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Dear Martin:

I have read with interest and pleasure the Ph.D. thesis of Jan Humplik. Without a doubt, this is a job well done, and I can easily recommend that the thesis is ready for defense.

The thesis approaches the physiological regulation of growth in tomato seedlings, with a focus on the role of the plant growth regulator abscisic acid. There are at least two clear results from the work: one is that abscisic acid promotes, rather than inhibits growth of the tissue investigated, the hypocotyl (seedling stem). Another result shows that growth regulation in this instance is related to endoreduplication of nuclear DNA. Both results are significant, as are a number of other findings presented in the thesis.

The comprehensive introduction to the thesis is informative and very well written, displaying the clear focused thinking of Jan Humplik. The literature review is scientifically well balanced, integrating metabolic, growth, and molecular genetic mechanisms. The question of the work is put forward simply – what is the role of the presumed growth inhibitor abscisic acid in early development of a dicot seedling, tomato. The choice of tomato seedlings is wise, as stems of this organism are large enough to provide enough tissue for metabolic/chemical and molecular investigations. J. Humplik more than adequately connects what is known for tomato and other dicot seedlings with the knowledge base developed for the model system Arabidopsis.

Jan Humplik's thesis work has been published in two journal articles which are presented as Chapters 6 and 7. A third body of work is presented as Chapter 8. Results were obtained from a variety of experimental and quantification methods: growth analyses, hormone level quantification, measurement of gene expression, quantification of cell cycle, photobiological treatments, hormone-analog and metabolite analyses, quantification of photosynthetic parameters. Most impressive, both in the literature review and especially in the discussion of the results, is J. Humplik's sophisticated integration of information.

In my view, it is difficult for a physiologist or organismal biologist to address a question as complicated as growth regulation or hormone action with diverse and contradictory data sets. Jan Humplik shows that he has wide knowledge in the relevant literature of plant

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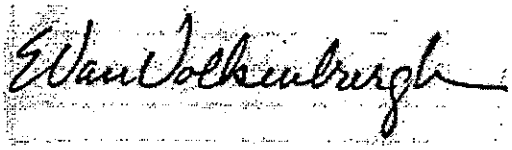
biology, and that he can cope with opposite findings in the literature. He does not pretend to offer solutions to the contradictions in others' findings, but he does push to find a coherent result in his own work. I commend him, and his mentors, for their contributions in this challenging arena.

In addition to the three chapters presenting his thesis research, appended to the thesis are two publications first-authored by J. Humplik. These are more recently published and present a different approach to analyzing plant growth behavior, by high-throughput phenotyping methods.

The thesis work itself is sufficiently broad, in terms of approach, and complete, in terms of findings, to be ready for defense. The additional publications reinforce that J. Humplik is a mature scientist, now able to complete and publish his research.

I look forward to hearing Jan Humplik defend his thesis, and discuss his results. I'm sure he will be happy to address questions about it, and consider different conclusions than he might have thought of or written in this work. I will return by air mail my marked up copy of the thesis in which I changed the usage of "the" from a Czech to an English form – this amused me, and is not a serious problem with the thesis as is. Minor other comments and edits are included.

Sincerely,



Přírodovědecká
fakulta

Posudek disertační práce Mgr. Jana F. Humplíka

Studijní program P1527 Botanika

A role of abscisic acid in tomato (*Solanum lycopersicum* L.) early seedling development.

The thesis focuses on early stage of seedling development, the switch from heterotrophic to autotrophic, photosynthetic development of tomato seedling. Specifically on etiolated growth (skotomorphogenesis) to study the role of ABA in hypocotyl elongation. To address this question wild type and two tomato mutant lines (*sitiens* and *notabilis*) defective in last steps of ABA biosynthesis were used as models.

The **literature review** provides an overview on abscisic acid discovery, biosynthesis, catabolism, signalling and related water status. It provides also information on role of ABA as plant growth regulator, being investigated via the use of various mutants. It challenges the most commonly viewed action of ABA as growth and physiological processes inhibitor. The role of ABA in seed maturation, dormancy, germination and fleshy fruit ripening are classical textbook examples. It is clear that plant phytohormones interaction network is still far from comprehensive understanding. Particularly negative relationship to gibberellic acid in seed dormancy and germination. Than author goes more into study relevant topic of ABA role in seedling development particularly effect in photomorphogenesis. This part is well covered on total of 26 pages. What I possibly miss is some brief description of experimental material in this part, e.g. what are the benefits of using tomato instead of other models (tobacco, *Arabidopsis* etc.) particularly if there are tomato mutants used in the study. Also beneficial and useful would be graphical presentation of later studied genes (PLOS One paper).

Material and Methods section is largely taken from published papers and is sufficiently descriptive to conduct experiment repetition if necessary. On the other hand in my view thesis could/should present more detailed description mentioning also critical steps not commonly used in papers. Separately should be section on use of statistical methods, software.

The results are presented entirely in form of published impacted papers were author is first author of the work and fulfil thus Ph.D. criteria. This part has thus undergone rigorous reviewing process by experts in the field. Therefore I will concentrate only on selected parts. To me is unclear if it is known which genes are mutated in respective tomato mutants? Are these orthologues genes to *Arabidopsis* AtCED and ABA3, respectively? Table 1 of PLOS One paper provides primers and accession numbers, however all expect of AJ441249 of SlKR1 gene do not lead to NCBI deposited sequences, but to presented paper. Full gene names would be very helpful. *When performing BLAST analysis, it is clear that CYP707A1-4 genes are very homologues and care has to be taken to amplify them specifically. How this was verified*?

The work presented in PLOS One paper demonstrated that ABA promotes DNA endoreduplication via enhancement of genes encoding inhibitors of cyclin-dependent kinases and reducing cytokinin levels. This work demonstrate usefulness of ABA biosynthesis tomato mutants, which enables to view stimulatory effect of ABA at nanomolar concentrations. In this work I appreciate the combination of analytical, physiological and anatomical approaches. This is of course achieved by various co-authors which contribution is stated and Jan Humplík is listed in both experiment and data analysis.

Question related to Figure 5 of PLOS One paper. *What is possible explanation of peak of SlCYP707A3 gene expression at 96h under blue light conditions*? *What about the expression of LeNCED1, SlCYP707A3 and remaining genes in mutants*? The expression of only inhibitors of kinases (SlKRP1 and 3) and SlLOG2 transcripts in *sit* mutant is shown (Figure S1, Table C).

Second paper published at Plant Signalling and Behaviour 10: 2015 is providing spatio-temporal analysis of ABA changes in tomato seedlings. ABA accumulates in elongation zone of hypocotyl and apparently participates in cell expansion. What I appreciate is that this comparably short paper is followed by added results and discussion. Promotive effect of ABA at 1 to 100 nM concentrations on relative growth rate of etiolated tomato hypocotyl was shown. Further qRT-PCR of selected genes, involved in cell cycle regulation, SlCycD cyclins and active cytokinin synthesis (SlLOG1, 2, 4 and 6) was analysed. Number of tables presenting detailed cytokinin measurements clearly demonstrated the effort. Particularly clear is presentation in form of "heat map" as shown in table 8. Section 9, page 103 provides

one page conclusions and perspectives. It challenges the common view of ABA as growth inhibitor. Here I have a question. *If the cross talk to cytokinins and cell division was demonstrated, are there any tomato mutants in these pathways known*? I would drive attention of authors to existing and available tomato TILLING mutant collection created and maintained at INRA, France (http://www-urgv.versailles.inra.fr/tilling/tomato.htm) or others like TOMATOMA (http://tomatoma.nbrp.jp/index.jsp). With completed tomato genome this provides great platform to test various hypothesis.

Eleven pages of references demonstrate author´s theoretical background in relevant published work. Finally, as appendixes are added two published papers, again as first authorship. These do not directly relate to thesis topic e.g. ABA and tomato seedling growth, but are focused on nowadays highly popular and important high throughput phenotyping. One is as review article of plant shoot imaging analysis in relation to stress responses (Plant Methods 11: 2015, impact factor 3.10). Case study of cold tolerance of pea is presented in last published paper (Plant Methods 11, 2015).

In summary, the thesis is well written and present comprehensive view on ABA role during early stages of tomato seedling development, nicely using suitable mutants. Is presented in form of commented published papers and clearly demonstrates author´s scientific capabilities.

Taken together I am recommending thesis for defence and suggest it the entitlement for granting Ph.D. degree.

In Olomouc 11 November 2015

Ing. Petr Smýkal Ph.D.

Department of Botany, Faculty of Sciences, Palacký University in Olomouc

REVIEW

Ph.D. thesis:
"A role of abscisic acid in tomato (*Solanum lycopersicum* L.) early seedling development"

Author: Jan F. Humplík

The Ph.D. thesis of Jan Humplík is focused on the elucidation of the function of abscisic acid (ABA) during skotomorphogenesis and de-etiolation. I highly appreciate that Jan Humplík devoted his study to the physiological effects of ABA in plant development, namely in seed imbibition, germination, etiolated and de-etiolated growth of tomato seedlings. He avoided the simplified generalization of ABA as the stress hormone, which exhibits only growth inhibition. Jan Humplík clearly showed that in low concentrations, which are physiological in non-stress, well-watered conditions, ABA is necessary for the elongation of hypocotyls of etiolated plants. He performed spatio-temporal analysis on ABA content and found that ABA promotes hypocotyl growth via the stimulation of CDK inhibitor family SlKRPs and down-regulation of the content of active cytokinins. He elucidated the mechanism of ABA mode of action, which is based on stimulation of endoreduplication and cell expansion. Upon de-etiolation, which can be stimulated by blue light, endogenous ABA content decreased, while cytokinins, which stimulate plastid differentiation and thus the transition from heterotrophy to autotrophy, were up-regulated. Simultaneously, cytokinins promoted cell division, stimulating cotyledon growth and greening. Thus, Jan Humplík significantly contributed to the elucidation of hormonal cross-talk during seedling development.

Jan Humplík is the first author of four impacted papers. Two of them are devoted to the parallel area of Jan Humplík's research – development and optimization of phenotyping platform. In his thesis he presented a lot of non-published data, which will definitely be used for at least two other papers.

The PhD. thesis consists of English and Czech summary, introduction, brief methodological part, published papers concerning the main topic of Ph.D. thesis, description of unpublished results, discussion, conclusions, extensive list of references and papers focused on phenotyping. The Ph.D. thesis has been written in good English and includes very informative schemes.

I have several questions or comments.

1) p. 17 – Isopentenyl diphosphate is mentioned as precursor of ABA and gibberellins. Do you know other hormones which have this compound in their biosynthetic route?

2) p. 20 – When ABA signalling pathway is described, "activation of SnRK2 kinases which in turn phosphorylate bZIP transcription factors..." is mentioned. This signalling occurs in nucleus. But de-repression of SnRK2 kinases takes place also in cytoplasm. Do you know their target? For examples of OST1?

3) p. 40 – "... and GA-induced transcription factor DELLA..." DELLA proteins are important part of GA signalling pathways, however, they are repressors. Could you briefly describe mechanism of GA – DELLA interaction?

4) p. 91 – When up-regulation of cytokinins during de-etiolation was studied, expression of *LOG* genes was determined. Did you measure also expression of some *isopentenyltransferase* or *cytokinin oxidase/dehydrogenase* genes?

Minor comments:

The list of abbreviations contains ABRE and iP twice.

The iPR is usually called isopentenyladenosine (not isopentenyladenine riboside).

Conclusion: I consider the presented Ph.D. thesis as very valuable. According to my opinion it fulfils all the necessary requirements. Thus, I recommend the thesis for the PhD. defence.

26. 11. 2015

RNDr. Radomíra Vaňková, CSc.