IST AUSTRIA
Institute of Science and Technology

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Eva Benkova
Professor

Phone + 43 (0)2243 9000-5301
eva.benkova@ist.ac.at
Bertalanffy Foundation Building
Am Campus 1
3400 Klosterneuburg
Austria

Doctoral thesis assessment submitted by Mgr. Barbora Parizkova

PhD thesis entitled "Biological activity of novel auxin synthetic derivatives" submitted by Mgr. Barbora Parizkova implemented multidisciplinary approaches to perform biological and chemical-physical characterisation of new synthetic auxin analogues with aim to gain novel insights into mechanisms underlying regulation of auxin distribution and activity *in planta*. Overall, aims and objectives addressed in the PhD thesis are fundamental and highly relevant for in depth understanding basic principles of hormonal regulations in plant growth and development.

Introductory chapter of the thesis brings excellent overview of the biological role of auxin with focus on methods and techniques developed for detection, identification and quantification of this plant hormone. In this state-of-art review, Mgr. Parizkova closely discusses strengths and weakness of established approaches and reflects on perspectives of their improvements.

Results and major achievements of the PhD work are presented in form of research articles and manuscript in preparation with Mgr. Parizkova being either first or co-author. In these studies Mgr. Parizkova performed extensive in depth functional and biological characterisation of several fluorescently labelled auxin analogues. To achieve objectives of her work Mgr. Parizkova successfully implemented large spectrum of techniques including chemical genomics, mass spectrometry based methods, reverse genetics, and advanced confocal microscopy, all properly

described in material and methods. Significance of her work in context of the current research and future perspectives are critically discussed in the final chapter of the thesis.

Mgr. Parizkova presents in her PhD work extensive set of data of high scientific quality. She is first and co-author on articles published in *International Journal of Molecular Sciences*, *New Biotechnology*, P*NAS* and *PLoS One*, and results of manuscript in preparation will be certainly of interest to the broad research community, and have great potential to be published in renowned scientific journal.

The PhD thesis clearly shows that Mgr. Parizkova is an excellent experimenter, and very talented researcher. To conclude, the PhD thesis is of the high scientific quality and I recommend it for public defence.

Eva Benkova

Professor IST Austria

Questions to the presented PhD thesis:

1. Where do you see main obstacles and challenges in developing tools and techniques for detection and visualisation of plant hormones? What are the futures trends in this area of research?
2. Where do you see specifics, differences, challenges in auxin analytics and biochemistry when compared to other plant hormones?
3. In the study Bieleszova et al., (2018) you have demonstrated that several auxin analogues (5b, 5c, 5d) act as anti-auxins and constrain activity of exogenously applied auxin. Did you observe any biological effects of these compounds that would indicate their impact on endogenously acting auxin? Please discuss possible mechanisms of their action.
4. In the study Parizkova et al., (in preparation) pattern of FluorAI and AII accumulation

in plant tissues and organs was examined. Please compare and discuss the observed pattern in light of other established auxin reporters. Does auxin perception and signalling pathway feedback on kinetics and pattern of accumulation of these auxin derivatives?

5. Figure 3G indicates different levels of FlourA uptake in Col when compared to Ler ecotype, please comment on this observation. Are there any indications that auxin uptake might vary among different ecotypes?
6. Where do you see main potential of auxin derivatives (you have characterised) for application in future research? Which biological questions could prospectively be assessed using these auxin analogues?

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PhD thesis opponent review
Barbora Pařizková, Mgr
Palacký University Olomouc, Faculty of Scienve, Study program Experimental Biology

Contact:
Hélène Robert Boisivon, PhD
+420 549 49 8421
helene.robert.boisivon@ceitec.muni.cz

Brno, July 17th, 2019

To whom it may concern,

In my quality of opponent, I carefully read the PhD thesis that B. Pařizková, Mgr., submitted to the PhD committee in order to defense her wok in view to obtain the PhD Doctoral degree.

The thesis consists in all the requested chapters (Introduction, Aims and scopes, Literature review of the topic, Materials and Methods, Survey of the published results, Conclusion and a list of references). Barbora Pařizková is first and co-first author in 3 publications (one review, and 2 research papers) published in IJMS, New Biotechnology and in preparation. She is also co-author in two other papers where she participated in view of her expertise and method development. All of these above-mentioned criteria are fulfilling the requirements of the PhD thesis. I would have had appreciated a CV summary stating her participation in conferences, especially with oral presentations and eventual invitations.

During her PhD studies, Barbora Pařizková developed and optimized methodologies for the purification and the detection of the synthetic auxin 2,4-D and its derivatives, as well as for the determination of the stability of auxin analogs in plants. Moreover, she designed new auxin (IAA and 2,4-D) fluorescent derivatives and performed bioassays to test their distribution, stability and biological functions in plants. The work of Barbora Pařizková clearly brought new knowledge and new tools in the field of auxin biology and analytic chemistry. In addition, I have knowledge that Barbora Pařizková is working on methods to precisely deliver hormones for local applications using micro-pipettes that are not described in this thesis. Therefore, the contribution of Barbora Pařizková is greatly appreciated in the field and clearly demonstrated great ability on the design, the performance, the analysis and the reporting of concrete biological questions. She is combining both methodology development, biochemistry expertise and the analysis of the biological relevance of the newly developed hormonal analogs. In that respect she has a broad view of the biological problems, as discussed in the thesis and the publications, and a handful expertise acquired during her doctoral studies.

Her publication activities during her doctoral studies are above average, 4 published articles (one review article, three research articles including one as co-first author). One more manuscript is in preparation and, according to the thesis report nearly, finalized. May I ask what is the current status of this manuscript?

The thesis starts with a literature review of the field of study, meaning auxins (structure and relation to its activity in plants, functions, bioassays and analytic methods for detection and quantification). Several natural auxins are present in the plant kingdom, IAA, IBA, 4-Cl-IAA and PAA. Their presence is not homogenous in all plant species. For example, pea produces, especially in seeds, 4-Cl-IAA, less present in Arabidopsis. The text questions the presence of

Hélène ROBERT BOISIVON, PhD
Research Group Leader
Mendel Centre for Genomics and Proteomics of Plant Systems
CEITEC – Central European Institute of Technology
Masaryk University
Kamenice 753/5, A26, office 2.17
625 00 Brno, Czech Republic
Phone: +420 549 49 8421
www.ceitec.eu
www.ceitec.eu/hormonal-crosstalk-in-plant-development-helene-robert-boisivon/rg47

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IBA as natural endogenous auxin analog because the current methods are not sensitive to detect IBA in Arabidopsis (pp 13, 20).

-But are there any particular conditions that would trigger the production of IBA by the plant?

-Or are there any plant species where IBA would be in greater levels that would allow the development of better detection methods?

Later in the text, the polar auxin transport is introduced with notably the PIN proteins, speaking of long and short PINs.

- What refers this "long and short" to?

PILs proteins are also mentioned, indicating that "both short PINs and PILS participate in the regulation of auxin uptake and consequently auxin signalling and downstream response", p 24.

- This statement requires clarifications: uptake from where to where? All in the same direction of transport? Signalling, if we refer to TIR-dependent signalling, occurs in the nucleus. So, how transport by the short PINs and PILS could affect signalling in the nucleus?

In the auxin perception and signalling (p 25), there are mention of three auxin receptors. One is commonly referred to (TIR1/AFB), another is nowadays controversial (ABP1). The last mentioned one is SKP2A.

- My question is: Is the fact that a protein binds to auxin enough to categorize it as auxin receptor? What would be the requirements to define an auxin-binding protein as auxin receptor?

The next part of the thesis is about Materials and Methods. I am not a (bio)chemist and I, therefore, do not feel qualified to judge the relevance and accuracy of the used chemicals and of the described methods for extraction and purification, UHPLC-MS/MS methods, in this part nor in the publications in annexes. I will focus on the methods for the bioassays. In general, the description of these methods could be more informative. I understood that specific details are given in the respective annexes. However, the text would have benefitted from information about, for example, root measurement and fluorescence measurement methods (software, statistical analysis), the traits analyzed in reverse genetics and about number of replicates, types of controls and analysis method for the RT-qPCR (instead of the details on the extraction method).

I have now further questions on the supplements (publications), that may or may not be answered during the defense.

The Supplement I is a review article in IJMS presenting a literature survey of the topic of the PhD thesis with an up-to-date literature in the field (indirect and direct visualization of auxins in the plant tissue and cells). The part on labeled auxinic molecules nicely introduces the subject and the methods presented in the PhD thesis.

The Supplement II is a copy of the published work (Barbora Pařizková as co-first author) in New Biotechnology. The methods are clearly and deeply described. This publication presents the production methods and the biological function of fluorescently labeled IAA molecules with NBD. It shows that N1-labeling affects the activity of the IAA molecules, i.e. its capacity to induce an auxin-type growth response and the expression of auxin response reporters. It shows that the length of the linker positively correlates with the fluorescence activity as well as the anti-auxin activity of the labelled molecule. My only question is:

- IAA is known to be rather instable (also describe in Supplemental V). Is the stability of the compounds 5a-d been tested?

Another comment related to the legend in Fig. S3, detailed indication of what each panel represent would be informative (corresponding to which treatment time and concentration?).

Hélène ROBERT BOISIVON, PhD
Research Group Leader
Mendel Centre for Genomics and Proteomics of Plant Systems
CEITEC – Central European Institute of Technology
Masaryk University
Kamenice 753/5, A26, office 2.17
625 00 Brno, Czech Republic
Phone: +420 549 49 8421
www.ceitec.eu
www.ceitec.eu/hormonal-crosstalk-in-plant-development-helene-robert-boisivon/rg47

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Supplement III present a work in preparation for publication, the development of fluorescent-labeled 2,4-D molecules as probes to detect the distribution of auxin in plants. This work is yet to be peer-reviewed and I have therefore more questions. I would like to also state my appreciation as for the detailed description of the methods. I have one minor comment on the pictures presented in the figures, as very dark. It would be beneficial to the reader to have grayscale pictures rather than green or red, when color is unnecessary for the presentation. The manuscript describes the design and generation of 2,4-D derived auxin analogs coupled with NBD via various linkers (FluorA).

- The stability of the compounds is tested, were any 2,4-D-Glu-NBD or 2,4-D-Asp-NBD identified (Supplement V)?

The authors end up working with only 3 of the 11 produced molecules based on bioassays of the auxin response of root growth and its induction of auxin signaling reporters. The goal is to identify compound/s that would be active auxins as for the transport to mimic natural auxin distribution, but anti-auxin or inactive for the auxin signalling. They further describe FluorAI to AIII, based on defined criteria.

- Why has FluorA IX not been selected as suitable candidate? It seems to me to have the same response in the bioassays as FluorA I-III.

- Legend of Figure S5 should be checked. It is there stated that NPA does not inhibit the fluorAs transport, but this conclusion is not different than presented in Figure 3 and in the text: "NPA pre-treatment of the hypocotyls to block active auxin efflux led to an increase signal close to the agar block, …" and "These FluorA accumulations in the apical hook were abolished by NPA treatment". So, are FluorA actively transported? Direct transport assays in heterologous systems may help to answer these questions,

- The authors show that FluorAs do not bind to TIR1. Is it simply because they don't localize to the nucleus?

- There is no mention about the pH stability of the fluorescence of NBD. Can you comment on the possibilities of localization of the FluorA in the apoplast or/and the vacuole?

- In the discussion, it is stated that "the distribution is thus more similar to that of the native auxin than that of free 2,4-D." Native auxin, IAA, has been localized using anti-IAA antibodies. Do we have any clue how localizes 2,4-D in plants?

In conclusion, these FluorAs are inactive in auxin signaling (also no effects on *tir1* mutant phenotype) but are still able to inhibit root growth and other defects, to induce the expression of some auxin reporter, to rescue some root growth in *wei2 wei7* seedlings. How can the molecules have physiological effects if they are inactive in the auxin signalling pathway?

Supplements IV and V present the publications in which Barbora Pařizková participated in view of her expertise. In Supplemental IV, she tested the stability of the RN compounds and in supplemental V, she developed a methodology to quantify 2,4-D and its conjugates.

The presented work is a great quality. Finally, **I do recommend** the presented PhD thesis of Barbora Pařizková for the doctoral defense.

Yours sincerely,

Hélène Robert Boisivon, PhD

Hélène ROBERT BOISIVON, PhD
Research Group Leader
Mendel Centre for Genomics and Proteomics of Plant Systems
CEITEC – Central European Institute of Technology
Masaryk University
Kamenice 753/5, A26, office 2.17
625 00 Brno, Czech Republic
Phone: +420 549 49 8421
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