The thesis submitted by Mr. Daniel Nedvěd for the fulfilment of PhD degree entitled "**Modulation of cytokinin distribution between roots and shoots**" addresses molecular mechanisms that contribute to the long-distance distribution of the plant hormone cytokinin. This is a very important and timely topic, knowledge of which is essential to our understanding of how cytokinins coordinate physiological and developmental processes across the various tissues and organs of multicellular plant organisms. These insights may lead to future advances in agricultural practices, facilitating improved plant growth and development, as well as offering potential strategies for enhanced crop stress resilience.

The dissertation presented is a manuscript-based compilation report formally consisting of **three results chapters** with a **clear thematic connection** between them. Daniel Nedvěd is either the first or joint-first author on these three publications/manuscripts.

The dissertation opens with a nice introductory chapter covering topics related to cytokinins; ranging from cytokinin chemistry, metabolism, signaling to membrane and long-range transport. The author provides a thorough introduction to the topic, and the literature presented is current and comprehensive.

The first chapter of the results section has been published as a review article in *International Journal of Molecular Sciences* (Nedvěd et al, 2021, Int. J. Mol. Sci. 22:3428), which is a peer-reviewed journal with IF~6.0 (Q1 category in the Journal Citation Reports ranking list). This review delves in more details into the current research in the field of membrane transport of cytokinins. It provides an excellent overview of recent developments, covering in detail different types of transmembrane protein transporters involved in the cytokinin transport, the molecular mechanisms of their activity, as well as inter- and intracellular distribution of the hormone. I very much appreciate the fact that the scope of this review article goes beyond a descriptive overview and offers many valuable theoretical predictions and concepts that are helpful for the further development in the field. For example, the article predicts and discusses how the physicochemical properties of different cytokinin molecules may affect their molecular transport. The issue of molecular hydrophobicity, for example, has been neglected in the past. The article also provides an excellent theoretical basis for cytokinin transport by discussing the basic kinetic and thermodynamic aspects of transmembrane cytokinin transport.

The second chapter of the results section is a manuscript of a research article titled "*Comprehensive Model of Cell-to-Cell Cytokinin Transport Reveals a Specific Mode of Cytokinin Riboside Influx*" and published as a preprint in bioRxiv (https://doi.org/10.1101/2024.06.04.597342). This manuscript addresses the role of the ribosylated forms of cytokinin and explores the molecular mechanism of their membrane transport. The manuscript first presents a characterization of the kinetics of uptake of different cytokinin molecules in tobacco BY-2 cells, which is a popular and very useful plant model system. The work reveals significant differences in the kinetics of cytokinin nucleobase and riboside transport and in the affinity of membrane-bound transporters for these two cytokinin forms. Interestingly, it is reported that ribosides can inhibit the uptake of cytokinin nucleobases in BY-2 cells, but not vice versa, suggesting that a membrane transport system specific for

cytokinin ribosides may exist. Given the very specific experimental context of this work (the use of BY-2 cells), it will be important to discuss the extent to which these findings can be extrapolated to different cell types of intact plant tissues.

The second part of the manuscript addresses the molecular and *in-planta* functions of Arabidopsis AtENT3, a member of the EQUILIBRATIVE NUCLEOSIDE TRANSPORTER family of nucleoside carriers, which are widespread in all domains of life but relatively poorly characterized in the model plant A. thaliana. First the transport activity of AtENT3 expressed in BY-2 cells is shown, demonstrating its affinity towards cytokinin nucleobases and ribosides, with a slightly higher preference for tZR compared to iPR. To assess the molecular interactions underlying the cytokinin binding to AtENT3, the authors performed molecular docking of tZR into a predicted structure of the protein. This elegant approach revealed amino acid residues likely to be responsible for the specific interaction with the cytokinin side chain. This conclusion was further supported by the notion that these residues are conserved in all AtENTs but not in the ENTs from non-plant species. This hypothesis remains to be confirmed experimentally in the future. The last part of the manuscript aims to explore the role of AtENT3 in shoot development by analyzing the corresponding gene mutation in the Arabidopsis.

Interestingly, it is shown that the *atent3* develops larger shoots, but unfortunately it is not clearly shown whether the mutation is causal for this developmental phenotype. The authors attempt to explain the observed phenotypic alterations by linking them to changes in the activity of genes involved in cytokinin homeostasis and meristem activity, however, the data provided are largely corelative and the conclusions drawn are hypothetical. For example, it remains unclear how reduced cytokinin import into the shoot could lead to reduced *WUSCHEL* activity and increased shoot growth. Therefore, although the role of root-borne tZR in maintaining shoot development has been clearly documented to date, the function of ENTs in this process will require further experimental validation. The work generated several good testable hypotheses.

The third chapter of the results section is a manuscript of a research article titled "*Cytokinin Dehydrogenase in Xylem Sap Reveals a Direct Link Between Cytokinin Metabolism and Long-Distance Transport*" and published as a preprint in bioRxiv (https://doi.org/10.1101/2023.11.06.565614). This part addresses the role of the cytokinin metabolic degradation in the xylem to regulate the root-to-shoot cytokinin distribution in plants. The manuscript begins with an impressively comprehensive large-scale bioinformatic analysis to address diversity of cytokinin oxidase/dehydrogenase (CKX) substrate specificities. Monocot CKX proteins were phylogenetically clustered and a specific sequence motif that can interact with cytokinin ligands was identified. The analysis suggests that that each CKX class comprises enzymes with similar substrate specificities. Further bioinformatic analyses presented in the manuscript suggest that the monocot CKX clustering partially correlates with sequence-based predictions of their subcellular localization. This is a very interesting conclusion, although it should be noted that sequence-based predictions of subcellular localization – especially in the different subcompartments of the endomembrane system – have limited resolution, and experimental validation will be inevitable.

In the following section, the authors provide direct experimental evidence that CKX proteins are secreted and active in the xylem sap of several different monocots. This is a very relevant, and to my knowledge, the first evidence of cytokinin degradation in the xylem. Focusing further

on the oat (*Avena sativa*) plant, the thesis shows that iP is the preferred substrate of the the CKX from the oat xylem sap, however similar substrate affinities were also detected in others tissues. It's a bit of a pity that the work didn't consider analyzing the substrate preferences of CKX in the xylem more broadly. Of particular interest would be the activity towards cytokinin ribosides, which would allow the authors to validate their hypothesis that the xylem-located CKXs preferentially cleave cytokinin nucleobases.

The authors of the manuscript further question which particular CKX isoform encoded in oat (which contains 36 putative *CKX* genes) contribute to the detected CKX activity in the xylem sap. Homology-based sequence comparison with maize apoplastic ZmCKX1 and further prediction based on gene expression analyses led to the prediction of three AsCKX1 isoforms as putative xylem-acting proteins. Unfortunately, the work does not provide a direct experimental confirmation of this theoretical assumption. However, it is clear, that such an experimental analysis (probably involving mass spectrometry of xylem proteins) is technically demanding and beyond the scope of the present thesis.

The last section of the manuscript addresses the very interesting question of the physiological relevance of the xylem sap CKX in adaptively regulating root-to-shoot cytokinin flux. Given that cytokinin activity in plants is closely related to nitrogen availability, changes in xylem CKX activity in response to differential nitrate availability were investigated. Interestingly, the data presented show that CKX activity in xylem sap correlates with cytokinin concentrations in roots and xylem and that both CKX activity and CK concentrations respond to changes in nitrate supply. This leads to a very exciting conclusion that CKX proteins may be a direct molecular component of the regulatory network that maintains cytokinin homeostasis by integrating environmental inputs and translating them into appropriate adaptive responses.

In Summary, Mr. Daniel Nedvěd submitted a comprehensive and carefully written documentation of his experimental work, including a very detailed theoretical introduction and well-defined experimental questions and goals. Experimental results are presented in a clear and logical fashion, and they are discussed critically and in depth. I believe that the results presented in the two unpublished manuscripts will be published in respected international research journals and will be of great interest to the scientific community.

Conclusions

The dissertation presents original research results and I have no serious criticism of the methods, evaluation of results or other technical aspects of the dissertation. The dissertation is written in very good English, very carefully and rigorously. Overall, the present thesis meets the standard requirements for dissertations in the field, and **I recommend the dissertation for defense**. I would like to congratulate both the candidate and the supervisor on a job well done.

Prof. Dr. Tomas Werner, University of Graz, 13.09.2024