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Review of the Ph.D. Thesis submitted in the Doctoral study program in Experimental Plant Biology at the Faculty of Natural Sciences of Charles University

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Thesis title: Modulation of Cytokinin Distribution Between Roots and Shoots.

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The PhD thesis of Daniel Nedved tackles several important aspects of cytokinin (CK) transport and homeostasis at the whole plant, cell and subcellular levels. The thesis represents a cumulative report of three papers. One of them is published and this is a review. Two other research manuscripts, are under revision and in all three Daniel Nedved is a first author. His contribution in each project is clearly defined. Taking into account the volume and complexity of the research it is frank to say that proposed report is well balanced. The introduction, despite being spacious, helps the reader better orientate in the main body/output of Daniel's achievement with the respect of the current state of art. I appreciate a concise description of the PhD aim additionally supplemented with a very useful scheme. The latter describing the biological importance of addressed questions. Discussion, as a summary of a complex research, is well written and this is the part which I would like to develop/talk over further during upcoming personal communication.

The review paper (J.Mol.Sci.2021,22,3428. https:// doi.org/10.3390/ijms22073428) covers an important (and unfortunately still underestimated) issue of the CK chemical properties impacting, not only transport, but more generally CK signaling and biological readout. It highlights different properties of naturally occurring free CK bases, which can be accounted to the alterations in their side chains composition, and importantly puts this into perspective of protonation status in various pH, enzymes, CK receptors and membrane-bound transporters. This is important and up to date perspective. Study of molecule/ligand interaction with respective proteins and proper matching of the interacting partners is a forefront of membrane transport research extending far beyond CKs. Recent work regarding, for instance ABC proteins has shown how meaningful understanding of molecular determinants behind ligand-protein interaction could be for e.g. transporter selectivity (Pakuła et al., 2023, Xia et al., 2024). The provided holistic view of thermodynamics, compartmentation and spatiotemporal CKs distribution underlines also a tight bound between transport and metabolism. This is, in my opinion, the key component not only of this paper but crucial perspective for further research aiming identification of new transporters, placing receptors in the signaling cascade and understanding source-sink power in a CK message readout.

The second publication (bioRxiv https://doi.org/10.1101/2024.06.04.597342) focuses on cell to cell CK transport and addresses the specific kinetics of the cellular uptake of CK ribosides and its impact on shoot development. Initially Authors by using tobacco BY2 cells found that BY2 can effectively discriminate between CK nucleobases and ribosides. This part is a nice combination of a simple biological setup (BY2) and biochemistry of transport (competition assay, inhibitors) both leading to affinities estimation of transporters present on the BY2 plasma membrane towards different CKs in terms of the IC_{50} values. The concept and the setup go along very well. BY2 cells represent easy to handle standard in such „transport oriented" research. Next authors conclude that CKs are translocated by at least two independent systems of membrane-bound carriers, of which one transports both CK nucleobases and ribosides and the other strictly CK ribosides. This

is an important finding not only because of potential carriers but also possible regulatory perspective. Finally, to look for an individual carrier, they focused on ribosides and took advantage of existing knowledge about ENT family, as some of this family members have been linked to the transport of such CKs. This is justified and allowed for intelligent guess/shortcut towards Arabidopisis and AtENT3. The choice of AtENT3 is rational with a hope for riboside transporter. However its heterologous expression in BY2 and transport implies that AtENT3 translocates both nucleobases and ribosides. It also does not discriminate based on the character of the CK N^6-bound side (tZ and iP) and mediates facilitated diffusion rather than active transport. From targeted riboside transporter research it may be disappointment but not in my opinion, it beautifully illustrates complexity of transport and CKs flow. Intriguingly, it rises also a possible question about impact of heterologous expression system choice/usage for transport assays as a potential challenge and obstacle. Again in this paper I would like to underline the attention to molecular determinants representing, not only particularities of plant ENTs but being of importance for ligand stability (Tyr61 and Asp129 as well as Gln62). For me this is not only an observation, I classify this as a new perspective. In the last part of work, authors address biological relevance of AtENT3 activity and provide a solid support for hypothesis that AtENT3 could be involved in the uptake of root-borne tZR in the shoot apex that is necessary for its LOG-catalysed activation. Useful is an observation that *WUS* (Wuschel transcription factor) expression is down-regulated in *atent3* shoots, which can be partially reversed by treating the mutant plants with tZR and that overexpression of *WUS* results in downregulation of *AtENT3* together with *AtLOG*s, as well as conversely, upregulation of *AtCKX7*, indicating negative feedback aimed to hinder the incoming CK signal.

The monocot CKXs (Cytokinin Dehydrogenase enzymes) are subject of the third paper (bioRxiv https://doi.org/10.1101/2023.11.06.565614). The story starts with bioinformatic overview (492 monocot CKXs) of sequences particularities leading to clustering and defining a specific composition of the variable triplet in the semi-conserved FLXRVXXXE motif. Yet again from our perspective I must say that motive search can be helpful in defining activity and/or protein - ligand relationship (Pengchao Hao et al., 2020, Banasiak and Jasinski 2021) which is the case here. I like the specialization context (different preferences N6/N9 substitutions) and not a simple redundancy. Authors correlate sequence based predictions with CKXs subcellular localization. The latter is a game changer especially for the so called class VI CKXs. Their extracellular localization, root origin and substrate specialization represent the major highlights here. Importantly increase in the CK root-to-shoot flux in response to the nitrate supply is followed by the increase of CKX activity in the xylem providing CK degradation in relation to the nitrogen status. All together could be a tongue of attention for a definition/importance of solely „transport impact" for root-shoot communication.

There are several general thoughts that came to my mind while reading the thesis that may be potentially developed during discussion:

1) transporters redundancy vs specialization/cooperation (between different types of transporters and transporters from the same family) as a regulatory context of signal transduction. For instance the AtENT3 story describes potentially different impact of this non selective facilitator in various places (organs) and the *atent3/WUS* phenotype complementation by tZR is well discussed (CPN/LOG). However can be hypothesize that complementation of WUS in *atent3* mutant by tZR possibly indicates also presence/involvement of other importers in such scenario (functional redundancy argument)? On the other hand in the view of recent findings there is a cell/tissue/scenario specific specialization of membrane transporters with a consequences in the CK message readout and the developmental processes (Tessi et al., 2020, Jarzyniak et al., 2021).

2) intriguing is an issue of being facilitator and/or dedicated transporter together with the impact of general physical properties e.g. pH for translocation/transport mechanism/possible activation but also precise post-translational modifications (e.g. phosphorylation) of well-defined carriers for CKs readout. Related to this is still an open question how we define readout? When transport is of physiological importance locally and when simply results from physicochemical conditions and is an element of more general flux having anyway a systemic impact. Can we separate these two scenarios?

3) how localization in different cellular membranes of both carriers and receptors, their turnover, and local pH variation impacts signal transduction but also buffers CK pools, balancing the levels of biologically active CKs in particular compartments. What we are missing in this big picture?

4) finally if and how the acquired knowledge about molecular determinants behind plant transporters particularities (e.g. selectivity) can be adopted for the new concepts. This concern not only extrapolation to non-plant transporters but designing of new class of modified molecules with dedicated properties.

Overall the concept, proposed experimentation, logic of presentation as well as discussion presented in the dissertation are consistent and bring a new quality to the understanding of the signaling cascade involving cytokinins with a special attention to transport. In my opinion, the reviewed thesis fulfills all requirements posed on theses aimed for obtaining PhD degree. Provided material fulfills all requirements for a PhD thesis according the rules of the Charles University in Prague. This thesis is ready to be defended in front of respective committee.

Poznań 10.08.2024

Michał Jasiński