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Ph.D. thesis of Mgr. Vedrana Marković entitled „Functional specialization of EXO70A and EXO70B paralogs of the EXO70 exocyst subunit in Arabidopsis" was elaborated within the Ph.D. study programme Experimental Plant Biology at Faculty of Science, Charles University in Prague, under supervision of renowned experts from Department of Experimental Plant Biology, Faculty of Science, Charles University and Laboratory of Cell Biology, Institute of Experimental Botany, The Czech Academy of Sciences. Ph.D. thesis encompassing 131 pages is arranged as a consistent file of 4 original scientific publications, which is nicely introduced by up-to-date summary of recent knowledge on 11 pages, and concluded by a comprehensive general discussion on 8 pages, finally wrapped up by a summary particular points.

The Introduction part, based on its content, topical validity, and particularly by the form of its presentation, performs very well as the outlining and justifying the aims of the work. It clarifies why it is so important to study the vesicle-tethering exocyst complex and its isoforms in cellular processes depending both on intensive polarized secretion and non-canonical and exocytosis non-related functions, using modern and complementary scientific methods.

It is known that the exocyst is an evolutionary conserved tethering protein complex mediating the initial interaction between transport vesicles and their target membranes within the last stages of exocytosis. Thus, the exocyst complex determines and mediates such cellular processes depending on polarized secretion, as cell polarity, morphogenesis, defence and autophagy. This is the first crucial pint of functional diversification of the whole exocyst complex that one should take into account in the effort to understand it. Secondly, however, it has been found that some exocyst subunits such as EXO70s can function also independently of the rest of the complex, and even in processes unrelated to regulated exocytosis. As a result, the exocyst whole complex or particular subunits might be functionally (and distinctly) specialized in different plant tissues, in different cell types, but also within the same cell. Of course, I take into account that each publication attached provide comprehensive and up-to-date introductory part, describing the motivation of the particular work. However, from the points of view mentioned above, the "generalized" Introduction part included in the thesis significantly contributes to a good understanding of the overall complexity of this topic, to selection of quite diversified aims of the thesis, but also to attached publications and interpretation of their results. Therefore, the introductory part of the work is very valuable, especially for readers who do not directly work in this area.

In the two publications, the Ph.D. student is the first author, in the other two publications she is the co-author. The contribution of the student in each publication is specified.

Publication No. 1, for which the doctoral student is the first author, is devoted to characterization of EXO70A2, the evolutionarily closest paralog to the main sporophytic isoform EXO70A1 of the EXO70.1 clade. Based on this similarity, and due to the fact that *EXO70A2* paralog is highly expressed in the male gametophyte, the EXO70A2 was selected as the best candidate out of the EXO70 isoforms that might function as a part of the exocyst complex in the regulation of polarized exocytosis in pollen, playing a role analogous to EXO70A1 in the sporophyte. Using sophisticated and complementary methodological approaches, this prediction was confirmed. Characterization of CRISPR-generated loss-of-function *exo70a2* mutant revealed that EXO70A2 is essential for efficient pollen maturation, pollen grain germination, and pollen tube growth. Tagged form (GFP-EXO70A2) was localized to the apical domain in growing pollen tube tips characterized by intensive exocytosis. Analysis of the transmission efficiency of the mutant allele through pollen in the progeny of heterozygous *exo70a2* mutant plants revealed a severe transmission defect, which was male specific. One of

the most interesting evidences was the fact that characterized pollen-specific EXO70A2 was the only EXO70 isoform able to substitute for the function of EXO70A1 in the sporophyte. Because this was not possible vice-versa, it indicates that these two closely related isoforms share only partial functional redundancy and pollen-related functions are highly specific.

Publication No. 2 describes how the plant plasma membrane identity supports the recruitment of the plant exocyst complex and promotes its plasma membrane interaction. It was known that the exocyst is targeted to the plasma membrane via EXO70A1 subunit and EXO70A1 binds the plasma membrane via interactions with specific phospholipids. However, this work shows that anionic phospholipids such as phosphatidic acid, phosphatidylserine and phosphatidylinositol 4-phosphate might be in plants more important than PIP2 (which is typical in animals or yeast) for defining plasma membrane - exocyst interaction. Thus, a unique plasma membrane-lipid signature in plants has been identified. Authors found that the C-terminal part of EXO70A1 is critical for this process. Using very elegant experimental approach, authors show localization of exocyst subunits SEC3a, SEC6, SEC8, SEC10a, SEC15b, and EXO84b in root epidermal cells lacking EXO70A1. It was revealed that all these tested subunits lost their plasma membrane localization in the *exo70a1* mutant.

Publication No. 3, for which the doctoral student is the first author, presents a thorough characterization of *exo70a1* and *exo70b1* mutants through their systematic cross-complementation analysis with the aim to reveal a functional specialization within the *EXO70* gene family in Arabidopsis. The results showed and confirmed that EXO70A1 was functionally substituted only by its closest paralog, EXO70A2. Conversely, EXO70B1 could not be substituted by none of the EXO70 isoforms tested, including EXO70B2, which is its closest relative paralog. Localization analysis of EXO70A2 showed that it localized identically to EXO70A1 in root epidermal cells of both *exo70a1* mutant and WT plants, in the cytoplasm and typically enriched in the plasma membrane at the outer lateral domain of cells. However, all other isoforms relocalized to distinct intracellular aggregates (with almost completely lost plasma membrane signal) in the *exo70a1* mutant.

Publication No. 4 provides an overview of recently known functions of exocyst related to unconventional secretion in plants connected to autophagy and defence. It is stated and summarized here that several exocyst subunits forming particular exocyst complex versions in Arabidopsis such as EXO70B1, EXO70B2, EXO70E2, SEC5, EXO84B and SEC6 might be involved in unconventional secretion involved in autophagy-related transport to the vacuole.

Results of the submitted dissertation of Mgr. Vedrana Marković are very impressive, which is also documented by the provided publishing activity. Not only from the attached publications, but also from the accompanying text of the dissertation, it is clear that the doctoral student fulfilled successfully the set goals. It can be stated without any doubt that the fulfillment of Ph.D. Thesis aims has been fully achieved. From the formal point of view, the thesis meets all the prerequisites for this type of work. The text parts are precisely written with a minimum of errors or typos. Only three points I would like to pinpoint are:

- citation as: „Drs M., unpublished data“ at the page 10 is unclear
- In publication No. 1, the analysis of unicellular, bicellular and tricellular pollen is convincing due to known developmental stages represented by „number of cells“. However, apart of „unicellular“ stage, the evidences for „cellularity“ are not evident from the Figure 6. More convincing would be referring to „uninuclear, binuclear and trinuclear“ stage. The approach for analysis of nuclear composition is correctly mentioned in Materials and Methods. In addition, the purpose of arrow in Figure 6A is not explained.
- In publication No. 3 (published version), Materials and Methods, part 4.5. Microscopy, there is information: „For visualization of cellular membranes, seedlings were labelled by FM4-64 (Invitrogen, http://www.invitrogen.com, accessed on 25 April 2021)“ the meaning is not clear

In the context of the attached publications and presented results, I would like to know the opinion of the doctoral student on the following questions:

1) Specific polarized localization of EXO70A1 at the plasma membrane in root epidermal cells was analysed by semiquantitative fluorescence ratio measurement between the outer lateral plasma membrane and a cortical cytoplasm beneath. In wild type the ratio is around 1.5 to 2, but in *exo70a1* and *exo84b* mutant cells with lost plasma membrane signal it looks unlikely that the ratio could give the numbers reaching 1. The information that measurements were done in a medial optical section of epidermal cells is provided only in the legend of the Figure 7. Which part of the images was taken as a background (control) level? Were these data somehow normalized?

2) In respect of *EXO70A2* expression, which is pollen-specific, it is stated a prediction in the Thesis that EXO70A2 might play some role in the sporophyte despite its minimal expression in sporophytic tissues. Is it known what is the level of *EXO70A2* gene expression (and protein abundance) during microsporogenesis and microgametogenesis? Because EXO70A1 is dominant isoform in sporophyte and EXO70A2 is dominant isoform during pollen development, what is the ratio of their expression at the transition stage, e.g. in microsporocytes (pollen mother cells)?

3) In the Thesis there are thoroughly described and characterized all phenotypes of some *EXO70* mutants such as *exo70a1* knock-out mutant. The phenotype of mutants under study has been rescued by stable expression of related EXO70 isoform under control of the own (*EXO70A1*) promoter and tagged with GFP. The ability to rescue the *exo70a1* mutant phenotype and the restoration of *exo70a1* mutant fertility was considered as a proof that the GFP-tagged version of the fusion protein is active. Is it however, known in each series of constructs for stable expression of *EXO70s* insertions employed if they were present in homozygous or heterozygous state in F2 generation? In light of all described phenotypical abnormalities of *exo70a1* knock-out mutant, what effects are achieved by *EXO70A1* overexpression in WT background?

4) Growth rate of pollen tubes in *exo70a2* mutant is lower and they have much wider diameter than wild-type pollen tubes. It suggests that secretion is delocalized in the apex of *exo70a2* pollen tubes and it might be due to altered cell wall biogenesis. Because pollen tubes of the mutant are shorter, maybe also the number of callose plugs was adequately lower. However in the case of normalization (to the units of pollen tube length) would show lower number of callose plugs, could be EXO70A2 involved in localized delivery of ß 1-3 glucan synthase for localized callose plug formation, similarly as EXO70H4 regulating polarized callose deposition during secondary thickening of the cell wall in Arabidopsis trichomes?

5) In pollen tubes of *exo70a2* mutant, but also in other mutants of several core exocyst subunits (*sec5a/b*, *sec6*, *sec8*, and *sec15a*), extremely short and wide pollen tubes are formed. It is most likely caused by an inefficient targeting of secretory vesicles carrying the cargo to the growing tip, but due to missing exocyst subunit(s) unable to tether. This might lead to over-accumulation of secretory vesicles at the tip of growing pollen tubes that are not able to fuse. Was something like that observed in your mutants?

In conclusion, I like to state that the dissertation of Mgr. Vedrana Marković is of high quality and meets all criteria according to the relevant law. I recommend the thesis for defense and after a successful defense I propose to award the title of Ph.D.

Olomouc, 23. 07. 2021

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