the authors from the Czech Republic are not present in the publications. Can the PhD candidate comment on this?

It is also worth highlighting that the research was conducted as part of the National Science Center project (Opus program; no. 2017/25/B/NZ9/00720) and the European Union's 7th Framework Program for research, technological development, and demonstration under grant agreement no. 621321, the co-funding allocated for education through project W26/7.PR/2015[GA 3113/7.PR/2] by the Polish Ministry of Science and Higher Education for the years 2015-2019. Participation in the OPUS project confirms the high quality of the research conducted. It also allowed the PhD candidate to work within a clearly defined research project, fostering the development of scientific and organizational skills.

The Dissertation is 109 pages long and includes: a list of publications constituting the scientific achievement, a table of contents, a list of abbreviations used (which makes it easier for the reader to follow the text), an English abstract, and an identical Polish translation. In my opinion, the abstract (and its translation) should not be treated as numbered chapters in the Dissertation, so the Introduction should not be considered Chapter 3. The abstract is a stand-alone summary of the entire work, while the Introduction is the first substantive chapter of any publication. Next, the PhD candidate included nine chapters in her Dissertation: "Introduction," "Research hypothesis and

objectives," "Materials and methods," "Results and discussion," "Summary and conclusions," "References," and "Supplementary information." The "Materials and methods" and "Results and discussion" sections are divided into subchapters, which I consider a positive feature. The final part of the Dissertation consists of attached copies of publications included in the monothematic cycle. Additionally, the PhD candidate included all essential declarations, funding information, and acknowledgments.

Substantive evaluation of the Dissertation

The research topic undertaken by the PhD candidate is current and important. Vector genetic transformation using *Agrobacterium* is among the most intensively used genetic engineering techniques. It is estimated that plant transformation using *A. tumefaciens* has been successfully applied to over 100 plant species across more than 30 botanical families. This method applies to both dicotyledons (where the efficiency is usually higher) and many monocotyledons, including cereals. To date, the proteome of plants in response to *A. tumefaciens* exposure has only been described for *Vitis vinifera* L. and *Triticum aestivum* L. The use of innovative techniques, such as liquid chromatography/tandem mass spectrometry (LC-MS/MS) and quantitative real-time PCR (qRT-PCR), allows for understanding the reasons for unsuccessful transformation attempts in St. John's wort and other plants resistant to *A. tumefaciens* infection. The work is innovative and interdisciplinary, combining classical plant biotechnology approaches (*in vitro* cultures, *in vivo* experiments) with the achievements of proteomics, mass spectrometry, and bioinformatics.

The ***title*** of the Dissertation is concise and reflects its content. It is desirable to understand the factors influencing the effectiveness of herbal plant transformation, particularly *Hypericum perforatum*. Therefore, I consider the choice of the topic to be interesting and to demonstrate innovative elements.

In a comprehensive ***Introduction*** (6.5 pages long), the PhD candidate clearly and unambiguously justifies the need for her own research. She also presents a synthetic overview of the current state of knowledge, including the mechanisms of plant genetic transformation mediated by *Agrobacterium tumefaciens*, with particular emphasis on T-DNA transfer, the role of bacterial virulence systems (Vir), plant factors, and environmental conditions that influence the effectiveness of the process. The plant defense response to *Agrobacterium* infection is also presented, including the activation of membrane receptors, the mitogen-activated protein kinase (MAPK) cascade, oxidative burst, and changes in protein expression. Particular attention was

focused on *Hypericum perforatum* as a species recalcitrant to transformation, highlighting the lack of proteomic studies on this species and the need for an integrated analysis of the proteome and physiological parameters to elucidate the mechanisms underlying its resistance to *Agrobacterium* infection. The schemes, presented in three aesthetic graphs, are well thought out, helping the reader understand the text.

The research ***hypothesis*** is a logical consequence of the literature review presented in the Introduction and assumes the involvement of plant proteins in *Agrobacterium*-mediated transformation. The ***aim*** of the study was clearly presented and comprised investigating the behavior of *A. tumefaciens* during co-cultivation with *H. perforatum* cell suspension and plant response at the molecular and physiological levels.

To verify the hypotheses, the PhD candidate conducted laboratory experiments, including antibacterial assays with different concentrations of *H. perforatum* cell suspensions, investigations of the proteome response and gene activity of plant cells during interaction with *A. tumefaciens*, and assessments of the physiological changes of *H. perforatum* in response to *A. tumefaciens*.

The ***methodology*** was described in adequate detail to allow for the understanding of the experimental process. I have no critical comments regarding the selection of methods. The PhD candidate thoroughly planned the research, combining both molecular (protein extraction, digestion, and identification of differentially expressed genes; expression of selected specific genes) and physiological (antibacterial assays, photosynthetic efficiency) aspects. The chosen analytical and methodological approach is correct and justified. It is worth emphasizing the advanced nature of the research techniques used (liquid chromatography with mass spectrometry and bioinformatic analyses).

There is only a minor inconsistency in the materials descriptions – sometimes the PhD candidate provides both the manufacturer's name and the country of origin, which is correct (e.g., for plant growth regulators). In contrast, sometimes she provides only the manufacturer's name (e.g., for the photosynthesis measurement device).

The statistical analysis demonstrated the PhD candidate's strong expertise in analytical methods, and the tools used enabled accurate interpretation of the results.

Question: please explain how the homogeneity of the plant cell suspensions was ensured/tested.

The ***results*** of the original research are presented synthetically in Chapter 6 (over six pages), while their full versions (including tables, graphs, and photographs) are included in the attached articles, that are the core of this Dissertation. These articles were thoroughly peer-reviewed and corrected by the editors during the publishing process, so I will only summarize the main achievements and ask a few questions.

The first two papers are review articles. The first summarizes the current state of the art on the genetic transformation of St. John's wort. It analyzes why *Hypericum perforatum* is so resistant to the introduction of foreign DNA into its genome by bacteria (describes the plant's defense mechanisms, including the production of reactive oxygen species and the release of compounds that kill bacteria). It suggests new, promising directions for further research.

The second paper summarizes knowledge on how to treat *H. perforatum* plants *in vitro* to induce the overproduction of medicinal substances. The authors describe the use of various elicitors, both biotic (e.g., fungal and bacterial) and abiotic (metal ions, nanoparticles, irradiation), to enhance the biosynthesis of, for example, hypericin for industrial purposes.

The preparation of these papers confirms that the PhD candidate has read, understood, and systematized the available international literature. These articles also highlight existing research gaps. In summary, these publications are valuable scientific contributions that introduce the reader to the Dissertation topic.

Publication 3 focuses on the impact of plant and plant cell contact with *A. tumefaciens* on bacterial survival, the plant's proteomic response, the expression of key defense genes, and physiological changes (photosynthetic activity). Contact of *Hypericum perforatum* cells and plants with *A. tumefaciens* has been found to induce a strong, early, and multilevel defense response, including activation of pathogen recognition receptors, antioxidant enzymes, the phenylpropanoid pathway, and the synthesis of xanthones and pathogenesis-related (PR) proteins, as confirmed both proteomically and at the transcript level. At the same time, energy metabolism is reprogrammed, cell wall remodeling occurs, and the synthesis of proteins involved in nuclear transport is reduced, potentially hindering T-DNA transfer. Physiological changes, such as decreased photosynthesis and dynamic stomatal regulation, further indicate stress-promoting recalcitrance to transformation.

Questions for this part:

1. Does the PhD candidate know any other examples of species resistant to transformation? Could the molecular mechanism proposed in her research also occur in these species?
2. In *in vitro* cultures, it is not uncommon for various cultivars within the same species to respond differently to the same culture conditions. Is it possible that the mechanisms you described are also so diverse in terms of bacterial infection?
3. Is the upregulation of PR-10, PAL, TXS7, and BPS proteins directly induced by bacterial signals, or does it result from general cellular stress?
4. Why does bacterial infection cause a decrease in photosynthetic activity?

The presented works raise no objections on merit. The entire work forms a coherent publication cycle. The PhD candidate uses her extensive knowledge of green biotechnology, emphasizing the research's original aspects. She thoroughly relates the obtained results to literature data and explains the mechanisms underlying the observed relationships. In the context of future research, I would like to point out that the International System of Units (SI) recommends notating units as products with exponents (e.g., $mg \cdot L^{-1}$), as this is unambiguous. However, the PhD candidate uses the concentration quotient form (e.g., mg/L).

It is somewhat unfortunate that the PhD candidate did not refer to the more current name of the bacterium being studied anywhere in the text. For decades, the bacterium responsible for crown gall disease and widely used in plant genetic engineering was known as *Agrobacterium tumefaciens*. However, in the early 2000s, DNA sequencing (specifically 16S rRNA analysis) revealed that the *Agrobacterium* genus is so closely related to *Rhizobium* that taxonomists proposed merging the two genera. Because the species epithet '*radiobacter*' had priority in naming, the newly classified organism became *Rhizobium radiobacter*. Of course, the name *A. tumefaciens* is still a standard in plant biotechnology, but it would be good to at least mention its synonym.

The research results allowed the PhD candidate to draw three ***conclusions*** consistent with her findings. They can be summarized as follows: *Hypericum perforatum* cells strongly inhibit the growth of *Agrobacterium tumefaciens* by triggering a complex cascade of defense responses, including changes in the expression of proteins associated with oxidative stress, primary and secondary metabolism, and physiological functions, which is responsible for plant resistance to the genetic transformation induced by this bacterium. These findings establish a comprehensive framework for future studies aimed at explaining the molecular mechanisms underlying *H. perforatum*'s resistance to *Agrobacterium*-mediated transformation.

The ***references*** used in the Dissertation comprise 51 items (all in English). They are relevant (especially considering the highly specialized nature of the subject matter) and up-to-date (21 articles were published within the last five years, another 13 within the last decade, while 10 publications fall within the 15 years). Their selection confirms a good knowledge of the available literature related to the topics addressed in the Dissertation. However, the non-uniform formatting of the bibliographic information is noteworthy, e.g., the lack of italics for some Latin names and inconsistent formatting of journal titles (full or abbreviated) and DOI numbers. A similar, somewhat careless presentation can be found in the information on the publication cycle on page 5 of the Dissertation.

The PhD candidate has enclosed the presentation of her articles with ***supplementary information*** containing three additional figures. Here, I would like to point out that Figure S3 refers to concentration; however, the units are presented solely as weight (no volume units are provided).

Regarding the entire Dissertation, I would like to emphasize that it is generally written correctly in terms of language. The main shortcoming is the first conclusion (p. 34). This sentence is stylistically incorrect and contains grammatical errors. The conclusion would be more readable if it were divided into two sentences: "The *H. perforatum* cell suspension exhibited strong antibacterial activity by suppressing the growth of viable *A. tumefaciens* cells. This intense defense mechanism contributes significantly to the plant's resistance to the pathogen".

Other minor errors occur rarely:

- p. 7 – The PhD candidate mixes the scientific names "*japonica*" and "*indica*" with the common name "rice",
- citations of Figures and Tables in the text should be capitalized (e.g., p. 8),
- missing a comma before "respectively" (p. 9),
- at the first mention of species names, the author citations (taxon authorities) are often missing, which is required in scientific writing (e.g., *Vitis riparia*, pp. 10, 11). On the other hand, these initials should not be written in italics (e.g., "L." on page 15),
- p. 19 – grammatical error "between in experimental repeats" (double preposition).

The structure of the Dissertation is generally logical. The formal remarks I have raised do not diminish its substantive value.

Unfortunately, the Dissertation lacks information regarding the PhD candidate's other academic achievements. I have checked that, apart from the publications included in the Dissertation, P. Shakyav, M.Sc., is a co-author of one more scientific paper published in a JCR-indexed journal (Scientific Reports; DOI: 10.1038/s41598-025-30002-8; IF_{2025}: 3.9; 140 points according to the Polish Ministry of Science and Higher Education). Has the candidate participated in any conferences or training courses? Does she have experience in organizational, teaching, or popularization activities, which candidates for the doctoral degree typically present? The Dissertation should enable the reviewer to become acquainted with the PhD candidate's full academic profile.

Summary and conclusion

In summary, I state that Preeti Shakya, M.Sc., has taken an innovative approach to developing a model of the interaction between *Agrobacterium tumefaciens* and the recalcitrant plant species *Hypericum perforatum*, which led to the identification of various up- and down-regulated plant proteins in response to bacterial infection. She has demonstrated solid theoretical knowledge of agricultural sciences (particularly modern approaches used in medicinal plant biotechnology), as well as appropriate methodological advancement. The submitted doctoral Dissertation demonstrates her ability to formulate research hypotheses correctly, plan and conduct experiments on plant material, interpret the obtained results, and draw accurate conclusions. The PhD candidate has made a significant contribution to expanding knowledge in the scientific discipline of agriculture and horticulture by obtaining several valuable results. These findings open new perspectives for further research on the genetic transformation of St. John's wort.

I therefore conclude that the Dissertation submitted for Review meets the criteria specified for doctoral theses, in accordance with Article 192, paragraph 1, and Article 192, paragraph 2, point 5 of the Act. I recommend to the Scientific Council of the Institute of Plant Genetics, Polish Academy of Sciences in Poznań, that the PhD candidate be admitted to the next stages of the procedure for awarding the academic degree of Doctor.

Dariusz Kulus