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Review of the doctoral dissertation of Deeksha Singh entitled "The Role of PHLOEM EARLY DOF transcription factors (PEARs) in *Plasmodiophora brassicae*-driven phloem reprogramming in *Arabidopsis thaliana* (L.) Heynh", prepared under the supervision of prof. dr hab. Robert Malinowski.

The goal of the study was to explore molecular, cellular, and developmental aspects of *Plasmodiophora brassicae* -induced formation of clubroot galls in *Arabidopsis thaliana* seedlings. Gall formation is associated with local cell over-proliferation and remodelling of vascular development, in particular, enhanced phloem formation and suppressed xylem development.

To achieve this goal, the study was structured around two main objectives, each corresponding to one published article. First, a protocol based on tissue clearing, vibratome sectioning, and fluorescence microscopy was developed and optimized for comprehensive analysis of cellular and genetic processes underlying pathogen-induced gall formation in *Arabidopsis*. Second, the study focused on the role of PHLOEM EARLY DOF (PEAR) transcription factors, central regulators of phloem differentiation, using a range of single and higher-order *pear* mutants in *Arabidopsis*. The findings allow for the conclusion that PEARs are not direct targets of the pathogen. Instead, PEAR upregulation appears to be an indirect downstream consequence of upstream developmental reprogramming triggered by the pathogen, including perturbations in auxin and cytokinin signalling, which together likely drive ectopic phloem proliferation. Chemical disturbance of auxin homeostasis (using inhibitors of auxin biosynthesis, transport, and signalling) led to changes in vascular patterning, but was unable to abolish DOF6 induction upon pathogen infection. This led to the conclusion that although the infection strongly correlates with upregulation of PEAR transcription factors, this represents an indirect effect of earlier upstream events controlling vascular cambium activity and phloem development.

The thesis itself is relatively concise, the main text spans 34 pages covering summary (in both Polish and English), introduction, main hypotheses and aims, materials and methods, results and discussion, conclusions, future perspectives, and references, with the two published first-author articles attached as the primary source of detailed methodology and results (Singh et al., *J. Vis. Exp.* 2022; Singh et al., *J. Exp. Bot.* 2025). The overall structure is logical and appropriate for a dissertation of this format, where the main text serves as a coherent synthesis and the attached publications provide the full experimental detail.

I have few comments and specific questions regarding this study.

1. Direct versus indirect effects of pathogen infection on xylem suppression.

The thesis reports a suppression of xylem formation in clubroot galls, which correlates with strong ectopic phloem proliferation. However, it remains unresolved whether the pathogen directly suppresses xylem development or whether xylem suppression is an indirect consequence of the ectopic phloem expansion resulting from imbalance between phloem and xylem fates. Thus, is xylem suppression a primary or secondary effect? Are there any experimental evidence that addresses it directly?

2. Reduced cytokinin signalling in galls. The thesis confirms, using both TCS::GFP and pARR5::GFP reporters, that cytokinin signalling responses are reduced in developing galls. This is an expected observation given that cytokinins are known as positive regulators of PEAR gene expression and phloem identity in undisturbed root development. The thesis offers an explanation that PEAR induction during gall formation may be independent of cytokinin, but does not include any experiment directly testing cytokinin effects on gall formation, for example by cytokinin treatment or the use of cytokinin biosynthesis or signalling mutants. Were such experiments performed or are planned to be performed in the future?

3. Signal quantification using ImageJ 3D surface plots. In the second paper (Singh et al. 2025), in the Fig. 3, the expression intensity of three PEAR promoter reporters (pHCA2::erRFP, pPEAR1::GFP, pDOF6::erVenus) is compared between mock and infected hypocotyl sections using ImageJ 3D interactive surface plots. Two methodological concerns arise here.

First, the sections are also stained with Calcofluor White (cell wall stain), and it is not clear whether the surface plots capture only the specific reporter signal or also pick up overlapping Calcofluor White signal, which could affect apparent signal intensity, for example in regions of dense or thick cell walls.

Second, the plots represent single ROIs per section. For conclusions about signal intensity and spatial distribution differences between mock and infected samples, particularly for cases where intensity differences are subtle (for example at Fig. 3C for DOF6, but also Fig. S3 for ARR5), quantification across several ROIs per section from several replicates, would strengthen the conclusions. The authors' conclusions about broader distribution of signal in infected plants are generally well-supported, but the claim of higher signal intensity in some cases (DOF6, ARR5 is less convincing from the presented plots alone.

4. Use of the classical DR5::GFP auxin reporter. The study uses the classical DR5::GFP reporter line (from Eva Benkova lab) to detect auxin responses in mock- and infected hypocotyls. This is a well-established tool and the results clearly show more broadly distributed DR5::GFP signals in the gall. For future studies, however, it would be worth considering the next-generation DR5v2 reporter with improved sensitivity to auxin (Brackmann et al 2018). This reporter could better express the differences in auxin signalling differences between mock and infected samples.

5. Possible involvement of other phloem regulators SMXL3/4/5. The finding that even higher-order *pear* mutants fail to suppress pathogen-induced phloem overproduction strongly suggests that PEAR transcription factors are not essential downstream effectors of the pathogen's action on phloem production, and that upstream or parallel mechanisms are responsible. The thesis proposes that changes in cell cycle progression and hormone imbalance underlie the observed phloem overproduction. However, is it possible that other phloem regulators might be involved, for example SMXL3/4/5 proteins which were shown as positive regulators of phloem differentiation in Arabidopsis roots (Wallner et al 2017, Curr Biol)?

Assessment of Theoretical Knowledge

The dissertation of Deeksha Singh demonstrates a solid and well-grounded theoretical foundation on plant development and plant-pathogen interactions appropriate for a doctoral study. The introduction chapters accurately and concisely cover the biology and life cycle of *the Plasmodiophora brassicae*, gall development, and the significance of clubroot disease in agriculture. In addition, it clearly identifies the key gaps in current understanding that motivated the study. The candidate shows deep and accurate knowledge of the genetic regulation of phloem identity, the PEAR transcription factor family, and the hormonal regulatory network involving auxin and cytokinin. The reference list is carefully selected and up to date, spanning most important

literature in the field. Overall, the theoretical knowledge presented in this dissertation is comprehensive and accurate.

Assessment of the Ability to Conduct Independent Research

The dissertation provides clear evidence of Deeksha Singh's ability to independently design and conduct scientific research at a high level. The study is structured around a logical and well-motivated experimental progression: from the development of an optimised protocol for visualization of gall anatomy and activity of genetic reporters, to series of experiments based on genetic and chemical disturbance of the system.

The choice of experimental tools is appropriate and well-justified, combining promoter-reporter lines, single and higher-order mutant analysis, fluorescence and light microscopy, GUS histochemistry, and chemical inhibition of auxin homeostasis to address the hypotheses.

The candidate demonstrates critical thinking in interpreting unexpected results, for example the failure of higher-order *pear* mutants to suppress pathogen-induced phloem overproduction. Conclusions are logically consistent and appropriately cautious proposing alternative explanations for her findings. The two first-authored publications in internationally recognised journals (*Journal of Visualized Experiments* and *Journal of Experimental Botany*), provide a strong argument to her independent research competence.

Assessment of Originality and Contribution to the Field

The dissertation makes a genuine and timely original contribution to two interconnected fields: plant vascular developmental biology and plant-pathogen interaction research. The optimised tissue-clearing protocol developed in the first publication represents a methodological advance of broad utility. The second and central contribution, the functional testing the role of PEAR transcription factors during gall formation, is novel and scientifically significant. The finding that *pear* mutants fail to suppress pathogen-driven phloem overproduction was unexpected and represents a meaningful advance in understanding of molecular mechanisms of pathogen action. This shifts the conceptual framework and opens new avenues for identifying the true direct targets of the pathogen manipulation strategy.

Beyond its fundamental significance, the work carries clear applied relevance: clubroot disease is a serious and growing threat to Brassicaceae crops, including oilseed rape, one of the world's most economically important crops. A mechanistic understanding of how *Plasmodiophora brassicae* affects host vascular development is an essential for the design of resistance strategies. Altogether, this dissertation advances fundamental knowledge, delivers a practically useful methodological tool, and generates well-grounded hypotheses that will guide future research in both plant developmental biology and crop protection.

I hereby state that the submitted doctoral dissertation meets the requirements specified in Article 187 of the Act of 20 July 2018 – Law on Higher Education and Science (consolidated text: Journal of Laws of 2024, item 1571, as amended). In view of the above, I hereby request that Deeksha Singh be admitted to the subsequent stages of the proceedings for the award of the doctoral degree.

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