ERC Starting Grant 2014
Research proposal [Part B2)]
(not evaluated in Step 1)

Part B2: *The scientific proposal* (max. 15 pages)

Section a: State-of-the-art and objectives

Plant hormone auxin plays an important morphoregulatory role during the development of sessile plant body[1]. The setting of auxin gradients in the plant tissues depends largely on the activity and localization of its influx and efflux plasma membrane transporters from AUX1/LIKE AUX1 (AUX1/LAX), PIN and ABCB protein families[2]. To the various, developmentally regulated extents, all of these carriers are recycled between plasma membrane and endomembrane compartments by endosomal recycling pathways[3,4]. The process of recycling that includes both exocytosis and endocytosis[5] determines the ability of particular carrier to be redistributed to another plasma membrane domain without *de novo* protein synthesis. Although the molecular machinery that regulate plant vesicle trafficking processes and the role of auxin in this process is intensively studied[6], the trafficking of individual auxin carriers is described only fragmentary. The best characterized is the trafficking of PIN auxin efflux carriers that includes small GTPases, clathrin-dependent endocytosis[6], exocyst-dependent exocytosis[7] and depends on the sterol composition of the plasma membrane[8,9]. Phosphorylations and de-phosphorylations of PINs regulate their recruitment into the various membrane domains[10,11]. The trafficking of AUX1/LAX and ABCB carriers also involve the activity of small GTPases and is dependent on the sterol composition of the plasma membrane, but details are mostly missing[2].

The movement of membrane vesicles and endosomes within the cell would not be possible without the assistance of actin filaments (AFs) and microtubules (MTs). In animal cells, dynamic AFs assist through the activity of motor proteins (myosins) in the trafficking of clathrin-coated vesicles from the plasma membrane, during endosomal trafficking as well as during the exocytosis[12,13,14]. Similarly, animal MTs provide mobility to endomembranes by motor proteins like dynein or kinesins. The role of highly dynamic plant AFs[15] in the endosomal trafficking is well-established through the activity of myosins[16] and some evidence suggests that AFs may mediate clathrin-dependent endocytosis[2]. The dynamics of both AFs and MTs is differentially regulated by Rop (Rho of Plants) GTPases, which act a common upstream factors signalling to both cytoskeletons[17].

It is obvious that thanks to their intensive research, auxin carriers might serve as the membrane cargo on which the mechanisms of intracellular trafficking of membrane proteins could be studied. Although clathrin has been established as the important element of the recycling machinery for some auxin carriers (PINs), it is not known how the clathrin-coated vesicle might be attached to acto-myosin and which myosins might influence the localization of auxin carriers in the plasma membrane. Similarly, the role of dynamins that perform membrane scission activity during the formation of vesicle is unclear. We have already shown by FRET that dynamin DRP1A[18] interacts with PIN2 on the growing ends of the cell plate, but there could be also activity of this protein on the plasma membrane[19,20]. Our preliminary microscopical data further show that even single mutation in the individual alpha- and beta-tubulin genes triggered changes in the membrane distribution of PIN1 and PIN2 in Arabidopsis roots **(Figure 1)**. The connection of PIN dynamics to MTs was recently shown to be assited by MTs plus end binding protein CLASP[21]. Besides that, PIN proteins are maintained in the plasma membrane in the association with cell wall[22, 23].

Altogether, one of the major information that is lacking these days is the information on the localization of individual auxin carriers on the sub-cellular and ultra-structural levels. Within the proposed project I suggest to concentrate our effort on the testing of "ultra-structural phenotypes" i.e. the localization of individual carriers in the set of mutants in cytoskeletal proteins. We will primarily employ methods of high resolution fluorescence and electron microscopy. By such a sub-cellular phenotyping, i.e. by the localization of individual auxin carriers in the set of *Arabidopsis thaliana* lines carrying mutations in genes coding for cytoskeletal and associated proteins and enzymes for cell wall biogenesis and sterol biosynthesis, it will be possible to fill the gap in our knowledge on the structural connection between auxin carriers and machinery that secures their dynamics.

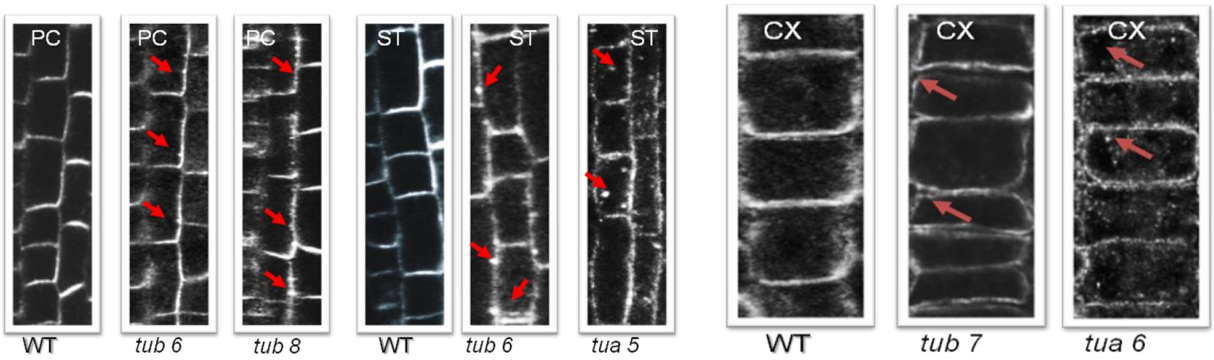

Figure 1: CLSM of PIN proteins in the tubulin mutants, preliminary data. Indirect immunofluorescence visualization of PIN1 **(A)** in pericycle (PC) and stele parenchyma (ST) and PIN2 **(B)** in the root cortex cells of 3-day-old *Arabidopsis thaliana* seedling. Homozygous mutations for individual alpha- (tua5, tua6) and beta-tubulin genes tub6, tub7, tub8) clearly triggered changes in the distribution of PIN1 and PIN2 (red arrows) in contrast to wild type (WT), although the overall morphology of the root was not altered. Jelínková et al. 2013 (unpublished).

One of the major hypotheses that could be experimentally challenged is that individual types of auxin carriers differ in their association with cytoskeleton and that this determines their behaviour. As the extra value that will come out of this project will be the creation of web-based database of ultra-structural images, determination of dynamics and function of auxin carriers with respect to structures of cytoskeleton, plasma membrane and cell wall. This database could be further extended in future and could serve as an invaluable source of information for the community of experimental plant biologists.

There are three main objectives of this project:

Objective 1: Searching for cell structure determinants of the localization and dynamics of auxin carriers

Objective 2: Identification of new determinants of auxin carrier dynamics

Objective 3: Generation of web-based catalogue of localizations, dynamics, function and interactions of auxin carriers

Section b: Methodology

Objective 1: Searching for cell structure determinants of the localization and dynamics of auxin carriers

Two work packages (WP) are associated with this part of the project to fulfil the objective 1. In the WP1/1 we will generate a molecular toolbox for indirect immunofluorescence stainings and *in vivo* localizations of auxin carriers and screen by several high-end microscopical approaches for the localizations of auxin carriers in the collection of insertional mutant lines of *Arabidopsis thaliana*. This collection is already available in the laboratory of PI and includes homozygous mutations in all individual alpha- and beta- tubulin genes, in all actin genes and in the set of genes coding for cytoskeleton-associated proteins. For MTs, the collection includes insertions in genes for *Arabidopsis thaliana* tubulin genes, microtubule-bundling proteins, plus-end binding proteins, microtubule stabilizing and severing proteins, nucleators and some other regulators of polymerization[24,25,26]. For AFs, we have collected mutations in genes for individual actins, actin nucleation factors and actin binding proteins[27,28,29,30]. The ultimate goal is to assemble the collection of mutants that would be complete in terms of up to date knowledge. Of course, there could be some obstacles in obtaining knock-out mutations. At the moment we have around 40 individual lines ready and plan to genotype and verify with qRT-PCR other 50 lines, including cell wall biogenesis mutants and sterol biosynthesis deposition mutants.

WP1/1: Screening for structural determinants of the localization and dynamics of auxin carriers

Generation of molecular toolbox for indirect immunofluorescence and *in vivo* microscopy

To search and screen for the localization of auxin carriers, specific antibodies for indirect immunofluorescence stainings or translation fusions of genes coding for auxin carriers with fluorescent proteins are needed. As the first step, we will start to generate primary antibodies against auxin carriers. At the moment, we already have functional, mostly commercial (Agrisera, NASC) polyclonal antibodies against Arabidopsis PIN1, 2, 3, 4. They are used regularly in the laboratory for the immunostainings in Arabidopsis seedlings using automated immunostaining station (Intavis Pro VSi). The protocol allows simultaneous

labelling of two auxin carriers using green and red Alexa-labelled secondary antibodies (Invitrogen). The antibodies against AUX1/LAXes and ABCBs will be produced commercially or we will kindly ask for these, which are already available in other laboratories (not all are available at the moment). We will also generate new gene constructs carrying genes for auxin carriers in translational fusions with photo-activable GFP (PA-GFP) for the photo-activated localization microscopy, PALM) as well as with other spectral variants of fluorescent proteins for co-localizations. We have very good experience with exchanging genes for fluorescent proteins in gene constructs. It will be also necessary to perform genotyping and qRT-PCR verifications of all mutants used in this work. For *in vivo* approaches, we have already started crossing and have collection of mutants in individual tubulin and actin genes crossed with all members of PIN family in fusion with fluorescent protein under natural promotor. In principle, there should not be any pitfalls during the generation of the set of antibodies and marker lines, but it could be that we will need to test more antibody versions generated using oligopeptides synthesized from various regions of the carrier. We have already good experience with using various antibodies.

Fluorescence scanning and wide field super-resolution microscopy of auxin carriers in the set of mutant lines

Firstly, based on our preliminary data that shows changed patterns in the root distribution of individual PINs **(Figure 1)**, we will screen for the localization of all auxin carriers using indirect immunofluorescence staining in wild type and mutant seedlings of *Arabidopsis thaliana*. We will concentrate on root, hypocotyl, cotyledons and eventually first young leaves to follow the expression domains for individual auxin carriers[2]. This primary screen will be performed by Ph.D. student (PS1) by confocal laser scanning microscopy, the attention will be concentrated on the cellular and subcellular level using immersion objectives with high magnification and numerical aperture. In parallel, *in vivo* data will be grabbed from crossings of mutants with fluorescent marker lines for all individual auxin carriers.

This mostly classical confocal fluorescence screen will be complemented with several techniques of super-resolution fluorescence microscopy. One of the main challenge of the modern fluorescence light microscopy is the improvement of the optical resolution that would break the diffraction limit down to the resolution that allows imaging of individual fluorescent molecules[31]. Within this project we plant to obtain high-resolution images using three wide-field super-resolution techniques that are already, with one exception, established in our laboratory. In particular, we will use structured illumination technique that could clearly identify fine

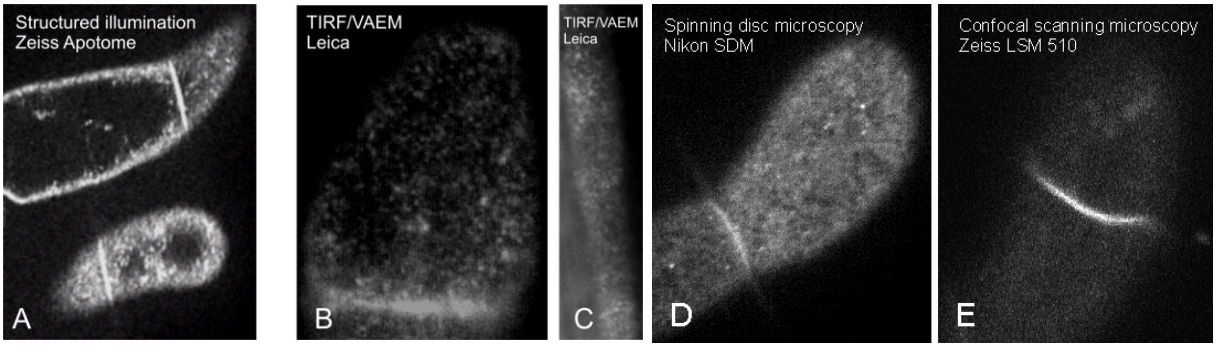

Figure 2: Distribution of PIN proteins in the plasma membrane and cortical cytoplasm *in vivo*. PIN1::PIN1:GFP in tobacco BY-2 cells **(A, B, D, E)** and PIN3::PIN3:GFP in the hypocotyl cells of *Arabidopsis thaliana* seedling. In contrast to CLSM imaging **(E)**, structured illumination on Zeiss Apotome **(A),** total internal reflection/variable angle microscopy (TIRF/VAEM) microscopy **(B, C)** or spinning disc microscopy **(D)** allows the visualization of a spectrum of endosomes and plasma membrane heterogeneity that might be tracked *in vivo* or their distribution quantified after fixation.

dotted patterns in the cortical cytoplasm of tobacco cells (**compare figure 2A and 2E**). We have experience with the Zeiss Apotome microscope, but for the purpose of the project, we will use Nikon N-SIM super-resolution system that is now installed in Prague and freely accessible for researchers from our faculty. In parallel, we have established the method for TIRF/VAEM and SDM observations in tobacco cells as well as Arabidopsis seedlings that shows even brighter spots thanks to the fact that the fluorescence is grabbed from wider axial space with very good xy resolution (**Figure 2B, 2C, 2D)**. As shown in **Figure 2** for PIN1-GFP, methods of structured illumination **(Figure 2A),** VAEM with TIRF illumination **(Figure 2B, C)** and SDM, they all show that the cortical layer of cytoplasm and plasma membrane is actually full of endosomes and structures carrying auxin carriers. These structures are normally hidden when doing classical CLSM. There is only one report on the localization of auxin carriers by scanning super-resolution technique stimulated emmision depletion (STED)[32] We speculate that using methods of super-resolution microscopy in the set of

Arabidopsis mutant described above will provide us with absolutely new quality of the information that could be subsequently quantified using image analysis and correlated with electron microscopy approaches.

To overcome possible pitfalls when using super-resolution approaches, we will pay attention on several aspects of this work. Firstly, because individual auxin carriers are distributed throughout the root, not being limited only to the surface tissues, we will combine TIRF/VAEM for scanning epidermal tissues in root, hypocotyl and leaves with N-SIM approach for grabbing images from internal tissues. SIM allows good penetration into the sample with very good speed. We will also combine this with SDM confocal technique that greatly improve the photostability of used fluorochromes and could be used for *in vivo* observations in crosses. Secondly, for fixed samples we will try to complement our observations using new method of stochastic optical reconstruction microscopy (STORM) or photo-activated localization microscopy (PALM), both techniques available in the campus. However, these techniques needs special photoactivable flurochromes that allow that only small portion of them would emit fluorescence. Therefore, we will perform this technique on several fixed samples, where we will choose proper photoswitchable secondary antibody and decide whether we will go further. N-SIM will be our first super-resolution method.

High resolution scanning electron microscopy (HR-SEM) of auxin carriers

High-resolution scanning electron microscopy (HR-SEM) is the method of choice for the detection of auxin carriers mainly from several reasons. As shown in **Figure 3A, B**, in our laboratory we are already able to prepare samples for high quality HR-SEM. The technique uses protoplasts isolated from the plant tissue (tobacco, Arabidopsis) and preparation of "membrane ghosts"[33] - the fraction of plasma membrane together with many associated proteins. Subsequent exposition of this fraction to the fixation procedure[34] give very good results that allow clear identification of many structures within the region below plasma membrane that could be easily identified based on their morphology (**Figure 3A, B**). Moreover, this technique could be combined with specific immunostainings using nanogold-labelled secondary antibodies (**Figure 3C)** or even fluoronanogold-labelled for correlative approaches combining fluorescence and electron microscopy. I am glad that Dr. Fišerová, the expert in these techniques[35] agreed to become a senior member of the team and will supervise Ph.D. student (PS2) for the immunolocalizations of auxin carriers. We plan to test the collection of Arabidopsis mutants described above for HR-SEM in the core faculty facility that is going to be opened in 2015. Besides that, we could take advantage to perform initial observation in the laboratory of Prof. Martin Goldberg (University of Durham), where Dr. Fišerová spent her postdoc.

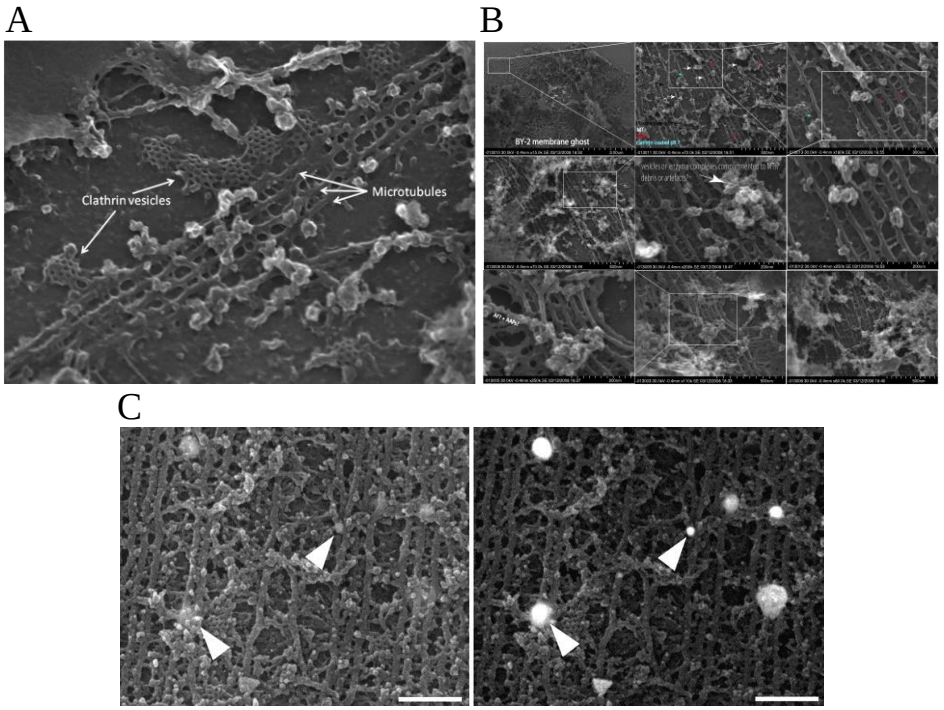

Figure 3: High resolution scanning electron microscopy (HR-SEM). (A, B) Samples from membrane ghosts isolated from tobacco BY-2 cells. Structures of cytoskeleton are well observable, including microtubules and associated clathrin vesicles **(A)**. The complexity of structures observable with HR-SEM **(B)**. (Fišerová, J., Goldberg, M. unpublished). **(C)** Immunogold staining of microtubule plus end binding protein EB1 on HR-SEM image from Tradescantia epidermal peels. Secondary electrons (left) and back-scattered electrons (right)[36].

Raster image correlation spectroscopy (RICS) of auxin carriers

To test then dynamics of auxin carriers, we will perform correlative spectroscopy in the roots of Arabidopsis thaliana mutants. Raster image correlation spectroscopy (RICS)[37] will be used for determination of the mobility of auxin influx and efflux carriers within the plasma membrane. 100 confocal images (256x256 pixels each) of plasma membrane with GFP-tagged auxin carriers (**Fig 4A**) will be collected. Fluorescence fluctuations arising from the diffusion of labeled particles at a given focal point are correlated with respect to a certain time delay. These fluctuations are correlated between different focal positions separated by the time, which the scanner needs to move from one spot to the other, i.e. the temporal shift is translated to the distance. Since the scanner samples different timescales by scanning pixels within a line, between lines, and between frames, timescales from tens of microseconds, up to seconds, can be accessed. This is characterized by to 2D autocorrelation function (**Fig 4 B**) that describe diffusion of fluorescent protein in 2D space (x, y). From this analysis it appeared that PIN1 moves faster than AUX1 in BY-2 cells (**Fig 4 B**). We have applied a model that considers transient binding of the protein molecules that interrupts the free diffusion. The model assumes that the binding time ***tau*** is significantly longer than the time a molecule would diffuse though the focal point. The correlation function was calculated and fitted with a home written macro in Matlab using Fast Fourier algorithm. Higher mobility of PIN1 was characterized by lower value of ***tau*** in PM of BY-2 cells (**Fig 4 C**). The same result was obtained for PIN2 that was more mobile (lower value of ***tau***) than AUX1 in epidermal cells of Arabidopsis roots (**Fig 4 D**). In other words, AUX1 was more transient bound (retained) than PINs (PIN1, 2) in both BY-2 and Arabidopsis model system (**Fig 4 D**).

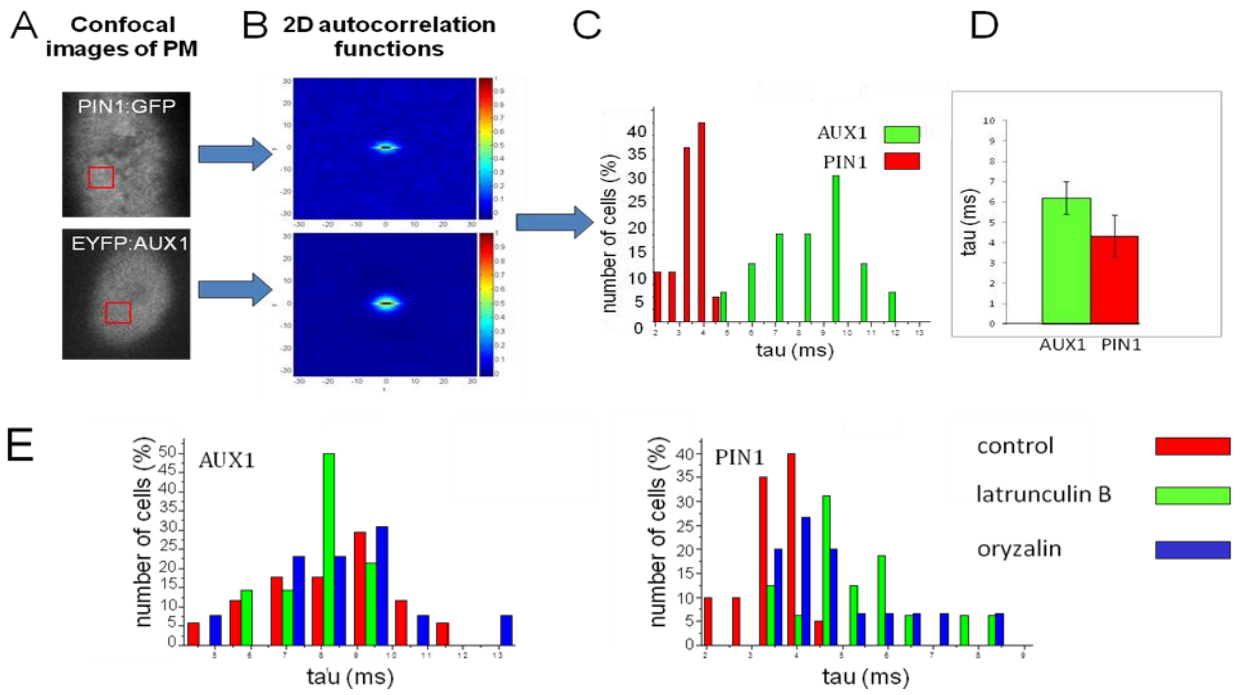

Figure 4: **Preliminary data showing the feasibility of RICS to determine mobility of auxin carriers within the region of plasma membrane**. RICS correlates fluctuations between pixels of one image in space (**A**). Higher mobility of PIN1 is here characterized by lower value of ***tau*** in the plasma membrane model BY-2 cells (**C**). The same result was obtained for PIN2 that was more mobile (lower value of ***tau***) than AUX1 in epidermal cells of Arabidopsis roots (**D**). In other words, AUX1 was more transient bound (retained) than PINs (PIN1, 2) in both BY-2 and Arabidopsis model system (**D**). Depolymerization of AFs with latrunculin B decreased the mobility of PIN1 (i.e. increased the binding time of PIN1 characterized by an increased *tau* values). The same response was observed after the depolymerization of MTs with oryzalin. In contrast, the mobility of AUX1 within the PM was not changed so remarkably after the application of latrunculin B or oryzalin (**E**). Already these results suggest that we could expect differential mobilities in our set of mutants.

The senior member of the team Dr. Jana Humpolíčková will supervise Ph.D. student (PS3) for this work. The major obstacle that might slow down our progression in this point is the fact that laser light used for RICS measurements must be coming perpendicularly to the plasma membrane. Therefore, we coudl measure easily on lateral plasma membrane or root epidermal cells, but we need to place the sample so that apical and basal

membranes will be accessible. We will solve it by special chamber or by inserting roots into agarose block that will allow orienting sample perpendicularly.

WP1/2: Testing the significance of interactions between cell structures and auxin transporters

Co-localization studies of the screened candidates in WP1/1 and auxin carriers with CLSM, SD, VAEM, SR and HR-SEM, auxin transport assays

To test how significant are changes in the localization of auxin carriers in all screened plants, we will continue in the microscopical approaches described above by testing the localization of proteins whose mutants showed remarkable differences in their localization. We do not intend to prove some tight co-localizations, they can not be expected in most cases, but rather we would like to co-immunostain these proteins with auxin carriers to see how they are organized in wild type situation. Antibodies against candidate proteins will be ordered commercially. Where possible, we will also use *in vivo* approach. Post-doc (PD1) experienced in the immunostainings will be recruited for this work.
Simultaneously, we will test the functionality of auxin efflux and influx machinery by measuring the accumulation of radiolabelled auxins[39] in the suspensions derived from individual mutants[38,39]. Measurements of auxin transport in *Arabidopsis thaliana* plants will be performed as described[40].

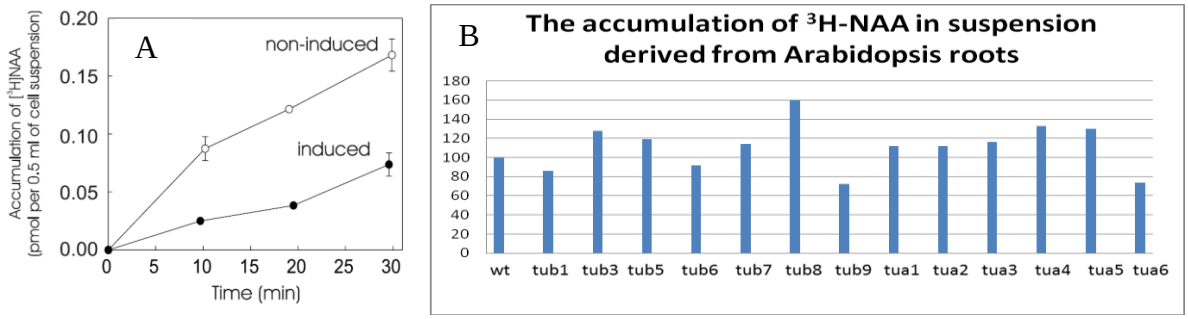

Figure 5: Auxin transport assay in suspensions derived from seedlings of *Arabidopsis thaliana* roots[38]. (A) Cells with induced PIN auxin efflux carriers shows decreased accumulations of activity thanks to the activity of auxin efflux machinery[39petrasek]. **(B)** Values for each individual knock-out mutants in individual alpha- and beta-tubulin genes are shown here to show that we are able to isolate large number of cell suspension lines from the spectrum of mutants and to determine auxin transport characteristics.

Objective 2: Identification of new determinants of auxin carrier dynamics

Immunoprecipitations in the membrane fractions from the set of mutant lines using antibodies against auxin carriers, MALDI

The same procedure starting from membrane ghosts as in HR-SEM will be used here to obtain fractions (protoplasts ghost) that will be used to isolate all proteins that are in association with plasma membrane within the cortical cytoplasm. Protoplasts will be isolated from the roots of *Arabidopsis thaliana* wild type seedlings using enzymatic digestion. The technique is already established in the laboratory[33]. We will also try to perform the same procedure with our set of mutants. By immunoprecipitations using specific antibodies against auxin carriers it will be possible to get fractions that will potentially contain interacting partners. Depending on the mutant, some of these interaction might be found to be missing or appearing. Therefore, it will be extremely important to recruite experienced post-doc that will under supervision of Dr. Schwarzerová identify interaction partners using matrix-assited laser desorption/ionization spectra (MALDI). We will also take opposite approach using antibodies against proteins that appeared to be important for the localization of auxin carriers and identify these spectra as well.

WP2/2 - Functional testing and localization of candidates identified with MALDI in WP2/1

Colocalization studies of the candidates from MALDI with auxin carriers, auxin transport assays in corresponding mutant lines.

The same approach as described in WP1/2 will be used, but here we will look specifically on the localization and effects on auxin accumulations in MALDI-identified candidates.

Objective 3: Generation of web-based catalogue of localizations, dynamics, function and interactions of auxin carriers

For this objective experienced post-doc (PD1) experienced in the immunostainings and image analysis will be recruited. Since the project will generate huge amount of images, the aim will be to improve our techniques for image analysis so that we will be able to quantify even subtle changes in the distributions of auxin carriers. We will also try to integrate data from CLSM, N-SIM and HR-SEM to perform, where possible, correlative measuremets. In addition, time-lapse in vivo data will be also quantified (**Figure 6**).

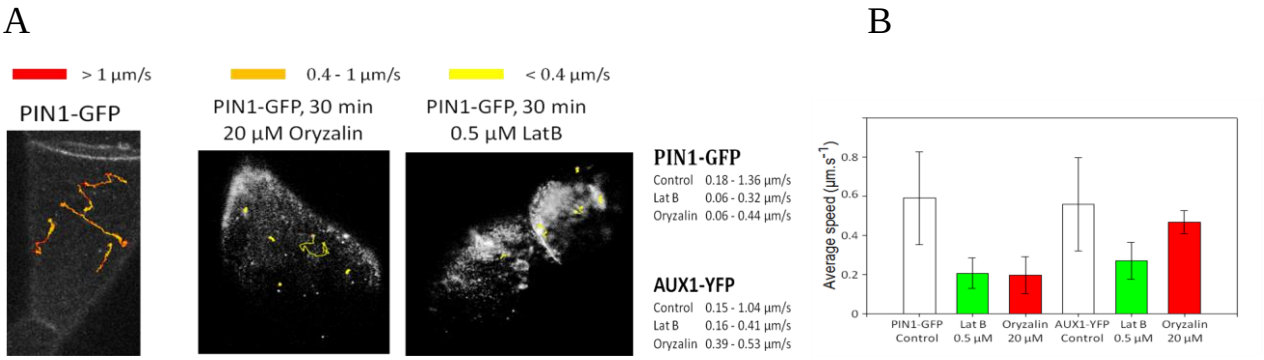

Figure 6: Preliminary results showing TIRF/VAEM microscopy of individual endosomes carrying PIN1-GFP and AUX1-YFP. (**A**) Image analysis software for particle tracking was used to follow individual trajectories of endosomes wit PIN1 and AUX1. Values describing both speed and trajectory of these endosomes decrease upon the addition of cytoskeletal drugs **(B).**

The database will allow future adding of other then auxin carriers and we will offer it for the scientific community.

Section c: Resources (including project costs)

c1: Infrastructure and equipment

The research team of the proposed project will be localized at the Department of Plant Experimental Biology, Faculty of Science, Charles University in Prague, Czech republic. I have received sufficient laboratory and office space and as an extra bonus the chance to move in several years to the newly established Bio-centre located in the campus. Starting from the second half of 2014, new laboratory devoted to cellular and developmental biology of transport of plant hormones is going to be formed under my leadership. The facilities and equipment necessary for the project are available, including greenhouse, media preparation room, laminar flow hoods and autoclaves, light and dark cultivation rooms with incubators, routine inverted and upright fluorescence microscopes, dissection microscopes, image analysis systems, equipment for molecular biology work (PCR cyclers, light cyclers for qRT-PCR) and cloning, preparative centrifuge and ultracentrifuge, refrigerators, deep-freezers, equipment for gel electrophoresis and immunoblotting, etc. The laboratory will be sharing facilities with departments performing research in biological sciences, including core facilities for microscopical imaging, flow cytometry, mass spectrometry, and gene sequencing. Departments of the Biological section at the Faculty of Science of the Charles University belong to leading research and teaching centres in the Czech Republic. Moreover, proposed project will be profiting from contacts with the Laboratory of hormonal regulations in plants in the Institute of Experimental Botany, ASCR, Prague, where I have been leading the group of auxin transport for several years. In particular, the technique of measuring auxin transport in suspension-cultured cells and Arabidopsis seedlings and spinning disc microscopy will be performed there.

This mostly microscopically-oriented project proposal benefits from the existence of high-end imaging facilities that are all located in Prague, either in the campus itself (CLSMs, PALM/STORM, SD) or at other places in Prague that belong to the joint imaging facility of Faculty of Science and Institute of Molecular Genetics and that is now associated with Eurobioimaging network (VAEM/TIRF, SR N-SIM, HR-SEM). RICS microscopy will be performed at Heyrovský Institute of Physical Chemistry ASCR in Prague. Members of the proposed team have already practical experience with the majority of all these equipments. I do not propose to buy any expensive equipment for this project.

c2: Team composition

The team of proposed project will consist of PI, 3 senior staff experienced researchers, 3 PhD students and 2 post-docs, both these categories recruited for 3 years to contribute to individual work packages. The involvement of individual members of the team in respective work packages (WP) is specified below and in the table 1 showing the schedule of the project. I will try to keep planned capacities according to the schedule, but depending on the progression of the project, modifications might be expected.

Jan Petrášek, Ph.D. (JP), **Principal Investigator (capacity for the project 80%).** My role will be in the scientific coordination and administration of the project. I will coordinate the research of all team members and supervise students and post-docs. I will be fully responsible for all kind of reporting and writing manuscripts.

Kateřina Schwarzerová, Ph.D. (KS), member of the team (capacity for the project 30%). Kateřina Schwarzerová is the internal member of Department of Plant Experimental Biology, Faculty of Science, Charles University in Prague. Her strong background in the molecular biology and biochemistry of cytoskeletal proteins, cell fractionations, immunoprecipitations, co-sedimetations and 2-D electrophoresis[33] will be used especially in WP2/1 and WP1/1. She will co-supervise students involved in WP1/1 and post-doc in WP2/1 (PD3).

Jindriška Fišerová, Ph.D. (JF), **member of the team (capacity for the project 20%)**. During her postdoctoral stay in the laboratory of Dr. Goldberg at Durham University Jindřiška Fišerová successfully optimized techniques for HR-SEM observations of preparations obtained from various materials including plant membrane ghosts[34] and the combination with immunogold stainings[35]. She is the member of the research group of Prof. Hozák in the Institute of Molecular Genetics, ASCR in Prague. She will be recruited for the purpose of the project for 20% to co-ordinate and co-supervise PhD student and post-docs (PS2, PD1) mainly in WP1/1, 1/2 and 2/2.

Jana Humpolíčková, Ph.D. (JH), **member of the team (capacity for the project 20%)**. Jana Humpolíčková is expert in the methods of advanced fluorescence microscopy and for the project she will guarantee the fluorescence spectroscopic techniques FCS and RICS[37]. She is the member of the research group of Prof. Hof at the J. Heyrovský Institute of Physical Chemistry ASCR in Prague. She will be recruited for the purpose of the project to process microscopical data (WP 1/1) and supervise Ph.D. students in WP1/1 (PS3).

Table 1: Schedule of the project

Year	**1**		**2**		**3**		**4**		**5**	
Month	**1-6**	**7-12**	**13-18**	**19-24**	**25-30**	**31-36**	**37-42**	**43-48**	**49-54**	**55-60**
Objective 1	**Searching for cell structure determinants of the localization and dynamics of auxin carriers**									
WP 1/1	**Screening for structural determinants of the localization and dynamics of auxin carriers**									
Generation of molecular toolbox for indirect immunofluorescence and *in vivo* microscopy of auxin carriers	JP/KS/ JF	JP/KS/ JF/PS1 PS2/ PS3	JP/KS/ JF/PS1 PS2/ PS3	JP/KS/ JF/PS1 PS2/ PS3	JP/KS/ JF/PS1 PS2/ PS3	JP/KS/ JF/PS1 PS2/ PS3				
Fluorescence scanning and wide field super-resolution microscopy of auxin carriers in the set of mutant lines	JP	JP/KS/ PS1	JP/KS/ PS1	JP/KS/ PS1	JP/KS/ PS1	JP/KS/ PS1	JP/KS/ PS1	JP/KS	JP	
High-resolution scanning electron microscopy (HR-SEM) of auxin carriers in mutant lines		JP/JF/ PS2	JP/JF/ PS2	JP/JF/ PS2	JP/JF/ PS2	JP/JF/ PS2	JP/JF/ PS2	JP/JF	JP/JF	
Raster image correlation spectroscopy (RICS) of auxin carriers in mutant lines	JP/JH	JP/JH/ PS3	JP/JH/ PS3	JP/JH/ PS3	JP/JH/ PS3	JP/JH/ PS3	JP/JH/ PS3	JP/JH		
WP 1/2	**Testing the significance of interactions between cell structures and auxin carriers**									
Co-localization studies of candidates from WP1/1 and auxin carriers with CLSM, SD, VAEM, SR and HR-SEM				JP/JF/ KS	JP/JF/ KS /PD1	JP/JF/ KS /PD1	JP/JF/ KS /PD1	JP/JF/ KS /PD1	JP/JF/ KS /PD1	JP/JF/ KS /PD1
Auxin transport assays in the set of mutant lines			JP/ PD2	JP/ PD2	JP/ PD2	JP/ PD2	JP/ PD2	JP/ PD2		
Objective 2	**Identification of new determinants of auxin carrier dynamics**									
WP 2/1	**Immunoprecipitation assays from the set of mutant lines**.									
Immunoprecipitations in the membrane fractions from the set of mutant lines using antibodies against auxin carriers, MALDI	JP/KS/ PD3	JP/KS/ PD3	JP/KS/ PD3	JP/KS/ PD3	JP/KS/ PD3					
WP 2/2	**Functional testing and localization of candidates identified with MALDI in WP2/1**									
Co-localization studies of the candidates from MALDI and auxin carriers with CLSM, SD, VAEM, SR and HR-SEM			JP/KS/ JF	JP/KS/ JF/ PD1	JP/KS/ JF/ PD1	JP/KS/ JF/ PD1	JP/KS/ JF/ PD1	JP/KS/ JF/ PD1	JP/KS/ JF/ PD1	JP/KS/ JF/
Auxin transport assays in MALDI-identified candidate mutant lines			JP/ PD2	JP/ PD2	JP/ PD2	JP/ PD2	JP/ PD2	JP/ PD2		
Objective 3	**Generation of web-based catalogue of localizations, dynamics, function and interactions of auxin carriers**									
WP 3/1					JP/KS/ JF /PD1	JP/KS/ JF /PD1	JP/KS/ JF /PD1	JP/KS/ JF /PD1	JP/KS/ JF /PD1	JP/KS/ JF
Image analysis of immunostained and in vivo samples										
WP3/2							JP/KS/ JF /PD1/ PD2	JP/KS/ JF /PD1/ PD2	JP/KS/ JF/JH/ PD1	JP/KS/ JF/JH
Generation and maintenance of the database and web										

Post-doc 1 (PD1, 3 years, months 19-54) – post-doc with strong backgrounds in microscopy and image analysis, colocalizations and in vivo particle tracking will be recruited. He/she will be involved in the quantification of images obtained in this project and WP 1/2, 2/2 and 3/1.

Post-doc 2 (PD2, 3 years, months 13-48) – mainly for WP 1/2 and 2/2 this post-doc will be recruited to perform auxin transport assays in cell suspension and seedlings and mutant phenotype analysis. He/she should be very strong in plant physiology and hormonal regulations.

Ph.D. student 1 (PS1, 3 years, months 7-42) - this Ph.D. student will be involved in the WP1/1 and will search for the cytoskeletal, cell wall and sterol determinants of localization of auxin carriers in

Arabidopsis seedlings using fluorescence scanning and wide field super-resolution microscopy.
Ph.D. student 2 (PS2, 3 years, months 7-42) – this Ph.D. student will perform HR-SEM microscopy of auxin carriers in membrane ghosts and epidermal peels from the set of cytoskeletal mutants and mutants with affected biogenesis of cell wall and sterol biosynthesis (WP1/1).
Ph.D. student 3 (PS3, 3 years, months 7-42) – this Ph.D. student will study the localization of auxin carriers with fluorescence spectroscopic methods (RICS) in the set of cytoskeletal, cell wall and sterol biosynthesis mutants.
Laboratory technician/manager (5 years) – the technician will be responsible for running the laboratory, ordering, keeping laboratory protocols and procedures and databases in good shape. He/she will be under direct supervision of the PI.

c3: Project costs

Direct costs of the project are planned according to the table 2. Personnel costs are calculated according to rules valid in the Czech Republic and reflects capacities planned for PI (80%) and senior staff (KS 30%, JF 20%, JH 20%). Ph.D. students, post-docs and technician/lab manager are planned for their full capacities. Travel costs are planned to cover regular participations of PI and team members on the scientific conferences and seminars and visits of collaborating laboratories (5000,-€/year), costs for equipment are planned for necessary small laboratory equipment (shakers, mini-centrifuge, pipettes, etc.; 2000,-€/year). Other goods and services cover consumables (chemicals, radio-chemicals, molecular biology kits, enzymes, inhibitors, laboratory plastic and glass, etc.; calculated as 14000,-€/year/PI and senior staff), fees connected with publication in open access journals and printing posters (1200,-€/year) and other costs are primarily costs of buying scientific monographs (500,-€/year). Other Direct Costs with no overheads are planned for covering expenses connected with the work on microscopes of collaborating institutions (6000,-€/year).

Table 2: Budget table

Cost Category			**Total in Euro**
Direct Costs	**Personnel**	PI (80%)	140,000
		Senior Staff	115,000
		Postdocs	250,000
		Students	216,000
		Other (lab manager)	70,000
	i. Total Direct costs for Personnel (in Euro)		791,000
	Travel		25,000
	Equipment		10,000
	Other goods and services	Consumables	280,000
		Publications (including Open Access fees), etc.	6000
		Other (scientific books)	2500
	ii. Total Other Direct Costs (in Euro)		323,500
A – Total Direct Costs (i + ii) (in Euro)			1,114,500
B – Indirect Costs (overheads) 25% of Direct Costs (in Euro)			278,625
C1 – Subcontracting Costs (no overheads) (in Euro)			0
C2 – Other Direct Costs with no overheads (in Euro)			30,000
Total Estimated Eligible Costs (A + B + C) (in Euro)			1,423,125
Total Requested EU Contribution (in Euro)			1,423,125

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