



To
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Re: Report on the PhD Thesis of Mgr. Edita Holaskova
Study program Biochemistry

Dear Prof. Kubala,
dear members of the PhD committee,

It is my pleasure to provide my report on the PhD thesis entitled „Generation of new transgenic barley lines expressing recombinant antimicrobial peptides“.

This thesis addresses the recombinant production of antibacterial peptides in cereal plants. The subject addresses a timely and innovative area of contemporary biotechnology, given the widespread resistance of bacteria against antibiotics and the resulting need to explore alternative agents to control bacterial pathogens.

The use of plants as bioreactors to produce antibacterial peptides is sensible and offers the potential for cost-effective production of these compounds. However, there are remaining technical bottlenecks that need to be overcome with respect to production levels, product isolation and biological activity. The present study thus represents a considerable advance in the field of molecular farming as it integrates important questions of applied science and underpinning basic research to optimize a platform for the production of recombinant peptides and proteins.

The objectives of the research work are logical, well-defined and clear, with remarkable achievements in several lines of research. Firstly, the fusion of antimicrobial peptide sequences to



protein moieties and tags for increased production, stabilization and purification were explored in a systematic and methodical manner. Secondly, transgenic barley seeds were chosen for stable transgene expression, and to this end the sequence elements of the recombinant constructs were optimized and transgenic plants were generated and carefully analysed. The subcellular localization of the recombinant peptides was determined by immunolabeling and microscopy, and peptide extraction was optimized and quantified. Remarkably, the addition of a maltose-binding protein was found to increase stability in the desiccated grains. Incorporation of the recombinant peptides into endosperm-specific storage organelles was achieved for further stabilization and facilitated purification. Finally, biological activity of recombinant peptide LL-37 was confirmed. Overall, the results of the thesis represent a very significant advance for the production of recombinant peptides and proteins in seed crops.

The work was scientifically and technically demanding and obviously involved a variety of techniques including the biochemical analysis of proteins, tissue culture, microscopy and protein purification methods. The results are presented in a very clear and logical manner, closely following the experimental steps, and there is no doubt for the reader that new and original results have emerged from the project. Mgr. Holaskova is first author on two papers that are based on the results of the thesis. One concentrates on barley as a production system for antimicrobial peptides, and the second reviews the plant-based production and application of antimicrobial peptides in more general terms. Mgr. Holaskova is also involved in a further review reflecting the use of barley seed as bioreactor. All the papers are of high quality.

The thesis itself is well written and includes a very comprehensive, well-documented and informative presentation of the state of the art in the field of molecular farming technologies. This part of the thesis shows that Mgr Holaskova has a good grasp of her field of research and also a sound knowledge outside the subject matter she specifically worked on. For example she reflected various plant expression systems and described the various methods for transforming plants, including general strategies to increase yield of recombinant proteins.

The introduction is followed by a chapter describing Materials and Methods, which includes a lot of detail and certainly sufficient information to reproduce experimental setups. The thesis is completed by a very nice and thoughtful discussion viewing the results in a wider context and perspective.



To sum up, Mgr. Holaskova has fulfilled entirely the purpose of the thesis since she succeeded in obtaining antimicrobial peptides from barley seeds, and she could confirm their biological functionality. This proof of principle opens the possibility of generating seed-based industrial production platforms for this type of compounds. In the course of the work, Mgr Holaskova has proven her ability to design, conduct and discuss highly valuable and novel research in a successful manner.

I strongly agree that the thesis should be accepted for public discussion and I look forward to the presentation of the work.

Should you require any further information, please do not hesitate to contact me (+43 1 47654-94111).

Yours sincerely,



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Reviewer report on the dissertation thesis "Generation and analysis of new transgenic barley lines expressing recombinant antimicrobial peptides" by mgr. Edita Holásková.

The issue of antimicrobial peptides belongs to interesting topics of both human and veterinary infectious medicine. The increase in bacterial resistance to antibiotics, including those of the last resort, is an important impetus for seeking alternative biocidal options. The therapeutic potential of antimicrobial peptides is considerable, due to their diverse mechanisms of action, low toxicity to eukaryotic cells and minimal risk antibacterial resistance development. Therefore, I appreciate the innovative approach of the author of this work in terms of seeking an alternative approach to the preparation of these interesting and promising biomolecules.

The submitted dissertation is written on 185 text pages and has a classic arrangement usual for this kind of work, ie introduction, material and methods, results and conclusion. The literature review contains a large number of mostly recent references. The work is supplemented with 39 pictures and tables to increase the clarity of the text.

The literature review documents that the author is well acquainted with the solved problems and can formulate the acquired knowledge in a professional text. In this part of the thesis, the author deals mainly with the available knowledge about the properties of AMP but also about the possibilities and strategy of expression of recombinant proteins in plants. A separate part of the literature review is then devoted to the possibilities of AMP expression in plant cells, specifically in barley.

The goals of the work are clearly set out and can be considered as fully fulfilled.

In the chapter material and methods, the author deals with the procedures used in the experimental part of her work, ie. selection of suitable promoters allowing expression in barley, construction of binary vectors necessary for combination of these promoters and other functional components of expression vectors, further isolation of recombinant AMP from plant material, their analysis by western blot and mass spectrometry, and ultimately testing their antimicrobial activity. The author therefore had to master an impressive number of molecular biological methods to achieve experimental goals.

The results obtained demonstrate the successful expression of three different antimicrobial peptides (human cathelicidin LL-37, human beta-defensin and pexigan derived from *Xenopus laevis*). The most significant level of expression was achieved in the case of LL-37, with the level of expression highly dependent on the type of promoter used and the final intracellular localization of the resulting recombinant peptide due to the presence of the KDEL leader peptide allowing retention in the endoplasmic reticulum.

The author tested the anti-bacterial activity of LL-37 in an *E.coli* cell model and demonstrated significant inhibition of bacterial cell proliferation in relation to LL-37 concentration, even partially in the presence of a poly-His tag.

I have the following notes or questions to answer:

Expression optimization for beta-defensin and pexignan was significantly simplified compared to that of LL-37. Could this be the reason for significantly lower yields of the two mentioned peptides?

What are the differences of posttranslational modifications between plant and animal cells? The author mentions insignificant differences in the text, but they may also have a considerable impact on the resulting AMP conformation.

Can recombinant proteins expressed in plants be used without their purification, for example, for oral immunization of farm animals by feeding these GMOs?

Finally, I can state that the dissertation thesis meets all the professional requirements required for this type of work and therefore I recommend that Edita Holásková be awarded the corresponding Ph.D. degree.

Brno, 22. 11. 2019



prof. MVDr. Vladimír Celer, Ph.D.